Long-Term Effects Of Juvenile Fluoxetine Exposure On Autophagy Induction And Phosphorylated Tau In The Hippocampus And Prefrontal Cortex Of Male Mice

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LONG-TERM EFFECTS OF JUVENILE FLUOXETINE EXPOSURE ON AUTOPHAGY
INDUCTION AND PHOSPHORYLATED TAU IN THE HIPPOCAMPUS AND
PREFRONTAL CORTEX OF MALE MICE

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Para Susana y Rafael, los quiero mucho.
LONG-TERM EFFECTS OF JUVENILE FLUOXETINE EXPOSURE ON AUTOPHAGY INDUCTION AND PHOSPHORYLATED TAU IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX OF MALE MICE

by

MINERVA RODRIGUEZ

THESIS

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Abstract

Fluoxetine (FLX) represents the antidepressant of choice for the management of pediatric mood-related illnesses. Accumulating preclinical evidence suggests that ontogenic FLX exposure leads to deregulated affect-related phenotypes in adulthood. Mood-related symptomatology constitutes a risk-factor for various neurological disorders, including Alzheimer’s disease (AD), making it possible for juvenile FLX history to exacerbate the development of neurodegenerative diseases. Because AD is characterized by the pathological accumulation of hyperphosphorylated Tau – which can result from impaired function of protein degradation pathways, such as autophagy and the ubiquitin-proteasome system (UPS), this thesis evaluated the potential long-term effects of adolescent FLX exposure on these pathways, using mice as a model system. Specifically, C57BL/6 adolescent male mice were administered FLX (20 mg/kg/day) during adolescence (postnatal days [PD] 35-49). After a 21-day washout period, mice were euthanized (PD70) and, using immunoblotting analysis, we evaluated protein markers of autophagy (Beclin-1, LC3-II, p62) and the UPS (K48-pUb), as well as AD-associated forms of phosphorylated Tau, within the hippocampus and prefrontal cortex. We found that FLX exposure altered protein expression of autophagy and UPS markers in both the hippocampus and prefrontal cortex. Specifically, that juvenile FLX exposure facilitated autophagy activation mostly in the adult prefrontal cortex (increased LC3-II and decreased p62). Lastly, while K48-pUb was not affected, antidepressant history mediated a long-lasting increase of phosphorylated Tau in both brain regions assessed, suggesting that exposure to FLX during adolescence may increase the expression of neurodegenerative markers in adulthood.
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Introduction

Affective disorders, including depression and anxiety, are a leading cause of disability, affecting over 300 million people worldwide [Bernaras et al., 2019]. This is further compounded by their chronic and recurrent nature, comorbidity with other psychiatric conditions [Salcido et al., 2022; Saccaro et al., 2021], as well as the limited efficacy of available pharmacological treatments, which primarily consist of selective serotonin reuptake inhibitor (SSRI) medications [Jacob, 2012; Kantor et al., 2015]. In the pediatric population, depression can have devastating effects, such as poor academic performance, substance abuse, and suicide [Mullen, 2018]. Indeed, the incidence of depression in adolescents has increased in recent years, and consequently, prescription of antidepressant medications in this vulnerable group has also increased [Meng et al., 2014; Mojtabai et al., 2016; Sparks & Duncan, 2013; Trujillo & Iniguez, 2020].

Fluoxetine (FLX), a SSRI antidepressant medication, is approved by the Food and Drug Administration (FDA) for the treatment of both depression and anxiety disorders in children and adolescents [Perez-Caballero et al., 2014; Garland et al., 2016]. FLX’s primary mechanism of action is enhancing serotonin (5-HT) transmission - mostly by blocking the uptake activity of the serotonin transporter (SERT) [Perez-Caballero et al., 2014; Fuller, 1995]. Indeed, competitive binding assays have shown that FLX displays a strong affinity for the SERT and weaker affinity for dopamine and noradrenaline transporters, indicating that it is mostly serotonin selective. Few reports also indicate that FLX binds directly to serotonin receptors with relatively poor affinity [Wong et al., 1983]. Because FLX increases global levels of serotonin, it is not clear which of the serotonin receptors may be responsible for its antidepressant properties. Interestingly, preclinical evidence suggests that the 5-HT$_{2B/2C}$ receptors play a significant role in mediating FLX’s therapeutic effects [Albert et al., 2014]. Rat studies have shown that FLX decreases the number of
5-HT$_{2B}$ receptor sites while reversing behaviors associated with depression-related memory dysfunction [Meneses, 2007]. Likewise, prior work has corroborated that the therapeutic effects of FLX can be potentiated by pharmacological inhibition of 5-HT$_{2B}$ receptors [Diaz et al., 2012]. However, the specific serotonin receptor-mediated downstream molecular mechanisms that induce an antidepressant response [Iniguez et al., 2019], following FLX treatment, remains poorly understood.

Clinical studies have provided evidence for the successful use of FLX in the pediatric population due to its long half-life and fewer side effects when compared to older tricyclic antidepressant agents (i.e., indigestion, weight changes, anxiety) [Bridge et al., 2007; Hopkins & Gelenberg, 1995]. However, factors such as safety, limited , as well as long-term neurodevelopmental effects [Bowman & Daws, 2019; El Marroun et al., 2014; Amitai et al., 2020], along with increases in suicidal ideation and behavior in young populations, are still of concern. For this reason, in 2004, the FDA issued a “black-box” warning label when administering this medication to adolescents regarding the risk of suicidal ideation [Newman, 2004; Olivier et al., 2011].

When assessing the long-term effects of adolescent antidepressant treatment, preclinical studies have uncovered that developmental exposure to FLX results in undesired neurobehavioral consequences in adulthood [23]. For example, Iniguez and colleagues found that exposing adolescent male mice with FLX (10 mg/kg, for 15 days) changed responses to aversive stimuli in adulthood (PD70). Specifically, FLX history suppressed despair-like behavior (in forced swim and social defeat stress paradigms) while increasing anxiety-like responses in the elevated plus-maze [Iniguez et al., 2014; Iniguez et al., 2010; Flores-Ramirez et al., 2021]. Because changes in responses to stress are highly associated with memory performance, researchers have also
evaluated how adolescent exposure to FLX influences memory in adulthood. Here Sass and colleagues found that spatial memory performance in a Morris water maze task was impaired in adulthood as a function of juvenile FLX history in male Wistar rats (Sass & Wortwein et al., 2012). More recently, our research team found supporting evidence of spatial memory impairment following FLX history in male C57BL/6 mice (Flores-Ramirez et al., 2019). Interestingly, FLX mediates long-term changes in both gene expression [Iñiguez et al., 2021; Kroeze et al., 2015] as well as protein expression and phosphorylation [Flores-Ramirez et al., 2021; Sherstha et al., 2014] within the hippocampus, a brain region highly associated with spatial memory – suggesting that adolescent FLX pre-exposure results in long term memory impairment along with neurobiological alterations within the hippocampus. However, the precise cellular and molecular mechanisms underlying the long-term neurobehavioral effects of developmental FLX exposure are not fully understood (Olivier et al., 2011).

Protein homeostasis (proteostasis) is a highly complex network of molecular mechanisms responsible for strictly regulating cellular processes controlling proteomic stability, including protein synthesis, folding, trafficking, localization, and degradation [Balchin et al., 2016; Klaips et al., 2018]. As part of the proteostasis network, two major pathways participate in the degradation of proteins: autophagy and the ubiquitin-proteasome system (UPS) [Lim & Yue, 2015]. Macropath, also known as autophagy, is a cellular degradative system that employs double-membrane vesicles, known as autophagosomes, that deliver cytoplasmic cargo into the lysosome for degradation [Levine & Kroemer, 2018]. The UPS is a protein degradation pathway that uses highly specific ubiquitin signals to tag proteins for degradation via the 26S proteasome, a multicatalytic complex with endoprotease activity [Murata et al., 2019; Diaz-Villanueva et al., 2015]. While autophagy is responsible for the degrading of long-lived, membrane-bound, and
aggregated proteins, as well as large protein complexes and even whole organelles [Menzies et al., 2015], the UPS degrades short-lived, soluble, and misfolded proteins [Diaz-Villanueva et al., 2015]. Proper function of protein degradation pathways is particularly important for neurons, as it has been demonstrated that impairment of autophagy and the UPS leads to accumulation of neurotoxic, misfolded, and aggregated proteins, which are the hallmark of several neurodegenerative disorders, such as Alzheimer’s disease (AD) [Plassman et al., 2007; Ciechanover & Kwon, 2015; Kundra et al., 2020; Tanaka & Matsuda, 2014]. Accumulation of Tau, a cytoskeletal protein, constitutes one of the major neuropathological markers in the brains of AD patients, as well as in other neurological disorders collectively termed Tauopathies [Arendt et al., 2016; Orr et al., 2017]. Under physiological conditions, Tau binds to and stabilizes the microtubule cytoskeleton, facilitating axonal transport and neuronal polarity [Mietelska-Porowska et al., 2014]. Tau function is regulated by phosphorylation, and in the diseased brain, hyperphosphorylation impairs its normal role, causes its accumulation and aggregation into insoluble structures, and ultimately becomes neurotoxic [Hanger et al., 2009; Xia et al., 2015]. Importantly, both normal and pathological variants of Tau are degraded via autophagy and the UPS [Caccamo et al., 2010; Dantuma & Bott, 2014].

Epidemiological evidence indicates that a lifetime history of affect-related illnesses significantly increases the risk of developing AD [Byers & Yaffe, 2011]. For example, postmortem studies have shown that neurofibrillary tangles, pathological structures composed of hyperphosphorylated Tau, are present in greater numbers in the brains of AD patients with co-morbid depression [Rapp et al., 2016; Rapp et al., 2008; Brown et al., 2016], suggesting that accumulation of hyperphosphorylated Tau could be a common pathological factor between both disorders. Furthermore, emerging data points to a probable link between impairment of protein
degradation pathways and the pathology of depression [Gassen et al., 2015; Minelli et al., 2015; Rein, 2019] and SSRI antidepressants have been shown to modulate autophagic flux [Gulbins et al., 2018], regulate expression of proteasomal components [Park et al., 2017], and decrease Tau phosphorylation [Yang et al., 2014]. Nevertheless, how developmental FLX can alter protein degradation pathways that impact the accumulation of abnormal proteins in specific brain regions has not been previously addressed. Therefore, the current thesis aimed to determine whether adolescent FLX exposure could cause persistent alterations in the autophagy and UPS protein degradation pathways that are accompanied by accumulation of abnormal forms of Tau in the hippocampus and prefrontal cortex of adult C57BL/6 mice – given that these brain regions regulate affect-related behavior, as well as SSRI antidepressant response [Duric et al., 2013; Sierra-Fonseca et al., 2019; Elsayed et al., 2012; Duman & Duman, 2015; Iñiguez et al., 2019]. To accomplish this, we employed immunoblotting analysis to evaluate the levels of several autophagic protein markers that represent distinct steps along the degradative pathway, namely LC3-II, p62, and beclin-1 [Tanida et al., 2008; Nascimento-Ferreira et al., 2011; Sahani et al., 2014], as well as the presence of lysine 48-linked polyubiquitinated (K48-pUb) proteins, a highly specific signal for proteasomal-mediated degradation [Hill et al., 2016]. Furthermore, we also assessed the accumulation of two forms of phosphorylated Tau; at threonine 231 (pTau-T231) and at serine 262 (pTau-S262) given their implication in the pathology of AD [Bertrand et al., 2010; Hampel et al., 2010]. Since previous work from our lab indicates that juvenile exposure to FLX impairs spatial memory in adult male mice [Flores-Ramirez et al., 2019] we hypothesized that antidepressant exposure during adolescence, a critical period of development, would increase autophagy protein marker activation and increase Tau accumulation in both the hippocampus and the prefrontal cortex of the brain.
2.1 Animals: Male C57BL/6 mice (Charles River, Hollister, CA) were utilized in this study. Mice were housed in a colony room under a 12-h light/dark cycle (lights on at 7:00 A.M.), with the temperature between 21–23 $^{\circ}$C, and with access to food and water ad libitum. Experiments were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals [Council, 2003], the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines [Percie et al., 2020], and with the approval of the Institutional Animal Care and Use of Committee at The University of Texas at El Paso (Protocol #955664-14).

2.2 Drug treatment and experimental design: Fluoxetine hydrochloride (FLX) was purchased from Spectrum Chemicals (#F1200-25G; Gardena, CA) and dissolved in distilled sterile water [vehicle (VEH)]. Animals were administered VEH or FLX via intraperitoneal (IP) injections, using a volume of 2 mL/kg. Specifically, male C57BL/6 mice (N=20; 10 animals per group) received either distilled sterile water (VEH) or FLX during the adolescent period of development (postnatal days [PD] 35-49). Following antidepressant treatment, mice had a 21-day washout period where no injections were administered and were subsequently euthanized on PD70 (see Figure 1 for experimental timeline). The age window for drug exposure (PD35-49) was selected because it is roughly equivalent to adolescence in humans [Andersen, 2003; Abreu-Villaca et al., 2010]. Lastly, the 20 mg/kg/day dosage of FLX was selected because our prior work [Iniguez et al., 2010; Flores-Ramirez et al., 2019; Iniguez et al., 2015] has demonstrated that this dose results in an antidepressant-like effect in animal models for the study of affect-related behaviors [LaPlant et al., 2010; Surget et al., 2011; Krishnan & Nestler, 2011; Warren et al., 2020; Duque-Wilkens et al., 2020].
2.3 Electrophoresis and Western Blotting: Immunoblotting was conducted as previously described (Kroeze et al., 2015; Warren et al., 2020). Specifically, samples with equal amounts of protein (~10μg) were dissolved in Laemmli buffer containing 5% Beta-mercaptoethanol (BioRad, #1610737) and subjected to sodium dodecyl sulfate-polyacrilamide gel electrophoresis (8-16% gradient) using pre-cast gels (BioRad, #1610710; Hercules, CA). Following this step, the precast gels were transferred onto polyvinylidene difluoride membranes (BioRad, #1704159). The membranes were then incubated in blocking buffer containing 5% non-fat dry milk dissolved in TBST (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 hr at room temperature, followed by an overnight incubation at 4°C with primary antibodies dissolved in blocking buffer. Membranes were washed with TBST and incubated with appropriate HRP-conjugated secondary antibodies for 1 hr at room temperature. Chemiluminescent technique using the Clarity Western Substrate kit (BioRad, #1705061) was employed to visualize protein bands in a ChemiDoc XRS+ imaging system (BioRad). Next, the membranes were subsequently stripped with Restore Stripping Buffer (ThermoFisher Scientific, #46430; Rockford, IL) and re-probed with anti-α-tubulin or anti-actin antibodies to serve as loading control. Densitometric analysis of protein bands were performed with the ImageJ software (National Institutes of Health).

2.4 Tissue collection and homogenization: Brain samples were homogenized in Tissue-Protein Extraction Reagent buffer (ThermoFisher Scientific, #78510; Rockford, IL) supplemented with commercially available protease and phosphatase inhibitor cocktail tablets (Roche, #04693116001, #049068445001; Indianapolis, IN), using an ultrasonic homogenizer. Homogenates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C, and the supernatant was collected as total homogenate and stored at 80°C. Protein concentration in the samples was
determined with bicinchoninic acid method (ThermoFisher Scientific, #23225; Rockford, IL), using bovine serum albumin as standard.

2.5 Antibodies: Primary antibodies for Western blotting included anti-LC3 (#3868), anti-beclin-1 (#3495), anti-K48-linkage specific polyubiquitin (#8081), and anti-total Tau (D1M9X; #46687) from Cell Signaling Technology (Danvers, MA); anti-p62 (#ab56416), anti-pTau-T231 (ab151559), and anti-pTau-S262 (#ab131354) from Abcam (Cambridge, MA); anti-actin (#MA5-11869) from ThermoFisher Scientific (Rockford, IL), and anti-α-tubulin (DM1A; #T9026) from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated anti-rabbit (#7074) and anti-mouse (#7076) secondary antibodies were from Cell Signaling Technology.

2.6 Statistical analysis: Differences in the expression of protein markers were determined using two-tail Student’s t tests. Significance was considered as p<0.05.
Chapter 3: Results

3.1 Adolescent FLX exposure caused different autophagic responses in the hippocampus and prefrontal cortex of adult male mice

Figure 2 displays the effects of adolescent FLX exposure on the expression of autophagy protein markers in the hippocampus (N=20; 10 per group) and prefrontal cortex (N=19; 9 in VEH group, 10 in FLX group). In the adult hippocampus, a two-tailed Student’s $t$ test indicated that adolescent FLX exposure did not influence the expression of the autophagy initiator beclin-1 ($p>0.05$; Fig. 2B). However, adolescent FLX history significantly increased LC3-II ($t_{18} = 2.90$, $p<0.05$; Fig. 2C) but not p62 ($p>0.05$; Fig. 2D) in the hippocampus of adult male mice. In the prefrontal cortex (Fig. 2E-H), adolescent FLX pretreatment did not alter beclin-1 expression in adulthood ($p>0.05$; Fig. 2F) but led to a significant increase of LC3-II ($t_{17} = 4.00$; $p<0.05$; Fig. 2G) while significantly decreasing p62 ($t_{17} = 3.259$; $p<0.05$; Fig. 2H). No differences were observed in actin between experimental groups in either brain region ($p>0.05$).

3.2 Adolescent FLX exposure did not affect k48-pUb proteins in the hippocampus and prefrontal cortex of adult mice

Figure 3 displays the effects of adolescent FLX exposure on a highly specific signal for proteasome degradation, K48-pUb. A two-tailed Student’s $t$ tests indicate accumulation of proteins tagged with the K48 linkage signal were not affected by FLX history in either hippocampus ($p>0.05$; Fig. 3A, B) nor prefrontal cortex ($p>0.05$; Fig. 3C, D). No differences were observed in tubulin between experimental groups in either brain region ($p>0.05$).
3.3 Adolescent FLX exposure induces accumulation of phosphorylated Tau in the hippocampus and prefrontal cortex of adult male mice

**Figure 4** displays the effects of adolescent FLX exposure on accumulation of phosphorylated Tau forms in the hippocampus and prefrontal cortex. A two-tailed Student’s *t* tests indicate that adolescent FLX exposure led to significant increased levels of pTau-231 (*t*<sub>18</sub> = 3.51; *p*<0.05; Fig. 4B) without affecting pTau-S262 (*p*>0.05; Fig. 4C) or total Tau levels (*p*>0.05; Fig. 4D) in the adult hippocampus. In the prefrontal cortex, when normalized to tubulin, adolescent FLX exposure increased phosphorylation of pTau-T231 (*t*<sub>17</sub> = 2.197; *p*<0.05; Fig. 4F) and pTau-S262 (*t*<sub>17</sub> = 2.518; *p*<0.05; Fig. 4G) while also increasing the levels of total Tau (*t*<sub>17</sub> = 3.541; *p*<0.05; Fig. 4H). However, when normalized to total Tau, levels of phosphorylated Tau did not change as a function of adolescent FLX pretreatment in the hippocampus (*p*>0.05, respectively; Fig. 4B-C, right panels) or prefrontal cortex (*p*>0.05, respectively; 4F-G, right panels). Importantly, no differences in tubulin were noted between the experimental groups in the hippocampus (*p*>0.05) or prefrontal cortex (*p*>0.05).
Chapter 4: Discussion

4.1 Summary

The goal of the current investigation was to evaluate the enduring effects of adolescent FLX exposure on two major protein degradation pathways, autophagy, and the UPS, within the hippocampus and prefrontal cortex of adult male mice. A secondary goal was to determine if adolescent FLX pretreatment leads to an enduring abnormal accumulation of AD-associated forms of Tau within these two brain regions. We exposed adolescent C57BL/6 male mice to FLX (PD35–49) and then gave them a 21-day resting period (Fig. 1). Once these mice reached adulthood (PD70), hippocampal and prefrontal cortex tissue was collected for immunoblotting analysis. We found that adolescent FLX treatment differentially altered autophagic protein markers, while not affecting the accumulation of polyubiquitinated proteins targeted for UPS-mediated degradation and increasing levels of phosphorylated Tau in the hippocampus and prefrontal cortex of adult C57BL/6 male mice.

4.2 The long-term effects of adolescent FLX exposure on autophagy protein markers in adult male mice

To evaluate the long-term effects of juvenile FLX exposure on autophagic function, we examined the expression of protein markers representative of different steps of the pathway within the adult hippocampus and prefrontal cortex. First, we measured the levels of beclin-1, an adaptor protein expressed during autophagy initiation [Kang et al., 2011]. However, we did not detect any differences in adult beclin-1 expression because of juvenile FLX exposure in either the hippocampus (Fig. 2B) or prefrontal cortex (Fig. 2F). Next, we measured levels of the type II form of LC3 (LC3-II), which is known to be specifically associated with the autophagosomal
membrane, and thus serves as a reliable marker of autophagosome formation [Hansen & Johansen, 2011; Runwal et al., 2019; Mizushima & Yoshimori, 2007]. Here, we found that LC3-III expression was increased because of juvenile FLX in both the hippocampus (Fig. 2C) and prefrontal cortex (Fig. 2G), an observation that is consistent with autophagy activation. We also assessed levels of p62, a prototypical autophagy receptor protein (and substrate of the autophagy pathway) and found that adolescent FLX exposure exerted effects in only one brain region assessed. While hippocampal p62 was not affected (Fig. 2D), p62 was significantly decreased in the prefrontal cortex as a function of adolescent FLX exposure (Fig. 2H)—an indication of autophagy induction, given that p62 is engulfed inside the autophagosomes and degraded by the lysosome when autophagy proceeds to completion, and thus, decreased p62 expression is generally suggestive of successful autophagic flux [Mizushima et al., 2010].

4.3 The long-term effects of adolescent FLX exposure on accumulation of phosphorylated Tau markers in adult male mice

To determine if the observed alterations of the autophagy pathway induced by juvenile FLX were accompanied by accumulation of abnormal proteins into adulthood, we measured two AD-associated forms of phosphorylated (p) Tau, pTau-T231 and pTau-S262. In the hippocampus, FLX pretreatment significantly increased levels of pTau-T231 (Fig. 4B, left panel) without affecting Tau phosphorylation at S262 (Fig. 4C). However, in response to adolescent FLX, we observed that the prefrontal cortex of adult male mice accumulated significantly higher amounts not only of both forms of phosphorylated Tau (Fig. 4F, G, left panels), but also levels of total Tau (Fig. 4H), which have also been shown to be linked to AD [Barton et al., 1990; Sjogren et al., 2001]. The observed profile of sustained increase of phosphorylated and total Tau in the adult
prefrontal cortex because of FLX pre-exposure is consistent with our evidence of autophagy induction in this same brain region, as supported by the observed increase in LC3-II (Fig. 2G) and decrease in p62 (Fig. 2H). Interestingly, in an inducible cell model of tauopathy that develops toxic Tau aggregation, toxicity can be rescued by clearing the aggregates via activation of the autophagy pathway and not the UPS [Wang et al., 2010], which is consistent with our findings of increased Tau phosphorylation with autophagy induction and absence of UPS involvement. In addition, pharmacological activation of autophagy in a transgenic mouse model of human Tauopathy was shown to significantly decrease Tau aggregation [Schaeffer et al., 2012].

4.4 The long-term effects of adolescent FLX exposure on k48-pUb proteins in adult male mice

To assess a possible alteration of the UPS function in response to juvenile FLX treatment, we also measured the levels of K48-pUb proteins, a highly specific signal that targets proteins for degradation via the 26S proteasomal complex [Hill et al., 2016] in the hippocampus and prefrontal cortex of adult male mice. Our results indicate that UPS function does not appear to be sensitive to the enduring effects of juvenile FLX in these two brain regions, given that adult levels of proteins targeted for UPS-mediated degradation were not affected by the treatment (Fig. 3). While it is well established that proteasomal inhibition can lead to accumulation of toxic levels of abnormal Tau [Keller et al., 2000], our data shows that the increased levels of phosphorylated Tau observed in response to juvenile FLX exposure are not accompanied by significant accumulation of proteins targeted for proteasomal degradation, therefore indicating normal proteasomal function. A possible explanation for these observations comes from in vitro evidence demonstrating that levels of phosphorylated Tau need to reach a certain threshold to inhibit the
UPS, and moderate levels of phosphorylated Tau do not cause proteasomal function impairment [Ren et al., 2007].

Preclinical evidence indicates that juvenile FLX exposure is linked to dysregulated affect-related phenotypes in adulthood, as highlighted by the presence of a complex behavioral profile that includes memory impairment [Sass & Wortein, 2012; Flores-Ramirez et al., 2019] enhanced sensitivity to anxiety-like environments [Iñiguez et al., 2012], changes in despair-like behavior [Boulle et al., 2016; Homberg et al., 2011], and altered reward sensitivity [Iñiguez et al., 2015]. In our model of adolescent FLX exposure, we observed an effect of sustained autophagy activation and increased Tau phosphorylation into adulthood after a 3-week period without FLX treatment. In humans, mood-related disorders are chronic and are potentially a lifelong condition. Therefore, patients are commonly prescribed with antidepressant medications, such as SSRIs, for long periods of time, with intermittent periods of no treatment [Fava et al., 2006]. Furthermore, epidemiological evidence has established that SSRI treatment may delay the onset of AD symptoms, while preclinical studies have established that SSRI exposure is associated with neuroprotective effects [Sun et al., 2017; Zhou et al., 2019; Mdawar et al., 2020]. Thus, it is reasonable to speculate that the observed accumulation of phosphorylated Tau is a consequence of FLX absence after experiencing long-term treatment, and the increase in autophagic marker expression is the result of FLX induced sustained autophagic activity that acts to decrease levels of abnormal Tau, as it has been shown with other genes [Iñiguez et al., 2021] and proteins [Iñiguez et al., 2014]. Interestingly, while treatment with antidepressants (including FLX) is generally associated with positive outcomes in psychiatric patients [Han et al., 2011; Xie et al., 2019] a growing body of literature also indicates that antidepressant use is linked to progression of AD and other forms of dementia in vulnerable populations [Rosenberg et al., 2012; Moraros et al., 2017]. Along with this
prior work, the present data suggest that ontogenic FLX treatment may render the brain vulnerable to the development of AD-related neurodegenerative processes later in life. Importantly, given that the protein degradation pathways analyzed in this study, as well as Tau function, are all critical processes for the normal development and function of the nervous system [Baptista et al., 2012; Kimura et al., 2014; Fiock et al., 2020; Wang et al., 2019] it is very likely that the observed alterations contribute to the appearance of the complex neurobehavioral profile observed in animals with a history of juvenile FLX exposure [Iñiguez et al., 2014; Iñiguez et al., 2021; Flores-Ramirez et al., 2018; Sharp et al., 2019].

4.5 Limitations and future directions

Inclusion of female subjects in future studies is critical, given that the incidence of mood-related disorders and AD is higher among women than men [Vina & Lloret, 2010; Albert, 2015] and sex differences in the activity and expression of several components of the autophagy and UPS pathways have been previously reported [Du et al., 2019; Hansen et al., 2012; Rodriguez et al., 2014; Demarest et al., 2016]. Likewise, future work is needed to evaluate if the present molecular changes observed in young adults (PD70), as a function of juvenile FLX exposure (PD35–49), are observed in much older rodents (> 35 weeks), given that most individuals suffering from AD are over 60 years of age. Furthermore, while the selected markers for protein degradation pathways are representative of specific steps along the autophagy pathway, as well as a specific signal for proteasomal degradation, they do not provide a measurement of the dynamic nature of the autophagic flux, nor the specific catalytic activities of the proteasome, and thus warrant further functional study in a brain-region specific manner. Of note, the increased accumulation of AD relevant forms of phosphorylated Tau detected in our study were observed when levels of
phosphorylated Tau were normalized by those of tubulin and not total Tau. This approach was employed given that FLX pretreatment significantly increased levels of total Tau in the adult prefrontal cortex (Fig. 4H) and we therefore sought to normalize levels of phosphorylated Tau to a stable control (tubulin), which expression remained constant throughout experimental conditions. Noteworthy, in transgenic mice models of AD, overexpression of wild-type murine Tau has been shown to be sufficient to cause neuropathological changes, including aberrant accumulation of hyperphosphorylated Tau [Adams et al., 2009; Hoover et al., 2010]. Given our findings, where adolescent FLX increased phosphorylated and total Tau in adulthood, it is possible that ontogenic FLX treatment may influence both translational and post-translational regulation of Tau in adult individuals; an important finding with clinical implications, given that FLX is commonly prescribed for numerous pediatric chronic illnesses, including depression and anxiety [Hengartner, 2020].

It should be noted that even though our pharmacological approach did not incorporate AD-related behavioral endpoints, previous work has demonstrated that juvenile FLX exposure leads to impaired memory performance later in life [Sass & Wortwein, 2012; Flores-Ramirez et al., 2019; Sharp et al., 2019], modeling a key symptom of AD, while increasing AD-associated molecular markers in adulthood (Fig. 4). For this reason, future studies should examine whether adolescent FLX exposure, in AD-transgenic mice, will normalize both amyloid beta plaques and Tau later in life. This approach will better capture whether FLX alone in normal mice, versus FLX exposure in a transgenic AD model, leads to the exacerbation or reversal of proteins associated with neurodegenerative diseases. For example, Zhou et al., 2019, treated 8-month-old male double transgenic mice expressing mutations associated with AD, specifically a human amyloid precursor protein and a mutant human presenilin 1 (APP/PS1), for 10-weeks with FLX (10 mg/kg/day). They
found that FLX improved cognitive function when evaluated on the Morris water maze. This highlights that FLX treatment in adult APP/PS1 mice results in neuroprotective effects shortly after antidepressant exposure. As such, it is possible that adolescent FLX history in APP/PS1 mice may have potentially long-term protective effects on memory performance later in adulthood.

Another caveat of this study is that the animals utilized in this study were not exposed to stress, a known risk factor for the development of mood-related disorders – which may be necessary to model FLX treatment within a preclinical paradigm of mood-related illnesses [Iñiguez et al., 2014; Iñiguez et al., 2018]. This approach may be necessary, given that studies exposing adolescent male mice with ketamine, a fast-acting antidepressant, along with social stress, does not alter responses to behaviors associated with anhedonia in adulthood [Garcia-Carachure et al., 2020]. In other words, when mice were exposed to the antidepressant medication, while undergoing stress, did not result in long-term side effects later in life. Accordingly, it is possible that concomitant FLX and stress exposure may not alter AD associated proteins within the brain of C57BL/6 mice. Lastly, future pharmacological studies will be needed to help dissect the mechanism by which FLX is mediating its long-term effects on neurodegenerative-associated proteins within the hippocampus and prefrontal cortex. For example, researchers can evaluate whether FLX and escitalopram, the two most prescribed SSRIs for juvenile MDD, mediate similar or different long-term effects on AD-associated proteins. Although both SSRIs have a strong affinity for the serotonin transporter, FLX also binds to 5HT2 receptors [Perez-Caballero et al., 2014] whereas escitalopram displays more affinity for 5HT1 receptors [Ceglia et al., 2004; Rossi et al., 2008]. In this case, if escitalopram does not result in long-term increases of autophagy and Tau, it would suggest that FLX is mediating its enduring increases in neurodegenerative proteins via overactivation of 5HT2 receptors during adolescence. Overall, this line of
pharmacological studies will be needed to uncover the mechanism by which juvenile FLX exposure increases Tau and autophagy markers within brain regions associated with mood and memory performance.

4.6 Conclusion

This thesis shows that adolescent exposure to the antidepressant FLX leads to alterations in components of the autophagy pathway (LC3-II, p62) along with increases of AD-relevant phosphorylated Tau, within the hippocampus and prefrontal cortex of adult male C57BL/6 mice. These findings provide first line evidence that the enduring memory impairment previously reported as a function of antidepressant history [Sass & Wortein et al., 2012; Flores-Ramirez et al., 2019] is likely the result of FLX-induced abnormal autophagy and accumulation of phosphorylated Tau within brain regions associated with AD.
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Figure 1. Experimental design. Male C57BL/6 mice (N = 20; 10/group) were administered with vehicle (VEH; distilled water) or fluoxetine (FLX) for 15 consecutive days during the adolescent period of development (Postnatal Day [PD] 35–49), followed by a 21-day break without FLX exposure (washout period). Tissue was collected on PD70 (adulthood) for western blotting analysis.
Figure 2. Long-term effects of adolescent fluoxetine (FLX) treatment on autophagy markers in the hippocampus and prefrontal cortex of male mice. A) Representative immunoblots displaying the enduring effect of adolescent FLX exposure (postnatal day 35–49) on hippocampal autophagy markers in adulthood (postnatal day 70). B-D) Densitometric analysis of beclin-1, LC3-II, and p62 normalized by actin. E) Representative western blots displaying FLX effect on autophagy markers in the prefrontal cortex. F-H) Densitometric analysis of beclin-1, LC3-II, and p62 normalized by actin. Adolescent FLX pretreatment increased LC3-II levels in hippocampus and prefrontal cortex, and decreased p62 in prefrontal cortex of adult male mice. *p < 0.05 when compared with control (VEH). For hippocampal samples, N= 20; 10/group. For prefrontal cortex samples, N= 19; 9/VEH, 10/FLX.
**Figure 3.** Long-term effects of adolescent fluoxetine (FLX) treatment on K48-pUb proteins in the hippocampus and prefrontal cortex of adult male mice (postnatal day 70). **A)** Representative Western blot displaying the enduring effects of FLX on hippocampal K48-pUb proteins. **B)** Adolescent FLX history did not influence the accumulation of K48-pUb proteins in the hippocampus of adult male mice. Densitometric analysis as normalized by tubulin. **C)** Representative western blots displaying FLX effect on K48-pUb proteins in the prefrontal cortex. **D)** Adolescent FLX did not influence the accumulation of K48-pUb proteins in the prefrontal cortex of adult male mice. Densitometric analysis as normalized by tubulin. For hippocampal samples, N= 20; 10/group. For prefrontal cortex samples, N= 19; 9/VEH, 10/FLX.
Figure 4. Adolescent fluoxetine (FLX) exposure induces abnormal accumulation of phosphorylated Tau in the hippocampus and prefrontal cortex of adult mice. A) Representative western blots displaying the long-term effects of adolescent FLX exposure (postnatal day 35–49) on hippocampal pTau-T231, pTau-S262, and total Tau, in adulthood (postnatal day 70). B-D) Densitometric analysis of the different forms of hippocampal Tau normalized by tubulin or total Tau. FLX pretreatment increased pTau-T231 when normalized to tubulin, but not total Tau. E) Representative western blots displaying the enduring effects of juvenile FLX exposure on pTau-T231, pTau-S262, and total Tau in the prefrontal cortex of adult mice. F-H) Densitometric analysis of the different forms of Tau in the prefrontal cortex normalized by tubulin or total Tau. When normalized by tubulin, adolescent FLX pre-exposure increased the accumulation of pTau-T231, p Tau-S262 and total Tau in the prefrontal cortex of adult male mice. *p < 0.05 when compared to VEH. For hippocampal samples, N= 20; 10/group. For prefrontal cortex samples, N= 19; 9/VEH, 10/FLX.
Vita

Minerva Rodriguez was born in El Paso, Texas, USA. The youngest daughter of Susana Salazar and Rafael Rodriguez, she graduated from the University of Texas at El Paso, in the spring of 2018, with a Bachelor of Science in Psychology. She began the doctoral program in Psychology at the University of Texas at El Paso in the fall of 2019 under the mentorship of Professor Sergio D. Iñiguez.