

AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF CIPROFLOXACIN ON SENSORY  
PROCESSING, COGNITION, AND HIPPOCAMPAL  
NEUROGENESIS IN HEALTHY RATS

by  
JUDITH JEAN NADDOUR

A thesis  
submitted in partial fulfillment of the requirements  
for the degree of Master of Science  
to the Department of Anatomy, Cell Biology, and Physiological Sciences  
of the Faculty of Medicine  
at the American University of Beirut

Beirut, Lebanon  
August 2022

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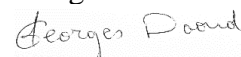
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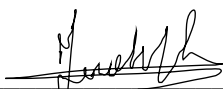
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## ACKNOWLEDGEMENTS

"Nothing is Impossible, the word itself says, I'm Possible!" –Audrey Hepburn

I would like to thank Dr. Nada Lawand for giving me the opportunity to work in her lab. I was able to learn and to broaden my knowledge through her constant guidance throughout the project. Not only was she a great academic mentor, but Dr. Lawand believed in my abilities and always thrived to show the best out of me.

I would like to extend my gratitude to Ms. Maya Jammoul and to Ms. Batoul Darwish for their continuous help and advice throughout the project. I had the privilege to learn from their experience and that everything is achievable through hard work, patience, and confidence.

I would like to thank Dr. Wassim Abou-Kheir for supervising the project while Dr. Lawand was abroad. He followed up on every step of the project and he was always available whenever I needed guidance. Dr. Abou-Kheir taught me to never give up and to believe in myself. No matter where I go, I will always remember his life advice.

This project would not have been possible without the generous funding by the Diana Tamari Sabbagh Scholars Program (DTSSP).

Last but not least, no words can describe the amount of appreciation and gratitude I have for my family and friends. Therefore, I would like to dedicate this project to them.

ABSTRACT

OF THE THESIS OF

Judith Naddour

for

Master of Science

Major: Neuroscience

Title: Effect of Ciprofloxacin on Sensory Processing, Cognition, and Hippocampal Neurogenesis in Healthy Rats

Ciprofloxacin (CPFX), a broad-spectrum antibiotic in the fluoroquinolone class, is one of the most widely prescribed agents for uncomplicated cases of urinary tract infections (UTI). However, over the past decade, the safety of CPFX was questioned due to reported cases regarding its adverse side effects on the nervous system.

Hence, our aim is to test the effect of CPFX on cognition, hippocampal neurogenesis, and pain sensitivity in healthy one-month old male Sprague-Dawley rats.

One-month old male Sprague-Dawley rats were randomly and equally divided into two groups: control (vehicle) and ciprofloxacin-treated (44mg/kg, orally for 14 days) rats. A battery of cognitive and sensory behavioral tests (sequential learning, T-maze, Y-maze, novel-object recognition, mechanical and thermal sensitivity tests) were conducted before, and weekly for two weeks after treatment. The rats also received 3 injections of BrDU (200mg/kg) where the total dosage was equally distributed over the first three days of treatment to label proliferating cells and immature neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. At the end of the experiment, fixed brain tissue were collected and processed for immunofluorescence staining using BrDU and NeuN as neuronal markers for neurogenesis. The data was analyzed and plotted using Graphpad prism.

Our data has shown that CPFX did not impair cognitive functions such as episodic, recognition, reference, and spatial-working memories. This was consistent with the immunofluorescence staining which demonstrated that CPFX did not alter hippocampal neurogenesis. However, CPFX affected the peripheral nervous system (PNS) by decreasing the sensitivity threshold to thermal and mechanical stimuli over time.

The mechanism underlying the development of pain in the CPFX-treated group is not fully understood and requires further investigations. We speculate that CPFX may have triggered a pain-related behavior by inducing a hyper-excitability state in the dorsal horn of the spinal cord through inhibition of GABAergic interneurons.

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## ABBREVIATIONS

- AHN: Adult Hippocampal Neurogenesis
- BBB: Blood-Brain Barrier
- BrdU: 5-Bromo-2'-deoxyuridine
- CNS: Central Nervous System
- CSF: Cerebral Spinal Fluid
- COPD: Chronic Obstructive Pulmonary Disorder
- CPFX: Ciprofloxacin
- DAPI: 4', 6-Diamidino-2-Phenylindole
- DG: Dentate Gyrus
- DNA: Deoxy-ribonucleic Acid
- ESRD: End-Stage Renal Disease
- FDA: Food and Drug Administration
- GABA: Gamma-Aminobutyric Acid
- GCL: Granular Cell Layer
- GCN: Granule Cell Neuron
- IPC: Intermediate Progenitor Cell
- ML: Molecular Layer
- NeuN: Neuronal Nuclear Antigen
- NPCs: Neural Progenitor Cells
- NSAIDS: Non-Steroidal Anti-Inflammatory Drugs
- NSCs: Neural Stem Cells
- pH: Potential of Hydrogen
- RMS: Rostral Migratory Stream

SGZ: Sub-Granular Zone

SVZ: Sub-Ventricular Zone

UTI: Urinary Tract Infection

WHO: World Health Organization

# CHAPTER I

## INTRODUCTION

### **A. Ciprofloxacin**

#### *1. Overview*

Ciprofloxacin (CPFX) belongs to the family of fluoroquinolones, which are among the most commonly prescribed antibiotics due to their broad-spectrum activity against gram-negative and gram-positive bacteria (Grada & Bunick, 2021). They are mainly recommended as a first-line therapy for gastrointestinal tract, genitourinary tract, community-acquired respiratory tract, and dermatological infections that are resistant to the standardized antibiotic treatments (Kushner, Peckman, & Snyder, 2001). In fact, the first quinolone was discovered in 1962 under the name of “Nalidixic Acid”, a non-fluorinated drug, which was derived from “1, 8-naphtyridine” molecule that possesses an antibacterial activity (Talebi Bezmin Abadi, Rizvanov, Haertlé, & Blatt, 2019; Tillotson, 1996). Minor modifications to the quinolone nucleus and to its side chains led to the development of the first 4-quinolone “naphtyridine carboxylic acid”; however, it was restricted for the treatment of urinary tract infections (UTI). The substitution of the hydrogen atom with a fluorine atom at positions C-6 or C-7 created the first fluoroquinolone “flumequine”. This development led to a broader spectrum of antibacterial activity, higher bioavailability, and better tissue distribution (Tillotson, 1996). Further structural modifications to the nucleus and side chains resulted in the development of several generations of fluoroquinolones that improved antimicrobial coverage (Talebi Bezmin Abadi et al., 2019).

## ***2. Bactericidal Action***

Most of the FDA (Food and Drug Administration)-approved fluoroquinolones are lipophilic small molecules that are available in our body in their zwitterionic forms at physiological pH (Blokhina, Sharapova, Ol'khovich, Volkova capital Te, & Perlovich, 2016). These chemical properties enable them to penetrate the bacterial outer membrane through porin proteins, and the cytoplasmic membrane by passive diffusion. Once in the cytoplasm, the fluoroquinolones kill bacterial DNA (Deoxy-ribonucleic acid) by targeting bacterial topoisomerases II (gyrase) and IV; hence the name bactericidal agents. Fluoroquinolones bind to DNA gyrase to form a complex that will block the initiation and the progression of the replication fork while DNA topoisomerase IV separates the duplicated chromosomes. In fact, DNA gyrase has demonstrated a more important role than DNA topoisomerase IV due to the latter's location behind the replication fork, which allows it to reach the replication fork after several cycles of DNA replication. As a result, the CPMX-DNA gyrase-DNA complex kills the bacteria while DNA topoisomerase IV inhibits their growth (Ojkic et al., 2020).

## ***3. Therapeutic Use***

As previously mentioned, CPMX is recommended in the US and other countries to treat complicated and uncomplicated cases of UTIs and to treat cases of acute pyelonephritis. It is widely prescribed since it targets a wide range of uropathogens whose resistance to the first-line therapy, cotrimoxazole, exceeds 10%. In addition to ciprofloxacin's broad spectrum of antibacterial activity, the antibiotic has achieved a great clinical outcome in pathogen eradication (Blondeau, 2004).

Women are significantly more likely than men to develop UTIs, with the majority of women becoming infected at least once in their lifetime (Blondeau, 2004). The higher infection rate in women leads to higher consumption of Ciprofloxacin (CPFX) and consequently to higher exposure. Compared to males, females have a lower muscle mass to body fat ratio and less water (Overholser, Kays, Forrest, & Sowinski, 2004). These physiological aspects result in slower tissue distribution of CPFX in women, leading to a higher serum concentration of CPFX and total drug exposure (Overholser et al., 2004).

Interestingly, the sub-populations that are at higher risk of developing UTIs are also those that are more likely to be exposed to CPFX. These include immunocompromised patients, patients with catheters, patients with underlying urologic abnormalities, elderly patients, pregnant women, and infants. Even though infants are partly treated with CPFX “off-label” due to the lack of pharmacokinetic studies, this antibiotic is prescribed in severe cases of infections when infants are at a major risk of developing meningitis or secondary cerebral abscess (W. Zhao et al., 2014). CPFX is the drug of choice to treat nervous system infections due to its high penetration ratio across the BBB and due to its distribution across the cerebral spinal fluid (CSF) (Tomé & Filipe, 2011).

Often, ciprofloxacin was shown to be highly efficient in treating chronic obstructive pulmonary disorders (COPDs) such as community-acquired pneumonia and acute bronchitis (Bishai, 2002). In fact, this drug established an overall clinical outcome of 93.7% for general and undifferentiated respiratory tract infections, along with a bacteriological eradication outcome of 93.4% that is quite similar to the responses achieved while administering the standardized antibiotics (Ball & Tillotson, 1995). This

achievement is attributed to ciprofloxacin's antimicrobial activity against antibiotic-resistant, gram-negative respiratory tract pathogens, such as *Haemophilus influenza* and *Moraxella catarrhalis* and its increased access into respiratory tract tissues (Bishai, 2002).

#### ***4. Adverse Side Effects***

Throughout the years, researchers became skeptic regarding the safety of fluoroquinolones. In fact, temafloxacin, trovafloxacin, grepafloxacin, clinafloxacin, and gatifloxacin were withdrawn from the global market due to their adverse side effects such as: hemolytic and aplastic anemia, liver failure, photo-toxicity, tendon rupture, prolonged QTc interval, and cases of hypo-/hyperglycemia, respectively (Tomé & Filipe, 2011). Recently, concerns have been raised regarding the neurotoxic effects of these drugs. A study conducted by Blokhina et al. (2016) showed that fluoroquinolones have the ability to penetrate the blood-brain barrier (BBB) and interact with GABA receptors due to structural similarity with GABA (Blokhina et al., 2016; Tomé & Filipe, 2011). In addition, several case reports demonstrated an association between CPFY consumption and the onset of psychosis, peripheral axonal neuropathy, headaches, dizziness, confusion, anxiety, manic episode, and epileptic seizures (Francis & Higgins, 2014; Grimm & Alm, 2011; Tan & Teo, 2021). However, despite clinical evidence of neurotoxicity, the fluoroquinolones remain widely prescribed.

#### **B. Ciprofloxacin-Associated Neurotoxicity**

In 2013, the FDA warned about the potential neurotoxic consequences associated with ciprofloxacin (Rossi & Mazoki, 2018). In fact, neurological

manifestations are among the most commonly reported side effects upon administration of CPFX. The most frequently reported symptoms are mild headache, insomnia, and minor dizziness that occur in about 1% to 2% of the population (Reeves, 1992).

Although neuropsychiatric manifestations are rare, they have been reported in 1% to 4% of patients throughout treatment (Mulhall & Bergmann, 1995). The most common psychiatric sequelae associated with ciprofloxacin include acute psychotic reactions (Ranjan & Praharaj, 2014), seizures (Tan & Teo, 2021), and manic episodes (Ibiloglu, Atli, Asoglu, & Ozkan, 2017). It has been suggested that the ciprofloxacin-induced neurotoxic effects could be due to its antagonistic action on GABA<sub>A</sub> receptors, and the resulting overstimulation of the central nervous system (Hondebrink et al., 2015).

Tables 2-6 summarize the results of 23 case reports revealing the neurotoxicity-related symptoms as reported by patients treated with CPFX.

### ***1. Ciprofloxacin-Induced Psychiatric Disorders***

According to the World Health Organization (WHO), CPFX was the causative agent of 14.4% of antimicrobial-induced mania (Abouesh, Stone, & Hobbs, 2002; Sahoo, Aneja, & Basu, 2016). Clinical reports have demonstrated that patients who are at risk of developing any type of personality disorders and those who suffer from any psychiatric illnesses, should be monitored throughout the course of therapy to diminish CPFX-induced psychiatric imbalances (Portillo et al., 2010; Reeves, 1992; Sohn, 2009). Meanwhile, other case reports highlighted the importance of adopting an individualized drug dosing approach rather than opting for a uniform dosage to minimize the development of any psychiatric disorders associated with CPFX intake (Agbaht, Bitik,

Piskinpasa, Bayraktar, & Topeli, 2009; Mulhall & Bergmann, 1995; Tattevin, Messiaen, Pras, Ronco, & Biour, 1998).

**Table 1: Ciprofloxacin-induced mania case reports.**

<b>Case report</b>	<b>Clinical findings</b>	<b>Reference</b>
28-year old man with cholangitis	Violence, agitation, irritability, grandiosity, and delusions	("Ciprofloxacin: First report of mania: case report," 2007)
85-year old man with UTI	Euphoria, hard to interrupt in conversations, delusions, and difficulty in engaging in discussions	(Sohn, 2009)
75-year old woman with pyelonephritis	Euphoria, agitation, psychomotor restlessness, great sensation of well-being, and auditory hallucinations	(Portillo et al., 2010)
62-year old woman with end-stage renal disease (ESRD) and UTI	Delirium, increased energy, mood irritability, paranoia, pressured speech, and insomnia	(Heaton & Heinrich, 2017)
32-year old woman with UTI	Insomnia, increased energy, grandiosity, and mood irritability	(Ibiloglu, Atli, Asoglu, & Ozkan, 2017)

Thus far, the specific mechanisms responsible for CPF<sub>X</sub>-induced psychosis are not fully understood. However, based on preclinical and clinical studies, it can be suggested that the emergence of psychosis following CPF<sub>X</sub> could be due to altered glutamatergic transmission in the prefrontal cortex triggered by the antagonistic action of CPF<sub>X</sub> on GABA<sub>A</sub> receptors. In fact, several lines of evidence have shown that

perturbation of the glutamate system impacts dopaminergic transmission and contributes to the pathophysiology of psychosis (Grimm & Alm, 2011).

**Table 2: Ciprofloxacin-induced psychosis case reports.**

<b>Case report</b>	<b>Clinical findings</b>	<b>Reference</b>
32-year old woman with multidrug-resistant tuberculosis	Anxiousness, agitation, disorientation, and restlessness Visual and acoustic hallucinations Cold pressure sensation in the head	(Norra et al., 2003)
45-year old female with cystitis	Incoherent thinking and concentration deficits Aggressive and labile mood Nervous breakdown Auditory hallucinations and illusions	(Grimm & Alm, 2011)
22-year old single woman with gastroenteritis	Poor self-care, fearfulness, and psychomotor retardation Visual and auditory hallucinations	(Ranjan & Praharaj, 2014)
64-year old male with COPD exacerbation and bronchiectasis	Disorientation, confusion, and visual delusions Unaware of time and location	(Ben-Chetrit, Rothstein, & Munter, 2013)
36-year old African American male with epididymitis	Depression, anxiousness, disorientation, agitation, confusion, and paranoia	(Rossi & Mazoki, 2018)

## ***2. Ciprofloxacin-Induced Seizures***

Many studies warned about the interaction of CPF<sub>X</sub> with other medications, mainly nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, naproxen, and indomethacin. NSAIDs were shown to potentiate the interaction of CPF<sub>X</sub> with the benzodiazepine-binding sites on GABA<sub>A</sub> receptors by 30,000 times

(Tomé & Filipe, 2011), and lead to overstimulation of the CNS and development of seizures (Sutter, Rüegg, & Tschudin-Sutter, 2015). The possible mechanisms of interaction between ciprofloxacin and NSAIDs are not clear; however, given that these anti-inflammatory medications inhibit prostaglandins and decrease renal blood flow rate, it is reasonable to suggest that the decreased renal clearance would increase the concentration of CPMX in the blood and prompt neurotoxicity (Agbaht et al., 2009). Nevertheless, case reports (Bader, 1992; Kushner et al., 2001), illustrated in Table. 3, have shown that metabolic disturbances along with treatment with CPMX are associated with the onset of epileptic seizures.

**Table 3: Ciprofloxacin-induced seizures case reports.**

<b>Case report</b>	<b>Clinical findings</b>	<b>Reference</b>
25-year old African American female with UTI	Two episodes of recurrent convulsions with agitation	(T. Darwish, 2008)
24-year old female with sinusitis and UTI	Generalized tonic-clonic seizures, hyper-salivation, tongue-biting, confusion, nausea, and urinary inconsistency	(Agbaht et al., 2009)
83-year old woman with septic shock and UTI	Myoclonic convulsion	("Ciprofloxacin: Myoclonic seizure: case report," 2018)
26-year old man with UTI	Tonic-clonic convulsions	("Ciprofloxacin: Tonic-clonic seizures: case report," 2018)
48-year old man with UTI with diabetic ketoacidosis	Generalized seizures	("Ciprofloxacin/imipenem : Generalised convulsive seizures: case report," 2021)
85-year old woman with pneumonia	Tonic-clonic convulsions, facial twitching, nausea, confusion, and up-rolled eyes	(Tan & Teo, 2021)

### ***3. Ciprofloxacin-Induced Peripheral Neuropathy***

The incidence of CPFX-induced peripheral neuropathy accounts for approximately 80% of all fluoroquinolone-induced neurotoxicity (Francis & Higgins, 2014). Sensory peripheral neuropathies are the most prevalent and they occur mainly due to demyelination of the nerves and may be accompanied by axonal degeneration in advanced stages (Vilholm, Christensen, Zedan, & Itani, 2014). In a study submitted to the Swedish Adverse Drug Reactions Advisory Committee, 81% of patients complained of paraesthesia, 51% suffered from hypoaesthesia (numbness), 27% complained of hyperaesthesia (pain), and 11% reported having muscle weakness. Symptoms were initiated within one week upon administration of CPFX in 68% of patients. Additionally, 71% of patients recovered within two weeks upon cessation of treatment (Jumma, Dick, Marshall, & Mellor, 2013).

**Table 4: Ciprofloxacin-induced peripheral neuropathy case reports.**

<b>Case report</b>	<b>Clinical findings</b>	<b>Reference</b>
49-year old male with UTI	Severe burning in the right foot more than the left one	(Jumma et al., 2013)
57-year old Caucasian female with UTI	Entire body burning sensation	(Francis & Higgins, 2014)
57-year old woman with UTI	Weakness, numbness, and pain in her upper and lower extremities	("Ciprofloxacin: Sensorimotor polyneuropathy: case report," 2017)

### ***4. Ciprofloxacin-Induced Cognitive Impairment***

There is little clinical evidence on the effect of CPFX on cognitive functions since the prevalence of CNS adverse reactions upon CPFX is around 1-2%, with the

majority of cases being attributed to psychiatric disorders (Tomé & Filipe, 2011).

However, it is worth mentioning that cognitive disorders mainly memory loss, lack of focus, and moodiness have been associated to fluoroquinolone administration, especially levofloxacin ("Ciprofloxacin/levofloxacin/moxifloxacin," 2015). (Table. 5)

**Table 5: Ciprofloxacin-induced cognitive impairment case reports.**

<b>Case report</b>	<b>Clinical findings</b>	<b>Reference</b>
28-year old woman with sinus infection	Treatment with levofloxacin CNS problems that extended to mood, sleep, cognition, and confusion In addition to gastrointestinal disturbances, peripheral neuropathy, and tendinopathy	("Ciprofloxacin/levofloxacin/moxifloxacin," 2015)
46-year old man with an unconfirmed diagnosis of epididymis	Treatment with levofloxacin CNS problems that extended to mood and cognition In addition to gastrointestinal disturbances, peripheral neuropathy, and autonomic disturbances	("Ciprofloxacin/levofloxacin/moxifloxacin," 2015)
55-year old woman with urinary tract infection (UTI)	Treatment with levofloxacin CNS problems that extended to cognitive impairment which was expressed with confusion, memory loss, and moodiness In addition to peripheral neuropathy and tendinopathy	("Ciprofloxacin/levofloxacin/moxifloxacin," 2015)
23-year old woman with traveler's diarrhea	Treatment with ciprofloxacin Nausea, dizziness, light-headedness, tachycardia, and cognitive impairment which resulted in memory loss and lack of concentration	("Ciprofloxacin/levofloxacin/moxifloxacin," 2015)

## **C. Neurogenesis**

### ***1. History of Neurogenesis***

In 1913, Santiago Ramon y Cajal described the human brain as fixed and non-regenerative (Kumar, Pareek, Faiq, Ghosh, & Kumari, 2019).

Then, in the 1960s, Joseph Altman contradicted the held dogma towards the brain's regenerative properties by showing the first evidence of adult brain neurogenesis in rats. Altman and his colleagues used radioactive thymidine analogs ([<sup>3</sup>H]-thymidine) to label dividing cells during the S-phase of mitosis and therefore track their fates (Altman & Das, 1965). As a result, they were able to observe the production of new neurons in two main regions: the olfactory bulb (Altman, 1969) and the hippocampus (Altman & Das, 1965). Unfortunately, the lack of neuronal markers and the reliability on morphology to identify the newly proliferating cells as neurons led most biologists to reject Altman's experimental discovery (Suh, Deng, & Gage, 2009).

Later on, in the 1980s, Nottebohm and his team were able to link the morphological evidence of adult brain neurogenesis to the physiological implication of such process in songbirds. They achieved so by setting several criteria to be met such as: incorporation of [<sup>3</sup>H]-thymidine in the DNA of dividing cells, observation of the morphological characteristics of neurons and the expression of neuronal markers, and evidence for the integration of the newly synthesized cells in the brain circuitries (NOTTEBOHM, 2004).

## ***2. The Neurogenic Niche***

Neurogenesis is defined as the persistent birth of new neurons (G. Kempermann, Song, & Gage, 2015). This process is responsible for the formation of the CNS in embryos (Urbán & Guillemot, 2014). Meanwhile, throughout postnatal development and in the adult brain, neurogenesis becomes limited and restricted to two main regions: the sub-ventricular zone (SVZ) which lines the lateral ventricles, and the sub-granular

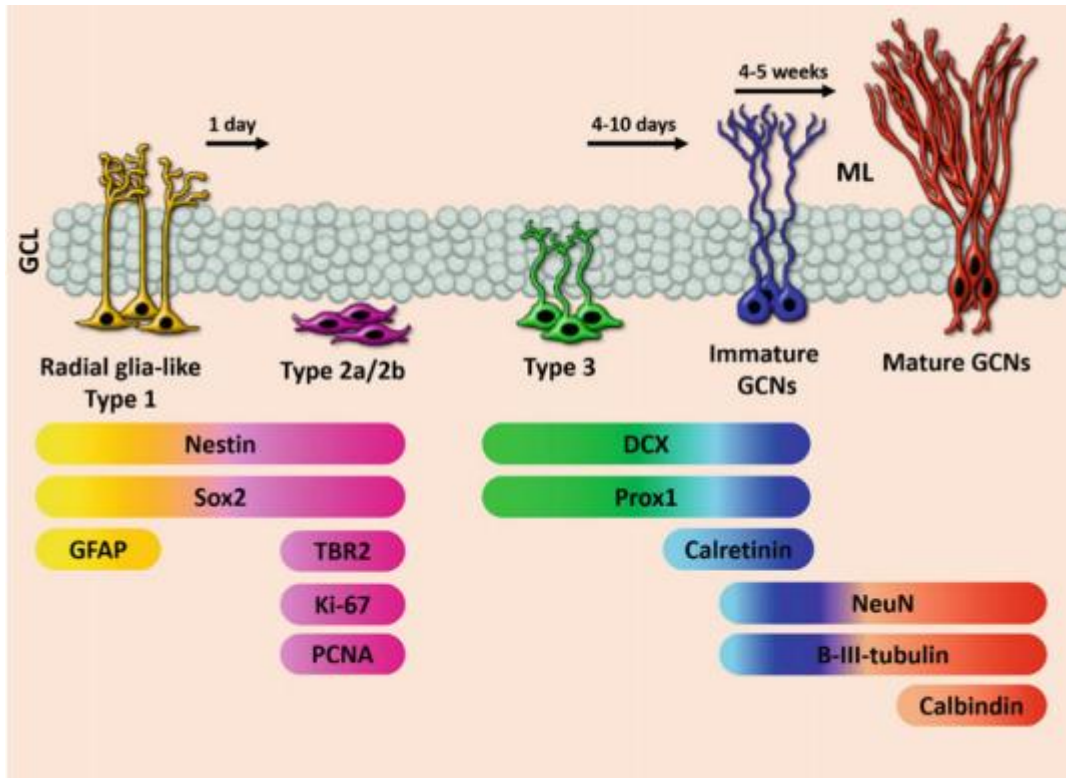
zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Moreno-Jiménez, Terreros-Roncal, Flor-García, Rábano, & Llorens-Martín, 2021). The two regions are referred to as the "*neurogenic niche*" because they provide a suitable microenvironment for housing neural progenitor cells (NPCs) (Matsubara, Matsuda, & Nakashima, 2021). Activated neural stem cells (NSCs) in the SVZ migrate along the rostral migratory stream (RMS) and differentiate into local circuit interneurons in the olfactory bulb, meanwhile activated cells in the SGZ give rise to mature and functional granular neurons (Suh et al., 2009). In humans, adult neurogenesis in the SVZ is robust. It has been shown that the number of neural stem cells in the SVZ and along the RMS decline drastically since birth until 18 months of age (Yang, Ming, & Song, 2011). However, throughout the years, scientists were able to build a bulk of evidence on human adult hippocampal neurogenesis (AHN) based on immunohistochemistry (Moreno-Jiménez et al., 2021). These supporting evidence were built on postmortem samples (Bayer et al., 2015; Boldrini et al., 2018; Boldrini et al., 2009; Flor-García et al., 2020) and biopsies (Blümcke et al., 2001; D'Alessio et al., 2015; Liu et al., 2018; Seki, Hori, Miyata, Maehara, & Namba, 2019). Therefore, the human hippocampal neurogenic niche is an adequate and unique milieu for the generation of new functional neurons, since it consists of an extracellular matrix that is rich in humoral factors and cell-cell interactions (G. Kempermann et al., 2015), in addition to the presence of astrocytes that were found to be key-inducers of neurogenesis (Lledo, Alonso, & Grubb, 2006).

### ***3. The Dentate Gyrus of the Hippocampus: The Sub-Granular Zone***

AHN mainly occurs in the SGZ of the DG in the hippocampus (Moreno-Jiménez et al., 2021). The DG consists of a germinal layer that encompasses a neurogenic niche

of stem cells. This layer provides an adequate environment for the proliferation, differentiation, and maturation of radial glia-like type 1 stem cells into mature, functional, and newly integrated granule cells (Lledo et al., 2006). Throughout the distinct developmental stages of neurogenesis, each stage is classified based on the different neuronal markers that each cell expresses and on their morphological changes (Kronenberg et al., 2003). (Fig. 3)

The process is initiated once radial glia-like type 1 stem cells are activated to produce non-radial proliferating transit-amplifying cells (TAPs) which are needed to amplify the neurogenic pool. Type 1 cells in the granular cell layer (GCL), expressing the stem-cell markers GFAP, Nestin, and Sox2, can extend their apical processes throughout the layer to reach the molecular layer (ML) (Bonaguidi, Song, Ming, & Song, 2012). They can also undergo asymmetrical and infrequent divisions to produce two types of cells: self-renewed radial glia-like type 1 stem cells and intermediate progenitor cells (IPC). The latter are also referred to as type 2 cells that have short processes and express the protein markers: Nestin, Sox2, and TBR2 (G. Kempermann et al., 2015). They proliferate rapidly to yield type 3 cells where short vertical processes appear and these cells express the immature neuronal markers: Prox1 and DCX. At this point, the newly proliferated cells become committed to the neuronal lineage. Type 3 cells then differentiate into mature granule cells that branch their dendrites into the ML and extend their axons to the CA3 region of the hippocampus, they also express the mature neuronal markers: Calbindin and NeuN (Chamaa, Darwish, Saadé, & Abou-Kheir, 2021). In brief, the whole neurogenesis process may be divided into three phases: proliferation, differentiation, and maturation.



**Figure 1: Maturation of radial glia-like type 1 cells into mature granule cell neurons (GCNs).**

Type 1 cells express Nestin, Sox2, and GFAP. They produce type 2 cells that express TBR2, Ki-67, and PCNA. The latter proliferate rapidly to yield type 3 cells that express DCX and Prox1. These type 3 cells migrate short distances across the GCL to differentiate into immature GCNs that express Calretinin. These cells eventually mature into functional GCNs that extend their processes into the ML. Adopted and modified from (Chamaa et al., 2021)

The estimated time for the entire maturation process from a NPC to a functional granular neuron is around 4-5 weeks (Lucassen et al., 2010; C. Zhao, Deng, & Gage, 2008). This process is dynamically and systematically regulated by the milieu of neuronal activity and cognitive experience (Dupret et al., 2007), since not all cells are destined to reach the maturation phase (Biebl, Cooper, Winkler, & Kuhn, 2000; Gerd Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003). In fact, during the first few weeks, 50% or even more cells are eliminated through active apoptosis (Sierra et al., 2010; Sun et al., 2004). Meanwhile, the rest of the cells enhance their chance of

survival by extending their processes to form synapses and interconnections with pre-existing networks (Chamaa et al., 2021). With that being said, the newly integrated neurons may influence the hippocampal functions of learning, memory, spatio-motor performances, and acquisition of new experiences (Deng, Aimone, & Gage, 2010).

#### ***4. Effect of Broad-Spectrum Antibiotics on Neurogenesis***

A study (Möhle et al., 2016) conducted on adult C57BL/6 mice showed that treatment with broad-spectrum antimicrobial agents decreased adult hippocampal neurogenesis. The findings of Möhle et al. (2016) showed that gut flora dysbiosis through antibiotics has a long-term effect on neurogenesis. Moreover, a recent study demonstrated that treatment of a UTI with fosfomycin, a broad-spectrum antibiotic, had a detrimental impact on AHN where the total number of BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells significantly decreased when compared to the untreated group (B. Darwish et al., 2022). Interestingly, CPFY is also a broad-spectrum antimicrobial agent; however, an *invitro* study on the stem cell model of neurogenesis showed that CPFY did not impact AHN (Cao, Livesey, & Halliwell, 2015).

#### **D. Aim of the Study**

CPFY is a broad-spectrum antimicrobial agent with wide therapeutic uses (Grada & Bunick, 2021). While several clinical studies have reported the neurotoxic effects associated with CPFY administration ("Ciprofloxacin/levofloxacin/moxifloxacin," 2015; Francis & Higgins, 2014; Ibiloglu et al., 2017; Rossi & Mazoki, 2018; Tan & Teo, 2021), none had systematically investigated its impact on the peripheral and central nervous system. Therefore, the purpose of this study is to evaluate the effects of oral CPFY on sensory and cognitive

functions in healthy male Sprague-Dawley rats. Behavioral and double-labeling immunofluorescence studies were conducted to determine whether CPF<sub>X</sub> alters sensory transmission, impairs memory, and modifies structural plasticity in the hippocampus.

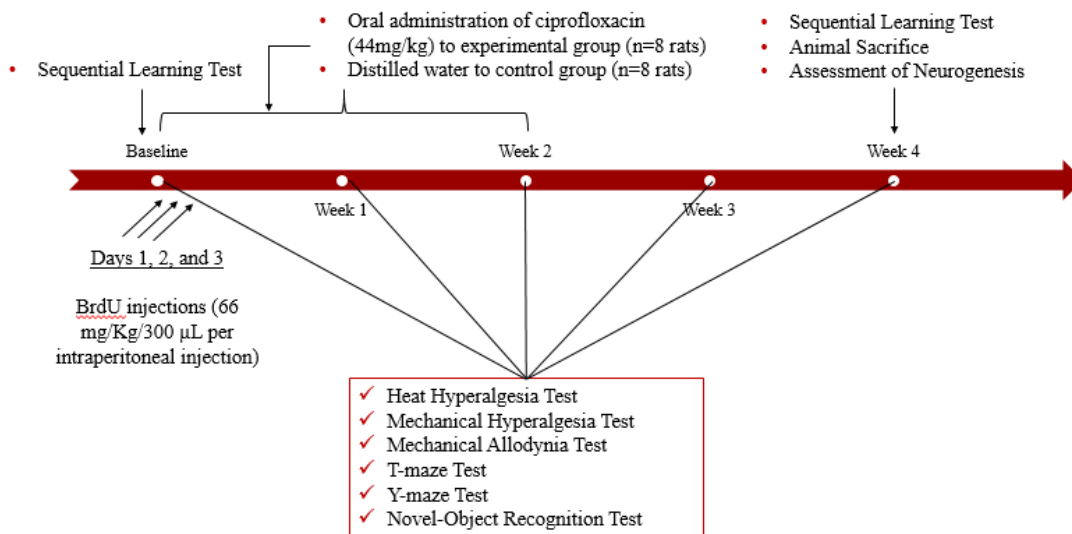
## CHAPTER II

### MATERIAL & METHODS

One-month old male Sprague Dawley rats, weighing 150-200 g, were used in this study. All experimental procedures were performed in accordance with the ethical guidelines set by the Institutional Animal Care and Use Committee at the American University of Beirut. All animals were housed in a room at standard conditions at a constant temperature of 20-22°C on a 12 h light/dark cycle, along with standard rodent chow and water provided ad libitum.

#### **A. Experimental Groups**

A total of 16 rats were randomly divided into two experimental groups (n=8/group). The first group received oral administration of CPF<sub>X</sub> (44mg/kg) for 14 consecutive days, while the second group received distilled water and acted as control.



**Figure 2: Experimental timeline.**

A total of 16 rats were divided into 2 groups: experimental and control groups. The experimental group consisted of 8 healthy rats who received CPMX at a dosage of 44 mg/kg for 14 consecutive days. The control group consisted of 8 healthy rats who received distilled water for 14 consecutive days. To assess neurogenesis, BrdU injections were given at days 1, 2, and 3. Behavioral tests were also performed over a period of 4 weeks.

## **B. BrdU Administration**

Bromodeoxyuridine (BrdU) is a synthetic thymidine analog that is incorporated into the DNA during the S-phase cycle of mitosis. This signal was used to label newly proliferating/immature neurons to study neurogenesis. BrdU powder (5'-bromo-2-deoxyuridine, Boc Sciences) was weighed and dissolved in 0.9% warm saline. Once the solution was properly dissolved, each rat, within each group, received 3 intraperitoneal BrdU injections (66 mg/Kg/300 µL per intraperitoneal injection). Hence, each rat received a total of 200 mg/Kg of BrdU. The 3 intraperitoneal BrdU injections were delivered for 3 consecutive days upon initiation of CPMX, where 1 injection was given per day to ensure maximum availability.

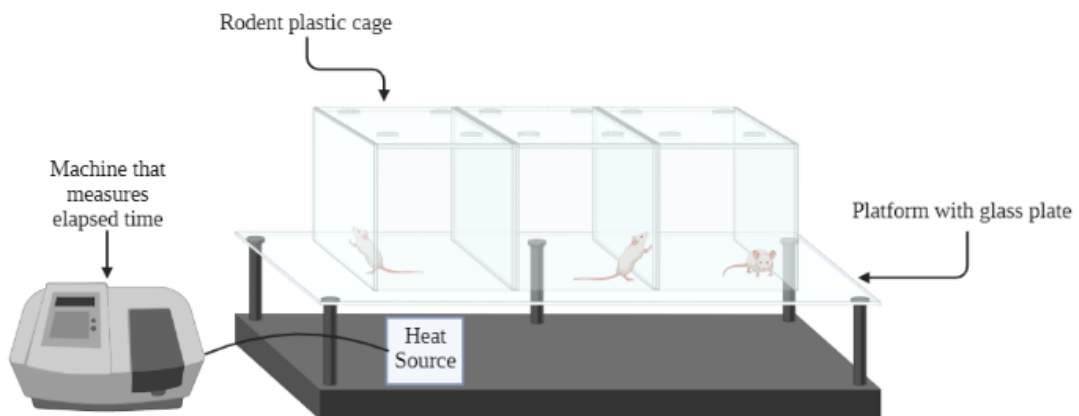
## C. Behavioral Tests

Behavioral tests were performed during the light phase of the circadian cycle. Animals were moved to the experimental room at least 1 hour before testing to adapt and familiarize with the new environment. All tests, except the sequential learning test, were conducted in both experimental groups at baseline before CPFY treatment, at weeks 1 and 2 during CPFY treatment, and at weeks 3 and 4 post-treatment. The sequential learning test was performed on both experimental groups at baseline before CPFY treatment and at week 4 before animal sacrifice.

### 1. *Pain Tests*

#### a. Heat Hyperalgesia (Plantar Test)

Heat hyperalgesia was assessed in CPFY and vehicle-treated groups using the behavioral apparatus illustrated in Fig. 5. Rats were individually placed in clear plastic chambers on a 3-mm thick glass plate. After 30 minutes of accommodation, a radiant heat stimulus (intensity of 35 infrared units) was applied to the plantar surface of the hind paw of rats. The same procedure was applied three times with a resting period of 10 minutes between each trial to avoid conditioning paw withdrawal. Withdrawal latency was defined as the elapsed time, in seconds, from stimulus onset to paw withdrawal.



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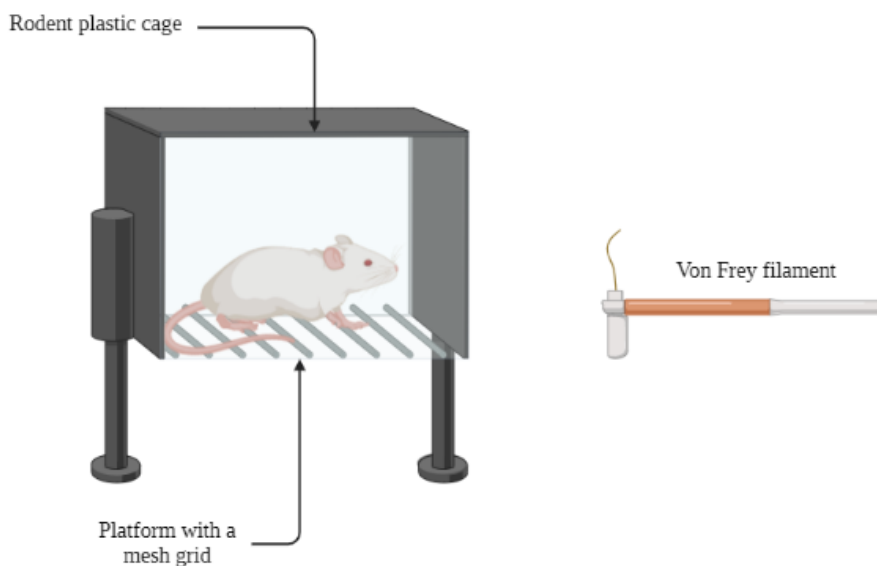
**Figure 3: Heat hyperalgesia apparatus.**

Heat hyperalgesia was assessed by measuring the paw withdrawal latency to a radiant heat stimulus (intensity of 35 infrared units) applied to the plantar surface of the hind paw of rats. The test was performed using the following equipment: Ugo Basile machine (Gemonio VA, Italy; Serial number 0462 U16), Ugo Basile platform with a glass plate (Gemonio VA, Italy; Length 90 cm, Width 14 cm, Height 6.5 cm), and heat generator (Ugo Basile; Gemonio VA, Italy; Model 37,370–002; Serial number 046216).

b. Mechanical Hyperalgesia and Mechanical Allodynia Tests

Mechanical hyperalgesia and mechanical allodynia were assessed in both experimental groups using the behavioral apparatus illustrated in Fig. 6. The rats were placed individually in clear plastic chambers on a metal wire mesh floor and left for 30 minutes to accommodate to the new testing environment. For the mechanical hyperalgesia test, a Von-Frey filament with a bending force of 15 g (noxious stimulus) was applied to the plantar surface of each hind paw to stimulate both mechanoreceptors and nociceptors. For the mechanical allodynia test, a Von-Frey filament with a bending

force of 2 g was used to stimulate low threshold mechanoreceptors. In both tests, the medial plantar surface of the hind paw was poked by the tip of the filament from below the mesh grid until bending of the filament occurred. Both tests followed the same experimental procedure, which consists of five successive applications per trial with a resting period of 5 minutes to avoid conditioning paw withdrawal. The paw withdrawal frequency (PWF) to each force was calculated from five applications. The results of the three provided the average PWF.



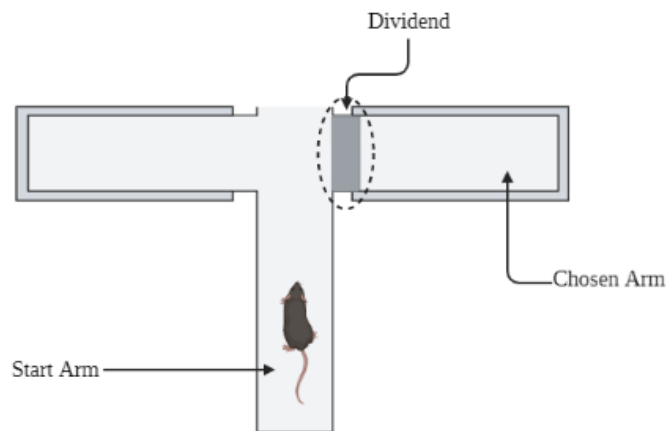
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**Figure 4: Mechanical hyperalgesia and allodynia apparatus.**

Mechanical hyperalgesia and allodynia were assessed by measuring the paw withdrawal frequency to a 15 g mechanical stimulus (noxious) and to a 2 g mechanical stimulus (non-noxious) when applied to the plantar surface of the hind paw of rats. The tests were performed using the following equipment: Ugo Basile platform with a mesh grid (Gemonio VA, Italy; Length 90 cm, Width 14 cm, Height 10 cm) and aesthesio filaments: von Frey Filament with a bending force of 2 g and 15 g (USA, Patent number 5823969–8512259).

## 2. Spontaneous Alternation T-maze Test

After 15 minutes of habituation, the rats were placed in the start arm of the T-maze (Fig. 7) where they alternated between the right and left arms. Once the rat entered into one arm, a barrier was used to entrap the animal in the chosen arm for 30 seconds. Then the rat was placed back at the initial position in the start arm and was allowed to choose to alternate between the right and left open arms (Deacon & Rawlins, 2006). Three trials were recorded, and the percentages of successful trials were compared among both experimental groups.



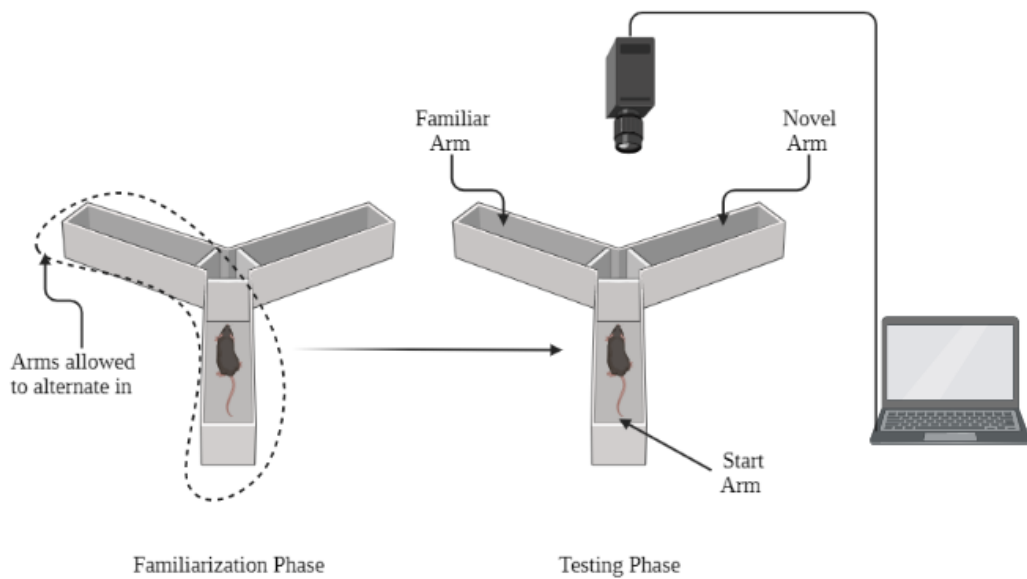
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**Figure 5: T-maze apparatus.**

The aim is to test the working memory. The animal is placed in the start arm of the apparatus and is allowed to alternate between the right and left arms. The animal is entrapped in the chosen arm for 30 seconds and is placed back in the start arm for it to alternate between both arms again.

### ***3. Spontaneous Alternation Y-maze Test***

The apparatus (Fig. 8) consists of three identical arms (10 cm wide and 40 cm long) that are equally spaced (120° apart). The test constituted of two phases: an acquisition phase and a testing phase. During the acquisition phase, the Novel (N) arm is locked with a barrier and the animal was placed in the Start (S) arm. The rodent was allowed to explore for 10 minutes the S and the Familiar (F) arms. An inter-phase separation time of 1 hour was achieved where the animal was returned to its cage and the apparatus was cleaned with 70% ethanol to avoid any odor cues. During the testing phase, the N arm was opened and the animal was allowed to alternate between the three arms (S, N, and F arms) for 5 minutes. The testing phase was recorded and was analyzed via the software ANY-maze Video Tracking System (Stoelting Co.) for the number of entries to the novel arm; in addition to the time spent. The data collected for the number of entries and for the time spent in the novel arm was analyzed, examined separately, and then compared between the experimental and control groups.



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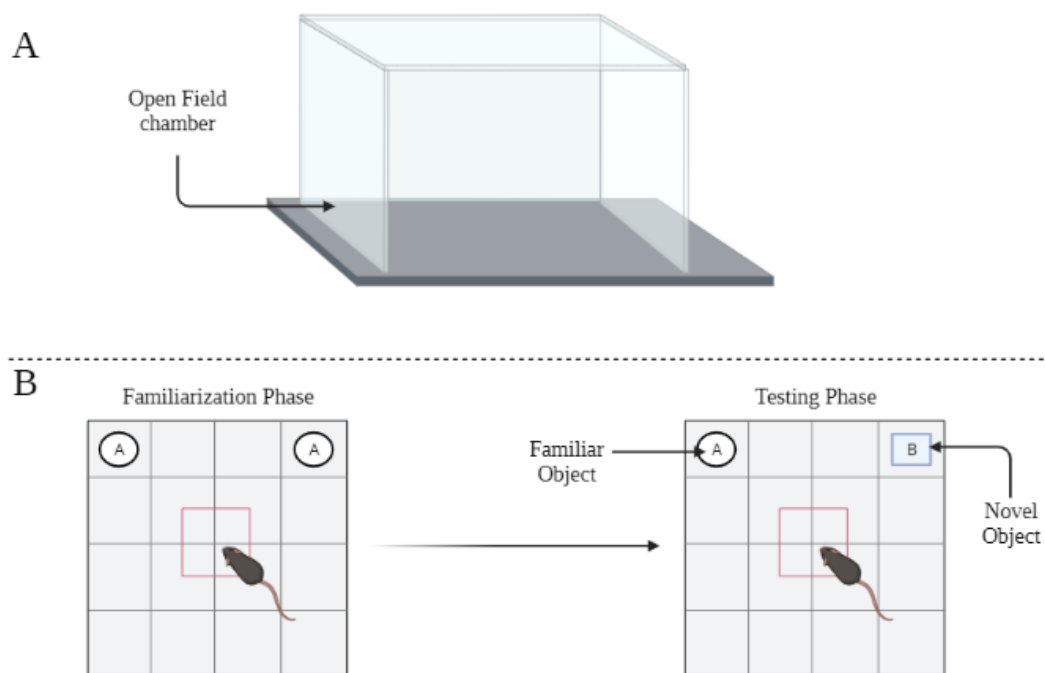
**Figure 6: Y-maze apparatus.**

The purpose is to test the reference memory and the test is divided into two phases: acquisition and testing. In the acquisition phase, the Novel (N) arm is locked. The animal was placed in the Start (S) arm and was allowed to alternate between its initial position and the Familiar (F) arm. In the testing phase, the N arm is unlocked. The animal was placed again in the S arm and was allowed to explore all three arms (S, N, and F) for 5 minutes.

#### ***4. Novel Object Recognition Test (NOR)***

The NOR test was performed in the Open Field apparatus (Fig. 9) in both experimental groups. The test consisted of two phases: a familiarization phase and a testing phase. During the familiarization phase, two identical cubes are placed adjacently at the corners of the open field where the rats were placed facing the objects and were allowed to explore for 5 minutes. An inter-phase separation time was 5 minutes where the animal was returned to its cage and the apparatus was cleaned with 70% ethanol to avoid any odor cues. During the testing phase, one of the objects was

replaced with a novel object and was kept at the same location; then the animal was allowed to explore for 5 minutes (Leger et al., 2013). The testing phase was recorded and analyzed via the software ANY-maze Video Tracking System (Stoelting Co.) for the number of entries to the novel zone; in addition to the time spent in it. The data collected for the number of entries and for the time spent in the novel zone was analyzed, examined separately, and then compared between the experimental and control groups.



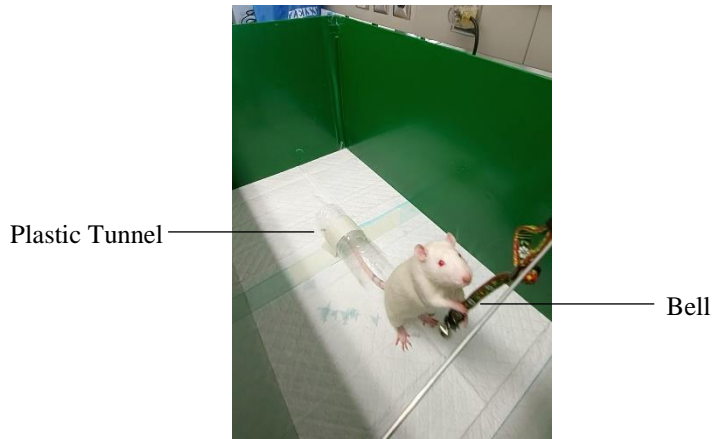
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**Figure 7: Novel-object recognition apparatus.**

The purpose is to test the recognition memory and the test is divided into two phases: familiarization and testing. In the familiarization phase, two identical objects are placed at the periphery of the open-field facing the animal. The latter was allowed to explore both objects for 5 minutes. In the testing phase, one of the objects was replaced with a novel one. The animal was allowed again to explore the familiar and novel objects.

### ***5. Sequential Learning Test***

In this test, the animals were trained to perform two tasks in sequence: to ring a bell following their entrance into a plastic tunnel. The whole training process takes 8 consecutive days, where the rats were trained for 1 hour daily. A day prior to the initiation of the training, each rat is placed in the behavioral apparatus (Fig. 10) for 1 hour to adapt to the new environment. In addition, each rat is conditioned to the sound of 2 clicks by receiving a piece of cake. On the first 4 days of the training, each rat is placed in the behavioral apparatus at a specific position facing the plastic tunnel that is open on both ends. Each rat is taught for 1 hour to enter the plastic tunnel from the same initial position. On days 5 and 6 of the training, each rat is placed in the behavioral apparatus at a specific position facing the bell, without the plastic tunnel. Each rat is taught for 1 hour to ring the bell with its forepaws. On days 7 and 8 of the training, each rat is taught for 1 hour to perform both tasks in sequence upon placing the rat in the behavioral apparatus facing both the tunnel and the bell. Upon successful completion of the learned task, the rat is rewarded with a piece of cake along with the sound of 2 clicks. On day 9, all rats are tested to perform both tasks in sequence and upon successful completion, the rats will only hear the sound of 2 clicks. Three trials were recorded with a resting period of 5 minutes between each trial. The test is repeated again at week 4 before animal sacrifice. The percentages of successful trials and the time spent to complete the tasks in sequence were analyzed and compared among the two groups at baseline level before CPFX treatment and at week 4 before animal sacrifice.



**Figure 8: Sequential Learning Test.**

The aim is to test the episodic memory. Animals are trained for 8 consecutive days to perform two tasks in sequence: to enter a plastic tunnel, then to ring a bell. The animals were conditioned to a sound of two clicks when they received a piece of cake. Throughout the training process, the rodents were rewarded with a piece of cake and with the sound of 2 clicks upon successful completion of a task. During test day, once the animal performed both tasks in sequence, it was rewarded only with the induction of the sound of 2 clicks.

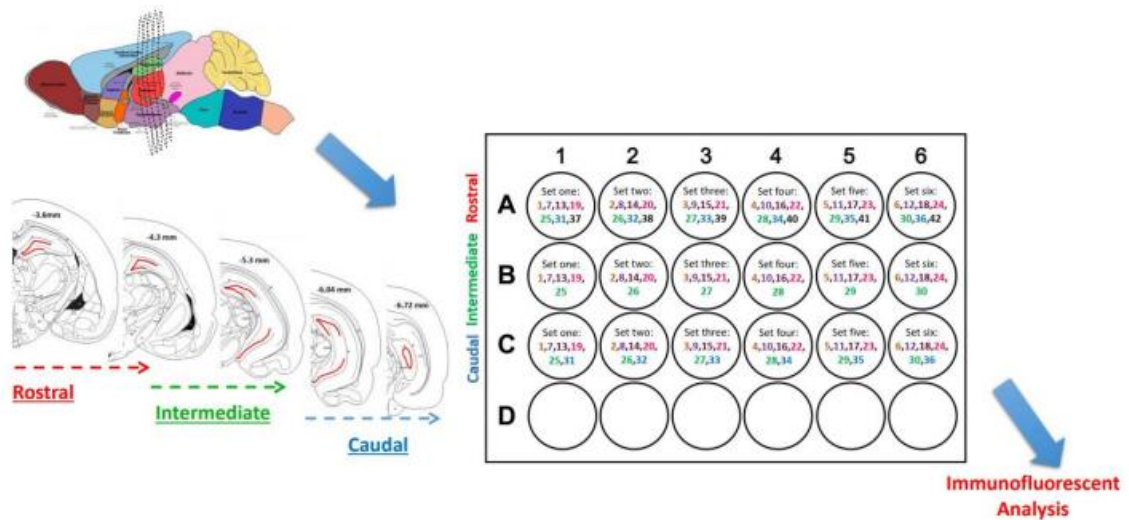
#### **D. Animal Euthanasia: Perfusion and Brain Extraction**

At week 4, all animals were deeply anesthetized via an intraperitoneal injection of ketamine (80 mg/Kg, Ketalar, Panpharma, Luitré, France) and xylazine (10 mg/Kg, Xyla; Interchemie, Harju County, Estonia). They were perfused trans-cardially with 200 mL of 0.9% saline followed by 200 mL of 4% formalin. The chest cavity was opened to expose the heart and a perfusion cannula was introduced into the left ventricle to allow 0.9% saline to flow in the circulation; the right ventricle was cut to clear the blood from the body. Once cleared, 4% formalin was allowed to flow in to fix the tissues. The skull was exposed at the midline through an incision and opened with a bone cutter. The brain was carefully extracted and placed in a conical tube containing 15 mL of 4% paraformaldehyde. The brains were fixed overnight at room temperature with 4% paraformaldehyde. The next day, they were transferred to a 30% sucrose solution in 0.1

M of phosphate-buffered saline (PBS) solution and were stored at 4°C for 3 days until full impregnation.

### **E. Brain Sectioning**

The entire region of the DG of the hippocampus was collected and divided topographically into three regions: rostral (coordinates: -2.12 to -3.7 rostral to bregma), intermediate (coordinates: -3.7 to -4.9 intermediate to bregma), and caudal (coordinates: -4.9 to -6.3 caudal to bregma) (Paxinos & Watson, 1983). The perfused brains were sectioned coronally at a thickness of 40  $\mu\text{m}$  on a freezing microtome. Sections were sliced serially from the rostral to the caudal extent of the DG of the hippocampus following the rostro-caudal coordinates of -2.12 to -6.6 mm relative to bregma. For unbiased cell stereology, the fractionating method was adopted for systematic-random sampling of the brain sections (Gundersen, Jensen, Kiêu, & Nielsen, 1999) (Fig. 6). The sections were collected in a 24-well plate as six sets where each set contained seven rostral, five intermediate, and six caudal sections. All sets contained 15 mM sodium azide dissolved in PBS. The first brain section was placed in the first set, the fourth section was placed in the fourth set, and the sixth section was placed in the sixth set. The process was repeated such that the seventh section was placed in the first set; hence, both sections would be 300  $\mu\text{m}$  apart (Chamaa et al., 2021). With that being said, each set serves as a random topographical representation of the hippocampal region of interest.



**Figure 9: The Fractionator Method.**

Free-floating coronally sectioned brains at a thickness of 40 $\mu$ m are collected in a 24-well plate based on the topographical regions of the dentate gyrus of the hippocampus: rostral, intermediate, and caudal. Each number in each set represents the number of the brain section that was placed while the brain was cut on the freezing microtome. Adopted and modified from (Chamaa et al., 2021).

## F. Immunofluorescence Staining

One representative well was randomly chosen. Thus, the total number of brain sections stained per rat was around 18-20 sections. The free-floating sections were first washed three times, for 5 minutes each, with 0.1 M PBS in a 24-well plate. For the detection of BrdU-positive cells, the tissues were incubated at 37°C with 2N HCl for 30 minutes to denature the DNA so that the primary anti-BrdU antibody will have access to the previously incorporated BrdU from the administered animal injections. After DNA denaturation, the tissues were washed with 0.1 M PBS for 5 minutes. To neutralize the acidity, a solution of sodium borate (0.1 M, pH 8.5) was added for 10 minutes at room temperature. The samples were again washed with 0.1 M PBS three times for 5 minutes each and they were later transferred to a 10% blocking solution (10% NGS, 10% BSA, and 0.1% Triton X diluted in 0.1 M PBS) for 1 hour at 4°C to decrease the chances of non-specific bindings. The tissues were then directly incubated overnight at 4°C with

primary mouse BrdU antibody (1:250; Santa Cruz) and with primary rabbit NeuN antibody (Neuronal Nuclear Antigen, 1:1000; Neuromics). On the next day, the samples were washed with 0.1 M PBS three times, for 5 minutes each. They were later incubated in the dark at room temperature with secondary goat anti-rat 568 (1:500; Abbexa) and with secondary goat anti-rabbit 488 (1:500; Abbexa) on a shaker for 2 hours. All primary and secondary antibodies were diluted in a 3% blocking solution (3% NGS, 3% BSA, and 0.1% Triton X). The tissues were then washed three times with 0.1 M PBS for 5 minutes, with the third wash being in the mounting plate. Finally, the brain sections were mounted on slides with anti-fade mounting media with DAPI (4', 6-Diamidino-2-Phenylindole, Abcam, USA) and covered with thin glass coverslips.

### **G. Cell Quantification and Confocal Microscopy**

To quantify the number of stem cells in the SGZ that have undergone neurogenesis, BrdU and NeuN-labeled cells were counted using the confocal microscope (Zeiss LSM 710) on the 40X-oil objective. The count was achieved on the brain sections that were chosen from the representative set of each topographic region (rostral, intermediate, and caudal) of the DG of the hippocampus. The final number of BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells is multiplied by 6 (the number of representative sets in each topographic region) to obtain the total count of labeled cells in each of the rostral, intermediate, and caudal regions. To obtain the total number of BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells in the whole DG of the hippocampus, the sum of labeled cells in the rostral, intermediate, and caudal regions were added. The data was presented as the % of BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells in the DG of the hippocampus and compared among the experimental and control groups.

Images were acquired using the confocal microscope (Zeiss LSM 710) on the 40X-oil objective. Serial Z-stack was used to capture the BrdU-positive cells distributed within the 40  $\mu$ m thick section and tile scan was used to capture the DG of the hippocampus. The acquired images were analyzed using the Zeiss ZEN 2009 image-analysis software and were processed with maximal intensity projection. To achieve consistency, BrdU and NeuN-labeled cells were counted by a single-blinded experimenter, and the images were captured under the same microscopic and laser parameters.

#### **H. Statistical Analysis**

The behavioral tests and the BrdU-labeled cell counts were analyzed at every time point and were expressed as the mean ( $\bar{X}$ )  $\pm$  the standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was conducted for comparison between the control and the CPFY-treated groups, with Sidak's multiple comparisons test. Repeated measure ANOVA was conducted for comparison between the control and the CPFY-treated groups in the behavioral tests within the tested time points, with Dunnett's multiple comparisons test. A P value  $< 0.05$  was considered significant. The plotting of figures and all statistical analyses were performed using the GraphPad Prism 7 package (GraphPad Software, Inc., CA, USA).

## CHAPTER III

### RESULTS

#### A. Effect of CPFY Administration on Sensory Functions: Behavioral Studies

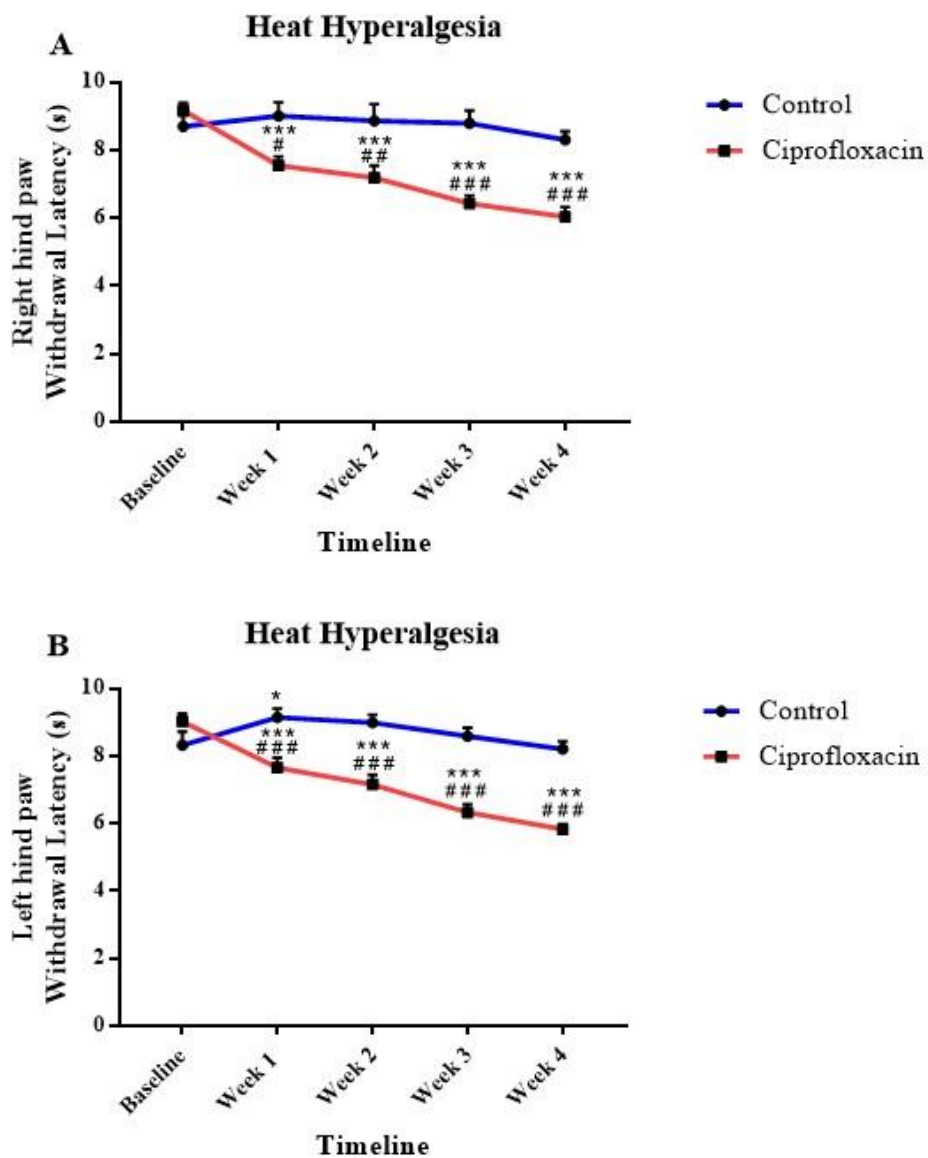
##### 1. CPFY-Treated Rats Developed Heat Hyperalgesia

The heat hyperalgesia test was conducted on all rats of both groups to assess the animals' response to a thermal stimulus. A decrease in paw withdrawal latency indicates an increased sensitivity to the noxious heat stimulus.

In the right paw, the CPFY-treated group ( $7.55 \pm 0.27$  at week 1;  $7.19 \pm 0.36$  at week 2;  $6.43 \pm 0.23$  at week 3;  $6.04 \pm 0.28$  at week 4) exhibited a statistically significant reduction in paw withdrawal latency when compared to the control group at weeks 1 ( $9.01 \pm 0.4$ ;  $P = 0.0116$ ), 2 ( $8.86 \pm 0.5$ ;  $P = 0.0028$ ), 3 ( $8.79 \pm 0.38$ ;  $P < 0.0001$ ), and 4 ( $8.31 \pm 0.24$ ;  $P < 0.0001$ ), respectively. When compared to baseline ( $9.17 \pm 0.22$ ), the CPFY-treated group showed a statistically significant decline in paw withdrawal latency at weeks 1 ( $P = 0.0001$ ), 2 ( $P = 0.0001$ ), 3 ( $P = 0.0001$ ), and 4 ( $P = 0.0001$ ), respectively.

In the left paw, the CPFY-treated group ( $7.65 \pm 0.3$  at week 1;  $7.15 \pm 0.29$  at week 2;  $6.33 \pm 0.24$  at week 3;  $5.83 \pm 0.15$  at week 4) showed a statistically significant decrease in paw withdrawal latency when compared to the control group at weeks 1 ( $9.15 \pm 0.26$ ;  $P = 0.0007$ ), 2 ( $8.99 \pm 0.23$ ;  $P < 0.0001$ ), 3 ( $8.59 \pm 0.25$ ;  $P < 0.0001$ ), and 4 ( $8.2 \pm 0.23$ ;  $P < 0.0001$ ) respectively. When compared to baseline ( $9.02 \pm 0.23$ ), the CPFY-treated group exhibited a statistically significant reduction in paw withdrawal latency at weeks 1 ( $P = 0.0003$ ), 2 ( $P = 0.0001$ ), 3 ( $P = 0.0001$ ), and 4 ( $P = 0.0001$ ) respectively. Moreover, when compared to baseline ( $8.33 \pm 0.4$ ), the control group ( $9.15$

$\pm 0.26$ ;  $P = 0.0419$ ) illustrated a statistically significant increase in paw withdrawal latency at week 1.



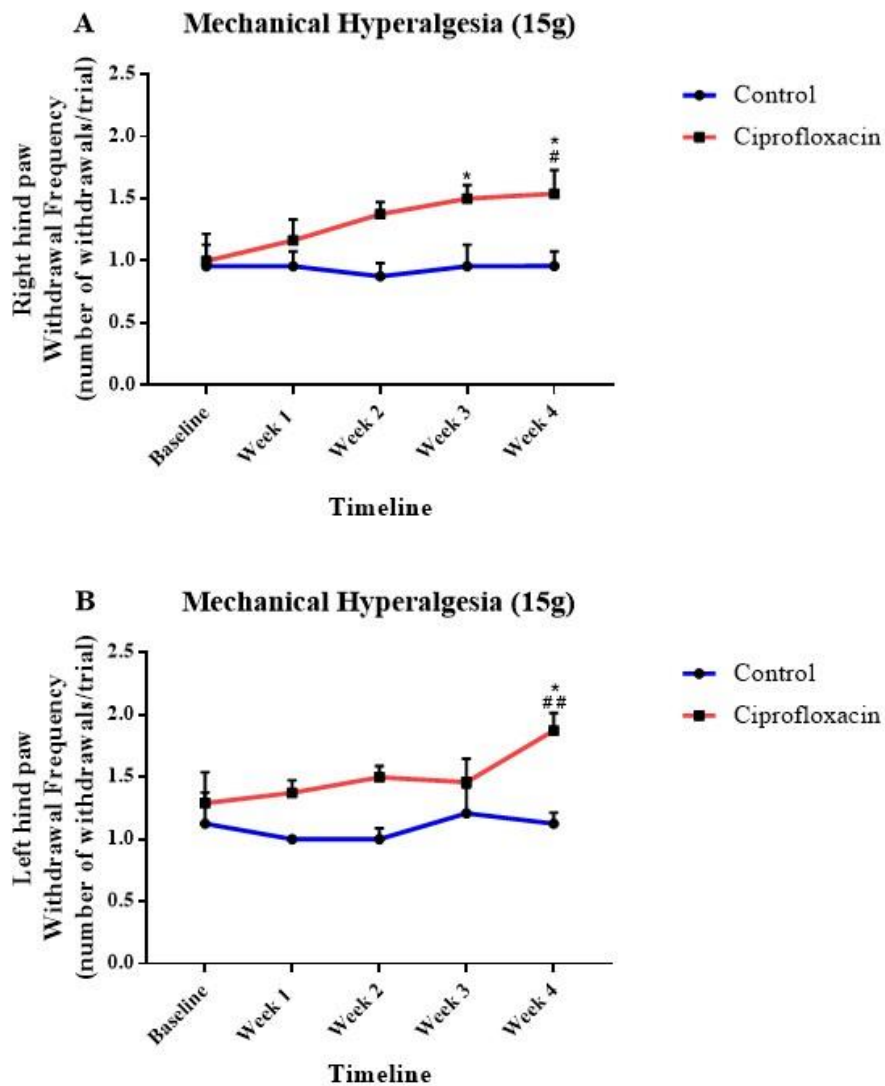
**Figure 10: Heat hyperalgesia test results.** **A)** Effect of CPF administration on heat hyperalgesia in the right hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*\*\*:  $P < 0.001$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (#:  $P < 0.05$ ; ##:  $P < 0.01$ ; ###:  $P < 0.001$ ). **B)** Effect of CPF administration on heat hyperalgesia in the left hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*:  $P < 0.05$ ; \*\*\*:  $P < 0.001$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (###:  $P < 0.001$ ). \*: indicates significance from baseline. #: indicates significance from control group

## ***2. CPMX-Treated Rats Developed Mechanical Hyperalgesia***

The mechanical hyperalgesia test was conducted on all rats of both groups to assess the animals' response to a noxious mechanical stimulus. An increase in paw withdrawal frequency (PWF) indicates an increased sensitivity to the harmful mechanical stimulus.

In the right paw, the CPMX-treated group ( $1.54 \pm 0.19$ ) showed a statistically significant increase in PWF when compared to the control group ( $0.96 \pm 0.12$ ;  $P = 0.0407$ ) at week 4. When compared to baseline ( $1 \pm 0.16$ ), the CPMX-treated group exhibited a statistically significant rise in PWF at weeks 3 ( $1.5 \pm 0.11$ ;  $P = 0.0453$ ) and 4 ( $1.54 \pm 0.19$ ;  $P = 0.0266$ ), respectively.

In the left paw, the CPMX-treated group ( $1.88 \pm 0.14$ ) at week 4 illustrated a statistically significant increase in PWF when compared to baseline ( $1.29 \pm 0.25$ ;  $P = 0.0233$ ) and to control group ( $1.13 \pm 0.088$ ;  $P = 0.0073$ ).



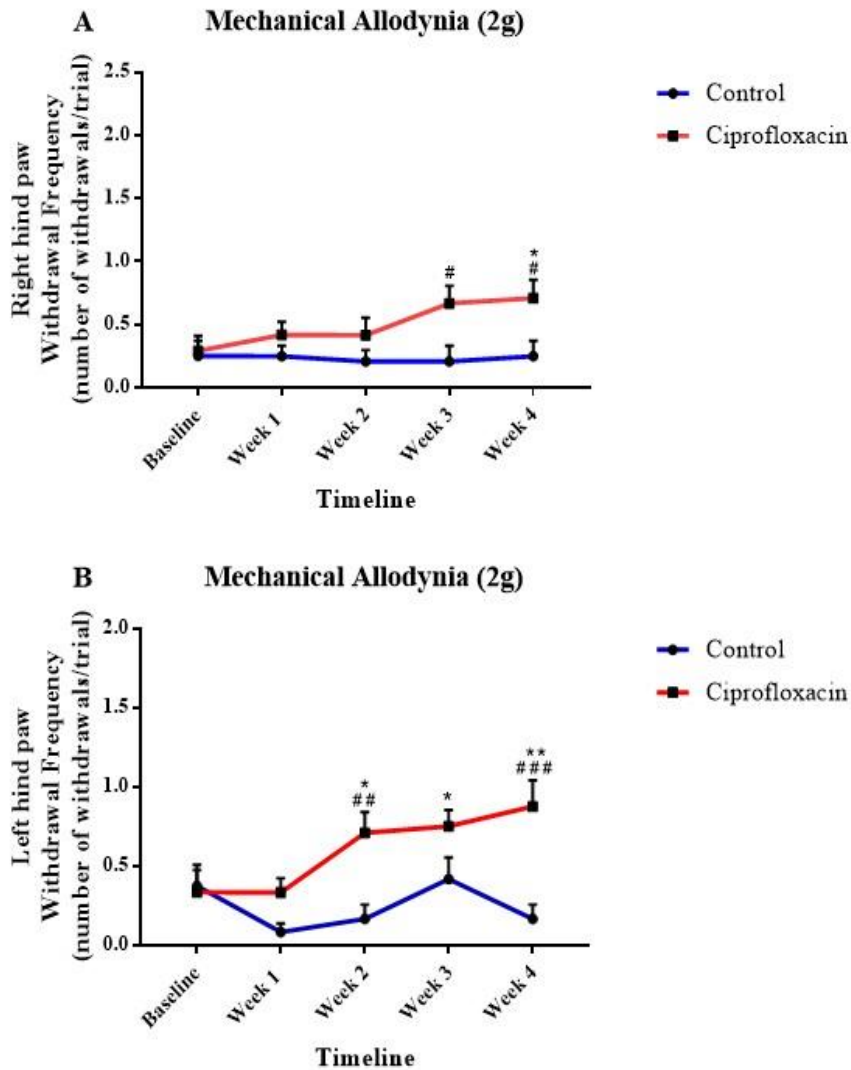
**Figure 11: Mechanical hyperalgesia test result.** **A)** Effect of CPMX administration on mechanical hyperalgesia in the right hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*:  $P < 0.05$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (#:  $P < 0.05$ ). **B)** Effect of CPMX administration on mechanical hyperalgesia in the left hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*:  $P < 0.05$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (##:  $P < 0.01$ ). \*: indicates significance from baseline. #: indicates significance from control group.

### ***3. CPFY-Treated Rats Developed Mechanical Allodynia***

The mechanical allodynia test was conducted on all rats of both groups to assess the animals' response to a purely tactile stimulus. A rise in PWF indicates an increased sensitivity to the non-noxious tactile stimulus.

In the right paw, the CPFY-treated group ( $0.7 \pm 0.14$  at week 3;  $0.71 \pm 0.15$  at week 4) showed a statistically significant increase in PWF when compared to the control group at weeks 3 ( $0.21 \pm 0.13$ ;  $P = 0.0435$ ) and 4 ( $0.25 \pm 0.12$ ;  $P = 0.0435$ ), respectively. At week 4, the CPFY-treated group ( $0.71 \pm 0.15$ ) exhibited a statistically significant rise in PWF when compared to baseline ( $0.3 \pm 0.12$ ;  $P = 0.0329$ ).

In the left paw, the CPFY-treated group ( $0.71 \pm 0.13$  at week 2;  $0.88 \pm 0.17$  at week 4) exhibited a statistically significant rise in PWF when compared to the control group at weeks 2 ( $0.17 \pm 0.09$ ;  $P = 0.0089$ ) and 4 ( $0.17 \pm 0.09$ ;  $P = 0.0003$ ), respectively. When compared to baseline ( $0.33 \pm 0.14$ ), the CPFY-treated group showed a statistically significant increase in PWF at weeks 2 ( $0.71 \pm 0.13$ ;  $P = 0.0375$ ), 3 ( $0.75 \pm 0.1$ ;  $P = 0.0176$ ), and 4 ( $0.88 \pm 0.17$ ;  $P = 0.0014$ ), respectively.

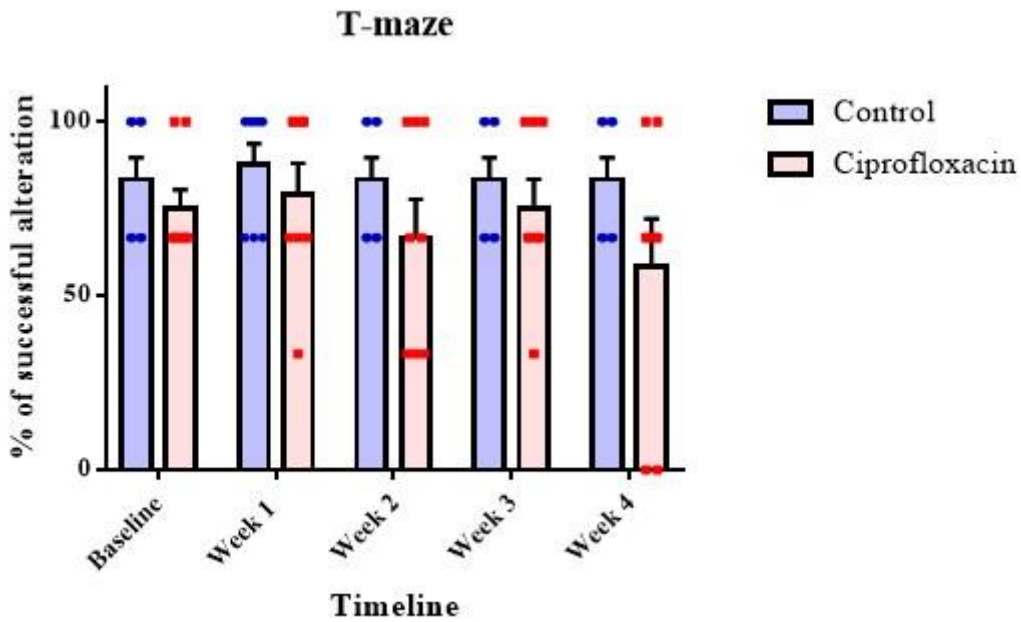


**Figure 12: Mechanical allodynia test result.** **A)** Effect of CPF administration on mechanical allodynia in the right hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*:  $P < 0.05$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (#:  $P < 0.05$ ). **B)** Effect of CPF administration on mechanical allodynia in the left hind paw. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ). Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (##:  $P < 0.01$ ; ###:  $P < 0.001$ ). \*: indicates significance from baseline. #: indicates significance from control group.

## B. Effect of CPFY Administration on Cognitive Functions: Behavioral Studies

### 1. Effect of CPFY Administration on Spatial Working Memory

The spontaneous alternation T-maze test was conducted on all rats of both groups to assess spatial working memory. A slight decrease in spatial working memory was observed in the CPFY-treated group at weeks 2 ( $66.67 \pm 10.9$ ) and 4 ( $58.34 \pm 13.73$ ) when compared to baseline ( $75 \pm 5.45$ ) and to control group ( $83.34 \pm 6.3$  at week 2;  $83.34 \pm 6.3$  at week 4); however, no statistical significance was detected.



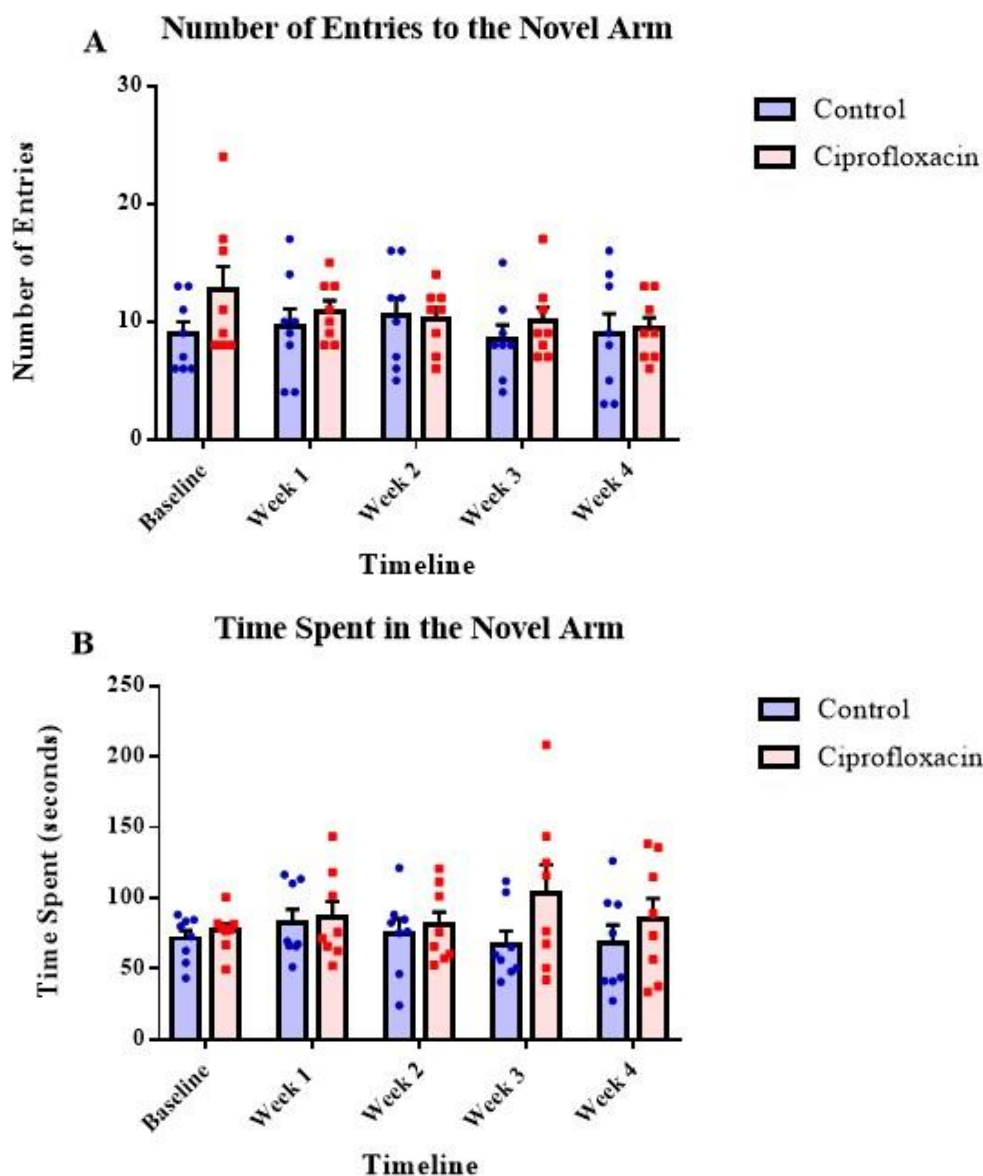
**Figure 13: Spontaneous alternation T-maze test result.**

Effect of CPFY administration on spatial working memory in the T-maze test. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups.

## ***2. Effect of CPF<sub>X</sub> Administration on Reference Memory***

The spontaneous alternation Y-maze test was conducted on all rats of both groups to examine the effect of CPF<sub>X</sub> on reference memory. The rodents' exploratory behavior prompts them to alternate more frequently and to explore the novel arm (N) rather than the familiar one (F). Thus, this test will measure the number of entries and time spent in the N arm.

Our results have shown no statistical significance for the number of entries to the N arm when both groups were compared at each time point and when compared to baseline. At week 3, the CPF<sub>X</sub>-treated group ( $76.4 \pm 9.44$ ) exhibited a slight increase in the time spent in the N arm when compared to the control group ( $58.1 \pm 5.05$ ); however, no statistical significance was recorded.

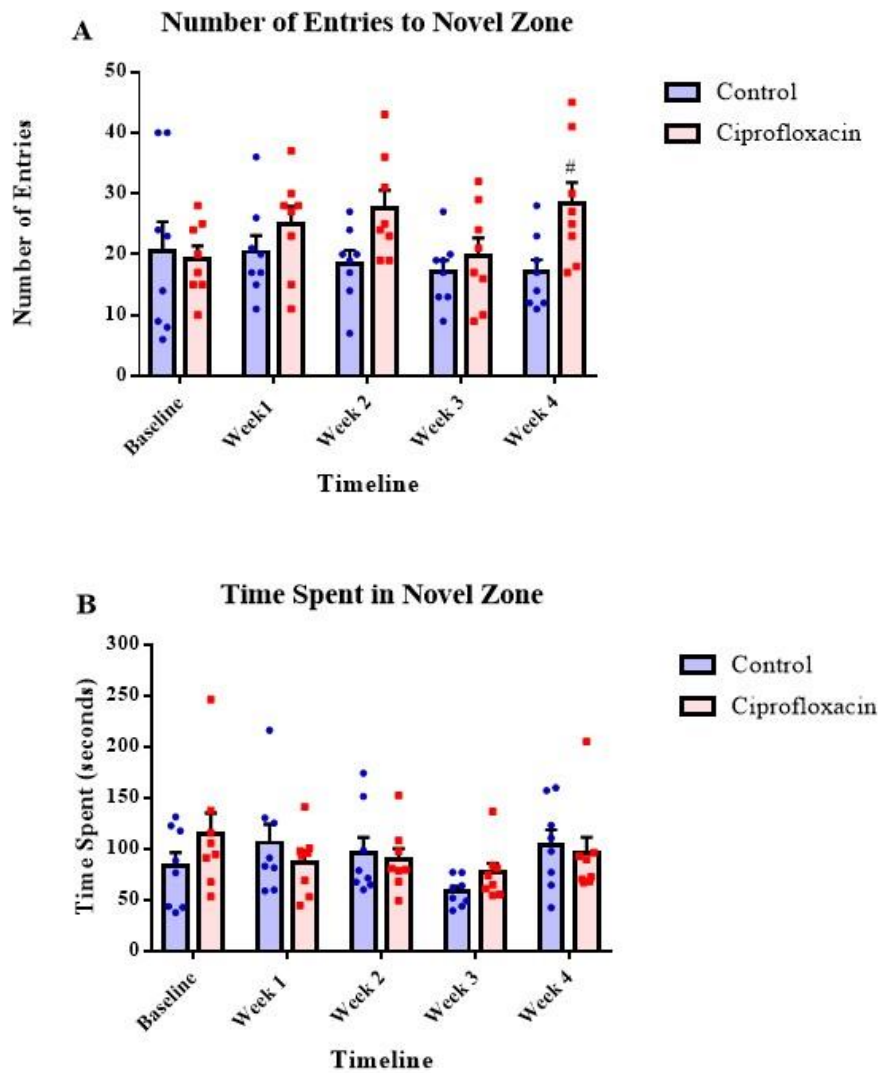


**Figure 14: Spontaneous alternation Y-maze test result.** **A)** Effect of CPF administration on the number of entries to the N arm in the Y-maze test. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups. **B)** Effect of CPF administration on the time spent in the N arm of the Y-maze test. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups.

### ***3. Effect of CPF<sub>X</sub> Administration on Recognition Memory***

The novel-object recognition (NOR) test was conducted on all rats of both groups to assess the effect of CPF<sub>X</sub> on recognition memory. Healthy rodents have clear tendency to explore their surroundings, thus when put in an open field with a novel object, they will attempt to discover the novel (N) zones and to alternate more frequently in them rather than familiar (F) ones.

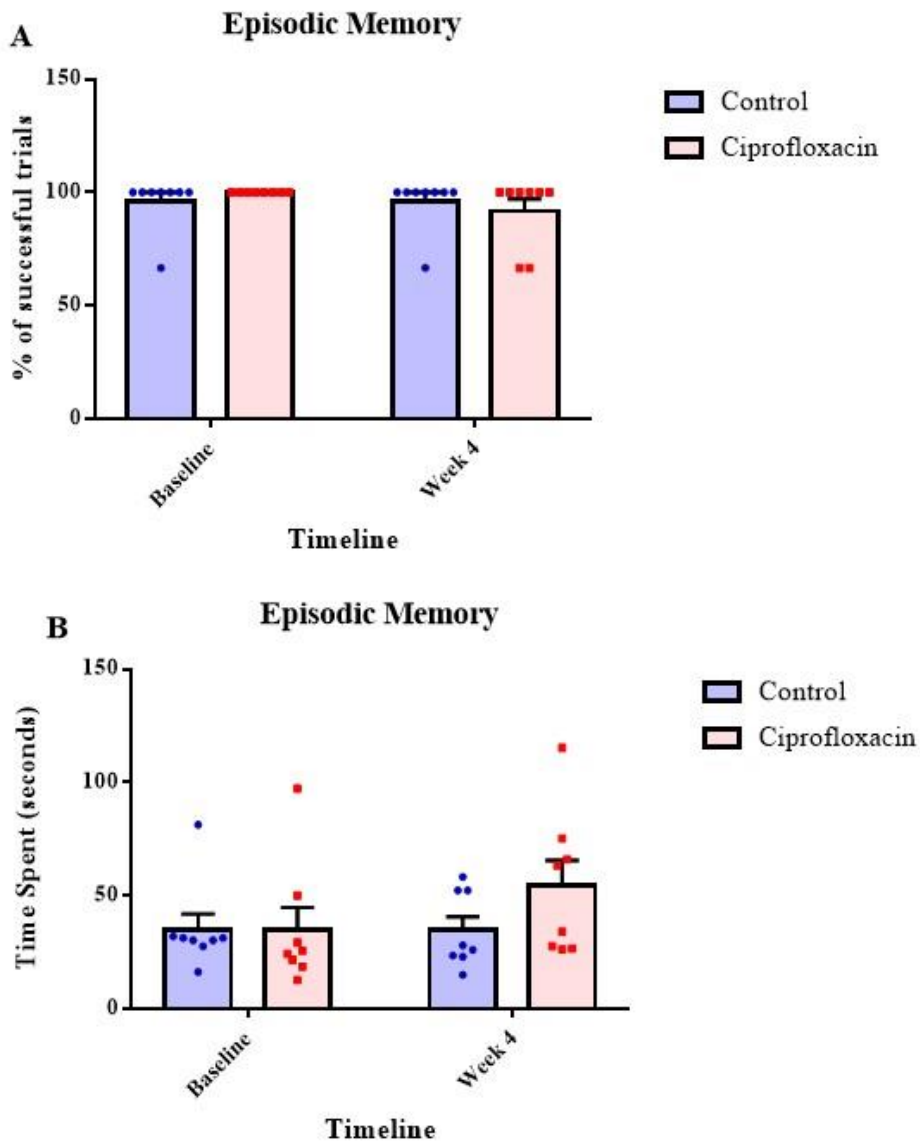
Our findings have demonstrated that the CPF<sub>X</sub>-treated group showed a decrease in the number of entries to the zone at week 3 ( $19.75 \pm 2.95$ ) when compared to the control group ( $17.13 \pm 1.95$ ); followed by a statistically significant increase when compared to the control group ( $17 \pm 2.14$ ) at week 4 ( $28.25 \pm 3.58$ ;  $P = 0.0449$ ). For the time spent in the N zone, no statistical significance was recorded when both groups were compared at each time point and when compared to baseline.



**Figure 15: Novel-object recognition (NOR) test result. A)** Effect of CPF<sub>X</sub> administration on the number of entries to the novel zone. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups (#:  $P < 0.05$ ). **B)** Effect of CPF<sub>X</sub> administration on the time spent in the novel zone. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups. #: indicates significance from the control group.

#### ***4. Effect of CPFY Administration on Sequential Learning***

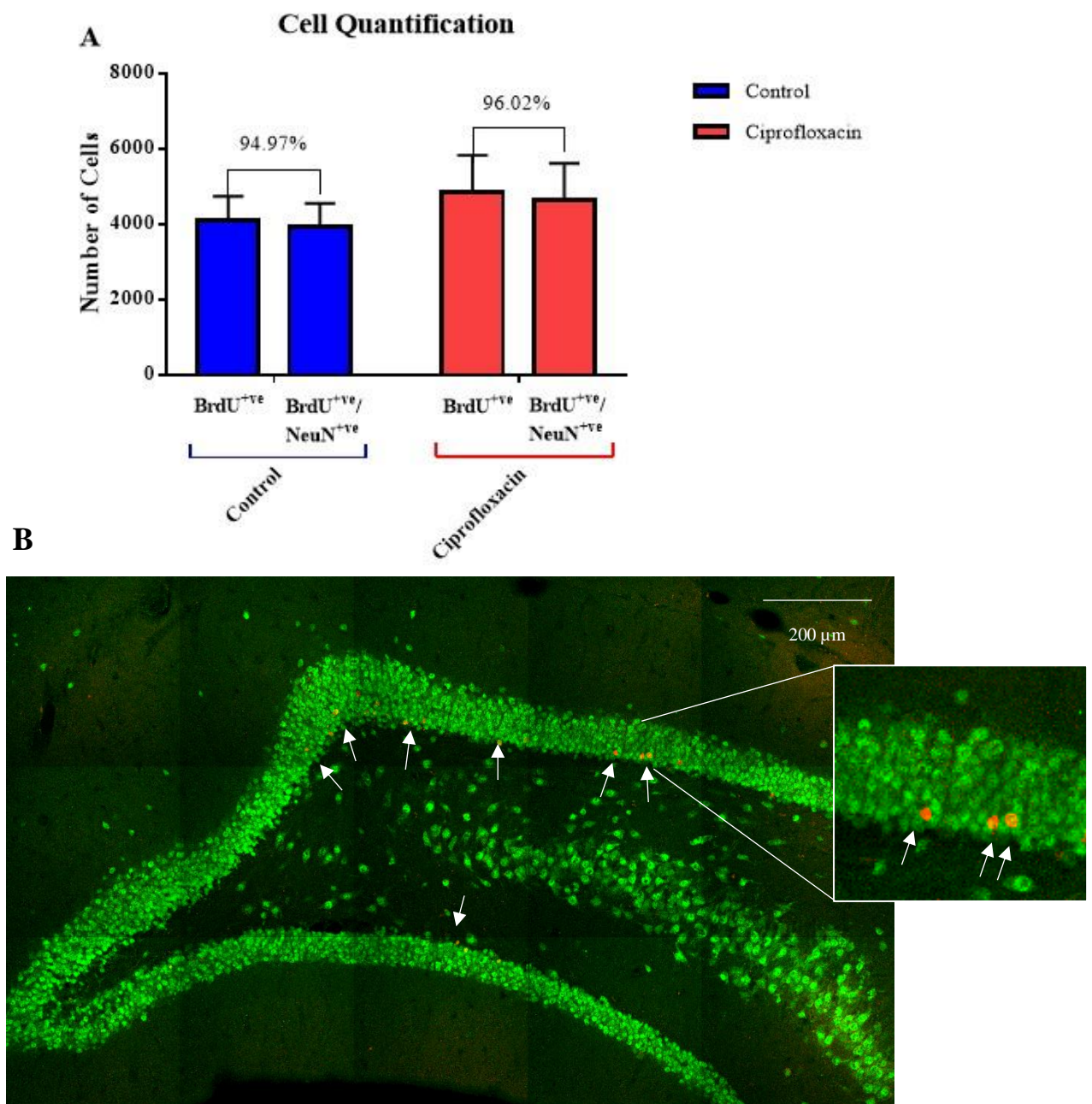
All rats were able to learn and execute the two assigned tasks in a sequential manner prior to treatment. Rats exposed to CPFY performed comparably to those in the control group when tested at week 4 post-treatment. No statistically significant difference was found when the CPFY-treated group was compared to the control group or to baseline. Nonetheless, the CPFY-treated group ( $54.33 \pm 11.22$ ) showed an increase in the time spent to perform both tasks sequentially when compared to the control group ( $34.81 \pm 5.9$ ) and to baseline ( $34.96 \pm 9.72$ ); however, no statistical significance was recorded.



**Figure 16: Sequential learning test result.** **A)** Effect of CPMX administration on episodic memory. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups. **B)** Effect of CPMX administration on the time spent to perform both tasks in sequence. Data are represented as mean  $\pm$  SEM. Two-way ANOVA test followed by Dunnett's multiple comparisons test were used to compare the mean at each time point with the baseline. Two-way ANOVA test followed by Sidak's multiple comparisons test were used to compare the means at each time point between both groups.

### **C. Effect of CPF<sub>X</sub> Administration on Hippocampal Neurogenesis**

To assess the effect of CPF<sub>X</sub> on neurogenesis in the DG of the hippocampus, double-labeled BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells were counted after animal sacrifice on week 4. No statistical significance was observed in the number of co-labeled BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cells between the control group ( $3871.2 \pm 600.47$ ) and the CPF<sub>X</sub>-treated group ( $4645.2 \pm 967.89$ ). In addition, no statistical significance was detected in the number of newly-proliferating cells that have committed to the neuronal lineage between the control group ( $94.97\% \pm 0.83$ ) and the CPF<sub>X</sub>-treated group ( $96.02\% \pm 0.28$ ). A representative confocal image of the newly-born neurons in the intermediate region of the DG of the hippocampus of the control group is illustrated in Figure 18, *B*.



**Figure 17: Cell quantification and confocal microscopy results. A)** The total number of co-labeled BrdU<sup>+</sup>ve/NeuN<sup>+</sup>ve cells (colored in yellow) in the DG of the hippocampus of both groups. The percentages of BrdU<sup>+</sup>ve cells that have committed to the neuronal lineage by expressing NeuN in both groups. **B)** Confocal image of the intermediate region of the DG of the hippocampus of the control group. BrdU<sup>+</sup>ve cells (indicated by white arrows) were integrated into GCL of the DG of the hippocampus. Tile scan image was taken with a 40X oil objective. Scale bar is at 200  $\mu$ m. Mature granule cell neurons are colored in green since they express NeuN, while newly-proliferating cells are colored in red since they express BrdU.

## CHAPTER IV

### DISCUSSION

This study was conducted to assess the safety profile of CPF<sub>X</sub> by examining its effect on cognitive and sensory functions in healthy male rats. Our findings have demonstrated that oral treatment with CPF<sub>X</sub>, under normal conditions, did not impact cognitive performance or adult hippocampal neurogenesis. However, CPF<sub>X</sub> did alter sensory processing by inducing an increased sensitivity to heat and mechanical stimulation over time.

CPF<sub>X</sub>, the most widely used fluoroquinolone, is a lipophilic antibacterial agent capable of crossing the BBB into the brain parenchyma and CSF under physiological conditions. In the CSF, CPF<sub>X</sub> can be present in concentrations about 10% of those in plasma. However, this concentration would significantly increase when the meninges are inflamed, intensifying the severity of CNS toxicity. The neurotoxic effects, such as psychosis, delirium and confusion, have been reported in 1-2% of patients treated with CPF<sub>X</sub> (Tomé & Filipe, 2011). In this pre-clinical study, oral administration of CPF<sub>X</sub> for 2 weeks did not cause any changes in cognitive behaviors. Rats subjected to the novel-object recognition and Y-maze tests, showed no statistically significant differences from control.

In the novel-object recognition test, CPF<sub>X</sub>-treated animals were able to distinguish the novel object from the familiar one. The number of entries to the novel zone, as well as the time spent in it, were similar to that of the control group when measured over a period of three weeks following treatment. While a statistical significance was noted at week 4, this increase may not be attributed to the effect of

CPFEX on recognition memory, because by then, the effect of CPFEX had worn off, given that the serum half-life of CPFEX is approximately 4 hours (Schaeffer, 2003).

In the Y-maze test, animals in the CPFEX-treated group were also able to distinguish the novel arm from the familiar one, a performance that was comparable to that of the control group over a period of 4 weeks. Our results are consistent with the findings of a previous study done on mice, showing that CPFEX does not affect spatial working or reference memory in rodents (Alhowail, 2019). However, it is worth noting that the CPFEX-treated mice in their study demonstrated a significant increase in total time spent in the open arms of the Elevated-Plus maze indicating the development of anxiety, a behavior that was detected in our rats based on observation only (Alhowail, 2019).

Unsurprisingly, CPFEX treatment had also no significant effect on spatial working memory or episodic memory when rats were tested at week 4 post-treatment. Animals in both groups were able to learn two tasks and to execute them in a sequential manner.

The lack of significant effect of oral administration of CPFEX on cognitive functions in healthy rats was inconsistent with clinical reports that have shown CPFEX-associated cognitive impairment ("Ciprofloxacin/levofloxacin/moxifloxacin," 2015) in 0.4-4.4% of patients (Abdalla, Abdalla, & Tsang, 2014). However, it should be pointed out that the cognitive changes seen in these patients were reversible once the drug was discontinued ("Ciprofloxacin/levofloxacin/moxifloxacin," 2015). Furthermore, the magnitude of CPFEX effect in clinical studies differs greatly among different populations, an observation that could be noted in animals had we substantially increased the sample size (Overholser et al., 2004).

To confirm the lack of CPF<sub>X</sub> impact on learning and memory, we further evaluated adult hippocampal neurogenesis, a mechanism that plays an important role in regulating cognitive abilities, particularly memory recognition and spatial working memory (Fanselow & Dong, 2010). Our results have demonstrated that the BrdU<sup>+ve</sup>/NeuN<sup>+ve</sup> cell counts in all regions of the hippocampus were comparable in both groups suggesting that CPF<sub>X</sub> did not alter the level of neurogenesis, an effect that is consistent with that of cognitive behaviors. Furthermore, our findings are in agreement with those of a previously published in-vitro study showing no effect of CPF<sub>X</sub> on cellular proliferation using the stem cell model of neurogenesis (Cao et al., 2015). Therefore, based on our observations, one can speculate that CPF<sub>X</sub> may be considered as a safe alternative to other antimicrobial agents that proved to negatively impact neurogenesis (B. Darwish et al., 2022; Möhle et al., 2016).

With respect to sensory processing in the peripheral and central nervous system, our behavioral data have provided evidence implicating CPF<sub>X</sub> in the development of heat and mechanical hyperalgesia. Rats treated with CPF<sub>X</sub> developed increased sensitivity to noxious thermal stimulation as well as to innocuous and noxious mechanical stimuli when measured over a period of 4 weeks. Symptoms started within 1 week after the administration of CPF<sub>X</sub> and lasted until animal sacrifice. These observations are consistent with previously published clinical reports raising concerns about the adverse effects of CPF<sub>X</sub> and its effect on the development of sensory peripheral neuropathy in treated patients ("Ciprofloxacin: Sensorimotor polyneuropathy: case report," 2017; Francis & Higgins, 2014; Jumma et al., 2013). Pain manifestations started as early as 24 hours upon administration of CPF<sub>X</sub> and were

mitigated upon cessation of therapy; however, symptoms persisted for over a year in 58% of patients (Francis & Higgins, 2014).

In this study, the development of hyperalgesia and allodynia in CPFEX-treated rats, suggests that CPFEX may have induced sensitization of nociceptive neurons in the spinal cord through inhibition of GABAergic interneurons. Owing to the particular configuration of CPFEX, the presence of unsubstituted piperazinyl ring at the C-7 position (Tillotson, 1996) enables it to strongly bind to and block GABA<sub>A</sub> receptors (Hondebrink et al., 2015). A broad array of evidence points to the prevalence of GABA<sub>A</sub> receptors in nociceptive circuits and to the significance of these receptors in modulating synaptic release of excitatory neurotransmitters from the central terminals of primary afferents. This raises speculation that CPFEX could trigger pain-related behaviors by driving a hyper-excitability state in the dorsal horn of the spinal cord and inducing central sensitization (Shehla, 2019). Yet still, further investigation is needed to dissect the signaling pathways and determine the mechanisms that underlie pain behaviors associated with CPFEX treatment. Overall, the results of this study corroborate the clinical findings and highlight the possible neurotoxic effects of oral administration of CPFEX on the sensory system. More generally, our findings reinforce the necessity of limiting CPFEX use in patients with pre-existing neuropathies.

To the best of our knowledge, this is the first pre-clinical study that investigates the effect of CPFEX on sensory and cognitive behaviors in healthy rats; however, despite the novel findings, it still has some limitations. The sample size used is relatively small, which makes it harder to detect statistically significant results. Hence, increasing the sample size will increase the statistical power and effect size. Furthermore, additional nociceptive behavioral and electrophysiological experiments are needed to identify the

type of sensory fibers impacted by CPFX, and to unfold new mechanisms of CPFX action in the spinal cord that paved the way towards defining the origin of its neurotoxicity.

In conclusion, this study evaluated the safety profile of oral CPFX administration by assessing its effect on cognitive functions, hippocampal neurogenesis, and sensory functions in healthy rats. Our findings demonstrated that CPFX did not impair hippocampal functions such as reference, episodic, recognition, and spatial-working memories; in addition to neurogenesis. However, CPFX significantly altered sensory responses leading to the development of heat hyperalgesia, mechanical hyperalgesia, and mechanical allodynia. The mechanism behind the development of pain is still not fully understood and requires further investigation; however, we suggest that CPFX may have inhibited GABAergic transmission in the dorsal horn of the spinal cord leading to over-excitation of nociceptive neurons. These results call for further investigation into the receptor mechanisms underlying CPFX-induced neurotoxicity.

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