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LONG-TERM EFFECTS OF ADOLESCENT FLUOXETINE EXPOSURE ON HIPPOCAMPAL GENE EXPRESSION IN MALE C57BL/6 MICE

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Anapaula Themann

2021

Dedication

Para mis abuelos, Alicia y Eduardo.

LONG-TERM EFFECTS OF ADOLESCENT FLUOXETINE EXPOSURE ON HIPPOCAMPAL GENE EXPRESSION IN ADULT MALE C57BL/6 MICE

by

ANAPAULA THEMANN

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Abstract

Mood-related disorders, including depression and anxiety, are prevalent among children and adolescents. This poses a public health challenge, given their adverse impact on these young populations. Treatment with the selective serotonin reuptake inhibitor fluoxetine (FLX) is the first line of pharmacological intervention in pediatric patients suffering from affect-related illnesses. Although the use of this antidepressant has been deemed efficacious in the juvenile population, the enduring neurobiological consequences of adolescent FLX exposure are not well understood. For this reason, we explored for persistent molecular adaptations, in the adult hippocampus, as a function of adolescent FLX pretreatment. To do this, we administered FLX (20 mg/kg/day) to male C57BL/6 mice during adolescence (postnatal day [PD] 35-49). Twenty-one days later (PD70), whole hippocampal tissue was dissected. We then incorporated the quantitative real-time reverse transcription polymerase chain reaction (qPCR) approach to assess changes in the expression of genes associated with major intracellular signal transduction pathways, including the extracellular signal-regulated kinase (ERK), the phosphatidylinositide-3-kinase (PI3K)/AKT pathway, and the wingless (Wnt)-dishevelled-GSK3B signaling cascade. Our results show that FLX treatment results in long-term dysregulation of mRNA levels across numerous genes from the ERK, PI3K/AKT, and Wnt intracellular signaling pathways, along with increases of the transcription factors CREB, ΔFosB, and zif268. Lastly, FLX treatment resulted in persistent increases of transcripts associated with cytoskeletal integrity (β-actin) and caspase activation (DIABLO), while decreasing genes associated with metabolism (fucose kinase) and overall neuronal activation (c-Fos). Collectively, these data indicate that adolescent FLX exposure induces persistent alterations in hippocampal gene expression in adulthood, thus, questioning the safety of early-life exposure to this antidepressant medication.

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Chapter 1: Introduction

Depression and anxiety disorders are major health problems worldwide, given their association with significant disability and mortality, as well as reduced quality of life (Bandelow & Michaelis, 2015; Kessler & Bromet, 2013). The occurrence of mood-related illnesses is highly prevalent in younger populations, with an incidence of depressive disorders increasing from 1% in childhood up to 8% in adolescence, and anxiety disorders affecting up to 20% of the pediatric population (Birmaher et al., 2003; Merikangas et al., 2010). To make matters worse, if left untreated, these neuropathologies increase the risk of substance abuse, suicide attempts, impaired social function, and the development of comorbid neuropsychiatric conditions in adulthood (Pine et al., 1998; Thapar et al., 2010; Tiller, 2013; Wehry et al., 2015).

Despite the high incidence and negative health impact of mood-related disorders in the pediatric population, pharmacological treatment options are severely limited (Birmaher et al., 2003; Bylund & Reed, 2007). Fluoxetine (**FLX**), a selective serotonin reuptake inhibitor (**SSRI**), is widely prescribed for the management of both depression and anxiety, and is approved by the Food and Drug Administration for use in children and adolescents (Jane Garland et al., 2016; Perez-Caballero et al., 2014). However, the prescription of SSRIs, particularly FLX, in young patients remains controversial, as several studies have demonstrated limited efficacy, as well as age-related differences in treatment response (Bowman & Daws, 2019; Bridge et al., 2007; Kronenberg et al., 2007). Furthermore, because brain development continues into adolescence (Konrad et al., 2013), exposure to psychotropic medications during this sensitive period may have enduring consequences (Alcantara et al., 2014; Garcia-Carachure et al., 2020; Olivier et al., 2011). Indeed, accumulating evidence from animal studies indicate that exposure to FLX results in adverse neurobehavioral effects that persist into later adulthood, including memory

impairment (Flores-Ramirez et al., 2019; Sass & Wortwein, 2012), altered drug-seeking behavior (Flores-Ramirez et al., 2018; Iñiguez et al., 2015), and decreased reactivity to inescapable stress (Airan et al., 2007; Iñiguez, Alcantara, et al., 2014; Iñiguez et al., 2010; Karpova et al., 2009).

The primary mechanism of action of SSRI medications is to block the uptake activity of the serotonin transporter, indirectly increasing global levels of serotonin, an effect that occurs rather quickly (Bowman & Daws, 2019; Trivedi et al., 2006). However, therapeutic response to SSRI treatment in patients usually takes several weeks, indicating that intracellular signaling molecules downstream of serotonergic receptors underlie this delayed effect (Kroeze et al., 2015). Experimental evidence from adult normal animals indicate that brain-derived neurotrophic factor (**BDNF**), and several of its intracellular targets such as the mitogen-activated protein kinase (**MAPK**) extracellular signal-regulated protein kinase (**ERK**)-1/2, phosphoinositide-3 kinase (**PI3K**)/protein kinase-b (**AKT**) signaling molecules, and members of the wingless (**Wnt**) and phospholipase-c-gamma-1 (**PLCϒ1**) cascades, play a role in mediating the therapeutic effects of SSRIs (Bjorkholm & Monteggia, 2016; Duman & Monteggia, 2006; Freitas et al., 2013; Zhou et al., 2016). Nevertheless, there is a dearth of research addressing the status of intracellular signaling pathways as a function of early-life FLX exposure in adulthood. In order to address this gap in the literature, the goal of this thesis is to examine the potential long-lived molecular impact of adolescent FLX exposure on hippocampal gene expression in adulthood, using C57BL/6 male mice as a model system. We focus on the hippocampus, given that this is a brain region that is implicated in the etiology of affect-related illnesses (Airan et al., 2007; Cha et al., 2016; Persson et al., 2014), the expression of drug-seeking behavior (Hitchcock & Lattal, 2018; Meyers et al., 2006), and in mediating the therapeutic action of SSRIs (Dale et al., 2016; Duric et al., 2010).

Specific Aim of Master's Thesis:

Aim 1: Evaluate whether adolescent FLX exposure alters gene expression in the hippocampus of adult male mice. To accomplish this, we will evaluate mRNA from three major intracellular signaling pathways, namely the ERK1/2, PI3K-AKT, and Wnt cascades, as well as additional molecular players with diverse cellular functions including metabolism, cell death, and overall membrane integrity (see **Table 1** for full list of genes).

Hypothesis: Previous work has shown that chronic FLX exposure increases mRNA of members in the nerve growth family, namely BDNF, its receptor Tropomyosin receptor kinase B (**TrkB**), as well as cAMP response element binding protein (**CREB**), in the rat hippocampus (Nibuya, Nestler, & Duman, 1996). Additionally, signaling molecules downstream of BDNF have also been shown to be upregulated by chronic antidepressant treatment (First et al., 2011; Li & Jope, 2010). Yet, the developmental ramifications of this antidepressant-mediated mRNA expression remain unknown. Therefore, our central hypothesis is that adolescent FLX exposure will lead to dysregulated gene expression of intracellular signaling cascades and transcription factors in the hippocampus of adult male mice.

Chapter 2: Materials and Methods

2.1 Animals

Postnatal day (**PD**)-28 male C57BL/6 mice were obtained from Charles River Laboratories (Hollister, CA). Mice were maintained in an animal facility under controlled humidity and temperature conditions (21-23°C). Mice were housed in clear polypropylene boxes (3-4 per cage) containing wood shaving bedding, maintained on a 12:12 hr cycle (lights on at 700 hr), and were provided with water and food *ad libitum*. Experiments were conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 2003), and with approval of the Institutional Animal Care and Use Committee at The University of Texas at El Paso (Protocol: A-201609-1).

2.2 Drug Treatment

Fluoxetine hydrochloride (FLX) was purchased from Spectrum Chemicals (Gardena, CA), and was dissolved in distilled sterile water (vehicle; **VEH**). Animals received intraperitoneal (**IP**) injections of either FLX or VEH using a volume of 2 ml/kg.

2.3 Experimental Design

A total of 24 male C57BL/6 mice were randomly assigned to receive VEH or FLX treatment (n = 12 per group). Specifically, mice were injected with VEH or FLX (20 mg/kg/day) for 15 consecutive days during PD35-49. Animals were then allowed a 21-day period without drug administration and were subsequently euthanized once they reached adulthood (on PD70). The selected timeframe of FLX administration (PD35–49) was chosen because it closely resembles the human adolescent period (Abreu-Villaca et al., 2010; Andersen, 2003), while the

FLX dose (20 mg/kg/day) was selected due to its well-established antidepressant-like response in animal models commonly adopted for the study of mood-related disorders (Englander et al., 2005; Flores-Ramirez et al., 2019; Iñiguez et al., 2010; LaPlant et al., 2010; Surget et al., 2011). A timeline of the experimental design is provided in **Figure 1**.

2.4 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Gene expression is the biological process by which nuclear deoxyribonucleic acid (DNA) transcribes into messenger ribonucleic acid (mRNA), and ultimately translates into functional proteins (Crick, 1970). Measuring genetic expression can provide new information about the amount of gene product (i.e., mRNA) present in a cell. For example, this approach can be used to determine if an experimental manipulation (i.e. FLX pretreatment) has the potential to increase or decrease the expression of genes of interest, in a specific brain region (i.e. the hippocampus). One of the most precise gene quantification tools used in molecular biology is the quantitative real-time reverse transcriptase Polymerase Chain Reaction (qPCR) method.

The qPCR technique analyzes the gene transcripts present in a sample of interest through reverse transcription. First, isolated mRNA is purified and then homogenized with reverse transcriptase (DNA polymerase enzyme that transcribes mRNA into DNA) and deoxyribonucleotides (nucleotides specific to DNA). This synthesis yields a single strand of DNA complementary (cDNA) to the original mRNA template. Then, with the help of (1) primers (short pieces of single-stranded DNA from target genes, that bind to the cDNA sequences in a sample), (2) deoxynucleoside triphosphates (dNTPs; substrates of DNA nucleotides), (3) DNA polymerase (DNAP; an enzyme that generates DNA molecules by assembling nucleotides), and (4) fluorophore (a fluorescent chemical compound that binds to sample DNA and emits light

upon light excitation), single-stranded cDNA is reverse transcribed into a double-stranded DNA (dsDNA) template of the target genes of interest. Lastly, qPCR is performed to amplify and measure the dsDNA templates. Read-outs (SYBR Green fluorescence emitted by the dsDNA) from the qPCR reaction are analyzed and fold-change (a change in expression of genes between a control and an experimental group) is calculated (see section 2.4.3 below).

2.4.1 Strengths and Limitations of qPCR

A strength of the qPCR technique is the ability to quantify and measure specific sequences of transcripts (i.e., specific gene sequences). During reverse transcription, gene-specific primers can be utilized in place of random primers to increase the specificity of the cDNA strand. The specific-primer approach also reduces the amount of unspecific cDNA in a sample, thereby improving the read-outs of subsequent qPCR amplification. Yet, another advantage of the qPCR method can be its ability to increase cDNA yield with the use of random primers. Random priming maximizes the number of cDNA templates produced, thereby increasing the number of subsequent qPCR reactions that can be processed, making this a more economically viable option for exploratory analysis. Overall, the results from qPCR analysis increase our understanding of the transcriptional mechanisms of genes in a cell. However, conclusions regarding a cell's post-translational mechanisms cannot be made using this molecular technique.

A limitation of the qPCR method is the inability to detect and measure protein quantities. Proteins, the byproducts of gene translation, carry out a diverse range of functions within a cell (i.e., differentiation). Hence, protein detection is considered a crucial step in determining the characteristics and molecular processes of specific cells.

While several molecular techniques, such as Western Blotting, can measure the total protein levels of a sample, protein quantification cannot be achieved using qPCR in part because of its starting material. Given that the purified mRNA (starting material) is reverse transcribed into cDNA, the only end-product possible is dsDNA, not protein. Moreover, those results can prove the existence of a gene in a tissue but cannot lead to direct conclusions about levels of total or phosphorylated protein. Therefore, interpretations about which specific proteins are present in a cell are limited to protein quantification tools, such as immunoblotting techniques.

2.4.2 Experimental Procedure

Whole hippocampus was microdissected on dry ice and stored at -80°C until assayed (Iñiguez et al., 2012). RNA isolation was carried out with RNEasy Micro kits according to the manufacturer's instructions (Qiagen; Austin, TX). RNA was then reverse transcribed into cDNA, using the iScript cDNA synthesis kit (Bio-Rad; Hercules, CA). qPCR was then conducted using a commercially available kit (RealMasterMix, Eppendorf; Westbury, NY), running duplicate samples from each animal. Cycle threshold (Ct) values were determined and changes in gene expression were analyzed by the ΔΔCt method (Vialou et al., 2010), using GAPDH as housekeeping reference. The primer sequences for the analyzed genes are displayed in **Table 2**.

2.4.3 ΔΔCt Method of Analyses for qPCR

The ΔΔCt method determines relative fold change. A ΔΔCt value is calculated using Ct values obtained from the qPCR reaction. A Ct value represents the cycle number at which the fluorescence emitted by the qPCR product from gene sample, is above background levels. The Ct values of a housekeeping gene sample, such as GAPDH, is also included in the qPCR analysis as a control, because its stably expressed across all cell types and their gene expression is often not affected by pharmacological treatment such as FLX.

The first step in a ΔΔCt calculation is to acquire the Ct value of every target gene and housekeeping gene sample in a group. Also, since samples are loaded into the qPCR plate in duplicates or triplicates, an average Ct value for each gene (target and housekeeping), across experimental groups (VEH and FLX), needs to be calculated. Ultimately, leading to four Ct group averages [target genes Ct (VEH), Ct (FLX) and housekeeping genes Ct (VEH), Ct (FLX). Next, normalized (variation is removed via the housekeeping gene values) Ct values (ΔCt) in the VEH and FLX groups are calculated using the following equations: $\Delta Ct_{(VEH)} = Ct_{(TARGET\ GENE)} - Ct_{(GAPDH)}$, and $\Delta Ct_{(FLX)} = Ct_{(TARGET\ GENE)} - Ct_{(GAPDH)}$. The difference between a normalized VEH and FLX group is calculated using the following equation: $\Delta\Delta Ct = \Delta Ct_{(FLX)} - \Delta Ct_{(VEH)}$. Finally, fold change or changes in level of gene expression are calculated as followed: $2^{-\Delta\Delta Ct}$. The target genes analyzed are displayed in **Table 1**.

2.4.4 Troubleshooting qPCR

qPCR is highly prone to cross-contamination. To prevent this, working surfaces should be sanitized with a DNA decontamination solution (such as DNAzap™) before and after every qPCR protocol.

Negative controls, such as "No Template Controls" (NTC), can be also incorporated in qPCR plate analysis to rule out any random and/or reagent contamination. NTC wells are comprised of the qPCR master mix, RNAase free water, and primers, but no DNA or mRNA templates. Therefore, amplification should not occur in an NTC sample during the qPCR reaction. However, if for example the qPCR master mix happens to be contaminated with an amplicon (a gene-specific sequence of nucleotides that elongate cDNA) or any other DNA contaminant then amplification will be detected, ultimately leading to false positive results. To resolve this, a new mixture of the reagents should be prepared in a separate and decontaminated working space.

2.5 Data Analysis

Experimental animals were randomly assigned to receive VEH or FLX during adolescence. Data were analyzed using two-tail Student's t tests. Data are presented as ± standard error of the mean (**SEM**), and statistical significance was defined as $p<0.05$.

Chapter 3: Results

3.1 Adolescent FLX exposure increases gene expression of the MAPK pathway in the adult hippocampus

Figure 2 displays the effects of juvenile FLX exposure (PD35-49) on the expression of multiple genes within the MAPK signaling pathway in adulthood (PD70). When compared to VEH-pretreated controls (n = 12) adult mice pretreated with FLX during adolescence (n = 12) displayed increases in MEK1 ($t_{22} = 3.60$, $p = 0.0016$; Fig. 2B), MEK2 ($t_{22} = 6.48$, $p<0.0001$; Fig. 2C), ERK1 ($p = 0.06$; Fig. 2D), ERK2 ($t_{22} = 5.40$, $p<0.0001$; Fig. 2E), and p90RSK ($t_{22} = 6.11$, $p<0.0001$; Fig. 2F). However, no differences in BDNF ($p>0.05$; Fig. 2A) mRNA were noted between FLX and VEH pretreated mice.

3.2 Adolescent FLX exposure increases gene expression of transcription factors in the adult hippocampus

Figure 3 shows the effects of juvenile FLX treatment (PD35-49) on adult hippocampal expression (PD70) of transcription factors, on which several signaling pathways are known to converge. When compared to VEH-pretreated controls (n = 12), adult mice pretreated with FLX during adolescence (n = 12) displayed significant increases in CREB ($t_{22} = 6.40$, $p<0.0001$; Fig. 3A), Zif268 ($t_{22} = 5.14$, $p<0.0001$; Fig. 3B), and ΔFosB ($t_{22} = 3.16$, $p = 0.0045$; Fig. 3C).

3.3 Adolescent FLX exposure increases gene expression of the IRS2/PI3K/AKT pathway in the adult hippocampus

Figure 4 shows the effects of adolescent FLX treatment (PD35-49) on hippocampal expression of genes from the IRS2/PI3K/AKT pathway in adult male mice (PD70). We found

that when compared to VEH-pretreated controls (n = 12), adult mice pretreated with FLX during adolescence (n = 12) displayed significant increases in IRS2 (t_{22} = 4.18, p = 0.0004; Fig.4A), PI3K (t_{22} = 2.48, p = 0.0212; Fig. 4B), PDK (t_{22} = 3.62, p = 0.0015; Fig. 4C), AKT1 (t_{22} = 4.25, p = 0.0003; Fig. 4D), and GSK3β-1 (t_{22} = 2.75, p = 0.0114; Fig. 4E).

3.4 Adolescent FLX exposure alters genes from the Wnt signaling pathway in the adult hippocampus

Figure 5 displays the effects of juvenile FLX treatment (PD35-49) on hippocampal expression of genes within the Wnt signaling pathway in adulthood (PD70). When compared to VEH-pretreated controls (n = 12), adult mice pretreated with FLX during adolescence (n = 12) displayed significant decreases in Wnt1 (t_{22} = 3.63, p = 0.0014; Fig. 5A), without changes in Wnt5a (p>0.05; Fig. 5B). Conversely, adolescent FLX pretreatment increased mRNA levels of DVL2 (t_{22} = 5.56, p<0.0001; Fig. 5D), but not DVL1 (p>0.05; Fig.5C) or DVL3 (p>0.05; Fig. 5E). Likewise, adolescent FLX history increased the expression of β-catenin (t_{22} = 6.73, p<0.0001; Fig. 5F), PLCγ1 (t_{22} = 4.71, p = 0.0001; Fig. 5G), and CaMKIIα (t_{22} = 2.33, p = 0.0291; Fig. 5H) in the hippocampus of adult mice.

3.5 Adolescent FLX exposure alters the expression of hippocampal genes with diverse cellular functions in adulthood

Figure 6 displays the effects of adolescent FLX treatment (PD35-49) on the expression of hippocampal genes with various intracellular functions in adulthood (PD70). Here, when compared to VEH-pretreated controls (n = 12), adult animals pretreated with FLX during adolescence (n = 12) displayed increased gene expression of DIABLO (t_{22} = 2.35, p = 0.0279;

Fig. 6A) and β-actin (t_{22} = 4.69, p = 0.0001; Fig 6B). Conversely, adolescent FLX history significantly decreased expression of fucose kinase (FUK; t_{22} = 2.35, p = 0.0278; Fig. 6C) and the immediate early-gene c-Fos (t_{22} = 3.38, p = 0.0027; Fig. 6D), without affecting mRNA levels of either BAD (p>0.05; Fig. 6E) or sonic hedgehog (SHH, p>0.05; Fig. 6F).

Chapter 4: Discussion

4.1 Summary

Accumulating preclinical evidence suggests that juvenile exposure to FLX leads to complex behavioral side effects in adulthood; wherein rodents display attenuated responses to inescapable stress (Airan et al., 2007; Iñiguez, Alcantara, et al., 2014; Karpova et al., 2009) along with enhanced drug-seeking behavior (Iñiguez et al., 2015), among other phenotypes (Olivier et al., 2011). Collectively, these enduring FLX induced alterations suggest that ontogenic exposure to SSRIs may render the organism in need of subsequent antidepressant re-exposure in later life to normalize behavior (Iñiguez et al., 2010; Karpova et al., 2009). Thus, the goal of this thesis was to explore the persistent molecular alterations that may result as a function of adolescent SSRI exposure in the adult hippocampus, given that this brain region modulates responses to stress and reward-seeking behavior under normal conditions (Duric et al., 2010; Meyers et al., 2003). Specifically, we evaluated whether FLX administration during adolescence exerts long-term changes in adult hippocampal expression of genes belonging to several major intracellular signaling pathways involved in neuronal growth and survival, including the ERK1/2, IRS2/PI3K/AKT, and Wnt cascades, as well as genes with varied cellular functions, including modulation of calcium signaling (Ca^{++}/calmodulin-dependent protein kinase II [**CamKIIα**]), mitochondrial homeostasis (Direct IAP binding protein with low pI [**DIABLO**], and Bcl2-associated agonist of cell death [**BAD**]), metabolism (fucose kinase [**FUK**]), neuronal survival (sonic hedgehog [**SHH**]), and cytoskeletal assembly (β-actin).

4.2 Adolescent FLX exposure increases MAPK-related gene expression in the adult hippocampus

BDNF is a central component of several intracellular signaling cascades, given its ability to initiate signal transduction of different pathways, including those mediated by MAPK, AKT, and Wnt-disheveled (DVL)-phospholipase C gamma (PLCγ1) signaling (Bjorkholm & Monteggia, 2016). Previous studies have established BDNF as a key molecule in mood-related pathologies, and thus, signaling pathways modulated by this neurotrophic factor can be influenced by the actions of antidepressant drugs, including SSRIs (Autry & Monteggia, 2012; Castren & Rantamaki, 2010). Acute FLX has been shown to alter multiple intracellular signaling pathways (Alcantara et al., 2014; Warren et al., 2011; Yan et al., 2020), and early-life FLX exposure induces long-term increases in the expression of hippocampal BDNF and its main receptor TrkB (Karpova et al., 2009). Thus, we evaluated BDNF mRNA levels within the adult hippocampus as a function of adolescent FLX exposure. Surprisingly, we did not find changes in hippocampal BDNF mRNA (Fig. 2A) – likely due to the differences in the age window of FLX pre-exposure (prepubertal [PD4-21] vs. adolescence [PD35-49]) as well as the promoter specificity of BDNF assessed between the studies. Yet, we found an overall upregulation in mRNA levels of the downstream MAPK signaling pathway (MEK1/2-ERK1/2-p90RSK) 21-days post FLX exposure (Fig. 2B-F). This is an intriguing finding that now bridges hippocampal neurobiological alterations with the persistent FLX-dependent behavioral effects previously reported (Homberg et al., 2011). For example, adult male mice pre-exposed with FLX during adolescence display enhanced preference for rewarding substances like sucrose (Iñiguez et al., 2010) and cocaine (Iñiguez et al., 2015), mimicking the functional role of hippocampal ERK signaling that is observed in adult animals displaying drug-seeking behavior (Tropea et al.,

2008). Further supporting the relationship between ERK and facilitated reward, we found persistent FLX induced elevations of CREB, ΔFosB, and Zif268 (Fig. 3A-C); transcription factors downstream of ERK that have been associated with cocaine-seeking behavior (Gajewski et al., 2019; Hearing et al., 2010; Tropea et al., 2008). Interestingly, while psychological and/or physical stress precipitates drug preference (Fosnocht et al., 2019; Garcia-Carachure et al., 2020; McLaughlin et al., 2006), in a paradoxical manner, juvenile FLX history leads to persistent decreases in responsivity to inescapable stress challenges – since FLX pretreated rodents do not exhibit the characteristic social avoidance induced by repeated social defeat stress (Iñiguez, Alcantara, et al., 2014) or enhanced immobility on the forced swim test (Iñiguez et al., 2010; Karpova et al., 2009). Thus, the enduring FLX induced increases of hippocampal MAPK signaling (Fig. 2), and its downstream transcription factors (Fig. 3), capture both the facilitated drug-seeking phenotype, as well as the resilient-like properties, that these molecules induce in adult animals under normosensitive conditions (Duric et al., 2010; Iñiguez et al., 2019). Yet, here, we report that juvenile SSRI pre-exposure leads to hippocampal MAPK upregulation in adulthood, a critical finding that provides a potential molecular mechanism for the behavioral alterations observed as a function of adolescent FLX history. Interestingly, previous work from our laboratory shows decreases in ERK-signaling within the ventral tegmental area of the midbrain in adult male rodents pre-exposed to FLX during adolescence (Iñiguez, Alcantara, et al., 2014). Along with this earlier work, we now show that juvenile antidepressant exposure changes MAPK signaling differentially across different brain regions in adulthood; with adolescent FLX exposure resulting in long-term decreases of ERK in the ventral tegmental area, while increasing it in the hippocampus (Fig. 2) – an important finding that uncovers long-term

molecular circuit-based alterations between reward related regions (i.e., ventral tegmental area) and the hippocampal formation.

4.3 Adolescent FLX exposure alters AKT- and Wnt-related hippocampal gene expression in adulthood

Given that insulin receptor substrate (**IRS**)-2 modulates synaptic plasticity within the hippocampus (Martin et al., 2012), as well as responses to antidepressant medications and drugs of abuse (Glombik et al., 2017; Iñiguez et al., 2008; Russo et al., 2007), we further evaluated the enduring impact of adolescent FLX on IRS2 and its downstream signaling components, including PI3K, PDK, AKT, and glycogen synthase 3 beta-1 (**GSK3β-1**). Here, adolescent FLX history increased the mRNA levels of these genes in the adult hippocampus (Fig.4). This prolonged FLX induced upregulation of the AKT signaling cascade is consistent with previous work demonstrating that adult male rodents pre-exposed to FLX during adolescence display long-lasting prophylactic phenotypes (Iñiguez et al., 2010). In other words, the persistent increases in AKT signaling, as a function of FLX history, mediates resilient-like behavioral responses on preclinical tests of despair, as well as in postmortem tissue of depressed patients that were taking antidepressants at the time of death (Krishnan et al., 2008). Likewise, we evaluated molecular markers related to the Wnt pathway, given that deregulation of this signaling cascade has been proposed to underlie aspects of major depression and antidepressant efficacy (Wilkinson et al., 2011), as well as responses to cocaine (Dias et al., 2015). In this case, adolescent FLX pre-exposure decreased Wnt1, but not Wnt5a (Fig. 5A-B) hippocampal mRNA levels in adulthood. Conversely, we found that adolescent FLX pretreatment resulted in a persistent upregulation of several downstream components of the Wnt canonical (β-catenin; Fig.

5F) and noncanonical (PLCγ1 and CaMKIIα; Fig. 5G-H) pathways. Specifically, FLX exposure resulted in a lasting increase of DVL2 (but not DVL1 or DVL3; Fig. 5C-E), β-catenin (Fig.5F), PLCγ1 (Fig.5G) and CaMKIIα (Fig.5H). Of note, PLCγ1 and CaMKIIα are both signaling markers that crosstalk with the protein kinase C (**PKC**) cascade, and thus, it is likely that FLX history de-regulates additional signaling pathways involved in plasticity, cell migration, and neurogenesis (Abdolmaleki et al., 2020).

4.4 Adolescent FLX exposure alters hippocampal transcripts associated with neuronal function/structure in adulthood

One of the interesting things about FLX is that it increases plasticity/neurogenesis markers within the hippocampus, along with decreases in immobility on the forced swim test, three weeks post antidepressant exposure (Airan et al., 2007) – thus, matching the persistent resilient-like profile commonly reported in adult male mice and rats with juvenile FLX history (Iñiguez, Alcantara, et al., 2014; Iñiguez et al., 2010; Karpova et al., 2009). Because the integrity of the hippocampus plays a central role in affect-related disorders (Cha et al., 2016), we further evaluated whether SSRI history would result in long-term changes of hippocampal genes associated with neuronal growth (SHH; (Gonzalez-Reyes et al., 2019)), apoptosis (DIABLO, BAD; (Troy et al., 2002)), cytoskeletal assembly (β-actin; (Chung, 2015)), metabolism (FUK; (Angenstein et al., 1992; Popov et al., 1983)) as well as overall neuronal activation (c-Fos; (Gao & Ji, 2009)). While no differences in SHH or BAD were noted between the groups, we found a persistent increase in DIABLO and β-actin, along with decreases in FUK and c-Fos mRNA expression (Fig. 6). Interestingly, these long-lived FLX induced transcriptional changes mimic those induced by stress/injury insults; wherein rodents display increases in β-actin (P et al.,

2012) and the release of proapoptotic mitochondrial intermembrane space proteins, like DIABLO (X et al., 2005). Moreover, stress/injury insults impair memory performance, and since FUK activity (Popov et al., 1983) and c-Fos expression (J et al., 2002) are positively correlated with hippocampus-dependent memory performance, the long-term FLX-induced downregulation of FUK and c-Fos, respectively, potentially contributes to the spatial memory impairment observed in adult rodents with a history of FLX exposure during adolescence (Flores-Ramirez et al., 2019). Collectively, these findings suggest that aberrant transcription of DIABLO, FUK, c-Fos, β-actin, as well as multiple intracellular pathways (i.e., ERK, AKT, Wnt) caused by adolescent FLX exposure, may lead to altered neuronal plasticity/survival, and overall activation of the adult hippocampus (Cowen, 2007; Varela-Nallar & Inestrosa, 2013); resulting in an imbalance of information processing within brain circuits that modulate responses to rewards, memory performance, and stress (Fig. 7). Although correlational, the results of this thesis may provide a molecular signature to the complex behavioral profile exhibited by adult rodents previously exposed to FLX during adolescence (Homberg et al., 2011; Iñiguez et al., 2015; Iñiguez et al., 2010).

4.5 Limitations

A limitation of the present work is the exclusion of female rodents in our experimental design, consequently reducing the interpretability of our data to the clinical setting – wherein women, when compared to men, represent most of the patients prescribed with FLX for the management of numerous illnesses including depression, eating disorders, anxiety, pain, and pre-menstrual dysphoric disorder (Hoffmann et al., 2014). As such, future investigations using female rodents will be needed to assess whether similar or different patterns of hippocampal

gene alterations, when compared to the present results in males, are expressed in adulthood as a function of FLX pretreatment. Particularly, because juvenile FLX history results in differential behavioral responses in adulthood to reward-related stimuli between the sexes – wherein males display enhanced preference for drug rewards like cocaine (Iñiguez et al., 2015) while females display a decrease in preference for the stimulant (Flores-Ramirez et al., 2018). Another caveat is that the animals utilized in this investigation were not exposed to stress, a known risk factor for the development of mood-related disorders. As such, future work is needed where adolescent mice undergo similar FLX treatment along with stress models for the study of affect-related illnesses (Iñiguez, Riggs, et al., 2014; N et al., 2020; Warren et al., 2020). Lastly, given that we only evaluated mRNA expression in this study, additional experiments are needed to specifically assess whether the gene expression findings translate to respective changes in hippocampal protein levels.

4.6 Conclusion

We report that juvenile FLX exposure results in persistent gene expression changes across several intracellular signaling cascades (ERK, AKT, and Wnt) that are implicated in growth, plasticity, and the survival of neurons in the adult hippocampus of male C57BL/6 mice (Iñiguez et al., 2020). These long-term FLX induced transcript changes provide a molecular link to the complex behavioral phenotypes that result from early-life SSRI exposure, such as enhanced drug-seeking behavior (Iñiguez et al., 2015) along with lower reactivity to inescapable stress (Iñiguez, Alcantara, et al., 2014) and memory deficits (Flores-Ramirez et al., 2019; Sass & Wortwein, 2012). Importantly, this work provides novel insight about the persistent hippocampal

molecular consequences of adolescent exposure to the antidepressant FLX on adult behavior – thus, questioning the safety of SSRI exposure during early stages of development.

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Table 1. Genes

Metabolism Factors:

- **β-actin** (*Beta-actin*) – a member of the actin family; involved in cell motility, structure, and integrity (RefSeq, 2015).
- **BAD** (*Bcl2-Associated Agonist of Cell Death*) – positively regulates cell apoptosis by reversing cell's death repressor activity. It is known to be regulated by protein kinases AKT and MAP kinase (RefSeq, 2019).
- **DIABLO** (*Direct IAP Binding Protein with Low pI*) – mitochondrial protein that is released into the cytosol in response to apoptotic stimuli (Verhagen et al., 2000)
- **FUK** (*Fucose Kinase*) – this enzyme catalyzes the first step in the utilization of free L-fucose (thought to mediate inflammation, as well as metastasis) in glycoprotein and glycolipid synthesis (Miller et al., 2005).
- **GAPDH** (*Glyceraldehyde 3-Phosphate Dehydrogenase*) – a housekeeping gene that plays an essential role in cell glycolysis (Barber et al., 2005).
- **SHH** *(Sonic Hedgehog)* – important in the maintenance of the structure and overall integrity of neuronal cells (Herzog et al., 2003)

Mitogen Activated Protein Kinase (MAPK) Pathway:

- **BDNF** (*Brain Derived Neurotrophic Factor*) – involved in the growth, differentiation, and survival of specific types of developing neurons both in the central- and peripheral-nervous system. Involved in regulating synaptic plasticity in the CNS (RefSeq, 2015).
- **ERK1** (*Extracellular Signal-Regulated Kinase 1*) – also known as Mapk3, regulates various cellular processes, including proliferation, differentiation, and cell cycle progression in response to a variety of extracellular signals (RefSeq, 2008)
- **ERK2** (*Extracellular Signal-Regulated Kinase 2*) – also known as Mapk1, is involved in cell proliferation, differentiation, transcription regulation, and development (RefSeq, 2014)
- **MEK1** *(Mitogen-Activated Protein Kinase Kinase 1)* – involved in the phosphorylation of MAPK3/ERK1 and MAPK1/ERK2 and aids in the binding of extracellular ligands (i.e., growth factors) to their cell-surface receptors (RefSeq, 2008)
- **MEK2** *(Mitogen-Activated Protein Kinase Kinase 2)* – an essential component of the MAPK signaling pathway, known to play a role in mitogen growth factor signal transduction (RefSeq, 2008)
- **p90RSK** *(phospho-90 kDa Ribosomal Kinase; MAPK Activated Protein Kinase 2)* – part of the MAPK signaling cascade as a direct downstream effector of ERK1/2 (Lin, White & Hu, 2019)

Nuclear Transcription Factors:

- **c-Fos** (*FBJ Murine Osteosarcoma Viral Oncogene Homolog*) – an immediate early gene involved in cell activation, proliferation, differentiation, and survival (RefSeq 2008).
- **CREB** (*cAMP Response Element-Binding Protein*) – a cellular transcription factor that plays a role in neuronal plasticity, long-term memory formation, and is integral in the formation of spatial memory (Silva et al., 1998).
- **ΔFosB** (*DeltaFosB*) – a member of the Fos family of transcription factors whose role is commonly implicated in the development addiction- and depression-related behaviors (Nestler, Barrot, & Self, 2001)
- **Zif268** *(Zinc Finger Protein 268)* – a transcriptional regulator protein, important for cell differentiation and the modulation of apoptotic process (RefSeq, 2014)

Phosphoinositide 3-Kinase (PI3K)/ Protein Kinase B (AKT) Pathway:

- **AKT1** (*Thymoma Viral Proto-oncogene 1/ Protein Kinase B*) – a major downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway that mediates the effects of various growth factors (RefSeq, 2009).
- **GSK3β1** (*Glycogen Synthase Kinase 3 Beta 1*) – involved in energy metabolism, inflammation, ER-stress, mitochondrial dysfunction, and apoptotic pathways (Plyte et al., 1992).
- **IRS2** (*Insulin Receptor Substrate 2*) – a cytoplasmic signaling molecule that activates neuronal growth and survival signaling pathways, such as PI3K/AKT, and modulates synaptic plasticity within the hippocampus (Schubert et al., 2003; Martin et al., 2002)
- **PDK** *(Pyruvate Dehydrogenase Kinase)* – mitochondrial regulatory enzyme known to mediate the oxygen metabolism of the cell, as part of the PI3K/EKT pathway (Cerniglia et al., 2015)
- **PI3K** *(Phosphoinositide 3-Kinase)* – activates protein kinase B (AKT) as part of the PI3K/AKT pathway. Also plays an important role in modulating several neuronal functions, such as cell growth, survival, and intracellular trafficking (Cuesto et al., 2011)

Canonical Wingless/ Integrated (Wnt) Pathway:

- **β-catenin** (*Beta-catenin*) – a subunit of the cadherin protein complex, acts as an intracellular signal transducer in the Wnt signaling pathway, which controls cell growth and differentiation during normal development (RefSeq, 2009).
- **CaMKIIα** (*Calcium/calmodulin-dependent Protein Kinase 2 Alpha*) – this alpha subunit of the CaMKII family is required for hippocampal long-term potentiation (LTP), spine structural LTP, and spatial learning (Silva et al., 1992).

Canonical Wingless/ Integrated (Wnt) Pathway:

- **DVL1** (*Dishevelled-1*); **DVL2** (*Dishevelled-2*); **DVL3** (*Dishevelled-3*) – members of the Dishevelled (Dsh) family, implicated in the regulation of canonical (involving β-catenin) and non-canonical Wnt signaling, specifically the transduction of the Wnt signal to down-stream effectors (Komiya & Habas, 2008).
- **PLCγ1** *(Phospholipase-C Gamma 1)* – cell growth factor important for cell growth, cell migration, and apoptosis. In neuronal cells, it is involved in actin cytoskeleton organization and synaptic plasticity (Kang et al., 2020)
- **Wnt1** *(Wingless-Type MMTV Integration Site Family, Member 1)* – extracellular protein, part of the Wnt/canonical signaling pathway, involved in cytokine-, frizzled-binding, and protein domain specific binding activity (Castelo-Branco et al., 2003)
- **Wnt5a** *(Wingless-Type MMTV Integration Site Family, Member 5A)* – part of the Wnt signaling pathway involved in several processes, including the generation of neurons and hormonal response (Lange et al., 2006)

Table 2. Primer sequences

Gene	Forward primer	Reverse primer
AKT1	5'-ATGAACGACGTAGCCATTGTG-3'	5'-TTGTAGCCAATAAAGGTGCCAT-3'
BAD	5'-AAGTCCGATCCCGGAATCC-3'	5'-GCTCACTCGGCTCAAACTCT-3'
BDNF	5'-GAAGAGCTGCTGGATGAGGAC-3'	5'-TTCAGTTGGCCTTTTGATACC-3'
β-actin	5'-AGTGTGACGTTGACATCCGTA-3'	5'-GCCAGAGCAGTAATCTCCTTCT-3'
β-catenin	5'-ATGGAGCCGGACAGAAAAGC-3'	5'-CTTGCCACTAGGGAAGGA-3'
CaMKIIα	5'-GTTCTCCGTTTGCACTAGG-3'	5'-TTCCCAGTTCCTCAAAGAGC-3'
c-Fos	5'-AACCGCATGGAGTGTGTTGTTCC-3'	5'-TCAGACCACCTCGACAATGCATGA-3'
CREB	5'-AGTGACTGAGGAGCTTGTACCA-3'	5'-TGTGGCTGGGCTGAAC-3'
DIABLO	5'-TCCTGTACCTGTGACTTCACC-3'	5'-TCCTGTACCTGTGACTTCACC-3'
DVL1	5'-GGCGGAGACCAAAATCATC-3'	5'-GGACTTGAAGAAGAATTTGTAGGC-3'
DVL2	5'-GGCGGAGACCAAAATCATC-3'	5'-GGACTTGAAGAAGAATTTGTAGGC-3'
DVL3	5'-GCGAGACCAAGATCATCTACC-3'	5'-TCGTCGTCCATAGACTTGAAGA-3'
ERK1	5'-TCCGCCATGAGAATGTTATAGGC-3'	5'-GGTGGTGTTGATAAGCAGATTGG-3'
ERK2	5'-GGTTGTTCCCAAATGCTGACT-3'	5'-CAACTTCAATCCTCTTGTGAGGG-3'
ΔFosB	5'-AGGCAGAGCTGGAGTCGGAGAT-3'	5'-GCCGAGGACTTGAACTTCACTCG-3'
FUK	5'-CTGGAGGTAAGGCAGAGACG-3'	5'-TGTGCAAGATGAGGATCCAG-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-GTAGACCATGTAGTTGAGGTCA-3'
GSK3β-1	5'-GACAAGCATTTAAGAACCGAGA-3'	5'-ACCAGGTAAGGTAGACCTACATC-3'
IRS2	5'-CTGCGTCCTCTCCCAAAGTG-3'	5'-GGGGTCATGGGCATGTAGC-3'
MEK1	5'-AAGGTGGGGGAACTGAAGGAT-3'	5'-CGGATTGCGGGTTTGATCTC-3'
MEK2	5'-GTTACCGGCACTCACTATCAAC-3'	5'-CCTCCAGCCGCTTCCTTTG-3'
PDK	5'-TCCTGGACTTCGGAAGGGATA-3'	5'-GAAGGGCGGTTCAACAAGTTA-3'
PI3K	5'-ACACCACGGTTTGGACTATGG-3'	5'-GGCTACAGTAGTGGGCTTGG-3'
PLCγ1	5'-ATCCAGCAGTCCTAGAGCCTG-3'	5'-GGATGGCGATCTGACAAGC-3'
p90RSK	5'-CCATCACACACCACGTCAAG-3'	5'-TTGCGTACCAGGAAGACTTTG-3'
SHH	5'-AAAGCTGACCCCTTTAGCCTA-3'	5'-TTCGGAGTTTCTTGTGATCTTCC-3'
Wnt1	5'-CTCGCCACTCATTGTCTGTG-3'	5'-TTCCCAGGCTGGCTCTAATA-3'
Wnt5a	5'-CAACTGGCAGGACTTTCTCAA-3'	5'-CATCTCCGATGCCGGACT-3'
Zif268	5'-TCGGCTCCTTTCCTCACTCA-3'	5'-CTCATAGGGTTGTTCGCTCGG-3'

AKT1 – thymoma viral proto-oncogene 1/protein kinase b; BAD – Bcl2-associated agonist of cell death; BDNF – brain derived neurotrophic factor; β-actin – beta-actin; β-catenin – beta-catenin; CaMKIIα – calcium/calmodulin-dependent protein kinase 2 alpha; c-Fos - FBJ Murine Osteosarcoma Viral Oncogene Homolog; CREB – cAMP response element-binding protein; DIABLO - Direct IAP binding protein with low pI; DVL1 – dishevelled-1; DVL2 – dishevelled-2; DVL3 – dishevelled-3; ERK1 – extracellular signal-regulated kinase 1; ERK2 – extracellular signal-regulated kinase 2; ΔFosB – DeltaFosB; FUK – fucose kinase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; GSK3β-1 – glycogen synthase kinase3 beta 1; IRS2 – insulin receptor substrate 2; MEK1 – mitogen-activated protein kinase kinase 1; MEK2 – mitogen-activated protein kinase kinase 2; PDK – pyruvate dehydrogenase kinase; PI3K – phosphoinositide-3 kinase; PLCγ1 – phospholipase C, gamma 1; p90RSK – MAPK-activated protein kinase 2; SHH – sonic hedgehog; Wnt1 – Wingless-Type MMTV Integration Site Family, Member 1; Wnt5a – wingless-type MMTV integration site family, member 5A; Zif268 – zinc finger protein 268.

Figure 1. Timeline of experimental procedures. Adolescent male C57BL/6 mice (N=24; 12/group) were exposed to fluoxetine (0 or 20 mg/kg/day) for 15 consecutive days (postnatal day [PD] 35-49). Twenty-one days later (rest period), animals were euthanized (PD70) and hippocampal tissue was collected for qPCR (quantitative real-time Polymerase Chain Reaction) analysis.

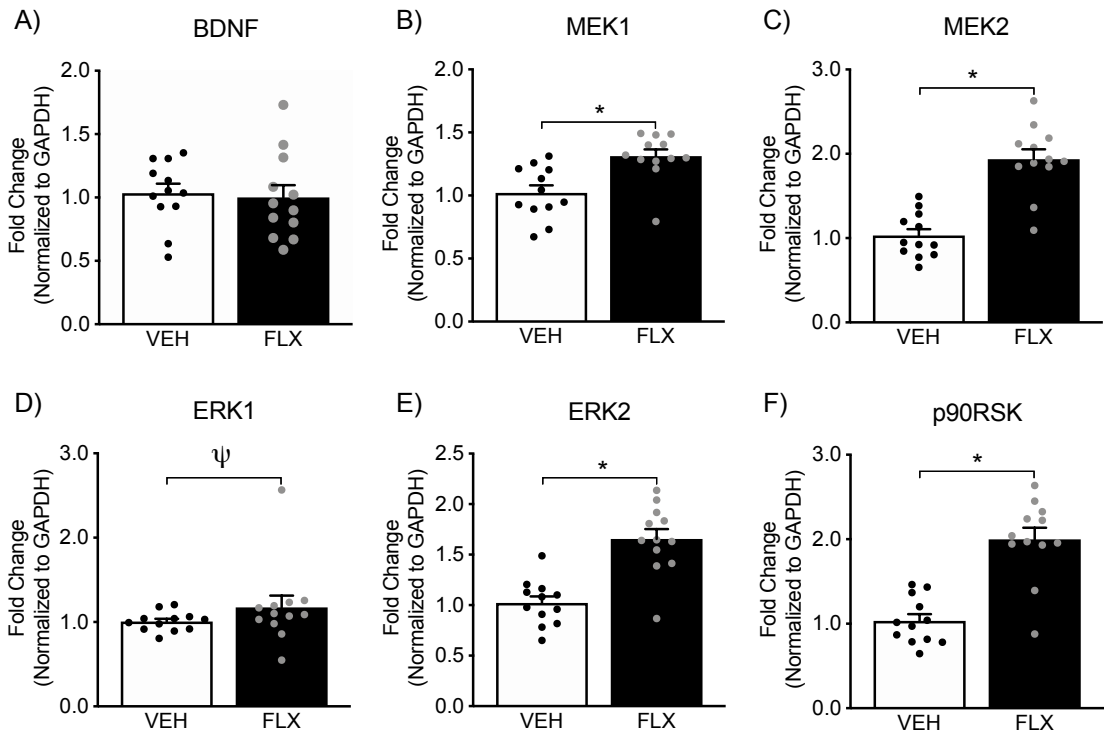

Figure 2. Effects of adolescent fluoxetine (FLX) exposure on the expression of hippocampal genes from the intracellular MAPK signaling pathway in adulthood. FLX exposure resulted in increased hippocampal expression of MEK1, MEK2, ERK1, ERK2, and p90RSK, but did not alter BDNF levels, in adult mice. Data are presented as mean ± SEM. *$p<0.05$, $^{\Psi}p=0.06$ when compared with control (VEH).

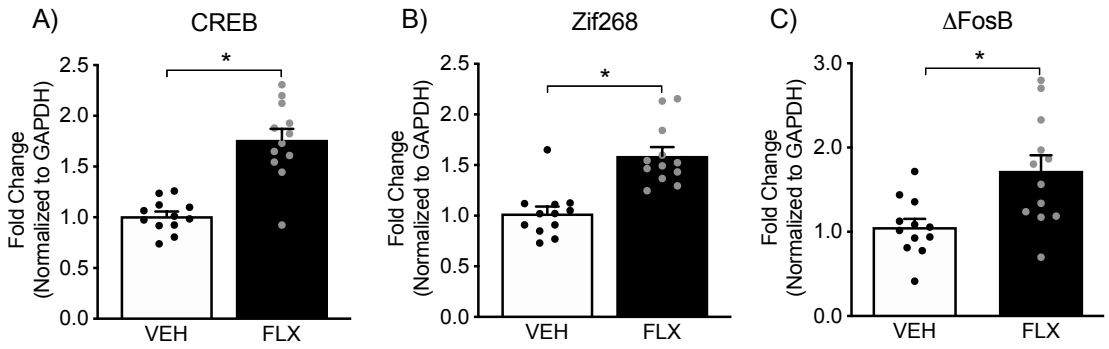

Figure 3. Effects of adolescent fluoxetine (FLX) exposure on the expression of hippocampal transcription factors in adulthood. FLX pretreatment induced significant increases in hippocampal expression of CREB, Zif268, and ΔFosB. Data are presented as mean ± SEM. *$p<0.05$ when compared to control (VEH).

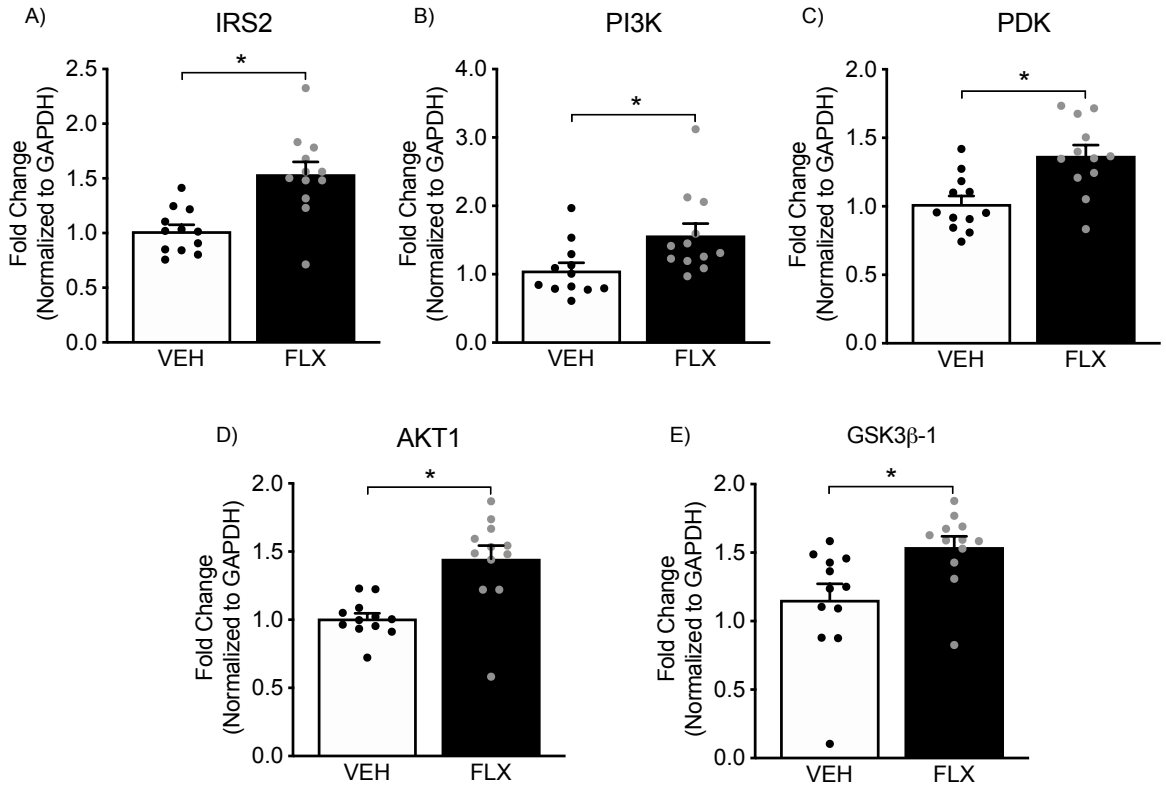

Figure 4. Effects of adolescent fluoxetine (FLX) treatment on the expression of hippocampal genes from the IRS2/PI3K/AKT pathway in adulthood. Adolescent FLX pretreatment mediated significant increases in hippocampal expression of IRS2, PI3K, PDK, AKT1, and GSK3β-1. Data are presented as mean ± SEM. *$p<0.05$ when compared to control (VEH).

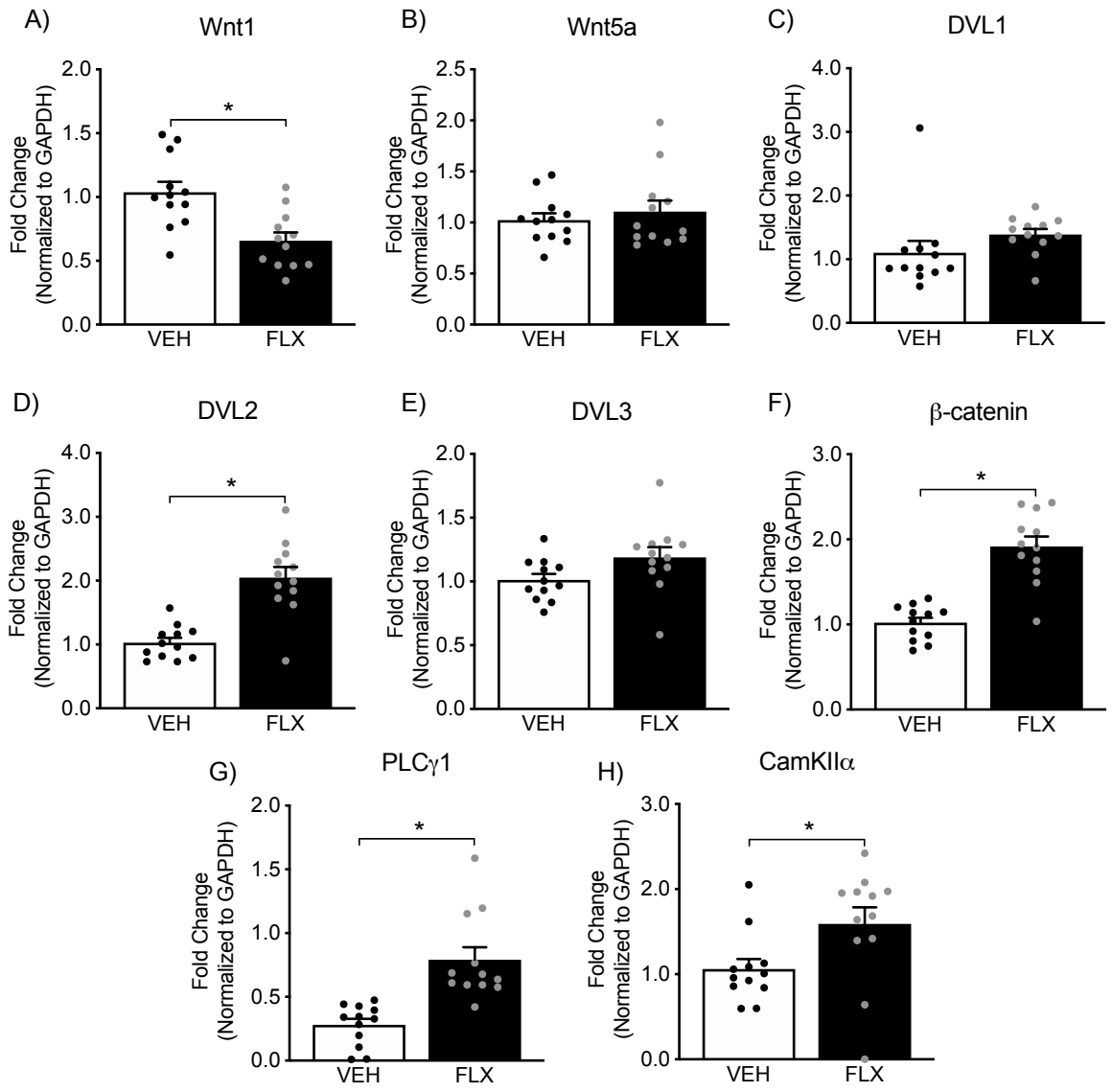

Figure 5. Effects of adolescent fluoxetine (FLX) treatment on the expression of hippocampal genes from the Wnt pathway in adulthood. Adolescent FLX pretreatment downregulated Wnt1, while increasing DVL2, β-catenin, PLCγ1, and CaMKIIα. No changes in Wnt5a, DVL1, or DVL3 were noted between the groups. Data are presented as mean ± SEM. *$p<0.05$, when compared with control (VEH).

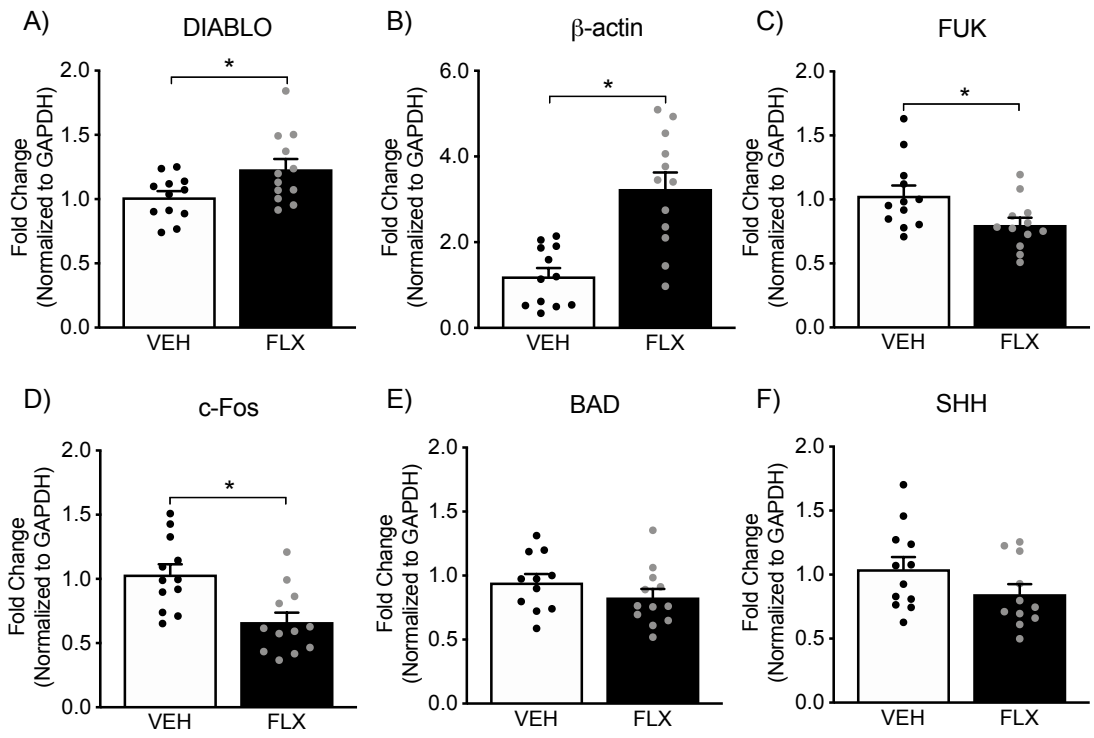

Figure 6. Effects of adolescent fluoxetine (FLX) treatment on the expression of hippocampal genes with diverse cellular functions in adulthood. Adolescent FLX treatment increased DIABLO and β-actin, while decreasing FUK and c-Fos expression, without altering BAD or Sonic hedgehog (SHH). Data are presented as mean ± SEM. *$p<0.05$, when compared with control (VEH).

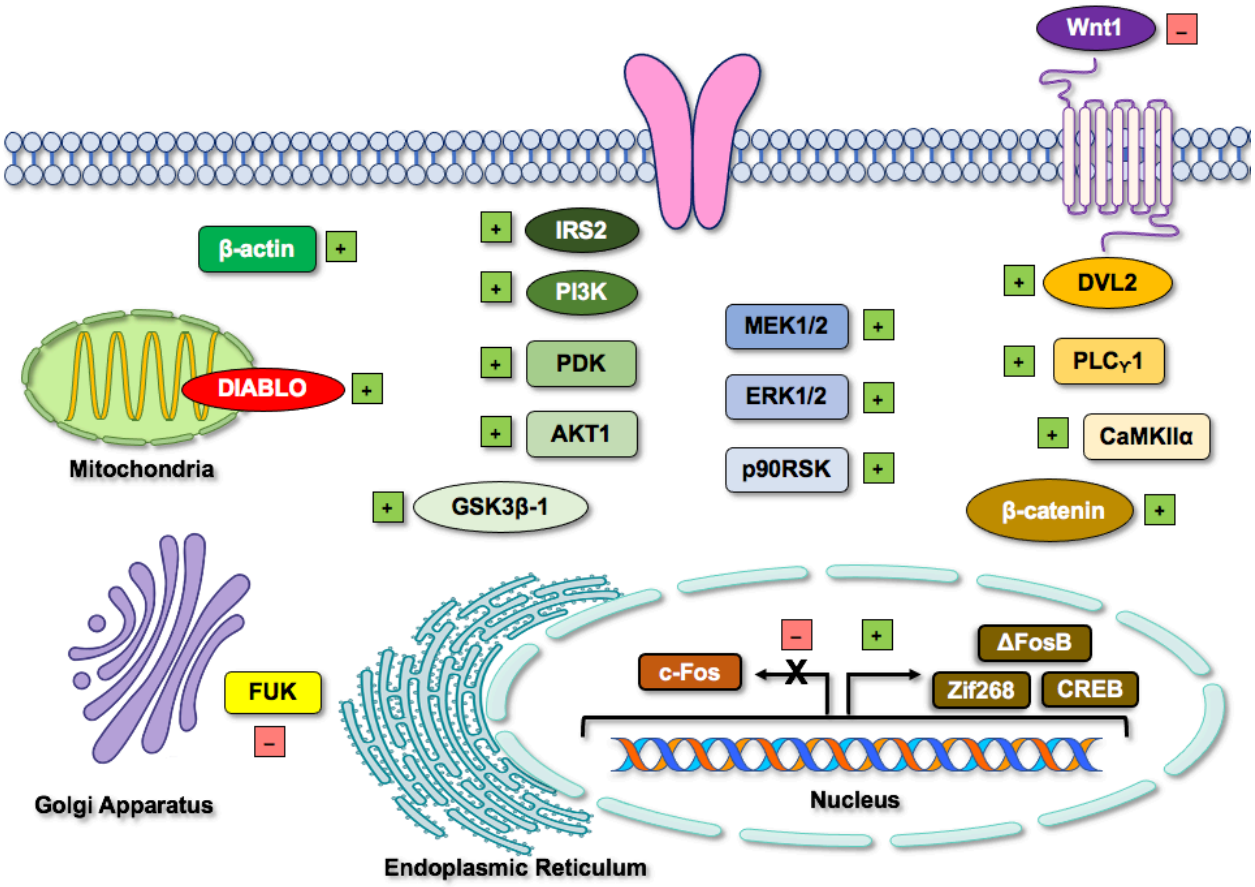

Figure 7. Schematic representation of the long-lived impact of adolescent fluoxetine exposure on hippocampal mRNA expression in adulthood. Exposure to fluoxetine during adolescence (postnatal days 35-49) altered genes associated with major intracellular signaling cascades (AKT/ERK/Wnt), transcription factors (ΔFosB, Zif268, CREB), apoptosis (DIABLO), metabolism (FUK), as well as neuronal structure (β-actin) and activation (c-Fos) in adulthood (postnatal day 70). [+] Significantly higher when compared to controls. [-] Significantly lower when compared to controls.

Vita

Anapaula Themann was born in Chihuahua, Mexico. The eldest daughter of Ana Alicia Rios Saldaña and Joshua Todd Themann, she graduated from the University of Texas at El Paso, in the Spring of 2018, with two Bachelor of Science – Psychology and Cellular & Molecular Biochemistry. She joined the Master's program in Experimental Psychology at The University of Texas at El Paso in the Fall of 2019. The following year (Fall 2020) she was accepted into the Behavioral Neuroscience Ph.D. Program under the mentorship of Dr. Sergio D. Iñiguez.