Journal of Alzheimer's Disease xx (2021) x–xx
DOI 10.3233/JAD-210475
IOS Press

Autophagy Induction and Accumulation of Phosphorylated Tau in the Hippocampus and Prefrontal Cortex of Adult C57BL/6 Mice Subjected to Adolescent Fluoxetine Treatment

Jorge A. Sierra-Fonseca[a], Minerva Rodriguez[a], Anapaula Themann[a], Omar Lira[a], Francisco J. Flores-Ramirez[a], Javier Vargas-Medrano[b], Bharathi S. Gadad[b] and Sergio D. Iñiguez[a,*]
[a]*Department of Psychology, The University of Texas at El Paso, El Paso, TX, USA*
[b]*Department of Psychiatry, Paul L. Foster School of Medicine, Texas Tech University Health Science Center, El Paso, TX, USA*

Accepted 23 July 2021
Pre-press 20 August 2021

Abstract.
Background: 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.
Objective: 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), we evaluated the long-term effects of adolescent FLX exposure on these pathways, using mice as a model system.
Methods: We subjected C57BL/6 adolescent male mice to FLX (20 mg/kg/day) from postnatal day (PD) 35 to PD49. Twenty-one days after the last FLX injection (i.e., adulthood; PD70), mice were euthanized 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.
Results: Juvenile FLX pre-exposure mediated long-term changes in the expression of protein markers (increased LC3-II and decreased p62) that is consistent with autophagy activation, particularly in the prefrontal cortex. Furthermore, FLX history induced persistent accumulation of AD-associated variants of tau in both the hippocampus and prefrontal cortex
Conclusion: Adolescent FLX treatment may have enduring effects in the neuronal protein degradation machinery, which could adversely influence clearance of abnormal proteins, potentially predisposing individuals to developing AD in later life.

Keywords: Abnormal protein accumulation, Alzheimer's disease, antidepressant, drug safety, juvenile, LC3-II, long-term effects, p62, proteostasis, SSRI

INTRODUCTION

Affective disorders, including depression and anxiety, are a leading cause of disability, affecting over 300 million people worldwide, with incidence

*Correspondence to: Sergio D. Iñiguez, PhD, Department of Psychology, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA. Tel.: +1 915 747 5769; Fax: +1 915 747 6553; E-mail: sdiniguez@utep.edu.

expected to surge in the upcoming decades [1]. This is further compounded by its chronic and recurrent nature, comorbidity with other clinical conditions, and the limited efficacy of available pharmacological treatments, which primarily consist of selective serotonin reuptake inhibitor (SSRI) medications [2, 3]. In younger populations (children and adolescents), depression can have devastating effects, such as poor academic performance, substance abuse, and suicide [4]. The incidence of depression in adolescents has increased in recent years, and consequently, the prescription of antidepressants in this vulnerable group has also increased [5–8].

Fluoxetine (FLX), a SSRI antidepressant medication, is approved by the Food and Drug Administration to treat both depression and anxiety disorders in children and adolescents [9, 10]. Clinical studies have provided evidence for the successful use of FLX in pediatric populations [11]. However, factors such as safety, limited efficacy, as well as long-term neurodevelopmental effects are still of concern [12–14]. Preclinical studies have been useful to uncover that developmental exposure to FLX results in enduring neurobehavioral consequences in adulthood, including changes in response to aversive and rewarding stimuli [15–18], memory and cognitive performance [19–21], despair- and anxiety-inducing environments [16, 22, 23], while altering hippocampal gene expression [23, 24] as well as protein expression and phosphorylation [16, 25, 26]. However, despite widespread use, the precise cellular and molecular mechanisms underlying the long-term neurobehavioral effects of developmental FLX exposure are not fully understood.

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 [27, 28]. As part of the proteostasis network, two major pathways participate in the degradation of proteins: autophagy and the ubiquitin-proteasome system (UPS) [29]. Macroautophagy, hereafter referred to simply as autophagy, is a cellular degradative system that employs double-membrane vesicles, known as autophagosomes, that deliver cytoplasmic cargo into the lysosome for degradation [30]. 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 [31, 32]. While autophagy is in charge of degrading long-lived, membrane-bound, and aggregated proteins, as well as large protein complexes and even whole organelles [33], the UPS degrades short-lived, soluble, and misfolded proteins [32]. Proper function of protein degradation pathways is particularly important in neuronal cells, 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, including Alzheimer's disease (AD) [34–37]. Accumulation of abnormal forms 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* [38, 39]. Under physiological conditions, tau binds to and stabilizes the microtubule cytoskeleton, facilitating axonal transport and neuronal polarity [40]. Tau function can be 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 [41, 42]. Importantly, both normal and pathological variants of tau are degraded via autophagy and the UPS [43, 44].

Epidemiological evidence indicates that a lifetime history of affect-related illnesses significantly increase the risk of developing AD later in life [45]. 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 [46–48], thus suggesting that accumulation of hyperphosphorylated tau could be a common pathological factor between both neurological disorders. Furthermore, data point to a link between impairment of protein degradation pathways and the pathology of depression [49–51]. Also, SSRI antidepressants have been shown to modulate autophagic flux [52], regulate expression of proteasomal components [53], and decrease tau phosphorylation [54]. Nevertheless, how developmental FLX can alter protein degradation pathways that negatively impact the accumulation of abnormal proteins in specific brain regions has not been previously addressed. Therefore, the current investigation aimed to determine whether adolescent FLX exposure can 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 [55–59]. To accomplish this, we employed immunoblotting analysis to measure the levels of several autophagic protein markers that represent distinct steps along the degradative pathway, namely LC3-II, p62, and beclin-1 [60–62], as well as the presence of lysine 48-linked polyubiquitinated (K48-pUb) proteins, a highly specific signal for proteasomal-mediated degradation [63]. Furthermore, we also assessed the accumulation of two forms of phosphorylated tau, specifically, tau phosphorylated at threonine 231 (pTau-T231) and serine 262 (pTau-S262), given their implication in AD pathology [64, 65].

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (purchased from Charles River Laboratories, Hollister, CA) were kept in an animal facility with controlled humidity and temperature (23–25°C). Mice were housed in clear polypropylene boxes containing wood shavings, *ad libitum* access to food and water, and placed on a 12:12 h light/dark cycle (lights on at 7:00 a.m.). All procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [66], the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines [67], and the Institutional Animal Care and Use Committee at The University of Texas at El Paso.

Drug treatment

Fluoxetine hydrochloride (FLX; Spectrum Chemicals, Gardena, CA) was dissolved in distilled sterile water and administered via intraperitoneal injections, using a volume of 2 mL/kg/day. Separate groups of male C57BL/6 mice (N = 20; 10 animals per group) received either VEH (distilled sterile water) or FLX (20 mg/kg) injections during the adolescent period of development (postnatal days [PD] 35–49). Mice in both experimental groups were then allowed a 21-day break period during which no injections occurred and were subsequently euthanized on PD70 (see Fig. 1 for experimental timeline). The selected period of FLX exposure (PD35–49) was chosen because it is roughly equivalent to adolescence [68, 69]. The 20 mg/kg/day dose of FLX was selected because of its previously characterized antidepressant-like effects [15, 20, 70, 71] in animal models for the study of affect-related behavior [72–74].

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), and 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 until use. Protein concentration in the samples was determined with bicinchoninic acid method (ThermoFisher Scientific, #23225; Rockford, IL), using bovine serum albumin as standard.

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)

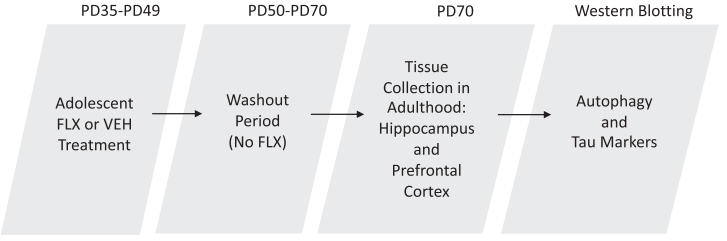

Fig. 1. Experimental design. Male C57BL/6 mice (N = 20; 10/group) were administered with vehicle (VEH; distilled water) or fluoxetine (FLX) for 15 consecutive days during the adolescent period of development (Postnatal Day [PD] 35–49), followed by a 21-day break without FLX exposure (washout period). Tissue was collected on PD70 (adulthood) for western blotting analysis.

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 (Danvers, MA).

Electrophoresis and western blotting

Immunoblotting was conducted as previously described [25, 75]. Specifically, samples with equal amounts of protein (~10 μg) were dissolved in Laemmli buffer containing 5% β-mercaptoethanol (Bio Rad, #1610737) and subjected to sodium dodecyl sulfate-polyacrilamide gel electrophoresis (8–16% gradient), using pre-cast gels (BioRad, #1610710; Hercules, CA), followed by protein transfer onto polyvinylidene difluoride membranes (BioRad, #1704159). The membranes were incubated in 5% blocking buffer (BioRad, #1706404) dissolved in TBST (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies dissolved in blocking buffer. Membranes were then washed with TBST and incubated with appropriate HRP-conjugated secondary antibodies for 1 h 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). 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 the loading control. Densitometric analysis of protein bands was performed with the ImageJ software (National Institutes of Health).

Data analysis

Differences in the expression of protein markers were determined using two-tail Student's *t* tests. In all cases, a value of $p < 0.05$ was chosen to define statistical significance. The Sigma Plot 12.5 software (Systat, Chicago, IL) was used for all statistical calculations. During tissue extraction, we accidentally damaged and lost one prefrontal cortex sample from the VEH group, resulting in unequal group numbers between the two brain regions collected (hippocampus $N = 20$; prefrontal cortex $N = 19$).

RESULTS

Adolescent FLX exposure caused different autophagic responses in the hippocampus and prefrontal cortex of adult male mice without affecting K48-pUb proteins

Adolescent FLX pretreatment induced persistent changes in 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 (Fig. 2A-D), a two-tailed Student's *t* test revealed that adolescent FLX 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), juvenile 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 decreasing p62 ($t_{17} = 3.259$, $p < 0.05$; Fig. 2H). No differences in actin were noted between the experimental groups in either brain region ($p > 0.05$, respectively).

In addition to evaluating the long-term effects of adolescent FLX on autophagic markers, the levels of K48-pUb, a highly specific signal for proteasome degradation was assessed (Fig. 3A-D). Two-tail Student's *t* tests revealed that 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 in tubulin were noted between the experimental groups in the hippocampus ($p > 0.05$) or prefrontal cortex ($p > 0.05$).

FLX treatment during adolescence induces accumulation of phosphorylated tau in the hippocampus and prefrontal cortex of adult male mice

Adolescent FLX pretreatment induced abnormal accumulation of phosphorylated tau forms in the hippocampus (Fig. 4A-D) and prefrontal cortex (Fig. 4E-H) in adulthood. Immunoblotting analysis (normalized to tubulin) revealed that, adolescent FLX pretreatment significantly increased levels of pTau-T231 ($t_{18} = 3.51$, $p < 0.05$; Fig 4B, left panel), without affecting pTau-S262 ($p > 0.05$; Fig. 4C, left panel) or total tau levels ($p > 0.05$; Fig. 4D) in the adult

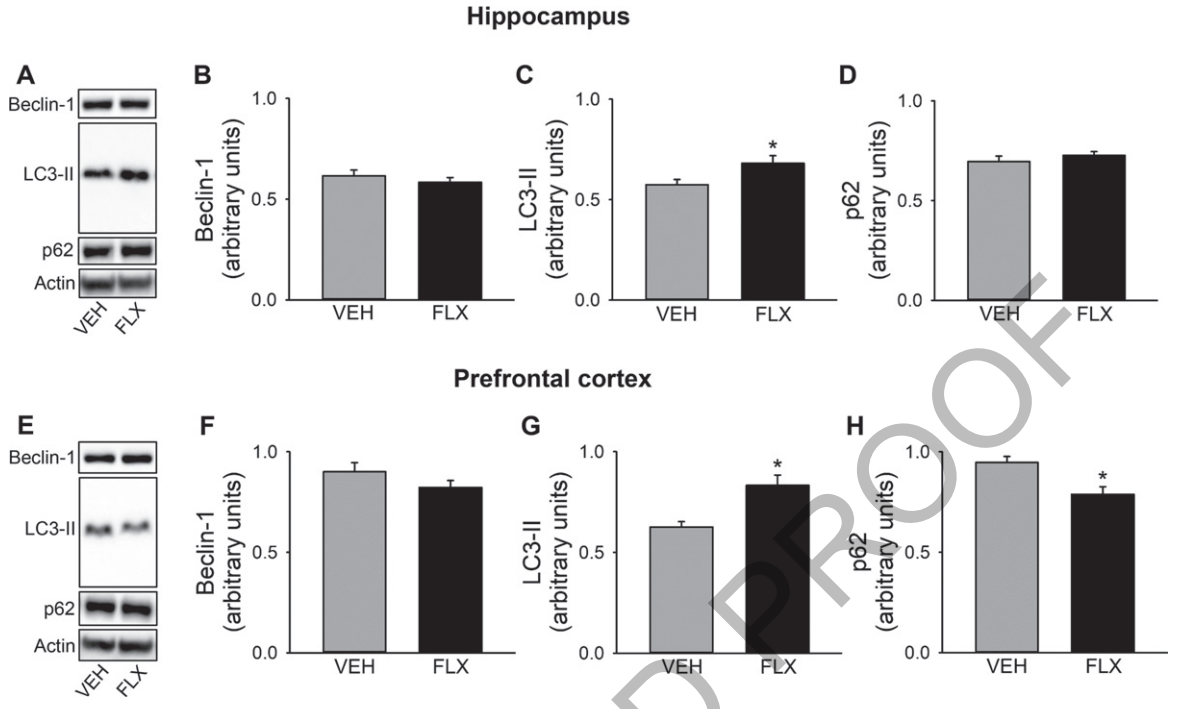

Fig. 2. Long-term effects of adolescent fluoxetine (FLX) treatment on autophagy markers in the hippocampus and prefrontal cortex of male mice. A) Representative immunoblots displaying the enduring effect of adolescent FLX exposure (postnatal day 35–49) on hippocampal autophagy markers in adulthood (postnatal 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.

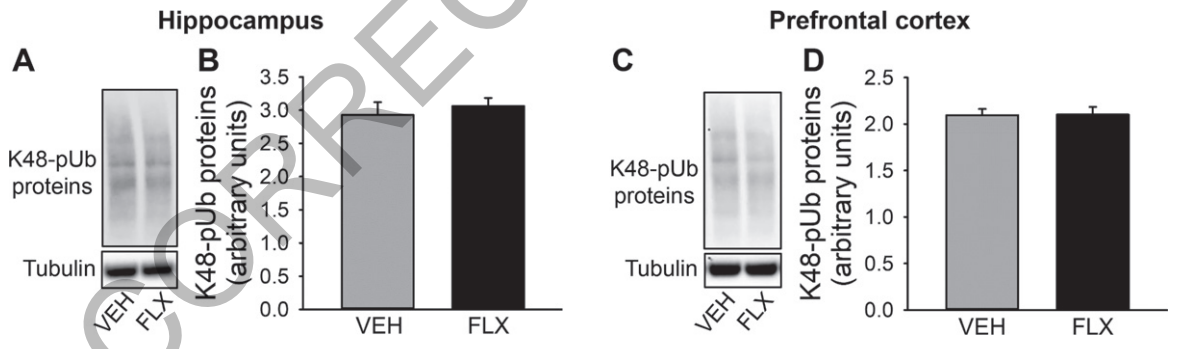

Fig. 3. Adolescent FLX exposure (postnatal day 35–49) does not affect K48-pUb proteins in the hippocampus or prefrontal cortex of adult male mice (postnatal day 70). A) Representative Western blot displaying the enduring effects of FLX on hippocampal K48-pUb proteins. B) Adolescent FLX history did not 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.

hippocampus. In the prefrontal cortex, when normalized to tubulin, juvenile FLX pretreatment increased phosphorylation of pTau-T231 ($t_{17} = 2.197$; $p < 0.05$; Fig. 4F, left panel) and pTau-S262 ($t_{17} = 2.518$; $p < 0.05$; Fig. 4G, left panel), while also increasing the levels of total tau ($t_{17} = 3.541$; $p < 0.05$; 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

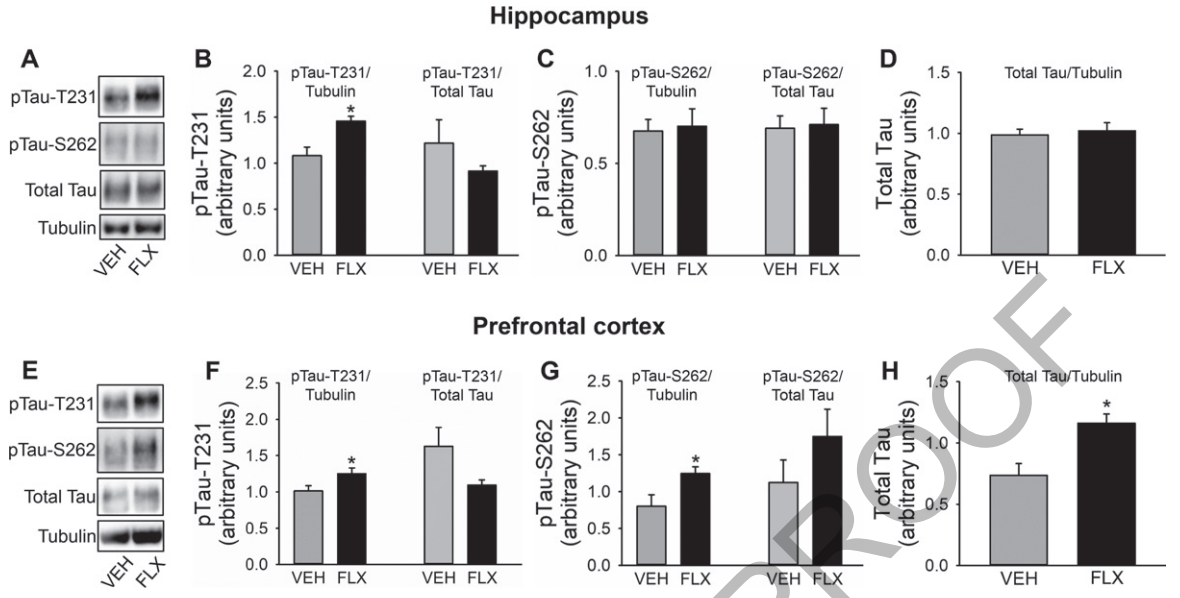

Fig. 4. Juvenile fluoxetine (FLX) pretreatment induces abnormal accumulation of phosphorylated tau in the hippocampus and prefrontal cortex of adult mice. A) Representative western blots displaying the long-term effects of adolescent FLX exposure (postnatal day 35–49) on hippocampal pTau-T231, pTau-S262, 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, pTau-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.

noted between the experimental groups in the hippocampus ($p > 0.05$) or prefrontal cortex ($p > 0.05$).

DISCUSSION

The goal of the current investigation was to assess the persistent 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. Also, 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.

In order to assess 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 [76]. However, we did not detect any differences in adult beclin-1 expression as a result 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 [77–79]. Here, we found that its expression was increased as a result 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 differential effects on p62 expression across the brain regions 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 [80].

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 [81, 82]. The observed profile of sustained increase of phosphorylated and total tau in the adult prefrontal cortex as a result 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 [83], 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 [84].

In order 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 [63], 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 [85], 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 [86].

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 [19, 20], enhanced sensitivity to anxiety-inducing situations [15], changes in despair-like behavior [16, 22, 87], and altered reward sensitivity [17, 18]. 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 can be exposed to antidepressant drugs, such as SSRIs, for long periods of time, with intermittent periods of no treatment [88]. 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 [89–91]. 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 [23] and proteins [16]. 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 [19–21], modeling a key symptom of AD, while increasing AD-associated molecular markers in adulthood (Fig. 4). Interestingly, while treatment with antidepressants (including FLX) is generally associated with positive outcomes in psychiatric patients [92, 93], a growing body of literature also indicates that antidepressant use is linked to progression of AD and other forms of dementia in vulnerable populations [94, 95]. 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 [96–99] 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 [16, 18, 20, 21, 23].

Inclusion of female subjects in future studies is necessary, given that the incidence of mood-related disorders and AD is higher in women [100, 101] and sex differences in the activity and expression of several components of the autophagy and UPS pathways have been previously reported [102–105]. 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 [106, 107]. 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 implactions, given that FLX is commonly prescribed for numerous pediatric chronic illnesses, such as depression and anxiety [108].

To the best of our knowledge, this is the first study to address the enduring effects of juvenile FLX exposure on the adult expression of hippocampal and prefrontal cortex markers associated with autophagy and the UPS, two major protein degradation pathways. Importantly, we show that the alterations in components of the autophagy pathway (LC3-II, p62), but not the UPS (K48-pUb), are accompanied by increased levels of AD-relevant phosphorylated tau, therefore establishing altered autophagy and abnormal accumulation of phosphorylated tau as persistent molecular consequences of juvenile FLX exposure.

ACKNOWLEDGMENTS

SDI acknowledges support from the National Institute of General Medical Sciences (SC2GM109811 and SC3GM130467). The funding agencies were not involved in the experimental design of the current study, data collection, interpretation of the results, nor the writing of this manuscript for publication. JAS-F and FJF-R were supported by a postdoctoral training contract from the National Institute on Drug Abuse (NIDA; HHSN271201600057C). MR was supported by a *Doctoral Excellence Fellowship* from The Graduate School at UTEP. Lastly, the authors would like to thank Alejandra Gonzalez for technical assistance.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/21-0475r1)

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