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Impact of individual differences in the development of opioid dependence

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Alla mia famiglia

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Abstract:

Opioid use disorder (OUD) is a chronic relapsing brain disease characterized by compulsion to seek and take the drug despite adverse consequences that has recently risen epidemic proportion becoming a global health problem. Opioids belong to a class of highly addictive narcotics used for pain management in a number of acute and chronic medical conditions; however their abuse leads to tolerance, dependence and risk of overdose. By binding opioid receptors in many areasof the brain involved in the reward circuitry, they are able to induce an increased release of dopamine into the Nucleus Accumbens creating positive reinforcements and pleasurable feelings.

Not all people who experienced drugs become addicted: in fact it is a pathological response generated only in a subset of individuals with a vulnerable phenotype that pre-exists the first exposure to the addictive substance. This intrinsic predisposed state derives from a combination of biological, genetic and environmental factors that, taken together, make an individual more prone than others, to precipitate into the addiction cycle.

Following the presentation of the research background in Chapter 1, in Chapter 2 my study is focused on the role of stress in facilitating heroin seeking and taking. The Marchigian Sardinian alcohol preferring (msP) rats is a validated animal model selected for excessive alcohol drinking and high sensitivity to stress due to the dysregulation of the HPA axis and an over expression of the opioid receptor system: considering their vulnerable phenotype, we set out to compare heroin taking and motivation between male and female msP rats and their non-selected Wistar counterparts. Results of the heroin/response and heroin/breakpoint curve showed that msP line consumed a higher amount of heroin and a higher motivation to work for the drug compared with controls. We had demonstrated that the msP rat line could be a valid preclinical model of stress-induced vulnerability to poly-drug abuse.

Stress, however, is only one factor that contributes to enhancing the risk to develop OUD: in Chapter 4 I extend my investigation on addiction vulnerability taking into consideration all the individual differences that could be involved in red to better characterize the heroin-addictive behavior of vulnerable and resilient phenotypes. For this purpose we selected as animal model

the NIH_ Heterogeneous stock rats, an outbred line that best mimics the individual variability of the human population. Data collected from the behavioral screening allow us to clusterize the overall population in three different phenotype (vulnerable, intermediate and resilient) and also compare their heroin predisposition with innate behavioral trait (basal locomotor activity, anxiety, pain and heroin analgesic sensitivity) in order to investigate if they could be predictive of a vulnerable or resilient phenotype.

In Chapter 3 and 5, I approached the OUD study from a pharmacological point of view:nowadays, approved OUD treatment are based on the maintenance therapy with the use of long-acting opioid agonists, like methadone and buprenorphine whose efficacy is limited by adverse side effects like abuse liability, tolerance and respiratory depressions.

I investigate the role of the MOP and NOP concomitant stimulation in reducing heron seeking and taking with a promising candidate Cebranopadol, already in clinical trials for the treatment of chronic and acute pain, that is characterized by a nano molar affinity for both these receptors. The NOP agonism is not only responsible for its reduced abuse potential but has also anxiolytic effects and made this drug particularly interesting in coping with stressful conditions, acting as a functional antagonist of the CRF1 receptor system. For these reasons, I explored the effects of Cebranopadol pretreatment in reducing heroin self-administration and motivation for heroin in male and female msP rats. From the results emerged that Cebranopadol succeeded in counteracting heroin related behaviors both in male and female, but also that gender is a factor that has to be taken into account in the valuation of the effectiveness of a therapy approach. On this point, in the last Chapter, I investigated how the pharmacological response can vary depending on the individual. For this purpose, I used part of the previously characterized HS rats that were treated with Cebranopadol to evaluate its effect on heroin self-administration and cue-induced reinstatement. Results allow us to identify a subgroup of non-responder animals, that reflect the necessity also in human society, to fine tune individualized therapy depending on the genetic traits of each patient.

Keywords: Opioid Use Disorder, alcohol-preferring rats, heroin self-administration, addiction vulnerability, stress, individual differences, clustering, Cebranopadol, NOP/opioid receptor agonist

List of papers:

1. Benvenuti F, Cannella F, Stopponi S, Soverchia L, Ubaldi M, **Lunerti V**, Vozella V, Cruz B, Roberto M and Ciccocioppo R (2021*)*. Effect of Glucocorticoid Receptor Antagonism on Alcohol Self-Administration in Genetically-Selected Marchigian Sardinian Alcohol-Preferring and Non-Preferring Wistar Rats. *International Journal of Molecular Sciences* 22(8):4184 .

2. Allen C, Kuhn BN, Cannella N, Crow AD, Roberts AT., **Lunerti V**, Ubaldi M, Hardiman Ga, Solberg Woods LC., Ciccocioppo R, Kalivas PW, Chung D (2021). Network-Based Discovery of Opioid Use Vulnerability in Rats Using the Bayesian Stochastic Block Model. *Frontiers in Psychiatry*, 12:745468.

3. Domi A, **Lunerti V**, Petrella M, Domi E, Borruto AM, Ubaldi M, Weiss F, Ciccocioppo R (2021). Genetic deletion or pharmacological blockade of nociceptin/orphanin FQ receptors in the ventral tegmental area attenuates nicotine-motivated behaviour. *British Journal of Pharmacology. (Online ahead of print).*

4. **Lunerti V**, Li H, Benvenuti F, Shen Q, Domi A, Soverchia L, Di Martino MRC,Bottegoni, Haass-Koffler CL, Cannella N (2021). The multitarget FAAH inhibitor/D3 partial agonist ARN15381 decreases nicotine self-administration in male rats. *The European Journal of Pharmacology*. (*Manuscript under revision*).

5. Kuhn BN, Cannella N, Crow AD, Roberts AT, **Lunerti V** , Allen C, Nall RW, Hardiman G, Solberg-Woods LC, Chung D, Ciccocioppo R, and Kalivas PW (2022). Novelty-induced locomotor behavior predicts heroin addiction vulnerability in male, but not female, rats. *Psychopharmacology*. (*Submitted under revision*)

Chapter 1:

GENERAL INTRODUCTION

1. Drug addiction

Drug addiction, also known as substance use disorder (SUD), is considered a complex chronic brain disorder and a mental illness, characterized by three major focal points: (1) compulsive drug seeking and craving that persist despite all adverse consequences, (2) loss of control in limiting drug intake and (3) emergence of a negative emotional state , (like dysphoria, anxiety and irritability) that reflects a motivational withdrawal syndrome, when the drug is not available (Koob GF and Le Moal M, 1997; Koob GF and Volkow ND, 2010). Substances such as tobacco, cannabis, hallucinogens, inhalants, opioids, sedatives, hypnotics, anxiolytics, stimulants, and alcohol have a high probability of leading to addiction (American Psychiatric Association 2013).

However, the recreational and occasional use of a rewarding and abusable substance have not to be compared with its repeated and chronic use that characterize addiction in which drug procurement and administration start to rule the subject's behavior and motivational status, disrupting decision-making ability at expense of engaging in behaviors to seek natural rewards (like sex or also social relationship). The replacement of natural reward with drug reward underlies that the neuropathology of addiction could involve the same neural systems that mediate the detection and acquisition of natural rewards (Kalivas PW, 2002). But, most drug users do not become abusers or drug-dependent: this pathological behavior appears only in a small minority of people who experienced drugs and only in those ones it takes on the characteristics of a chronic disease. There are many different factors that could make an individual more vulnerable to develop an addicted phenotype like genetics, history of drug use, stress, certain mental illness, social environment and life events.Koob GF and Volkow ND (2010) identify a complex addiction cycle composed of three recurring stages that interact with each other (**Figure 1**): *binge* and *intoxication* , *withdrawal* and *negative affect*, *preoccupation* and *anticipation* (also known as *craving*). Each one of these stages leads to allostatic changes and neuroadaptations in three key neurobiological circuits that, at the end, lead to the pathological and compulsive state of addiction: basal ganglia, extended amygdala and prefrontal cortex, respectively (Koob GF and Volkow ND,2010; Volkow ND et al, 2016) .

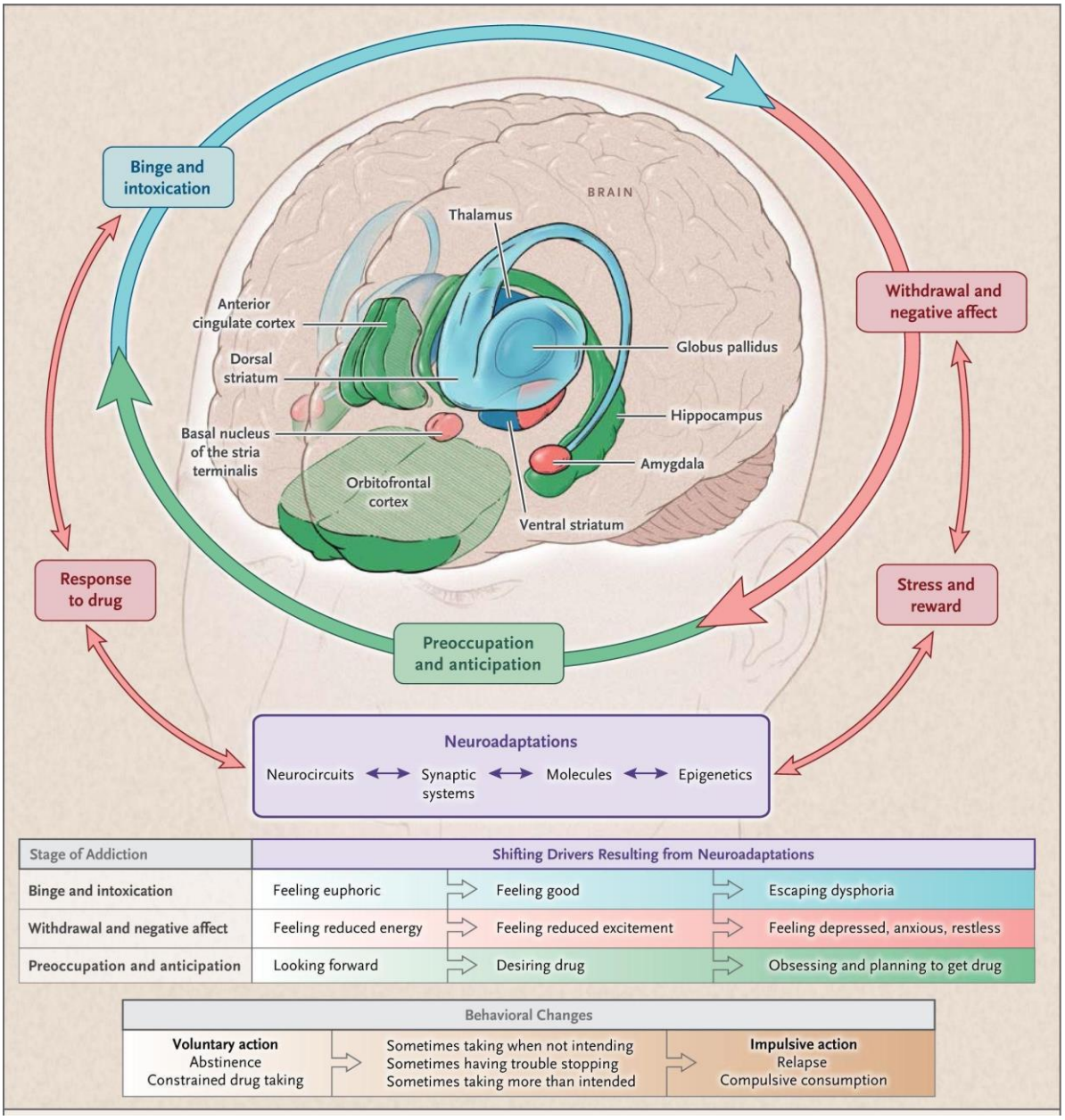

Figure 1. Adapted from Volkow et al (2016) and Wise RA et al (2014).

Diagram shows the three recurring stages of the addiction cycle color coded with the corresponding neural circuits involved in each stage: the binge/intoxication phase (blue), the withdrawal/ negative affect phase (red), the preoccupation and anticipation or craving stage (green)

The binge/intoxication phase is characterized by a high release of dopamine in the basal ganglia (in particular in the ventral striatum) suggesting that this increase could be related with the hedonic value of the addictive drug (that acts as positive reinforcer). According with the Pavlovian learning theory, the repeated exposure to the rewarding effects of the drug become associated with the environmental stimuli paired with them (called “cues”): As a consequence, the dopamine neurons stop to fire in response to the drug itself and instead start to respond to the earliest cue that predicts the future reward.

The withdrawal/ negative affect phase refers to many neuroadaptations that occur in the circuitry of extended amygdala (that encompasses central nucleus of amygdala, CeA, the bed nucleus of stria terminalis , BNST, and a transition area in the shell of nucleus accumbens) and cause a person's reactivity to stress and negative emotions, like chronic irritability, emotional pain, malaise and dysphoria. The compromised brain reward functions, caused by prolonged and repeated drug exposure, make the “anti-reward” system overactive when drug is withdrawn and decrease reactivity of dopamine cells in the brain's reward regions. So, people continue to take drugs in order to escape from this distress and alleviate the aversive condition associated with abstinence (negative reinforcement) falling in a vicious cycle in which the hedonic homeostasis of the reward system is completely dysregulated and far from the normality. A new, pathological, set-point is already established . This “allostatic state” has been defined as a chronic deviation of the regulatory system from the normal state of operation, in which the decreased activity of brain reward system is counterbalanced with the recruitment of the brain anti-reward or stress system, both of which lead to the compulsivity of drug seeking and taking (Koob GF, 2008, Koob GF and Le Moal M, 2001): the organism produces tonic adaptations at all levels of organization and the “price” that the body have to pay for being forced to adapt to a chronic adverse or deleterious situation was called “allostatic load” and “reflects the accumulation of damage that can lead to pathological states" (Koob GF, 2015).

In other words, drugs progressively shift from being strongly wanted to strongly needed (Piazza PV et al, 2013)

The preoccupation and anticipation or craving stage is a consequence of the dysfunction of the reward circuitry that extends into the prefrontal cortex causing cognitive and executive

impairment, affecting also decision making, self-regulation, error detection, working memory and attention. The learned association of drug relief from an aversive mental state and negative affects, either pre-existing or created by withdrawal, drives craving and could be considered a sort of disease prime. In this point of view, addiction could be considered as an associative learning disorder, with parallels to the post traumatic stress disorder (PTSD) (**Figure 2**, Evans CJ., Cahill CM., 2016)

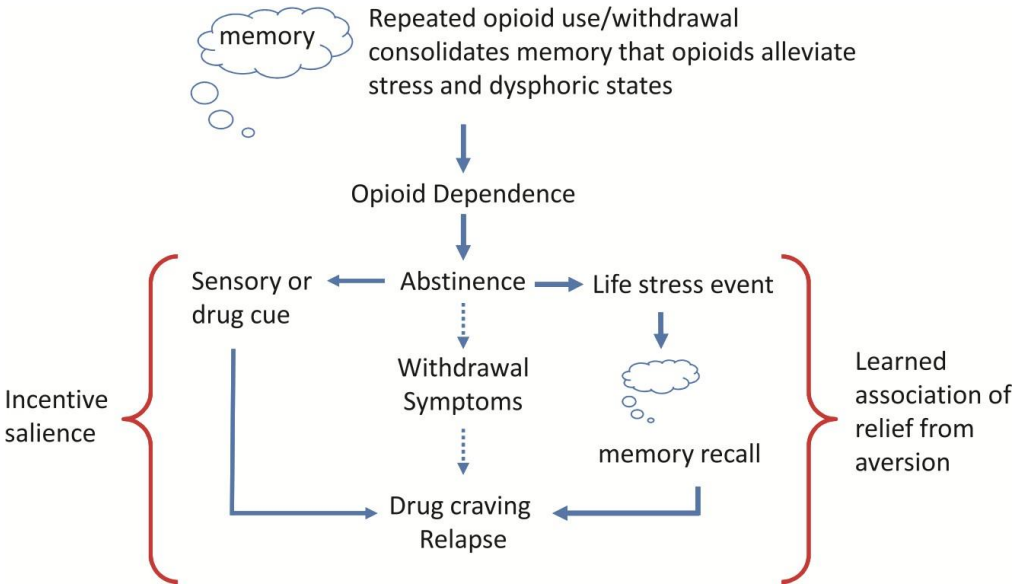

Figure 2. Adapted from Evan JC., Cahill MC (2016)

The initial stage is based on the positive reinforcement of euphoria and positive mood, promoting further drug use. However, the motivation changes with repeated use, where positive reinforcing effects of the drug wane in comparison to the drive to alleviate withdrawal effects (negative reinforcement). With repeated use, drug dependence develops and the learned association with relief of the aversive withdrawal state is reinforced. Following abstinence, the risk of relapse can be driven by three paths. The first is by direct negative reinforcement and relief of withdrawal. The second path would be sensory or drug cues (e.g., drug paraphernalia, familiarity of location to previous drug use, scent, etc.) and drug access (left side of figure), where incentive salience drives craving and loss of inhibitory control drive relapse. The other

(right side of figure) is the trigger of life stress events that recall the memory of learned association between drug taking and aversion relief.

When we talk about the "reward circuitry" (**Figure 3**), we are referring specifically to the mesocorticolimbic dopamine pathway, which project from the ventral segmental area (VTA) to the limbic subcortical areas, that are the nucleus accumbens (NaC), olfactory tubercle and amygdala, and to the frontal cortex, each of them implicated in the acute reinforcing actions of both natural rewards (food, drink and sex) and addictive drugs.

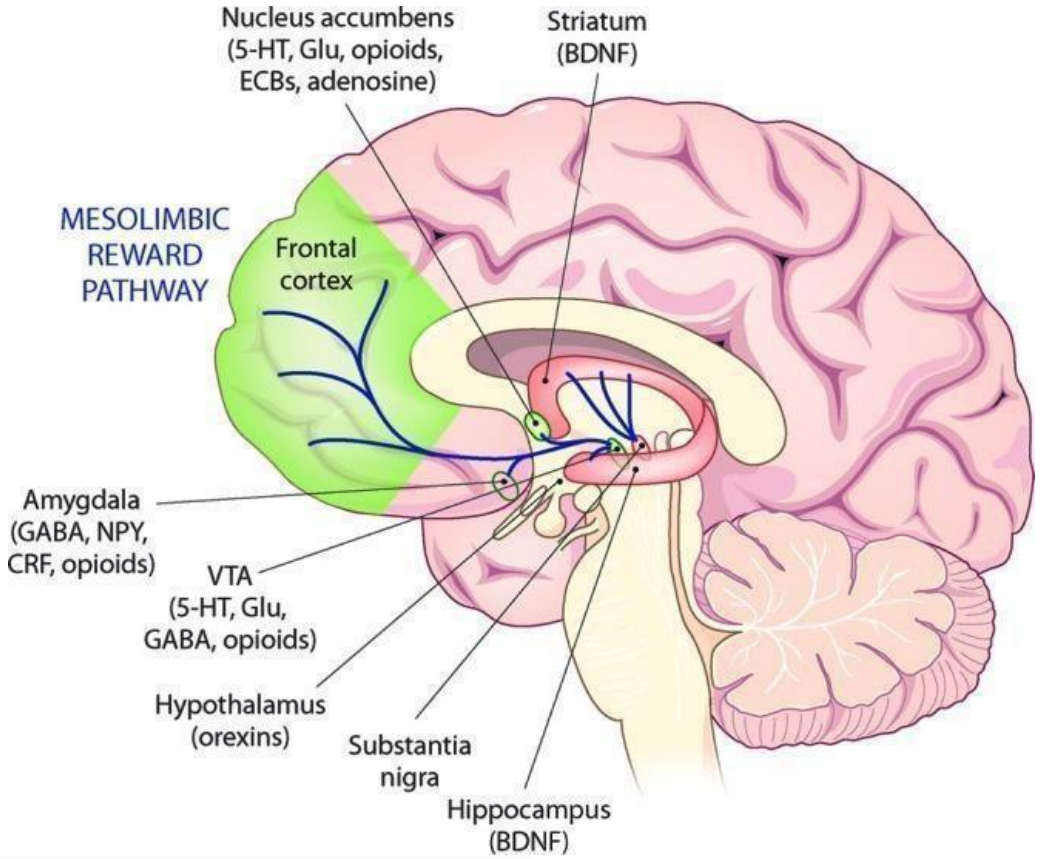

Figure 3. Adapted from Gass JT et al, 2012.

Schematic of the mesolimbic reward pathway in the human brain. The reinforcing effects of an addictive drug are believed to be mediated by the activation of dopamine-containing cell bodies in the ventral tegmental area (VTA) that project rostrally to the nucleus accumbens and frontal cortex. This activation occurs primarily at the level of the VTA, with an increased release of endogenous opioid peptides which hyperpolarize local inhibitory GABAergic neurons, thereby

disinhibiting mesolimbic dopamine neurons. In addition, the drug promotes the release of glutamate in the VTA and also affects various ionic currents intrinsic to VTA dopamine neurons that regulate the excitability of these cells. Abbreviations: Glu, glutamate; 5-HT, 5-hydroxytrypamine (serotonin), ECBs, endocannabinoids; NPY, neuropeptide Y; CRF, corticotropin releasing factor, BDNF, brain-derived neurotrophic factor.

It is well known that all drugs of abuse can initially elicit the supra-physiological release of dopamine in the NaC and can eventually trigger the neuroadaptation that leads to tolerance and withdrawal.

In fact, with repeated administrations, the occurring neuroadaptations in the mesocorticolimbic system, reduce the dopamine firing in response to the drug itself, while firing in an anticipatory response to the cues paired with the drug (Schulteis and Koob, 1996), so that drug cues acquire incentive salience with the development of the addiction. But when the drug use become almost chronic, the drop of the dopamine response could be read as a symptom of an homeostatic adaptation called tolerance: in other words, addicted people no longer experience the same degree of euphoria as when they started to take it and need to increase the dose to re-experience the initial intensity of the effect. In addition to tolerance, these homeostatic neuroadaptations can also lead to the development of dependence: Dependence is defined as " a state produced by the repeated administration of a drug, which necessitates its continued administration in order to prevent the rising of a withdrawal syndrome" (Jaffe J.H. 1990).

Another important pathophysiological mechanism largely involved in transition to the compulsive drug use is the interaction between the dopaminergic system and the recruitment of the stress system mediated by corticotropin-releasing factor (CRF) and the consequent dysregulation of the hypothalamic-pituitary-adrenal(HPA) axis. The glucocorticoid tone is one of the most crucial regulators of the dopaminergic transmission activity in the NaC (Deroche et al, 1997). Compulsive-like drug taking increases CRF levels in the amygdala , prefrontal cortex and VTA , contributing to stress-like response and negative emotional state providing the driving

force for sustaining compulsivity in taking drugs via negative reinforcement (Uhl GR and al, 2019). Acute withdrawal from all drugs of abuse produces an anxiety-like state that can be reversed by CRF antagonists, and CRF antagonists also block the increased intake of drugs associated with dependence (Koob GF and Le Moal M, 2008). Thus, CRF activation may be a common element in the development of drug dependence and may contribute to subjective symptoms like increased stress and negative affect. (Koob GF, 1996).

Increased stress-sensitivity and anxiety-like behavior have been shown to persist many weeks into abstinence in animals with a history of dependence, resulting in a negative emotional state, also called protracted abstinence. This negative feeling associated with a residual deficit in the reward system or sensitization of the reward system to stimuli that predict drug effects, that persist even months or years after detoxification, could be responsible to the vulnerability to reinstatement of drug-taking behavior (Koob GF, Le Moal M,1997). Much of the

Factors that could trigger relapse and craving include: acute re-exposure to the drug or drug-priming, exposure to environmental stimuli previously paired with drug use or conditioned drug cues, and exposure to environmental stressor (Camì J, Farré M,2003). In contrast to the acute stimulation of dopamine release produced by drug use, the enduring vulnerability to relapse arise from long-lasting neuroadaptations in the corticostriatal glutamatergic circuitry (in particular the efferent from prefrontal cortex to the nucleus accumbens) due to an imbalance of the glutamate homeostasis. The impairment is associated with several drug-induced changes in pre- frontal glutamatergic synapses in the NAc that have the potential to reduce effective regulation of drug seeking (Kalivas PW, 2009).

The reinstatement model of relapse has been developed in order to mimic human craving in experimental animals: animals were trained to self-administer drugs and then, they are subsequently put on extinction training to reduce responding. When levels of responding are minimal, animals are presented with a stimulus that causes a “reinstatement” of the drug contingent response. This model, for example, made possible to discover that infusion of AMPA agonists into NaC core causes reinstatement whereas antagonism of AMPA receptors was able to prevent cocaine cue and primed induced reinstatement (Knacksted LA, Kalivas PW, 2009).

2. Opioid pharmacology and dependence

"Among the remedies which Almighty God has pleased to give to man to relieve his sufferings, none is so universal and so efficacious as opium", as the famous 17th-century physician Thomas Sydenham put it. For more than 4000 years in medicinal opium has been used to blunt feelings of pain and today, physicians write more than 200 million prescriptions for opioid painkillers in the USA each year.

Opioids belong to a class of highly addictive narcotics used for pain management in a number of acute and chronic medical conditions including chronic non-cancer pain, anesthesia, post-surgical care and musculoskeletal pain; however their abuse often leads to tolerance, dependence and overdose. In fact, as good as the other drugs used to relieve pain, opioids also arouse the reward network in the human brain, eliciting strong feelings of euphoria that can drive addiction-like behavior and craving.

Opium is extracted from the seeds of poppies plant, the *Papaver somniferum,* and its most active ingredient, the morphine, was first isolated in 1805 and remains, until now, the most potent painkiller in modern medicine, despite severe adverse consequences and a strong abuse liability. (Darcb E, Kieffer BL, 2018).

Opioid use disorder (OUD) is recognized as a chronic relapsing disorder with serious potential consequences including disability, relapses, and death. The Diagnostic and Statistical Manual of Mental Disorders, 5th Edition describes opioid use disorder as a problematic pattern of opioid use leading to problems or distress, with at least two of the following occurring within a12-month period:

1. Taking larger amounts or taking drugs over a longer period than intended.
2. Persistent desire or unsuccessful efforts to cut down or control opioid use.
3. Spending a great deal of time obtaining or using the opioid or recovering from its effects.
4. Craving, or a strong desire or urge to use opioids
5. Problems fulfilling obligations at work, school or home.

6. Continued opioid use despite having recurring social or interpersonal problems.
7. Giving up or reducing activities because of opioid use.
8. Using opioids in physically hazardous situations.
9. Continued opioid use despite ongoing physical or psychological problems likely to have been caused or worsened by opioids.
10. Tolerance (i.e., need for increased amounts or diminished effect with continued use of the same amount)
11. Experiencing withdrawal (opioid withdrawal syndrome) or taking opioids (or a closely related substance) to relieve or avoid withdrawal symptoms (American Psychiatric association)

Prescription pain relievers include oxycodone (OxyContin®) hydrocodone (Vicodin®), codeine, morphine, and others. Synthetic opioids include fentanyl, methadone, pethidine, tramadol and carfentanil. (American Psychiatric association)

Heroin, the diacetylated form of morphine, was originally marketed as over-the-counter cough suppressant in 1898 by Bayer. However, contrary to Bayer's advertising as a "non-addictive morphine substitute", very soon heroin showed one of the highest rates of addiction liability among the users.

Opioids are characterized by their ability to bind the opioid receptors μ (mu or MOR), δ (delta o DOR), 1 (kappa o KOR), that belong to the family of 7-transmembrane domain G protein-coupled receptors (GPCR) and they are widespread in both central nervous system(CNS) and peripheral nervous system. Their distribution into the brain depends on their classification, but all receptors are found in high concentration in the amygdala, NAc, and the caudate putamen (CP). These areas, like also the ventral tegmental area (VTA), contain gamma-aminobutyric acid(GABA)-interneurons and dopaminergic neurons that are an active part of the reward circuitry of the dependence (Darcq E., Kieffer BL, 2018; Kalivas PW, Volkow

ND, 2005).All three types of receptor are involved in this process, although with very different contributions. MORs mediate the pleasurable properties of therapeutic or abused opiates and has an essential role in mediating natural rewards and it is necessary for the rewarding effects of other drugs of abuse (like alcohol, THC and nicotine). In contrast to MOR agonists, KOR agonists are strongly aversive inducing an acute dysphoric state and negative mood. Infact KOR and dynorphin, its preferred endogenous ligand, form an "anti-reward system" that is highly reactive to stress and cause the inhibition of dopamine release in the NaC: considered together, MOR and KOR oppositely regulate hedonic homeostasis. By contrast DOR activity reduces anxiety and depressive states and regulate learning and memory (Darcq E, Kieffer BL, 2018; **Figure 4**)

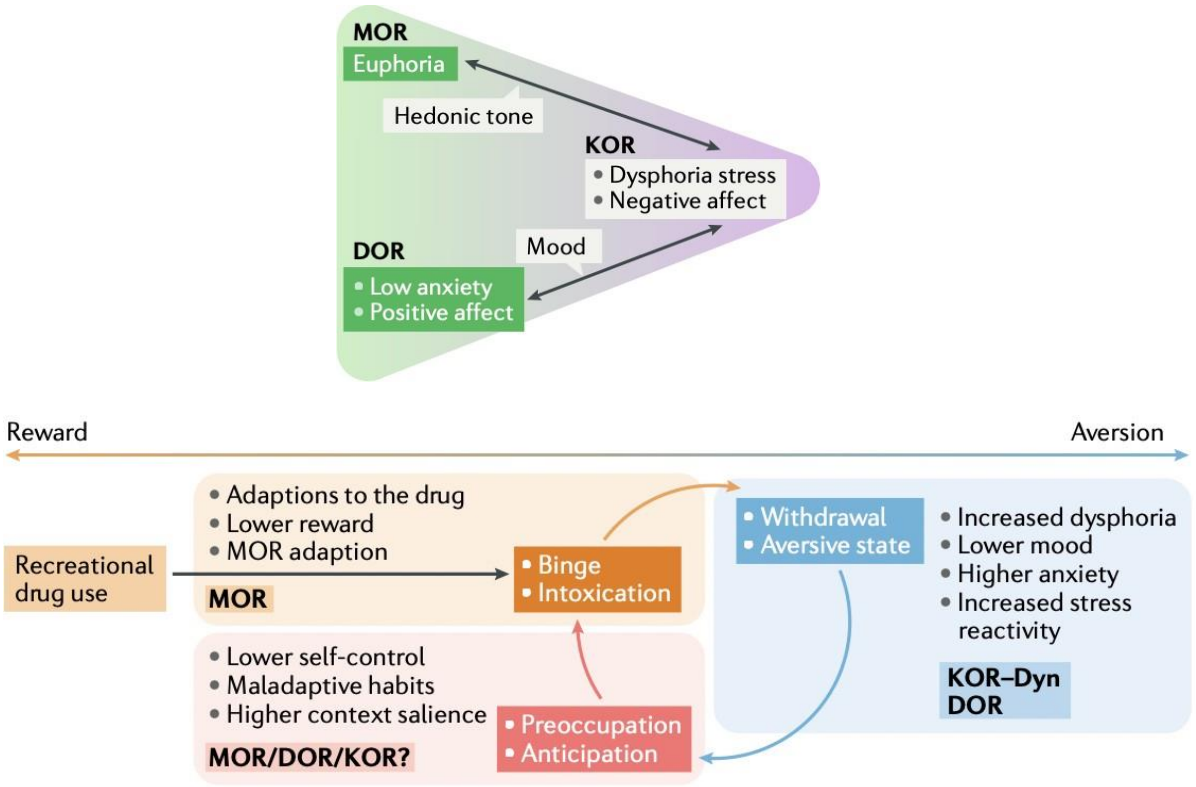

Figure 4. Adapted from Darcq E., Kieffer BL (2018)

Opioid receptors differently contribute to regular reward and aversion: physiologically they promote reward elicited by natural stimuli (food, sex and social interaction), regulate mood states, facilitate efficient coping with pain and stress. Animal study suggested that MOR drives

rewarding properties of opioids drugs and repeated activation of this receptor leads to tolerance and compensatory adaptations (dependence and withdrawal symptoms). KOR activation is important in negative affect that characterize withdrawal whereas DOR should limit the development of the aversive state.

The cell bodies of dopamine neurons that project into the NAc originate in the ventral tegmental area. When an opioid agonist binds the mu-presynaptic receptor on the GABA interneurons into the reward pathway, it causes an increase in K conductance that hyperpolarizes the membrane and decreases GABA secretion with inhibition of their tonic firing. This event allows dopaminergic neurons to release more dopamine into the Nucleus Accumbens creating positive reinforcements and pleasurable feelings (Johnson SW, North RA, 1992; Kalivas PW et al, 2009; **Figure 5**).

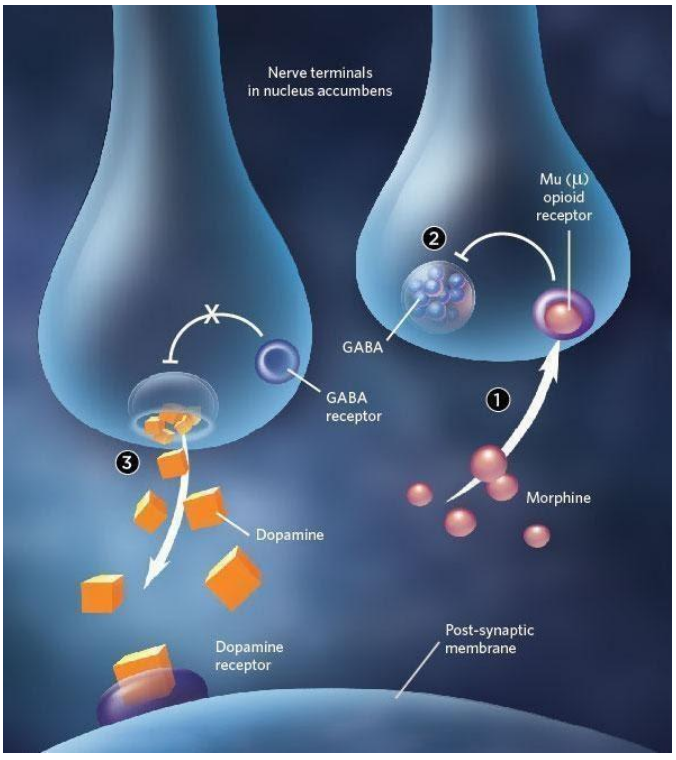

Figure 5. Adapted from Science Magazine (2014).

Opioid binding to opioid receptors in the VTA that caused an increase of dopamine release into the reward pathway by hyper-polarization of GABA interneurons.

Opioid receptors are normally stimulated by endogenous peptides, including enkephalins, β-endorphines and dynorphines, produced for example, in response to noxious stimulation.

Following repeated therapeutic doses of morphine or its surrogates, a gradual loss of effectiveness occurs and to contrast this tolerance a larger dose of drug must to be administered in order to achieve the same analgesic effect: clinically, more than 10-fold dose escalations of opioid dose in chronic pain management are common (Morgan MM, Christie MJ,2011).

Neurobiological mechanisms of tolerance range from opioid receptor desensitization and downregulation to cellular and circuitry allostasis. Physical dependence develops along with tolerance. Physical dependence is defined as a characteristic withdrawal or abstinence syndrome that appears when the drug is not on board or when an opioid antagonist is administered (Katzung GB et al, 2012). Some withdrawal symptoms, like sweating, shaking and diarrhea, resolve within days, while others, like dysphoria, insomnia and anxiety, can linger for a long time, and some adaptations, such as learned associations, may be established for life. It is not difficult to envisage that after time a learned association of opioid drug taking with the relief of dysphoric states becomes engraved into brain motivational memory circuitry. Thus, when the addict experiences a future life event (maybe after years of abstinence) inducing negative affect (e.g., sadness, disappointment, failure, and apathy), the learned association of drug taking with relief of similar symptoms (albeit created by drug withdrawal) will trigger drug craving and relapse (Evans CJ et al, 2016).

2.1 Heroin

Heroin is an illegal, highly addictive semisynthetic drug processed from morphine: it was first marked in 1898 as diacetylated form of morphine (**Figure 6**) by Bayer Pharmaceutical as an over-the- counter cough suppressant under the trademark name of “heroin”.

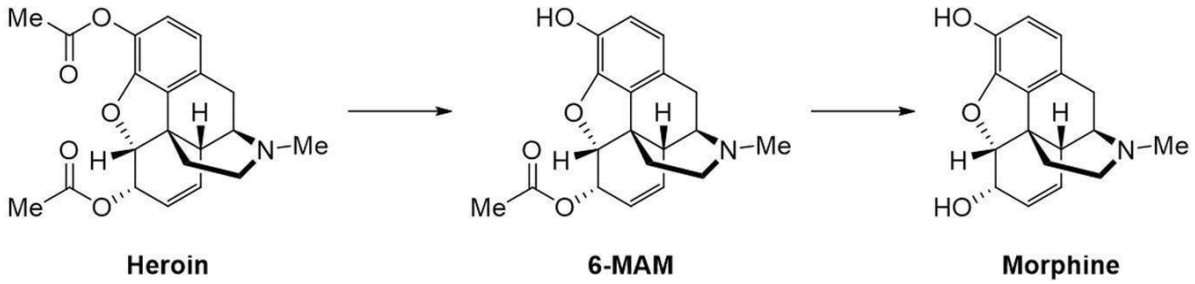

Figure 6. Adapted from Zhang T, et al, 2018

The metabolic pathway of heroin to morphine: once administered, heroin was rapidly metabolized by cholinesterases, which hidrolize the acetyl groups, to 6-monoacetylmorphine (6-MAM) first, and then to morphine. Both 6-MAM and morphine are responsible for physiological and also, toxic, effects of the heroin. In fact, heroin is at least 10-fold more toxic than morphine but the binding affinity of heroin itself with the mu receptors is significantly lower than that of morphine. The most toxic metabolite is 6-MAM which has the highest binding affinity with the μ-opiate receptors. 6-MAM (with a relatively shorter biological half-life compared to morphine) is mainly responsible for the acute toxicity, whereas morphine (with a much longer biological half-life) is mainly responsible for the long-term toxicity of heroin.

Bayer's initial idea was to get a more active drug of codeine to improve respiratory function and to calm cough; in fact in those years pulmonary tuberculosis was a widespread disease and this worldly problem made heroin very profitable on the market for pharmaceutical industries. Between 1899 and 1905 about 180 clinical papers on heroin were published around the world.

However, contrary to Bayer's advertisement as a " non-addictive morphine substitute", very soon heroin showed one of the highest rates of addiction among the users. It was soon noticed that heroin was a pro-drug quickly metabolized to morphine and, consequently, shared with this one the same risk of addiction, depression of the central nervous system, respiratory depression and also overdose and death (Kanouse AB et al, 2015).

Heroin is highly lipophilic and, therefore, rapidly crosses the blood-brain barrier. All routes of administration lead to the rapid absorption of heroin. Peak serum levels in each of these routes are five to ten minutes subcutaneously, three to five minutes intranasally and intramuscularly, and less than one minute intravenously: once in the serum, heroin reaches the brain in 15 to 20 seconds.

Heroin acts as agonist in the CNS binding all the opioid receptor subtypes **μ**, ð, ι:

- μ1-receptor effects account for both the analgesic effects while μ2-receptors are responsible for respiratory depression, delayed gastrointestinal motility, miosis, euphoria and physical dependence;

- ț-receptor activation causes some degree of analgesia and play part in producing miosis, respiratory depression and dysphoria;

- δ-receptors are more involved in spinal analgesia phenomena, but are also found in cortical regions.

Nowadays heroin has no FDA-approved indications for medical use whereas in the United Kingdom the use of heroin, known under the generic name of diamorphine, was allowed for diverse analgesic indications such as postoperative pain, chronic pain, palliative care, and even post-cesarean section (Huecker MR et al, 2017).

2.2 The opioid epidemic in USA

In November 17, 2021 CNN released a dreadful article entitled : “Drug overdose deaths top 100.000 annually for the first time, driven by fentanyl, CDC data show” (**Figure 7**).

Opioids continue to be the driving cause of drug overdose deaths. Synthetic opioids, primarily fentanyl, caused nearly two-thirds (64%) of all drug overdose deaths in the 12-month period ending April 2021, 49% more with respect to the previous year, according to CDC's National Center for Health Statistics.

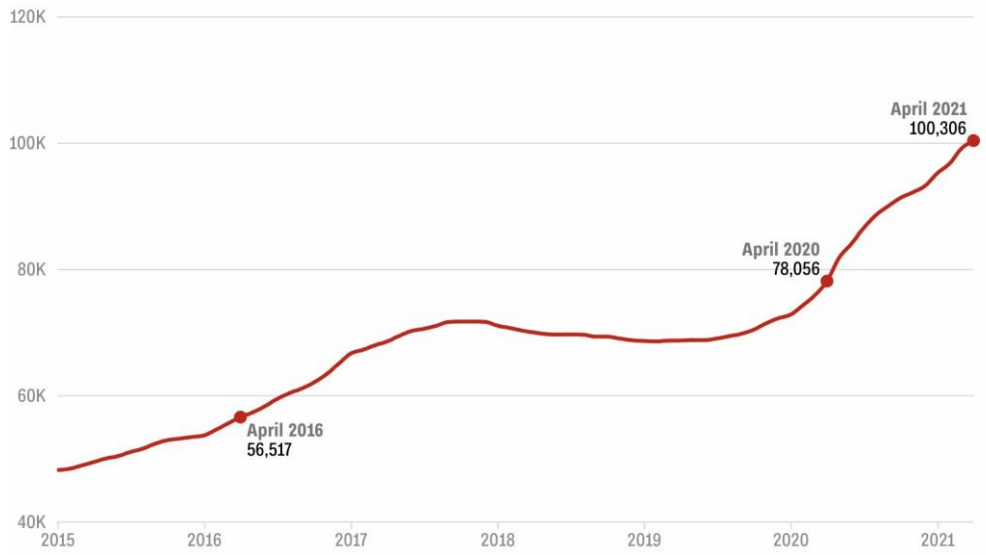

Figure 7. Adapted from National Center for Health and Statistics (2021)

Drug overdose deaths soared during the pandemic: more than 100.000 people died from overdose in the USA between April 2020 and April 2021, up from 78.000 the years before and nearly double the deaths five years ago.

This dates back about 20 years. Over the course of these two decades, the US spread a severe opioid epidemic. This record was exacerbated by the Covid 19 pandemics in the latest years.

Which are the roots of the dramatic resurgence of this serious public health concern?

In 1992 the Agency of Healthcare Quality Research asserted that more than half of patients do not receive adequate post-surgical analgesia, depriving them of a fundamental right to have pain

management, and that the fears of the abuse liability of opioid, if used under correct conditions, are largely unfounded (Kanouse AB et al, 2015). So, few years later, the American Pain Society instituted the "pain as the fifth vital sign" in the effort to improve pain medical care. A consequence of this approach was the liberalization of opioid prescription as pain relievers (OPRs) for chronic, non cancer pain. This was the first act that contributed to the increase in opioid misuse to the point that, recently, the American Medical Association (AMA) recommended removing pain as the fifth vital sign in professional medical standards (Anson P, 2016).

Another point to consider is that, as the prediction opioids increased, they were perceived by abusers to be safer due to they legality and readily apparent brand and dose specificity, which could help to avoid overdoses (Cicero TJ et al, 2017)

Also considering the lack of effective analgesic to treat chronic nonmalignant pain, clinicians began prescribing opioids (POs) like never before: between 1998 and 2007 the number of prescription written for hydrocodone increased by 198%, 588% for oxycodone and 933% for methadone. For example, in those years, Vicodin (Hydrocodone with acetaminophen) became the most commonly prescribed pain medicine in the US.

Another factor that could be considered a potential contribute to this upsurge in opioid abuse is the increasing access to opioid, not only for the great number of prescriptions written in US each year, but also the online pharmacies that have opened a new and very available access to these drugs: anyone with a credit card can get access to prescription opioids and that these drugs may be taken without the supervision of a physician. It is also recognized that some of the opioid analgesics available over the internet are manufactured by sites that do not have proper quality controls (Compton Wl et al, 2006)

Not absolved from responsibility is the aggressive marketing by the pharmaceutical industries and opioid manufacturers that work to minimize the risks of OPRs, exaggerating the benefits of long-term OPR use. In fact, high-quality, long-term clinical trials demonstrating the safety and

efficacy of OPRs for chronic non-cancer pain have never been conducted. (Kolodny A. et al, 2015)

The most famous is the case of Purdue Pharma and its new Oxycontin.

Purdue Pharma promoted its new sustained-release formulation of oxycodone (Oxycontin) with the indication that the delayed absorption is believed to reduce the abuse liability of the drug. However things did not go exactly in this way: within 5 years prescription for Oxycontin to treat non cancer pain increased about 10 fold but this new delayed formulation could be crushed and consequently snorted or injected. It means that Oxycontin became also the most misused and abused PO (Skolnick P, 2018).

In an effort to deter abuse via injection and snorting, Purdue Pharma presented a tamper-resistant new formulation of Oxycontin that prevents the tablet from being chewed, crushed, smoked or injected and it was approved by the US Food and Drug Administration (FDA).

Even this strategy was doomed to fail because the consequence was a resurgence in the abuse of less expensive and more available alternatives like heroin or other illicit synthetics like fentanyl.

According to the federal government's national Survey on Drug Use and Health, 4 of 5 current heroin users report that they have first been addicted to POs before heroin initiation and then, switch to heroin that is cheaper and easy available on the black marked (much of it come from Mexico) (Kolodny A. et al, 2015). As users switched from high-quality pharmaceuticals to street drugs of unreliable composition and quality, deaths mounted. Mortality rates surged further as potent illicit synthetic drugs - such as fentanyl - were mixed in with heroin (Burke DS, 2016) due also to the imprecision in estimating a correct dose, its purity and the potential adulterant (Cicero TJ et al, 2017).The opioid epidemic is not only a health crisis for the US, but it has also a tremendous economic and social costs to OUD: heroin users are less productive due to their premature death or drug-related hospitalizations, absenteeism and unemployment. High rates of criminal activity and incarceration among heroin users exacerbate the economic burden as well as the productivity loss during incarceration. Additionally, heroin use, in particular via injection, is associated with several chronic infection disease like Hepatitis C (HCV), B (HBV) HIV-AIDS and tuberculosis (TB) (**Figure 8** Jiang R et al, 2017) and also an increase in the number of

infants born dependent on opioids as result of mother's opioid use (also called neonatal abstinence syndrome)

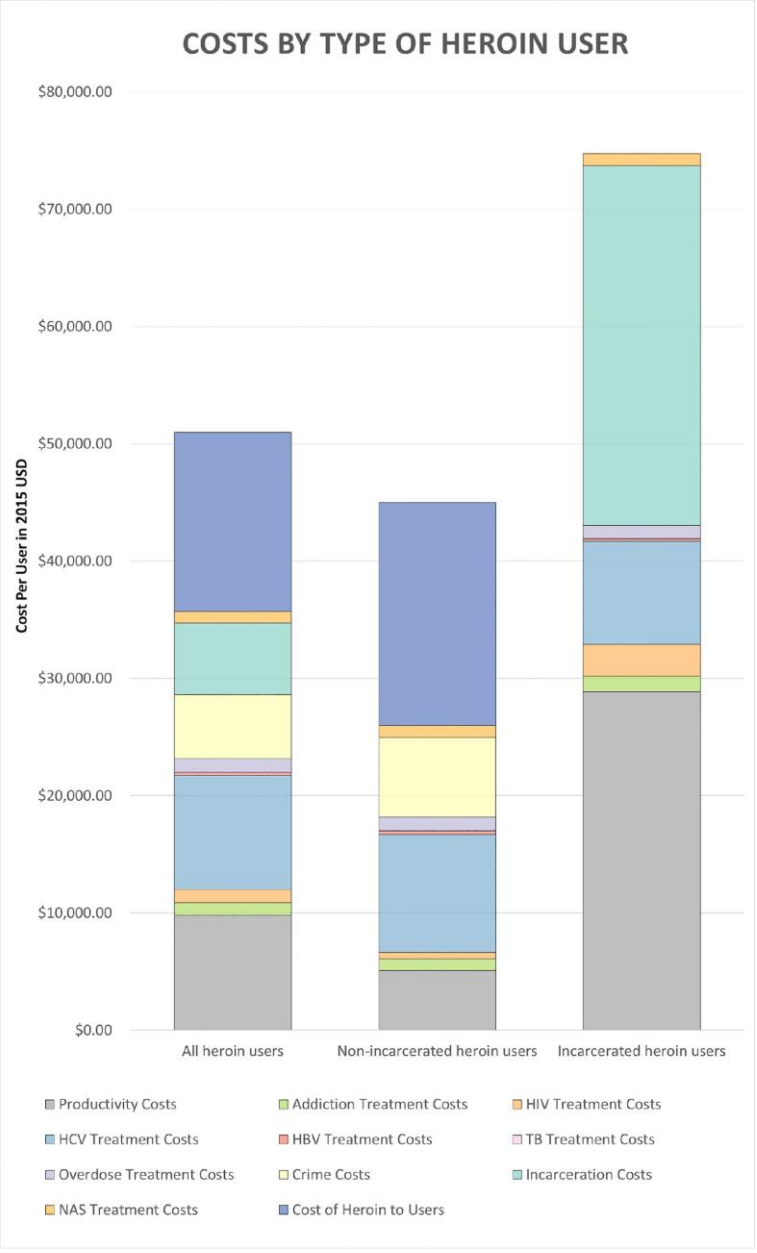

Figure 8. Adapted from Jiang R et al, 2017

The estimated total cost of heroin use disorder by type of heroin user (dividing non- incarcerated people from the incarcerated ones). This study reported that the estimated total cost in the United States in 2015 was 51,2 billion dollars for 1 million heroin addicts, with an average of

50.799 per heroin user, that is very much higher compared with the annual societal costs of some other chronic illness like chronic obstructive pulmonary disease (2.567 dollars per patient) or diabetes (11.148 dollars per patient).

Nowadays, the COVID-19 pandemic is having a dramatic impact on addicted patients. There is strong evidence that chronic opioid use, like many other substances of abuse, have long been linked with immunosuppressive effects resulting in greater susceptibility to infection (Baillargeon G, et al 2021). People with OUD may also be at increased risk for the most adverse consequences due to the coronavirus infection and, on the other hand, the respiratory functions compromised by COVID-19 could increase risk of fatal overdose death in people with OUD (Volkow ND, 2020).

2.3 Opioid Use Disorder Treatment

Effective treatment for OUD has been identified as a national priority to reduce the rates and societal costs of individual disability associated with the dependence, the infectious disease associated with intravenous opioid use and the escalating number of overdose deaths (Connery HS, 2015).

Nowadays, medications are the gold standard to treat opioid use disorder; FDA had approved three medication for preventing opioid relapsed for stabilization/ maintenance treatment of OUD, all of which target the μ opioid receptor (**Figure 9**):

- Methadone and buprenorphine have agonist effects, affecting craving and withdrawal symptoms but avoiding euphoria caused by heroin, they are used for long-term maintenance therapy;
- Naltrexone is a μ receptor antagonist that prevents the possibility of an illicit opioid to take its effect directly blocking the receptor site.

In the USA, medications are required to be given in conjunction with some form of counseling or behavioral therapy, called medication-assisted treatment (MAT) considering also that, often heroin abusers have some psychiatric comorbidity, which play an important role in precipitating their deteriorated physical and mental state. Nevertheless, as Nora Volkow want to underlie on The Lancet (January 2018), many addicted people had no real access to these treatments because of the popular stigma against the therapy for OUD arising from the belief that these medication simply replace one addiction with another : the scientist put in evidence that when an opioid user receives methadone or buprenorphine , the doses used are not able to produce euphoria or craving, but reduce only withdrawal syndrome, improve mood and restore the physiological balance (Volkow ND, 2018). On the contrary, even if drug use continues to be penalized, the punishment does not ameliorate SUD or related problems, rather imprisonment actually leads to much higher risk of drug overdoses upon release: in fact relapse, in case of an untreated OUD, can be fatal due to the loss of opioid tolerance that may be occurred while the person was incarcerated (Volkow ND, 2021).

In fact , there is strong evidence that the correct use of medications markedly improves outcomes, facilitates recovery, protects against overdoses and reduces risks of other infections and criminal behaviors.

Methadone, the first medication developed for OUD, is less expensive than the other medications and it is the most frequently prescribed globally since the 1950s. It is a *full μ opioid receptor agonist* and NMDA receptor antagonist, that is effective when administered orally as a liquid or pills, once a day. It has an onset of action within 30 minutes and an average duration of action of 24 to 36 hours. Due to its long half-life and its tendency to accumulate with repeated doses, initiation of treatment begins with low doses and escalates slowly. A Sufficiently high dose of methadone produces greater reductions in use of heroin and other non-prescribed opioids. It has to be administered under direct daily supervision in licensed outpatient treatment programs, separate from the regular healthcare system, and not by office-based clinicians(Strong J et al, 2020; Krantz MJ and Mehler PS, 2004; Blanco C and Volkow ND, 2019). Methadone

discontinuation requires careful tapering to avoid severe withdrawal associated with the abrupt termination.

Buprenorphine was approved for opioid treatment in the USA in 2002: In this country, differently from methadone, it can be prescribed by a qualified physician in an office-based setting instead of only being dispensed in federally authorized opioid- maintenance clinics (Ling W et al, 2012). Buprenorphine is a *partial μ opioid receptor agonist* and *antagonist of the k receptor:* as a partial agonist, it has a "ceiling effect", that is, after a certain point taking more will not increase any of the effects of the drug reducing the potential for overdose and conferring low toxicity also at high doses. In addition, buprenorphine has a high affinity for the μ receptor, which means that it reduces the effects of additional opioid use. As a potent kappa antagonist, buprenorphine has less dysphoric effects than methadone and, therefore, may be better tolerated (Whelan PJ, Remski K, 2019).

Buprenorphine is available in a tablet form for sublingual administration and also in parental form but it is most commonly prescribed as a tablet o film containing buprenorphine hydrochloride mixed (4:1) with naloxone (BUP-NX) that is a short-acting opioid agonist and is combined with buprenorphine in order to avoid its misuse by injection (in fact, naloxone is able to block buprenorphine effect if injected). Extended-release formulations of buprenorphine (6-month implants) were developed to improve patient compliance.

Naltrexone is the only *μ and k opioid receptor antagonist* approved by the FDA to treat opioid use disorder, available since the 1980s. Naltrexone has the advantage that it can be prescribed by any provider in the USA and does not cause euphoric effects, physical dependence, withdrawal if it is stopped or respiratory depression and therefore avoid the risk of diversion and overdose (Volkow ND, 2018).

Despite its high efficacy in blocking heroin effects, oral naltrexone is rarely prescribed because of problems with initiation of the therapy and the poor treatment compliance. In fact, prior to treatment with naltrexone, the patient has to be fully detoxified to prevent the precipitation

withdrawal symptoms (so called “detox hurdle”).These medication's instructions require at least 7 to 10 days without using opioids to avoid triggering withdrawal symptoms.

In 2010 the FDA approved a monthly depot injection of naltrexone (Vivitrol) for relapse prevention: long-acting depot naltrexone is injected intramuscularly and is effective for 1 month removing the burden on patients to take medication daily. This new formulation has the potential to be particularly useful in the criminal justice system, where the majority of incarcerated individual with a past of OUD are already abstinent and poor access to medication-assisted therapies, putting them at high risk of relapse and overdose upon release (Skolnick P, 2017; Comer SD et al, 2007).

Another dramatic problem to deal with by the healthcare system is the opioid overdose death. Opioid overdose is now one of the main causes of premature death in many countries.

Risk of overdose is increased when:

- a higher dose than prescribed was used;
- opioids are mixed with illicit opioids such as fentanyl or other drugs like alcohol or benzodiazepines,
- they are used after a period of abstinence that lead to decrease dose tolerance,
- they are used by individuals with comorbid disorders,
- they are used by people with a history of overdose.

The acute treatment of overdose is immediate administration of **Naloxone**: Naloxone is a competitive mu-opioid receptor antagonist historically used only by trained clinical professionals for the reversal of opioid overdose in an emergency or inpatient setting through intravenous, intramuscular or subcutaneous route. Parental naloxone has been approved to treat overdose for over 45 years but the recent availability of auto-injectable naloxone device (Evzio) and a naloxone spray formulation (Narcan) has greatly facilitated the utilization to layperson for

out-of-hospital administration and consequently, this resulted in a substantial decrease in legal opioid overdoses (Skolnick P, 2017; Blanco C and Volkow ND, 2019).

Although sometimes a single dose of this antidote is not enough to revert completely overdoses and more than one is necessary to restore spontaneous breathing, in particular if the abused opioid has an high potency like fentanyl: in fact, because of its high affinity to mu-receptor, fentanyl is able to display naloxone and reoccupy the receptor, triggering the recurrence of overdoses symptoms (Blanco C, Volkow Nd, 2019).

However, it is also important to remember that naloxone has a shorter half-life than the most part of opioids that could have caused the overdose and, therefore, there is a risk of return of overdose symptomatology which requires further medical attention (Strong J et al, 2020).

Despite the great and well established efficacy of selective mu-receptor agonists, like methadone, in treating opioid abuse, the adverse side effects and their abuse liability, connected with their similar profile with morphine or heroin, limit their security and always keep the risk of misuse, tolerance development and respiratory failure. This dark side of their profile has led researchers to search alternative agents that could have similar efficacy but reduced abuse liability.

	Type	Dosage	Provider	Clinical management
Opioid use disorder				
Methadone*	Full μ-opioid receptor agonist	Daily dose 80–160 mg	Dispensed mainly by so-called methadone clinics	Discontinuation requires slow tapering to avoid withdrawal; reduces illicit opioid use and overdoses and improves other outcomes
Buprenorphine*	Partial μ-opioid receptor agonist and κ-opioid receptor antagonist	Daily sublingual dose 8–24 mg	Dispensed by physicians or nurses	As a partial μ-opioid receptor agonist, some patients might experience withdrawal when treated with buprenorphine; extended release formulations (eg, 1-month, 6-month) might facilitate adherence; reduces illicit opioid use and overdoses and improves other outcomes; κ-opioid receptor antagonist properties might improve mood
Naltrexone*	μ-opioid receptor antagonist that interferes with the binding of opioid drugs, thus inhibiting reward and analgesia	Daily oral dose of 50 mg or one monthly injection of 380 mg	Dispensed by physicians	Patients need to have medically supervised opioid withdrawal before induction to avert withdrawal symptoms; evidence still limited, but studies suggest that the drug reduces opioid use and might prevent overdoses; κ-opioid receptor antagonist properties might improve mood
Overdose				
Naloxone*	μ-opioid receptor agonist that displaces opiod drugs (eg, heroin, fentanyl, or morphine) interfering with their respiratory depressant effects	Autoinjector: 2 mg per 0·4 mL naloxone for intramuscular or subcutaneous injection;[41] nasal spray: 4 mg for intranasal dosing;[42] intravenous injection: 0·4 mg/mL[43]	Dispensed by physicians; in many jurisdictions, naloxone can be dispensed through a so-called standing order signed by a health official; the order covers the whole population and negates the need for prescriptions for individuals	Indicated for overdose reversal, not for maintenance treatment; triggers an acute withdrawal syndrome in individuals who have recently taken (prescribed or illicit) full or partial μ-opioid receptor agonists; can be administered by non-professionals (eg, bystanders or first responders)

*Approved by the US Food and Drug Administration for opioid use disorder management, opioid withdrawal, or overdose reversal.

Figure 9. Adapted from Blanco C, Volkow ND (2019)

Synthesis of actual opioid medication available for treatment of OUD (opioid use disorder) and overdoses.

A new strategy adopted in recent years by researchers and that has yielded promising results, is the use of "mixed-ligand" that means a single drug with multiple targets. Buprenorphine is one example but another one that has the potential to be an excellent candidate in term of opioid abuse treatment, is a novel compound, the **Cebranopadol** (**Figure 10**)

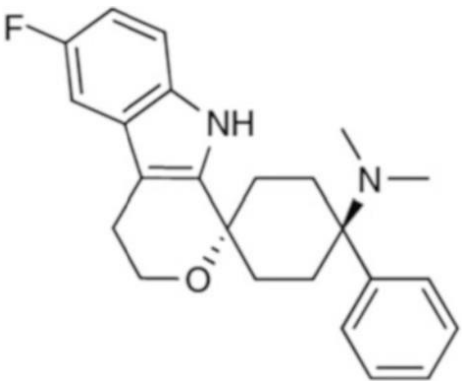

Figure 10. Adapted from Tzschentke TM et al, 2019

Chemical structure of Cebranopadol.

Cebranopadol is a pan-opioid agonist that activates MOP and NOP receptor with similar potency and efficacy; radioligand binding studies revealed sub-nanomolar affinity to both human and rat NOP and MOP receptor, 20 times higher than human KOP receptor and a partial agonism on DOP receptor. (Linz K. et al, 2014).

The growing interest in the efficacy on pain management and OUD treatment for compounds able to simultaneously activate nociceptin/orphanin FQ peptide (NOP) and opioid receptors (particularly the mu opioid one) was suggested by converging evidences on the mechanism of action of buprenorphine: this drug is classically presented as a mu-receptor agonist and kappa-antagonist but more recent studies demonstrated that buprenorphine also acts as a low

affinity - NOP partial agonist and this latter characteristic seems to be responsible of its ability to reduce the rewarding effect of heroin and of its lower addiction potential and respiratory depression compared with morphine or classical MOP agonists . The ability of NOP agonism to block opioid reward was further demonstrated in conditioned place preference (CPP) experiments in which the intracerebroventricular (ICV) injection of N/OFQ peptide blocked the acquisition and the expression of morphine CPP (Ciccocioppo R et al, 2000)

Kallupi M. and colleagues demonstrated that Buprenorphine induces also reduction of alcohol drinking and cocaine self-administration but only at highest doses due to its low affinity in binding NOP receptor and that this action on cocaine consumption was prevented only by simultaneously blocking of both MOP and NOP receptors (Kallupi M et al, 2018).

So, the combination of the analgesic effect of MOP agonism and the concomitant anti-rewarding effect and low abuse potential and development of tolerance due to the NOP agonism, associated to its prolonged half-life (24 hours) make Cebranopadol a interesting candidate pain management as a promising alternative to traditional opioids: currently this drug is under clinical trials for treatment of a variety of pain condition, including acute postoperative pain, chronic osteoarthritis, chronic low back pain, cancer-related pain and diabetic peripheral neuropathy (Schunk S et al, 2014).

First preclinical data about the efficacy of Cebranopadol in treating drug dependence focused on cocaine: in particular De Guglielmo et al (2017) reported that it reversed the escalation of cocaine intravenous (IV) self-administration in rats given extended access (6h) to cocaine whereas it did not affect self-administration of sweetened condensed milk or lever press on inactive lever, confirming that the efficacy on cocaine intake is specific and not related to motor impairment.

The study showed also that Cebranopadol blocked cue-induced reinstatement of cocaine seeking: keeping together, these data suggest that it may be not only effective to reduce cocaine intake but it may be also able to prevent relapse to cocaine-seeking during abstinence (De Guglielmo G et al, 2017). These results find further confirmation in preclinical experiment performed, in the same years, in our laboratory by Shen Q et al (2017) that demonstrated the ability of Cebranopadol in reducing also motivation for cocaine but not for saccharine and in a

place-conditioning test, the researchers found that in contrast with morphine, Cebranopadol did not elicit significant expression of place preference (Shen Q et al, 2017).

No preclinical data are already published related to the efficacy of Cebranopadol in treating opioid dependence: considering the well consolidated usage and efficacy of Buprenorphine and the similar pharmacodynamic profile with Cebranopadol but its lower abuse potential, during myPhD program, we have tested this drug in heroin self-administration paradigm to analyze its effect on heroin intake and motivation, in cue-induced and priming-induced relapse, in different preclinical model of addiction-like behaviors in both sex, male and female rats:

- Wistar rats (inbred rat's strain);
- Marchigian Sardinian genetically selected alcohol-preferring rats;
- NIH_Heterogenous stock rats (outbred rat's strain)

In order to evaluate how gender and genetic/individual differences can impact on the development of heroin addiction-like behavior and on the response to pharmacological treatment of OUD.

3. Effect of individual differences in addiction-like behavior

The classical definition of drug addiction, that dominated for decades in laboratory research, is centered on the role of prolonged drug taking as a major cause of the transition to addiction and the psychopharmacological changes consequent to chronic drug use.

This is a sort of “drug-centered theory” that focus on the mechanism of drug-induced sensitization, tolerance, dependence, withdrawal and, according to this approach, the addiction

state depends on the type and amount of substance consumed or administered with the aim to counteract the drug's effects with other pharmacological strategies.

But, currently, this perspective appears to have a big limitation that is consider individual differences as a sort of outlier result hidden under statistical standard error or an artifacts of the research protocol (Piazza PV and LE Moal M, 1996; Swendsen J and Le Moal M, 2011; **Figure 11**)

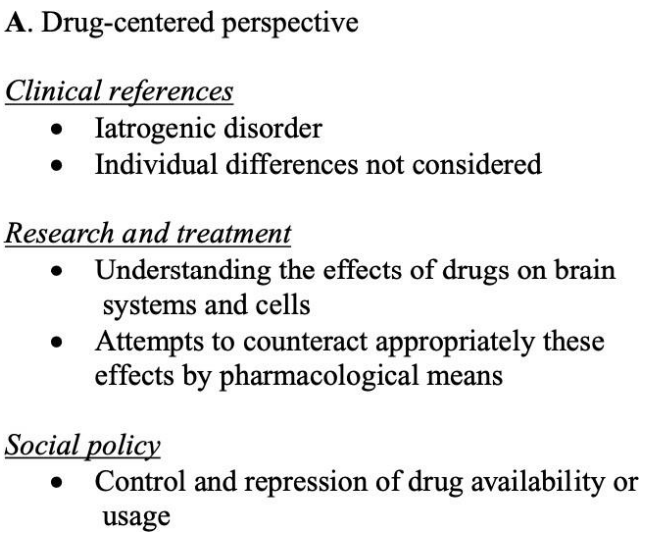

Figure 11. Adapted from Swendsen J and Le Moal M, 2011

The "drug-centered paradigm" of addiction considers the drug as the principal cause of addiction and it is aimed to understand the pharmacological properties of psychoactive substances that can explain the transition from initial drug use to compulsive behavior and loss of control over drug intake. In this perspective the subjects are not considered for their individual characteristics but they are considered equal or similar and addiction is approximated to an iatrogenic disorder that is a disease resulting from the acute or chronic intake of prescribed/therapeutic drug and its side effects.

This theory ignores a great evidence in the human society, as well as, also in the laboratory animals: given equal exposure to a specific substance of abuse, not all individuals will develop

an addiction, but only a small subset of them, due to a particular vulnerable state , experiences a singular effect of the drug that promotes a shift from recreational and occasional use to abuse.

This new individual-centered theory of addiction is well summarized by Charles P. O' Brien who stated: "*Some addicts go for months or years using heroin or cocaine only on weekends before becoming a daily, addicted, user. Others report that they had such an intense positive response that they became addicted with the first dose…"* (O'Brien CP et al, 1986).

According to this approach drug is not the main cause that leads to addiction: drug is necessary, obviously, but it is not a sufficient condition for the development of the dependence. Addiction results from a pathological response to drug exposure that is generated in a few individuals by a vulnerable biological phenotype that pre-exist the first exposure to the psychoactive substance. This is an intrinsic predisposed state that derives by a combination of biological, genetic and environmental factors, that , taken together, make an individual more prone than others (that we define "resilient phenotype") to precipitate into the addiction cycle. Patients, as well as laboratory animals in preclinical research, are considered different and more or less vulnerable according to their past, their behavior and also the genetic background (Le Moal M. 2009; **Figure 12**).

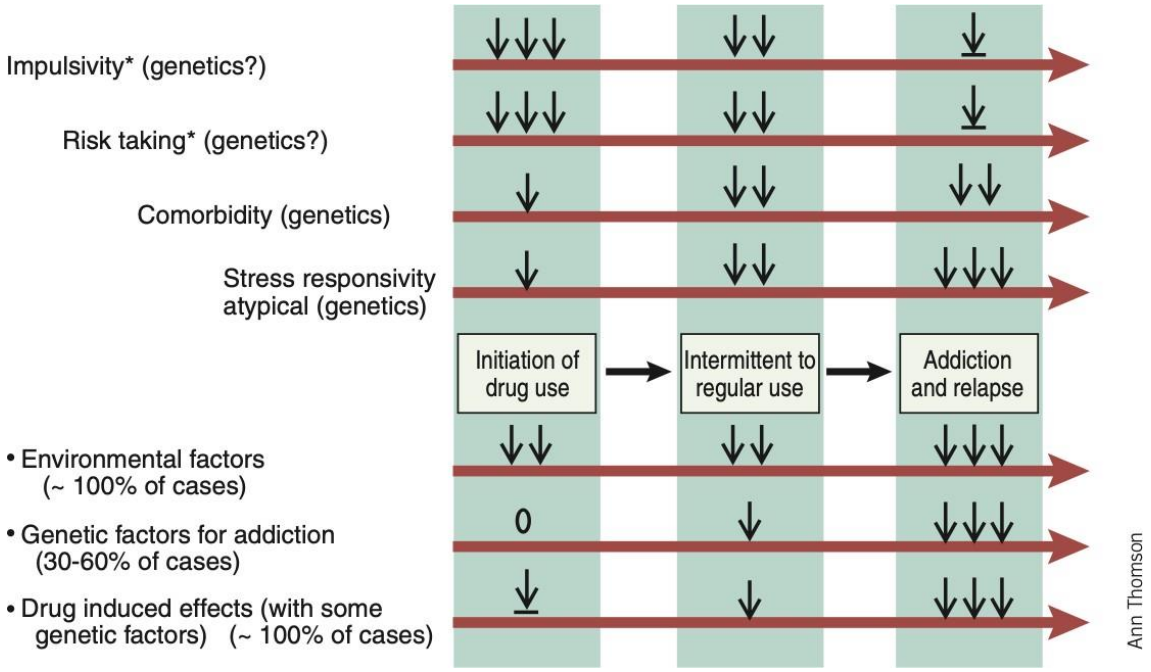

Figure 12. Adapted from Kreek MJ et al, 2005.

Diverse contributions of genetic differences and environmental factors to initial drug use, abuse and impulsivity. The researchers hypothesized that impulsivity and risk taking have a major role in facilitating the initiation of drug use whereas the stress response and the environmental factors influence the progression to addiction and relapse.

Greatest relative influence. *Medium relative influence.* *Small relative influence.*

Epidemiological data confirm this theory : in fact even if approximately 15,6% of the US population will go to engage in non-medical or illicit drug use at some times in their life, only 3,1% of them going on to drug abuse and still less going on to addiction cycle (Le Moal M, 2009).

Preclinical studies provide a number of advantage over human studies for investigate individual variability in addiction (in particular in opioid addiction) vulnerability: a basic requirement for this kind of investigation is that the genetic background/ biological/behavioral features that could be implicated in the determination of a vulnerable /resilient phenotype, must be characterized before the exposure in a controlled and stable environment that minimize the numbers of external variables, that could represent a bias in the analysis, and offer an equal access to the

drug at all individual under the same environmental conditions (Swain Y et al, 2021; Piazza PV et al, 1996).

In pre-clinics, many behavioral models have been developed in order to stay and mimic the human different traits implicated in addiction vulnerability (like anxiety, impulsivity, novelty-seeking, etc.) but intravenous (IV) drug self-administration is often evaluated as the one with the major degree of translation validity first of all, because many other animal model of addiction (like conditioned place-preference or locomotor sensitization) require that drug is administered by the experimenter while the IV self-administration involves the volitional drug taking , as occur in human. Secondly, but not less important, the self-administration procedure allows to characterize many traits involved in addiction: initiation of the drug use (acquisition phase), loss of control of drug intake (escalation phase), motivation to drug procurement (progressive ratio schedule of reinforcement), relapse induced by environmental cue or stressors (reinstatement model) (Swain Y et al, 2021).

However, it is also important to choose the best rat model that could mimic the individual differences of human population: the standardized use of inbred rat's strain in preclinical research that guarantee the experimental reproducibility and a constant and uniform animal model, for this purpose, is a limitation due to their genetic homogeneity. If the aim of the study is to characterize the individual difference involved in the vulnerability or resilience to develop addiction-behavior, the better choice is to use an out-bred rat line in order to guarantee the most close approximation to the genetic and phenotypic variability characteristic of the human population (Parker CC et al, 2014) showing a significant individual differences both in drug self-administration and its behavioral predictors.

Within these predictors, previous studies had identified some traits that are strongly correlated with the vulnerability to loss of control and sustained drug use, in particular locomotor response to novelty, to stressors and anxiety-like behavior.

Piazza PV and colleagues identified a positive correlation between the locomotor response to novelty (a locomotor behavioral test in a novel, inescapable environment) and the amount of

drug received in the first days of self-administration (during the acquisition phase), and consequently they divided rats in two subgroups considering they locomotor activity: the HRs (high responders) with the activity score above the median and in contrast the LRs (low responders). All rats learnt to self-administer drugs during the first days but only some vulnerable rats, the HR group, escalate their drug intake, whereas in most rats (LRs) drug intake decrease: also in the human world people who tried drugs can be similarly divided into those who do or do not shift to escalated drug use (Piazza PV et al, 2013). The assertion that high locomotor activity could be a predictor of individual variability to rapid escalation in drug intake and maintenance high level of consumption, was confirmed by the same research group showing that HR rats had a higher preexisting dopaminergic activity and high corticosterone secretion in response to stress, than did LR rats (Piazza PV et al, 1991).

The interaction between stress, glucocorticoids system and the dopaminergic transmission in the mesolimbic pathway is also a crucial point that seems to be involved in the transition from recreational to escalated and sustained drug use. It has been demonstrated that many stressor events , like food restriction, social isolation or electric foot shock increased opioid self-administration and that the breakpoint for heroin in the progressive ratio schedule of reinforcement is consistently higher in stress than control rats (Shaham Y and Stewart J, 1995). It is well known that glucocorticoid secretion by adrenal gland is the principal biological response to stress and also that mesolimbic dopaminergic neurons express corticosteroid receptors . So, the link between these three factors appear to be evident: stress raises the level of glucocorticoid hormones that increase the dopaminergic activity in the mesolimbic system causing an higher vulnerability to drugs in stressed subjects (Piazza PV and Le Moal M, 1996; **Figure 13**). Levels of corticosterone measured 2 hours after exposure to stressor are positively correlated with the amount of drug consumed when it is presented for the first time in the HR subjects.

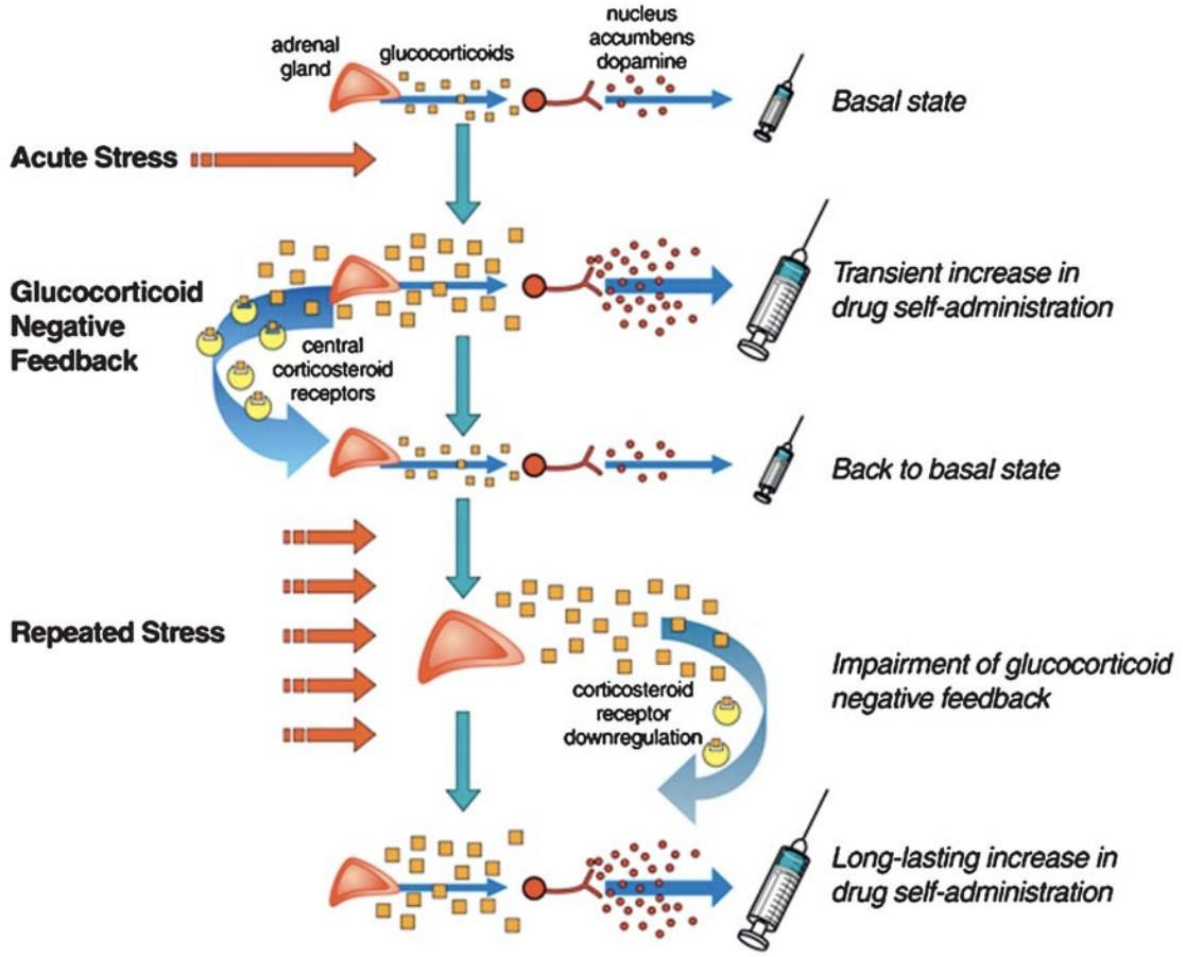

Figure 13. Adapted from Le Moal M, 2009.

Stress exposure causes the activation of the hypothalamic pituitary-adrenal axis and the secretion of corticotropin-releasing factor (CRF) and of glucocorticoid hormones; the concentration of glucocorticoids determine the level of dopamine release in the nucleus accumbens increasing the sensitivity to the reinforcing effects of drug of abuse. A direct consequence can be an increase in self-administration. Chronic or repeated stress exposure can disrupt the negative feedback mechanism that controls glucocorticoid level and maintain it at the homeostatic levels resulting in a long-lasting increase in the secretion of these hormones and so, in the sensitivity to the development of drug intake.

In an epidemiological study (Mills et al, 2006) , 88% of people that abused opioid had been exposed to traumatic stress and the highest prevalence of PTSD was among the individuals with

OUD. Conversely, not every individual with this kind of adverse experiences in his past exhibit an addiction disorder.

According to this evidence, it could be hypothesized that a preclinical animal model of stress will have a higher demand for heroin and will be more prone to heroin abuse compared to a non-stressed control rat line.

3.1 Marchigian-Sardinian Alcohol Preferring rats

The genetically selected Marchigian Sardinian alcohol-preferring (msP) rat line is a well consolidated animal model to study alcohol use disorder (AUD) in term of binge-like ethanol drinking and relapse in which anxiety and depression-like traits have co-segregate with alcohol preference during the selection leading to the generation of useful preclinical model of genetic susceptibility to alcohol abuse linked to self-medication of negative affective states (Ciccocioppo R et al, 1999; Ciccocioppo R et al, 2006).

This rat line has been selected for its high ethanol preference for about 18 years starting from the 13th generation of Sardinian alcohol-preferring (sP) rats originally selected at the Department of Neuroscience in Cagliari, Italy (Colombo G et al, 2006) from Wistar rats. In 1998, after 20 generations of selective breeding, at the Department of Experimental Medicine and PublicHealth of Camerino, Italy, these animals were re-named msP (Ciccocioppo R et al, 2006).

Converging evidence proposed that long-term up-regulation of CRH transmission acquiredthrough a prolonged history of alcohol use is central to developing and maintaining alcoholism (Valdez GR and Koob GF, 2004).
In the study published in 2006, Hannson and colleagues (Hannson AC et al, 2006) confirmed that the elevated behavioral sensitivity to stress in the msP line is the main cause that leads to a lowered threshold for stress-induced reinstatement of alcohol-seeking behavior. Analyzing the genetic roots of this behavioral conditions, the researchers found that the msP rats carry a unique mutation driven by two single nucleotide polymorphisms at the CRF1 locus, encoding the corticotropin-releasing hormone receptor 1(CRH-R1), leading to over-expression of the CRF system in areas of the brain associated with negative affect such as the amygdala (Hannson AC

et al, 2006). They showed there that genetic variation associated with increased CRH-R1 expression can emulate this state even in the absence of a long drinking history. Infact this mutation causes innate hyperactivity of the CRF/CRF1 system, which correlates with excessive alcohol drinking, heightened stress sensitivity, potentiated negative affect, and behavioral alterations that possibly resemble post-traumatic stress disorder (PTSD) traits. In behavioral tests, msP rats had showed many behavioral traits such as low locomotor activity and percent time spent into the central area of the Open field test, and a higher level of freezing during fear conditioning test, that markedly resemble an anxiogenic-like phenotype (Hannson AC et al, 2006).

Ethanol drinking in msP rats is thought to be motivated by negative reinforcement, modeling the drinking behavior of a subpopulation of individuals who drink for tension relief and self-medication purposes (Vozella V et al, 2021; Benvenuti F et al, 2021).

It is also well known that alcohol consumption in humans often co-occurs with other drugs of abuse disorder, suggesting that innate predisposing factors may confer vulnerability to polydrug abuse (Bifone A et al, 2018). So, into the attempt to evaluate which individual factors that pre-exist to the drug exposure, could be related with the vulnerability or reliance to develop OUD, the msPs and their genetic hypersensitivity to stress could be a valid preclinical animal model of stress and consequently, an example of heroin addiction-prone phenotype compared to the non-selected Wistar counterpart.

From a pharmacological point of view, we have also investigated the effect of Cebranopadol on heroin dependence: previous studies had demonstrated that direct ICV administration of nociceptin/OFQ in msP rats attenuated voluntary two-bottle choice alcohol drinking while in non-selected Wistar rats tested under the same experimental conditions N/OFQ did not alter ethanol consumption (Ciccocioppo R et al,1999; Ciccocioppo R et al, 2004); finding that was then replicated also with NOP selective agonists.

In the study cited above, Hansson and colleagues (Hansson et al,2006) have first demonstrated that antagonism on CRH_R1receptors is able to prevent stress-induced reinstatement of alcohol seeking, bringing out the idea to block this system to cope with stress. Considering the possibility

that activation of NOP receptors mediates a potent anxiolytic and anti-stress effect and that nociceptin acts as a functional antagonist of the CRF1 receptor system (Ciccocioppo et al. 2001; 2002), it is possible that in msP rats the effect on alcohol drinking produced by N/OFQ was due to its ability to alleviate the negative affective state (responsible to trigger excessive drinking) associated with heightened CRF1R transmission.

The *in situ* hybridization results showed higher expression of N/OFQ and NOP receptor mRNAs in several stress-regulatory brain regions, including the central amygdala (CeA), compared to Wistar rats, resulting in a significant increases in NOP receptor binding capacity (Economidou D et al, 2008). Based on these findings, the upregulation of the N/OFQ/NOP system occurs in msP rats in response to, and in an attempt to compensate for the up-regulation of CRF1 receptorsin this line of rats (Hansson AC et al, 2006). This adaptive response may be one of several compensatory functional adaptations that occur in msP rats and this is the reason why msP rats were more sensitive to the effects of a NOP agonist.

Taking into consideration these genetic predisposing factors to alcohol dependence and the frequent co-occurrence between alcohol abuse and others substances, like nicotine and psychostimulants, Bifone and collegueas had demonstrated that msP rats exhibited a higher propensity to escalate cocaine intake following long access (6 hrs) suggesting that neurobiological and genetic mechanisms that convey vulnerability to excessive alcohol drinking also facilitate the transition from psychostimulants use to abuse (Bifone A et al,2018). In our laboratory then, NOP agonist (Ro.

As illustrated above, Cebranopadol is a pan-opioid agonist with a particular affinity to MOP and NOP receptor: basis upon the up regulation of NOP system in msP rats, we want to compare the effect of the drug in reducing heroin addiction and related behavior between this vulnerable rat model and the Wistar controls.

3.2 The NIH Heterogeneous Stock Rats

One of the major aims of my study is to investigate which could be the individual traits, in terms of genetic makeup or innate behaviors, that predict OUD-vulnerability or resilience in order to prevent the development of addiction-like behavior or to identify a priori which phenotypes resulted in a high risk to fall into addiction.

The consequent implication is that we need a particular preclinical animal model that conserves and best mimics the individual variability typical of the human population in order to identify genetic and phenotypic variants associated with OUD.

The NIH_Heterogeneous Stock rats (NIH_HS) is an outbred population that represents a random mosaic of the eight inbred founder strains, with each animal being genetically and phenotypically distinct in order to more closely resembling the variation found in natural population, including human (Solberg Wood LC and Palmer AA, 2019).

The NIH_HS rats was first established at the National Institute of Health (NIH) in 1984 and was derived from eight inbred progenitor strains by a random rotational breeding strategy for 60 generations, to minimize the extent of inbreeding: Agouti (ACI/N), Brown Norway (BN/SnN), Buffalo (BUF/N), Fischer 344 (F344/N), Maudsley Reactive (MR/N), M520/N, Wistar Nettleship (WN/N) and Wistar Kyoto (WKY/N) **(Figure 14**).

As a result, it is possible to precisely map the genetic variants that contribute to phenotypic differences; because each animal is genetically and phenotypically unique, it is not possible to have biological replicates in this outbred population. In 2006, the HS colony was transferred to two locations: Dr. Solberg Woods at the Medical College of Wisconsin and Dr. Alberto Fernando Teruel at the Autonomous University of Barcelona in Spain.

Currently, the HS rat colony is a national resource funded through a NIDA Center of Excellence for genome-wide association studies (GWAS) in outbred rats which aim to identify genetic loci underlying drug abuse behaviors (Solberg Woods LC and Palmer AA, 2019).

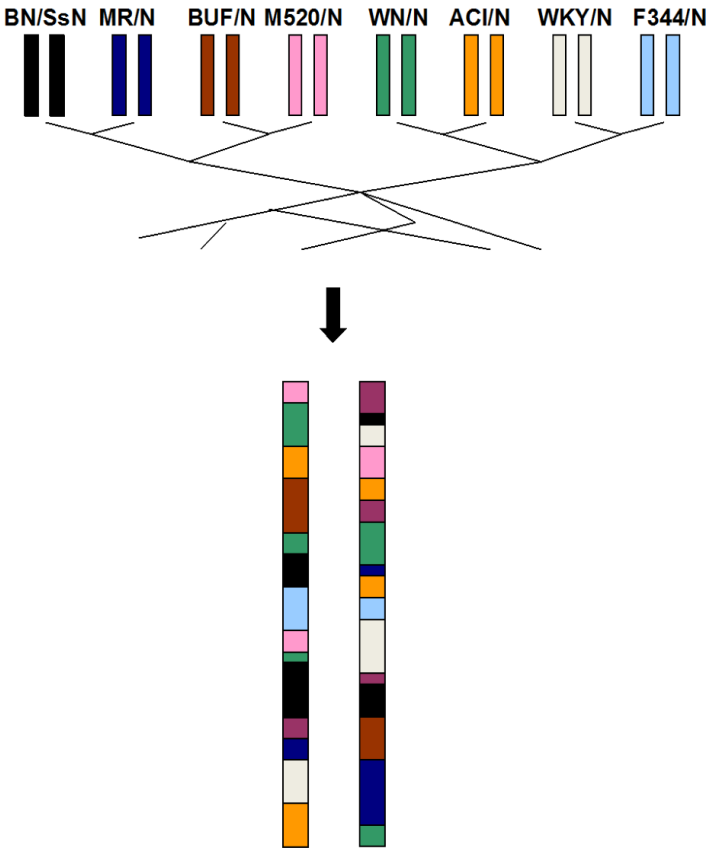

Figure 14. Adapted from Baud A et al (2014)

Schematic diagram of the development of Heterogeneous stock rats from 8 inbred progenitor strains through more than 60 generations of outbreeding. As result, each of these HS rats represents a unique, genetically random mosaic of founding animal chromosomes due to recombination that have accumulated over many generations (Alam I et al, 2011).

4. Research Objectives and Significance

Opioid use disorder (OUD) is a neuropsychiatric disease that arises in a subset of individuals with a vulnerable phenotype that pre-exist the first exposure to the drug (Le Moal M, 2009). Buthow individual variations in multiple behavioral traits may interact and contribute to shape an OUD-vulnerable or resilient phenotype is a challenging question to deal with and represent the major research objective of the present dissertation.

With this aim, in **Chapter 2** we will investigate the role of stress innate vulnerability in enhancing heroin dependence using a validated animal model of comorbidity between stress sensitivity and alcohol binge-drinking, the Marchigian Sardinian alcohol preferring rats (msP). We will compare the heroin taking and motivation to work for heroin in self-administration between male and female msP rats and their non-selected counterpart, the Wistar rats. Rats were exposed to self-administer 4 increasing heroin doses in order to evaluate their propensity to develop heroin addiction-behavior, also comparing gender-related differences.

In **Chapter 4** we will broaden our investigation on opioid-addiction vulnerability selecting for the study an heterogeneous rat line, the NIH heterogeneous stock rats that best mimic the individual variability of the human population in order to identify different clusters with varying degree of opioid vulnerability and to characterize their heroin-addictive behavior. With this purpose HS rats were characterized in terms of innate locomotor activity, anxiety-like behavior and pain/analgesic sensitivity prior to exposing them to heroin long-access self-administration sessions to screen their heroin-related behavior in terms of heroin taking, refraining and heroin seeking. The correlation between their innate traits and the heroin-related cluster allocation couldhave a predictive role in determining the individual predisposition to develop OUD.

Then we will investigate OUD from a pharmacological point of view: in fact the current clinical approach based on the use of opioid agonists like methadone and buprenorphine often failed due to their abuse liability and adverse side effects. In **Chapter 3** we will test the efficacy of a novel pan opioid agonist, Cebranopadol, with a nanomolar affinity for both MOP and NOP receptor, in reducing heroin taking and motivation in male and female msP rats, whose vulnerability to

develop OUD we had demonstrated in Chapter 2. We will also explore how sex-differences influence the response to the pharmacological treatment.

Assessed the efficacy of Cebranopadol in reducing heroin taking and seeking, in **Chapter 5** we will benefit from the great individual variability of HS population, to investigate their different pharmacological responses to treatment and detect the presence of a non-responder subpopulation: our purpose is to look into an eventual correlation between the genetical or behavioral factors associated with the OUD-vulnerability or resilience and the responsiveness to the related drug therapy.

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Chapter 2:

“OPIOID ABUSE VULNERABILITY IN GENETICALLY SELECTED MARCHIGIAN SARDINIAN ALCOHOL-PREFERRING RATS”

1. ABSTRACT

Opioid use disorder (OUD) is a chronic relapsed disease characterized by compulsion to seek and take the drug that is generated in a subset of individuals with a vulnerable phenotype. Many risk factors have been identified, associated with genetic, environmental and social background. Among these, stress plays a crucial role in contributing to enhancing the susceptibility to develop OUD. Our hypothesis is that a preclinical animal model of stress/addiction interaction could be suitable to study heroin reinforced behavior in stressful phenotype.

The Marchigian Sardinian alcohol preferring rats (msP) is a validated animal model genetically selected for excessive alcohol drinking and high sensitivity to stress due to a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and an over-expression of the opioid receptor system. Considering this vulnerable phenotype, we set out to compare heroin taking and motivation for heroin between msP rats and non selected Wistar counterparts. Heroin dose/response curve and heroin dose/breakpoint curve were evaluated in male and female, msP and Wistar rats giving them access to self-administer 4 heroin doses (1, 7, 20, 60 µg/inf) in 2h daily sessions under FR1and PR schedule of reinforcement, respectively.

Our results demonstrated that both msP male and female consumed a higher amount of heroin compared with Wistar controls and these differences increased proportionally with heroin doses. However female rats, independently by line, showed a statistically significant tendency to self-administer more heroin than males. When tested under PR schedule of reinforcement to evaluate the motivation to work for obtaining the drug, msP rats showed a higher breakpoint at middle doses with respect to Wistars. There is an evident gender-difference: contrary to the literature, female rats (independently by line reached a lower breakpoint compared with males atall doses tested (with the only exception of 1 µg heroin/inf). In conclusion, we had demonstratedthe validity of the msP rat line as a model of stress-vulnerability to poly drug abuse, including opioid and also that gender is a non-negligible factor in determining the susceptibility to addiction.

Keywords: Opioid Use Disorder, alcohol-preferring rats, heroin self-administration, addiction vulnerability, stress, sex differences

2. INTRODUCTION

Opioid use disorder (OUD) is a chronic relapsing disease that has recently risen epidemic proportions, particularly in the USA and Canada. Approved medications show only limited efficacy, as the patient population can be stratified in treatment responder and non-responder subjects (Crist RC et al, 2018). For this reason, in recent years drug development research has focused its attention on individualized treatment approaches compared to those that are currently available.

In fact, preclinical and clinical studies reported that drug addiction results from a pathological response to drug exposure that is generated only in a subset of individuals with a vulnerable phenotype that pre-exist the first exposure to the substance of abuse (Le Moal M. 2009).

Patients, as well as laboratory animals in preclinical research, are considered different and more or less prone to precipitate into the addiction cycle according to their past experiences , their behavior and also their genetic background (Le Moal M., 2009). In particular, it is known that stress is an important factor in the development of addiction disorders: stress, past traumatic experiences and mental trauma (that could also lead to psychiatric pathologies like PTSD) are strongly identified as risk factors for substance use and addiction. Epidemiological studies indicate that stress increases the risk to use and abuse heroin. For example, Mills et al (2006) reported that trauma exposure and PTSD were highly prevalent across all substance use disorders and the highest rates of PTSD were among those with opioid dependence (37% of total PTSD and SUD comorbidity). Nevertheless, there is only limited preclinical evidence that has investigated the correlation between stress sensitivity and susceptibility to develop OUD. Shaham Y. and colleagues demonstrated that daily immobilization stress increased oral consumption of both fentanyl and heroin (Shaham et al, 1993) and intermittent foot-shock stress increased intravenous self-administration of heroin (Shaham and Stewart, 1994). More recently, Stafford NP and colleagues demonstrated that rats's individual response to stress, also measured as level of corticosterone response, predicted the behavioral economic value of heroin.

Stress exposure causes the activation of the hypothalamic pituitary-adrenal axis and the secretion of corticotropin-releasing factor (CRF) and of glucocorticoid hormones; the concentration of glucocorticoids determine the level of dopamine release in the nucleus accumbens increasing the

sensitivity to the reinforcing effects of drug of abuse. A direct consequence can be an increase in self-administration. Chronic or repeated stress exposure can disrupt the negative feedback mechanism that controls glucocorticoid level and maintains it at the homeostatic levels resulting in a long-lasting increase in the secretion of these hormones and so, in the sensitivity to the development of drug addiction (Le Moal et al,2006).

According to this evidence, we thought that preclinical models of stress/addiction interaction would be suitable to study increased motivation for heroin at preclinical level .

The Marchigian Sardinian alcohol preferring (msP) rats is a validated animal model of co-morbidity between increased sensitivity to stress, elevated anxiety state and increased propensity to consume alcohol (Ciccocioppo R et al, 1999; Ciccocioppo R et al, 2006; Cippitelli A et al, 2015). MsP is a genetically selected rat line that closely mimics the fundamental features of alcohol use disorder, such as ethanol binge drinking, escalating alcohol intake after abstinence and high vulnerability to stress-induced reinstatement of alcohol seeking (Hansson AC et al, 2006). Considering also that in humans alcohol consumption often co-occurs with other drugs of abuse, the msP rats were recently shown to have increased response to psychostimulants (Bifone A et al, 2018). Therefore, this rat line, originally selected for excessive alcohol drinking, could also represent a model of vulnerability to other drugs of abuse.

First of all, in behavioral tests, msP rats showed many behavioral traits such as low locomotor activity and percent time spent into the central area of the Open field test, and a higher level of freezing during fear conditioning test (Hannson AC et al, 2006); also in our laboratory we demonstrated that male and female msP rats, exposed to elevated plus maze, forced swimming test, foot shock stress response, exhibited higher anxiety-like behavior compared with Wistar rats (Borruto AM et al, 2021). Their enhanced stress sensitivity and anxiety-like behavior is caused by a unique genetic mutation driven by two single nucleotide polymorphism at the CF1 locus that lead to an innate overexpression of the CRF1 receptor system in extra-hypothalamic areas associated with negative affect and dysphoric state (i.e. amygdala)(Hannson AC et al, 2006). In fact , in msP rats, the high ethanol drinking is motivated by negative reinforcement and the necessity to cope with their stressful phenotype, modeling the drinking behavior of the subpopulation of individuals who drink for tension relief and self-medication purpose (Vozella V

et al, 2021). It was already demonstrated that, enhanced CRF1R- transmission not only drive excessive alcohol drinking in msP rats, but also caused a huger cocaine intake in self-administration procedure and facilitates escalation in heterogeneous rat lines (Cippitelli A et al , 2015) ; mRI data also confirmed that msP rats present an increased reactivity to D-amphetamine in prelimbic cortex and extended amygdala, compared to Wistar counterpart(Bifone A et al, 2018).

Based on these premises, we set out to compare heroin reinforced behavior between msP and non selected Wistar counterparts. We hypothesized that msPs would show higher propensity to self-administer heroin and higher motivation than Wistars. To support our hypothesis there are also gene expression studies showing that msP rats are also characterized alteration of the opioid system, in particular by an innate over-expression of the NOP receptor system in several stress regulatory areas of the brain, including central amygdala (Ciccocioppo et al, 2019) making them potentially more prone to develop opioid dependence. To support the involvement of the NOP system into facilitating SUD, a previous study assessed that NOP receptor knockout ratsself-administered less cocaine, alcohol, and heroin compared with wild-type controls (Kallupi M et al, 2017).

Among the risk factors that make an individual vulnerable to develop addiction-like behavior and so more prone to progress from a recreational drug use to a rapid escalation of drug taking and continuous use despite adverse negative consequences, sex differences play an important role: in a vulnerable group of individuals, females exhibit a greater rate of escalation of drug use than males (Becker JB et al, 2016). Furthermore when investigating around sex differences in addiction, it is necessary to take into account the hormone condition of women because the phases of the menstrual and estrous cycle and the associated reproductive hormones release (progesterone and estradiol) highly affect drug taking behavior. There were many papers published assessing that female rats acquire morphine and heroin self-administration faster and will work harder to get cocaine, morphine and heroin than will males (Carroll ME et al, 2002).

Similarly, preclinical studies have reported that, as obvious for their genetically selection, msP rats consume more alcohol compared with their non-selected Wistar counterpart, but , among them, female msP rats consume higher amounts of alcohol when compared to males (Borruto

AM et al, 2021). This is the reason why we carried out our experiments on heroin-addiction vulnerability between msP and Wistar control rats, also comparing male and female's behaviors among each line.

3. MATERIAL AND METHODS

3.1 Animals

Male and female Wistar and genetically selected alcohol-preferring msP rats (Ciccocioppo et al, 2006) were employed for these experiments.

Wistar rats, male (n=7) and female (n=11), were purchased from Charles River (Calco, Italy). Rats' body weight ranged between 260 and 320 (male) and between 200 and 240g (female) atthe beginning of experimental procedures,

MsP rats , male (n=7) and female (n=12), were bred at the School of Pharmacy of the University of Camerino (Italy). At the beginning of the experimental procedures, the rat's body weight ranged between 260 and 300g (male) and between 200 and 220g (female).

Rats were pair-housed in plexiglass home-cages in a room with artificial 12/12h reverse light/dark cycle (light off at 7:00 am), constant temperature (20-22°C) and humidity (45-55%). All animals were handled once daily for one week before the beginning of the experiments. During their permanence in the animal facility , rats were offered free access to tap water and food pellets (4RF18 Mucedola, Settimo Milanese, Italy).

Experiments were performed during the dark phase of the light/dark cycle. All procedures were conducted in adherence to the *European Community Council Directive for Care and Use of Laboratory Animals* and the *National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals*.

3.2 Drugs

Heroin (Diacetylmorphine hydrochloride, purchased by SALARS, Como, Italy) was dissolved in sterile physiological saline (0,9% NaCl) and administered intravenously by the rats.

3.3 Intravenous surgery

Animals were anesthetized with isoflurane anesthesia: 5% induction and 2% maintenance. A single catheter made from micro-renathane tubing (ID = 0.020", OD = 0.037"; Braintree Scientific) was implanted in the right jugular vein and subcutaneously positioned between the vein and the back between the shoulders. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk sutures. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle. The cannula was inserted in a support made of dental cement and covered with a plastic cap (Kallupi M.et al, 2017).

Immediately after surgery, rats were allowed to recover for 1 week before self-administration training. During recovery, they received antibiotic prophylaxis with Enrofloxacin (Baytril®, Bayer) through the drinking water dissolved at the concentration of 25 mg/ml . Throughout the self-administration training and tests, catheters were flushed daily with 0.1-0.2 ml of heparinized saline solution (Nadroparin calcium 3800 U.I.; Italfarmaco S.p.A, Milan, Italy) containing 1mg/ml of Enrofloxacin. Body weights were monitored on a weekly base At the end of the experiments catheter patency was confirmed with an injection of 0.2-0.3 ml of ThiopentalSodium solution (Pentothal Sodium, 1g/50 ml, MSD Animal Health S.r.l), immediate loss of reflexes was taken as a positive sign of patency.

3.4 Self-administration apparatus

Heroin self-administration was performed in rat operant conditioning chambers (Med Associate St Albans, VT) enclosed in sound-attenuating ventilated environmental cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber with two stimulus lights placed above each lever, and a house light plus a tone generator on the opposite wall. The heroin solution was delivered through a Tygon tube that connected the catheter with an infusion pump. The pump was activated by responses on the right (active) lever and resulted in a delivery of 0.1 ml of fluid. Responses on the left (inactive) lever were recorded but had no programmed consequences. A windows compatible computer controlled the delivery of heroin solution and recorded the behavioral data.

3.5 Heroin self-administration procedures

Rats were trained to heroin self-administration (HSA) in two-hours daily sessions five days per week. Rats were trained to self-administer heroin under fixed-ratio 1 (FR1) schedule of reinforcement at the training dose of 20 µg/infusion (infusion volume 0.1 ml delivered over 5 seconds) for fifteen consecutive sessions . Contingent with heroin infusion, a 20s time-out period (TO) started. During TO active lever presses were not reinforced with additionalinjections.. An intermittent beep tone (1sec ON/OFF) was active throughout the self-administration sessions, functioning as contextual stimulus.

3.6 Experimental procedures

3.6.1 Heroin dose-response curve in male and female, msP and Wistar rats under fixed ratio 1 schedule of reinforcement

At the end of the training phase, the heroin dose response curve in Wistar and msP rats of both sexes was evaluated. To assess the heroin D/R curve, each rat had access to four heroin doses (1,7, 20 and 60 µg/infusion), that could be self-administered under FR1 contingency for four consecutive days. The experiment lasted for four weeks until rats experienced all heroin doses. The four doses of heroin were presented in counterbalanced order following a latin-square design.

3.6.2 Heroin dose-response curve in male and female, msP and Wistar rats under progressive ratio (PR) schedule of reinforcement

Each week, following the four days in FR1 SA described in section 3.6.1, the fifth day rats were subjected to a self-administration session run under a progressive ratio (PR) schedule of reinforcement. During PR session the number of active lever response required to receive a single infusion of heroin increased after every infusion according to the following order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268 etc.. (adapted from de Guglielmo et al, 2015; Richardson and Roberts, 1996). The session stopped if the required

number of responses was not achieved within one hour from the last reinforced response. The last ratio completed is defined as the break point (BP) and is considered as a direct measure of rat's motivation for the drug (Sanchis-Segura C and Spanagel R, 2006).

3.7 Statistical analysis

Heroin infusions and intake (µg/kg) earned under FR1 schedule of reinforcement, as well as the BP reached under PR contingency were initially analyzed by three-way ANOVA with heroin doses as within subjects factor and rats line and sex as between subjects factors. Male and female data were then analyzed separately by two-way ANOVA with rat lines as between subjects factor and heroin doses as within subjects factor.

ANOVA was followed by Bonferroni's post-hoc analysis for multiple comparisons when appropriate. Significant difference was conventionally set at $*p<0.05$.

4. RESULTS

4.1 Heroin dose-response curve in male and female, msP and Wistar rats under fixed ratio 1 (FR1) schedule of reinforcement

We set out by investigating the interaction between three factors: line, sex and heroin doses. Three-way ANOVA of heroin infusions earned revealed an overall effect of dose and [$F(3, 99)=13.76$, $p<0.0001$] and line [$F(1, 33)=7.68$, $p<0.01$], and no effect of sex [$F(1, 33)=2.57$, $p>0.05$]; none of the interactions were significant: dose by sex [$F(3, 99)=2.2$, $p>0.05$], dose by line [$F(3, 99)=0.8$, $p>0.05$], sex by line [$F(1, 33)=1.54$, $p>0.05$], dose by sex by line [$F(3, 99)=1.18$, $p>0.05$]. These results are consistent with higher amount of infusions observed atlower doses independently of sex and of a higher amount of infusions earned by msP rats independently of dose and sex (**Figure 1A**).

We noticed that msP and Wistars, and male and females differed in body weights, and therefore we analyzed heroin intake also as normalized by body-weight. This analysis confirmed anoverall effect of heroin dose [$F(3, 99)=60.85$, $p<0.0001$] and rat line [$F(1, 33)=9.72$, $p<0.01$]. However, there was an overal effect of sex [$F(1, 33)=27.52$, $p<0.0001$], and heroin dose by sex interaction [$F(3, 99)=13.79$, $p<0.0001$], with all other interaction remaining not significant: dose by line [$F(3, 99)=1.99$, $p>0.05$], sex by line [$F(1, 33)=0.72$, $p>0.05$], dose by sex by line [$F(3, 99)=0.72$, $p>0.05$]. These results confirmed that msP rats consumed more heroin than wistar and they indicated that female rats consume more heroin than male, independently by rat line(**Figure 1B**).

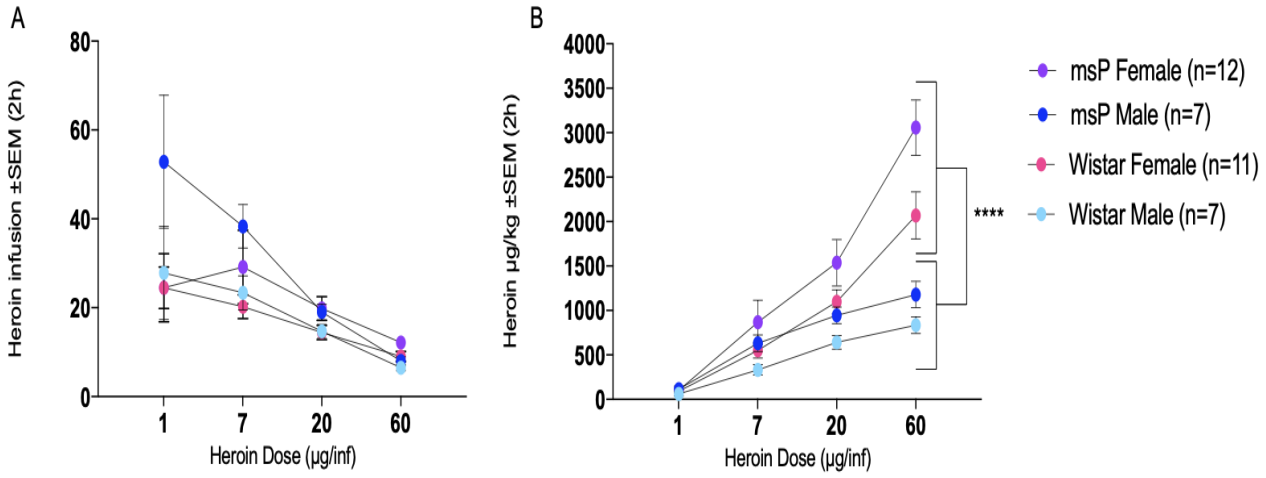

Figure 1: *Heroin self-administration of different doses of heroin in male and female msP and Wistar rats.* ***A)*** *The four groups of rats adapted the number of infusions to heroin dose, self-administering higher amounts of infusions at lower doses. msP rats tended to self-administera higher number of infusions than Wistars independently of sex.* ***B)*** *When intake was normalized by body weight, female rats consumed a higher amount of heroin independently of the rat line. Data are expressed as mean ± SEM of heroin infusions/intake averaged over the four days of SA. Statistical significance: ****p<0.0001 indicates overall effect of sex.*

To further evaluate line difference in heroin dose/response within each sex, we analysed the two sex independently. Both msP and Wistar male rats adapted the number of infusions to the heroin dose self-administered, earning a higher amount of infusions at lower doses as confirmed by an overall effect of doses [$F(3, 36)=9.197$, $p<0.001$]. we also observed an overall effect of rat line [$F(1, 12)=5.312$, $p<0.05$] but no dose by line interaction [$F(3, 36)=1.1229$, $p=0.3135$], which is consistent with a higher amount of infusions self-administered by msP rats independently of doses, with the difference between the two lines getting larger at decreasing doses (**Figure 2A**). We noticed that the two lines showed different body-weight and therefore we analyzed heroin intake also as normalized by body-weight. Two-way ANOVA confirmed an overall effect of heroin dose [$F(3, 36)=95.73$, $p<0.0001$], as the total heroin intake increased at increasing doses,

the overall effect of line [$F(1, 12)=6.420$, $p=0.0262$] as well as the lack of dose by line interaction [$F(3, 36)=2.634$, $p=0.0646$] were also confirmed. This was consistent with a higher amount of heroin self-administered by msP independently of the heroin dose; the difference between the two lines got larger at increasing doses (**Figure 2B**).

The analysis of infusions earned by female msP and Wistar rats revealed an overall effect of heroin dose [$F(3, 63)=4.039$, $p<0.05$], however, there was no line effect [$F(1, 21)=1.768$, $p>0.05$]and dose by line interaction [$F(3, 63)=0.3127$, $p>0.05$]. This is consistent with a decrease in heroin infusions observed at increasing doses in both lines (**Figure 2C**). Also in females, we observed a difference in body weight between the two lines, and therefore we normalized the heroin intake to rat's body weights. Two-way ANOVA confirmed an overall effect of doses [$F(3,63)=56.41$, $p<0.0001$], but in this case we found an overall effect of line [$F(1, 21)=7.485$, $p<0.05$], and still no dose by line interaction [$F(3, 63)=2.122$, $p>0.05$]. This analysis is consistent with a higher amount of heroin consumed at increasing doses by both lines, and with msP rats earning more heroin than Wistars (**Figure 2D**).

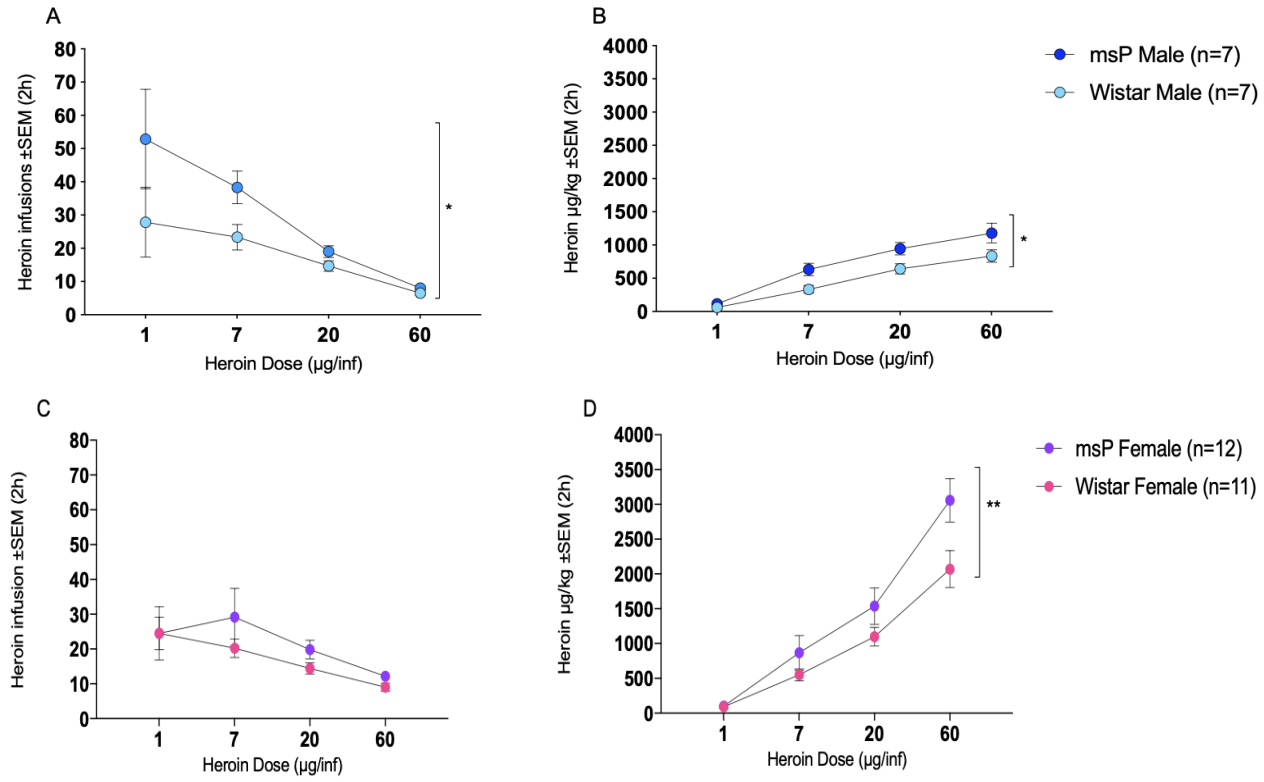

Figure 2: *Heroin dose/response curves in male and female msP and Wistar rats.* ***A****) Both msP and Wistar male rats self-administered increased number of infusions at decreasing heroindoses. MsP self-administered a higher number of infusions than wistar independently of heroin dose, with the fork between the two lines getting larger at lower doses.* ***B****) The heroin intake in msP and Wistar male rats normalized by rat's body weight increased at increasing doses in both lines. sPs showed higher intake independently of the heroin dose, with the fork between the two lines getting larger at higher doses.* ***C****) The number of infusions earned by msP and Wistar female rats decreased at increasing doses, with no difference observed between the two lines.* ***D****) The heroin intake in msP and Wistar female rats normalized by rat's body weight increased at increasing doses in both lines, withsP showing higher intake independently of the heroin dose, and the fork between the two lines getting larger at higher doses. Data are expressed as mean ± SEM of heroin infusions or intake averaged over the four days of HSA. Statistical significance:*
**p<0.05 and **p<0.001 expresses the line overall effect indicated by ANOVA.*

4.2 Heroin dose-response curve in male and female, msP and Wistar rats under progressive ratio (PR) schedule of reinforcement

One Wistar female rat was excluded from the analyses because the BP reached for the doses of 20 and 60 µg/infusion was identified as outlier by ROUT analysis (Q=0.1%), therefore only 10 female Wistar rats were considered for further analyses.

Three-way ANOVA revealed an overall effect of the doses [$F(3, 96)=7.77$, $p<0.0001$], sex [$F(1, 32)=23.16$, $p<0.0001$] and line [$F(1, 32)=11.31$, $p<0.01$]. There was an overall sex by dose interaction [$F(3, 96)=4.27$, $p<0.01$], dose by line interaction approached but did not reached statistical significance [$F(3, 96)=2.48$, $p=0.06$]. sex by line [$F(1, 32)=0.92$, $p>0.05$] and sex by line by dose interactions were not significant [$F(3, 96)=2.16$, $p>0.05$]. These results are consistent with a different trajectory of dose related break-points in male and female rats and with a higher break point expressed by male rats (**Figure 3**).

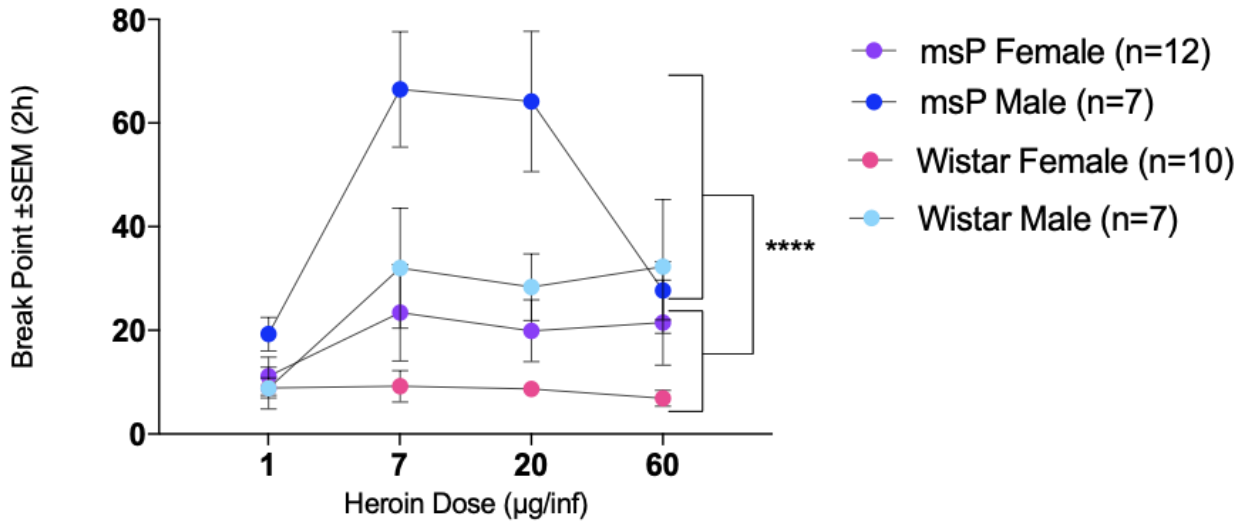

Figure 3: *Dose/BP curve in PR sessions at different doses of heroin in male and female msP and Wistar rats. Male rats tended to show a higher motivation for heroin than females, independently by line. Within each gender, the msPs express a stronger motivation for heroin compared with Wistar controls, consistently with results obtained in experiment 4.2 Statistical significance : ****p<0.0001 indicates overall effect of sex*

To further evaluate line difference in heroin dose/break point within each sex, we analysed the two sex independently.

ANOVA of the Breakpoint reached by male msP and Wistar rats revealed an overall effect of the dose [$F(3, 36)=9.5$, $p<0.0001$], the effect of line was not significant [$F(1, 12)=3.9$, $p=0.07$], however, there was a dose by line interaction [$F(3, 36)=3.467$, $p<0.05$] Post-hoc comparison indicated that msP showed higher BP at the dose of 7 and 20 µg/infusion ($p< 0.05$) (**Figure 4A**). Analysis of the dose/BP curve in female msP and Wistar rats revealed an overall effect of line [$F(1, 20)=8.96$, $p<0.01$] but no effect of heroin dose [$F(3,60)=0.4$, $p>0.05$] and no dose by line interaction [$F(3, 60)=0.5$, $p>0.05$]. These results are consistent with a higher BP reached by msP female rats independently of heroin dose (**Figure 4B**)

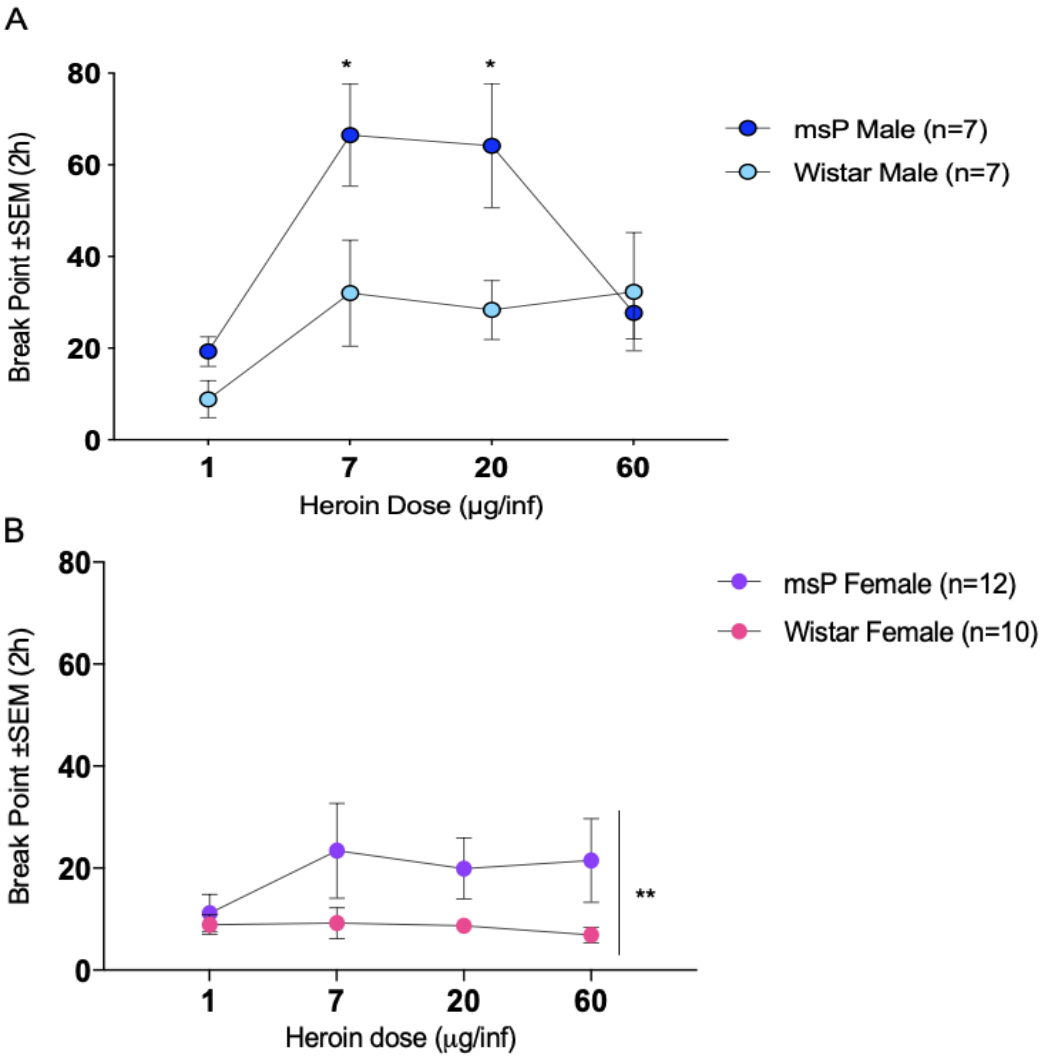

Figure 4: *Heroin dose/BP curves in male and female msP and Wistar rats. (**A**)Male msP rats showed a higher breakpoint compared with Wistar controls in particular at middle doses, following a classical inverted U-shape. (**B**) Female msP rats, consistently with males, reached a higher breakpoint at all doses tested and the difference appears to remain constant across increasing doses. Data are expressed as mean ± SEM of Breakpoint reached . Statistical significance:*p<0.05 expresses the dose by line interaction, **p<0.001 indicates the overall effect of the line set out by ANOVA.*

DISCUSSION

In here we hypothesized that individual susceptibility to stress is an important risk- and predictor - factor for the development of OUD.

Marchigian Sardinian alcohol preferring rats are genetically selected for binge alcohol drinking associated with many behavioral traits that well fit with the human alcoholic condition like anxious phenotype and depressive-like symptoms, with substantial predictive, face and construct validity (Ciccocioppo R et al, 2006; Hannson C et al, 2006; Ciccocioppo R, 2012).

Alcoholism is often associated with other forms of drug abuse, suggesting that common innate predisposing factors may confer vulnerability to addiction to diverse substances. It suggests that animals genetically more prone to excessive alcohol drinking could be also more vulnerable to other addictive drugs. Previous study of Bifone A. and colleagues (Bifone A. et al, 2018) had demonstrated that msP rats showed an high propensity to escalate cocaine intake following long-access conditions (6 hours) compared with Wistar controls. Under psychostimulant challenge, msP rats line showed also an high reactivity of the dopamine system in the extended amygdala, a network innervated by the mesocorticolimbic dopamine system, also known as reward circuitry, suggesting that this mechanism could make them more responsive to many addictive substances. Also the hypothesis that the high stress vulnerability could have a causal role in their propensity to develop many form of OUD has been demonstrated using other formof stressed animal model: Shaham and colleagues demonstrated that rats subjected to daily immobilization stress and presented fentanyl in their home cage drinking water, increased their fentanyl preference compared with unstressed rats (Shaham Y et al, 1992)

In light of this evidence, and for the first time, we want to use msP as a model of OUD vulnerable phenotype comparing their hypothesized heroin addiction propensity with the one of the non selected counterparts, the Wistar rats.

At first we had compared heroin taking between the two lines using an intravenous self-administration procedure: in comparison with other models of addiction (based on forced consumption, like dissolving drug in tap water), this procedure provides the most direct point-to-point correspondence with addictive behavior that occurs in the natural environment. For this reason, these methods have a high degree of face validity and it is considered aprocedure in which the operant response of the animal is reinforced by the rewarding effect ofthe drug (Panlilio LV. Goldberg SR, 2007).

To investigate the sensitivity of the two lines at the heroin rewarding and addictive effects, the use of a single heroin dose (generally the classical training dose) could result insufficient to capture line and gender specific differences. So, we had decided to expose animals to different doses in order to assess a dose/response curve of heroin self-administration. The range of doses chosen (1,7, 20 and 60 µg/infusion) is reported in literature as able to maintain operant responding in rats in short-access self-administration sessions in a dose-dependent manner (Martin TJ et al, 1996; Wade CL et al, 2015). When data are reported as the number of infusions earned versus heroin doses, the curve obtained is an inverted U-shape, typical of many opioids inself-administration procedure. It has been demonstrated by Wade CL and colleagues in a study in which they exposed rats to self-administer increasing doses of heroin, fentanyl, oxycodone and buprenorphine in order to determine the range of doses that support self-administration: the animals reduced responding at higher doses, consistent with a titration of opioid dosing to maintain similar brain levels. Considering our results expressed as number of rewards, the rangechosen appears to be already on the descending limb of the curve that is similar to what MartinTJ reported in a study (Martin TJ et al, 1996): he compared the infusions earned by rats in a within versus between dose-response curve of heroin self-administration in the range from 18 to
100 µg/kg/infusion and found a descending curve; if we report our dosage expressed in µg/infusion to µg/kg/infusion the dose interval detected is quite similar. The only exception was represented by the female msP rats whose dose/response curve maintained an inverted U-shape form with the middle dose of 7 µg/infusion supporting the highest level of responding.

Both msP males and females self-administered a higher number of infusions compared with Wistar controls independent by heroin doses even if, in females, this difference did not reach the statistical significance.

However, considering the differences in body weight between the two lines we had normalized the heroin intake to rat's bodyweight and the results obtained confirmed our hypothesis that, in both genders, msP rats consumed a higher amount of heroin compared with Wistar controls and these differences increased proportionally with the doses. So their stress vulnerability seems to play a crucial role in making them more prone to self-administer heroin.

Plotting together data of both genders merge a statistically significant tendency of females to self-administer more heroin compared with male, independently by line indeed female Wistar rats consume a higher amount of the drug compared also by msP males. In this case, the innate stress reactivity of the genetically selected line had to cope with the influence of the sex on develop SUD. The combination of the two predisposing factors is, surely, the reason why female msP turned out to be the subgroup that showed the higher intake at all doses.

Surprisingly there are few preclinical data related about the influence of gender differences in the abuse liability of the opiates. More in general, from a neurobiological point of view, among female rats and also humans there is the tendency to experience the shift in loss of voluntary control to compulsive drug use, more rapidly than males (Becker et al., 2012; Perry et al., 2016).

There is a reduction in nucleus accumbens dopamine release that is thought to be what allows the dorsal striatum to assume control of the addict's behavior, thereby transforming the drug taking into a compulsive behavior. Lynch WJ end colleagues (Lynch WJ et al, 1999) demonstrated that female rats acquired both heroin and cocaine self-administration more rapidly,than did males.

Cicero TJ in his laboratories deeply investigated this focal point and demonstrated that (Cicero TJ et al, 2003) comparing male and female heroin dose-response curve in Sprague Dawley rats. Our results are consistent with his findings : in fact he demonstrated that females took a much larger absolute amount of heroin as intake, at all doses tested but , in his study, the magnitude of this difference is striking at the lowest dose.

Conversely in the dose/response curve that we obtained, the sex difference seem to be amplified at the highest dose at which msP female rats took more than threefold as much as heroin compared with males. We can reasonably suppose that the reason is a combination of the genetically determined stress vulnerability, innate in msP line, the sex-related higher behavioral response to environmental stressor and hormonal related factor, in particular gonadal and ovarianhormones.
The lack of great sex difference in the amount of heroin taken at lower doses suggested that isnot a matter related to enhanced ability in detecting drug but probably the differences is in the response to the rewarding effects of the heroin, that in msPs act as functional antagonist of the hyper activated CRF1 receptor system, and the hormonal dimorphic state between male and females.
Cicero TJ in fact in the conclusion of his paper underlines that there were sex-differences in the number and in the distribution of opioid receptors in sexually dimorphic brain areas and also that sex steroid are involved in the modulation of opioid receptor populations and in the development of these gender-specific brain regions (Cicero TJ et al, 2002).
In here, we had also investigated if the msP addiction-like behavior also includes an increased motivation to take the drug, in other words if they are able to work harder to get a dose than non selected Wistar controls.
The appropriate protocol to observe this behavior is there the progressive ratio schedule of reinforcement, an operant procedure that allows to measure the maximum amount of work an animal is willing to carry out in order to obtain a reward. In our experiment we wanted to evaluate in the motivation for the drug changed in a dose-depended manner and depending on sex/rat line.
When using a within session PR schedule, the most common index of performance is the so-called "breaking point" (BP) , defined as the highest response rate accomplished to obtain a single reinforce (Sanchis-Segura C and Spanagel R, 2006): the higher is the breakpoint , the stronger is the motivational property or the rewarding potency of the drug. The dose-response procedure allows us to assess the relative responding efficacy of each dose.
In AUD study, male msP rats show a higher motivation for alcohol compared with Wistar counterparts (Domi A et al, 2019).

Heroin doses are the same tested under FR1 contingency: also in term of motivation to obtain the reward, msP male rats showed at high response following an inverted U-shape with an increased BP at the middle doses of the dose-effect function compared with Wistar controls whereas at the dose of 1 µg/inf. The difference in motivation is canceled suggesting that it is too low to sustain motivation in lever pressing to obtain too little reward and the same at the highest dose, in which the animals could obtain an enough rewarding effect also with few numbers of infusion.

As hypothesized, the vulnerability of the msP phenotype conferred them a higher motivation to take the drug, both in male and in females, compared with non-selected Wistar controls.

Also in the analysis of the motivation to work for heroin there is an evident gender-difference but unexpectedly and contrary to the literature, female rats (independently by line) reached a lower breakpoint compared with males at all doses tested (with the only exception of 1 µg/inf that results to much low reinforcing to sustain high number of lever press and so to detect some differences between groups).

In literature, there were some studies assessing that females acquire morphine and heroin self-administration faster and show a higher motivation to self-administer morphine and heroin than do males, with the more evident differences revealed at the higher doses (Lynch and Carroll,1999; Cicero TJ et al., 2003). To justify this contradictory result, it is important to remember thatfor laboratory animals there are behavioral, experimental and procedural effects that interact with biology response of the two sex with different affect.

In here, we cannot give a clear explanation of this discrepancy that require further investigations; we cannot also exclude that the different results obtained are also affected by different experimental protocol applied in these 2 study: in his study Cicero Tj used Sprague Dawley rats and a different progressive ratio scale (4,8,16,32,64,128…) with an heroin SA training phase under FR4 ratio. Due to this latter procedure applied in the training phase, it could be possible that rats of that study were already used to press more than one time to obtain a single reward than ours that are trained under FR1 contingencies and also the different ratio applied during the PR could create some bias in the interpretation of the discrepancy of results obtained.

An other caveat could be the N of the sample: Cicero TJ used a very large number of rats (N=24/sex) for his experiment and so, given the overlap in the frequency distribution of the breakpoint, it could be reasonable to suggest that using fewer animals, as us, is difficult to detect the differences observed by that previous work between the two sexes.

It is obvious that to better understand how gender differently affects the heroin taking and the motivation to work for the drug further investigations are needed but in here we had clearly demonstrate that, in comparison with a non selected line, msP rat could be a validated model of vulnerability to poly-substance abuse, due, in particular, to their innate propensity to self-medicate a negative affective state (linked to anxiety- and depressive-like symptoms co-segregate with their ethanol binge drinking).

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Chapter 3:

"EFFECT OF CEBRANOPADOL ON HEROIN SELF-ADMINISTRATION AND MOTIVATION IN MALE AND FEMALE MSP RATS"

1. ABSTRACT

The gold-standard pharmacological treatment of opioid use disorder (OUD) is the maintenance therapy with long-acting opioid agonists such as methadone and buprenorphine: despite the well consolidated efficacy, their adverse side effects and their abuse potential limit their security and keep the risk of misuse, tolerance development and respiratory failure. A new strategy in opioid addiction therapy is the use of multi-target drugs: a promising candidate is Cebranopadol, a pan opioid agonist with sub-nanomolar affinity for MOP and NOP opioid receptors already inclinical trials for the treatment of chronic and acute pain. Previous studies had demonstrated its efficacy in reducing cocaine addiction and its low abuse potential. The co-activation of NOP receptor could have a key role also to cope with the stress system: NOP agonists act as functionalantagonists of the CRF1- receptor system. Marchigian Sardinian alcohol preferring (msP) rats is a genetically selected animal model characterized by excessive alcohol drinking co-segregated with hyper stimulation of the CRF1r system and over-expression of NOP receptor in several stress regulatory areas of the brain. Our hypothesis is that this vulnerable phenotype made msP rats potentially more responsive to Cebranopadol treatment. In this study we want to explore the effect of oral administration of Cebranopadol (0.0, 25.0, 50.0 µg/kg) in reducing heroin taking and motivation for heroin at different heroin doses (1, 7, 20, 60 µg/inf) in male and female msP rats. Our results demonstrated that Cebranopadol, at the highest dose, significantly attenuated heroin self-administration (independently by the dose) in both sexes but the dose of 25 µg/kg resulted in more effectiveness in female msP rats. In addition, Cebranopadol is able in decreasingthe motivation for heroin as detected by the reduction of breakpoint measured in the progressive-ratio paradigm in both genders but, contrary to what is seen under FR1 contingency, females were less responsive to the treatment. These results are consistent with the different sensitivity to heroin reinforced behavior previously detected between male and female msP rats and also suggest that Cebranopadol could be a valid pharmacological choice in case of stress and heroin abuse comorbidity.

Keyword: Opioid Use Disorder, alcohol-preferring rat, Cebranopadol, NOP/opioid receptor agonist, stress

2. INTRODUCTION

The gold-standard pharmacological treatment of opioid use disorder (OUD) is the maintenance therapy with long-acting opioid agonists such as the well-known methadone: despite the well consolidated efficacy, their adverse side effects and their abuse potential connected with the similar profile of morphine or heroin, limit their security and keep the risk of misuse, tolerance development and respiratory failure.

More recently, researchers and clinicians focused the attention on the multifunctional "mixed ligand" or "multi-target" drug: one of these, already approved for the treatment of OUD, is buprenorphine. Buprenorphine is a long-acting partial MOP agonist and antagonist of DOP and KOP opioid receptors. For a long time the efficacy of buprenorphine in reducing heroin dependence was obviously attributed to its MOP agonism but many preclinical studies had later demonstrated that this drug is also efficacy in reducing alcohol and cocaine intake but only when given at very high doses : given its high ligand affinity for MOP receptor and that its effect on cocaine is not blocked by naltrexone, the conclusion was that its efficacy on cocaine or alcohol cannot be mediated by mu receptor and is independent from that on heroin (Montoya et al, 2004). In fact, previous work assessed that at high concentration buprenorphine is also able to activate NOP receptor where it acts as a low-affinity agonist: so, the concomitant activation of NOPr not only could explain the mechanism with which it reduce cocaine (Kallupi M et al, 2018) and alcohol (Ciccocioppo R et al, 2007) consumption at high concentration but it is also the reason of its enhancer safety pharmacological profile and lower abuse potential compared with morphine and methadone.

These findings point the attention to new possible strategies in opioid addiction therapy involving NOP receptor stimulation: an interesting candidate was a new pan opioid agonist that activates MOP and NOP receptor with similar potency is Cebranopadol.

Radio-ligand binding assays revealed a subnanomolar affinity for both MOP and NOP receptor and an additional 20 times lower affinity for DOP and KOP receptors (Linz K et al, 2014) conferring it a favorable pharmacological profile with fewer side effects and lower rewarding properties compared with classical opioid agonists.

The risk of abuse-liability of Cebranopadol is associated with its high affinity for the MOP receptor that confer the potential to produce physical dependence and specific withdrawal symptoms after abrupt cessation. But non-clinical data in literature indicate the NOP receptor activation may attenuate some of the MOP side effects, like tolerance development, physical dependence, addiction potential, and respiratory depression conferring a sort of "anti-abuse and protective" effect. (Lutfy K et al, 2001; Ciccocioppo R et al, 2000).

Previous study performed in our laboratory to assess the abuse-liability of Cebranopadol had underlined that it produce a lower conditioned place preference compared with morphine and that, whereas Cebranopadol is able to maintain operant responding in self-administration under FR1 contingency, due to its MOP-receptor affinity, it did not support operant self-administration under progressive ratio contingency meaning that the motivation to work for obtaining the drugis very low.

There is an other published preclinical study carried out both in mice and rats by Tzschentke and colleagues to measure the development of physical dependence and OUD-like withdrawal symptoms after chronic treatment with Cebranopadol, demonstrated that Cebranopadol induced fewer signs of withdrawal compared to morphine (Tzschentke TM et al, 2018).

Taken together, these findings bolster the use of Cebranopadol for pain management in order to avoid opioid physical dependence responsible of the still current opioid epidemic (i.e. fentanyl use as OPR) and also for opioid maintenance therapy to support or replace methadone or buprenorphine treatment.

Cebranopadol is a compound already tested and in ongoing phase II and III clinical trials for cancer pain management and preclinical studies had also assessed its efficacy in reducingcocaine self-administration and motivation (Shen Q et al, 2017) and in blocking conditioned reinstatement of cocaine seeking (De Guglielmo G et al, 2017) in Wistar rats.

The co-activation of NOP receptor could have a key role also in order to coping with the stress system: in fact the activation of NOP by its endogenous ligand or by a synthetic NOP agonist produces anxiolytic-like effects (Jenck F et al, 1997) that appears particularly strong under stressful conditions. This effect depends on its ability to act as functional antagonist of the CRF1 receptor system: antagonism or genetic deletion of CRF1R attenuated stress-induced reinstatement of morphine and heroin seeking (Wang J et al, 2008; Shaham Y et al, 1998). Cebranopadol, by activating NOP receptors, may indirectly antagonize CRF1 mediated actions. Nowadays no preclinical data are published about the efficacy of Cebranopadol in opioid dependence but previous unedited studies of our laboratory had already investigated the effect of Cebranopadol on heroin taking, motivation and seeking in non-selected Wistar male rats demonstrating that oral Cebranopadol pretreatment (0.0, 25.0, 50.0 µg/kg) significantly attenuated drug self-administration independent by heroin doses (1, 7, 20, 60 µg/inf) and it was also able to prevent yohimbine stress-induced reinstatement of heroin seeking after extinction paradigm.

Based on these experimental evidences and considering the stress sensitivity due to hyper-activation of CRF1 receptor system of the msP rat line, we hypothesize the msP rats could represent a valid preclinical model to investigate the effect of Cebranopadol in a vulnerable OUD phenotype represented by msP rat line, as we had also confirmed in the previous Chapter of my PhD dissertation. To support our hypothesis there are also gene expression studies showing that msP rats are also characterized alteration of the opioid system, in particular by an innateover-expression of the NOP receptor system in several stress regulatory areas of the brain, including central amygdala (Ciccocioppo et al, 2019) making them potentially more prone to respond at the effect of Cebranopadol pretreatment.

In the previous experiment we have already compared the heroin dose/response curve in male and female rats, comparing the different sensitivity to the effect of the drug between the two rat lines, msP and Wistar. Statistical analysis revealed that msP rats, both male and female, appeared to be more prone, compared with the non selected counterpart, to develop heroin addiction either in terms of drug taking or of motivation for heroin and that, within the line, female rats are more prone to self-administer greater amount of heroin despite low motivation to work for it (More detailed results in Chapter 2).

In this study, we had partially replicated the experimental procedure and the drug doses previously tested on the Wistar rats: here, we explored the effects of Cebranopadol in reducing heroin self-administration and motivation for increasing dose of heroin (1, 7, 20, 60 µg/inf) in male and female msP rats.

3. MATERIAL AND METHODS

3.1 Animals

Male (n=18) and female (n=24) msP rats bred at the animal facility of the University ofCamerino (Italy)were employed for this work. Male rats weighed 350-390g and female rats weighed 200-240g at the beginning of the experiments.

Rats were pair-housed in plexiglass home-cages in a room with artificial 12/12h reverse light/dark cycle (light off at 7:00 am), constant temperature (20-22°C) and humidity (45-55%). All animals were handled once daily for one week before the beginning of the experiments. During the entire permanence in the vivarium, rats were offered free access to tap water and foodpellets (4RF18 Mucedola, Settimo Milanese, Italy).

Experiments were performed during the dark phase of the light/dark cycle. All procedures were conducted in adherence to the *European Community Council Directive for Care and Use of Laboratory Animals* and the *National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals*.

3.2 Drugs

Heroin (Diacetylmorphine hydrochloride, purchased by SALARS, Como, Italy) was dissolved in sterile physiological saline (0,9% NaCl) and administered intravenously by the rats.

Cebranopadol (Biochempartner Co., Ltd., China) for operant tests was dissolved in 5% DMSO and 95% of glucose (5%) and administered *per os* (p.o.) by gavage at the dose of 25 µg/kg and 50 µg/kg, one hour before self-administration session (Shen Q. et al, 2017). We chose oral administration to mimic the most common administration route in humans in order to have the best translational validity.

MsP rats were trained to the gavage administration procedure for three consecutive days before starting experimental tests, during which they received distilled water to familiarize with this kind of procedure.

3.3 Intravenous surgery

Animals were anesthetized with isoflurane anesthesia: 5% induction and 2% maintenance. A single catheter made from micro-renathane tubing (ID = 0.020", OD = 0.037"; Braintree Scientific) was implanted in the right jugular vein and subcutaneously positioned between the vein and the back between the shoulders. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk sutures. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle. The cannula was inserted in a support made of dental cement and covered with a plastic cap (Kallupi M.et al, 2017).

Immediately after surgery, rats were allowed to recover for 1 week before self-administration training. During recovery, they received antibiotic prophylaxis with Enrofloxacin (Baytril®, Bayer) through the drinking water dissolved at the concentration of 25 mg/ml . Throughout the self-administration training and tests, catheters were flushed daily with 0.1-0.2 ml of heparinized saline solution (Nadroparin calcium 3800 U.I.; Italfarmaco S.p.A, Milan, Italy) containing 1mg/ml of Enrofloxacin. Body weights were monitored on a weekly base At the end of the experiments catheter patency was confirmed with an injection of 0.2-0.3 ml of ThiopentalSodium solution (Pentothal Sodium, 1g/50 ml, MSD Animal Health S.r.l), immediate loss of reflexes was taken as a positive sign of patency.

3.4 Self-administration apparatus

Heroin self-administration was performed in rat operant conditioning chambers (Med Associate St Albans, VT) enclosed in sound-attenuating ventilated environmental cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber with two stimulus lights placed above each lever, and a house light plus a tone generator on the opposite wall. The heroin solution was delivered through a Tygon tube that connected the catheter with an infusion pump. The pump was activated by responses on the right (active) lever and resulted in a delivery of 0.1 ml of fluid. Responses on the left (inactive) lever were recorded but had no

programmed consequences. A windows compatible computer controlled the delivery of heroin solution and recorded the behavioral data.

3.5 Heroin self-administration procedures

Rats were trained to heroin self-administration (HSA) in two-hours daily sessions five days per week. Rats were trained to self-administer heroin under fixed-ratio 1 (FR1) schedule of reinforcement at the training dose of 20 µg/infusion (infusion volume 0.1 ml delivered over 5 seconds) until reaching a stable baseline. A 20s time-out period (TO) started contingently with heroin infusion. During TO active lever presses were not reinforced with additional injections.

3.6 Experimental procedures

After self-administration training, Considering their heroin intake in the last three days of heroin self-administration, msP rats were divided in two balanced cohorts in which Cebranopadol was tested under FR1 (n=10)or PR schedule of reinforcement (n=8). The same experimental protocol was applied in a separate group of female msP rats (n=24) obtaining two balanced cohorts for testing the effect of Cebranopadol under FR1 and PR schedule of reinforcement.

3.6.1 Effect of Cebranopadol treatment on heroin self-administration in male and female msP rats

Ten male and twelve female msP rats were employed in this experiment. After rats had acquired a stable self-administration training, they were divided into four sex-balanced subgroups, each allocated to one heroin self-administration dose (1, 7, 20, and 60 µg/inf). After one week of baseline the effect of cebranopadol (0.0, 25.0, 50.0 µg/kg) on heroin self-administration was tested. One hour before tests, rats received gavage administration of one dose of Cebranopadol or its vehicle in counterbalanced order. Tests were repeated every third day: on the first intervening day rats remained in their home-cage and on the second day they were subjected to a baseline heroin SA session. When rats had received all the doses of cebranopadol and its vehicle,the heroin dose self-administered was changed and the effect of cebranopadol on the new heroin

dose was tested as described above. Tests continued until cebranopadol was tested on each heroin dose in all rats.

3.6.2 Effect of Cebranopadol on motivation for heroin in male and female msP rats.

Eight male and twelve female msP rats were employed in this experiment. The procedure was identical to as described in section 3.6.1 except that on Cebranopadol test day rats performed the heroin self-administration session under a progressive ratio (PR) schedule of reinforcement. In PR sessions, the response requirements necessary to receive a single heroin infusion increased after every infusion according to the following order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268 etc.. (adapted from de Guglielmo et al, 2015; Richardson and Roberts, 1996). The session stopped if the required number of responses was not achieved within one hour from the last reinforced response. The last ratio completed is defined as the break point (BP) and is considered as a direct measure of rat's motivation for the drug (Sanchis-Segura C and Spanagel R, 2006).

3.7 Statistical analysis

The effect of Cebranopadol on heroin infusions earned under FR1 contingency and on the break point under PR contingency were analyzed by two way ANOVA with doses as within-subjects factor and sex as between subjects factor. ANOVA was followed by Dunnett's post hoc analysis when appropriate significant difference was conventionally set to $p<0.05$. Heroin doses were analyzed separately.

4. RESULTS

4.1 Effect of Cebranopadol treatment on heroin self-administration in male and female msP rats

Three males and two females lost catheter patency before completion of tests, and they were therefore excluded from the analyses of the heroin doses that they did not complete. Therefore 7 males and 10 females were included in the analyses of heroin 7 and 1 µg/infusion doses.

4.1.1 Effect of cebranopadol on 1µg/infusion heroin self-administration.

ANOVA revealed an overall effect of Cebranopadol treatment [$F(2, 30)=38.94$, $p<0.0001$] but no sex [$F(1,15)=4.036$, $p>0.05$] and no treatment by sex interaction [$F(2, 30)=0.3454$, $p>0.05$]. These results are consistent with a dose dependent decrease in heroin infusions observed in both male and female rats (**Figure 1A**).

4.1.2 Effect of Cebranopadol on 7µg/infusion heroin self-administration.

ANOVA found an overall effect of Cebranopadol treatment [$F(2, 30)=50.62$, $p<0.0001$] and no sex effect [$F(1,15)=1.94$, $p>0.05$], but there was a significant treatment by sex interaction [$F(2, 30)=8.95$, $p<0.001$]. Dunnett's post hoc analysis revealed that both doses of Cebranopadol reduce heroin infusions in female rats whereas only the higher dose was effective in male ($p<0.0001$) (**Figure 1B**).

4.1.3 Effect of Cebranopadol on 20µg/infusion heroin self-administration.

ANOVA found an overall effect of Cebranopadol treatment [$F(2, 40)=28.37$, $p<0.0001$] and no sex effect [$F(1,20)=1.66$, $p>0.05$], and no treatment by sex interaction [$F(2, 40)=2.65$, $p>0.05$]. These results are consistent with a dose dependent decrease in heroin infusions observed in both male and female rats (**Figure 1C**).

4.1.4 Effect of Cebranopadol on 60µg/infusion heroin self-administration.

ANOVA found an overall effect of Cebranopadol treatment [F(2, 40)=28.58, p<0.0001] and no sex effect [F(1,20)=0.46, p>0.05], and no sex by dose interaction [F(2, 40)=3.15, p>0.05]. These results are consistent with a dose dependent decrease in heroin infusions observed in both male and female rats. However, data observation suggested that the dose of 25µg/kg of Cebranopadol could have been not effective in male, and the sex by treatment interaction was only slightly higher than the conventional threshold (p=0.054). Therefore, although a lack of sex by treatment interaction would not normally be followed by a post-hoc analysis, we decided to run Dunnett's post hoc test anyway. In line with our impression, Dunnett's analysis confirmed that 25µg/kg of Cebranopadol did not decrease 60µg/infusion heroin self-administration (**Figure 1D**):

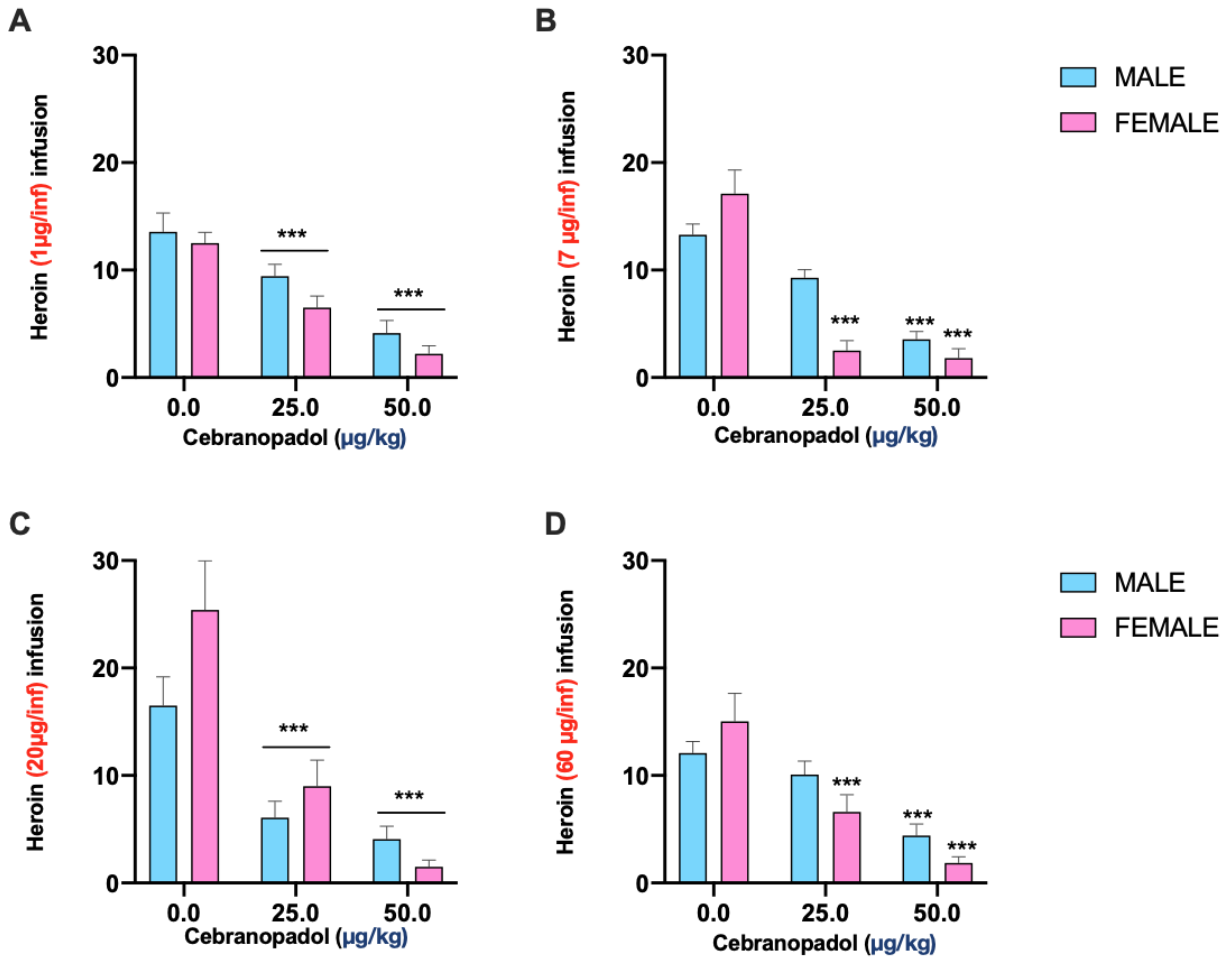

Figure 1. *Effect of Cebranopadol on self-administration of four different doses of heroin **A)** Cebranopadol dose dependently decreased 1µg/infusion heroin self-administration in both male*

and female msP rats. ***B****) both doses of Cebranopadol decreased 7µg/infusion heroinself-administration in female msP rats while only the higher dose was significantly effective in male rats.* ***C****) Cebranopadol dose dependently decreased 20µg/infusion heroin self-administration in both male and female msP rats.* ***D****)Cebranopadol dose dependently decreased 60µg/infusion heroin self-administration in female msP rats; only the higher dose wassignificantly effective in male. Data are expressed as mean ± SEM of heroin infusion. Significantdifferences: : ***p<0.001 vs vehicle same sex* ***p<0.0001 ANOVA overall effect of treatment.*

4.2 Effect of Cebranopadol on motivation for heroin in male and female msP rats.

After the exclusion of rats whose catheter lost patency before completion of the experiments, analyses included 11 females in the analysis of heroin 20µg/infusion and 10 females in each of the other heroin doses. Males included in the analyses were 5 for heroin 1 µg/infusion and 20µg/infusions doses and 6 for the remaining doses.

4.2.1 Effect of Cebranopadol on BP for 1µg/infusion heroin.

ANOVA revealed an overall effect of Cebranopadol treatment [$F(2, 26)=30.57$, $p<0.0001$] and no overall effect of sex [$F(1,13)=0.14$, $p>0.05$] or treatment by sex interaction [$F(2, 26)=1.34$, $p>0.05$]. These results are consistent with a decrease in BP for heroin observed in induced by both Cebranopadol doses in both male and female rats (**Figure 2A**).

4.2.2 Effect of Cebranopadol on BP for 7µg/infusion heroin.

ANOVA found an overall effect of Cebranopadol treatment [$F(2, 28)=34.73$, $p<0.0001$] and no sex effect [$F(1,14)=1.59$, $p>0.05$], but there was a significant treatment by sex interaction [$F(2, 28)=5.77$, $p<0.01$]. Dunnett's post hoc analysis revealed that both doses of cebranopadol reduced BP for heroin ($p<0.001$) (**Figure 2B**). We run further investigation by Bonferroni's to understand

what caused the sex by treatment interaction, and we found than male rats reached a significantly higher break point than males under vehicle treatment (**Figure 2B**).

4.2.3 Effect of Cebranopadol on BP for 20µg/infusion heroin.

ANOVA found an overall effect of Cebranopadol treatment [$F(2, 28)=11.36$, $p<0.0001$] and sex [$F(1,14)=13.33$, $p<0.15$], and treatment by sex interaction [$F(2, 28)=4.54$, $p<0.05$]. Dunnett'spost hoc analysis revealed that the dose of 50µg/infusion decreased BP for heroin in male rats ($p<0.001$). Despite a clear trend to decrease BP in females can be observed, this effect was not significant according to Dunnet's. (**Figure 2C**). We further investigated male-female differences by Bonferroni's and we found that males showed higher BP than females both under vehicle and Cebranopadol 25µg/kg treatments (**Figure 2C**).

4.2.4 Effect of Cebranopadol on BP for 20µg/infusion heroin.

ANOVA revealed an overall effect of Cebranopadol treatment [$F(2, 28)=7.38$, $p<0.001$] and no overall effect of sex [$F(1,14)=0.53$, $p>0.05$] or treatment by sex interaction [$F(2, 28)=1.71$, $p>0.05$]. These results indicate a decrease in BP for heroin induced by both Cebranopadol doses in both male and female rats. However, data observation suggested that the 25µg/kg Cebranopadol dose was not effective in females. Therefore, although a lack of sex by treatment interaction would not normally be followed by a post-hoc analysis, we decided to run anyway Dunnett's test anyway, and in line with observed data we found that only the dose of 50µg/kg decreased BP in female rats (**Figure 2D**).

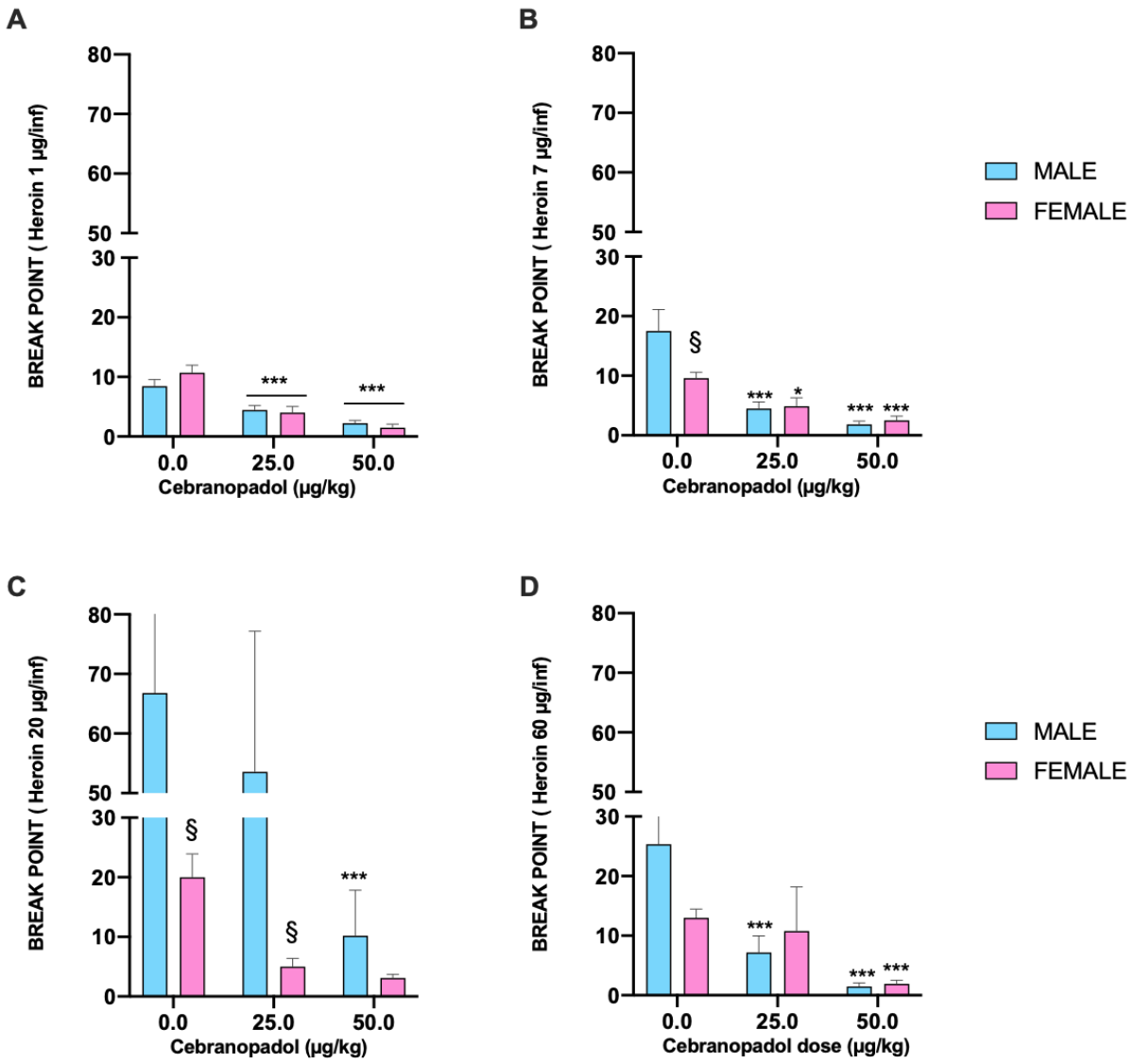

Figure 2: *Effect of Cebranopadol on motivation for heroin express as Breakpoint reached under Progressive ratio schedule of reinforcement.* ***A****) Both doses of Cebranopadol reduced BP for 1µg/infusion heroin dose in both male and female rats.* ***B****) Female rats showed lower BP for 7µg/infusion heroin dose than male. Both doses of Cebranopadol reduced BP in both sexes.* ***C****) Female rats showed lower BP for 20µg/infusion heroin dose than male. 50µg/kg of Cebranopadol reduced BP in male rats. The reduction observed in females was not statistically significant.* ***D****) 50 µg/kg of Cebranopadol reduced BP for 20µg/infusion heroin dose in bothsexes, while 25µg/kg of Cebranopadol was effective only in males. Data are expressed as mean ±SEM of Data are expressed as mean ± SEM of BP. Significant differences: ***p<0.001 ANOVA overall effect of doses; *p<0.05 and ***p<0.001 vs vehicle same doses;. §p<0.05 vs male same dose.*

5. DISCUSSION

In the previous chapter, we had demonstrated that the msP rat line could represent a validated preclinical model of vulnerability to develop OUD. Here, we want to test a promising molecule, Cebranopadol, that is a pan opioid agonist with nanomolar affinity for both MOP and NOP receptor, in heroin taking and motivation for heroin in this vulnerable rat line.

Our hypothesis is supported by literature: converging evidences assess that a NOP agonist (like Cebranopadol) acts as functional antagonist on the CRH-R1 system (Jenck F et al, 1997; Wang J et al, 2018; Shaham Y et al, 1998) and so could have a great efficacy in reducing the innate hyper activation of the CRH-R1 detected in msP rats (Hannson AC et al, 2006); moreover their NOP system over-expression (Ciccocioppo R et al, 2019) suggests that this line has also the potential to be very responsive to the effect of Cebranopadol treatment. Indirectly, our findingsare also confirmed by Kallupi M et al (2017) that affirmed that the genetic deletion of theN/OFQ receptor in rats causes aversion to self-administering many substances of abuse, like cocaine and heroin.

We tested Cebranopadol on male and female msP rats under FR1 and PR contingency at the four heroin doses that, in the previous Chapter, we have demonstrated to support heroin self-administration in msP ratline.

Results demonstrated that the high dose of the drug is able in reducing heroin taking both in male and female msP rats at all heroin doses; in the female group the treatment is so efficacious that it almost gets to block the active lever presses. However the selectivity of the compound for the addictive drug has already been demonstrated in previous works: De Guglielmo G. (De Guglielmo et al, 2017) observed that Cebranopadol did not impair locomotor activity in the conditioned place preference test and also it did not reduce sweetened condensed milk self-administration; concomitantly in our laboratory Shen Q (Shen Q et al, 2017) confirmed the substance-specific action of the drug testing it in saccharin self-administration and the lack of motor impairment with CPP. In both cases Cebranopadol doesn't reduce the natural reward intake, indeed it showed a tendency to increase the consumption of sweet solution due to its ability to activate MOP receptors.

These findings gave us the support to assess the specificity of the effect of Cebranopadol on the reduction of heroin consumption. Moreover it represent another advantage with respect to other treatment now approved for OUD: in fact this selectivity is not shown in Buprenorphine that slightly decreased both saccharin and glucose self-administration in rats (Carroll ME and Lac,1992) and monkeys (Carroll ME et al, 1992) and is often associated with a decrease in locomotor activity, like methadone too (Marquez P et al, 2007).

In male msP rats Cebranopadol (at the dose of 50 µg/kg) markedly reduced heroin intake at lower and intermediate heroin doses but its efficacy decreased at increasing doses (in particular at dose of 60 µg/inf). These findings replicate results of a previous experiment performed in our laboratory testing Cebranopadol on heroin self-administration at the same doses but on male Wistar rats: in both cases the highest dose of heroin resulted less affected by Cebranopadol pretreatment. The possible explanation is that the doses of Cebranopadol chosen in our experiments have not been high enough to reach a sufficient level of MOP receptor occupancy tosurmount the effect of the highest dose of heroin. This justification finds support in literature: there were a similar clinical result (Comer SD et al, 2005) related to the effect of buprenorphine on heroin-dependent volunteers that lost its efficacy at high dose of intranasal heroin tested (100 mg): in this study authors affirmed that it is necessary the occupancy of almost 80/90% of the µuopioid receptor in order to obtain a significant reduction in heroin-induced effects.

The efficacy of Cebranopadol on female msP rats is strongly more pronounced and also the lower dose of 25 µg/kg reduced heroin intake at all doses tested and without dose-dependency. One reasonable explanation can be found in their higher propensity to self-administer great amount of heroin that we have previously shown in Chapter 2, and that is here confirmed by the high number of infusions earned by the female vehicle group , compared with the male one, that replicate as well, the dose-response curve presented above: considering the high affinity of Cebranopadol for µu receptor, it make sense that female rats are also more responsive to the treatment and not only to the addictive drug, compared with the male counterpart. The lack of dose-dependent effect of Cebranopadol on female msP rats suggests testing lower dose of the drug (i.e 12.5 µg/kg) on them to better individuate a dose/response curve of this drug.

In here we also evaluated the effect of Cebranopadol on motivation for heroin express as reduced BP: as discussed above, the efficacy of the drug seems to be reduced at increasing heroin doses. Unlike the highest sensitivity to the treatment in reducing heroin intake at all doses tested, female msP rats appear to be less responsive the effect of Cebranopadol in reducing their motivation for heroin : the effect of the treatment is reduced with increasing heroin dose and was statistically significant only when tested at the highest dose (50 µg/kg) instead under FR1 contingency all Cebranopadol doses were able to suppress heroin taking. Basing on our previous finding, we can hypothesize that this pharmacological limited efficacy is directly related with the lower motivation of female msP rats, compared with their male counterpart, demonstrated in the heroin dose/BP curve and that is confirmed here by comparing the Breakpoint reaches by themale and female vehicle groups of the present experiment.

A caveat to discuss is its partial agonistic activity on KOP receptor : at higher concentration in fact, Cebranopadol activate KOP and DOP receptor, differently to buprenorphine that is a KOP and DOP antagonist (Linz K et al, 2014; Huang P et al, 2001). Activation of the KOP receptor is associated with dysphoria , enhanced anxiety and pro-addictive behaviors (Grella SL et al, 2014). However Cebranopadol, in the CPP, neither produced significant place preference, suggesting its low abuse liability, nor caused place aversion unlike pure KOP agonists (Shen Q et al, 2017; Tzschentke TM et al, 2019). The risk using a n animal model of stress-vulnerability for this study, like the msP rat lines, is the the KOP agonism of Cebranopadol could exacerbate their anxious phenotype counteracting the positive effect in reducing heroin SA. Results obtainedconfirm that Cebranopadol appears to be devoid of negative affective properties.
There were no studies available in literature that investigated the effect of other treatments for OUD, like for example buprenorphine, on female rats and so we had no possibility to compare our data with other preclinical evidence.

All the studies on buprenorphine, either on heroin or on cocaine dependence, were carried out only on male rats and before us no one had investigated the effect of these treatments in both genders. Based on now available literature, compared to already approved medications formaintenance therapy in OUD (i.e., buprenorphine, methadone), Cebranopadol may offers same important advantages such as lower abuse liability, no impairment in locomotor activity or respiratory depression, reduced side effects , a long half-value duration (14-15 h) (Kleideiter E et al, 2018): considering our finding we may speculate that it will be a valid pharmacological choice in case of stress and heroin abuse comorbidity and we also investigate for the first timeits efficacy on both sexes.

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Chapter 4:

INDIVIDUAL VARIABILITY IMPACTS ON THE DEVELOPMENT OF OPIOID USE DISORDER: behavioral characterizationof the NIH_Heterogeneous stock rats on heroin dependence

1. ABSTRACT

Opioid use disorder (OUD) is a neuropsychiatric disease that arises in a subset of individuals with a vulnerable phenotype that pre-exist the first exposure to the drug. But how individual variations in multiple behavioral traits may interact and contribute to shape an OUD-vulnerable or resilient phenotype is a challenging question to deal with. For this purpose we had characterized the heroin addiction behavior of 800 NIH_Heterogeneous stock (HS) rats that is anoutbred rat line so that each individual rat is genetically and phenotypically distinct from the others in order to best mimic the individual variability of human population and to identify different subpopulation with varying degrees of opioid vulnerability.

All the experiments were performed in two geographically distinct laboratories: first of all, rats were subjected to a battery of behavioral test to scored their basal locomotor activity (with open field test), anxiety-like behavior (with elevated plus maze test), reactivity to acute thermal pain and sensitivity heroin analgesic effect (with tail flick test). The heroin-related behavioral screening includes heroin self-administration under long-access condition, progressive ratio,heroin-primed reinstatement and cue-induced reinstatement after an extinction phase. From thesemultivariate measurements we selected seven parameters most representative of the OUD (heroin taking-refraining-heroin seeking) in order to establish how these factors interact with each other determining a heroin vulnerable or resilient phenotype.

A rat-rat similarity network was constructed and the Bayesian stochastic block model (SBM) was applied to identify, in this network of data, behaviorally distinct clusters with different levelsof opioid vulnerability. Setting the number of clusters at K=3 we obtained the most parsimonious representation of the data without overlapping between the subpopulations.

Analyzing how each cluster differed in each of the seven behavioral parameters selected, we were able to identify cluster 1 as vulnerable to OUD-like behavior because its scores, in all the behavioral measures, are higher compared to cluster 2, defined as intermediate, and cluster 3, defined as the resilient one. The analysis of the gender distribution into each cluster revealed that, independently by cluster allocation, female rats reached a higher score of total heroin intakeand cued active lever press than males.

In conclusion, we had compared the pre-heroin behavior of the rats with their cluster allocation in order to detect some correlation that could be predictive of the vulnerability or resilience to develop an OUD-like phenotype: we are so far to be able in identifying which are the innate predisposing factors that determine addiction vulnerability but the results of the ongoing GWAS analysis could provide insights into the genetical underpinning at the basis of the OUD vulnerability.

Keywords: Opioid use disorder, clustering, Bayesian model, stochastic block model, network analysis, community detection, individual differences, addiction vulnerability, heroin dependence.

2. INTRODUCTION

Current predominant theories define addiction as a pathological response to drug exposure that is generated in a few individuals by a vulnerable biological phenotype that pre-exist the first exposure to the addictive substance. This is an intrinsic predisposed state that derives from a combination of multiple variable traits with biological, genetic and environmental roots that taken together, contribute to shape a vulnerable phenotype that results more prone than others to precipitate into the addiction cycle (Le Moal M. 2009). However, relatively little is known aboutfactors that drive or protect people from developing addiction: i.e., confer resilience. The term “resilience” refers to protective factors that help individuals to successfully cope with or overcome exposure to significant risk, adversity, or potentially harmful environments. These factors may include personality traits, attitudes and neural systems that are able to compensatefor adverse exposures (Ersche KD et al, 2020).

From this perspective, the standardized use of an inbred rat's strain in preclinical research, that guarantee the experimental reproducibility of the study, is not a good choice to analyze the individual differences in the development of addiction behavior because these lines are characterized by genetic homogeneity derived from inbreeding (Parker CC et al, 2014). In chapter 2 we have tried to analyze the role of the stress vulnerability in enhancing the risk to develop some of the factors associated with OUD, like heroin taking, sensitivity to different heroin doses, and motivation to obtain the drug, using a selected rat line, the Marchigian Sardinian alcohol preferring rats (msP) that had an innate predisposition for binge drinking and in which genetic traits, associated with their predisposition to high alcohol consumption, were co-segregate with a stressful and depressive-like phenotype (Ciccocioppo R, 2006) .

Animal models like this one, for sure, could be useful to focus separately on the role of one or two genetic or behavioral factors that could influence the vulnerability to develop drug addiction,but still remain a limited approach to mimic the real multifaceted aspect of a OUD-prone or resilient phenotype. It is also true that the diagnosis of OUD had to match neither with only one neither with everyone of the diagnostic criteria listed in DSM-V, but to be defined addicted a

person has to respond to a sub-cluster of these parameter that could differ between one person to another, despite the fact that both of them resulted addicted (APA, 2013).

In order to extend the number of variables considered in the analysis of opioid addiction vulnerability and to take into account the great role of the individual differences that could be predictable of a vulnerable or resilient phenotype, we have chosen a preclinical model that could best mimic the variability of human population and so, could have a significant translational validity also in the perspective of personalized therapies (we will take a deeper look into this topic in the next Chapter).

The choice fell on the NIH heterogeneous stock rats , an heterogeneous rat population that represent a random mosaic of the eight inbred progenitor strain and was obtained by a random rotational breeding strategy for 60 generations, to minimize the extent of inbreeding: Agouti (ACI/N), Brown Norway (BN/SnN), Buffalo (BUF/N), Fischer 344 (F344/N), Maudsley Reactive (MR/N), M520/N, Wistar Nettleship (WN/N) and Wistar Kyoto (WKY/N). As a result each animal is genetically and phenotypically distinct from the others in order to closely resemble the variation found in the human population (Solberg Wood LC and Palmer AA, 2019).

This behavioral characterization of 800 individual rats in term of multidimensional traits associated with addiction was carried out in two distinct laboratories: our laboratory under the supervision of the professor Ciccocioppo R. in the University of Camerino (Unicam), and the other at the Medical University of South Carolina (MUSC) in the USA of the professor Kalivas P. This strategy is adopted in order to keep in consideration the role of the environment on the individual behavior. In fact the response of an animal to an experimental protocol often depends on the phenotypic state that is the result of the interaction between its innate genotype and the environmental conditions. It means that the phenotypic plasticity caused by gene x environment mutual influence determine the range of variability of the animals' behavior (Voelkl B et al,2018; Schooner TW et al, 2011); for example microbiota is highly affected by environmental conditions. In our study the heterogeneity is not only related to the animal model used but also tothe multi-laboratory experimental design. Once the data has been collected, the statistical analysis has to take into account the site-specific effect to avoid confounding bias.

The experimental protocol, performed in parallel in the two laboratories over the last 3 years, consisted of a battery of behavioral tests carried out before and after heroin self-administration period in order to evaluate locomotor activity, anxiety-like behavior and pain sensitivity and how these ones could be affected by heroin exposure or if could the correlated with some addiction parameters. The heroin self-administration protocol explores the main important paradigm in order to characterize an addicted behavior: the long-access self-administration , the progressive ratio schedule of reinforcement, the extinction phase and reinstatement of heroin seeking inducedby heroin priming dose and by representation of environmental cues. The results of the analysis of these multidimensional data has been already published in a paper by Carter A. et al(December 2021) in *Frontiers in Psychiatry* and was used to identify subpopulations of individuals with different degrees of addiction vulnerability. Seven behavioral traits most representative of heroin use and seeking in 451 outbred rats of this study, were selected to a network-based clustering approach, the Stochastic Block model (SBM) that creating a rat-rat similarity network and identifying discrete clusters of animals either in term of number of cluster(in our case, n=3) either within each cluster, rats share high similarity in their addictive behaviorthat significantly differ from the one of the other clusters. The predictive validity of these clusterizations could be better highlighted by investigating the distribution of the relevant variables selected before across the three inferred clusters in order to detect if each cluster represents a different and separated subpopulation corresponding to a vulnerable/ intermediate / resilient phenotype for heroin use, refraining and seeking. The aim of this clusterization is to propose a promising translational and predictive validated model for future genetical studies. Each cluster, within which there is high degree of similarity in behavioral addiction phenotype, will have to be explored in terms of genetic traits shared by its members and that differs between the other clusters with the purpose to identify some genetic variances that predict a phenotype more prone or resilient to heroin taking rather than seeking, for example. With this purpose we had tried to correlated this clusterization analysis to the behavioral data related to the baseline locomotor activity, anxiety-like behavior and pain sensitivity collected before heroin exposure inorder to investigate if there were some pre-existing behavior shared by individual of the same cluster or that stand out members of different clusters.

3. MATERIAL AND METHODS

3.1 Animals

A total of 800 NIH Heterogeneous Stock (HS) rats were obtained from Wake Forest University (currently NMcwiWFsm:HS; Rat Genome Database number 13673907) and shipped in batches of 40 (20 males and 20 females per site) to either the Medical University of South Carolina (MUSC, USA) or to the University of Camerino (UCAM, Italy) at approximately 5 weeks of age.

Upon arrival, animals were pair-housed and left undisturbed in a climate-controlled colony room with a standard artificial 12-hour light:dark cycle (light on at 7:00 am), room temperature 20-22 °C, humidity 45-55%. During the entire period of the experimental phase, rats were offered free access to tap water and standard chow pellets .

Males and Females will be housed in the same room but in separate cages. The week of arrival and the following week, rats were left undisturbed in their home-cage, the third week they were habituated to operator handling, the fourth week, when animals had aged 8 weeks, experimental protocols started.

All procedures abided by the National Institute of Health Guide for the Care and Use ofLaboratory Animals and the Assessment and Accreditation of Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of Laboratory Animals.

3.2 Drugs

Heroin hydrochloride supplied by the National Institute on Drug Abuse (Bethesda, MD) was dissolved in sterile physiological saline (0,9% NaCl) and self-administered intravenously by rats at dose of 20 µg/kg/0.1 ml infusion adapted to the body weight recorded every week for each animal.

3.3 Experimental protocol

An overview of the complete experimental protocol followed in parallel MUSC and UCAM laboratories is shown in the **Figure 1**. From day 1 to 7, the innate level of locomotor activity, anxiety and pain sensitivity of heroin naive rats were screened by the following behavioral tests:

- Open field test (OF)
- Elevated plus maze test (EPM)
- Tail flick (TF) test

Subgroups of 10 rats (5 males and 5 females) were subjected first to OF, then to EPM and finally to TF test on the same day. All forty rats of each cohort were screened within four days.

From day 8 to 14 rats were implanted with an intra-jugular catheter.

From day 14 to 48 heroin-related behavioral screening was conducted, this screening included:

- Heroin self-administration under 12h long access (LgA) conditions,
- Heroin-primed reinstatement,
- Cue-induced reinstatement.

Fecal pellets, blood and other tissues including liver, spleen, kidneys and brain were collected at the time points indicated in **Figure 1** to generate a tissue bank for microbiome, GWAS and epigenetic analyses.

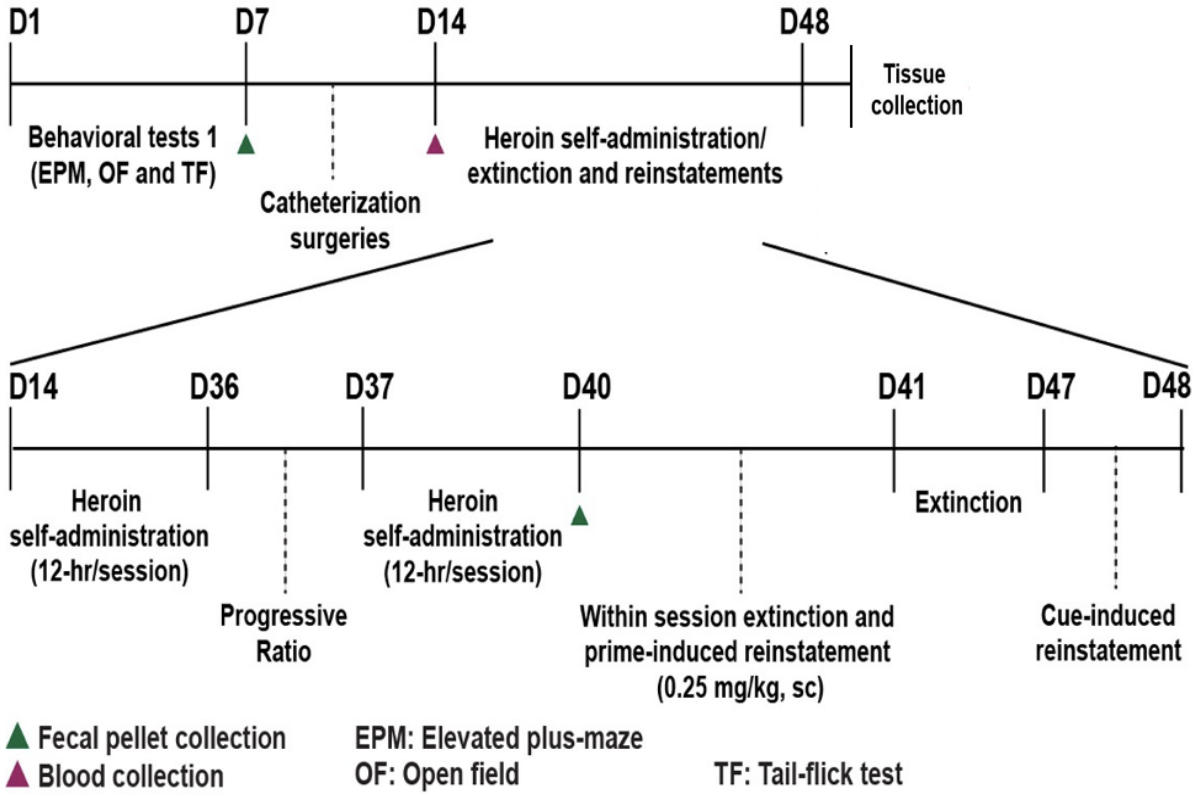

Figure 1: *Behavioral timeline protocol to obtain a multiple addiction phenotype: this protocol was applied in the two laboratories for each cohort of rats received.*

3.4 Pre-heroin behavioral screening

- **Elevated plus maze test**

The elevated plus maze test (EPM) was performed in order to evaluate anxiety behavior in rodents based on their natural aversion to spending time in open space. The elevated plus maze (EPM) apparatus consisted of two black wooden open arms crossed by two enclosed arms (50 cm-high walls), arranged so that the similar arms were opposite to each other. The EPM tests were conducted in a sound-attenuated dimly illuminated room. In order to avoid the formation ofshadow cones that could affect the rat's behavior, the two red light sources were placed on two opposite walls of the room, below the maze level (75 cm from the ground). The 5-minute trial begins when the animal is placed in the center of the maze, facing a closed arm. An arm entry was defined as the presence of all four paws inside it. The number of open-arm and closed-arm entries and the time spent in each arm were video-tracked and scored by a trained experimenter.

The percent time spent in the open arms (TOA) is considered as index of generalized anxiety-like, the higher the the TOA the lower the anxiety, whereas the number of total arm entries is used as a measure of locomotor activity (Cruz APM. et al, 1994)

- **Open field test**

The open field is used to test locomotor and behavioral activity levels of rats in a novel environment. The test is also widely used to assess anxiety-like and exploratory behaviors(Tatem KS et al, 2014). Locomotor activity was scored for 1 hour in the same light condition of the housing room with automated locomotor activity boxes (43x43x30cm; MedAssociated, VT05478).

Data collected included the total distance traveled, the ambulatory time, as a measure of locomotion, the time spent in the central area that could be considered a parameter of anxiety-like behavior, the numbers of rears and stereotypes.

At the end of the test, fecal pellets were collected and placed into sterile, 2ml Eppendorfs for microbiome analysis and stored at -20°C.

- **Tail flick test**

The Tail Flick test is a nociceptive assay frequently used to evaluate thermal acute pain (Deuis JR et al, 2017). The tail of each animal is positioned upon a sensor and irradiated with a light beam placed 2cm above the sensor. The application of the light beam (I.R. 50) heats the tail skin provoking the withdrawal of the tail by a sudden vigorous movement. The latency between the beginning of the irradiation and the tail withdrawal, defined as the tail flick reaction time, is recorded by an electronic device connected to the apparatus used for the test (Ugo Basile, Varese, Italy). Maximum latency time is fixed at 10 sec (cut-off) to prevent damages to rats' skin due to overheating. Animals were subjected to two sessions of four consecutive trials. Fifteen minutes before the first session, rats received a subcutaneous injection of 1 ml/kg of saline. The second session took place 1 hour after the first. Fifteen minutes before the second session, rats received a subcutaneous injection of 0.75 mg/kg of heroin (injection volume 1 ml/kg) to verify their response to heroin analgesic effects.

3.5 Heroin self-administration and reinstatement protocols

- **Intravenous surgery**

Animals were anesthetized with isoflurane anesthesia: 5% induction and 2% maintenance. A single catheter made from micro-renathane tubing (ID = 0.020", OD = 0.037"; Braintree Scientific) was implanted in the right jugular vein and subcutaneously positioned between the vein and the back between the shoulders. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk sutures. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle. The cannula was inserted in a support made of dental cement and covered with a plastic cap (Kallupi M.et al, 2017).

Immediately after surgery, rats were allowed to recover for at least three days before self-administration training. During recovery, they received antibiotic prophylaxis with Enrofloxacin (Baytril®, Bayer) through the drinking water dissolved at the concentration of 25 mg/ml . Throughout the self-administration training and tests, catheters were flushed daily with 0.1-0.2 ml of heparinized saline solution (Nadroparin calcium 3800 U.I.; Italfarmaco S.p.A, Milan, Italy) containing 1mg/ml of Enrofloxacin. Body weights were monitored on a weekly basis. At the end of the experiments catheter patency was confirmed with an injection of 0.2-0.3 ml of Thiopental Sodium solution (Pentothal Sodium, 1g/50 ml, MSD Animal Health S.r.l), immediate loss of reflexes was taken as a positive sign of patency.

- **Self-administration apparatus**

Heroin self-administration was performed in rat operant conditioning chambers (Med Associate St Albans, VT) enclosed in sound-attenuating ventilated environmental cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber with two stimulus lights placed above each lever, and a house light plus a tone generator on the opposite wall. The heroin solution was delivered through a Tygon tube that connected the catheter with an infusion pump. The pump was activated by responses on the right (active) lever and resulted in a delivery of 0.1 ml of fluid. Responses on the left (inactive) lever were recorded but had no

programmed consequences. A windows compatible computer controlled the delivery of heroin solution and recorded the behavioral data.

- **Heroin self-administration procedures**

Long-access (LgA) heroin self-administration (HSA) sessions were performed during the dark phase of the light/dark cycle, for 4 days per week with one random break-day. Rats were trained to self-administer of heroin (20 µg/kg/0.1ml infusion over 3s)under fixed-ratio 1 (FR1) schedule of reinforcement. At the start of the infusion, the house light also turned off for 20-s signaling a time-out period during which additional presses on the active lever were recorded but without consequence. Presses on the inactive lever were recorded but without consequence. Self-administration occurred Monday-Friday, with one session off per week, for a total of four sessions/week.

- **Heroin overnight self-administration under PR schedule of reinforcement**

Following 12 self-administration sessions rats underwent a progressive ratio test whereby the number of presses p(t) required to receive an infusion increased exponentially after each infusion t = 1, …, T according to the function $p(t) = 5e^{0.2t}-5$ (reinforced ratio progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 304 etc.). The last ratio completed is defined as the break point (BP) and is considered as a direct measure of rat's motivation for the drug (Sanchis-Segura C and Spanagel R, 2006). After the PR session, rats had three more days of self-administration training to re-establish baseline heroin-taking behavior prior to tests for reinstatement.

- **Priming-induced reinstatement of heroin seeking**

At the conclusion of heroin self-administration training, rats underwent a within-session extinction-prime test that lasted for 6 h.

The first 4 h were under extinction training conditions during which presses on both the active and inactive levers were recorded but without consequence (i.e., active lever presses no longer result in presentation of the light/ tone cues or heroin infusion). With 2 h left in the session, rats were administered a subcutaneous injection of heroin (0.25 mg/kg), and continued testing under extinction conditions.

- **Cue-induced reinstatement of heroin seeking**

Then, daily extinction training sessions (2 h) commenced for 6 consecutive days prior to a testfor cue-induced reinstatement. During this test, presses on the active lever resulted in presentation of the light/tone cue and turning off of the house light, but no heroin infusions.

At the end of all tests: blood, liver, spleen, kidneys and brain were collected to produce a tissue bank to be used in future GWAS and epigenetic analysis.

3.6 Clusterization of NIH_HS rats based on individual heroin-related phenotype

We used heroin self-administration and seeking data to cluster rats into three groups as we previously described (Allen C et al, 2021). Briefly:

Seven behavioral measures were selected for clustering analyses to reflect three behaviorally distinct phases of drug addiction: drug-taking (drug reinforced behavior), refraining (drug non-reinforced behavior), and seeking behaviors (both drug reinforced and non-reinforced).Heroin-taking behaviors include total heroin consumption (total µg/kg heroin consumed across the first 12 self-administration training session), escalation of intake (total heroin consumed the first 3 days of self-administration subtracted from the last 3 days), and break point achieved during the progressive ratio test. Refraining behavior consisted of active lever presses during the first 2 h of the within-session extinction-prime test (extinction burst) and the last day of extinction training prior to the test for cue-induced reinstatement (extinction day 6). Two extinction training time points were used to capture refraining behavior immediately after heroin

taking, and following several sessions of non-reinforced seeking prior to cue-induced reinstatement. Heroin-seeking behavior is represented by active lever presses during the heroin-prime and cue-induced reinstatement tests. Active lever presses were used for all variables to maintain continuity in measured behavioral output for each behavior.

Next, to analyze the MUSC and UCAM cohorts simultaneously, we first performed a visual inspection of possible batch effects between the two study sites. Specifically, we began by concatenating the raw data matrices from each site into an integrated data matrix, where rows correspond to individual rats and columns correspond to behavioral measures. Then, to facilitate visualization, we applied the Uniform Manifold Approximation and Projection (UMAP) (McInnes L et al, 2018) algorithm to compute 2-dimensional embeddings for each rat.

To correct for the apparent batch effect between study sites, we *z*-score transformed each behavioral measure *within study site*. This allowed for analysis of each behavioral measurement on a standardized scale, and, in effect, regressed out unwanted site-specific effects (exemplified in **Figure 2**).

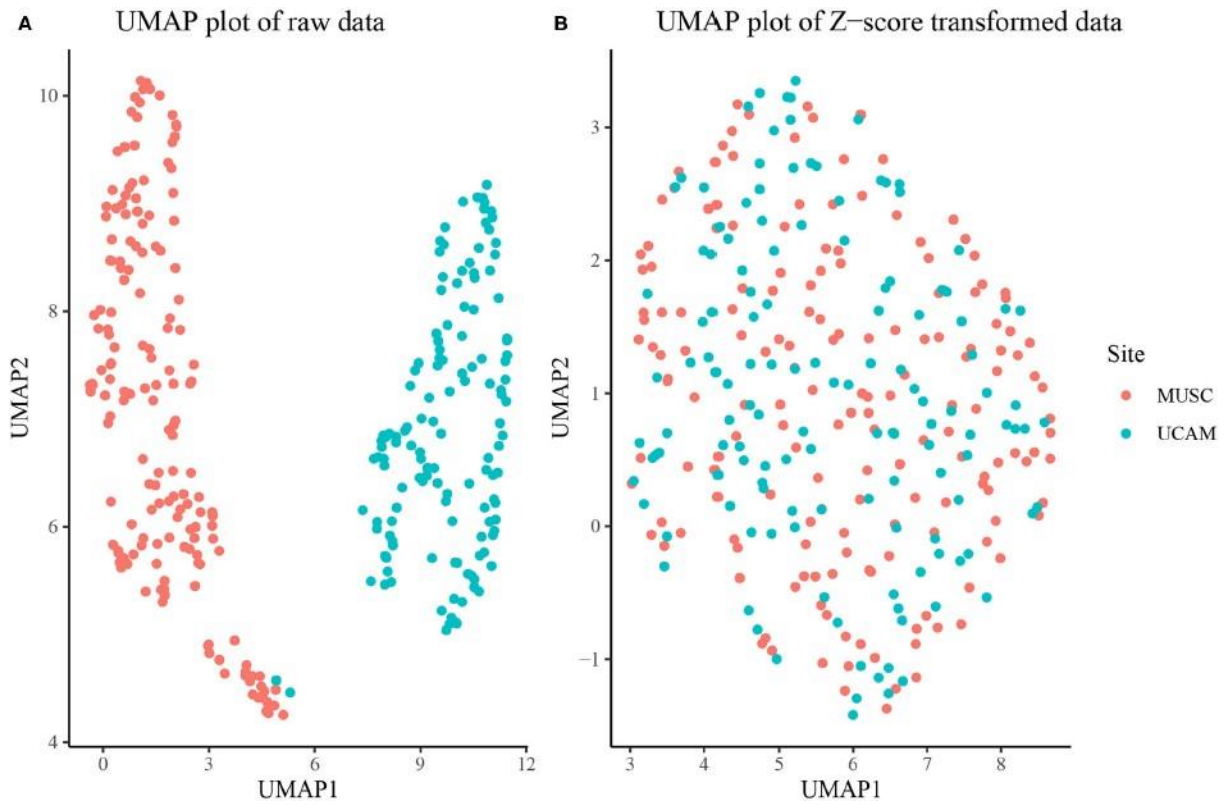

***Figure 2**: (**A**) UMAP dimension reduction of behavioral measures before site-specific z-scoring shows significant batch effect of study site (MUSC vs. UCAM). (**B**) UMAP dimension reduction after site-specific z-scoring shows adjustment for study site batch effect. Figure adapted from our published work: Allen et al, Frontiers in Psychiatry (2021), Vol 12 Art.745468.*

Next, we constructed a rat-rat similarity network as follows. We used the subset behavioral measures described above to compute the Euclidean distance between each pair of rats.

We then formed a rat-rat similarity network, i.e., a collection of nodes and edges, where nodes in the network represent individual rats and edges represent similarities between rats. We placed an edge from each node to its *R* closest other nodes based on the rat-rat distance measures. Here, the number of neighbors *R* is a tuning parameter that controls the density of edges in the similarity network. By default, we adopt the widely used heuristic $R=N^{1/2}$ (Stork DG et al, 2001).

To detect communities within the overall rat-rat similarity matrix that might correspond to behaviorally distinct sub-populations, we adopted the Bayesian stochastic block model (SBM), a generative model for network data (Snijders TA et al,1997). Finally, we used Bayesian Information Criterion (BIC) (Schwarz G et al, 1978) in conjunction with biological knowledge to choose the most appropriate number of clusters *K*. We fitted the SBM to the adjacency network for a range of *K* from $K = 2, \ldots, 10$. We ran each model for 10,000 MCMC iterations and discarded the first 1,000 iterations as burn-in. Using BIC, we found that $K = 3, 4, 5$ provided approximately equal goodness of fit, with $K = 2$ or $K > 5$ provided relatively poor fit (**Figure3A**). As such, we chose $K = 3$ to provide the most parsimonious representation of the data and to assess the vulnerable, intermediate, and resilient sub-types.

The network composed by the three SBM estimated clusters is depicted in **Figure 3B**.

Figure 3C shows the SBM estimated cluster labels on UMAP space.

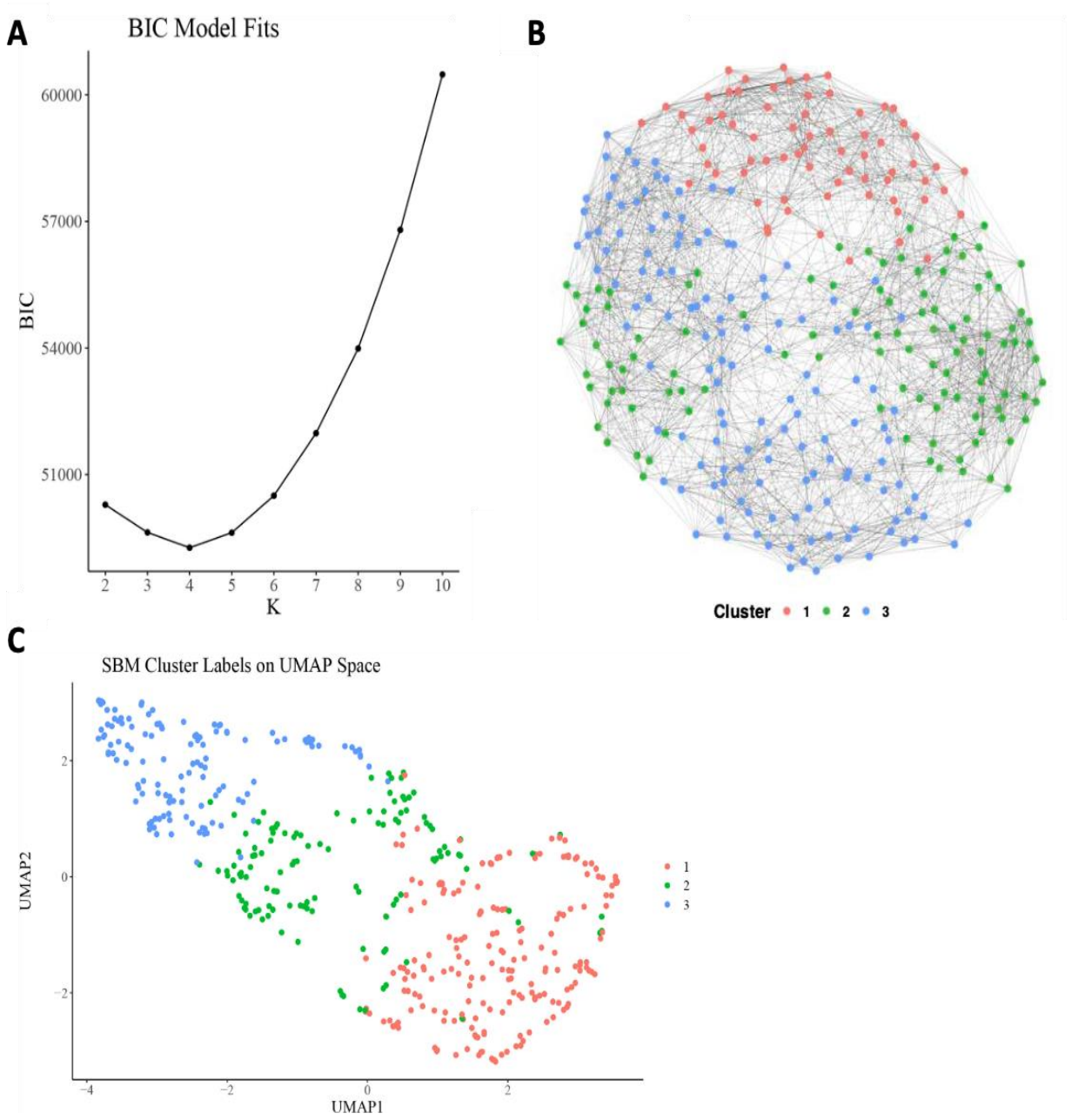

Figure 3: A) *Bayesian Information Criterion (BIC) from SBMs fit with a range of K. K = 3, 4, 5 seem to provide similarly optimal fit in terms of BIC.* ***B****) Rat-rat similarity network in the three-dimensional space obtained with SBM cluster analysis using K=3 clusters. Each rat (node) connected to each of its* $N^{1/2}$ *closer rats. Each discrete cluster is indicated with a different color.* ***(C****) UMAP reduction of the behavioral measurement colored by inferred cluster labels that shows a clear separation between each subgroup identified. Figure adapted from our published work: Allen et al, Frontiers in Psychiatry (2021), Vol 12 Art.745468.*

3.7 Statistical analysis

The performance of clusters in each of the seven Z-scored heroin-related behavioral measures used to define them was analyzed by one-way ANOVA with clusters (sexes matched) as between-subjects factor. Once clusters were defined as OUD-like vulnerable, resilient and intermediate, sex was considered as an independent factor and analyses were made by two-way ANOVA with clusters and sex as between-subjects factors. ANOVAs were followed by Tukey's post hoc analysis when appropriate. Correlation analyses were done by Pearson's two-tailed test. All analyses were run on Z-scored values to correct for the apparent batch effect between study sites. Statistical significance was set to conventional $p<0.05$ for ANOVAs, and $p<0.05$ (uncorrected) then corrected by Bonferroni for correlation.

4. RESULTS

Of the 800 rats included in this study, 320 were screened at MUSC and 480 at UCAM. 80 rats were subjected to saline yoked procedure to be used as naive controls in future epigenetic studies and were therefore excluded from the present analyses. 106 rats were excluded for one of the following reasons: loss of catheter patency, bug in data recording, or died before the end of the procedures. The analyses presented here were therefore executed on 243 rats screened at MUSC and 371 rats screened at UCAM

4.1 Characterization of heroin-related phenotype clusters

The 614 rats analyzed were composed of 316 males and 298 females: the composition of the three clusters is summarized in **Table 1**.

Table 1: composition of clusters derived from SBM analysis				
Cluster	**N (% of total)**	**UCAM/MUSC**	**Female (% of cluster)**	**Male (% of cluster)**
Cluster 1	239 (38.9%)	131/108	147 (61.5%)	92 (38.5%)
Cluster 2	217 (35.4%)	153/64	87 (40.1%)	130 (59.9%)
Cluster 3	158 (25.7%)	87/71	64 (40.5%)	94 (59.5%)

Next, we analyzed how the three clusters differed in each of the seven heroin-related Z-scored behaviors used for the clusterization. As expected, ANOVAs found an overall effect of cluster in each behavior; statistical details are reported in **Table 2**.

Table 2: summary of between clusters ANOVAs of heroin-related Z-scored behaviors			
Behavioral phase	Behavioral measure	F value (df_n, df_d)	p value
Drug reinforced behavior	Escalation of intake	117.6 (2, 611)	<0.0001
	Total intake	306.8 (2, 611)	<0.0001
	Break point	131.9 (2, 611)	<0.0001

Refraining (extinction)	Extinction burst	61.41 (2, 611)	<0.0001
	Extinction day 6	99.0 (2, 611)	<0.0001
Heroin seeking (relapse)	Prime active	58.2 (2, 611)	<0.0001
	Cued active	106.4 (2, 611)	<0.0001

Tukey's post-hoc analyses revealed that clusters 2 and 3 showed lower escalation of intake than cluster 1 and did not differ between each other (**Figure 4A**). In all other behavioral measures, clusters 2 and 3 scored lower than cluster 1 and cluster 3 scored lower than both cluster 1 and cluster 2 (**Figure 4B-G**). Based on these analyses, cluster 1 was henceforth defined as Vulnerable to OUD-like behavior, cluster 3 as Resilient to OUD-like behavior and cluster 2 as Intermediate between them.

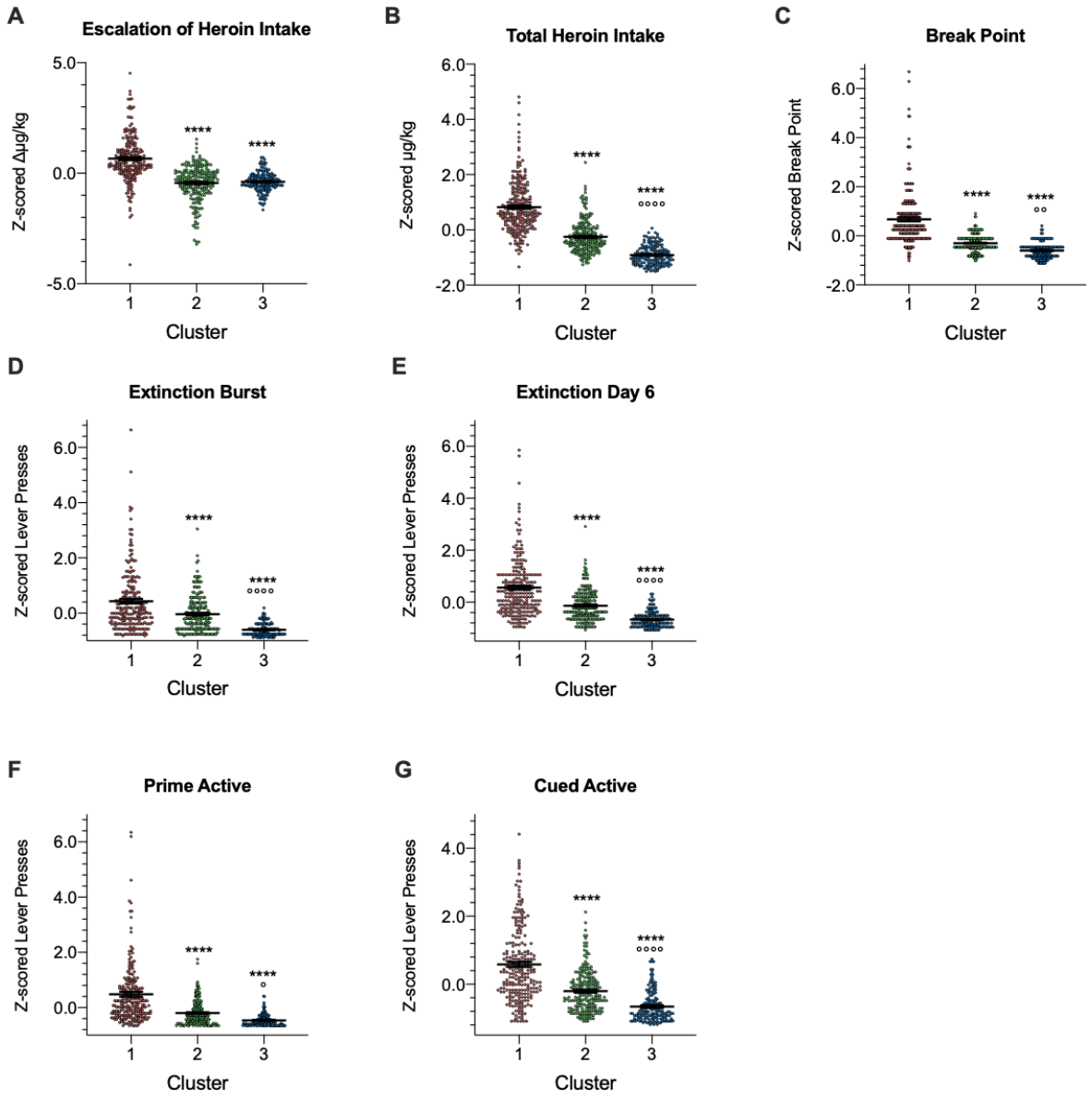

Figure 4*: scatter plot distributions of the seven relevant behavioral measures (Z-scored) in each cluster.* ***A)*** *Clusters 2 and 3 showed lower escalation of intake than cluster 1. Graphs* ***B-G*** *show (in order of appearance): Total Heroin Intake (****B****), Break Point (****C****); Extinction Burst, expressed by the number of active lever presses during the first two hours of extinction in the within-session extinction/priming test (****D****); Extinction Day 6, expressed by the number of active lever presses during the last day of extinction before the cued reinstatement test (****E****); Prime Active, expressed by the number of active lever presses primed by heroin in the within-session extinction/priming test (****F****), Cued Active, expressed by the number of active lever presses produced during the cued*

*reinstatement test (**E**). Whiskers indicate mean ± SEM. Statistical significance: ****p<0.0001 vs cluster 1, °p<0.05 and °°p<0.01 and °°°°p<0.0001 vs cluster 2.*

4.2 Heroin self-administration and seeking in male and female OUD-like vulnerable, intermediate and resilient rats

Having defined OUD-like vulnerable, resilient and intermediate clusters, we wanted to study the behavior of male and female rats within the three clusters. Two-way ANOVAs confirmed an overall effect of clusters in each of the seven behavioral measures. We found no difference between male and female rats within the same cluster except in two cases, Total Heroin Intake and Cued Active, in which there was a significant effect of sex; in this two behavioral measures females scored higher than males independently of clusters. None of the analyses reported a significant cluster by sex interaction. Statistical details are summarized in **Table 3**.

Table 3: summary of sex by clusters ANOVAs of heroin-related Z-scored behaviors

Behavioral phase	Behavioral measure	Sex	Cluster	Interaction
Drug reinforced behavior	Escalation of intake	$F_{(1,608)}=2.0$; $p>0.05$	$F_{(2,608)}=115.8$; $p<0.0001$	$F_{(2,608)}=1.0$; $p>0.05$
	Total intake	$F_{(1,608)}=58.0$; $p<0.0001$	$F_{(2,608)}=281.6$; $p<0.0001$	$F_{(2,608)}=1.5$; $p>0.05$
	Break point	$F_{(1,608)}=0.4$; $p>0.05$	$F_{(2,608)}=130.4$; $p<0.0001$	$F_{(2,608)}=1.6$; $p>0.05$
Refraining (extinction)	Extinction burst	$F_{(1,608)}=1.3$; $p>0.05$	$F_{(2,608)}=57.22$; $p<0.0001$	$F_{(2,608)}=0.6$; $p>0.05$
	Extinction day 6	$F_{(1,608)}=0.01$; $p>0.05$	$F_{(2,608)}=93.0$; $p<0.0001$	$F_{(2,608)}=0.2$; $p>0.05$
Heroin seeking (relapse)	Prime active	$F_{(1,608)}=1.9$; $p>0.05$	$F_{(2,608)}=52.6$; $p<0.0001$	$F_{(2,608)}=0.3$; $p>0.05$
	Cued active	$F_{(1,608)}=8.8$; $p<0.01$	$F_{(2,608)}=93.6$; $p<0.0001$	$F_{(2,608)}=0.3$; $p>0.05$

Scatter plot of male and female performance in each behavioral measure within the three clusters is presented in **Figure 5**:

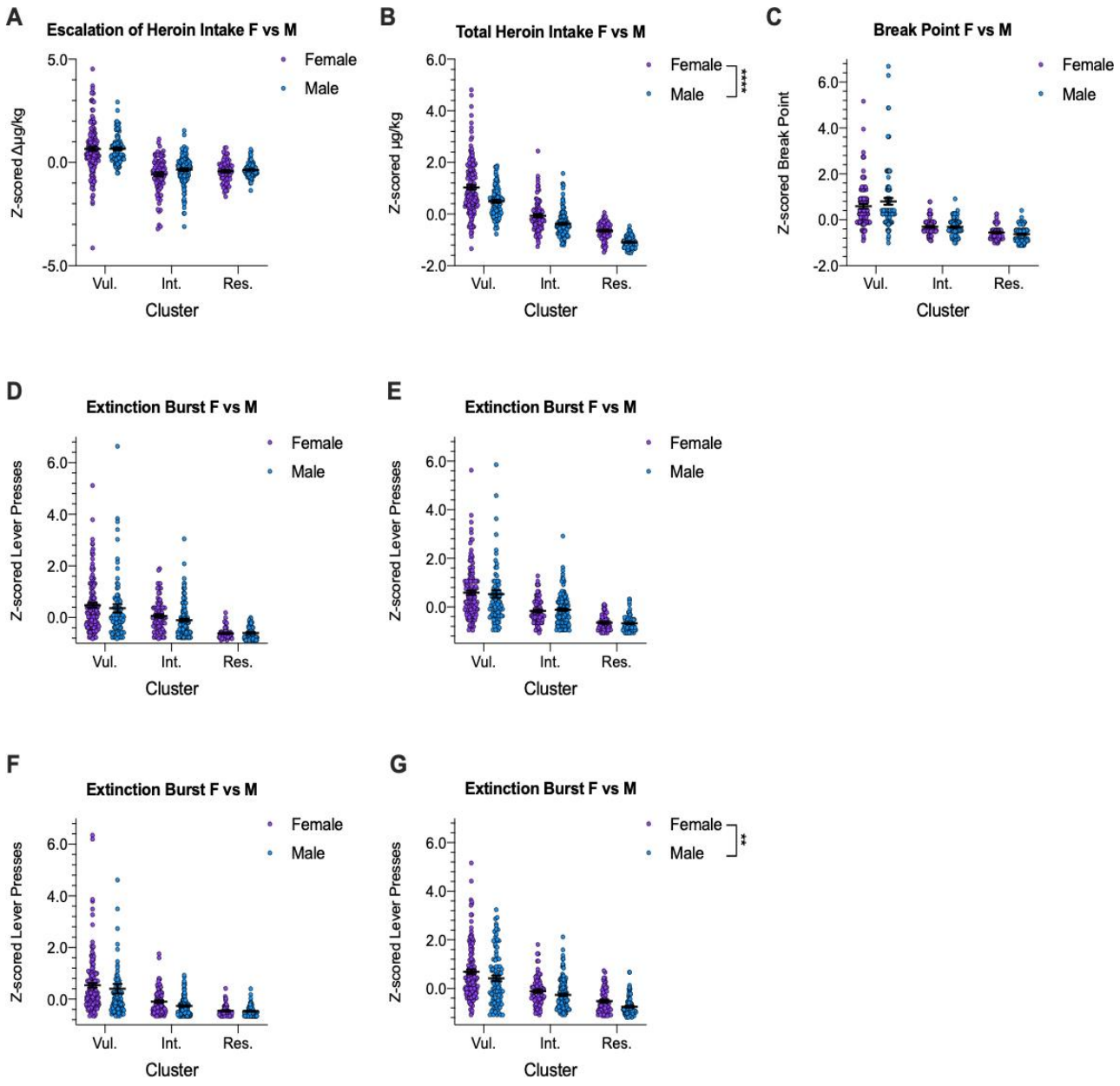

Figure 5*: scatter plot distributions of the performance (Z-scored) expressed by male and female rats of the Vulnerable (Vul) Intermediate (Int) and Resilient (Res) clusters in the seven relevant behavioral measures. Graphs* ***A-G*** *show (in order of appearance): Escalation of heroin intake (****A****); Total Heroin Intake (****B****), Break Point (****C****); Extinction Burst, expressed by the number of active lever presses during the first two hours of extinction in the within-session extinction/priming test (****D****); Extinction Day 6, expressed by the number of active lever presses*

*during the last day of extinction before the cued reinstatement test (**E**); Prime Active, expressed by the number of active lever presses primed by heroin in the within-session extinction/priming test (**F**), Cued Active, expressed by the number of active lever presses produced during the cued reinstatement test (**E**). Female showed higher response than male in total heroin intake (**B**), and cued Active (**C**) measures independently of clusters. Whiskers indicate mean ± SEM. Statistical significance: brackets with ****p<0.0001 and **p<0.01 indicate sex overall effect.*

4.3 Characterization of innate locomotor activity anxiety level and pain sensitivity in male and female OUD-like vulnerable, intermediate and resilient rats

Before heroin self-administration, rats were screened for their innate levels of locomotor activity, anxiety, pain sensitivity, and sensitivity to the analgesic effect of heroin. Therefore we retrospectively analyzed these behavioral traits in the three OUD-like clusters.

- **Innate Locomotor Activity**

Two animals were removed from this analysis because open field recording stopped unexpectedly during the locomotor activity test.

Analysis of Z-scored distance traveled revealed an overall effect of clusters [$F(2, 606) = 5.2$; $p<0.01$] and sex [$F(1, 606) = 60.9$; $p<0.0001$], but no sex by cluster interaction [$F(2, 606) = 0.3$; $p>0.05$]. As we were specifically interested in cluster difference, being in lack of a significant sex by cluster interaction, we run Tukey's post hoc analysis within the main cluster factor (i.e. without differentiating sex subgroups) and we found that vulnerable rats showed higher locomotor activity compared to the other two clusters; females showed higher locomotor activitythan male independently of clusters (**Figure 6A**).

- **Innate Anxiety Level**

Anxiety is expressed as the percent of time spent (%TOA) in the open arm of the EPM, the higher the %TOA the lower the anxiety.

One rat was excluded from this analysis as they jumped out of the maze during the test. Analysis of Z-scored %TOA revealed an overall effect of clusters [$F(2, 607) = 6.6$; $p<0.01$] and sex [$F(1, 607) = 49.1$; $p<0.0001$], but no sex by cluster interaction [$F(2, 607) = 0.3$; $p>0.05$].
Again, being in lack of a significant sex by cluster interaction, we run Tukey's post hoc analysis within the main cluster factor to assess between clusters difference independently of sex. Tukey's test for multiple comparison revealed that both vulnerable and resilient showed higher %TOA (i.e. lower anxiety) compared to the intermediate cluster; females showed higher %TOA (i.e. lower anxiety) than male independently of clusters **(Figure 6B).**

- **Pain Sensitivity**

We tested the rat's sensitivity to (termal) pain by tail flick latency in the tail flick test. Analysis of Z-scored tail flick latency found no overall effect of clusters [$F(2, 608) = 0.4$; $p>0.05$], sex [$F(1, 608) = 0.3$; $p>0.05$] or sex by cluster interaction [$F(2, 608) = 0.4$; $p>0.05$] (**Figure 6C**).

- **Sensitivity to the analgesic effect of heroin**

We tested the rat's sensitivity to the analgesic effect of heroin as the difference between the tail flick latency in a tail flick trial with heroin onboard, and the tail flick latency scored in the tail flick test for pain sensitivity (innate pain sensitivity), i.e. the higher the Δ Tail Flick Latency the higher the sensitivity to heroin's analgesic effects. Analysis of Z-scored Δ Tail Flick Latency found an overall effect of clusters [$F(2, 608) = 4.2$; $p<0.05$] and sex [$F(1, 608) = 17.4$; $p<0.0001$], but no sex by cluster interaction [$F(2, 608) = 1.5$; $p>0.05$]. Here as well, due to the lack of a significant sex by cluster interaction, we explore the difference between clustersindependently of sex by Tukey's post hoc analysis within the main cluster factor, and we found that vulnerable rats showed lower Δ Tail Flick Latency than resilient rats, i.e. they were less sensitive to heroin's analgesic effect (**Figure 6D**).

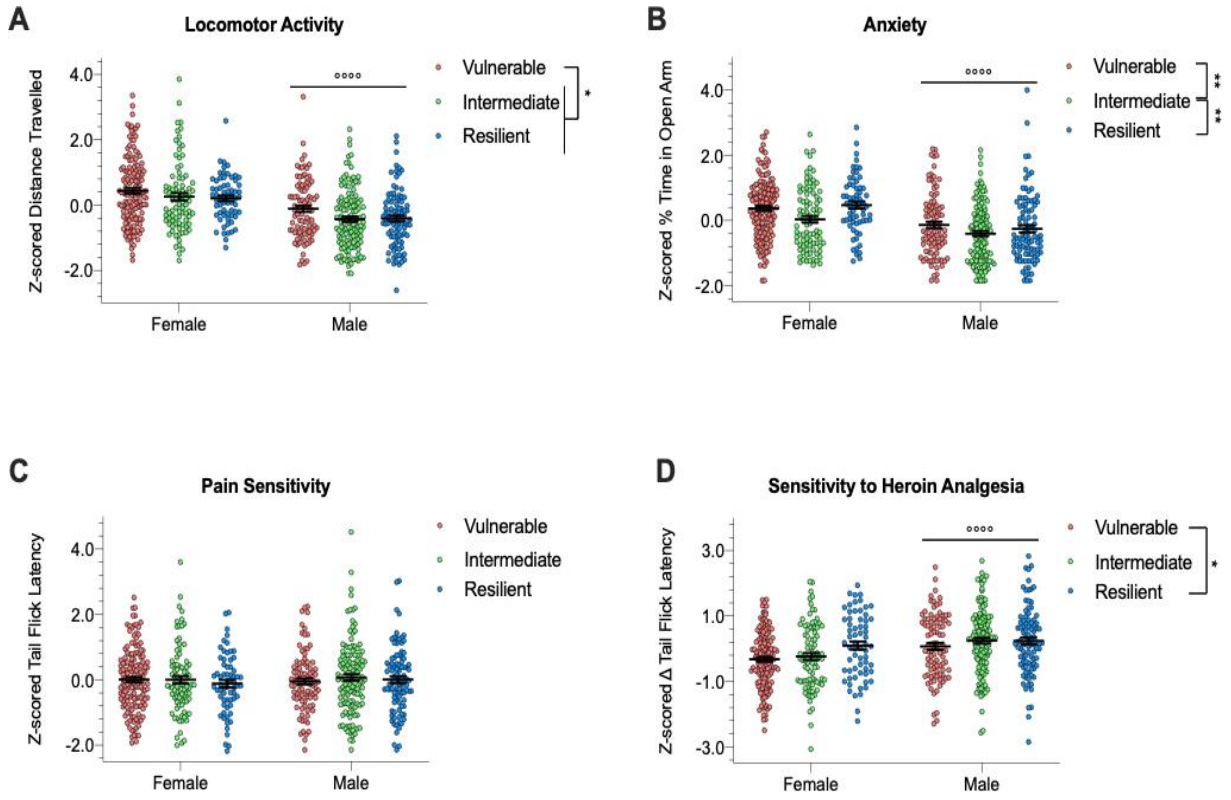

Figure 6: *Scatter plots distributions of levels of locomotor activity, anxiety, pain sensitivity, and sensitivity to the analgesic effect of heroin in OUD-like Vulnerable, Intermediate and Resilient clusters: (**A**) Female rats showed a higher locomotor activity compared to males independently of clusters; vulnerable rats showed higher locomotor activity than intermediate and resilient rats independently of sex. (**B**) Male rats showed a higher innate anxiety, i.e. lower %TOA than females independently of cluster allocation; intermediate rats showed higher anxiety. i.e. lower %TOA then the other two clusters independently of sex. (**C**) There was neither cluster nor sex differences in pain sensitivity expressed by the latency in tail flick (**D**) Females showed lower sensitivity to heroin induced analgesia, i.e. lower A tail flick latency, than males independently of clusters. OUD-like Vulnerable rats showed lower sensitivity to heroin induced analgesia, i.e. lower A tail flick latency, than resilient rats independently of sex . Whiskers indicate mean ± SEM. Statistical significance: °°°°p<0.0001 vs females independently of clusters; *p<0.05, **p<0.01 between the clusters indicated by the brackets independently of sex.*

4.4 Correlations between heroin seeking behaviors and innate phenotypic behaviors

Finally we wanted to explore in detail the relationship between the seven heroin seeking behaviors that we used to define clusters and the innate locomotor activity, anxiety, pain sensitivity and sensitivity to heroin's analgesic effects. The significant correlations before Bonferroni's correction are reported in **Figure 7**. Specifically, we found that distance traveled in the positively correlated with total heroin intake, break point, extinction burst, extinction day 6 and cued active, i.e. the higher the spontaneous locomotion the higher the heroin seekingmeasured by these parameters. Percent TOA positively correlated with escalation of intake, total intake and break point; i.e. the lower the innate generalized anxiety, the higher the heroin seekingmeasured by these parameters. Tail flick latency showed no correlation with none of the heroin seeking parameters. Heroin analgesic ratio showed negative correlations with escalation ofintake, total intake, break point and extinction day 6; i.e. the lower the sensitivity to heroin's analgesic effects the higher the heroin seeking measured by these parameters.

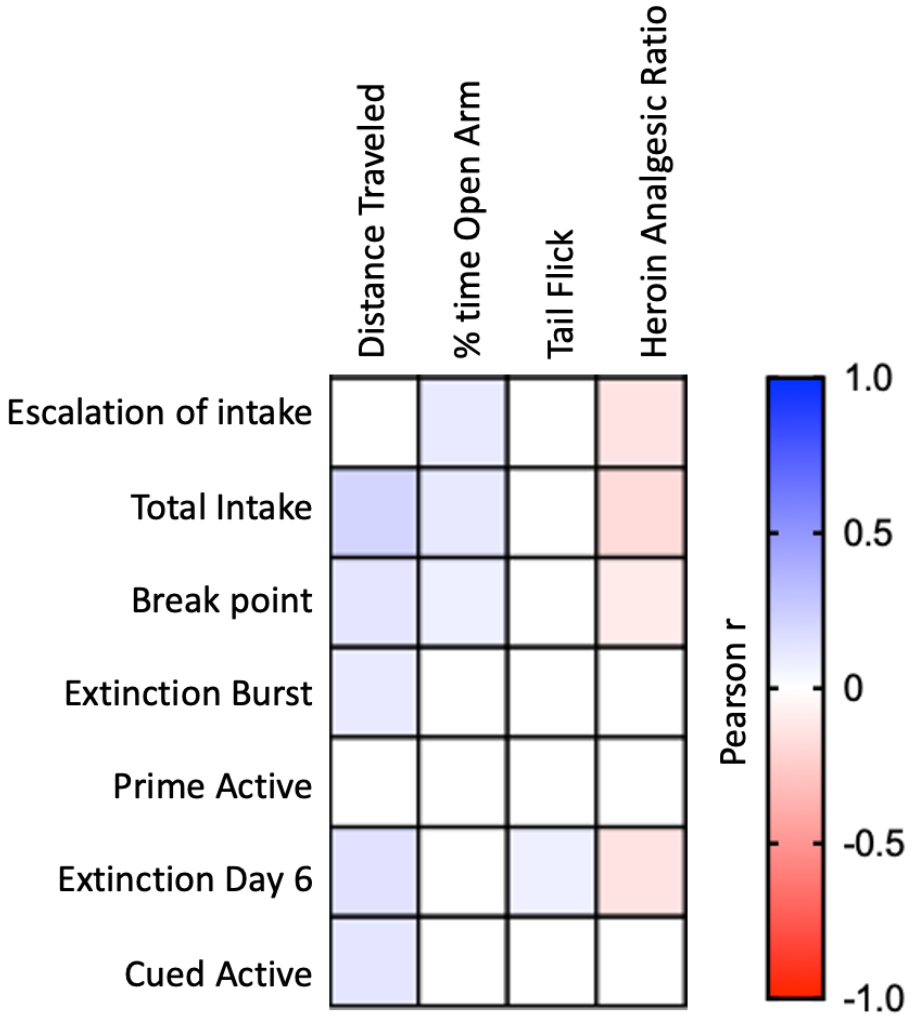

Figure 7*: Heatmap of correlation analysis between innate phenotypes (columns) and heroin seeking parameters used to define vulnerable, intermediate and resilient clusters. Only interactions showing significant correlations (p<0.05 uncorrected) are colored.*

Bonferroni's correction was then applied to the correlation results. **Figure 7** shows 28 interactions (7 heroin seeking parameters by 4 innate phenotypic traits). However to run the correlation analyses we used GraphPad Prisma8, in which the worksheet included all 11 variables that are by default all correlated with each-other by the software (11 x 11 =121 interactions). Therefore, our Bonferroni corrected p value was p_{corr}= 0.5/121 = 0.00413. The positive correlation between distance traveled and total heroin consumption (**Figure 8A**), the positive correlation between distance traveled and extinction day 6 (**Figure 8B**), and the negativecorrelation between heroin intake and heroin analgesic ratio (**Figure 8C**), survived Bonferroni's correction.

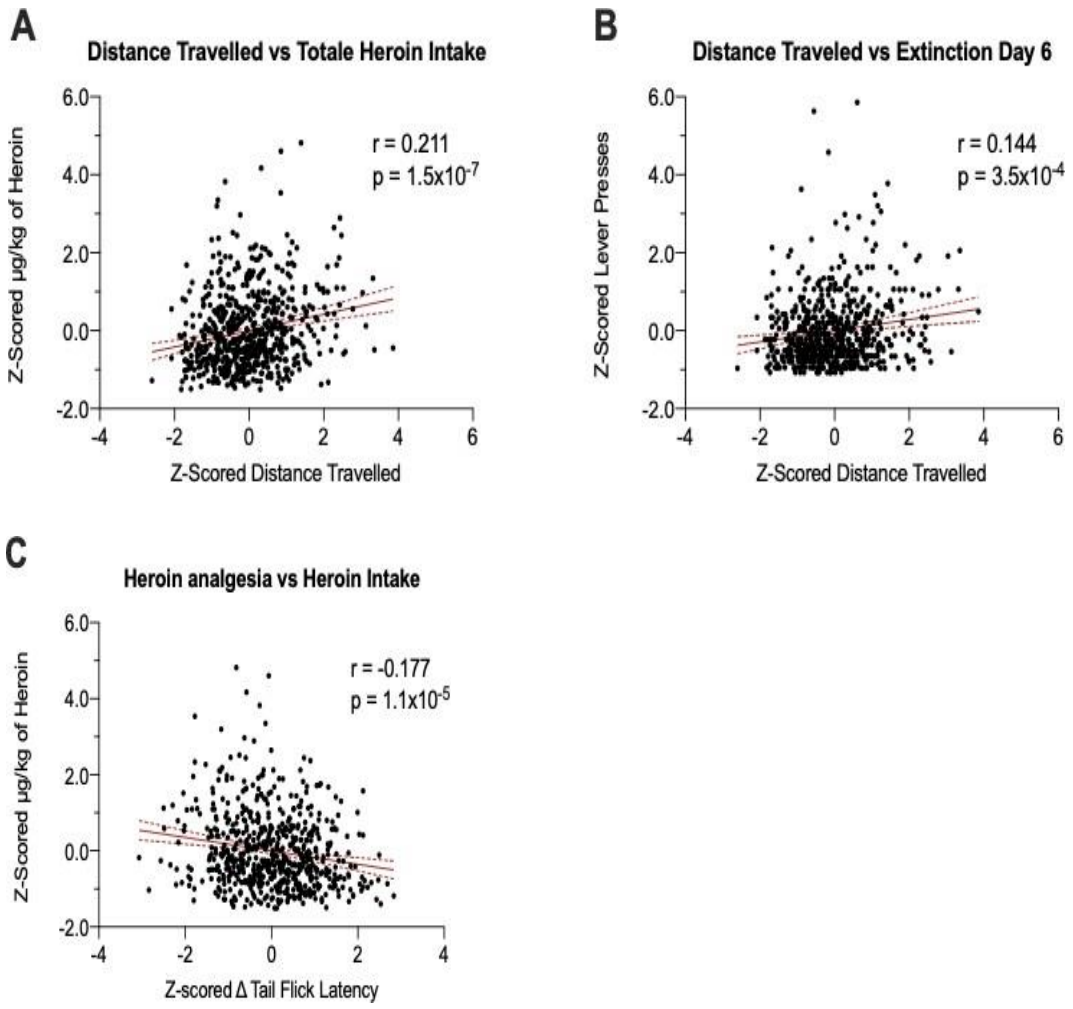

Figure 8*: Scatter Plot of correlation analyses between: (**A**) Distance traveled and Total Heroin Intake; (**B**) Distance traveled and Extinction Day 6; (**C**) Heroin-induced analgesia and total heroin intake. Continuous and dotted red lines represent best fit linear regression and 95% interval respectively.*

5. DISCUSSION

Although thousands of studies have been published on genetics of addictive disorders in humans and thousands more studies have been published on animal models of addictive diseases, the major part of them was focusing on the isolation of a single risk factor that seems to be predictive of a vulnerable phenotype that was segregate in an inbred animal strain in order to better investigate its impact on the addictive behavior, without confounding of additional genetics variables (like, i.e, genetic variant of the μ receptor, the deletion of an opioid receptor, the overexpression of the CRH1 receptor system to cite only some possible genetic manipulations).

However OUD has a multi-traits nature so that many behavioral characters can differentially contribute to vulnerability or resilience to develop drug addiction depending on the individual phenotype. It is also true that the presence in an individual of a predisposing factor did not necessarily predict the presence of the other traits generally associated with a vulnerable phenotype.

It means that if we want to better investigate how genetic predisposition and associated behavior could influence the development of the OUD, we need to characterize a great number of different phenotypes that allow to analyze simultaneously multiple traits differently spared in the population.

This is the goal of our study, and this is the reason why we carried out, for the last three years, a behavioral characterization of 800 heterogeneous rats (NIH_HS rats) characterized by a great individual variability that could mimic the one of the human population, creating the precondition for this study to have a translational validity.

To manage with all the behavioral data collected and try to characterize a possible vulnerable or resilient phenotype, we have to choose only a discrete number of these traits that taken together quantify the most important aspects of the dependence: the heroin taking, the refraining and the drug seeking behavior.

There are many evidences to support and validate this selection that portrait exactly the three recurring stage of the addiction cycle largely described in literature (Koob GF and Volkow ND, 2010; Volkow ND et al, 2016; Wise RA et al, 2014; Evans CJ and Cahill CM.,2016) : the binge/intoxication phase is related to the heroin taking (reinforced behavior: escalation, heroin intake and motivation to take heroin); the withdrawal/negative affect correspond to our refraining that resemble the extinction phase in which drug is not available (non-reinforced behavior); the craving stage is represented by the heroin seeking behavior that lead to relapse (reinforced and non-reinforced behavior).

All the variables selected are related to the heroin self-administration phase and are reported as the number of active lever presses in order to maintain a continuity in the measure of the behavioral output for each behavior.

We have already discussed the necessity, in order to analyze MUSC and UCAM cohorts simultaneously, to z-score normalize the data due to the batch site effect that affected them. The z-score normalization allows us to standardize the measurement regressing the site-specific effect. This manipulation is needed from a statistical point of view to avoid that site-related differences could be confounded with the effect of the individual differences but is also symptomatic of the influences of the environmental conditions on determining the range of variability of the animals' behavior (Voelkl B et al, 2018; Schooner TW et al, 2011).

The Stochastic Bayesian block model (SBM) was used to investigate the presence of distinct behavioral subpopulations within the overall network and it succeeded in identifying three distinct clusters with significant separation. To give a behavioral significance to this clusterization, it was applied to each one of the seven parameters previous selected in order to identify which cluster match with a vulnerable, intermediate, resilient phenotype with an high interval of confidence (95%): the lack of overlap between them indicates that they really represent three different phenotypes in term of addiction behavior and the differences between each of them always reached a statistically significant value.

Our results indicate that cluster 1 is the one with a higher active lever pressing across all the behavioral tasks compared with cluster 2 and 3. Conversely cluster 3 results the one with the lower score for each parameter: based on these results we can identify cluster 1 as Vulnerable, cluster 2 as Intermediate and cluster 3 as Resilient to develop heroin addiction-like behaviors.
However belonging to a cluster does not necessarily mean to best fit with the z-score profile of all the variables: not all vulnerable rats will have a high lever press (high responder) in all the features to be considered vulnerable. This latter condition finds a good correlation also in human society: when we identify an addicted profile following the DSM-V diagnostic criteria, a person never meets neither one of them nor all of them but only a subgroup sufficient to be classified as addicted. It means that, among each cluster, persists a certain degree of heterogeneity that suggests the possibility to identify different subpopulations with some different behavioral traits even if belonging to the same cluster.

The sex difference founded in cluster composition with females more present in the vulnerable cluster (61.5%) and male in the resilient one (59.5%), confirms the translational value of this analysis : also in human, among a vulnerable population, females acquire and maintain highlevel of drug intake and are more prone to relapse after a period of abstinence or under maintenance therapy than male (Becker JB et al, 2017). This unbalanced distribution is also consistent with what we had previously noticed in msP rats (see for more details Chapter 2) : also in this ratline, characterized by high stress vulnerability, female rats are more inclined to self-administer a higher amount of heroin compared with male counterparts. To reinforce the importance of the gender factor we found that, within each cluster, female rats reached a high score in heroin taking and seeking. It means that also in the resilient animals the sex still represents a vulnerable factor: resilient females are however more incline than males to self-administer the addictive substance.
The gender-dependent vulnerability to develop, more in general, addiction-like behavior and in particular to shift from voluntary drug intake to loss of control over it and compulsive behavior, will linger across species, across different substances of abuse and also across different animal models and also encompass the cluster allocation.

The biggest goal of this model to assess OUD vulnerability with a great level of translational validity is to be able to identify which are the behavioral or genetic traits that pre-exist the first heroin exposure and could predict OUD vulnerability in humans.

Some SUD-like predictive animal models have already been characterized in literature focused on one or two behavioral traits correlated with propensity to seek and take the drug: one of the most cited in literature is the High Responder/Low Responder classification presented by Piazza PV and colleagues (Piazza PV et al, 1989; Piazza PV et al, 1998) based on the locomotor activity in a novel environment with HR rats that show greater locomotion compared with LRs. Piazza PV identify a correlation between the degree of locomotor reactivity to novelty and the acquisition of drug-taking behavior, demonstrated specifically in the psychostimulants like cocaine (Piazza PV et al, 2000) and amphetamine (Piazza PV et al 1989, 1990), and nicotine self-administration (Suto N et al, 2001): this correlation appear to be founded on a higher basal dopamine level in the mesolimbic dopamine system in HRs compared to LRs, also associated with a high corticosterone secretion in response to stress exposure (Piazza PV et al, 1991).

Another validated predictive model is the Sign-tracker(ST)/Goal-tracker (GT) model used to predict the motivational value of the reward-paired cue under a Pavlovian conditioning approach (Flagel SB et al, 2007). Sign-tracker rats, that attribute both predictive and incentive values to conditioned cues, resulted more impulsive (Flagel SB et al, 2010), will work harder to obtain a single dose of the drug, are more prone to reinstate the drug-seeking behavior, compared with GTs rats (who attribute only predictive value to the conditioned stimulus)(Saunders BT et al, 2013).

The usefulness of these predictive models coincides also with their own limit: isolating one or two traits made possible and easier to characterize their influence on addictive behavior, but this made them not replicable into human population since the multi-traits nature of the SUD.

The advantage of our study is in the use of an heterogeneous animal model of individual variability and that many vulnerable factors are kept simultaneously in analysis to obtain the presented clusterization. However, we are still far from being able to predict the vulnerability of an individual based only on his pre-existing behavioral traits: at the moment we had tried to perform a retrospective analysis to investigate if the behavioral parameters screened prior to heroin exposure could be correlated with the cluster the rat will enter.

With this aim, we had compared the behavioral data collected before heroin exposure with the heroin-related cluster allocation: the basal locomotor activity, the anxiety-like behavior, the response to acute thermal pain and the consequent sensitivity to heroin analgesic effect.

The interaction between locomotor activity and anxiety with the addiction vulnerability has been previously discussed ; investigating the pain sensitivity is also important considering that opioid , at first, are used in clinics as pain reliever and, as consequences, a reduced sensitivity to their analgesic effect need to increase the treatment dose and could lead to tolerance and dependence.

Based upon the HR/LR model presented by Piazza PV and previously discussed (Piazza PV et al, 1989), a high level of locomotor activity is associated with enhanced drug taking behavior: our data confirmed this theory because we found that all female rats, independently by cluster allocation, showed a higher basic level of locomotion compared with males. Converging evidence in literature has demonstrated this gender difference: females tend toambulate more and defecate less than males when placed in an open field (Archer J et al,1975;Tropp J and Markus EJ, 2001). Among male, the high degree of locomotor activity is reached bythe vulnerable cluster that well fit with the HR's behavior . From the correlation analysis, it emerged that rats with a higher heroin taking, motivation and craving showed the highest value of distance traveled.

Our data confirmed the precedent HR/LR theory and sketched locomotor activity as a behavioral task with a consistent predictive validity.

Locomotor activity levels are often interpolated with data obtained from the Elevated plus maze because both tests give information about exploratory functions, reactivity to a novel environment and anxiety-like behavior.

This behavioral test is based on the well-consolidated knowledge that rats that spend more time in open arms (%TOA) are characterized by less anxious phenotype linked to a high activity and exploratory level.

The statistical analysis of our results suggests that there is no interaction between sex and cluster allocation but, however, we can infer some interesting correlations between gender: in fact females rats, independent by the cluster they belonged, enter the open arm of the apparatus more readily and spent more time in it than male rats, that result in a more anxious phenotype. This results are consistent with their higher exploratory behavior shown in the Open field task: many studies support our findings assessing that there is sex differences in both activity and exploration for the initial exposure to an environment and in particular males may be less active during an exploration task because they are more anxious when placed in an open environment, that is exactly what we found with our data (Tropp J and Markus EJ, 2001; Johnston AL et al, 1991).

Also in clinical studies are assessed the comorbidity of OUD and anxiety disorder: in the review of McHugh RK and colleagues, the researchers underlined that also within addicted people, the more anxious were the ones with the higher severity progression of the misuse, in particular with an increase in motivation for heroin seeking as relief to distress (McHugh RK et al, 2021). The relationship between the anxiety-like behavior and the heroin vulnerability is also confirmed by correlation analysis: the more a phenotype is anxious the higher is its escalation of heroin taking, the amount of heroin taken and the motivation for obtaining the drug .

We have also analyzed the sensitivity to acute thermal pain and to the analgesic effect of opioid using the Tail flick test: even if there were no differences neither between clusters nor in the basal reactivity to peripheral acute thermal pain, male rats showed an increased latency in response to application a thermal stimulus under heroin pretreatment, resulting more sensitive to heroin analgesic effects.

This results are confirmed by converging preclinical and clinical evidences, in which the majority of the studies assessed that morphine is more efficacious in modulating pain in males than females (Loyd DR et al, 2014; Craft RM, 2003); indeed researches have shown that morphine's median effective dose in female rodents is approximately twice the concentration of the dose needed for males to achieve comparable levels of pain relief (Fullerton EF et al, 2018).

The sex differences in opioid-analgesia sensitivity is not due to the pharmacokinetics of the drug (Cicero TJ et al, 1997) but to sexual dimorphic expression of MOP receptors in rat's periaqueductal gray (PAG), that is considered an essential neural substrate at the basis ofopioid-mediated analgesia (Loyd DR et al, 2008)

We also found that vulnerable rats showed a lower sensitivity to heroin induced analgesia and that there is a negative correlation between heroin intake and heroin analgesic ratio: it means thatlower is the sensitivity to heroin analgesia, higher is the heroin seeking.

A possible explanation is associated with the expression of the μu receptor (MOPR) that mediates either the opioid analgesic effects or the rewarding and addictive ones. Clinical evidences assessed that the gene coding for the human MOPR (OPRM1) has an important functional single nucleotide polymorphism (SNP), A118G (Bond C et al, 1998; Kreek MJ et al, 2005) and patients that are carriers of this 118G allele have lower sensitivity to opioid analgesic effects requiring a higher opioid dose to obtain analgesia (Zhen-Yu Ren et al, 2015).

Woodcock EA (Woodcock EA et al, 2017) and colleagues demonstrated that 118G carriers, compared with 118AA homozygous heroin-users, experienced more “severe” opioid dependence: he suggested that 118G allele may be less physiologically so that heroin may result less potent or have a lower intrinsic efficacy in 118G carriers who therefore need to self-administer larger doses to achieve rewarding effects, enhancing overdose risk. Behavioral preclinical studies demonstrated that A112G male and female mice harboring a functionally equivalent SNP in OPRM1, self-administered more heroin in extended-access sessions and had ahigher escalation over sessions compared their wild type littermates (Zhang Y et al, 2015). Takentogether this preclinical and clinical evidence support our results and give further translational validity to our study.

The great amount of data collected during our study still has much to reveal about the predictive validity of individual behavior in terms of propensity to develop OUD and need more in-depth investigations: GWAS analysis was the next step to discover the genetic underpinnings that determine a vulnerable or resilient phenotype.

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Chapter 5:

EFFECT OF CEBRANOPADOL ON HEROIN SEEKING AND TAKING IN AN ANIMAL MODEL OF INDIVIDUAL VARIABILITY, THE NIH_HS RATS

1. ABSTRACT

The inter-individual variability of the human population is at the basis of the different vulnerability to develop substance use disorder (SUD) but is also the reason why well validated medications failed in having success in some resistant patients. With the attempt to focus to a more individualized medical approach, the use of an outbred animal model in preclinical studies could be useful to investigate the different pharmacological responses to treatment due to individual variability.In this study we want to investigate the effect of Cebranopadol, a pan opioid agonist with nano-molar affinity for MOP and NOP receptor, in reducing heroin taking and seeking on the NIH_ heterogeneous stock rats that is a ratline characterized by a strong individual variability that allow us to detect the presence of a non-responder subpopulation. We evaluated the effect of Cebranopadol (0.0, 12.5, 25.0, 50.0 µg/kg p.o.) in alleviate heroin (20 µg/kg/infusion) taking in long-access self-administration session and cue-induced reinstatement of heroin seeking after an extinction phase in HS rats: results demonstrated that Cebranopadol succeeded in reducing both the number of heroin infusions in SA sessions and active lever presses in reinstatement test compared with vehicle, at all doses tested in males and females.

Despite the great efficacy of the treatment from an overall analysis, when we moved to visualize the individual response of the rats by a scatter plot of the data, results showed that in some rats Cebranopadol at doses of 25.0 and 12.5 µg/kg was not able in reducing heroin taking and seeking, independently by the cluster (vulnerable, intermediate, resilient the heroin dependence) allocation of the non responder subjects that are widespread in all the three behavioral cluster without predominance of one of them. These results let assume that the sensitivity to theCebranopadol treatment could be not related with the same predisposing factors that determine the vulnerability or resilience to develop heroin dependence and this is not related with gender differences. Preclinical models like this one could be useful to identify which genetic mutationsor traits are shared by non-responder individuals to fine tune personalized and more efficient therapies.

Keyword: individualized treatment, cluster, opioid use disorder, Cebranopadol

2. INTRODUCTION

Over the course of these two decades, the US spread a severe opioid epidemic. This record was exacerbated by the Covid 19 pandemics in the latest years. There was a parallel increase in both prescriptions of opioids as opioid pain relievers (OPRs) and abuse of illicit opioids, like heroin, fentanyl and oxycodone, as the majority of heroin addicts reports that they started usingprescribed opioid prior to switch to heroin that is less expensive and easy available on the black market (Cicero TJ et al, 2014; Compton WM et al, 2016).

Agonist maintenance therapy to treat OUD with long acting opioid agonists, like methadone and buprenorphine, are today the most effective strategy against heroin dependence (Kreek MJ et al, 2002; Ling W et al, 2003). One of the major limitations of these treatments is the poor patient compliance that is the reason why in recent years many extended-release formulations or monthly depot injection of these drugs were developed.

But, despite these new formulations available, buprenorphine and methadone in some individuals failed to obtain optimal response. For sure the side effects (similar to morphine in terms of withdrawal symptoms, respiratory depression and dysphoria state) or the high risk of abuse-liability (in particular related to methadone use) had a significant role in this therapeutic concern. One response to this problem was the development of new treatment characterized by low abuse potential and less impactful side effect : Cebranopadol (to deeper information see Chapter 3) appears to be a good candidate in term of low abuse liability, efficacy in relapse prevention, possible faster tapering in patients that are motivated to transition into a medication free state, also thanks to its long half-life.

A further factor to consider is that, even if a treatment appears overall efficacious in the great part of the population sampled, there will always be some patients, probably a little subgroup, that do not respond to classical and approved medications. There are inter-individual differences in drug metabolism for example, that is influenced by many factors like sex, environment, drug, as well as individual genetic profile (León-Cachón RB et al, 2012).

Many studies have demonstrated that genetic factors account for 20% and 40% of these differences between patients and in many cases they are most important for the outcome of drug therapy (Ingelman-Sundberg M, 2001).

Nowadays, has become more and more popular the individualized approach proposed by the Personalized Medicine (PM) that has the potential to tailor therapy to the best patient response and to develop agents targeted to groups for whom do not respond to traditional medications (Vogenberg FR et al, 2010).

NIH heterogeneous stock rats represent a valuable resource of genetic variability due to their heterogeneity in terms of genome and phenotype: each rat is genetically and phenotypically unique and it is not possible to have a biological replicate working with this outbred population. So they could be used not only to identify genetic loci underlying drug abuse behavior, that isone of the purposes of the study I presented in the previous Chapter, but this rat line could be useful also to investigate the different pharmacological responses to treatment due to theirindividual variability.

In Chapter 3 I have investigated the role of the stress vulnerability in enhancing the effect of Cebranopadol on msP male and female rats, underlying a significant gender difference in the response to this drug. Now, using 4 of the 15 cohorts that took part at the OUD-behavioral characterization discussed in Chapter 4, we want to investigate the efficacy of Cebranopadol ina population characterized by strong inter-individual differences that mimic the one of human population and could have a significant translational validity with the aim to improve the personalized treatment also in the field of the opioid use disorder.

Previous studies performed in our laboratory have already demonstrated the effectiveness of Cebranopadol in reducing heroin taking in 2h-self administration sessions and motivation for heroin in Wistar and msP rats (for more details about this latter experiment see Chapter 3 of this thesis) .

Here we want to investigate the effect of Cebranopadol pretreatment under FR1 contingency in HS rats in 12hour long-access self-administration sessions to evaluate not only different individual response to the drug but also its potency for a longer period, compared to classical 2h short access sessions, made possible thanks to its long half-life.

Then, after an extinction phase, we evaluated its effects in reducing reinstatement of heroin seeking induced by the representation of environmental cues: no data were published about it,but there were some evidences in preclinical study, published by De Guglielmo et colleagues (De Guglielmo et al, 2017), confirming that Cebranopadol blocked conditioned reinstatement of cocaine seeking.

Finally, we applied the clusterization of HS phenotypes carried out by Allen C. (Allen C et al, 2021), to the results obtained in order to better understand if there was a relationship between the predisposition to develop heroin dependence, indicated by the cluster allocation of each rat , and the individual response to Cebranopadol pretreatment: our purpose is to look into an eventual correlation between the genetical or behavioral factors associated with the OUD-vulnerability or resilience and the responsiveness to the related drug therapy.

3. MATERIAL AND METHODS

3.1 Animals

After the completion of experimental procedures described in Chapter 4 and confirmation of catheter patency with an IV injection of of 0.2-0.3 ml of Thiopental Sodium solution (Pentothal Sodium, 1g/50 ml, MSD Animal Health S.r.l), NIH Heterogeneous Stock (HS) rats were selected to test the effect of Cebranopadol on heroin self-administration and on cued reinstatement of heroin seeking. . Rats were maintained in the same housing conditions described in Chapter 4. . This work was conducted exclusively in NIH-HS rats trained at the University of Camerino.

3.2 Drugs

Heroin hydrochloride supplied by the National Institute on Drug Abuse (Bethesda, MD) was dissolved in sterile physiological saline (0,9% NaCl) and self-administered intravenously by rats at dose of 20 µg/kg/0.1 ml infusion adapted to the body weight recorded every week for each animal.

Cebranopadol (Biochempartner Co., Ltd., China) for operant tests was dissolved in 5% DMSO and 95% glucose (5% concentration dissolved in distilled water) and administered *per os* (p.o.) by gavage at the dose of 25 µg/kg and 50 µg/kg, one hour before self-administration session (Shen Q. et al, 2017). We chose oral administration to mimic the most common administration route in humans in order to have a better translational validity.

NIH_HS rats were trained to the gavage administration procedure for three consecutive days before starting experimental tests, during which they received distilled water to familiarize with this kind of procedure.

3.3 Heroin self-administration training

At the conclusions of the experimental procedures described in Chapter 4, 12 hours long access (LgA) heroin self-administration baseline was restored. Self-administration sessions were identical to as described in Chapter 4.

3.4 Experimental procedures

3.4.1 Effect of Cebranopadol on LgA heroin self-administration in male and female NIH_HS rats

Forty-four male and 42 female NIH_HS rats were employed in this study. Once the heroin self-administration baseline was re-acquired, the effect of Cebranopadol (0.0, 12.5, 25.0, 50.0 µg/kg) on LgA heroin self-administration was tested. Rats received the three doses of Cebranopadol and its vehicle in a Latin-square counterbalanced order. Tests were repeated every third day: on the first intervening day rats remained in their home-cage and on the second day they were given a baseline heroin SA session. Test sessions were repeated until the Latin square design was completed, i.e. all rats received all doses of Cebranopadol.

3.4.2 Effect of Cebranopadol on cued reinstatement of heroin seeking in in male and female NIH_HS rats

Fifty-Nine males and 56 female NIH-HS rats were employed in this study. After heroin self-administration was re-baselined, rats were subjected to two-hours daily extinction sessions. During extinction sessions active lever press was no longer reinforced by either heroin infusions or presentation of environmental cues previously paired with heroin infusion. Extinction sessions were performed for six continuous days.

The day after the last extinction session, the effect of Cebranopadol (0.0, 12.5, 25.0, 50.0 µg/kg) on cue-induced reinstatement of heroin seeking was evaluated. Cued reinstatement sessions were identical to heroin self-administration sessions, except that they lasted 2 hours and active lever presses resulted in presentation of the light/tone cue and turning off the house light associated with heroin but not in heroin delivery. Rats received the three doses of Cebranopadol and its vehicle in a Latin-square counterbalanced order. Tests were repeated every third day, during the two intervening days rats remained in their home cages. Test sessions were repeated until the Latin square design was completed, i.e. all rats received all doses of Cebranopadol.

3.7 Statistical analysis

The effect of Cebranopadol on heroin self-administration was analyzed by two-way ANOVA with sex as independent factor and Cebranopadol doses as repeated measures.

Data from the cued reinstatement test were analyzed twice. First, to verify that heroin paired cues had reinstated heroin seeking, lever response on the last day of extinction and on the cued reinstatement session under vehicle treatment were analyzed by two-way ANOVA with sex as independent factor and extinction/cue conditions as repeated measure. Then, the effect of cebranopadol on cued reinstatement was analyzed by two-way ANOVA with sex as independent factor and cebranopadol doses as repeated measures.

Active and inactive levers were analyzed separately.

The relative change in response induced by cebranopadol was calculated independently for each cebranopadol dose as follow: [(response under dosed treatment / response under vehicle treatment)-1]*100. The variables used to compute cebranopadol-induced change were heroin infusions in the heroin self-administration study and active lever response in the cued reinstatement study.

ANOVAs were followed by Bonferroni's or Dunnett's post hoc test when appropriate. Statistical significance was set to conventional $p<0.05$.

4. RESULTS

4.1 Effect of Cebranopadol on LgA heroin self-administration in male and female NIH_HS rats.

ANOVA found an overall effect of Cebranopadol dose [$F(3, 252) = 83.52$, $p<0.0001$], sex [$F(1, 84) = 15.29$, $p<0.0001$], and dose by sex interaction [$F(3, 252) = 13.67$, $p<0.0001$]. Dunnett's test for multiple comparisons revealed that each dose of Cebranopadol decreased heroin self-administration in both female and male rats (**Figure 1, upper panel**). We further analyzed between sex differences by Bonferroni's and we found that female rats earned a higher amount of heroin than males under vehicle treatment (**Figure 1, upper panel**).

Analysis of inactive lever found an overall effect of dose [$F(3, 252) = 12.45$, $p<0.0001$], but no effect of sex [$F(1, 84) = 2.99$, $p>0.05$], and no dose by sex interaction [$F(3, 252) = 0.3$, $p>0.05$] (**Figure 1, lower panel**).

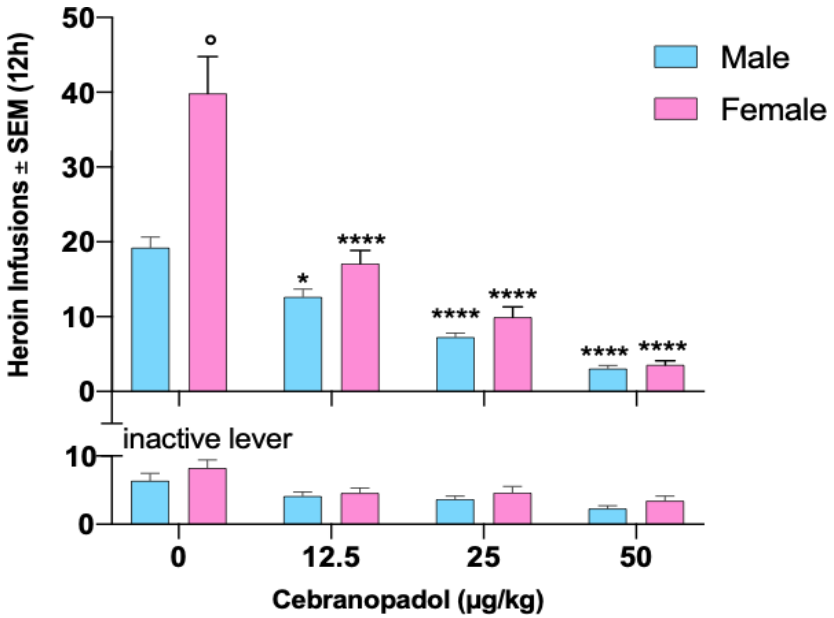


Figure 1: *Effect of Cebranopadol on LgA heroin self-administration in male and female NIH_HS rats. Female rats showed higher heroin self-administration than males. Cebranopadol decreased heroin self-administration in both male and female rats (upper panel). Inactive lever responses were very low and slightly decreased by Cebranopadol treatment. Data are expressed as Mean ±*

*SEM. Statistical Significance: °°°°p<0.0001 vs male same dose, *p<0.05 and ****p<0.0001 vs vehicle same sex.*

The significant sex by dose interaction described above indicate that male and female rats responded differently to Cebranopadol. Indeed, the effect of Cebranopadol relative to vehicle baseline seems stronger in female and in male rats (**Figure 1 upper panel**). In addition, observation of raw data indicated that individual rats showed different responses toCebranopadol. Therefore, for each rat we computed the relative change in infusion induced by each dose of Cebranopadol with respect to the vehicle treatment. This analysis revealed that 13 out of the 44 male rats showed no change or an increase in heroin infusion earned when treated with 12.5µg/kg of Cebranopadol and were defined as Non-Responder rats (30.9% NR of the male population). Five out of these 13 rats were also non-responder at the 25µg/kg dose (**Figure 2A**).

Within the 42 female rats we found 5 non-responder to the 12.5µg/kg dose, 2 of which were Non-Responder (NR) to the 25µg/kg dose as well (11.9% NR of the female population) (**Figure 2B**). All rats that showed a decrease in heroin infusion at each dose of Cebranopadol were defined as Responder (R).

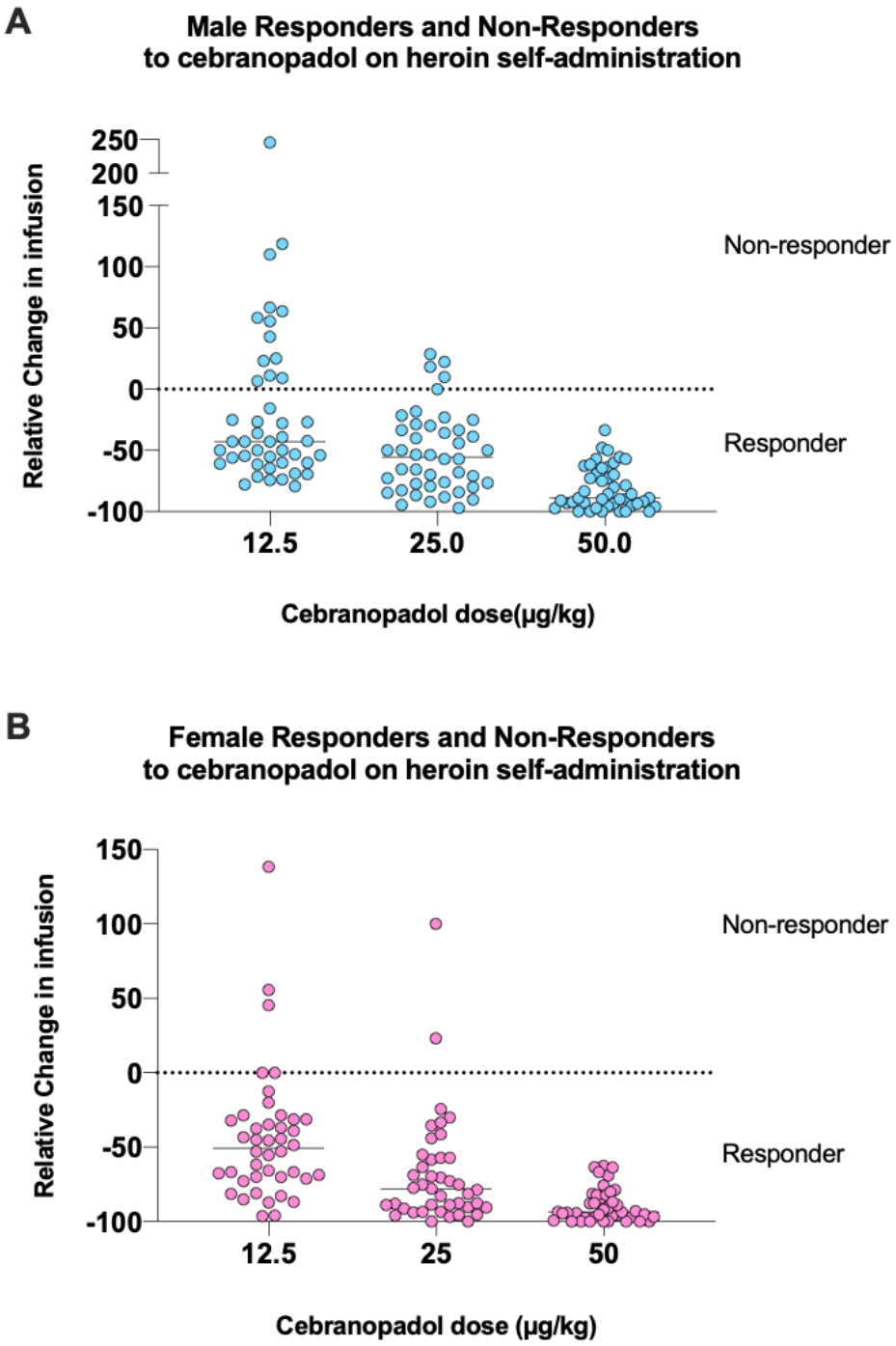
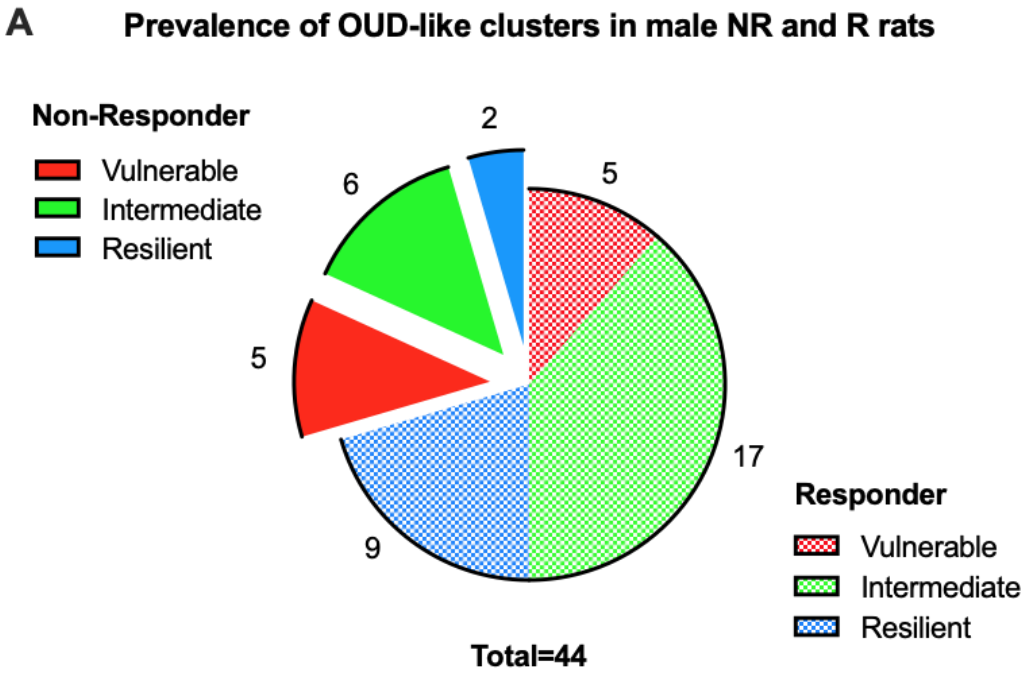


Figure 2: *Scattered plots of relative change induced by Cebranopadol on heroin self-administration in male (**A**) and female (**B**) rats. Rats with a relative change ≥0 were defined as non-responder whereas those with a relative change <0 were defined as responder.*

The rats included in this study were a subset of those used in Chapter 4, therefore they were characterized for their individual vulnerability to opioid use disorder (OUD)-like behavior, and they were clustered into Vulnerable, Resilient and Intermediate rats (See Chapter 4 for detail). Therefore we analyzed how OUD-like clusters were distributed within Cebranopadol R and NR rats.

We found that all three clusters were represented in both NR and R male (**Figure 3A**) and female (**Figure 3B**) rats :



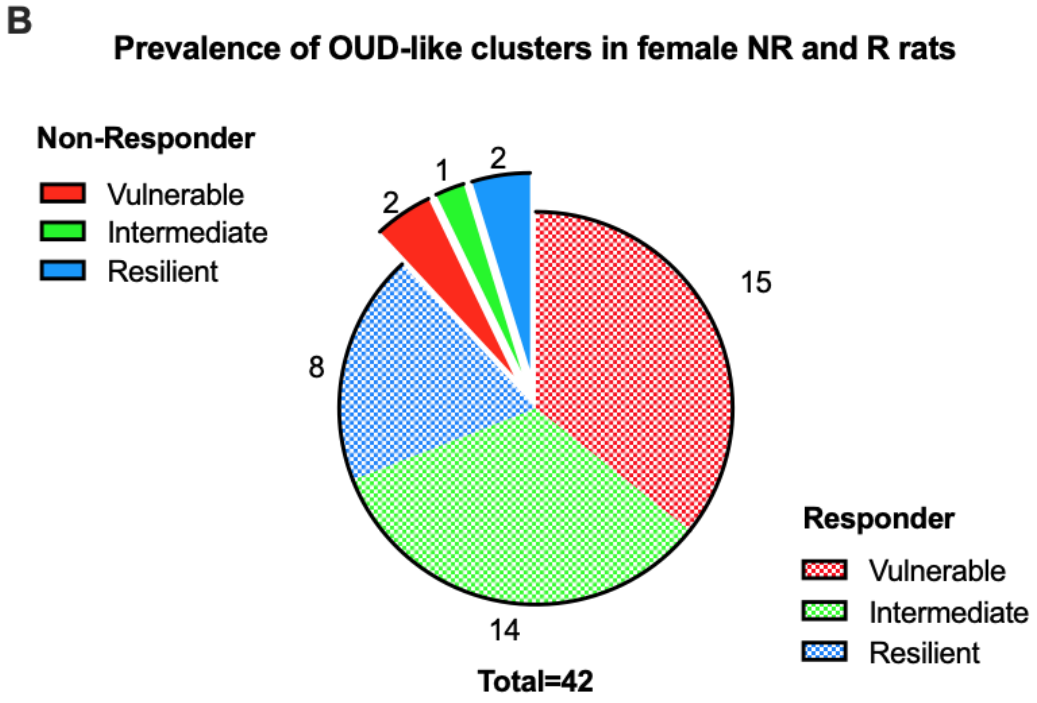


Figure 3: *Pie charts representing the prevalence of Vulnerable, Intermediate and Resilient OUD-like rats within male (**A**) and female (**B**) rats characterized as Responder (hashed color slices) and Non-Responder (full color slices) to the effect of Cebranopadol on heroin self-administration.*

4.2 Effect of Cebranopadol on cued reinstatement of heroin seeking in male and female NIH_HS rats

We first verified that representation of heroin-paired cued after extinction reinstated heroin seeking by comparing the number of lever presses produced on the last extinction day to the number of lever presses produced on the cued reinstatement session in which the rats received Cebranopadol's vehicle. ANOVA of active lever presses found and overal effect of sex [($F(1, 113) = 10.0$; $p<0.01$] and cue [($F(1, 113) = 169.3$; $p<0.0001$] but no cue by sex interaction [($F(1,113) = 2.1$; $p>0.05$]; consistent with a reinstatement of extinguished lever presses induced by cues under vehicle conditions in both male and female rats and a higher number of responses expressed by female rats both in extinction and reinstatement sessions (**Figure 4, upper panel**). Analysis of inactive lever presses found an overall effect of sex [($F(1, 113) = 8.4$; $p<0.01$] but noeffect of cue [($F(1, 113) = 0.7$; $p>0.05$] and cue by sex interaction [($F(1, 113) = 0.02$; $p>0.05$] (**Figure 4, lower panel**).

These analyses confirmed that heroin-paired cues increased selectively active lever presses and therefore that there was a reinstatement of heroin seeking, therefore we proceeded to analyze the effect of Cebranopadol on cued reinstatement. ANOVA of active lever presses found an overal effect of sex [($F(1, 113) = 10.9$; $p<0.01$], treatment [($F(3, 339) = 162.8$; $p<0.0001$] and treatment by sex interaction [($F(3, 339) = 4.0$; $p<0.01$]. Dunnett's test for multiple comparisons revealed that each dose of Cebranopadol decreased reinstatement of heroin seeking in both female and male rats (**Figure 4, upper panel**). We further analyzed between sex differences by Bonferroni'sand we found that female rats produced a higher amount of lever presses than males under the vehicle condition (**Figure 4, upper panel**). Analysis of inactive lever also found an overal effect of sex [($F(1, 113) = 7.0$; $p<0.01$], treatment [($F(3, 339) = 17.2$; $p<0.0001$] and treatment by sex interaction [($F(3, 339) = 3.1$; $p<0.05$] (**Figure 4, lower panel**).

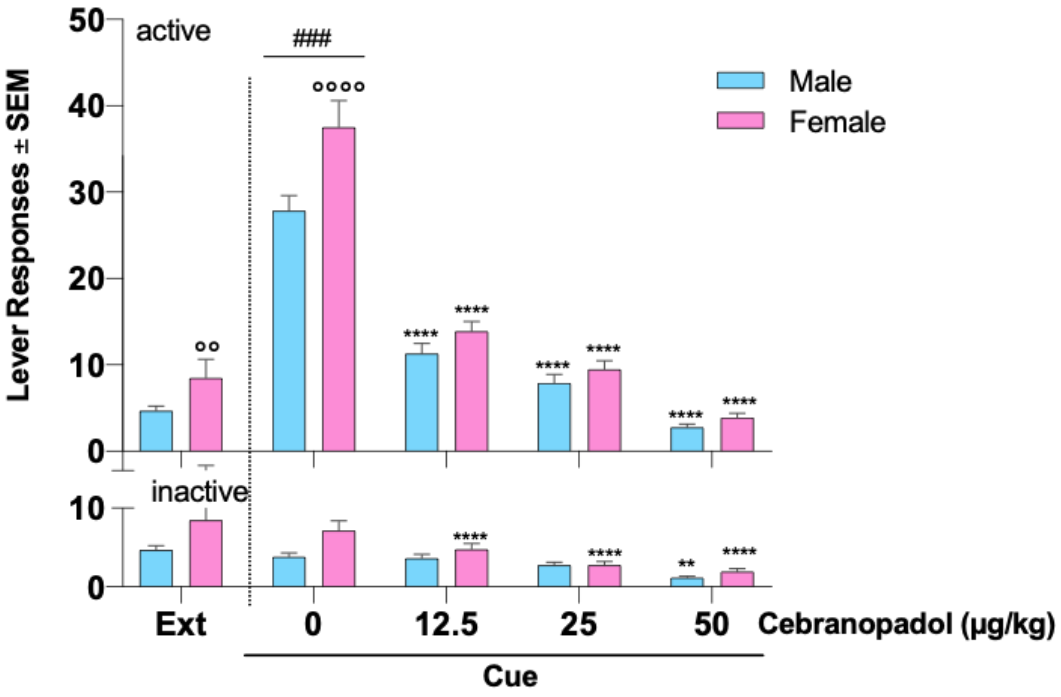


Figure 4: *Effect of Cebranopadol on cued reinstatement of heroin seeking in male and female NIH_HS rats. Heroin paired cues increased the number of presses on the active but not on the inactive lever, indicating reinstatement of heroin seeking. Female rats showed higher reinstatement than males. Cebranopadol decreased reinstatement of heroin seeking in both male and female rats. Inactive lever responses were very low and slightly decreased by cebranopadol treatment. Data are expressed as Mean ± SEM. Statistical Significance: ### p<0.001 vs extinction (Ext); °°°°p<0.0001 vs male same dose, *p<0.05 and ****p<0.0001 vs vehicle same sex.*

Also in this case we explored the existence of non responder rats computing the relative change on active lever presses induced by Cebranopadol, and again we found 7 NR out of the 59 male (11.8%)rats and 8 NR out of the 56 female rats (14.3%) (**Scatter plots in Figure 5 A and B respectively**).

Two males and three females were also NR to the 25µg/kg cebranopadol dose.

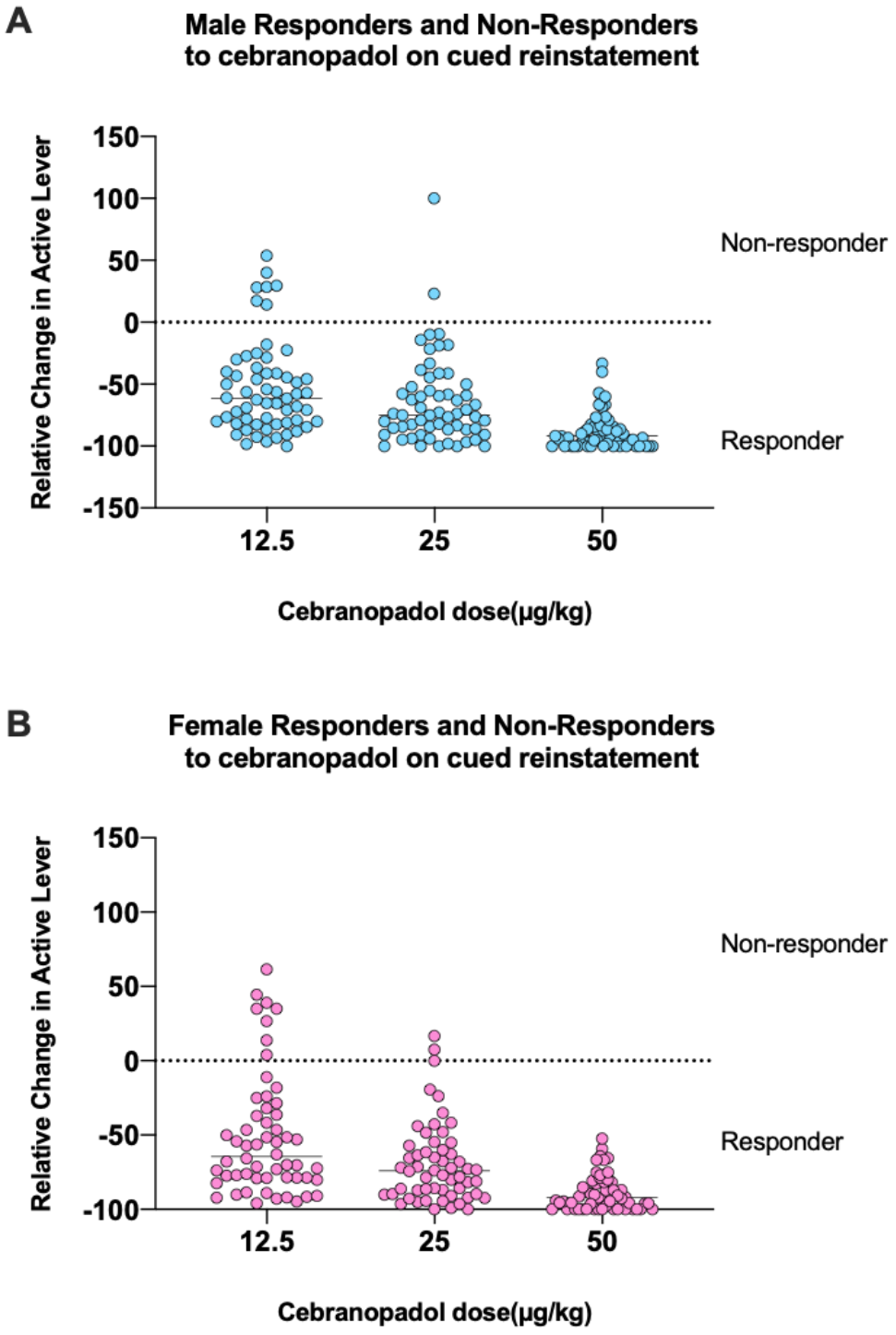


Figure 5: *Scattered plots of relative change induced by Cebranopadol on cued reinstatement of heroin seeking in male (**A**) and female (**B**) rats. Rats with a relative change ≥0 were defined as non-responder whereas those with a relative change <0 were defined as responder .*

Also in this case we analyzed how OUD-like clusters were distributed within cebranopadol R and NR rats. Again, we found that all three clusters were represented in both NR and R male (**Figure 6A**) and female (**Figure 6B**) rats

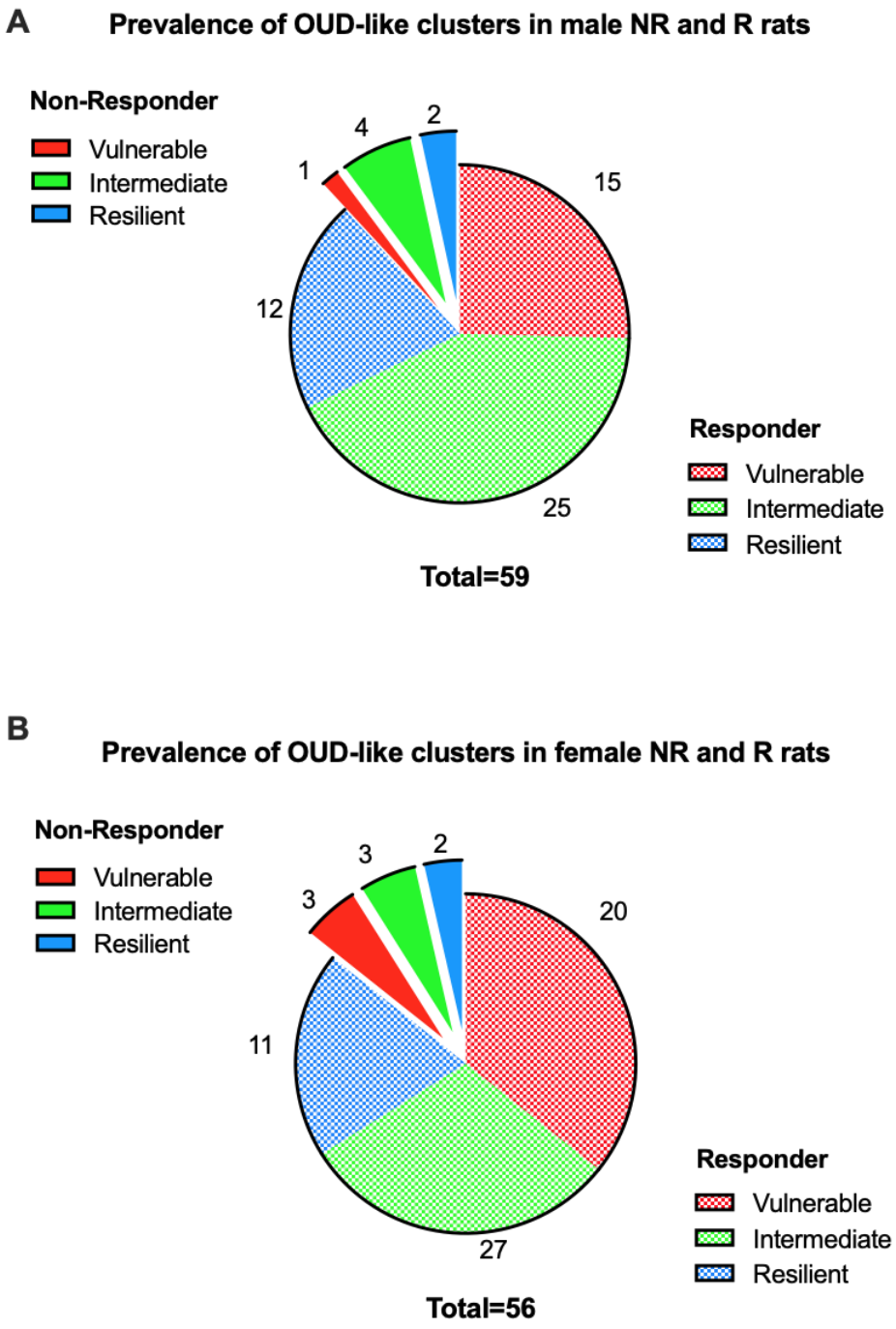


Figure 6*: Pie charts representing the prevalence of Vulnerable, Intermediate and Resilient OUD-like rats within male (**A**) and female (**B**) rats characterized as Responder (hashed color*

slices) and Non-Responder (full color slices) to the effect of cebranopadol on cued reinstatement of heroin seeking.

As detected above as regard to heroin taking (Figure 3), there was no prevalence of a particular cluster allocation in the non-responder group but the individuals are scattered indifferently on all the three clusters.

5. DISCUSSION

The NIH_ HS rats population has been initially created with the purpose to serve as a source of genetic diversity that more closely resemble the variations found in human population (Solberg Woods LC and Palmer A, 2018): in the previous Chapter, HS rats has been described as a model of individual variability useful to investigate genetic and phenotypic traits involved in producing vulnerability or resilience to develop OUD-like behavior. But there is more. We have hypothesized that their unique genetic and phenotypic makeup could be useful to study how these different OUD-like phenotypes could differently influence the response to pharmacologicaltreatment. If this assumption holds true, our model proves to have an important and useful translational validity also in the field of new more personalized therapies for the treatment of addiction disorders.

It is now well known that, even though the efficacy of a drug has been approved and largely efficacious in most of the population, some people do not respond to the therapy. These clinical occurrences are due to inter-individual variability in drug response.

All patients do not respond to the same medicine in the same way.

In addition to environmental, sexual, age, general medical conditions factors, today the differences in patient genetic make-up have been recognized to play an important role in the individual response to drugs and in determining the outcome of a pharmacological therapy (Vogenberg FR et al, 2010; Mini E. et al, 2009).

This is because many drug responses appear to be genetically determined and the relationship between genotype and drug response may have a very valuable diagnostic value (Shastry B, 2006). In many cases the differences are related to polymorphisms in drug metabolizer enzymes often leading to challenges in optimizing the dosage regimen for a particular patient (Wilkinson GR, 2005).

We had tested Cebranopadol, whose effectiveness in reducing heroin taking has been previously demonstrated (see Chapter 3 for more details), in HS rats in order to investigate how it works ina heterogeneous population and if there is a correlation between the OUD vulnerability or resilience and the responsiveness to this pharmacological treatment.

At first, we reconfirmed that Cebranopadol succeeded in reducing both heroinself-administration and heroin cue-induced reinstatement, considering the population as a whole just separating male and female behavioral responses: from an overall outlook results seem to replicate what we had previously demonstrate with msP rat line, so that in female rats Cebranopadol was able to minimize the number of heroin reward without dose-dependence effect. Even if in this study we added a lower dose of Cebranopadol (12.5 µg/kg) than those previously tested on msP rats, also this one did not differ in its potency compared with the higher ones.

In male rats, heroin taking is significantly reduced by Cebranopadol but in a dose-dependent manner. Taken together this data confirms precedent findings related to the gender-different sensitivity to Cebranopadol pretreatment that we have already discussed in Chapter 3 with females resulting more susceptible.

The number of heroin infusion earned by female HS rats in long-access self-administration session as control are in line with data collected for Wistar and msP female rats so that there is a statistically significant sex-differences that is maintained not only across different strains , but also across species because epidemiological studies confirmed the same trend also in human society. Despite the high degree of variability of this animal model, HS rats preserve thisgender-dependent trait so that females resulted in higher amounts of heroin taken independently by species.

As well, female HS rats resulted also more prone to relapse after representation of environmental cues previously paired with heroin, considering the number of active lever presses when treated with Cebranopadol's vehicle : taken together this results account female population more vulnerable either to heroin taking or to heroin seeking, resulting also less prone to extinguished their addictive behavior as result from the higher residual lever press at the end of the extinction phase, compared with male. These are the three variables taken into account, in our paper published by Allen C.. in 2021 (Allen C et al, 2021), to classify the different addiction-like phenotypes, and in this case, females resulted positive to all the three, confirming that they are the more vulnerable gender.

The selectivity in the action of Cebranopadol is suppressing only heroin taking/seeking and not locomotor activity of the animals is confirmed by the analysis of number of the inactive lever press that was only slightly affected by the drug in addition to the studies of De Guglielmo G. and Shen Q. (De Guglielmo G et al, 2017; Shen Q et al, 2017) that concomitantly demonstrated that Cebranopadol did not reduce saccharine and sweetened condensed milk self-administration and did not significantly reduce locomotion in CPP.

We have been the first to test Cebranopadol in long-access (12 hours) heroin self-administration demonstrating that is efficacy last at least for all the duration of the overnight session, based on pharmacokinetics data already published: phase 1 pharmacokinetic studies have demonstrated that this drug is characterized by a late time to reach maximum concentration (Cmax =4 to 6 hours) and a half-life of 24 hours (Linz K et al, 2014). These features made Cebranopadol a good candidate for chronic therapies because it is suitable for once-daily administration even without an extended-release formulation (Tzschentke TM et al, 2019).

In literature there were no data about the efficacy of the Cebranopadol on reinstatement of heroin seeking induced by representation of environmental cues: all the studies published are related to cocaine seeking and, in our laboratory, Cebranopadol has been tested in Wistar rats (data not published) only on a stress (yohimbine) induced reinstatement. However, we demonstrated that Cebranopadol is really effective in reducing active lever press in cue-induced reinstatement test at all the three doses and without gender differences.

Despite Cebranopadol succeeded in reducing either heroin taking or heroin seeking from an overall analysis, when we computed the relative change in infusion induced by each dose of Cebranopadol, we have noticed that some rats, both male and female, appeared to be resistant to the treatment and, conversely, increase active lever press with respect to when they are treated with vehicle , even if the overall result is not affected by these contrary responses.

But that's not all: the individual rats classified as non-responder to Cebranopadol pretreatment under FR1 contingency are not the same that result in the reinstatement test. This finding suggests that an individual could be not completely resistant to a pharmacological treatment but only to one of its effects depending on its own genetic makeup.

The heterogeneous responses to a drug treatment shown by HS rats reinforce the translational validity of this animal model to mimic human population and also the existence of littlesubgroups of patients that are resilient to some pharmacological therapies. Preclinical models like this one could be useful to identify which genetic traits, mutations or polymorphism are shared by non-responder individuals to fine tune personalized therapies or personalized dosage (considering that all the rats respond to the highest dose) and to avoid, in people with these resilient traits, the use a priori of the classical pharmacological therapies.

These are the assumptions on which the recent development of the branches of pharmacogenetics and pharmacogenomics is based: while the former one is largely focused to genes that determine different drug metabolism, the latter encompasses all genes in the genome that may determine drug responses (Pirmohamed M, 2001, Evans WE et al, 1999). The advantage in the use of HS population to identify the genetic loci responsible of the difference in drug response is that the genome of the founders of all extant HS have been fully sequenced and these data are available in public database easily accessible because of each rat is assigned a unique ID associated with its genome informations, so that it is possible to identify markers, coding variants and structural differences in each of their genomes (Solberg Wood LC and Palmer AA, 2018).

We have also analyzed the distribution of the non-responder subgroups among clusters, and we have found that they did not belong to a particular one, rather, they were spared in all the three clusters, without a particular predominance of one of them.

This suggests that the behavioral phenotype associated with the propensity to take and seek the drug, at least in the case of heroin dependence, cannot be considered predictive of the responsiveness to the corresponding treatment. Most importantly, in view of future clinical trials, we have to remark that only a portion of vulnerable subjects, that are the key targets of a OUD therapy because are the ones that in the human society will have a high risk to fall into addiction, result in reducing their heroin intake follow treatment and the ones who do not respond to Cebranopadol upon substance abuse are not the same non-responder under relapse condition. Translating these findings from preclinics to clinics, this component assumes a particular significance in the evaluation of personalized pharmacological strategies: Cebranopadol could be a good choice for a patient to stop heroin taking but could completely fail in the very same individual to prevent relapse.

Among our sample of treated, the non-responder subgroup in term of heroin taking behavior, showed a differentiate composition not only as regard the phenotypical cluster allocation, but also concerning the sex: the percentage of non-responder females (30.9%) is about triple that of males (11.9%). One more time, gender appear a trait with a valuable influence in all the fields of addiction, including OUD therapies failure.

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Conclusive remarks

In conclusion, the main findings of this work can be summarized as follows:

- Stress is one of the vulnerability's factors that enhances heroin taking and motivation. Having demonstrated a higher propensity to OUD-like behavior in msP rats, already selected for their alcohol preference and hyper-activation of the stress system, we further validated the existent comorbidity between stress and polydrug abuse, including opioids.
- HS rats represent a valid preclinical model to investigate the different vulnerability or resilience to develop OUD due to their genetic heterogeneity. The three clusters identified by the SBM model result in a great translational validity because they biologicallyresemble the behavioral heroin-related traits of a vulnerable, intermediate and resilient phenotype.
- The gender is a variable that cannot be excluded from preclinical study because has a strong impact in determining addiction vulnerability: both msP and HS rats displayed a sex-dependent response to heroin exposure. Female rats, as well as in human, resulted more prone to fall into the heroin dependence.
- There were some innate behavioral traits, like locomotor activity, anxiety-like behavior, sensitivity to opioid analgesics that could have a predictive role in determining the vulnerability to develop OUD.
- In the field of pharmacological treatment for OUD, Cebranopadol results to be a promising strategy due to its low abuse potential and high selectivity for the addictive substances: either in a model of stress vulnerability (msP rats) and in a model of individual variability (HS rats) Cebranopadol succeeded in reducing heroin intake, motivation for heroin and cue-induced reinstatement.

- The use of an outbred ratline has allowed to identify the presence of a subpopulation that does not respond to Cebranopadol despite the overall efficacy of the treatment, among which there are also some vulnerable individuals with a higher risk to develop OUD: this finding give another important translational value to our study supporting the importance to fine-tune personalized therapy.

Appendix:

Scientific Contributions

International Journal of
Molecular Sciences

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Article

Effect of Glucocorticoid Receptor Antagonism on Alcohol Self-Administration in Genetically-Selected Marchigian Sardinian Alcohol-Preferring and Non-Preferring Wistar Rats

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Abstract: Alcoholism is a chronically relapsing disorder characterized by high alcohol intake and a negative emotional state during abstinence, which contributes to excessive drinking and susceptibility to relapse. Stress, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and alterations in glucocorticoid receptor (GR) function have been linked to transition from recreational consumption to alcohol use disorder (AUD). Here, we investigated the effect of pharmacological antagonisms of GR on alcohol self-administration (SA) using male and female Wistar and Marchigian Sardinian alcohol-preferring (msP) rats, a rodent line genetically selected for excessive alcohol drinking and highly sensitive to stress. Animals were trained to self-administer 10% (*v*/*v*) alcohol. Once a stable alcohol SA baseline was reached, we tested the effect of the GR antagonists mifepristone (0.0, 10, 30 and 60 mg/kg; i.p.) and CORT113176 (0.0, 10, 30 and 60 mg/kg) on alcohol SA. To evaluate whether the effects of the two compounds were specific for alcohol, the two drugs were tested on a similar saccharin SA regimen. Finally, basal blood corticosterone (CORT) levels before and after alcohol SA were determined. Systemic injection with mifepristone dose-dependently reduced alcohol SA in male and female Wistars but not in msPs. Administration of CORT113176 decreased alcohol SA in male and female Wistars as well as in female msPs but not in male msP rats. At the highest dose, mifepristone also reduced saccharin SA in male Wistars and female msPs, suggesting the occurrence of some nonspecific effects at 60 mg/kg of the drug. Similarly, the highest dose of CORT113176 (60 mg/kg) decreased saccharin intake in male Wistars. Analysis of CORT levels revealed that females of both rat lines had higher blood levels of CORT compared to males. Alcohol consumptionreduced CORT in females but not in males. Overall, these findings indicate that selective blockade of GR selectively reduces alcohol SA, and genetically selected msP rats are less sensitive to this pharmacological manipulation compared to heterogeneous Wistars. Moreover, results suggest sex differences in response to GR antagonism and the ability of alcohol to regulate GR transmission.

Keywords: alcohol use disorder; stress; alcohol preferring rats; glucocorticoids; mifepristone; alcohol self-administration

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1. Introduction

Alcohol use disorder (AUD) is a complex psychiatric condition characterized by excessive drug use, loss of control over its consumption and emergence of a negative emotional state during withdrawal that contribute to relapse [1]. AUD is a major public health problem, and alcohol represents a significant disability and morbidity factor responsible of about three million deaths per year [2].

Stress and dysregulation of related hormones of the hypothalamic-pituitary-adrenal (HPA) axis have been proposed as important factors affecting disease progression [3,4].

The HPA axis represents the primary neuroendocrine network controlling stress response, and its activation in response to external or internal perturbation culminates in the production and release of cortisol in humans and corticosterone (CORT) in rodents [5]. Once released, glucocorticoids act through either the high affinity mineralocorticoid or the low affinity glucocorticoid receptor (GR). GR is highly expressed in several brain regions of the limbic system, in the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary gland [6]. Once released, glucocorticoids produce an array of physiological effects to adjust the organism to stressor exposure and are also responsible for termination of their actions via negative feedback inhibition at HPA level [5].

The motivation to drink alcohol is initially driven by positive reinforcement mechanisms, and its consumption is usually linked to recreational purposes. Studies in rodent models mimicking the early stages of alcohol consumption demonstrated that CORT administration increased alcohol self-administration (SA) [7–9] whereas adrenalectomy decreased it [10]. Noteworthy is that alcohol drinking was recovered by corticosterone replacement, suggesting that glucocorticoids facilitate alcohol reinforcement [10]. As a result of chronic alcohol drinking, the excessive and protracted activation of the HPA axis may lead to its dysregulation. This contributes to the surge of compulsive alcohol drinking motivated by the need to self-medicate to attenuate the negative symptoms associated with alcohol withdrawal [4,11,12]. Earlier studies demonstrated that alcohol-dependent rats exhibited significant downregulation of GR during acute withdrawal, and GR upregulation during protracted abstinence in several stress/reward related brain areas, suggesting that the GR system may contribute to the progression of AUD [13].

The genetically selected Marchigian Sardinian alcohol-preferring (msP) rat line is a well consolidated animal model to study AUD. In this rat line, anxiety and depressive-like traits have been cosegregated with high alcohol preference during the selection process. Hence, it is possible that their innate propensity to consume high amounts of alcohol is driven by the attempt to self-medicate from an innate negative affect, specifically mimicking the subpopulation of humans with alcoholism that consume alcohol for tension relief purposes [14,15]. Consistent with this view, earlier studies showed that msP rats carry two single nucleotide polymorphisms in the promoter region of the CRF1 receptor (CRF1-R) leading to hyperactivation of the system that is attenuated by voluntary alcohol consumption [16–18]. These mutations have been also associated with a decreased threshold for stress-induced alcohol-seeking and conferred to msP rats higher sensitivity to CRF1-R antagonists [16,19,20]. Noteworthy is that these gene polymorphisms are conserved in the human CRF system and have been correlated with the diagnosis of AUD [21,22]. We also reported that male msP rats displayed dysregulated GABA and glutamate signaling [23–25]. Recently, it has been found that male msP rats displayed diminished stress-induced GR phosphorylation at the serine site 232 in the PVN and a constitutive increase in phosphorylated GR levels in the central nucleus of the amygdala (CeA) [26]. The elevation of GR phosphorylation was also observed in the CeA of alcohol-dependent rats during acute withdrawal [27]. In postdependent rats, systemic and intra-CeA administration of mifepristone, a nonselective glucocorticoid and progesterone receptor antagonist, reduced alcohol intake and yohimbine-induced reinstatement of alcohol seeking [27,28].

Currently, it is unknown whether the constitutive alteration of GR levels of msP rats might contribute to their excessive alcohol-drinking phenotype. However, considering that this rat line shows features resembling postdependent rats, we thought it important to explore the effect of pharmacological antagonism of GR on alcohol self-administration by comparing the msP rat line with its Wistar counterpart. Moreover, considering that several sex differences have been described in response to stress and to alcohol, and that the HPA axis function is greater in female rats, in the present study we tested males and females separately [15,29–33].

2. Results

2.1. Experiment 1.1: Effect of Mifepristone on Alcohol Self-Administration in Male and Female msP and Wistar Rats

We tested the effect of mifepristone on alcohol SA under Fixed Ratio 1 (FR1) schedule of reinforcement in male and female msP (N = 10/sex) and Wistar (N = 9–10/sex) rats. Experimental subjects received mifepristone (10, 30 and 60 mg/kg) or its vehicle in a counterbalanced within subject Latin square design. A three-way ANOVA revealed an overall effect of treatment [$F_{(3,35)} = 7.5$; $p < 0.001$], sex [$F_{(1,35)} = 55.8$; $p < 0.0001$] and strain [$F_{(1,35)} = 41.2$; $p < 0.0001$]. There was a significant sex x strain interaction [$F_{(1,35)} = 10.2$; $p < 0.01$], but no other significant interactions. These results reflect higher SA levels in msP, a higher number of rewards by male msP rats and a general reduction of alcohol SA induced by mifepristone. To further evaluate the effect of mifepristone, we carried out single ANOVAs to independently analyze the drug effect on male and female msPs as well as on male and female Wistars. In msP rats no overall effect of treatment in male [$F_{(3,9)} = 0.4$; $p > 0.05$] or in female rats [$F_{(3,9)} = 1.1$; $p > 0.05$] was detected. Conversely, an overall significant effect of treatment was detected in male [$F_{(3,8)} = 4.0$; $p < 0.05$] and female [$F_{(3,9)} = 7.5$; $p < 0.01$] Wistars. Dunnett's post hoc analysis showed a significant decrease in the number of alcohol-reinforced responding at doses of 30 mg/kg and 60 mg/kg of mifepristone in both male and female Wistar rats ($p < 0.05$; Figure 1A, upper panel).

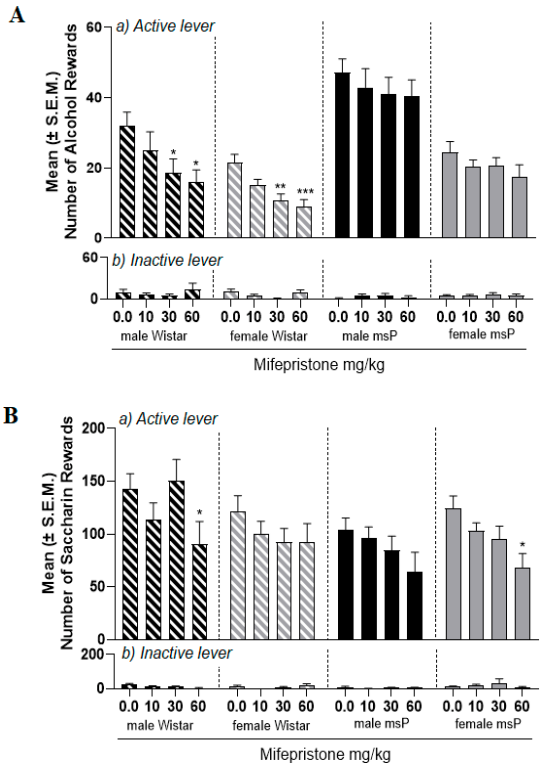

Figure 1. Effect of mifepristone on alcohol and saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with mifepristone (0.0, 10, 30 and 60 mg/kg) i.p., 90 min prior to test session. (**A**) Mifepristone treatment significantly reduced the number of alcohol rewards in male and female Wistars. Drug treatment did not decrease alcohol SA in male and female msPs. (**B**) At the dose of 60 mg/kg, mifepristone significantly reduced saccharin SA in male Wistars and in female msPs. Data are expressed as the mean ± SEM of number of: (**a**) reinforced responses (rewards) at the active lever and (**b**) total responses at the inactive lever. Significant difference from vehicle (0.0 mg/kg): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A three-way ANOVA applied to inactive lever responding showed no overall effect of treatment [$F_{(3,35)} = 0.9$; $p > 0.05$], sex [$F_{(1,35)} = 0.05$; $p > 0.05$] or strain [$F_{(1,35)} = 2.3$; $p > 0.05$]. Neither interaction was detected (Figure 1A, lower panel).

2.2. Experiment 1.2: Effect of Mifepristone on Saccharin Self-Administration in Male and Female msP and Wistar Rats

To control for the selectivity of mifepristone effect on alcohol SA, other groups of male and female msP (N = 6–8/sex) and Wistar (N = 7–8/sex) rats were tested for the effect of mifepristone (10, 30 and 60 mg/kg) or its vehicle on saccharin SA. A three-way ANOVA found a significant effect of treatment [$F_{(3,25)}$ = 8.8; p = 0.0001], no effect of sex [$F_{(1,25)}$ = 0.3; p > 0.05], no effect of strain [$F_{(1,25)}$ = 0.4; p > 0.05] and no interactions. To further explore the effect of mifepristone, data from male and female msPs and male and female Wistars were analyzed separately by single ANOVAs. Results revealed an overall effect of treatment in male Wistars [$F_{(3,6)}$ = 4.7; p < 0.05] and female msPs [$F_{(3,7)}$ = 6.4; p < 0.01]. Conversely, no overall effect was found in female Wistars [$F_{(3,7)}$ = 1.4; p > 0.05] and male msPs [$F_{(3,5)}$ = 1.2; p > 0.05]. Dunnet's post hoc tests showed that 60 mg/kg of mifepristone reduced saccharin SA in both male Wistars and female msPs (p < 0.05) (Figure 1B, upper panel).

Analysis of inactive lever responding found no significant overall effect of treatment [$F_{(3,25)}$ = 0.7; p > 0.05], sex [$F_{(1,25)}$ = 1.4; p > 0.05], strain [$F_{(1,25)}$ = 0.005; p > 0.05] and no interactions (Figure 1B, upper panel).

2.3. Experiment 2.1: Effect of CORT113176 on Alcohol Self-Administration in Male and Female msP and Wistar Rats

Mifepristone is a GR antagonist that also has activity on the progesterone receptor. To confirm that effects observed were specifically mediated by GR antagonism, we tested CORT113176, which is another more selective GR antagonist [27]. Once a stable baseline of alcohol SA was reached, male and female msP (N = 9–10/sex) and Wistar (N = 10/sex) rats were treated with CORT113176 (10, 30, 60 mg/kg) or its vehicle. A three-way ANOVA revealed an overall effect of treatment [$F_{(3,35)}$ = 11.1; p < 0.0001], sex [$F_{(1,35)}$ = 16.04; p < 0.001], strain [$F_{(1,35)}$ = 24.6; p < 0.0001] and sex x strain interaction [$F_{(1,35)}$ = 6.3; p < 0.05], but no other significant interactions (Figure 2A, upper panel). At this point we conducted single ANOVAs to further determine the effect of CORT113176 on male and female msPs and male and female Wistars. Results showed an overall effect of treatment in male Wistars [$F_{(3,9)}$ = 4.4; p < 0.05], female Wistars [$F_{(3,9)}$ = 4.5; p < 0.05] and female msPs [$F_{(3,9)}$ = 4.0, p < 0.05]. No effect was found in male msP [$F_{(3,8)}$ = 1.9; p > 0.05] rats. Dunnet's post hoc tests revealed that at 60 mg/kg, CORT113176 decreased alcohol SA in male (p < 0.01) and female Wistars (p < 0.05) as well as female msPs (p < 0.01).

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,35)}$ = 0.8; p > 0.05] and strain [$F_{(1,35)}$ = 3.7; p > 0.05] but an overall effect of sex [$F_{(1,35)}$ = 7.7; p < 0.01], treatment x strain [$F_{(1,35)}$ = 5.6; p < 0.01] and sex x strain interaction [$F_{(1,35)}$ = 5.5; p < 0.05] was observed (Figure 2A, lower panel).

2.4. Experiment 2.2: Effect of CORT113176 on Saccharin Self-Administration in Male and Female msP and Wistar Rats

We next verified the specificity of action of CORT113176 by testing its effect on saccharin SA in male and female msP (N = 9–10/sex) and Wistar (N = 9–10/sex) rats. Three-way ANOVA demonstrated a significant effect of treatment [$F_{(3,34)}$ = 5.2; p < 0.01], strain [$F_{(1,34)}$ = 10.3; p < 0.01] and treatment x sex interaction [$F_{(3,102)}$ = 3,4; p < 0.05] (Figure 2B, upper panel). When single ANOVAs were carried out, we found an overall effect of CORT113176 on saccharin SA only in male Wistar rats [$F_{(3,8)}$ = 4.5; p < 0.05]. No drug effect was detected in female Wistars [$F_{(3,9)}$ = 0.7; p > 0.05], male msPs [$F_{(3,8)}$ = 1.7; p > 0.05] and in female msPs [$F_{(3,9)}$ = 0.4; p > 0.05].

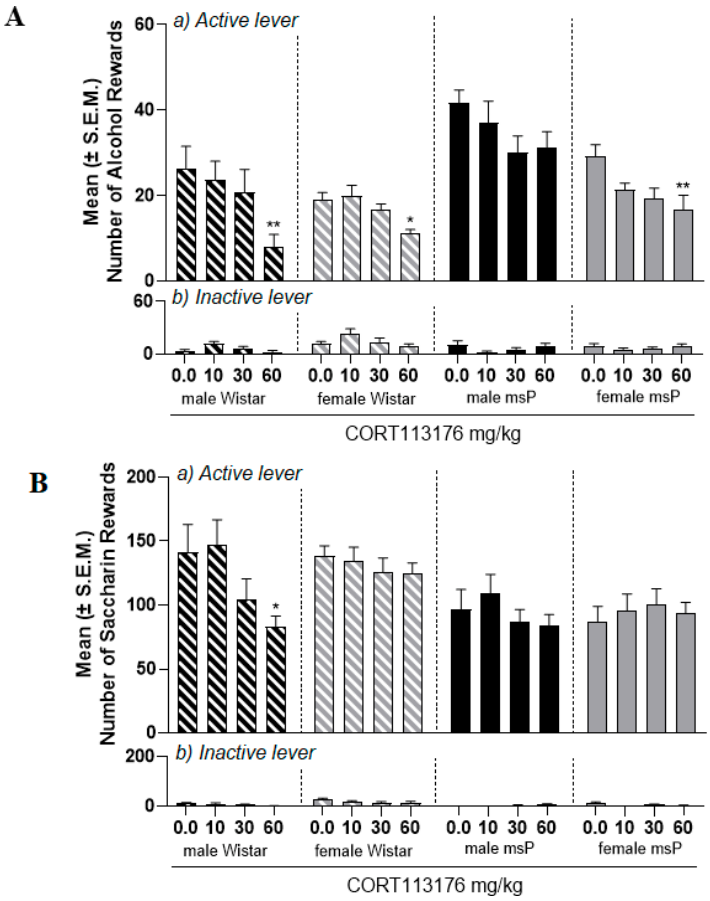

Figure 2. Effect of CORT113176 on alcohol and saccharin self-administration in male and female msP and Wistar rats. Male and female msP and Wistar rats were treated with CORT113176 (0.0, 10, 30 and 60 mg/kg) i.p., 90 min prior to test session. (**A**) CORT113176 treatment significantly reduced the number of alcohol rewards in male and female Wistars and in female msP rats. (**B**) CORT113176 at the dose of 60 mg/kg significantly reduced saccharin SA in male Wistar rats only. Data are expressed as the mean ± SEM of number of: (**a**) reinforced responses at the active and (**b**) total responses at inactive lever. Significant difference from vehicle (0.0 mg/kg): ** $p < 0.01$; * $p < 0.05$.

Analysis of the inactive lever found no significant overall effect of treatment [$F_{(3,34)} = 2.7$; $p > 0.05$], but there was a significant effect of sex [$F_{(1,34)} = 9.3$; $p < 0.01$], strain [$F_{(1,34)} = 15.4$; $p < 0.01$] and treatment x strain interaction [$F_{(3,102)} = 2.9$; $p < 0.05$] (Figure 2B, lower panel).

2.5. Experiment 3: Blood CORT Levels Following Alcohol Self-Administration in Male and Female msP and Wistar Rats

Finally, we assessed the blood CORT levels under basal conditions and after alcohol SA in male and female msP (N = 6/sex) and Wistar rats (N = 8–7/sex). Three-way ANOVA revealed a main effect of sex [$F_{(1,23)} = 84.5$; $p < 0.0001$], alcohol condition [$F_{(1,23)} = 19.5$; $p < 0.001$], strain [$F_{(1,23)} = 13.8$; $p < 0.01$], sex x alcohol condition interaction [$F_{(1,23)} = 18.3$; $p < 0.001$] and sex x strain interaction [$F_{(1,23)} = 4.4$; $p < 0.05$]. Female rats from both genotypes displayed persistently higher levels of CORT compared to male rats in both conditions. Female Wistar rats showed higher CORT levels than female msPs ($p < 0.001$). Alcohol consumption in a SA session decreased CORT levels only in female animals ($p < 0.001$). In male rats, blood CORT concentrations were not affected by alcohol SA (Figure 3).

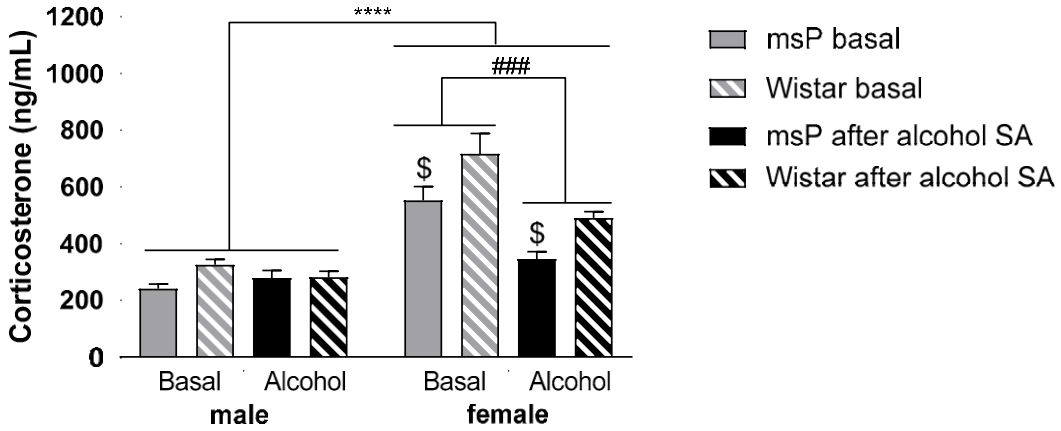

Figure 3. Blood corticosterone (CORT) levels under basal conditions and after alcohol SA sessions in male and female msP and Wistar rats. Females displayed significantly higher blood CORT levels than males independently of rat strain. Female Wistars had higher CORT levels than female msPs. Alcohol consumption decreased basal CORT levels in female animals only. In both rat lines, CORT levels of male rats remained unchanged following alcohol SA. Data are presented as mean ± SEM. Main effect of sex: **** $p < 0.0001$; main effect of sex x alcohol condition: ### $p < 0.001$; $ $p < 0.05$ vs. msP same condition and sex (sex x strain interaction).

3. Discussion

The present study investigated the effect of glucocorticoid receptor antagonism on alcohol drinking in genetically-selected msP rats in comparison with nonselected Wistar rats. To summarize, we found that mifepristone administration reduced alcohol SA in both male and female Wistar rats, but not msPs, at similar dose ranges utilized in previous studies measuring alcohol SA in dependent Wistar rats [27]. The ability of mifepristone to reduce alcohol SA was apparent at the intermediate dose of 30 mg/kg, while higher doses (60 mg/kg) appeared to produce nonselective reductions of saccharin SA, suggesting the occurrence of nonspecific effects. Given the nonselectivity of mifepristone in antagonizing progesterone receptors also, we tested the selective CORT113176 compound that targets GR to confirm whether reducing alcohol SA requires specificity for the GR. Consistent with results with mifepristone, CORT113176 significantly reduced alcohol SA in male and female Wistars as well as female msP rats. As for mifepristone, male msPs did not respond to CORT113176 treatment. Furthermore, administration of CORT113176 at thehighest dose reduced saccharin SA only in male Wistar rats. Taken together, we suggest that our drug regimen is specific to alcohol SA, since the number of saccharin rewards was not modified in the other groups of rats. However, at high doses, nonspecific inhi- bition of motivated behavior may emerge. Earlier work demonstrated that mifepristone decreases alcohol consumption in a limited-access two-bottle choice paradigm [34], andintra-CeA infusion of mifepristone reduces alcohol-seeking behavior following a yohimbine challenge [28]. Noteworthy is that it has been also demonstrated that chronic adminis- tration of mifepristone in alcohol vapor-exposed rats prevented the escalation of alcoholintake [13]. Consistently, acute mifepristone administration selectively reduced alcohol in- take in alcohol-dependent but not in nondependent rats [27]. Moreover, both mifepristone and CORT113176 selectively reduced binge-like ethanol intake in mice selectively bred for high ethanol concentration using drinking in the dark procedures [35]. Finally, it was shown that in nondependent Wistar rats, GR antagonism was more efficacious in female than in male rats [36]. Our results are consistent with these earlier works and confirmed that GR antagonists also reduced alcohol intake in nondependent animals, an effect more robust in female versus male rats [35,36].

Msp rats have long been proposed as an innate phenocopy of a subpopulation of patients that drink excessive amounts of alcohol for tension relief and self-medicating purposes [14]. Earlier studies have demonstrated that this rat line is characterized by two single-nucleotide polymorphisms at the CRF1-R receptor locus, leading to an enhanced

expression of CRF1R in different brain regions [16]. Because of this overexpression, they are highly sensitive to stress and show anxious and depressive-like symptoms that are relieved by alcohol consumption [14,16,17]. Recent findings have proved that negative feedback processes regulating HPA responsiveness are impaired in msP versus Wistar rats. Notably, male msP rats showed an innate increase in phosphorylation at the serine site 232 in the CeA, a marker of GR nuclear localization and transactivation [26]. Considering these constitutive alterations in their stress system, and the role of GR in the transition to alcohol dependence, we initially hypothesized that administration of GR antagonists would attenuate alcohol SA more efficaciously in msP rats versus Wistar controls.

In fact, msP rats have long been proposed as a phenocopy of postdependent animals, since they display comorbid symptoms of alcohol preference, high anxiety-like traits and hypersensitivity to stress. Consequently, we proposed that GR antagonism would attenuate the negative affect state that may drive their high alcohol consumption. However, contrary to our expectations, GR antagonists appeared more efficacious in Wistars than in msP rats. Furthermore, we recently reported the GR antagonism also does not alter the innate anxiety-like behaviors in msP rats [30].

There are few possibilities to explain the limited efficacy of GR antagonists in msPs. For instance, in an earlier study we found that male msPs had higher adrenocorticotropic hormone levels but lower circulating CORT, whereas in females, msP rats displayed larger elevation of CORT levels in response to restraint stress versus Wistars. In line with this suggestion, in response to a dexamethasone challenge, msP rats showed a lower reduction in CORT compared to Wistar controls [26]. These findings suggest that msP rats have a different regulation of the HPA axis, and the negative feedback processes modulating its responsiveness are diminished in this rat line. Hence it is possible that an acute injection of GR antagonist is not sufficient to normalize the hormonal equilibrium and to prevent the high alcohol drinking of msP rats. Future studies are needed to evaluate the effects of GR antagonists following chronic administration. A second possibility is that the higher innate GR phosphorylation observed in msP rats may lead to a differential regulation of the intracellular signaling pathways associated with the GR, an effect that may impair binding activity following mifepristone and CORT113176 administration. Thus, it is important to evaluate if transcriptional changes associated with GR activation are different in msPs versus Wistars.

In this study, we also measured plasma CORT levels prior to and after alcohol self-administration. Consistent with the results of earlier work, we found higher basal CORT levels in female compared to male rats [26,37,38]. The highest concentration was detected in female Wistars followed by female msPs. Moreover, we observed that alcohol SA markedly reduced CORT levels in females of both strains, whereas no changes were observed in males. These data are consistent with earlier studies showing that females displayed enhanced glucocorticoids secretion both at baseline and following stress, and after an alcohol challenge [37–40]. The motivational factors contributing to drinking in males and females may be different, and whether circulating corticosteroid levels may contribute to these discrepancies is unclear. However, it is worth noting that our results indicate that the higher the basal circulating CORT levels, the stronger the inhibitory effect of GR antagonists on alcohol drinking.

Since stress enhances the motivation for alcohol, particularly in female rats, we speculate that their drinking is reduced by GR antagonists via processes that suppress HPA axis function and possibly reduce negative mood associated with steroid hormones dysregulation [41,42].

In summary, our results showed that GR antagonism attenuates alcohol SA, particularly in female rats. Moreover, despite the observation that msPs are more vulnerable to stress and are highly motivated to drink alcohol for tension relieving purposes, they showed a poorer response to GR antagonists.

4. Materials and Methods

4.1. Animals

Male (N = 25–26/line) and female (N = 28/line) msP and Wistar rats, bred at the animal facility of the University of Camerino, Italy, weighed 250–300 g (male) and 160–200 g (female) at the beginning of the experiments. Rats were housed three per cage in a temperature (20–22 °C) and humidity (45–50%) controlled room with a reverse 12 h light/dark cycle (lights off at 8 AM). During the entire residence in the facility, animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). Before the beginning of training, for three days rats were handled 5 min daily by the same operators who performed the experiments. Experiments were conducted during the dark phase of the light/dark cycle. All the procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Italian Ministry of Health approval 1D580.24.

4.2. Drugs

The alcohol drinking solution 10% (*v*/*v*) was prepared by diluting 95% alcohol (F.L.Carsetti, Camerino, Italy) with tap water. Saccharin (Sigma-Aldrich, Milan, Italy) was diluted to 0.2% (*w*/*v*) with tap water. The glucocorticoid and progesterone receptors antagonist mifepristone (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in propy- lene glycol (Sigma-Aldrich, Milan, Italy). Mifepristone was administered intraperitoneally (i.p.) at the doses of 0.0, 10, 30 and 60 mg/kg in a volume of 1 mL/kg, 90 min before tests. The selective glucocorticoid receptor antagonist CORT113176 (Corcept Therapeutics Incorporated, Menlo Park, CA, USA) was suspended in a vehicle containing 10% dimethylformamide (Sigma-Aldrich, Milano, Italy), 10% Cremophor EL (Sigma-Aldrich, Milano, Italy) and 80% saline. The drug was administered at the doses of 0.0, 10, 30 and 60 mg/kg (i.p). in a volume of 3 mL/kg, 90 min prior the test session. Drug doses were chosen based on published data [24,30].

4.3. Self-Administration Apparatus

Self-administration (SA) sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT, USA) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with two retractable levers located in the front panel of the chamber with a drinking reservoir placed in between and connected with a syringe pump. A house-light was located on the wall opposite to the levers. Behavioral sessions were controlled and recorded by a windows compatible PC equipped with Med-PC-5 software (Med Associates).

4.4. Self-Administration Training

Animals were trained to self-administer 10% (*v*/*v*) alcohol or saccharin 0.2% (*w*/*v*) for five days a week, in 30 min daily sessions under a fixed-ratio 1 (FR1) schedule of reinforcement. Operant sessions started with lever insertion and ended with lever retraction. Responses at the right (active) lever were reinforced with 0.1 mL of fluid (alcohol or saccharin solution) delivered in the drinking reservoir. Rats were trained to alcohol SA using a saccharin-fading procedure [43]. Briefly, during the first five days of training, active lever responses were reinforced with 0.2% (*w*/*v*) saccharin. Next, 8% (*v*/*v*) alcohol was added to saccharin to familiarize rats with alcohol and then alcohol concentration was stepwise increased to 10% (*v*/*v*) and saccharin removed. Starting with alcohol 10% (*v*/*v*) SA, reinforcement delivery was followed by a 5 s time-out (TO), during which the house light was contingently illuminated. During the TO, active lever responses were recorded but not reinforced. Throughout the sessions, responses at the left (inactive) lever were recorded but had no scheduled consequences.

Drug treatments began once a stable SA baseline was established. Approximately three weeks (five SA sessions per week) were necessary to reach a stable baseline of responding.

4.5. Experiment 1.1: Effect of Mifepristone on Alcohol Self-Administration in Male and Female msP and Wistar Rats

On test days, male and female msP (N = 10/sex) and Wistar (N = 9–10/sex) rats were injected with mifepristone (10, 30 and 60 mg/kg, i.p.) or its vehicle 90 min before the SA session in a within-subject counterbalanced design. Tests were conducted every fourth day until each rat had received all doses of mifepristone. During the first of the three intervening days, rats remained in their home cage, whereas during the second and third days they performed baseline alcohol SA sessions.

4.6. Experiment 1.2: Effect of Mifepristone on Saccharin Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 6–8/sex) and Wistar (N = 7–8/sex) rats. The procedure was identical to experiment 1.1 except that the SA fluid was saccharin 0.2% (*w*/*v*).

4.7. Experiment 2.1 Effect of CORT113176 on Alcohol Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 9–10/sex) and Wistar (N = 10/sex) rats. The procedure was identical to experiment 1.1 except that the selective GR antagonist CORT113176 (0.0, 10, 30 and 60 mg/kg) was used.

4.8. Experiment 2.2: Effect of CORT113176 on Saccharin Self-Administration in Male and Female msP and Wistar Rats

This experiment was conducted on male and female msP (N = 9–10/sex) and Wistar (N = 9–10/sex) rats. The procedure was identical to experiment 2.1 except that the SA fluid was saccharin 0.2% (*w*/*v*).

4.9. Experiment 3: Blood Corticosterone Levels Following Alcohol Self-Administration in Male and Female msP and Wistar Rats

The effect of alcohol SA on blood corticosterone levels in male and female msP (N= 6/sex) and Wistar (N = 7–8/sex) rats was evaluated. Rats were trained to self-administer alcohol as described above. When a stable alcohol SA baseline was established, blood for corticosterone analysis was collected under a basal alcohol-free condition and immediately after the alcohol self-administration session. The experiment was conducted in a within-subject design and animals were subjected to two blood samplings, one under the basal condition and the other immediately after the self-administration session. At least three days passed between the two blood samplings and sampling order was counterbalanced. Blood was collected by tail nicking. The hypothalamic stress response induced by this sampling procedure is detectable after 3 min [44]; to avoid this confounding factor, we completed sampling within 2 min. Blood was sampled in lithium-heparinized tubes (Sars EDT, Nümbrecht, Germany). Samples were centrifuged at 1500 rcf for 10 min at 4 °C and plasma was collected, aliquoted and stored at 20 °C until further use. Plasma corticosterone levels were determined using enzyme-linked immunosorbent assay (ELISA) (RE52211, IBL International GmbH, Hamburg, Germany) following manufacturer instructions.

4.10. Statistical Analysis

All behavioral experiments were analyzed by three-way analysis of variance (ANOVA) with treatment as a repeated measure, and strain and sex as between-subject factors. Active and inactive lever responses were analyzed separately. Behavioral performances of each independent strain/sex group were further analyzed by one-way with factor ANOVAs with treatment as a repeated measure. ANOVAs were followed by Dunnet's post hoc analysis when appropriate. Significance was conventionally set at $p < 0.05$.

CORT ELISA standards were used to generate an optimalfit 4-parameter standard curve from which sample values were extrapolated. CORT data were analyzed via three-way ANOVA with condition (basal vs. alcohol condition) as the within-subject factor

and strain and sex as between-subject factors. Significant effects were explored with Newman-Keuls multiple comparison test. Significance was conventionally set at $p < 0.05$.

All statistical analyses were performed using GraphPad Prism v8.

Author Contributions: Conceptualization, R.C., M.R., N.C.; formal analysis, F.B., N.C., M.U.; investigation, F.B., V.V., B.C., V.L., M.R., R.C., S.S., M.U., L.S.; resources, M.R., R.C.; data curation V.L., V.V., B.C., F.B.; writing—original draft preparation, V.V., N.C., R.C.; writing—review and editing, F.B., N.C., V.V., B.C., M.R., R.C.; funding acquisition, M.R., R.C. All the authors approved the submitted version and agree to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All the procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Italian Ministry of Health approval 1D580.24.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request to the corresponding author.

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Network-Based Discovery of Opioid Use Vulnerability in Rats Using the Bayesian Stochastic Block Model

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Opioid use disorder is a psychological condition that affects over 200,000 people per year in the U.S., causing the Centers for Disease Control and Prevention to label the crisis as a rapidly spreading public health epidemic. The behavioral relationship between opioid exposure and development of opioid use disorder (OUD) varies greatly between individuals, implying existence of sup-populations with varying degrees of opioid vulnerability. However, effective pre-clinical identification of these sub-populations remains challenging due to the complex multivariate measurements employed in animal models of OUD. In this study, we propose a novel non-linear network-based data analysis workflow that employs seven behavioral traits to identify opioid use sub-populations and assesses contributions of behavioral variables to opioid vulnerabilityand resiliency. Through this analysis workflow we determined how behavioral variables across heroin taking, refraining and seeking interact with one another to identify potentially heroin resilient and vulnerable behavioral sub-populations. Data were collectedfrom over 400 heterogeneous stock rats in two geographically distinct locations. Rats underwent heroin self-administration training, followed by a progressive ratio and heroin-primed reinstatement test. Next, rats underwent extinction training and a cue-induced reinstatement test. To enter the analysis workflow, we integrated data from different cohorts of rats and removed possible batch effects. We then constructed a rat-rat similarity network based on their behavioral patterns and implemented community detection on this similarity network using a Bayesian degree-corrected stochastic block model to uncover sub-populations of rats with differing levels of opioid vulnerability. We identified three statistically distinct clusters corresponding to distinct behavioral sub-populations, vulnerable, resilient and intermediate for heroin use, refraining and seeking. We implement this analysis workflow as an open source `R` package,named `mlsbm`.

Keywords: clustering, community detection, Bayesian model, opioid use disorder, network analysis, stochastic block model

INTRODUCTION

Opioid addiction is a chronic neuropsychiatric disorder characterized by compulsive drug taking and relapse, despite efforts to remain abstinent. Opioid use disorder (OUD) has risen substantially in the United States over the past two decades, for both prescription drugs (1), as well as illicit opioids, notably heroin (2). The parallel rise in both prescription and illicit opioid use and abuse are related to one another, as a majority of heroin users report using prescription opioids prior to heroin use (2–4). Death due to an overdose is also positively correlated between these two opioid classes (2), posing an additional obstacle in addressing the current opioid epidemic. Furthermore, heroin use since 2000 has increased in all demographics, regardless of age, sex or socio-economic status (2, 4), suggesting factors independent of these are contributingto the escalation in OUD. This ubiquitous increase in heroinuse and dependence across disparate populations highlights the need to assess how individual variation in multiple behavioral traits may be interacting to contribute to an OUD resilient vs. vulnerable phenotype.

OUD remains such a critical social and personal problem in part because we are limited by current animal models that predict neurological pathologies for OUD. Though animal models capturing individual variation in addiction-related behaviors have greatly contributed to our understanding of drug addiction, most focus on one or two behavioral phenotypes, then apply the power of animal experimentation to uncover circuitry and cellular mechanisms for individual phenotypes. While this approach has greatly enhanced our understanding of how brain circuits and cell signaling mechanisms contribute to specific behavioral phenotypes, OUD is a disorder containing many behavioral traits that may contribute differentially to resilience and vulnerability to drug addiction depending on individual genetics and sociology (5–7). Indeed, the DSM-V diagnostic criteria for OUD is neither meeting a single behavioral criterion nor meeting all criteria, but rather a person needs to meet a subcluster of criteria to be considered diagnostic (5). This diagnostic protocol is employed because of individual differences resulting from the presence of one diagnostically positive trait does not necessarily predicting the presence of another trait. In an effort to more accurately portray the multi-trait nature of substance use disorders (SUDs), some studies have created composite scores consisting of a few traits that are generally summed in a linear manner to create an addiction score(8, 9). Here we propose a different approach to analyzing multiple traits and explore a multidimensional data clustering strategy of seven behavioral traits potentially characteristic of heroin use and seeking in 451 outbred rats, examined in two distinct laboratories, one at the Medical University of South Carolina (MUSC) in the USA and the other at the University of Camerino (UCAM) in Italy. This approach allows for non- linear relationships between multiple traits to be simultaneously quantified, resulting in clusters of animals that may correspond to overall resilient and vulnerable subgroups.

Various clustering algorithms are available, including k-means clustering (10), hierarchical clustering (11), and finite mixture models (12), among others. However, behavioral studies generate complex multivariate measurements which can make clustering difficult using standard algorithms. Recently, network-based clustering approaches have become popular across multiple disciplines due to their flexibility and applicability to high-dimensional data. For example, in high dimensional single cell genomics studies, these algorithms are employed in multiple software packages for identifying latent cell types such as T andB cells (13). In general, these network-based clustering approaches first construct a similarity network based on observations and then implement a community detection algorithm on this similarity network to identify underlying clusters. As a result, these approaches are less affected by violations of underlying assumptions, such as Gaussianity.

In this paper, we adopt the stochastic block model (SBM), which has strong and rigorous theoretical foundation in statistics literature (14, 15). In essence, the SBM allows for identification of latent communities using a probabilistic model that describes interconnectivity between nodes within and between clusters. In this sense, the SBM may be used as a descriptive tool to assess the presence of distinct latent populations in a data set. The biological utility of such populations may then be determined by investigating the distributions of relevant variables (e.g., heroin consumption) across clusters. While we do not seek to propose a predictive model for opioid vulnerability, the sub-populations identified from our approach may be correlated with data from future studies (e.g., genetic studies) to assess the predictive ability of characteristics that define the identified sub-populations.

Due to its probabilistic nature, the SBM has multiple strengths over deterministic approaches. First, it provides a natural framework for deriving uncertainty measures for identified clusters, which are critical to understanding latent community structure, e.g., understanding gradual changes across multiple latent clusters. Second, using goodness-of-fit measures, the SBM helps selection of the number of clusters, which is a long-standing problem in clustering methodology and not straightforward to address in deterministic algorithmic approaches. Finally, the SBM fits naturally into the Bayesian framework, allowing for incorporation of prior expert knowledge to guide the clustering and the ability to make posterior probability statements about all model parameters (15).

MATERIALS AND METHODS

Experimental Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at MUSC and by the Italian Ministry of Health (approval 1D580.18). Procedures abided by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of Laboratory Animals.

A total of 600 heterogeneous stock (HS: originally n/NIH-HS) rats bred at Wake Forest University (currently NMcwiWFsm:HS; Rat Genome Database number 13673907) were obtained for these studies. Of these rats, 149 were excluded from final analyses due to death following surgery ($n = 21$), death over the course

of training ($n = 77$) or undergoing saline, not heroin, self-administration training ($n = 51$). Final analyses were performed on 451 rats (males, n = 238; females, $n = 213$). HS rats were outbred from eight inbred strains and maintained in a way to minimize inbreeding (16), allowing genetic fine-mapping to relatively small intervals (17). Animals were shipped in batches of 40 (20 males and 20 females per site) to either MUSC (USA) or UCAM (Italy) at approximately 5 weeks of age. Upon arrival, animals were pair-housed and left undisturbed in a climate-controlled colony room with a standard 12-h light:dark cycle for 3 weeks prior to the start of testing. Throughout training, rats had *ad libitum* access to food and water. Testing occurred during the dark cycle, between 18:00 and 6:00 h. Heroin hydrochloride supplied by the National Institute on Drug Abuse (Bethesda, MD) dissolved in 0.9% sterile saline was used in these studies.

Following the 3-week acclimation period, rats underwent surgery under isoflurane anesthesia for the implantation of an indwelling jugular catheter. An analgesic (Ketorolac, 2 mg/kg, sc; or Meloxicam, 0.5 mg/rat, sc), and antibiotic (Cefazolin, 0.2 mg/kg, sc; or enrofloxacin, 1 mg/kg, iv), were administered pre-operatively. Rats were given a minimum of 3 days of recovery prior to heroin self-administration training commencing. All testing occurred in standard behavioral testing chambers (MED Associates, St. Albans, VT, USA). Presses on an active lever resulted in presentation of a light and tone cue for 5-s and an infusion of heroin (20 µg/kg/100 µg infusion over3 s) ona fixed-ratio 1 schedule of reinforcement. At the start of the infusion, the house light also turned off for 20-s signaling a time-out period during which additional presses on the active lever were recorded but without consequence. Presses on the inactive lever were recorded but without consequence. Sessions lasted for 12 h or until 300 infusions were earned. Self-administration occurred Monday-Friday, with one session off per week, for a total of four sessions/week. Following 12 self-administration sessions rats underwent a progressive ratio test whereby the number of presses $p(t)$ required to receive an infusion increased exponentially after each infusion $t = 1, \ldots, T$ according to the function $p(t) = 5e^{0.2t} - 5$ (18). Rats then had three more days of self-administration training to re-establish baseline heroin-taking behavior prior to tests for reinstatement.

At the conclusion of heroin self-administration training, rats underwent a within-session extinction-prime test that lasted for 6 h. The first 4 h were extinction training conditions during which presses on both the active and inactive lever were recorded but without consequence (i.e., active lever presses no longer result in presentation of the light/ tone cues or heroin infusion). With 2 h left in the session, rats were administered an injection of heroin (0.25 mg/mg, sc), and continued testing under extinction conditions. Daily extinction training sessions (2 h) then commenced for 6 consecutive days prior to a test for cue-induced reinstatement. During this test, presses on the active lever resulted in presentation of the light/tone cue and turning off of the house light, but no heroin infusions.

At the conclusion of training, several behavioral measures were selected for clustering analyses to reflect three behaviorally distinct phases of drug addiction: drug-taking (drug reinforced behavior), refraining (drug non-reinforced behavior), and seeking behaviors (both drug reinforced and non-reinforced). Heroin-taking behaviors include total heroin consumption (total µg/kg heroin consumed across the first 12 self-administration training session), escalation of intake (total heroin consumed the first 3 days of self-administration subtracted from the last 3 days; see **Supplementary Figure 2** for heroin self-administration acquisition curve), and break point achieved during the progressive ratio test. The break point is the total number of active lever presses the rat is willing to perform in order to receive an infusion of heroin. Refraining behavior consisted of active lever presses during the first 2 h of the within-session extinction-prime test (extinction burst) and the last day of extinction training prior to the test for cue-induced reinstatement (extinction day 6). Two extinction training time points were used as to capture refraining behavior immediately after heroin taking, and following several sessions of non-reinforced seeking prior to cue-induced reinstatement. Heroin-seeking behavior is represented by active lever presses during the heroin-prime and cue-induced reinstatement tests. Active lever presses were used for all variables to maintain continuity in measured behavioral output for each behavior.

Data Pre-processing

Batch Correction for Multi-Site Samples

To analyze the MUSC and UCAM cohorts simultaneously, we first performed a visual inspection of possible batch effects between the two study sites. Specifically, we began by concatenating the raw data matrices from each siteinto an integrated data matrix, where rows corresponded to individual rats and columns correspond to behavioral measures, as described in section Experimental Methods. Then,to facilitate visualization, we applied the Uniform Manifold Approximation and Projection (UMAP) (19) algorithm to compute 2-dimensional embeddings for each rat. To correct for the apparent batch effect between study sites, we z-score transformed each behavioral measure *within study site*. This allowed for analysis of each behavioral measurement on a standardized scale, and, in effect, regressed out unwanted site-specific effects. Distributions of raw behavioral measures (i.e., before z-scoring) are shown in **Supplementary Figures 5**, **6**.

Similarity Network Construction

After integrating the behavioral data from each study site as described in section Batch Correction for Multi-Site Samples, we constructed a rat-rat similarity network as follows.First we defined a single parsimonious subset of relevant behavioral measures from the experiments discussed in section Experimental Methods using expert knowledge. Here, the goal was to choose variables that reflected the behavioral propensity of each rat for opioid dependence. Next, we computed the Euclidean distance between each pair of rats using this single parsimonious variable subset. We then formed a rat-rat similarity network, i.e., a collection of nodes and edges, where nodes in the network represent individual rats and edges represent similarities between rats. We placed an edge from each node to its R closest other nodes based on the rat-rat distance measures. Here, the number of neighbors R is a tuning parameter that controls the density of

edges in the similarity network. By default, we adopt the widely used heuristic $R = \sqrt{N}$ (20).

Stochastic Block Model

To detect communities within the overall rat-rat similarity matrix that might correspond to behaviorally distinct sub-populations, we adopted the Bayesian stochastic block model (SBM), a generative model for network data (15). Let **A** be an $n \times n$ adjacency matrix encoding the rat-rat similarity network among n total rats, with $A_{ij} = 1$ if rat i shares an edge with rat j ($i \neq j$), and $A_{ij} = 0$ otherwise. For a fixed and pre-specified number of communities, K, the SBM assumes

$$A_{ij} | \mathbf{z}, \Theta \overset{ind}{\sim} \text{Bernoulli}(\vartheta_{z_i, z_j}) \text{ for } i < j = 1, \ldots, n, \quad (1)$$

where $z_i \in \{1, \ldots, K\}$ is a categorical indicator variable that denotes the community membership of rat i, $\mathbf{z} = (z_1, \ldots, z_n)$, and Θ is a $K \times K$ connectivity matrix with elements ϑ_{rs} described in detail below. Equation (1) implies that the probability of an edge occurring between two nodes depends only on the community membership of each node. Thus, all rats belonging to the same sub-population are regarded as *stochastically equivalent*.

While our primary object of inference is the vector of latent community indicators **z**, an advantage of the SBM over other community detection algorithms is its ability to conduct statistical inference on the edge probability parameters ϑ_{rs}, for $r \leq s = 1, \ldots, K$. By encoding these parameters in a symmetric connectivity matrix Θ, we obtain a useful summary of community structure. Here, diagonal elements of Θ are within-community edge probabilities, and off-diagonal elements of Θ are between-community edge probabilities. In most cases, we expect to find an *assortative* community structure, in which within-community connections are more likely than between-community connections, though the model is capable of detecting *dissortative* community structures as well (21). Thus, in addition to the community labels, the SBM allows us to characterize the global relationships between communities.

Commonly, the SBM as formulated in model (1) is refined to accommodate heterogeneous degree distributions, i.e., *degree correction* (22). Since model (1) assumes that the probability of an edge being place between two nodes only depends on the community membership of the nodes, it is not suitable for networks in which each node may have varying degree, that is, the number of edges connected to it. However, as described in section Similarity Network Construction, our workflow relies on construction of a nearest neighbors network, in which each node, by definition, will have exactly R edges, thus degree correction is not necessary.

We estimate parameters of the SBM using a fully Bayesian approach by assigning prior distributions to all unknown model parameters. We select conjugate priors to obtain closed-form full conditional distributions of all model parameters, which in turn allows for straightforward Gibbs sampling. First, for the cluster indicators $z_1, \ldots, z_n$, we assume a conjugate multinomial-Dirichlet prior with $z_i \overset{iid}{\sim} \text{Categorical}(\pi)$ for $i = 1, \ldots, n$, and $\pi \sim \text{Dirichlet}(\alpha_1, \ldots, \alpha_K)$, where $\pi = (\pi_1, \ldots, \pi_K)$ control the number we adopt a conjugate beta-Bernoulli prior for Θ by letting $\vartheta_{rs} \overset{iid}{\sim} \text{Beta}(\beta_1, \beta_2)$ for $r < s = 1, \ldots K$. By default, we opt for weakly informative priors by setting $\alpha_1 = \alpha_2 = \ldots = \alpha_K = 1$ and $\beta_1 = \beta_2 = 1$ (23).

Posterior Inference

We implement parameter estimation using Gibbs sampling, as detailed in the **Supplementary Material**. A critical step of our proposed workflow for identifying behavioral sub-populations in rats is the choice of K, i.e., the number of communities. Since the choice of K should consider both expert knowledge and evidence from the data, we refrain from proposing a "one size fits all" globally optimal method for choosing of K. Instead, in section Results we discuss how Bayesian Information Criterion (BIC) (24) can be used in conjunction with biological knowledge to make informed choices for K.

Label switching is an issue encountered in Markov chain Monte Carlo (MCMC) methods, such as the Gibbs sampler proposed above, wherein the model likelihood is invariant to permutations of a latent categorical variable such as **z**. As a result, we may observe natural permutations of **z** overthe course of the MCMC sampling that cause the estimatesof all other community-specific parameters to be conflated,thereby jeopardizing the accuracy of model parameter estimates. This problem is exacerbated when communities are not well-separated. Previous works have attempted to address the issue by re-shuffling posterior samples after the sampling has completed (25). However, these post-sampling methods rely on prediction and thereby are fallible to prediction error. To address label switching, we adopt the canonical projection of **z** proposed by (26) in the context of Bayesian SBMs, in which we restrict samples of **z** to the canonical sub-space $\mathcal{Z} = \{\mathbf{z} : \text{ord}(\mathbf{z}) = (1, \ldots, K)\}$. In other words, we permute **z** at each MCMC iteration such that community 1 appears first in **z**, community 2 appears second in **z**, *et cetera*. Finally, we choose as our final estimate of **z** the maximum *a posteriori* (MAP) estimate of **z** across all post-burn MCMC samples (23).

Continuous Phenotyping

While the SBM presented thus far assumes that the overall experimental cohort can be decomposed into a fixed number of discrete communities, where each experimental unit (e.g., rat) is assigned to exactly one community, often interest lies in further differentiating members within a community in a more continuous fashion. Indeed, a core benefit of the Bayesian SBM is that the discrete model structure may be augmented using uncertainty measures, i.e., a quantification of our inferred level of confidence in each estimated model parameter. For instance, let $\hat{\mathbf{z}} = (\hat{z}_1, \ldots, \hat{z}_n)$ be the posterior estimate of the true community labeling vector **z** obtained from the MCMC estimation procedure described in the **Supplementary Material**. Letting $s = 1, \ldots, S$ index the post burn-in MCMC iterations, we may quantify the uncertainty in each estimate $\hat{z}_i$ as

$$u(\hat{z}_i) = 1 - \frac{1}{S} \sum_{s=1}^{S} \mathbb{I}\left(\hat{z}_i^{(s)} = \hat{z}_i\right) \text{ for } i = 1, \ldots, n, \quad (2)$$

of nodes in each community, i.e., the community size. Similarly, $S_{s=1}^{i}$ ^{i} ^{i}

where $\hat{z}_i^{(s)}$ is the estimate of z_i at MCMC iteration s, and $\mathbf{I}(\hat{z}_i^{(s)} = \hat{z}_i)$ is the indicator function equal to 1 if $\hat{z}_i^{(s)} = \hat{z}_i$ and 0 otherwise. In words, $u(\hat{z}_i)$ represents the proportion of MCMC iterations where the estimate of z_i was not the posterior MAP estimate $\hat{z}_i$. For nodes that share many edges with other nodes within their respective community, i.e., those that are highly typical of their community, the uncertainty measure should be low. Meanwhile, for nodes that share edges with nodes outside of their respective community, the uncertainty measure should be high, as these nodes will likely be assigned to other communities intermittently over the course of the MCMC estimation. In this way, we may augment the cluster labels obtained by the SBM with quantification of our level of confidence in them—a significant advantage over other non-model-based clustering methods.

In addition to uncertainty quantification, we may similarly use the MCMC draws $\hat{\mathbf{z}}^{(1)}, \ldots, \hat{\mathbf{z}}^{(S)}$ to conduct *continuous phenotyping*, or the ranking of subjects based on their affinity toward a certain phenotype. For example, in our context of assigning rats to vulnerable and resilient phenotypes using the SBM, we may also provide a continuous measure of affinity toward the vulnerable phenotype for each rat that can be used to rank rats within clusters. In this setting, let cluster $k_v \in \{1, 2, \ldots, K\}$ be the cluster annotated as vulnerable for opioid dependence. For each rat $i = 1, \ldots, n$, we define the continuous phenotype vulnerability score $v(i)$ as $\sum_{s=1}^{S} \mathbf{I}(\hat{z}_i^{(s)} = k_v)/S$, i.e., the proportion of MCMC iterations in which rat i is assigned to cluster k_v.

Software Implementation

For convenient implementation of the workflow proposed throughout section Materials and Methods, we developed "`mlsbm`," an efficient and user-friendly `R` package for the identification of sub-populations in network data (27). The `mlsbm` package is freely available for download from the Comprehensive `R` Archive Network (28) (https://cran.r-project.org/package=mlsbm). The `mlsbm` package includes robust documentation to facilitate applications to a variety of clustering tasks.

Comparison to Alternative Approaches

We sought to assess the performance of the SBM clustering workflow relative to alternative clustering approaches, we applied five popular clustering algorithms, namely the Louvain, walktrap, hierarchical clustering, K-means, and DBSCAN algorithms. The Louvain (29) and walktrap (30) algorithms, like the SBM, are network-based methods that operate on the nearest neighbors network described in section Similarity Network Construction. The Louvain algorithm seeks to maximize the modularity of the graph, a measurement of the strength of clustering structure of a graph relative to randomly generated graphs. The walktrap algorithm uses random walks on the nearest neighbors graph to find the most densely connected sub-graphs, i.e., clusters, within the graph. Hierarchical clustering (11) is a "bottom up" approach that iteratively merges the most similar observations into clusters to form a tree structure that can be used to produce cluster labels for a pre-specified value of K. K-means (10) and DBSCAN (31) seek to place boundaries around observationsin high-dimensional space such that the data points within boundaries, i.e., clusters, are more similar than those across boundaries. While these approaches are commonly used, they lack the inferential benefits of the SBM such as the ability to choose K using model fit criteria and provide uncertainty quantification in addition to cluster labels.

RESULTS

The overall sample was composed of $N_m = 238$ males and $N_f = 213$ females. The MUSC study site contributed 243 rats, while the UCAM study site contributed 208. As seen in **Figure 1A**, the MUSC and UCAM cohorts exhibit clear separation on the 2-dimensional UMAP space, indicating the potential of study site to act as a confounding variable in our analysis, and preventing simultaneous analysis of rats from both cohorts. The site difference is also apparent in **Supplementary Figure 6**, where in spite of substantially overlapping populations, the MUSC site shows higher mean values than the UCAM site in each of the traits quantified, except for escalation, suggesting a location shift batch effect present between study sites. In **Figure 1B**, we present the 2-dimension UMAP embedding of the concatenated z-score transformed data set, in which no distinguishable separation exists between the MUSC and UCAM rats. Hence, the site-specific z-scoring approach detailed in section Batch Correction

for Multi-Site Samples was able to effectively remove the site-specific batch effect from the data.

To construct the rat-rat similarity network, we computed the Euclidean distance between each pair of rats using the 7 variables discussed in section Experimental Methods and then formed anadjacency network where each rat was connected to its 21 most similar rats. We applied the SBM clustering analysis described in section Stochastic Block Model to the analysis of $N = 451$ rats. To choose the most appropriate number of clusters K, we fit the SBM to the adjacency network for a range of K from $K = 2, ..., 10$. We ran each model for 10,000 MCMC iterations and discarded the first 1,000 iterations as burn-in, resulting in atotal run time of under 4 min for each model using a single 4.7 GHz Intel i7 processor. Using BIC, we found that $K = 3, 4, 5$ provided approximately equal goodness of fit, with $K = 2$ or $K > 5$ provided relatively poor fit (**Figure 2A**). As such, we chose $K = 3$ to provide the most parsimonious representation of the data and to assess the vulnerable, intermediate, and resilient sub-type hypothesis discussed in section Introduction. An adjacency matrix with rows and columns sorted by inferred cluster indicators from the 3 cluster model is shown in **Figure 2B**. **Figure 2C** shows the SBM estimated cluster labels on UMAP space. In **Table 1**, we present the distribution of two covariates of interest across the three inferred clusters, namely sex and study site. We find a significantly skewed distribution of sex across clusters, with a female bias in cluster 1 and a male bias incluster 3 (3-sample normal proportion test $p < 0.0001$), while the distribution of study site across inferred clusters is more uniform(3-sample normal proportion test $p = 0.601$).

Figure 3 shows empirical means and 95% z confidence intervals for each of the 7 selected behavioral measures across each of the inferred clusters from the SBM. Notably, each clusterappears to show clear separation in most of the behavioral

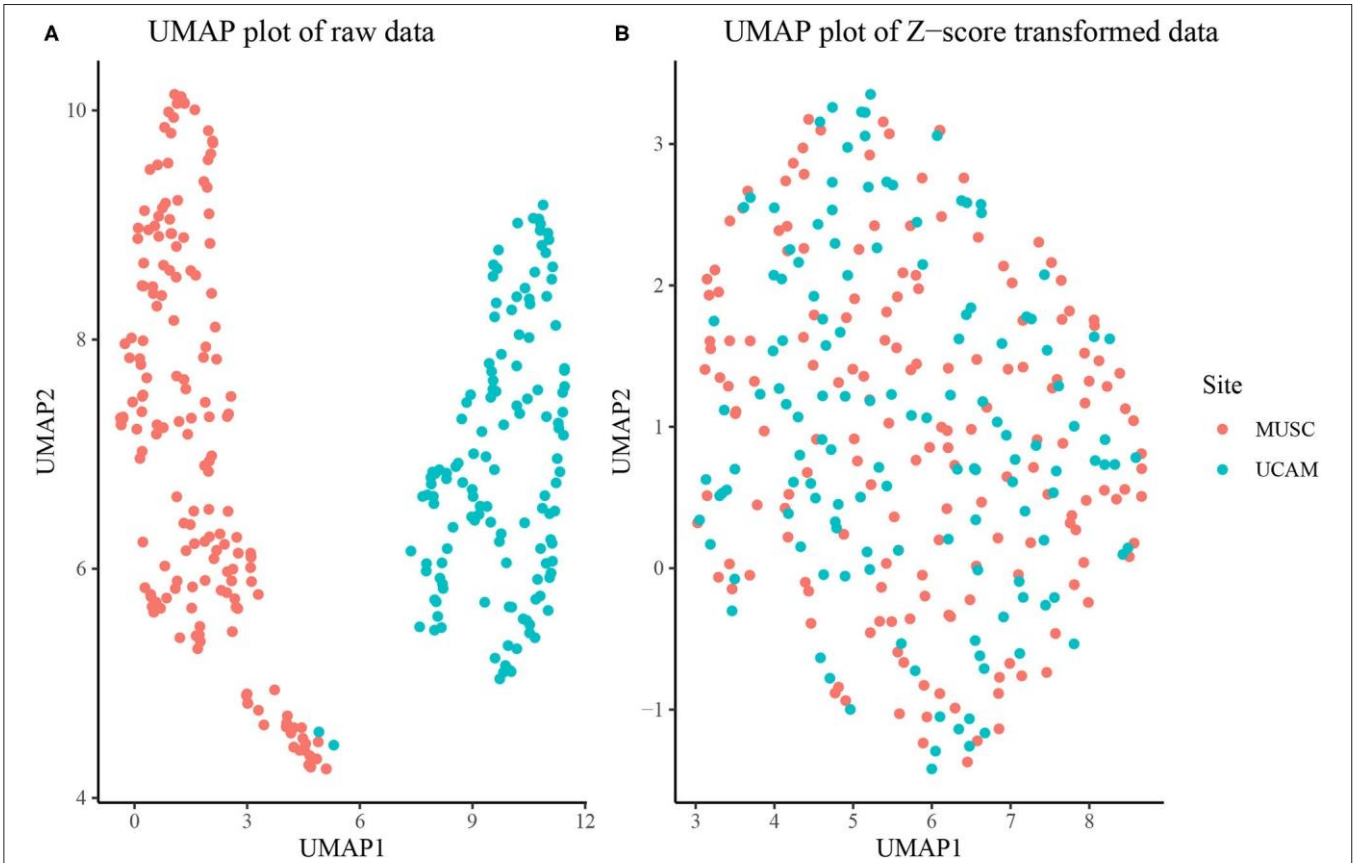

FIGURE 1 | (A) UMAP dimension reduction of behavioral measures before site-specific z-scoring shows significant batch effect of study site (MUSC vs. UCAM). (B) UMAP dimension reduction after site-specific z-scoring shows adjustment for study site batch effect.

variables. For instance, the total heroin consumption was highest in cluster 1 and lowest in cluster 3, with cluster 2 falling in between clusters 1 and 3, and all 95% confidence intervals not overlapping. Similarly, cluster 1 demonstrated a more rapid escalation of heroin intake relative to clusters 2 and 3. We quantified the difference between clusters byfitting a one-way ANOVA for each of the 7 behavioralmeasures vs. the SBM cluster indicators. We conducted a global *F*-test for mean differences among groups. F-statistics and associated *p*-values are displayed in **Table 2**. Distributions of raw behavioral measures in each cluster are shown in **Supplementary Figure 1**, where the same pattern persists as with standardized variables. We observed qualitatively consistent results in site-specific analyses (**Supplementary Figure 4**). We quantified this observation through use of the adjusted Rand index (ARI) between each site-specific analysis and the integrated analysis, which revealed high correspondence between each site-specific analysis and the integrated analysis (MUSC ARI = 0.43; UCAM ARI = 0.54).

To further investigate the vulnerable, intermediate, and resilient sub-type hypothesis, we leveraged the inferential abilities of the Bayesian SBM to infer the similarity among rats from each cluster. Specifically, by investigating the posterior distribution of the elements of the matrix $\boldsymbol{\vartheta}$, we may characterize the similarity among rats within and between each of the three clusters. In **Figure 4**, we show a heatmap of posterior means and 95% Bayesian credible intervals for ϑ_{11}, ϑ_{22}, ϑ_{33}, ϑ_{12}, ϑ_{13}, and ϑ_{23}. We found that the estimated values of the within-cluster connectivity parameters ϑ_{11}, ϑ_{22}, ϑ_{33} were found to be significantly higher than those of the between-cluster parameters ϑ_{12}, ϑ_{13}, and ϑ_{23}. In fact, cluster 1, which had the weakest estimated within-cluster connectivity ($\hat{\vartheta}_{11} = 0.116$), was still over four times more densely connected than the highest between-cluster connection, which was shared between clusters 2 and 3 ($\hat{\vartheta}_{23} = 0.025$). This is indicative of strong assortative community structure in the rat-rat similarity network, in which rats of the same community are more likely to be correlated in terms of behavioral measurements than rats of differing communities. Further, **Figure 4** shows that clusters 1 and 3 were the most dissimilar, with cluster 2 serving as an intermediate cluster.

In **Figure 5**, we plot results from the uncertainty measure and continuous phenotyping analysis presented in section Continuous Phenotyping. **Figure 5A** plots the cluster assignments on UMAP space, where each point is sized proportionally to its uncertainty measure of cluster assignment (larger points imply higher uncertainty). We label the ID ofeach rat that featured an uncertainty measure above 0.10, corresponding to rats that spent at least 10% of the post burn-in

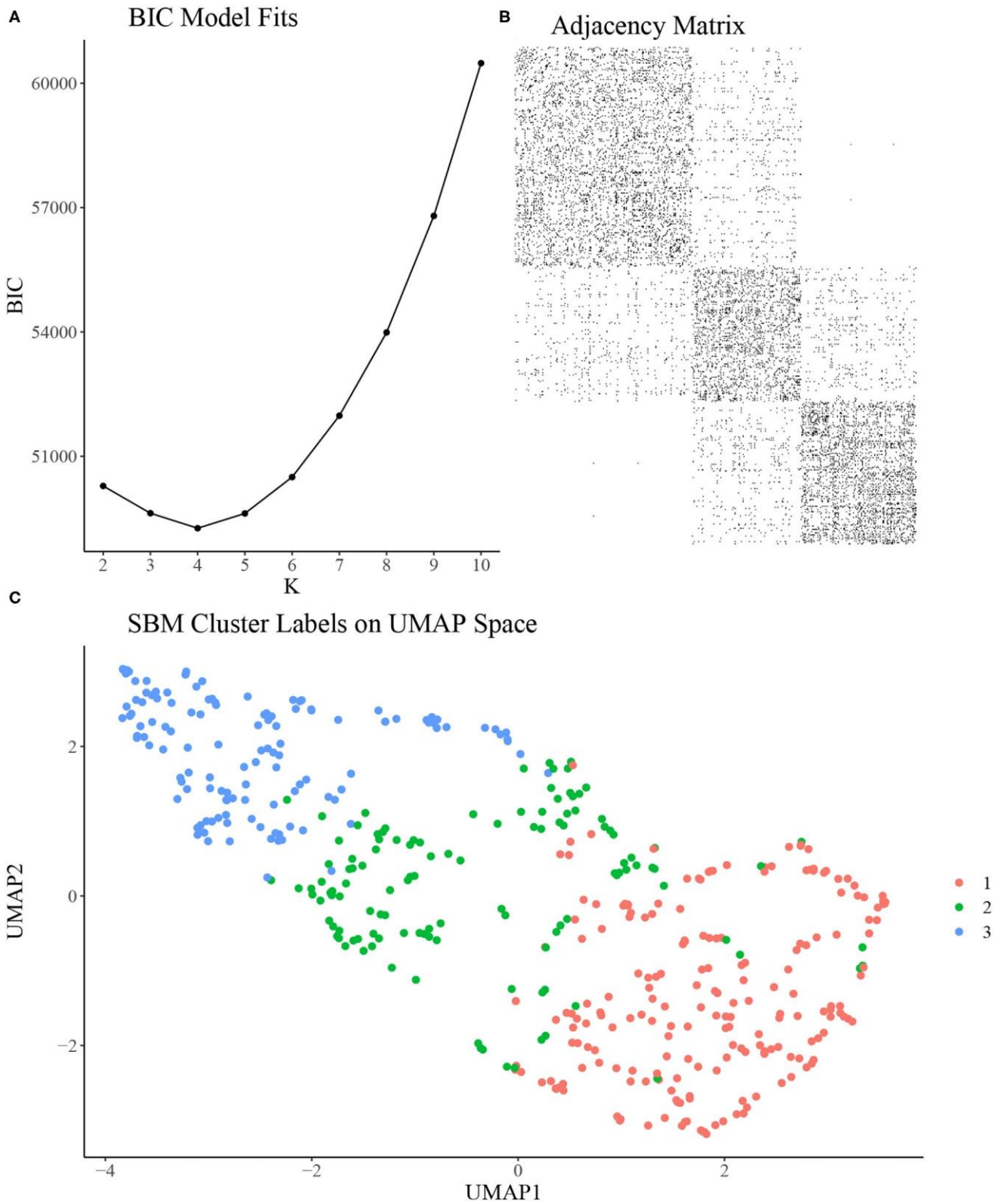

FIGURE 2 | (A) Bayesian Information Criterion (BIC) from SBMs fit with a range of K. $K = 3, 4, 5$ seem to provide similarly optimal fit in terms of BIC. (B) Adjacency matrix of inferred clusters from the SBM using $K = 3$ clusters. (C) UMAP reduction of behavioral measurements colored by inferred cluster labels from the SBM using $K = 3$ clusters.

MCMC iterations from the $K = 3$ SBM in a cluster other than the cluster it was assigned to by the MAP estimate $\hat{\mathbf{z}}$. A number of interesting patterns emerge from this uncertainty analysis. First, we find that rats with higher uncertainty tend to be located near borders between clusters on the UMAP space. Interestingly, rat 101, which was assigned to cluster 2 but is surrounded in UMAP space by rats in cluster 3, featured high uncertainty. Meanwhile, several cluster 2 rats were surrounded by cluster 1 rats in the UMAP space but featured low uncertainty.

Figure 5B displays results from the continuous phenotyping analysis, wherein cluster 1 was annotated as the vulnerable cluster (**Figure 3**) and chosen as the phenotype of interest. We computed the vulnerability score of each rat as the proportion of post burn-in MCMC iterations from the SBM that were spent in cluster 1. We labeled the IDs of the most interesting rats: those with uncertainty measures above 0.10 but vulnerability measures less than 0.90. These rats were located on the border between the intermediate cluster 2 and the vulnerable cluster 1, indicating higher propensity toward opioid dependence than other rats in cluster 2. These results demonstrate the ability of continuous phenotyping to augment the clustering results of the SBM to allow for disambiguation of within-cluster differences between subjects.

Figure 6 displays results from alternative clustering methods as described in section Comparison to Alternative Approaches.

TABLE 1 | Distribution of sex and study site across clusters.

Cluster	% Female (*N*)	% UCAM (*N*)
1: Vulnerable ($N = 200$)	58.5 (117)	44.5 (89)
2: Intermediate ($N = 122$)	47.5 (58)	50.0 (61)
3: Resilient ($N = 129$)	29.5 (38)	45.0 (58)

TABLE 2 | ANOVA global F-statistics and associated *p*-values for each behavioral measure.

Variable	F-statistic	*P*-value
Total consumption	283.8	<0.0001
Escalation of intake	220.7	<0.0001
Break point	221.6	<0.0001
Extinction burst	94.78	<0.0001
Extinction day 6	77.12	<0.0001
Prime reinstatement	72.36	<0.0001
Cued reinstatement	200.6	<0.0001

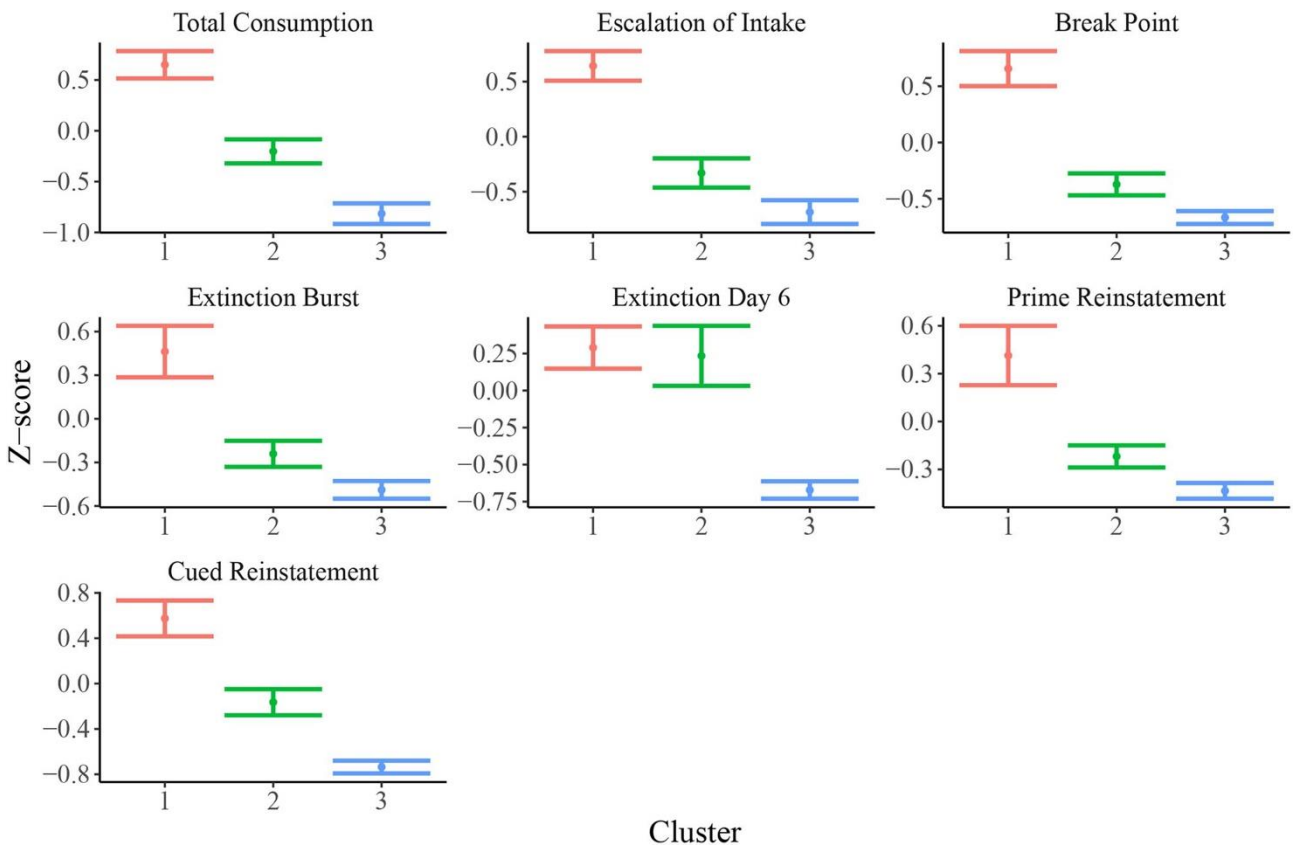

FIGURE 3 | Means and 95% confidence intervals for relevant behavioral measures (z-scored) in each cluster. Distributions of z-scored behavioral variables indicate evidence for vulnerable (cluster 1; $N = 200$), intermediate (cluster 2; $N = 122$), and resilient (cluster 3; $N = 129$) sub-populations.

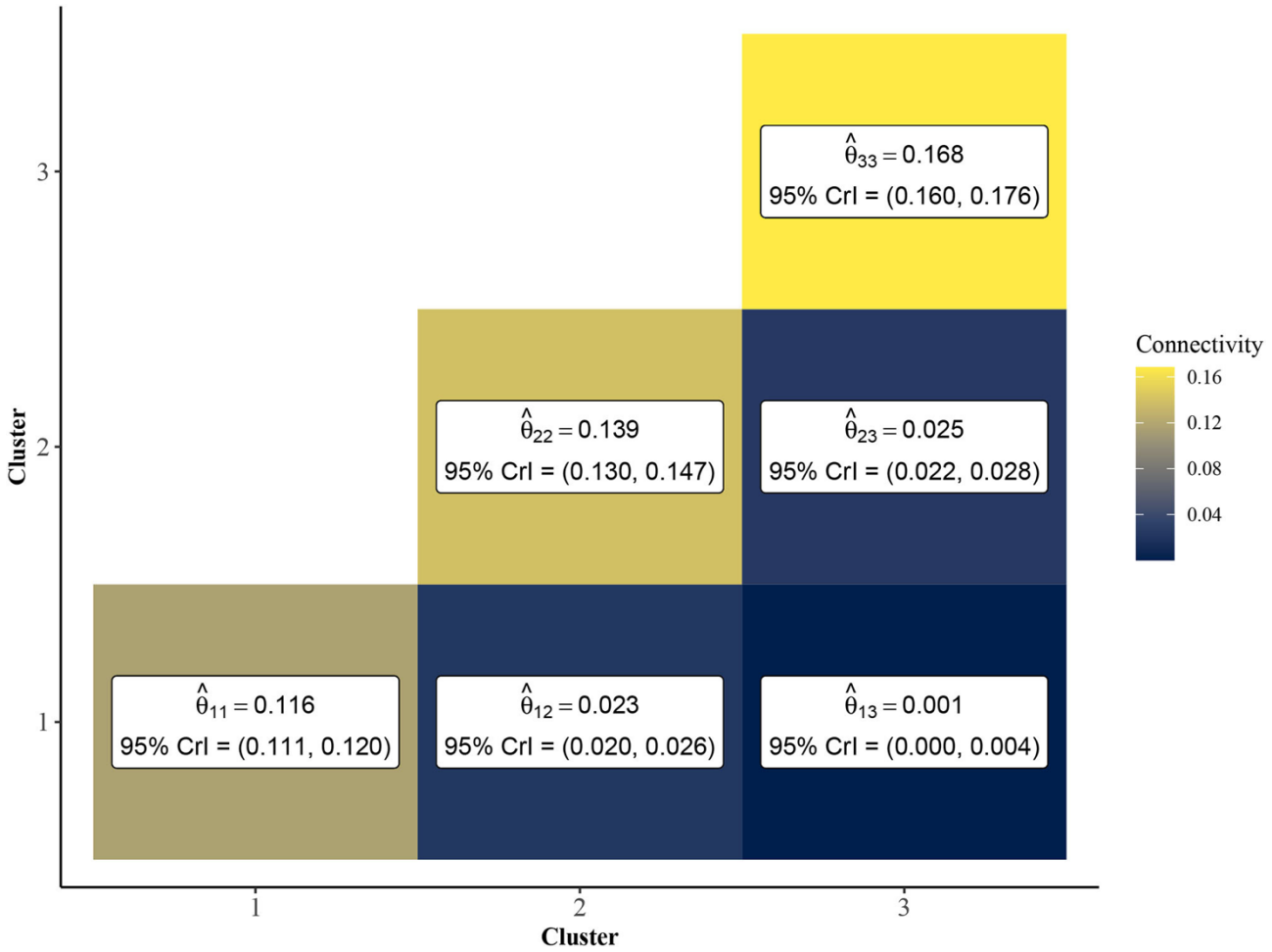

FIGURE 4 | Point estimates and 95% credible intervals of cluster connectivity parameters ❗. The SBM estimates higher values for within-cluster connectivity parameters, ϑ_{11}, ϑ_{22}, and ϑ_{33}, which is indicative of an assortative community structure. Thus, rats within the same community are expected to have significantly higher similarity than rats of different clusters. Clusters 1 and 3 are most dissimilar as evidenced by lower values of $\hat{\vartheta}_{13}$ relative to $\hat{\vartheta}_{12}$ and $\hat{\vartheta}_{23}$.

The network-based clustering algorithms such as Louvain and walktrap algorithms tended to produce a larger number of clusters, each smaller in size relative to the SBM. Due to this, the agreement between the results from these methods and those from the SBM is low (ARI $<$ 0.30). Both the hierarchical clustering method using squared Ward dissimilarity (32) and the K-means algorithm resulted in moderate agreement with the SBM (ARI $=$ 0.343 and 0.374, respectively), while the DBSCAN algorithm yielded a 4 cluster result using default parameters, two of which were sparsely populated. These results suggest the SBM is best suited to addressing the research question at hand.

In addition to validating the capacity of the SBM to create three sub-populations of rats with high, intermediate and low responding for seven heroin associated behavioral traits, we evaluated how the sub-populations compare in terms of weight, site and cohort differences. **Supplementary Figure 7** shows that between sites proportionally equivalent numbers of rats were assigned to each sub-population between the two testing site, and when analyzing between cohorts of rats within each site we found that assignment into sub-populations was equivalent across cohorts at the MUSC site, but that differences existed at the UCAM site. Also, because all the behavioral traits involved the same operant response (active lever pressing), we examined whether any traits within each sub-population were correlated using a Pearson's linear correlation statistic. **Supplementary Figure 3** shows the Pearson's coefficient for each trait comparison within each sub-population, which reveals that only Extinction Day 6 and Cued reinstatement were linearly correlated within each cluster. Otherwise, there was no consistent trait correlation across the three sub-populations. The lack of linear relationship between traits within the clusters is also revealed in **Supplementary Figure 8**, which shows the z-scored behavioral responses for all rats in cluster 1 with a selection of rats highlighted for descriptive purposes. Note that rats need not be high responders in all traits to be identified in the cluster 1 sub-population. These differences between clusters and the overall

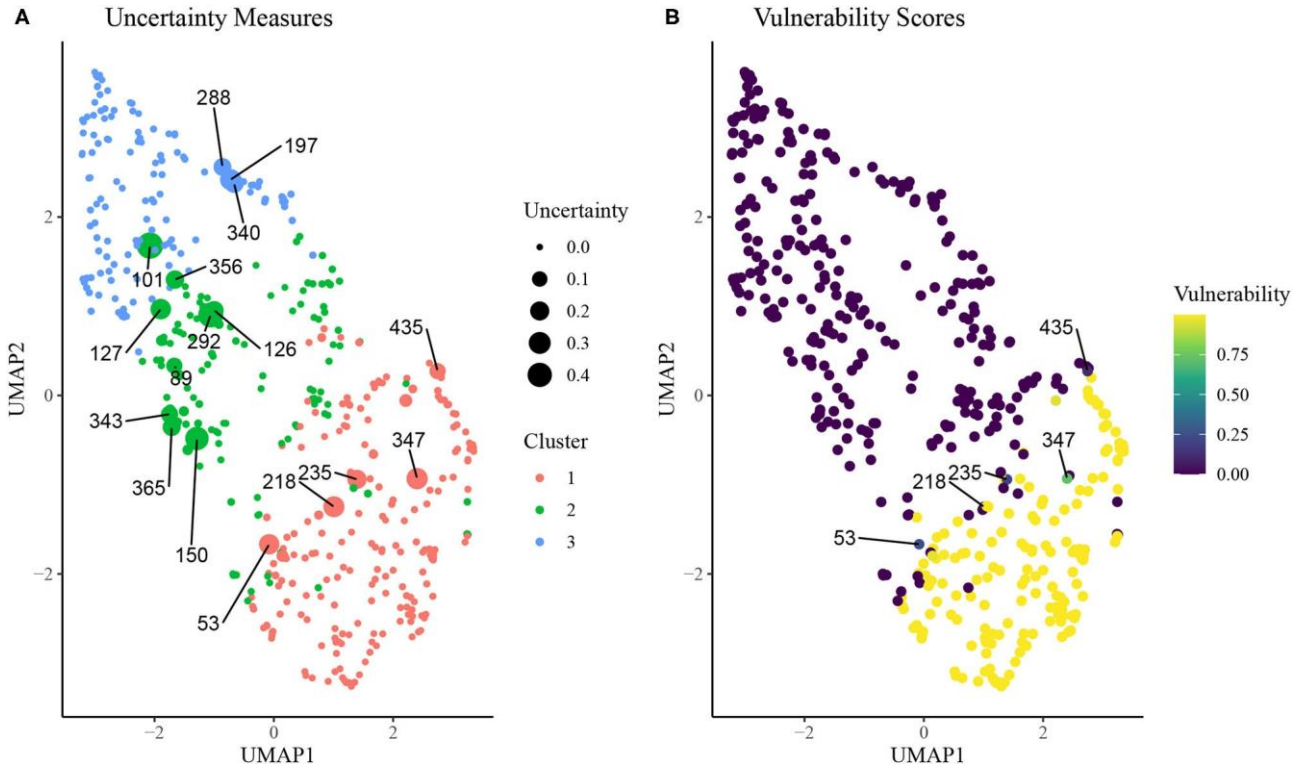

FIGURE 5 | (A) Uncertainty scores of cluster assignment for each rat on UMAP space. Animal IDs are given for subjects with uncertainty measure above 0.10, which is indicative of at least 10% of MCMC iterations spent in a cluster other than the final inferred cluster. (B) Vulnerability scores for each rat on UMAP space. Animal IDs were shown for subjects with uncertainty above 0.10 and vunerability <0.90.

low levels of linear correlation between traits supports exploring the SBM non-linear clustering approach described here as a means to identify non-linear relationships between multiple traits and thereby identify high (vulnerable) and low (resilient) heroin responding sub-populations. Finally, **Supplementary Figure 9** shows that equivalent weight gains occurred before and after completing the behavioral testing between each sub-cluster.

DISCUSSION

In this paper, we developed a comprehensive framework for the descriptive analysis of behavioral sub-populations, and applied it to the cohort of 451 outbred rats subject to heroin self-administration exposure. We discovered the presence of batch effects between the two study sites that contributed to this cohort, and we corrected for these effects using study-site specific z-scoring. Seven behavioral measures were chosen to characterize the vulnerability of each rat to forming opioid dependence. Taken together, these measures quantified three important aspects of dependence: drug-taking, refraining and seeking behaviors. Using these measures, we then converted the multidimensional behavioral data into a rat-rat similarity network, which allowed for investigation of distinct communities within the overall network.

We chose the Bayesian stochastic block model, a statistical model for network data, for investigation of behavioral sub-populations within this cohort. We used the model fit criterion BIC to choose a subset of best fitting models in terms of number of communities. Of this best fitting subset, we chose the three cluster model as it offered the best balance between optimizing statistical and biological criteria. Using ANOVA global *F*-tests, we found significant separation between clusters in terms of each of the seven behavioral measures. Additionally, investigation of average trends across clusters in each behavioral measure allowed us to annotate vulnerable, resilient, and intermediate sub-groups with high confidence. Using the community connectivity parameters inferred by the SBM, we described the relative similarity between clusters, with the vulnerable and resilient clusters each displaying similarity to the intermediate cluster but very little similarity to one another.

To augment the discrete community labels obtained from the SBM, we developed an uncertainty measure, which uses samples from the posterior distribution of the cluster labels to estimate our confidence in the inferred community structure. We also implemented continuous phenotyping to investigate heterogeneities within clustersin terms of vulnerability to opioid dependence. We found a subset of intermediate vulnerability animals who featured relatively high affinity toward the vulnerably cluster,

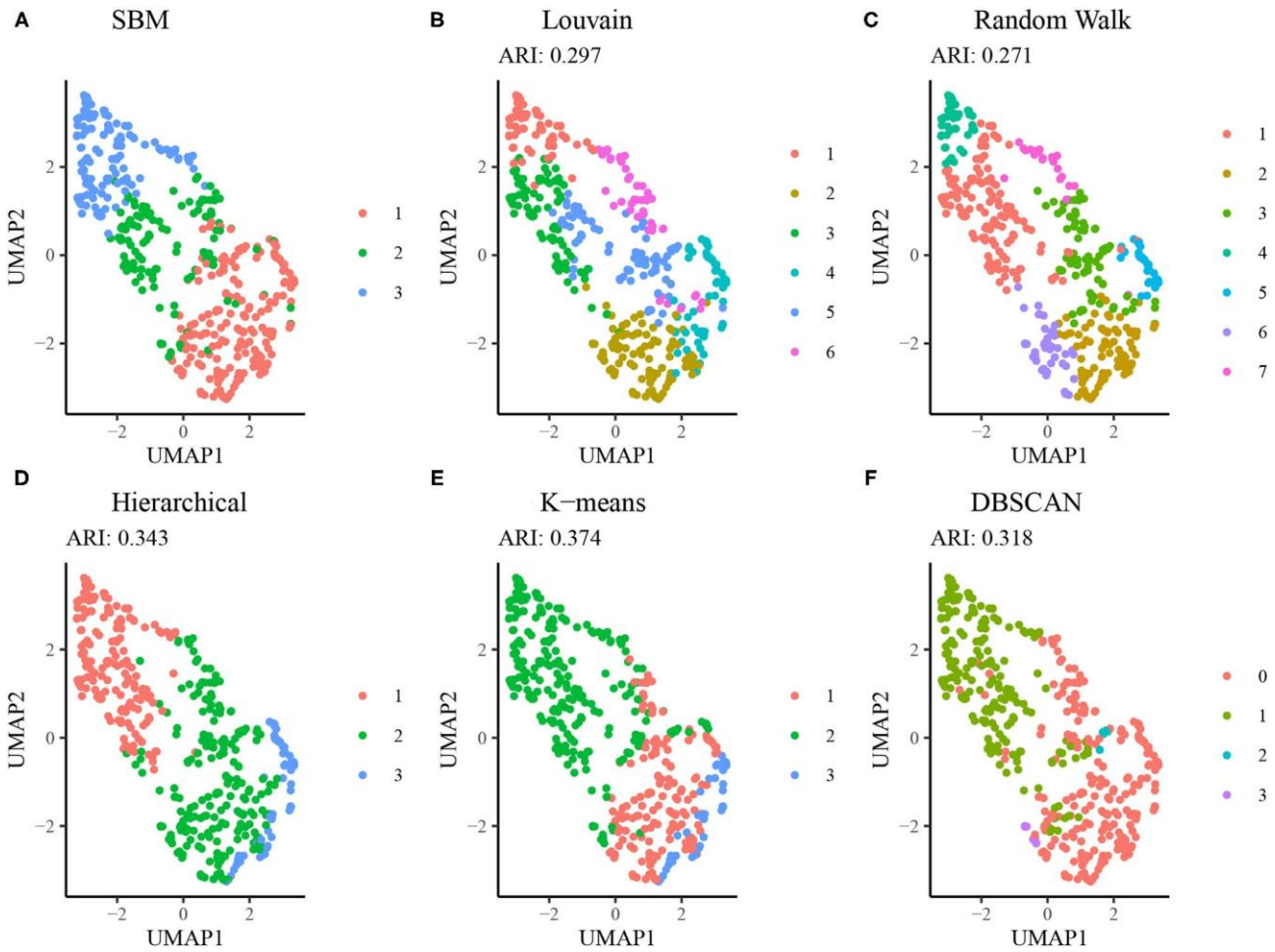

FIGURE 6 | Comparison of SBM performance relative to alternative methods. (A) Clustering results from the SBM using $K = 3$. (B) Clustering results from the Louvain algorithm (no tuning parameters available). (C) Clustering results from the Walktrap algorithm using random walks of length 4. (D) Hierarchical clustering results using a dendrogram cut at $K = 3$. (E) K-means clustering results using the Hartigan-Wong method and $K = 3$. (F) DBSCAN clustering results using a radius of 0.8 and minimum neighborhood size of 5.

providing candidate animals for further investigation of the differences between vulnerable and resilient animals. Finally, we developed "`mlsbm`," an efficient and robust `R` packagefor implementation of our proposed clustering workflow. The `mlsbm` package is publicly available through CRAN (https://cran.r-project.org/package=mlsbm) for use in future behavioral studies.

The SBM analysis identified three behaviorally distinct populations of rats that varied based on their apparent vulnerability to OUD. OUD is a complex and multi-symptomatic disorder, making it imperative to understand how various behaviors over the course of addiction interact with one another to confer vulnerability vs. resiliency. Results indicate that individuals more vulnerable to OUD exhibit higher lever pressing across the behavioral tasks, but largely not in a linear manner (**Supplementary Figure 3**). Thus, in the SMB, it is the non-linear interaction between several variables that ultimatelyresults in differences between clusters. This is illustrated in **Supplementary Figure 8**, showing how all animals in cluster 1 (vulnerable cluster) vary across the seven traits we used for modeling. Highlighted are examples of three rats each showing a distinct high and low z-score profile depending on the traits. For example, not all rats in the vulnerable clusterhad high heroin consumption, although the mean consumptionfor this cluster was greater than for the other two clusters(**Figure 3**).

Both males and females were used in this study, and we found sex differences in cluster composition with females more represented in Cluster 1, and males in Cluster 3. These data align with what is observed in humans, as females both acquireand maintain higher levels of drug use, and relapse more often,than males across several classes of drugs, including heroin (33).This finding further bolsters the potential translational validity of this model in assessing OUD vulnerability. However, a deeperanalysis of translational validity requires future studies where traits determined prior to heroin exposure that predict OUD

vulnerability in humans can be evaluated to determine if they predict which cluster a rat will enter. For example, levels of impulsivity, novelty-induced locomotor behavior and attributing incentive salience to a reward-paired cue have all been show to predict relapse propensity [for review see (34)]. Moreover, measuring behaviors of drug seeking after obtaining the heroin measures can be used as covariates to further validate cluster allocation by the SBM model. For example, the model would predict that cluster 1 rats would more compulsively seek heroin in the presence of punishment than cluster 3 subpopulations. Also, identifying these three distinct phenotypes using this model allows for further characterization of individual variation in the neurobiological mechanisms and genetic background underlying OUD vulnerability. Finally, we plan to develop an interactive web application using the SBM model to analyze a variety of network-based data sets without the need for programming experience in R, thereby allowing other laboratories to evaluate a variety of network-based data sets for subpopulations of animals and humans that may be more vulnerable or resilient to developing SUDs or other neuropsychiatric disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The R package mlsbm is publicly available from the Comprehensive R Archive Network (https://cran.r-project.org/package=mlsbm). The behavioral data used in this paper are not readily available due to ongoingdata collection, which is implemented as part of the ongoing NIH-funded research project. Please contact the corresponding author for any inquiry related to the behavioral data.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and by the Italian Ministry of Health (approval 1D580.18). Procedures abided by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

Statistical modeling, software development, and data analyses were conducted by CA and DC. The behavioral experiments were designed by NC, BK, MU, LW, GH, RC, and PK. All behavioral experimental procedures were conducted by BK, NC, VL, AC, and AR. This manuscript was written by CA, BK, NC, RC, PK, and DC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.745468/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RES EARCH ARTICLE

Genetic deletion or pharmacological blockade of nociceptin/orphanin FQ receptors in the ventral tegmental area attenuates nicotine-motivated behaviour

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Background and Purpose: The nociceptin/orphanin FQ (N/OFQ)-nociceptin opioid-like peptide (NOP) receptor system is widely distributed in the brain and pharmacological activation of this system revealed therapeutic potential in animal models of substance use disorder. Studies also showed that genetic deletion or pharmacological blockade of NOP receptors confer resistance to the development of alcohol abuse. Here, we have used a genetic and pharmacological approach to evaluate the therapeutic potential of NOP antagonism in smoking cessation.

Experimental Approach: Constitutive NOP receptor knockout rats ($NOP^{-/-}$) and their wild-type counterparts ($NOP^{+/+}$) were tested over a range of behaviours to characterize their motivation for nicotine. We next explored the effects of systemic administration of the NOP receptor antagonist LY2817412 (1.0 & 3.0 $mg \cdot kg^{-1}$) on nicotine self-administration. NOP receptor blockade was further evaluated at the brain circuitry level, by microinjecting LY2817412 (3.0 & 6.0 $\mu g \cdot \mu l^{-1}$) into the ventral tegmental area (VTA), nucleus accumbens (NAc) and central amygdala (CeA).

Key Results: Genetic NOP receptor deletion resulted in decreased nicotine intake, decreased motivation to self-administer and attenuation of cue-induced nicotine reinstatement. LY2817412 reduced nicotine intake in $NOP^{+/+}$ but not in $NOP^{-/-}$ rats, confirming that its effect is mediated by inhibition of NOP transmission. Finally, injection of LY2817412 into the VTA but not into the NAc or CeA decreased nicotine self-administration.

Conclusions and Implications: These findings indicate that inhibition of NOP transmission attenuates the motivation for nicotine through mechanisms involving the VTA and suggest that NOP receptor antagonism may represent a potential treatmentfor smoking cessation.

KEYWORDS
nicotine, NOP, reinforcement, relapse, reward, VTA

Abbreviations: CeA, central amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area.

1 | INTRODUCTION

Nicotine is the major reinforcing component of tobacco responsible for addiction in cigarette smokers (Stolerman & Jarvis, 1995). Despitea general decline in cigarette smoking, more than 8 million deaths are predicted from tobacco use worldwide each year by 2030 (World Health Organization [WHO], 2016). While wealthy countries aim for smoke-free future generations, the public health burden of tobacco persists in medium-low-income countries (Jha & Peto, 2014). The introduction of electronic cigarettes into the market place as a proposed tool for smoking cessation has, reportedly, increased initiation of cigarette smoking among adolescents, prompting calls for regulation of tobacco/vaping products (Hammond et al., 2017; Leventhal et al., 2015; Primack et al., 2018). Pharmacological interventions to induce smoking cessation include a variety of Food and Drug Administration (FDA) approved pharmacotherapies, including nicotine, as a replacement therapy, varenicline. that acts as a partial agonist at nicotinic (nACh) receptor and bupropion, that works through blockade of the dopamine transporter (DAT; SLC6A3) (Elrashidi & Ebbert, 2014). Nevertheless, these therapies appear to be efficaciousonly in a proportion of patients seeking such treatment. Thus the development of more efficacious cures remains an important priority. Pharmacological and genetic studies support a critical role of the endogenous opioid system in shaping the rewarding and motivational properties of nicotine on the different stages of the addiction process (for reviews, see Berrendero et al., 2010; Hadjiconstantinou & Neff, 2011). The nociceptin/orphanin FQ (N/OFQ)-nociceptin opioid-like peptide (NOP) receptor system is the fourth member of the opioid family and a growing body of evidence indicates that it has an important role in substance use disorders (for review, see Ciccocioppo et al., 2009; Schank et al., 2012; Witkin et al., 2014). The N/OFQ peptide and the NOP receptors are highly expressed in the mesocorticolimbic system where they modulate dopamine, GABA and glutamate transmission (Kallupi et al., 2014; Roberto & Siggins, 2006). Previous work has demonstrated therapeutic potential of NOP agonists in the treatment of psychostimulant and alcohol use disorders (for review, see Ciccocioppo et al., 2019; Zaveri, 2011). However, recent findings point to the possibility that not only NOP agonism but also NOP antagonism attenuates the motivation for alcohol (Borruto et al., 2020; Brunori et al., 2019; Cippitelli et al., 2016; Rorick-Kehn et al., 2016). Finally, in a study in which rats were trained to lever press in a model of concurrent alcohol and nicotine self-administration, the NOP antagonist SB612111 reduced nicotine consumption whereas the NOP agonist AT-202(SR16835) increased it (Cippitelli et al., 2016). In contrast, another study has demonstrated that mice lacking the NOP receptor show increased hippocampal acetylcholine (ACh) release, higher voluntary drinking of a nicotine solution and increased sensitivity to nicotine compared with wild-type mice (Sakoori & Murphy, 2009; Uezu et al., 2005).

To clarify the role of the N/OFQ-NOP system in nicotine abuse, we examined nicotine-motivated behaviour in a line of rats ($NOP^{-/-}$) carrying a constitutive deletion of NOP receptors in comparison with that of a wild-type ($NOP^{+/+}$) control line. We next employed these two rat lines to establish the effects of systemic administration of the selective NOP receptor antagonist LY2817412 on nicotine self-administration. Finally, using $NOP^{+/+}$ rats, we evaluated the effects of site-specific microinjections of LY2817412 into the ventral tegmental area (VTA), the nucleus accumbens (NAc) and the central amygdala (CeA) on nicotine self-administration.

What is already known

- NOP receptors are pivotal role for the reinforcement and motivational aspects of drugs of abuse.
- NOP receptor agonism enhances nicotine consumption in rats.

What does this study add

- NOP receptor blockade attenuates the motivation for nicotine through mechanisms involving the ventral tegmental area.

What is the clinical significance

- NOP receptor antagonism maybe, potentially, a new approach for smoking cessation.

2 | METHODS

2.1 | Animals

Experiments were performed in adult male $NOP^{-/-}$ and Wistar Han $NOP^{+/+}$ control rats to enable comparison with prior literature on the role of the NOP system in operant drug self-administration in the $NOP^{-/-}$ rat line (Kallupi et al., 2017). Animals were bred at the University of Camerino. The $NOP^{-/-}$ rat line was originally generated at the Hubrecht Institute (the Netherlands) by target-selected ENU-induced mutagenesis on a Brown Norway background. Heterozygous mutants were then outcrossed onto a Wistar Han line to eliminate confounding effects from other mutations that may have been introduced by the ENU mutagenesis. Biochemical characterization revealed that the NOP receptor is completely absent in homozygous knockout rats, and no adaptive changes in other opioid receptor levels and their distribution have occurred (Homberg et al., 2009). The $NOP^{-/-}$ rats used in the experiments were obtained from mating homozygous male and female mutants. Rats (225-250 g) were housed in a temperature- and humidity-controlled environment under a reverse light cycle (lights off at 8:00 AM) with food and water *ad libitum*. Rats were habituated to the facility and handled prior to experiments. Animals were treated in accordance with the guidelines of the European Community Council

Directive for Care and Use of Laboratory Animals (2010/63/EU). Formal approval was obtained from the Italian Ministry of Health and Internal Ethical Committee for Laboratory Animal Protection and Use of the University of Camerino (protocol no. 1D580.22). All efforts were made to minimize rats' suffering and distress. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.2 | Catheter implantation

Animals were anaesthetized with isoflurane anaesthesia: 5% induction and 2% maintenance. A single catheter made from micro-renathane tubing (ID = 0.020^{00}, OD = 0.037^{00}; Braintree Scientific) was implanted in the right jugular vein and subcutaneously positioned between the vein and the back as previously described (Shen et al., 2017). During recovery from anaesthesia rats were maintained in a separate cage, kept warm with a heated pad and observed for at least 30 min. For the subsequent 3 days, rats were treated subcutaneously with 10 mg·kg^{-1} of enrofloxacin (50 mg·ml^{-1} , Baytril, Germany) and allowed 1 week of recovering before self-administration training. Catheters were flushed daily throughout the experiment with 0.1-0.2 ml of sterile saline mixed with heparin (20 U·ml^{-1}; Italfarmaco S.p.A, Milan, Italy) to maintain patency that was confirmed by loss of reflex occurring in less than 5 sec following intravenous injection of 150 μl per rat of sodium pentothal (25 mg·ml^{-1}, Intervet, Italy) at the end of experimental procedures.

2.3 | Intracranial surgery and histological analysis

For intracranial surgery, rats were anaesthetized as described above and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). During surgery the body temperature was maintained with the aid of an heated pad positioned under the animal. Animals were bilaterally implanted with stainless-steel guide cannulas (0.65 mm outside diameter) using the following coordinates (in mm): incisor bar at —3.3 mm; NAc shell, anteroposterior (AP) +1.4, lateral (L) ±1.1 and ventral (V) —6.0; CeA, AP —2.5, L ±4.3 and V —6.5; VTA, AP —5.6, L ±2.2 and V —7.4, angle 12°. The guide cannulas were fixed to the skull with dental cement and three anchoring screws. All coordinates were based on the rat brain atlas (Paxinos & Watson, 2013) and were adjusted for the body weight of the animals. Following surgery and for the subsequent 3 days, animals were treated subcutaneously with 10 mg·kg^{-1} of enrofloxacin (50 mg·ml^{-1}, Baytril, Germany). Rats were allowed to recover 1 week after surgery. For the intracranial injections, LY2817412 was administered at a volume of 0.5 μl per side via a 10-μl Hamilton syringe over 60 s per side. The stainless-steel injector, 1.5 mm longer than the guide cannula, was allowed to remain in the brain an additional 60 s per side before being retracted. Cannula placements were verified with injection of black India ink (0.5 μl per site) into the VTA, NAc and CeA after completion of the experiments. Histological verification of ink diffusion into the region of interest was evaluated. Animals then were deeply anaesthetized with isoflurane and killed with an anaesthetic overdose. The brains removed from the skull, were quickly frozen in isopentane and stored in a —80°C freezer until sectioning. Coronal sections of 40 mm obtained in the cryostat were mounted on slides and stained with cresyl violet. Placements of the injector were determined using a light microscope and mapped onto coronal sections of a rat brain stereotaxic atlas (Paxinos & Watson, 2013).

2.4 | Self-administration apparatus

Nicotine self-administration was performed in rat operant conditioning chambers (29.5 × 32.5 × 23.5 cm; Med Associates, St. Albans, VT) enclosed in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with two 4-cm-wide retractable levers (8 cm above the grid floor and 12 cm apart) located in the front panelwith two stimulus light placed above each lever, a house light at the top of the opposite panel and a tone generator. Nicotine solution was delivered by Tygon tubing connected to a swivel and then to the catheter before the beginning of each session. A syringe pump (3.33 RPM, Med Associates, St. Albans, VT) was activated by responses on the right (active) lever and resulted in a delivery of 0.1-ml nicotine solution, while responses on the left (inactive) lever were recorded but did not result in any programmed consequences. The operant chambers were controlled and data collected with MED-PC® IV windows-compatible software.

2.5 | Experimental procedures

2.5.1 | Nicotine self-administration

Acquisition and maintenance under fixed ratio

After 1 week of recovery from i.v. surgery, NOP$^{+/+}$ and NOP$^{-/-}$ rats were trained in 2-h daily nicotine (30 μg·kg^{-1}·0.1 ml of infusion) self-administration sessions (5 days·week^{-1}). The response requirement for each infusion was incremented from a fixed ratio 1 (FR1) to fixed ratio 3 (FR3) until baseline was reached. Pressing the active (right) lever resulted in the infusion of 0.1 ml of nicotine followed by the activation of the cue light above the lever and a 20-s time-out period during which responses at the active lever had no programmed consequences. Inactive lever presses were recorded but resulted in no reinforcer delivery.

Progressive ratio

NOP$^{+/+}$ and NOP$^{-/-}$ rats previously trained to self-administer nicotine under a fixed ratio schedule were subsequently challenged under a progressive ratio (PR) schedule of reinforcement. During progressive ratio sessions, the response requirements necessary to receive a single dose of nicotine increased according to the following scale: '3,6,9,12,15,20,25,32,40,50,62,77,95,118,145,178,219,268 etc.' (adapted from Richardson & Roberts, 1996). The maximal

number of responses that a rat performed which was obtained for one infusion was referred to as the break point. The progressive ratio session lasted 4 h or ended if the required ratio was not achieved within 1 h.

Withdrawal and cue-induced reinstatement

Following the progressive ratio experiment, $NOP^{+/+}$ and $NOP^{-/-}$ rats underwent a withdrawal period of 21 days during which they were housed in 2-3 per cage in the animal facility and handled three times per week. After this period, animals were returned to the self-administration boxes to be tested for cue-induced reinstatement of nicotine seeking. During 2-h reinstatement sessions, responding at the active lever led to the activation of the cue light previously paired with nicotine infusion but nicotine was no longer delivered. The total number of responses at the previously active lever was considered a measure of reinstatement. Inactive lever presses were also recorded as a measure of non-specific responding.

2.5.2 | Effect of LY2817412 on nicotine self-administration in $NOP^{-/-}$ and $NOP^{+/+}$

After the progressive ratio test, to evaluate the effect of the NOP antagonist LY2817412 on nicotine consumption, $NOP^{+/+}$ and $NOP^{-/-}$ rats were re-trained to fixed ratio 3 nicotine self-administration until stable baseline of lever pressing was reached. At this point, LY2817412 (0.0, 1.0 and 3.0 $mg \cdot kg^{-1}$) was given p.o. in a within-subject Latin square using William's design 1 h before the beginning of the self-administration session. The fixed ratio 3 nicotine self-administration baseline was re-established between drug tests. Rats were familiarized with the intragastric gavage procedure for three consecutive days before drug tests.

2.5.3 | Effect of LY2817412 microinjection into the ventral tegmental area (VTA), nucleus accumbens (NAc) or central amygdala (CeA) on nicotine self-administration in $NOP^{+/+}$rats

New cohorts of $NOP^{+/+}$ rats were used to test the effect of LY2817412 on fixed ratio nicotine self-administration following infusion into the VTA, NAc or CeA. Rats were trained to self-administer nicotine (30 $\mu g \cdot kg^{-1} \cdot 0.1$ ml of infusion) as previously described until a stable baseline was reached. At this point, LY2817412 (0.0, 3.0 and 6.0 $\mu g \cdot \mu l^{-1}$) was microinjected into the brain areas of interest according to a Latin square using William's design. Drug infusion occurred 15 min before beginning of the self-administration session. The fixed ratio 3 nicotine self-administration baseline was re-established between tests. Prior to drug tests, rats were familiarized with the intracranial injection procedures. Upon completion of the experiments, rats were anaesthetized with isoflurane, and black India ink (0.3 μl per site) was injected into the targeted brain areas. Rats then were killed to remove their brains for histological verification of cannula placements. Only data from rats with correct cannula placements were included in the statistical analysis.

2.6 | Materials

The (—)-nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile physiological saline and administered intravenously at a concentration of 30.0 $\mu g \cdot kg^{-1} \cdot 0.1$ ml of infusion. The NOP receptor antagonist LY2817412 [2^0-chloro-4^0,4^0-difluoro-1-{[1-(3-fluoropyridin-2-yl)-3-methyl-1*H*-pyrazol-4-yl]methyl}-4^0,5^0-dihydrospiro [piperidine-4,7^0-thieno[2,3-C]pyran]2,3-dihydroxybutanedioate] was synthesized at Lilly Research Laboratories (Indianapolis, IN, USA) and kindly provided to us. LY2817412 was dissolved in a formulation consisting of a 1:1 mixture of distilled water and 1-M H_3PO_4 (pH 3) and was administered orally via gavage (p.o.). For intracranial microinjections, the compound was dissolved in 3% DMSO (Merck, Milano, IT), 10% Tween (Merck, Milano, IT) and 87% distilled water. Drug doses were chosen based on earlier studies in our laboratory (Borruto et al., 2020, 2021).

2.7 | Blinding and randomization

The laboratory animals were randomly assigned to the different experimental groups considering their nicotine responding baseline and the treatments were assessed blindly. Correct cannula placement was assessed in a blinded manner.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). In the first experiment, the same groups of $NOP^{-/-}$ and $NOP^{+/+}$ rats were subjected to fixed ratio, followed by progressive ratio (Figure 1a,b) and with systemic LY28177412 (Figure 2). The reinstatement study (Figure 1c) and brain microinjection experiments (Figure 3) were carried out in a different group of rats. Group size of $n \geq 5$ was employed for statistical evaluation, and using randomization, the experimental groups were designed accordingly. The declared group size represents the number of independent values, and the statistical analysis was performed using these independent values. The sample sizes and animal numbers were determined by power analysis of pre-existing data. Behavioural data were analysed by ANOVA followed by post hoc tests when appropriate. In particular, the difference between the $NOP^{+/+}$ and $NOP^{-/-}$ in the number of infusions during the acquisition and maintenance of nicotine self-administration was analysed by two-way ANOVA with one factor between (rat line) and one factor within (sessions) and the lever presses by three-way ANOVA with one factor between (rat line) and two factors within (lever and ses- sions). The progressive ratio data were analysed by unpaired

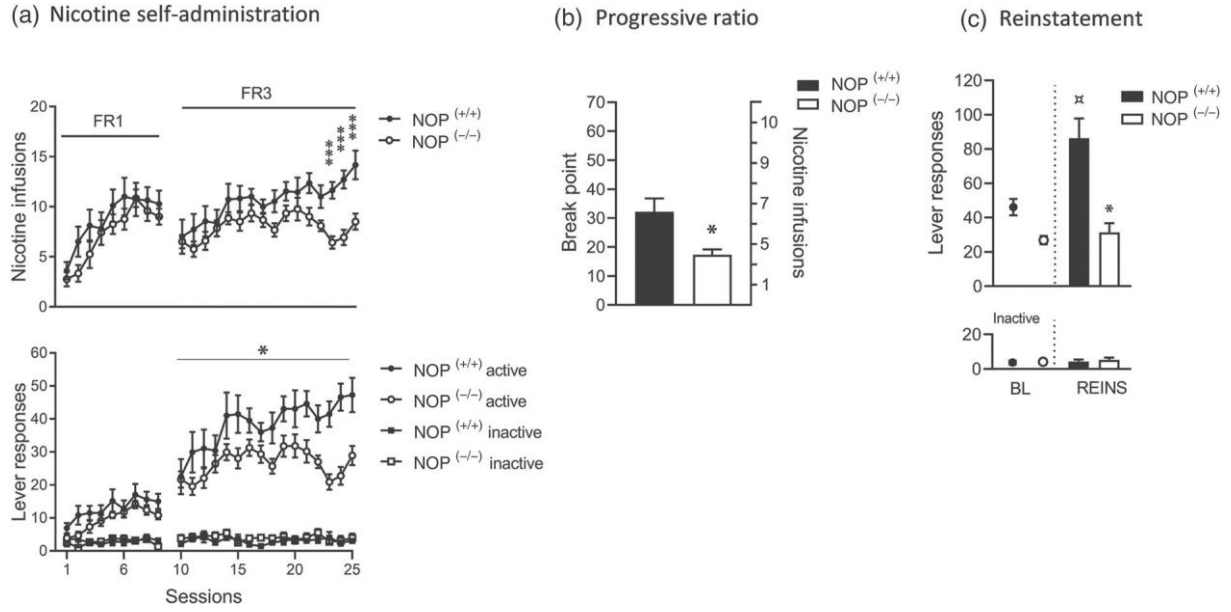

FIGURE 1 Nicotine addictive-behaviours in NOP$^{+/+}$ and NOP$^{-/-}$ rats. (a) Acquisition pattern of nicotine self-administration in NOP$^{+/+}$ (n = 11) and NOP$^{-/-}$ (n = 12) rats. The number of nicotine infusions and total number of active and inactive lever presses are shown for fixed ratio 1 (FR1; Days 1–9) and fixed ratio 3 (FR3; Days 10–25) conditions. *P < 0.05 versus NOP$^{+/+}$ and NOP $^{-/-}$ rats nicotine infusions on Days 23, 24 and 25. *P < 0.05 versus NOP$^{+/+}$ and NOP$^{-/-}$ rats active lever presses in fixed ratio 3. (b) Motivation for nicotine as measured by the break point on a progressive ratio (PR) schedule of reinforcement and corresponding nicotine infusions earned in NOP$^{+/+}$ (n = 11) and NOP$^{-/-}$ (n = 12) rats. *P < 0.05 versus NOP$^{+/+}$ and NOP$^{-/-}$ rats. (c) Cue-induced reinstatement of nicotine seeking after 21 days of withdrawal in NOP$^{+/+}$ (n = 8) and NOP$^{-/-}$ (n = 9) rats compared with their respective baseline (average of active lever presses and inactive lever presses during the last 4 days of training). *P < 0.05 versus NOP$^{+/+}$ and NOP$^{-/-}$ rats. ¤P < 0.05 versus NOP$^{+/+}$ baseline responding and NOP$^{+/+}$ reinstatement responding. Values represent mean (±SEM)

Student's t-test. Cue-induced reinstatement of nicotine seeking was analysed by two-way ANOVA with one factor between (rat line) and one factor within (sessions). The effects of systemic and central administration of LY2817412 on nicotine self-administration were analysed using one-way ANOVA with repeated measures using 'drug dose' as a within-subject factor. Post hoc comparisons were carried out by Dunnett's or by Newman-Keuls test only when the F value attained P < 0.05 and there was no significant inhomogeneity of variances. The data are presented as the mean ± SEM. Statistically significant difference was set at P < 0.05. Prior to ANOVA, we examined for significant violations for assumptions of homogeneity of variance by using Levene's test. Mauchly's test of sphericity was used to testif assumption of sphericity had been violated when using repeated measures ANOVA. Data were analysed using STATISTICA, Stat Soft 13.0 (RRID:SCR_014213).

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Mathie,et al., 2021).

3 | RESULTS

3.1 | NOP$^{-/-}$ rats self-administer less nicotine compared with NOP$^{+/+}$ counterparts

NOP$^{+/+}$ (n = 11) and NOP$^{-/-}$ (n = 12) were trained to self-administer nicotine for 9 consecutive days under fixed ratio 1 schedule of reinforcement followed by 16 consecutive days of fixed ratio3 (Figure 1a). Analysis of the nicotine self-administration data under the fixed ratio 1 condition revealed no difference in the number of nicotine infusions and lever responding between NOP$^{+/+}$ and NOP$^{-/-}$ rats. Under the fixed ratio 3 schedule of reinforcement, NOP $^{-/-}$ rats administered significantly less nicotine compared to the NOP$^{+/+}$ controls and had significantly lower active lever presses. Inactive lever responses were very low and not significantly different between NOP$^{+/+}$ and NOP $^{(-/-)}$ rats on the fixed ratio 1 and fixed ratio3 schedule of reinforcement.

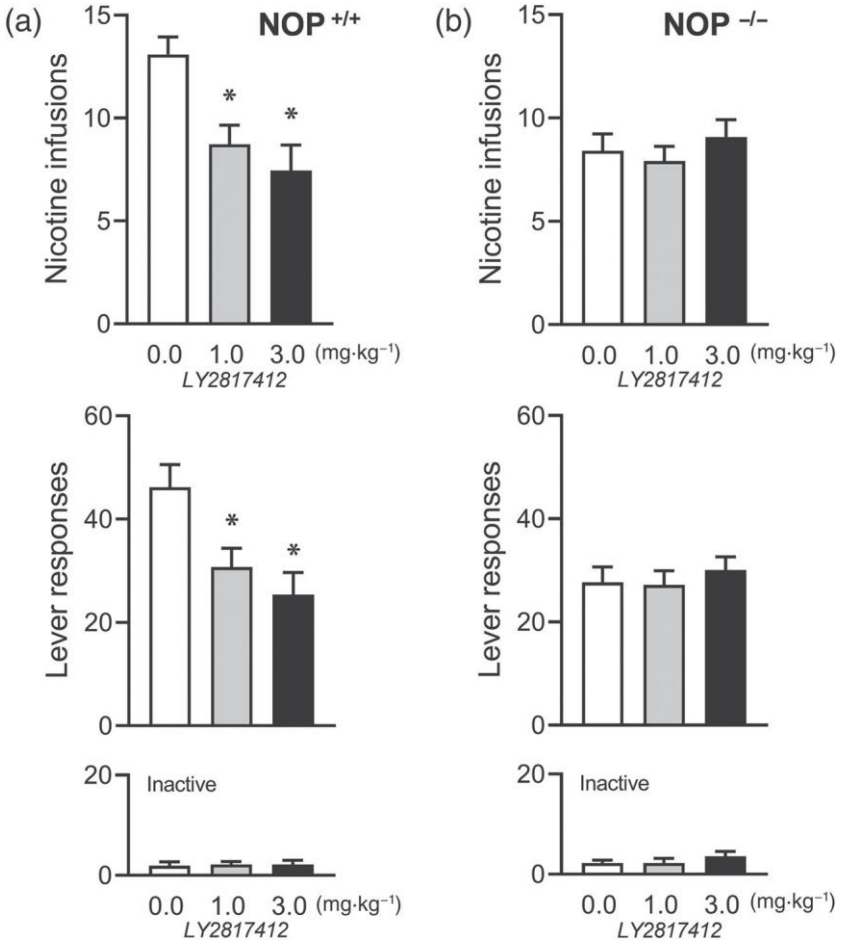

FIGURE 2 Systemic effects of the NOP antagonist LY2817412 on nicotine self-administration in NOP$^{+/+}$ and NOP$^{-/-}$ rats. (a) Number of responses (infusions and total number of active and inactive lever presses) following systemic LY2817412 administration (0.0, 1.0 and 3.0 mg·kg^{-1}) in NOP$^{+/+}$ (n = 11) and (b) NOP$^{-/-}$ (n = 12) rats. *P < 0.05 versus 0.0 mg·kg^{-1} versus 1.0 and 3.0 mg·kg^{-1}. Values represent mean (±SEM)

3.2 | NOP$^{-/-}$ rats show lower break point for nicotine compared with NOP$^{+/+}$ counterparts

Under the progressive ratio contingency, the number of lever presses required was increased exponentially as the session progressed. The break point reached was significantly lower in NOP$^{-/-}$ (n = 11) compared with NOP$^{+/+}$ (n = 12) rats, suggesting that NOP$^{-/-}$ rats have lower motivation to self-administer nicotine compared with their NOP$^{+/+}$ counterparts (Figure 1b).

3.3 | Environmental conditioning factors reinstate nicotine seeking in NOP$^{+/+}$ controls but not in NOP$^{-/-}$ rats

NOP$^{+/+}$ (n = 8) and NOP$^{-/-}$ (n = 9) rats previously trained to self-administer nicotine were tested for cue-induced reinstatement of nicotine seeking after 21 days of withdrawal. When compared with nicotine self-administration baseline (average of nicotine-related lever presses during the last 4 days of self-administration), a significant reinstatement was reached in NOP$^{+/+}$ but not in NOP$^{-/-}$ rats. When evaluating the differences in reinstatement levels, NOP$^{+/+}$ rats showed higher levels of reinstatement compared with the NOP$^{-/-}$ line. Inactive lever responses were very low and there were no differences observed between NOP$^{+/+}$ and NOP$^{-/-}$ (Figure 1c).

3.4 | The NOP antagonist LY2817412 reduces nicotine self-administration in NOP$^{+/+}$ but not in NOP$^{-/-}$rats

When the efficacy of LY2817412 on fixed ratio 3 nicotine self-administration was tested in NOP$^{+/+}$ rats (n = 11), the compound when compared with vehicle significantly reduced nicotine self-administration and the number of active lever presses at both doses (1 and 3 mg·kg^{-1}) tested (Figure 2a). Analysis of the effects of LY2817412 on nicotine self-administration in NOP$^{-/-}$ rats (n = 12) failed to confirm a significant effect of treatment (Figure 2b). Inactive lever presses were very low and not affected by treatment in NOP$^{+/+}$ or NOP$^{-/-}$ rats (Figure 2a,b).

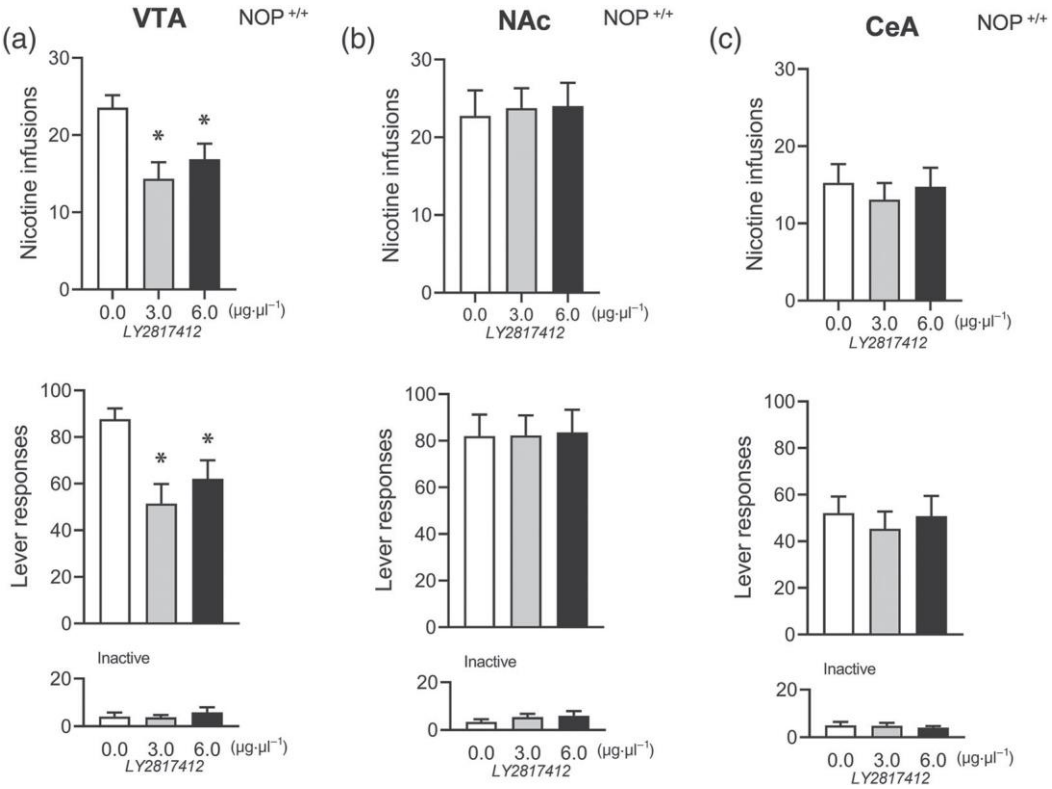

FIGURE 3 Central effects of the NOP antagonist LY2817412 on nicotine self-administration in ventral tegmental area (VTA), nucleus accumbens (NAc) and central amygdala (CeA) in NOP$^{+/+}$ rats. (a) Number of responses (infusions and total number of active and inactive lever presses) following central LY2817412 injection (0.0, 3.0 and 6.0 $\mu g \cdot \mu l^{-1}$) in VTA ($n = 9$), (b) NAc ($n = 8$) and (c) CeA ($n = 11$) in NOP$^{+/+}$ rats. *$P < 0.05$ versus 0.0 versus 3.0 $\mu g \cdot \mu l^{-1}$ and *$P < 0.05$ versus 0.0 versus 6.0 $\mu g \cdot \mu l^{-1}$. Values represent mean (±SEM)

3.5 | Microinjection of LY2817412 in the ventral tegmental area (VTA) but not nucleus accumbens (NAc) or the central amygdala (CeA) reduces nicotine self-administration in NOP$^{+/+}$ rats

The lack of efficacy of LY2817412 in NOP$^{-/-}$ after peripheral administration prompted us to investigate the effects of central administration of LY2817412 in NOP$^{+/+}$ rats only. Following LY2817412 microinjection into the VTA ($n = 9$), the number of nicotine infusions and active lever presses were significantly reduced at both doses tested (3.0 and 6.0 $\mu g \cdot \mu l^{-1}$) (Figure 3a). When the NOP antagonist was administered into the NAc ($n = 8$) or CeA ($n = 11$), no significant drug effects on the nicotine response were observed (Figure 3b,c). Inactive lever pressing was very low and not affected by the drug treatment in each brain region. Following histological analysis, four rats belonging to the VTA group, one to the NAc, and three to the CeA groups were excluded from the statistical analysis due to incor- rect cannula placement (Figure S3).

4 | DISCUSSION

Results demonstrated that genetic deletion or pharmacological blockade of the NOP receptor by the selective antagonist LY2817412 blunted the motivation for nicotine in the rat. Following intracranial administration, LY2817412 reduced nicotine self-administration when injected into the ventral tegmental area (VTA) but not into the central amygdala (CeA) or the nucleus accumbens (NAc).

4.1 | Genetic deletion of the NOP receptor supresses nicotine-motivated addictive-behaviours

During the early acquisition phase of nicotine self-administration, there were no differences between the NOP$^{+/+}$ and NOP$^{-/-}$ lines when tested to a fixed ratio 1 schedule of reinforcement. However, when the response requirement was increased to fixed ratio 3, responding initially decreased in both genotypes, but in NOP$^{+/+}$ rats it rapidly returned to fixed ratio 1 level and then further increased, an effect not observed in NOP$^{-/-}$ rats. The implication of these findings is twofold. First, genetic deletion of NOP does not affect the ability of rats to learn operant responding as suggested by the fact that under the fixed ratio 1, both genotypes equally acquired nicotine-reinforced responding. This is particularly relevant because ithas been shown previously that modulation of central N/OFQ-NOP transmission modifies reward-related learning. For instance, in socially defeated rats, reward learning was inversely correlated with N/OFQ mRNA expression levels in the VTA, NAc and striatum (Der-Avakian

et al., 2017). Secondly, the lower rate of nicotine infusions in the NOP$^{-/-}$ line, when operant responding was increased to fixed ratio 3, indicates that NOP deletion blunts the motivating effects of nicotine when the effort required to obtain the drug increases. This reduced motivation for nicotine of NOP$^{-/-}$ was confirmed in the progressive ratio experiment, where the break point in the knockout was significantly lower compared with wild-type controls. In one of our earlier studies, we demonstrated that NOP$^{-/-}$ rats also self-administered less cocaine, heroin and alcohol compared with the control line, while operant responding for saccharin was the same in both lines (Kallupi et al., 2017). A possible explanation of this finding is that NOP$^{-/-}$ rats have reduced motivation for substances of abuse in general while reward processing for natural reinforces is unaltered. However, this interpretation contrast with conditioned place preference data showing that NOP$^{-/-}$ are more sensitive to the rewarding effects of morphine (Rutten et al., 2011). On the other hand, in our pilot study (see Figure S2), we found that, compared with NOP$^{+/+}$, NOP$^{-/-}$ self-administered less nicotine independently of concentration, suggesting that these two genotypes most likely differ in the motivation for nicotine rather than in their sensitivity. If taken together, these data may suggest that the NOP receptor system playsa different role depending on the substance of abuse under examination. Noteworthy, the fact that NOP$^{+/+}$ and NOP$^{-/-}$ did not differ in saccharin-reinforced responding indicates that the low rate of responding for drugs of abuse in the NOP$^{-/-}$ is not secondary to an impairment in motor performance. Moreover, when we evaluated drug seeking evoked by presentation of cues previously paired with nicotine self-administration, we found that NOP receptor deletion resulted in a complete loss of relapse-like behaviour. This provides additional evidence that in NOP$^{-/-}$ rats, the motivation for nicotine is low and further reduced following a period of abstinence. Based on this observation, it is tempting to hypothesize that high basal NOP transmission facilitates relapse. Although unproven, this hypothesis is indirectly supported by earlier findings with alcohol showing thatbrain expression levels of N/OFQ and NOP transcripts are higher in post dependent and genetically selected alcohol preferring rats, in which relapse propensity is higher compared with Wistar controls (Aujla et al., 2013; Economidou et al., 2008; Hansson et al., 2006). Apossible confounding factor in the present set of experiment consists of the possibility that for NOP$^{+/+}$ and NOP$^{-/-}$ littermates, parental differences might have affected animals response to nicotine. To mitigate this risk, we paid careful attention to maintaining the two genotypes under identical breeding and environmental conditions throughout the study.

4.2 | NOP receptor antagonism attenuates nicotine self-administration in NOP$^{+/+}$ but not NOP$^{-/-}$ rats

Having established that NOP deletion reduces the motivation for nicotine, we sought to further confirm this finding by testing the effects of LY2817412, a potent and selective NOP antagonist on nicotine-motivated behaviour. As expected, LY2817412 significantly reduced nicotine self-administration in NOP$^{+/+}$ but not NOP$^{-/-}$ rats. This provides important proof of concept for the potential efficacy of selective NOP antagonists as a treatment for smoking cessation. Moreover, this finding demonstrates that the effects of LY2817412 are mediated specifically by NOP receptors. The therapeutic potential of this pharmacological approach is further supported by the results of an earlier study in which SB612111, another NOP antagonist, showed efficacy in reducing nicotine intake in rats trained to concurrently self-administer alcohol and nicotine (Cippitelli et al., 2016). Moreover, NOP antagonists also have been shown to reduce alcohol drinking and relapse (Borruto et al., 2020, 2021; Brunori et al., 2019; Koizumi et al., 2004; Rorick-Kehn et al., 2016). This action of NOP antagonists was observed also in a preliminary study in depressed alcoholic patients (Post et al., 2016). From the clinical perspective, the ability of LY2817412 to attenuate the motivation for both nicotine and alcohol is particularly significant as these two substances are usually co-abused (Anton et al., 2018; Domi et al., 2021; McKee et al., 2007; McKee & Weinberger, 2013).

4.3 | Microinjection of LY2817412 into the VTA but not NAc or CeA reduces nicotine self-administration

Nicotine reward is thought to be mediated by its facilitation of mesocorticolimbic fixed ratio transmission via complex mechanisms involving both postsynaptic and presynaptic modulation of VTA dopamine neurons (Mao et al., 2011; Marti et al., 2011; Pontieri et al., 1996; Tolu et al., 2013; Yan et al., 2019). We hypothesized therefore that the inhibitory effects of LY2817412 on nicotine self-administration were mediated by its interference with nicotine-induced activation of VTA dopamine transmission. This hypothesis also was driven by our earlier findings on LY2940094, an NOP antagonist analogue of LY2817412, that reduces alcohol-induced increases of dopamine release in the NAc shell (Rorick-Kehn et al., 2016). These effects appear to be specific for drug reinforcers in view of reports that site-specific ablation of NOP receptors in the VTA enhances the motivation for sucrose and injection of N/OFQ in the same brain region decreases binge eating (Hernandez et al., 2021; Parker et al., 2019). While selective deletion of the NOP receptor in the CeA attenuates the hedonic value of palatable food (Hardaway et al., 2019). Moreover, we have found earlier that site-specific microinjection of LY2817412 into the VTA and CeA, but not NAc, reduces alcohol drinking in genetically selected alcohol preferring rats (Borruto et al., 2020). Guided by these earlier observations, we sought to establish whether the effects of LY2817412 on nicotine self-administration are mediated via blockade of NOP receptors in one of these regions. The microinjection experiments identified the VTA as the critical site for this effect in that nicotine self-administration was significantly reduced following administration of LY2817412 into this area but not when injected into the CeA or NAc. These findings raise two issues: - the first, is that the effects of LY2817412 on nicotine self-administration seem to be

mediated selectively by VTA NOP receptors, whereas secondly, as per our previous findings with alcohol, both the VTA and the CeA seem to mediate LY2817412-induced reductions in alcohol intake. It is likely, therefore, that the mechanisms through which NOP blockade attenuates the motivation for alcohol and nicotine do not fully overlap and differ with regard to the relevance of the CeA. Secondly, considering that NOP antagonism reduces alcohol-induced dopamine release in the NAc, one may speculate that LY2817412 attenuates nicotine reward by blunting the ability of nicotine to stimulate mesolimbic dopamine transmission. However, this hypothesis contrasts with earlier evidence that facilitation of VTA N/OFQ transmission acts as a stop signal to terminate reward-related responses and that activation of mesolimbic dopamine transmission by substances of abuse is also prevented by NOP agonists (Di Giannuario et al., 1999; Parker et al., 2019; Vazquez-DeRose et al., 2013).

However, with regard to this inconsistency, it is important to consider that the VTA contains two populations of NOP positive neurons (Figure 4); the first one co-express tyrosine hydroxylase and its activation negatively regulates dopamine transmission (Norton et al., 2002; Zheng et al., 2002). The second population is negative to tyrosine hydroxylase and is composed of GABA and glutamate cells that are located presynaptically and, by impinging onto dopamine neurons, regulate the activity of the VTA dopamine system (Driscoll et al., 2020). It is known that N/OFQ acting at presynaptic level inhibits GABA release onto dopamine neurons that may potentially result in their disinhibition (Zheng et al., 2002). Conceivably, such dis-inhibition is prevented by simultaneous activation of NOP receptors, that are located on dopamine cells and hyperpolarize them. However, in condition when the role of presynaptic NOP is prevalent, receptor antagonists by blocking the inhibitory effect of N/OFQ on VTA GABA cells can enhance their activity consequently inhibiting dopamine neurotransmission. Based on this conceptualization, it is possible therefore to propose a heuristic mechanism according to which modulation of N/OFQ system by NOP agonist and antagonists may both oppose the activation of VTA dopamine transmission by substances of abuse.

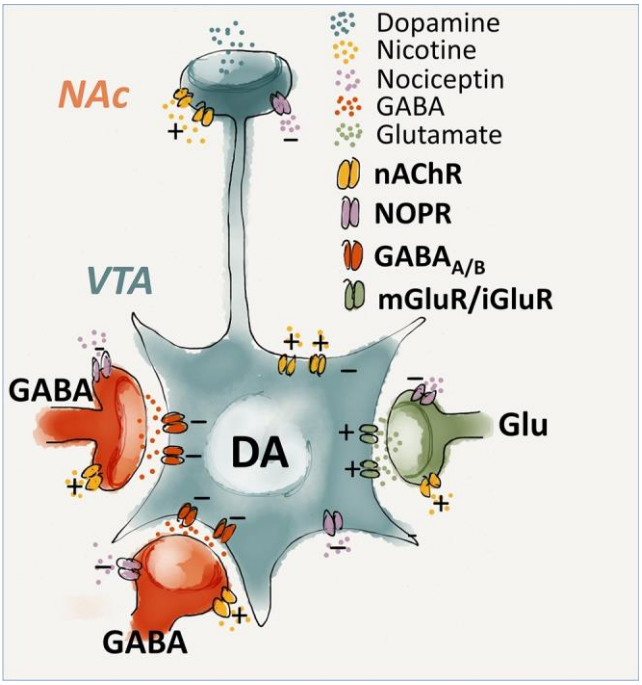

FIGURE 4 Schematic drawing of N/OFQ-NOP system in the ventral tegmental area (VTA)-nucleus accumbens (NAc). Within the VTA, NOP positive neurons are expressed in dopamine (DA), GABA and glutamate cells. The figure illustrates the possible mechanism through which NOP signalling influences the cellular components affecting the mesolimbic dopamine transmission. N/OFQ exerts an inhibitory role in the activity of both GABA interneurons and dopamine neurons within the VTA. At a presynaptic lever, activation of the NOP receptors inhibits GABA release onto dopamine neurons, with subsequent disinhibition and increase in dopamine release. However, simultaneous activation of NOP receptors that are located in dopamine cells prevent such disinhibition resulting in a final attenuation of the dopamine neurotransmission

In conclusion, the present findings confirm that genetic deletion or pharmacological blockade of NOP attenuates the motivation for nicotine and, by extension, suggest that selective receptor antagonists such as LY2817412 may prove effective as smoking cessation agents. One analogue of LY2817412, BTRX-246040 (LY2940094), is currently under clinical development for the treatment of depression (ClinicalTrials.gov Identifier: NCT03193398). Given the high co-occurrence of depression and nicotine abuse, it may be of particular clinical relevance to test the therapeutic potential of NOP antagonists not only in nicotine-dependent patients but also particu- larly in these comorbid patient populations.

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AUTHOR CONTRIBUTIONS

A.D. and R.C. designed the project. A.D. designed and performed the experiments, analysed the data and wrote the manuscript.
V.L. performed the experiments. R.C. and F.W. supervised the project and contributed to writing the manuscript. E.D. contributed to writing the manuscript. M.P., E.D., A.-M.B., L.M.R.-K. and M.U. provided critical comments and assisted with data interpretation. All authors reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical

research as stated in the *BJP* guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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The multitarget FAAH inhibitor/D3 partial agonist ARN15381 decreases nicotine self-administration in male rats.

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Abstract.

Tobacco use disorder is a worldwide health problem for which available medications show limited efficacy. Nicotine is the psychoactive component of tobacco responsible for its addictive liability. Similar to other addictive drugs, nicotine enhances mesolimbic dopamine transmission. Inhibition of the fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of the endocannabinoid anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), reduces nicotine-enhanced dopamine transmission and acquisition of nicotine self-administration in rats. Down-regulation of dopamine transmission by antagonists or partial agonists of the dopamine D3 receptor (DRD3) also reduced nicotine self-administration and conditioned place preference. Based on these premises, we evaluated the effect of ARN15381, a multitarget compound showing FAAH inhibition and DRD3 partial agonist activity in the low nanomolar range, on nicotine self-administration in rats. Pretreatment with ARN15381 dose dependently decreased self-administration of a nicotine dose at the top of the nicotine dose/response (D/R) curve, while it did not affect self-administration of a nicotine dose laying on the descending limb of the D/R curve. Conversely, pretreatment with the selective FAAH inhibitor URB597 and the DRD3 partial agonist CJB090 failed to modify nicotine self-administration independent of the nicotine dose self-administered. Our data indicates that the concomitant FAAH inhibition and DRD3 partial agonism produced by ARN15381 is key to the observed reduction of nicotine self-administration, demonstrating that a multitarget approach may hold clinical importance for the treatment of tobacco use disorder.

Keywords: Tobacco use disorder, endocannabinoid, dopamine transmission, self-administration

1. Introduction

Tobacco use disorder (TUD) is an unmet medical emergency for which new and more efficacious therapies are needed due to the limited efficacy of approved medications (Garcia-Rivas et al., 2017; Xi et al., 2009). Nicotine is the main psychoactive component of tobacco, responsible for the development and maintenance of TUD (Garcia-Rivas and Deroche-Gamonet, 2019; Le Foll and Goldberg, 2006). In rats, nicotine reinforces self-administration behavior (Corrigall and Coen, 1989) and induces conditioned place preference (CPP) (Le Foll and Goldberg, 2005).

The endocannabinoid system plays a role in the reinforcing effects of nicotine, representing a potential pharmacological target for the treatment of TUD (Muldoon et al., 2013; Scherma et al., 2016). This system comprises two receptors - CB1 and CB2 - and their endogenous ligands - anandamide (AEA) and 2-arachidonoylglycerol (2-AG) - that are inactivated by the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase respectively (Rodriguez de Fonseca et al., 2005). The selective FAAH inhibitor URB597 reduces nicotine-induced dopamine mesolimbic transmission (Melis et al., 2008; Scherma et al., 2008), and it blocks the acquisition of nicotine self-administration and CPP in rats (Forget et al., 2009; Scherma et al., 2008). Beside AEA, FAAH inhibition also increases the levels of palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Melis et al., 2008; Panlilio et al., 2013), two endogenous agonists at the a-subtype peroxisome proliferator-activated receptor (PPAR-a). The PPAR-a antagonist MK886 reduced the effects of FAAH inhibition on nicotine self-administration, but not the effects of URB597 on cued nicotine seeking in squirrel monkeys (Justinova et al., 2015), suggesting that FAAH may modulate the primary reinforcing effects of nicotine through mechanisms independent of the endocannabinoid system. As such, FAAH inhibition may represent a promising target to help smoking cessation.

The endocannabinoid system has shown to modulate the mesolimbic dopamine transmission (Melis and Pistis, 2012), and the inhibition of FAAH activity to counteract nicotine-induced dopaminergic release (Luchicchi et al., 2010; Melis et al., 2008; Scherma et al., 2008); except for Pavon et al. (2018). The dopamine D3 receptor (DRD3) is predominantly expressed within the mesolimbic system (Levesque et al., 1992), and striatal levels of DRD2/DRD3 negatively correlated with TUD (Okita et al., 2016). Selective antagonism at DRD3 attenuated nicotine self-administration (Ross et al., 2007), while the partial agonist BP-897 and the antagonist SB-277011A blocked the expression of nicotine induced CPP in rats (Le Foll et al., 2005; Pak et al., 2006). These findings suggest that molecules able to tone down DRD3 activity may have potential as treatments for TUD (Le Foll et al., 2007; Sokoloff and Le Foll, 2017).

Based on these premises, we hypothesized that the concurrent inhibition of FAAH activity and activation of DRD3 by a partial agonist would contrast the primary reinforcing effects of nicotine and we developed the novel dual FAAH inhibitor/DRD3 partial agonist ARN15381 accordingly (De Simone et al., 2017). To test our hypothesis, we compared the ability of ARN15381 to reduce nicotine self-administration in rats with that of the selective FAAH inhibitor URB597 and the DRD3 partial agonist CJB090.

2. Materials and Methods

2.1. Animals

Fortyeight male Wistar rats (Charles River, Calco, Italy), weighing 225-250g the day of their arrival, were housed two per cage on a 12-hour light/dark cycle (lights off at 8.00am) in a temperature (21-22°C) and humidity-controlled room (45-55%). During the experiments, animals were given *ad libitum* access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). The week after their arrival, animals were

allowed to acclimate to the housing room. The second week they were handled daily for 5 minutes by the same operator who carried out the experiment. All behavioral procedures were performed during the dark phase of the light/dark cycle.

All procedures were conducted in adherence with the guidelines of the *European Community Council Directive for Care and Use of Laboratory Animals and National Institutes of Health, and were approved by the local ethical commission (1D580.21).*

2.2. Drugs

For self-administration training, (-)-nicotine hydrogen tartrate salt was dissolved in sterile physiological saline at the free-base dose of 30 µg/0.1ml and 15 µg/0.1ml per infusion (pH adjusted to 7.4 ± 0.1). The solutions were filtered through a 0.2 µm filter and given intravenously (i.v.). ARN15381, corresponding to the hydrochloride salt of compound **2** (**Figure 1**, derivative **33** in (De Simone et al., 2017)), was synthetized at the Italian Institute of Technology (IIT) (Genova, Italy) as described in supplementary information (SI). ARN15381 synthesis (**Figure 1)** was performed following the procedure reported by De Simone et al. (2017). ARN15381 was dissolved in a vehicle consisting of 5% DMSO, 20% PEG 400 and 75% distilled water. The FAAH inhibitor URB597 was dissolved in 20% DMSO and saline. The dopamine DRD3 receptor partial agonist CJB090 was dissolved in 5% β-cyclodextrin. Excluding ARN15381, all drugs and solvents were purchased from Sigma-Aldrich (Milan, Italy).

The molecular structures ARN15381, CJB090 and URB597 are represented in **Figure 2**.

2.3. Catheter implantation

Animals were anesthetized with isoflurane diluted in oxygen (5% for the induction of anesthesia and 2-2.5% for its maintenance). Incisions were made to expose the right jugular vein and in the intra-scapular region. A catheter made of micro-renathane tubing (I.D.=0.020 in., O.D.=0.037 in.) was subcutaneously positioned between the vein and the back. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle that protruded from the back of the animal. The cannula was embedded in a support made by dental cement. One week of recovery was allowed before starting self-administration training. During this week, rats received the antibiotic enrofloxacin (Baytril®, Germany) dissolved in the drinking water (25 mg/100ml). For the duration of the experiments, catheters were flushed daily with 0.2 ml of heparinized saline solution containing 1 mg/ml of enrofloxacin. After experiments, catheter patency was confirmed by administration of 0.2 ml of a thiopental sodium (20 mg/ml) solution. Catheter patency was assumed when an immediate loss of reflexes was observed.

2.4. Self-administration apparatus

Experiments were performed in MedAssociate operant conditioning chambers (ENV-008CT) enclosed in sound attenuating and ventilated environmental cubicles. Each chamber was equipped with two retractable levers located on the front panel, a cue-light above each lever and a tone generator. Nicotine was delivered by a plastic tube that was connected to the catheter before the beginning of the session. An infusion pump was activated by the response on the active lever according to the programmed schedule, while responses on the inactive lever were recorded but had no scheduled consequence. Activation of the pump resulted in the

delivery of 0.1 ml of fluid. A Windows-compatible Med-PC-IV software controlled the delivery of nicotine solution and recorded behavioral data.

2.5. Nicotine self-administration training

Rats were trained to self-administer nicotine i.v. in two-hours daily sessions, five days a week. Half of the rats were trained to self-administer 30 µg/infusion and the other half 15 µg/infusion of nicotine (infusion volume was 0.1 ml delivered over 5 seconds).The first week, rats were trained under Fixed Ratio 1 (FR1) schedule of reinforcement. The second week, reinforcement contingency was increased to FR2 and the third week to FR3, which was maintained for the remainder of training and tests. A 20-second time out period (TO) started contigently with nicotine infusion. During the TO, the cue-light positioned above the active lever was illuminated and responses at the active lever were not reinforced. A 2.9 kHz intermittent beep-tone (1s ON/1s OFF) was generated by a SC628 tone generator (MedAssociate) and was presented throughout the session. Pharmacological tests started when a stable baseline of nicotine infusion was achieved (a minimum of ten infusions and ±20% variation over the last three days). For each nicotine dose, rats were divided into three groups (*N* = 7-9 each): one receiving ARN15381, one receiving CJB090 and the third receiving URB597.

2.6. Effect of ARN15381 on nicotine self-administration

2.6.1 Nicotine 30µg/infusion: The effect of ARN15381 (0.0, 3.0, 10.0 mg/kg (De Simone et al., 2017)) on voluntary nicotine (30µg/infusion) self-administration was tested in rats (*N*=8) trained as described above, using a within subject Latin-square design, in which each rat received all doses of ARN15381 in a counterbalanced order. ARN15381 was administered orally by gavage 60 minutes prior to the test session at

the volume of 1 ml/kg (De Simone et al., 2017). Animals were acclimated to the treatment procedure for three days before tests. Test sessions were carried out every fourth day. The first day between test sessions, rats remained in their home-cage, whereas the second and third day they were subjected to nicotine self-administration baseline.

2.6.2 Nicotine 15µg/infusion: The effect of ARN15381 (0.0, 3.0, 10.0 mg/kg) on voluntary nicotine (15µg/infusion) self-administration was tested in a separate group of rats (*N*=9) using the same protocol described for nicotine 30µg/infusion dose.

2.7 Effect of CJB090 on nicotine self-administration

2.7.1 Nicotine 30µg/infusion: The experimental design described in session 2.6 was also used to test the effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine (30 µg/infusion) self-administration in a separate group of rats (*N*=7). CJB090 was administered i.v. through the same catheter used for self-administration at the volume of 1 ml/kg immediately before the test session. Considering the plasma volume estimated by our rats' body weights at the time of experiment (Bijsterbosch et al., 1981; Lee and Blaufox, 1985) and the brain/plasma concentration ratio of CJB090 (Mason et al., 2010), we estimated that 3.0 mg/kg i.v. dose of CJB090 would yield a brain concentration more than one thousand times higher than CJB090 EC_{50} (6.3 nM) (Newman et al., 2003) and therefore adequate to effectivelly engage the target.

2.7.2 Nicotine 15µg/infusion: The same protocol was used to test the effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine (15 µg/infusion) self-administration in a separate group of rats (*N*=8).

2.8 Effect of URB597 on nicotine self-administration

2.8.1 Nicotine 30µg/infusion: The experimental design described in session 2.6 was used to test the effect of URB597 (0.0, 0.3 and 1.0 mg/kg) on nicotine (30 µg/infusion) self-administration in an independent group of rats (*N*=9). URB597 was administered intraperitoneally (i.p.) 30 minutes before the test session at the volume of 1ml/kg. These doses, administration route, and time was previously demonstrated to fully inhibit FAAH activity in the rat brain (Fegley et al., 2005).

2.8.2 Nicotine 15µg/infusion: To test the effect of URB597 (0.0, 0.3, and 1.0 mg/kg) on nicotine (15 µg/infusion) self-administration a separate group of rats (*N*=7) was trained as described above.

2.9. Statistical analyses

Acquisition of nicotine self-administration was analyzed independently for the two doses of nicotine by two-ways ANOVA with both lever and time as repeated measures. The infusions earned after vehicle treatment of the three drug tests for each nicotine dose were analyzed by two-tailed *t*-test for independent samples. The effects of ARN15381 (0.0, 3.0 and 10.0 mg/kg), and URB597 (0.0, 0.3 and 1.0 mg/kg), on nicotine self-administration were analyzed using a one-way within subjects analysis of variance (ANOVA) with dose as a repeated measure. The effect of CJB090 (0.0 and 3.0 mg/kg) on nicotine self-administration was analyzed by a two-tailed paired *t*-test. Statistical significance was conventionally set at $p<0.05$, ANOVAs were followed by Dunnett's *post-hoc* test when appropriate. Experiments run on different doses of nicotine were analysed separately. Infusions earned, active and inactive-lever responses were analyzed separately.

3. Results

Self-administration acquisition training of the rats subjected to ARN15381 treatment are presented as typical self-administration acquisition curve for the two doses of nicotine (30µg/infusions and 15µg/infusions; **Figure 3**)

Rats trained to self-administer 30µg/infusions of nicotine increased the number of infusion earned over time, which became stable starting from the fourth session. Rats responded at increasing reinforcement schedule (from FR1 to FR2 and finally to FR3) by increasing the number of active lever presses produced, in order to maintain a stable number of infusions; conversely, inactive lever responses remained very low and stable (**Figure 3A**; Lever [$F(1,7)=142.5$; $p<0.0001$], Time [$F(16,112)=27.7$; $p<0.0001$], Lever by Time interaction [$F(16,112)=25.0$; $p<0.0001$]).

Also rats trained to self-administer 15µg/infusions adapted their active lever responding to increasing reinforcement schedule while inactive lever responses remained very low and stable (**Figure 3B**; Lever [$F(1,8)=115.8$; $p<0.0001$], Time [$F(16,128)=8.4$; $p<0.0001$], Lever by Time interaction [$F(16,128)=13.1$; $p<0.0001$]).

Comparison of 15 and 30µg nicotine infusions earned after vehicle treatment in the three drug tests indicated that the 30µg nicotine dose laid on the descending limb of the nicotine D/R curve (**Supplementary Figure 1S**).

3.1. Effect of ARN15381 on nicotine self-administration

3.1.1 Nicotine 30 µg/infusion: ANOVA of nicotine 30µg-infusions earned after ARN15381 treatment found no overall effect of doses on nicotine self-administration [$F(2,14)=0.77$, $p=0.44$] (**Figure 4A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$F(2,14)=1.05$;

p=0.35] (**Figure 4B, upper panel**). Inactive lever responses were very low and also not affected by treatment [F(2,14)=1.56, p=0.25] (**Figure 4B, lower panel**).

To exclude the possibility that the 10 mg/kg ARN15381 was not sufficently high to observe an effect on nicotine 30µg/infusion self-administration, we tested the effect of 30 mg/kg of ARN15381 in an independent group of rats, but we found no increased efficacy ([t(6)=0.96; p=0.37]; **Figure 4A inset**). Inactive lever response was also not affected by ARN15381 30 mg/kg treatment [t(6)=0.3; p=0.77].

3.1.2 Nicotine 15µg/infusion: ANOVA of nicotine 15µg-infusions earned after ARN15381 treatment found an overall effect of doses on nicotine self-administration [F(2,16)=6.95, p=0.008]. Dunnett's *post-hoc* analysis revealed that the 10 mg/kg dose of ARN15381 decreased the number of infusions earned compared to vehicle (p<0.05; **Figure 4C**). ANOVA of active lever responses found an overall effect of ARN15381 treatment [F(2,16)=7.72; p=0.005]. Dunnet's *post-hoc* test indicated that both doses of ARN15381 decreased active lever presses compared to vehicle (**Figure 4D, upper panel**). Conversely, inactive lever responses was very low and not affected by treatment [F(2,16)=1.04, p=0.37] (**Figure 4D, lower panel**).

These results indicated that concomitant inhibition of FAAH activity and partial agonistic activity at DRD3 produced by ARN15381 selectively reduced 15µg/infusion nicotine self-administration.

Next, we wanted to test whether the partial agonism at DRD3 and the selective inhibition of FAAH activity alone would affect nicotine self-administration.

3.2. Effect of CJB090 on nicotine self-administration

One rat from the 30µg/infusion group and one rat from the 15µg/infusion group were excluded from analyses because of catheter failure.

3.2.1 Nicotine 30µg/infusion: 3.0 mg/kg of CJB090 did not affect nicotine 30µg/infusion self-administration [$t(5)=0.3$, $p=0.77$] (**Figure 5A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$t(5)=0.04$; $p=0.97$] (**Figure 5B, upper panel**). Inactive lever responses were very low and were also not affected by treatment [$t(5)=1.4$, $p=0.22$] (**Figure 5B, lower panel**).

3.2.2 Nicotine 15µg/infusion: CJB090 did not affect nicotine 15µg-infusions self-administration [$t(6)=0.23$, $p=0.82$] either (**Figure 5C**). Analysis of active lever responses was consistent with infusions and reported no effect of treatment [$t(6)=0.3$, $p=0.8$] (**Figure 5D, upper panel**). Inactive lever responses were very low and were also not affected by treatment [$t(6)=1.3$, $p=0.23$] (**Figure 5D, lower panel**).

3.3. Effect of URB597 on nicotine self-administration

3.3.1 Nicotine 30µg/infusion: When the effect of the FAAH inhibitor URB597 on nicotine 30µg-infusions earned was evaluated, ANOVA found no effect of URB597 doses on nicotine self-administration [$F(2,16)=1.29$, $p=0.3$] (**Figure 6A**). Analysis of active lever responses was consistent with infusions and reported no overall effect of treatment [$F(2,16)=1.08$; $p=0.35$] (**Figure 6B, upper panel**). Inactive lever responses were very low and also not affected by treatment [$F(2,16)=0.21$, $p=0.74$] (**Figure 6B, lower panel**).

3.3.2 Nicotine 15µg/infusion: When the effect of the FAAH inhibitor URB597 on nicotine 15µg-infusions earned was evaluated, ANOVA found no effect of URB597 doses on nicotine self-administration [$F(2,12)=1.42$, $p=0.28$] (**Figure 6C**). Analysis of active lever responses was consistent with infusions and

reported no overall effect of treatment [$F(2,12)=0.21$, $p=0.80$] (**Figure 6D, upper panel**). Inactive lever responses were very low and also not affected by treatment [$F(2,12)=0.20$, $p=0.77$] (**Figure 6D, lower panel**).

4. Discussion

Based on the hypothesis that a multitarget mechanism of action towards FAAH (inhibition) and DRD3 (down-regulation of transmission) would decrease the primary reinforcing properties of nicotine, we set out to evaluate the effect of the FAAH inhibitor/DRD3 partial agonist ARN15381 (De Simone et al., 2017), on nicotine self-administration in rats. ARN15381 selectively decreased self-administration of 15 µg/infusion nicotine dose. Whereas neither the selective inhibition of FAAH by URB597, nor DRD3 modulation by the partial agonist CJB090 significantly decreased nicotine self-administration. These findings demonstrate that the concomitant inhibition of FAAH activity and modulation of DRD3 transmission by partial agonism exerted by ARN15381 was efficacious where the two mechanisms alone failed.

ARN15381 decreased nicotine self-administration of 15 µg/infusion of nicotine but was not effective at 30 µg/infusion dose. Normalizing nicotine doses according to the rats' body weight at the time of the self-administration tests corresponded to doses of 35 and 61 µg/kg/infusions, falling on the top and on the descending limb of the nicotine dose/response (D/R) curve respectively (Watkins et al., 1999). In other words, ARN15381 decreased nicotine self-administration on the top of the D/R curve, and it failed to affect response for nicotine on the descending limb. Our results are consistent with previous reports in non-human primates using the FAAH inhibitors URB597 and URB694, in which both FAAH inhibitors decreased nicotine self-administration on the top but not on the descending limb of the nicotine D/R curve (Justinova et al., 2015).

The lack of ARN15381 effect on the higher dose of nicotine can be explained by three alternative interpretations. A first interpretation would be that the 30µg nicotine infusion dose (≃ 60 µg/kg) fell within the aversive range of the nicotine D/R curve, and rats were not sufficiently engaged to self-administer nicotine to observe a significant decrease. For instance, Markou and colleagues reported that rats could self-administer 60µg/kg/infusion of nicotine only after tolerance had developed (Watkins et al., 1999), suggesting an intrinsic aversive effect of this dose. However, in our experiments rats readily acquired self-administration of this dose of nicotine and they adapted their active lever response in order to maintain a stable level of infusion when FR contingency was increased from FR1 to FR3. This indicates that the 30µg/infusion dose of nicotine was devoid of aversive effects, and it was rather experienced by rats as a positive reinforcer, which prompts us to deem them unlikely that possible aversive effects of nicotine affected ARN15381 efficacy.

A second interpretation could be that the dose of ARN15381 was not sufficiently high. However, notwithstanding a 21% oral bioavailability, the brain concentration (391 nM) following oral administration of 10 mg/kg of ARN15381 is more than adequate to effectively engage the targets (FAAH IC_{50}=0.9 nM, DRD3 EC_{50}=18 nM) (De Simone et al., 2017). Yet, to exclude this possibility, and to generate a maximal dose response, in an independent group of rats, we tested the effect of 30 mg/kg of ARN15381 on nicotine 30µg/infusion self-administration and, as expected, there was no increased efficacy of ARN15381 with a dose three times as high.

A third and more appealing, though speculative at this time, mechanicistic interpretation can be proposed. ARN15381 exerts a double action by inhibiting FAAH activity, and therefore increasing AEA, PEA and OEA levels (Panlilio et al., 2013; Rodriguez de Fonseca et al., 2005), and down-toning DRD3 transmission. Inhibition of FAAH is expected to counteract dopamine release induced by nicotine (Imperato et al., 1986;

Melis et al., 2008), and the partial agonism at DRD3 to prevent the reinforcing effects of possible residual dopamine release. The inhibition of nicotine-induced dopaminergic firing could be obtained through a PPAR-a mediated inactivation of β2 nicotinic acetylcholine receptors (β2nAChR) promoted by the increase in OEA levels (Melis et al., 2010; Melis et al., 2008) induced by the FAAH inhibition (Panlilio et al., 2013). Indeed, activation of PPAR-a was reported to decrease self-administration of 30µg/kg of nicotine, a dose similar to the one on which ARN15381 was effective here (15µg/infusion = 35µg/kg/infusion). To reconcile the lack of effect on nicotine 30µg/infusion, one could consider that the reinforcing effects of nicotine are mediated by β2nAChR, as this subtype of nAChR promotes burst firing of DA neurons and thus the switching from tonic to phasic activity (Mameli-Engvall et al., 2006). Nicotine has been reported to increase the relative expression and membrane availability of β2nAChR in high affinity state (Moroni et al., 2006; Vallejo et al., 2005; Wecker et al., 2010). Therefore, the possibility exists that the dose of 30µg/infusion was sufficiently high to surmount the inactivation of nAChR induced by ARN15381 through OEA, thereby promoting enough DA release to displace ARN15381 from DRD3. As a consequence, 30µg/infusion would have maintained its reinforcing effects. Future studies should be directed to verify this hypothesis.

One target of ARN15381 is the inhibition of FAAH activity (De Simone et al., 2017). Justinova et al. (2015) investigated the effects of the FAAH inhibitors URB597 and URB694 on nicotine self-administration in squirrel monkeys and demonstrated that enzyme inhibition reduces nicotine self-administration for a dose of nicotine on the top, but not for doses on the descending limb, of the D/R curve. On the one hand, our results with ARN15381 are in line with those of Justinova et al. (2015), but on the other hand, we also demonstrated that URB597 only showed a trend to reduce nicotine self-administration in the rat. The lack of a significant

effect due to URB597 is not to be attributed to a weak pharmacological action because we chose doses that were previously demonstrated to fully inhibit FAAH activity in the rat (Fegley et al., 2005). In addition, Scherma et al. (2008) demonstrated that the FAAH inhibitor URB597 prevents the acquisition of nicotine self-administration at a dose within the range tested here. However, Scherma et al. (2008) administered URB597 throughout the acquisition phase of nicotine self-administration, starting from the first operant responding session, and an effect appeared only after twelve training sessions. On the contrary, to the best of our knowledge we are the first to report the effect of acute URB597 on nicotine self-administration under FR contingency. It should also be emphasized that, in line with our results, in the work by Forget et al. (2009) neither acute nor chronic URB597 modified nicotine self-administration under PR contingency. Altogether, these findings suggest that URB597 may reduce nicotine self-administration in the rat only if given chronically from the beginning of the acquisition phase. However, since Justinova and colleagues, like us, tested URB597 after the acquisition of operant responding (Justinova et al., 2015), the more likely explanation of the inconsistency with our results relies on inter-species differences between the two studies.

It is worth noting that the fact that URB597 induced a non-significant decrease of self-administration where ARN15381 was instead fully effective emphasizes the importance of the double action of ARN15381 on FAAH and DRD3. As proposed above, ARN15381 could have been efficacious where URB597 failed because the partial agonist action on DRD3 would have toned down possible residual dopamine transmission after inactivation of β2nAChR by the FAAH inhibitory activity.

The other target of ARN15381 is DRD3, which ARN15381 selectively activates as a partial agonist (65% efficacy, EC_{50} 18 nM (De Simone et al., 2017). Here, we report for the first time data on the effect of selective

DRD3 partial agonism either alone (CJB090) or in combination with FAAH inhibition (ARN15381) on operant nicotine self-administration under FR contingencies. Partial agonists have lower intrinsic activity at receptors than full agonists (Hoyer and Boddeke, 1993), allowing them to act as agonists or antagonists depending on the levels of the endogenous neurotransmission. Similar to other addictive drugs, the primary reinforcing effects of nicotine are mediated by the release of mesolimbic dopamine (Di Chiara and Imperato, 1988; Imperato et al., 1986). In fact, earlier studies showed that the DRD3 antagonist SB-277011A reduced nicotine self-administration under progressive ratio contingency (Ross et al., 2007). However, in our experiments, CJB090 did not reduce nicotine self-administration under FR contingency. This might indicate that to reduce self-administration by exclusively targeting DRD3 an antagonist rather than a partial agonist would be necessary, or in alternative that observations made on this target under PR contingency does not translate to FR contingency. Within the interpretation of the effect of ARN15381 on nicotine self-administration, the lack of effect of CJB090 alone further corroborates the view proposed above that the action on DRD3 co-operate with the inhibitory effects on FAAH to reduce nicotine self-administration.

This work was conducted exclusivelly in male rats. Female rats show differences in nicotine self-administration compared to males (Flores et al., 2019). In addition, estrogen has been demonstrated to modulate dopamine transmission (Peart et al., 2022; Yoest et al., 2014) and the activity of FAAH (Grimaldi et al., 2009). This suggests that females might respond differently to ARN15381 than males and dedicated studies should be conducted before generalizing the present results to both sexes.

In summary, we tested the effect of ARN15381, a compound exerting both FAAH inhibitory and DRD3 partial agonism activity on nicotine self-administration in male rats. Our results demonstrated that ARN15381 decreases nicotine self-administration. Importantly, the FAAH inhibitor URB597 and the DRD3 agonist CJB090 alone failed in significantly affecting nicotine seeking, demonstrating that the concomitance of the two mechanisms of action is the major advantage of ARN15381 and represents a novel promising line within TUD research and development. From a translational perspective, our preclinical results cast clinical importance as they suggest that targeting multi-pharmacological sites may represent a valuable approach to treat TUD.

Supplementary Materials: Chemicals, materials, and methods for the synthesis of ARN15381; Figure S1, self-administration training; Figure S2, effect of ARN15381 (30 mg/kg) on nicotine (30µg/infusion) self-administration.

Author Contributions: VL, HL, QS, FB, and AD, performed the experiments; LS assisted with the project implementation, RMCDM synthesized ARN15381; GB designed ARN15381; NC supervised the project and analyzed data; NC, CLH-K, FB, RMCDM, VL, and GB wrote the manuscript.

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FIGURE LEGENDS

Figure 1. ARN15381 synthesis scheme. i) dry CH_3CN, K_2CO_3, 85 °C, 6 h, yield: 99%; (ii) hydrazine monohydrate, CH_3OH, 80 °C, 2 h, then HCl (2 N), 1 h, yield: 90%; (iii) 4-phenylphenol, $(Boc)_2O$, DMAP, dry CH_3CN, rt, 23 h, yields: 23%; (d) HCl in CH_3OH (1.25 M), room temperature, 2 h, yield: 100%.

Figure 2. Chemical structures of the**:** selective FAAH inhibitor URB597, DRD3 partial agonist CJB090, and the FAAH inhibitor/ DRD3 partial agonist ARN15381.

Figure 3. Self-administration trainings of rats of rats subjected to ARN15381 treatment are presented as typical nicotine self-administration acquisition ratio obtained with our protocol. **A**) Acquisition of nicotine 30µg/infusion self-administration. The number of nicotine infusions increased over time and became stable starting from the forth session. When reinforcement schedule was increased from FR1 to FR2 and finally to FR3, rats increased their response at the active lever in order to maintain a stable nicotine self-administration, while inactive lever responses remained very low and stable. **B**) Acquisition of nicotine 15µg/infusion self-administration. the number of infusions of this dose of nicotine became readily stable. When reinforcement schedule was increased from FR1 to FR2 and finally to FR3, rats increased their response at the active lever in order to maintain a stable nicotine self-administration, while inactive lever responses remained very low and stable. Data are expressed as mean ± *SEM* of nicotine infusion (black dots), active lever responses (black squares) and inactive lever responses (white squares).

Figure 4. Effect of the FAAH inhibitor/DRD3 partial agonist ARN15381 on nicotine self-administration (SA) under FR3 schedule of reinforcement. A) Pretreatment with ARN15381 (0.0, 3.0 and 10.0 mg/kg) did not affect nicotine 30µg/infusion self-administration. *Inset*: 30.0 mg/kg of ARN15381 did not affect nicotine 30µg/infusion self-administration. **B)** Active (upper panel) and inactive (lower panel) lever responses were also not affected by ARN15381 treatment. **C**) ARN15381 (0.0, 3.0 and 10.0 mg/kg) dose dependently reduced nicotine 15µg/infusion self-administration. **D**) Active (upper panel) but not inactive (lower panel) lever responses were reduced by both doses of ARN15381. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session. Statistical significance: **p*<0.05 vs vehicle (0.0 mg/kg).

Figure 5. Effect of the DRD3 partial agonist CJB090 on nicotine self-administration under FR3 schedule of reinforcement. A) Pretreatment with CJB090 (0.0 and 3.0 mg/kg) did not affect nicotine 30µg/infusion SA. **B)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 30µg/infusions were also not affected by CJB090 treatment. **C)** Pretreatment with CJB090 (0.0 and 3.0 mg/kg) did not affect nicotine 15µg/infusion SA. **D)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 15µg/infusions were also not affected by CJB090 treatment. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session.

Figure 6. Effect of the FAAH inhibitor URB597 on nicotine self-administration under FR3 schedule of reinforcement. A) Pretreatment with URB597 (0.0, 0.3, and 1.0 mg/kg) did not significantly affect nicotine 30µg/infusion SA. **B)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 30µg/infusions were also not affected by URB597 treatment. **C)** Pretreatment with URB597 (0.0, 0.3, and 1.0 mg/kg) did not affect

15µg/infusion nicotine SA. **D)** Active (upper panel) and inactive (lower panel) lever responses for nicotine 15µg/infusions were also not affected by URB597 treatment. Data are expressed as mean ± *SEM* of number of nicotine infusions earned during a 2-hours SA session.

Figure 1

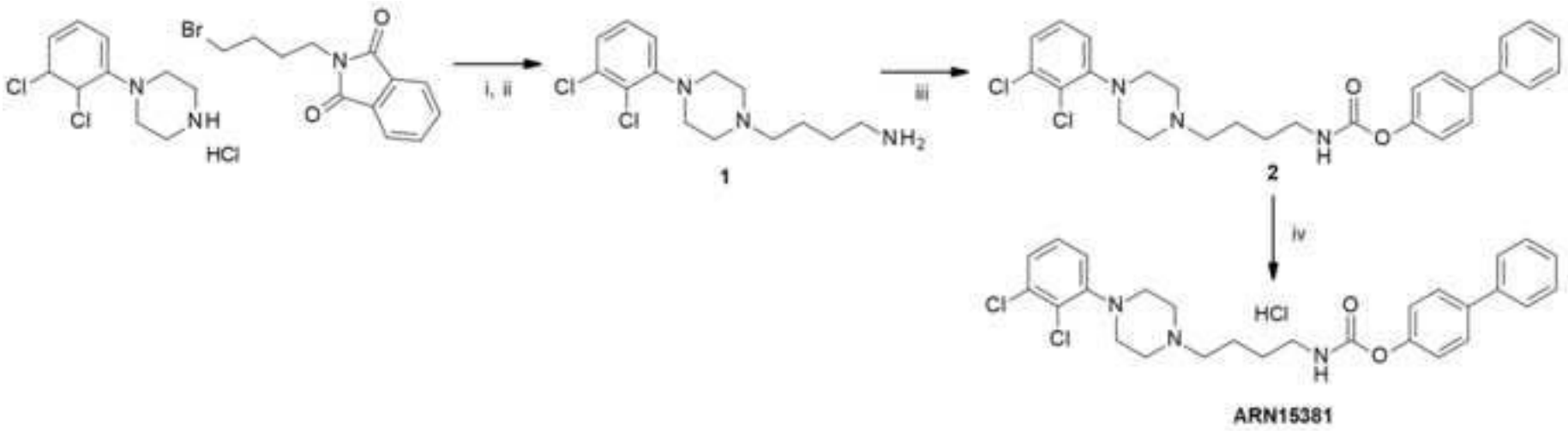
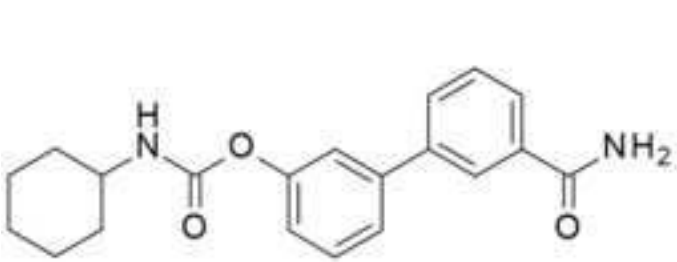
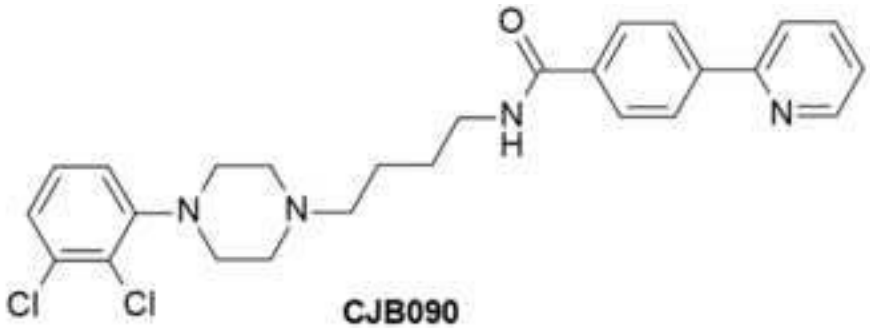


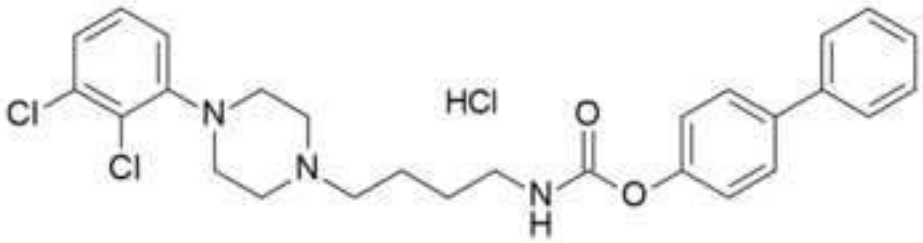
Figure 2



URB597



CJB090



ARN15381

Figure 3

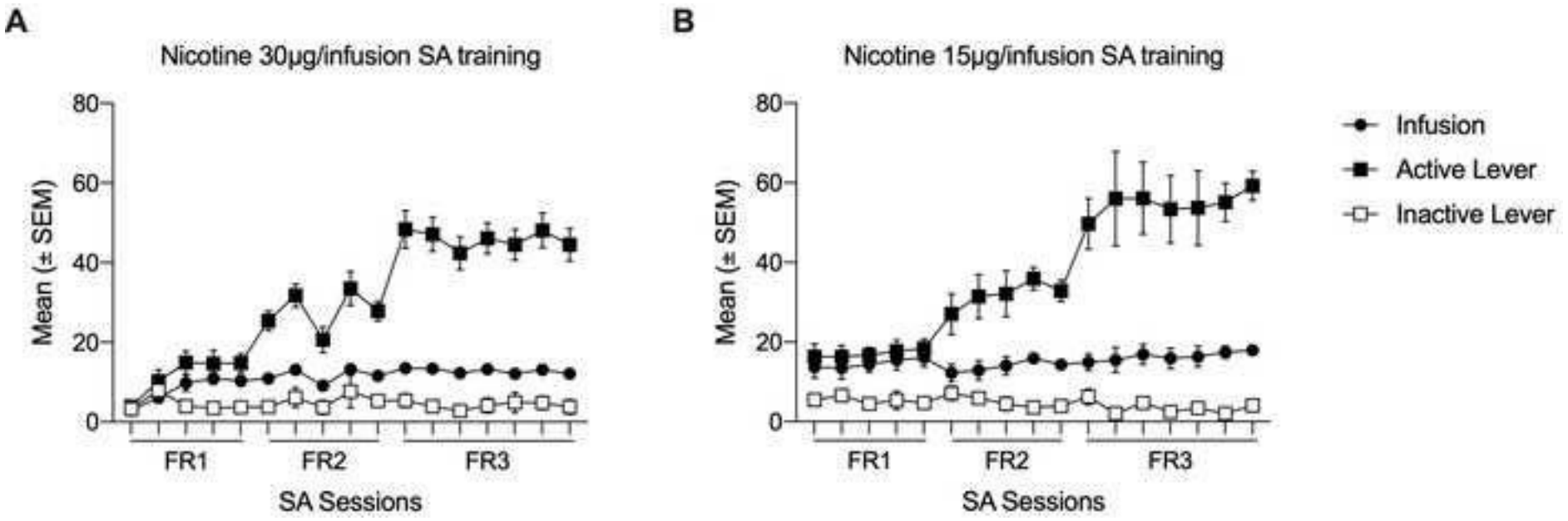


Figure 4

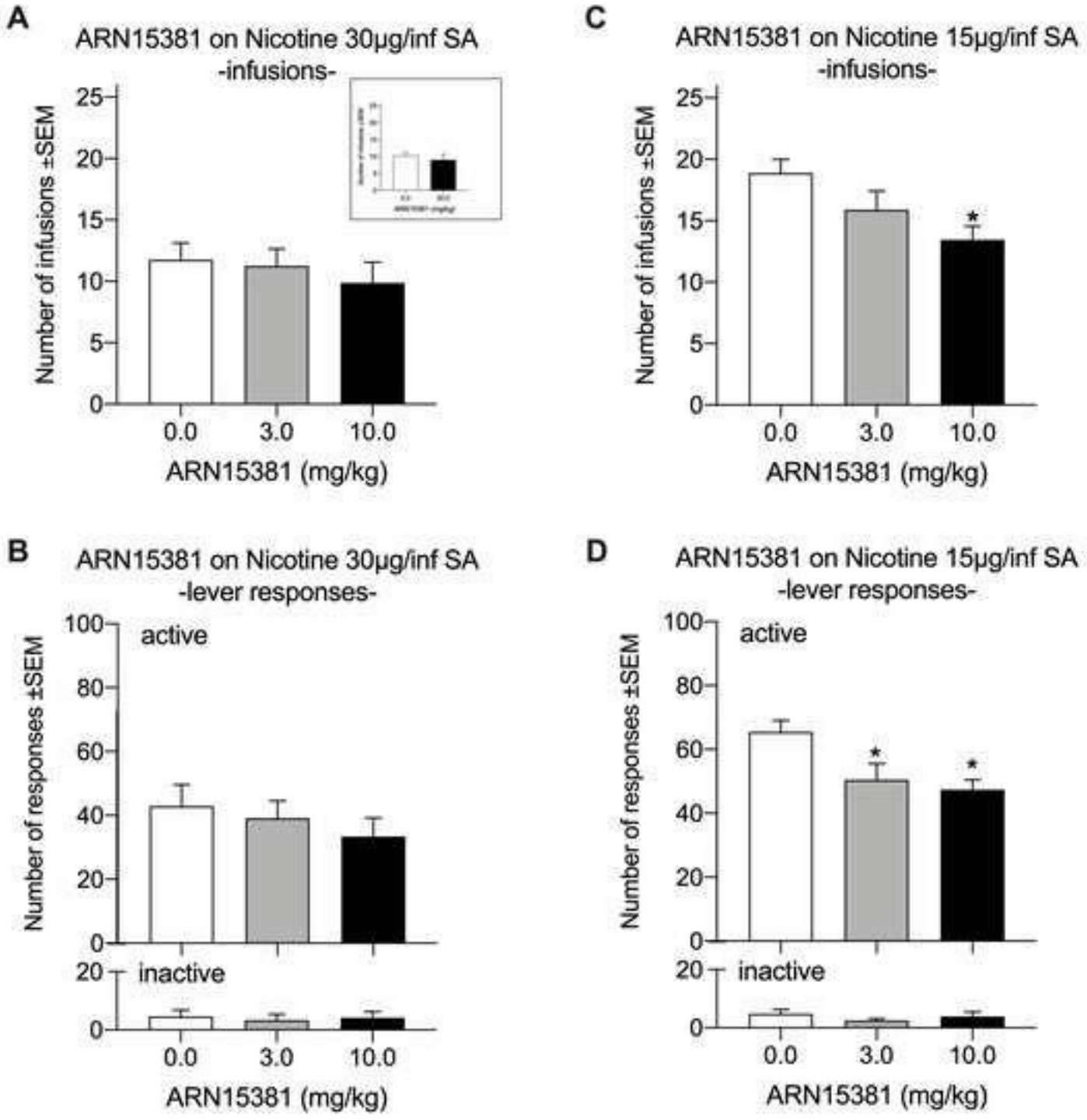


Figure 5

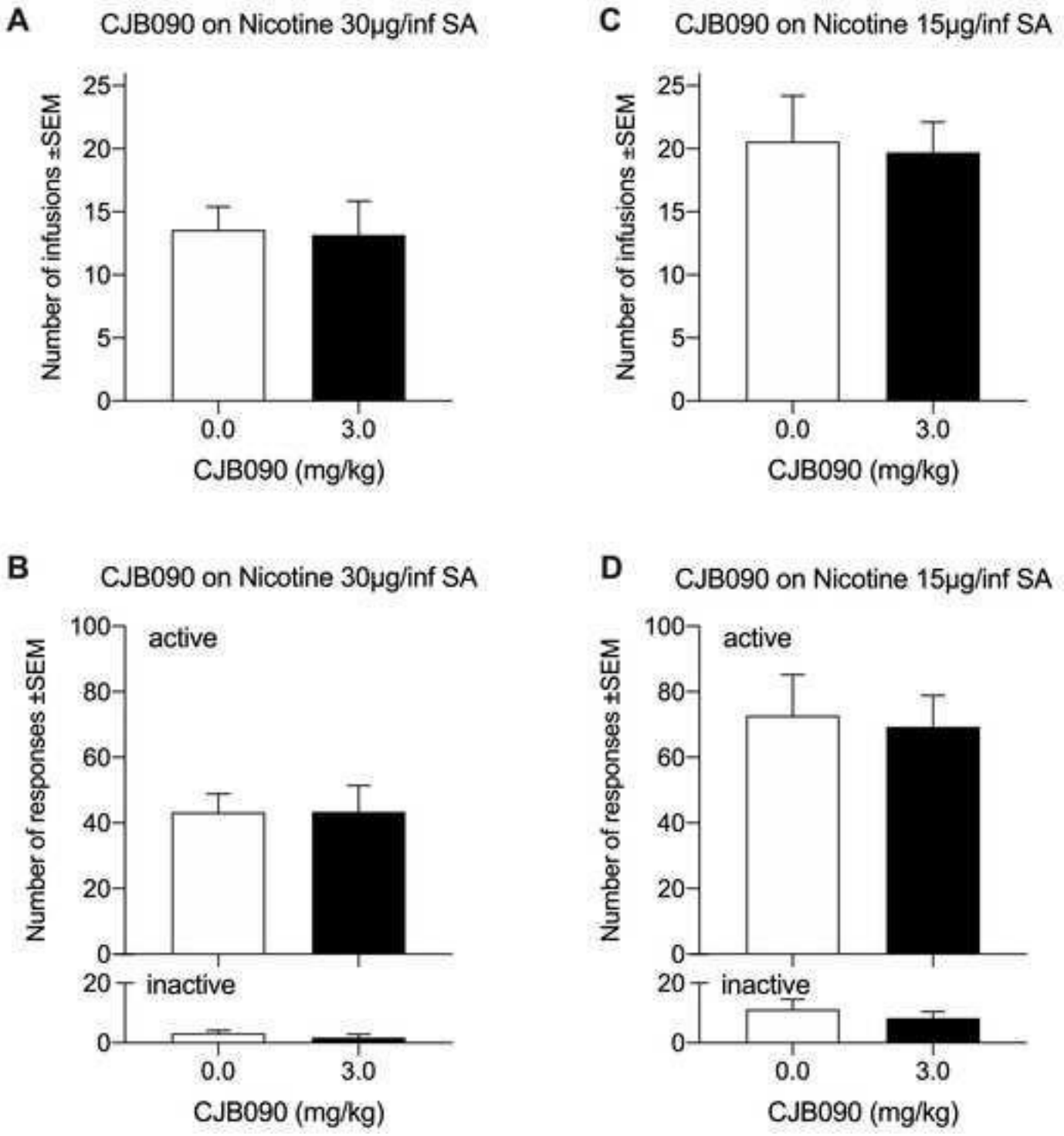


Figure 6

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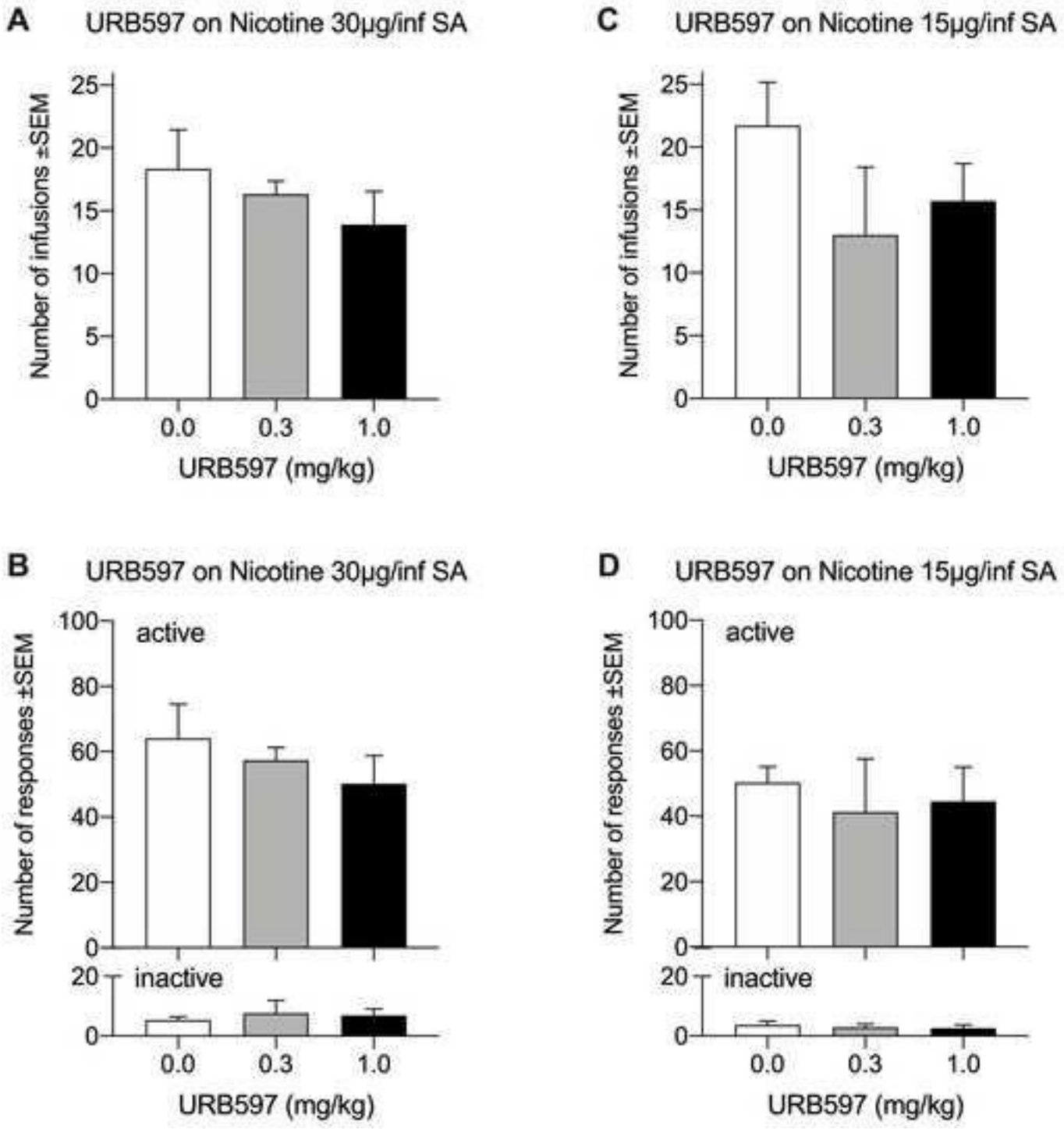

Novelty-induced locomotor behavior predicts heroin addiction vulnerability in male, but not female, rats

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Abstract (249/250)

Rationale: The ongoing rise in opioid use disorder (OUD) has made it imperative to better model the individual variation within the human population that contributes to OUD vulnerability. Using animal models that capture such variation can be a useful tool. Individual variation in novelty-induced locomotion is predictive of substance use disorder (SUD) propensity. In this model, rats are characterized as high-responders (HR) or low-responders (LR) using a median split based on distance travelled during a locomotor test, and HR rats are generally found to exhibit a more SUD vulnerable behavioral phenotype.

Objectives: The HR/LR model has commonly been used to assess behaviors in male rats using psychostimulants, with limited knowledge of the predictive efficacy of this model in females or the use of an opioid as the reward. In the current study, we assessed several behaviors across the different phases of drug addiction (heroin taking, refraining and seeking) in over 500 male and female heterogeneous stock rats run at two geographically separate locations. Rats were characterized as HRs or LRs within each sex for analysis.

Results: Overall, females exhibit a more OUD vulnerable phenotype relative to males. Additionally, the HR/LR model was predictive of OUD-like behaviors in male, but not female rats. Furthermore, phenotypes did not differ in anxiety-related behaviors, reacquisition of heroin-taking or punished heroin-taking behavior in either sex.

Conclusions: These results emphasize the importance of assessing females in models of individual variation in SUD and highlight limitations in using the HR/LR model to assess OUD propensity.

Introduction

The prevalence of opioid use disorder (OUD) has increased in the past two decades, with an over six-fold increase in opioid overdose deaths [1]. The rise in OUD makes it imperative to gain a better understanding of the behavioral characteristics underlying opioid use vulnerability. A key barrier in assessing addiction liability is the substantial amount of individual variation within the human population that contributes to addiction vulnerability. Using animal models that inherently account for such variation in addiction-related behaviors is one approach that may improve capturing variability in human drug addiction, leading to more efficacious treatment options.

Separating rats into high-responder (HR) and low-responder (LR) subgroups based on cumulative locomotor movements in a novel inescapable environment has been widely used to account for individual variation in addiction-related behaviors [2]. In this model, HRs more rapidly learn to self-administer nicotine [3], amphetamine [2, 4-8], methamphetamine [9], and cocaine [10-12] relative to LRs. Additionally, HRs exhibit greater locomotor sensitization to repeated amphetamine [13] and nicotine injections [14], and greater motivation to take cocaine compared to LRs using a behavioral economics approach [15]. Augmenting the potential translational value of the HR/LR model, novelty-induced locomotor behavior has been associated with increased vulnerability to addiction across several classes of drugs in humans [16].

There are several applications of the HR/LR model that have yet to be fully explored. For example, previous studies have assessed differences between HR and LR rats using psychostimulants with only a few studies focused on opioids [17-20]. Also, with the exception of two studies, all work using this model have examined male rats only [21, 22]. Moreover, only a few studies have assessed whether HR/LR distinctions are reflected in other behaviors such as cue- or context-induced drug seeking, or compulsive drug taking in the presence of adverse consequences [20, 23-25].

To further substantiate the use of the HR/LR model in capturing individual variation in addiction-related behaviors, we assessed the predictive validity of the model for OUD propensity. To best capture the genetic and phenotypic variability observed in humans, 507 male and female heterogeneous stock rat littermates shipped to two distinct laboratories were assessed for multiple behaviors that contribute to OUD liability; including heroin use, rewarded and unrewarded motivation to seek heroin, and learning to refrain from heroin seeking. Analgesic threshold and anxiety-like behavior were also assessed prior to heroin experience. Data were first assessed for behavioral differences between sexes, to which we found female rats exhibited a more vulnerable OUD behavioral phenotype compared to males. Next, data were analyzed within the scope of the HR/LR mode. In parallel with studies using psychostimulants, we hypothesized HR male and female rats would exhibit a more vulnerable OUD phenotype relative to LR rats. However, we found the HR/LR model successfully predicted OUD vulnerability in male rats, but had no predictability in females, emphasizing the necessity to account for sex differences in models of individual variation in addiction-related behaviors. HR and LR rats did not differ in reacquisition of heroin taking or punished heroin taking-behavior, cardinal features of OUD, regardless of sex, suggesting limits regarding the applicability of the HR/LR model in predicting OUD propensity.

Methods

All experimental procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee, and by the Italian Ministry of Health. Procedures abided by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of Laboratory Animals. The experimental timeline is shown in Figure 1 with greater detail on each procedure in the following sections.

Subjects

A total of 680 heterogeneous stock (N/NIH-HS) rats bred at Wake Forest University were used in these studies. Animals were shipped to either the Medical University of South Carolina (MUSC; USA) or the University of Camerino (UCAM; Italy) in batches of 40 (20 males and 20 females per site) at approximately 5 weeks of age. Upon arrival, animals were pair-housed and left undisturbed in a climate-controlled vivarium with a standard 12-hr light:dark cycle for 3 weeks prior to testing. Animals had ad libitum access to food and water over the course of training. All behavioral testing occurred during the dark cycle, between the hours of 18:00 and 6:00 h. To minimize site differences in behavioral output, all experimental procedures were standardized across the two sites. Of the 680 rats entering the study, a total of 100 rats were excluded from analyses due to death (surgery, n=21; illness, n=79), 14 rats were excluded due to technical issues regarding data collection, and an additional 59 were excluded as they underwent saline and not heroin self-administration training. Final analyses consisted of 507 rats (male, n=264; females, n=243).

Drugs

Heroin hydrochloride supplied by the National Institute on Drug Abuse (Bethesda, MD) dissolved in 0.9% sterile saline was used in these studies.

Locomotor test

Following the acclimation period, rats underwent a 60-min locomotor test in a novel inescapable environment (i.e., open field test). Testing chambers were composed of clear Plexiglas within a metal frame (Omnitech Electronics, Columbus, OH; 16” L x 16” W x 12” H) with photocell beams that captured both lateral and vertical movements. All activity was recorded and analyzed using Versamax (Omnitech Electronics, Columbus, OH; version 1.80-0142). Ten animals, counterbalanced by sex, were run per day Monday-Thursday. At the conclusion of testing, animals were returned to their home cage in the vivarium.

Elevated-plus maze

Approximately 1 hour following the completion of the OFT, rats underwent a 5-min elevated plus maze (EPM) test to assess anxiety-related behaviors. Testing apparatus were composed of black plexiglass (San Diego Instruments) and comprised of four arms (43.5" long and 4" wide) with two having enclosed walls along the arm (12" high walls; "closed" arms) and two without walls ("open" arms). The maze was elevated approximately 19.5" off the ground. The maze flooring was interchangeable based on rat color to optimize detection of each animal for analysis. ANY-maze behavioral tracking software (Stoelting, Wood Dale, IL; version 6.17) was used for automatic detection and quantification of the animal movement throughout the maze. To be considered in an arm, a minimum of 85% of the rat's body had to be within it.

Tail flick test

A minimum of 1 hour after the EPM test, analgesic threshold for each rat was assessed using a tail flick (TF) test. The TF apparatus (Ugo Basile S.R.L., Gemonio, Italy) consisted of a flat platform with a mounted sensor that is irradiated by an infrared light beam below the platform to heat the rat's tail. The light beam automatically turned off once the animal moved its tail, or after 10 seconds have passed, and the reaction time was indicated on the display screen. Fifteen minutes prior to the baseline session rats received an injection of saline (1 mg/kg, sc). One hour after baseline testing, rats received an injection of heroin (0.75 mg/kg heroin, sc) to assess potential changes in analgesic threshold with heroin present, and were tested 15 minutes later. Testing consisted of 4 trials, with the location on the rat's tail being adjusted each subsequent trial by 1 cm to prevent issue damage. Data from all 4 trails were averaged to compute the overall latency to remove tail from the sensor (i.e., reaction time).

Heroin self-administration

Approximately 1 week after locomotor testing, rats underwent surgery for the implantation of an indwelling jugular catheter. Isoflurane anesthesia was used, and an analgesic (Ketorolac, 2 mg/kg, sc; or

Meloxicam, 0.5 mg/rat, sc) and an antibiotic (Cefazolin, 0.2 mg/kg, sc; or enrofloxacin, 1 mg/kg, iv) were administered post-operatively. Animals were given a minimum of three days of recovery prior to testing. All training occurred in standard behavioral testing chambers (Med Associated, St. Albans, VT). Chamber were outfitted with a house light and speaker on one wall, and two levers with lights above them on the opposite wall. During a session, presses on the active lever using a fixed-ratio 1 schedule of reinforcement resulted in presentation of a light and tone cues for 5-seconds and an infusion of heroin (20 µg/kg/100 µl infusion over 3 seconds). The house light turned off at the start of the infusion for 20-seconds to signal a timeout period whereupon additional presses on the active lever were recorded but without consequence. Throughout testing, presses on the inactive lever were recorded but without consequence. Sessions lasted 12 hr or until 300 infusions were earned. Training occurred Monday-Friday, with one session off per week resulting in a total of four sessions/week. After 12 sessions were complete, rats underwent a progressive ratio test to assess motivation to continue taking heroin as the effort for an infusion increased. During this test, the number of active lever presses needed to receive an infusion of heroin exponentially increased after each infusion according to the following formula: $(5 \times e^{0.2n})-5$ [26]. Sessions terminated after 12 hr or 1 hr of no earned infusions. Animals then underwent three more days of heroin self-administration training to re-establish heroin-taking behavior prior to additional testing.

Extinction training and reinstatement tests

Following heroin self-administration training, rats underwent a 6 hr within-session extinction-prime test. Rats were under extinction training conditions for the first 4 hr of testing whereupon presses on both the active and inactive lever were recorded but without consequence (i.e., no cue presentations or heroin infusion). Rats received a 0.25 mg/kg (sc) injection of heroin [27-29] with two hours left in the session and continued testing under extinction conditions (e.g., heroin-prime reinstatement). At the conclusion of this test, rats underwent daily 2 hr extinction training sessions for 6 consecutive days preceding a test for cue-

induced reinstatement. During the 2 hr cue-induced reinstatement test, active lever presses resulted in cue presentation and pump activation, but no heroin infusion.

Punishment training

Following training and approximately 3 weeks after heroin experience, a subset of rats (males, n=15; females, n=14) underwent three additional days of heroin self-administration training to re-establish taking behavior. Chambers were then outfitted with a shock floor grid, and on the next day of training there was a 50% probability of foot shock delivery (0.40 mA) with each infusion.

Data analysis and statistics

Once testing was complete, several behavioral measures were selected for analysis in order to best capture the different phases of drug addiction: heroin-taking, refraining and seeking behaviors. Heroin-taking behavior was comprised of the following: total heroin consumption (µg/kg) across the first 12 training sessions; escalation of heroin intake (µg/kg; average consumption days 1-3 subtracted from average days 10-12); break point achieved during the progressive ratio test. The break point was the number of active lever presses an animal was willing to expend in order to receive an infusion of heroin. Refraining, or withholding from seeking, behavior included active lever presses made during the first 2 hr of the within-session extinction-prime test (extinction burst) and the last day of extinction training (extinction day 6). Seeking behaviors included active lever presses made during the heroin-prime and cue-induced reinstatement tests.

Raw data were first analyzed for sex differences using a non-parametric Mann-Whitney test. Next, data were analyzed using a 2-way ANOVA, with site (MUSC vs UCAM) and sex (male vs female) as independent variables. Results showed several site and sex differences (see Table 1). Accordingly, all data were standardized using z-score transformation within site and sex and males and females were analyzed as independent groups. A 2-way repeated-measures ANOVA with sex (male vs female) and session (baseline

vs test) was used to assess behavioral differences between sessions during the tail flick test. Differences between a high-responder and low-responder behavioral phenotypes within each behavior, or session for tail flick test, was evaluated using either a t-test (normally distributed) or Mann Whitney U test (non-normally distributed). Phenotype composition between males and females when data were combined were assessed via a Chi-squared test. Behavior during heroin reacquisition was analyzed using a repeated-measures ANOVA, and punishment training was assessed using a Mann-Whitey U test. Analyses were performed using GraphPad Prism version 9.2.0 (San Diego, CA). Statistical significance was set to $p<0.05$.

Results

Raw data

Behavioral differences between females and males for selected behaviors was first analyzed using the raw data. Females exhibited less anxiety-like behavioral relative to males in both the OFT (Mann-Whitney U= 20077, $p<0.0001$; Fig. 2a) and the EPM (Mann-Whitney U= 20978, $p<0.0001$; Fig. 2b). Sexes did not differ in analgesic threshold under baseline conditions (Mann-Whitney U= 31625, $p=0.78$; Fig. 2c). However, following administration of heroin, males showed a greater heroin-induced analgesic threshold relative to females (Mann-Whitney U= 23978, $p<0.0001$; Fig. 2d), suggesting differences in how an opioid affects pain processing in males and female rats.

Several sex differences existed for both heroin reinforced and non-reinforced behaviors across measure of heroin taking, refraining and seeking. Compared to males, females showed augmented levels of heroin consumption (Mann-Whitney U= 19069, $p<0.0001$; Fig. 2f), motivation to work for an infusion of heroin (Mann-Whitney U= 25907, $p=0.0002$; Fig. 2g), refraining behavior both at the start (extinction burst; Mann-Whitney U= 26498, $p=0.0007$; Fig. 2h) of extinction training and at the end (extinction day 6; Mann-Whitney U= 26328, $p=0.0005$; Fig. 2j), as well as heroin-seeking behavior during the heroin-induced (Mann-Whitney U= 22939, $p<0.0001$; Fig. 2i) and cue-induced (Mann-Whitney U= 23988, $p<0.0001$; Fig. 2k) reinstatement tests. Females and males did not differ in the escalation of heroin intake across training (Mann-Whitney U= 29858, $p=0.18$; Fig. 2e), suggesting females start at and maintain a higher level of heroin consumption throughout training, but that escalation patterns between the two sexes are similar. These data suggest that females exhibit a more vulnerable OUD behavioral phenotype.

Locomotor test

Rats were designated as either high-responders (HR) or low-responders (LR) using a median split based on total distance travelled during the OFT creating two non-overlapping subpopulations of equal size.

When sexes were combined for analysis, female rats predominated the HR group while males were more represented in the LR phenotype ($x^2(1, 507)= 13.54$, p= 0.0002; Fig. 3a), suggesting female rats were more prone to higher levels of novelty-induced locomotor behavior. This finding, along with the substantial sex differences within the raw data reported above, resulted in sexes being analyzed separately and median splits for HR/LR characterization within sex (males: Mann-Whitney U= 0, p<0.0001, Fig. 3b; females: Mann-Whitney U= 0, p<0.0001, Fig. 3c).

Elevated-plus maze

Possible differences in anxiety-like behavior prior to heroin experience was assessed using the EPM test. Time spent in the open arms, an indicator of a less anxious phenotype, did not differ between HR or LR rats in either males (Mann-Whitney U= 8046, p=0.33, Fig. 4a) or females (Mann-Whitney U= 7146, p=0.67, Fig. 4b).

Tail flick test

Phenotypic differences in analgesic threshold was established using the TF test. As expected, all rats showed a greater latency to remove their tail from the noxious stimuli during the test session relative to the baseline session (sexes combined: $F_{1,503}= 1544.50$, p<0.0001; males: $F_{1,262}= 1006.92$, p<0.0001; females: $F_{1,241}= 574.18$, p<0.0001) with no phenotypic differences present (sexes combined: $F_{1,503}= 0.14$, p= 0.71; males: $F_{1,262}= 0.16$, p= 0.69; females: $F_{1,241}= 0.73$, p= 0.39). However, when analyzing sexes together, an interaction between sex and session was present ($F_{1,503}= 27.63$, p<0.0001), with males showing a potentiated heroin-induced analgesic threshold relative to females (p<0.0001).

Data within each session were then assessed. Male HR and LR rats did not differ in latency to remove their tail from the noxious stimuli under baseline (i.e. saline injection; Mann-Whitney U= 8132, p=0.35, Fig. 4c) or testing (i.e. heroin injection; Mann-Whitney U= 8515, p=0.75, Fig. 4e) conditions. In contrast, female HR and LR rats differed under baseline conditions, with HR rats exhibiting a greater analgesic threshold

compared to LR rats (t(241)= 2.14, p= 0.03; Fig. 4d). However, phenotypic differences were no longer present following an injection of heroin (Mann-Whitney U= 6609, p=0.16, Fig. 4f).

Capacity of HR/LR model in predicting heroin addiction-related behaviors in male and female rats

Akin to previous studies with psychostimulants [2, 4-8, 10-12], male HRs showed greater total heroin consumption across training (Mann-Whitney U= 7079, p= 0.01, Fig. 5a). Additionally, relative to male LRs, HRs exhibited greater motivation in heroin rewarded drug seeking in a progressive ratio task (Mann-Whitney U= 6764, p= 0.002, Fig. 5b) and greater cue-induced heroin seeking compared to LRs (Mann-Whitney U= 7403, p= 0.03, Fig. 5c). However, the HR/LR phenotype did not predict differences in escalation of heroin intake (Mann-Whitney U= 8291, p= 0.50, Fig. 5d), extinction burst (Mann-Whitney U= 7642, p= 0.08, Fig. 5e), extinction day 6 (Mann-Whitney U= 8059, p= 0.29, Fig. 5f), or heroin-prime reinstatement (Mann-Whitney U= 8690, p= 0.97, Fig. 5g). Together, these data suggest the HR/LR model successfully predicts some behaviors associated with OUD in male rats.

In females, HRs and LRs did not differ in any heroin reinforced behaviors (consumption: t(241)= 0.82, p= 0.42, Fig. 6a; break point: Mann-Whitney U= 7116, p= 0.63, Fig 6b; escalation: Mann-Whitney U= 6966, p= 0.045, Fig. 6d), extinction-related behaviors (extinction burst: Mann-Whitney U= 6477, p= 0.07, Fig. 6e; extinction day 6: Mann-Whitney U= 7125, p= 0.64, Fig. 6f) or reinstated heroin-seeking behaviors (prime reinstatement: Mann-Whitney U= 7289, p= 0.87, Fig. 6g; cued reinstatement: Mann-Whitney U= 7359, p= 0.97, Fig. 6c). These data show that in contrast to males, the novelty-induced locomotor trait is not predictive of OUD-associated behaviors in females.

Heroin reacquisition and punished heroin-taking behavior

HR and LR rats exhibited potentiated heroin taking on day 1 or reacquisition, and then decreased consumption over training (Sexes combined: $F_{1.53,42.78}$=27.39, p=0<0.0001, Fig. 7a; Males: $F_{1.40,19.62}$=21.17, p<0.0001, Fig. 7b; Females: $F_{1.43,17.20}$=9.67, p=0.003, Fig. 7c). However, phenotypes did not differ in

reacquisition of heroin self-administration training following an abstinence period when sexes were analyzed together ($F_{1,28}$=1.09, p=0.31; Fig. 7a), or separately (Males: $F_{1,14}$=1.48, p=0.24, Fig. 7b; Females: $F_{1,12}$=0.67, p=0.43, Fig. 7c). During punishment training, HR and LR rats equally consumed heroin when analyzed together (Mann-Whitney U= 100, p= 0.67; Fig. 7d) or separately (Males: Mann-Whitney U= 16.50, p=0.11, Fig. 7e; Females: Mann-Whitney U= 20.50, p=0.82, Fig. 7f). When data were analyzed by the number of infusions per hour, analyses showed no phenotypic effects (Sexes combined: $F_{1,28}$=0.05, p=0.81; Males: $F_{1,14}$=4.01, p=0.06; Females: $F_{1,12}$=1.05, p=0.32). This analysis suggests time course of consumption also does not differ between HR and LR rats. Though there was a significant phenotype by hour interaction for males ($F_{11,154}$=2.13, p=0.02), no significant post-hoc analyses were present. These data suggest that the HR/LR model does not capture differences in the reacquisition of heroin taking or continued heroin taking in the presence of an adverse stimuli, important features of substance use disorder (SUD).

Discussion

In an attempt to account for the extensive individual variation in addiction-related behaviors in humans, we employed the HR/LR model to assess how novelty-induced locomotor behavior predicted OUD vulnerability in male and female rats. Heterogeneous stock (HS) rats were used for these studies as they exhibit considerably more behavioral and genetic variation than commonly used laboratory inbred lines [30-32], resulting in diversity more akin to the human population. HS rats have been used to model such variation in several disorders, including SUD-related behaviors [33-37]. Over 500 HS rat littermates underwent behavioral testing at two distinct locations (MUSC, Charleston, South Carolina, USA and UCAM, Camerino, Italy) in an effort to account for potential environmentally imposed epigenetic changes that may occur due to testing site. We assessed how multiple OUD behaviors across the phases of SUD (i.e., heroin taking, refraining and seeking) differed between male and female rats, and found female rats exhibit a greater OUD vulnerable phenotype relative to males. Next, rats were characterized as high or

low locomotor responding in a novel open field test, a trait associated with SUD vulnerability [2, 38]. We showed that the HR/LR model successfully predicted OUD vulnerability only in male rats, with no predictability in female rats. Furthermore, phenotypes did not differ in heroin reacquisition or punished heroin-taking behavior, highlighting the theoretical limitations of this model for assessing OUD propensity.

Sex differences in anxiety, analgesic threshold and heroin OUD behaviors

Anxiety-related behaviors have been shown to differ between male and female rodents, and our results align with previous work showing female rats spend more time in the open arm of the EPM [39, 40] and exhibit greater locomotion during an OFT [40]. These data infer females have less anxiety-like behaviors compared to males as measured using the EPT and OFT. In contrast, males appear to be more sensitive to the analgesic effects of heroin, as latency to remove the tail from a noxious stimulus was higher in males than females. Clinically, opioids have been shown to be less efficacious in females than males [41-44], with females requiring higher doses to attain the same biological outcome [41]. This effect is mirrored in rodent models, with opioids producing a greater analgesic effect in male rats compared to female rats, and sexual dimorphisms in the engagement of pain processing pathways being implicated for this difference [for review see 45]. Our results further support these findings and given the genetic and behavioral heterogeneity inherently captured in the HS rat line, future studies assessing sexual dimorphism in pain processing would benefit from using the HS rat.

Compared to males, we showed females exhibited a more vulnerable OUD phenotype for heroin taking, refraining and seeking behaviors. In humans, females stabilize at a higher drug dose and relapse more often than males across several classes of drugs, including opioids [46], and reach criteria for an OUD diagnosis at a faster rate [47]. Work using rodent models have found that female rats consume more opioids [48-52] and do so at a faster rate [48, 49], and show more seeking behavior relative to males [52-54]. Our results support these findings, and also show that female rats are less able to refrain from non-

reinforced drug seeking, and are more motivated to work for an infusion of heroin than males are. One explanation for these sex differences may be fluctuations in hormones as a result of different estrous cycle phases. Behaviors associated with drug use in humans are impacted by estrous cycle phase [46], and delivery of estradiol can potentiate opioid and cocaine self-administration in ovariectomized female rats [46]. However, similar to female rats, male rats exhibit large variability in observed behaviors so while hormonal fluctuations during estrous cycle may contribute to differences within females, animals are behaving similarly between each sex.

Elevated plus maze and tail flick

HR and LR rats differ in several behavioral and endocrine measures associated with anxiety [for review see 55], as well as differential engagement of the hypothalamic-pituitary-adrenal axis [6, 56]. Work using outbred male Sprague-Dawley rats showed HR rats spend more time in the open arm of the EPM relative to LR rats, suggesting they exhibit a less anxious phenotype [57]. Here we show that male and female HS rats do not differ in anxiety-related measures as assessed by the EPM, suggesting that in a rat line capturing more genetic and behavioral variability, the HR/LR model is not efficacious for assessing the relationship between anxiety and addiction-related behaviors. We also evaluated analgesic threshold prior to heroin experience, and found no phenotypic difference in males. However, in females HR rats were more resistant to a painful stimulus relative to LR rats. Behavior during this test did not predict any subsequent OUD-related behaviors, implying this phenotypic finding is not relevant to OUD, but rather may be a pertinent model for studying individual variation in the neurobiology of pain in a rodent model.

HR/LR behavioral phenotype in male rats

Novelty-induced locomotor behavior has been shown to correlate with morphine self-administration in male rats [17]. Similarly, and in alignment with previous findings using psychostimulants [2, 4-8, 10-12, 15], HRs consumed more heroin than LRs during heroin self-administration. Interestingly, the two phenotypes did not differ in escalation of intake, suggesting both groups were increasing at similar rates,

but that HRs started at and maintained a higher rate of intake. This behavior has been observed in male HR rats during cocaine self-administration, with HRs consuming more drug when cost is low compared to LR rats [15]. Relative to LRs, HRs also showed greater motivation to work for an infusion of heroin following self-administration training, implying these phenotypes differ not only in the acquisition of heroin-taking behavior, but also in more complex motivational behaviors like rewarded drug seeking.

We also show male HR rats exhibited greater cue-induced reinstatement of heroin-seeking behavior compared to LRs, a trait that has not been observed in outbred rats for any drug [20, 24, 58, 59]. However, the two phenotypes did not differ in heroin-prime reinstatement, suggesting HRs and LRs differ in discrete cue-reward motivated behaviors, but not in contextual or interoceptive cue motivated behaviors. One explanation is that HRs show greater cue-induced reinstatement of heroin-seeking behavior because they consume more heroin than LRs during self-administration, thus receiving more cue-reward pairings thereby producing a stronger association between the action, cue and reward. It is then possible that for heroin, but not for stimulants, the extent to which an individual acquires drug taking behaviors has long term effects on overall addiction liability in males, including relapse propensity, in the HR/LR model. An alternative explanation for the phenotypic difference in cue-induced reinstatement is variation in the motivational properties attributed to the reward-paired cues. However, the relationship between novelty-induced locomotor behavior and incentive salience attribution [assigning intense motivational value to reward-paired cues; for review see 60] has not been observed consistently in outbred rat lines for cocaine [33, 59, 61] or an opioid [20]. Lastly, our findings may not align with work using psychostimulants due to neurobiological differences imposed by drug choice [62-64]. Regardless, to further understand the phenotypic differences present during cue-induced reinstatement, future studies can include cue removal tests or devaluation procedures to assess the motivational value of the heroin-paired cues or standardize the number of infusions earned during daily self-administration sessions to clarify if differences in consumption affects cue-induced reinstatement behavior.

Contrary to our findings, recent work showed no HR/LR phenotypic differences in male rats during self-administration of remifentanil, a short acting opioid [20], or subsequent cued reinstatement. Differences are likely due to choice of opioid used, as remifentanil is quickly removed from circulation [half-life of 0.3-0.7 min, 65], whereas heroin remains in the bloodstream for substantially longer [half-life of 7.6 min, 66], with its active metabolites persisting even longer [morphine: half-life of 2-3 hr, 6-acetylmorphine: half-life of 22 min; 67]. It is plausible the duration of the interoceptive effects of the opioid affect HR/LR phenotypic differences in male rats, supporting the necessity to account for drug pharmacokinetics when assessing individual variation in OUD propensity. Alternatively, discrepancy in these findings may be due to the many methodological differences between the current study and that by Chang and colleagues [20]. For example, we employed long-access training sessions (12 hr versus <3 hr) and frequent brief abstinence periods during training, both of which likely impact the neurobiological mechanisms underlying drug taking and seeking behaviors [68-70].

HR/LR behavioral phenotype in female rats

While the HR/LR model had predictive validity for heroin addiction vulnerability in males, it did not for females. Though novelty-induced locomotion is not a predictive trait of OUD vulnerability in females, additional models of individual variation in SUD propensity should be employed to further understand sexual dimorphism of SUD predictive traits.

Punished heroin-taking behavior

Following several weeks of forced abstinence from heroin, HR and LR male and female rats did not differ in the reacquisition of heroin-taking behavior. These data suggest phenotypic differences, at least for male rats, in heroin self-administration is only present in the acquisition, and not long-term maintenance and compulsive taking of heroin. Phenotypes also did not differ in punished heroin-taking behavior for either sex. Compulsive drug taking in the presence of an adverse stimuli is an important feature of human SUD

[24, 71, 72], and the lack of phenotypic differences in this assay expose limitations of the HR/LR phenotypes in modeling human OUD.

Conclusion

These results emphasize the advantages of accounting for both sex differences and individual variation in addiction-related behaviors when assessing heroin addiction vulnerability. We showed that relative to male rats, females show less anxiety-like behavior, lower levels of heroin-induced analgesia, and a more vulnerable OUD phenotype across several heroin taking, refraining and seeking measures. Next, we demonstrated that novelty-induced locomotion, a trait associated with human SUD vulnerability, is predictive of heroin addiction vulnerability in male, but not female, rats. These results highlight the necessity to assess sex differences in addiction-related behaviors and address the limitations associated with the HR/LR model when predicting OUD vulnerability in a heterogeneous population of rats.

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