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"DEVELOPMENT OF A RAT MODEL FOR GAMING DISORDER: REWARD SYSTEM ANALYSIS AND VALIDATION WITH FLUOXETINE TREATMENT"

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Abstract

Gaming Disorder (GD) is a psychopathological condition that primarily affects adolescents, characterized by compulsive gaming behavior, loss of control, and psychopathological symptoms such as social anxiety and depression. Despite the recognition of the disorder by the International Classification of Diseases-11th Revision (ICD-11) in 2018, a specific animal model for GD is still lacking.

This thesis proposes a novel animal model of GD, developed using Wistar Kyoto rats trained for five to six weeks on a touch-screen platform designed to simulate typical behaviors associated with the disorder. The results show that rats exposed to the GD protocol exhibit behavioral traits similar to those observed in humans, such as hyperactivity and loss of control, which are consistently maintained over time.

Neurobiological analysis revealed significant alterations in neuronal activity in key brain regions, such as the prelimbic cortex, orbitofrontal cortex, and amygdala, suggesting dysfunctions in reward and decision-making circuits. Furthermore, the examination of neurotransmitters, including orexin, dopamine, and serotonin, showed alterations both at the brain and peripheral levels, indicating involvement of reward systems and emotional regulation. Fluoxetine administration, a selective serotonin reuptake inhibitor, significantly reduced compulsive behaviors in GD rats, suggesting its effectiveness in improving control over gaming behavior and emotional dysregulation associated with the disorder.

In summary, the proposed animal model provides an innovative tool to explore the neurobiological mechanisms underlying GD and to test potential therapeutic interventions, such as Fluoxetine, paving the way for future clinical research on the treatment of this emerging disorder.

Key words (EN)

Gaming Disorder (GD), Animal model, Neurobiological alterations, Fluoxetine, Compulsive behavior.

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CHAPTER 1

General introduction

Video games have emerged as one of the most popular and influential forms of entertainment in modern times. From humble beginnings as technological experiments in researchers' laboratories in the 1950s, they have evolved into an artistic and interactive medium with a profound impact on contemporary culture (*Reynaldo et al., 2021*). These interactive experiences have sparked our imagination, offering excitement, challenges, and stimulation to millions of players worldwide. In 1958, William Higinbotham's "Tennis for Two," a simple game run on an analog computer, marked the beginning of an extraordinary journey that led to the creation of a far-reaching entertainment industry (*Jo et al., 2019*). Over time, video games have undergone incredible evolution, both technologically and narratively, transforming into immersive experiences (*Jo et al., 2019).*

In the 1970s and 1980s, the video game industry experienced remarkable growth with the advent of arcade consoles and early home video game systems (*H. S. Kim et al., 2022a*). Iconic titles such as "Pac-Man" and "Space Invaders" became cultural phenomena, attracting players of all ages and creating a passionate community around arcade games. The success of these games helped establish video games as one of the most profitable entertainment industries, often surpassing the revenues of other forms of entertainment such as movies and music (*Larche & Dixon, 2021*).

New technologies and enhanced console hardware capabilities enabled the development of increasingly complex and immersive games, allowing players to engage in incredibly realistic virtual worlds (*Reynaldo et al., 2021).* The continuous evolution of consoles and gaming platforms has allowed developers to create increasingly sophisticated games that address profound themes and evoke empathy in players. The gaming experience can be incredibly rewarding, offering benefits such as improved cognitive abilities and mental well-being. According to Paulus et al. (2018), play is considered an essential part of human behavior development and the consolidation of new experiences. Another positive aspect of video game use is the potential for improving learning and developing cognitive skills in patients with learning disorders (*Granic et al., 2014).*

However, overuse can become a risk factor for adolescents, who may develop a common pathology among gamers: Gaming Disorder (GD).

Gaming Disorder

Despite its many benefits, excessive use of video games, associated with certain risk factors, can lead to the development of a mental disorder known as GD. GD was only recently included in the section on disorders related to addictive behaviors in the International Classification of Diseases (ICD) (Jo et al., 2019).

According to the definition by the ICD (*Jo et al., 2019*), excessive video game use can interfere with daily activities and interpersonal relationships, creating a downward spiral that can be difficult to break. The American Psychiatric Association (*APA, 2013*) defines GD as a pattern of persistent or repetitive gaming behavior that lasts for at least 12 months. This mental disorder is characterized by several symptoms, such as a loss of control over gaming, increasing priority given to gaming, and

continued gaming despite negative consequences. These symptoms are often accompanied by behaviors such as compulsiveness, hyperactivity, and withdrawal, which are also observed in substance addiction (*Jo et al., 2019*).

In addition to the shift in priorities toward video games, individuals with GD often experience emotional changes, including heightened anxiety, social isolation, and the development of depression (*H. S. Kim et al., 2022a*). Although GD is a relatively new mental disorder, it is estimated that about 3.67% of the global population suffers from it, with 60 million adolescents among those affected *(H. S. Kim et al., 2022b).* Following the COVID-19 pandemic, the demand for treatment for this disorder has increased, suggesting a rise in its incidence rate (Larche & Dixon, 2021).

Impact of Gaming Disorder on young people

Gaming Disorder has a significant impact on young people, affecting various aspects of their lives. From a mental health perspective, it can cause anxiety and depression due to social isolation and loss of control over gaming (*H. S. Kim et al., 2022a*). Additionally, excessive video game use, especially at night, can lead to sleep disturbances, insomnia, and chronic fatigue. Some studies also suggest an increase in impulsivity and aggression among individuals affected by GD (*Jo et al., 2019*). Regarding academic performance, time spent gaming reduces the time available for studying, leading to declining grades, difficulty concentrating, and a tendency to procrastinate. From a social standpoint, young people with GD may develop social isolation, difficulty in communication skills, and family conflicts due to gaming addiction (*H. S. Kim et al., 2022a*). Finally, physical well-being can be compromised by a sedentary lifestyle, increasing the risk of obesity and cardiovascular problems, postural issues caused by long gaming sessions, and eye strain due to prolonged screen exposure *(H. S. Kim et al., 2022b)*. Beyond these personal and social consequences, GD also has a significant economic impact on young individuals and their families. Excessive gaming can lead to financial strain due to in-game purchases, microtransactions, and subscription fees that may accumulate over time. Some young players spend large sums on virtual items, leading to financial stress and potential conflicts within families. Furthermore, families may need to bear additional expenses for psychological therapy and rehabilitation programs aimed at treating GD (*Larche & Dixon, 2021*). On a broader scale, the disorder can negatively affect academic and professional performance, leading to reduced productivity and potential job loss, ultimately impacting the economy. Healthcare systems may also experience increased costs due to the rising demand for mental health services related to gaming addiction (*Larche & Dixon, 2021*).

In addition to these factors, numerous studies have highlighted the role of gender, game type, and gaming platform in the onset and progression of GD (*Evren, C. et al., 2021*). The disorder appears to be more prevalent among males than females, with an estimated ratio of approximately 3:1. This difference is reflected in distinct gaming preferences: males are more likely to engage in First-Person Shooters (FPS), Multiplayer Online Battle Arena (MOBA) games, and Massively Multiplayer Online Role-Playing Games (MMORPG), which emphasize competition, skill progression, and social interaction in virtual worlds (*Zendle, D., & Cairns, P., 2020; King, D. L., & Delfabbro, P. H., 2022*). In contrast, females tend to prefer games with strong social and narrative components. Additionally, the type of gaming device can influence both the severity and the persistence of GD. Computer and console games are often linked to more severe forms of addiction due to their immersive nature and prolonged gaming sessions, whereas mobile games, despite being less intense, are more accessible and can foster a fragmented yet persistent pattern of gaming. Microtransactions and loot boxes,

particularly in mobile and free-to-play games, further contribute to compulsive gaming behaviors by leveraging reward-based mechanisms similar to gambling (*Zendle, D., & Cairns, P., 2020; King, D. L., & Delfabbro, P. H., 2022; Lemmens, J. S., & Hendriks, V., 2024; Evren, C. et al., 2021*). Understanding the interaction between gender, game genre, and gaming platform is crucial for developing effective prevention strategies and targeted interventions for individuals affected by GD.

Human clinical studies and neurobiology of Gaming Disorder

The triggers leading to the development of GD are not yet fully understood. However, numerous studies indicate a higher incidence of GD in individuals with mental health disorders, such as social anxiety and low self-esteem (*H. S. Kim et al., 2022a*).

Despite the potential influence of these factors, some individuals may have a genetic or neurobiological predisposition that increases their risk of developing video game addiction *(Kuss et al., 2018*). These predispositions could include traits such as hyperactivity, emotional dysregulation, and impulsivity (*Ko, 2014; Weinstein et al., 2017*). Such traits may elevate the risk of video game addiction by affecting the ability to regulate behavior, including emotional states and cognitive processes (*Ko, 2014*).

Understanding the benefits and risks associated with video game use can help mitigate the negative effects while maximizing the benefits this technology can offer (*Jo et al., 2019*).

Although there is no conclusive evidence regarding the incidence of GD development in either sex, some studies suggest gender differences in the timing of the disorder's onset. According to a study by *Lopez-Fernandez et al. (2019),* males are more likely to develop GD during early adolescence, coinciding with higher video game use, while females are more likely to develop GD at a later stage, often linked to social network usage (*Lopez-Fernandez et al., 2019*).

At the neurobiological level, human studies have revealed significant differences in brain structure and activity between men and women with GD (*Han et al., 2012*). For example, *Han et al. (2012)* conducted a study examining regional differences in gray matter between men and women with GD, revealing some notable findings. The study found that men with GD showed significant reductions in gray matter in the prefrontal cortex and parietal lobes compared to women. The prefrontal cortex is involved in regulating executive functions, planning, and decision-making, while the parietal lobes process visual-spatial and sensory information. Decreased gray matter in these areas could indicate impaired cognitive abilities and decision-making in men with GD (*Han et al., 2012*).

In contrast, women with GD had reductions in gray matter in the inferior frontal gyrus, which is associated with emotional regulation and impulsive responses (*Han et al., 2012*). These differences in gray matter reduction in specific brain regions suggest that men and women may develop unique addictive mechanisms, likely due to differing cognitive and emotional engagement during gaming.

Reward system and its implication in Gaming Disorder

The reward system is a large brain network consisting of a set of brain areas that regulate each other. This system is involved in multiple functions, including the regulation of emotional state, social behavior, sexual behavior, and food or substance intake. Key areas involved in the reward system include the nucleus accumbens (NAc), which plays a central role in processing rewards and reinforcement; the ventral tegmental area (VTA), responsible for dopamine production and the initiation of reward-driven behavior; and the prefrontal cortex, which is involved in decision-making

and controlling impulses. Additionally, the amygdala contributes to emotional processing and the hippocampus is involved in memory formation, both of which can influence the processing of rewards and motivation (Alonso-Alonso et al., 2015; Koob, 2009). Due to the strong involvement in regulating several functions, its dysfunction is often studied in the context of addictive behaviors. Just as in substance addiction, the reward system in patients with GD shows alterations in the regulation of compulsive behaviors associated with gaming (*Li et al., 2015*).

A crucial aspect that has been the subject of several investigations, including in GD, is the sex difference in the reward circuit. Some researchers have demonstrated sex dimorphism, showing greater sensitivity to reward in male GD patients (*Li et al., 2015*). This finding suggests that males may be more likely to pursue rewards associated with pathological gaming than females (*Li et al., 2015*). One possible explanation could be a greater increase in dopamine (DA) during play in males compared to females, as DA plays a crucial role in modulating motivation, attention, and pleasure (*Koepp et al., 1998*).

Additionally, male GD patients show a stronger connection between the ventral tegmental area (VTA) and the basal terminal stria nucleus (BNST) (*Wang et al., 2019*), a brain area involved not only in reward circuitry but also in regulating emotional state and responses to stressful situations (*Avery et al., 2016*).

However, GD patients also show sex differences in BNST activation (*Avery et al., 2016*). GD patients exhibit increased levels of anxiety and stress, with greater BNST activation in women than in men (*Hsu et al., 2014)*. BNST activity is regulated by circulating gonadal hormone levels, with estradiol acting more rapidly than testosterone, which may result in greater activation of this brain area in women than in men.

In addition to the BNST, the VTA sends projections to, and receives projections from other brain areas connected to reward circuits, such as the NAc, orbitofrontal cortex (OFC), and caudate-putamen (Cpu) (*Weinstein et al., 2017*). These regions are closely involved in reward perception, motivation, and the maintenance of rewarding behaviors. Pathological gaming can alter reward perception and decision-making, leading to the formation of maladaptive behaviors such as impulsivity and compulsivity, which are typical of addiction (*Wang et al., 2019*).

The development of harmful and maladaptive behaviors is often associated with dysfunction in brain areas involved in assessing dangerous situations, such as the basolateral amygdala (*Avery et al., 2016*). GD patients show decreased impulse control and an increased tendency to make poor choices, which is linked to changes in basolateral amygdala activity. This suggests that pathological gaming may lead to dysfunction in these areas by involving circuits related to reward processing and the perception of potentially dangerous situations (*Avery et al., 2016*).

In addition to these changes, GD patients show increased brain activity in the medial prefrontal cortex (mPFC) and posterior cingulate cortex (PCC), two areas implicated in future planning, self-management, and decision-making—processes that are altered in individuals with GD (*Sharaev et al., 2016; Weinstein et al., 2017*). Recently, research on reward circuitry and addiction has increasingly emphasized the role of social components in reducing typical addictive behaviors. GD patients exhibit a reduction in social engagement, and restoring this component has been shown to help patients reduce video game use and alleviate symptoms of anxiety, depression, and addiction (*Falade-Nwulia et alsupp., 2022*).

Neurotransmitters involved in Gaming Disorder

In addition to behavioral and activation changes in specific brain areas of GD patients, several hypotheses suggest alterations in the production and activity of neurotransmitters involved in regulating motivational circuits, such as DA, serotonin (5-HT), oxytocin (OXT), and corticotropin releasing hormone (CRH).

For example, DA, known for its role in regulating reward and emotion circuits, seems to be more reactive in pathological gamers. In fact, it appears that in GD patients, DA responds disproportionately to stimuli produced by video games, contributing to the loss of control and the maintenance of compulsive gaming behaviors (*Ariatama et al., 2019; Wang et al., 2019*).

5-HT plays a key role in modulating impulse control and emotional states through various excitatory and inhibitory mechanisms. Alterations in 5-HT function can lead to increased susceptibility to addiction (*Zoratto et al., 2017*) and the development of mental disorders, including GD (*Tian et al., 2014*). GD patients exhibit changes in 5-HTreceptor density and neurotransmitter availability in regions involved in cognitive decision-making and emotional processing (*Zoratto et al., 2017*).

OXT is a hormone involved in several social and cognitive functions, such as understanding and interpreting the mental states of others, as well as the development and maintenance of emotional bonding (Sarnyai & Kovács, 2014). OXT appears to promote different mechanisms depending on the context. In positive social situations, OXT enhances emotional sensitivity and social interaction (Menon & Neumann, 2023). In contrast, in negative or threatening situations, OXT seems to reduce aggression and the stress response (*Olff et al., 2013; Sarnyai & Kovács, 2014*). In addition to regulating the social component, OXT modulates cognitive and decision-making processes, such as minimizing effort, maximizing rewards, and preventing the development of harmful behaviors, such as addiction (*Baskerville & Douglas, 2010*). GD patients exhibit alterations in serum OXT levels and epigenetic changes to its receptor (*Choi et al., 2020; Liang et al., 2023; Sindermann et al., 2021*).

CRH is a steroid hormone produced by the hypothalamus in response to stress and regulates various brain functions and behaviors (*Kovács, 2013*). During adolescence, the brain undergoes significant changes, and new experiences or activities, such as video games, can become particularly appealing to young people. Exposure to stressful situations, such as long gaming sessions, increases the production of cortisol, which, along with various genetic factors, can affect adolescents' brain function, making them more vulnerable to developing mental disorders, including GD (*Geisel et al., 2018; J. Park et al., 2018; Paulus et al., 2018*). A prolonged increase in corticosterone levels can negatively impact the regulation of the reward system, affecting motivation and gratification associated with gaming, and promoting the maintenance of pathological gaming behaviors (*Koot et al., 2014; J. Park et al., 2018*). Importantly, cortisol and other neurotransmitters do not act in isolation on addiction but as part of a complex network of neurobiological interactions.

GD is thus influenced by a combination of genetic, neurobiological, environmental, and social factors, which may vary at different stages of the disorder.

Drug Treatments

GD is a mental disorder that has become more prevalent over the past 20 years. Symptoms include altered emotional states, with increased levels of anxiety, the development of depression, and social isolation. These mood changes are accompanied by a loss of control over gaming, as well as the development of compulsive and hyperactive behaviors. Additionally, GD is strongly comorbid with

other mental disorders such as depression (*Liu et al., 2018*), psychotic disorders (*Huot-Lavoie et al., 2023*), affective disorders, schizophrenia, and neurodevelopmental disorders like attention-deficit hyperactivity disorder (ADHD) (*Han et al., 2017*) and obsessive-compulsive disorder (*M. Kim et al., 2017*). Since no specific medications are approved for GD, treatment typically involves using drugs already known to treat mental illnesses (e.g., bupropion and escitalopram) and neurodevelopmental disorders (e.g., methylphenidate and atomoxetine) (*Zajac et al., 2020*).

Escitalopram and Fluoxetine is an antidepressant in the selective serotonin reuptake inhibitor (SSRI) class, primarily used to treat major depression, anxiety disorders, and obsessive-compulsive disorder (*Kirino, 2012; Pelissolo, 2008; Stein et al., 2008*).

Bupropion is an antidepressant that inhibits the reuptake of DA and norepinephrine. It is used to treat major depression, obesity, smoking cessation, and asthenia (*Foley et al., 2006).*

Methylphenidate hydrochloride (MPH) is a psychostimulant with a 2-benzylpiperidine structure. Like bupropion, MPH inhibits DA reuptake and is commonly used to treat ADHD (*Van der Oord et al., 2008*).

Atomoxetine is a selective inhibitor of the pre-synaptic norepinephrine transporter used to treat neurodevelopmental disorders such as ADHD; unlike methylphenidate it does not directly affect serotonin (5-HT) or DA transporters (*Fu et al., 2022*).

Typically, these medications are combined with behavioral therapy (*Zajac et al., 2020*). The duration of drug treatment varies: 6 to 12 months for bupropion, escitalopram, and atomoxetine, and at least 8 to 12 months for methylphenidate (*Han et al., 2009; J. H. Park et al., 2016*). All of these medications, either individually or in combination, help reduce video game use and alleviate GD-induced behaviors alterations. Escitalopram appears to reduce social isolation and improve interactions (*Zajac et al., 2020)*.

Limitation of clinical studies in patients with Gaming Disorder

Despite significant advances in the study of neurobiology and brain circuits associated with GD, understanding of the condition remains limited due to the non-invasive techniques used in human studies. Functional Magnetic Resonance Imaging (fMRI) and Electroencephalogram (EEG) are used to study functional changes in the brains of GD patients, and while important, they appear less comprehensive in identifying neural changes. Moreover, these studies have additional limitations, such as the large variability of subjects included, the failure to consider both sexes, and differences in electronic devices used (*Kuss et al., 2018*). These critical issues could be addressed by using a specific animal model, which would allow for a more detailed neurobiological analysis of the changes induced by GD (*Marraudino et al., 2022*). In the field of substance and gambling addiction research, several animal models exist, but they only reflect some of the characteristics of this mental disorder.

Existing animal models

Existing animal models that may partially reflect some characteristics of GD are those used to study Gambling Disorder. Gambling is a recreational activity that involves betting money or goods of material value on events with uncertain outcomes, with the goal of gaining a financial or material reward (van den Bos et al., 2006). This definition emphasizes the crucial element of uncertainty in the outcome and the intent to obtain a reward. The analogy between gambling and gaming becomes

evident when considering the existence of a "reward" system in digital games, similar to that found in gambling activities. For example, in video games, players may be rewarded for passing a difficult level, completing a mission, or accumulating a certain number of play hours. These rewards can take the form of virtual coins, points, items, or gadgets within the game, which the player can use to gain advantages or improvements in the game (*Orsini et al., 2022*). Just like gambling, the use of video games activates the reward system, inducing a sense of pleasure and satisfaction in the player. The uncertainty of reward and activation of reward circuits are common aspects shared by both gambling and GD (*Bélanger-Lejars, 2015; Raneri et al., 2022*).

One of the first animal models developed is the Iowa Gambling Task (IGT), in which *Ruud van den Bos et al. (2006)* attempted to replicate as closely as possible the IGT used in humans. The IGT is a widely used paradigm for studying decision-making and gambling behavior in humans. In the IGT, participants must make choices from four virtual decks of cards, each associated with different rewards and losses. Two decks are considered “advantageous” because they result in a net gain over the long run, while the other two are “disadvantageous” due to immediate gains but long-term losses (*Bechara et al., 1994*). During the game, participants receive feedback on the outcomes of their choices in the form of monetary gains and losses. Over time, participants should learn to avoid the disadvantageous decks and prefer the advantageous ones, demonstrating adaptive behavior. The researchers worked to adapt this task to the context of rats, attempting to recreate the risk-reward decision-making process experienced by humans during the IGT. Specifically, rats are exposed to a maze with six arms, two of which are defined as "starting arms" where the animal is placed, while the remaining four are associated with different rewards and punishments. The rats are thus required to make choices in order to obtain rewards (***Figure 1***).

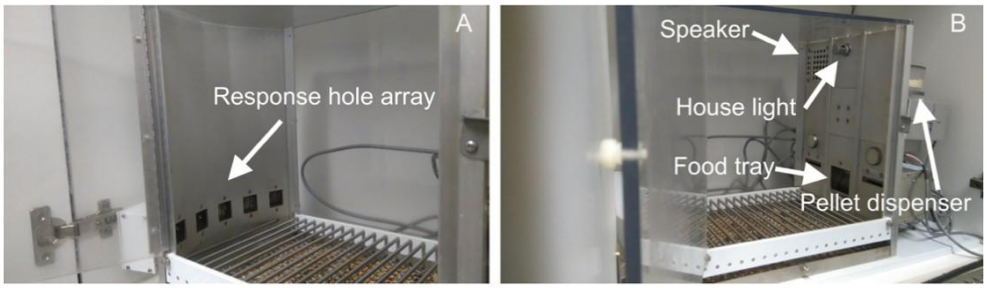


Figure 1 *Iowa Rat Gambling Task Apparatus (Ruud Van Den Bos et al., 2006)*

An additional model developed in 2017 is the Cued Rat Gambling Task (CrGT) (*Barrus & Winstanley, 2017*), in which the authors aimed to create a behavioral study to investigate reward- and risk-based choice evaluation in rats. In the CrGT, the key difference compared to the Rat Gambling Task (rGT) is the addition of a “cue” or stimulus, in this case an audiovisual one, associated with the pellet reward. This is similar to what happens in slot machines when a certain amount is won; indeed, these cues increase in complexity as the “payoff” increases. In the CrGT, rats are presented with four options, each associated with a different pellet reward, a distinct audiovisual stimulus, and a specific punishment period (pause time). This model is designed to enhance our understanding of how environmental cues influence choice and behavior in animals, and how these mechanisms may relate to decision-making processes observed in humans (***Figure 2***).

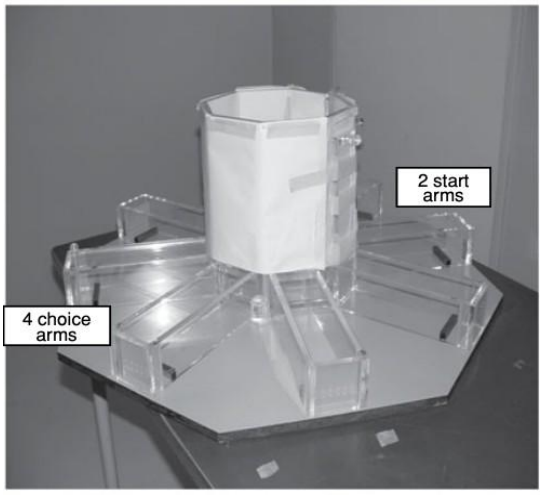


Figure 2 *Cued Rat Gambling Task apparatus (Michael M. Barrus and Catharine A. Winstanley., 2017)*

Another rat model is the "Rat Slot Machine Task" (*Rafa et al., 2016*). This model is used to investigate the effects of optimism on gambling behavior. In this paradigm, rats are trained to press levers to receive rewards under different conditions, understood as probabilities. The researchers aimed to examine how the degree of optimism, defined as the tendency to positively evaluate the chances of receiving a reward, influenced the rats' gambling behavior (***Figure 3***).

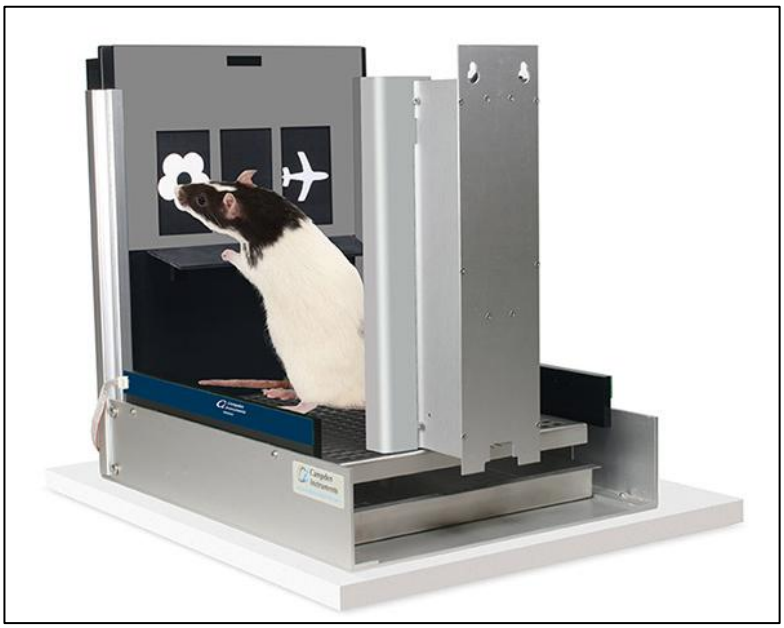


Figure 3 *Rat Apparatus Slot Machine Task (Dominik Rafa et al., 2016)*

Limitations of existing animal models

As mentioned earlier, several gambling models have been developed in rats using electronic equipment, such as dispensers or screens (*Barrus & Winstanley, 2017; Rafa et al., 2016; Winstanley et al., 2011*). Typically, these models involve an association between a visual stimulus and a reward delivered by pressing a lever. However, there is no direct interaction with the screen, which merely represents the visual component of the task. These rat models primarily focus on the animal's ability to discriminate between more advantageous and less advantageous choices, while neglecting aspects such as loss of control and the development of hyperactive behaviors. The main objective of these models is to understand the mechanisms underlying the loss of rational control over gaming in the presence of random choices, which mimic the use of slot machines in rats (*Barrus & Winstanley, 2017; Rafa et al., 2016*). Loss of control over gaming is also a key feature of GD, alongside the underexplored hyperactivity and compulsivity observed in rat models of gambling (*Barrus & Winstanley, 2017; Rafa et al., 2016; Winstanley et al., 2011*).

These two parameters are partially assessed by quantifying the frequency of lever pressing. However, there is no estimation of the duration of “play” or an assessment of the type of play (hyperactive or non-hyperactive). Quantifying the duration and type of play is crucial for evaluating the loss of control and hyperactivity components in GD. Another aspect that is either underexplored or completely absent in gambling models is the assessment of the social context, which is significantly impaired in individuals with GD. In recent years, social context and interaction have been studied more extensively, particularly for their positive effect on reducing self-administration or relapse in animal models of substance dependence (*Venniro et al., 2018*).

CHAPTER 2

Aim of the thesis

In 2018, the International Classification of Diseases (ICD-11) classified GD as a mental disorder. GD primarily affects adolescents, who, after developing a compulsive and hyperactive attachment to video games, exhibit psychopathological traits such as anxiety, depression, social isolation, and attention deficits (*Jo et al., 2019*).

The various studies conducted thus far on humans have focused on analyzing peripheral biological markers and brain activity. These studies reveal alterations in the connectivity of different brain areas, which are correlated with changes in circulating hormone levels. Among the various hypotheses regarding the development and maintenance of symptoms associated with GD, there is the potential alteration of the reward system, particularly the serotonergic and dopaminergic systems. These two neurotransmitters play crucial roles in regulating several brain functions, including information processing, working memory, impulse control, and the regulation of emotional states. GD patients show behavioral alterations in these functions, along with changes in peripheral levels of DA and 5-HT, as well as changes in the methylation levels of genes related to receptors or transporters of these neurotransmitters. Indeed, the use of DA and 5-HT reuptake inhibitor drugs has been shown to reduce GD symptoms, such as altered emotional states and excessive video game use.

However, while important, human studies have significant limitations, including the variability in the duration of video game exposure, the type of device used, and the failure to consider both sexes. These limitations could be addressed with an animal model, which would allow for a more thorough exploration of the neurobiological changes associated with GD and facilitate the development of specific drugs for treating this mental disorder.

The purpose of this thesis is to develop a rat model for the study of GD, to analyze the possible involvement of the reward system, and to evaluate the predictive validity of this model by testing a drug currently used to treat GD, potentially validating both the model itself and its predictive value for future interventions.Specifically, this thesis is divided into three main goals:

1. Development of a rat model for studying GD: characterization of the behavioral phenotype, quantification of changes in brain activity, and comparison with studies in humans;
2. Analysis of the possible central and peripheral involvement of 5-HT, DA, and orexin (ORX) in the regulation of reward circuits;
3. Validation of the GD rat model using fluoxetine, a drug already used for the treatment of GD patients, and the validation of other animal models for the study of mental disorders.

CHAPTER 3

AIM 1

Novel rat model of Gaming Disorder: assessment of social reward and sex differences in behavior and c-Fos brain activity

INTRODUCTION

Play is an important part of developing human behavior and consolidating new experiences (*Paulus et al., 2018).* Over the past 20 years, recreational activities have changed since the increased availability and use of computer technology have greatly raised (*Paulus et al., 2018*). Computer games, internet use, and social media have become common activities for children and adolescents (*Paulus et al., 2018*) engaging experience and producing a sense of accomplishment as they acquire new skills (*Reynaldo et al., 2021*), underestimating the negative aspects, probably due to the influence of "social desirability" (*Jo et al., 2019*). Recent studies showed that children and adolescents spend more than 8 to 10 hours per day using various electronic media, such as television, computers, smartphones, and social media (*Nobre et al., 2020*). This phenomenon was amplified even more in 2020, right in the first phase of COVID-19 (*Wijman, 2020*), when the number of players globally reached 2.6 billion people and video game sales were record-breaking (*J. Schreier, 2020; Porter, 2021*).

However, the term 'gamers' include individuals with various intra- and inter-personal risk factors who use computer games as a coping strategy for various issues (*Kuss et al., 2014*). Excessive use becomes a negative factor that limits real-life experiences (*Kuss et al., 2014*), so much so that it assumes a solid resemblance to addictive disorders. Therefore, GD was included in the ICD-11 by the World Health Organization (WHO) (*American Psychiatric Association, 2013*) and recently (on May 25th, 2019) recognized as a medical disorder (*WHO, 2018*).

GD is characterized by a repetitive or persistent gaming behavior pattern over a period of at least 12 months (*WHO, 2018*). Specifically, the diagnosis of GD involves three symptoms: *(i)* impaired control over the game, *(ii)* increasing priority given to the game, and *(iii)* continuation or escalation of the game despite the occurrence of negative consequences (*WHO, 2018*). Although GD is a clinically identifiable disorder, today it is not easy to understand the risk of developing it due to the lack of clear predictive signals and many potential contributing factors.

Interestingly, GD shows a higher prevalence in males compared to females (*Mihara & Higuchi, 2017*). Furthermore, boys have greater gaming addiction prevalence during adolescence, when the time spent playing video games increases, while girls develop greater use of the game at an older age, when they generate an addiction to social networks (*Lopez-Fernandez et al., 2019*). Currently, the studies available on GD present a strong limitation in the sampling of subjects, which appear to be almost all males and Asians (*Bouna-Pyrrou et al., 2018*). This underlines a serious problem in the study of the GD as very few data relating to females, as well as the lack of demographic heterogeneity. Further, dimorphism is associated with psychiatric comorbidities (*Tang & Koh, 2017*) and the development of poor mental health (*Ciarrochi et al., 2016*), which appears to be greater in female gamers. Probably, this is because there are important sex differences in the circuits that control attention, addictions, and psychiatric behaviors, both in their structure and in differentiated responses. The poor consideration of sex, the different ages of the players, the different types of games, and the mode of recruitment of subjects are the limiting factors of these studies that could be overcome using an animal model (*Barrus & Winstanley, 2017; Marraudino et al., 2022; Rafa et al., 2016*).

Animal models that reflect some of the features described in GD patients could be a valuable tool for studying the behavioral and neurobiological characteristics of the disorder. However, at present, no GD-specific models have been developed so far. On the contrary, in the literature, there are several gambling models developed in rats that use electronic equipment, such as dispensers or screens (*Barrus & Winstanley, 2017; Rafa et al., 2016; Winstanley et al., 2011*). Usually, there is an association of visual stimulus with a reward released by pushing a lever. Thus, there is no direct interaction with the screen, which represents only the visual part of the task.
The main purpose of these models is to understand the mechanisms underlying the loss of rational control over the game in the presence of random choices, that mimic slot machine use. Loss of control over gaming is also one of the main features of GD, as well as the hyperactivity and compulsivity, which are under-studied in the known rat models of gambling (*Barrus & Winstanley, 2017; Rafa et al., 2016; Winstanley et al., 2011*). These two parameters are partially assessed by quantifying the frequency of lever pushing. Thus, there is neither an estimation of the duration of "play" nor an assessment of the type of play (compulsive or non-compulsive). Quantification of duration of play and type of play are very important behavioral parameters for assessing the compulsive and hyperactive components in GD.
Another aspect that is little or completely absent in gambling models is the assessment of social context, another aspect that is highly compromised in GD patients. In recent years, social context or social interaction has been more studied, especially the positive effect it has on reducing self-administration or relapse in animal models of substance dependence (*El Rawas et al., 2012; Fritz et al., 2011; Venniro et al., 2018*). Thus, taking advantage of a new apparatus consisting of a touch-screen platform, the main purpose of this work is to propose a new rat model that closely reflects the behavioral phenotype observed in GD patients, allowing to evaluate the hyperactive and compulsive components and to investigate the possible positive effects of social context on the reduction of parameters associated with GD.

MATERIALS AND METHODS

Animals

Adult male (n=4) and female (n=8) Wistar Kyoto rats were purchased from Charles River (Charles River Laboratories Italia s.r.l., Milan, Italy). Rats were housed in standard conditions at 22 ± 2 °C, under a 12:12 light-dark cycle (lights on at 08:00 AM). Food (standard chow diet, VRF1, SDS - Charles River Laboratories) and water were provided *ad libitum* throughout the study. One male and two female rats (3-month-old) were housed together to achieve a successful mating. Obtained pups (37 males and 41 females) were sexed and weaned at postnatal day 28 (PND28) and were then housed into separated cages containing 4 same-sex rats.

Experimental groups

After the selection phase (described below), selected rats (n= 30 males / 34 females) at PND40 were randomly divided into the following experimental groups:
- Control males (CON-M, n=12);
- Control females (CON-F, n=13);
- Male rats subjected to GD protocol (GD-M, n=18);
- Female rats subjected to GD protocol (GD-F, n=21).

The control groups underwent a 10min/day period of adaptation to the apparatus one week before the beginning of the testing phase. The GD group underwent the pre-training and training phase as described below. A schematic representation of the experimental timeline is reported in ***Figure 1A***.

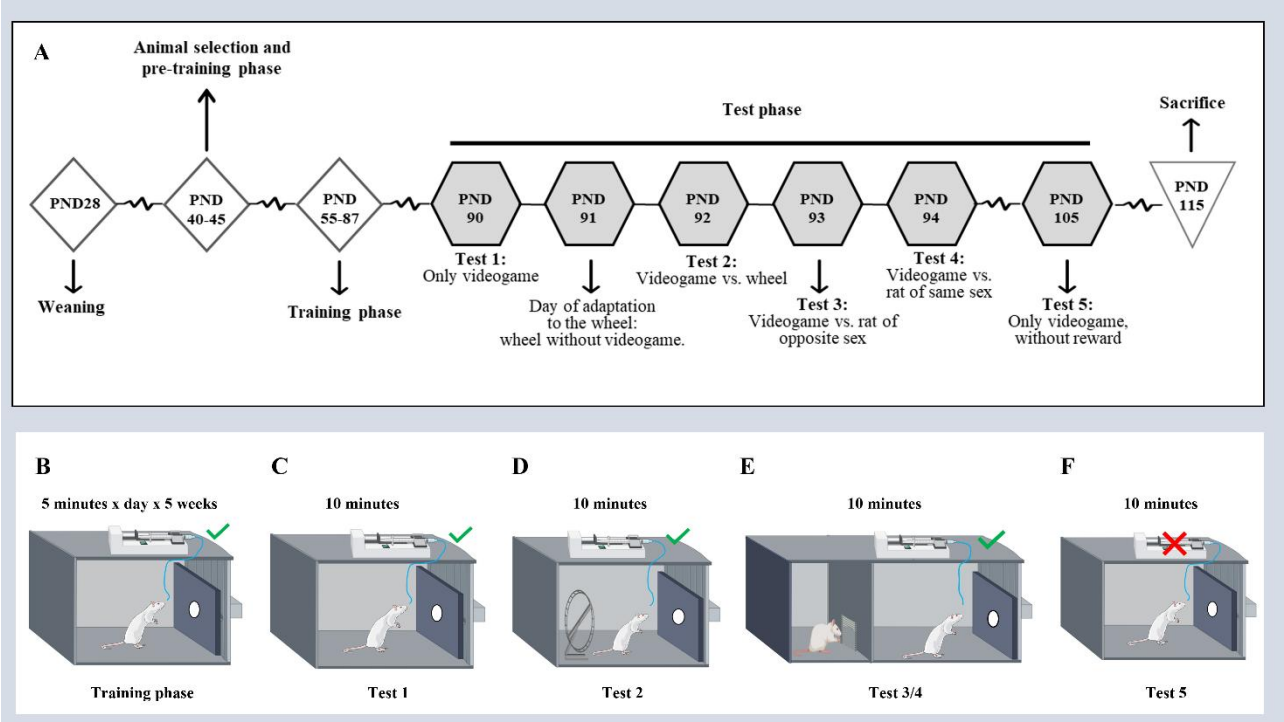


Figure 1. Experimental timeline. *Schematic representations of* ***(A)*** *experimental procedures' timeline,* ***(B)*** *training and different stimuli during* ***(C-F)*** *Test 1 and Test 5 (videogame alone),* ***(D)*** *Test 2 (videogame vs new object), and* ***(E)*** *Test 3 (videogame vs sexual stimulus) and Test 4 (videogame vs social stimulus). PND=Postnatal day (Casile et al., 2024).*

GD protocol

Behavioral Apparatus

The GD protocol took place in a rectangular planned apparatus (50 x 55 x 50 cm) with a plastic base and plywood walls (***Figure 2***). The apparatus was positioned 50 cm above the floor in a room illuminated with medium-low light using a house light, tempered to 22 ± 2 °C, and with a camera (Basler GenICam, acA 1300-60 gm) fixed on the roof to register (Media Recorder, Noldus, Wageningen, the Netherlands) the sessions.

In the center of the apparatus, there was a removable panel that allowed it to be divided into two areas: on the one hand, the play area (50 x 55 x 50 cm), on the other one (20 x 50 x 50 cm) an area in which different competitive stimuli were placed during the test 2, 3 and 4 (detailed below).

In the play area, a 50 x 30 cm touchscreen tablet (developed by UX experts from SCAI DOO.IT Group, Turin, Italy) was fixed on the shorter wall of the apparatus, with which the rats could interact freely. The area in front of the touchscreen tablet was defined as the play area (5 x 30 cm) (***Figure 2***).

The game involved the fixed or random appearance of a white dot (4.5 cm in diameter) on the black screen. When the animal touched the dot correctly, it got a reward of 0.5 g of yogurt (Strawberry yogurt – no added sugar). Yogurt delivery followed a fixed ratio (FR) for the first 3 weeks of the training phase, and then it moved to a random ratio (RR) for the last 2 weeks of the training and for the Test phase.

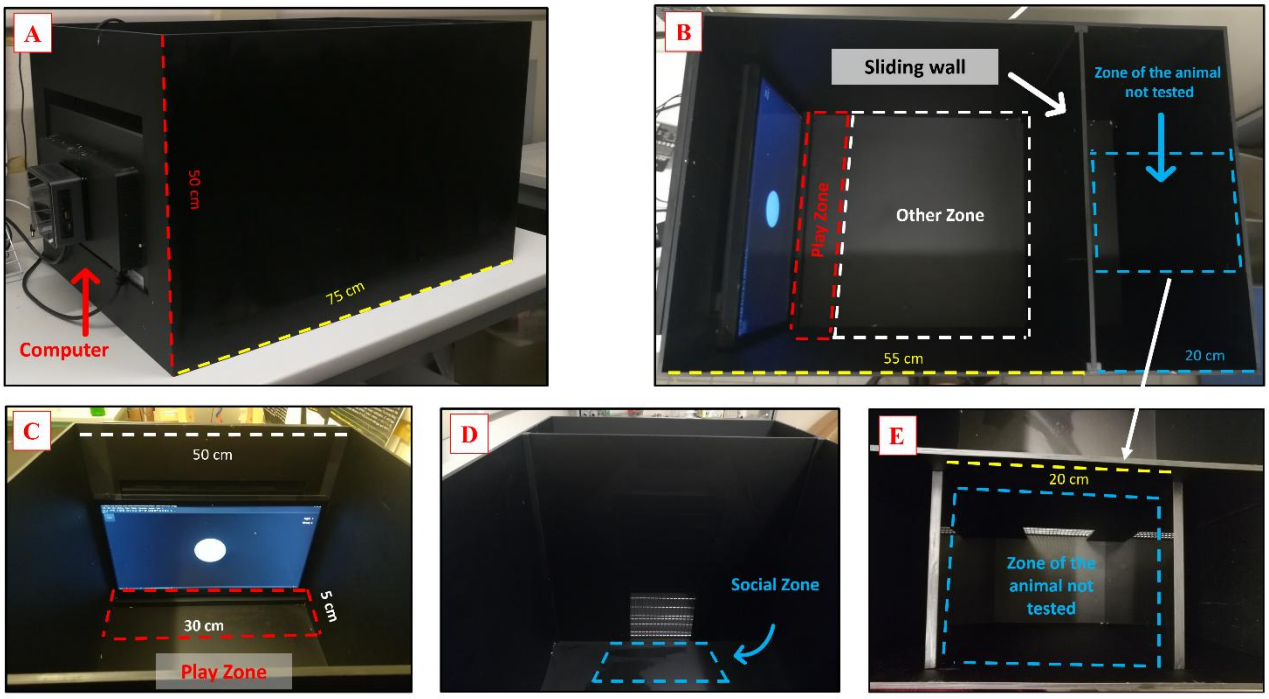


Figure 2. Behavioral apparatus. *Photos of* ***(A)*** *the apparatus with* ***(B-C-D-E)*** *a graphic representation of the different zones (play zone, other zone, and social zone). The apparatus has* ***(B)*** *a movable panel that was replaced with* ***(D)*** *an open one during tests where social interaction is planned (Test 3-4). The red rectangle represents the play zone, the blue rectangle represents the social zone, and finally, the white rectangle represents the remaining zone of the arena (Casile et al., 2024).*

Selection of animals and pre-training phase

Starting from PND40, rats underwent the selection, pre-training, and training phases.

All animals participated in the selection phase and will be evaluated on various parameters to exclude those that are too fearful or anxious. From PND40 to PND45, the spontaneous behavior of the animals was observed for 20 minutes in their home cages after 1 hour of habituation in the behavior room. The assessment consisted of evaluating exploratory ability (movement inside the cage), fear of the operator, behaviors implemented to get the yogurt (leaning out of the cage) and freezing. Daily, each behavior was scored (0 = never, 1 = little, 2 = enough; 3 = a lot; 4 = always). Animals with a five-day mean score of less than 17 were excluded from the study (M = 2, F = 3). The other animals, which passed the selection phase, were randomly divided into the following groups: controls (M=12, F=13), which were not subjected to the next pre-training and training phases, and animals subjected to GD protocol (M=18, F=21).

In the pre-training phase, from PND48 to PND52, the animals underwent a period of adaptation to the apparatus of 5 minutes per day. The spontaneous behaviors of the animals were also observed in this phase. As before, the assessment consisted of evaluating exploratory ability, fear of the operator, behaviors implemented to get the yogurt, and freezing, assigning to each behavior a daily score (0 = never, 1 = little, 2 = enough; 3 = a lot; 4 = always). Animals that were frightened by the apparatus explored little or nothing and did not come close to the reward and displaying a five-day mean score of less than 17 were excluded from the experiment (M = 5, F =4).

Training phase

The training phase lasted 5 weeks (from PND55 to PND87), 5 days per week. The sessions took place always between 8:00 a.m. and 1:00 p.m.

In the first training week, during the first three days, the tablet was in OFF mode. On the last two days of the first week, the tablet was switched to ON mode with the videogame running, and the

reward was dispensed with FR1. In the second week, the reward was dispensed with FR2, whereas for week 3 dispensing was with FR3. In the 4th and 5th week of training, the reward was dispensed in RR.
Each day, the rats were evaluated for the different game-related parameters: time spent in front of the screen, interaction with the screen (both correct touch or not), following the dot, undertraining the connection between correct touch and reward, the number of touches made, and obtained reward. A form was filled daily with reached scores in all evaluated parameters for each rat. In this form were indicated the different behaviors related to the game. Depending on the number of correct touches, a different score was assigned to each game-related behavior performed by the animal (time spent in front of the screen, interaction with the screen, following the dot, understanding the dot-reward link). The score assigned to each behavior (from the minimum of 1 to the maximum of 5) was related to the number of correct touches (0=0 correct touches; 1=1-5 correct touches; 2=6-10 correct touches; 3=11-15 correct touches; 4=16-20 correct touches; 5≥20 correct touches). The daily score was the sum of the scores obtained in each behavior plus the sum of the correct touches. In this way, it was also possible to evaluate the daily progression of the performance. Last, each week, a mean weekly score was assigned to each animal.

Test
The test phase, lasting 6 consecutive days, from PND90 to PND95, aimed to understand if the animals have developed game addiction, testing their ability and interest in the videogame even in the absence of reward and in the presence of new stimuli (exploratory, sexual, or social). Thus, the tests were performed as follows (***Figure 1A***):

- PND90: **Test 1**
The tablet was in ON mode with the videogame running (***Figure 1C)***. The animal was allowed to choose whether to interact with the videogame to obtain the reward or to explore the apparatus. Tested animal was assessed for attachment (time spent in the play zone), duration (time spent interacting with the video game), and compulsivity (number of correct touches, speed, and distance traveled in the play zone) shown during the play session.

- PND91: **Day of adaptation to the new object (wheel)**
The animal could explore the apparatus in the presence of the wheel, a hitherto unknown stimulus, with the tablet in OFF mode.

- PND92: **Test 2**
The condition was similar to the previous day (the day of adaptation to the wheel), but the tablet was switched ON with the videogame running. The animal was free to choose whether to play or to interact with the stimulus, and the same parameters of Test 1 were evaluated with the addition of all those behaviors expressed towards the wheel (exploration time and duration of the run on the wheel) (***Figure 1D***).

- PND93: **Test 3**
The tablet was in ON mode with the videogame running. In addition, an unknown animal of the opposite sex (socio-sexual stimulus) was present in the apparatus (***Figure 1E***). The animal was able to choose whether to interact with the video game or with the sexual stimulus. The time spent in the sexual zone (zone adjacent to the sexual stimulus), number, and total duration of sniffing, in addition to the same parameters evaluated in Test 1, were recorded.

- PND94: **Test 4**
The test condition was similar to test 3, but a co-specific of the same sex (social stimulus) was present in the apparatus (***Figure 1E***). The parameters assessed were the same as in Test 3.

- PND95: **Test 5**
Differently from the previous tests, the animal was evaluated as in Test 1 but did not receive a reward, when it made the correct touches (***Figure 1F***).

All females (both tester and no-tester ones) were tested during the estrus phase of the ovarian cycle in Test 3 or 4, when social interaction with a conspecific was planned. Thus, if the animal was not in the estrus phase, the test was postponed until the next day. The estrus phase was assessed by vaginal smear (Cora et al., 2015).

All tests lasted 10 minutes and were recorded using the camera attached to the roof at a distance of 2.5 meters from the apparatus. The reward was dispensed in RR.
The following parameters were evaluated during the tests and were subsequently analyzed with EthoVision 8 (Noldus Information Technology; Noldus, Spink, & Tegelenbosch, 2001):
- Time (s) spent in the different zones of the arena (play zone and arena);
- Distance (cm) traveled (in different zones or total arena);
- Speed (cm/s) achieved (in different zones or total arena);
- Game-related behaviors: interaction with games (cumulative duration), correct touches (*i.e.*, number of goals; frequency);
- Non-game-related behaviors: grooming (cumulative duration), protected rearing (frequency), and unprotected rearing (frequency);
- Wheel-related behaviors: time (s) spent exploring the wheel, time (s) spent running on the wheel;
- Behaviors related to socio-sexual or social interaction: time (s) spent sniffing.

Fixation and tissue sampling
At PND115, a subset of rats, with 5 animals selected from each group (5 control males, 5 control females, 5 GD males, and 5 GD females), underwent a 10-minute play session. After the session, they were sacrificed 90 minutes later by deep irreversible anesthesia (intraperitoneal injection of Zoletil 100 mg/kg - Rompum 20 mg/kg) and transcardially perfused with a 4% paraformaldehyde (PFA) solution (*Tronel & Sara, 2002*). Females were sacrificed during the estrus phase, assessed by vaginal smears (*Alboni et al., 2017; Cora et al., 2015; Micioni Di Bonaventura et al., 2017*). Females were selected and, prior to the test, were assessed by vaginal smears to determine if they were in the estrus phase before being sacrificed (*Alboni et al., 2017; Cora et al., 2015; Micioni Di Bonaventura et al., 2017*).
Brains were removed and stored in 4% PFA solution for 24 hours, followed by several washes in 0.01 M phosphate-buffered saline (PBS). Finally, they were stored in 30% sucrose solution in PBS at +4°C, frozen in pre-cooled isopentane on dry ice at -35°C and stored in a deep freezer at -80°C until sectioning.
Brains (n = 5/group) were serially cut in the coronal plane at 30 μm thickness using a cryostat in three series. The sectioning plane was oriented to match the corresponding patterns to the coronal sections of the rat brain atlas (Paxinos & Watson, 1998). Sections were collected in a cryoprotective solution and stored at -20°C.

c-Fos immunohistochemistry
The presence of c-Fos was detected by immunohistochemistry performed on free-floating sections from one series. Briefly, the sections were washed overnight in PBS at pH 7.3. The following day, sections were first incubated with a citrate buffer (citric acid 10 mM, 0.05% Tween, pH 6.0) previously heated at 95°C for antigen retrieval and then washed three times in PBS. Next, the sections were washed in PBS containing 0.5% Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of PBS containing methanol/hydrogen peroxide for 20 min. Sections were incubated for 30 min with blocking solution containing normal goat serum (Vector

Laboratories, Burlingame, CA, USA) and bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) diluted in PBS containing 0.2% Triton, and then incubated two overnight at +4 °C with polyclonal anti-c-Fos antibody (Cell Signaling Technology, Danvers, Massachusetts, USA; 9F6, Cat. #2250; Rabbit, 1:3.000) diluted in blocking solution. A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in PBS, pH 7.3–7.4, containing 0.2% Triton X-100 was then used at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin–peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was visualized with a solution containing 0.400 mg/ml 3,3-diamino-benzidine (Sigma-Aldrich, Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris–HCl buffer at pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene, and cover-slipped with New-Entellan mounting medium (Merck, Milano, Italy). This antibody was successfully used in previous studies (*Cho et al., 2020; Netser et al., 2020; Zhou & Jia, 2021*). The specificity of this antiserum was previously assessed (*Kovary & Bravo, 1991*), but, as a further control, we omitted the primary antiserum or the secondary biotinylated one and replaced it with PBS. Positive cell bodies were totally absent.

c-Fos quantitative analysis

For quantitative analysis, selected standardized sections of brain areas summarized in **Table 1,** were chosen according to the rat brain atlas (Paxinos & Watson, 1998). Sections of comparable levels (see ***Table 1***) for each nucleus were acquired with Slide-Scanner Axioscan Z1 (ZEISS, Oberkochen, DE) both at low and high magnification (5x and 20x, respectively). Digital images were processed and analyzed by ImageJ (version 2.10/1.53c; Wayne Rasband, NIH, Bethesda, MD, USA). Measurements were performed within predetermined fields (region of interest, ROI), boxes of fixed size and shape that are inserted inside each labeled considered nucleus (see ***Table 1***). In particular, we counted the number of c-Fos-positive cells in all analyzed nuclei.

Table 1. Neuroanatomical regions analyzed for c-fos immunoreactivity*. The table reports the Bregma coordinates, the total number of levels, and the mean ROI area of each analyzed nucleus. ROI: region of interest (Casile et al., 2024)*

Nucleus	Bregma	N° of levels	Mean ROI area (μm²)
Cortex	2.70/-2.56 mm	10/15	434560.725
Prelimbic cortex (PrL)	2.70/2.20 mm	3	121406.056
Primary motor cortex (M1)	2.70/2.30 mm	10 / 12	45459.095
Orbitofrontal cortex (OFC)	2.70/-2.56 mm	8/12	22571.449
Cingulate cortex (Cg)	1.60/0.70 mm	4	61477.015
Nucleus Accumbens	2.70/0.70 mm	6/8	54080.231
Nucleus Accumbens core (AcbC)	2.70/0.70 mm	6/8	21035.513
Nucleus Accumbens shell (AcbSh)	1.20/0.70 mm	3	33044.718
Caudate putamen (CPu) / Striatum	1.60/0.70 mm	3	92327.774
Medial Septal Nucleus (MS)	1.60/0.70 mm	4	31648.192
Bed nucleus of stria terminalis (BNST)	- 0.92 mm	1/3	38892.460
Lateral hypothalamic area (LH)	- 0.92 mm	1/3	19208.670

Supraoptic nucleus (SON)	- 0.92 mm	1/3	1147.705
Suprachiasmatic nucleus (SCh)	- 0.92 mm	1/3	844.809
Paraventricular thalamic nucleus (PVT)	-1.80/-2.30 mm	3/5	29641.310
Amygdala	-1.88/-2.30 mm	2/5	44761.803
Central amygdaloid nucleus (CeM)	-1.88/-2.30 mm	2/5	17322.898
Basolateral amygdaloid nucleus (BLA)	-1.88/-2.30 mm	2/5	18964.800
Medial amygdaloid nucleus (Me)	-1.88/-2.30 mm	2/5	8474.105
Ventral hippocampus	-5.80/-6.04 mm	2/3	164484.941
Ventral tegmental area (VTA)	-5.60/-6.04 mm	1/3	63042.950
Substantia nigra (SN)	-5.60/-6.04 mm	1/3	38121.337
Peripeduncular nucleus (PP)	-5.60/-6.04 mm	2	388.601
Dorsal raphe nucleus (DR)	-8.00/-8.72 mm	3	821.354
Median raphe nucleus (MnR)	-8.00/-8.72 mm	3	701.500
Pontine nucleus (Pn)	-8.00/-8.72 mm	3	678.587
Caudal Dorsal raphe nucleus (DRC)	-9.16/-9.75 mm	3/4	326.327

Statistical analysis

Quantitative behavioral and immunohistochemical data were analyzed with SPSS 27 statistic software (SPSS Inc, Chicago, IL, USA) via two-way analysis of variance (ANOVA), with sex and condition (GD vs CON) considered as independent variables. To understand whether the behavior of the GD groups was maintained across the different tests, we compared the behavioral parameters performed in Test 1 with the other tests, using three-way ANOVA (test, sex, and condition as independent variables) for repeated measures.

For the data from the last week of training, we used two-way ANOVA (day and sex as independent variables) for repeated measures to compare the behaviors of the GD groups on different days.

If the ANOVA was significant, the *post hoc* analysis was performed using Tuckey's HSD (honestly significant difference) test.

Finally, to find out whether there was a significant correlation between time spent in the play zone and other behaviors (the speed and distance traveled in the play zone, duration of interaction with the tablet, and number of correct touches) during training and in Test 1, we used Person R Correlation.

The data are presented as mean ± SEM, and the differences between groups are considered significant for values of $p<0.05$.

RESULTS

Training

To determine whether animals undergoing the protocol-maintained game-related behaviors, we evaluated the last five days of training. The GD groups of both sexes, during the training phase, spent almost all the time in the play zone reducing the time spent in the remaining part of the apparatus ($p<0.001$). Excluding some small daily variation, when comparing the different parings assessed, the rats of both sexes in the GD group kept both the distance traveled in the play zone, the time spent interacting with the game, and the number of correct touches constant.

Performing Person R Correlation, it was possible to find a significant correlation among different behavioral parameters. Increasing time spent in the play zone was positively correlated with increasing distance traveled ($p<0.001$ for males; $p=0.041$ for females) and velocity in the play zone ($p<0.001$, for males; $p=0.005$, for females), game interaction ($p=0.002$ for males; $p=0.005$ for females), and a number of correct touches ($p<0.001$ for males; $p=0.013$ for females).

Test

Behavioral results obtained from the analysis of the different tests are summarized in **Table 2.**

Table 2. Results obtained from the analysis of different behaviors within the Test phases.

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold (Casile et al., 2024).

Test 1						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(5.58)}$	p
Frequency in play zone (N)	12.580±0.67	13.380±0.89	11.67±0.81	11.67±0.81	0.95	0.424
Cumulative duration in play zone (s)	102.92±10.18	145.19±13.12	352.71±10.18	363.34±13.12	151.60	**<0.001**
Latency to enter in Play zone (s)	19-76±4.53	4.44±0.58	6.38±2.26	2.19±0.32	11.59	**0.001**
Cumulative duration in the other zone (s)	377.74±10.15	335.76±13.13	128.25±11.19	117.49±8.70	152.27	**<0.001**
Distance traveled in play zone (cm)	251.09±23.94	339.03±25.50	1238.34±49.21	1700.06±49.10	24.87	**<0.001**
Distance traveled in the other zone (cm)	1243.62±64.05	1095.43±72.61	667.24±61.00	700.24±91.92	97.41	**<0.001**
Velocity in play zone (cm/s)	2.49±0.18	2.41±0.15	3.52±0.15	4.74±0.30	23.04	**<0.001**
Cumulative duration to game interaction (s)	0.21±0.21	0.53±0.53	147.8±7.39	167.92±5.70	253.51	**<0.001**
Correct touch (N)	1.33±0.31	1.38±0.24	36.00±2.05	41.62±1.84	160.77	**<0.001**
Protecting rearing (N)	37.25±2.96	34.15±3.35	14.78±1.50	16.48±1.62	26.16	**<0.001**
Un-protecting rearing (N)	8.25±1.94	1.38±0.69	1.44±0.32	1.10±0.35	14.63	**<0.001**
Grooming (s)	7.97±1.89	21.29±3.61	9.37±3.82	8.56±2.78	3.24	**0.028**
Adaptation to wheel						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(5.58)}$	p
Frequency in play zone (N)	8.17±1.01	6.46±0.72	8.68±0.58	10.55±0.78	4.88	**0.004**

Cumulative duration in play zone (s)	63.65±7.67	39.14±3.97	54.44±7.21	61.94±6.44	2.34	**0.082**
Frequency in wheel zone (N)	11.58±1.92	15.69±1.25	21.32±1.84	21.32±1.84	6.29	**0.001**
Cumulative duration in wheel zone (s)	88.87±19.81	149.65±13.69	135.64±12.17	135.64±12.17	2.86	**0.044**
Distance traveled In Arena (cm)	1692.81 ±83.45	1731.74±55.99	1771.29±50.78	2061.55±39.14	10.33	**<0.001**
Distance traveled in play zone (cm)	223.73±23.54	191.84±18.18	231.48±29.18	339.46±42.11	3.98	**0.012**
Velocity in play zone (cm/s)	3.75±0.31	4.80±0.36	4.84±0.28	5.54±0.26	5.61	**0.002**
Cumulative duration to game interaction (s)	0.77±0.77	0.14±0.14	7.88±3.59	12.54±3.40	3.79	**0.015**
Correct touch (N)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.48	0.230
Grooming (s)	25.66±16.91	25.66±3.10	5.07±0.83	3.93±3.32	2.06	0.114
Cumulative duration to exploration wheel (s)	237.79±17.77	331.67±9.53	351.69±1.45	313.48±0.83	14.40	**<0.001**
Protecting rearing (N)	26.92±2.34	20.31±1.81	12.63±1.67	25.60±2.02	11.67	**<0.001**
Un-protecting rearing (N)	0.92±0.34	0.62±0.27	0.68±0.30	1.20±0.42	0.60	0.617
Test 2						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(5.58)}$	p
Frequency in play zone (N)	8.17±0.59	6.38±0.89	7.63±0.69	10.71±0.59	7.47	**<0.0001**
Cumulative duration in play zone (s)	45.80±5.31	38.38±7.28	121.55±15.72	245.01±12.57	59.05	**<0.0001**
Frequency in wheel zone (N)	18.42±1.64	17.23±1.49	18.42±1.79	17.23±1.77	3.54	**0.020**
Cumulative duration in wheel zone (s)	175.82± 14.410	232.65±27.69	125.38±1.77	76.37±11.31	17.96	**<0.0001**
Distance traveled In Arena (cm)	1712.82±103.13	1596.69±120.72	1710.85±65.11	2376.82±105.93	14.61	**<0.0001**
Distance traveled in play zone (cm)	174.82±20.48	162.79±31.67	514.74±79.97	1064.19±74.32	39.68	**<0.0001**
Velocity in play zone (cm/s)	3.87±0.32	4.25±0.37	3.72±0.16	4.44±0.29	1.58	0.202
Cumulative duration to game interaction (s)	0.00±0.00	0.00±0.00	16.90±10.65	62.31±0.86	22.54	**<0.0001**
Correct touch (N)	1.00±.0.30	1.23±0.77	18.68±16.27	113.09±28.04	156.24	**<0.0001**
Grooming (s)	13.65±3.11	23.88±9.41	10.41±6.12	12.60±8.08	2.90	**0. 042**
Cumulative duration to exploration wheel (s)	285.05±12.08	313.74±14.92	286.00±18.24	154.18±8.46	29.28	**<0.0001**
Protecting rearing (N)	24.00±1.51	19.31±2.92	11.00±0.99	12.10±1.29	12.83	**<0.0001**

Un-protecting rearing (N)	0.75±0.37	0.31±0.17	0.57±1.36	0.46±1.03	12.83	0.548
Test 3						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(5.58)}$	p
Frequency in play zone (N)	5.92±1.07	6.46±1.16	6.00±0.90	14.40±0.94	19.54	**<0.0001**
Cumulative duration in play zone (s)	21.20±3.20	42.120±13.39	59.60±12.59	291.70±14.23	108.09	**<0.0001**
Latency to enter in Play zone (s)	86.28±37.77	116.86±41.05	30.61±8.88	20.72±8.88	3.91	**0.013**
Frequency in Soci-exual zone (N)	19.83±1.62	13.38±1.76	17.61±1.27	16.45±1.30	2.76	**0.050**
Cumulative duration in Socio-sexual zone (s)	367.24±21.78	328.97±23.02	335.21±22.02	154.72±11.90	27.78	**<0.0001**
Distance traveled In Arena (cm)	1257.92± 92.83	1069.54± 132.65	1184.29±81.44	2891.92±98.65	12.48	**<0.0001**
Distance traveled in play zone (cm)	82.51±12.81	136.49±27.42	251.32±49.85	1703.87±92.44	153.49	**<0.0001**
Velocity in play zone (cm/s)	3.87±0.22	3.73±0.15	4.94±0.31	5.79±0.25	12.48	**<0.0001**
Cumulative duration to game interaction (s)	0.09±0.09	0.72±0.72	23.92±3.70	125.22±5.79	215.39	**<0.0001**
Correct touch (N)	0.50±0.19	0.54±0.39	7.17±1.40	39.65±2.10	157.80	**<0.0001**
Grooming (s)	62.78±2.15	24.67±4.76	56.64±7.80	16.87±2.15	14.17	**<0.0001**
Protecting rearing (N)	19.75±0.08	19.31±0.10	9.50±6.05	18.40±7.50	7.81	**<0.0001**
Un-protecting rearing (N)	0.08±1.50	0.15±2.51	0.17±0.12	0.60±0.17	1.71	0.175
Cumulative duration to socio-sexual interaction (s)	215.79±14.19	185.21±21.71	173.05±1.43	81.83±1.68	23.42	**<0.0001**
Test 4						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(5.58)}$	p
Frequency in play zone (N)	5.50±0.75	7.31±1.00	10.33±0.97	13.38±0.84	14.10	**<0.0001**
Cumulative duration in play zone (s)	25.11±4.92	45.94±8.58	173.82±21.24	348.73±13.48	97.73	**<0.0001**
Latency to enter in Play zone (s)	100.47±41.93	48.81±13.61	27.11±7.78	9.13±2.53	4.81	**0.005**
Frequency in Social zone (N)	14.33±1.53	12.54±1.12	14.33±0.82	11.19±0.54	2.98	**0.038**

Cumulative duration in Social zone (s)	373.13±29.64	347.46±24.24	292.80±25.43	122.53±11.78	28.83	**<0.0001**
Distance traveled In Arena (cm)	1142.02±129.80	1167.84±113.78	1930.33±149.22	3088.67±115.50	53.79	**<0.0001**
Distance traveled in play zone (cm)	104.31±15.93	166.26±30.33	902.81±132.18	2189.43±105.06	95.59	**<0.0001**
Velocity in play zone (cm/s)	3.87±0.32	3.70±0.28	4.94±0.19	6.31±0.25	24.79	**<0.0001**
Cumulative duration to game interaction (s)	0.65±0.62	0.20±0.20	73.73±10.12	152.96±8.0	91.07	**<0.0001**
Correct touch (N)	0.08±0.08	0.15±0.10	23.00±3.05	41.19±1.87	90.32	**<0.0001**
Grooming (s)	5.17±6.41	2.38±4.12	5.39±4.73	8.14±3.70	2.72	**0.034**
Protecting rearing (N)	15.33±1.88	20.46±2.64	13.06±1.68	18.33±0.90	3.64	**0.018**
Un-protecting rearing (N)	0.08±0.08	0.15± 0.10	0.50±3.05	1.05±1.87	2.82	**0.046**
Cumulative duration to Social interaction (s)	241.96±13.01	185.97±19.82	152.23±11.37	67.82±6.55	39.27	**<0.0001**
Test 5						
Parameters	**CON-M**	**CON-F**	**GD-M**	**GD-F**	**ANOVA**	
					$F_{(x,y)}$	p
Frequency in play zone (N)	12.58±0.67	13.38±0.89	12.63±0.50	14.00±0.97	0.64	**0.590**
Cumulative duration in play zone (s)	102.92±10.18	150.43±12.12	303.87±11.91	349.87±6.90	136.70	**<0.0001**
Latency to enter in Play zone (s)	18.19±4.79	4.48±0.48	5.40±0.81	3.06±0.46	13.12	**<0.0001**
Cumulative duration in the other zone (s)	377.74±10.15	335.76±13.12	237.21±11.90	162.16±8.93	78.36	**<0.0001**
Distance traveled In Arena (cm)	1142.02±105.98	1167.84±113.78	1930.33±149.22	3020.32±104.56	55.82	**<0.0001**
Distance traveled in play zone (cm)	127.57±21.99	263.71±114.34	902.81±132.18	2152.90±99.38	78.00	**<0.0001**
Velocity in play zone (cm/s)	3.91±0.89	3.68±0.99	4.94±0.19	6.30±0.21	28.32	**<0.0001**
Cumulative duration to game interaction (s	0.23±0.21	0.00±0.00	188.42±10.57	201.26±8.96	150.56	**<0.0001**
Correct touch (N)	1.42±0.29	2.683±1.37	43.61±2.38	51.81±2.70	108.27	**<0.0001**
Grooming (s)	7.35±1.78	20.88±	9.87±1.78	7.23±1.78	7.56	**<0.0001**
Protecting rearing (N)	36.50±2.87	34.50±3.59	25.33±1.78	23.04±2.43	5.79	**<0.0001**
Un-protecting rearing (N)	7.67±2.87	1.50±3.33	1.78±2.17	1.48±2.43	11.34	**<0.0001**

Test 1

In Test 1, male and female GD animals spent significantly more time in the play zone, reducing that spent in the remaining part of the apparatus compared with same-sex control animals (***Figure 3A***; CON-M vs. GD-M $p<0.001$; CON-F vs. GD-F $p<0.001$). Interestingly, while spending time in the play zone, GD groups of both sexes interacted more with the videogame compared to same-sex CON groups (***Figure 3B***; CON-M vs. GD-M $p<0.001$; CON-F vs. GD-F $p<0.001$). This increase in interaction with the videogame of GD was correlated with an increase in the number of correct touches (***Figure 3C***; CON-M vs. GD-M $p<0.001$; CON-F vs. GD-F $p<0.001$), the distance covered (***Figure 3D*** CON-M vs. GD-M $p<0.001$; CON-F vs. GD-F $p<0.001$), and, only in females, also to a greater speed in the play zone (***Figure 3E***; $p<0.0001$) related to the same-sex CON group. Finally, in GD animals, males and females displayed a similar number of correct touches (***Figure 3C***; $p=0.068$). Conversely, GD animals displayed sexual differences in the game interaction (***Figure 3 B***; $p=0.036$), the distance traveled (***Figure 3D***; $p<0.0001$), and the speed (***Figure 3E***; $p<0.0001$) in the play zone, with females displaying higher values compared to males.

In addition, the Person R Correlation showed a significant correlation both between time spent in the play zone and game interaction ($r=0.797$, $p<0.0001$ for males, $r=0.491$, $p<0.024$ for females) and with the number of touches ($r=0.815$, $p<0.0001$ for males, $r=0.505$, $p<0.020$ for females) in the GD groups.

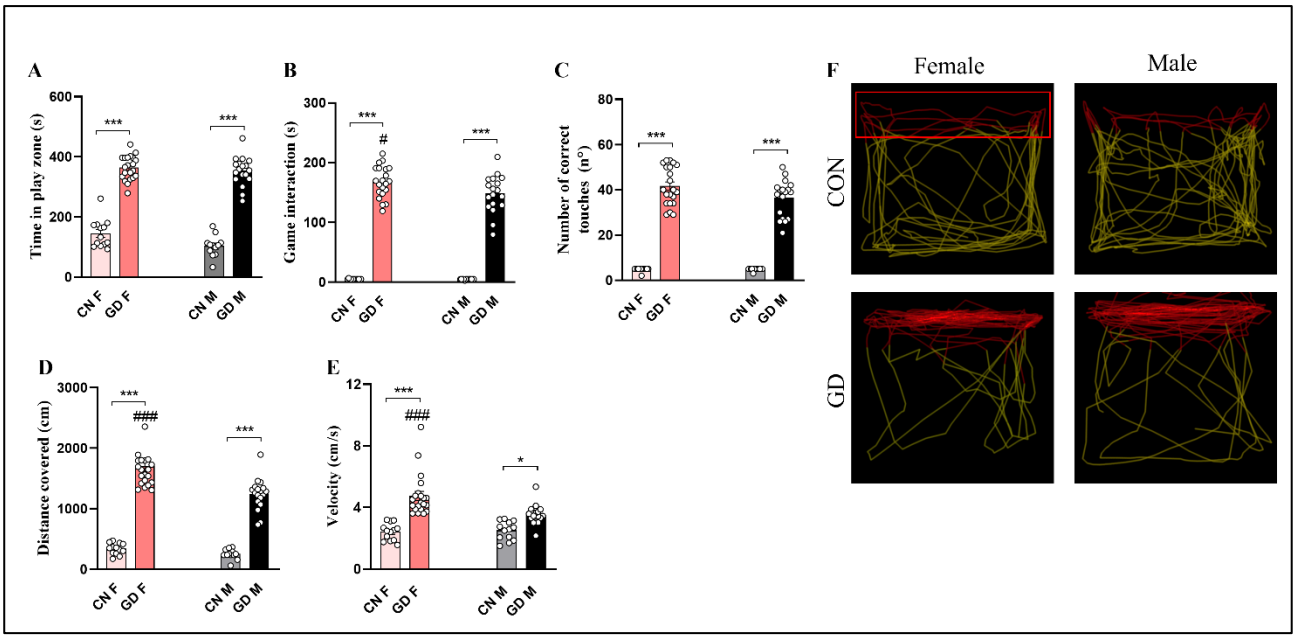


Figure 3. Analysis of the addictive behavior during Test 1 (in the presence of the reward). *The upper histograms show* ***(A)*** *the time spent in the play zone,* ***(B)*** *the time spent in game interaction, and* ***(C)*** *the number of correct touches performed by the different groups. The lower histograms show* ***(D)*** *the distance traveled in the play zone, and* ***(E)*** *the mean velocity.* ***(F)*** *Representative images of the distance traveled by the different experimental groups in the total arena and within the play zone (red rectangle). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for $p\leq0.05$ (* comparison between different groups; # comparison between sexes) (Casile et al., 2024).*

Test 2

On the second day, the wheel fit took place. During the adaptation to the wheel, the test always lasted 10 minutes, the tablet was in OFF mode, and the animal could explore and interact with the wheel. CON animals showed a sex difference in wheel exploration (evaluated as time spent sniffing): females explored more than males (***Figure 4A***; p<0.0001), and this sex difference was not maintained in the GD groups (***Figure 4A***; p=0.071). Comparing same-sex animals from the different experimental groups, significant differences was found only in males. In fact, CON-M spent less time on the exploration of the wheel than GD-M (***Figure 4A***; p<0.0001). Despite the videogame being in OFF mode, the GD-F group interacted more with the screen than the CON-F group (***Figure 4A***; p=0.272). However, no differences were found among the GD groups (***Figure 4A***; p=0.626). In addition, all experimental groups spend more time interacting with the wheel than the tablet (***Figure 4A***; p<0.0001).

During Test 2, when the tablet was switched to the ON mode and the videogame was running, the GD groups increased the time spent interacting with it compared to same-sex CON groups (***Figure 4B;*** CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001). Excluding the GD-F group (p=075), all groups preferred to interact with the wheel to the game (***Figure 4B***; p<0.0001).

In Test 2, compared to the previous day of wheel habit, the video game was in ON mode. This had a different effect on the assessments analyzed. The GD animals spent more time in the play zone than the same-sex CON groups (***Figure 4C***; CON-M vs. GD-M, p=0.001; CON-F vs. GD-F, p<0.0001). Comparing the two sexes, CON groups showed no difference in the time spent in the play zone (***Figure 4C***; p=0.991), while GD-F spent more time in this zone than GD-M (***Figure 4C***; p<0.0001). The video game in ON mode also increased the interaction time with the screen compared with the day of the adaptation when the tablet was switched OFF in both GD groups compared to same-sex CON (***Figure 4D;*** p<0.0001). Moreover, the GD-F showed more interaction with the game both in terms of time spent playing (***Figure 4D***; p<0.001) and number of correct touches than the GD-M (***Figure 4E;*** p<0.001). This sex difference was not present in the CON groups (***Figure 4D-E;*** p=1.000).

Comparing Test 1 and Test 2, the presence of the new object (*i.e.*, the wheel) influenced all parameters assessed in the GD groups. GD groups of both sexes showed a significant reduction in the time spent in the play zone (CON-M vs. GD-M, p<0.001; CON-F vs. GD-F, p<0.001) along with a reduction in both the time spent interacting with the tablet (CON-M vs. GD-M, p<0.001; CON-F vs. GD-F, p<0.001) and the number of correct touches (CON-M vs. GD-M, p<0.001; CON-F vs. GD-F, p<0.008).

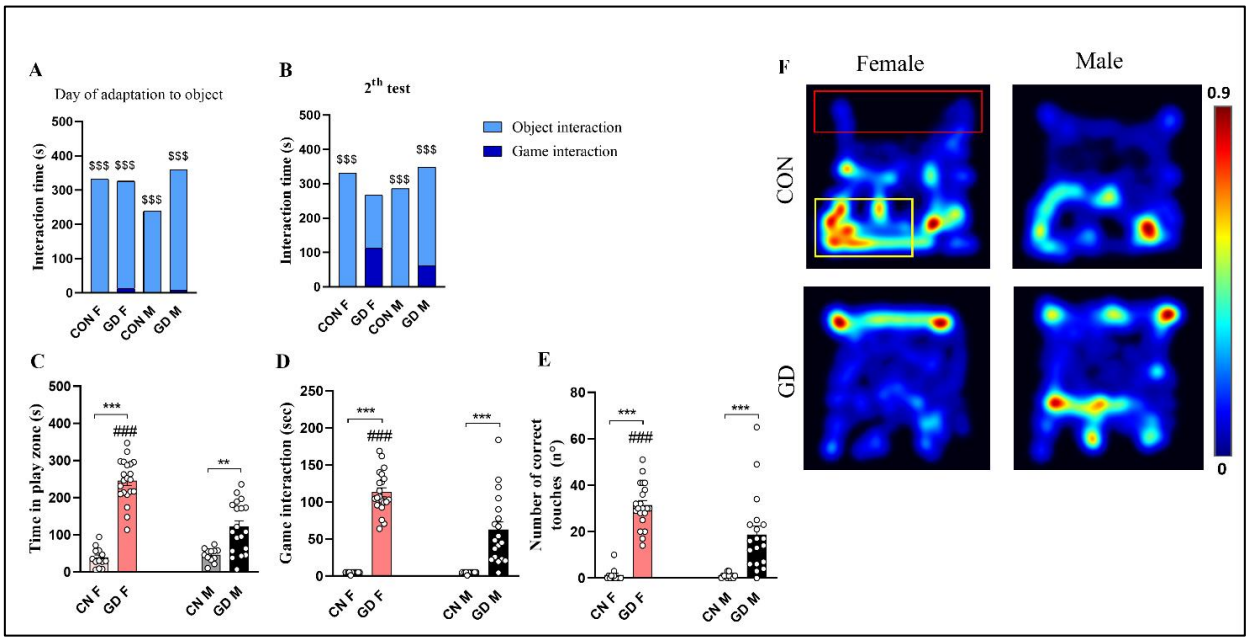


***Figure 4**. **Analysis of behavioral data during the wheel habituation phase and Test 2.** Graph in **(A)** shows the comparison between the time spent interacting with the wheel (evaluated as time spent sniffing the wheel) and the time spent interacting with the tablet, in OFF mode, on the wheel habituation day of the different groups, while graph in **(B)** depicts that obtained on Test 2 day when the tablet was in ON mode and the videogame was running. The histograms in the lower panel show **(C)** the time spent in the play zone, **(D)** the time spent in game interaction, and **(E)** the number of correct touches performed by CON and GD animals during Test 2. **(F)** Representative Heatmaps of the time spent by the different experimental groups in the different zones of the arena (play zone delimited by the red rectangle and area of the wheel delimited by the yellow one). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect of protocol for p≤0.05 (*comparison between different groups; # comparison between sexes). Person R Correlation significant for p≤0.05 (**$** comparison between Test 1 and Test 4). CON = control; GD = gaming disorder (Casile et al., 2024).*

Test 3

The presence of a sexual stimulus (opposite-sex no-tester rat) in the apparatus influenced differentially males and females during the test session. In many of the parameters evaluated, GD-M in the presence of a female behaved similarly to CON-M. In fact, time spent in the play zone (***Figure 5A***; p= 0.195)**,** distance traveled in the play zone (p= ***Figure 5B***; 0.321), and socio-sexual interaction (***Figure 5C***; p= 0.107) did not differ compared to the CON-M. Instead, in females, the GD protocol produced a different effect among the different experimental groups.

GD-F showed a significant reduction in comparison to CON-F in socio-sexual interaction (**Figure 5C**; p<0.0001) but a significant increase in time spent in game interaction (***Figure 5D***; p<0.0001) and time spent in the play zone (***Figure 5A*** p<0.0001). Furthermore, sex also influenced the performance in the duration of the game as well as for the correct number of touches in GD groups, but not in CON animals. Comparing males and females in the same group, except for the CON group (p=0.738), GD-F spent more time in the play zone (***Figure 5A,*** p<0.0001) and game interaction (***Figure 5D,*** p<0.0001) than males, accumulating even a greater number of correct touches (***Figure 5E,*** p<0.0001).

Furthermore, when comparing time spent game interaction to that spent interacting with a conspecific of the opposite sex, CONs and GD-M groups preferred socio-sexual interaction (***Figure 5F,*** p<0.0001), while GD-F preferred playing (***Figure 5F,*** p=0.002).

Comparing the time spent in gaming interaction obtained in this test to that observed in Test 1, revealed that the presence of the socio-sexual stimulus reduced play-related behaviors regardless of sex in GD groups. In fact, compared to Test 1, the GD groups reduced both times spent in the play zone (***Figure 5G***; p<0.0001, for males; p<0.0001, for females) and game interaction (***Figure 5H***; p<0.0001, for males; p<0.0001, for females). Interestingly, GD-M group significantly reduced the number of correct touches performed in Test 3 compared to Test 1 (***Figure 5I***; p<0.0001), while the GD-F group did not show any same difference (***Figure 5;*** p= 0.995). The GD-F group did not show any difference in the distance traveled in the arena between Test 1 and 3 (***Figure 5L***; p=1.000). However, they displayed an increase of the mean velocity in Test 3 (p=0.016). In contrast, the GD-M group significantly reduced both parameters in Test 3 compared to test 1 (***Figure 5L***; p<0.0001, for distance; p<0.0001, for velocity).

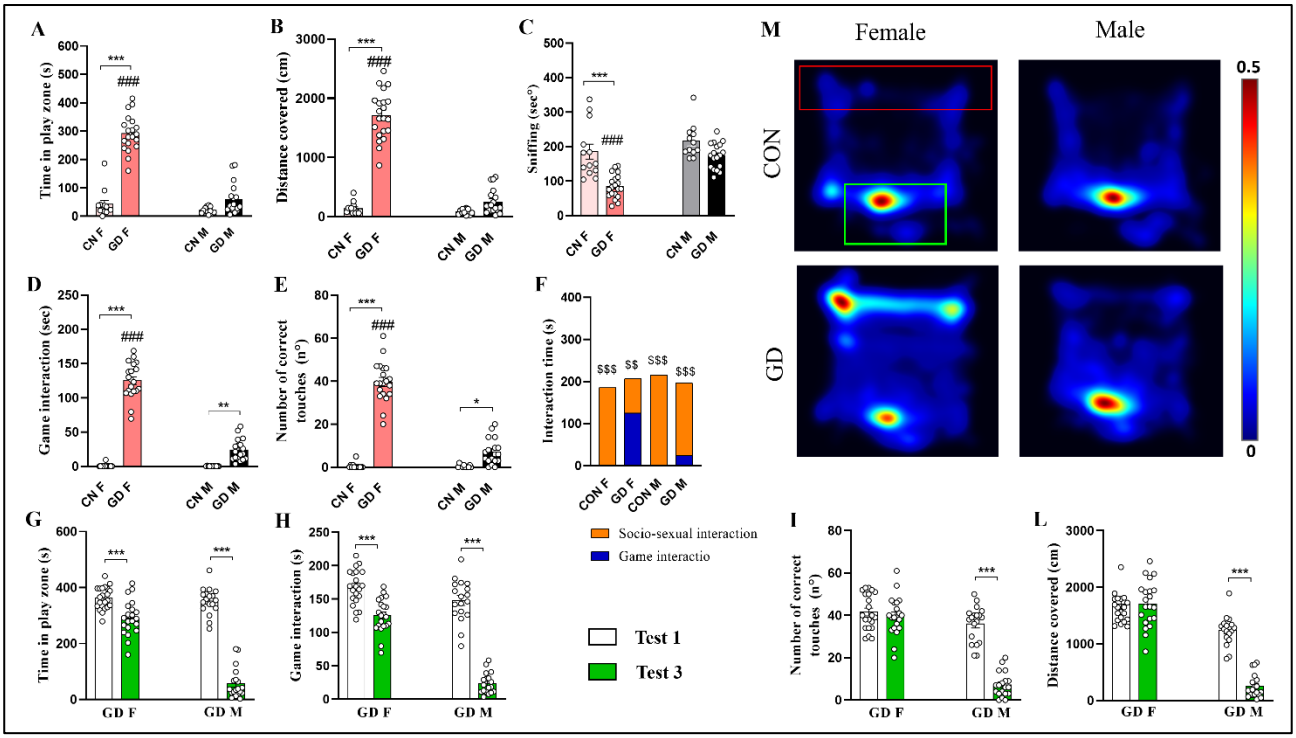


Figure 5. Analysis of behavioral data during Test 3. *Histograms show* ***(A)*** *time spent in the play zone,* ***(B)*** *distance traveled in the play zone,* ***(C)*** *time spent in sniffing behavior,* ***(D)*** *time spent in game interaction,* ***(E)*** *the number of correct touches during play, and* ***(F)*** *the time (expressed as cumulative duration) spent in sniffing behavior on tester animal vs play time during Test 3 (i.e., in the presence of an animal of the opposite sex).* ***(G)*** *Representative Heatmaps of the time spent by the different experimental groups in the different zones of the arena (play zone delimited by the red rectangle and social zone delimited by the green one). Comparison of* ***(G)*** *time spent in the play zone,* ***(H)*** *time spent in game interaction,* ***(I)*** *number of correct touches, and* ***(L)*** *distance traveled between Test 1 and Test 3.* ***(M)*** *Representative Heatmaps of the time spent by the different experimental groups in the different zones of the arena (play zone delimited by the red rectangle and social zone delimited by the green one). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect of protocol for p≤0.05 (*comparison between different groups; # comparison between sexes). Person R Correlation significant for p≤0.05(**$** comparison between Test 1 and Test 3). CON = control; GD = gaming disorder (Casile et al., 2024).*

Test 4

In Test 4, the presence of a social stimulus (same-sex no-tester rat) had a less strong effect with respect to sexual stimulus on gaming. During this test, as before, GD-rats spent more time in the play zone compared to same-sex controls (***Figure 6A***; CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001). In particular, GD-F spent more time in the play zone **(*Figure 6A*;** p<0.0001**)**, game interaction **(*Figure 6B*;** p<0.0001**)** and performed better in terms of correct touches **(*Figure 6C*;** p<0.0001**)** than GD-M. As found previously in Test 3, also in this test, the new stimulus mainly interferes with gaming behavior. In fact, GD rats spent less time in sniffing behavior compared to same-sex controls (***Figure 6D***; CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001). Interestingly, GD-F spent significantly less time in sniffing behavior compared to GD-M (***Figure 6D***; p<0.0001). Indeed, when comparing time spent playing to that spent in social interaction ***(Figure 6E*)**, the CONs (p<0.0001, for both males and females) and the GD-M (p<0.0001) groups preferred interaction with same-sex conspecifics. On the contrary, GD-F spent more time game interaction (***Figure 6E***; p<0.0001)**.**

Differently to Test 3, in Test 4 the social stimulus produced a different sex-dependent effect.

Comparing Test 4 with Test 1, the presence of a rat of the same sex reduced game-related behavioral parameters only in the GD-M group. In fact, GD-M displayed a significant decrease in the time spent in the play zone (***Figure 6G;*** p<0.0001), time spent playing (***Figure 6I;*** p<0.0001), and the number of touches (***Figure 6L;*** p<0.0001) in Test 4 compared to Test 1. In contrast, the GD-F group shows no difference between the two tests (***Figure 6G-I-L***; p=0.9871, for the time spent in the play zone; p=0.622, for the time spent playing; p=1.000, for the number of correct touches).

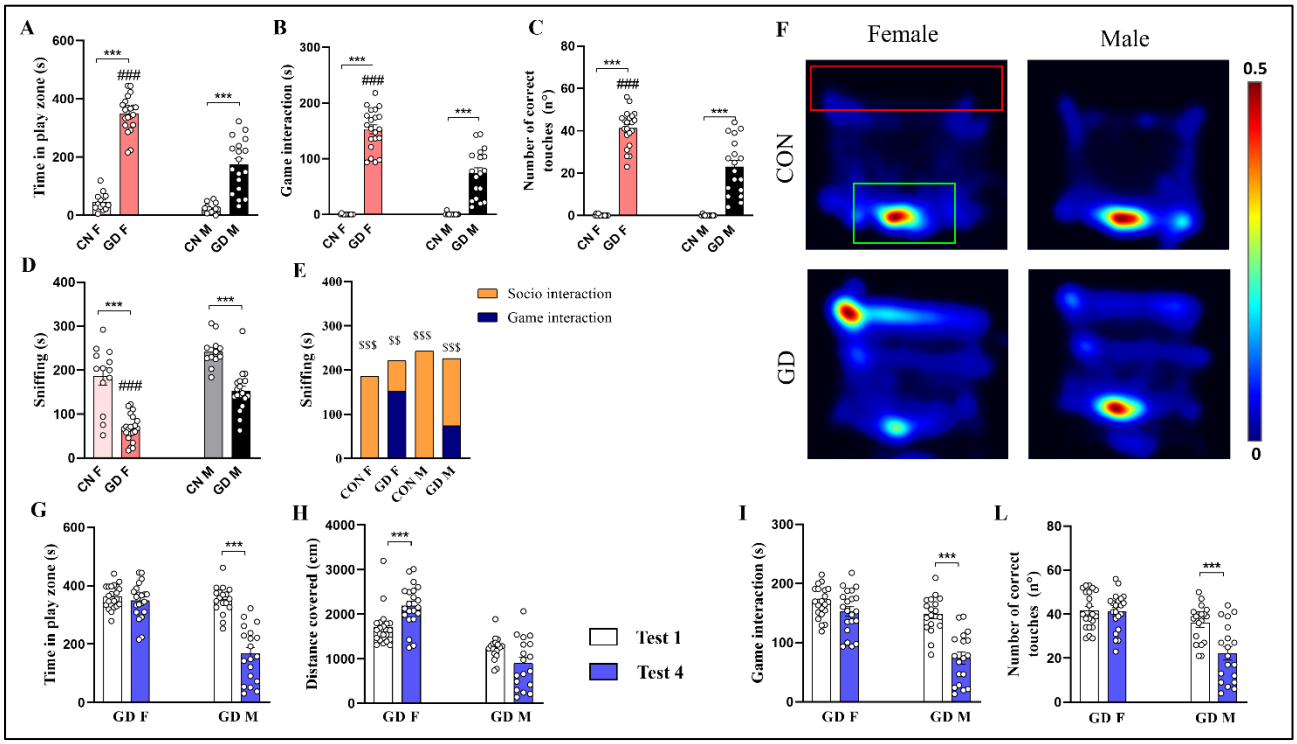


Figure 6. Analysis of behavioral data during Test 4. *Histograms show* ***(A)*** *time spent in the play zone,* ***(B)*** *time spent in game interaction,* ***(C)*** *the number of correct touches during play, and* ***(D)*** *the time spent sniffing behavior towards no-tester rat.* ***(E)*** *Comparison between the time (expressed as cumulative duration) spent in sniffing behavior towards no-tester animal vs time spent in game interaction during Test 4 (i.e., in the presence of an animal of the same sex).* ***(F)*** *Representative Heatmaps of the time spent by the different experimental groups in the different zones of the arena (play zone delimited by the red rectangle and social zone delimited by the green one). Comparison of* ***(G)*** *time spent in the play zone,* ***(H)*** *distance traveled,* ***(I)*** *time spent in game interaction, and* ***(L)*** *number of correct touches between Test 1 and Test 4. Data are expressed as mean ± SEM. Tukey's test revealed a significant effect of protocol for p≤0.05 (*comparison between different groups; # comparison between sexes). Person R Correlation significant for p≤0.05(\$ comparison between Test 1 and Test 4). CON = control; GD = gaming disorder (Casile et al., 2024).*

Test 5

In this last test, we analyzed interaction with the videogame in the absence of the reward. During Test 5, as before in Test 1, GD rats spent more time in the play zone (***Figure 7A;*** CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001) and game interaction (***Figure 7B;*** CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001) than the CON groups. Moreover, they also increased the number of correct touches compared to same-sex CONs (***Figure 7C;*** CON-M vs. GD-M, p<0.0001; CON-F vs. GD-F, p<0.0001). In addition, the GD-F group showed a difference in the time spent in the play zone (***Figure 7A***; p=0.002) and in the number of correct touches (***Figure 7C;*** p<0.0001), but not in the time spent game interaction (***Figure 7B;*** p=0.689) compared with GD-M. Instead, comparing Test 1 with Test 5, it is interesting to note that the absence of reward induced a significant increase in game interaction only in GD groups ***(Figure 7E***; for males, p=0.020, for females, p=0.013) and only GD-F, in the number of correct touches (***Figure 7F,*** p=0.027).

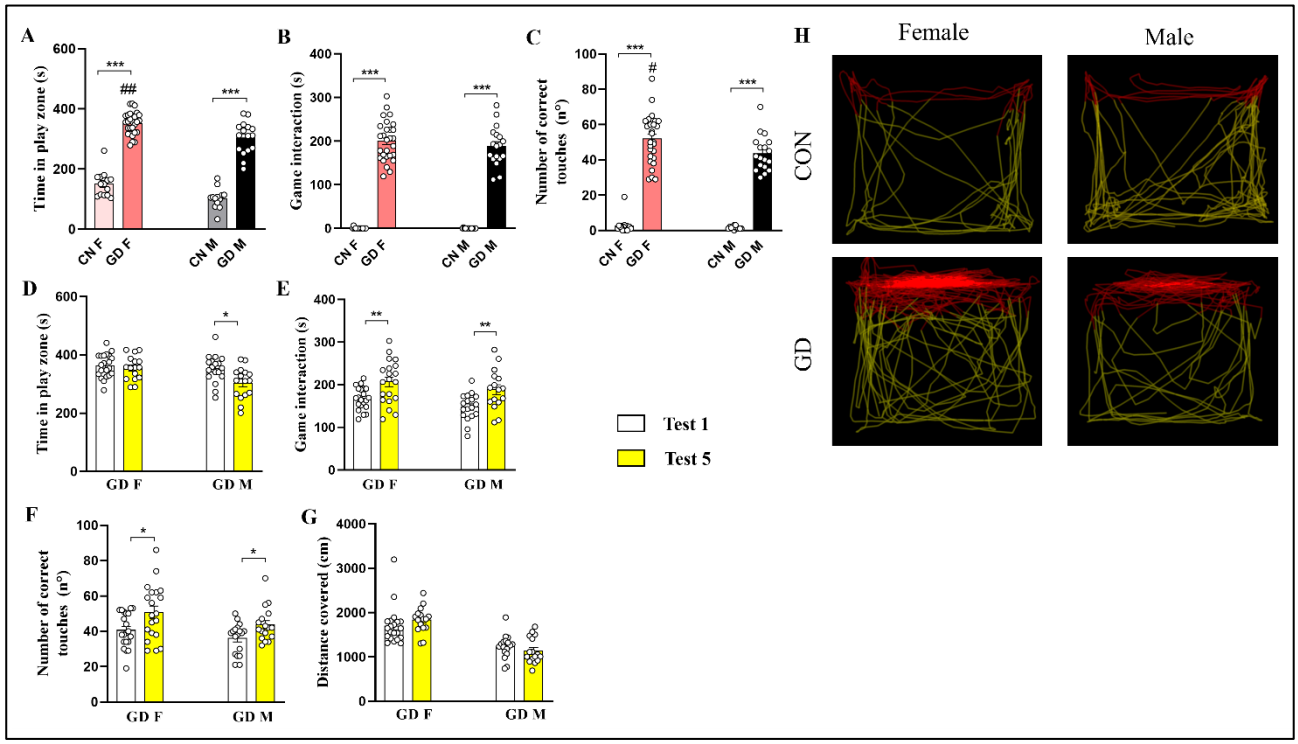


Figure 7. Analysis of the addictive behavior during Test 5 (in absence of the reward). *The upper histograms show* ***(A)*** *the time spent in play zone,* ***(B)*** *the time in game interaction, and* ***(C)*** *the number of correct touches performed during play. The lower histograms show the comparison between Test 1 (i.e., in presence of the reward) and Test 5 (i.e., in absence of the reward) in terms of* ***(D)*** *the time spent in play zone,* ***(E)*** *time spent in game interaction,* ***(F)*** *the number of correct touches, and* ***(G)*** *distance travelled in the play zone. (****H****) Representative images of the distance travelled by the different experimental groups in the different zones of the arena (red path for distance travelled in the play zone and yellow path for that travelled in the remaining part of the arena). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect of protocol for p≤0.05 (* comparison between different groups; # comparison between sexes). Person R Correlation significant for p≤0.05($ comparison between Test 1 and Test 5). CON = control; GD = gaming disorder (Casile et al., 2024).*

c-Fos immunoreactivity

The analysis of the immunoreactivity (ir) for c-Fos is summarized in ***Table 3***. Here we show some relevant results.

Table 3. Results obtained from the analysis of c-fos-ir in all selected nuclei. *Data (number of c-fos positive cells) are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold. OFC = orbitofrontal cortex; PrL= prelimbic cortex; Cg = cingulate cortex; Acb = nucleus accumbens; M1 = primary motor cortex; VTA = ventral tegmental area; CPu = caudate-putamen; PVT = periventricular nucleus of the thalamus; BNST = bed nucleus of stria terminalis; MS = medial septal nucleus; LH = lateral hypothalamic area; SON = supraoptic nucleus; SCh = suprachiasmatic nucleus; SN = substantia nigra; PP = peripeducular nucleus ; DR = dorsal raphe nucleus; MnR = median raphe nucleus; Pn = pontine nucleus. LC = locus coeruleus; DRC = causal dorsal raphe nucleus. (Casile et al., 2024).*

Nucleus	Bregma (mm)	Comparable levels	CON M	CON F	GD M	GD F	ANOVA	
							$F_{(3.16)}$	p
Decision-making circuits								
OFC	2.20	1	1179.86±146.73	556.97±43.28	2151.75±102.42	1604.30±136.55	34.59	**<0.001**
PrL	2.20	1	2748.20±370.48	1435.20±110.71	4387.20±297.98	5935.80±1003.24	12.30	**<0.001**
Cg	0.70	1	462.00±56.76	622.00±87.65	1330.00±100.10	335.25±27.911	32.44	**<0.001**
Mesocorticolimbic Reward System								
OFC	2.20	1	1179.86±146.73	556.97±43.28	2151.75±102.42	1604.30±136.55	34.59	**<0.001**
Acb	1.00	1	777,98±64,37	877,97±54,72	1246,38±85,81	401,75±37,23	30.36	**<0.001**
AcbC	1.00	1	409,3333±73,69	393,3438±80.33	644,0000±63,08	140,6250±35,57	9.87	**0.001**
AcbSh	1.00	1	276,75±45,14	484,63±36,24	602,38±35,67	261,13±41,14	17,40	**<0.001**
Motor learning circuit								
M1	2.20	1	1495.60±42.50	1458.70±184.15	3141.30±135.17	1859.93±75.91	49.12	**<0.001**
VTA	5.60/-6.04	3	456.71±14.59	256.06±26.10	330.46±29.05	108.73±10.68	45.65	**<0.001**
Striatum - CPu	1.00	1	365.30±27.38	587.90±47.24	1146.70±34.38	230.60±28.35	131.32	**<0.001**
Emotional and motivated behaviors								
PVT	2.30	3	97.12±20.86	349.12±23.96	188.50±14.56	52.75±4.85	55.117	**<0.001**
BNST	0.92	3	182.76±20.89	274.80±25.14	343.76±19.60	101.96±10.86	28.424	**<0.001**
Amygdala	2.30	3	885.17±61.71	260.33±26.89	1235.08±43.50	1037.96±66.52	65.44	**<0.001**
CeA	2.30	3	336,60±24,60	88,80±10,41	475,20±22,78	403,60±44,09	35,54	**<0.001**
BLA	2.30	3	391,40±28,21	114,20±7,41	546,00±44,28	446,00±26,46	38,94	**<0.001**
Me	2.30	3	139,20±9,32	56,60±9,79	215,40±9,24	188,60±23,54	23,68	**<0.001**

Other brain regions								
Cortex	1.88/-2.56	5	18596.62±1113.98	9708.20±834.90	23593.00±1488.38	19440.00±1570.84	12,43	**<0.001**
MS	1.00	1	316.75±30.56	290.75±32.01	568.00±66.35	126.00±10.48	20.58	**<0.001**
LH	0.92	3	240.06±11.64	259.70±14.22	172.10±15.44	188.16±21.71	6.60	**0.004**
SON	0.92	3	26.73±1.84	36.06±0.80	29.96±3.37	73.86±5.97	37.26	**<0.001**
SCh	0.92	1	41.10±4.93	65.60±7.256	73.80±8.379	110.20±12.49	10.80	**<0.001**
Ventral hippocampus	5.80	2	1193.91±159.33	1190.78±87.24	1854.250±87.00	643.2±71.70	21.48	**<0.001**
SN	5.60/-6.04	3	232.92±26.16	99.77±18.48	335.433±26.93	247.13±24.88	15.954	**<0.001**
PP	5.60	1	112.95±7.46	104.70±4.26	65.30±3.36	48.70±1.35	43.81	**<0.001**
DR	8.72	1	236.60±206.94	457.26±384.52	348.20±323.75	28.60±14.42	148.29	**<0.001**
MnR	8.72	1	190.60±12.09	284.00±19.85	254.00±11.41	16.60±3.80	83.944	**<0.001**
Pn	8.72	1	340.00±49.99	438.90±38.84	545.30±38.81	269.00±22.14	9.630	**0.001**
DRC	9.16	1	43.20±11.40	54.80±7.50	93.00±5.67	9.60±1.80	21.33	**<0.001**

Inter-individual decision-making circuits

The decision-making process is highly complex and inter-individually variable (Rivalan et al., 2011). It needs the integration of a wide set of executive functions, which involve different brain regions (Rivalan et al., 2011). Particularly relevant for this process are the orbitofrontal cortex (OFC), the prelimbic (PrL), and the cingulated (Cg) cortex (Rivalan et al., 2011). Within the control groups, only in the OFC, the c-Fos-ir was sexually dimorphic, with higher expression in males compared to females (***Figure 8C***; p=0.007). No sexual differences were found in the PrL (p=0.654) or in the Cg (p=0.943) (***Figure 8C***). The dimorphism in OFC was maintained in GD groups (p=0.018). However, the c-Fos-ir in GD rats increased in comparison to same-sex CON groups ***(Figure 8C***; CON-M vs. GD-M, p<0.001; CON-F vs. GD-F, p<0.001). Also, within the PrL, c-Fos-ir was differently affected in GD males and females compared to the controls, showing an increase in the males (p<0.001) and a decrease in the females (p<0.001). Last, in the Cg, GD-F displayed a decreased ir compared to CON-F (p=0.004), whereas GD-M showed an increased ir compared to CON-M (p=0.001). This led to a sexually dimorphic expression of c-Fos among GD-animals, with males showing greater ir compared to females (p<0.0001).

Mesocorticolimbic Reward System

An important circuit for GD is the mesocorticolimbic reward system, which includes the nucleus accumbens (Acb) and the OFC (Chen et al., 2016). The c-Fos analysis showed that, while in the CON groups, the immunoreactivity in NAc did not show sexual dimorphism (***Figure 8D***; p=0.843), in GD animals, males presented greater c-Fos-ir than females (***Figure 8D***; p<0.0001). Moreover, GD-M showed an increased c-Fos-ir (p<0.0001) compared to CON-M, while GD-F displayed a decreased c-Fos-ir in comparison to CON-F (p=0.008) (***Figure 8D***). c-Fos expression in OFC was sexually dimorphic in control animals, with higher ir in males compared to females (p=0.007). This

dimorphism was maintained in GD groups (p=0.018). However, the c-Fos immunoreactivity in GD animals increased in comparison to same-sex CON groups (***Figure 8C***; CON-M vs. GD-M, p<0.001; CON-F vs. GD-F, p≤0.001).

Motor learning circuit

Motor learning is considered the ability to adapt movement to changing environmental stimuli (Peters et al., 2017). The central component of motor-related circuits is the motor cortex, while particularly important in learning are its connections with the striatum and its modulation by VTA neurons (Peters et al., 2017). GD animals learned how to play with the tablet, acquiring the movement of touch to achieve goals. Interestingly, we found that in the primary motor cortex (M1) GD animals, unlike controls, displayed a sexual dimorphism, with males showing greater activation compared to females (p=0.009). Furthermore, both GD groups displayed higher c-Fos-ir compared to same-sex control (***Figure 8E***; CON-M vs. GD-M p<0.001; CON-F vs. GD-F p<0.015).

In the VTA, c-Fos-ir is sexually dimorphic in CON animals, with males showing higher ir compared to females (p<0.001). This difference is maintained among the GD groups (p<0.001). However, GD caused a reduction in c-Fos-ir in both GD-M (p=0.004) and GD-F (p=0.001) compared to same-sex controls (***Figure 8E***). Lastly, in the CPu, we observed higher c-Fos-ir in CON-F compared to CON-M (p=0.002), but this sexual dimorphism was completely reversed in the GD groups (p=0.018). In fact, GD-M displayed an increased ir compared to CON-M (p<0.0001), while GD-F showed a decreased ir compared to CON-F (p<0.0001) (***Figure 8E***).

Regions related to anxiety, stress, and emotional and motivated behaviors

Response to stress stimuli and emotional and motivated behaviors are highly controlled by the paraventricular thalamic nucleus (PVT) and its connections with the BNST and amygdala (Kirouac, 2021). Interestingly in these regions, we found a sexually dimorphic expression of c-Fos, higher in females compared to males, which was completely reversed by GD (***Table 3***). Specifically, in the PVT, we observed higher c-Fos-ir in CON-F compared to CON-M (p<0.0001), but the sexual dimorphism was reversed in the GD groups (p<0.0001). In fact, GD-M displayed an increased ir compared to CON-M (p=0.010), while GD-F showed a decreased ir compared to CON-F (p<0.0001) (***Figure 8F***). Within the BNST, CON-F showed higher c-Fos-ir compared to CON-M (p=0.022), but the sexual dimorphism was reversed in GD groups, where GD-M displayed higher ir compared to GD-F (p<0.0001). In fact, GD-M showed increased expression compared to the same-sex CON group (p<0.0001), whereas in GD-F c-Fos-ir was decreased compared to CON-F (p<0.0001) (***Figure 8F***). In the amygdala, CON-M showed higher expression compared to CON-F (p<0.0001), while among the GD animals, males displayed higher ir compared to females (p<0.0001) (***Figure 8D***). In particular, in GD animals, c-Fos-ir was increased compared to CON (***Figure 8D***; GD-F vs. CON-F p<0.0001; GD-M vs. CON-M p=0.038).

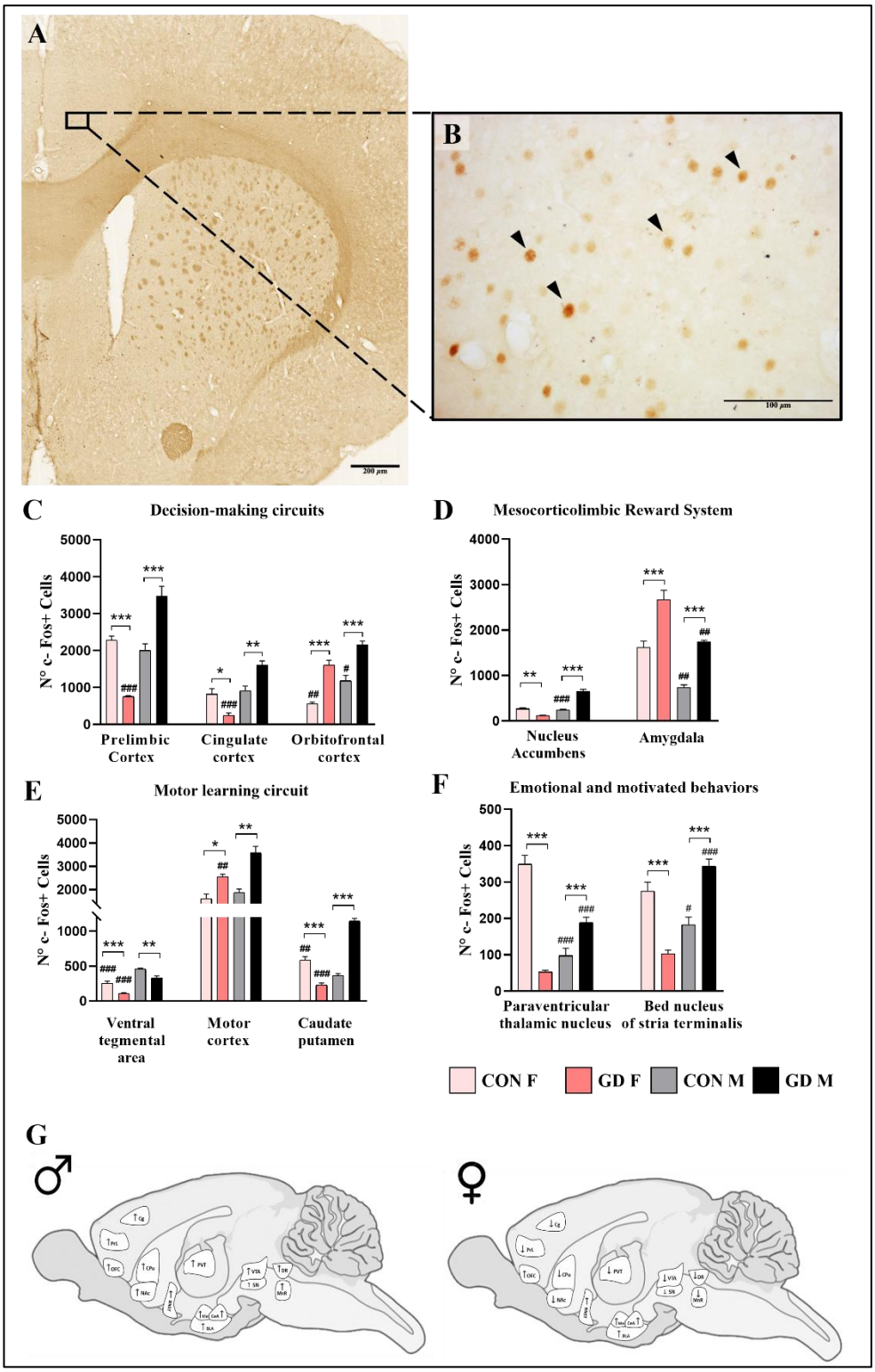


Figure 8. Analysis of c-Fos immunoreactivity in the brain circuits related to addictive behavior in the control and GD animals. *Representative images of the c-Fos immunoreactivity in a coronal brain section from a control male rat at* ***(A)*** *20x magnification (scale bar = 200µm) and* ***(B)*** *40x magnification (scale bar =100µm). Quantification of the number of c-Fos-positive cells in* ***(C)*** *decision-making circuit,* ***(D)*** *mesocorticolimbic reward system,* ***(E)*** *motor learning circuit, and* ***(F)*** *emotional and motivational circuit.* ***(G)*** *Images show the different areas involved in GD, that display higher (↑) or lower (↓) c-Fos-ir in the GD animals compared to control animals of the same sex. Data are expressed as mean ± SEM. TWO-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes). CON = control; GD = gaming disorder; ir = immunoreactivity (Casile et al., 2024).*

DISCUSSION

GD is a recognized mental health condition that urgently needs new therapeutic approaches. However, the research is limited and presents some bias. The possibility of being able to take advantage of an animal model could help the understanding of the core behavioral and neurobiological features present in GD, including compulsivity and sex differences.

The new model proposed here resembles some of the specific behavioral characteristics observed in GD patients, such as loss of control over play accompanied by compulsive and hyperactive behavior (Lopez-Fernandez et al., 2019; Marraudino et al., 2022).

After 5 weeks of training, the rats subjected to GD protocol develop these patient-like traits, and the behavioral phenotype is maintained consistently over time. In fact, it does not decrease after stop periods and increases in the absence of reward (yogurt delivery). The hyperactivity and compulsive components, measured by the number of correct touches, speed, and distance traveled in the play zone, are more strongly observed when the animals are in the play zone (near the tablet). Moreover, they are positively associated with the time spent in the play zone and the behaviors performed during the task. These aspects are completely absent in the control groups. During the task, this pattern does not show a sex difference except when rats are exposed to the social context. In fact, in the presence of a social stimulus (same-sex no-tester rat), males show a reduction in behaviors associated with play, while females continue to prefer play to social interaction, possibly due to the alteration of reward circuits which are closely related to both social behavior and mental disorders.

At present, GD lacks an experimental model, and our schedule to induce GD in rats shows several advantages compared to other addiction-related models:

a. no stressogenic deprivation (*i.e.*, no caloric restriction or social isolation is used), differently from others (Barrus & Winstanley, 2017; Di Ciano & Le Foll, 2016; Ishii et al., 2018; Rafa et al., 2016; Rygula et al., 2012; Winstanley et al., 2011);

b. developing a propensity to use electronic devices, this is similar to other gambling models (Barrus & Winstanley, 2017; Ishii et al., 2012; Rafa et al., 2016; Rygula et al., 2012; van den Bos et al., 2006), but unlike others, the used task prompts the rats to interact as much as possible with the screen, because obtaining the reward is directly related to the correct touch of the moving stimulus;

c. the use of a much larger apparatus (50 x 55 x 50 cm) than those used in gambling models (30.5 x 25.9 x 30.5 cm) (Barrus & Winstanley, 2017) allows the development and quantification of the motor component in relation to the task, which is not entirely counted in other studies;

d. the use of both sexes. Excluding the work of Ishii et al. (Ishii et al., 2018), this aspect is almost absent in most of the research done on gambling using rat models. The choice to exclude the female sex is justified by the higher incidence of addiction in men (Gartner et al., 2022). In recent years, numerous works showed that the incidence of the development of these mental disorders is similar between the sexes and that females manifest stronger symptoms than men (Lucas et al., 2023; Quigley et al., 2021). Enhancing the construct validity of our GD model, we observed that female rats, like male ones, show the same behavioral phenotype that remains stable even in different settings;

e. the evaluation of the social component, another unexplored feature in other gambling models.

The addition of this last component strongly characterizes and differentiates our model from others. The GD group presents a reduction in social interaction in Test 3 (competitive socio-sexual stimulus) and 4 (competitive social stimulus) compared to controls, thus maintaining an inclination towards playing a videogame. However, GD rats display a sex-dependent reduction in addiction-related behaviors, demonstrating that, as it happens in rat models of substance abuse, also in our protocol compulsivity and seeking behaviors are reduced in the presence of a social reward (El Rawas et al., 2012; Fritz et al., 2011; Venniro et al., 2018).

Also, it is important to underline that, in addition to the establishment of harmful behaviors, GD patients present changes in brain activity within different circuits involved in the control of different aspects of addiction. For this reason, in our work, besides the behavioral evaluations, the brain areas activated during play were investigated, focusing on nuclei belonging to GD-relevant circuits, *i.e.*,

decision-making (Rivalan et al., 2011), mesocorticolimbic reward system (J.-T. Zhang et al., 2016), motor learning (Peters et al., 2017), and regions related to anxiety, stress, and emotional and motivated behaviors (Kirouac, 2021). Our results support the idea that the development of addiction led to changes in the activation of all the circuits mentioned above. Moreover, the sexual dimorphism in c-Fos-ir described could reflect some of the behavioral differences previously discussed.
There are only two works evaluating the expression of c-Fos in gambling (Ishii et al., 2018) or gambling-related (Koot et al., 2014) rat model. The first considers only the PrL and OFC of male rats, whose c-Fos expression is increased (Ishii et al., 2018), as we observed in our GD-male rats. The second assesses c-Fos expression only in the PV in both sexes (Koot et al., 2014), showing a sex difference in line with our findings.

Translational perspectives: comparison between the GD rats and GD patients
There are numerous studies of brain activity performed on GD patients. GD patients present changes in brain activity within different circuits involved in the control of different aspects of addiction. *i.e.*, decision-making (Rivalan et al., 2011), mesocorticolimbic reward system (J.-T. Zhang et al., 2016), motor learning (Peters et al., 2017), and regions related to anxiety, stress, and emotional and motivated behaviors (Kirouac, 2021). Although there are different types of analysis, our model induces GD in rats and demonstrates several similarities with GD patients.
The OFC, which plays an important role in maintaining and controlling attention (Menon & Uddin, 2010), the PrL, involved in the cognitive control and regulation of the reward system (J.-T. Zhang et al., 2016), and the Cg, implicated in the desire induced by the game (Ko et al., 2013), are key regions of the decision-making process (Rivalan et al., 2011). Male patients with GD have increased functional connectivity in the resting state within OFC, PrL (Chen et al., 2016), and Cg (Ko et al., 2013), while nothing is reported about women with GD. In our GD rats, we observed alterations of c-Fos-ir in the same regions. In particular, we highlighted an enhancement of c-Fos-ir in both sexes in the OFC, while we observed opposite effects in the two sexes in the PrL and in the Cg, revealing an increased ir in the males and a decrease in the females. The OFC also participates in the mesocorticolimbic reward system, receiving inputs from the NAc (Koob & Volkow, 2010), which is required for the consolidation and mediation of the reward-gratifying effects of addictions (Koob & Volkow, 2010). In GD male patients, the NAc exhibits a reduction in functional connectivity (Chen et al., 2016). However, in our model, male GD rats show an increase in c-Fos-ir, while we observed a diminution in the females.
Fundamental regions participating in the motor learning circuit are the VTA (Peters et al., 2017), whose dopaminergic projections reach the PrL, the OFC, and the NAc, implied in the elaboration of reward and motivation (Volkow et al., 2017), and the CPu (Peters et al., 2017), essential for conditioning processes and habit formation in addiction, which receives dopaminergic projections from the VTA and substantia nigra (SN) (Drui et al., 2014). The analysis of the functional connectivity in the resting state indicated a reduction of the activity within the VTA of the GD male patients, while an increase was observed in the CPu and the SN (R. Wang et al., 2019). Our current data resembles these features. In fact, GD rats revealed a decrease in c-Fos-ir in the VTA and an increase in the SN in both sexes. Otherwise, an increase in the SN of males was observed, while a decrease in females.
Lastly, the circuit involving the regions related to anxiety, stress, and emotional and motivated behaviors was analyzed, including the amygdala, linked to the processing and regulation of emotions (Kirouac, 2021), the PVT, involved in the motivation, drug-seeking, and drug taking (Millan et al., 2017), and the BNST for its critical role in stress response and anxiety (Avery et al., 2016). In our model, c-Fos-ir was increased in the amygdala of both GD males and females, while in the PVT and in the BNST, it was increased in the males but decreased in the females, reflecting what happens in humans as far as PVT is concerned. Research performed in GD patients describes a reduction in the connectivity of those nuclei to other brain areas (M. Wang et al., 2022), except for the PVT, which displays greater activation among male, but not female, GD patients (J. Zhang et al., 2020). PVT is considered a key region that regulates approach/avoidance behavior in the emotional response (Choi

& McNally, 2017; Hsu et al., 2014; Kirouac, 2015). Moreover, PVT, when the reward is omitted in an unpredictable way (as happens in our experiment, specifically in Test 5), seems to modulate the expression of the exploratory foraging behavior, while if the result was expected, the PVT was not recruited (Do-Monte et al., 2017). The same sexually dimorphic expression of c-Fos in PVT (higher in male than female rats), observed in our GD group, was also found in another animal model of gambling, in which PVT was identified as a candidate region contributing to sex differences in risky decision making (Ishii et al., 2018).

CONCLUSIONS

In the last decade, a wide range of epidemiological studies focused on neurobiological changes in GD patients (Casale et al., 2021; H.-Y. Wang & Cheng, 2022). However, these studies are almost entirely performed in men since they are considered more susceptible to this disorder (Marraudino et al., 2022). The current clinical data is highly heterogeneous as a result of not uniform inclusion criteria (*i.e.*, lack of demographic heterogeneity and equal representation of age and differences in the type of game and the follow-up periods) and of the various research methodologies applied (Kim et al., 2022). In this scenario, the new GD rat model, here described, could represent an innovative tool to investigate, in both sexes, the behavioral and neurobiological features of this disorder, the possible role of external factors, such as the social context, the predisposition and susceptibility, and the development of new pharmacological therapies.

CHAPTER 4

AIM 2

Central and peripheral monoamine changes in a rat model of Gaming Disorder

INTRODUCTION

Adolescents are increasingly immersed in surfing the Internet or using new equipment, such as smartphones and computers. Faster communication, faster access to information, or the ability to find new sources of entertainment and amusement are among the motivations that drive individuals to use these new technologies (*Bediou et al., 2018*). In particular, video games are certainly a very popular form of entertainment among young people (*Cudo et al., 2022*).

However, the excessive use of video games becomes a risk factor for young people, as it interferes with the individual's own normal psychological and social functioning. The possibility of developing a dysfunctional emotional attachment to this activity is high, and it is related both to the psychological condition of the players and to the excessive and prolonged use of the device *(Ostinelli et al., 2021)*. Nonetheless, only in 2013 *(Diagnostic and statistical manual of mental disorders: DSM-5™, 5th ed, 2013*), the World Health Organization (WHO) recognizedGD and then included it in the ICD-11, finally defining it as a mental disorder in 2019 ("The ICD-11 Classification of Mental and Behavioral Disorders: DIagnostic Criteria for Research," 2018).

In the ICD-11, individuals with GD are defined as gamers who, in addition to persistent and recurrent use of video games for at least 12 months, present other symptoms, such as loss of control over the game, little interest in other activities, increased anxiety, accompanied by the development of depressive symptoms (*Petti et al., 2022)* (*Archibald & Tuddenham, 1965; Asken et al., 2007*).

Previous studies show that GD occurs at a higher probability during adolescence *(Nobre et al., 2020; Paulus et al., 2018*). An imbalance in impulse control ability and an alteration in reward circuits are among the various neurobiological hypotheses implicated in this disorder (*Li et al., 2020)*. Multiple studies conducted in adolescents diagnosed with Gender Dysphoria have demonstrated a decrease in cognitive control functions and an increase in behaviors analogous to addiction-seeking (*Li et al., 2020*). These two symptoms are associated with changes in the activity of the serotonergic and dopaminergic systems, two of the most important systems regulating emotion and cognitive functions *(Cools et al., 2019)*.

At the cortical level, 5-HT modulates the function of various brain regions through its receptors, exerting inhibitory effects via 5-HT1A receptors and excitatory effects via 5-HT2A receptors. The regions affected include the anterior cingulate cortex (ACC), which is involved in the regulation of reward-seeking behavior; OFC, which integrates sensory and emotional information; and the PrL, which is responsible for the regulation of decision-making processes (*Kranz et al., 2010*).

DA regulates the activity of the NAc and striatum, which are involved in the control of decision-making and motivational behaviors, via its D1, D2, and D3 receptors. These nuclei project to the ACC and PrL, regulating their functions. Additionally, DA is linked to the functioning of other limbic regions that regulate the processing of emotional information, such as the amygdala and ventral hippocampus (*Arias-Carrión et al., 2010; Assadi et al., 2009)*.
GD patients show alterations in these brain areas and, particularly in the expression of DA and 5-HT receptors and transporters *(Hong et al., 2018; Tian et al., 2014)*. Disruption of these two systems in GD patients is also associated with the onset of other mental disorders that have been described in co-morbidity with GD *(Jorgenson et al., 2016; Schou Andreassen et al., 2016),* including anxiety, depression, and bipolar disorder (*Furuyashiki & Kitaoka, 2019; Hong et al., 2018; Tian et al., 2014)*. Indirect evidence of the involvement of the DA and 5-HT systems in GD relies on fact that drugs that increase cerebral levels of 5-HT and DA, such as Fluoxetine or Bupropion, respectively, reduce the symptoms of GD including impulsivity, addiction toward gambling, and depression *(de Sá et al., 2023)*.
Another important neurotransmitter that seems to be dysfunctional in GD patients is ORX. Individuals with GD display an increase in the blood levels of ORX correlated with enhanced symptoms typical of this mental disorder (*Choi et al., 2020; Zhou et al., 2021*). ORXs are hypothalamic neuropeptides that modulate several physiological functions, such as motivated behavior, feeding, mood, sleep, and stress *(James, Campbell, et al., 2017; Sargin, 2019)*. Dysfunction in the ORX system has been linked to increased addictive behavior *(James & Aston-Jones, 2022; James, Mahler, et al., 2017)* and mood *(Khairuddin et al., 2020)* dysregulation, and predisposes more in general to the development of mental disorders *(Akça Ö et al., 2020; Katzman & Katzman, 2022)*. ORX is a potent regulator of DA function *(Calipari & España, 2012)*. Increased ORX activity leads to increased DA activity by influencing the perception of pleasure and reinforcement of addiction-related experiences *(Cason et al., 2010)*. Like DA *(Esposito et al., 2008),* ORX is also regulated and, in turn, regulates 5-HT activity *(Muraki et al., 2004; Wang et al., 2005)*. These two neurotransmitters are interconnected and form a dynamic balance in regulating the functions of sleep/wake *(Saito et al., 2018; Sakurai et al., 2010),* stress reduction *(James, Campbell, et al., 2017),* and regulation of emotional states *(Mavanji et al., 2022; Tsujino & Sakurai, 2013)*.

Finally, GD and mental disorders that often occur in co-morbidity *(Dullur et al., 2021; Furuyashiki & Kitaoka, 2019; Hong et al., 2018; Koncz et al., 2023; Tian et al., 2014)* are also related to the activation of the immune system and cytokine production under stress conditions *(Benedetti et al., 2017; Comai et al., 2020)*. Among the pathways activated by proinflammatory cytokine and stress there is the kynurenine (KYN) pathway of tryptophan (Trp) *(Comai et al., 2022)*. Increased conversion of Trp into KYN not only reduces 5-HT availability in the brain, but also increases the formation of quinolinic acid (QA), a neurotoxic molecule N-methyl-D-aspartate (NMDA) agonist, affecting the glutamatergic system *(Comai et al., 2022; Schwarcz et al., 2012)*. The excitotoxicity of QA also has a direct effect on 5-HT, DA, and ORX neurons by reducing their numbers and activity *(Cummings & Walker, 1996; Katsuki & Akaike, 2004; Stepanova et al., 2020)*, increasing depressive symptoms, and predisposing individuals with increased susceptibility to stress to more likely develop psychiatric disorders *(Firk & Markus, 2007; Grafe & Bhatnagar, 2018; Moghaddam, 2002)*.

The possibility of investigating changes in the DA, 5-HT, and ORX systems in humans is limited to the analysis of peripheral biomarkers, for which relevance of what is happening in the brain is still debated *(Marraudino et al., 2022)* and to analysis of the brain activity using functional magnetic resonance imaging. However, these techniques have some limitations in that they do not provide data on the functioning of specific neurons.
Recently, in our laboratory, we have developed an animal model characterized by main features present in GD patients, including changes in brain activity in areas related to cognitive control functions, an increase in compulsive and seeking behaviors, and alterations of social *skills (Casile et al., 2024)*. Taking advantage of this new animal model, here, we investigate the potential correlation between the development of GD symptoms and the alteration of the DA, 5-HT, and ORX systems at a central and peripheral level. In particular, by immunohistochemical techniques, we quantified the immunoreactivity of DA, 5-HT, and ORX neurons in the VTA, in the nucleus of the Raphe, and in the lateral hypothalamus (LH), respectively. The analysis of these neurotransmitters in the brain was paralleled by the quantification at the peripheral level, in plasma samples, of DA and Trp metabolites via both 5-HT and KYN pathways.

MATERIALS AND METHODS

Materials.

Winstar Kyoto rats from our colony at the Neuroscience Institute Cavalieri Ottolenghi (originally purchased from Charles River - Charles River Laboratories Italia s.r.l., Milan, Italy) were housed in standard conditions at 22 ± 2 °C, under a 12:12 light-dark cycle (lights on at 08:00 AM). Food (standard chow diet, VRF1, SDS -Charles River Laboratories) and water were provided *ad libitum* throughout the study.
Specifically, at weaning (*i.e.,* postnatal day 28, PND28), 64 rats (30 males and 34 females) were housed in separate cages containing 4 same-sex rats.
The rats were randomly subdivided into four experimental groups:
- Control males (CON M, n=12);
- Control females (CONF F, n=13);
- Males subjected to the GD protocol (GD M, n=18);
- Females subjected to the GD protocol (GD F, n = 21).

GD Protocol
Rats belonging to the GD group were subjected to the GD protocol as described in the previous chapter.
Briefly, after weaning, all rats were habituated to the operator handling and to the apparatus (from PND44 to PND55). From PND55 to PND87 only the GD group of both sexes were subjected to the GD protocol for 5 weeks. The protocol involved training the rat to touch a visual stimulus (white circle) on a touch screen to receive a reward. At the end of the 5 weeks of training, in which the rats were allowed to interact with the screen for 5 minutes a day for 5 days a week, they were subjected to various tests. The purpose of the tests was to find out whether the rats developed compulsive and hyperactive behavior toward the game, and whether they maintained it in the presence of other stimuli (a new object, an unknown individual of the opposite sex, and an unknown individual of the same sex), or in the absence of reward after a period of interruption of play. All these tests were used to

positively assess the development of the GD behavior (*data not shown, for details see Casile et al.,*) (Casile et al., 2024).
At the end of the tests, rats were sacrificed after a 10-minute session for brain and blood collection as described below.

Fixation and tissue sampling for immunohistochemical analysis
Part of the animals (6 CON M, 6 CON F, 11 GD M, and 10 GD F) were sacrificed by deep irreversible anesthesia (intraperitoneal injection of Zoletil 80 mg/kg/ Rompum 10 mg/kg) and transcardially perfused with a 4% paraformaldehyde (PFA) solution. Females were sacrificed in the estrus phase and assessed by vaginal smear (Cora et al., 2015).
Brains were removed and stored in 4% PFA solution for 24 hours, followed by several washes in 0.01 M phosphate-buffered saline (PBS). Finally, they were stored in 30% sucrose solution in PBS at +4°C, frozen in pre-cooled isopentane on dry ice at -35°C and stored in a deep freezer at -80°C until sectioning.
Brains (n=6-11/group) were serially cut in the coronal plane at 30 μm thickness using a cryostat in three series. The sectioning plane was oriented to match the corresponding patterns to the coronal sections of the rat brain atlas (Paxinos & Watson, 1998). Sections were collected in a cryoprotective solution and stored at -20°C. Different areas from one series were selected and processed, using the free-floating technique, for ORX, DA, and 5-HT immunoreactivity, respectively.

Immunohistochemistry
The presence (*i.e.*, immunoreactivity, ir) of ORX, DA (evaluated as Aromatic L-Amino Acid Decarboxylase, AADC), and 5-HT were detected by immunohistochemistry performed on free-floating sections.
First, all sections were washed overnight in 0.01 M PBS pH 7.3-7.4. Before starting, the sections selected for the 5-HT analysis were first incubated in citrate buffer (10 mM citric acid, 0.05% Tween, pH 6.0) that was previously heated to 95 ◦C for antigen retrieval. All sections were washed in PBS pH 7.3-7.4 containing 0.5% Triton X-100 for 30 min and then treated with a solution of PBS containing methanol/hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. Nonspecific binding was blocked by incubating the sections for 30 minutes at room temperature in a blocking solution prepared diluting 1.5% normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) in PBS pH 7.3–7.4, for the ORX-ir and the DA-ir sections, and diluting 1.5% normal horse serum (NHS, Vector Laboratories, Burlingame, CA, USA) and 0.2% bovine serum albumin (BSA, Sigma-Aldrich, Milan, Italy) in PBS pH 7.3–7.4 and 0.5% Triton X-100, for the 5-HT ones. The sections were then incubated two overnights at +4 ◦C with primary antibodies diluted in PBS pH 7.3–7.4 containing 0.2% Triton (*i.e.*, anti-AADC antibody, Rabbit, 1:30000, Cat. No. TE102, Eugene Tech International, New Jersey, USA; anti-ORX-A, Rabbit, 1:1000, Cat. No. D55078, Merck-Millipore, Milan, Italy) or in blocking solution (*i.e.*, anti-5-HT antibody, Goat, 1:5000, Immunostar, #20079). Next, sections were incubated in biotinylated secondary antibody (*i.e.*, goat anti-rabbit or horse anti-goat, Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS pH 7.3–7.4 containing 0.2% Triton X-100, for 60 minutes at room temperature. The antigen-antibody reaction was revealed after a 60-minutes incubation with avidin–peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was visualized with a solution containing 0.400 mg/ml 3,3-diamino-benzidine (Sigma-Aldrich, Milan, Italy) and 0.004% hydrogen

peroxide in 0.05 M Tris–HCl buffer at pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene, and cover-slipped with New-Entellan (Merck, Milano, Italy).
These antibodies were successfully used in previous studies *(Aspesi et al., 2021; Bonaldo et al., 2024).* As a further control, we omitted the primary antisera or the biotinylated secondary ones and replaced them with PBS. In both cases, positive cell bodies and fibers were totally absent.

Plasma levels of tryptophan, tryptophan-metabolites, and dopamine

Blood was taken directly from the heart before the sacrifice procedures began and collected in EDTA-treated vials. After collection, the blood was centrifuged for 10 min at 3000g to separate the plasma. 42 rats (CON M = 8; CON F = 9; GD M = 13; GD F = 12) were sacrificed to quantify plasma levels of DA and the following Trp metabolites along the 5-HT and Kyn pathways: Trp, 5-HT, MLT, Kyn, 3-Hydroxykynurenine (3-HK), kynurenic acid (KYNA), and quinolinic acid (QA) In brief, the biomarkers were quantified by LC-MS/MS using a routine method in the lab *(Sapienza et al., 2024; Tassan Mazzocco et al., 2023)* on a Varian system consisting of a binary Prostar pump, a 410 autosampler, and an MS320 triple quadrupole mass spectrometer equipped with an Electro Spray ion source, and using alpha-methyl tryptophan as an internal standard. The instrument operated in multiple reaction monitoring mode, working in positive ion mode, except for quinolinic acid, which was analyzed in negative mode. LC analysis was performed using an Agilent Eclipse XDB C8 column (3 × 150 mm, 3.5 μm) and gradient elution with (A) water 1% formic acid and (B) acetonitrile (0 min: 95% A; 5 min: 30% A; 8.3 min: 10% A; 10 min: 10% A; 11 min: 95% A; 15 min: 95% A) at a flow rate of 400 μL/min.
The following ratios were also calculates as a proxy of the activity of some of the enzymes involved in the metabolic steps of the Kyn pathway: KYN/Trp ratio as an index of the activity of the indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) activities; 3-HK /KYN as an index of kynurenine 3-monooxygenase (KMO) activity; KynA/3–HK as an index of kynurenine aminotransferase (KAT) activity. Finally, the KYNA/QA ratio was calculated as an index of neuroprotection *(Comai et al., 2020).*

Quantitative analysis of DA-, 5-HT-, and ORX-ir

For quantitative analysis, standardized sections of the brain areas, summarized in ***Tables 1***, ***2***, and ***3*** and selected according to the rat brain atlas (Paxinos & Watson, 1998), were acquired with the Axioscan Z1 scanner (ZEISS, Oberkochen, DE) at both low and high magnification (5x and 20x, respectively). The digital images were processed and analyzed with ImageJ (*version 2.10/1.53c; Wayne Rasband, NIH, Bethesda, MD, USA).* Measurements were performed within predetermined fields (region of interest, ROI), boxes of fixed shape and size inserted within each considered labeled nucleus (see ***Tables 1***, ***2***, and ***3*** for details). Specifically, we counted the number of positive cells, and we evaluated the ir of cell bodies, dendrites, and fibers in all the selected nuclei as area fraction (AF) covered by immunopositive material *(Aspesi et al., 2021; Bonaldo et al., 2024).*

Table 1. Neuroanatomical regions analyzed for ORX immunoreactivity. *The table reports a representative image of the brain areas and the respective ROIs, the Bregma coordinates, the mean ROI area of each analyzed nucleus, and the total number of levels analyzed. ORX = orexin; ROI: region of interest.*

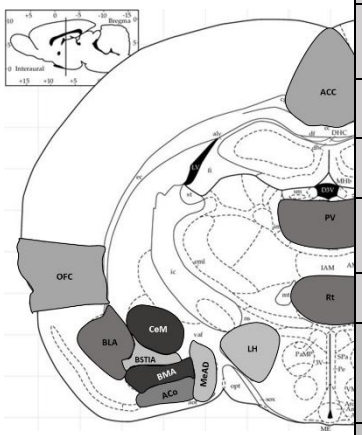
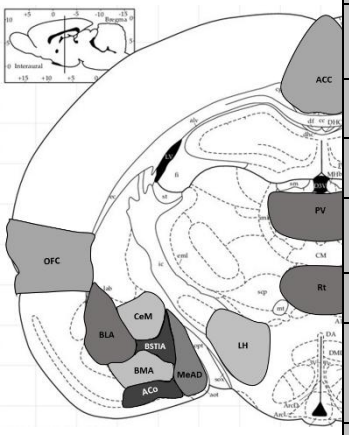
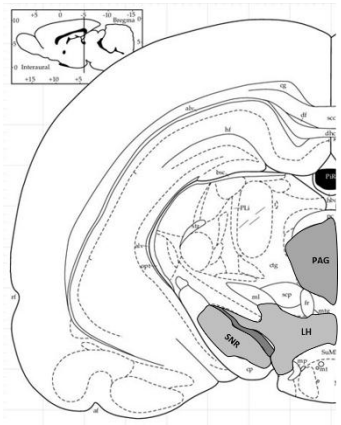
Orexin					
1st and 2nd LEVEL	**Bregma: -2.12/-2.30 mm**		**3rd and 4th LEVEL**	**Bregma: -2.56/-2.80 mm**	
	N°of comparable levels: 2			**N°of comparable levels: 2**	
	Nucleus	**Mean ROI area (mm^2)**		**Nucleus**	**Mean ROI area (mm^2)**
	**PV** - paraventricular thalamic	1.359.227.471.372		**PV** - paraventricular thalamic	1.359.227.471.372
	Rt - reticular thalamic nucleus	1.973.485.517.832		**Rt** - reticular thalamic nucleus	1.973.485.517.832
	LH - Lateral Hypothalamic	3.591.305.802.052		**LH** - Lateral Hypothalamic	3.591.305.802.052
	OFC - orbitofrontal cortex	3.428.722.360.655		**OFC** - orbitofrontal cortex	3.428.722.360.655
	Cg - cingulate cortex	4.423.819.036.770		**Cg** - cingulate cortex	4.423.819.036.770
	BLA - basolateral amygdaloid nucleus	2.935.084.005.343		**BLA** - basolateral amygdaloid nucleus	2.935.084.005.343
	CeM - central amygdaloid nucleus	1.083.232.686.905		**CeM** - central amygdaloid nucleus	1.083.232.686.905
	MeAD - medial amygdaloid nucleus, anterodorsal part	1.060.680.508.344		**MeAD** - medial amygdaloid nucleus, anterodorsal part	1.060.680.508.344
	BMA - basomedial amygdaloid nucleus, anterior part	808.189.307.303		**BMA** - basomedial amygdaloid nucleus, anterior part	808.189.307.303
	Aco - anterior cortical amygdaloid nucleus	584.696.538.186		**Aco** - anterior cortical amygdaloid nucleus	584.696.538.186
	BSTIA - bed nucleus of the stria terminalis, intraamygdaloid division	696.175.946.890		**BSTIA** - bed nucleus of the stria terminalis, intraamygdaloid division	696.175.946.890

Table 2. Neuroanatomical regions analyzed for DA immunoreactivity. *The table reports a representative image of the brain areas and the respective ROIs, the Bregma coordinates, the mean ROI area of each analyzed nucleus, and the total number of comparable levels analyzed. DA = dopamine; ROI: region of interest.*

Dopamine

1ST LEVEL

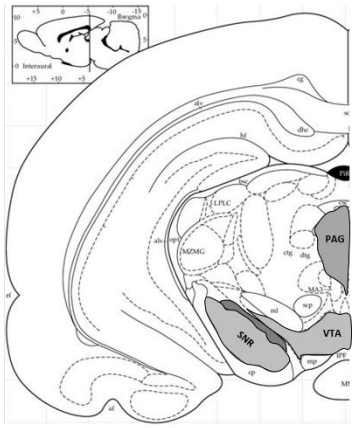


Bregma: -4.80 mm

N°of comparable levels: 2

Nucleus	Mean ROI area (mm^2)
LH - Lateral Hypothalamic	64.061.363
SNc - substantia nigra, compact part	21.088.135
SNr - substantia nigra, reticular part	5.842.293
Arc - Arcuate hypothalamic nucleus	377.771
PAG - periaqueductal gray	1.448.934

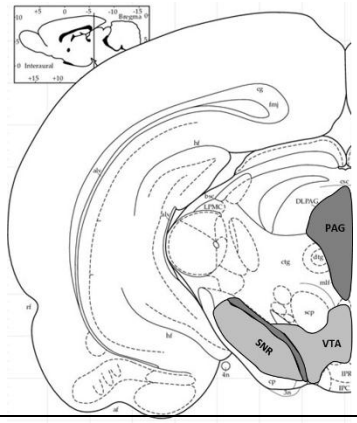
2nd LEVEL



Bregma: -5.30 mm

N°of comparable levels: 2

Nucleus	Mean ROI area (mm^2)
VTA - ventral tegmental area	4.989.219
SNc - substantia nigra, compact part	5.523.147
SNr - substantia nigra, reticular part	664.114
PAG - periaqueductal gray	866.649

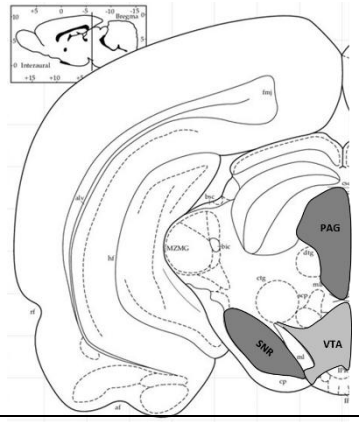


Bregma: -5.80 mm

N°of comparable levels: 2

Nucleus	Mean ROI area (mm^2)
VTA - ventral tegmental area	4.892.776
SNc - substantia nigra, compact part	663.583
SNr - substantia nigra, reticular part	1.010.188
PAG - periaqueductal gray	532.069

4th LEVEL

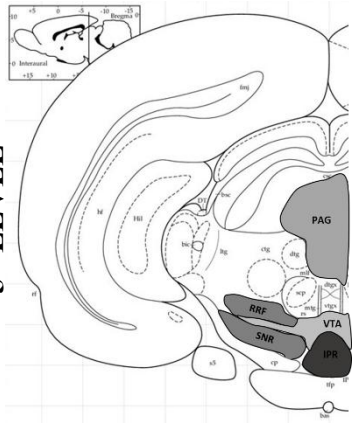


Bregma: -6.04 mm

N°of compaable levels: 2

Nucleus	Mean ROI area (mm^2)
VTA - ventral tegmental area	1.182.928
SNr - substantia nigra, reticular part	711.468
PAG - periaqueductal gray	524.365

5th LEVEL

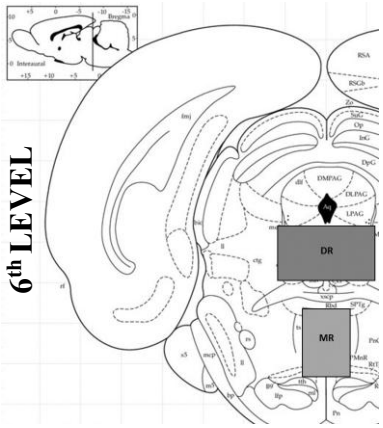


Bregma: -6.30 mm

N° of Levels: 2

Nucleus	Mean ROI area (mm^2)
VTA - ventral tegmental area	163.002
SNr - substantia nigra, reticular part	262.037
RRF - retrorubral field	265.047
IPR - interpeduncular nucleus	247.154
PAG - periaqueductal gray	554.370

6th LEVEL

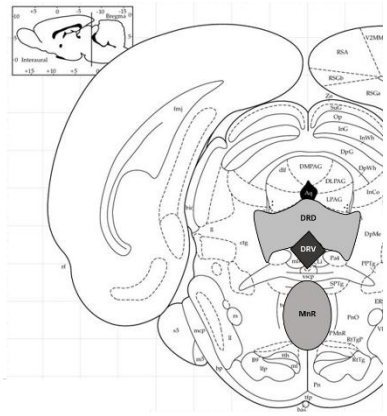
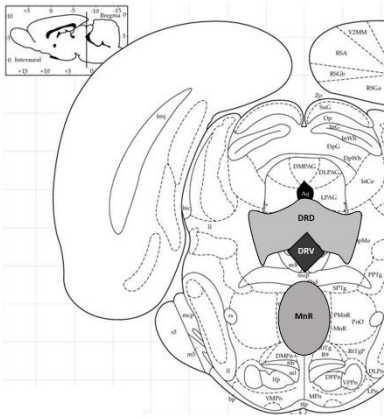


Bregma: -7.64 mm

N° of Levels: 2

Nucleus	Mean ROI area (mm^2)
DR - dorsal raphe nucleus	2.353.706
MnR - median raphe nucleus	403.664

Table 3. Neuroanatomical regions analyzed for 5-HT immunoreactivity. *The table reports a representative image of the brain areas and the respective ROIs, the Bregma coordinates, the mean ROI area of each analyzed nucleus, and the total number of levels analyzed. 5-HT = serotonin; ROI: region of interest.*

<table>
<tr><th colspan="4">Serotonin</th></tr>
<tr><td colspan="2">1st LEVEL
  </td><td colspan="2">Bregma: -7.64 mm
N°of comparable levels: 2
<table><tr><th>Nucleus</th><th>Mean ROI area (mm²)</th></tr><tr><td>DRD - dorsal raphe nucleus, dorsal part</td><td>506.441</td></tr><tr><td>DRV - dorsal raphe nucleus, ventral part</td><td>224.935</td></tr><tr><td>MnR - median raphe nucleus</td><td>276.438</td></tr></table></td></tr>
<tr><td colspan="2">2nd and 3th LEVEL
  </td><td colspan="2">Bregma: -7.80/-8.00 mm
N°of comparable levels: 4
<table><tr><th>Nucleus</th><th>Mean ROI area (mm²)</th></tr><tr><td>DRD - dorsal raphe nucleus, dorsal part</td><td>506.441</td></tr><tr><td>DRV - dorsal raphe nucleus, ventral part</td><td>224.935</td></tr><tr><td>MnR - median raphe nucleus</td><td>276.438</td></tr></table></td></tr>
</table>

Statistical analysis

Quantitative data were analyzed with SPSS 27 statistical software (SPSS Inc, Chicago, IL, USA) by two-way analysis of variance (ANOVA), considering sex and treatment as independent variables. If the ANOVA was significant, the *post hoc* analysis was performed using Tuckey's HSD (honestly significant difference) test. Data is presented as mean ± SEM, and differences between groups are considered significant for p values < 0.05.

RESULTS

The complete analysis of the ir for ORX, AADC, and 5-HT is summarized in ***Tables 4, 5, and 6***. Here we focus on some particularly relevant results.

Table 4. Results obtained from the analysis of ORX-ir in all selected nuclei. *Data is expressed both as number of ORX+ cells and AF and reported as Mean ± SEM. Two-way ANOVA (sex and protocol as independent variables) revealed a significant effect for p ≤ 0.05, highlighted in bold. + = positive; ORX = Orexin; AF= Area fraction; Nuclei: Aco = anterior cortical amygdaloid nucleus; BLA = basolateral amygdaloid nucleus; BMA = basomedial amygdaloid nucleus, anterior part; BSTIA = bed nucleus of the stria terminalis, intra-amygdaloid division; CeM = central amygdaloid nucleus; Cg = cingulate cortex; LH = Lateral Hypothalamic; OFC = orbitofrontal cortex; PV = paraventricular thalamic; Rt = reticular thalamic nucleus.*

Levels		CON M	CON F	GD M	GD F	ANOVA	
						$F_{(3,27)}$	p
ORX AF(%)							
-2.12 mm	**PV**	6.43±0.19	7.30±0.61	10.27±2.20	10.27±0.45	4.07	**0.017**
	Rt	0.74±0.21	2.20±0.27	0.79±0.14	0.32±0.07	21.01	**<0.001**
	LH	10.80±0.07	5.53±0.07	4.79±0.63	2.67±0.63	29.87	**<0.001**
	OFC	1.39±0.10	1.27±0.09	1.27±0.11	1.27±0.14	29.87	**0.043**
	Cg	0.63±0.08	0.63±0.10	0.73±0.11	0.63±0.16	0.20	0.894
	BLA	1.02±0.15	1.23±0.09	1.23±0.16	01.23±0.09	4.32	**0.013**
	CeM	1.88±0.15	1.81±0.15	1.81±0.22	0.89±0.19	5.19	**0.006**
	MeAD	2.21±0.30	1.92±0.22	2.81±0.26	1.77±0.17	4.25	**0.014**
	BMA	2.06±0.11	01.05±0.09	2.21±0.23	1.05±0.23	14.09	**<0.001**
	Aco	2.52±0.04	2.52±0.04	2.93±0.12	2.93±0.19	26.46	**<0.001**
	BSTIA	2.53±0.19	2.53±0.19	2.60±0.19	2.60±0.18	22.47	**<0.001**
-2.30 mm	**PV**	7.60±0.95	7.93±0.61	9.90±1.40	3.54±0.41	7.86	**0.001**
	Rt	0.71±0.12	0.71±0.09	0.71±0.15	0.18±0.04	7.86	**0.002**
	LH	11.50±0.87	6.33±0.15	6.33±0.18	3.46±0.29	66.61	**<0.001**
	OFC	3.46±0.07	0.92±0.12	1.61±0.12	1.21±0.09	66.61	**0.001**
	Cg	0.42±0.10	0.42±0.14	0.42±0.07	0.55±0.14	5.18	**0.006**
	BLA	0.86±0.08	0.87±0.07	1.21±0.11	0.72±0.10	5.43	**0.005**
	CeM	1.71±0.03	1.44±0.12	1.71±0.25	0.85±0.11	7.77	**0.001**
	MeAD	2.09±0.11	2.58±0.10	2.66±0.26	2.66±0.26	2.80	**0.059**
	BMA	2.66±0.21	1.06±0.21	1.82±0.21	1.27±0.21	3.27	**0.037**
	Aco	2.27±0.13	0.99±0.15	2.49±0.09	2.05±0.15	22.55	**<0.001**
	BSTIA	2.80±0.22	1.59±0.15	2.72±0.17	1.72±0.12	14.39	**<0.001**
-2.56 mm	**PV**	14.67±1.24	7.55±0.58	9.33±0.83	3.85±0.37	30.76	**<0.001**
	Rt	0.96±0.06	0.39±0.06	0.39±01.82±0	0.22±0.03	50.55	**<0.001**
	LH	11.35±0.03	5.28±0.03	5.28±0.03	2.97±0.03	35.93	**<0.001**
	OFC	1.67±0.08	1.61±0.11	1.19±0.12	1.39±0.12	3.30	**0.036**
	Cg	0.35±0.04	0.35±0.06	0.31±0.06	0.51±0.09	2.24	0.106
	BLA	1.36±0.10	01.36±0.05	1.36±0.15	0.80±0.06	6.91	**0.001**
	CeM	1.95±0.06	1.82±0.13	1.82±0.20	0.94±0.20	9.24	**<0.001**
	MeAD	2.37±0.15	2.37±0.15	2.68±0.33	1.47±0.28	5.72	**0.004**
	BMA	1.90±0.50	1.05±0.12	1.57±0.14	1.74±0.13	2.43	0.087
	Aco	1.57±0.13	1.22±0.09	2.16±0.14	1.22±0.22	15.76	**<0.001**
	BSTIA	3.44±0.40	2.15±0.40	2.85±0.37	2.85±0.11	9.35	**<0.001**
-2.80 mm	**PV**	8.90±1.36	11.05±1.17	11.52±0.94	3.33±0.94	18.59	**<0.001**
	Rt	1.21±0.94	0.67±0.02	0.67±0.02	0.24±0.05	25.94	**<0.001**
	LH	8.37±0.81	8.37±0.81	8.37±0.54	8.37±0.16	18.50	**<0.001**
	OFC	8.37±0.10	1.03±0.03	1.03±0.03	1.44±0.13	2.60	0.073
	Cg	0.44±0.09	0.44±0.09	0.44±0.05	0.47±0.13	0.37	0.773
	BLA	1.54±0.05	0.84±0.03	1.25±0.14	1.25±0.10	9.35	**<0.001**
	CeM	2.98±0.09	1.22±0.09	1.74±0.20	0.89±0.10	31.35	**<0.001**
	MeAD	3.07±0.03	3.07±0.08	2.52±0.16	1.70±0.22	31.35	**<0.001**
	BMA	2.08±0.19	1.03±0.07	1.03±0.22	1.38±0.16	7.37	**0.001**
	Aco	2.34±0.02	1.32±0.07	1.32±0.26	1.32±0.29	3.63	**0.025**
	BSTIA	1.32±0.16	1.47±0.16	2.38±0.15	1.39±0.14	17.44	**<0.001**
ORX+ cells							
-2.12 mm	**LH**	105.60±6.77	60.20±9.63	129.55±26.11	93.93±14.45	2.09	0.125
-2.30 mm	**LH**	203.00±12.88	115.50±10.26	155.90±8.89	169.56±14.58	7.04	**0.001**
-2.56 mm	**LH**	248.40±18.45	174.90±11.28	150.25±12.11	149.17±15.36	9.03	**<0.001**
-2.80 mm	**LH**	156.40±8.79	160.40±12.21	136.15±12.70	124.94±14.82	1.51	0.234
TOT.	**LH**	713.40±25.63	511.00±14.17	571.85±30.64	521.28±26.82	9.19	**<0.001**

Table 5. Results obtained from the analysis of AADC-ir in all selected nuclei. *Data is expressed both as number of AADC+ cells and AF and reported as Mean ± SEM. Two-way ANOVA (sex and protocol as independent variables) revealed a significant effect for p ≤ 0.05, highlighted in bold. + = positive; AADC=Aromatic L-amino acid decarboxylase; AF=area fraction. Nuclei: Arc = Arcuate nucleus of the hypothalamus; DRN = dorsal raphe nucleus; IPR = interpeduncular nucleus; LH = lateral hypothalamus; MnR = median raphe nucleus; PAG = periaqueductal gray; RRF = retrorubral field; SNcp = Substantia nigra compact part; SNpr = Substantia nigra pars reticulata; VTA = ventral tegmental area.*

Levels		CON M	CON F	GD M	GD F	ANOVA	
						$F_{(3.27)}$	p
AADC AF(%)							
-4.80 mm	**LH**	7.53±0.55	4.82±0.37	3.52±0.23	5.04±0.60	12.29	**<0.001**
	SNc	3.47±0.32	4.52±0.29	2.07±0.31	2.72±0.48	7.11	**<0.001**
	SNr	8.70±0.48	9.42±1.10	4.37±0.68	3.22±0.47	18.62	**<0.001**
	Arc	9.83±0.30	11.16±0.66	8.53±0.87	13.00±0.65	7.93	**<0.001**
	PAG	6.76±0.53	5.85±0.51	3.87±0.32	3.07±0.37	16.47	**<0.001**
-5.30 mm	**VTA**	34.61±1.88	36.76±2.10	26.63±1.10	20.35±2.00	17.97	**<0.001**
	SNc	22.09±1.51	28.57±1.13	11.83±0.75	11.81±1.30	47.47	**<0.001**
	SNr	47.86±1.52	41.22±1.65	40.53±2.23	25.23±1.86	23.09	**<0.001**
	PAG	7.12±0.51	12.40±0.64	4.82±0.63	5.91±1.28	12.18	**<0.001**
-5.80 mm	**VTA**	29.01±1.27	32.17±1.54	16.99±0.97	17.63±1.30	36.78	**<0.001**
	SNc	19.64±1.33	19.20±1.95	6.86±0.32	6.21±0.68	52.48	**<0.001**
	SNr	34.28±2.19	33.95±1.09	23.47±2.76	15.84±2.19	13.16	**<0.001**
	PAG	4.27±0.49	6.66±0.58	1.93±0.19	1.62±0.14	48.04	**<0.001**
-6.04 mm	**VTA**	14.77±0.80	11.74±1.18	6.86±0.36	4.78±0.66	39.06	**<0.001**
	SNr	5.90±0.46	3.91±0.25	3.72±0.22	2.62±0.36	16.07	**<0.001**
	PAG	1.87±0.09	1.51±0.05	1.57±0.12	1.32±0.08	4.92	**0.007**
-6.30 mm	**VTA**	6.23±1.08	1.74±0.21	0.78±0.31	0.18±0.08	29.89	**<0.001**
	SNr	4.23±0.50	3.66±0.26	2.31±0.38	2.10±0.21	8.31	**<0.001**
	RRF	0.00±0.00	3.88±0.57	0.00±0.00	0.83±0.31	36.51	**<0.001**
	IPR	7.97±0.50	8.28±1.33	3.48±0.52	3.19±0.36	16.00	**<0.001**
	PAG	3.21±0.39	2.40±0.08	2.30±0.27	1.47±0.34	5.19	**0.006**
-7.64 mm	**DR**	8.06±0.45	12.95±0.57	8.83±0.91	8.38±1.12	4.92	**0.007**
	MnR	0.92±0.16	2.10±0.14	0.35±0.14	0.53±0.19	20.21	**<0.001**
AADC + cells							
-4.80 mm	**LH**	1263.00±34.75	1147.27±42.52	970.68±65.83	1223.75±68.73	4.83	**0.008**
	SNc	9.75±3.39	16.00±3.21	3.72±1.15	9.83±2.37	4.45	**0.012**
	SNr	170.05±7.63	112.90±7.52	124.45±8.02	65.43±10.61	21.37	**<0.001**
	Arc	261.55±19.58	185.42±9.90	250.33±19.29	190.31±15.62	4.74	**0.009**
	PAG	882.10±33.13	573.50±31.83	596.90±44.34	301.81±32.98	34.94	**<0.001**
-5.30 mm	**VTA**	3044.20±95.83	2515.30±67.20	2430.64±74.23	1747.33±142.75	24.19	**<0.001**
	SNcp	28.08±3.94	35.25±4.87	15.63±3.44	29.08±2.46	5.72	**0.004**
	SNpr	181.65±11.75	188.55±9.12	169.35±8.65	163.75±15.58	0.81	0.499
	PAG	752.30±39.57	534.05±9.99	413.31±26.95	290.96±17.53	53.88	**<0.001**
-5.80 mm	**VTA**	1626.54±79.87	1476.30±32.15	1416.09±58.85	1216.61±102.23	4.51	**0.011**
	SNc	79.05±6.04	72.60±3.00	52.53±4.30	60.29±4.08	6.91	**0.001**
	SNr	222.57±14.25	175.35±12.57	176.35±6.58	161.15±24.95	2.16	0.115
	PAG	523.08±23.10	378.54±19.59	264.66±29.92	264.66±11.92	45.41	**<0.001**
-6.04 mm	**VTA**	1324.40±35.42	1082.52±70.89	787.39±60.38	652.42±35.79	28.92	**<0.001**
	SNr	198.20±10.63	180.10±31.93	121.03±10.21	138.72±17.38	3.97	0.018
	PAG	338.90±29.25	349.68±16.43	236.76±16.65	137.12±11.53	29.45	**<0.001**
-6.30 mm	**VTA**	209.06±32.90	214.00±13.96	161.31±13.96	119.89±8.31	6.19	**0.002**
	RRF	243.10±3.35	297.80±22.81	208.16±13.54	182.06±20.26	7.72	**0.001**
	IPR	178.70±19.32	270.20±16.83	118.53±11.19	181.15±16.48	15.40	**<0.001**
	PAG	359.70±47.78	351.90±43.77	274.33±16.27	167.07±11.13	10.40	**<0.001**
-7.64 mm	**DRN**	837.00±23.50	858.80±22.17	858.80±30.38	735.67±46.68	2.22	0.109
	MnR	421.80±29.95	386.44±30.30	378.37±27.77	268.56±38.84	4.08	**0.016**

Table 6. Results obtained from the analysis of 5-HT-ir in all selected nuclei. *Data is expressed both as number of 5-HT+ cells and AF and reported as Mean ± SEM. Two-way ANOVA (sex and protocol as independent variables) revealed a significant effect for p ≤ 0.05, highlighted in bold. + = positive; 5-HT = serotonin; AF=area fraction. Nuclei: DRD=dorsal raphe nucleus, dorsal part; DRV=dorsal raphe nucleus, ventral part; MnR=median raphe nucleus.*

Levels		CON M	CON F	GD M	GD F	ANOVA	
						$F_{(3.27)}$	p
5HT AF(%)							
-7.64 mm	**DRD**	18.32±1.04	22.38±1.27	13.33±0.92	12.27±1.12	17.16	**<0.001**
	DRV	19.53±0.57	27.98±1.25	14.74±1.42	13.29±0.94	27.20	**<0.001**
	MnR	5.12±0.36	7.98±0.40	3.78±0.37	1.82±0.25	50.87	**<0.001**
-7.80 mm	**DRD**	28.85±0.72	35.52±0.73	23.46±1.43	31.52±0.51	23.86	**<0.001**
	DRV	23.02±0.71	26.86±0.62	19.55±0.86	17.22±1.49	13.55	**<0.001**
	MnR	6.96±0.47	10.67±0.53	5.18±0.43	3.70±0.71	24.98	**<0.001**
-8.00 mm	**DRD**	24.98±1.47	31.23±0.90	25.77±2.13	27.98±1.15	2.32	0.098
	DRV	19.89±0.22	22.26±1.21	19.38±0.42	17.27±1.00	6.17	**0.002**
	MnR	6.24±0.24	6.85±0.28	6.12±0.20	2.44±0.31	61.57	**<0.001**
5HT+ cells							
-7.64 mm	**DRD**	894.50±22.20	755.94±42.18	551.30±65.09	332.33±32.27	22.89	**<0.001**
	DRV	272.28±11.30	244.78±16.62	214.20±19.64	183.50±9.43	5.45	**0.005**
	MnR	469.92±35.74	558.10±12.86	371.66±26.37	237.11±16.82	30.05	**<0.001**
-7.80 mm	**DRD**	1015.60±42.50	856.90±27.85	733.85±48.08	659.21±31.80	13.00	**<0.001**
	DRV	239.57±9.54	236.38±27.28	162.00±10.81	177.78±11.14	7.22	**0.001**
	MnR	421.33±16.85	414.62±25.17	332.34±28.09	219.56±23.75	13.10	**<0.001**
-8.00 mm	**DRD**	908.02±15.70	924.75±68.78	694.30±48.85	544.25±19.16	17.05	**<0.001**
	DRV	187.84±4.08	240.39±17.89	183.17±2.45	152.39±2.45	20.63	**<0.001**
	MnR	557.38±17.54	419.66±15.18	386.44±31.45	195.83±18.32	34.88	**<0.001**

Reduction of the ORX immunoreactivity in GD rats

ORX neurons are mainly located in the LH, but their fibers extend into different brain regions (Marcus & Elmquist, 2005). The GD protocol caused an overall reduction in ORX-ir in all nuclei analyzed (***Table 1***) compared to CON rats, as shown by the coronal brain sections depicted in ***Figure 1A.*** In particular, quantitative analysis performed in LH (Bregma -2.56 mm) showed a sex difference in ORX-ir. CON M showed more ORX+ cells than CON F (p=0.004). In contrast, the GD protocol abolished this sex difference in these rats (p=0.999). Furthermore, the GD M showed a reduction compared with the same-sex controls (males, p<0.0001), but no differences were found between CON and GD females (p=0.628) ***(Figure 1B***).

The analysis of the AF in the LH revealed further differences. In fact, both CON and GD groups showed a sex difference, with a higher AF in males than females (CON, p<.0001; GD, p=0.003). Furthermore, in line with the number of ORX+ cells, it was possible to observe a reduction in AF in both GD groups compared with the same-sex control group (males, p<0.0001; females, p=0.040) ***(Figure 1C***).

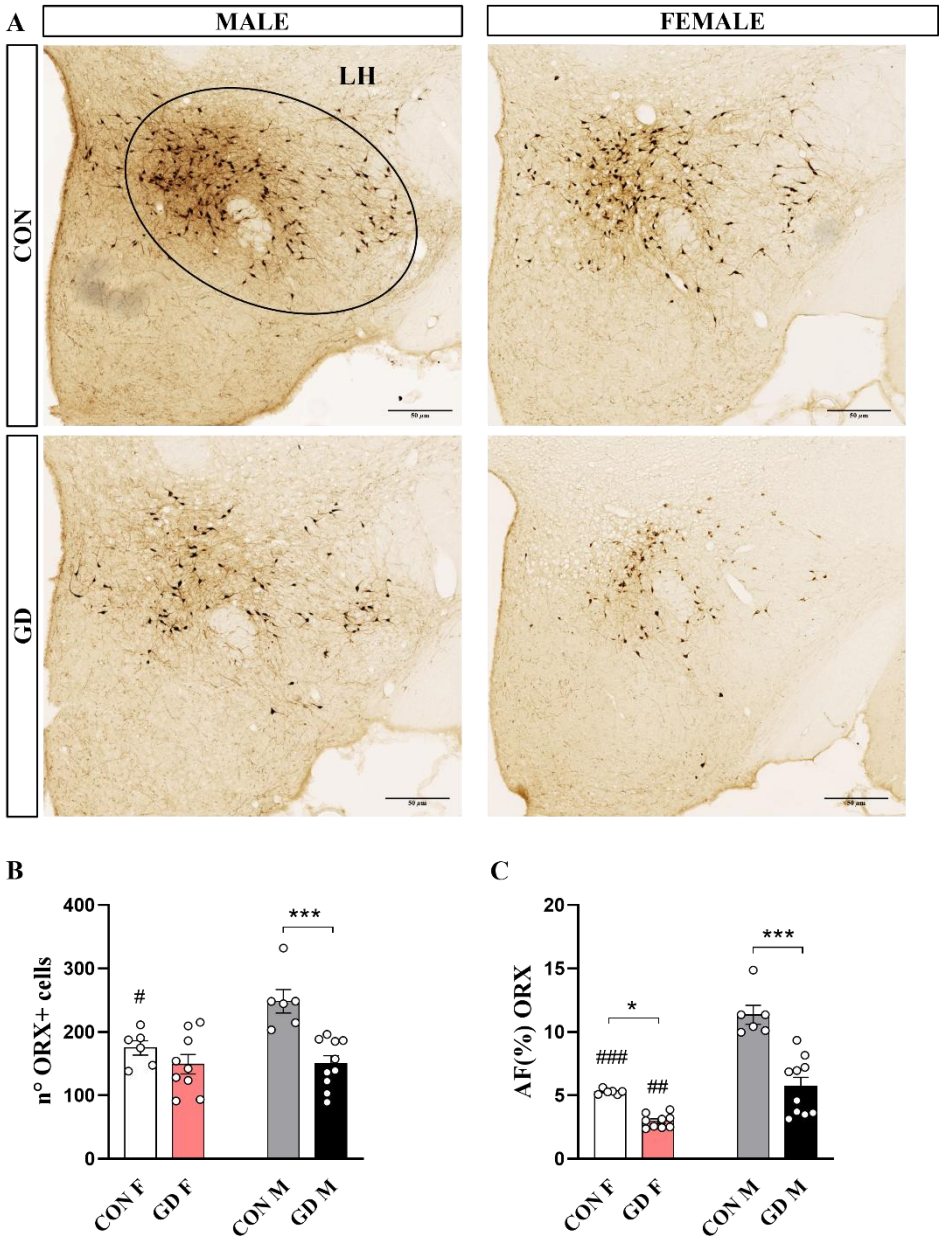


Figure 1. Analysis of ORX immunoreactivity in the LH. ***(A)*** *Representative images of ORX-ir in coronal sections of rat LH. Quantification of* ***(B)*** *number of ORX+ cells and* ***(C)*** *AF in the LH. Data is expressed as mean ± SEM. Statistical analysis revealed a significant effect for p≤0.05 (*comparison between different groups; #comparison between sexes). +=positive; ORX=Orexin; AF=area fraction; LH=lateral hypothalamus; CON=control; GD=gaming disorder.*

Reduction of the DA immunoreactivity in GD rats

Most dopaminergic neurons reside in the VTA and SN, sending their projections to different brain regions *(Bayer et al., 1995)*. After the development of the GD model, rats of both sexes show an alteration of the dopaminergic system with a consequent reduction in DA-ir in different nuclei (***Table 5***). First, as shown by images of coronal sections of rat brain (***Figure 2A***), the AADC-ir within the VTA was sexually dimorphic (Bregma -5.80 mm). In fact, CON F showed fewer AADC+ cells than males (p=0.022), and this difference was also maintained in the GD group (p=0.022). The GD protocol resulted in a reduction in the AADC+ cells in both sexes, compared with the controls (males, p=0.002; females, p<0.0001) (***Figure 2B***). The analysis of the AF, on the other hand, revealed no sex

differences in the control groups (p=0.859). In contrast, GD females showed a lower AF compared with GD males (p=0.020) due to the overall effects of the GD protocol that led to a significant reduction of AF in both sexes compared to controls (males, p= 0.016; females, p<0.0001) (***Table 5***).

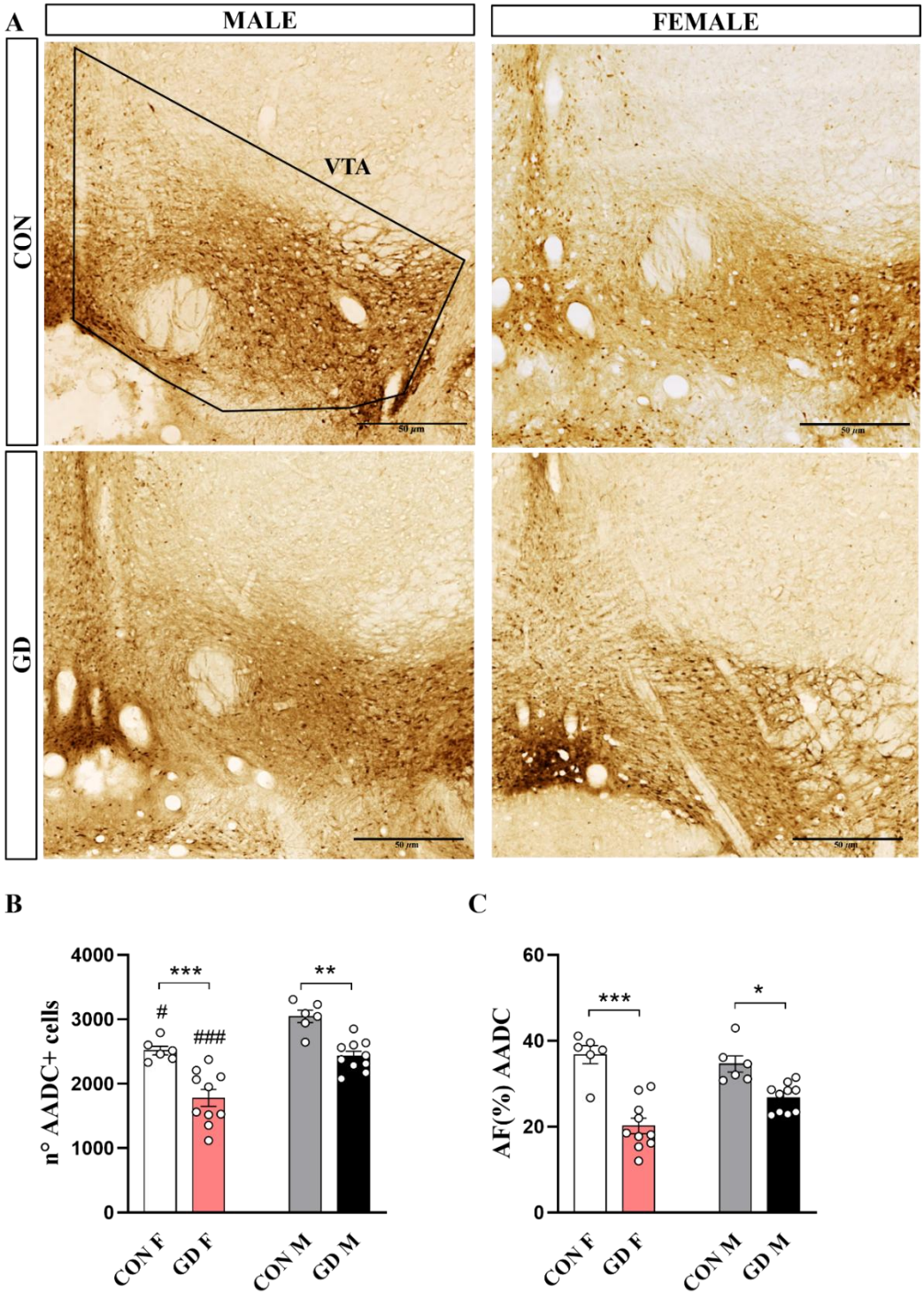


Figure 2. Analysis of AADC immunoreactivity in the VTA. ***(A)*** *Representative images of AADC-ir in coronal sections of rat VTA. Quantification of* ***(B)*** *the number of AADC+ and* ***(C)*** *AF in the VTA. Data is expressed as mean ± SEM. Statistical analysis revealed a significant effect for p≤0.05 (***** comparison between different groups; **#** comparison between sexes). Scale bar=50µm. +=positive; AADC=Aromatic L-Amino Acid Decarboxylase; VTA= ventral tegmental area; AF = area fraction; CON=control; GD=gaming disorder.*

The analysis also highlighted a reduction in AADC+ cells also in the substantia nigra pars compacta (SNc). This subnucleus displayed a sexual dimorphism, maintained also in the GD group: males showing more AADC+ cells than females (CON, $p<0.0001$; GD, $p=0.005$). The GD protocol reduced the number of AADC+ cells in GD groups compared with same-sex controls (males, $p<0.0001$; females, $p<0.0001$) ***(Figure 3B***). The reduced AADC-ir in the SNc is also reflected by a decrease in AF in the GD groups compared with the same-sex controls (males, $p=0.005$; females, $p<0.0001$). In addition, the reduction in AF abolished the sex difference among the GD group ($p=0.994$) (***Table 5***). In the substantia nigra pars reticulata (SNr), the ANOVA revealed a difference in the AF ($p<0.0001$), (***Figure 3D***) but not in the number of AADC+ cells ($p=0.834$) (***Figure 3C***) between groups, since the GD protocol reduced AF in females, resulting in a sex difference ($p<0.0001$) not present in the controls ($p=0.154$). In addition, both groups exposed to the GD protocol showed a reduction in the AF compared with CON groups of the same sex (males, $p=0.023$; females, $p<0.0001$) ***(Figure 3C***).

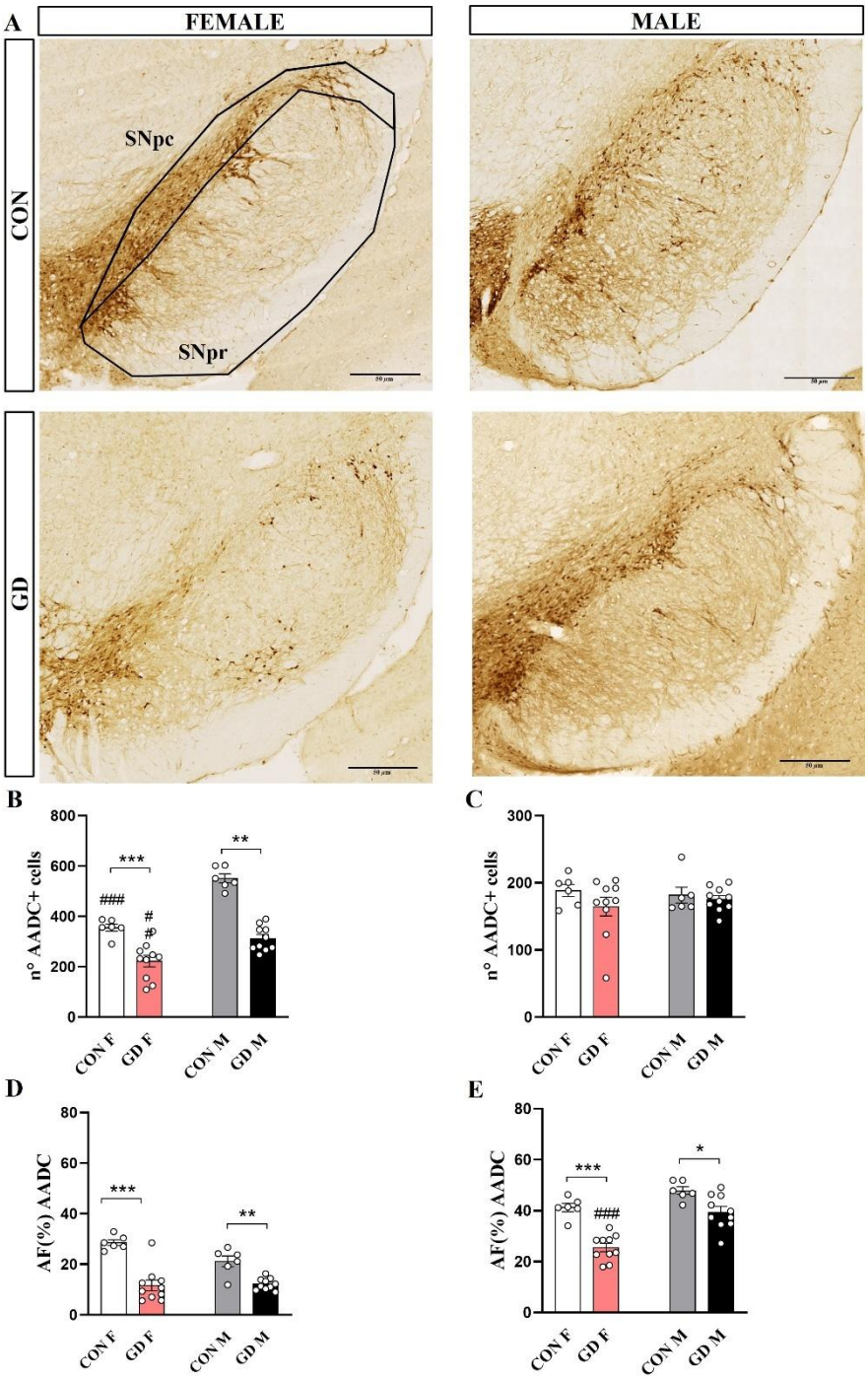


Figure 3. Analysis of AADC immunoreactivity in the substantia nigra. *(**A**) Representative images of AADC-ir in coronal sections of rat SN. Quantification of the number of AADC+ in **(B)** SNc, **(C)** SNr, **(D)** AF SNc, and **(E)** AF in SNr. Data is expressed as mean ± SEM. Statistical analysis revealed a significant effect for p≤0.05 (*comparison between different groups; #comparison between sexes). Scale bar=50µm.+ = positive; SN=substantia nigra; AADC=Aromatic L-Amino Acid Decarboxylase; AF=Area Fraction; SNc=Substantia nigra compact part; SNr= Substantia nigra pars reticulata; CON=control; GD=gaming disorder.*

Reduction of the 5-HT immunoreactivity in GD rats

The serotonergic neurons are mainly located in the most caudal area of the brain, more specifically in the dorsal (DRN) and median (MnR) raphe nuclei (Molliver, 1987). At the end of the GD protocol, rats of both sexes show a strong reduction of 5-HT-ir (***Table 6***) throughout the rostro-caudal development of these nuclei. Alterations were particularly significant in the central portion of the nuclei (Bregma -7.80 mm) (***Figure 4A***) and are described more in detail below.

In the DRN, the statistical analysis of 5-HT-ir highlighted a sex difference in the dorsal part of the nucleus (DRD). In fact, CON males showed more 5-HT+ cells than CON females ($p=0.033$). This sex difference is maintained among the GD groups ($p=0.045$). However, the GD rats showed an overall reduction in the number of 5-HT+ cells in both sexes, compared with the same-sex control group (males, $p<0.0001$; females, $p<0.0001$) ***(Figure 4B***). In the ventral part of the dorsal raphe nucleus (DRV), there are no sex differences between CON ($p=0.999$) or GD ($p=0.832$) groups.

Interestingly, the GD protocol induced a reduction of 5-HT+ cells compared to same-sex controls in both the DRV subnucleus (males, $p=0.005$; females, $p=0.049$) (***Figure 4C***) and the DRN totality (males, $p<0.0001$; and females, $p=0.0007$) (***Figure 4D***). The analysis of the AR showed the same results in the DRD. However, in contrast to the quantification of the number of 5-HT+ cells in the DRV, only the female GD group showed a reduction in the AF compared with the same-sex control group (males, $p=0.136$; and females, $p<0.0001$) (***Table 6***).

Similar outcomes are shown in the MnR. However, unlike the control groups, the GD groups showed a sex difference in the MnR, with more 5-HT+ cells in males than females (GD, $p=0.003$; CON, $p=0.997$). In addition, GD protocol produced a reduction in the number of 5-HT+ cells in GD groups compared to same-sex controls (males, $p=0.039$; females, $p<0.0001$) ***(Figure 4E).*** Comparing the AF, the control group showed a sex difference, with higher values in males than females ($p=0.001$). The GD protocol abolished this sex difference in GD groups ($p=0.270$) and caused a significant AF reduction in both sexes compared to controls (males, $p=0.030$; females, $p<0.0001$) (***Table 6***).

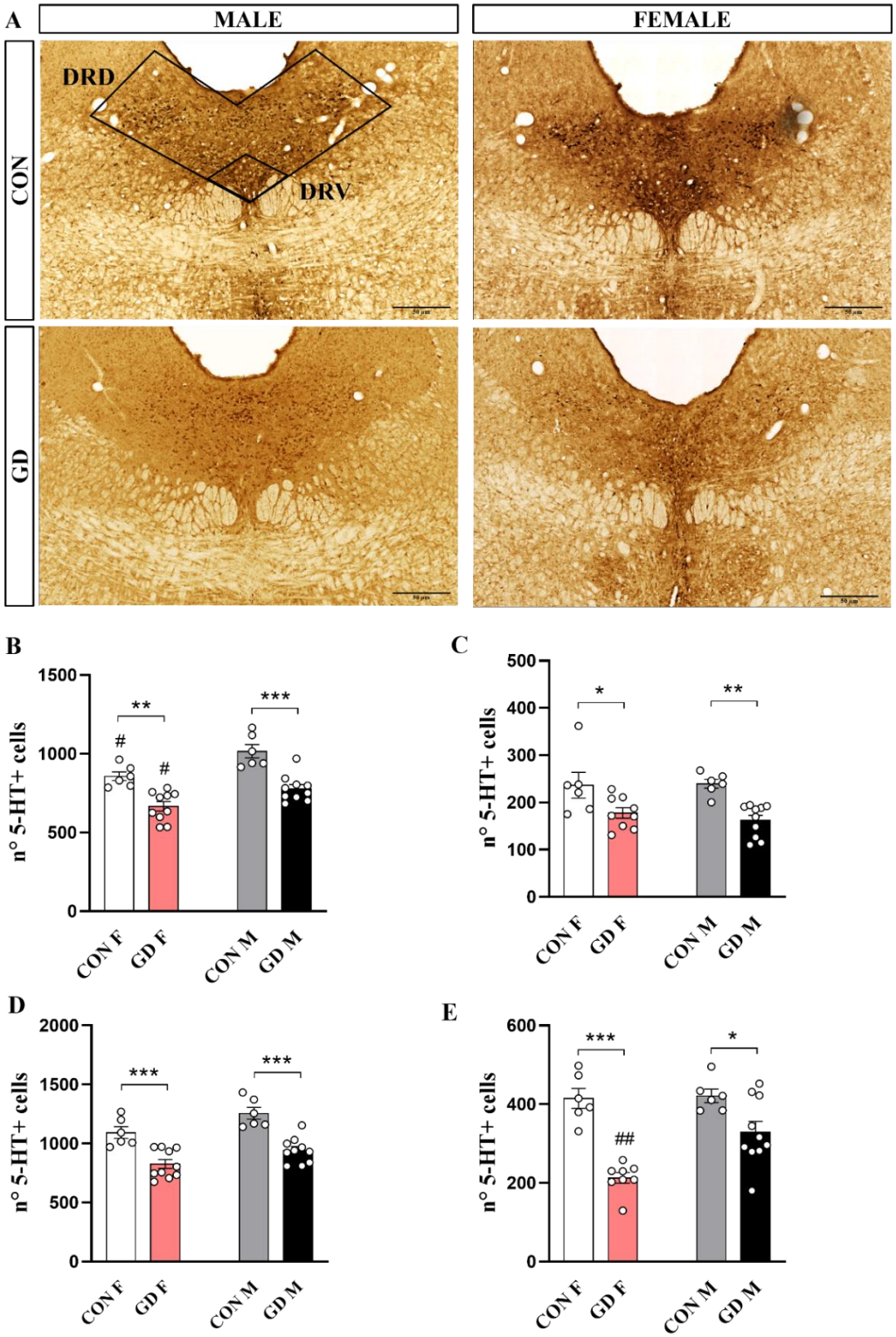


Figure 4. Analysis of 5-HT immunoreactivity in theDRN. ***(A)*** *Representative images of 5-HT-ir in coronal sections of rat DRN. Quantification of the number of 5-HT+ cells in* ***(B)*** *DRD,* ***(C)*** *DRV, (**D**) DRN, and* ***(E)*** *MnR. Data is expressed as mean ± SEM. Statistical analysis revealed a significant effect for p≤0.05(*****comparison between different groups; #comparison between sexes). Scale bar=50µm. +=positive; 5-HT=serotonin; DRN=dorsal raphe nucleus; DRD=dorsal raphe nucleus, dorsal part; DRV= dorsal raphe nucleus, ventral part; MnR=median raphe nuclei; CON=control; GD=gaming disorder.*

GD induces changes in plasma levels of Trp and its metabolites via 5-HT and KYN

Rats subjected to the GD model showed significant changes in plasma levels of Trp and its metabolites along the 5-HT and Kyn pathways.

GD groups of both sexes showed an increase in plasma Trp levels after the game session compared with sex-matched control groups (males, p=0.028; females, p=0.045) (***Figure 5B***). The GD groups displayed a strong reduction in plasma 5-HT levels compared to controls (males, p=0.0002; females, p=0.003). Interestingly, while 5-HT plasma levels were higher in males than in females control animals (p=0.002), this sex difference was not present in GD animals (p=1.000) (***Figure 5C***). In both sexes, the control groups, despite having a lower Trp level than the GD groups, showed higher Trp to 5-HT conversion than the GD groups (males, p=0.001; females, p=0.049) (***Figure 5D***).

Similar to 5-HT, GD rats had a strong reduction in KYN plasma levels compared to sex-matched controls (males, p=0.0007; females, p=0.017). However, while CON males had higher KYN levels than CON females (p=0.002), this difference due to sex was not present (p=0.939) in GD rats (***Figure 5E).***

Regarding downstream metabolites of KYN, both 3-HK and QA plasma levels were higher in CON males than in CON females (3-HK, p=0.291; QA, p<0.0001) ***(Figure 5F, G***). This sex difference was not present in GD rats (3-HK, p=0.879; QA, p=0.684). Interestingly, while GD males showed a reduction in plasma 3-HK (males, p=0.045) and QA (males, p<0.0001) compared to CON male rats, no difference for these two metabolites was present comparing GD versus CON females (3-HK, p=0.691; QA, p=0.432) ***(Figure 5F, G***).

Higher plasma levels of KYNA were present in CON females than in CON males (p=0.048), and in GD females than in GD males (p=0.001). In addition, in both sexes, KYNA levels increased significantly in GD compared to sex-matched CON rats (males, p=0.048; females, p=0.005) (***Figure 5H***).

We also studied indirectly the activity of the main enzyme involved in the KYN pathway. We observed a significantly lower KYN/Trp ratio in GD than in sex-matched CON (males, p=0.001; females, p=0.007) rats (***Figure 5I***).

In contrast, the KYNA/KYN ratio was higher in GD than in sex-matched CON rats (males, p=0.001; females, p=0.047) (***Figure 5L***). Comparing the control and GD groups of both sexes, the latter had a higher conversion index of KYN into its neurotoxic catabolites, KYNA and 3-HK (males, p=1.000; females, p=0.036) (***Figure 5LM***). Finally, comparing the *ratio* of KYN acid to QA, the GD groups of both sexes showed a greater presentation of QA in plasma than the control groups (males, p=0.029; females, p=0.029), with a higher index in GD females than males (p=0.037) (***Figure 5N***).

Finally, we examined plasma levels of DA. Similarly to 5-HT, we found higher DA levels in CON males than in CON females (p=0.0003), and no sex-difference between male and females GD rats. Moreover, both male and female GD rats had lower plasma DA levels than their respective CON male (p=0.0007) and female (p=0.049) rats (***Figure 5O***).

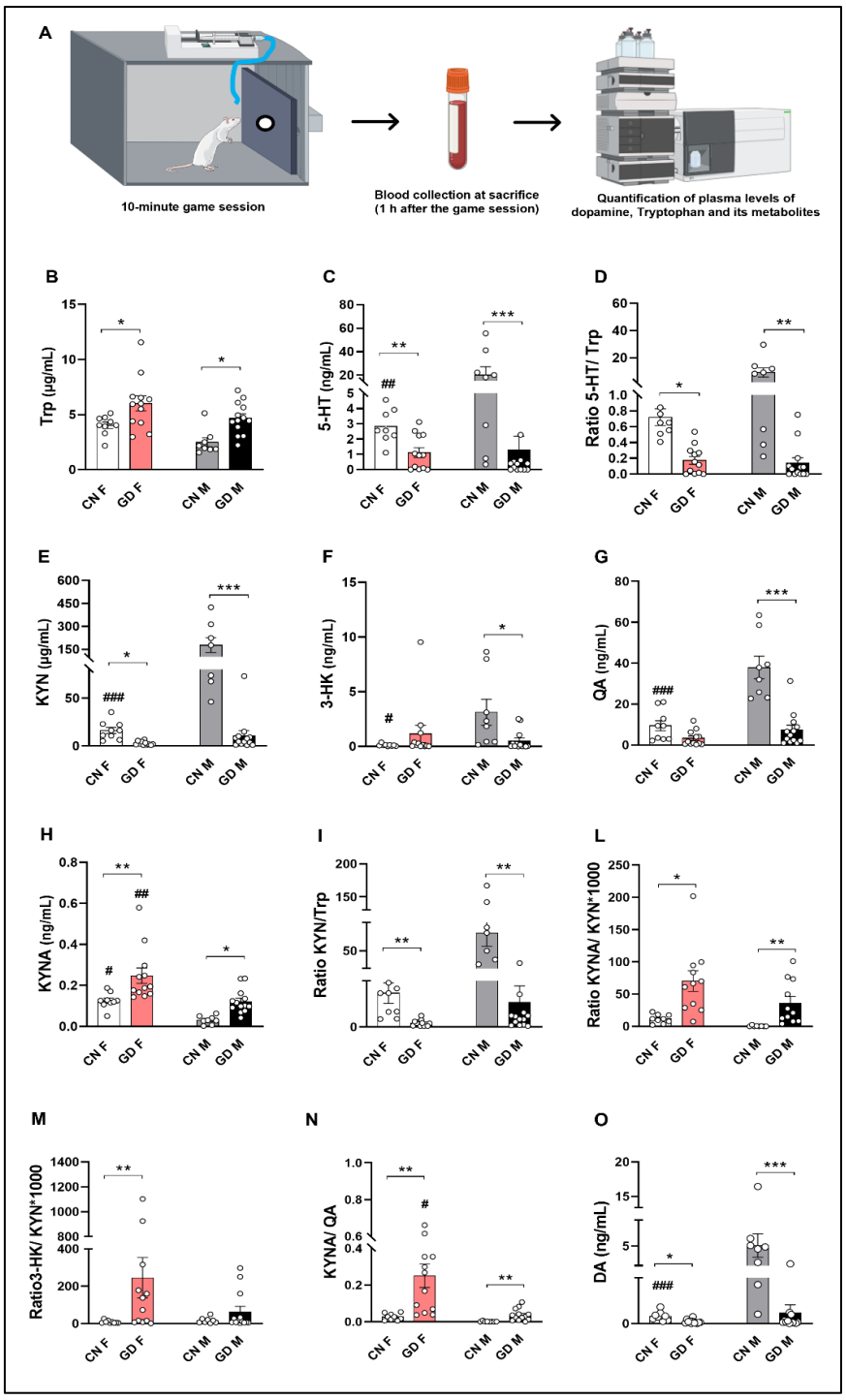


Figure 5. HPLC-mediated determination of plasma levels of DA, Trp, and its metabolites. *(**A**) Schematic representation of the timing of sample collection and processing. Quantification of (**B**) Trp, (**C**) 5-HT, (**D**) 5-HT/trp ratio, (**E**) KYN, (**F**) 3-HK, (**G**) QA, (**H**) KINA, (**I**) KYN/TRP ratio, (**L**) KYNA/ KYN ratio, (**M**) 3-HK/TRP ratio, and (**N**) KYNA/QA, and (O) DA in rat plasma samples. Data is expressed as mean ± SEM. Statistical analysis revealed a significant effect for p≤0.05 (*comparison between different groups; #comparison between sexes). Trp=Tryptophan; 5-HT=serotonin; KYN=kynurenine; 3-HK=3-hydroxykynurenine; QA=quinolinic acid; KYNA=kynurenine acid; DA=dopamine.*

DISCUSSION

GD is a mental disorder that primarily affects adolescents. Patients with GD show several symptoms, among the best known being loss of control over play, increased compulsive and hyperactive behaviors, anxiety, and social isolation (Harrison et al., 2021; "The ICD-11 Classification of Mental and Behavioral Disorders: DIagnostic Criteria for Research," 2018). These symptoms are associated with a reduction in neural activity in areas suitable for inhibitory control or regulation of emotional state *(Li et al., 2020).*

GD is a currently under-explored mental disorder whose causes are still poorly understood. Among the possible causes of the development and maintenance of this mental disorder is the potential alteration of the dopaminergic, serotonergic and orexinergic systems *(Ariatama et al., 2019; Choi et al., 2020; Hong et al., 2018; Li et al., 2020; Tian et al., 2014).* These three systems are fundamental for the proper functioning of different brain areas, regulating decision-making functions, as well as the perception and processing of emotional stimuli, including reward processing *(Arias-Carrión et al., 2010; Assadi et al., 2009; Homberg, 2012).*

Patients with psychiatric disorders or mental illnesses have decreased expression of DA, 5-HT, or ORX transporters and receptors *(Cummings & Walker, 1996; Katsuki & Akaike, 2004; Stepanova et al., 2020).* Impairment of these systems predisposes the development of some mental disorders that show high comorbidity with GD (*i.e.*, ADHD, anxiety, depression, and bipolar disorders) *(Dullur et al., 2021; Furuyashiki & Kitaoka, 2019; Hong et al., 2018; Koncz et al., 2023; Tian et al., 2014).* The administration of drugs that increase cerebral levels of DA and 5-HT reduces typical symptoms of GD, such as loss of control over play, hyperactivity, and depressive symptoms *(Sá et al., 2023).*

In a previous work, we developed a rat model that mimics some behavioral characteristics found in GD patients. The rats subjected to the GD protocol are trained to play with a touch screen platform, toward which they develop imperative and compulsive behaviors *(Casile et al., 2024).* We also demonstrated that GD behavior led to changes in the activation of specific brain circuits in rats, mainly related to the control of decision-making processing, emotional, motivated, and reward-seeking behaviors *(Casile et al., 2024).* These alterations in brain activity could reflect the behavioral change present in the GD rats, who develop a hyperactive and compulsive behavioral phenotype when exposed to the task, accompanied by a loss of interest in social novelty and impaired social interaction. These changes recall some brain dysfunctions already described in GD patients *(Li et al., 2020).*

The main purpose of this work was to investigate the possible involvement of the dopaminergic, serotonergic, and orexinergic systems in the development of GD, using this rat model. We have shown that male and female GD rats exhibit central and peripheral impairment of these neurotransmitters.

GD rats showed a reduction of 5-HT-ir in the DRN and MnR like that found in rats developing alcohol dependence *(Ohta et al., 2010).* Alterations in this system may contribute to impaired signal transduction, leading to reduced 5-HT release. Reduction in cell number or amount of circulating serotonin causes persistent alteration of neurocognitive pathway involved in emotion processing, reward mechanism, and executive control *(Adjimann et al., 2021; Choi et al., 2020; Malave et al., 2022).* GD rats showed an dysregulated reward mechanism during play, which is not restored in the presence of a social stimulus with a positive valence *(Casile et al., 2024).* These behavioral modifications are common in psychiatric disorders and make individuals more susceptible to the development of depression, anxiety, and bipolar disorder *(Liu et al., 2019).*

Dopaminergic neurons in the VTA are involved in several cognitive functions and they are required in motivated learning, reward prediction, and the propensity to initiate or stop an action *(Bromberg-*

Martin et al., 2010; Kaufling, 2019). GD rats exhibited a reduction in the number and fibers of dopaminergic neurons in the VTA. This could be associated with a loss of control during play and a reduced predictive ability to obtain rewards *(Casile et al., 2024).* Numerous animal models miming neuropsychiatric disorders, such as depression or schizophrenia, show the same cognitive and behavioral alterations *(Douma & de Kloet, 2020; Kaufling, 2019; Morikawa & Paladini, 2011).* In addition, different studies demonstrate a reduction of the dopaminergic system in rodent models exposed to substances of abuse, such as alcohol or drugs *(Bass et al., 2013; Bocklisch et al., 2013; Matthews & German, 1984)*. Also, in SN, GD rats showed a reduction in the number of DA+ neurons. Specifically, the GD group of both sexes exhibited a decrease in AF and the number of DA+ cells in the SNr and SNc compared with the same-sex controls. In psychiatric diseases, such as schizophrenia, it has been described that a reduction in the number of DA+ cells in the SN *(Birtwistle & Baldwin, 1998; Rice et al., 2016)* is often correlated with a reduction in brain-derived neurotrophic factor (BDNF), which regulates its survival and differentiation and synaptic plasticity *(Baquet et al., 2005).* ORX plays an important role in learning, memory acquisition, and cognitive functions through the activation of monoaminergic systems *(Al-Kuraishy et al., 2020; James, Campbell, et al., 2017).* Compared to control rats, GD groups showed a reduction in AF and the number of ORX+ cells in the LH. Repeated or chronic stress can lead to a decrease in the functionality of the ORX system generating a behavioral phenotype of low motivated arousal or depressive-like behavior *(James, Campbell, et al., 2017; Murgatroyd et al., 2015).*

In fact, the diminish of ORX pathway is typical in depression or anxiety and it is associated with deficits in DA and 5-HT production *(Abbas et al., 2015; James et al., 2014; Johnson et al., 2012).*Trp is an essential amino acid required for protein biosynthesis, but also the biochemical precursor of several metabolites that regulate immune, metabolic, and nervous system functions *(Comai et al., 2020).* The two major routes of Trp metabolism are the 5-HT/MLT and the KYN pathways which are actively studied as potential pharmacological targets for different peripheral and central disorders *(Modoux et al., 2021).* Variations in the levels of Trp metabolites, along with high levels of pro-inflammatory factors, are involved in the development of depression and bipolar disorder (Pisanu et al., 2022) but also of other psychiatric disorders *(Comai et al., 2020).* We have observed that the GD protocol induces an alteration in the metabolism of Trp along both the 5-HT and the KYN pathways. Specifically, GD rats exhibited high levels of Trp likely as the result of reduced conversion of the amino acid into 5-HT and KYN. Within the context of the KYN pathway, in which its activation could lead to an imbalance between the neurotoxic (formation of QA and 3-HK) and the neuroprotective (formation of KYNA) branches, we observed that GD rats compared to CON rats exhibit on one side low conversion of Trp into KYN (lower KYN/Trp ratio), on the other side they showed increased conversion of KYN into 3-HK (higher 3-HK/KYN ratio), the precursor of QA. Looking at the KYNA/QA ratio as a measure of NMDA functioning, GD rats showed significantly higher KYNA/QA ratio than CON rats, especially in females. These findings are in clear agreementwith those present in Schizophrenia patients, in whom high levels of KYNA lead to a hypofunction of NMDA, which produces impaired cognitive function, increasing the positive and negative symptoms typical of this mental disorder *(Adell, 2020; Comai et al., 2022).* This may be suggestive of overall NMDA hypofunction occurring in GD animals, which exhibit increased KYNA and decreased QA.

Consistent with these results, there is also the reduction in 5-HT conversion from Trp in GD groups compared with CONs. The decrease in peripheral 5-HT conversion coincides with a reduction in the amount and number of 5-HT cells in the brains of GD rats. In addition, the GD groups show a similar

diminution in DA and ORX. Another possible explanation could be increased QA at the central level, which, in addition to reducing the number and fibers of these neurotransmitters, could lead to altered cognitive processes and emotional state *(Cummings & Walker, 1996; Katsuki & Akaike, 2004; Stepanova et al., 2020)*. Decrement of these neurotransmitters or their production predisposes subjects to the depressive symptoms, increased susceptibility to stress, and development of psychiatric disorders *(Firk & Markus, 2007; Grafe & Bhatnagar, 2018; Moghaddam, 2002)*.

The development of harmful behaviors, such as compulsive behaviors, typical of addiction and mental disorders, chronically affects the reward and stress centers. Functional imbalance of these centers occurs as a consequence of a pathological adaptation of the brain to these abject behaviors, which reduces the functionality of DA and 5-HT, leading to a form of anhedonia *(Dresp-Langley, 2023; Prakash et al., 2020)*.

These two systems are mutually connected and exert an inhibitory and excitatory action between them to balance their activity (Esposito et al., 2008). In the same way, both DA and 5-HT stimulate and are stimulated by ORX, which exerts an excitatory role of these two neurotransmitters *(Calipari & España, 2012; Mavanji et al., 2022)*. In psychiatric diseases, this balance of interactions is disrupted, resulting in dysfunction of the system. One possible cause of this dysfunction is the direct action of CRH system, which in chronic conditions reduces the release of DA, 5-HT, and ORX *(Dedic et al., 2018; Fox & Lowry, 2013; Sargin, 2019)*. Chronic stress conditions are common in mental disorders such that they may predispose individuals to the development of those diseases *(Babenko et al., 2015; Cattaneo & Riva, 2016)*. For example, there is growing evidence indicating that stressogenic conditions alter Trp metabolism, reducing its conversion to 5-HT and increasing the KYN metabolic pathway *(Nazzari et al., 2020)*.

Thus, the reduction of these neurotransmitters in GD patients and in our rat model could be the result of the chronic action of the CRH system, which could either directly act on the neurotransmitter or indirectly by altering the Trp metabolic pathway.

CONCLUSIONS

GD is a mental disorder that is still poorly understood. Current evidence is based on epidemiological and neuroimaging studies of human patients. Possible causes of the onset and progression of this mental disorder include disruptions in the dopaminergic, serotonergic, and orexinergic systems. These three systems are important for the proper functioning of various brain pathways, for the regulation of decision-making functions, and for the perception and processing of emotional stimuli, including reward processing. The rat model developed in our laboratory exhibits these behavioral alterations and supports the hypothesis of neurotransmitter impairment.

Taking advantage of our experimental model, we could not only investigate the neurobiological basis that underlies GD disorder, but also better understand common mechanisms shared with other mental disorders. Widening the understanding of these disorders will help to design and test more specific pharmacological therapies.

CHAPTER 5

AIM 3

Assessing the Efficacy of Fluoxetine in a Gaming Disorder Animal Model: Study on predictive validity

INTRODUCTION

The excessive use of video games has become an increasingly relevant topic in both public debate and the scientific community. This phenomenon has led to the identification of a condition known as GD. In 2018, the WHO included GD in the *ICD-11*, defining it as a mental disorder characterized by a loss of control over gaming, along with the development of other negative symptoms such as anxiety, depression, social isolation, and hyperactivity (*Blashfield & Fuller, 2016; Ostinelli et al., 2021*). GD can have a significant impact on an individual's mental health, interpersonal relationships, academic or work performance, and overall well-being. It is important to note that GD is not simply about spending excessive time playing games, but rather about dysfunctional behaviors associated with excessive video game use (*Bediou et al., 2018*). GD primarily develops among young people aged 10 to 19. The WHO estimates that approximately 4 percent, or 40 million adolescents, are affected by GD, with a higher incidence in males than females (*Fam, 2018; Feng et al., 2017*). Patients with GD exhibit an imbalance in impulse control and impaired reward circuits (Tian et al., 2014), and their symptoms resemble those of other mental disorders such as depression, schizophrenia, and substance dependence *(Furuyashiki & Kitaoka, 2019; Tian et al., 2014*). These behavioral alterations are linked to changes in the serotonergic and dopaminergic systems. In fact, the use of antidepressant drugs such as fluoxetine and bupropion, which increase cerebral levels of 5-HT and DA, has been shown to reduce the symptoms and behaviors associated with GD (*Sá et al., 2023*). Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is widely used to treat various mental disorders, including depression, anxiety disorders *(Kryst et al., 2022*), and obsessive-compulsive disorder (*Tollefson, 1994*). Despite numerous studies on GD patients, these studies have limitations, such as insufficient consideration of female participants, varying player ages, and differences in the types of games used.

To address these limitations, we recently developed a rat model for studying GD in our laboratory (*Casile et al., 2024*). Like GD patients, this animal model exhibits a loss of control over play, a reduction in social interactions, and the development of an anxious and hyperactive phenotype. The development of this behavioral phenotype in rats is associated with changes in neural activity that resemble those seen in GD patients (*Casile et al., 2024*). Furthermore, after developing the model, GD rats show a reduction in the number of dopaminergic and serotonergic cells, along with alterations in the tryptophan pathway, confirming the hypothesis of disturbances in these systems in GD. Currently, the pharmacological treatments used for GD patients involve DA and 5-HT reuptake inhibitors. These drugs reduce the symptoms of GD and improve patients' emotional states (*Sá et al., 2023*).

To further confirm the translatability of this model, we administered fluoxetine in an acute form.

Fluoxetine is commonly used in GD treatment as it reduces the loss of control over gaming and helps regulate emotional states (*Sá et al., 2023*). Additionally, fluoxetine is used to validate animal models of mental disorders such as anxiety (*David et al., 2009; Lecci et al., 1990*), depression (*Dulawa et al., 2004; Rodríguez-Gaztelumendi et al., 2009*), and obsessive-compulsive disorder (Arora et al., 2013). Despite being a potent humoral regulator, fluoxetine administered in high doses (greater than 10 mg/kg) has been shown to reduce social interaction in animal models (*Dulawa et al., 2004; Grieb et al., 2022; Payet et al., 2018*). Several studies in the literature highlight the positive effect of acute fluoxetine administration at low doses (2.5 or 5 mg/kg) in regulating emotional states (*Drapier et al., 2007; Greenwood et al., 2008; Sekine et al., 2007*), but none have analyzed its effect on social interaction. To avoid impairing social behavior in this animal model, we chose to administer two low doses of fluoxetine—2.5 and 5 mg/kg—both of which regulate emotional states without significantly impairing social interaction.

MATERIALS AND METHODS

Animals

Adult female (n=9) and male (n=3) Wistar Kyoto rats were purchased from Charles River (Charles River Laboratories Italia s.r.l., Milan, Italy). The rats were housed under standard conditions at 22 ± 2 °C, with a 12:12 light-dark cycle (lights on at 08:00 AM). Food (standard chow diet, VRF1, SDS - Charles River Laboratories) and water were provided ad libitum throughout the study. One male and three female rats (3 months old) were housed together to facilitate successful mating. The resulting pups (38 males and 41 females) were sexed and weaned on postnatal day 28 (PND28), after which they were housed in separate cages, each containing four same-sex rats.

Experimental

At PND40, rats were randomly assigned to the following experimental groups:
Control males (CON-M, n=11);
Control females (CON-F, n=10);
Male rats subjected to GD protocol (GD-M, n=18);
Female rats subjected to GD protocol (GD-F, n=31).
One week prior to the test phase, the control groups underwent a 10-minute daily adaptation period to the apparatus. The GD rats followed the GD protocol described below.
Animal care and handling were conducted in accordance with the European Union Council Directive of 22nd September 2010 (2010/63/EU). All procedures outlined in this study were approved by the Italian Ministry of Health (authorization no. 1035/2020-PR) and by the Ethical Committee of the University of Torino (Project no. 290132). The experimental design adhered to the ARRIVE guidelines as originally published by Kilkenny et al. in 2010 (*Kilkenny et al., 2010*).

GD protocol

A schematic representation of the experimental timeline is shown in ***Figure 1A***.

Behavioral Apparatus

Throughout the GD protocol, the experiment was conducted in a rectangular apparatus (50 x 55 x 50 cm) with a black plastic base and walls, as described in our previous article. The apparatus was positioned 50 cm above the floor in a room illuminated with medium-low light (20 lx) provided by a house light, maintained at a temperature of 22 ± 2°C. A camera (Basler GenICam, acA 1300-60 gm) was mounted on the ceiling to record the sessions using Media Recorder (Noldus, Wageningen, the

Netherlands). The apparatus consists of a removable panel that divides it into two areas: one side, the play area (50 x 55 x 50 cm), and the other, a 20 x 50 x 50 cm area where social competitive stimuli were placed during test phases 2 and 3 (described below).
The game involved the fixed or random appearance of a white dot (4.5 cm in diameter) on the black screen. When the animal correctly touched the dot, it received a reward of 0.5 g of yogurt (strawberry flavor, no added sugar). Yogurt delivery followed a fixed ratio (FR) schedule for the first 3 weeks of the training phase, then transitioned to a random ratio (RR) schedule for the final 2 weeks of training and the Test phase (*Casile et al., 2024*).

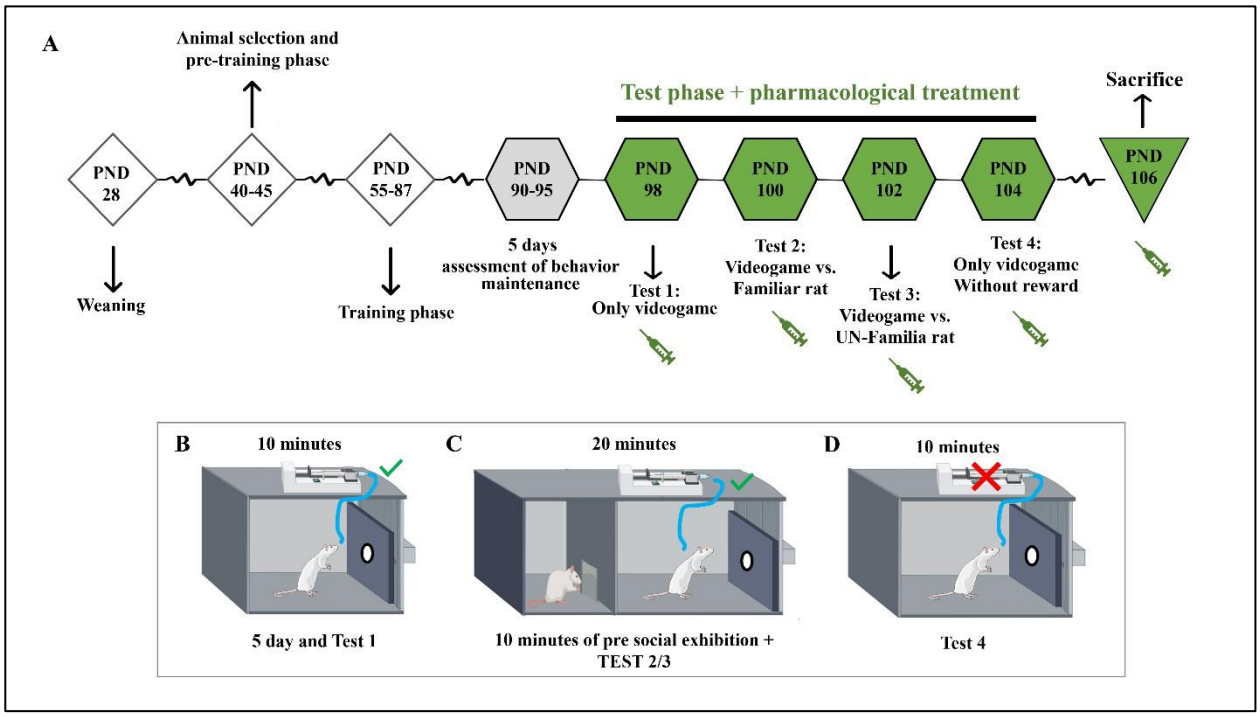


Figure 1. Experimental timeline. *Schematic representations of* ***(A)*** *experimental procedures' timeline,* ***(B)*** *5 days assessment of behavior maintenance and Test 1(videogame alone),* ***(C)*** *Test 2 (videogame vs familiar social stimulus) and Test 3 (videogame vs UN-familiar social stimulus), and* ***(D)*** *Test 4 (videogame without reward). PND=Postnatal day.*

Training phase

The training phase lasted for 5 weeks (PND55 to PND87). During this phase, the rats were exposed to the platform touch screen for 5 days a week, with each session lasting 5 minutes. The sessions always took place between 8:00 a.m. and 1:00 p.m. The rats were placed in the room with soft lighting (20 lx) 1 hour before the beginning of the test.
During the first three weeks, the reward was delivered according to a fixed ratio that changed each week. In the first week, the FR was 1:1 (1 correct touch = 1 reward), in the second week it was 2:1 (2 correct touches = 1 reward), and in the third week, it was 3:1 (3 correct touches = 1 reward). In the fourth and fifth weeks of training, the reward delivery ratio transitioned from fixed to RR.
Each day, the rats were evaluated based on different game-related parameters: time spent in front of the screen, interaction with the screen (both correct and incorrect touches), following the dot, and reinforcing the connection between correct touches and rewards. The number of touches made, and rewards obtained were also recorded.
The evaluation included assigning a daily score based on the number of correct touches made. The score for each behavior (ranging from a minimum of 1 to a maximum of 5) was based on the number

of correct touches as follows: 0 = 0 correct touches; 1 = 1–5 correct touches; 2 = 6–10 correct touches; 3 = 11–15 correct touches; 4 = 16–20 correct touches; 5 = ≥20 correct touches. The daily score was the sum of the scores for each behavior, plus the total number of correct touches. This method allowed for evaluating the daily progression of performance. Finally, each week, a mean weekly score was assigned to each animal (*Casile et al., 2024*).

Evaluation of GD Pattern Maintenance

Rats undergoing training to develop the GD model will undergo a 10-minute play session daily for 5 consecutive days (PND85 to PND91). Model development and maintenance will be assessed by quantifying the following behavioral parameters: time spent in the play zone, distance and speed traveled in the play zone, duration of interaction with the screen (game interaction), and the number of correct touches made.

Test

Model Validation Using Fluoxetine

The GD model was validated through the administration of Fluoxetine. Several tests were conducted to assess the effects of Fluoxetine on the model. All tests were performed from 8:00 a.m. to 1:00 p.m. in a room illuminated by medium-intensity light (20 lux). Prior to testing, rats were randomly assigned to three experimental groups based on sex:

- GD Vehicle: 11 females and 9 males
- GD FLX2.5: 10 females and 9 males (treated with 2.5 mg/kg Fluoxetine)
- GD FLX5: 10 females and 9 males (treated with 5 mg/kg Fluoxetine)

To ensure there were no significant differences between the groups, a 10-minute game session was conducted to compare several parameters, including: time spent in the play zone, distance and speed in the play zone, game interaction, and the number of correct touches.

PND94: Test 1

The tablet was in ON mode with the video game running (***Figure 1***). The rat was allowed to choose whether to interact with the video game to obtain a reward or to explore the apparatus. The rat was assessed for attachment (time spent in the play zone), duration (time spent interacting with the video game), and loss of control (compulsivity), as indicated by the number of correct touches, speed, and distance traveled in the play zone during the play session.

PND96: Test 2

The tablet was in ON mode with the video game running. Additionally, a familiar rat of the same sex was present in the apparatus (***Figure 1***). The rat could choose whether to interact with the video game or the social stimulus. The time spent in the sexual zone (area adjacent to the social stimulus), the number and total duration of sniffs, and the same parameters assessed in Test 1 were recorded.

PND98: Test 3

The test conditions were similar to Test 2, but in this case, an unfamiliar rat was used for the social context (***Figure 1***). The parameters assessed were the same as in Test 2.

PND106: Test 4

Unlike the previous tests, the rat was evaluated as in Test 1, but it did not receive a reward for correct touches (***Figure 1***).

All females (both tester and non-tester rats) were tested during the estrus phase of the ovarian cycle in either Test 3 or 4, when social interaction with a conspecific was planned. If a female was not in the estrus phase, the test was postponed until the next day. The estrus phase was determined by vaginal smear (*Cora et al., 2015*).
All tests lasted 10 minutes and were recorded using a camera attached to the roof, positioned 2.5 meters from the apparatus. The reward was dispensed using a random ratio (RR).

Parameters Evaluated During the Tests
The following parameters were evaluated and subsequently analyzed using EthoVision 8 (*Noldus Information Technology; Noldus, Spink, & Tegelenbosch, 2001*):

- Time (s) spent in different zones of the arena (play zone and arena)
- Distance (cm) traveled (in different zones or the total arena)
- Speed (cm/s) achieved (in different zones or the total arena)
- Game-related behaviors: interaction with games (cumulative duration), correct touches (i.e., number of goals; frequency)
- Non-game-related behaviors: grooming (cumulative duration), protected rearing (frequency), and unprotected rearing (frequency)
- Behaviors related to socio-sexual or social interaction: time (s) spent sniffing

Drug Treatment
Sixty minutes before the start of each test, including the Elevated Plus Maze (EPM), Open Field (OF), and sacrifice, rats received an intraperitoneal (IP) injection of vehicle (saline), Fluoxitine (2.5 and 5 mg/kg, Sigma).

Sacrifice and Tissue Sampling
At PND108, after drug treatment, rats underwent a 10-minute play session and were sacrificed 90 minutes later via deep irreversible anesthesia (intraperitoneal injection of Zoletil 100 mg/kg and Rompum 20 mg/kg). Following this, rats were transcardially perfused with 0.9% NaCl and then with a 4% paraformaldehyde (PFA) solution (*Tronel & Sara, 2002*). Females were sacrificed during the estrus phase, as determined by vaginal smears (*Alboni et al., 2017; Micioni Di Bonaventura et al., 2017*).

Statistical Analysis
Quantitative behavioral data were analyzed using SPSS 27 statistical software (SPSS Inc, Chicago, IL, USA) with a three-way analysis of variance (ANOVA), considering sex, condition, and treatment as independent variables. For comparison of data obtained from the five consecutive days of testing to assess the retention of the GD model, we used two-way ANOVA (with day and sex as independent variables) for repeated measures.

Behavioral Tests to Assess Anxiety and Hyperactivity
Behavioral tests were conducted at PND100 (Elevated Plus Maze - EPM) and PND102 (Open Field - OF) between 9:00 a.m. and 1:00 p.m., following a one-hour room adaptation period. A medium-intensity light (20 lux) was maintained in the room for the entire duration of the tests (Aspesi et al., 2021).

EPM
This test is based on the conflict between the rat's exploratory drive and its innate fear of open areas.

Less time spent exploring the open arms indicates greater anxiety-related behavior. The apparatus consists of two closed arms (50 × 10 cm), each with 30 cm high walls, and two open arms (50 × 10 cm) without walls. All arms are positioned 50 cm above the floor. At the start of the test, each rat was placed on the central platform, facing an open arm, and allowed to explore the maze freely for 5 minutes (*Spink et al., 2001; van den Bos et al., 2006*). The entire session was recorded using a camera positioned 1.5 meters above the apparatus. Using EthoVision 8 software, the following parameters were evaluated: frequency, time spent, and distance traveled in the different arms, along with exploratory behaviors such as protected rearing, unprotected rearing, and grooming.

OF

The Open Field (OF) test is typically used to assess both anxiety and hyperactivity in rodents. The apparatus consists of a 70 × 70 cm black plexiglass arena with 40 cm high walls. The center of the arena is defined as a square, 16.5 cm from all four walls. The test began when the animal was placed inside the apparatus, and it was allowed to explore freely for 5 minutes. The entire session was recorded using a camera positioned 2 meters away from the apparatus. The video was then analyzed using EthoVision 8 software (*Spink et al., 2001*). The following parameters were measured: time spent in the different zones of the arena (center and edges), distance traveled (total, in the center, and along the edges), speed, and exploratory behaviors (protected rearing, unprotected rearing, and grooming) (*Aspesi et al., 2021*).

RESULTS

To determine whether animals subjected to the protocol-maintained game-related behaviors, rats were evaluated over five consecutive days before the test phase (***Figure 2).*** Throughout all five days of evaluation, rats of both sexes exhibited high game-related behavioral parameters, peaking on day 3. Indeed, comparison across the different days showed significant differences in time spent (***Figure 2A;*** p=0.009 for females, p=0.002 for males), distance traveled (***Figure 2B;*** p<0.0001 for females, p<0.0001 for males) in the play zone, game interaction (Figure ***2C;*** p<0.0001 for females, p=0.007 for males), and the number of correct touches (***Figure 2D;*** p=0.0003 for females, p=0.008 for males), as indicated by the three-way ANOVA.

Behavioral results from the analysis of the different tests are summarized in the ***tables 1, 2, 3, 4***, *and* ***5***. The p-values presented in the results are based on Tukey's HSD test.

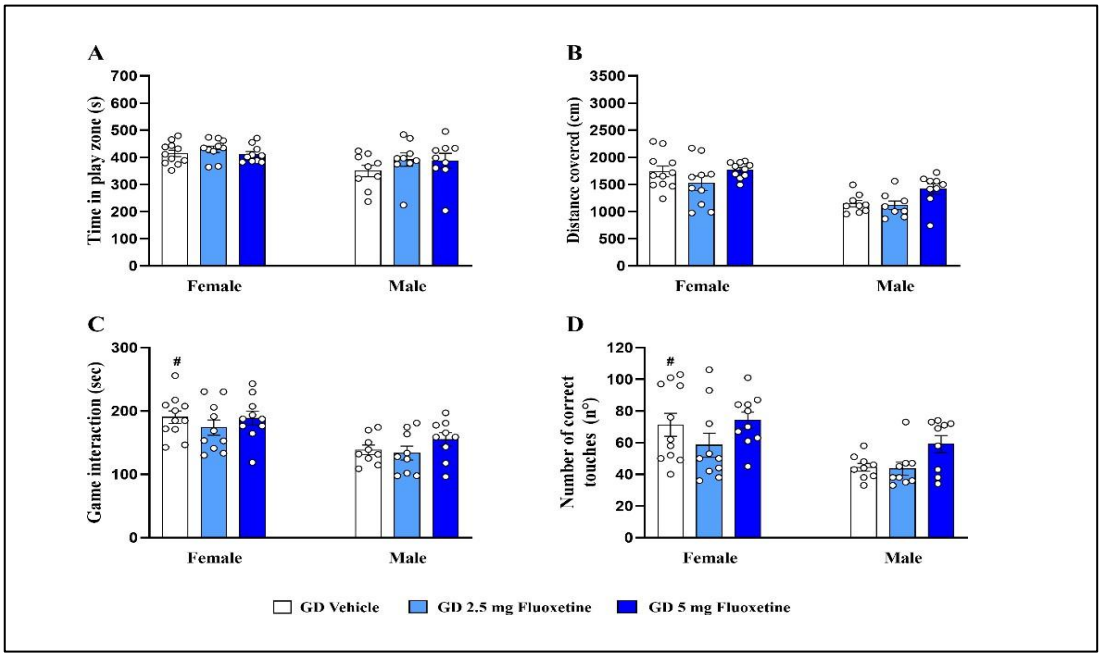


Figure 2. Analysis of the addictive behavior during 6th week (in the presence of the reward). *The upper histograms show (A) the time spent and (B) the distance traveled in the play zone, (C) the time spent in game interaction, and (D) the number of correct touches performed by the different groups. Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes). GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Table 1. Results obtained from the analysis of different behaviors within the test without pharmacological treatment.

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Test without pharmacological treatment								
Parameters	**GD M vehicle**	**GD M 2.5 mg/kg Fluoxitine**	**GD M 5 mg/kg Fluoxitine**	**GD F vehicle**	**GD F 2.5 mg/kg Fluoxitine**	**GD F 5 mg/kg Fluoxitine**	**ANOVA**	
							$F_{(5,55)}$	p
Frequency of entry into the Play Zone (N)	15.44±0.68	11.11±1.27	16.44±1.36	17.36±1.41	11.90±1.33	14.70±1.09	4.20	**0. 003**
Time in the Play Zone (s)	349.44±19.21	392.02±22.05	387.64±24.29	414.72±12.18	429.97±12.03	411.35±9.90	2.62	**0. 034**
Time in Other zone (s)	188.83±19.01	147.85±21.99	152.16±24.44	125.54±12.20	110.35±12.03	128.96±9.90	2.49	**0. 042**
Total distance traveled (Arena) (cm)	2159.62±71.02	1943.70±63.50	2175.31±115.49	2684.95±104.73	2240.00±154.31	2577.18±89.82	7.32	**<0.001**
Distance traveled in the Play Zone (cm)	1145.97±50.95	1119.57±63.92	1419.80±85.83	1738.65±99.33	1522.79±133.73	1769.36±46.05	10.63	**<0.001**
Distance traveled in the Other Zone (cm)	1003.51±68.82	815.35±84.25	756.13±90.24	946.30±91.10	717.21±66.69	807.82±62.47	2.00	0,093
Frequency of Grooming (N)	0.80±0.20	2.11±0.53	0.78±0.47	1.00±0.30	1.50±0.37	0.50±0.22	2.56	**0.037**
Cumulative duration of Grooming (s)	4.18±1.17	8.69±2.53	5.28±3.42	4.01±1.36	4.35±0.93	1.19±0.49	1.58	0.182
Cumulative Game Interaction (s)	138.47±6.77	133.63±9.73	155.15±9.47	190.28±9.89	173.80±12.13	188.56±10.95	6.17	**<0.001**
Number of correct Touch (N)	44.44±2.23	43.86±3.57	59.11±4.90	71.27±7.23	58.40±7.61	74.20±5.09	5.41	**<0.001**
Protected rearing (N)	17.67±2.25	12.67±2.16	7.33±1.17	11.64±2.35	9.50±1.07	10.10±1.15	3.77	**0.005**
Un- Protected rearing (N)	0.89±0.43	0.11±0.10	0.22±0.20	0.82±0.82	0.00±0.00	2.40±0.65	3.30	**0.011**

Table 2. Results obtained from the analysis of different behaviors within the Test 1.

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Test 1								
Parameters	**GD M vehicle**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 5 mg/kg Fluoxetine**	**GD F vehicle**	**GD F 2.5 mg/kg Fluoxetine**	**GD F 5 mg/kg Fluoxetine**	**ANOVA**	
							$F_{(5,55)}$	p
Frequency of entry into the Play Zone (N)	15.67±0.71	7.89±1.25	12.33±1.25	17.36±1.41	8.70±0.98	10.90±0.87	10.46	**<0.001**
Time in the Play Zone (s)	341.31±19.91	307.63±38.36	228.36±17.78	414.72±12.18	340.85±29.76	251.73±23.61	7.89	**<0.001**
Time in Other zone (s)	196.96±19.77	232.62±38.39	311.72±17.69	125.54±12.20	199.49±29.76	288.44±23.64	7.91	**<0.001**
Total distance traveled (Arena) (cm)	2328.49±102.02	1043.18±82.83	1336.01±126.61	2788.28±151.66	1577.84±141.65	1471.22±49.14	32.78	**<0.001**
Distance traveled in the Play Zone (cm)	1180.42±64.19	452.88±39.74	532.78±57.31	1738.65±99.33	897.49±94.70	584.63±45.51	47.41	**<0.001**
Distance traveled in the Other Zone (cm)	1148.07±106.56	590.29±61.30	803.23±85.31	1049.63±94.17	680.34±65.12	886.58±48.02	7.06	**<0.001**
Frequency of Grooming (N)	0.78±0.22	2.00±0.69	1.56±0.53	1.00±0.30	2.40±0.34	2.6±0.52	2.74	**0.029**
Cumulative duration of Grooming (s)	4.18±1.30	21.70±6.74	14.05±5.71	4.01±1.36	24.12±5.77	17.82±3.98	3.68	**0.006**
Cumulative Game Interaction (s)	138.47±7.56	45.21±5.34	17.71±3.66	190.28±9.89	102.94±11.44	21.78±4.06	81.80	**<0.001**
Number of correct Touch (N)	44.44±2.49	10.33±1.41	5.44±1.20	71.27±7.23	22.60±2.57	8.30±1.70	49.33	**<0.001**
Protected rearing (N)	17.67±2.52	9.33±3.03	14.56±2.72	11.64±2.35	7.10±1.36	15.40±2.17	2.75	**0.028**
Un- Protected rearing (N)	0.89±0.48	1.33±1.33	6.78±1.29	0.82±0.82	1.00±0.60	7.00±2.17	5.85	**<0.001**

Table 3. Results obtained from the analysis of different behaviors within the Test 2 (Videogames VS Social Familiar stimulus).

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Test 2 (Videogames VS Social Familiar stimulus)										
Parameters	**CN M**	**GD M vehicle**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 5 mg/kg Fluoxetine**	**CN F**	**GD F vehicle**	**GD F 2.5 mg/kg Fluoxetine**	**GD F 5 mg/kg Fluoxetine**	**ANOVA**	
										p
Frequency of entry into the Play Zone (N)	11.64±0.88	6.22±1.48	2.44±0.84	3.56±0.87	6.13±0.68	6.91±0.93	4.60±1.13	2.50±0.56	10.67	**<0.001**
Time in the Play Zone (s)	108.88±10.64	209.58±39.97	85.86±38.57	69.84±21.64	167.35±52.73	368.47±25.80	113.58±27.93	24.47±6.76	13.68	**<0.001**
Frequency of entry into the Social Zone (N)	19.36±1.52	7.13±1.01	7.85±1.02	10.00±2.45	11.37±1.48	5.00±0.62	7.10±0.85	7.30±1.34	11.98	**<0.001**
Time in the Social Zone (s)	257.29±8.09	170.39±28.06	246.37±37.09	249.36±24.72	213.29±29.28	77.94±15.71	261.04±41.26	162.79±15.86	6.38	**<0.001**
Total distance traveled (Arena) (cm)	1767.40±82.22	954.97±86.87	389.55±33.67	593.77±82.09	1059.01±93.36	1482.28±127.87	865.49±120.26	562.46±85.09	25.68	**<0.001**
Distance traveled in the Play Zone (cm)	269.86±50.30	440.48±53.50	57.48±6.25	79.85±15.70	222.39±28.11	1147.64±78.15	403.11±81.30	81.87±13.03	51.32	**<0.001**
Frequency of Grooming (N)	2.82±0.38	2.38±0.37	3.62±0.39	3.89±0.68	3.44±0.50	1.36±0.28	3.50±0.98	2.10±0.71	2.40	**0.029**
Cumulative duration of Grooming (s)	39.14±12.18	65.20±14.63	87.56±19.43	52.84±15.96	89.56±22.23	26.71±7.62	58.51±12.43	48.72±10.66	2.30	**0.036**
Cumulative Game Interaction (s)	0.00±0.00	62.34±6.71	10.25±1.23	5.04±1.88	0.00±0.00	170.27±11.57	47.90±9.07	3.29±1.41	98.83	**<0.001**
Number of correct Touch (N)	0.00±0.00	15.00±1.96	5.22±1.50	1.11±0.42	0.00±0.00	48.36±3.23	11.80±3.10	0.90±0.41	81.24	**<0.001**
Cumulative social interaction (s)	124.44±12.84	25.29±4.33	25.46±6.09	56.29±4.29	79.47±12.73	10.22±1.97	40.08±8.57	24.80±5.08	23.52	**<0.001**
Protected rearing (N)	18.18±1.73	5.00±1.11	2.89±0.45	6.00±1.67	11.88±2.91	4.18±0.74	5.10±1.16	7.50±1.24	11.95	**<0.001**
Un- Protected rearing (N)	0.64±0.31	1.63±0.71	0.44±0.24	3.11±1.17	2.38±1.21	1.91±0.72	2.60±1.40	4.40±1.55	1.64	0.138

Table 4. Results obtained from the analysis of different behaviors within the Test 3 (Videogames VS Social UN-Familiar stimulus).

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Test 3 (Videogames VS Social UN-Familiar stimulus)										
Parameters	**CN M**	**GD M vehicle**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 5 mg/kg Fluoxetine**	**CN F**	**GD F vehicle**	**GD F 2.5 mg/kg Fluoxetine**	**GD F 5 mg/kg Fluoxetine**	**ANOVA**	
									$F_{(7,70)}$	p
Frequency of entry into the Play Zone (N)	6.45±0.76	5.56±0.84	3.67±1.43	2.38±0.58	4.78±1.02	8.70±0.88	5.90±1.67	2.40±0.40	4.64	**<0.001**
Time in the Play Zone (s)	79.97±14.93	205.70±35.33	52.03±21.74	21.82±5.93	36.02±10.34	366.26±28.78	157.98±29.58	26.66±4.15	31.61	**<0.001**
Frequency of entry into the Social Zone (N)	12.40±1.40	10.11±1.68	8.44±0.87	10.13±1.34	12.33±1.78	6.70±1.09	8.10±0.67	8.80±1.15	2.60	**0.019**
Time in the Social Zone (s)	255.10±32.77	174.59±18.67	221.07±27.08	259.69±23.27	331.07±36.56	62.95±10.41	193.11±23.29	228.72±35.31	8.58	**<0.001**
Total distance traveled (Arena) (cm)	1067.79±155.99	953.39±98.52	602.03±113.45	606.29±108.85	1085.91±128.38	1687.61±100.32	1070.36±105.22	587.57±74.95	11.02	**<0.001**
Distance traveled in the Play Zone (cm)	269.86±50.30	486.99±49.72	169.31±48.10	73.77±19.33	150.16±41.68	1168.49±103.52	472.01±88.08	114.74±34.05	34.77	**<0.001**
Frequency of Grooming (N)	2.73±0.79	3.11±0.42	4.11±0.81	2.33±0.76	1.78±0.60	1.09±0.46	2.20±0.33	2.70±0.88	1.86	**<0.001**
Cumulative duration of Grooming (s)	69.18±23.79	67.35±9.60	79.71±14.18	38.49±10.80	27.61±8.27	11.95±6.31	38.36±10.00	22.40±6.49	3.72	**0.002**
Cumulative Game Interaction (s)	0.00±0.00	79.82±7.17	4.11±0.81	3.63±1.62	0.00±0.00	176.15±15.09	62.95±14.65	4.69±1.62	59.81	**<0.001**
Number of correct Touch (N)	0.00±0.00	19.11±2.29	4.33±2.17	3.67±2.24	0.00±0.00	52.09±5.54	12.60±3.08	1.30±0.45	43.79	**<0.001**
Cumulative social interaction (s)	94.24±17.21	32.23±5.69	47.81±3.48	35.20±8.33	130.71±23.70	23.02±6.88	44.34±4.58	31.41±7.21	10.23	**<0.001**
Protected rearing (N)	9.00±2.08	6.38±1.52	2.89±0.48	8.43±2.39	10.67±1.91	4.91±0.88	5.70±1.41	7.70±1.63	2.22	**0.042**
Un- Protected rearing (N)	0.36±0.24	0.63±0.29	1.22±0.57	1.62±0.50	0.11±0.11	2.18±0.74	3.40±1.07	2.00±0.79	3.04	0.008

Table 5. Results obtained from the analysis of different behaviors within the Test 4 (No reward).
Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Test 4 (No reward)								
Parameters	**GD M vehicle**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 5 mg/kg Fluoxetine**	**GD F vehicle**	**GD F 2.5 mg/kg Fluoxetine**	**GD F 5 mg/kg Fluoxetine**	**ANOVA**	
							$F_{(7,70)}$	p
Frequency of entry into the Play Zone (N)	11.11±0.95	6.22±0.60	7.22±0.97	15.45±1.40	12.10±1.46	8.80±1.12	8.97	**<0.001**
Time in the Play Zone (s)	347.97±18.81	212.11±30.74	117.27±26.09	383.86±17.35	173.50±21.33	79.01±13.92	33.71	**<0.001**
Time in Other zone (s)	190.30±18.91	328.14±30.74	422.81±26.02	156.40±17.34	366.84±21.33	461.16±13.95	33.88	**<0.001**
Total distance traveled (Arena) (cm)	1840.44±101.15	1125.93±96.25	1055.17±87.08	2239.98±74.95	1655.78±115.10	1252.89±125.78	21.69	**<0.001**
Distance traveled in the Play Zone (cm)	1055.31±46.78	387.22±32.73	245.61±35.61	1375.05±31.80	576.29±61.49	275.56±44.85	117.04	**<0.001**
Distance traveled in the Other Zone (cm)	785.12±76.53	738.71±84.95	809.56±63.26	864.93±68.48	1079.49±71.69	977.33±93.58	2.78	**0.027**
Frequency of Grooming (N)	1.22±0.57	3.00±0.58	2.44±0.50	3.36±0.45	2.80±0.42	2.50±0.75	1.73	0.145
Cumulative duration of Grooming (s)	5.84±3.04	29.27±5.22	37.10±15.27	22.01±6.72	19.84±5.45	23.72±5.45	1.72	0.147
Cumulative Game Interaction (s)	152.99±8.61	42.21±10.58	12.72±3.20	192.27±7.20	60.04±9.29	21.50±6.09	93.42	**<0.001**
Number of correct Touch (N)	36.00±2.57	8.67±1.82	2.11±0.51	52.27±2.11	16.20±2.16	6.50±1.96	103.08	**<0.001**
Protected rearing (N)	11.44±2.14	15.00±1.84	13.22±2.09	13.36±2.25	18.20±3.08	17.60±1.91	1.35	0.260
Un- Protected rearing (N)	0.44±0.24	0.33±0.33	1.78±0.60	2.27±0.68	5.10±1.47	4.50±0.75	6.02	**<0.001**

Test 1

In Test 1, the highest dose of fluoxetine reduced play parameters in both sexes. Specifically, when comparing the time spent in the play zone across the different GD groups, the GD FLX5 groups of both sexes showed a reduction compared with the vehicle GD group (***Figure 3A***; p=0.041 for males; p=0.002 for females), but not when compared to the GD FLX2.5 groups (***Figure 3A;*** p=0.300 for males, p=0.118 for females). As the dose of fluoxetine increased, the FLX2.5 (***Figure 3B***; p<0.0001 for males; p<0.0001 for females) and GD FLX5 (***Figure 3B***; p<0.0001 for males; p<0.0001 for females) groups showed a reduction in the distance traveled in the play zone compared with the vehicle GD group. Similarly, as the fluoxetine dose increased, both the GD FLX2.5 (***Figure 3C***; p<0.0001 for males; p<0.0001 for females) and GD FLX5 (***Figure 3C***; p<0.0001 for males; p<0.0001 for females) groups exhibited reduced game interaction compared with the vehicle GD group. In addition to the reduction in game interaction, fluoxetine also decreased the number of touches made in the GD FLX2.5 (***Figure 3D***; p<0.0001 for males; p<0.0001 for females) and GD FLX5 (***Figure 3D***; p<0.0001 for males; p<0.0001 for females) groups compared to the vehicle GD group.

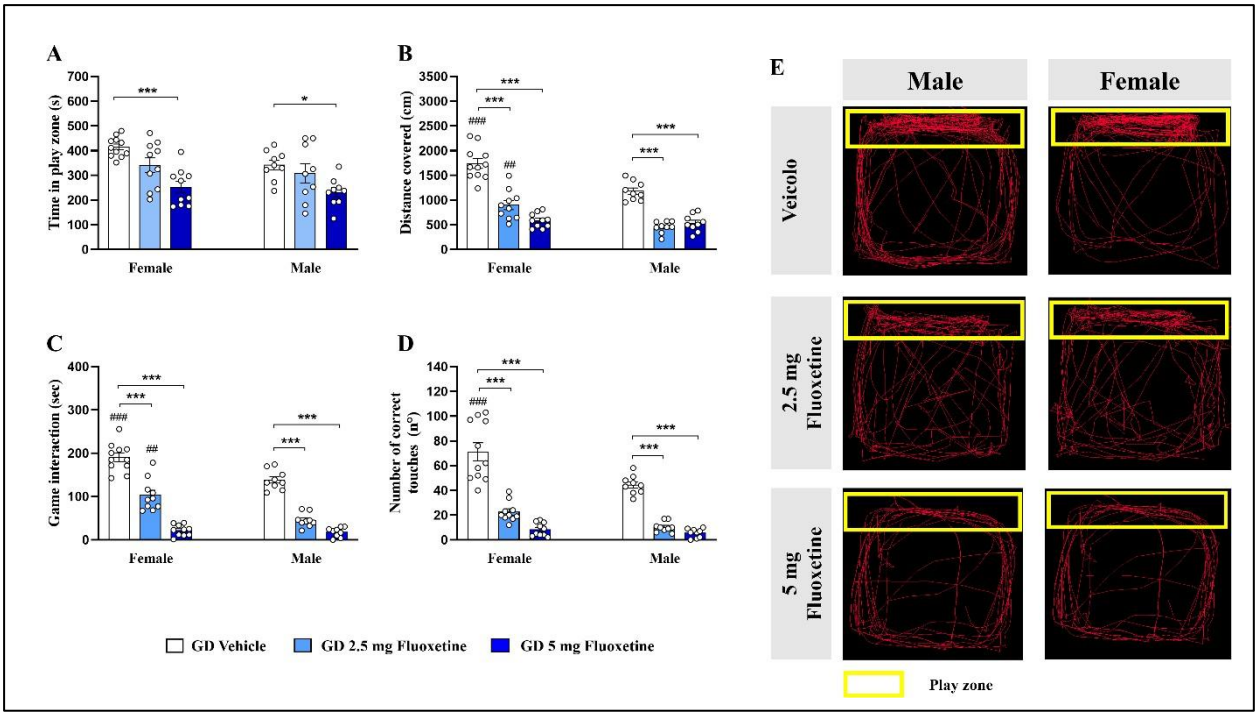


Figure 3. Analysis of the addictive behavior during Test 1 (in the presence of the reward). (*The upper histograms show (A) the time spent and (B) the distance traveled in the play zone, (C) the time spent in game interaction, and (D) the number of correct touches performed by the different groups. **E)** Representative images of the distance traveled in the play zone (yellow rectangle). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes). GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Test 2

The presence of a familiar social stimulus (cage mate) in the apparatus affects males and females differently during the play session. Specifically, the GD M vehicle group shows a reduction in the time spent in the play zone (***Figures 4A***; p=0.002), distance traveled (***Figures 4B***; p<0.0001), game interaction (***Figures 4C***; p<0.0001), and the number of correct touches (***Figures 4D***; p<0.0001) compared with females in the same group. However, this sexual difference is eliminated by Fluoxetine. Both doses of the drug reduce the sexual difference, with no significant difference observed in the time spent in the play zone (***Figures 4A***; GD FLX2.5, p=0.982; GD FLX5, p=0.870), distance traveled (***Figures 4B***; GD FLX2.5, p=0.0005; GD FLX5, p=1.000), game interaction (***Figures 4C***; GD FLX2.5, p=0.252; GD FLX5, p=1.000), or in the number of correct touches (***Figures 4D***; GD FLX2.5, p=0.316; GD FLX5, p=1.000).

The 2.5 mg/kg Fluoxetine dose reduced the time spent in the play zone (***Figure 4A***; p=0.047 for males; p<0.0001 for females), distance traveled (***Figure 4B***; p=0.0002 for males; p<0.0001 for females), game interaction (***Figure 4C***; p=0.014 for males; p<0.0001 for females), and the number of touches (***Figure 4D;*** p=0.044 for males; p<0.0001 for females) compared with the same-sex vehicle group. More pronounced results were observed when comparing the vehicle groups with those treated with 5 mg/kg Fluoxetine: time spent in the play zone (***Figure 4A***; p=0.017 for males; p<0.0001 for females), distance traveled (***Figure 4B***; p=0.0005 for males; p<0.0001 for females), game interaction (***Figure 4C***; p<0.0001 for males; p<0.0001 for females), and the number of touches (***Figure 4D***; p=0.001 for males; p<0.0001 for females).

Rats subjected to the GD protocol reduced interaction time with conspecifics regardless of drug treatment or sex. When comparing social interaction time, both males (***Figures 4E***; GDM vehicle, p<0.0001; GDM FLX2.5, p<0.0001; GDM FLX5, p<0.0001) and females (***Figures 4E***; GDF vehicle, p<0.0001; GDF FLX2.5, p=0.022; GDF FLX5, p=0.0003) show a reduction in social interaction compared to the control groups of the same sex.

However, when comparing the two different stimuli (game vs. social interaction), the vehicle GD groups prefer interaction with the game (***Figures 4F***; male, p=0.0004; female, p<0.0001), the FLX2.5 GD groups show no preference (***Figures 4F***; male, p=0.842; female, p=0.538), while the FLX5 GD group prefers social interaction to the video game (***Figures 4F***; male, p<0.0001; female, p=0.002).

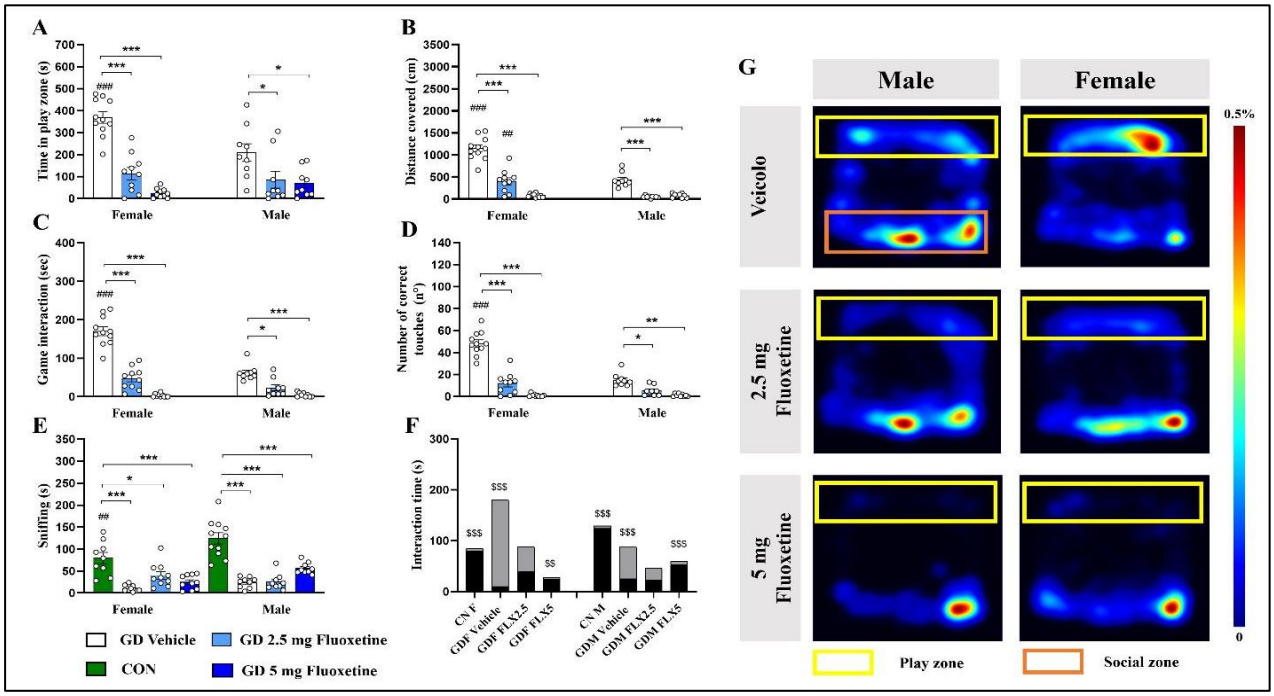


Figure 4. Analysis of the addictive behavior during Test 2 (In the presence of a familiar rat). *The upper histograms show* ***(A)*** *the time spent and* ***(B)*** *the distance traveled in the play zone,* ***(C)*** *the time spent in game interaction, and* ***(D)*** *the number of correct touches performed by the different groups. (****E****) Time of interaction with an familiar conspecific.* ***(F)*** *Comparison of time spent interacting with familiar conspecific versus gaming interaction time.* ***(G)*** *Representative images of the distance traveled in the play zone (yellow rectangle). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (***** comparison between different groups; **#** comparison between sexes; $ social interaction vs gaming interaction). CN = Control; GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Test 3

The presence of an unfamiliar social stimulus produces effects like those observed with the familiar social stimulus. In the presence of an unfamiliar social stimulus, the vehicle GDM group shows a reduction in time spent in the play zone (***Figures 5A***; p=0.003), distance traveled (***Figures 5B***; p<0.0001), game interaction (***Figures 5C***; p<0.0001), and the number of correct touches (***Figures 5D***; p<0.0001), in contrast to the vehicle GDF group, which does not exhibit a similar reduction. Fluoxetine treatment at both doses reduces these behaviors in both sexes similarly: time spent in the play zone (***Figures 5A***; GD FLX2.5, p=; GD FLX5, p=), distance traveled (***Figures 5B***; GD FLX2.5, p=0.046; GD FLX5, p=0.998), game duration (***Figures 5C***; GD FLX2.5, p=0.087; GD FLX5, p=1.000), and the number of correct touches (***Figures 5D;*** GD FLX2.5, p=0.488; GD FLX5, p=0.995).

As the drug dose increased, the GD FLX2.5 groups showed further reductions in time spent in the play zone (***Figure 5A***; p=0.001 for males; p<0.0001 for females), distance traveled (***Figure 5B***; p=0.031 for males; p<0.0001 for females), game interaction (***Figure 5C***; p=0.005 for males; p<0.0001 for females), and the number of touches (***Figure 5D***; p=0.035 for males; p<0.0001 for females) compared with the same-sex vehicle group.

The effects were even more pronounced when the dose of Fluoxetine was increased: time spent in the play zone (***Figure 5A***; p<0.0001 for males; p<0.0001 for females), distance traveled (***Figure 5B***; p=0.002 for males; p<0.0001 for females), game interaction (***Figure 5C***; p=0.001 for males; p<0.0001 for females), and the number of touches (***Figure 5D;*** p=0.024 for males; p<0.0001 for females) were all reduced in the drug-treated groups compared with the same-sex control groups.

Rats subjected to the GD protocol exhibited reduced interaction time with conspecifics, regardless of drug treatment or sex. When comparing social interaction time, both males (***Figures 5E***; GDM vehicle, p=0.007; GDM FLX2.5, p=0.103; GDM FLX5, p=0.012) and females (***Figures 5E***; GDF vehicle, p<0.0001; GDF FLX2.5, p<0.0001; GDF FLX5, p<0.0001) showed a reduction in social interaction compared to the same-sex control groups.

However, when comparing the two different stimuli (game vs. social interaction), the vehicle GD groups preferred game interaction (***Figures 5F***; male, p<0.0001; female, p<0.0001), unlike the control groups, which preferred social interaction. Fluoxetine administration reduced play time and increased social interaction time (sniffing time). Both GD FLX5 groups (***Figures 5F***; male, p=0.005; female, p=0.004) spent more time interacting with conspecifics than engaging in play (game interaction). The 2.5 mg/kg dose of Fluoxetine increased interaction time in males compared to game interaction (***Figure 5F***; p=0.031). However, the same dose had a different effect in females, as they showed no preference between the two stimuli (***Figure 5F***; p=0.251).

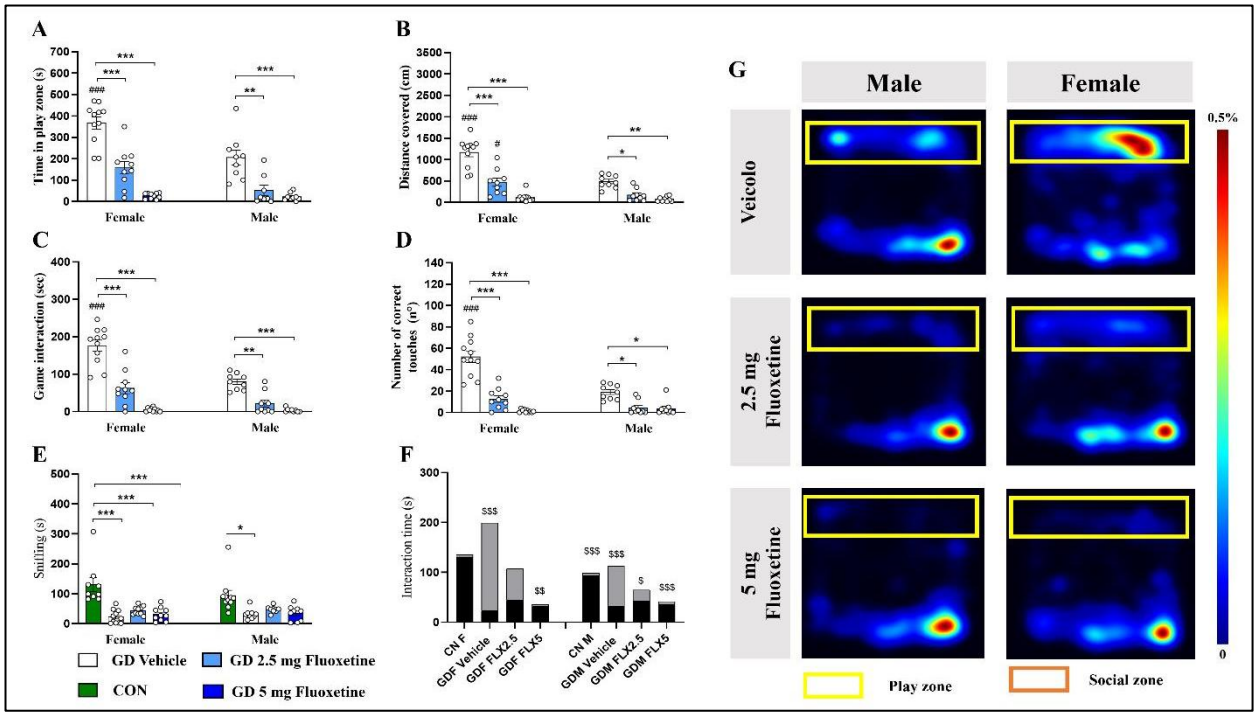


Figure 5. Analysis of the addictive behavior during Test 3 (In the presence of a UN-familiar rat). *The upper histograms show* ***(A)*** *the time spent and* ***(B)*** *the distance traveled in the play zone,* ***(C)*** *the time spent in game interaction, and* ***(D)*** *the number of correct touches performed by the different groups.* ***(E)*** *Time of interaction with an unfamiliar conspecific.* ***(G)*** *Representative images of the distance traveled in the play zone (yellow rectangle).* ***(F)*** *Comparison of time spent interacting with unfamiliar conspecific versus gaming interaction time. Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes; $ social interaction vs gaming interaction). CN = Control; GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Test 4

In Test 4, even in the absence of a reward, the vehicle GD groups showed similar results to those observed in Test 1. The vehicle GD groups exhibited sex differences. Females did not spend more time in the play zone (***Figure 6A***; p=0.839) but traveled a greater distance (***Figure 6B***; p<0.0001). Additionally, compared with the same-sex group, females spent more time in game interaction (Figure 4C; p=0.009) and performed more correct touches (***Figure 6D***; p<0.0001).

This sexual dimorphism was almost completely abolished by the administration of both doses of Fluoxetine. Specifically, the GDF FLX2.5 and GDF FLX5 groups showed no differences in time spent in the play zone (***Figures 6A***; GDM FLX2.5, p=0.808; GDM FLX5, p=0.814), time spent engaged in game interaction (***Figures 6C***; GDM FLX2.5, p=0.607; GDM FLX5, p=0.969), or the number of correct touches (***Figures 6D***; GDM FLX2.5, p=0.103; GDM FLX5, p=0.637) when

compared with males in the same group. The only remaining sexual difference was in the distance traveled in the play zone between the GD FLX2.5 groups (***Figures 6B***; GDM FLX2.5, p=0.041; GDM FLX5, p=0.996).

When comparing the groups across conditions, Fluoxetine administration reduced hyperactivity and attachment to the play zone in rats that underwent GD. The 2.5 mg/kg Fluoxetine dose reduced time spent in the play zone (***Figures 6A***; male, p=0.010; female, p<0.0001), distance traveled (***Figures 6B***; male, p<0.0001; female, p<0.0001), game interaction (***Figures 6C***; male, p<0.0001; female, p<0.0001), and the number of correct touches (***Figures 6D***; male, p<0.0001; female, p<0.0001) compared to the same-sex vehicle GD group. These effects were more pronounced at the higher Fluoxetine dose, where comparing the GD FLX5 groups to the same-sex vehicle groups revealed a substantial reduction in time spent in the play zone (***Figures 6A***; male, p<0.0001; female, p<0.0001), distance traveled (***Figures 6B***; male, p<0.0001; female, p<0.0001), game interaction (***Figures 6C***; male, p<0.0001; female, p<0.0001), and the number of correct touches (***Figures 6D***; male, p<0.0001; female, p<0.0001).

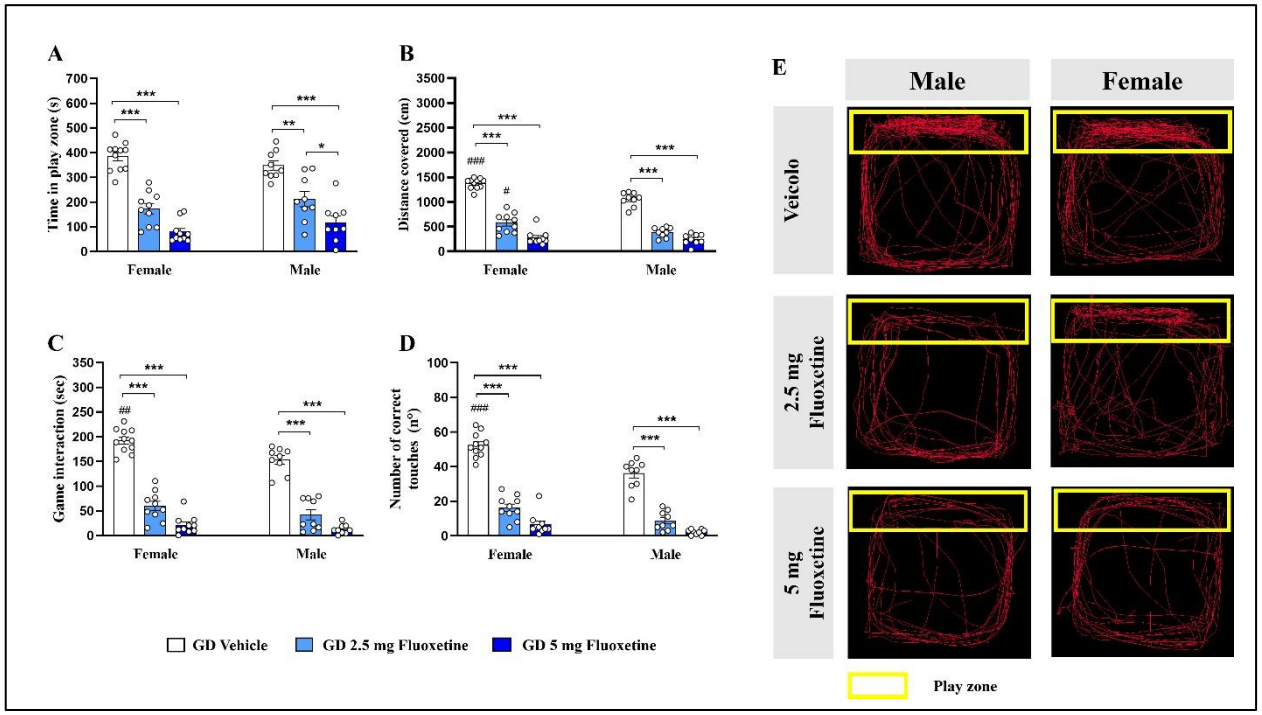


Figure 6. Analysis of the addictive behavior during Test 4 (in the absence of the reward). *The upper histograms show* ***(A)*** *the time spent and* ***(B)*** *the distance traveled in the play zone,* ***(C)*** *the time spent in game interaction, and* ***(D)*** *the number of correct touches performed by the different groups.* ***(E)*** *Representative images of the distance traveled in the play zone (yellow rectangle). Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (***** comparison between different groups; **#** comparison between sexes). GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

OF

Statistical analysis of the OF test revealed an increase in motor activity following the development of the GD model (***Table 6***. The p-values presented in the results were based on Tukey's HSD test). The GD F vehicle group showed an increase in the distance traveled compared to the same-sex control group **(*Figure 7A; p=0.045***). However, this hyperactivity was not observed in the GD M vehicle group, which showed no significant difference compared to the CN M group (***Figure 7A;*** *p=0.999*).

Hyperactivity induced by the GD model in females was reduced by the lower dose of Fluoxetine (***Figure 7A***; GD F vehicle vs GD F FLX2.5, p=0.0003), showing no significant difference from the CN F group (***Figure A;*** p=0.960). In contrast, the GD F FLX5 group exhibited increased motor activity in the arena, but this increase was only seen when compared to the same-sex control group (***Figure 7A;*** CN F, p=0.046; GD F vehicle, p=1.000; GD F FLX2.5, p=0.491).
More specifically, the highest dose of Fluoxetine (FLX5) reduced anxiety in the GD F FLX5 group, resulting in an increase in the distance traveled (***Figure 7B;*** CN F, p<0.0001; GD F vehicle, p=0.001; GD F FLX2.5, p<0.0001) and time spent in the center of the apparatus (***Figure 7C***; CN F, p<0.0001; GD F vehicle, p=0.0002; GD F FLX2.5, p<0.0001) compared to all same-sex groups. As distance and time in the center increased, the GD F FLX5 group showed a reduction in time spent at the edges of the arena (***Figure 7D;*** CN F, p<0.0001; GD F vehicle, p=0.002; GD F FLX2.5, p<0.0001) compared to the same-sex groups.
Hyperactivity induced by the GD model was not observed in the GD M vehicle group, which showed no significant difference in the total distance traveled compared to the other groups (***Figure 7A;*** CN M, p=0.999; GD M FLX2.5, p=0.494; GD M FLX5, p=0.999). Similar to the GD F FLX5 group, the GD M FLX5 group, treated with the highest dose of Fluoxetine, showed an increase in the distance traveled (***Figure 7B;*** CN M, p=0.003; GD M vehicle, p=0.004; GD M FLX2.5, p=0.001) and time spent in the center (***Figure 7C;*** CN M, p=0.005; GD M vehicle, p<0.0001; GD M FLX2.5, p<0.0001) compared to the other same-sex groups. Just like the GD F FLX5 group, the GD M FLX5 group also showed a reduction in the time spent at the edges of the arena compared to the same-sex groups (***Figure 7D;*** CN M, p=0.003; GD M vehicle, p=0.0001; GD M FLX2.5, p<0.0001).

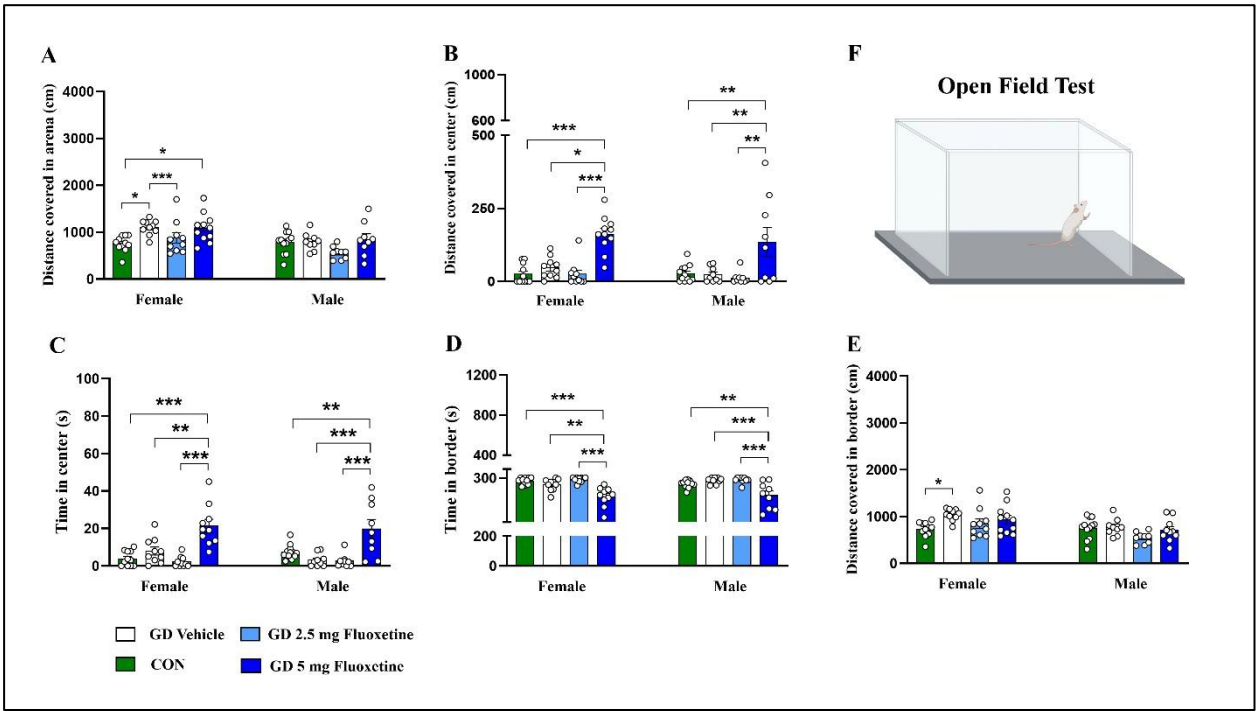


Figure 7. Analysis of hyperactivity during the Open Field Test. *The upper histograms show* ***(A)*** *the distance covered in Arena,* ***(B)*** *the distance covered in the center,* ***(C)*** *the time spent in center, (D) the the time spent in border, and* ***(E)*** *the distance covered in the border performed by the different groups.* ***(F)*** *Representative images of the Open Field apparatus. Data are expressed as mean ± SEM. Two- way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes; $ social interaction vs gaming interaction). CN = Control; GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Table 6. Results obtained from the analysis of different behaviors within the Open Field Test.
Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Open Field Test										
Parameters	**CN M**	**CN F**	**GD M vehicle**	**GD F vehicle**	**GD M 2.5 mg/kg Fluoxitine**	**GD M 2.5 mg/kg Fluoxitine**	**GD M 5 mg/kg Fluoxitine**	**GD F 5 mg/kg Fluoxitine**	**ANOVA**	
									$F_{(7.72)}$	p
Frequency of entry into the Center (N)	2.50±0.47	1.73±0.43	1.13±0.35	4.78±1.27	1.63±0.53	1.33±0.67	6.00±1.09	6.00±0.74	7.76	**<0.001**
Time in the Center (s)	7.44±1.09	3.95±1.20	3.09±1.14	7.89±2.13	2.62±1.16	2.19±0.91	19.89±4.36	21.39±3.15	12.11	**<0.001**
Frequency of entry into the Border (N)	3.33±0.51	2.73±0.43	2.13±0.35	5.78±1.27	2.63±0.53	2.22±0.68	6.33±1.28	6.50±0.70	5.77	**<0.001**
Time in the Border (s)	293.36±1.17	296.48±1.20	297.30±1.14	292.45±2.14	297.77±1.16	298.20±0.91	280.25±4.43	279.00±3.15	12.03	**<0.001**
Total distance traveled (Arena) (cm)	783.22±67.33	756.74±51.56	796.05±61.59	1095.27±47.31	560.38±44.72	874.31±111.59	845.45±105.49	1093.23±92.56	4.21	**<0.001**
Velocity in the Arena (cm/s)	2.61±0.22	2.65±0.22	2.65±0.21	3.60±0.22	1.87±0.15	2.91±0.37	2.82±0.36	3.59±0.30	5.05	**0.001**
Distance traveled in the Border (cm)	756.19±64.44	731.45±49.51	772.90±60.69	1048.43±38.87	547.90±40.58	848.62±98.88	709.67±76.11	930.79±93.09	4.47	**<0.001**
Velocity in the Border (cm/s)	2.57±0.22	2.60±0.22	2.60±0.20	3.43±0.18	1.84±0.14	2.86±0.34	2.53±0.28	3.30±0.31	3.71	**0.002**
Distance traveled in the Center (cm)	27.02±8.37	25.29±10.04	23.15±8.58	46.84±11.40	12.48±6.89	25.69±13.55	135.77±44.09	162.44±18.75	9.56	**<0.001**
Velocity in the Center (cm/s)	4.46±1.12	3.04±1.09	4.49±1.32	7.51±1.53	4.22±1.43	5.91±1.87	6.00±1.31	8.10±0.66	1.81	**0.098**
Frequency of Grooming (N)	0.83±0.27	4.13±0.70	0.88±0.31	0.90±0.23	0.75±0.22	0.67±0.21	1.11±0.41	2.00±0.50	8.64	**<0.001**
Cumulative duration of Grooming (s)	13.35±4.58	38.20±6.04	10.59±3.67	19.13±6.24	10.50±4.13	7.36±2.44	8.81±3.77	19.97±5.75	4.09	**0.001**
Protected rearing (N)	3.50±0.83	2.38±0.80	5.75±1.22	6.00±0.84	2.50±0.58	5.89±1.57	4.33±1.22	9.40±1.56	4.15	**0.001**
Un- Protected rearing (N)	0.25±0.18	2.00±0.90	0.13±0.11	0.30±0.30	0.63±0.23	0.44±0.30	1.11±0.78	1.40±0.49	1.85	**0.091**

EPM

Statistical analysis of the EPM revealed an increase in anxiety in the groups subjected to the GD model, which was reduced by Fluoxetine administration (***Table 7.*** The p-values presented in the results were based on Tukey's HSD test). In terms of time spent in the open arms, the GD vehicle group (for both sexes) spent less time in the open arms than the GD FLX5 group (***Figure 8A***; Males, p=0.049; Females, p=0.040). However, although the CN and GD FLX2.5 groups spent more time in the open arms than the vehicle GD group, the statistics showed no significant differences in males (***Figure 8A;*** CN M, p=0.999; GD M FLX2.5, p=0.999) or females (***Figure 8A;*** CN F, p=0.999; GD F FLX2.5, p=0.116).

Similarly, regarding the distance traveled in the open arms, the GD F vehicle group showed a reduction compared to the group treated with the highest dose of Fluoxetine (***Figure 8B;*** CN F, p=0.776; GD F FLX2.5, p=0.116; GD F FLX5, p=0.035). Among males, although there was a reduction in motor activity in the open arms in the GD M vehicle group, this group showed no significant differences compared to the other same-sex groups (***Figure 8B;*** CN M, p=0.990; GD M FLX2.5, p=1.000; GD M FLX5, p=0.375).

Reduced time spent in the open arms was associated with increased time spent in the closed arms. The GD vehicle (***Figure 8C;*** p<0.0001) and GD FLX2.5 (***Figure 8C;*** p<0.0001) groups showed a sex difference, with females spending less time in the closed arms compared to males within the same group. However, this sexual difference was not observed among the control groups (***Figure 8C;*** p=0.746) or in those treated with the highest dose of Fluoxetine, GD FLX5 (***Figure 8C;*** p=0.766). As the pharmacological dose increased, the time spent in the closed arms by males decreased. In fact, the GD M FLX5 group spent less time in the closed arms than all other same-sex groups (***Figure 8C***; CN M, p=0.010; GD M vehicle, p<0.0001; GD M FLX2.5, p=0.0008). In females, only the GD F FLX5 group spent less time in the closed arms than the same-sex control group (***Figure 8C;*** GD F vehicle, p=0.721; GD F FLX2.5, p=0.437; GD F FLX5, p=0.010).

Although there were significant differences in the time spent in the closed arms, the ANOVA revealed no significant differences in the distance traveled in the closed arms between the different groups (***Figure 8D;*** p=0.937).

Looking at the data on the time spent in the center, it showed an increase in anxiety in the GD M vehicle group, which spent less time in the center compared to the CN M group. As the pharmacological dose increased, anxiety in the GD M groups decreased. However, only the GD M FLX5 group spent more time in the center than the vehicle group (***Figure 8E;*** p<0.0001). In contrast, the GD F groups spent more time in the center than the CN F group (***Figure 8E;*** GD F vehicle, p=0.038; GD F FLX2.5, p=0.440; GD F FLX5, p=0.005).

The reduction in anxiety was associated with an increased frequency of head protrusions outside the apparatus. The GD M vehicle group reduced the frequency of head protrusions compared to the CN M group (***Figure 8F***; p=0.034) and the GD M FLX5 group (***Figure 8F;*** p=0.002), but not compared to the GD M FLX2.5 group (**Figure 8F;** p=0.221). Similarly, as the pharmacological dose increased, GD females protruded more frequently compared to the GD F vehicle group (***Figure 8F;*** GD F FLX2.5, p=0.014; GD F FLX5, p=0.103), but not compared to the CN F group (***Figure 8F;*** GD F

FLX2.5, p=0.362; GD F FLX5, p=0.790).

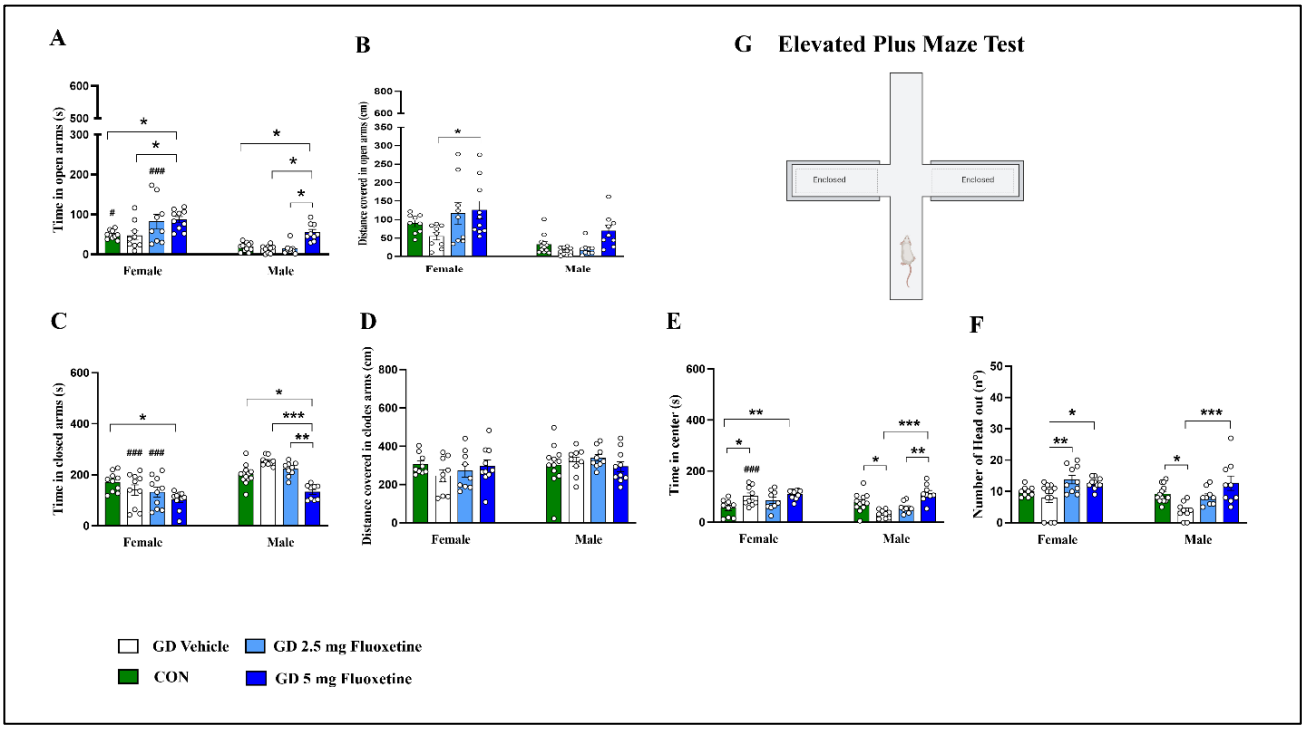


Figure 7. Analysis of hyperactivity during the OF Test. *The upper histograms show* ***(A)*** *the distance covered in Arena,* ***(B)*** *the distance covered in the center,* ***(C)*** *the time spent in center, (D) the the time spent in border, and* ***(E)*** *the distance covered in the border performed by the different groups.* ***(F)*** *Representative images of the Open Field apparatus. Data are expressed as mean ± SEM. Two- way ANOVA followed by Tukey's HSD test revealed a significant effect for p≤0.05 (* comparison between different groups; # comparison between sexes; $ social interaction vs gaming interaction). CN = Control; GD Vehicle = Gaming Disorder treated with vehicle; GD 2.5mg Fluoxitine = Gaming Disorder treated with 2.5 mg/kg of Fluoxitine; GD 5mg Fluoxitine = Gaming Disorder treated with 5 mg/kg of Fluoxitine.*

Table 7. Results obtained from the analysis of different behaviors within the Elevated Plus Maze.

Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$ highlighted in bold.

Elevated Plus Maze										
Parameters	**CN M**	**CN F**	**GD M vehicle**	**GD F vehicle**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 2.5 mg/kg Fluoxetine**	**GD M 5 mg/kg Fluoxetine**	**GD F 5 mg/kg Fluoxetine**	**ANOVA**	
									$F_{(7.72)}$	p
Frequency of entry into the Center (N)	11.92±1.69	14.44±1.07	10.13±1.69	18.33±1.29	15.43±1.89	14.44±1.42	17.11±0.81	19.00±2.11	3.90	**0.001**
Time in the Center (s)	86.64±17.15	55.84±9.86	33.38±5.26	102.24±11.24	58.11±7.78	85.74±11.24	113.34±10.06	110.44±5.08	6.56	**<0.001**
Frequency of entry into open arms (N)	8.42±1.38	8.44±0.75	7.00±1.07	10.00±1.40	11.43±1.81	7.44±1.28	10.67±0. 76	10.70±1.77	1.43	**0.206**
Time in the open arms (s)	192.57±17.77	170.26±12.03	253.69±6.16	139.88±20.91	228.64±7.59	131.73±19.38	132.43±8.81	101.64±10.46	12.43	**<0.001**
Frequency of entry into closed arms (N)	3.50±0.47	5.89±0.77	4.13±0.89	8.78±0.68	4.43±0.62	7.33±0.91	7.56±1.04	9.00±0.73	7.95	**<0.001**
Time in the closed arms (s)	18.85±2.94	50.20±3.24	13.20±3.13	47.12±10.37	13.59±5.00	82.89±17.13	55.17±6.44	87.04±7.15	12.43	**<0.001**
Total distance traveled (Arena) (cm)	425.66±27.78	464.97±23.60	376.94±23.36	387.08±19.92	413.15±23.09	474.97±36.64	471.36±22.78	532.83±42.78	3.17	**0.006**
Distance traveled in the closed arms (cm)	302.48±32.73	304.88±16.19	319.82±23.53	246.30±28.46	339.62±20.59	271.48±29.69	293.75±26.81	296.45±28.81	1.00	**0**.436
Distance traveled Center (cm)	71.62±7.80	67.91±8.60	43.86±10.01	79.79±5.27	53.98±8.35	86.44±8.46	108.13±8.31	113.44±7.07	8.88	**<0.001**
Distance traveled in the open arms (cm)	32.57±7.44	89.48±7.71	15.11±3.48	54.78±8.99	18.01±7.09	117.05±26.29	69.48±13.32	125.78±21.67	8.53	**<0.001**
Frequency of Grooming (N)	0.42±0.19	0.63±0.16	0.75±0.22	0.40±0.22	0.57±0.26	0.30±0.15	0.22±0.13	0.82±0.23	1.17	**0.39**
Cumulative duration of Grooming (s)	3.33±1.47	7.49±2.48	3.19±1.08	2.50±1.29	8.43±3.52	0.84±0.46	1.28±0.78	3.05±1.16	2.55	**0.021**
Protected rearing (N)	8.92±1.43	6.25±1.66	8.25±1.82	9.70±2.74	8.00±1.70	9.07±1.34	9.67±1.76	9.20±2.58	0.31	**<0.001**
Un- Protected rearing (N)	0.00±0.00	3.62±1.50	0.00±0.00	0 .10±0.10	0.00±0.00	0 .20±0.13	0 .11±010	0 .10±0.09	6.16	**0.946**
Frequency head out of the open arms (N)	9.42±0.81	9.38±0.60	3.75±0.91	7.70±1.75	8.43±1.02	13.88±1.34	12.67±2.08	12.20±0.67	6.47	**<0.001**

DISCUSSION

GD is a mental disorder that primarily affects adolescents. It is estimated that approximately 4-6% of gamers develop symptoms of GD after prolonged and persistent exposure to video games (Stevens et al., 2021). Symptoms of GD include loss of control over gaming, reduced social interaction, and increased levels of anxiety and hyperactivity (*Wartberg et al., 2019*).

Research into the mechanisms underlying GD has been ongoing for several years. However, studies conducted so far have limitations in terms of the neurobiological investigation of this mental disorder. To address these limitations and complement human research, we developed an animal model to study GD (*Casile et al., 2024*). This model replicates key characteristics observed in GD patients, such as loss of control over gaming, increased hyperactivity and anxiety, and reduced social engagement (*Casile et al., 2024*). One hypothesis for the development of GD involves alterations in DA and 5-HT (*Tian et al., 2014*), two neurotransmitters critical for mood regulation, reward circuitry, and decision-making (*Furuyashiki & Kitaoka, 2019; Tian et al., 2014)*. In line with GD patients, this rat model also exhibits alterations in these neurotransmitters, confirming the hypothesis of reduced DA and 5-HT activity (*UNDER REVIEW: Casile et al. Central and peripheral monoamine changes in a rat model of Gaming Disorder*). Pharmacological treatment with inhibitors of these neurotransmitters has been shown to reduce video game use in GD patients and improve their emotional state by decreasing hyperactivity and anxiety (*Sá et al., 2023*). To further validate the model, we administered Fluoxetine and analyzed its effects on loss of control over gaming, anxiety, and hyperactivity.

Just as in humans *(Sá et al., 2023*), Fluoxetine administration in rats of both sexes reduced attachment to video gaming and hyperactivity, eliminating sex differences. Both doses of Fluoxetine reduced the distance traveled in the play zone, the duration of interaction with the game, and the number of correct touches. Another key aspect of this model, which mirrors that of GD patients, is the prioritization of play over other activities, including social interaction (*Giardina et al., 2021; Marino et al., 2020*). Both doses of Fluoxetine reduced the rats' preference for video gaming, increasing their interaction with a social stimulus in both familial and non-familial conditions. However, only the highest dose of Fluoxetine prompted the rats to prioritize social interaction over video games.

The attachment to video games and hyperactivity observed in this model are stable conditions, maintained even in the absence of rewards and over time (*Casile et al., 2024*). Fluoxetine appears to disrupt this pattern by reducing the time spent and distance traveled in the play zone, as well as the duration of game interaction and the number of correct touches. The reduction in these parameters across different tests seems to correlate with the amount of drug administered.

GD patients experience heightened anxiety and social anxiety (Bonnaire & Baptista, 2019; Marino et al., 2020), which often leads to self-isolation (*Giardina et al., 2021*). This rat model also shows increased levels of anxiety, as evidenced by tests in both the EPM and OF. Compared to rats treated with the vehicle alone, GD groups exposed to Fluoxetine doses exhibit lower anxiety levels, spending more time in the open arms, increasing the distance traveled, and the frequency of head-out behaviors. In contrast, the vehicle-exposed groups tend to stay longer in closed arms. A similar effect is observed in the OF, where the GD groups treated with the highest dose of Fluoxetine showed lower anxiety levels than the other groups, spending more time in the center of the arena and traveling a greater distance. In contrast, the other groups, including those treated with the lower Fluoxetine dose, preferred to stay at the edge of the apparatus.

Fluoxetine is a potent humoral regulator; its direct mechanism of action involves the inhibition of 5-HT reuptake (*Walker, 2013*), while its indirect mechanism increases the activity of serotonergic

neurons (*Czachura & Rasmussen, 2000*). Both mechanisms elevate postsynaptic serotonin levels, regulating the function of multiple brain regions (Walker, 2013) that control working memory (*Cano-Colino et al., 2014*), decision-making (Homberg, 2012), impulse control (*Winstanley et al., 2005*), and emotion processing (*Kranz et al., 2010*). Additionally, 5-HT is a potent regulator of DA, which partially performs functions similar to those of 5-HT serotonin (*Laviolette, 2007; Voon et al., 2010; Winstanley et al., 2005*).

GD patients display impairments in these functions (*Ko et al., 2017; Lin et al., 2020; Zha et al., 2022*), along with the development of an anxious and hyperactive phenotype (*Bonnaire & Baptista, 2019; Dullur et al., 2021*). This model exhibits these behavioral alterations, and like GD patients undergoing Fluoxetine treatment (Sá et al., 2023), the rats show a reduction in typical GD symptoms, including excessive video game use. Therefore, the restoration of these alterations following Fluoxetine treatment, both in GD patients and the rat model, may be the result of a combined effect: an increase in circulating 5-HT levels and the restoration of its activity (*Sá et al., 2023*). Moreover, acute administration of low doses of Fluoxetine reversed play preference in GD rats and improved their social, hyperactive, and anxious behaviors.

CONCLUSION

GD is a currently underexplored mental disorder, with existing evidence primarily based on epidemiological and neuroimaging studies of human patients. The potential use of an animal model that replicates some of the neural and behavioral changes seen in GD could further enhance our understanding of this mental disorder. By using a drug that has already been employed to validate animal models, we were able to validate this model and investigate its effects on reducing attachment to play, anxiety, hyperactivity, and the restoration of social interaction. Future use of this animal model could provide insights into the neurobiological alterations underlying the development of GD and aid in the design and testing of more targeted therapies.

CHAPTER 6

GENERAL CONCLUSIONS

GD is a mental disorder that primarily affects adolescents (*H. S. Kim et al., 2022a*). It is estimated that approximately 60 million individuals develop this disorder after prolonged and persistent exposure to video games (*M. Kim et al., 2017*). In addition to the loss of control over gaming, GD patients exhibit behavioral and emotional changes, such as hyperactivity and compulsive behavior, which are associated with depressive symptoms, increased anxiety, and social isolation (*H. S. Kim et al., 2022a*). One possible explanation for these behavioral and emotional changes is the alteration of reward circuits (*Avery et al., 2016; Wang et al., 2019*), particularly involving DA (*Ariatama et al., 2019; Wang et al., 2019*) and 5-HT (*Zoratto et al., 2017*), in response to increased stress levels (*Park et al., 2018; Paulus et al., 2018*). These hypotheses are consistent with clinical studies in GD patients, which showed changes in brain activity within reward circuits, as well as reduced levels of DA and 5-HT transporters and receptors (*Ariatama et al., 2019; Wang et al., 2019; Zoratto et al., 2017*).

These two neurotransmitters are involved in the regulation of cognitive functions and emotional states, and their reduction is associated with the development of depressive, anxious, compulsive, and hyperactive behaviors (*Dunlop & Nemeroff, 2007; Lin et al., 2014; OADES, 2008; Sinopoli et al., 2017; Voon et al., 2010*), which are common in GD patients (*Jo et al., 2019*). In fact, the use of drugs that reduce DA and 5-HT reuptake has been shown to reduce the typical symptoms of GD (*Sá et al., 2023*).

Currently, the exact causes of GD are not fully understood, and research remains limited by the minimally invasive techniques used in clinical trials. These studies have limitations that could be addressed and complemented by using animal models that replicate this mental disorder *(Marraudino et al., 2022*). The goal of this thesis was to develop a rat model that mimics the behavioral symptoms and neural activity changes seen in GD patients, to investigate the involvement of the reward system in this mental disorder, and to validate this animal model using a drug already employed in human GD treatment.

In the first experiment (**Chapter 3**) (*Casile et al., 2024*), we developed a new rat model for the study of GD. This model shows that GD rats exhibit behavioral changes similar to those seen in GD patients, revealing a high degree of face validity. Specifically, during the video game interaction session, rats exposed to a 5-week experimental protocol show increased hyperactive behaviors and a loss of control over the game, compared to control rats. Hyperactivity was expressed as increased interaction with the video game, more correct touches, and greater motor activity (distance and speed traveled). In addition to the hyperactive behavioral phenotype, GD rats also show increased attachment to the video game, accompanied by a reduction in social interaction. Like GD patients, rats exposed to this protocol show changes in the activity of several brain regions involved in reward circuits, motor learning, emotional and motivated behaviors, and decision-making circuits (*Casile et al., 2024*).

In the second experiment (**Chapter 4**), we further investigated the involvement of DA and 5-HT in GD (*Ariatama et al., 2019; Choi et al., 2020; Wang et al., 2019; Zoratto et al., 2017*) and explored the possible regulation by another neurotransmitter, ORX, which is co-involved in reward circuits, stress regulation, and emotional states (*Arias-Carrión et al., 2010; Assadi et al., 2009; James & Aston-Jones, 2022; Kranz et al., 2010*). Rats exposed to the GD protocol showed a reduction in both

the number and fibers of DA and 5-HT, and only the fibers of ORX. The CNS-level reduction in DA and 5-HT was also associated with peripheral changes in their levels. As in other mental disorders, in addition to changes in neurotransmitter levels, alterations in the Trp metabolic pathway were observed (*Adell, 2020; Comai et al., 2022*). Specifically, GD rats exhibited an increased conversion of Trp to KYN and QA at the peripheral level, which could partly explain the reduction in cell and fiber numbers and suggest a possible alteration of the glutamatergic system in this mental disorder.

In the third experiment (**Chapter 5**), we focused on validating this animal model using a drug already used in GD patients. The aim was to determine whether this model, like GD patients, exhibited a reduction in symptoms associated with the disorder in response to the administration of the SSRIfluoxetine (*Sá et al., 2023*). The rationale for using fluoxetine is twofold: first, it has been used to validate other animal models of mental disorders (*Arora et al., 2013; David et al., 2009; Lecci et al., 1990; Rodríguez-Gaztelumendi et al., 2009*); second, GD itself is characterized by emotional dysregulation, particularly increased anxiety and depression *(H. S. Kim et al., 2022b*), often accompanied by social self-isolation (*Liang et al., 2023; Sindermann et al., 2021*). In clinical trials, fluoxetine has been shown to reverse these conditions, restoring emotional regulation, increasing social interaction, and reducing gaming behaviors. This rat model, in addition to mimicking the behavioral changes typical of GD, shows a reduction in social interaction (*Sá et al., 2023*).

Although fluoxetine is widely used, doses greater than 10 mg/kg tend to reduce social interaction in rodents (*Payet et al., 2018; Sekine et al., 2007*). To address this issue, we used two lower doses of fluoxetine (2.5 and 5 mg/kg) to reduce hyperactive behaviors during play and promote social interaction. As the dose of fluoxetine increased, play-related behaviors decreased, along with the distance and speed traveled in front of the screen. GD vehicle rats, in a choice situation, showed a preference for playing over social interaction. Only the highest dose of fluoxetine reversed this preference, increasing social interaction time over play time. Furthermore, just as in GD patients, this rat model exhibited increased hyperactivity (*Dullur et al., 2021*), but only the highest dose of fluoxetine reduced hyperactivity in both the EPM and OF tests. Similar to GD patients, administration of fluoxetine in rats reduced play behaviors and restored social interaction, thereby validating the model (*Sá et al., 2023*).

In conclusion, the rat model described in this thesis represents a valid tool for the study of GD, as it partially reproduces the cerebral and behavioral changes observed in GD patients. It also reveals one possible mechanism underlying this mental disorder: the alteration of the reward circuit. This model provides a foundation for further investigation into the neurobiological changes induced by GD and offers a potential means for the development of specific treatments for this novel mental disorder.

Furure prospects

GD is an expanding phenomenon that raises significant concerns due to its individual and social implications. Despite advances in understanding the underlying biological mechanisms, many questions remain regarding the exact causes of the disorder and the most effective treatment approaches. Future research prospects on GD are multifaceted and focus on several key areas, including the development of more comprehensive animal models, the improvement of pharmacological therapies, and the exploration of targeted psychological interventions. Regarding animal models, research could evolve to include more complex variables, such as genetics and environment, using transgenic models to better understand the genetic factors that predispose some

individuals to develop the disorder. Additionally, by combining behavioral experiments with advanced brain imaging techniques, it will be possible to visualize in more detail the changes in the brains of individuals affected by GD. On a neurobiological level, it will be important to further investigate how alterations in neurotransmitters such as DA, 5-HT, and ORX influence compulsive behavior and emotional responses, aiming to identify changes in the activity of these neurotransmitters in vivo using advanced Calcium Imaging techniques during the different stages of GD model development. Furthermore, while the use of SSRIs has shown promising results, future research should focus on identifying more targeted pharmacological treatments that can specifically intervene in the altered brain circuits without causing negative side effects, such as reduced social interaction. In summary, future prospects for the study of GD are promising and are expected to provide crucial answers for the treatment and management of this emerging disorder, with the goal of developing more personalized and effective interventions, reducing the negative impact of this disorder on the lives of young people and their families. A multidisciplinary approach, combining biological, psychological, and social sciences, will be essential to successfully address the challenges posed by GD and improve patients' long-term well-being.

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