Post-natal deficiency of omega-3 fatty acids: its role on cocaine-seeking behaviors and drug withdrawal severity

By

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DOCTORAL DISSERTATION

Presented to the Faculty of the Graduate School of Biology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR IN PHILOSOPHY

In

Biology

August 21, 2019

University of Puerto Rico

Rio Piedras Campus

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ACKNOWLEDGEMENTS

I will never have the appropriate words to express my gratitude to all the collaborators who have given me their full confidence in the elaboration and culmination of this work. First, I would like to thank my family—my parents, brothers & aunts—for their patience and their unconditional support during this arduous journey. I would also like to thank Dr. Carmen Maldonado-Vlaar, my mentor and the director of this research, for giving me the opportunity to work in her laboratory, and for her flexibility, patience, supervision, professionalism, talent and knowledge.

I must offer my distinctive appreciation to Dr. José E. García-Arrarás, whom I also consider to be a mentor and a friend. I will always admire him for his trust, objectivity, admonishment, motivation, and suggestions. Dr. Carlos A. Jiménez-Rivera, thank you for your advice, humbleness, integrity, availability and your unwavering passion for science and research. I should also recognize the professionalism, experience and wisdom of Dr. Carlos I. González and Dr. Abel J. Baerga Ortiz for the realization of this dissertation.

Recognition is due to all the members of the Maldonado's Laboratory, especially Dr. Jean Roussel, Dr. Arlene Martínez and Natalia Rodríguez, who contributed to my development, both personal and professional. Moreover, my sincere gratitude to the animal house facility personnel and the administration of the biology program; Aidamarie Perez and Millie Viera. I would furthermore like to thank all of the students from Julio Garcia Diaz (JGD), who entered this program

with me and became my friends during the past six years. Last, but not least, I would like to express my appreciation for the financial support I received from Research Initiative for Student Enhancement (RISE) program (5R25GM061151) and the DEGI (Decanato de Estudios Graduados e Investigación) office.

I appreciate that everyone has given me a new perspective on what science and research is. The doctoral program in biology at the University of Puerto Rico (UPR) has been an enriching process. I will always carry beautiful memories that will stay with me for the rest of my days. I will conclude by saying; most likely I'm not the best, and who knows if I'm the worst, nonetheless with all certainty and with my head held high, I can say that I always gave my best, THANK YOU ALL!

This work is dedicated to my parents, Migdalia Torres-Velez and to the memory of my father, Sergio Serrano-Luciano (1948-2015), who had the noblest and humblest heart that I have ever known. Though his life has ended, my love, reverence, and appreciation for him never will.

THESIS SUMMARY

Adolescence is a transitional stage characterized by unique neurobiological changes in the brain. The brain's maturation during this stage, particularly in the frontal cortex and the limbic system, is responsible for cognitive control and reward-seeking behaviors. Adolescence is also characterized by an enhanced vulnerability to external factors: Nutritional deficiencies, for example, can lead to long-lasting neurochemical changes that may persist into adulthood. Research studies have highlighted the adverse effects of omega-3 (ω-3) on brain function, which result in cognitive impairments and modified reward processing.

It has been noted that deficiencies in ω-3 play an essential role during adolescence, predisposing individuals to behavioral and cognitive deficits. Molecular studies in rodents have indicated that deprivation of ω-3 can dysregulate neurotransmitters, such as the dopaminergic, serotoninergic, glutamatergic and endocannabinoids, involved in the pathophysiology of addiction. While several mechanisms underlying the effects of dietary ω-3 deficiency have been described, those specifically linked to adolescence are limited and poorly understood. The modulatory effect triggered by such a deficiency may influence addictive drug behaviors and neurotransmitters associated with motivation and reward.

Research studies have demonstrated that low levels of omega-3 (ω-3) in the brain can alter the dopaminergic system of the brain and, as a result, increase the risk of developing addictive disorders as well as the severity of the disorder. Studies have additionally shown the detrimental effects of ω-3 deficiency in the treatment of cocaine abuse: ω-3 deficiency can aggravate withdrawal symptoms

such as anxiety, anger, cravings, and depression. Conversely, it has been demonstrated that dietary supplementation reverse these symptoms. Together, this evidence suggests that insufficient levels of ω-3 may influence addictive behaviors. The precise mechanisms by which ω-3 modulate addiction, however, remain uncertain. To the best of our knowledge, evidence linking ω-3 deficiencies and cocaine use disorder within the brain's reward regions was non-existent.

Thus, the aim of this dissertation was to examine whether adolescence may represent a critical period during which ω-3 deprivation may contribute to a higher vulnerability of future cocaine abuse in adulthood. Moreover, we analyzed subsequent predisposition to depression and anxiety at several timepoints. We hypothesized that deficiency on the intake of ω-3 starting in adolescence would have a direct effect on cocaine consumption, intensifying cue-induced and cocaine-seeking behaviors in adulthood.

Based on the preceding, **Chapter 1** addresses general biochemical concepts about fatty acids and reviews the literature regarding the harmful consequences of ω-3 deficiency and its impact on neurodevelopment, reward-related neurocircuitry, and the likelihood of its influence on addictive behaviors. **Chapter 2** evaluates how ω-3 deficiency alters the development of cocaine-seeking behaviors using the cocaine self-administration paradigm with a fixed-ratio schedule. Furthermore, Chapter 2 includes evidence about the etiology of anxiety-like behaviors after a short abstinence period. Our results show that prolonged dietary deprivation of ω-3 did not exert any significant effect in lever-pressing activity through the acquisition and extinction phases of the cocaine seeking

behavior paradigm. However, DEF group demonstrated a substantial decrease in lever pressing during cue-induced reinstatement. Moreover, n-3 PUFA-deficient animals showed enhanced anxiety-like behaviors after 14 days of cocaine abstinence. Furthermore, we discover that protein levels of the dopaminergic receptor-1 (DR1) were increased within the PFC. **Chapter 3** considers how ω-3 deprivation and chronic cocaine consumption disturb cue-induced reinstatement behaviors using the incubation of cue reactivity between ID1 (Incubation Day 1) and ID40 after forced abstinence. In addition, anxiety-like and depression-like behaviors were evaluated after 10 weeks using the elevated plus maze test (EPM) and the Forced Swimming Test (FST). On ID40, the DEF group showed lower cue-induced cocaine-seeking behavior compared to the CON group. In the EPM, the DEF group showed an enhanced anxiety based on a gradual reduction in the time spent on the open-arm and an increased time in the closed-arms. During the FST test, the DEF group demonstrated greater immobility time compared to the CON group which is interpreted as an enhanced depressive-like behavior. Finally, **Chapter 4** summarizes results, evaluates limitations, and recommends future experiments.

We concluded that dietary absence of ω-3 could intensify the symptoms of anxiety and depression during withdrawal periods after extended access of cocaine self-administration. Furthermore, we suggested that this deficiency could modify sensitivity to the rewarding effects of cocaine. We can speculate that these dietary-induced perturbations in PUFA homeostasis can deregulate the dopaminergic systems, leading to behavioral mood changes and impaired

responsiveness to positive events. Future studies will further address this statement, evaluate other neurotransmitter systems and brain areas that might also be involved to achieve a more definite conclusion.

LIST OF ABBREVIATIONS

5-HT	Serotonin
AA	Arachidonic Acid
AC	Adenylate Cyclase
ACTH	Adrenocorticotrophic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
ALA	A-Linolenic Acid
AMI	Myocardial Infarction
AMPH	Amphetamine
BBB	Blood-Brain Barrier
BChE	Butyrylcholinesterase
BE	Benzoylecgonine
Ca2+	Calcium
CAMP	Cyclic Adenosine Monophosphate
GC	Gas Chromatography
Cm	Centimeter
CNS	Central Nervous System
CON	Control
CPP	Conditioned Place Preference
CRF	Corticotrophin Releasing Factor/Hormone
CSF	Cerebral Spinal Fluid
CVD	Cardiovascular Disease

D1	Day 1
D35	Day 35
D40	Day 40
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine Transporters
DEF	Deficient
DHA	Docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DPA	Docosapentaenoic Acid
DR1	Dopamine Receptor 1
DR2	Dopamine Receptor 2
E	Experimental
ECS	Endocannabinoid System
EFA	Essential Fatty Acids
EMCDDA	European Monitoring Center for Drugs and Drug Addiction
EME	Ecgonine Methyl Ester
EPA	Eicosapentaenoic Acid
EPM	Elevated Plus Maze
FO	Food Only
FR	Fixed Ratio
FST	Forced Swimming Test
h	Hour

hCE-1	Human Carboxylesterase
HPA	Hypothalamus Pituitary Axis
HPC	Hippocampus
HRP	Horseradish peroxidase
i.v.	Intravenous
IACUC	Institutional Animal Care and Use Committee
i.p.	Intraperitoneal
LA	Linoleic acid
MGluRs	Metabotropic glutamate receptors
MPFC	Medial prefrontal cortex
NA	Norepinephrine
NAcc	Nucleus Accumbens Core
NAcs	Nucleus Accumbens Shell
NME	Norecgonine methyl ester
P21	Post-Natal Day 21
PFC	Prefrontal Cortex
Phe	Phenylalanine
PR	Puerto Rico
PR	Progressive Ratio
PSM	Ponce School of Medicine
PUFA's	Polyunsaturated Fatty Acids
RISE	Research Initiative for Student Enhancement
SA	Paradigm of Self-Administration

SD	Sprague-Dawley
SEM	Standard Error of the Mean
SN	Substantia Nigra
Str	Striatum
Thr	Threonine
TO	Time Out
Tyr	Tyrosine
U.S.A	United States of America
UN	United Nations
UNGDR	United Nations Global Drug Report
VP	Ventral Pallidum
VS	Ventral Striatum
Vs	Versus
VTA	Ventral Tegmental Area

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CHAPTER I

Review of Literature

1.1 DRUG ADDICTION

1.1.1 History of drugs of abuse in antiquity

Drug addiction is one the most complex and challenging neuropsychiatric disorders in today's society that adversely affects the lives of millions across all geographic borders and cultures (F Ali et al., 2011). At the same time, it should be noted that the use of psychoactive substances has not always been perceived as a socio-cultural issue (Crocq, 2007). In fact, these drugs have played roles in many aspects of various societies since the beginning of humanity. It is possible to trace drug use back thousands of years in different cultures as a means of relaxation, mind-transforming experience, and for medical applications (Goldberg, 2013). Numerous drugs have been used, from opioids in the Asian continent (3,400 years), to alcohol in ancient Babylon (3,000 years), to cocaine in the Inca empire (8,000 years) (Ghodse, 2010). Their intake was a significant part of everyday life without representing a social complication. Nonetheless, the purpose of the consumption of drugs between ancient times and contemporary culture differs significantly (Crocq, 2007).

With valid arguments, the battle against drugs and their production is intensifying. However, the continual focus by the media on this war gives an erroneous impression as if drugs, including both licit and illicit, were discovered recently and neglects the fact that the consumption of psychoactive substances goes back to ancient times. Their ingestion for religious rituals or other purposes, such as mitigating hunger and thirst, energizing combatants in wars or modifying

levels of consciousness was a custom for natives of diverse regions from across continents (Kamienski, 2017). Moreover, psychotropic preparations were made from vegetable or animal origin that naturally contained a negligible drug concentration. In contrast, throughout the centuries, advances in the elaboration and manipulation of chemicals has permitted societies to obtain stronger psychoactive compounds of higher purity. Along with this, there has been a spread in their use leading to an epidemic of enormous proportions (Marion & Oliver, 2014). Thus, despite their original historical motive, psychoactive substances have transformed into a public health issue that affects millions of individuals worldwide.

1.1.2 Epidemiology of Drug Addiction: United States, Europe, Latin America and Puerto Rico

Substance use disorders (SUDs) are still one of the most prevalent types of mental health disorder globally (Whiteford, Ferrari, Degenhardt, Feigin, & Vos, 2015). In addition to its obvious repercussions on the physical and psychological health of consumers, drug abuse has substantial cultural, political, and economic consequences, representing a complex social phenomenon (Blühdorn & Welsh, 2007). It is responsible for a high proportion of all crimes costing nations billions of dollars each year in health care, prevention, and rehabilitation programs, as well as law enforcement and other economic costs (NIDA, 2017). According to the Substance Abuse and Mental Health Services Administration (SAMHSA, 2016), current direct and indirect financial costs in the US deriving from substance abuse

surpass $740 billion annually in expenses related to health, criminal justice, and rehabilitation (NIDA, 2017). This exceeds the cost per year for other chronic diseases such as cancer ($290 billion; Bloom et al., 2012), diabetes ($322 billion; Association, 2013), and cardiovascular diseases ($555 billion; Nelson, Whitsel, Khavjou, Phelps, & Leib, 2016). In 2018, SAMHSA requested a budget of $3.9 billion dollars to address substance abuse and mental health efforts to advance the behavioral health of the nation. Despite the economic investment of the US government and other countries to combat addiction, drug abuse trends remain relatively stable and are far from being solved.

In 2016, the United Nations Global Drug Report (UNGDR) estimated that around 272 million people aged 15–64 had used illegal drugs on at least one occasion (UNODC, 2017). Even more alarming is the fact that about 29.5 millions of those drug users succumbed to the repercussions of chronic drug use (UNODC, 2017). In 2016, tobacco consumption was directly responsible of 7.1 million deaths worldwide in both men and woman (Mackay, Eriksen, & Eriksen, 2002). Alcohol misuse, on the other hand, is responsible for nearly six percent of all global deaths per year (or 3.3 million people). Finally, illicit drugs accounts for another 200,000 (UNODOC, 2014). These data justify additional effort and basic clinical research to improve preventive interventions and effective treatments.

Excessive consumption of psychoactive substances has grown steadily throughout the world, and Latin America is no exception. Present information indicates that the consumption of drugs is increasing, especially synthetic substances (Rech, Donahey, Cappiello Dziedzic, Oh, & Greenhalgh, 2015). The

most used substance in the region is cannabis, and a fraction of Latin Americans consume cocaine in powder form or its derivates. There is also a growing market for ecstasy and there are relatively few users of heroin(Bergman, 2018a). Although the majority of previous data on the prevalence of illegal drug use in Puerto Rico is more than 20 years old, a recent publication established patterns similar to those of the US. Furthermore, drug use was shown to affect about 1 in 6 adults in the population, with cannabis being the primary illicit drug of choice in the country(Caetano, Vaeth, & Canino, 2018).

Recent national overdose death records demonstrate the worst drug epidemic in US history involving opioids (Rudd, Aleshire, Zibbell, & Gladden, 2016). In the United States, during 1999–2015, 568,699 people died from drug overdoses (Seth, Scholl, Rudd, & Bacon, 2018). These reports highlight that deaths related to overdose are disseminating geographically, across races, ethnic characters, and socioeconomic status. Moreover, an in-depth analysis made in 2016 show that deaths have increased proportionately over the years in the United States (Cicero & Ellis, 2017). In 2014, more than 47,055 people died from lethal overdoses; this tendency remained in 2015, with more than 52,000 deaths (Rudd, 2016). In the last report, published in 2016, a higher number of fatalities were shown, 63,632 (CDC, 2018), suggesting a worsening of the epidemic. Opioids were involved in nearly two-thirds (42,249) of deaths in 2016—five times higher compared to 1999 and higher than any other period in US history (Rudd, Aleshire, Zibbell, & Gladden, 2016). Moreover, the opioids crisis is affecting other countries besides the United States, like Canada (Tyndall, 2018).

The opioid epidemic has received considerable attention in the last year due to opioid misuse and related overdoses (Cicero & Ellis, 2017). Nevertheless, another drug-related problem is simultaneously growing in our country. The Drug Enforcement Administration (DEA) indicated that cocaine use and availability in the United States is outpacing the level of 2007 (DEA, 2017a). These findings correlate with the upsurge in coca production from Colombia, the primary supplier of cocaine in the US. The use of cocaine is a concern for the agency since it has steadily increased from 2013. Moreover, NSDUH data has shown a significant increase in domestic cocaine usage since 2009 (Hughes et al., 2016). Further, data analysis between 2009 and 2014 demonstrated cocaine use among people older than 12 years of age fluctuated between 1.4 and 1.7 million (Lipari, Ahrnsbrak, Pemberton, & Porter, 2017). That number increased to 1.9 million individuals in the year 2015, the highest figure since 2008 (Drugs & Crime, 2010).

While much of this evidence emphasizes cocaine use, in many cases, a large percentage of fatal overdoses are the result of poly-drug use. It can be anything from combining cocaine with opioids to drinking alcohol while smoking marijuana. These combinations are used to catalyze or enhance a specific substance, thereby intensifying its effects, consequently dangerously heightening another drugs' risk of overdose. McCall Jones, Baldwin, and Compton (2017) examined drug overdose death data and found that cocaine-related overdose involving opioids increased from 2000 to 2006, declined in 2010, and then rose again in 2015. NIDA (2017) published similar results showing similar trends with and without opioids. Notably, they mentioned that cocaine use alone has increased

by nine percent. Cocaine use is still a threat to public health worldwide, and to the well-being of our country.

1.1.3 Substance use disorders (SUDs)

Substance use disorders (SUDs) are defined as chronic and recurrent brain diseases characterized by the transition from controlled drug use to compulsive intake (Sinha, 2007; Baler & Volkow, 2006). Repeated drug exposure triggers a series of neurological dysfunctions of specific brain regions and/or circuits changing the way people think, feel, and behave (Brick & Erickson, 2013). Psychoactive drugs have the capacity to influence nerve impulses by acting on how neurotransmitters operate at the synapses, depending on a drug's unique characteristics (Petersen, 2005). Among the most remarkable features of SUDs is the enhanced saliency value of drugs, which manifests in a strong desire or craving responses leading to a compulsive search and inability to control-related conducts (Sinha, 2007; Baler & Volkow, 2006). Hence, individuals are unable to maintain abstinence. This behavior generally causes deterioration of the quality of life of individuals, as it interferes with daily life activities or causes health issues Venturelli, 2015). Neurological changes can persist in the brain for a prolonged period. Hence, individuals are unable to maintain abstinence and regulate their self-destructive behavior for weeks, months, or even years (Galanter & Castaneda, 1985).

It is important to understand addiction as a chronic disease. Although initially, the consumption of a substance is voluntary, with each consumption, acute changes occur in the brain. Their acute administration produces temporary changes, which regress when their pharmacological outcome concludes (Meyer & Quenzer, 2005). However, chronic use can leave enduring changes on the emotional memory and rewire neural connections and pathways, producing permanent or long-lasting changes, leaving the person more vulnerable to the reinitiation of drug intake (Hyman et a., 2006). Repeated consumption of substances can attain pre-eminence over other daily life interests and activities, and at the same time, a progressive diminished control over drug intake (Reynolds, Collado-Rodriguez, MacPherson, & Lejuez, 2013). In addition, there are deviations in the way of thinking, judgment, and motivations that are more favorable towards the consumption of the drug, minimizing the ability to recognize behavioral problems, interpersonal relationships, and dysfunctional emotional responses (Akikur Mohammad, 2016). Also, predisposing factors such as biological, psychological, social, and environmental factors can influence the development and maintenance of drug addiction (Saah, 2005). Although the view of addiction as a chronic brain disease has gradually been consolidated, the disease model concept is still being questioned(Hammer et al., 2013).

During the eighteenth and early nineteenth centuries, drug addiction was not considered as a brain disease, and society saw addicts as morally weak or a reflection of the addict's moral qualities. Since then, the field of addictions has undergone dramatic changes in the medical and scientific community on what to

include under this classification (Peele, 1987). There are two internationally accepted systems of classification to organize mental pathologies. One of them is the International Classification of Diseases (ICD), which is sponsored by the World Health Organization, and the other is the Diagnostic and Statistical Manual of Mental Disorders (DSM). The DSM approach to defining drug addiction has evolved since its previous first editions.

Addictive disorders were listed in the first edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-1) in 1952. However, the first two versions of the DSM (DSM-I and DSM-II) stigmatized addiction by categorizing it with similar deviant behaviors condemned by society. Substance dependence in DSM-III and IV put greater emphasis on biology, namely, the physiological and behavioral symptoms induced by substance dependence (Nathan, Conrad, & Skinstad, 2016). The World Health Organization (WHO) and the American Psychiatric Association (APA) once used the terms “dependence” or “abuse”, rather than “addiction,” until the publication of the DSM-V. The transition from DSM-IV to DSM-V preferred the word ‘dependence’ since a robust social stigma surrounded the word "addiction"(American, 2013). This, however, confused clinicians and experts in the field. The revisions for DSM-V aimed to overcome these problems, thereby providing an improved approach (O’Brien, 2011). Alternatively, specific criteria for "substance use disorder" or SUDs, were created by describing the mild to severe state of compulsive and continual recurrent use. Furthermore, they stated that all drugs taken in excess have the direct or indirect activation of the brain's reward system in common.

1.1.4 Drug Abuse Neurobiology: The Reward Circuit

Drug addiction is a chronic and relapsing brain disorder characterized by a state of compulsive drug seeking and use, incapability to stop taking the drug, and the emergence of a negative emotional state when the drug is discontinued (e.g. dysphoria, anxiety) (American, 2013). Addiction has a key transition from impulsive to compulsive behaviors. This impulsive-compulsive transitioning grounds the cycle, which has three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (craving) (Koob & Volkow, 2010). These three stages interact in the substance-consuming individual, becoming more intense until they reach the pathological state known as drug addiction. Moreover, studies with animals have shown that the reinforcing effects of psychoactive drugs have a common neurobiological substrate that is the effect of dopamine release within the nucleus accumbens (NAcc) (Koob & Volkow, 2010). Indeed, the first contacts with drugs produce pleasurable effects in the people who consume them, and these effects have been associated with the activation of the dopaminergic system. The reinforcement pathway consists of the mesocorticolimbic system, which contains dopaminergic neurons. Natural reinforcers such as food, water, and sex activate this reward pathway. When a reinforcer is presented, DA is released into the NAcc, generating a feeling of pleasure. It is well known that specific brain regions within the dopamine mesocorticolimbic circuit including the NAcc and ventral tegmental area (VTA) are responsible for the reinforcement and pleasure effects of many of drugs of abuse. The NAcc receives dopamine (DA) projections from the VTA within

the midbrain. These VTA dopaminergic neurons also make connections to the frontal cortex and limbic system (Berlanga et al., 2003).

The activation of the mesocorticolimbic DA system is a common neuroanatomical mechanism for the reinforcing actions in drug addiction. Moreover, the release of dopamine into the frontal cortex and NAcc provoke a pleasurable experience that causes recurrence of drug seeking behavior (Berridge & Kringelbach, 2015). While naturally-rewarding experiences can release some amount of dopamine, certain drugs can produce a robust elevation creating a more pleasurable experience (Koob & Franz, 2004). Five different receptor subtypes mediate this action and belong to both the D1-like subfamily and D2-like subfamily. D1-like receptors exert a crucial role in the development of addiction because they influence the sensitivity to drugs. By contrast, dopamine stimulation of dopamine D2 receptors is not sufficient for drug reward in the midbrain (Norman et al., 2011).

Using the same physiological mechanisms as natural reinforcers, psychoactive substances act directly or indirectly on this system causing large release of DA (Koob & Volkow, 2016; Volkow, Baler, & Goldstein, 2011). However, addictive drugs act on different neurotransmitter systems in the brain. For example, nicotine has its direct effects on the cholinergic system, heroin acts on the opiate system, and cocaine has its effects on the dopaminergic and noradrenergic systems (Yücel, Lubman, Solowij, & Brewer, 2007). These substances can act directly, activating the dopaminergic neurons in the VTA, or indirectly, inhibiting GABAergic interneurons of the VTA in the mesencephalon. Opioids, alcohol, and

cannabinoids produce an inhibition of the GABA interneurons of the VTA, thus disinhibiting it producing a greater release of DA in the NAcc. Nicotine directly activates DA neurons, both in the VTA and in its NAcc terminals. Some anesthetics and alcohol have an antagonistic effect on glutamatergic receptors of the NMDA type that may also be related to its enhancing effect. Psychostimulants such as cocaine block the reuptake of monoamines (DA, norepinephrine (NA) and serotonin-(5-HT)), moreover produce the depletion of monoamines within their presynaptic terminals (Meyer & Quenzer, 2005).

Addictive substances elicit a withdrawal syndrome defined by irritability and emotional distress (Koob, 2015). In laboratory animals, the withdrawal/negative state is provoked by elevations in reward thresholds during withdrawal periods (Markou & Koob, 1991). Previous research suggested that different substances of abuse shared the same neural system involved in positive reinforcement (Koob & Simon, 2009). Another important feature to note is that most substances of abuse have in common that their withdrawal may involve the development of dysphoria and anhedonia (lack of pleasure) and an intense desire for the drug (craving) (Hatzigiakoumis, Martinotti, Di Giannantonio, & Janiri, 2011).

Chronic drug exposure triggers neurochemical changes within-system neuroadaptations where neuronal cells adapt to compensate for the drug's effects Koob & Volkow, 2010). These adaptations affect different aspects of neurons ranging from changes in the morphology and excitability of the cell membranes (Edwards & Koob, 2010). This is probably related to the downregulation of the reward function influenced by the ventral striatum and to the involvement of the

brain's stress response (Edwards & Koob, 2010; Koob & Le Moal, 2008). Other neurotransmitter systems involved in the emotional deregulation of the motivational effects of abstinence are norepinephrine, substance P, vasopressin, neuropeptide Y, endocannabinoids, dynorphin among others (Koob et al., 2014a).

Chronic exposure to addictive leads to the rewiring of higher brain regions that control motivation, and the subject becomes preoccupied and feels an intense desire to obtain the drug (Bourzac, 2015). Glutamatergic projections that go to the extended amygdala and the NAcc, especially from the prefrontal cortex (PFC) and the basolateral amygdala (ABL) adapt to chronic drug use (Koob & Le Moal, 2008). Cognitive deficits have also been associated with craving and the involvement in it of the orbitofrontal cortex, the prefrontal cortex and the hippocampus (Koob & Volkow, 2010). Preoccupation and craving are more pronounced with stimulants addiction in comparison to other substances.

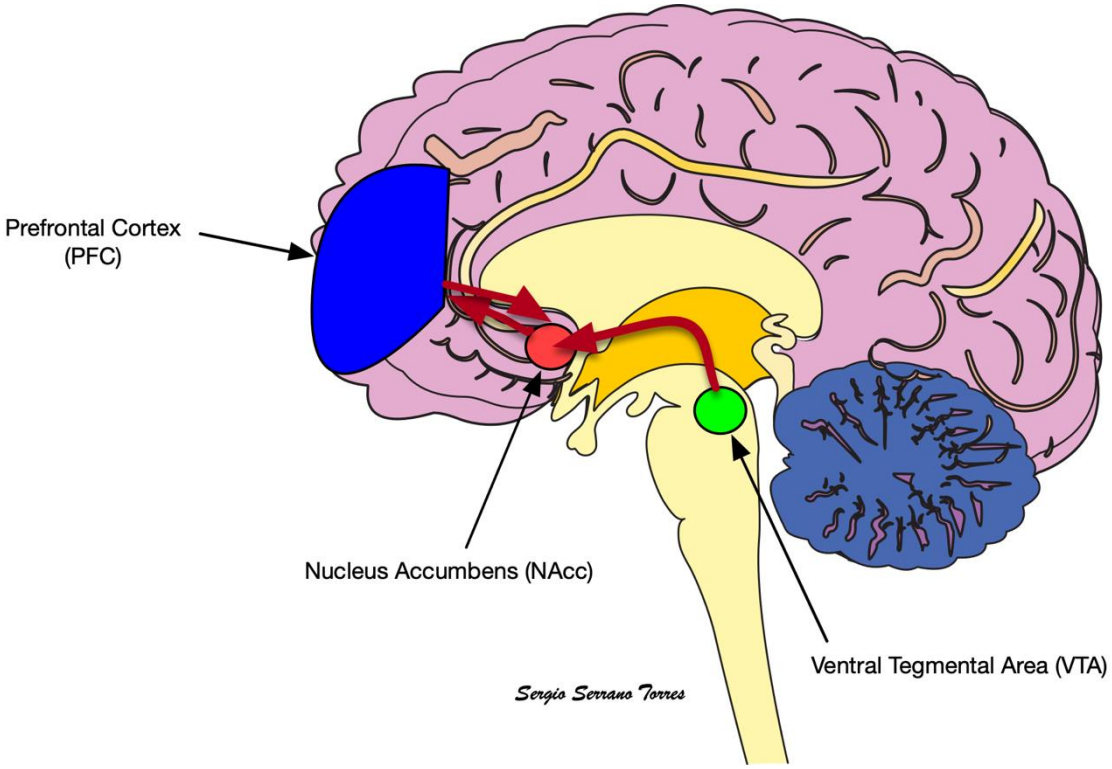

Figure 1.1: Drug Abuse Neurobiology: The Reward Circuit

1.1.5 Theoretical approaches of the transitional process to addiction

As mentioned, the transition to SUDs is a process of neuroadaptation that deregulates specific nerve pathways and neurotransmitter systems. Based on experimental data, different theoretical approaches have been proposed to explain this transitional process (Koob & Le Moal, 2008). According to the theories centered on drugs, chronic drug use results in drug-induced neurobiological alterations that include molecular, cellular, synaptic, and pathway levels (Koob & Volkow, 2016). There are four major theories: the opponent process theory, the aberrant learning theory, the incentive-sensitization theory, and the frontocortical dysfunction theory.

The Opponent Process Theory

This theory is based on the assumption that the brain contains control mechanisms capable of balancing any affective state that moves away from equilibrium (reward or aversive) (Solomon & Corbit, 1974). Furthermore, it proposes that even if the initial use of a substance is controlled by the intensity, quality, and duration of the reinforcing stimulus, a neuroadaptation of the brain occurs eventually which reduces the strength of the hedonic feelings. Subsequently, neuroadaptation caused by chronic drug use leads to a decrease in the reward, and a negative process is triggered, opposite to the initial one. This generates unpleasant withdrawal symptoms on the cessation of use which represents a lasting change in the usual reward threshold. Typically, after periods of drug abstinence, these symptoms diminish; however, the removal of signs does

not prevent drug users from future relapse. Subsequently, Koob and Le Moal (2008) proposed an “allostatic model” to explain this vulnerability of relapse in addiction, which theorizes that repeated drug use recruits related stress systems.

The Aberrant Learning Theory

Researchers have suggested that SUD is a pathological learned behavior that is mediated by the same neural pathways that underlie learning and memory processes associated with natural rewards (Kelley & Berridge, 2002). Drug-taking and drug-seeking conduct progress from controlled behavior, triggered by the reinforcing effects of drugs, to an automatic or habit stage in which stimulus–response associations take over. This model interprets the dopamine release in the VTA to the NAcc as essential to promote stimulus–reward learning Koob & Le Moal, 2008). Moreover, addictive drugs not only enhance normal learning systems involved in seeking rewards but also inhibit the ability to control their use (Everitt et al., 1999; Jentsch & Taylor, 1999). Thus, within the context of this theory, mesolimbic dopamine release is thought to function as incentive salience to drug cues and as a guide and motivator of appropriate behavioral responses.

The Incentive-Sensitization Theory

The theory of incentive awareness elaborates explanations from the perspective of neuroadaptations resulting from drug abuse as being responsible for the development of an addictive state. Everitt and Robbins (2005) postulated that in the early stages of addiction, there is predominant selection for the pleasure of drugs. However, when the addiction has been consolidated, an urgency to

consume (craving) predominates. Repeated use of the drug causes hypersensitization of the neurological substrate responsible for the positive incentive, which leads to compulsive behavior. In this theory, the authors explain that the brain circuits involved include the NAcc, the amygdala, and the prefrontal cortex (CPF). In 2016, this hypothesis was reconsidered and elaborated, and refinements to the comprehension of ventral and dorsal striatal mechanisms underlying goal-directed and habitual drug seeking were made (Everitt and Robbins, 2016).

The Frontocortical Dysfunction Theory

It is widely accepted that PFC is an essential contributor to decision-making, assignment of value, and the maintenance of goal-directed behaviors (Rushworth, Noonan, Boorman, Walton, & Behrens, 2011). The theory proposes that chronic exposure to drugs produces long-lasting changes in two complementary systems. On the one hand, the overwhelming motivational relevance of reinforcers makes an exaggerated assessment of the reinforcing properties of drugs. The lack of executive control over behavior and withdrawal-related emotions and the absence of modulation of limbic structures decreases the ability to control the desire to obtain drugs (Davidson, Jackson, & Kalin, 2000). Simultaneously, the PFC is sensitized to stimuli predicting drug availability, resulting in a supraphysiologic glutamatergic drive in the NAcc. Therefore, the salience attribution of drug-related cues and dysfunction of prefrontal regions could make significant deficits, such as loss of inhibitory control and decision-making processes (Volkow, Fowler, & Wang, 2003).

1.1.6 Classification of Psychoactive Substances

Psychoactive substances are chemicals that alter the functioning of the brain and hence produce changes in behavior, perceptions, mood, and thinking processes. These substances are categorized as licit (legal) and illicit (illegal). Some commonly used legal drugs include alcohol, caffeine, and nicotine. Despite having a lower level of consumption compared to licit drugs, illicit drugs include marihuana, cocaine, methamphetamine, LSD, and ecstasy (Goodman, Sherratt, & Lovejoy, 2014). Moreover, in some instances, prescription and over-the-counter medications may become drugs of abuse when misused, as in the case of opioids.

Despite drugs being considered legal or illegal, the damage caused by licit drugs such as alcohol and tobacco is indeed more prevalent and costly all over the world than that resulting from the use of illicit drugs. In 2016, an estimated 51.3 million people aged 12 or older were current cigarette smokers, and 136.7 million Americans within the same age group reported the use of alcohol (SAMHSA, 2016). Among those, 33 million involved binging alcohol and 16.3 million reported heavy usage. Although illicit drug use is significantly less common than either alcohol or tobacco use, in 2016, nearly 28.6 million people used an illicit drug, which corresponds to 10.6 percent of the total population (SAMSHA, 2016). Certainly, people are motivated to use substances, and this is common practice in the country.

The drug classification established by the DMS, ICD, and WHO is widely accepted. In addition, according to Koob, Arends, and Moal (2014b), drugs can be classified depending on their behavioral, pharmacodynamics, and legal outcomes. Regarding SUDs, drug classifications often used are the five classes regulated by the controlled substances act (CSA): narcotics, depressants, stimulants, hallucinogens, and anabolic steroids. These drugs can be further classified depending on the effects on the CNS. Furthermore, there is a legal classification of drugs based on their potential for abuse and lawful medical usage. According to the US (DEA) (2017b), most of the drugs included in this category may lead to the development of SUDs. The different categories are summarized in Table 1.

Abused drugs or controlled substances are classified into two types, according to their susceptibility to creating dependence: psychological, physical, or both (Hanson, Venturelli, & Fleckenstein, 2011). The first consists of licit drugs that can be bought either over the counter or with a medical prescription. They are not punishable by law and are accessible in the market. Controlled drug substances —such as amphetamines, opioids, ketamine, among others — are one example. Despite their high abuse potential, they can be taken in proper doses and under direct medical supervision. However, unmonitored or unbridled use, or use for purposes other than medical treatment may make them illegal. The second type of illicit drugs are substances that are penalized by law and are prohibited for sale and consumption such as heroin, cocaine, and ecstasy (Morgese et al., 2016). These substances possess a high potential for abuse, an absence of known

medical purpose and do not comply with the safety regulations established by the FDA.

SCHEDULE	DESCRIPTION	COMMON EXAMPLES
I	No accepted medical use; high potential for abuse; Most dangerous drugs of all the drug schedules; produce severe psychological or physical dependence.	Heroin Lysergic acid diethyl amide (LSD) Marijuana (Cannabis) Methylenedioxymethamphetamine
II	High potential for abuse; produce severe psychological or physical dependence.	Cocaine Phencyclidine (PCP) methamphetamine Methadone Adderall Ritalin
III	Moderate to low potential for physical and psychological dependence; abuse potential is less than Schedule I and Schedule II drugs but more than Schedule IV.	Ketamine Anabolic steroids Testosterone
IV	Low potential for abuse and low risk of dependence.	Xanax Valium Ativan Ambien Tramadol
V	Lower potential for abuse than Schedule IV	Codeine lomotil Motofen Parepectolin

Table 1.1: Chemical and Legal Classifications of Drugs.

1.1.7 Psychostimulants

Psychostimulants, also referred as sympathomimetics agents, had numerous uses during the last century. In ancient history, stimulants were traditionally used in almost every culture to combat fatigue, discouragement, and hunger, especially softer stimulants like coffee, mate, and tea and coca leaves. In addition, in antique medicine, they were given to treat medical illnesses, such as asthma, obesity, and a variety of other diseases (Avois et al., 2006). Although the consumption of these substances in indigenous communities was a common tradition, later, the isolation of these substances by chemical advances contributed, in part, to the establishment of abusive conducts.

Stimulants come in a variety of forms, including nicotine, caffeine, amphetamines, methamphetamines, and cocaine, among others. Some are extracted from natural sources, while others are created synthetically (Gorelick & Cornish, 2009). Some stimulate slightly, while others have robust physiological effects. Although they are chemical relatives and share similar mechanisms of action—central nervous system, increasing energy and improving mood—stimulants vary tremendously in their sources and strengths (Iversen, 2016). Additionally, stimulant consumption differs mostly in whether the substance used is legal, illegal or socially accepted (Franke, Lieb, & Hildt, 2012).

Psychostimulants are a group of drugs with a high potential for abuse. As the name implies, stimulants are psychoactive substances that accelerate or stimulate the functioning of the CNS, especially the brain. This type of drug makes

the individual feel hyperstimulated, euphoric, energized, and mentally alert after consuming the substance (King & Ellinwood, 1997) Although stimulants are one of the most used types of drugs worldwide, not all of them share the same legal status. In fact, many soft socially accepted stimulants are not considered drugs by vast sectors of society. Astoundingly, the damage caused by licit drugs such as nicotine in tobacco is indeed more prevalent and costly all over the world than that resulting from the use of illicit drugs (Gowing et al., 2015).

The consumption of mild stimulants is considered a usual and accepted practice in societies around the world (Hanson, Venturelli, & Fleckenstein, 2011). Two of the most used stimulants are nicotine (derived from *Nicotiana tabacum*) and caffeine (from many sources like plants, coffee, tea, and soft drinks). Low-to-moderate doses can improve mood, intensify alertness, decrease fatigue, and increase motor activity. On the contrary, strong stimulants are taken clandestinely due to the disapproval of society and its illegality in many countries. This latter group includes cocaine and amphetamines, stimulants that are potentially dangerous with limited medical applications (Hasan, 2009). Their abuse can lead to a variety of secondary effects such as repetitive motor activity, convulsions, hyperthermia, coma, and death (Rich & Singer, 1991).

The continued consumption of large doses of stimulants can lead to altered states of consciousness, hostility, and paranoia, facilitated by the high flow of information at the neuronal level resulting from increased dopaminergic and noradrenergic activity (Wisor et al., 2001). Besides, the use of high doses of a stimulant may cause an increased body temperature and arrhythmias.

Furthermore, drug tolerance can lead to rapid and abrupt cessation of drug use, which can cause depression, anxiety, cravings, and extreme fatigue (McCrady & Epstein, 2013). Although most psychostimulants have the potential for abuse, many of them have reasonable therapeutic purposes. Treatment of medical conditions, such as narcolepsy, attention deficit hyperactivity disorder (ADHD), and for cases of depression that have not responded to other treatments (Malhi et al., 2016).

Drug Enforcement Administration Controlled Substances for Psychostimulant Drugs	
Cathinone	Class I
Methcathinone	Class I
Cocaine	Class II
Methamphetamine	Class II
Methylphenidate	Class II
Amphetamine	Class III
pseudoephedrine	Class IV
Pvrovalerone	Class V

Table 1.2: Controlled Substances for Psychostimulant Drugs (DEA, 2010).

1.2 COCAINE USE DISORDER (CUD)

1.2.1 Epidemiology of Cocaine: U.S.A, Europe and Puerto Rico

Cocaine (benzoylmethylecgonine) is a powerful addictive stimulant that is present in coca plants (*Erythroxylon coca*) containing approximately 2% by weight of cocaine. Coca leaves, the material needed to produce refined cocaine, is grown in parts of Central and South America. Coca is a flowering bush that is mostly cultivated in South America (Ehleringer, Casale, Lott, & Ford, 2000). Archeological data show that chewing coca leaves or brewing leaves for tea was a well-established practice in Ecuador by 2500 BC (Gahlinger, 2003). Ancient Incas in the Andes were the pioneers of chewing coca leaves to accelerate their heartbeats and breathing to counteract the high-altitude effects of living in the mountains. The Peruvian natives of 600 A.D. chewed coca leaves for their euphoric properties and their ability to decrease fatigue, hunger, and increase physical endurance during religious ceremonies (Boghdadi & Henning, 1997). Coca has also been used for centuries to relieve headaches and various gastrointestinal problems. It was not until 1880 that it began to become popular in the medical community for its local anesthetic properties. Although the consumption of coca leaves in these indigenous communities was well known, the extracts of this plant did not begin to cause a socioeconomic problem until the 1970s and 1980s, when it regained popularity as a recreational drug, resulting in more incidences of additive-related pathologies (Kuhar, 1998).

Cocaine was first isolated from the leaves in the mid-1800s and gained popularity at the beginning of the 19th century. Between 1998 and 2014, the prevalence of cocaine users remained globally stable. The global incidence of cocaine use among the population (aged 15-64) remained mostly steady over the period from 1998–2014, while the number of cocaine users has increased by over 30 percent; however, as the population augmented, the number of cocaine users similarly increased, from 14 million in 1998 to 18.8 million in 2014 (UNODC, 2017). In the United States, cocaine abuse is the primary cause of consultation in the emergency room. The United States accounted for 15 percent of global cocaine seizures over the period from 2009–2014 and was second only to Colombia. In Europe, the Annual Report published by the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) pointed out that cocaine is the most widely used illegal stimulant drug. Its estimated consumption is of about 2.2 million users who are between the ages of 15 and 34 (European Drug Report, EMCDDA, 2014). The prevalence of cocaine use in Europe appears to have declined from a peak in 2007 and is now rather (UNODC, 2017). In the case of Latin America, cannabis is the most commonly used substance followed by cocaine (either in powder or in a smokable form). In fact, Latin America produces all the world's cocaine, being Bolivia, Peru, and Colombia, the primary supplier of cocaine products (Bergman, 2018b). Interestingly, the UNODC (2017) reported that the number of people in treatment for cocaine use disorders is significantly high in Latin America and the Caribbean, where nearly half of the people in treatment for drug use disorders are cocaine takers. In Puerto Rico, cocaine/crack consumption prevalence rates of the

population are between 2% and 3%. In this study, however, data collection was based on self-reports, which may lead to underreporting. Moreover, it was not possible to estimate the specific drug use (e.g., cocaine and crack) because the way in which the study data were gathered did not allowed to obtain this information.

1.2.2 Pharmacotherapies in treatments for Cocaine Addiction

The abuse of cocaine has expanded in such a way that it has led to considerable efforts toward developing treatments programs for cocaine addicts (Kampman, 2005). Although knowledge about how the pharmacodynamics of cocaine that affect the reward mechanism is expanding significantly, at present, there is no effective pharmacological treatment approved by the FDA (Kosten, Domingo, Orson, & Kinsey, 2014). In attempts to find a medication, scientists have focused on a variety of receptor agonists and antagonists of different neurotransmitters. These agents have the purpose of increasing the levels of the brain's stimulation of the synthesis, release, or inhibition of enzymes, for example, dopaminergic agents (Levodopa-Carbidopa, and Dextroamphetamine), noradrenergic agents (Disulfiram and Nepicastat/SYN 117), GABAergic agents (Topiramate, Baclofan, Tiagabine, Vigabatrin and its analogs), and agents with a novel or mixed mechanism of action, such as Modafinil (Shorter, Domingo, & Kosten, 2015). Also, antidepressants (Levin, Evans, McDowell, Brooks, & Nunes, 2002) and stimulants such as amphetamines (Negus & Mello, 2003) have been

tested. However, until today, drug trials have demonstrated inconsistent levels of efficacy.

Vaccines for cocaine use disorder have been tested (Kosten, Domingo, Orson, & Kinsey, 2014). Laboratories have tried to create vaccines to control the effects of cocaine, but, at least so far, the results have not been satisfactory. This strategy promises optimal results in cocaine addicts by preventing the entry of the substance into the CNS. The antibodies produced after administration of vaccine bind to cocaine for later sequestration by the blood vessels. It is theorized that cocaine then does not cause weak pleasurable effects for stimulating cocaine-seeking behavior. Nonetheless, health status, genetic makeup, patterns of drug usage, or low molecular weight cocaine, can lead to significant variation in antibody levels on individuals (Kinsey, Kosten, & Orson, 2010; Kuppili, Manohar, & Menon, 2018). However, more clinical and animal studies are needed to validate the effectiveness of this therapy.

Neurobiology of Cocaine Addiction

1.2.3.1 Cocaine molecule, production and routes of administration

Cocaine ($C_{17}H_{21}O_4N$) is an alkaloid molecule due to its nitrogenous base that can form salts in organic and inorganic acids. It belongs to the group of tropic alkaloids due to its tropane ring (bicyclic amine) (Fig. 1.2). It contains two ester moieties: one with benzoic acid (hydrophobic region) and with methylecognine (hydrophilic region). Chemists have focused on these structural features to produce synthetically derived drugs with improved anesthetic properties and thus, decreased adverse effects (William, Brent, Eric, & Christopher, 2013).

Coca leaves can be transformed into cocaine chemical derivatives, including cocaine hydrochloride or powder, base paste or coca paste, and cocaine base or crack. When extracted from coca leaves, cocaine paste is then converted into a fine white powder called cocaine hydrochloride. Specifically, cocaine hydrochloride production involves three refining steps: the production of paste to extract cocaine alkaloids; the removal of impurities and concentration of alkaloid chemicals, and finally the transformation of coca paste into a soluble powder (International, 2011). Cocaine hydrochloride is often adulterated with other substances to increase its weight, increase bitterness or to produce anesthetic effects (Broséus, Gentile, & Esseiva, 2016). The final concentration depends on the degree of adulteration and origin of production. It can also be mixed with other psychoactive substances, for example, when it is combined with heroin, the so-

called “speedball.” The user gets an atypical greater euphoric response compared to cocaine alone, which makes the combination highly addictive.

People can administer cocaine in many ways: snorted, injected, smoked, or orally taken (Hanson, Venturelli, & Fleckenstein, 2011). First, cocaine powder can be inhaled intranasally or, more appropriately named, insufflated. Second, it may be injected intravenously since cocaine hydrochloride is water-soluble. Third, the cocaine base can be smoked. Fourth, cocaine can also be taken orally; however, this method is uncommon.

Different routes of consumption produce different patterns and concentration levels of cocaine in plasma, followed by an extreme feeling of pleasure (MD, Steven B. Karch & Drummer, 2015). The route of administration determines how quickly the drug reaches the brain. This effect can vary in intensity and duration (Minogianis, Lévesque, & Samaha, 2013). Generally, the faster the absorption, the more intense the sensation is. In other words, the more quickly the substance enters the bloodstream and reaches the brain, the more intense the euphoria created is. For this reason, cocaine users smoke or snort the drug rather than taking it orally in order to maintain the euphoric effects (Wills, 2005). In addition to the fast-acting action, such methods have the advantage of eliminating the process and risks involved in administering the drug intravenously (i.v.). The i.v. administration route is associated with a significantly higher risk of contracting other severe health illnesses such as HIV and/or other infectious diseases (Des & Friedman, 1987). When inhaled, it passes through the blood vessels of the nasal membranes and then easily penetrates the blood–brain barrier (BBB). The

intranasal route has a variable bioavailability of 20% to 60%. Due to its ionized characteristics and vasoconstricting properties, poor penetration through the nasal mucosa is achieved. The onset of activity is within 3–5 minutes and fades between 45–60 minutes (Lowinson, 2005). In contrast, i.v. provides an almost complete bioavailability (~100%), having an onset of activity within 30 seconds and a duration of action of between 10–20 minutes. Finally, it should be mentioned that these effects could vary depending on the pureness of the cocaine (Cunha-Oliveira, Rego, Carvalho, & Oliveira, 2013).

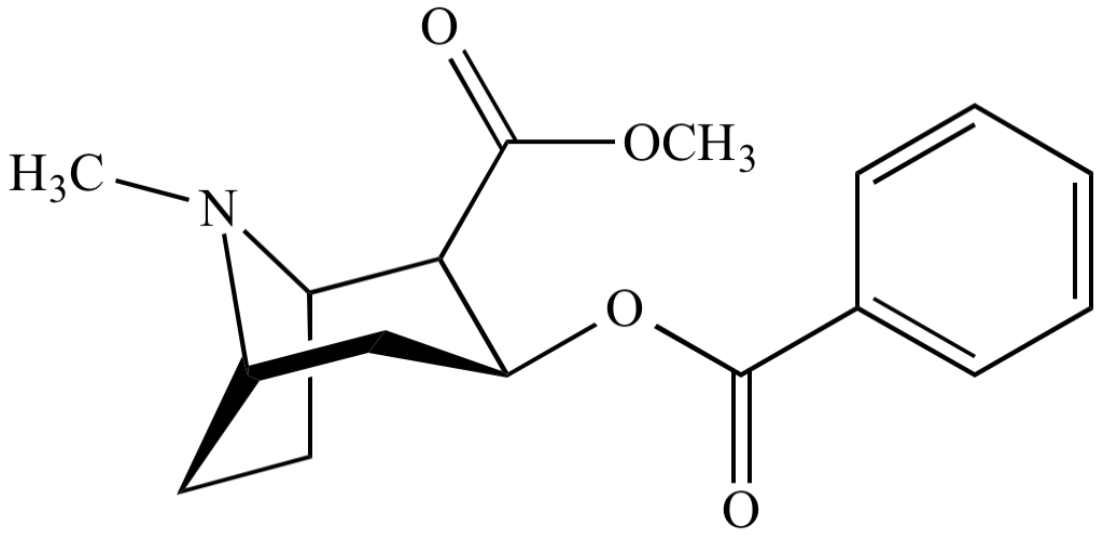

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Figure 1.2 Molecular Structure of Cocaine

1.2.3.2 Cocaine metabolism and urinary excretion

The pharmacokinetics of cocaine is multifactorial, involving complex interactions depending on the route of administration, genetics, concurrent consumption, and physical/chemical form (Masters & Coplan, 1999). Cocaine is rapidly metabolized, usually by enzymatic and spontaneous activity. Approximately 90% of cocaine is metabolized by spontaneous hydrolysis and de-esterification by enzymatic action in the liver, plasma, and other organs. Two distinct pathways in humans characterize the catabolic fate of cocaine, which are the major transformation caused by hydrolysis and minor transformation produced by oxidative reactions primarily in the liver (Kovacic, 2005).

Cocaine Hydrolysis

The cocaine molecule is hydrolyzed at the methyl ester by human carboxylesterase (hCE-1), while the benzoyl ester is either hydrolyzed by hCE-2 or by butyrylcholinesterase (BChE). These major cocaine-metabolizing enzymes produce various inactive metabolites that include ecgonine methyl ester (EME), benzoylecgonine (BE), and norecgonine methyl ester (NME). When ingested, irrespective of the route of administration, cocaine is subject to widespread and rapid attack by these enzymes, hence its short half-life span in the human body. Other minor metabolites of cocaine have been identified, including p-hydroxycocaine, m-hydroxycocaine, p-hydroxybenzoylecgonine, m-hydroxybenzoylecgonine, and norbenzolecgonine, among others (Kolbrich et al.,

2006). Nevertheless, cocaine metabolites as well as their potential toxicological effects have been fully characterized.(Dinis-Oliveira, 2015).

hCE-1 and hCE-2 are broad-spectrum serine hydrolyzes that are essential for drug metabolism. hCE-1 is primarily expressed in the endoplasmic reticulum of liver cells, with smaller amounts distributed throughout the body. In contrast, hCE-2 is highly expressed in the intestine with relatively low expression in the liver(Laizure, Herring, Hu, Witbrodt, & Parker, 2013). hCE enzymes de-esterify cocaine (~45%) to produce low-toxicity EME and BE, which are then excreted by the kidneys.

On the other hand, human BChE is a nonspecific cholinesterase that hydrolyzes ester functional groups. It is widely distributed throughout the body in humans but is particularly prevalent in blood serum. BChE is synthesized primarily in the liver and then secreted into the bloodstream. The endogenous activity in plasma substantially influences the rate at which cocaine is metabolized (~45%). People with atypical BChE variants may experience the risk of severe or fatal cocaine toxicity (Hoffman et al., 1992). Such observations have led to studies about re-engineering human BChE and enhancing the metabolic conversion of cocaine to less toxic levels, promoting it as a possible therapy for detoxification (Sun, Shen, Pang, Lockridge, & Brimijoin, 2002).

Cocaine Oxidation

In addition, cytochrome P450 3A4 (CYP3A4) is responsible for the oxidation of ~10% of the cocaine consumed by users (Chen et al., 2016). CYP3A4 is

abundant in the liver and is known to oxidize small organic molecules, such as toxins or drugs, so that they can be degraded and excreted at the renal level. This metabolic route leads to the N-demethylated product Norcocaine (Nor). Neither EME, BE, nor NME have significant biological activities in humans. In contrast, Nor is considered to be a neurotoxic, hepatotoxic, and potent vasoconstrictor (Madden & Powers, 1990) as well as a hormonal and hemodynamic modulator. Nevertheless, since only a small amount is produced, it is considered inconsequential (Hoskins et al., 2010).

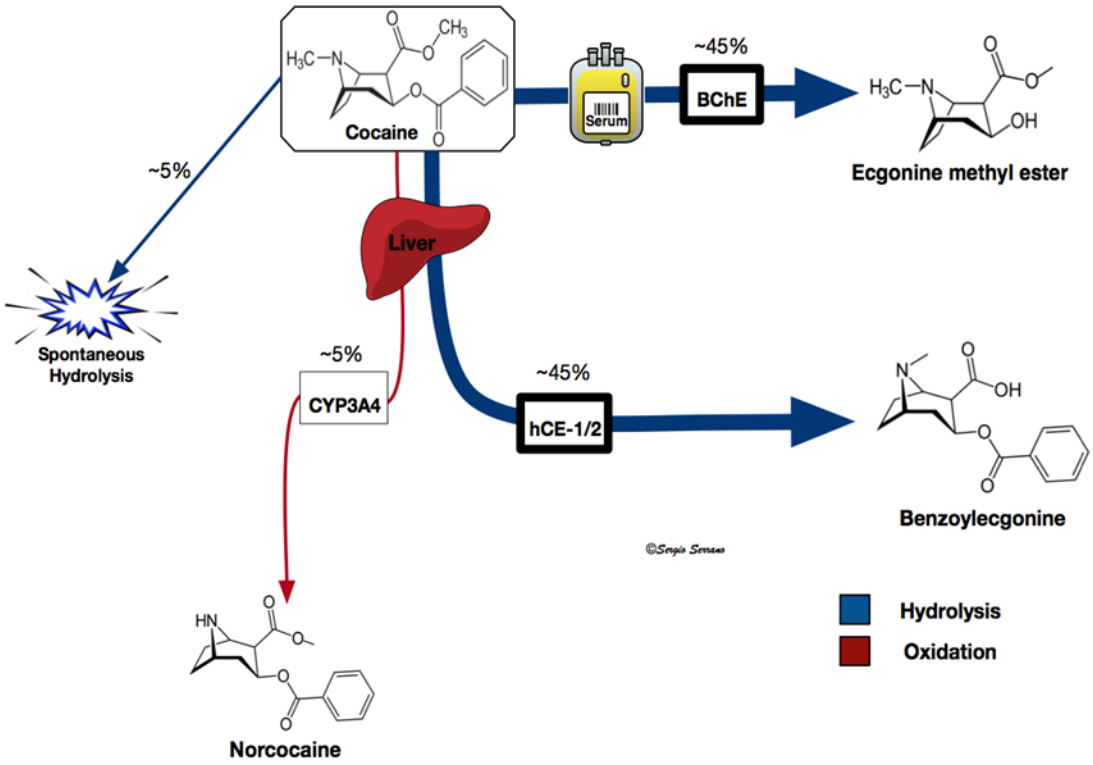


Figure 1.3 Principal Metabolic Pathways of Cocaine

1.2.3.3 Short- & Long-Term Physiological & Behavioral Effects of Cocaine

The abuse of cocaine can affect the entire human body, and more importantly, its toxicity primarily affects the CNS and cardiovascular system (Gillis et al., 1995). Nevertheless, as mentioned previously, the clinical symptomatology depends on the dose consumed, the pureness, the route of administration, and distinctive reactions from each individual. Moderate doses of cocaine give rise to psychomotor stimulation and locomotive and verbal hyperactivity (Meyer & Quenzer, 2005). When high dosages of cocaine are taken, stereotyped behaviors appear, that is, repetitive body movements or repetitive movement of objects. At high doses, cocaine behaves as a convulsive agent. Moreover, since cocaine has a short half-life, consumers need recurrent doses to sustain its euphoric effects. This form of compulsive intake is responsible for overdose episodes (Nestler, 2005).

This powerful sympathomimetic agent can have immediate and long-term health effects. The short-term physiological effects of cocaine include the contraction of blood vessels, dilation of the pupils, and increases in body temperature, heart rate, and blood pressure. Consecutive or high concentrated doses can produce tremors, dizziness, and muscle spasms. In rare cases, sudden death may occur due to cardiac arrest or seizures followed by respiratory complications (Heard, Palmer, & Zahniser, 2008).

In long-term consumers, acute myocardial infarction (AMI) is the most frequent cardiac complication secondary to cocaine abuse (Goldfrank & Hoffman,

1991). Loss of smell, bleeding from the nose, and perforation of the nasal septum are also frequent. Overstimulation of the alpha-adenogen receptors of the gastric and mesenteric vessels can initiate gastrointestinal ischemia leading to ulcers and perforations (Linder et al., 2000). Also, abnormalities in hepatic biochemical parameters and acute pancreatitis can also be seen (Chapela, de los Angeles Paz, & Ballestero, 2017).

The behavioral effects of short-term cocaine use are also wide-ranging. The consumption of a small amount generally causes individuals to be euphoric, energetic, talkative, and mentally alert, particularly to the sensations of sight, sound, and touch (White & Bates, 1993). Cocaine may also decrease the desire to eat and sleep (Schierenbeck, Riemann, Berger, & Hornyak, 2008). Some users feel more motivated to perform some physical and intellectual tasks. Using larger quantities can intensify the effects, leading to extravagant, erratic, and violent behaviors; this includes feeling restless, irritable, and anxious (Miller, Gold, & Mahler, 1991).

In long-term consumers, drug tolerance changes rapidly, and a higher concentration of the drug is required to reach the same euphoric state (Wills, 2005). Moreover, neuropsychological alterations like sudden mood changes, fatigue, anhedonia, anxiety, and depressive symptoms can appear (Lago & Kosten, 1994). Likewise, cognitive deficits and difficulty in performing tasks can be observed in these individuals.

1.2.3.4 Cocaine Molecular Mechanism on the Brain Reward Circuitry

The cocaine molecule due to its lipophilic properties can rapidly cross the blood-brain barrier (MD, Steven B. Karch & Drummer, 2015). Inside the brain, cocaine blocks the monoamine transporter at the synaptic terminal, which inhibits the transportation of dopamine (DA), serotonin (5HT) and, to a lesser extent, noradrenaline (NA), increasing the synaptic concentration of these monoamines at the synaptic junctions (Zhang et al., 2003; Schmidt & Weinshenker, 2014; Müller & Homberg, 2015). Cocaine acts as an unselective monoamine transporter blocker for dopamine (DAT), norepinephrine (NET) and serotonin (SERT) (Johnson, 2010). Furthermore, cocaine upsurges the release of dopamine into the NAcc and the amygdala through direct actions (Weiss et al., 2000). Acute and repetitive elevation of these monoamine neurotransmitters leads to both immediate and long-term changes in function and expression of the monoamine receptors. Stimulants cause long-term neuronal depression in NAcc and reduce glutamatergic postsynaptic responses. Thus, psychostimulant-induced changes in neuronal morphology and synaptic restructuring, which ultimately causes the physiological and behavioral changes (Golden & Russo, 2012).

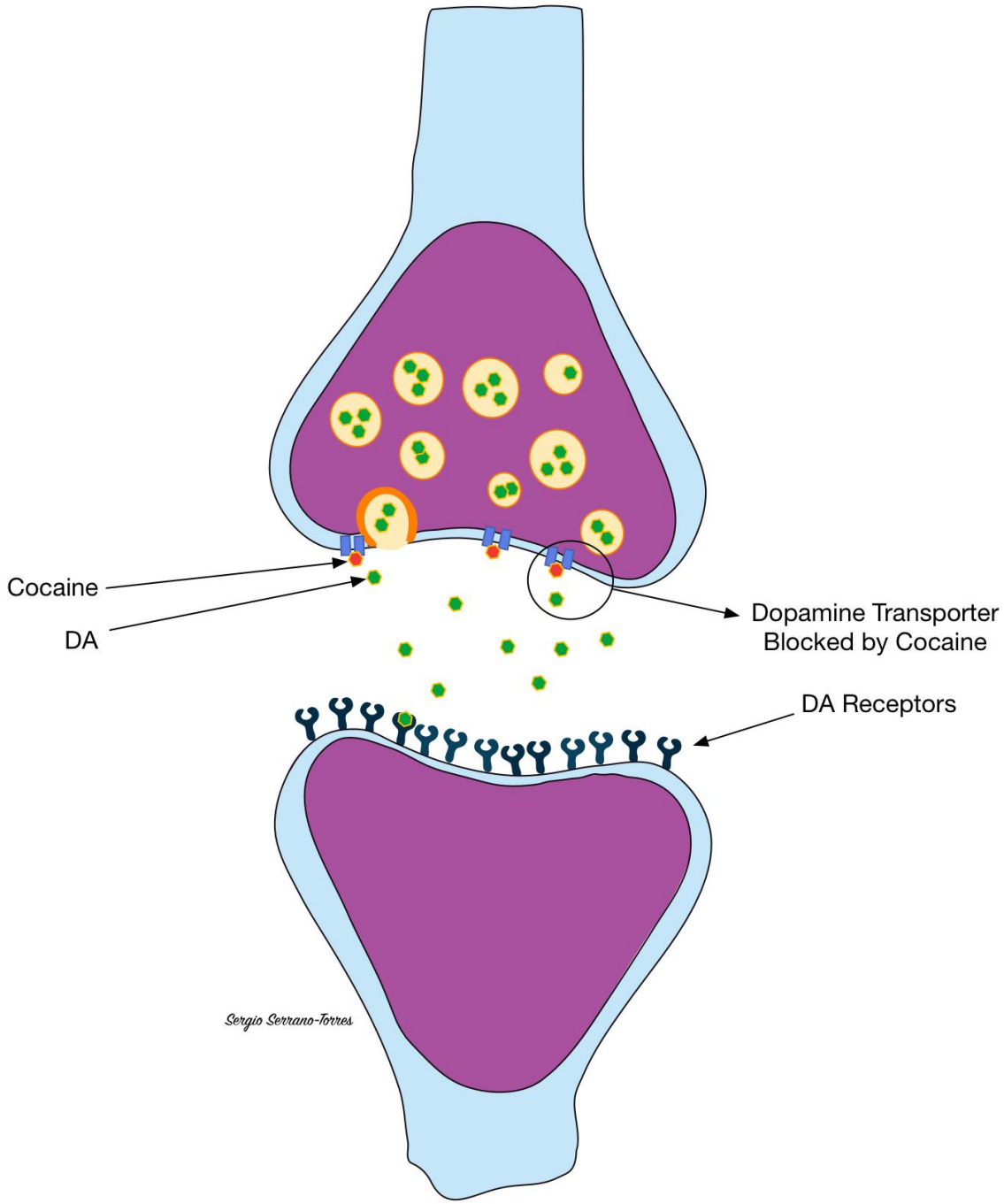


Figure 1.4 Cocaine Mechanism of Action

1.2.3.5 Animal Models of Drug Reward and Addiction

Most of the progress made in the study of addictive disorders and their neurobiological bases, comes from work developed by animal models. In this way, research in animals has contributed to the understanding of the neurobiological processes of SUDs and how the brain reward system is functionally and biologically interconnected. This is an effective tool since the direct evaluation of how drugs affect human subjects brings multiple ethical conflicts and impediments to the performance of experiments (Sloboda, 2006). Moreover, studies conducted on cells, tissues, or computer simulations have failed, to some degree, to replicate the complexity of living organisms (Hock, 2008). The utilization of simplified methods has allowed investigators to study isolated interactions in simulated environments; however, the extent of the validity in extrapolating, interpreting, and translating the data to a more complex organism is a challenging task.

Though substance addiction is a human phenomenon, some of the behavioral traits of this disease can be replicated in animal models (Lynch, Nicholson, Dance, Morgan, & Foley, 2010). Besides, humans do not provide a feasible alternative to animal models, given that clinical trials are time-consuming, expensive, and risky (Fischman & Foltin, 1998). Numerous manipulations directed at investigating neuropharmacological mechanisms involved in psychoactive drug effects can be applied in animal models but not in humans. Certain invasive procedures and dangerous experimental drugs used in humans would be considered non-ethical due to the high risk of irreversible adverse effects. Animal testing prevents these harmful sequelae from affecting human individuals

There is a considerable limitation concerning the reproducibility and control of external variables in understanding addictive cycles in humans. Animal research allows control over environmental factors, genetic influences, and the degree of drug exposure. It is expected that animal experiments to predict human reactions replicate what happens in the human brain with a high fidelity; however, there are cases where a neurological sequel identical to those observed in humans is not triggered (Bracken, 2009). Although obvious differences exist between the human and the nonhuman brain, general organization and the functioning of basic neural circuits throughout mammalian appears to be analogous to humans. Thus, the use of animal models allows us to make inferences and to unravel the pathophysiology and etiology of neurobiological mechanisms responsible for SUDs, and to design potential therapeutic approaches for the mitigation of addictive behaviors.

Since animal models do well in predicting drug effects in humans, neuroscientists have been using animal models to make inferences about how the brain changes during drug abuse. Not only do research advances depend on animal models, but also, governmental regulatory agencies will not approve any treatments for clinical testing without extensive animal research data that has complied with federal regulations and policies (Eifler & Thaxton, 2011). After having the concrete scientific evidence required by the FDA about the safety and efficacy of the procedures involved, investigators may begin pre-clinical studies using animals (Miller, Strang, & Miller, 2010). In the United States, the government strictly regulates animal testing. Two federal agencies monitor academic and industrial animal research: the U.S. Department of Agriculture (USDA) and the

Public Health Service (PHS). These agencies assure that there might be beneficial and a reasonable probability of improving a disorder in humans (Prus, 2017).

Since the disease of addiction is complex and multifactorial, until now, there has been no equivalent animal model of the addictive process as it develops in humans. Nevertheless, animals demonstrate similar addictive behaviors allowing us to investigate specific elements of the addictive pattern (Koob et al., 2014b). For example, repetitive behavioral testing, as in operant conditioning, establishes a pattern of behaviors similar to humans. From this perspective, the theory of learning through operant conditioning attempts to resemble and explain how addictive behaviors are initiated, maintained, influenced, and terminated under well-controlled experimental conditions.

1.2.3.5.1 Rodents as an Animal Model

The use of animals as biological reagents in the context of neuroscientific research has directly contributed to the understanding of the biological bases of different neurodegenerative diseases, regeneration, and the repair of nervous tissue, pain mechanisms, and therapeutic strategies, among others (Van der Worp et al., 2010). A wide variety of animal models have now been developed and perfected in order to meet the demands of researchers to carry out more sophisticated experiments. While small animals are commonly used in basic research due to their cost-efficient characteristics, large animals have also been reported as potential models for the behavioral and molecular mechanism of SUDs (Danek, Danek, & Araszkiewicz, 2017). While different species can produce dissimilar results, impeding direct translation of findings from animal research to humans, common outcomes in similar tests using a broad scientific animal classification, such as rodents or non-human primates, would give stronger conclusions, supporting their applicability

A review of the literature on animal models of addiction showed that rodents, mainly rats and mice, are the standard species for SUDs research (Sanchis-Segura & Spanagel, 2006). Rodents have long served as the ideal mammalian order for biomedical research due to the similarity of their anatomical, physiological, genetic and behavior pattern characteristics to humans (Bryda, 2013). To date, rodents have proved to be essential for understanding the neurobiological mechanisms involved in SUDs. This is because rodents share some neurobiological systems, both structurally and functionally, with humans,

thus allowing a detailed description of the circuitry underlying addictive behavior. In addition, these animals have general characteristics which make them very suitable for use in the laboratory, such as their relatively small size, low-cost, short life cycle, ease of handling and maintenance and, more importantly, similarities to human diseases. As result, rodents are the most used animal model in pre-clinical studies (Sitzia, Erratico, Farini, Torrente, & Meregalli, 2014).

The use of rodents to perform drug-dependence tests is an indispensable tool in biomedical research due to its accessibility. The rat is traditionally the animal of choice in nutritional research and the preferred laboratory mammal for behavioral investigations (Andersen, e Costa, & e Costa, 2016). Decades ago, researchers first tested rodents as an animal model for specific addictive traits, such as a high preference for certain drugs (Sanchis-Segura & Spanagel, 2006). The protocols implicated the repeated administration of psychostimulants to replicate the chronic administration in human addicts. There are two methods for animals to receive drugs of abuse, which are the non-contingent method (in which the investigator administers the drug), and the contingent method (in which drug taking is controlled by the animal). Contingent or non-contingent administration paradigms can elicit different neuroadaptations and behavioral responses (Lecca et al., 2007). Having the option of whether the animal chooses to consume the drug or not, and controlling the length, amount of drug, and the route of administrations are significant advantages to animal research. More importantly, human addictions are most effectively recapitulated using contingent models due to molecular and behavioral differences in noncontingent administration (Markou, et al., 1999).

1.2.3.5.2 Paradigm of Self-administration (SA)

Self-administered (SA)-based methods are probably the most commonly employed models for measuring reward value in animal models (Panlilio & Goldberg, 2007). Drug SA procedures have been found to be valid and trustworthy methods for determining the abuse accountability of drugs in humans. Laboratory animals will readily self-administer drugs of abuse, intravenously or orally, analogous to those used by humans. In addition, this paradigm is meant to model voluntary intake, resembling the voluntary response to obtain the drug, mimicking the behavior of substance users (Koob, Kandel, Baler, & Volkow, 2015). Over the years, SA has undoubtedly proven to be one of the most used and predictive models for the study of addiction. It has improved our understanding of the reinforcing effects of drugs, its evolution, and the neuropharmacological mechanisms involved.

Psychoactive drugs can generate similar reward behaviors in animals, which can be analyzed through self-administration protocols. It has been demonstrated that animals can self-administer most abuse substances consumed by humans (Stitzer, 2005). Indeed, drugs like cocaine, amphetamines, caffeine, opiates, ethanol, sedative-hypnotics, anesthetics, and other abuse substances self-administered by humans serve as reinforcers in animals supporting its use as a model of abuse liability. Moreover, it can also be applied to evaluate the motivation for natural reinforcements such as water, sex, or food (Perry, Westenbroek, & Becker, 2013). Operant conditioning has been used to explain the self-management behavior of abused drugs, as well as to decipher the different

molecular changes produced by voluntary drug intake through a self-administration paradigm (contingent) compared to experimenter-administered (non-contingent) (Chen et al., 2008). That may offer a further comprehensive picture about the development of addiction in humans, which involves voluntary responses and learned associations between the drug and its context (Leshner, 1997).

In drug SA, operant behavior is reinforced and maintained by drug delivery. Animals are trained to perform a specific task, such as pressing a lever or using a nose poke to acquire the drug of interest. SA protocols can use a variety of administration routes, although the intravenous method is the most prevalent among researchers. This implicates the surgical insertion of an intravenous catheter —typically a vein, such as the external jugular or femoral vein, allowing infusion of the drug solution (Angela, 2007). When drugs are positive reinforcers, drug injections alone are entirely capable of initiating SA responses. The accessibility of a drug is typically concurrent with an environmental stimulus, for example, light or sound. Schedule parameters and stimulus conditions vary depending on the purpose of the study, although the basic principles are consistent. This technique demonstrates that animal behavior can be controlled depending on the operant schedules of drug SA.

Producing a particular action, such as pressing a lever, provides a measure of the intake and effort an animal will do to get the drug. This correspondence gives researchers the option to adjust the protocol and concentrate on specific phases of SUDs. These changes involve manipulating the correlation between the number

of responses and the delivery of the drug. This relationship is known as the schedule of reinforcement (SR). Specific parameters are used, such as the response to demand an infusion (e.g., lever presses or nose pokes), whether any cues (sound or visual) are linked with the availability of the drug, how many responses are expected for receiving the drug, and how long the interval is programmed before subsequent doses (Howell & Wilcox, 2001). One of the most commonly used schedules of reinforcement is the fixed ratio schedule. The operative behavior is employed in a fixed ratio (FR) program. The animal performs a determined number of responses throughout the procedure to achieve a drug infusion (Román, 2015). In that way, the number of valid responses to deliver the drug successfully is dependent on the reinforcing properties of the drug. In addition to the fixed ratio programs, there is the continuous reinforcement paradigm where the animal performs a single action to receive a rewarding stimulus, contrary to the progressive ratio paradigm where the number of responses required to obtain a reinforcer progressively increases throughout the session (Howell & Wilcox, 2001).

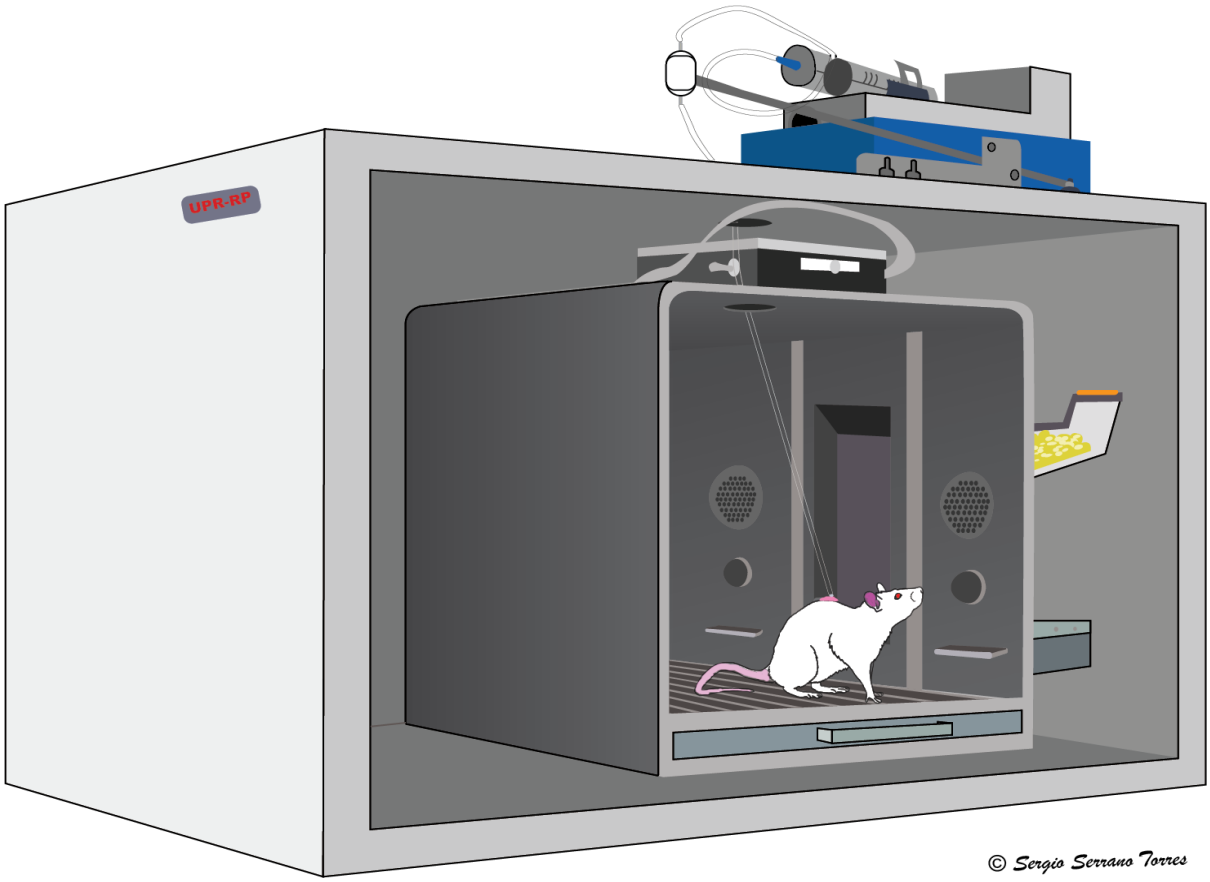


Figure 1.5 Self-Administration Box

1.2.3.5.3 SA: Incubation of Cocaine Craving Paradigm

In addition to the fixed ratio programs, schedules of cocaine SA have also been modified to evaluate elements of relapse conditions. Another aspect, relevant to human addiction behaviors is how the length of time following reward SA influences craving symptoms (Sayette et al., 2000). Individually, after several weeks of abstinence (either forced or voluntary abstinence) from a reward, rats respond robustly for the reward-paired cue upon returning to the reward SA environment. Studies in humans and non-human subjects have shown that drug-craving responses to drug-associated cues may have an amplified trend as the duration of abstinence progress (Sinha, 2011). These traits increase during the first weeks of abstinence and settle after months of withdrawal. This phenomenon is known as “drug craving incubation” and it has been observed not only with cocaine, but with several other drugs (i.e., heroin, nicotine, alcohol)

In the field of addictions, craving is considered to have a great influence on the maintenance of addictive behaviors, being responsible for the compulsive use of the drug, the difficulties associated in the period of abstinence and the high rate of relapse that follows (Tiffany, 1999).This recurrence is one of the main problems —after periods of forced or voluntary abstinence— in addicts (O’Brien, 2011). After prolonged-abstinence, users experience an escalation of uncontrollable cravings symptoms. These craving responses escalate as a function of time, intensifying the magnitude of cue-induced drug seeking (Grimm et al., 2001).

The term craving started from observing drug addicts continued drug consumption over time, even though they made tremendous efforts to suspend its usage (Sinha, 2011). The more intense the reinforcing effects of a substance, the more persistent the memories and the more urgent the craving to experience them again are. An imperious desire that can trigger in certain situations and that can also initiate automatic behaviors of search and consumption of that substance. This led to the belief that the cause that drives a person to have self-destructive behaviors in a compulsive way must be the existence of an uncontrollable and irresistible desire to consume the drug.

Research on the craving phenomenon emerged in the 1950s, and later recognized as a central component of addiction (Jellinek et al., 1955). It was in the nineties, when the interest for the craving phenomenon reappeared and the research was retaken. In 2013, the DSM-V added craving as a diagnostic criterion for addictive disorders and defining it as a strong desire to take drugs. However, craving symptoms can also be experienced by natural rewards such as food, sex, and money. This led researchers to distinguish between two kinds of cravings: tonic or background craving, and phasic or provoked craving that is also known as cue-induced craving (Ferguson & Shiffman, 2009). The main distinction between them is that phasic craving appears soon after suspending the drug and can be maintained for an extended period of time; while tonic craving has a more limited duration (Tiffany & Wray, 2012). There is also evidence that tonic craving is produced solely by abstinence, whereas phasic craving can be induced by substance-related cues, drug withdrawal states, drug priming and emotional states

(Tiffany & Wray, 2012). Therefore, during the last 15 years, investigators have incorporated these aspects of human abstinence into an alternate model of drug relapse in which abstinence is not achieved by extinction training

This phenomenon is involved in cocaine dependence (Kilts et al., 2001). These symptoms can endure by many regular consumers and it is a complex and incompletely known phenomenon. In cocaine abusers, craving progresses very quickly and is triggered by elements of the environment that activate the limbic system (the amygdala and the cingulate cortex). Animal studies similarly have shown that cue-induced craving responses intensify progressively after several weeks of abstinence (Grimm et al., 2001) The time-dependent amplification of these responses are known as “incubation of cocaine cravings.” This effect has been proposed to reflect a behavior that can emerge in humans after a prolonged period of cocaine withdrawal (Gawin and Kleber, 1986).

A key issue in the study of craving is to decipher the brain structures responsible for the onset of this powerful desire. In addition, to gain a greater understanding of the craving phenomenon and the psychological processes affected, in order to develop pharmacological treatments that act on precise structures (Kalivas & Volkow, 2005; Lecca, Cacciapaglia, Valentini, Acquas, & Di Chiara, 2007). Also, the identification of these regions as cue-responsive are important since their activation may relate to treatment successfulness.

In the last ten years, the development of functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) has allowed us to identify

the structures involved in the generation of craving in humans (Gloria et al., 2009). These studies have pointed out the existence of an extensive neural network involved in craving, which varies according to the paradigm and the drug used. These studies contained consistent findings across them demonstrating an increase in activation of the VS, amygdala, and ACC/ vmPFC in response to drug-related cues. This may be part of the core circuit that underlies the experience of drug craving, at least for nicotine, alcohol, and cocaine. These results suggest that the craving phenomenon involves both cortical and subcortical structures.

1.2.4 Psychiatric Comorbidity in Cocaine Addiction: Depression & Anxiety

Co-occurring disorders, also called dual pathology, are defined by the simultaneous existence of the substance-use disorder and another mental illness, or the intersection of both dysfunctions (Fisher & Roget, 2009). People with addictions are often more susceptible to a wide range of mental health problems depending on the drug being abused (Regier et al., 1990). Drugs such as alcohol, nicotine, cannabis, amphetamines, and cocaine, among others, can produce significant pathological mental disorders. In the case of cocaine, psychiatric disorders may occur during the period of intoxication or withdrawal period. Comorbidity of psychiatric disorders has been shown to affect the duration and quality of abstinence, and the prognosis among cocaine addicts (Levin et al., 2008). The symptoms vary in intensity and severity depending on multiple factors.

The abuse of cocaine, compared to other substances, increases the risk of many psychiatric disorders, in which anxiety bipolar and depressive disorders prevail (Rounsaville et al., 1991; Rounsaville, 2004). These psychiatric disorders often accompany cocaine addicts and their subsequent withdrawal from long-term use. Similarly, in rats, depressive-like symptoms and anxiety-like behaviors (Magalhaes et al., 2002; Blanchard & Blanchard, 1999) have been observed following chronic drug use and withdrawal. It has been shown that the hedonic state induced by cocaine withdrawal is proportionate to the amount of cocaine consumed (Markou & Koob, 1991). While these studies suggest that cocaine can produce depression- and anxiety-like states, determining the etiology of comorbidity is challenging; thus, additional studies are needed for a more decisive conclusion.

1.2.4.1 Paradigms for Evaluating Comorbidity in Cocaine Addiction: Depression & Anxiety

There are several examples in the clinical and basic research that demonstrate that various forms of stress are involved in the etiology and maintenance of drug dependence. Moreover, abstinence from most drugs of abuse is associated with an increase in negative affective symptoms, such as anxiety and depression (Magalhaes et al., 2002; Blanchard & Blanchard, 1999). In addition to the evaluation of the reinforcing effect of drugs, other tests have been developed to study aspects relevant to the addictive process. The discontinuation of drug intake

in cocaine abusers commonly produces a variety of adverse withdrawal symptoms among which anxiety and depression-related behavior prevail during abstinence (El Hage et al., 2012). Human studies have shown that the abuse of cocaine and its subsequent withdrawal can trigger both psychiatric symptoms (Perrine, Sheikh, Nwaneshiudu, Schroeder, & Unterwald, 2008b). In rats, the effect of cocaine on anxiety has been studied in cases of acute and prolonged cocaine use and/or withdrawal, offering similar results.

Elevated plus maze (EPM) can be used as a behavioral assay to study anxiety-related behaviors. The EPM well-characterized behavioral paradigm popular for its inexpensiveness, ease of replication, and reliability. Due to this particularity, it is one of the most widely used methods in today's research. It was first developed as a Y-shaped apparatus for evaluating fear induced by novel stimulation. Years later, it was transformed into the X-maze that we know now, with alternating open and enclosed arms (Handley & Mithani, 1984). These authors also correlated the anxiety behavior of animals by using the ratio of time spent on the open arms to the time spent on the closed arms. This test takes advantage of the natural tendency of rodents to explore, placing them in an elevated labyrinth with two open opposing arms and two closed opposite arms (Walf & Frye, 2007). Under natural conditions, animals spend less time in open arms since the lack of protection and the height of the labyrinth produce greater anxiety. According to the level of anxiety of the animals, they will invest more or less time in the open arms of the labyrinth, understanding that a greater time in open arms is indicative of an anxiolytic effect. EPM is often presented as a test or model of anxiety for the evaluation of

substance-induced anxiety effects of new pharmacological agents, drugs of abuse, and hormones, among others. Furthermore, it is a system that offers reliable data about the anxiogenic or anxiolytic power without the need for any previous training, as it can be seen on Figure 1.6 (Calabrese, 2008).

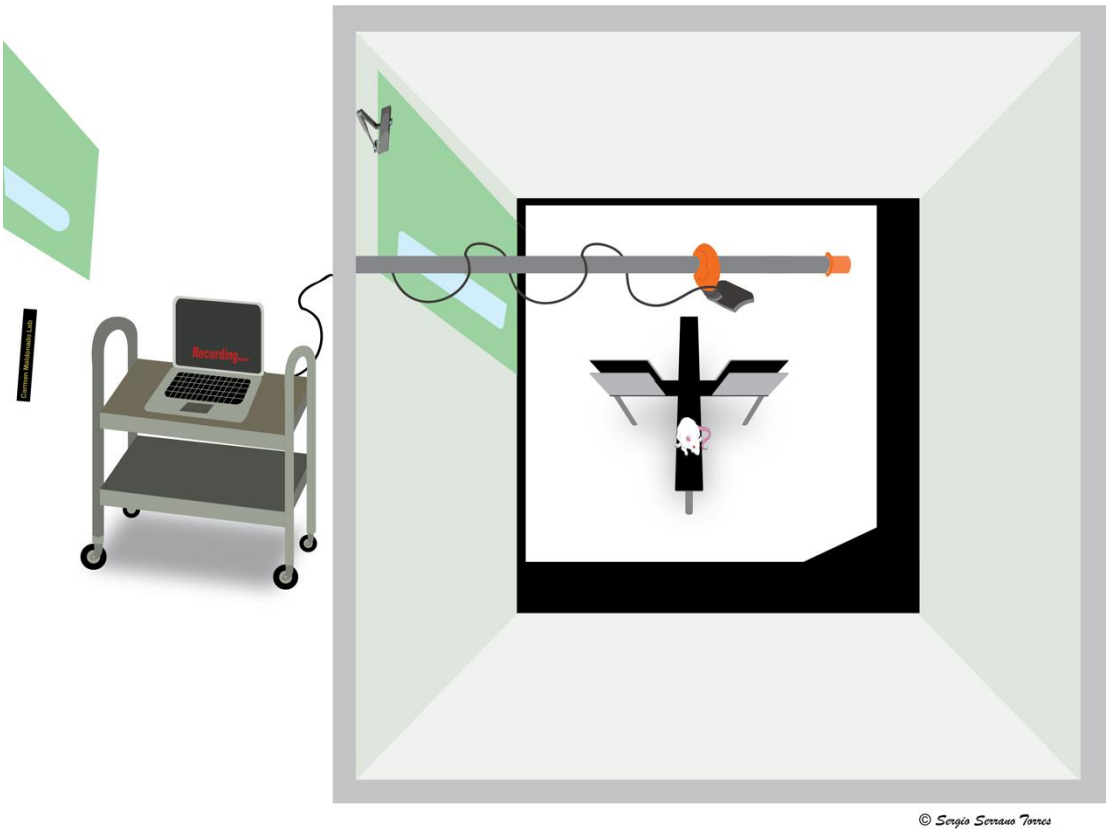


Figure 1.6 Elevated Plus Maze Test

The behavioral despair test (BDT), more commonly known as the forced swim model (FST) is one of the most used animal models for assessing depression-like behaviors. This includes evaluating substances with antidepressant potential and/or properties or trying to reproduce depression characteristics in experimental models. The FST was developed in the late 1970s (Porsolt, Le Pichon, & Jalfre, 1977); however, the test was later modified and the neurotransmitters responsible for the active behaviors were identified (Detke, Rickels, & Lucki, 1995). The FST is based on the induction of despair, a behavioral pattern in which the animals abandon any attempt to struggle and escape to solve a situation. The subject is forced to swim in a restricted space in which they cannot touch the bottom or escape Figure 1.7

The FST consists of a pretest in which the rat is placed in a glass cylinder filled with water to a certain height (40 cm) and temperature (22 ± 2 °C) for 15 minutes. After 24 hours, the official 5-minute test is performed and behaviors are then recorded (Slattery & Cryan, 2012). In the first minutes, the rat shows exploratory activities and an escape conduct where the animal swims, dives, and tries to climb the walls. After a few minutes, these activities cease, and the rodent adopts the characteristic of an immobile floating position—erected on the hind legs and tail, keeping the head raised just above the surface of the water. Immobility behavior is considered a state of hopelessness, which is proposed to be analogous to despair or abandonment actions shown by depressed individuals (Porsolt, Anton, Blavet, & Jalfre, 1978). This behavior is easy to quantify and the most important parameter of this test. A decrease in latency time and an increase in the

immobility time of the animals are indicative of depressant activity. Antidepressant drugs have been shown to have the ability to decrease the duration of the immobility period (Slattery and Cryan, 2012).

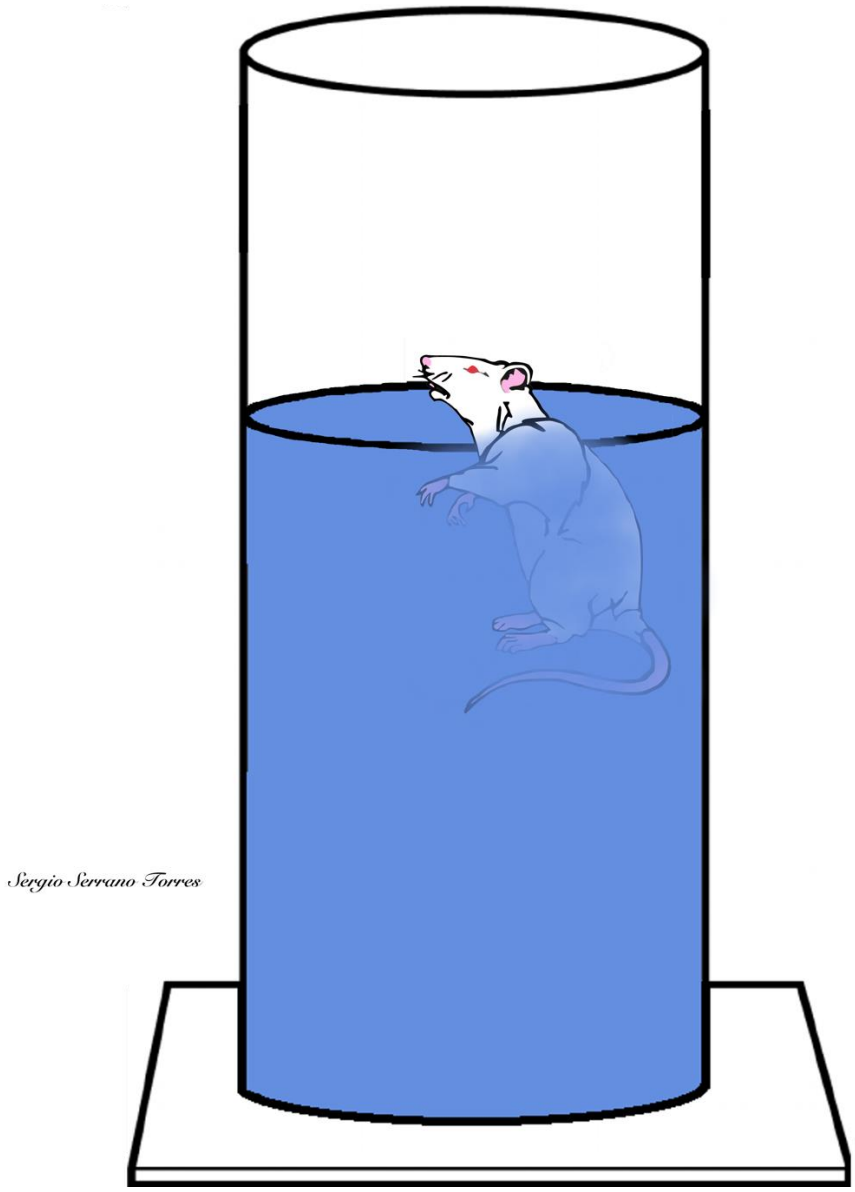


Figure 1.7 Forced Swimming test

1.2.5 Environmental Factors That Enhance Vulnerabilities Towards Cocaine Addiction

Although millions of people are exposed to addictive substances each year, the clear majority do not become addicted (Ducci & Goldman, 2012). The study of risk for drug use is a way to recognize the vulnerable factors involved in the onset, use, and abuse of consumption, which are related to individual biological status—such as psychological and genetic influences—and environment (Bryda, 2013). Vulnerability factors are those characteristics or variables that, if present, increase the likelihood that a person will develop addictive behaviors. These factors, under certain disadvantaged conditions, can facilitate the beginning, continuity, or worsening of addiction.

Research in the field of drug addiction has confirmed that several environmental circumstances predispose individuals to drug abuse (Brewerton & Dennis, 2014). The study of external factors in recent years has sought to analyze the interaction between genetic and environmental variables to explain the risk of dependence on psychostimulants. Nevertheless, it should be noted that environmental factors might have direct and determining roles in the causes of drug dependence. External risk factors include personal characteristics or circumstances that increase the likelihood of a person engaging in drug use or abuse problems. Although it is difficult to establish the relative importance of the different factors involved in drug use—since this depends on each case, it is likely that these factors can facilitate the beginning, continuity, or worsening of drug addiction for a particular group of people.

Along with the characteristics of the substance and the individual, drug use can have other origins, such as the characteristics of their social environment. Social influence, which is an essential modulatory force of drug use behavior, is unusually intense when it comes to people or groups that are part of the social environment closest to them (Davis & Tunks, 1991). Thus, the family or school environment plays an essential role in the onset of drug use. Other environmental factors that have been consistently associated include low socioeconomic status, physical and psychological abuse, drug availability, and nutrition among others.

Over the last three decades, a growing body of research has provided evidence that adequate nutrition plays a vital role in the development and functioning of the human CNS (Bourre, 2006). These studies highlight the significance of diet in the growth and development of the body, as well as its influence on several mental and psychological diseases, including depression, anxiety, and addiction (Lakhan & Vieira, 2008). In the latter, there is compelling evidence that nutrients are a potent environmental factor concerning the high prevalence and incidence on these disorders. Although the determinants of substance abuse disorders are multifaceted and complex, rigorous studies have made outstanding contributions towards understanding this link.

Evidence has demonstrated that dietary composition may contribute to the potency and/or efficacy of psychostimulant drugs (Davis et al., 2008; Jenney et al., 2016). Variations in nutritional constituents, such as carbohydrates, proteins, and lipids may contribute to the strength and/or efficiency of psychostimulant function in the brain. Animal studies have demonstrated that the actions of restriction of

specific nutrients can modulate the acquisition and maintenance phases of cocaine self-administration (Campbell & Carroll, 2000). For example, Wellman, Nation, and Davis (2007) found that administration of a diet high in fat inhibits the acquisition of cocaine self-administration in rats. In addition, varied diets, such as high-protein or high-carbohydrate diets, can influence the severity of cocaine withdrawal symptoms (Loebens & Barros, 2003). The differences in withdrawal symptoms observed among animals fed diets varying in macronutrient content may reflect dietary-induced alterations in the neurotransmitter system.

1.3 Possible Effects of Nutrition in Cocaine Addiction

1.3.1 Nutrients: Health and the Nervous System

Food intake is an everyday act that is part of the basic needs of human life, exerting a powerful influence on the structure and functioning of an organism. Dietary elements constitute an environmental factor that can directly disturb the general health status and the well-being of individuals. Proper nutrition is fundamental for satisfying the needs for growth, energy and good health (Food and Agriculture Organization of the United Nations (FAO), 2001). It has a crucial role in the development of human beings from the moment of conception, providing the necessary macro- and micro-nutrients. Research studies have acknowledged that nutrient deficiencies can either influence long-term health or precipitate the emergence of life-threatening illnesses across different stages of life (World Health Organization, 2003). These findings have not only provided valuable information but also triggered further investigations whether nutritional-factors are determinants for disease prevention, control, and the improvement of health. However, this is not a simple conclusion to reach due to its multifactorial and complex nature.

Nutrient-related diseases have been covered in different areas of knowledge (Bourre, 2006; Georgieff, Ramel, & Cusick, 2018). Public health experts such as scientists, nutritionists, and physicians, among others, have emphasized the importance of nutrients in improving and maintaining health. Moreover, the intake of appropriate amount of nutrients that support daily body

needs helps people maintain optimal health, while protecting them from many diseases and disorders (Coulston, Boushey, Ferruzzi, & Delahanty, 2017). Diets rich in fruits, vegetables, cereals, whole grains, and fish can add a protective factor. Conversely, poor nutrition characterized by food rich in saturated fats, trans-fats, sugars, and cholesterol, is related to an increased risk of cognitive, motor, and emotional impairment (Mozaffarian, 2016). Any situation of imbalance due to deficiency will compromise the nutritional status and its vital functions. The dietary contribution is also influenced by external factors, such as eating behavior, emotional and physical state, cultural influences, and socioeconomic status (Council, 2013). Furthermore, nutritional deficiencies are not confined to undeveloped countries because nations in the developed world also experience the consequences of nutritional deficiencies as a result of unhealthy foods consumption.

While there have been significant changes in human nutrition throughout history (Cordain, Eaton, & Sebastian…, 2005), diet components in the United States (U.S.) and most Western countries have been gaining attention as potential contributors to the increase in diet-related chronic diseases. Since the beginning of the 18th century, along with advancing technology after the industrial revolution, the Western diet has undergone a remarkable shift in its traditional food. Countries like the U.S. have adopted a style of nutrition called the "Western diet" which typically consists of high intakes of refined and high-energy foods that includes high-calorie meals, high content of saturated fat and processed carbohydrates (Cordain et al., 2005). In addition to this shift, the modern Western diet has carried

an imbalanced nutrient intake associated with the increased prevalence of diet-related chronic diseases, particularly in cardiovascular disease (CVD), stroke, cancer, diabetes, and obesity (Willett et al., 2006).

It was reported that a deficient diet triggers damage (Bourre, 2006; Georgieff, Ramel, & Cusick, 2018). One of the systems severely affected by nutritional deficiencies is the central nervous system (CNS) (Alvarez, et al.,2017). Just like any other system, the CNS requires a constant supply of nutrients. Nutritional inadequacies, particularly of vitamins, minerals, and lipids, can exert adverse effects on this system (Kumar, 2010). If, for any reason, the supply is affected, the integrity of the central and the peripheral nervous system become compromised (Kumar, 2010). This ranges from the involvement of the PNS causing sensory deficits and neuropathies to complex lesions of the CNS, primarily the brain, that leads to mental retardation, psychosis, seizures, and cognitive dysfunctions (Román, 2015).

1.3.2 Nutrients Deficiencies: Brain and Behavior

The human brain is considered extraordinarily complex due to its function and structure (Sporns, 2010). The brain is made up of an inconceivable number of nerve cells that are interconnected to ensure a whole series of functions. The brain is a complex organ that is capable of perceiving and responding to internal or external stimuli, allowing it to interpret and respond to experiences transforming our thoughts. It is also the organ of the cognitive faculties, whether voluntary or

involuntary encompasses processes, such as attention, emotions, memory, and intellect (Malenka, et al., 2009). The brain is also an extremely demanding organ that depends on nutrients for its optimal cognitive performance. Neuroscientists have identified key nutrients—amino acids, vitamins, fatty acids, minerals, among others—that we obtain from food as the primary raw materials needed for the composition of the nerve cells, gene regulation, metabolism, and antioxidant protection (Gómez-Pinilla, 2008). Extensive evidence has also demonstrated the decisive role of nutrients in neurotransmitter syntheses, such as GABA, norepinephrine, dopamine, and serotonin (Unger, Hegde, & Connor, 2011). Scientists have shown great interest in deciphering how specific macro- and micro-nutrients can alter their synthesis, production, and storage, thereby supporting a probable involvement with many psychiatric disorders (Kaplan, et al., 2007). Furthermore, evidence suggests that nutrition may have a causal role in the worsening and the onset of cognitive and emotional responses (Lakhan & Vieira, 2008b; Alvarez, Gomez, Fonzo, Sanchez, & Giménez, 2017; Kumar, 2010).

Indeed, epidemiological evidence supports the hypothesis that many constituents of the diet influence brain function. Diet, in particular, has become the object of intense research in relation to its effects on the brain. The brain requires energy and molecular components provided by the diet to develop, mature, function, and prevent diseases. A constant dietary supply of appropriate nutrients, including glucose, amino acids, fatty acids, vitamins, and minerals, required for normal brain function (Gómez-Pinilla, 2008). These nutrients can affect multiple neural physiology by regulating neurotransmitter synthesis, synaptic transmission,

membrane fluidity, and complex interaction between neurons that influence behavior and cognitive performance (Gómez-Pinilla, 2008). They also uphold the integrity of different regions of the brain, such as the prefrontal cortex and hippocampus, which is directly associated with learning and memory, and mood-related behavior (Bach et al., 2014; Larrieu et al., 2014). Changes in dietary patterns and nutritional status, such as decreased omega-3 intake, have been shown to impact brain volume (Titova et al., 2013) Other nutritional factors, such as vitamin B niacin, folate, and vitamin D, have effects on mental health (Haan et al., 2007; Lakhan & Vieira, 2008).

Dietary factors may predispose individuals to the development of certain mental health diseases, including negative repercussions on processes such as cognitive or emotional well-being (Gómez-Pinilla, 2008). Current epidemiological studies show a relationship between the intake of total fat, saturated fat, refined sugar, and cholesterol with deficits in cognitive flexibility and memory that depends on the hippocampus in humans (Kalmijn, 2000; Francis and Stevenson, 2011). Evidence also supports the role of a poor diet in the promotion of mental disorders, including depression and dementia (Lakhan & Vieira, 2008a). Also, an adequate nutrient supply is vital through periods of embryogenesis, fetal growth, and childhood for the appropriate development of the brain, supporting the advancement of cognitive and motor skills during childhood and into adulthood (Gibson & Makrides, 2000; Georgieff et al., 2018).

1.3.3 Nutrients Deficiencies: Brain and Development

Nutrition plays a vital role in the growth and development of the brain (Gibson & Makrides, 2000; Georgieff et al., 2018). In recent decades, there has been a growing interest in how the diet may affect the functioning of the brain throughout the entire lifecycle. Studies involving experimental animals and humans have demonstrated the importance of appropriate nutrition during crucial periods of brain growth (Jenney et al., 2016; Gibson & Makrides, 2000; Georgieff et al., 2018). The CNS is more vulnerable to nutritional influences during periods in which growth, development, and plasticity are taking place.

Previous research has shown that there are significant windows of susceptibility during prenatal and early postnatal development (Georgieff et al., 2018; (FAO), 2001; Makrides, Anderson, & Gibson, 2013). Dietary compounds are involved in the complex interactions and activity of the brain, from neurogenesis in the infant's brain through to adulthood, to metabolic pathways, such as glucose regulation and inflammation, which are involved in disorders (Barberger-Gateau, 2014; Schenk, Saberi, & Olefsky, 2008). Moreover, diet can provoke long-lasting or permanent neurotransmission abnormalities. These changes can impact the future cognitive development of a person. Thus, the effects of nutrient deficiencies are determined by the magnitude, duration, timing, and the precise requirement of the brain for a particular nutrient during the course of a deficit.

Evidence derived from animal studies as well as human investigations suggests that sensitive periods for specific nutrient exposures are indispensable for CNS integrity (Georgieff et al., 2018). Previous investigations that have been completed at various stages throughout the human lifecycle have ratified that

nutrient deficit negatively influence optimal brain function. Nutritional deficiencies are a problem that affects several transitional stages, especially in the most vulnerable individuals: fetuses, children, and adolescents (DeLong, 1993). Stipulating the timing of these phases of nutrient obligation is necessary to ensure nutritional interventions can occur to avoid permanent damage to brain development.

The consequences of malnutrition are an expanding area of interest due to the increasing prevalence and incidence of (i.e., fetal and infant) nutritional deficiencies (Jenney et al., 2016; McNamara et al., 2009; Imhoff-Kunsch, Briggs, Goldenberg, & Ramakrishnan, 2012). This interest includes the diet of the pregnant mother and nutrition during the early years when the brain is rapidly growing. Mounting evidence suggests that a dietary insult on the developing brain can incite long- or short-lasting deficits in learning and behavior. These environmental factors may harm a developing brain even more drastically than a mature brain. There is also interest in nutrition during the early years after conception.

During pregnancy, the three trimesters represent an intense period of neurodevelopment and growth (DeLong, 1993; Raygada, Cho, & Hilakivi-Clarke, 1998). Neurons and glia are formed during the (Wright & Kutcher, 2016; Johnson, Munakata, & Gilmore, 2008). In late pregnancy, marked axon development and the formation of connections between neurons result in substantial brain growth. Brain weight at birth is about 400 g, increasing during the first year of life to about 1000g or 71% of the adult brain weight (approximately 1400g) (Kinney, Brody,

Kloman, & Gilles, 1988). Given the dramatic changes in brain development that occur before birth, the prenatal period is both a window of opportunity and a period of great vulnerability. Early malnutrition also affects processes involved in brain maturation, such as neurogenesis, neuronal and glial migration, the number of synapses, and the degree of myelination. These changes are mainly irreversible and cause permanent cognitive deficiencies (Morley & Lucas, 1997; Benton, 2010). Similarly, throughout childhood and adolescence, the diet plays an essential role in the development of the adult brain.

Like prenatal nutrition, food intake in children is of similar importance for the brain and cognitive development (Benton, 2010). Differences in specific nutrients in the diets of infants and children have been shown to affect their cognitive behavior. During childhood, significant neurological development and brain growth occur. In humans, brain size begins to increase considerably, reaching 80–90% of adult volume by the age of two. Throughout this process, the developing brain is particularly sensitive to dietary factors. The brain continues to proliferate after conception and into early adulthood, suggesting an additional role of diet during childhood and adolescence (Isaacs & Lucas, 2011).

As previously mentioned, most studies involving nutritional deficiencies have focused on the last trimester of pregnancy and the first two years of life, as this is the period when the brain develops rapidly. However, recently, it has been concluded that brain maturation is not complete until early adulthood, and a growing body of evidence has demonstrated that adolescence is a period of continued brain growth and change. A lack of specific dietary nutrients, such as

essential omega-3 fatty acids, may significantly contribute to cognitive decline and increased risk and severity of brain injury (Weiser, Butt, & Mohajeri, 2016).

Adolescence represents a period of transition between childhood and adult life. Its onset corresponds to the appearance of secondary sexual characteristics and its termination with the cessation of growth. It is a stage marked by abrupt and sequential changes that allows growth and physical maturation as well as cognitive development (Wright & Kutcher, 2016). It begins with an increase in the production of sex hormones, such as estrogen, progesterone, and testosterone, and is characterized by the external appearance of secondary sexual characteristics. The increases in lean body mass, skeletal mass, and body fat observed during puberty demand greater energy and nutrient needs than at any other time in life (Das et al., 2017). Furthermore, changes in cognitive and emotional functioning allow adolescents to acquire greater independence as they mature (Das et al., 2017). This represents the passage from concrete operational thinking to logical operations.

In adolescence, brain changes occur and brain maturation affects behavior and emotions during this phase (Spear, 2013). According to studies where brain structures have been observed, the last area of maturation is the frontal lobes (Brown & Jernigan, 2012). In the frontal lobes, rapid changes in synaptic density are observed from birth to 15 months. These increases occur until two years of age and then decrease until around the age of 16 (Brown & Jernigan, 2012). These structural changes seem to correspond to the development of behaviors that mediate the frontal lobes, such as language, executive functions, and emotional

functions. However, this process of myelination is not complete until the third decade of life.

1.4 Lipids Basic Structures and Terminology

Food lipids exhibit unique physical and chemical properties, such as composition, structure, melting properties, and the ability to associate with other non-lipid molecules (Fennema & Parkin, 2008). The body uses dietary lipids for storage and immediate metabolic energy. Also, lipids perform several biological functions by acting as structural components of membranes, for transport and storage of energy, a protective cover and for cell communication (Khosroshahi, 2017).

Lipids are organic biomolecules that are composed of a diverse group of organic substances, which are extensively distributed in animal and plant tissues and thus, are a key source of energy in foods (Akoh, 2017). Lipids, oil, and fat are common terms that are often confused or inadequately used interchangeably. Lipids have the fundamental characteristic of being insoluble or poorly soluble in water, but miscible in non-polar solvents such as acetone, chloroform, hexane, and ethanol. Lipids comprise a wide variety of substances including phospholipids, fatty acids (FA), triglycerides (TG), fat-soluble vitamins, steroids, waxes, and other less commonly known materials, such as prostaglandins (Berg, Tymoczko, & Stryer, 2007). Furthermore, oils and fats are a subset of lipids called triacylglycerols. In fats, the fatty acids attached to the glycerol backbone are predominantly saturated or trans fatty acids, making it solid at room temperature. However, oils are composed of mainly unsaturated fatty acids providing a liquid consistency at room temperature (25˚C) (Nelson & Cox, 2017).

The chemo-physical properties of fatty acids, in addition to their nutritional qualities, depend greatly on the number of carbons, the number and positions of the double bonds and the isomerism that the molecule presents (Geissler & Powers, 2017). Fatty acids are the basis of lipids, and, from a chemical point of view, they are straight chains of hydrocarbons that have a carboxy-terminal (COOH) at one end and a methyl terminal (CH3) at the other. These molecules can vary in the number of carbon atoms, ranging from short (C4–C6), medium (C10–C14), long (C14–C20) and very long (C20 or more) fatty acids (Chow, 2007). Fatty acids are organic acids that usually contain an even number of carbon atoms. However, there are organisms that are capable of generating fatty acids with odd numbers of carbon atoms. The melting point increases with the length of the chain; therefore, C4–C8 are liquid at room temperature (25°C), while those greater than C10 are solid at room temperature (25°C) (Akoh, 2017). Also, the mechanisms of digestion, absorption, and metabolism vary depending on the number of carbon atoms.

The fatty acids present in oils and fats are classified by their degree of saturation or the presence or absence of double bonds (Berg, Tymoczko, & Stryer, 2007). Saturated fatty acids (SFA) are completely surrounded or “saturated” with hydrogen atoms and are unable to accept any additional molecules, making them less chemically reactive. SFA are common in meats and dairy products. Moreover, humans and other animals can produce SFA, and those fatty acids are classified as non-essential (Berg, Tymoczko, & Stryer, 2007). Unsaturated fatty acids are characterized by the presence of a C=C double bond. This provides high chemical

reactivity, due to the propensity of saturation, oxidation, and isomerization. In the formation of a C=C double bond, fewer hydrogen atoms are present, making the molecule unsaturated. The degree of unsaturation determines the rigidity of the molecule and provides cis-trans isomerism, notably changing its chemical and physical properties (Gropper & Smith, 2012).

Among the unsaturated fatty acids are compounds with one, two, or three double bonds (Whitney & Rolfes, 2015). Depending on the degree of unsaturation, unsaturated fatty acids can be classified as either monounsaturated or polyunsaturated. Monounsaturated fatty acids (MUFA) are molecules that have one double bond. Oils with high contents of MUFA are typically liquids at room temperature (25˚C) (Whitney & Rolfes, 2015). They are abundant in certain oils derived from plants, such as olive, canola, safflower, avocado, almond, and palm oils. Most of the lipids in this category have a chain length of 16–22C and a double bond with the cis configuration at the 9-position. One of the main MUFAs is oleic acid, which has been shown to have a positive effect on human health by reducing the levels of bad cholesterol (i.e. low-density lipoprotein) and increasing the supposed “good” cholesterol (i.e., high-density lipoprotein) (Hark, Ashton, & Deen, 2012).

Conversely, when a fatty acid has more than one double bond, it is called polyunsaturated or 'polyenoic'. Most naturally occurring polyunsaturated fatty acids (PUFA) have a long chain of between 20–24 carbon atoms, while others have a long chain of more than 25 carbon atoms (Whitney & Rolfes, 2015). These PUFAs are classified according to the position of the first double bond of the chain, called

the omega bond, counting from the methyl end. According to this, there are three families of polyunsaturated FAs: n-9 (first double bond on carbon 9), n-6 (first double bond on carbon 6), and n-3 (first carbon double bond) (Whitney & Rolfes, 2015). Omega-6 fatty acids are the most common and abundant in vegetable oils, such as flaxseed, corn, soy, and sunflower. Omega-3 fatty acids are common in fish and vegetable oils such as flaxseed, soybeans, and canola. These fatty acids are essential fatty acids since the organism itself cannot synthesize it de novo, which makes them a necessary part of a well-balanced diet. In addition, its derivatives are important substrates for the biosynthesis of more complex lipids such as EPA and DHA (Cruz & Alarcón, 2011).

1.4.1 Omega-3 Fatty Acids: Classification & Metabolism

Polyunsaturated fatty acids (PUFAs) are divided into groups corresponding to their derivation from particular biosynthetic precursors. PUFAs constitute an extensive family of aliphatic molecules whose primary structure involves a linear hydrocarbon chain containing at least two double bonds (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). If the FA comprises only one unsaturation, it is known as a monounsaturated acid (MUFA); a FA holding more than one unsaturation is termed a PUFA. Furthermore, PUFAs are classified according to the length of their chain and by the location of the double bonds. The carboxyl group (-COOH) is considered to be the beginning of the string (alpha), and the methyl group ($-CH_3$), is classified as the tail or the end of the chain. Nevertheless, the positions of double

bonds in PUFAs are often classified using the methyl end, which is called the omega carbon (ω-1) (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001).

PUFAs, such as omega-3 (ω-3), omega-6 (ω-6) and omega-9 (ω-9), are organized considering the location of the first double bond counted from the methyl terminal end of the chain (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). The different unsaturated sites in the chain confer different physiological properties to the PUFAs, which makes the association between omega-6 and omega-3 in the diet more relevant. These qualities, together with isomerization (cis or trans), define their nomenclature and physical-chemical characteristics in addition to the nutritional properties (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). Moreover, these essential FA are components of triacylglycerols and phospholipids in certain plants and fish that are eaten by humans. The presence of PUFAs in triacylglycerol protects the cell membrane from damage, and in phospholipids, they increase fluidity (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). Moreover, they are precursors of several fatty messengers, including the eicosanoids, docosatrienes, and resolvins (Spite, Clària, & Serhan, 2014). Therefore, ω-3 not only has a structural and molecular function but also synthesizes vital substances that are necessary to maintain cellular homeostasis (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001).

PUFAs can be classified based on the length of their chain. Short chain ω FAs contain less than 18 carbon atoms in their molecular structure (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). Linoleic acid (LA, 18: 2n-6) and α-linolenic (ALA, 18: 3n-3) constitute the most influential precursors of the two ω-3 and ω-6 families.

Once short chain ω-3 FAs are ingested, they can be metabolized giving rise to other FAs with greater complexity and functionality. ALA and LA are converted in the body into three other EFAs: the ω-3 FA eicosapentaenoic acid (EPA: 20: 5 n-3) and docosahexaenoic acid (DHA: 22: 6 n-3), and arachidonic acid (AA) (Gurr, 2016; Mostofsky, Yehuda, & Jr., 2001). The bioconversion of LA, ALA, and AA occurs due to the action of several elongases and desaturases, particularly in the liver (Nakamura and Nara, 2003). These enzymes are responsible for the insertion of double bonds during their formation. Although humans can synthesize longer derivatives using EFAs, they are not efficiently produced by the body and, thus must be obtained from food. All mammals, particularly humans, are unable to synthesize LA and ALA. Animals, whether terrestrial, or aquatic, cannot introduce the cis double bond that is necessary for their synthesis. They can only get LA and ALA through the diet since they lack the enzymes (desaturases Δ12 and Δ15) necessary to carry out the double bond formation (Abedi and Sahari, 2014).

Oils containing these FAs originate primarily from certain plant sources, as well as fish and unicellular organisms (Nichols, McManus, Krail, Sinclair, & Miller, 2014). The animal sources rich in ω-3 are found mainly in fatty fish, such as sardine, salmon, and tuna, among others. The primary origins of these FAs are microscopic algae, phytoplankton, and crustaceans found at the base of the food chain (Schuchardt et al., 2011). The ω-3 content of fish varies considerably depending on the species, fat content, and geographical location (Larsson et al., , 2004). Thus, deep-sea and cold-water fish have higher EPA and DHA contents. As for vegetable sources rich in ω-3, ALA is found in vegetable oils of chia seeds,

flaxseed, olive, and soybeans. Other sources are nuts such as almonds, walnuts, pecans, peanuts, among others (Ros, 2010).

In addition, LA and ALA use the same metabolic pathways, leading to competitive enzymatic availability. Therefore, an increase in the intake of a specific precursor leads to higher production of the same family. On the contrary, it causes a decrease in the formation of the other PUFAs of n-3, n-6, and n-9 series that compete for Δ6-desaturase. Thus, there is a competitive interaction between FAs. The biochemical processes of elongation and desaturation of LA and ALA are performed by enzymes located in the endoplasmic reticulum and the peroxisomes of liver cells. In addition, several factors decrease the enzymatic activity leading to the decrease in the conversion of EPA and DHA. Within these factors are the intake of saturated fats, cholesterol, and trans-fats (Liu et al., 2017). Other factors such as age and sex can contribute to the action of the desaturases (Lauritzen et al., 2016). This highly regulated or rate-limiting enzyme is under the control of many dietary and hormonal factors and is believed to be important in the synthesis of docosahexaenoic acid (DHA).

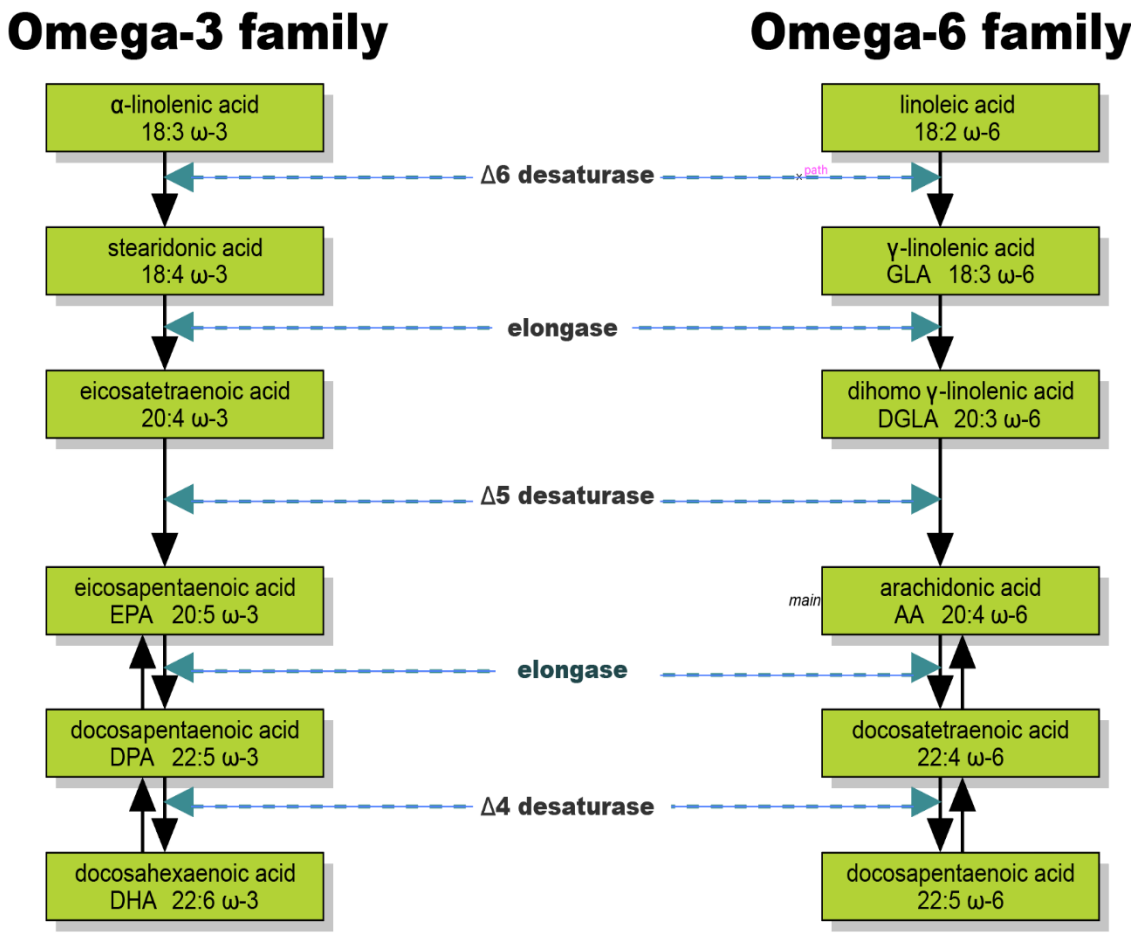


Figure 1.8 Metabolic Stages of n-6 and n-3 FA Biosynthesis from Their Precursors.

1.4.2 Omega-3 Deficiency and Neurological Health

The benefits of ω-3 PUFAs for human health are widely known, especially those arising from EPA and DHA. Today, we comprehend that DHA plays an essential role in the proper growth and development of the CNS, and in the prevention of coronary artery disease, diabetes, cancer and other inflammatory and autoimmune disorders (Simopoulos, 2008). The health-promoting effects of EPA and DHA excel in several vital organs, particularly those with cells that have electrical properties such as the neurons and cardiomyocytes. ω-3 are essential for maintaining the fluidity of membranes, which influences ion channels and the signal transduction processes of excitable cells (Farooqui, 2009). This effect is particularly significant in the formation and function of the nervous and visual system of mammals.

A unique aspect of the lipid composition of all mammalian neurological tissues is the extraordinarily high concentration of DHA and AA. Although DHA is found throughout the body, it accumulates almost exclusively in neural tissue. DHA is the most abundant in the CNS where it comprises the phospholipid of the grey matter as a component of the glycerophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) which are abundant in synapses and gametes(Mitchell et al., 1998). PUFA's are essential constituents of the myelin sheath, its synthesis and the myelin compound per se. Injury or rupture of myelin, which is fundamental in neuronal connections, can lead to the disintegration of many functions of the NS. The integrity of myelin is essential for the proper, faster, and accurate communication between neurons.

The brain is the most complex and sophisticated organ in the human body. It presents unique properties regarding its composition, organization, function and physical structure. From a biochemical standpoint, the lipid content of the brain demonstrates distinctive characteristics compared to other organs. The brain is the organ with the higher amount of lipids after the adipose tissue where these compounds represent approximately the 50-60% of its dry weight (Tobin, 1992). Furthermore, around 35% of the dry weight is in the form of PUFAs, mainly AA and DHA. A large percentage of brain lipids are classified as PUFAs from the ω-3 and ω-6 families. Within the ω-3 series, DHA and EPA appear to have the most significant impact on brain function. DHA accounts for 30%–40% of all lipids in the gray matter of the brain. DHA is an abundant constituent in the cortical gray matter volumes, more specifically, ∼15% of its total is heavily concentrated in the human prefrontal cortex (PFC) (McNamara, 2010). Meanwhile in the photoreceptors of the retina the DHA represents around a third of the PUFAs found (Makrides, Anderson, & Gibson, 2013). Astrocytes and oligodendrocytes have a much lower proportion of both DHA and AA compared to neurons (Moore et al., 1991).

The fluidity of the neuronal membranes is linked to the function of DHA in brain tissue as it facilitates the formation of axonal growth cones, the establishment of synapses and the interaction of dendrites, thus improving the plasticity of the cerebral tissue (Jumpsen et al.,1997). DHA also participates in neurogenesis, in the migration of neurons, in myelination and in synaptogenesis, which is characteristic of the development of the NS (Makrides, et al., 2010). In this way, the greater fluidity of the neuronal membranes is linked to the function of DHA in

brain tissue facilitating the formation of axonal growth cones, the establishment of synapses and the interaction of dendrites, thus improving the plasticity of the cerebral tissue (Jumpsen et al., 1997). DHA also participates in neurogenesis, in the migration of neurons, in myelination and in synaptogenesis, which is characteristic of the development of the NS (Makrides et al., 2010). Therefore, continued storage and dietary supply are needed to maintain sufficient levels for optimal brain levels and functionality.

DHA is considered critical for synaptic membranes and normal neuronal function (Farooqui, 2009). For example, it has been shown that both DHA and EPA affect the activities of membrane-bound enzymes, ion channels, and gene expression, which can in turn influence signal transduction and neuronal transmission (Kitajka et al., 2002). Furthermore, these modulations of cell membrane affect brain function and hence behavior. It appears that the level of DHA is crucial for normal cognitive functions, and any deviation from its recommended physiological levels is associated with cognitive impairment (Farooqui, 2009). Different studies have shown that omega–3 deficient rats exhibited more working memory errors and poorer memory retention performances in a variety of tests, such as the radial arm maze (Lim et al., 2008). Regarding the activity of neurotransmitters, PUFAs may influence several pathways of different neurotransmitters such as serotonin, endocannabinoids, noradrenaline, glutamate, dopamine, and acetylcholine. Moreover, that may explain the reported effects on learning and memory, mood stability, and other important cognitive functions (Belzung et al., 1998b; Delion et al.,1996; McNamara et al., , 2017b;

Takeuchi et al., 2002; Yoshida et al., 1997b; Zamberletti et al., 2017; Zimmer et al., 2002).

1.4.3 Omega-3 Deficiency: The Dopaminergic System and Other Neurotransmitters

As previously mentioned, the evidence suggests that insufficient ingestion of PUFAs might result in structural changes at cellular and molecular levels. These changes can modulate the physicochemical properties of neurons such as membrane fluidity, permeability, viscosity and neuronal synapses (Jumpsen et al., 1997). These adaptations can alter neurotransmitters in the synapse or neurotransmission itself. Several studies have investigated the effects on the neurotransmission using animal models during gestational, perinatal and postnatal n-3 deficiency. Some of these neurotransmitters have received more attention from the scientific community, for example, dopamine, serotonin, and glutamate.

Manipulation of brain DHA content can produce a number of effects on the brain dopaminergic system. Substantial decreases in brain DHA content using multigenerational deficiency model resulted in higher dopamine receptor expression in both the NAcc and the frontal cortex, a decreased vesicular monoamine amine transporter (VMAT2) density, and decreased tyramine-stimulated dopamine release (Zimmer et al., 2000b; Zimmer et al., 2002; Zimmer et al., 2000a; Chalon et al.,, 2001; Delion et al., 1994; Chalon et al., 1998a). Additionally, deficiencies can significantly reduce dopamine concentration in the

frontal cortex, their metabolites as well as the concentration of the dopamine D2 receptors (Zimmer et al., 2002; Chalon et al., 2001). Finally, it has been seen an increase in both, dopamine concentrations and in D2 receptors density in the NAcc in rats exposed to omega-3 deficient diet (Zimmer et al., 2000b; Zimmer et al., 2002). However, these studies suggest that the nutritional status may conditionally impact this system to the timing and magnitude of the status of DHA.

Changes in the serotonergic system have also been proved to depend on the omega-3 intake (Hibbeln et al., 2006; Delion et al., 1994; Delion, Chalon, Guilloteau, Besnard, & Durand, 1996) proposed that a decreased membrane fluidity leads to a lower receptor binding of serotonin, showing that there is a relationship between DHA deficiency and low serotonergic receptors and serotonin reuptake systems. Olsson and colleagues (1998) reported that a diet low in omega-3 FA decreased serotonin and 5-HIAA concentrations in several brain regions, including the cortex. Similarly, a deficiency in the diet can reduce the release of serotonin stimulated by fenfluramine in the hippocampus of rats (Aïd et al., 2003). In contrast, in a study using piglets, it was discovered that a diet deficient in omega-3 fatty acids for 18 days after birth decreased serotonin levels in the frontal cortex with no change in binding affinity, and without variation in serotonin levels (Owens & Innis, 1999). Moreover, 5-HT2 a higher receptor density is found in the frontal cortex of adult rats that were raised without omega-3 for two generations (Delion et al., 1996).

Emerging evidence suggests that DHA is required for hippocampal neuronal development and synaptic function of glutamatergic synapses (Cao et al., 2009). In the study performed by Yoshida and colleagues (1997a), it was found that glutamate synaptic vesicles of the hippocampus CA1 region were decreased in the deficient groups. Furthermore, DHA deficits are also associated with impaired astrocyte transport, activity, and maturation (Joardar et al., 2006; Champeil-Potokar et al., 2006; Champeil-Potokar et al., 2004). Dysregulation of glutamate homeostasis and deficits in the omega-3 fatty acid during development can promote anxiety, impair memory and exploratory patterns, and promote other behavioral performance in adulthood (Moreira et al., 2010).

1.4.4 Omega-3 Deficiency and Psychiatric Disorders: Anxiety and Depression

Low levels of omega-3 FA are reported in individuals suffering from different psychiatric disorders (Peet & Stokes, 2005; Freeman et al., 2006). Due to this trend, recent studies have questioned the relationship between a decreased intake of omega-3 FA and signs on individuals with mental disorders. Various chronic psychiatric diseases report an imbalance in terms of omega 6/omega 3 ratio (Simopoulos, 2008). Evidence suggests that the omega-3 deficits of conventional diets may be an influential variable in the pathophysiology of schizophrenia, attention-deficit hyperactivity disorder (ADHD), and neurodegenerative illness such as Alzheimer's (Buydens-Branchey & Branchey, 2006; Liu et al., 2013; Su et

al., 2015). Clinical and animal research findings have supported that the omega-3 FA depletion can impair cognitive and behavioral performances leading to impulsive violence, personality disorders, and substance abuse. Moreover, it may also represent a modifiable risk factor for disrupting moods and emotions (Lauritzen et al., 2016).

It is well established that DHA plays an imperative role in neurological development (Buydens-Branchey & Branchey, 2006; Liu et al., 2013; Su et al., 2015; Ross, 2009; Fedorova & Salem, 2006). Consequently, there are periods that depend on the consumption requirement of omega-3. The intake of a diet deficient in DHA during gestation, infancy, and adolescence increases the risk for depressive and anxiety-like behavior during adulthood and suggest the existence of molecular mechanisms that are involved (Buydens-Branchey & Branchey, 2006; Liu et al., 2013; Su et al., 2015; Ross, 2009; Fedorova & Salem, 2006). The detrimental effects of the deficits of omega-3 in promoting depression and anxiety are supported in pre-clinical studies of humans, animals, and cellular models. Considering these findings, it is suggested that early deficiency in omega n-3 can create a sub-optimal development contributing to impaired emotional and cognitive responses (Tesei et al., 2016; Weiser, Wynalda, Salem, & Butt, 2015; Zamberletti et al., 2017).

Selective dietary deprivation of the omega-3 over pre- or post-weaning has shown to induce depressive and anxiety-like behavior using the FST and EPM test, changing also locomotor activity in rodents; however, trials have provided mixed results. In a one-generational study done by Nakashima and colleagues (1993b),

a diet that was deficient in ALA, that is the precursor of DHA, did not significantly affect the baseline locomotor activity between n3 fatty acid adequate and deficient mice in the EPM test. Nonetheless, the expected increased locomotion induced by scopolamine injection was diminished in the deficient group. The effects of dietary fatty acids on the anxiety level in the EPM showed that time spent as well as the frequency of entry into the open arms tended to be higher in the deficient group. In the same line, Francès, (1995a) reported a higher percentage of entries into open arms in the male deficient group compared to the female groups (that had no significant difference with the control group). Furthermore, they did not find any difference in the time spent in the black compartment using the light-dark transition test. These results did not coincide with those reported by Carrié et al., (2000a), who found that that there were no differences in the percentage of entries into the open arms between deficient and control groups. However, in n-3 FA deficient mice, the time spent on open arms was significantly lower. Supplementation using a rich n-3 diet was capable of restoring behavior leading to a substantial reduction of the level of anxiety in the deficient group. The differences in the results between Francès, (1995a) and Carrié et al. (2000a) may be due to differences between the diet of the mice and environmental conditions. Nevertheless, in a more recent investigation, they found that a deprived DHA diet group showed significantly higher anxiety-like behavior compared to groups of animals consuming the 0.1% and 1.0% DHA (Jašarević et al., , 2014).

Many epidemiological studies support the bond that exists between dietary fish oil consumption and the prevalence of depression (Tesei et al., 2016; Chhetry et al., 2016; Weiser, Wynalda, Salem, & Butt, 2015; Morgese et al., 2016). In fact, omega-3 content in red blood cell (RBC) phospholipids is negatively correlated with several mental disorders, were depression and anxiety disorders has been highlighted. Investigations have shown a decreased in the omega-3 content on RBC of depressed patients (Carney et al., 2016); Even the severity of depression appears to correlate to total concentrations of omega-3 FA. Similarly, a low level of omega-3 in RBC in depressed patients was identified as a risk factor for suicide attempt Mingming et al. (2004). In the clinical study performed by Thesing et al. (2018) they used cross-sectional data of patients to examine the comorbidity of PUFAs with depressive and anxiety disorders. They concluded that patients with depressive episode combined with severe anxiety have lower circulating omega-3 compared to healthy controls (Thesing et al., 2018).

Deficits in omega-3 PUFA levels and mood disorders have also been reported in other groups, including mothers with postpartum depression, individuals with social anxiety disorder and elderly subjects. Raygada and colleagues (1998) reported that high n-6 PUFA diet during pregnancy alters behavior in the offspring by increasing both, the activity in the open field test the aggressive behavior, and diminishing the that the animals spend immobile in the FST. Similarly, Ferraz and colleagues (2011) indicated that the supplementation of omega n-3 counteracted the anxiogenic effects of stress as evidenced by FST, indicating that immobility and swimming were deeply influenced by PUFAs.

1.4.5 Omega-3 Deficiency: development & brain health

Based on three decades of research, we now recognize the susceptibility of the brain to an environmental stimulus such as nutrition, there are external factors, like nutrition, that profoundly influence the early brain development and in inadequate conditions confer a risk to the developing of the nervous system (NS) (Bourre, 2006; Alvarez, Gomez, Fonzo, Sanchez, & Giménez, 2017). In recent years, behavioral science and nutritional research have provided overwhelming evidence about the harmful effects of low PUFAs consumption on mental health (Peet & Stokes, 2005; Freeman et al., 2006; Bourre, 2005; Lakhan & Vieira, 2008a). Additionally, it has been shown that these effects depend greatly on the developmental stage. It is thought that omega-3 modulates the elasticity (Feller & Gawrisch, 2005) and fluidity of membranes regulating the activity of various membrane proteins including enzymes (Bourre et al., 1989) and ion channels (Vreugdenhil et al., 1996). In addition, researchers have precisely assessed the outcome of an omega-3 deficient diet on a variety of neurotransmitters and receptors including dopaminergic, serotonergic, glutamatergic, noradrenergic, among others (Chalon, 2006; Jones, Arai, & Rapoport, 1997; Kodas, Vancassel, Lejeune, & Guilloteau…, 2002; Zimmer, Delion-Vancassel, & Durand…, 2000). Moreover, EPA and DHA are important metabolites derived from omega-3 who give rise to anti-inflammatory eicosanoids, which means that they help to diminish and prevent inflammation (Saini et al. 2018).

Thus, during brain maturation, adequate quantities of EPA and, particularly DHA, are fundamental to the structure and function of the brain (Takeuchi,

Fukumoto, & Harada, 2002; McNamara & Carlson, 2006; Kodas, Vancassel, Lejeune, Guilloteau, & Chalon, 2002; Chen & Su, 2013; Larqué, Gil-Sánchez, Prieto-Sánchez, & Koletzko, 2012). Several studies demonstrate that the level and quality of dietary omega-3 influence the macro- and microstructure of the CNS as well as several neurotransmitters. There is also evidence linking nutritional deficits with several cognitive impairments and neurological disorders (Kidd, 2007a). An important issue is to identify the exact time in which an omega-3 FA deficiency affects the different developmental time windows, and this will allow health professionals to know the exact time in which these fatty acids can influence neural growth (Serfaty & de Velasco, 2014).

1.4.6 Omega-3 Deficiency: Pregnancy and Childhood

The requirement of PUFAs, especially DHA, has been established to be essential for the healthy development of the CNS in both humans and animals. It has been possible to explore the role of DHA in the maturation of visual and cerebral cortex thanks to the use of animal models with deficient n-3 FA diets. Experiments using both mammals (rodents, pigs, and monkeys) and fishes have shown that deficient diets during pregnancy markedly reduces the incorporation of DHA in brain cell membranes of newborns; and simultaneously increases n-6 PUFAs (Francès et al., 2000a; Guemez-Gamboa et al., 2015; Innis, 2008; Neuringer, Connor, Van Petten, & Barstad, 1984). In addition, many studies suggest that an inadequate amount of ω-3 FA during gestation is required for

normal cognitive processes after birth (Greenberg & Bell…, 2008). Thus, n-3 seem to be one of the primary determinants of PUFAs membrane composition, and its absence can trigger adverse physiological and behavioral effects.

Nutritional interventions aimed at pregnant women are based on a correct supply of ω-3 that ensures the proper fetal brain growth. ω-3 FA accumulates in the brain during the last trimester of gestation (Martinez, 1992). A minimum intake is required for adequate fetal neurodevelopment; 650 mg daily of EPA plus DHA, of which at least 300 mg per day must correspond from DHA (Greenberg & Bell…, 2008). The primary source of ω-3 FA depends on maternal supply, both through the placenta and during lactation after birth. Provision of these FA increases during these periods, as these are the stages of nerve cells proliferation, survival, and differentiation. Suboptimal fetal levels of DHA, either because the mother consumes low levels of ω-3 or consumes it in a disproportionate ω-6/ω-3 ratio, affects neuronal growth, synaptic pruning, and expression of genes that regulate cell differentiation(McNamara & Carlson, 2006). The buildup of cortical DHA and AA coincides with intense periods of neurogenesis, neural migration, neural arborization axonal myelination, and synaptogenesis (McNamara & Carlson, 2006). Furthermore, both maternal and fetal omega-3 FA status is negatively correlated with mental capacity after birth (Weiser, Butt, & Mohajeri, 2016).

Our knowledge of the impact that the omega-3 FA have upon cognitive function has been substantially extended by the investigation in animals, mainly rodents, deprived of any source of omega-3 FA during the perinatal development over many generations. Methods for inducing dietary DHA deficiency typically

involve removing all omega-3 FA, including the precursor ALA, from the diet throughout gestation and lactation thus leading to lower levels of DHA in the progeny. Rats of the second and third generation have significantly lower levels of DHA in the brain (Hauser, Stollberg, Reissmann, Kaunzinger, & Lange, 2018). At the anatomical level, researchers have reported that omega-3 deprived animals have a lower size in different brain regions that are related to memory, attention, and cognition such as the hippocampus, the parietal lobe, and the PFC respectively (McNamara et al., 2017b; McNamara, 2010).

Several investigations have exposed the important role of omega-3 in the shifting of cognitive states. Different reports have shown that DHA impact cognition; deficient animals having changes in the ratio of omega-6/omega-3 in areas of the brain that have a role in cognition and showed altered behavioral responses (Jackson, 2013). DHA deficiency can also be provoked by having an overabundance of n-6 compared to n-3 FA in the diet, so a proper balance between n-6 and n -3 PUFAs is required. Moreover, a omega-6/omega 3 low ratio diet can help prevent chronic inflammation as the metabolites derived from omega-3 can inhibit inflammatory cytokines while metabolites derived from omega-6 promote it (DiNicolantonio & O'Keefe, 2018).

On the other hand, deficiency of n-3 during prenatal development dramatically increases the probability of diminished cerebellar dysfunction, cognitive impairments, and other neurological disorders (Kuratko, Barrett, Nelson, & Salem, 2013; Jackson, 2013). Rodents subjected to a diet deficient in DHA showed reduced performance on a selection of learning and memory and olfactory

discrimination tasks (Umezawa et al., 1995; Jašarević et al., 2014; Bandeira, Lent, & Herculano-Houzel, 2009). Moreover, they showed anxiety, increased stress, and an altered attention and locomotor response (Song, Li, Leonard, & Horrobin, 2003; Belzung et al., 1998b; Bandeira et al., 2009).

Most of the studies on nutritional deficiencies in children evaluate the deficiencies of omega-3 in the last third of gestation (Greenberg, Bell, & Van Ausdal, 2008; Sable, Dangat, Joshi, & Joshi, 2012). Uptake is also crucial during the first two years of life, making postnatal periods equally decisive for maternal DHA consumption (Kuratko et al., 2013; Martinez, 1992; Neuringer et al., 1984; Imhoff-Kunsch, Briggs, Goldenberg, & Ramakrishnan, 2012; Cetin & Koletzko, 2008). After birth, brain growth depends on the kind of infant's nourishment. In the first two years, the brain develops rapidly, reaching 77% of the weight it will have as an adult (Benton, 2010). Observational studies demonstrate that maternal consumption of seafood, during pregnancy and breastfeeding, is correlated with infant neural health outcomes (Braarud et al., 2018). Studies have revealed breastfeeding is linked with higher scores assessments of neurodevelopment and cognition, indicating the influence of breast milk on neuronal growth (Belfort et al., 2016).

Breast milk DHA composition is remarkably responsive to dietary modification (Gibson & Makrides, 2000). Respectively, women are advised to ingest ample quantities in their diet during lactation to satisfy the demands of the child. Data addressing the question of whether dietary DHA is needed for optimal development during childhood have been generated by studies in human,

primates, and rodents (Kidd, 2007b). Investigations have compared the cognitive and behavioral performance with either unsupplemented or DHA-supplemented formulas. Evidence shows that infants consuming formulas without omega-3 had lower brain DHA than infants ingesting their mother's milk (Miller et al., 2010). The functional domains most consistently affected include the functions of attention span and planning. Low rates of seafood were associated with lower scores on verbal IQ tests and with suboptimal outcomes on tests of social development and fine motor skills (Tetens, 2009). Studies in rodents consistently reported that offspring of ALA-restricted mothers performed less well than controls in psychomotor tasks, maze learning tasks (McCann & Ames, 2005). Furthermore, delayed alternation processes have also been linked to DHA-deficient diets (Becker & Kyle, 2001; Greiner, Moriguchi, Hutton, Slotnick, & Salem Jr, 1999; Song et al., 2003; Lim et al., 2005). Thus, these investigations indicate that the deficiency of DHA in the infancy may have an effect on the brain causing cognitive decline and may be related to major psychiatric disorders.

1.4.7 Omega-3 Deficiency in Post-natal Development: Anxiety and Depression

Recently there is an increasing interest in the discovery of the harmful effects to the health of n-3 PUFAs deficiency, especially in the brain (McNamara, Schurdak, Asch, & Lindquist, 2017; Manduca et al., 2017). The prevailing idea is that there is a critical window in different aspects of the development of the brain

that n-3 PUFAs can modify. Nutritional deficiencies in this critical stage can permanently damage or cause long-lasting brain changes in adulthood. These periods comprise, for example, pregnancy, infancy, childhood, and adolescence. Even though a child's neurons and synaptic connections far exceed those of an adult, structural reforms remain throughout adolescence paralleling the brain's functional transitions which are exhibited in maturing of behavior.

Adolescence marks a time of unique neurocognitive development. Research studies have demonstrated that gray and white matter undertake vital transformation (Casey, Jones, & Hare, 2008). This stage is characterized by neuronal apoptosis, synaptic plasticity, and pruning essentially for neural network remodeling (Willing & Juraska, 2015). A reduction in gray matter and an increase in white matter is apparent between late adolescence and young adulthood (Arain et al., 2013; Giedd, 2004). As identified in structural MRI studies, there is a decrease in the cortical volume, thickness, and, to a lesser extent, the surface area of the cerebral cortex across this period (Tamnes et al., 2017). It has been concluded that the brain extends its maturation process until the late 20s (Johnson, Blum, & Giedd, 2009). These neural changes are associated with higher efficiency of neural conductivity, primarily in the frontal and subcortical brain regions, which are one of the final brain areas that complete development (Giedd, 2004). Moreover, neurobiological transitions allow cognitive and behavioral control from the frontal cortex. It has been suggested that these transformations, may leave the undeveloped brain defenseless against external factors such as n-3 PUFAs deficiency.

Despite the evidence shown, there are only a few investigations that have examined the role of n-3 deficiencies in adolescence and analyzed their repercussions in the adulthood. In a recent study conducted by Manduca and colleagues (2017), they developed a mouse model of n-3 PUFAs deficiency starting at early adolescence through adulthood, they demonstrated that this deficiency decreased n-3 PUFAs in both medial prefrontal cortex (mPFC) and NAcc leading to increased anxiety-like behaviors and decreased cognitive function in adulthood. In a recent study, also using a postnatal diet, it was determined that deficits in adolescent rats diminished rat forebrain white matter integrity (McNamara, Schurdak, Asch, Peters, & Lindquist, 2018). The same authors discovered that DHA levels in the PFC were inversely correlated with glutamate levels and positively correlated with astrocyte glutamate transporter (GLAST) expression (McNamara et al., 2017b). In another study, it was found that an increased proportion of n-6/n-3 PUFAs ratio produce enhanced inflammatory reaction creating spatial memory impairment when compared to the n-3 PUFAs balanced control (Delpech et al., 2015a). These findings suggest that a lower cortical DHA status is a major environmental risk factor during adolescent development, thus contributing to cognitive, behavioral, and emotional dysregulation.

Studies have reported that regulatory areas of cognition, such as the frontal cortex and the hippocampus, end up their development all the way into adulthood (Murty, Calabro, & Luna, 2016). Investigations have recognized that during are still emerging (Colver & Longwell, 2013). The prefrontal cortex is responsible for key

executive and higher-order cognitive abilities including abstract thinking, sustained attention, deductive reasoning, planning and problem-solving (Giedd, 2004; Crews, He, & Hodge, 2007). When missing necessary nutritional elements, alternative patterns of brain organization can emerge. In support of this, McNamara et al. (2010) randomly assigned healthy boys (8–10 years of age) to receive different doses of DHA and reported a positive correlation of DHA in RBC membranes and dorsolateral prefrontal cortex activation. Similarly, studies of deficits in dietary n-3 PUFAs during adolescence have shown a decreased DHA in both mPFC and NAcc (Manduca et al., 2017). Overall, low n-3 PUFAs acquisition during adolescence may predispose vulnerable individuals to a higher disordered network in crucial brain areas, diminishing their intellectual capability and enhancing their risk of neurological diseases (Åberg et al., 2009; McNamara et al., 2018; Robertson et al., 2017a).

Numerous studies using animal and human subjects have investigated the effects of dietary n-3 PUFAs deficiency throughout different stages of life. However, only a limited amount of studies have recognized the consequences of nutritional n-3 PUFAs deficiency during adolescence on mood-related behaviors (Manduca et al., 2017; Bondi et al., 2014; McNamara & Carlson, 2016). In one study using ALA-free diet during peri-adolescence, authors demonstrated that n-3 fatty acid deficiency is associated with abnormal elevations in climbing behavior in the FST. This response was linked to increases in presynaptic serotonin receptor (5-HT1A) and a decrease in postsynaptic serotonin (5-HT1A) mRNA expression (Able et al., 2014).

In contrast, Ferraz and colleagues (2011) demonstrated that n-3 PUFAs supplementation at the beginning of life prevented the occurrence of anxiety or depressive behaviors. A similar trend can be seen regarding the relationship between the n-3 fatty acids and the emotional status in rodents. In the study done by DeMar et al. (2006), male rat pups, weaned off, 21 days old were subjected to two treatments: a deficient or adequate n-3 PUFAs diet for 15 weeks. Rats of the deficient group demonstrated significantly elevated aggression and increased immobility time in the FST compared to the control group. Similarly, Weiser and colleagues (2015) reported that the animals of the omega adequate diet group spent significantly less time immobile and more time climbing compared to the deficient groups. Interestingly, and in accordance with the information reported above, Borsonelo, and others (2007) described a significant higher immobility time in rats supplemented with n-6 fatty acid.

Since depression and anxiety are behaviors that often occur together anxiety-like behaviors are also measured in these studies. Furthermore, chronic anxiety can also heighten the risk of suffering from depression (Kessler et al., 2008). The study published by Mizunoya and colleagues (2013) provided a diet composed of lard fat, and it was found to heighten anxiety-like behavior in mice. Therefore, adolescent nutritional n-3 PUFAs deficiency is a major environmental risk factor associated with neuropsychiatric disorders, including anxiety and depression. ω-3 deficiencies during adolescence may interfere with normal late development and increase the vulnerability to behavioral deficits during adulthood (McNamara et al., 2018; Messamore, Almeida, Jandacek, & McNamara, 2017;

Manduca et al., 2017; Bondi et al., 2014; Crews et al., 2007). They showed that n-3 PUFAs deficiency starting in adolescence is enough to exacerbate anxiety, anhedonia, and concluded that deprivation of n-3 alters emotional and cognitive-emotional behaviors later in life.

1.5 Omega-3 Deficiency & Addictions

Work has been done to investigate whether omega-3s deficiency has any direct or indirect effect on substance abuse disorders. In terms of behavior, deficiency has been associated with impulsivity states and compulsive behaviors in addicts (Buydens-Branchey, 2003; Buydens-Branchey et al., 2003b; Buydens-Branchey & Branchey, 2006a; Buydens-Branchey, Branchey, & Hibbeln, 2008b; Buydens-Branchey, et al., 2009). It is also well known that inappropriate omega-3 intake can worsen pre-existing addictive disorders and make reinstatement more likely (Rabinovitz, 2014; Borsonelo & Galduróz, 2008; Buydens-Branchey, Branchey, McMakin, & Hibbeln, 2003). Thus, a diet low in PUFAs could conceivably predispose some individuals to drug use and eventually addiction. Furthermore, that predisposition could be due to a modification in the composition of neural membranes of the brain.

In addition, omega-3 deficiency has also been associated with a reduction in the ability to cope with stress (Morgese et al., 2016), and it has been shown that an impairment in coping with stress is linked to the consumer behavior and the craving for drugs. A variety of mechanisms have been proposed to explain this behavior and the n-3 deficiency effects within the dopaminergic system have been

validated, however, the consequences of the changes concerning the progression from drug abuse to the onset of drug-depen dence have received relatively no attention this far (Schneider et al., 2017; Borsonelo & Galduroz, 2008).

There are several indications that omega-3 may interact with different kind of addictive drugs (Rabinovitz, 2014; Borsonelo & Galduróz, 2008; Buydens-Branchey, Branchey, McMakin, & Hibbeln, 2003). Previous studies have evaluated their effect regarding the consumption of nicotine in tobacco. Reports have shown that the omega-3 molecule is highly susceptible to oxidative stress and peroxidation caused by cigarette smoke (Rabinovitz, 2014; Zaparoli et al., 2015). PUFAs levels have been found to be different in the plasma of smokers suggesting influences to the processes related to metabolism, bioavailability, and inflammatory responses of the body, indicating a reduced the brain proportion of PUFAs (Simon et al., 1996; Abdolsamadi et al.,, 2011; Pasupathi et al.,, 2009; Pawlosky et al., 2007). In a study that aimed to investigate if the omega-3 PUFA supplementation reduces tobacco craving, the researchers found that omega-3 deficiency makes it harder for the smoker's body to deal with its craving compared to placebo treatment (Rabinovitz, 2014).

Similar results have been found in studies exploring the effects of alcohol on the intake, absorption, and metabolism of omega-3 (Fogaça, Santos-Galduróz, Eserian, & Galduróz, 2011), thus pointing to a deficiency state of omega-3 during periods of alcohol abuse(Simon et al., 1996). Epidemiological studies have shown that binge-drinking individuals tend to have lower omega-3 intakes compared to nondrinkers. Chronic alcoholics have their neuron membranes depleted of DHA by

an inhibition of the delta-5-desaturase and delta-6-desaturase enzymes, which are responsible for the synthesis and bioconversion of PUFAs (Reitz, 1984; Nervi, Peluffo, Brenner, & Leikin, 1980). Additionally, it has been found that DHA confers protection against the neurodegeneration caused by oxidative stress reducing ethanol-induced neuroinflammation, and cell death (Barbadoro, Annino, & Ponzio…, 2013; Brown, Achille, Neafsey, & Collins, 2009). In animal studies, n-3 PUFA EPA can alter the normal response behaviors and tolerance to alcohol of *Caenorhabditis elegans*, and further proposing PUFAs as a probable environmental modulator of the addictive behaviors (Raabe et al., 2014).

In contrast to previous works described, there are few studies on the effect of omega 3 on opioids. In the study done by Hakimian et al. (2017), supplemental n-3 PUFAs reduced glutamatergic plasticity and the proportion of Grin2BNMDARs caused by chronic morphine administration. Moreover, n-3 PUFA supplementation reversed the effect of the innervation of D2 neurons by the dorsomedial prefrontal cortex. They proposed that dietary n-3 PUFAs may reestablish the neuroadaptation induced by morphine. Furthermore, in the investigation of Escudero et al. (2015), supplementation of omega-3 attenuated the development of tolerance to morphine. Finally, the authors suggested that omega-3 might decrease the side-effects produced by morphine and enhance the analgesic effect using sub-therapeutic doses.

Studies related to stimulants have been developed in recent years. In the case of amphetamines (AMP), McNamara and others (2008a) demonstrated that in mice with omega-3 deficient diet there was an increase of AMP-induced locomotor

activity and sensitization. The authors suggested that the increase of AA:DHA ratio is responsible for sensitization and it is linked to greater amphetamine-induced mesolimbic dopamine activity, therefore, suggesting that deficiency leads to an abnormal dopamine neurotransmission. Moreover, they described a negative association between enhanced locomotor responses and decreased DHA content in the NAcc

In other stimulants such as cocaine, previous research has linked a high ratio of omega-6:omega-3 to relapse in cocaine addicts (Buydens-Branchey, 2003; Buydens-Branchey et al., 2003b), which may arise from enhanced anxiety accompanied with poor PUFAs intake (Buydens-Branchey & Branchey, 2006). The same author provided additional supporting evidence about the possible link between n-3 deficiency and aggression in cocaine addicts (Buydens-Branchey & Branchey, 2008).

Manipulation of brain DHA content can produce many effects on the brain dopaminergic system. Numerous molecular studies in young rodents have indicated that ω-3 PUFAs deficiency results in hypofunctioning of dopamine mesocorticolimbic pathways. Substantial decreases in brain DHA content using a multigenerational deficiency model resulted in dopaminergic repercussions consisting of altered dopamine receptor expression in the NAcc and the frontal cortex. Besides, it is reported that it causes a decrease on the vesicular monoamine amine transporter (VMAT2) density, the tyramine-stimulated dopamine release, and the vesicle pool in rat's frontal cortex (Zimmer et al., 2000b; Zimmer et al., 2002; Zimmer et al., 2000a; Chalon et al., 2001; Delion et al., 1994;

Chalon et al., 1998a). Moreover, DHA deficiencies can reduce dopamine concentration, their metabolites, and the number of dopamine D2 receptors in the frontal cortex (Zimmer et al., 2002; Chalon et al., 2001). Finally, increased dopamine concentrations and an increase in D2 receptors density are seen in NuAcc in rats exposed to omega-3 deficient diet (Zimmer et al., 2000b; Zimmer et al., 2002). Nevertheless, as it has been mentioned before, these studies suggest that nutritional status may impact this system depending on the stage of development and the DHA status.

This modulation of brain dopamine levels by ω-3 may affect dopamine-influenced behaviors related to motivation and reward, and perhaps potentiate the development of addictive disorders. Francès et al. (1996) demonstrated that morphine in mice induced an earlier and greater locomotor activity in the deficient ω-3 diet group. Moreover, McNamara et al. (2008a) reported that ω-3 deficiency in mice promoted the development of AMPH-induced behavioral sensitization. Similarly, Nakashima et al. (1993a) found that mice having an n-3 deficiency showed a greater sensitivity to pentobarbital. All these investigations manifest that ω-3 deficiency may play a role in drug abuse through their effect on the dopaminergic systems. Consequently, early development may represent a critical period where environmental factors can modulate brain development and predispose adolescents to initiate drug abuse or they may increase the risk of acquiring an addictive disorder in adulthood.

In conclusion, ω-3 deficiencies during adolescence may interfere with normal late development and increase the vulnerability to behavioral deficits during

adulthood. It has been suggested that these progressive neural transformations during this stage, may leave the undeveloped brain more vulnerable to external factors such as n-3 PUFAs deficiencies. Therefore, low omega-3 PUFAs availability during adolescence may predispose susceptible adolescents to a more disordered network in key brain regions increasing their risk of neurological diseases (Fu et al. 2013) especially in prefrontal cortex (mPFC) and NAcc (Manduca et al., 2017). Moreover, research studies have also mentioned the effects of PUFAs supplementation in the treatment of cocaine abuse, providing withdrawal relief such as decreased anxiety, anger, cravings, and depression. In addition, PUFAs can induced changes in neurotransmission, especially dopamine and serotonin, an indication of a direct impact on addictive-related behaviors, drug's addictive potential and/or withdrawal symptoms severity (Bondi et al., 2014). Moreover, these alterations may potentiate the development of drug dependency and craving responses to cocaine-cues. Consequently, it is relevant whether ω-3 deficient diets during postnatal development may promote a higher vulnerability later in life.

CHAPTER II

Omega-3 deprivation enhance anxiety-like behaviors after abstinence from cocaine seeking behavior: possible synergistic effects of cocaine and nutrition.

Abstract

Adolescence is a transition process that is vital for brain maturation due to many neurological modifications that take place. Insults during this period can permanently damage or alter the brain's regular structure and function in adulthood. Deficiencies of polyunsaturated fatty acids (PUFAs), especially omega-3 (ω-3), are proposed to play a critical role during these sensitive periods, predisposing individuals to behavioral and cognitive deficits. Numerous molecular studies in young rodents have indicated that sustained deficits of ω-3 results in the dysregulation of the dopaminergic neurotransmission. This modulation of neurotransmitters by ω-3 may affect drug-addictive behaviors related to motivation and reward and thus perhaps promote the development of dependence.

The main objective of the present study was to assess whether nutritional ω-3 deprivation from pre-puberty to adulthood would lead to changes in cocaine addictive behaviors. In addition, we evaluated the impact of ω-3 deprivation from pre-puberty in anxiolytic responses elicited during cocaine withdrawal. Male Sprague-Dawley rats at post-natal age 21 days (P21) were randomly assigned to one of two diet groups: standard rodent lab chow (n = 8) or low ω-3 rodent lab chow (n = 11). The groups began both diets upon their arrival to the animal house and continued it until the end of the experiment. Lever-pressing activity under a fixed-ratio schedule was recorded for statistical analysis. Following the self-administration experiment, anxiogenic-like behaviors were evaluated using the elevated plus maze test (EPM). Finally, we performed western blot analysis of dopaminergic receptors 1/2 (DR 1/2) within the PFC.

Our results show that prolonged dietary deprivation of ω-3 did not exert any significant effect in lever-pressing activity through the acquisition and extinction phases of the cocaine seeking behavior paradigm. However, DEF group demonstrated a substantial decrease in lever pressing during cue-induced reinstatement. Moreover, n-3 PUFA-deficient animals showed enhanced anxiety-like behaviors after 14 days of cocaine abstinence. Furthermore, DR1 protein levels were increased within the PFC. In summary, we can speculate that DR1 in the PFC is probably involved with the enhancement of motivational salience attribution, which plays a crucial role in substance use disorders (SUDs). This research also suggests that the down regulation of DR1 protein within this cortical region could be lessening cocaine-seeking behaviors. Thus, further studies are necessary to clarify this point to reach a more definitive conclusion.

Introduction

Nutrient deficiencies are often the result of the improper functioning of the human body (Jenney et al., 2016; McNamara et al., 2009; Imhoff-Kunsch, Briggs, Goldenberg, & Ramakrishnan, 2012). In fact, the lack of specific nutrients is associated with numerous peripheral and central neurological disorders (Alvarez et al., 2017). Proper nutrition is thought to be instrumental for obtaining persistent healthy homeostasis and a protective action against potential brain pathologies during development (Black & Ackerman, 2008). In the same way, diet can be a preventive measure. For instance, sufficient amounts of precise dietary factors such as ω-3, may contribute to the delay of onset and/or treatment of mental disorders(Peet & Stokes, 2005).

Polyunsaturated fatty acids (PUFAs) are one of the principal components of mammalian tissues (Edwards & Koob, 2010). They are abundant in the plasma membranes of central nervous system (CNS) cells such as neurons, astrocytes, and oligodendrocytes, as well as in myelin. PUFAs add to the stability of neural membranes and maintaining fluidity and flexibility (Bourre, 2004). They have also been shown to influence cellular processes, such as neuroinflammation, neurotransmission, neurogenesis, modulation of the immune system and production of lipid derivatives that include short-chain metabolites with neuroprotective properties (Calon & Cole, 2007). One PUFAs important for the proper CNS function is omega-3 FA, found in fish, algae and supplements (Schuchardt et al., 2011). ω-3 has been shown to regulate a diversity of neuronal

processes in both animal and humans studies and deficiencies can translate into changes in behavior, emotions, and learning abilities (Bhatia et al., 2011a).

Today, experts from many health-related fields recommend the consumption of ω-3. Evidence has demonstrated that alterations in the ω-3 profile of daily diet could affect proper neural growth and function (Swanson et al., 2012). Studies have suggested that high ω-3 consumption especially alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are associated with the reduction of mood and anxiety disorders (Su, Matsuoka, & Pae, 2015). In contrast, mounting evidence supports the detrimental effects of a decreased intake (Su et al., 2015). However, steady results have not necessarily been obtained. Differences in the stage of development, animal species, nutrients used in the diet, time of consumption, among others, are considered the grounds for inconsistent results (Kraguljac et al., 2009). The western diet is currently classified as deficient in ω-3, with a disproportion between omega-6 and omega-3. Assuring an adequate supply of omega fatty acids should be a priority for all.

Deficiencies in ω-3 FA consumption, especially EPA and DHA, can have an influence in all stages of development (i.e. fetal, neonate, infant, and adolescent) and into adulthood (Swanson et al., 2012). A balanced intake of ω-3 FA is needed for normal fetal and infant brain development (Swanson et al., 2012). Adolescence is a significant period of development where the fundamental physiological changes associated with puberty occur alongside psychological, cognitive and social changes (Blakemore & Choudhury, 2006). This transitional period represents the last fundamental stages of brain development, thus creating a

vulnerable stage for exerting a long-term impact on brain health. Research studies have demonstrated that gray and white matter undertake vital developmental changes during adolescence. Synaptic pruning and increased myelination result in a reduced gray matter and increased white matter (Swanson et al., 2012). Longitudinal neuroimaging studies have reported that, through adolescence, neurodevelopment continues, particularly the connection between the frontal lobe and the basal ganglia (Giorgio et al., 2010). Evidence has indicated that deficient consumption of fatty acids PUFA's by nutritionally poor foods during this critical period may have a detrimental functional impact on the still-developing adolescent brain (McNamara et al., 2017a). In addition, studies have reported regulatory areas of cognition, such as the frontal cortex and the hippocampus, develop all the way into adulthood, suggesting a life-long need for proper omega fatty acid consumption (Murty et al., 2016).

One of the neurotransmitter systems that have been recognized as a target of ω-3 FA deficiencies is the dopaminergic system (DA). Existing evidence suggest the engagement of these fatty acids in many neuropsychiatric disorders involving dopamine circuits, such as Parkinson's disease (PD), schizophrenia, attention deficit hyperactivity disorder (ADHD) and substance use disorder (SUD)(Shahidi & Miraliakbari, 2005), as well as a spectrum of anxiety diseases and mood disorders(McNamara, 2016; Shahidi & Miraliakbari, 2005; Kraguljac et al., 2009).These deficiencies have been implicated as affecting several aspects of DA neurotransmission, leading to hypofunctioning of the mesocorticolimbic system. Moreover, these results have generated suspicion as to how these deficiencies

could affect the dopaminergic system on different neurodevelopmental stages, thus impacting future cognitive processes (Schneider et al., 2017).

Manipulation of brain DHA content during development can produce a number of effects on the brain dopaminergic system can lead to substance abuse. Studies have implicated short-term omega-3 deprivation in affecting cocaine-induced behaviors by reducing D2R expression in the frontal cortex and striatum (Zimmer et al., 2000; Zimmer et al., 2002). Moreover, ω-3 deficiency augments AMPH-induced behavioral sensitization in mice compared to controls (McNamara et al., 2008b). In humans, clinical studies suggest deficiencies increase relapse vulnerability in addicts (Buydens-Branchey et al., 2003b; Buydens-Branchey et al., 2009; Buydens-Branchey, Branchey, & Hibbeln, 2009). At the moment, it is unknown if the effects of PUFAs are the same across all stages of development and if they are temporary, long-lasting, or permanent.

The primary objective of the present study was to study whether nutritional ω-3 deprivation from pre-puberty to adulthood would lead to addictive behavioral changes during the acquisition, extinction and reinstatement phases of cocaine seeking behavior paradigm. Moreover, we evaluated anxiety-like behaviors after a short period of cocaine abstinence. We speculated that that prolonged dietary deprivation of ω-3 would increase the lever-pressing activity through acquisition, extinction, or reinstatement phases. In addition, enhanced anxiety-like behaviors were measured. For this aim, we compared rats fed with n-3 PUFA deprive vs. n-3 PUFA balanced diet for two months, starting at weaning. Then, animals were trained in cue-induced cocaine seeking paradigm through acquisition, extinction,

or reinstatement phases. Finally, we also evaluated changes of anxiety-related responses after 14 days from the last session of SA.

Materials and Methods

Animals and Diet

Male Sprague-Dawley (SD) rats were weaned at post-natal day-21 (P21) and obtained from the animal house facility of Ponce Health Sciences University (PHSU; Ponce, PR). At the time of arrival, eleven animals were randomly assigned to the Control (CON) diet (TD.04285) and ten to the Omega-3-Deficient (DEF) diet (TD.04286) (Harlan-TEKLAD). See Supplemental Table I. Some rat's data were not included in the results because it had become ill or data were erratic. Both diets were continued until the end of the experiments. Weight gain, water, and food intake were monitored weekly since arrival and throughout the experimental period. During the first six weeks, rats were housed in clusters of six animals per cage in a temperature and humidity-controlled facility, on a 12 h light/dark cycle (lights on at 6 AM), with chow and water ad libitum; subsequently, they were housed individually. Differences in body weight were monitored weekly, before and throughout the experimental protocol. The nutrient levels and FA profile were provided by Harlan-TEKLAD in Supplemental Table I. Moreover, previous studies conducted by McNamara and others (2009), corroborated the FA content using Gas Chromatography (GC). Housing conditions and care of animals were approved by the Guide for the Care and Use of Laboratory Animals (National Council, 1996). All procedures were conducted according to the National Institutes of Health Guide (NIHG) and our Institutional Animal Care and Use Committee (IACUC).

To evaluate the possible effects of the diets on body weight throughout the study, rats were weighed weekly during the first 8 weeks. In the last week (week 8th), food and water consumption were measured daily as average per group, calculating the difference between the amount provided the previous day and the amount that remained in each animal cage the next day. Both CON and DEF diets were matched for all non-fat nutrients. The nutrient levels and FA profile were provided by Harlan-TEKLAD in Supplemental Figure I. Moreover, previous studies conducted by McNamara and colleagues (2009), corroborated the FA content using Gas Chromatography (GC). Animal were cared as recommended by the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All procedures were conducted according to the National Institutes of Health Guide (NIHG) and our Institutional Animal Care and Use Committee (IACUC).

Drugs

Cocaine hydrochloride (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline solution at a concentration of 0.25 mg/0.1. Pentobarbital (i.p. 50 mg/kg Sigma, St. Louis, MO) was purchased Sigma-Aldrich, St. Louis MO. Atropine Sulfate was acquired from Phoenix Pharmaceutical, MO, USA.

Catheter surgery

Rats were anesthetized with sodium pentobarbital and were surgically implanted with a chronic indwelling catheter made of silastic tubing (0.03 cm ID x 0.06 cm OD, Dow Corning, Midland, MI) as previously described (Morales-Rivera

et al., 2014). The patency of the catheter was maintained by flushing 0.1mL of sterile heparin/saline (1:25) solution before and after the self-administration training. Animals with diseased appearance, erratic behavior, or compromised catheters were excluded from the experiment.

Self-Administration Protocols (SA)

Cocaine SA sessions took place in standard operant conditioning chambers (30.5 cm wide, 30.5 cm high, and 25.5 cm deep). Each chamber was located in a second insulated-chamber to attenuate outside noise during testing (Coulbourn Instruments, PA, USA). They were equipped with 2 retractable levers, a cue light, and a reward receptacle located between them.

After five 5 days of recovery following surgery, animals were placed in a standard operant chamber for intravenous cocaine self-administration training. We followed our previous cocaine-seeking behavior paradigm (Morales-Rivera et al., 2014), which is composed of three phases: acquisition, extinction and reinstatement. During acquisition, animals were trained to lever press (LP) in a fixed schedule (FR) of FR1, FR5, and FR10. An FR1 means that the active lever should be pressed once in order to deliver the infusion of cocaine (0.5 mg/kg per infusion: 0.10 ml/infusion over 3 s). In the case of FR5 and FR10, the lever should be pressed for 5 and 10 times, respectively, to deliver the reinforcer. After each infusion, there were 20 seconds of inactivation (TO: Timeout period), thus decreasing the probability of a cocaine overdose and seizures. Moreover, active LP not only activated the infusion pump but simultaneously delivered a 5-s tone–

light cue (environmental stimuli). All the acquisition stages were conducted for three (3) hours/day. To move from one stage to the other, animals needed less than 10% difference in LP for three (3) consecutive sessions before starting both the next and the extinction phase.

During the extinction phase, learned responses are extinguished. The reinforcer and the tone–light cues are suppressed; thus, the animal learns to disassociate the behavior. One-hour sessions were conducted daily until the extinction criterion was reached (5 LP or less during five consecutive sessions). The reinstatement phase consisted of a 1h session for 2 consecutive days. Animals resumed the extinguished lever-pressing behavior upon re-exposure only to the cues (light and sound) previously paired with cocaine SA. Then, after 2 weeks of cocaine abstinence anxiety-like behaviors were analyzed.

Elevated Plus Maze (EPM)

In all EPM experiments, animals were tested in an apparatus consisting of four black Plexiglass arms with dimensions of 50 cm long × 10 cm wide (Coulburn Instruments). The EPM was composed of two open arms, while the other two were closed, making the shape of a plus sign. The apparatus was localized at the center of the testing room with normal lighting and quiet sound conditions. A digital camera was placed above the maze for recording purposes.

During testing, each animal was placed by the experimenter in the center of the apparatus facing an open arm. Rats were allowed to explore the maze freely for 5 minutes. At the end of each EPM session, the animals were returned to their

home cage. The following EPM behaviors were analyzed: (a) the total time spent in the open arms as a measurement of anxiety and (b) the total time spent in closed arms. The sessions were video recorded for evaluation by two blind experimenters and future references.

Western Blots (WB)

Animals from each group (CON and DEF) were euthanized by decapitation. The brains were removed, washed with a cold saline solution, and placed near dry ice for hardening consistency and precise dissection. For dissecting the PFC, we first removed the olfactory bulb using a sterilized scalpel and then moved the brain to an acrylic brain matrix. The tissues were then homogenized with a Polytron homogenizer in a prepared lysis buffer (20mM Tris base pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 100mM PMSF and 100uM of protease inhibitor cocktail). The homogenates were centrifuged in a refrigerated ultracentrifuge at 4°C for 20 minutes at a velocity of 12,000 RPM. Supernatants (cytosolic fraction) were removed and stored at -80 for future manipulation. Protein concentration was determined using the Bradford Assay Method.

We performed Western blot analysis for Dopamine receptors 1/2 using 10% Acrylamide/Bis SDS-PAGE gels. Equivalent amounts of total protein from the cytosolic fraction of each sample were loaded (10ug). A protein molecular weight standard (Precision Plus Protein All Blue, BioRad) was included in one gel lane as a reference. We heated the samples to 95 °C for 5 min and centrifuged it. Electrophoresis were run at a constant 100 Volts until the protein dye reaches the

bottom of the gel. Samples were transferred to a nitrocellulose membrane using the BioRad Mini Protean System at 4°C for 1 hour at 120V. Ponceau S staining was used to evaluate protein loading and transfer. The intensity of each band was then normalized to amounts of proteins in the respective Ponceau S stained lane. Non-specific binding was blocked using Tris-buffered saline／0.1% Tween 20 (TBS-T) with 5% non-fat milk at 4°C in a shaker for 1 h. Subsequently, the blots were washed with only TBS-T three times for 5 minutes. Later, the membrane was incubated overnight with polyclonal specific antibodies against DR1 (anti-D1, Millipore, 1:1000) or DR2 (anti-D2, Millipore, 1:2500) in whole-cell tissue extracts. The next day, blots were washed three times each for 10 minutes with TBS-T. After that, they were incubated with the secondary antibody (anti-Rabbit, 1:5000) (Li-cor Bioscience, #926-32213) for 1h at 4°C and washed three times with TBS-T (10 minutes each) and incubated with the secondary antibody horseradish peroxidase (HRP) for 60 min at 4°C in a shaker. We washed the membranes with TBS-T, chemo-labeled and visualized with the SuperSignal West Femto Maximum Sensitivity Kit (Thermo Scientific, USA). The blots were stripped and reprobed several times using a mild stripping buffer (Abcam, USA) to visualize other proteins or for optimizing detection of proteins.

Statistical Analyses

Analysis of the behavioral data was done using Two-factor analysis of variance (ANOVA). We used a student's t-test for results that compared only two groups. Post-hoc tests were conducted using Tukey when necessary. Western blot

and EPM data were analyzed using two-tailed Student's t-tests. Data was presented as mean ± SEM. A P-value of < 0.05 was considered to be significant. All statistics were performed using GraphPad software (7.0 v, GraphPad Prism, San Diego, CA, USA).

Results

Body weight, food and water intake were not affected between groups.

There were no significant differences in weight between groups at the beginning of the study (Figure 2.1). Following weaning, initial body weights of the 21-day rats averaged 55 g ± 0.024 g. Changes in body weight did not differ significantly between CON and DEF groups at any time point during the eight-week period that was measured ($P > 0.05$). In the DEF group, weekly body weight did not differ from the CON group ($P > 0.05$; Fig. 2.1A). Also, no differences were detected in food $F (1, 19) = 1.570$; ($P > 0.05$; Fig. 2.1B-C) or water intake $F (1, 19) = 1.362$; $P > 0.05$; Fig. 1; D-E). Collectively, these data indicate that the DEF diet, per se, did not affect body weight or water or food consumption.

Lever Press activity increased during acquisition and remained steady during extinction phases of cocaine SA.

Animals from each group acquired and maintained cocaine SA, and their response rates built up as the FR schedule parameter increased across days in a similar manner. Fig. 2.2 (A-B) depicts the average lever press (LP) during cocaine SA sessions throughout the sequential stages of acquisition and extinction. Nevertheless, LP activity in the last three sessions did not significantly differ between groups (DEF vs. CON) on the different schedules of reinforcements (FR1, FR5, and FR10) (Fig. 2.2A). Before shifting to the extinction phase, all animals required a stable LP on FR10 before starting the extinction protocol. Mean LPs

during extinction are presented in Fig. 2.2B. LP behavior remained steady throughout the extinction phase with no statistical difference between groups F (1, 21) = 0.1186

LP activity during cue-induce reinstatement phase of cocaine SA

We evaluated the effects of an omega-3 deficient diet on the reinstatement of cue-induced cocaine-seeking behaviors. Fig. 2.3 shows that there is a significant decrease in lever pressing during reinstatement in DEF animals ($F (1, 21) = 0.1186$; $p < 0.0002$) as compared to the CON group. Behavioral performance of the animals was consistent on each of the two days of testing for each treatment (Fig. 2.3). one-way ANOVA analysis was used to compare treatments for each session. Data were obtained from the first two days of the reinstatement phase of the cue-induced cocaine-seeking behavior paradigm. Thus, our reinstatement data reveal that the DEF diet significantly reduced the reinstatement of cue-elicited cocaine-seeking behavior in rats $F (3, 26) = 9.555$.

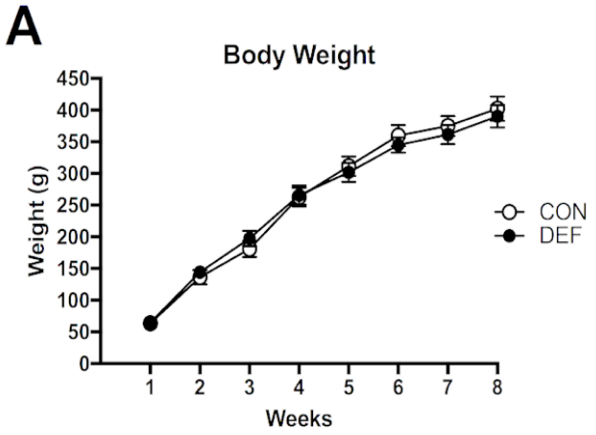
Anxiety-like behavior in the elevated plus-maze test after a short abstinence period

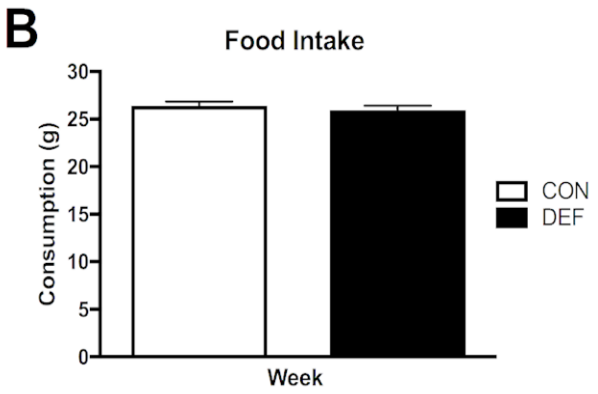
Differences were found between CON and DEF animals during the elevated plus maze test. Examination of the anxiety-like behaviors in the experimental animals revealed a significantly decreased percentage of time spent in the open arms ($F=12.96p < 0.0442$) in the DEF group when compared to the CON groups (Fig. 2.4A). In addition, figure 2.4B shows that DEF animals increased time spent

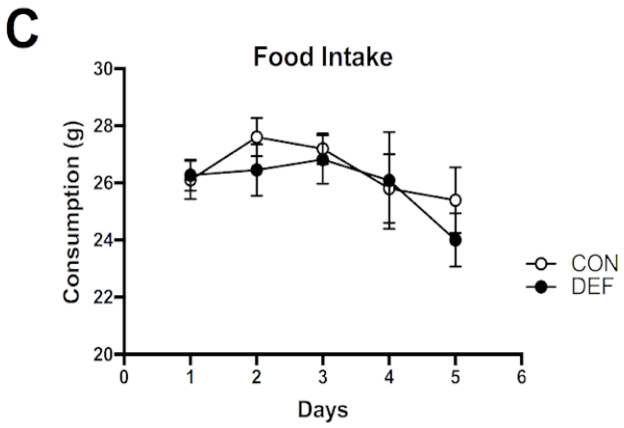
on closed arms when compared to controls F = 41.53p < 0.0090). These results indicate that exposure to the DEF diet elicited a greater anxiogenic-like behavior in rats after withdrawal.

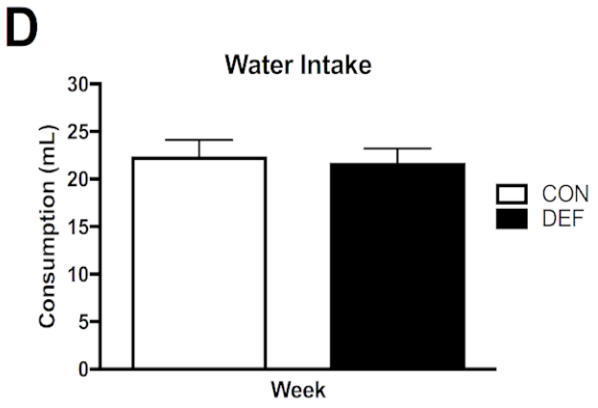
Dopaminergic receptors (D1/2) within the mPFC after a short abstinence period

We analyzed D1/2 receptors protein expression within the mPFC using western blots. The present results showed that the D1R protein levels were significantly increased (DEF vs CON p = 0.0286) in the DEF group when compared to the CON group (Fig. 2.5A), whereas protein levels of DR2 were similar (Fig. 2.5B) in both groups (DEF vs CON p = 0.3429). These results demonstrate that omega-3 deficient animals have a decreased DR1 density within the mPFC.


A
Body Weight
Weight (g)
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
Weeks
CON
DEF


B
Food Intake
Consumption (g)
30
25
20
15
10
5
0
Week
CON
DEF


C
Food Intake
Consumption (g)
30
28
26
24
22
20
0
1
2
3
4
5
6
Days
CON
DEF


D
Water Intake
Consumption (mL)
30
25
20
15
10
5
0
Week
CON
DEF

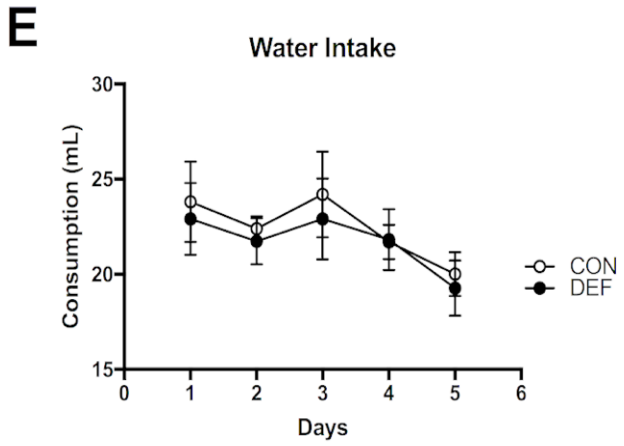
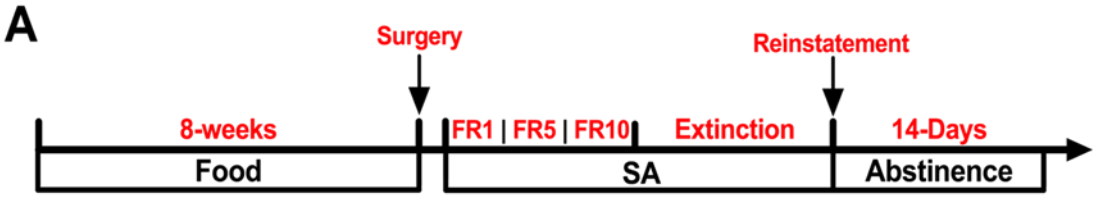
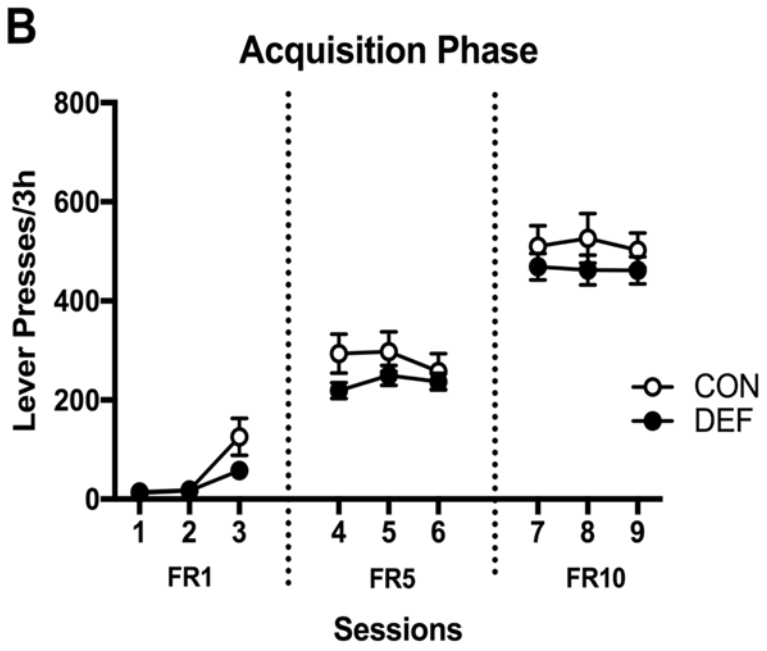

E
Water Intake
Consumption (mL)
30
25
20
15
0
1
2
3
4
5
6
Days
CON
DEF

Figure 2.1. Effects of diet on body weight, food and water intake between groups. (A) No difference ($F (1, 19) = 1.570$; $p > 0.05$) in body weight over fist 8 weeks of diet regimens between DEF and CON groups was found. **(B-E)** There was no change ($F (1, 19) = 1.362$; $p > 0.05$) in food and water intake averaged by day or over a week on their respective diets. Statistical analysis was performed using two-way ANOVA. Values are means ± SEM; Weight (g) of CON (n = 11) and DEF rats (n = 10) across weeks.


A
Surgery
Reinstatement
8-weeks
FR1 | FR5 | FR10
Extinction
14-Days
Food
SA
Abstinence


B
Acquisition Phase
Lever Presses/3h
800
600
400
200
0
1 2 3
4 5 6
7 8 9
FR1
FR5
FR10
Sessions
CON
DEF

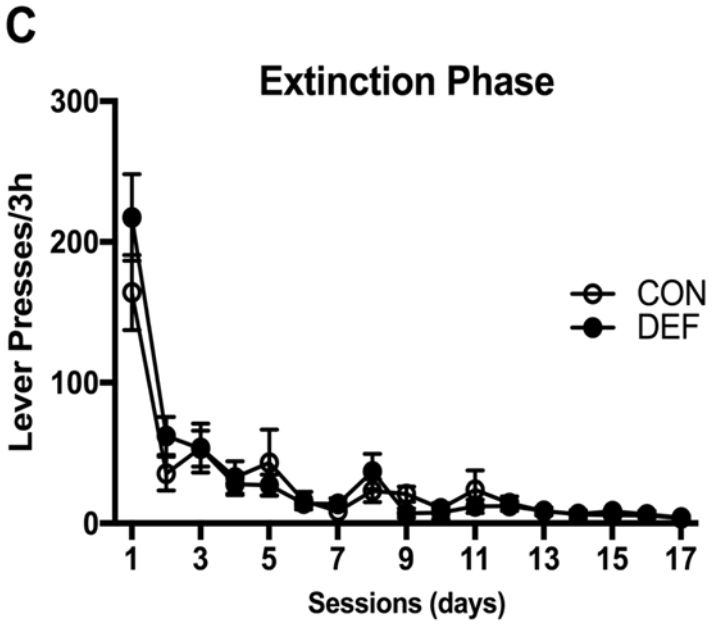

C
Extinction Phase
Lever Presses/3h
300
200
100
0
1 3 5 7 9 11 13 15 17
Sessions (days)
CON
DEF

Figure 2.2. LP Behavior during Acquisition and Extinction of Cocaine Self-Administration. (A) Schematic representation of the experimental procedure and timeline. LP during cocaine SA were recorded throughout acquisition and extinction phase. LP for cocaine during daily 3-hour sessions in CON (white circles) and DEF (dark circles). **(B)** The figure shows the number of LP in the last 3 days of acquisition phase under FR1, FR5, and FR10. No differences ($F(1, 22) = 1.838$; $p < 0.05$) were found in cocaine SA at a dose of 0.5 mg/kg. (**C)** LP rates during daily 60-min extinction sessions. Animals progressively decreased their seeking behavior over the extinction sessions without significant difference ($F(1, 21) = 0.1186$; $p < 0.05$) between groups. Values are means ± SEM; CON ($n = 11$) and DEF rats ($n = 10$) across weeks.

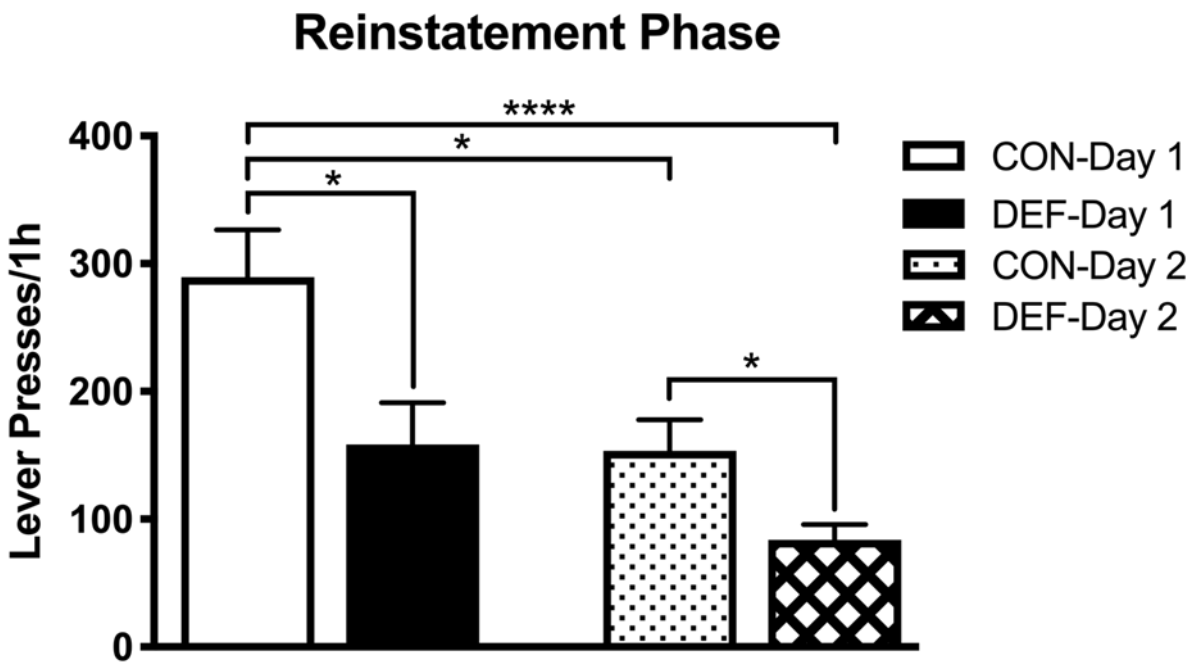
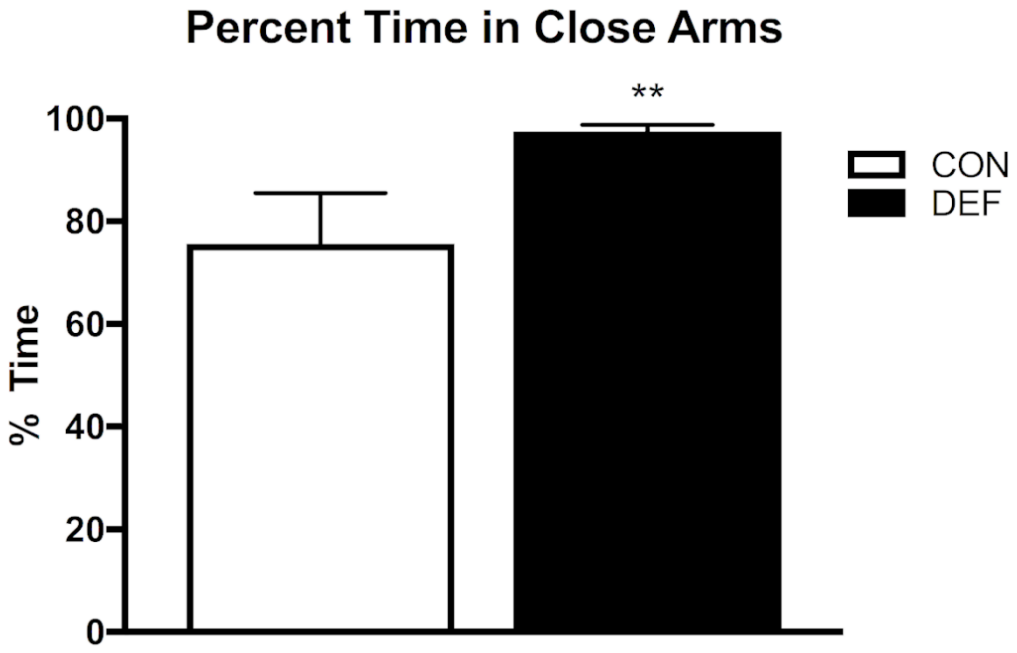

Reinstatement Phase
Lever Presses/1h
400
300
200
100
0
****
*
*
*
CON-Day 1
DEF-Day 1
CON-Day 2
DEF-Day 2

Figure 2.3. DEF diet significantly attenuated lever pressing triggered by cocaine-associated cues. **(A)** LP activity was recorded through the cue-induced reinstatement. Mean LP rates during a 60 -min reinstatement session for two consecutive days, during which responses resulted in presentation of the cue -paired CS. Data show that DEF treatment significantly decreased reinstatement of LP triggered by cocaine-associated cues. Values are means ± SEM; CON (n = 7) and DEF rats (n = 8) across weeks. F (3, 26) = 9.555; *p < 0.05, **** p < 0.0001.


Percent Time in Close Arms
**
100
80
60
40
20
0
% Time
CON
DEF

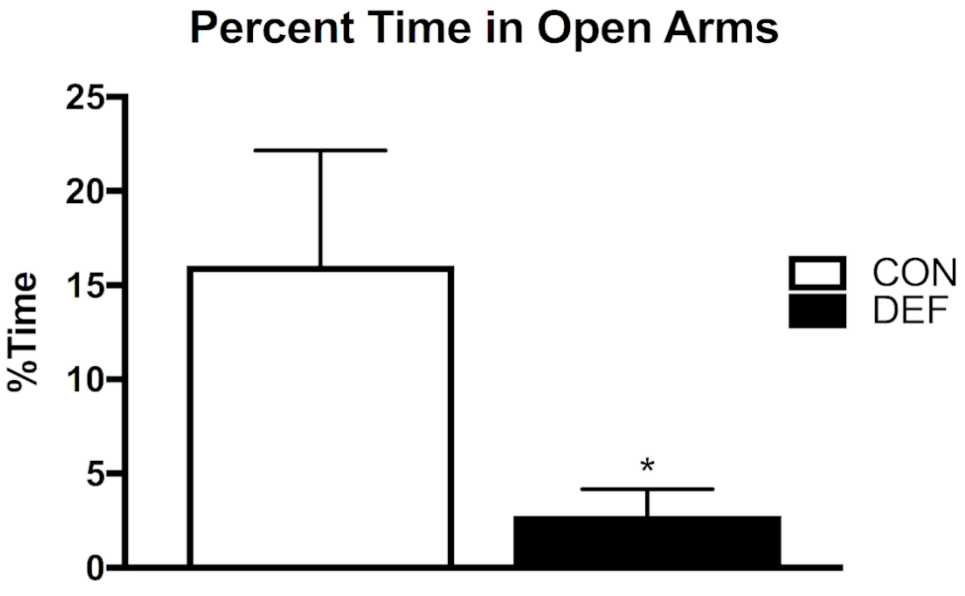

Percent Time in Open Arms
25
20
15
10
5
0
%Time
*
CON
DEF

Figure 2.4. The effect of omega-3 diet deficient on the anxiety-like behavior in the elevated plus-maze test. **(A)** percentage of time spent in the open arms and **(B)** the percentage time spent in the closed arms. Data show that DEF treatment significantly spent more time in the closed arm. Values are means ± SEM; CON (n = 7) and DEF rats (n = 8); ($F=41.53$; $p <0.05$) and less time in the open arm $F=12.96$; $p < 0.05$ compared to CON group. Values are means ± SEM; Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$.

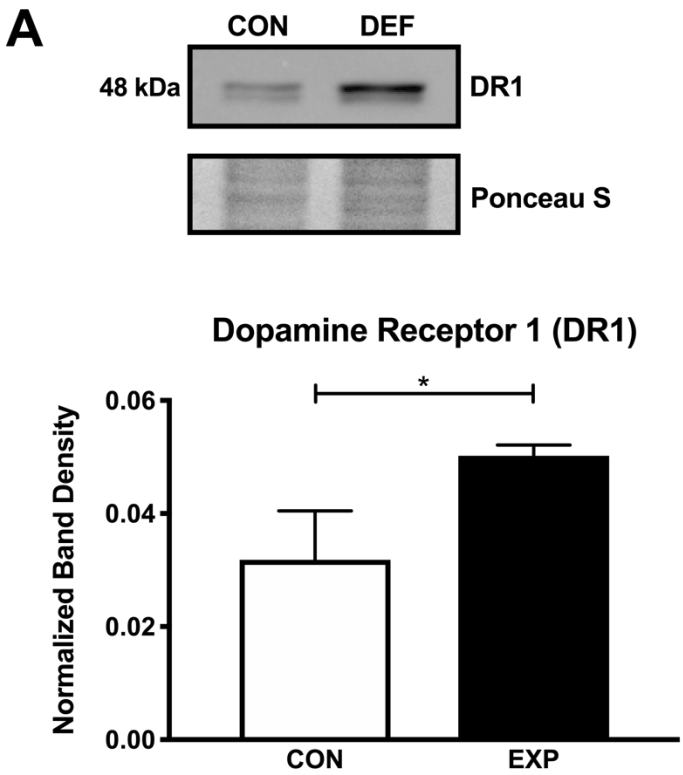

A
CON
DEF
48 kDa
DR1
Ponceau S
Dopamine Receptor 1 (DR1)
*
Normalized Band Density
0.06
0.04
0.02
0.00
CON
EXP
B
CON
DEF
48 kDa
DR2
Ponceau S
Dopamine Receptor 2 (DR2)
Normalized Band Density
0.06
0.04
0.02
0.00
CON
EXP

Figure 2.5. D1R and D2R protein levels within the PFC after 14 days of abstinence. Representative immunoblots **(A)** cumulative data and levels of D1R protein in the PFC **(B)** cumulative data and levels of D2R protein in the PFC. The protein bands were normalized using the total protein from Ponceau staining. Representative graphs for the total levels of D1R and D2R respectively. Values are means ± SEM; CON (n = 4) and DEF rats (n = 4); Mann-Whitney t-test; * $p < 0.05$

Discussion

The primary goal of this experiment was to investigate if the diet given to rats with limited access self-administration would lead to changes in cocaine-seeking and anxiogenic-like behaviors in adulthood after a short period of abstinence using the present adolescent diet deficient model. Moreover, this research adds new evidence of the potential interaction between ω-3 deficiencies and cocaine-related behaviors within the brain reward regions. In addition, this investigation is the first to combine effects of n-3 PUFA dietary deficiency starting in adolescence and cocaine addiction to evaluate if there is an existing association between them.

In addition, evaluation of the involvement of the dopaminergic system in these behaviors was tested. More specifically, the objectives were to evaluate the effect of the diet on the behavior of the animals evaluating the lever-pressing (LP) activity, the elevated plus maze test (EPM), as well as the expression levels of dopamine receptors 1 and 2 (D1 and D2) in the prefrontal cortex (PFC). First, deficiency starting early adolescence exerted a significant effect on LP activity during cue-induced reinstatement. Second, ω-3-deficient animals showed enhanced anxiety-like behaviors after 14 days of cocaine abstinence. Third, ω-3-deficient animals demonstrated decreased DR1 density in the PFC.

LP activity during acquisition, extinction and reinstatement phases of cocaine SA.

Previous experiments in the field suggest that there is a relation between n-3 deficiency and the rewarding effects of cocaine. Since weight can influence the sensitivity of brain reward circuitries (Johnson & Kenny, 2010b), we ruled out this different effect by evaluating weight periodically. Previously our results showed deficient diet did not alter body weight, water or food consumed in DEF rats, compared to CON group. In addition, prolonged dietary deprivation of ω-3 did not exert any significant effect in LP activity through the acquisition and extinction phases. However, during the cue-induced reinstatement, deficient animals showed a reduced LP activity. We can interpret these conducts as unmotivated behavioral patterns towards the search for cocaine. In other studies, applying variations in FA dietary constituents diminished acquisition of cocaine SA and reinforcement efficacy (Wellman et al., 2007). Moreover, studies with high-fat diets attenuated the rewarding aspects of amphetamines using the conditioned place preference (CPP) (Davis et al., 2008) and had increased brain stimulation reward thresholds in rodents (Johnson & Kenny, 2010a).

Our study demonstrated that DEF group had lower cue-induced cocaine-seeking behavior compared to the CON group. This result follows Frances and colleagues (2000) who showed that mice that received a n-3 deficient diet were less responsive to rewarding events than animals fed with a standard diet. They suggested that changes in brain dopamine by n-3 PUFA deficiency could be involved in weakening behavioral response to positive events. This finding is consistent with the induction of anhedonia—diminished interest or pleasure in rewarding activities— and is further supported by attenuation of cocaine seeking;

strengthening the association between anhedonia and cocaine use. Overall, the effects of n-3 deficient on reward function are consistent with human investigations that describe blunted subjective pleasure and motivation to obtain rewards. It has been suggested that dietary-induced perturbations in PUFA homeostasis can deregulate the dopaminergic systems, leading to mood behavioral changes and impaired receptivity to positive events (Messamore, Almeida, Jandacek, & McNamara, 2017; Su et al., 2015; Wani, Bhat, & Ara, 2015; Bondi et al., 2014; Ross, 2009; Kraguljac et al., 2009)

Effects of omega-3 diet deficient on the anxiety-like behavior in the elevated plus-maze test after a short abstinence period.

The EPM results revealed that rats raised on a diet that lacked n-3 starting adolescence and exposed to chronic consumption of cocaine were more anxious than control rats. Moreover, DEF rats spent more time in the closed arms and less time in the open arm compared to controls. These results mirror the previously described anxiogenic properties of n-3 deficient rats (Umezawa et al., 1995; Carrié, et al., 2000c; Takeuchi, et al., 2003b). Altogether, our findings suggest that dietary deficiency of n-3 is closely associated with anxiety-related behaviors.

Numerous animal and clinical studies have investigated the effects of dietary n-3 PUFAs deficiency or supplementation on behavioral function (Schneider et al., 2017; Borsonelo & Galduroz, 2008). A study where a PUFA-deficient diet was dispensed to mice (at the equivalent period of adolescence as our study) found that the n-3 deprived mice spent less time than controls exploring the center of a

Y maze (Caetano, Vaeth, & Canino, 2018; Patten, Sickmann, Dyer, Innis, & Christie, 2013). They also investigated whether heightened anxiety was accompanied by a decreased preference for sucrose, a hallmark of depression-like behavior and a putative sign of anhedonia. These data are consistent with our findings of the enhanced effects of dietary ω-3 deficiency on anxiety and depressive-like behaviors. Also, we developed a new rat model of omega-3 deficiency commencing in adolescence and continuing through adulthood. This contribution will provide a more thorough understanding of the consequences of nutritional imbalance during this period and how they influence mental Illness and behavioral disorders.

Effects of omega-3 diet deficient on dopaminergic receptors (D1/2) short abstinence period within the mPFC.

Studies analyzing the relationship between n-3 PUFAs deficiency in the brain and the dopaminergic system have proposed that an inadequate diet could affect densities of D1 and D2 receptors (Davis et al., 2010). Our immunoblots from the DEF group demonstrated a decreased receptor density in the mPFC. Alternatively, a decrease of D1R receptors in mPFC may inhibit the motivation for cocaine seeking behavior by lessening the reinforcing effects of cocaine. This interpretation, however, is problematic due to the fact that LP was unaltered between groups in cocaine SA sessions. Also, in our previous experiment using the cocaine incubation paradigm, locomotor activity was reduced in the DEF group, which is contrary to other stimulants (AMPH) in n-3 deficient rats where

sensitization was enhanced and movement activity increased (McNamara et al., 2008b).

Previous studies have shown that direct blockade of D2 receptors with the antagonist eticlopride in adolescents rats did not have an effect on CPP (Nazarian, Russo, Festa, Kraish, & Quinones-Jenab, 2004). Nevertheless, in adults blockade of the D1R within the mPFC attenuated the reinstatement of both CPP (Sanchez et al., 2003) and cocaine SA (Alleweireldt et al., 2002). Similarly, Sun and Rebec (2005) used a DR1 antagonist to decrease cocaine-primed reinstatement without affecting operant behavior maintained by food. They suggested that D1R in the PFC is involved in cocaine-primed drug-seeking behaviors. Results from Nakajima & McKenzie (1986) indicated that blockade of DR1 receptors reduced the rewarding effect of brain stimulation within the ventral tegmental area. In addition, Brenhouse and colleagues (2008) showed that peak levels of D1R were critically involved with an enhancement of motivational salience attribution, which plays a role in addiction. Taken together, these findings suggests that the availability of D1R receptors might be involved in emotional responses and decision-making processes. Based on our experiments, we further suggest that the down regulation of DR1 in the PFC might be responsible for the decrease in locomotor activity and cue-induce reinstatement.

Several lines of evidence have drawn attention to the pathophysiology of a wide range of psychiatric diseases associated with deficient n-3 intake (Peet & Stokes, 2005; Freeman et al., 2006). Studies have pointed out that n-3 PUFAs deficiency could modulate the dopaminergic system, which mediates the

reinforcement and reward functions that are fundamentally involved in the progression of substance abuse disorders. Manipulation of brain DHA content can produce many effects on the dopaminergic system. Numerous molecular studies in young rodents have indicated that ω-3 PUFA deficiency results in the hypofunction of dopamine mesocorticolimbic pathways (Zimmer et al., 2000b; Zimmer et al., 2002; Zimmer et al., 2000a; Chalon et al., 2001; Delion et al., 1994; Chalon et al., 1998a). Substantial decreases in brain DHA content using multigenerational deficiency models produced dopaminergic repercussions consisting of altered dopamine receptor expression in the NAcc and the frontal cortex (El Hage et al., 2012; Zimmer et al., 2000; Zimmer et al., 2002). Microdialysis has showed that perinatal deficits can create deficits of extracellular dopamine concentrations as well as their metabolites upon stimulation tyramine in the frontal cortex of adult rats (Kodas et al., 2002; Zimmer et al., 1998).

At the biomolecular level, it has been reported that decreased vesicular monoamine amine transporter (VMAT2) density, increased expression of tyrosine hydroxylase, and vesicle pool in the rat frontal cortex (Zimmer et al., 2000b; Zimmer et al., 2002; Zimmer et al., 2000a; Chalon et al., 2001; Delion et al., 1994; Chalon et al., 1998a). Additionally, alterations in dopaminergic receptors 1 and 2 (DR1 and DR2) expression and changes in binding affinity has also been described (Delion et al., 1996; Zimmer et al., 1998). It is presumed that the increase was generated to compensate for the low levels of dopamine in the synaptic clefts. Kuperstein, Eilam, and Yavin (2008) also described a robust increase in DR1 and DR2 levels in the cortex and striatum. At the neuronal level, Ahmad et al. (2008)

found an association between inadequate n-3 consumption and altered function, cell number and morphology of dopaminergic cells in the substantia nigra and the ventral tegmental area (VTA). Moreover, rats with n-3 deficiency also exhibited dendritic depletion and extensive arborization. They concluded that altered dopamine cells might be accountable for the behavioral variations observed in rats raised on n-3 deficient diets.

Not much is known about the effect of diet manipulation after post-weaning periods to examine changes in the dopaminergic system of specific brain areas. There are only a few studies that have assessed this role and predominantly in adult animals. The investigation performed by Owens and Innis (1999), showed that feeding piglets for 18 days after birth a formula deficient in linolenic acid results in lower frontal cortex concentrations of dopamine, monoamine oxidase products DOPAC and HVA, as well as serotonin and its product, 5-HIAA. The decrease in neurotransmitters and metabolites suggested a possible reduction in synthesis and metabolism. In contrast, adding DHA microemulsions for 60 days in adult rats revealed higher levels of dopamine and the serotonin in the brain compared to controls (Sugasini & Lokesh, 2014). In addition, Vancassel and colleagues (2008) postulated that n-3 supplementation could improve resistance to stress through action on monoaminergic neurotransmission. Although no statistical difference in dopamine and their metabolites levels was found, a trend manifested concerning the concentration of dopamine in animals exposed to stress compared to those that do not.

Adolescence is another post-weaning period in which n-3 PUFA's could have a significant effect on neurocognitive development (Casey, Jones, & Hare, 2008). Through this important developmental period, there is a shift in the balance of DA activity from the mesolimbic to mesocortical regions, especially in the PFC (El Hage et al., 2012; Zimmer et al., 2000; Zimmer et al., 2002). The mPFC plays a critical role in cognitive function that depends on dopamine signaling. These dopaminergic connections undergo refinements during adolescence, characterized by extensive synaptic pruning creating highly specific pathways as adults (D'Ardenne et al., 2012; Crone & Dahl, 2012). Consequently, D1R and D2R, are highly expressed in the mPFC and subcortical brain regions (NAcc and the amygdala) of adolescents compared to adults (Andersen et al., 2000). Thus, making this transition vital to the regulation of behaviors and emotions. Moreover, the maturation of the PFC sets the foundation for higher order functions, including choice behavior and decision-making (Mills et al., 2014). Animal and human neuroimaging studies have confirmed the prolonged development of the PFC as underlying changes in cognitive and impulse control (Casey et al., 2008). Moreover, the PFC is a brain region with a high-density composition of DHA that has been associated with changes in functional activity (McNamara & Carlson, 2006).

Data support the adolescent model where nutrition, in this case, n-3 PUFA's, is a major environmental factor influencing the remaining brain development that then transitions to adulthood. In a study conducted by Manduca and others (2017) specific deficits starting in adolescence decreased n-3 PUFAs in both medial

mPFC and NAcc. Adult n-3-deficient mice had abolished endocannabinoid/mGlu 5 -mediated LTD in these areas. Similarly, McNamara et al. (2017a) found that when post-weaning rats were fed a deficient diet beginning adolescence (21 days) until adulthood, magnetic resonance imaging (phMRI) showed that recruitment of frontostriatal regions by chronic escalating AMPH treatment was reduced. They hypothesized n-3 deficient rats might have an impaired regional alteration in dendritic spine density and synaptic remodeling following chronic AMPH exposure and that low brain DHA levels may compromise these processes. More recently using the same protocol, they showed that lacking DHA starting in adolescence negatively affected forebrain White Matter Microstructural Integrity (WMI) in adult rats. Further studies were suggested to determine if these results could indeed interrelate with other psychiatric disorders (McNamara et al., 2018).

Only a few studies have analyzed the effects of a diet deficient in omega-3 in adolescence and the relationship between the dopaminergic system and addictive behaviors (Kuhn et al., 2013; McNamara et al., 2008). Nevertheless, it should be pointed out that that research in particular analyzed other parameters outside the main focus of our study. For instance, Delpech and colleagues (2015b) studied mice that were given a n-3 deficient PUFAs diet for a 2-month period starting at post-natal day 21. Following an administration of bacterial endotoxin (LPS) in adulthood, they found an enhanced proinflammatory cytokine level in plasma and higher glucocorticoid levels that contributed to spatial memory impairment. The study undertaken by Oshima and colleagues (2018b) compared the effects of adding EPA and DHA to the diets of adolescent rats. Animals were exposed to

stress and fed the experimental diet for 14 days. EPA-enriched food suppressed the anxiety-like behavior in socially isolated rats. Other research demonstrated that consumption of an obesogenic diet (contained a more substantial amount of both omega-6 vs. omega-3) attenuate fear learning and fear extinction in rats (Vega-Torres et al., 2018). Evidence suggests that dietary n-3 deficiency during adolescence affects various behavioral patterns via changes in the FA composition of the brain, although the precise mechanisms remain to be elucidated.

There are major limitations to the present investigation that need to be addressed in the future. First, we did not explore other probable effects of DHA. It is conceivable that distinct mechanisms might also be involved. For example, various neurotransmitters, stress, neurodegeneration, neuroinflammation and active n-3 PUFA derived metabolites. A second limitation is the extent of n-3 deprivation; perhaps, using pre-weaning or transgenerational paradigms could enhance or produce different outcomes. Third, we did not analyze other brain areas, such as the amygdala and hippocampus confirmed to be affected by these FA (Conklin et al., 2007; Sopian et al., 2015). Future experiments should examine different drug categories, duration of diet consumption, different brain areas and developmental stages to reach a more definite conclusion.

Conclusion

In conclusion, our study presents evidence that n-3 PUFAs deficiency beginning at early adolescence diminished cue-induced reinstatement. This suggests that a 2-month exposure to deficiencies in this period is enough to desensitize dramatically the brain to discrete cues associated with cocaine. These findings complement our prior evidence were we highlighted hypoactivity, decrease emotional responses and perhaps alterations in dopaminergic transmission. Moreover, it was also evidenced that dietary n-3 FA negatively impacts anxiety performances in adult rats after 14 days of abstinence. We speculate that the mechanisms underlying these changes could also be directly associated with the modification of the dopaminergic systems, particularly with a decrease in D1R in the PFC based on the molecular experiments. The results of this study not only provide insight into how lipids can influence addictive responses but also suggest a role for n-3 PUFAs in the exacerbation of cocaine withdrawal symptom severity. In summary, these results suggest that DR1 in the mPFC could have an important role in the cocaine-seeking behavior and the sequelae of its chronic usage. Nevertheless, replication of experiments are needed to produce reliable conclusions. Moreover, we cannot exclude the possibility of different outcomes with other addictive drugs and/or behaviors that might also be influenced. Thus, future experiments should expand to alternative drugs and disorders that affect motivated behavior, such as ADHD, depression and anxiety disorders among others.

CHAPTER III

Nutritional omega-3 fatty acid deficiency intensifies anxiety- and depression-like behaviors after withdrawal in rats subjected to incubation of cocaine-craving paradigm

Abstract

In recent years, n-3 polyunsaturated fatty acids (PUFAs)—more commonly known as omega-3 (ω-3)— have gained attention due to their ability to modify the physiology of several neurotransmitter systems; and thus, supporting a potential involvement with cognitive and emotional processes. This modulatory role by ω-3 affects behaviors related to motivation and reward. Also, these PUFA-induced modulatory neurotransmissions may have a direct impact on addictive-related behaviors, drug addiction potential, and/or the severity of withdrawal symptoms.

To address this issue, we evaluated whether nutritional ω-3 deprivation from pre-puberty to adulthood would lead to changes in cocaine addictive behaviors. Moreover, emotional behavioral alterations of early and late withdrawal after chronic cocaine self-administration were investigated. Male Sprague-Dawley rats (P21) were fed with either a standard rodent lab chow (CON) or a deficient ω-3 rodent lab chow (DEF). Animals were trained to self-administer cocaine (6 h/ day with 0.5 mg.kg/ infusion) that was paired with a tone-light cue for 8 days. We observed the incubation of cue reactivity between ID1 (Incubation Day 1) and ID40 after forced abstinence. In addition, anxiety-like and depression-like behaviors were evaluated after 10 weeks of food/only consumption (FO), WD1 (Withdrawal Day 1) and WD35; using the elevated plus maze test (EPM) and the Forced Swimming Test (FST).

Interestingly, dietary ω-3 depletion reduced LP activity during the first two days and diminished movement episodes throughout the self-administration sessions. On ID40, the DEF group had lower cue-induced cocaine-seeking

behavior compared to the CON group. Conversely, there was a significant increase of cocaine seeking behavior in CON group at ID40 vs. ID1 compared to the DEF group, which did not have a substantial behavioral change. In the EPM, starting from WD1, the DEF group showed a gradual reduction in the time spent on the open-arm and an increased time in the closed-arms. During the FST test, the DEF group demonstrated greater immobility time on WD1 compared to the CON group, whereas no difference was observed in FO or WD35.

This study demonstrated that dietary absence of ω-3 could intensify the symptoms of anxiety and depression during withdrawal periods after extended access to self-administered cocaine. Furthermore, it also suggests that deficiency can modify sensitivity to the rewarding effects of cocaine. We can speculate that these dietary-induced perturbations in PUFA homeostasis can deregulate the dopaminergic systems, leading to behavioral mood changes and impaired responsiveness to positive events.

Introduction

In recent years, deficiencies of a range of nutrients in diets have drawn the attention of scientists due to substantial evidence on the profound impact on mental health and development Jenney et al., 2016; McNamara et al., 2009; Imhoff-Kunsch, Briggs, Goldenberg, & Ramakrishnan, 2012. Currently, low-nutritional content in food is perceived as an environmental factor that impacts health, more pronounced in individuals with ongoing neural growth (Gibson & Makrides, 2000; Georgieff et al., 2018). Pre-natal as well as post-natal stages, for example fetal, infant, childhood, and adolescence, are all time windows of enhanced sensitivity for nutrients to impact brain plasticity. These stages are not mere transformations but rather serve for changes in region-specific brain areas required for proper functioning (Arain et al., 2013). Studies have shown that specific nutritional deficiencies can have a range of long-lasting and even permanent-effects on the maturation of neural circuits.(Alvarez et al., 2017). Thus, depending on the developmental stage of a given brain region, specific nutritional deficiencies can negatively influence cognitive, functional, and behavioral outcomes later in life (Georgieff et al., 2018).

One such class of nutrients is polyunsaturated fatty acids (PUFAs), especially the omega-3 (ω-3) fatty acids (FA). In western countries, the intake of ω-3 has steadily decreased since the beginning of the industrial revolution (Simopoulos, 2009). In addition, excessive consumption of ω-6 FA has led to an upsurge in the ω-6/ω-3 ratio. Evolutionary data proposes that the original ratio in the human diet was 1:1; whereas today, it has come to be 20 times more ω-6 acids than ω-3 FA

(Georgieff et al., 2018). This means western culture has established a striking deficiency in ω-3 FA with potentially harmful neurological consequences.(Georgieff et al., 2018). Therefore, it has been speculated that FA is linked with a wide range of mental and emotional conditions (Schachter et al., 2005; Simopoulos, 2009).

Deficiencies in ω-3 have been implicated in neurocognitive deficits, elevated aggression, anxiety, and depression, along with hindered dopamine and serotonin transmission in animals and human models (Wani et al., 2015). One of the mental illness that has been prioritized is depression (Khalid et al., 2016). Studies suggest that deficiencies of ω-3 may contribute to the onset of depression, and dietary supplementation may play a useful role in the management of it. Clinical studies on patients with depression showed significant lower DHA (Docosahexaenoic Acid) and higher ω-6/ω-3, AA(Arachidonic acid)/DHA ratios, compared with non-depressed controls (Kiecolt-Glaser et al., 2007). This correlation has been reported in other sensitive populations, including postpartum depression, elderly patients, and social anxiety disorders (Ross, 2009). Furthermore, behavioral and neurochemistry symptoms of depression were reported during withdrawal from chronic administration of cocaine (Zilkha et al., 2008b). Based on these investigations, the influence of ω-3 has drawn attention within the scientific community.

Extensive research has been dedicated to discerning the potential significance of ω-3 PUFA's -either supplementing or having a deficiency- and disturbances in people's anxiety or mood states (McNamara, 2016; Shahidi & Miraliakbari, 2005; Kraguljac et al., 2009). Evidence supports ω-3 deficiencies

influencing stress-related disorders, particularly on the activity of the HPA axis (Larrieu et al., 2016). In addition, since depression and anxiety are often comorbid, anxiogenic outcomes caused by ω-3 deficiency has also been investigated (Oshima et al., 2018a). In fact, epidemiological analyses found that mood disorders present as a combination of anxiety and depression (Liu et al., 2013). Indeed, studies showed that intracellular ω-3 levels in subjects experiencing anxiety and depression are low and that anxiety can be ease by consuming ω-3 (Su et al., 2015). Another clinical trial reported that the severity of comorbid anxiety was associated with the lowest EPA and DHA levels. Liu and colleagues (2013) suggested that ω-3 PUFA might impact depression with severe cases of anxiety. Moreover, animals studies like the one done by Harauma and Moriguchi (2011), showed that mice with a decrease of 50% in brain DHA content, exhibited greater anxiety after being subjected to stressors. The severity of comorbid anxiety is related to low EPA and DHA levels. These researchers suggested that ω-3 might impact depression with severe cases of anxiety. A similar study showed that a reduction in dietary exposure across two generations increased anxiety-like phenotypes (Bondi et al., 2014). Although research investigating the link between PUFA deficiencies and anxiety in laboratory animals have reported increases anxiety and depression symptoms, inconsistent results have also been reported (Belzung et al., 1998a).

The implementation of an appropriate dietary supply of ω-3 has been recognized to have a preponderant role during fetal and early postnatal life. Studies indicate that the unique transitional period of adolescence may create an

additional window of vulnerability, since developmental changes are still occurring in the brain until the third decade of life (Blakemore & Choudhury, 2006). Recent studies have started to examine this by contrasting adolescence with other developmental stages and determining discrepancies in performance generated by ω-3 PUFA status (Weiser et al., 2016; Bondi et al., 2014; Weiser et al., 2015). Data indicated that low n-3 intake early in life can bring profound psychiatric abnormalities during adolescence related to and fear-induced memory (Joffre, Nadjar, Lebbadi, Calon, & Laye, 2014; Takeuchi, Iwanaga, & Harada, 2003). Furthermore, extended ω-3 deficiency into later life can induce further mental deficits and worsen behavioral outcomes associated with depression and social behavior in adulthood (Robertson et al., 2017b). Therefore, complex anatomical and rearrangement of neural connections that occur in the adolescent brain may not become apparent until adulthood.

Behavioral studies demonstrated that modulating ω-3 levels in adolescent rats may affect cognitive, emotional regulation and motivation (Manduca et al., 2017; Weiser et al., 2015). Deficiencies of ω-3 during adolescence causes specific neurobiological changes to reward systems that impact the development of frontal-striatal and frontal-temporal neurocircuitry. Dietary ω-3 deficiency is thought to influence behavior through DA neurotransmission in both adults and adolescents Buydens-Branchey & Branchey, 2006b; Buydens-Branchey et al., 2008b; Buydens-Branchey et al., 2009; McNamara et al., 2008a; Kuhn et al., 2013; Serafine et al., 2016; Eserian et al., 2012). Animal studies reported that deficits in brain DHA during different stages of development are associated with a significant

loss of dopamine neurons in the ventral tegmental area (VTA) (Ahmad, Park, Radel, & Levant, 2008). Also, deficits in mesolimbic DA function appear to be implicated in the pathogenesis of depression, especially manifestations of anhedonia and diminished motivation (Tremblay et al., 2005; Nestler & Carlezon Jr, 2006). Distinctly, decreased engagement in reward-oriented behaviors is observed in animals with reduced activity of DA projections (Morita et al., 2013). These findings suggest that pre and post-natal DHA are critical for the maturation of mesocorticolimbic DA pathways.

The modulatory role of DA by ω-3 has an effect on DA-influenced behaviors related to motivation and reward, and this, perhaps, potentiates the development of addictive disorders (Buydens-Branchey, 2003; Buydens-Branchey et al., 2003b; Buydens-Branchey & Branchey, 2006a; Buydens-Branchey, Branchey, & Hibbeln, 2008b; Buydens-Branchey, et al., 2009). Several studies related various aspects of drugs of abuse with deficiencies of ω-3 (Francès et al. 1996; McNamara et al. 2008a; Nakashima et al. 1993). For example, rodents given a deficient ω-3 diet group have greater locomotor activity and sensitization for drugs such as morphine, amphetamines and pentobarbital (McNamara et al., 2017a; McNamara et al., 2008a; Francès et al., 2000b; Nakashima et al., 1993a). Other studies assessed ω-3 supplementation on hostility in alcohol, cocaine, and heroin users and found that feelings of anger and anxiety that are strongly correlated with relapse among substance abusers were significantly decreased (Buydens-Branchey & Branchey, 2008; Buydens-Branchey et al., 2008b). The same authors showed that higher cumulative percentages of cocaine addicts who relapsed were

associated with lower PUFA status (Buydens-Branchey, 2003; Buydens-Branchey et al., 2003a). Together these results indicate that ω-3 deficiency may play a role in drug abuse through their effect on the dopaminergic systems. In addition to this, neural development may represent a critical period for ω-3 to modulate brain maturation and predispose individuals to initiate drug use or enhanced risk for acquiring an addictive disorder in adulthood.

Materials and Methods

Animals and Diet

Male Sprague-Dawley (SD) rats were weaned at post-natal day-21 (P21) and obtained from the animal house facility of Ponce Health Sciences University (PHSU; Ponce, PR). At the time of arrival, animals were randomly assigned to either Control (CON) diet (TD.04285) or Omega-3-Deficient (DEF) diet (TD.04286) (Harlan-TEKLAD). See Supplemental Table I. Both diets were continued until the end of the experiments. Weight gain, water, and food intake were monitored weekly since arrival and throughout the experimental period. During the first six weeks, rats were housed in clusters of 6 animals per cage in a temperature and humidity-controlled facility, on a 12 h light/dark cycle (lights on at 6 AM), with chow and water ad libitum; subsequently, they were housed individually. Differences in body weight was monitored weekly, before and throughout the experimental protocol. The nutrient levels and FA profile were provided by Harlan-TEKLAD in Appendix Table I. Moreover, previous studies conducted by McNamara et al. (2009), corroborated the FA content using Gas Chromatography (GC). Housing conditions and care of animals were approved by the Guide for the Care and Use of Laboratory Animals (Council, 1996). All procedures were conducted according to the National Institutes of Health Guide (NIHG) and our Institutional Animal Care and Use Committee (IACUC).

Experimental design

The SA protocol was an adaptation from the incubation of the cocaine-craving paradigm previously described in (Koya et al., 2009). The experiments consisted of four phases: food training, self-administration, forced abstinence period (1 or 40 days), and tests for cue-induced reinstatement.

Food Training

During the food training, rats were placed in operant chambers and received a sucrose pellet (45 mg) when they pressed a paired lever on an FR1 reinforcement schedule. All rats initially were food-restricted before receiving the training and across the duration of the training (4 days on average). Sucrose pellet (banana-flavored) delivery was paired with a cue light/tone cue for 5 s. We excluded chocolate-flavored sucrose pellet due to its nutritional fatty acid content. Animals were allowed to self-administer sucrose for a maximum of 15 pellets until the acquisition criterion was delivered for 3 consecutive test days. Once the operant behavior was acquired, the food was again made available ad libitum. Following food training, the animals went through the process of the intrajugular catheter surgery.

Catheter surgery

Rats were anesthetized using Ketamine/Xylazine mixture (100/6.7 mg/kg, ip; Sigma-Aldrich in St. Louis MO) and were surgically implanted with a chronic indwelling catheter made of silastic tubing (0.03 cm ID x 0.06 cm OD, Dow Corning, Midland, MI) as previously described (Morales-Rivera et al., 2014). Atropine sulfate

was given in small doses for inhibiting salivary and bronchial secretions, and increasing heart rate during surgery (Phoenix Pharmaceutical, MO, USA). After recovery, the patency of catheters was maintained by flushing 0.1mL of sterile heparin/saline (1:25) solution before and after the SA training for preventing the formation of blood clots. Animals with diseased appearance, erratic behavior or compromised catheters were excluded from the experiment. Five days after surgical implantations of i.v. catheters and recovering from surgery, the protocol for cocaine SA began.

Cocaine self-administration (SA)

Cocaine SA sessions took place in standard operant conditioning chambers (30.5 cm wide, 30.5 cm high and 25.5 cm deep). Each chamber was located inside a second ventilated, sound-attenuating box for mitigating outside noise during testing (Coulbourn Instruments, PA, USA). They were equipped with 2 retractable levers, a cue light and a reward receptacle located between them. The levers were extended through the whole session. Rats were subjected to daily, 6 h SA sessions on a fixed ratio of 1 (FR1) schedule for 8 days. Pressing on the active lever resulted in an infusion of cocaine hydrochloride (0.5 mg/kg per infusion; 0.10 ml/infusion over 5 s). Cocaine hydrochloride was dissolved in a sterile 0.9% saline solution at a concentration of 0.50 mg/0.1ml (Sigma-Aldrich, MO, USA). Also, cocaine infusions were paired with 5-s tone–light cue located above the left active lever, followed by a 40s time out period. Pressing on the right inactive LP were also recorded but had no programmed consequences. A timeout (TO) period of 40 s

was used during the entire sessions to decrease chances of cocaine overdoses. The forced-abstinence phase began immediately following the 8th day of the SA phase. That day will be referred to as the first day of forced abstinence (FA-D1).

Following cocaine SA, each group (DEF and CON) was divided into 2 groups. Rats in the Day 1 group underwent a cue-induced drug-seeking test on the first day of forced abstinence from cocaine SA. Rats in the Day 40 group underwent a cue-induced reinstatement test on day 40 of abstinence from cocaine SA. Rats from these groups remained undisturbed in their home cages during the forced abstinence period, aside from their usual bedding and food exchanges.

Cue-induced reinstatement

Following either 1 or 40 days of abstinence (depending on their assigned group) rats were subjected to cue-induced reinstatement. On the reinstatement phase, a 60-minute session with the presence of the cocaine-associated cues was conducted for two consecutive days. Rats returned to the same chamber where they previously self-administered cocaine. Active LP throughout testing resulted in contingent presentations of the tone–light cue previously paired with cocaine infusions. However, no cocaine was available during this session.

Motion Detection

Locomotor activity was monitored using an infrared activity monitor placed on the ceiling of the operant conditioning chamber. The ceiling mount activity monitors (The H24-61) that used infrared technology for precise collection of

locomotor activity data throughout each session. The sensor detects rats while rearing or leaning on the walls and senses movement at any elevation in the cage. Movement units option (brief pulses representing the minimum resolution of detection) was chosen for collection of movement responses (Coulbourn Instruments, Allentown, PA, USA). Data were collected using Graphic State (Graphic State 2.101, Coulbourn Instruments, Allentown, PA, USA).

Elevated Plus Maze (EPM)

In all EPM experiments, animals were tested in an apparatus consisting of four black Plexiglas arms with dimensions of 50 cm long × 10 cm wide (Coulburn Instruments). The maze was elevated 40 cm from the ground. The EPM was localized in the center of the testing room with normal lighting and quiet sound conditions. A digital camera was placed above the maze to record the behavior. One set of arms, opposing one another, were enclosed, while the other set was open, making the shape of a plus sign. During testing, each animal was placed by the experimenter in the center of the apparatus while facing an open arm. Rats were allowed to explore the maze for 5 minutes freely. At the end of each EPM session, the animals were returned to their home cages. Entries to the closed and open arms were manually coded from the videos. The following EPM behaviors were examined: (a) the total time spent in the open arms as a measurement of anxiety, (b) the total time spent in closed arms. The sessions were video recorded and later evaluated by two experimenters blinded to the treatments. Anxiety-like

behaviors were evaluated after 10 weeks of food/only consumption (FO), day 1 of forced abstinence (FA-D1) and FA-D35.

Forced Swimming Test (FST)

In order to verify whether there was a relationship between anxiety and depression, we accomplished the FST test. Subjects were placed in a glass cylinder (90 cm high and 25 cm in diameter) filled with 25°C water to a height of 40 cm. They first passed through a pretest session, which lasted 15-minute period. At the end of this pretest, each rat was removed, partially dried, illuminated with a heat lamp, and returned to the home cage. Twenty-four hours later, the test session consisted of the same previous procedure. At test time, the rat was again placed into the cylinder, and behavior was recorded for only 5 minutes, after which the rat was returned to its home cage. All swim sessions were video recorded for analyzing the animal's depressive state. Two distinct behaviors were scored: immobility and immobility frequency. Behavioral immobility was considered when the animal retains an immobile posture displaying only sufficient movement to keep the head above water and prevent sinking. The principal investigator and two trained raters, blind to the dietary treatment, analyzed the digital records. These experiments were carried out in different sets of rats to avoid inter-assay interference. Depression-like behaviors were evaluated after 10 weeks of food/only consumption (FO), day 1 of forced abstinence (FA-D1) and FA-D35.

Organ weights and Blood Samples

Rats were euthanized and both the brain and heart were immediately removed, cooled, and weighed. Then, those organs were stored at −80 °C for future dissection of targeted the regions. Blood was also collected immediately after decapitation. The blood was separated and stored in tubes modified for various analyses; (i) for serum analysis, blood was collected after centrifugation (4°C, 2500g, 10 minutes); and (ii) for plasma analysis, blood was collected in heparinized Eppendorf-tubes (15 i.u. ml−1). Plasma was immediately frozen in liquid nitrogen and stored at −80°C. Moreover, fecal samples were collected from each group at three different time points, after two months of consuming the diet, after SA protocols FA-D1, and on the last day FA-D40 before finishing the experiments.

Statistical Analysis

For results that compared only two groups, a student's t-test was employed. Analysis of the behavioral data was done using One-factor Analysis of Variance (ANOVA) Repeated Measures. Responding during the cue-induced reinstatement was analyzed by 2-way ANOVA with repeated measures on the lever, or across all 6 h with repeated measures using diet and time points as the main factors. Tukey's multiple comparison tests were used as a post-hoc analysis to identify differences with a significance level of $p < 0.5$ when necessary. In some cases, a one-tailed analysis was considered. For non-Gaussian distribution within the groups, data were transformed. A non-parametric test (Kruskal—Wallis KS with Dunn's correction) for multiple comparisons or Mann—Whitney U-test (MWU) were

used if the transformation was not possible. Data were tested for normality and then analyzed. In addition, data were presented as mean ± SEM. A p-value of < 0.05 was considered significant. All statistics were performed using GraphPad software (7.0 v, GraphPad Prism, San Diego, CA, USA).

Results

N-3 PUFA-Deficient Diet on body weight, cumulative food, and water intake.

Rats fed with a DEF or a CON diet were weighed weekly in order to monitor variations. There were no differences in weights between groups at the beginning of the study. Following weaning, initial body weights of the 21-day rats averaged 55 g ± 0.024 g, with no significant difference in weight gain between groups ($p > 0.05$; Fig. 3.1A). Moreover, we estimated the total food and water intake, and no significant differences were detected ($p > 0.05$; Fig. 3.1 B-D), and diet did not have had any effect on final heart and brain weight $p > 0.05$; Fig. 3.2 A-B.

N-3 PUFA-Deficient Diet on LP triggered by cocaine-associated cues.

Acquisition of cocaine SA was assessed using the FR schedule of reinforcement under a six-hour access procedure. Fig. 3.3 (B-C) illustrates average lever pressing for all animals during cocaine SA sessions of the paradigm. Rats acquired and maintained cocaine self-administration in a similar manner. However, the CON group showed a significant increase in active LP during the first two days of acquisition ($p < 0.0001$). Moreover, DEF revealed a significant decrease in inactive LP during the first day of acquisition ($p < 0.0001$). Both groups exhibited a robust preference for the active over the inactive lever, afterward. After the third day of SA, DEF and CON rats showed a non-significant difference ($p > 0.9999$) in LP activity until the last day (eighth day).

N-3 PUFA-Deficient Diet on locomotor activity during SA sessions.

Locomotor activity declined over the course of the six-hour assessment in DEF, Fig. 3.3 (D). Analysis of locomotor activity revealed a significant effect of diet ($F(7,111) = 1.239$, $p < 0.0001$) but no effect of time ($F(7, 111) = 1.239$; $p = 0.2875$). Also, the interaction between the two factors was not significant ($F(7, 111) = 0.6473$, $p = 0.7159$). Sidak's post-hoc analysis revealed that DEF showed significantly less activity than CON on days 1 ($p < 0.05$) and 5 ($p < 0.01$) activity sessions, but not on the other days, although there is a tendency for decreased locomotor activity in the DEF group.

N-3 PUFA-Deficient Diet on cue-induced reinstatement.

We also evaluated the effects of an omega-3 deficient diet on the reinstatement of cue-induced cocaine-seeking behaviors, Fig. 3.4. DEF animals did not exhibit the same magnitude of incubation of craving effect as compared to the CON group, evidenced by the lack of increase in lever response. Behavioral performance of the animals was consistent on each of the two days of testing. Figure 3.4 shows that there is a significant decrease in LP during reinstatement in DEF animals ($F(3, 25) = 14.03$; $p < 0.0001$) as compared to the CON group. Data were obtained from the first day of the reinstatement phase of the cue-induced cocaine-seeking behavior paradigm. At day 1, there was an expected significant increase in LP in the CON group ($p < 0.05$; CON day 1 vs. CON day 40) that does not appear in the DEF group (p = ns; DEF day 1 vs. DEF day 40) according to Tukey's multiple comparisons test. Also, there is a significant difference when

comparing day 1 ($p < 0.0001$) or day 40 ($p < 0.001$). Thus, our reinstatement data revealed that Omega-3 DEF diet significantly reduced the reinstatement of cue-elicited cocaine-seeking behavior in rats.

N-3 PUFA-Deficient Diet on anxiety-like behavior using the EPM test.

Tukey's post-hoc analyses revealed that DEF rats display significant differences in anxiety-related parameters in the EPM. As shown in Fig. 3.5(A), after 1 day of withdrawal DEF showed a statistically significant decrease in the time spent on the open arm ($p < 0.01$) and an increase in the closed arm ($p < 0.01$) Fig. 3.5(B), and this difference was more evident on day 35 ($p < 0.0001$) Fig. 3.5(A-B). Contrary, CON group showed enhanced anxiety-like behaviors only on day 35 of withdrawal Fig. 3.5(A-B). These results indicated that exposure to DEF diet elicited a greater anxiogenic-like behavior in rats after withdrawal. Analysis of anxiety-like behaviors revealed a significant effect of time ($F\ 2, 42 = 21.74$; $p < 0.01$), but not for diets ($F\ (1, 42) = 0.598$; $p = 0.4437$). Also, the interaction between the two factors was not significant ($F\ (2, 42) = 1.299$, $p = 0.2834$).

N-3 PUFA-Deficient Diet on depressive-like behaviors using the FST.

The behavior of rats during FST was recorded and scored to assess whether deficiency was able to influence emotional behavior after cocaine consumption. In the test session (24 h later), the DEF rats showed significantly higher immobility and higher frequency scores than the CON after chronic intake of cocaine (day 1)

(Fig. 3.6A–B). Analysis of depressive-like behaviors revealed a significant effect of diet ($F(1, 40) = 7.273$, $p < 0.01$) and time ($F(2, 40) = 20.19$; $p < 0.01$). Also, the interaction between the two factors was significant ($F(2, 40) = 5.579$, $p < 0.01$). Tukey's post-hoc analysis revealed that DEF showed significant high depressive-like behaviors on day 1 ($p < 0.01$). Moreover, the CON group exhibited significant depressive behaviors on day 1 ($p < 0.050$), but those symptoms were less intense compared with the DEF group (Fig. 3.6A). Interestingly, on day 35 those behaviors were decreased in both groups; however, the difference was significant in only the DEF group ($p < 0.0001$). Furthermore, immobility frequency increased significantly on day 1 and decreased on day 35 in DEF group, while the same parameter in the CON group was nonsignificant (Fig. 3.6B).

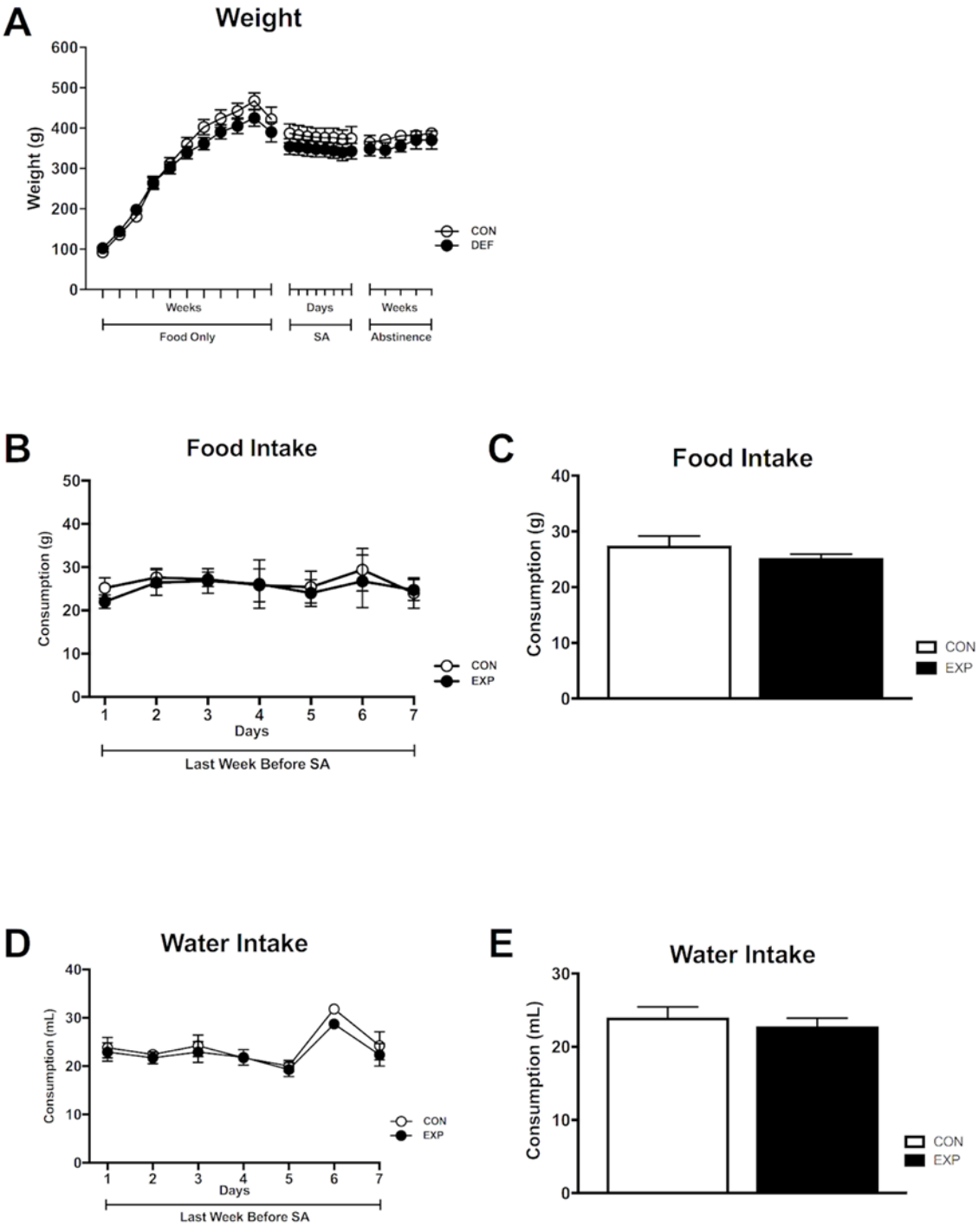

A
Weight
Weight (g)
600
500
400
300
200
100
0
CON
DEF
Weeks
Days
Weeks
Food Only
SA
Abstinence
B
Food Intake
Consumption (g)
50
40
30
20
10
0
1
2
3
4
5
6
7
Days
CON
EXP
Last Week Before SA
C
Food Intake
Consumption (g)
40
30
20
10
0
CON
EXP
D
Water Intake
Consumption (mL)
40
30
20
10
0
1
2
3
4
5
6
7
Days
CON
EXP
Last Week Before SA
E
Water Intake
Consumption (mL)
30
20
10
0
CON
EXP

Fig. 3.1. Effects on brain and heart weight of rat fed with an omega-3 deficient diet.

(**A**) There were no significant changes in body weight in DEF as compared to the CON group (**B-C**). Weight was plotted in the y axis vs. time (Days) in the x axis. Also, no change in food and (**D-E**) water intake averaged over a week on the respective diets were detected ($p > 0.05$); Consumption was plotted in the y axis vs. time (Days) in the x axis. Statistical analysis was performed using multiple comparison unpaired t-test with Holm-Sidak correction. Values were presented as mean ± SEM (n = 8-7). CON, control diet; DEF, deficient diet.

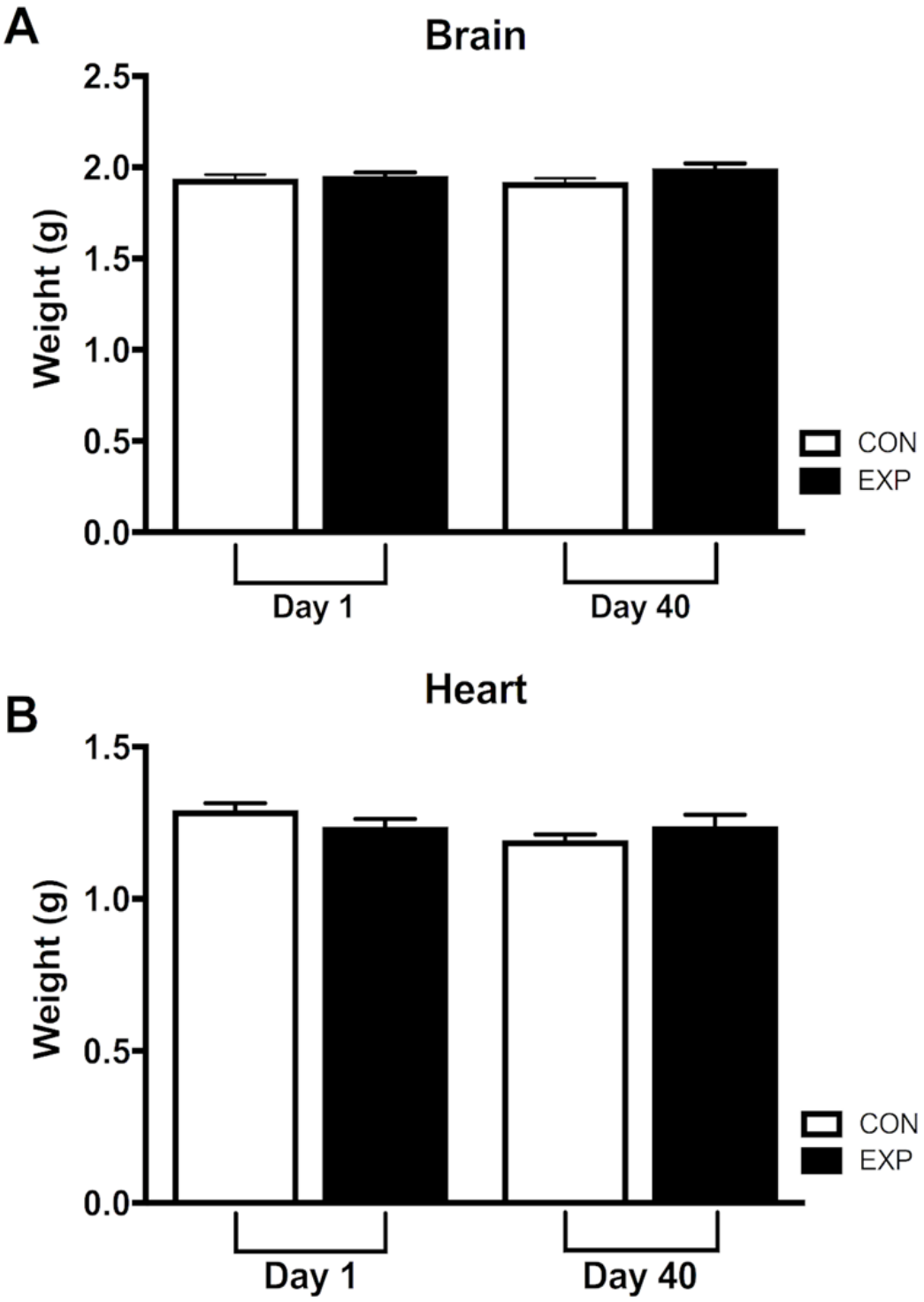

A
Brain
Weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
CON
EXP
Day 1
Day 40
B
Heart
Weight (g)
1.5
1.0
0.5
0.0
CON
EXP
Day 1
Day 40

Fig. 3.2. Effects on cumulative brain and heart weight of rat fed with an omega-3 deficient diet.

There were no significant changes detected in brain ($p = 0.0942$) (**A**) and heart ($p = 0.1201$) (**B**) weight following euthanization. Statistical analysis was performed using a nonparametric multiple comparisons Kruskal—Wallis KS with Dunn's correction. Values were presented as mean ± SEM (n = 8-7). CON, control diet; DEF, deficient diet.

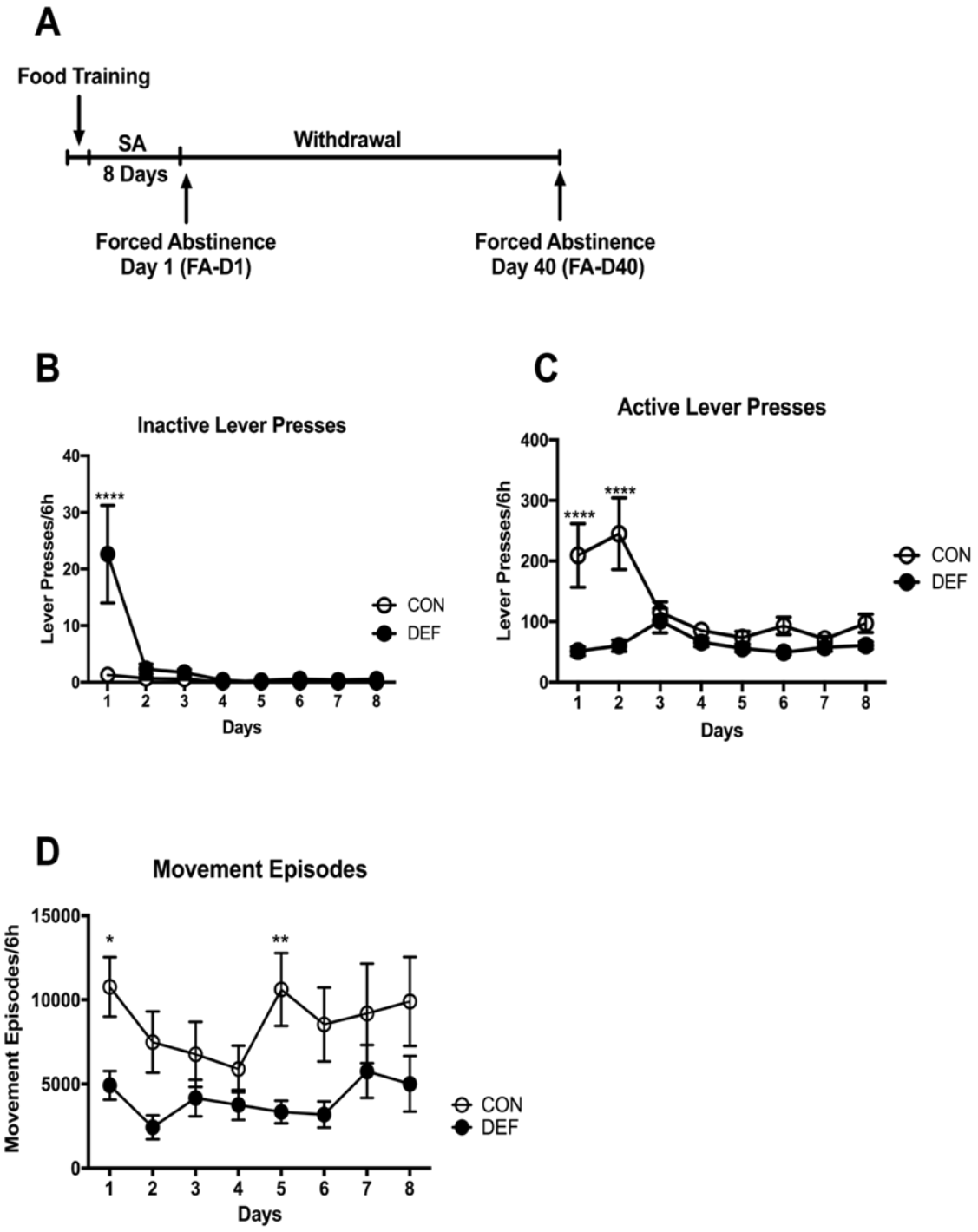
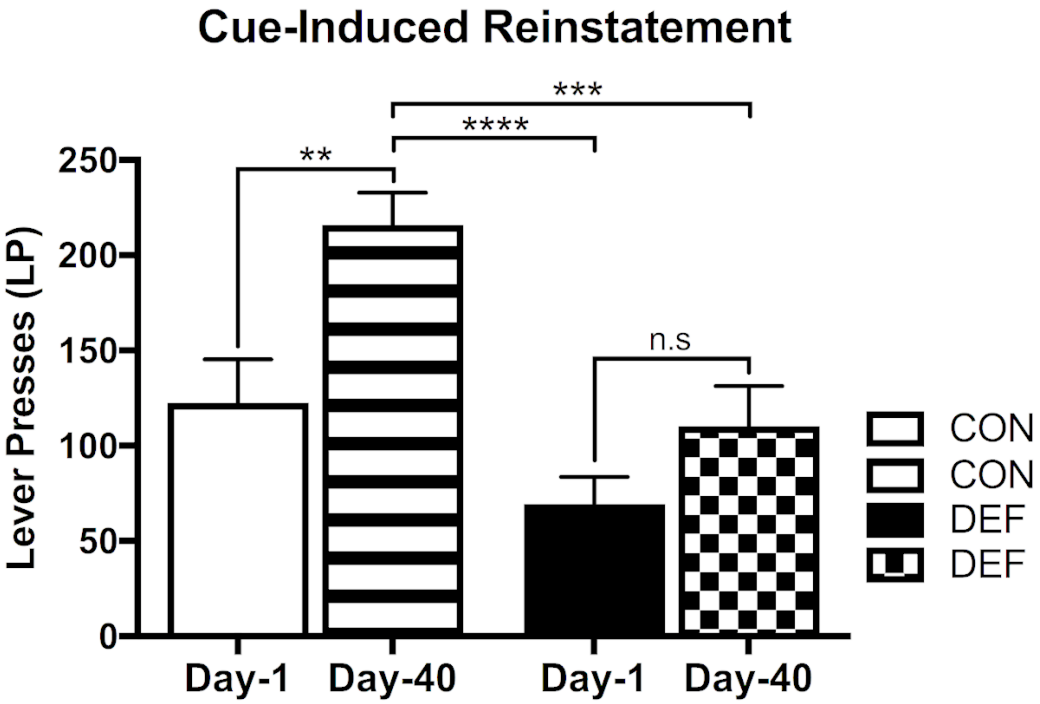

A
Food Training
SA
8 Days
Withdrawal
Forced Abstinence
Day 1 (FA-D1)
Forced Abstinence
Day 40 (FA-D40)
B
Inactive Lever Presses
Lever Presses/6h
Days
CON
DEF
****
C
Active Lever Presses
Lever Presses/6h
Days
CON
DEF
****
****
D
Movement Episodes
Movement Episodes/6h
Days
CON
DEF
*
**

Fig. 3.3. LP Behavior and locomotor activity during cocaine SA.

(**A**) Schematic representation of the experimental procedure and timeline. Rats self-administer cocaine over 8 days, before being assigned to 1 of 2 groups. Day 1 relapse rats were tested for cue-induced reinstatement on day 1 of forced abstinence. Day 40 rats were tested for cue-induced reinstatement after 40 days of forced abstinence. Total number of active (**B**), inactive and (**C**) active lever presses. (**D**) Mean locomotor activity of DEF recorded as counts during the 8-hour activity sessions during SA. The graphs show the daily levels of activity for 1 day thru day 8 after beginning SA. Values are presented as mean ± SEM across the 8 testing sessions, n = 6 (DEF) and 9 (CON), * $p < 0.05$, ** $p < 0.01$. CON, control diet group; DEF, deficient diet group.


Cue-Induced Reinstatement
***
****
**
250
200
150
100
50
0
Lever Presses (LP)
n.s
CON
CON
DEF
DEF
Day-1
Day-40
Day-1
Day-40

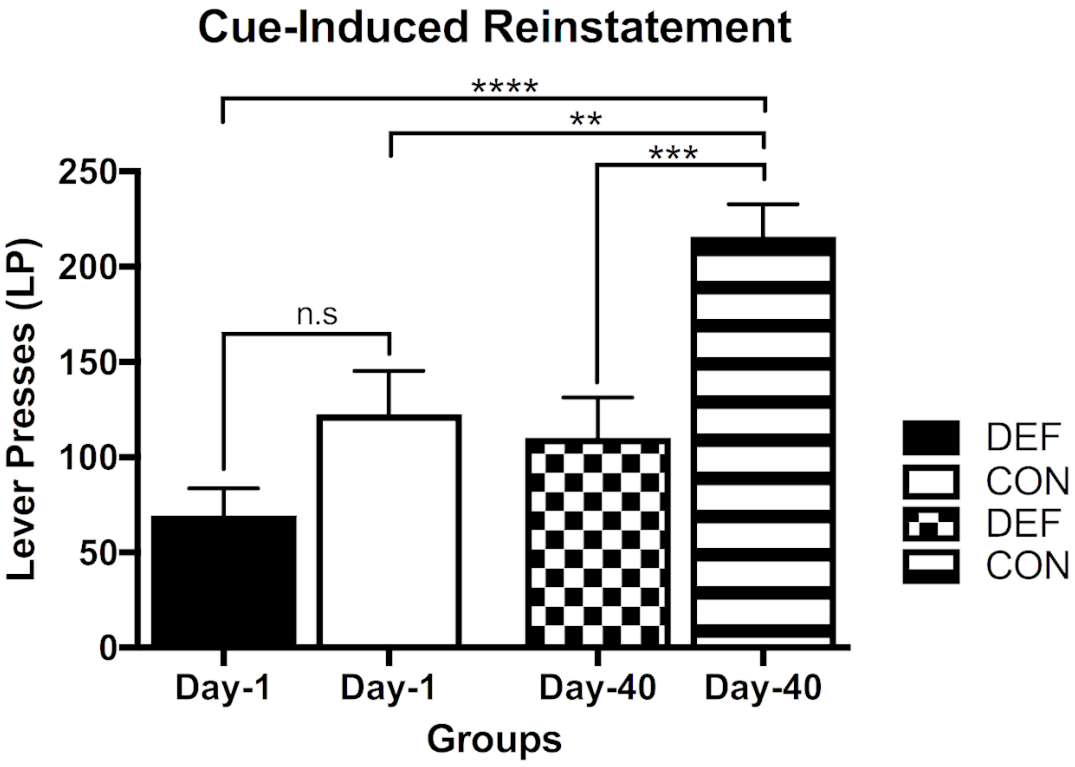

Cue-Induced Reinstatement
****
**
***
250
200
150
100
50
0
Lever Presses (LP)
n.s
DEF
CON
DEF
CON
Day-1
Day-1
Day-40
Day-40
Groups

Fig. 3.4. DEF diet significantly attenuated LP triggered by cocaine-associated cues.

Graphs shown are the number of LP in the previously active lever (a measure of cocaine-seeking behavior) during a 60-min test performed under reinstatement conditions (LP deliver cue but not cocaine) on day 1 or 40. DEF animals did not exhibit the same magnitude of incubation of craving effect as compared to the CON group, evidenced by the lack of increase in lever responding. This behavior was consistent on each of the two days of testing. At day 1, there was a significant increase in LP in the CON group ($p < 0.05$, Con day 1 vs. CON day 40). Conversely, in the DEF group at day 1, the LP was not significant ($p > 0.05$, DEF day 1 vs. DEF day 40). Also, there is a significant difference when comparing CON day 40 to DEF day 1 ($p < 0.0001$) or day 40 ($p < 0.001$). Values are presented as mean ± SEM, n = 6-8 (DEF) and 7-8 (CON), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

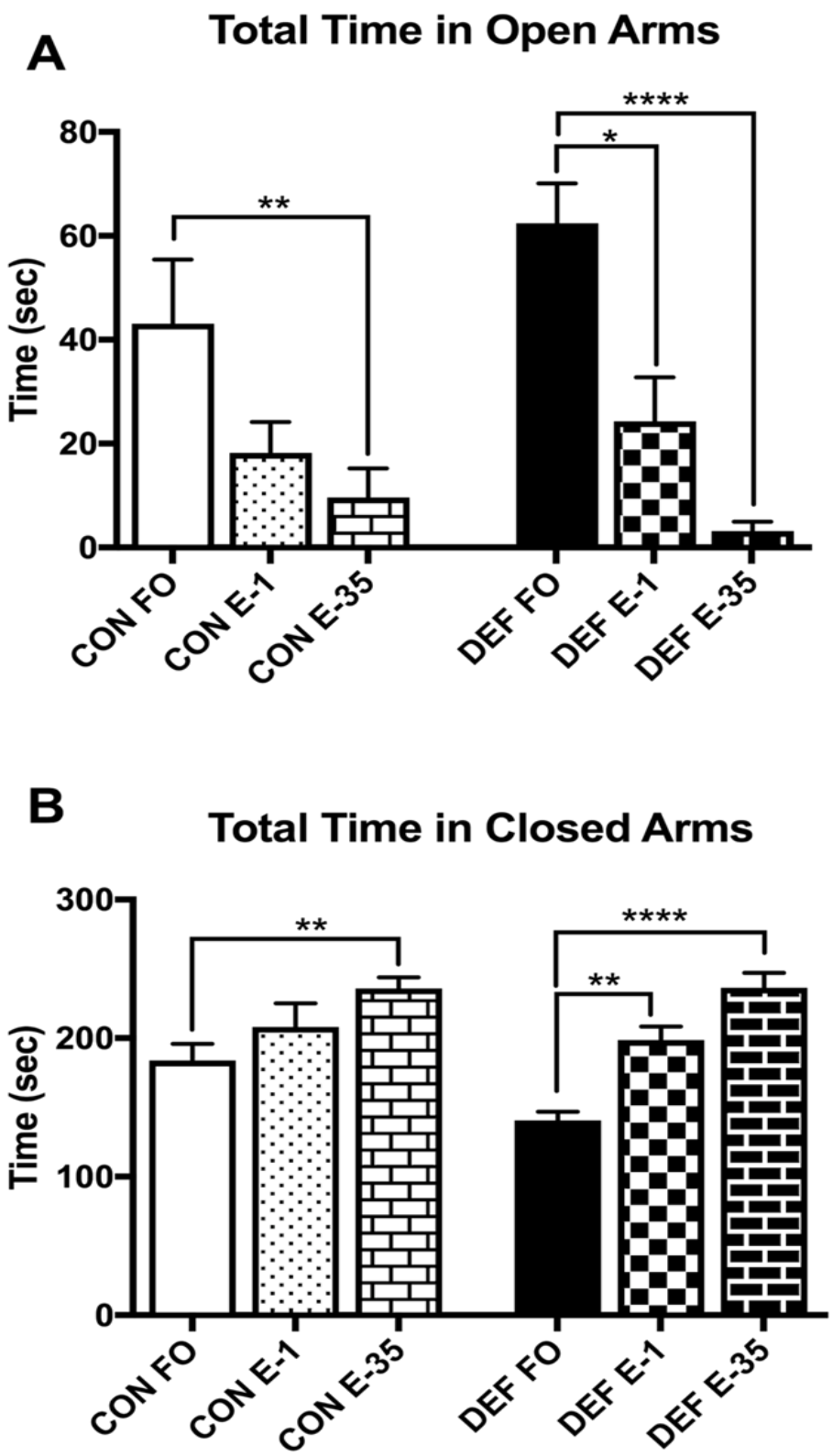
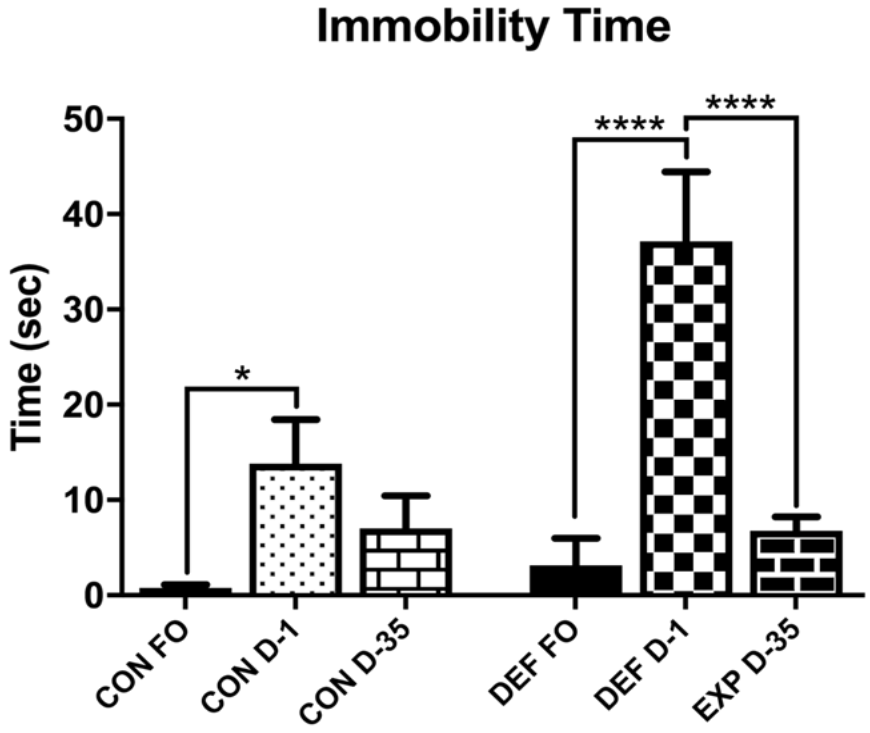

A
Total Time in Open Arms
Time (sec)
80
60
40
20
0
**
****
*
CON FO
CON E-1
CON E-35
DEF FO
DEF E-1
DEF E-35
B
Total Time in Closed Arms
Time (sec)
300
200
100
0
**
****
**
CON FO
CON E-1
CON E-35
DEF FO
DEF E-1
DEF E-35

Fig. 3.5. Effects of N-3 PUFA-Deficient Diet on anxiety-like behavior using the EPM test.

It can be seen the total time in open arms (**A**) and total time in closed arms (**B**) that rats spent after food only (FO) on day 1 and day 35 of withdrawal from chronic SA of cocaine. DEF and CON, rats were tested for 5 min duration on EPM. DEF rats spent significantly less time in open arms at day 1 ($p < 0.05$) and 35 ($p < 0.0001$). Likewise, DEF animals spent significantly more time in closed arms at days 1 ($p < 0.01$) and 35 ($p < 0.0001$) consistent with anxiety-like behavior. Contrary, CON group showed enhanced anxiety-like behaviors only on day 35 of withdrawal Fig. 3.5 (A-B). Values are presented as mean ± SEM, n = 8 (DEF) and 8 (CON), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.


Immobility Time
Time (sec)
50
40
30
20
10
0
*
****
****
CON FO
CON D-1
CON D-35
DEF FO
DEF D-1
EXP D-35

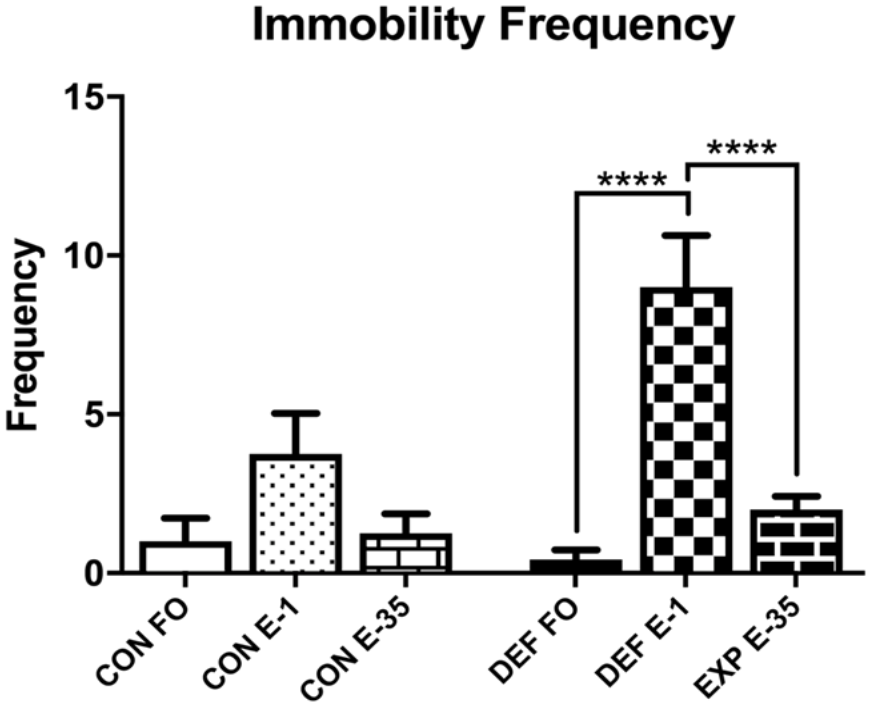

Immobility Frequency
Frequency
15
10
5
0
****
****
CON FO
CON E-1
CON E-35
DEF FO
DEF E-1
EXP E-35

Fig. 3.6. Effects of N-3 PUFA-Deficient Diet on depressive-like behaviors using the FST.

DEF rats showed significantly higher immobility and higher frequency scores than the CON after chronic intake of cocaine (Fig. 3.6 **A–B**). DEF group showed significant high depressive-like behaviors on day 1 ($p = 0.0001$); Similarly, CON group reported less robust but significant behaviors on day 1 ($p = 0.050$) (Fig. 3.6 **A**). On day 35 those behaviors were decreased in both groups. Furthermore, immobility frequency was significantly increased on day 1 and decreased on day 35 in DEF group, while there were no significant difference in the same parameter in the CON group (Fig. 3.6 **B**). One-way ANOVA followed by Tukey's multiple comparison test. Values are presented as mean ± SEM, n = 6-8 (DEF) and 7-8 (CON), * $p < 0.05$, **** $p < 0.0001$.

Discussion

The primary objectives of this series of experiments were to develop an adolescent diet model in rodents and test whether n-3 PUFAs deficient exposure affects cocaine addiction risks, reward-seeking behaviors and withdrawal symptoms in adulthood. This investigation is the first to employ this type of model to decipher if there is an association between factors. Here, we used the cocaine incubation paradigm, in which rats had extended access to the cocaine followed by cue-induced reinstatement. In addition, the effects of early (1 day) and late (40 days) withdrawal periods from chronic exposure to cocaine on mood and anxiety-related phenotypes were measured. Animal and epidemiological literature suggests a link between n-3 PUFAs deficiency and withdrawal symptoms severity. First, deficiency starting in early adolescence exerted a significant effect on LP activity during SA sessions. Second, locomotor activity was also extensively decreased thru SA sessions. Third, significant increases in depressive and anxiety-like behaviors after withdrawal were described. Fourth, deficiency diminished cue-induced reinstatement.

Effects of N-3 PUFA-Deficient Diet on LP triggered by cocaine-associated cues.

The first set of experiments suggested an actual interaction between deficiency of n-3 and the rewarding effects of cocaine. Since weight can influence the sensitivity of brain reward circuitries (Johnson & Kenny, 2010b), we ruled out this possibility by evaluating weight periodically. As formerly reported by Manduca

et al. (2017), deficient diet did not alter body weight in DEF rats, compared to CON group eating a standard chow. Fig. 3.3 shows the course of response output during the eight days of SA to be dissimilar with the DEF group, in disagreement with our hypothesis, manifesting a lower LP output than CON group. Moreover, DEF animals showed an unexpectedly high-level LP activity of the inactive lever compared to CON animals on the first day (see Fig. 3.3, day 1). One could suggest that n-3 deficiency impaired working memory as previous research has suggested (Plamondon & Roberge, 2008; Janssen et al., 2014), however, this interpretation is questionable since DEF group had a steady behavior after the first day. Previous studies have reported similar behaviors that are typically interpreted as non-directed activity and/or response generalization (Shalev et al., 2002; Nair et al., 2008). As no existing research have examined interaction between n-3 PUFAs deficiency and cocaine-seeking behaviors, we do not have the proper information to compare our results accurately and reliably. However, previous studies using variations in FA dietary constituents have reported diminished acquisition of cocaine SA and reinforcement efficacy (Wellman et al., 2007). In addition, studies using high-fat diet diets attenuated the rewarding aspects of amphetamines using the conditioned place preference (CPP) (Davis et al., 2008) and had increased brain stimulation reward thresholds (Johnson & Kenny, 2010a).

Effects of N-3 PUFA-Deficient Diet on N-3 PUFA-Deficient Diet on locomotor activity during SA sessions.

Locomotor activity inside the operant chambers was also assessed. In contrast to our hypothesis and previous investigations using similar methods, detrimental sensitivity to the locomotor stimulating effects of cocaine was decreased in the DEF group. Several authors have examined the impact of a reduced intake of n-3 fatty acids on general activity in rodents; nonetheless, results have been contradictory and diverse. One study by McNamara et al. (2008a) showed that dietary-induced DHA deficiency, with a concomitant elevation of AA:DHA ratio augmented induced amphetamine sensitization in mice. The elevated AA:DHA did not alter locomotor activity in response to acute amphetamine treatment. McNamara and colleagues (2008a), also stated conflicting results with a prior study conducted by Levant and co-authors (2004), finding different results in response to acute amphetamine doses. Analogous studies have equally found such inconsistent evidence. For example, Bourre and colleagues did not notice any change in the motor activity in a novel environment or an open field exploratory in n-3 deficient rats. Likewise, Enslen and colleagues (1991) did not observe any difference in locomotor activity using deficient diet in mice. These discrepancies may be due to differences in the stage of development, animal species, nutrients used in the diet, time of consumption, among others.

Results similar to our present study have shown that the intake of psychotropic chemicals by n-3 deficient animals triggered lesser movements than control groups. For instance, Nakashima and others (1993b) showed that an n-3 deficiency significantly attenuated the scopolamine-induced hyperlocomotion in mice as compared to with n-3 FA adequate group. Taken together, our results

suggest that pharmacological effects may be affected by dietary n-3/n-6 lessening the stimulating effects of cocaine; either, directly or indirectly affecting other neurotransmitter systems strongly associated with motivation and reward.

Effects of N-3 PUFA-Deficient Diet on cue-induced reinstatement.

A reinstatement model was used to explore changes in cocaine-seeking behaviors in n-3 deficient rats using the incubation model. Craving is acknowledged to have a considerable influence on 1) the conservation of addictive behaviors, 2) being liable for the compulsive use of drugs, 3) the complications accompanied in the course of abstinence and 4) the high rate of relapse that follows (Tiffany, 1999). After prolonged-abstinence, users encounter an escalation of uncontrollable cravings symptoms. These craving responses intensify as a function of time, strengthening the degree of cue-induced drug seeking (Lu et al., 2004). Animal studies similarly have revealed that cue-induced craving responses increase progressively after several weeks of abstinence (Grimm et al., 2001). This effect has been proposed to replicate behaviors that can manifest in humans after a prolonged time of cocaine withdrawal (Gawin & Kleber, 1986).

The present study demonstrates that DEF group had lower cue-induced cocaine-seeking behavior compared to the CON group. Similarly, Frances and colleagues (2000) showed that mice exposed to an-3 deficient diet appeared less responsive to rewarding events than animals fed with a standard diet. They suggested that brain DA by n-3 PUFA deficiency could be involved in impairing behavioral response to positive events. This finding is consistent with the induction

of anhedonia —diminished interest or pleasure in rewarding activities— and was further supported by attenuation of cocaine intake in our experiments. Previous research advocates for an association between anhedonia and withdrawal after cocaine use (Perrine et al., 2008). Overall, the effects of n-3 deficiency on reward function are consistent with human investigations that describe blunted subjective pleasure and motivation to obtain rewards. Dietary-induced perturbations in PUFA homeostasis can potentially deregulate the dopaminergic systems, leading to mood behavioral changes and impaired receptivity to positive events.

Effects of N-3 PUFA-Deficient Diet on depressive-like behaviors using the FST.

In addition to revealing the deficiencies of n-3 PUFAs and resulting disruption of reward-seeking behaviors, our findings showed that the 10-week n-3 PUFAs deficiency during adolescence is enough to provoke depressive-like states in early withdrawal and anxiety-like states behaviors. A n-3 PUFA-deficient diet increased immobility time in the FST, a test that reliably evaluates symptoms resembling depression

The effects of dietary fatty acids on the anxiety level in the elevated plus maze have been studied. In n-3 PUFAs deficient mice, anxiety was significantly increased compared to mice fed a diet containing both linoleic and a-linolenic acid (adequate group) (Carrié et al., 2000b). A similar study found that n-3 deficient rats spent less time in open arms compared to the adequate group (Takeuchi et al., 2003a). However, a recent study found that after a 10-week diet, rats spent less

time exploring the center of an open-field maze as compared to controls rats (Belzung et al., 1998b; Frances et al., 2000; Nakashima et al., 1993b). Moreover, it was found that rats who had a deficient diet during their adolescence spent less time exploring the center of an open-field maze compared to controls (Delpech et al., 2015a).

Evidence supports ω-3 deficiencies influence stress-related disorders, such as depression and anxiety, particularly on the activity of the HPA axis. Studies have shown emotional alterations through abnormal corticosterone secretion, altering neurons in the prefrontal cortex (PFC) (Larrieu et al., 2016). Also, since depression and anxiety are often comorbid, anxiogenic outcomes caused by ω-3 PUFA deficiency has also been investigated (Oshima et al., 2018a). Epidemiological studies have suggested that a lower n-3 fatty acid status is related to higher prevalence rates of major depression (Harris, 2008). Clinical studies have described abnormally low levels of DHA in the plasma and total erythrocyte fatty acids (Rizzo et al., 2012). In addition, animal studies have shown the same association with depression and anxiety (Weiser et al., 2014; DeMar et al., 2006). In fact, the beneficial effects of PUFAs supplementation on anxiogenic and depressive symptoms has been shown (Bhatia et al., 2011b).

On the other hand, concerning addiction, the symptoms of cocaine withdrawal are concomitant with more intense anxiety and depression in humans. Perrine and colleagues (2008b) found that one day of withdrawal from chronic administration of cocaine caused increased anxiety- and depression-like behaviors in rats. This finding has been corroborated by studies where similar depressive behaviors

following chronic drug use and withdrawal were observed (Anraku et al. 2001; Perrine et al. 2008). Furthermore, sub-chronic drug exposure precipitated similar depressive-like symptoms (Zilkha et al., 2014).

It is an open question whether or not omega-3s deficiency has any direct or indirect effect in the withdrawal of substance abuse disorders. Several studies suggest that DHA status may interact with withdrawal symptoms (Buydens-Branchey, 2003; Buydens-Branchey, Branchey, McMakin, & Hibbeln, 2003; Buydens-Branchey & Branchey, 2006; Buydens-Branchey, Branchey, & Hibbeln, 2008; Rabinovitz, 2014). Deficiencies are thought to lead to changes in drug cravings and mood behaviors, as well as, a reduction in the ability to cope with stress, characteristics whose are also linked to addicts' behaviors. For example, Buydens-Branchey and colleagues (2003b; 2003) linked low levels of omega-3 to relapse in cocaine addicts possibly due to the increased anxiety created by deficiencies. On the contrary, supplementary DHA has led to decreased distress symptoms (i.e., stress and anxiety), aggression, depression, and behaviors often coupled with relapse. Rabinovitz and colleagues (2014) found that omega-3 supplementation significantly lowered daily tobacco consumption and tobacco craving in regular smokers' use after one month of treatment. Moreover, high omega-3 can decrease basal cortisol secretion in abstinent alcoholics during withdrawal (Barbadoro et al., 2013). Other studies have found that n-3 PUFA supplementation lessens hostility in alcohol, cocaine, and heroin users.

More studies are required to fully understand the role of n-3 in stimulants addiction. Several clinical studies by several researchers (Buydens-Branchey &

Branchey, 2006b; Buydens-Branchey et al.,2008b; Buydens-Branchey et al., 2009), alluded to the relevance of PUFAs status (high proportion of n-6:n-3) and its influence on relapse in cocaine addicts in that low levels may have an impact on anger and anxiety. McNamara et al. (2008a) discovered that amphetamine (AMPH) sensitization is augmented in mice with diet-induced n-3 FA deficiency. In the same line, (Kuhn et al.,, 2013) evaluated the influence of different fats on preference parameters for AMPH and showed the detrimental effect on conditioned place preference paradigm and drug craving symptoms. Contrary, the administration of a diet rich in n-3 PUFAs prevented anxiety and oxidative damages in rats exposed to AMPH. Moreover, Serafine and colleagues (2016) reported greater locomotion and sensitization of cocaine in rats supplemented with fish oil. Nonetheless, studies using dietary supplementation of omega-3 have not always been consistent. Researchers have shown results evidencing no protective effect against cocaine-related behaviors (Eserian et al., 2012). Nonetheless, the majority of studies propose that the dopaminergic system is likely involved in the documented changes in addictive behaviors.

As stated before, there are major limitations to the present investigation that need to be addressed in the future. First, we did not explore other probable outcomes of DHA. It is conceivable that distinct mechanisms, processes, and compounds might also be involved like stress, neurodegeneration, neuro-inflammation, various neurotransmitters, and active n-3 PUFA derived metabolites. A second limitation is the extent of n-3 deprivation; perhaps, using pre-weaning or transgenerational paradigms could lead to different results. Third, we did not

analyze other brain areas, such as the amygdala and hippocampus which are affected by these FA omega-3 FA (Conklin et al., 2007; Sopian et al., 2015). Future experiments should examine different drug categories, duration of diet consumption, different brain areas and distinct developmental stages to reach a more definite conclusion.

Conclusion

In conclusion, our study reveals that chronic exposure to cocaine combined with an extensive n-3 PUFA deficiency starting in early adolescence can diminish cue-induced reinstatement and worsen withdrawal symptoms. A 10-week of exposure to n-3 PUFA dietary deficiency dramatically desensitizes the brain for cue-induced reinstatement based on diminished LP activity and reduced movement episodes throughout the SA sessions. Moreover, nutritional ω-3 deprivation triggered emotional, behavioral alterations of early and late withdrawal after chronic cocaine SA. The presented evidence showed that chronic daily exposure to cocaine could precipitate features of depression and anxiety in rats. Because omega-3 is implicated in the regulation of DA pathways and its sensitivity in the brain, we speculate on a possible mechanism by which n-3 PUFA may interfere on DA level.

These data suggest that low n-3 FA status may be an essential determinant of behavioral and neurochemical responses to addictive drugs. This environmental factor may contribute to illness progression, and to development of other major psychiatric disorders including mood or anxiety disorders after suspending the drug. Further studies should characterize the impact on other behavioral and cognitive performances using different drugs to determine whether the effects are attributable to the diet. These facts established that n-3 FA deficiency rat model worsens several factors associated with the pathophysiology of addiction and support further evaluation in future clinical trials.

CHAPTER IV

General Conclusions

General Conclusions

The stage of adolescence is a transitional process characterized by unique neurobiological changes in the brain. The maturational processes, especially in the frontal cortex and limbic system, are responsible for cognitive control and reward-seeking behaviors. Moreover, adolescence is characterized by an enhanced vulnerability to external factors. Insults during this postnatal period can lead to long-lasting neurochemical changes, which may persist into adulthood. Research studies have highlighted the adverse influence of specific nutrients on brain function, resulting in cognitive impairments and modified reward processing.

Deficiencies of ω-3 have been pointed out to play an essential role during this period, predisposing individuals to behavioral and cognitive deficits. Molecular studies in rodents have indicated that sustained deficits of ω-3 can dysregulate different neurotransmitters that are involved in the pathophysiology of addiction, such as the dopaminergic, serotoninergic and endocannabinoid systems. While several mechanisms underlying the effects of dietary ω-3 deficiency have been described, those specifically linked to pre-adolescence (post-weaning) are limited and poorly understood. This modulatory effect triggered might influence addictive drug behaviors and neurotransmitters associated with motivation and reward.

In recent years, ω-3 is recognized for controlling molecular mechanisms that can propagate psychiatric disorders including drug addiction. Research studies have demonstrated that low levels of n-3 PUFA in the brain alter the dopaminergic system of the brain and, because of this, increase the risk of developing addictive disorders and/or the severity of the disease. Around this,

studies have shown the detrimental aspects of n-3 PUFAs deficiency in the treatment of cocaine abuse, aggravating withdrawal symptoms like anxiety, anger, cravings, and depression. Moreover, as it has been mentioned before, dietary supplementation of n-3 PUFAs can reduce this symptoms. These pieces of evidence suggest that low consumption of ω-3 could influence addictive behaviors. Although causalities exist, the precise mechanisms by which ω-3 modulate drug effects remain poorly understood. To the best of our knowledge, evidence linking PUFAs deficiencies and cocaine use disorder within the brain reward regions was non-existent.

The present dissertation aimed to examine whether adolescent brain maturation may represent a critical period for n-3 PUFAs to contribute to a higher vulnerability for future cocaine abuse in adulthood. We also considered whether this deficit alters drug-reinforced behaviors and craving severity. Besides, we analyzed subsequent predisposition to depression and anxiety after chronic cocaine intake. In chapter 1 we discussed the foundation of the research question, general biochemical concepts about fatty acids, and reviewed the literature regarding the harmful consequences of ω-3 deficiency, and its impact on neurodevelopment, reward-related neurocircuitry and its possible influence in addictive behaviors. In chapter 2, we revealed that chronic exposure to cocaine combined with an extensive n-3 PUFA deficiency starting in early adolescence can diminish cue-induced reinstatement and worsen withdrawal symptoms. A 10-week of exposure to n-3 PUFA dietary deficiency dramatically desensitizes the brain for

cue-induced reinstatement based on diminished LP activity and reduced movement episodes throughout the SA sessions.

Moreover, nutritional ω-3 deprivation triggered emotional, behavioral alterations of early and late withdrawal after chronic cocaine SA. The presented evidence showed that chronic daily exposure to cocaine could precipitate features of depression and anxiety in rats. Because omega-3 is implicated in the regulation of DA pathways and is sensitivity in the brain, we speculated on a possible mechanism by which n-3 PUFA may interfere on DA level. These data suggest that low n-3 FA status may be an essential determinant of behavioral and neurochemical responses to addictive drugs. This environmental factor may contribute to illness progression, and to development of other major psychiatric disorders including mood or anxiety disorders after suspending the drug.

In chapter 3 we presented evidence that n-3 PUFAs deficiency beginning at early adolescence diminished cue-induced reinstatement. This suggests that a 2-month exposure to deficiencies in this period is enough to dramatically desensitizes the brain to discrete cues associated with cocaine. These findings complement our prior evidence where we highlighted hypoactivity, decrease emotional responses and perhaps alterations in dopaminergic transmission. This study also evidenced that dietary n-3 FA negatively impacts anxiety performances in adult rats after 14 days of abstinence. We speculated that the mechanisms underlying these changes could also be directly associated with the modification of the dopaminergic systems, particularly with a decrease in D1R within the PFC based on previous molecular experiments. The results of this study not only

provide insight into how lipids can influence addictive responses but also suggest a role for n-3 PUFAs in the exacerbation of cocaine withdrawal symptom severity. In summary, these results show that that DR1 in the PFC could have an important role in the cocaine-seeking behavior and the sequelae of its chronic usage.

Unfortunately, there were a few limitations to the study. First, our molecular analysis was not validated using sufficient brain samples to detect group differences, particularly for the western blots, in which only four brain samples were analyzed. Additionally, only two brain areas were assessed during this procedure. Due to these limitations, it is advisable to replicate this experiment for a more reliable interpretation. Second, fatty acid content was not tested. Previous studies have demonstrated that a cocaine-conditioning experiment can result in the remodeling of specific phospholipids in rat brain tissue in a region-specific manner (Cummings et al., 2015). Moreover, post-weaning dietary deficiency affect the overall FA composition of tissues that depends significantly on the timing and duration of the experiment, the location of the tissue, and the specific FA of interest (Manduca et al., 2017; Fedorova & Salem, 2006). Therefore, due to the influences of these two variables, makes our results difficult for comparisons with other related studies. Future investigations need to examine and consider this concurrent effect in a more comprehensive manner. Lastly, we did not assess other stages of development. Thus, we recommend for forthcoming research a thorough examination of the different stages of development.

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APPENDIX

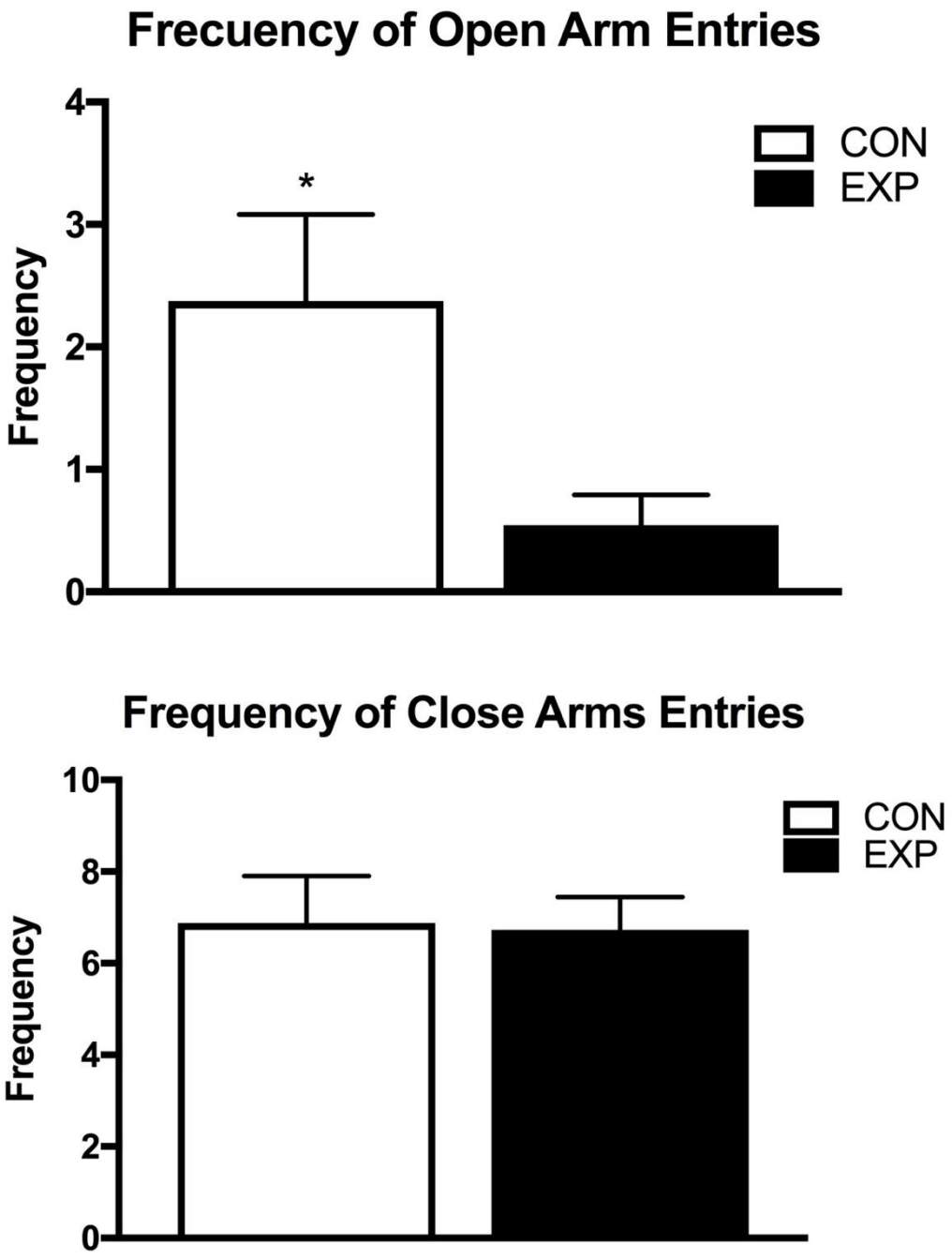
Appendix I

	Controls (TD.04285)	DEF (TD.04286)
Ingredients[1]		
Cornstarch	20	20
Sucrose	27	27
Dextrose	9.9	9.9
Maltose-dextrin	6	6
Cellulose	5	5
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Coconut oil	4.5	5.1
Safflower	1.9	1.9
Flaxseed	0.6	0
Fish oil	0	0
Fatty acid composition[2]		
C8:0	3.7	4.1
C10:0	3.3	3.7
C12:0	38	32.1
C14:0	11.5	12.9
C16:0	8.8	9.1
C18:0	10.7	11.7
16:1 n–7	n.d.	n.d.
18:1 n–9	6.7	5.1
18:1 n–7	n.d.	n.d.
18:2 n–6	22.5	21.3
20:4 n–6	n.d.	n.d.
18:3 n–3	4.9	n.d.
20:5 n–3	n.d.	n.d.
22:5 n–3	n.d.	n.d.
22:6 n–3	n.d.	n.d.

DEF, deficient (n–3-free); FO, fish oil; n.d., not detected. [1] g/100 g diet. [2] Weight % of total fatty acids.

Supplemental Table I. Nutritional and FA content of food sources

Appendix II



Supplemental Figure I. The effect of omega-3 diet deficient on the anxiety-like behavior in the elevated plus-maze test. (A) Frequency of entries to the open arms

and (B) the frequency entries to the closed arms. Data show that DEF diet enter significantly less to the closed arm ($p = 0.0164$) in comparison to the open arm ($p = 0.478$) compared to CON group. Values are means ± SEM; Mann-Whitney U test. * $p < 0.05$.

ABOUT THE AUTHOR

Sergio Serrano-Torres was born on September 17th, 1985, in Ponce, Puerto Rico (PR). He completed his elementary school at Escuela Herminia García de Cintrón and his middle & high school at Jardines de Ponce; all schools were in Ponce, PR. While in high school, he entered the Air Force JROTC for three years with the intention of joining the military. He ultimately decided not to enlist, but to pursue a college career instead. In 2003, he graduated with honors from the Pontifical Catholic University of Puerto Rico (PUCPR), where he obtained his bachelor's degree in Natural Science. In 2012, at the same university, he earned his master's degree in biotechnology and was awarded for obtaining the top GPA award in the biotechnology program and the entire graduate program. After receiving his master's degree, he became interested in research and decided to pursue a doctorate in biology. He was accepted to the graduate program in biology and began working with Dr. Carmen Maldonado-Vlaar laboratory focusing on the neurobiology of cocaine addiction. While discovering the world of research during graduate school, he always felt a greater inclination toward clinical investigations. Therefore, his future professional goal is to obtain a pharmacy degree (Pharm.D) from the University of Puerto Rico-Medical Science Campus. He firmly believes that this preparation will provide him with a foundation for conducting clinical research focused on the medical care of patients with mental health issues.