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Translating human drug use patterns into rat models: exploring interindividual differences via refined drug self-administration procedures

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Abstract

Drug users instrumentalize the drug dosing-timing relationship (i.e. preferred drug dose in the preferred time) to produce their desired effects (i.e. euphoria, withdrawal avoidance, etc.). This is achieved by harnessing drug type, dosage, route, and frequency of drug taking. Yet, preclinical addiction research often employs self-administration and choice procedures based on discrete, as opposed to continuous dimension strategies, characterized by pre-selected experimenter-imposed unit-doses spaced by timeouts. This approach imposes constraints on the dose-time relationship voluntary harnessed by individuals with drug-addiction in real-world.

This dissertation is devoted to the refinement of animal models of drug addiction. The considerations for the refinements stem from a detailed analysis of naturalistic patterns of drug taking in humans and are based on a strict pharmacokinetics and pharmacodynamics analysis of the drugs being investigated. The ambition is to guide preclinical researchers toward the self-administration procedure for neuropharmacological studies, tailored to the specific drug being investigated and is largely motivated by the limited advancements in available treatment options stemming from preclinical insights.

Keywords: addiction; pharmacokinetics; choice; patterns of drug taking; drug-seeking; social withdrawal; heroin; cocaine

Scientific contribution

Publications.

The present dissertation is based on the following publications:

1) Venniro, M., Reverte, I., Ramsey, L. A., Papastrat, K. M., **D'Ottavio, G.**, Milella, M. S., Li, X., Grimm, J. W., & Caprioli, D. (2021). Factors modulating the incubation of drug and non-drug craving and their clinical implications. Neuroscience and Biobehavioral Reviews, 131, 847–864. https://doi.org/10.1016/j.neubiorev.2021.09.050

2) Vincenzi M., Milella M.S., **D'Ottavio G.**, Caprioli D., Reverte I., Maftei D. (2022) Targeting Chemokines and Chemokine GPCRs to Enhance Strong Opioid Efficacy in Neuropathic Pain. Life, 12(3):398. https://doi.org/10.3390/life12030398

3) **D'Ottavio, G.**, Reverte, I., Ragozzino, D., Meringolo, M., Milella, M. S., Boix, F., Venniro, M., Badiani, A., & Caprioli, D. (2022). Increased heroin intake and relapse vulnerability in intermittent relative to continuous self-administration: Sex differences in rats. British Journal of Pharmacology. https://doi.org/10.1111/bph.15791

4) Milella M.S., **D'Ottavio G.**, De Pirro S., Barra M., Caprioli D., Badiani A. (2023) Heroin and its metabolites: relevance to heroin use disorder. Translational Psychiatry 13, 120 https://doi.org/10.1038/s41398-023-02406-5

Additional publications, which are not included in the experimental part of the present dissertation are listed below.

1) Reverte I., Marchetti C., Pezza S., Scaringi G., Ferrucci L., **D'Ottavio G.**, Pignataro A., Andolina D., Raspa M., Scavizzi F., Venniro M., Ramsey L. A., Gross C., Caprioli D., Ragozzino D. (2023) Microglia-mediated calcium-permeable AMPAR accumulation in the nucleus accumbens drives hyperlocomotion during cocaine withdrawal. Brain, Behavior, and Immunity. https://doi.org/10.1016/j.bbi.2023.11.007

2) Reverte, I., **D'Ottavio, G.**, Milella, M.S., & Caprioli, D. (2022). Sex differences in the immune system: implications for cocaine relapse. Brain, Behavior, and Immunity, S0889-1591(22)00133-7. https://doi.org/10.1016/j.bbi.2022.05.009

Abstracts.

1) **D'Ottavio G.**, Pezza S., Reverte I., Marchetti C., Zenoni S.F., Maftei D., Fabrizio F., Termine A., Milella M.S., Ragozzino D., Venniro M., Badiani A., Boix F., Caprioli D. The impact of discrete versus continuous dimensions strategies in heroin and cocaine self-administration on patterns of drug taking and social interaction. Poster presented at Neuroscience2023; Washington DC; November 2023

2) **D'Ottavio G.**, Pezza S., Reverte I., Marchetti C., Zenoni S.F., Maftei D., Fabrizio F., Termine A., Milella M.S., Ragozzino D., Venniro M., Badiani A., Boix F., Caprioli D. Evidence for heroin-induced social isolation in the rat. Poster presented at S4SN 2023, Virtual meeting; November 2023

3) Zenoni S.F., **D'Ottavio G.**, Marchetti C., Pezza S., Nicoletti F., Llebaria A., Notartomaso S., Shaham Y., Venniro M., Badiani A., Caprioli D. The role of Dorsal Raphe mGluR5 containing neurons on incubation of methamphetamine craving. Poster presented at EBPS Biennial Meeting 2023; Mannheim, DE; Aug 2023

4) **D'Ottavio G.**, Micheli C., Marchetti C., Zenoni S.F., Reverte I., Fabrizio C., Termine A., Milella M.S., Boix F., Venniro M., Caprioli D. Evidence for heroin-induced social isolation in the rat. Poster presented at INRC 2022; Valencia, ES; July 2022

5) Marchetti C., Reverte I., L.M. Rossi, **D'Ottavio G.**, Modoni J., Berretta N., Lo Iacono L., Miele R., Venniro M., Badiani A., Caprioli D., Effect of social choice-induced voluntary abstinence on incubation of methamphetamine craving and related expression of AMPA receptor in nucleus accumbens core. Poster presented at EBPS Biennal Workshop 'Spanning the spectrum of social behavior: towards more translationally relevant animal models', Rome, IT; June 2022

6) **D'Ottavio G.**, Reverte I., Ragozzino D., Meringolo M., Milella M.S., Boix F., Venniro M., Badiani A., Caprioli D. Intermittent access heroin self-administration increases drug intake in both sexes and relapse vulnerability in female rats. Poster presented at EBBS 2021; Lausanne, CH; September 2021

7) **D'Ottavio G.**, Reverte I., Ragozzino D., Meringolo M., Milella M.S., Boix F., Venniro M., Badiani A., Caprioli D. Intermittent access heroin self-administration increases drug intake in both sexes and relapse vulnerability in female rats. Poster presented at EBPS Biennial Meeting 2021; Maastricht, NL; July 2021

Oral communications.

1) **D'Ottavio G.** Heroin pharmacokinetics and their unexpected impact on heroin self-administration, relapse, and sociability. Special Behavioral Neuroscience Lecture at National Institute on Drug Abuse (NIDA); Baltimore, MD; November 2023

2) **D'Ottavio G.** The pharmacokinetic correlates of heroin-taking and their impact on sociability and relapse vulnerability at EBPS Biennial Meeting 2023; Mannheim, DE; Aug 2023

3) **D'Ottavio G.** Evidence for heroin-induced social isolation in the rat. Presented at EBPS Biennal Workshop 'Spanning the spectrum of social behavior: towards more translationally relevant animal models', Rome, IT, June 2022

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1) **D'Ottavio G.** Collaborator: Molecular and circuit mechanism underlying the protective effect of volitional social interaction on methamphetamine craving Fondi di Ateneo, Sapienza University of Rome Italy 2021-2022

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Awards.

1) **D'Ottavio G.** 2022 Selected speaker at the EBPS Biennial Workshop "Spanning the spectrum of social behavior: towards more translationally relevant animal models"

2) **D'Ottavio G.** 2022 "Vittorio Erspamer" Award (2000€), Sapienza, University of Rome

3) **D'Ottavio G.** 2022 British Pharmacological Society (BPS) Travel Award (312£), Valencia (ES)

4) **D'Ottavio G.** 2024 National Institute of Health (NIH) Center on Compulsive Behaviors (CCB) Fellowship (15000$)

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1. General introduction

In every culture, drugs are used to alter mental states (Siegel, 2005). Individuals display a remarkable ability to instrumentalize[1] their drug consumption, adopting patterns of drug taking to achieve specific subjective experiences (Heath, 2000; Müller & Schumann, 2011; Siegel, 2005; Zinberg, 1984). Nevertheless, in a subset of individuals – often considered "vulnerable" to developing drug addiction – instrumentalization of drug consumption can become excessive [i.e., "over-instrumentalization" (Müller & Schumann, 2011)], resulting in the emergence of severe and harmful patterns of drug taking. Ultimately, this can lead to the onset of drug addiction[2] (Anthony & Helzer, 2002; Koob, Kandel, Baler & Volkow, 2015).

Dole and colleagues (1966) proposed that addiction can be understood (and classified in terms of severity) via a systematic examination of the 'pharmacological state' of individuals using drugs. This referred to the drug experience sought, the dose-time relationship and patterns of drug taking adopted to maintain the drug state, the consequences of the drug taking regimen, and the withdrawal syndrome that might be experienced upon cessation of drug taking. As it will be later discussed (see **Chapter 2**), they proposed that restoring individuals' pharmacological state with medicine could have effectively mitigated drug use (Dole, Nyswander & Kreek, 1966). Notably, their in-depth behavioral and pharmacological analysis of opioid users led to the development of methadone maintenance therapy (Dole, Nyswander & Kreek, 1966; Kreek, 1992).

To understand the mechanisms underlying drug addiction, preclinical researchers have largely based their studies on Skinnerian assumptions, considering addictive drugs as positive reinforcers that,

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[1] In the original publication by Muller and Schuman, Müller CP, & Schumann G (2011). Drugs as instruments: a new framework for non-addictive psychoactive drug use. The Behavioral and brain sciences 34**:** 293-310. the term 'instrumentalization' was used to characterize the utilization of drugs as a functional adaptation to contemporary environments, dealing with particular situations, or merely altering their mental state. In this dissertation, however, this term is mainly used to denote the adaptable selection of dosage, administration route, and timing of drug administration with the goal of attaining the desired drug effects.

[2] In this dissertation, I mostly use the term 'addiction' rather than the more cumbersome 'severe substance use disorder' as defined in the DSM-5, APA APA (2013) *Diagnostic and statistical manual of mental disorders: DSM-5™, 5th ed*. American Psychiatric Publishing, Inc.: Arlington, VA, US..

regardless of their diverse pharmacological effects, activate a common biological mechanism associated with approach behaviors (Wise & Bozarth, 1987) and dopamine release (Di Chiara & Imperato, 1988). As we will discuss later, this perspective implicitly favored a convergent as opposed to a divergent view over drug addiction. Accordingly, the 'gold standard' self-administration procedure (to be described later, **Chapter 4**) was originally designed to achieve an extremely regular pattern of drug taking (Goldberg, 1973; Yokel & Pickens, 1973) (regardless of the drug under examination) similar to what can be observed for natural rewards.

The wide application of this procedure, regardless of the drug under examination, produced critical data supporting the current leading theories of addiction, which have contributed to our collective comprehension of the mechanisms underlying this medical condition (Everitt & Robbins, 2005; Koob & Le Moal, 2001; Piazza & Deroche-Gamonet, 2013; Robinson & Berridge, 1993c; Wise & Bozarth, 1987). Regrettably, the combination of these insights with modern neuroscience technologies has not yet led to limited advancements in available treatment options for individuals seeking help (Field & Kersbergen, 2020). There is recognition among addiction researchers that refinement of models of addiction may facilitate this goal (Ahmed, 2012; Ahmed, Badiani, Miczek & Müller, 2020; Roberts, Morgan & Liu, 2007; Venniro, Banks, Heilig, Epstein & Shaham, 2020). Below, I will briefly outline some of the refinement attempts, especially relevant to the present dissertation.

An initial attempt at refinement was made by Ahmed and Koob (Ahmed & Koob, 1998). They aimed to improve drug self-administration procedures to replicate aspects of human drug addiction in rats, such as the gradual increase in drug consumption (escalation) and reduced sensitivity to drug effects (tolerance). They accomplished this by providing rats with extended access to drugs in order to induce drug dependence (Ahmed & Koob, 1998; Ahmed, Walker & Koob, 2000) and argued that rats can only develop addiction when they expose themselves adequately to cocaine, surpassing the 'threshold of addiction' (Ahmed, 2011).

Adopting a similar refinement-focused strategy, the laboratory led by Prof. Roberts dedicated its efforts to developing an animal model that mirrors the patterns of drug taking exhibited by individuals with

addiction (Allain, Minogianis, Roberts & Samaha, 2015; Morgan, Liu, Oleson & Roberts, 2009; Roberts, Brebner, Vincler & Lynch, 2002; Roberts, Morgan & Liu, 2007; Ward, Morgan & Roberts, 2005; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012). This objective was accomplished by providing rats with intermittent-access to drugs, resulting in rapid fluctuations in drug brain levels. It is noteworthy that, contrary to what Dr. Ahmed asserted, rats do not display high drug intake with intermittent-access but still exhibit addiction-like behaviors. Subsequently, Belin et al. (2009) studied individual rats' patterns of drug taking and reported that rats which engage in repeated "bursts" of drug injections (i.e., brief periods of rapid, voluntary drug intake) also developed the most severe addiction-like behavior.

Notably, the studies presented above, and many related studies have primarily focused, and accordingly tuned the training parameters using cocaine as the prototypical drug (**Figure 1;** refer to **Chapter 4** for a historical excursus on animal model of addiction). This focus, coupled with the general assumption that all addictive drugs act as reinforcers, resulted in preclinical addiction research overlooking the impact of the distinct pharmacokinetics (PK) and pharmacodynamics (PD) properties of individual drugs on drug-related behaviors (such as patterns of drug taking, motivation to seek and take drugs, etc.) (Allain, Minogianis, Roberts & Samaha, 2015). This gap is noteworthy when considering that, in individuals using drugs, aspects concerning pharmacokinetics and pharmacodynamics play crucial roles in sustaining and shaping dose-time relationships and associates patterns of drug taking (Dole, Nyswander & Kreek, 1966; Müller & Schumann, 2011; Siegel, 1977; Siegel, 2005; Zinberg, 1984).

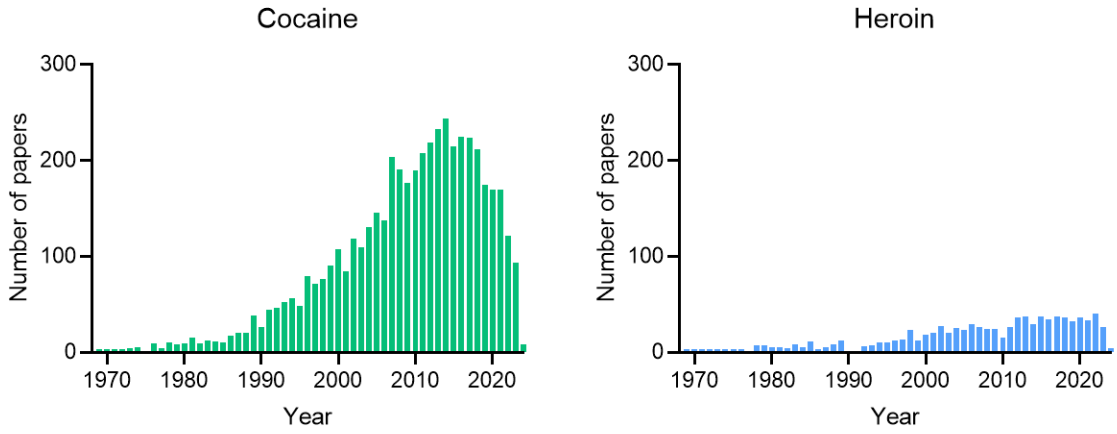

Figure 1. *Numbers of studies on cocaine (left) and heroin (right) self-administration in laboratory animals. Search query: 1) (cocaine) AND (self-administration) 2) (heroin) AND (self-administration). My NCBI filter: "other animals". The research was conducted on January 26, 2024.*

This is significant not just because studying dose-time relationships and the patterns of drug taking historically resulted in the development of treatment strategies for opioid addiction, but also because, as recently proposed by the Food and Drug Administration (FDA), patterns of drug taking serve as robust indicators of treatment effectiveness (Administration, 2020; Panlilio et al., 2020). They may even be used as clinical endpoints for evaluating the impact of opioid addiction treatment (Administration, 2020).

From this consideration, it follows that self-administration procedures should consider each drug under investigation by taking into account its unique pharmacokinetic and pharmacodynamic profile. The present dissertation aims to build on this framework. **Chapter 2** provides a thorough exploration of the similarities and differences in the careers of cocaine and heroin users, offering a detailed analysis of their distinct patterns of drug taking. **Chapter 3** offers an overview of the fundamental principles of pharmacokinetics and pharmacodynamics, accompanied by a detailed examination of the pharmacokinetic and pharmacodynamic profiles of cocaine and heroin. These two chapters will converge in providing the basis for a novel, pharmacology-based interpretation of the divergent patterns of drug taking observed between primarily-cocaine and primarily-heroin users. **Chapter 4**, describes historical developments in preclinical models of drug addiction, tracing the evolution of operant drug self-administration methodology in laboratory animals. I will also provide an overview of the various drug self-administration procedures that were

proposed over time to mimic human behaviors. This will include an in-depth examination of the potential shortcomings of these models. **Chapter 5** and **Chapter 6** are the core of this dissertation. In them, I present data from experiments designed to determine factors influencing patterns of drug taking, drug-seeking, and sociability in rats self-administering heroin or cocaine. I thoroughly investigate the effects of experimenter-imposed timeout and unit-dose on drug reinforcement and addiction-like behaviors. The importance of these factors for determining behavioral endpoints of interest will be considered in the general discussion section.

2. Similarities and differences across the multifaceted careers of cocaine and heroin users

2.1. Introduction

Epidemiological studies indicate that not all individuals who use cocaine and heroin develop addiction (Anthony & Helzer, 2002). As it will be later described, clinical studies indicate that a primary difference between individuals with addiction and those engaging in recreational drug use lies in their respective patterns of drug use (Siegel, 1977; Zinberg, Harding, Stelmack & Marblestone, 1978). Individuals with addiction typically exhibit more severe and harmful patterns of drug use compared to those using drugs recreationally, which often result in decreased functionality in managing their daily lives.

Accordingly, in clinical settings, the detailed analysis of patterns of drug use among individuals with addiction yielded valuable insights for the development of medication for this condition, including approaches like methadone maintenance or heroin-assisted treatment (Dole, Nyswander & Kreek, 1966; Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Kreek, LaForge & Butelman, 2002). Dole et al. (1966), by analyzing patterns of drug use and consequences of chronic drug use in heroin users, revealed that heroin addiction involves 'on-off' cycles, with rapid drug brain level rises and heroin ‘rush’ after injection (the 'on' period), followed by declining brain drug level, withdrawal symptoms, and craving (the 'off' period) (Kreek, LaForge & Butelman, 2002). Dole et al. (1966) leveraged this evidence and proposed methadone, an agonist of MORs with a long half-life ($t_{1/2}$), as a putative therapeutic strategy against opioid addiction, also referred to as gonist replacement therapy. The rationale was the following: enabling the attainment of a 'steady state'[3] while preventing the occurrence of the heroin ‘rush’ and emergence of withdrawal symptoms (**Figure 2**). Based on a similar strategy, individuals who do not respond to methadone maintenance therapy undergo heroin-assisted substitution treatment for opioid addiction (Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Rehm, Gschwend, Steffen,

[3] The term ‘steady state’ refers to a condition where continuous drug delivery leads to a balance between the rate of drug input and the rate of drug elimination. As a result, the concentration of the drug in the body remains constant over time.

Gutzwiller, Dobler-Mikola & Uchtenhagen, 2001). This treatment is tailored to the daily patterns of drug use of individuals with heroin addiction, including both the dosage of heroin provided and the timing of administration. Patients receive individually adjusted doses of injectable heroin, which they self-administer under direct medical supervision (Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Rehm, Gschwend, Steffen, Gutzwiller, Dobler-Mikola & Uchtenhagen, 2001).

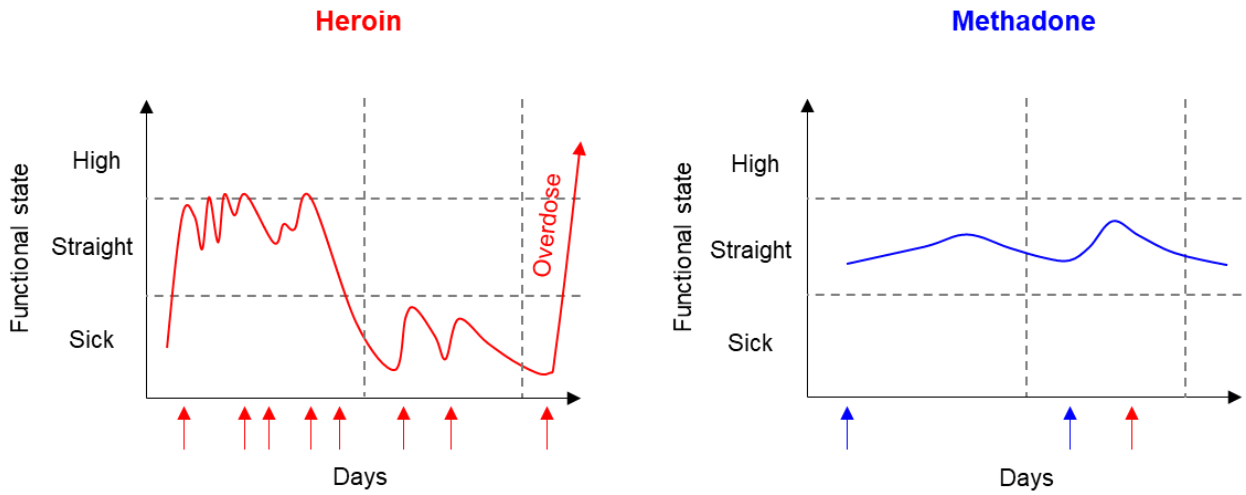

Figure 2. *A diagram illustrating the 'pharmacological state' of a typical heroin user in two scenarios: when consuming heroin (left) and when undergoing methadone maintenance treatment (right). Red arrows (↑) represent heroin injections (unknown dose), and blue arrows (↑) represent methadone doses. Adapted from Dole et al., 1966 (1966).*

Based on this evidence, the refinement of the animal models of drug addiction (the overarching goal of this dissertation) should consider the analysis of the naturally occurring pattern of drug use, that instead is often altered by experimenter-imposed stratagems. Here, I argued that the detailed analysis of a naturally occurring pattern of drug use along with a pharmacokinetic and pharmacodynamic analysis of the drug are critical steps to discovering the neural/molecular substrates which generate the underlying 'algorithms' that regulate behavior in addiction.

In the following sections, I will review the similarities and differences in patterns of drug use displayed by individuals with cocaine and heroin addiction. I will first provide a brief description of the career paths of individuals using these drugs, spanning from occasional and controlled drug use to the progression toward severe patterns of drug use and eventually addiction. Then, I will elucidate how heroin and

cocaine users uniquely instrumentalize their patterns of drug use, including drug dosage, route, and frequency of administration to achieve specific desired drug experiences, while responding to internal and external factors.

2.2. Patterns of drug use throughout cocaine careers

Despite the challenges in delineating the various types of cocaine use, longitudinal studies and analyses of frequency, quantity, and motivations behind cocaine use led to the identification of five routines of cocaine use characterized by distinct patterns of drug use (Siegel, 1977; Siegel, 1984; Siegel, 1985).

(1) Experimental users engage in cocaine use exclusively within social settings but do not purchase it for personal use.

(2) Recreational users acquire their own cocaine but primarily use it in social settings.

(3) Circumstantial users typically acquire their own cocaine and use it both in social settings and individually oriented situations, such as specific tasks or to cope with particular conditions (Müller & Schumann, 2011).

(4) Intensive users utilize cocaine to cope with prolonged or stressful issues or to maintain a certain level of performance. They engage in intensified individually oriented patterns of drug use, characterized by frequent and intense binge episodes lasting several months, rarely reverting to socially oriented patterns of drug use (Müller & Schumann, 2011; Siegel, 1977).

(5) Compulsive users represent (a relatively small subset) exhibiting highly intense and compulsive cocaine-use patterns. Arguably these patterns of drug use are linked to a significant reduction in individual and social functioning. Indeed, users in this category report social dysfunctions and consider themselves unable to regulate cocaine use and are impaired up to a point to warrant treatment ((SAMHSA), 1999; Erickson & Weber, 1994; Gawin & Kleber, 1985; Reinarman, Murphy & Waldorf, 1994; Siegel, 1977).

Of note, limited systematic and detailed studies explored the patterns of cocaine-use across different stages of a cocaine users' career. In most studies, descriptions of patterns of drug use were confined to documenting routes of administration and drug dosage, with more detailed descriptions available for users in treatment. These aspects will be summarized below.

Routes of administration

Individuals who use cocaine typically employ three main routes of administration: intranasal, smoking, and intravenous. Over time, significant shifts occurred in the preferred routes of cocaine administration, influenced by environmental factors and the availability of drug paraphernalia (Gossop, Griffiths, Powis & Strang, 1994). Initially, most users opted for intranasal administration, resulting in a moderate rate of drug delivery to the brain and relatively slow onset effects (Hatsukami & Fischman, 1996). However, with the widespread availability of crack pipes and hypodermic syringes, individuals began transitioning to intravenous injection or converting cocaine into crack cocaine, a smokable base form, through a process known as 'gourmet' cooking (Hatsukami & Fischman, 1996). These methods allow for more rapid delivery of cocaine to the brain compared to intranasal administration. These methods of administration necessitate less cocaine use to achieve similar effects, and with their distribution, cocaine became more affordable, leading to a significant increase in the number of cocaine users (Hatsukami & Fischman, 1996). In addition, while snorting cocaine hydrochloride is often perceived as non-addictive by users, and most occasional users employ this route (Prinzleve et al., 2004), intravenous and smoked cocaine are associated with greater toxicity and escalating patterns of drug use (Griffiths, Gossop, Powis & Strang, 1994; Siegel, 1984; Strang et al., 1998; Strang, Des Jarlais, Griffiths & Gossop, 1992). Consistently, it was reported that crack smoking and intravenous injections are linked to elevated levels of dependence compared to cocaine sniffing (Gossop, Griffiths, Powis & Strang, 1992; Gossop, Griffiths, Powis & Strang, 1994). Consistently, the transition to addiction is often associated with the transition to a faster route of administration (refer to **Chapter 3** for pharmacokinetic considerations on the matter). Notably, most individuals with a formal diagnosis of cocaine addiction utilize intravenous or smoking routes of administration.

Patterns of drug use

Despite different routes resulting in consistent differences in bioavailability and the rapidity of cocaine delivery into the brain, the predominant pattern of cocaine-use for all routes involves discrete episodes known as ‘runs’ or ‘binges’. These binge episodes, lasting from 1 to 96 hours, involve continuous smoking or injection of cocaine, with doses repeated every 10 to 30-min (with an average of 3 doses per hours) (Gawin, 1991; Siegel, 1977; Siegel, 1985). In certain severe instances, these episodes may endure for as long as 200 hours (Gawin, 1991). In these periods individuals consume around 1.5 grams (from 0.25 to 30.0 grams) (Siegel, 1985). Between binges cocaine users typically abstain for several days. In certain instances, the motivation behind these inter-binging patterns is a desire to instrumentally maximize the positive effects of the drug. Importantly, these patterns of drug use can be observed even among occasional cocaine users (Siegel, 1984).

During binge episodes, users experience many periods of intense euphoria, and they are motivated to continue compulsive levels of use to elicit the euphoria and stimulation of cocaine. However, euphoria during intoxication is typically contrasted with dysphoria in withdrawal, contributing to the development of cocaine craving, especially in the face of increasing tolerance (Gawin, 1991; Gawin & Kleber, 1985). The dysphoria experienced after cocaine use can drive individuals to consume cocaine by a desire to avoid the discomfort, the ‘crash’ and depression associated with withdrawal (Lerner & Klein, 2019). In some cases, to contrast the dysphoria, individuals alternate between ‘runs’ of intense cocaine smoking or intravenous use and days of lower use, mostly through intranasal administration (Gawin & Kleber, 1985) or they may instrumentally use other drugs such as alcohol, benzodiazepines, cannabis, or opioids, to induce and prolong sleep and avoid withdrawal symptoms ((SAMHSA), 1999). In other cases, physiological discomfort leads to several days of abstinence (Reinarman, Murphy & Waldorf, 1994). Notably, in some instances, individuals experience hallucinations (Siegel, 1985).

In cases of reduced cocaine availability, individuals may instrumentally use mixtures of legal powders as substitutes. These substitutes often include over-the-counter stimulants like caffeine, ephedrine, and

phenylpropanolamine. Additionally, they may contain easily accessible local anesthetics such as lidocaine, which can produce effects like cocaine, if smoked (Siegel, 2005).

2.3. Patterns of drug use throughout heroin careers

Zinberg (1978) was among the first ones to provide an empirical and pragmatic categorization of heroin users based on their patterns of drug use. This comprises three primary types of routines of drug use.

(1) Irregular/occasional controlled use, a 'hidden' population, referred to as 'chippers', who engage in controlled opioid use exclusively under specific circumstances and social rituals (Blackwell, 1983; Dorn & South, 1987; Powell, 1973; Siegel, 2005; Zinberg & Jacobson, 1976). Of note, a large portion of these 'chippers' are formerly individuals with heroin addiction who transitioned back to controlled heroin use, incorporating drug use into their daily routines (Blackwell, 1983).

(2) Regular and/or harmful use, these individuals define themselves, as 'psychologically' but not physically addicted to heroin (Johnson, 1984; Zinberg, Harding, Stelmack & Marblestone, 1978), indeed they exhibit withdrawal signs only to a limited extent (Dorn & South, 1987; Powell, 1973). Based on clinical interviews, these individuals purchase large quantities of heroin but take small amounts at a time. The ability to self-impose limits on drug use allows them to engage in daily heroin consumption while effectively maintaining functionality in their lives (Faupel, 1991; McAuliffe & Gordon, 1974).

(3) 'Near daily' use and/or drug addiction, are individuals that consume substantial quantities of heroin and find it difficult to quit without experiencing withdrawal symptoms.

Of note, this categorization is based on the degree of harmful patterns of heroin use, frequency, and quantity of heroin consumption, and the degree of physical dependence [that directly is related to the frequency and amount of drug used (Johnson, 1984)]. These three different routines are typically present in chronological order in the career history of people with heroin addiction. However, across their careers, they can also shift from one routine to another based on external or internal contingencies (Darke, 2011;

Faupel, 1991; Hser, Huang, Chou & Anglin, 2007; Siegel, 1984; Zinberg, 1984; Zinberg, Harding, Stelmack & Marblestone, 1978). This range of routines of drug use highlights the interindividual variability in responses to heroin. Understanding the determinants of these routines is propaedeutic for a complete comprehension of how heroin use can be successfully controlled and treated.

Routes of administration

Individuals who use heroin typically employ three main administration routes: intravenous, smoking and insufflation. In terms of the likelihood of developing addiction, some suggest that whether heroin is smoked or injected is comparable (Stewart, 1987). However, intravenous heroin injection is typically associated with social stigma (Strang, Des Jarlais, Griffiths & Gossop, 1992), it is correlated with more significant harm and poorer health (Griffiths, Gossop, Powis & Strang, 1994; Strang et al., 1998; Strang, Des Jarlais, Griffiths & Gossop, 1992) and is linked to more severe dependence compared to smoked heroin (Gossop, Griffiths, Powis & Strang, 1992). In addition, regular intravenous use increases the risk of overdose. While smokers and sniffers can still experience overdoses, their risk may be lower, even though in recent years, smoking has emerged as the most frequently reported method of consumption in cases of overdose fatalities (Tan Lauren J., 2024). Injecting delivers a concentrated dose directly to the brain, whereas smoking involves titrating the dose across multiple use episodes (refer to the next chapter for a pharmacokinetic account of this matter). Thus, injecting heroin is linked to a significantly higher risk (Strang et al., 1998).

Naturalistic investigations proposed that the shift from one route to exclusive or nearly exclusive use of another represents a ‘transition’ (Griffiths, Gossop, Powis & Strang, 1994; Strang, Des Jarlais, Griffiths & Gossop, 1992), which represents a significant event in the career of a heroin user (Griffiths, Gossop, Powis & Strang, 1994). Individuals may transition from one route to another for various reasons, with a predominant shift observed from inhalation to injection (Darke, 2011).

In the extensive examination of individuals with heroin addiction Faupel (Faupel, 1991) reported that in the initial phase, individuals gain fundamental knowledge about heroin use, and a key aspect is the skill of self-injection. Acquiring the skill of self-injection leads to independence, diminished dependence on

others, and the ability to take drugs whenever desired. Collectively, these aspects suggest that the transition may imply a shift toward more harmful patterns of heroin use (Darke, 2011). Direct injection into the bloodstream minimizes losses that may occur with alternative administration routes, resulting in faster and more substantial drug delivery to the brain (see **Chapter 3**). This enhances the speed of onset and intensifies the drug effects, known as the heroin 'rush' (Chessick, 1960; Comer, Collins, MacArthur & Fischman, 1999; Dorn & South, 1987; Gossop, Griffiths, Powis & Strang, 1992; Gossop, Stewart, Marsden, Kidd & Strang, 2004; Griffiths, Gossop, Powis & Strang, 1994; Seecof & Tennant, 1986; Siegel, 1985; Strang et al., 1998; Strang, Des Jarlais, Griffiths & Gossop, 1992). This intensely pleasurable 'rush' experienced during injection is typically referred to as a jeopardizing experience, making the shift back to routes involving smaller, repeated doses (smoking or inhaling) uncommon (Darke, 2011; Dorn & South, 1987). This happens only in rare cases (Darke, 2011). In most instances, when individuals who inject heroin encounter difficulty finding good veins, they may resort to riskier places like femoral and neck veins, or alternative methods like 'skin-popping' or 'muscling,' even though the effects of heroin are smaller (Ciccarone & Harris, 2015).

Patterns of drug use

As previously anticipated, individuals who develop heroin addiction typically display patterns of drug use characterized by high frequency of use (nearly daily) and high amounts of heroin. In addition, they develop tolerance and dependence and experience withdrawal symptoms. Of course, sustaining this lifestyle necessitates substantial funds, and can lead individuals to engage in multiple criminal activities (Darke, 2011; Faupel, 1991). In addition, the consequences of this lifestyle on individuals' psychophysiological state make heroin the center of their lives. Indeed, users typically adopt a routine characterized by obtaining heroin, using it, and recovering throughout the day (Darke, 2011; Faupel, 1991). Consequently, these individuals are frequently apprehended by law enforcement or, a small percentage, actively seek assistance.

Seasoned heroin users typically appreciate various aspects of the intoxication experience and its aftermath. They report the desire for a state of mind that combines experiencing euphoria, continuing

consciousness, and relaxed feelings (Dorn & South, 1987; McAuliffe & Gordon, 1974). As previously described, even in the context of extreme heroin use, individuals may retain the ability to instrumentalize their drug use behaviors to achieve a specific desired drug experience, while responding to internal and external factors (Zinberg, Harding, Stelmack & Marblestone, 1978; Zinberg & Jacobson, 1976). Depending upon the dose taken and the route of administration used, they may either continue to interact socially in a relaxed manner or experience a state of complete intoxication (Dorn & South, 1987).

The dose of heroin that they can inject is strongly related to the drug available in the environment. Despite some individuals exhibiting a stereotypical pattern of consuming as much of the drug as is available (McAuliffe & Gordon, 1974; Zinberg, Harding, Stelmack & Marblestone, 1978), the typical pattern adopted by these users is to administer heroin through intravenous injections and take heroin daily, following a typical pattern of 2–3 use episodes (Darke, 2011; Ross, McCurdy, Kilonzo, Williams, Leshabari & hygiene, 2008). They typically inject themselves with high doses through the fastest route of administration (intravenously) to experience the heroin ‘rush’, and then enter a state known as ‘the nod’, characterized by sedation (Crawford, Washington & Senay, 1983; Dorn & South, 1987). Of note, some users take cocaine or other psychostimulants to avoid sedation and the ‘nod‘ (Dorn & South, 1987). Due to the frequency of their drug use, these individuals often display physical and psychosocial indicators of opioid withdrawal syndrome (Alksne, Lieberman & Brill, 1967; Faupel, 1991; Kolb, 1925; McAuliffe & Gordon, 1974). While the primary motivation for using heroin is typically to attain euphoria (Martinez, Brandt, Comer, Levin & Jones, 2022), they also resort to heroin use to alleviate withdrawal symptoms (McAuliffe & Gordon, 1974).

In cases of reduced heroin availability, they often turn to substituting more economical functional alternatives. These substitutes typically involve various drugs with comparable chemical and pharmacological properties, used for a short period to manage through brief periods of scarcity. Methadone appears as the most common substitute for heroin, largely due to its easy accessibility from maintenance programs. Some individuals may also have access to Dilaudid® , fentanyl, oxycodone or other opioid drugs, enabling them to sustain their dosage level temporarily (Cicero, Ellis, Surratt & Kurtz, 2014; Crawford, Washington & Senay, 1983; Faupel, 1991; Mars, Rosenblum & Ciccarone, 2019).

Finally, Alksne et al. (1967) observed that seasoned heroin users can instrumentalize detoxification to maintain their drug habits when a marked tolerance is established. In other words, they instrumentally quit heroin use for a while to reduce their tolerance. In this manner, when they return to use, they experience a revival of the pleasurable effects of the drug, allowing them to experience a satisfying ‘rush’ once again (Alksne, Lieberman & Brill, 1967).

2.4. Summary

The analysis provided revealed that cocaine and heroin are instrumentalized in a distinct fashion.

Cocaine users, regardless of their chosen route of administration or stage of drug career, often engage in cyclic binging behavior (Gawin, 1991; Siegel, 1977; Siegel, 1985). This involves episodes of consuming small doses of cocaine repeatedly every 10-30 min over consecutive hours or days, separated by periods of abstinence (Gawin, 1991; Gawin & Kleber, 1985; Siegel, 1977; Siegel, 1984; Siegel, 1985). On the other hand, individuals with daily heroin consumption tend to concentrate their heroin use into a few doses per day (up to three), maintaining a consistent pattern across months/years (Darke, 2011; Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Ross, McCurdy, Kilonzo, Williams, Leshabari & hygiene, 2008).

These differences align with the perspectives of Zinberg (Zinberg, 1984) and Muller and Schumann (Müller & Schumann, 2011), who suggest that patterns of drug use are determined and instrumentalized based on the pharmacological characteristics of the chosen drug. Indeed, heroin and cocaine possess distinct pharmacological profiles that clearly account for the observed differences in patterns of drug use in humans.

Nevertheless, a notable convergence tends to emerge between the patterns of drug use displayed by individuals using these two drugs. In both instances, individuals commonly transition from slower to faster routes of administration with the aim of intensifying the drug's effects. In addition, they both display the ability to instrumentalize these patterns of drug use (including abstinence, dosage, route, and frequency

of administration) to achieve their desired drug effects, while responding to internal and external factors, such as drug availability, tolerance to drug effects or side effects of excessive drug consumption.

To corroborate the above-mentioned arguments and to better understand drug use dynamics, in the subsequent chapter, a deeper exploration of the pharmacological characteristics of heroin and cocaine will be undertaken.

3. General principles of pharmacokinetics and pharmacodynamics of heroin and cocaine[4]

3.1. Introduction

The patterns of drug use exhibited by drug users can be interpreted as a demonstration of skillfulness. Indeed, they leverage the interplay between the pharmacokinetics and pharmacodynamics properties of drugs (illustrated in **Figure 3**) to attain the desired subjective experience (Comer, Collins, MacArthur & Fischman, 1999; de Wit, Bodker & Ambre, 1992; Dole, Nyswander & Kreek, 1966; Kreek, LaForge & Butelman, 2002; Van Dyke, Ungerer, Jatlow, Barash & Byck, 1982; Zinberg, 1984). Notable evidence of this is reflected in the intentional 'instrumentalization' of the dosage, its route of administration, and frequency of administration (Csikszentmihalyi, 1992; Decorte, 2001; Dole, Nyswander & Kreek, 1966; Gawin & Kleber, 1986; McAuliffe & Gordon, 1974; Zinberg, Harding, Stelmack & Marblestone, 1978). An example is the sought-after euphoric experience known as the drug 'high' or 'rush' (Bornstein & Pickard, 2020; Dole, Nyswander & Kreek, 1966; Gawin & Kleber, 1986; Seecof & Tennant, 1986), often achieved by administering large drug doses through the fastest route of delivery (Allain, Minogianis, Roberts & Samaha, 2015; Gawin & Kleber, 1986; McAuliffe & Gordon, 1974; Mello & Mendelson, 1987). Of note, approaches such as increasing the drug dosage or choosing a faster route of administration can have a notable impact on the pharmacokinetics and pharmacodynamics profiles of the administered drug (Allain, Minogianis, Roberts & Samaha, 2015; Busto & Sellers, 1986; Marie, Canestrelli & Noble, 2019).

To provide a more in-depth comprehension of these aspects, the following sections will delve into the essential pharmacokinetic (PK) and pharmacodynamic (PD) properties of heroin and cocaine. I will first describe the pharmacokinetics and pharmacodynamics characteristics of cocaine and then of heroin.

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[4] Part of this chapter was extracted from Milella MS, D'Ottavio G, De Pirro S, Barra M, Caprioli D, & Badiani A (2023). Heroin and its metabolites: relevance to heroin use disorder. Transl Psychiatry 13**:** 120. that I coauthored: Milella M.S., D'Ottavio G., De Pirro S., Barra M., Caprioli D., Badiani A. (2023) Heroin and its metabolites: relevance to heroin use disorder. Transl Psychiatry 13, 120 https://doi.org/10.1038/s41398-023-02406-5

Subsequently, I will present a comprehensive description of the primary routes of administration adopted by individuals and their respective effects on drugs' metabolism.

For readers new to this field seeking a comprehensive understanding of pharmacokinetics and pharmacodynamics, authoritative volumes published in recent years [e.g., (Brunton & Knollmann, 2022; Vanderah, 2023)], are recommended. For the sake of brevity, the basic principles of pharmacokinetics and pharmacodynamics are concisely illustrated in **Box 1** and **Box 2**, respectively.

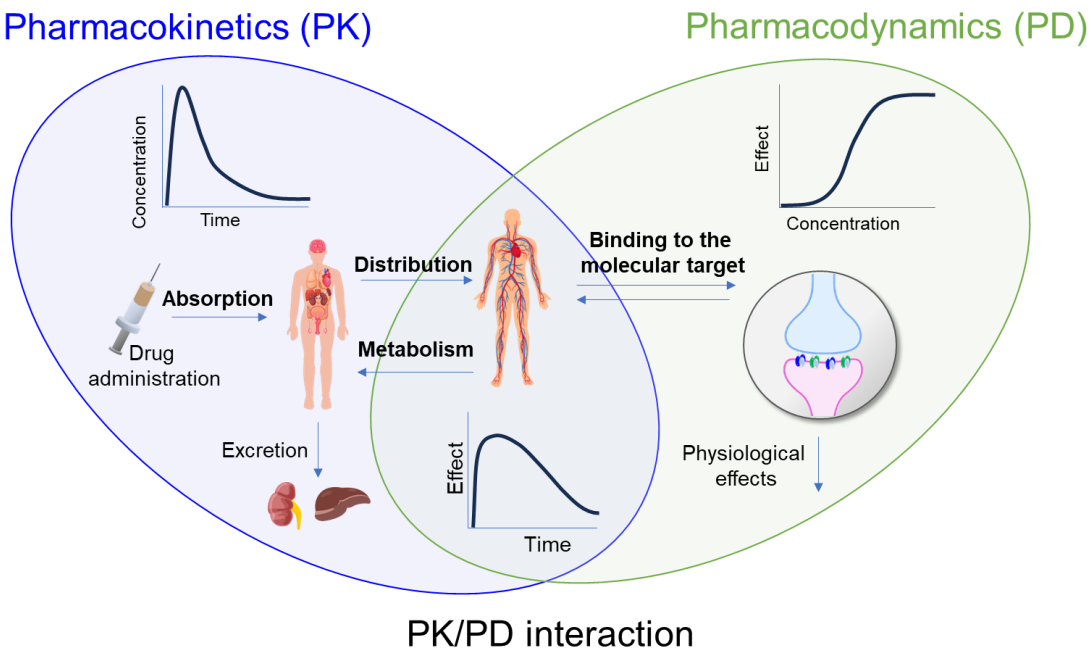

Figure 3. *Graphical description of the interaction between pharmacokinetics and pharmacodynamics.*

Box 1. Pharmacokinetics (PK)

Pharmacokinetics is a branch of pharmacology that studies how the body changes a specific substance after administration. It considers the process involving the absorption of drugs by the body, the distribution of the drugs in the tissues, the subsequent biotransformation, and the elimination of the drugs and their metabolites from the body over a specified duration. These processes are typically described using the acronym ADME (Absorption, Distribution, Metabolism, Elimination). Here are listed main factors that influence the ADME process, important for understanding the modalities of drug taking adopted by drug users. Note: the main pharmacokinetics metrics are described in **Glossary 1**.

Routes of drug administration. Different routes of drug administration result in varied pharmacokinetics profiles and distinct drug bioavailability. The intravenous (i.v.) route stands out as the fastest, ensuring rapid drug distribution throughout the body and complete bioavailability. Inhalation or absorption through the pulmonary epithelium and mucous membranes also provides quick drug delivery, with the added benefit of bypassing hepatic first-pass loss. On the other hand, routes like intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) injections exhibit slower absorption rates, contingent on blood flow to the injection site. Oral administration, being the slowest, involves drug metabolism by intestinal microbiota, mucosa, or liver enzymes before reaching the general circulation, resulting in a delayed delivery to the target site.

Dosing schedule. The timing and frequency of drug administration significantly impact pharmacokinetics profiles and drug bioavailability. In cases of repeated drug use, especially with drugs of abuse in humans, the pattern of administration plays a crucial role in determining the drug's response. Two primary strategies of drug administration exist: intermittent and continuous. Intermittent treatment involves dosing at specific intervals, creating fluctuating drug concentrations between peak and trough levels. In contrast, continuous drug administration establishes a steady state, maintaining a constant drug concentration by balancing input and elimination rates.

Glossary 1. Main pharmacokinetics metrics.

Bioavailability: the systemically available fraction of a drug.

$t_{1/2\alpha}$ (absorption half-life): the time required for 50% of a given dose of drug to be absorbed into the systemic circulation.

$t_{1/2b}$ (elimination half-life): the time required for a drug in a biological system to decrease by half due to biological processes, assuming an approximately exponential rate of removal.

k_a (absorption rate constant): the rate at which a drug enters the body for oral and other extravascular routes.

k_e (elimination rate constant): the rate at which a drug is removed from the body.

C_{max} (maximum serum concentration): highest concentration of a drug reached within a specific compartment, often referred to as the peak serum concentration.

T_{max} (minimum time for C_{max}): the time required for a drug to achieve its peak concentration (C_{max}) following the administration of an absorbable drug.

Box 2. Pharmacodynamics (PD)

Pharmacodynamics is a branch of pharmacology that studies the biochemical and physiological impacts of drugs. These effects may encompass responses observed in animals, including humans, as well as microorganisms, or interactions involving combinations of organisms, such as in the context of infections. The impact of drugs on biological systems is determined by their interaction with four primary protein targets: enzymes, membrane carriers, ion channels, and receptors.

A main foundation of pharmacodynamics is the dose–response relationship. This describes how the response of an organism changes (effects on the body) as a function dose of exposure to the drug over a specified exposure period.

The effects on the body are limited in time. The duration of effects can be divided into six parts:
1) total duration – refers to the period required for the effects of the substance to entirely diminish;
2) onset – time elapsed until the initial alterations in perception become apparent;
3) come up – interval from the onset to the highest subjective intensity;
4) peak – period of time during which the substance's effects are at their maximum intensity;
5) offset – duration between the conclusion of the peak effects and the return to a sober state;
6) after effects – any residual effects that may persist after the conclusion of the experience.

3.2. Cocaine

Cocaine is a tropane alkaloid isolated from the Erythroxylon coca plant, native to South America, by soaking leaves in organic solvents to form a thick paste sediment. The water-soluble hydrochloride cocaine form is most commonly administered intravenously, via nasal insufflation (i.e., snorted), or taken orally. The hydrochloride salt can be transformed into an alkaloid form suitable for smoking by introducing a base like sodium bicarbonate. This altered state of cocaine solidifies into a rock-like substance commonly known as *crack cocaine*.

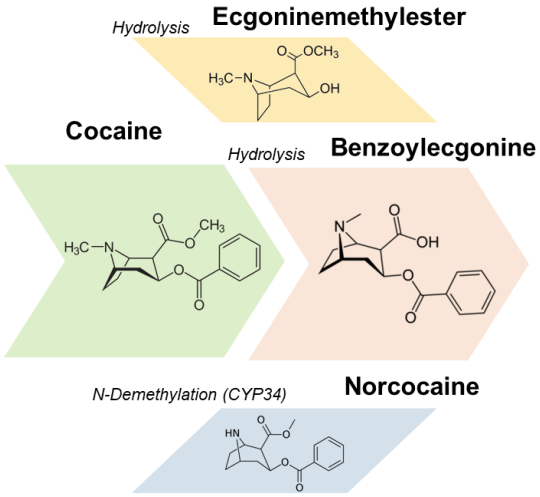

Figure 4. *Schematic representation of the metabolic pathway of cocaine with the sequential breakdown into the main metabolites. The involved enzymatic processes are listed in italics.*

3.2.1. Pharmacokinetics of cocaine and its metabolites

Cocaine is metabolized to benzoylecgonine (BZE), ecgoninemethylester (EME), and norcocaine (**Figure 4**). Cocaine undergoes predominant metabolism through four pathways: (1) liver carboxylesterase 1 breaks down the methyl ester linkage of cocaine, resulting in BZE ; (2) intestinal carboxylesterase 2 hydrolyzes the benzoate linkage, yielding EME; (3) serum butyrylcholinesterase generates EME; and (4) CYP450 3A4 demethylates cocaine, producing norcocaine. It was shown that cocaine can also undergo spontaneous hydrolysis in vitro at physiological temperature and pH, resulting in the formation of BZE and EME (Coe, Jufer Phipps, Cone & Walsh, 2018). After cocaine administration,

norcocaine is present in very low quantities in humans (Jatlow, 1988) and it is not detected following intravenous injections in rats (Sun & Lau, 2001). Among these metabolites, it was shown that direct administration of EME does not alter baseline behavior, whereas administration of BZE and norcocaine causes hyperactivity, indicating their pharmacological activity (Schuelke, Konkol, Terry & Madden, 1996). Furthermore, studies investigating cardiovascular effects suggest that EME and BZE do not induce cardiovascular effects and are unlikely to contribute to cocaine's overall cardiovascular effects (Schindler, Zheng & Goldberg, 2001). However, despite exhibiting some effects, norcocaine is produced only at low levels in vivo after cocaine administration (Ma, Falk & Lau, 1999; Walsh, Haberny & Bigelow, 2000). To the best of my knowledge, norcocaine is the only metabolite that was demonstrated to sustain self-administration (McKenna, Ho & Englert, 1979; Risner & Jones, 1980; Wang, Simpao, Sun, Falk & Lau, 2001). Therefore, it will be the sole metabolite discussed in detail below.

As I will discuss below, the metabolic rate of cocaine and its metabolites differ for each route of administration (**Figure 5**).

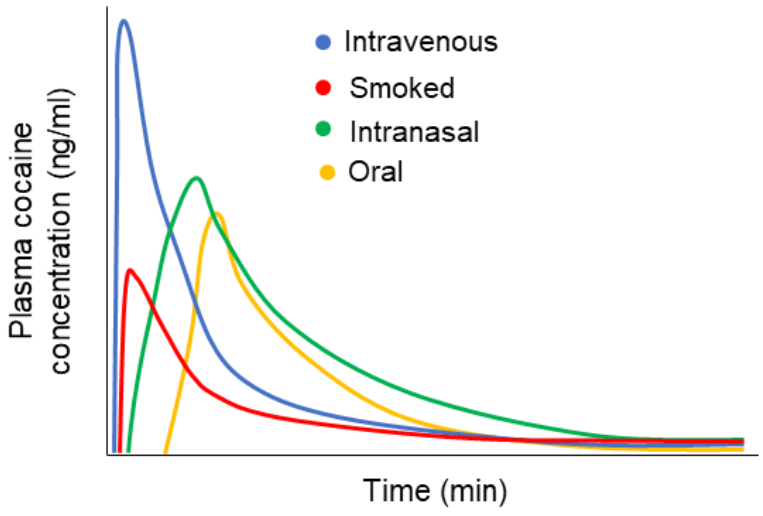

Figure 5. *Time course of plasma concentrations of cocaine in humans as a function of the route of drug administration. Intravenous (blue line), smoked (red line), intranasal (green line), and oral (yellow line). Adapted from (Allain, Minogianis, Roberts & Samaha, 2015; Jones, 1990).*

Pharmacokinetics of cocaine

Oral. Oral administration typically results in the lowest bioavailability due to the slower absorption of cocaine. Although cocaine is efficiently absorbed from the gastrointestinal tract, its bioavailability is reduced by gastric breakdown and intestinal metabolism (Van Dyke, Jatlow, Ungerer, Barash & Byck, 1978). After oral administration cocaine becomes detectable in plasma within 30-min of administration and achieves peak plasma concentrations within 50- to 90-min. This route of administration is often linked with a delayed onset of effects with respect to the others, indeed maximum subjective effects on the 'rush' occur after 45- to 90-min.

Insufflation. After inhaling intranasal doses of cocaine, plasma concentrations increase during the initial 20-30 min, reaching their peak levels before the 60-min mark. Subsequently, there is a gradual decline over the following hours (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977; Van Dyke, Barash, Jatlow & Byck, 1976; Van Dyke, Ungerer, Jatlow, Barash & Byck, 1982). Despite the onset of cardiovascular changes aligned with the rise in plasma cocaine levels, reaching peak values at approximately the same time, these results contrast with street cocaine users' reports. Indeed, subjects typically report their peak 'rush' 5-20 min after inhalation, and the return to pre-drug physiological and subjective levels occurred within 15-60 min, a more rapid decline than the decrease in cocaine plasma levels (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977; Van Dyke, Barash, Jatlow & Byck, 1976). It was suggested that the euphoria induced by cocaine might be associated with the rapidly rising concentration of cocaine in plasma rather than the peak values. In addition, because of the high lipophilicity of cocaine, the euphoria may have a stronger correlation with concentrations at brain receptor sites rather than with peak concentrations in plasma (Van Dyke, Barash, Jatlow & Byck, 1976).

Smoked. The bioavailability of smoked cocaine ranges between 60% and 70% when cocaine is vaporized as a base (Cone, 1995; Hatsukami & Fischman, 1996), although a significant portion of this bioavailability depends on the temperature at which the cocaine is heated. The peak of the 'rush' after cocaine smoking is similar to intravenous injection, indeed subjects report the maximal high around

minutes after consumption (Cone, 1995; Jeffcoat, Perez-Reyes, Hill, Sadler & Cook, 1989). This aligns with the observation that when smoked, the absorption of cocaine is very rapid with a T_{max} of 1.1-min (Jeffcoat, Perez-Reyes, Hill, Sadler & Cook, 1989). One study has shown that after either smoking or intravenous cocaine, maximal arterial cocaine concentrations occur within 15-s, while maximal venous cocaine concentrations occur within 4-min (Evans, Cone & Henningfield, 1996). The elimination half-life is approximately 60-min (Jeffcoat, Perez-Reyes, Hill, Sadler & Cook, 1989), and consistently, the duration of the effects is approximately 30-45 min (Hatsukami & Fischman, 1996).

Intravenous. Following intravenous injection, cocaine plasma concentrations reach their peak almost instantly (T_{max} ~ 5-11 min) (Coe, Jufer Phipps, Cone & Walsh, 2018; Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978), and the decline is gradual, with a half-life of disappearance from plasma ranging between 16 to 87-min (Cone, 1995). Following intravenous drug injection, subjects report that the peak of the 'rush' typically occurred around 3-5 min after injection, comparable to the timeframe when cocaine reaches its peak levels in the bloodstream. Subjects also report that the drug's effects diminished within 30-40 min, and if self-administering, they would be prepared for a second dose at that point (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977). In the rat, after intravenous administration, cocaine plasma concentrations peak rapidly both in the blood (T_{max} = 0.0-min in the venous circulation) and in the brain (T_{max} = 2.5-min in the accumbens extracellular fluid (Pan, Menacherry & Justice, 1991); T_{max} = 15-min in the whole brain (Nayak, Misra & Mulé, 1976). It is then hydrolyzed ($t_{1/2b}$ = 5-7 min in the venous circulation; $t_{1/2b}$ = 8-11 min in the accumbens extracellular fluid) and becomes undetectable after 1-6 hours (Nayak, Misra & Mulé, 1976; Pan, Menacherry & Justice, 1991).

Pharmacokinetics of norcocaine

Following intravenous cocaine administration, norcocaine is present in humans in very low quantities (Jatlow, 1988) and is not detected after intravenous injections in rats (Sun & Lau, 2001). In contrast, after oral cocaine administration, norcocaine concentration-time profiles increase proportionally with the dose of cocaine administered, in both humans and rats (Ma, Falk & Lau, 1999; Walsh, Haberny & Bigelow,

2000). Indeed, the majority of norcocaine is formed during the first-pass absorption (Sun & Lau, 2001). In rats, studies demonstrated that, after oral administration of cocaine, norcocaine levels peak around 30-min and dissipate within approximately 90-min (Ma, Falk & Lau, 1999). When directly administered, norcocaine pharmacokinetics in rats is like that of cocaine. After intravenous injections of norcocaine, the plasma concentrations increase in a few min, and its elimination half-life, is $t_{1/2\beta}$ = 28-33 min (Mets, Diaz, Soo & Jamdar, 1999).

3.2.2. Pharmacodynamics of cocaine and its metabolites

Pharmacodynamics of cocaine

Cocaine pharmacodynamics involves multiple complex mechanisms. Cocaine binds and blocks monoamine (dopamine, norepinephrine, epinephrine, and serotonin) reuptake transporters with similar potencies, and elevates extracellular concentrations of these monoamine neurotransmitters (Ritz, Lamb, Goldberg & Kuhar, 1987). However, it was shown that the main effects of cocaine, including cocaine's reinforcing properties, are mediated by its action on the dopaminergic system, particularly the dopamine transporter (DAT) (Chen et al., 2006). By blocking monoamine reuptake, monoamines accumulate in the synaptic cleft. However, the increase in monoamines correlates with feelings of euphoria, self-confidence, sexual arousal, increased energy, mental alertness, and instant relief from boredom or fatigue (Gold, Washton & Dackis, 1985). On the other hand, cocaine also induces anesthesia, vasoconstriction on the mucosa, elevates heart rate, systemic arterial pressure, and myocardial contractility. Acute intoxication results in tachycardia, hypertension, and agitation. Additional physical examination findings often include mydriasis, diaphoresis, hyperthermia, and tachypnea (Schwartz, Rezkalla & Kloner, 2010). Regarding other delayed effects of cocaine, they may be driven by its metabolites (benzoylecgonine, ecgoninemethylester, and norcocaine, **Figure 4**) (Hawks, Kopin, Colburn & Thoa, 1974; McKenna, Ho & Englert, 1979; Sun & Lau, 2001; Wang, Simpao, Sun, Falk & Lau, 2001). Additional research is required to elucidate this aspect further.

Pharmacodynamics of norcocaine

Only a few studies investigated the effects of norcocaine. Below, I summarize these findings.

Norcocaine has similar effects to cocaine, as it inhibits dopamine reuptake (Einhorn, Johansen & White, 1988). However, it exhibits approximately half the potency of cocaine in binding to DAT (Ritz, Lamb, Goldberg & Kuhar, 1987). Studies investigating the direct effects of norcocaine revealed that has a higher local anesthetic potency than cocaine (Just & Hoyer, 1977) and vasoconstrictive effects like cocaine (Madden & Powers, 1990). Regarding locomotor activity, studies report contrasting findings: locomotory activity is increased by norcocaine after oral and intracerebroventricular, but not the intravenous route of administration (Schuelke, Konkol, Terry & Madden, 1996; Wang, Simpao, Sun, Falk & Lau, 2001). In self-administration procedures, it was shown that norcocaine maintains self-administration in monkeys (Spealman & Kelleher, 1981) and dogs (Risner & Jones, 1980). In rats, norcocaine generalizes to the discriminative stimulus effects of cocaine (McKenna, Ho & Englert, 1979). However, according to Bedford et al. (1980), the lack of any stimulatory effect of norcocaine on locomotor activity and the absence of increased responding induced by intravenous norcocaine on fixed-interval behavior suggest qualitative differences between norcocaine and cocaine.

3.2.3. Distinct roles for cocaine and its metabolites in cocaine effects

Humans typically report that the effects of cocaine are almost immediate. The main desired effects include feelings of euphoria, energy, talkativeness, and social functioning. Indeed, most users take cocaine in social settings (see **Chapter 2**) (Siegel, 1977). Some people use cocaine in individually oriented settings, and they report using cocaine to enhance their ability to perform simple physical and cognitive tasks. Of note, even small amounts of cocaine can induce these effects. However, some users report that excessive cocaine consumption results in heightened mental alertness, hypersensitivity to sensory stimuli, and sometimes hallucinations. Additionally, cocaine temporarily reduces food intake and sleep (Advokat, Julien & Comaty, 2019).

After administration subjects report an immediate sensation of ‘rush’. However, the high given by cocaine and the duration of its effects varies depending on the route of administration. As reported above, individuals using cocaine intravenously report a peak of ‘rush’ around 3-10 min after cocaine administration, which diminishes within 30-40 min (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977). Notably, the time course of these effects aligns with the time course of cocaine metabolism (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977). The peak of the ‘rush’ after cocaine smoking is similar, (Evans, Cone & Henningfield, 1996), to intravenous injection. On the contrary, after snorting subjects typically report a peak of ‘rush’ after 5-20 min, a bit delayed relative to intravenous injections. The effects disappear within 15-60 min, a more rapid decline than the decrease in cocaine plasma levels (Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977; Van Dyke, Barash, Jatlow & Byck, 1976). When cocaine effects fade away (~30-min after administration) subjects report craving and readiness for another dose of cocaine (Jaffe, Cascella, Kumor & Sherer, 1989).

Norcocaine has conventionally been regarded as the most toxic metabolite of cocaine (Evans & Morarity, 1980). However, there remains limited available data regarding the implications of norcocaine's effects on both the immediate and after-effects of cocaine. Some studies suggested that norcocaine may contribute to cocaine's overall cardiovascular effects (Schindler, Zheng & Goldberg, 2001). Additionally, other research has proposed its involvement in cocaine's behavioral effects (Wang, Simpao, Sun, Falk & Lau, 2001). Nevertheless, to the best of my knowledge, no studies have thoroughly dissected the distinct effects of cocaine and norcocaine following cocaine administration, nor have they evaluated the levels of norcocaine and correlated them with the observed effects. Therefore, further investigation into this aspect is warranted. Future studies should aim to elucidate the specific contributions of norcocaine to the overall effects of cocaine, particularly focusing on its potential role in cardiovascular and behavioral outcomes.

3.3. Heroin

Heroin (3,6-diacetylmorphine or diamorphine) is a semi-synthetic derivative of morphine, a naturally occurring opiate contained, along with codeine, in the latex of the opium poppy (*Papaver somniferum*). Heroin is obtained by acetylation of morphine at both 3 and 6 positions. This double acetylation of morphine to heroin increases the lipophilicity and thus the blood-brain barrier permeability by approximately 100-fold compared to the parent compound morphine (Oldendorf, Hyman, Braun & Oldendorf, 1972).

3.3.1. Pharmacokinetics of heroin and its metabolites

The metabolism of heroin, and its metabolites (**Figure 6**), involves several biotransformation processes: (1) hydrolytic reactions, catalyzed by serum- or butyrylcholinesterase in the plasma, and by carboxylesterases 1 in the liver, brain, and other tissues; (2) synthetic reactions, mainly glucuronidation in the liver (but also in the brain, kidney, and intestine), and to a lesser extent sulfation; (3) oxidative reactions, yielding minor metabolites (Gianutsos et al., 1986; Lockridge, Mottershaw-Jackson, Eckerson & La Du, 1980; Way, Young & Kemp, 1965). The first hydrolytic reaction results in the loss of the acetyl group in position 3 and the origination of 6-monoacethylmorphine (6-MAM). Then the acetyl group in position 6 is removed through hydrolysis to the origination of morphine (Way, Young & Kemp, 1965). Then morphine undergoes glucuronidation in the liver, and this yields to production of morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G). In this dissertation, a comprehensive description of M6G and M3G will not be provided for two primary reasons: 1) M3G is mainly implicated in analgesia-related effects of morphine (Lewis et al., 2010; Lipkowski, Carr, Langlade, Osgood & Szyfelbein, 1994), and 2) the experiments conducted in this dissertation utilize rats as experimental subjects and only limited data are available on the pharmacokinetics profile of M6G in rats.

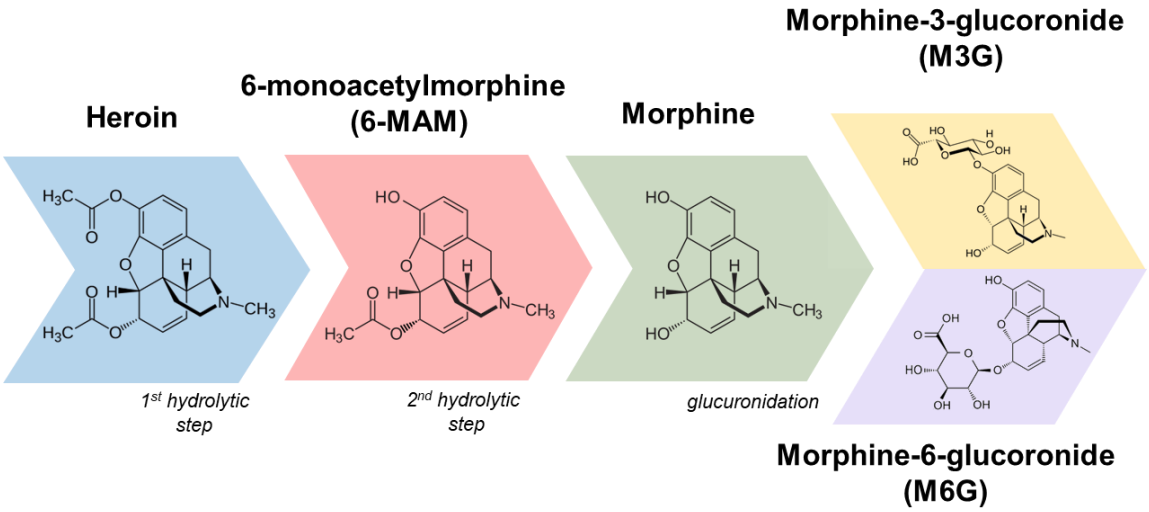

Figure 6. *Schematic representation of the metabolic pathway of heroin with the sequential breakdown into the main metabolites. The involved enzymatic processes are listed in italics. From Milella et al. (2023).*

The metabolic rate of heroin and its metabolites differs for each route of administration. Below the pharmacokinetics of heroin, 6-MAM, and morphine will be discussed separately for route of administration (**Figure 7**).

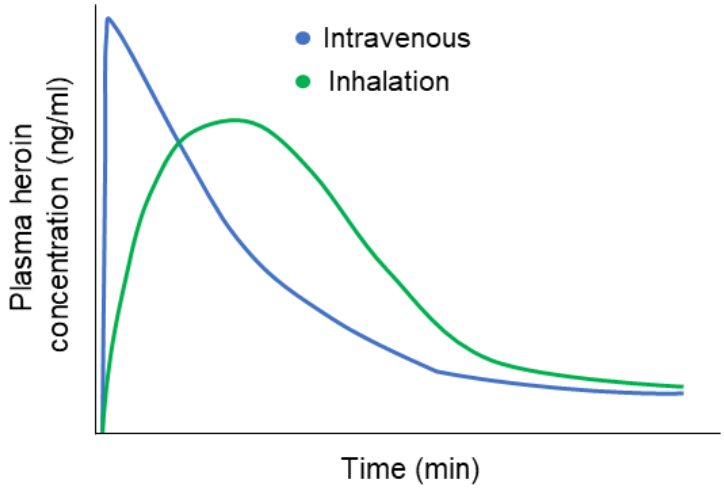

Figure 7. *Time course of plasma concentrations of heroin in humans as a function of the route of drug administration. Intravenous (blue line) and inhaled (green line). Adapted from Rook et al. (2006).*

Pharmacokinetics of Heroin

Intramuscular and subcutaneous injection. The subcutaneous and intramuscular injection of heroin is often due to poor injection practice or the inability to find a patent vein (Hope, Parry, Ncube & Hickman, 2016). However, heroin users who wish to lengthen the duration of drug effects and to experience a calm, warm ‘high’ rather than the ‘rush’ might deliberately inject the drug intramuscularly (Meyer, Eichenberger, Strasser, Dürsteler & Vogel, 2021). Heroin metabolism in the muscle is in fact negligible, and the C_{max} is twofold that seen after insufflation (Skoppl, Ganssmann, Cone & Aderjan, 1997). Furthermore, heroin is slowly released from the muscle into the general circulation, resulting in a half-life considerably longer [$t_{1/2b}$ = 7.8-min (Girardin et al., 2003)] than after intravenous injection.

Inhalation. Heroin can be consumed through inhalation such as 'chasing the dragon' (heating the drug over aluminum foil and inhaling the fumes) or by smoking tobacco mixed with heroin. The resulting ‘rush’ from these inhalation routes is comparable to intravenous administration (Alambyan et al., 2018). Bioavailability is estimated at 38–53% for 'chasing the dragon' and around 14% for smoking laced tobacco (Hendriks, van den Brink, Blanken, Bosman & van Ree, 2001; Jenkins, Keenan, Henningfield & Cone, 1994; Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a). Notably, the C_{max} is significantly lower than intravenous injection, with a T_{max} up to 5-min (Jenkins, Keenan, Henningfield & Cone, 1994). The half-life ($t_{1/2b}$) is approximately 3-4 min for both inhalation strategies (Jenkins, Keenan, Henningfield & Cone, 1994; Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a).

Insufflation. The rapid absorption of heroin after insufflation is facilitated by the rich submucosal venous plexus in the nasal region, characterized by fenestrated endothelia in its capillaries. However, only a fraction of the dose is absorbed, as heroin undergoes hydrolysis in the nasal cavity facilitated by various enzymes (Kendall & Latter, 2003)]. Consequently, the resulting C_{max} is notably lower, and the T_{max} is prolonged (approximately 4-5 min) compared to intravenous injection (Comer, Collins, MacArthur & Fischman, 1999; Cone, Holicky, Grant, Darwin & Goldberger, 1993; Skoppl, Ganssmann, Cone & Aderjan, 1997). This discrepancy elucidates why individuals who snort heroin, known as 'snorters,' do not achieve the same level of euphoria as 'mainliners' or even smokers (Comer, Collins, MacArthur &

Fischman, 1999). The $t_{1/2b}$ is slightly higher than that observed after intravenous injection (5-6 min) (Cone, Holicky, Grant, Darwin & Goldberger, 1993; Skoppl, Ganssmann, Cone & Aderjan, 1997).

Intravenous. As we described above the intravenous route of administration is the fastest route, indeed after intravenous infusions heroin penetrates the blood–brain barrier, and it is rapidly distributed into the bloodstream. This swift onset is commonly known among users as the 'rush' (or 'flash' or 'high') and represents a key characteristic of injection as a route of administration (Dorn & South, 1987). In humans, heroin plasma concentrations peak (C_{max}) almost immediately ($T_{max} \cong 30$ s in the arterial circulation; $T_{max} \cong 2$-min in the venous circulation), and then decline steeply with a half-life ($t_{1/2b}$) of 3-4 min (Inturrisi, Max, Foley, Schultz, Shin & Houde, 1984; Rentsch, Kullak-Ublick, Reichel, Meier & Fattinger, 2001; Rook et al., 2006). Within 10-45 min, heroin becomes undetectable in the blood (Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a; Rook, Huitema, van den Brink, van Ree & Beijnen, 2006b; Rook et al., 2006). Plasma cholinesterases and carboxylesterases swiftly transform heroin into 6-MAM through rapid hydrolysis. Spontaneous, non-enzymatic hydrolysis may also occur (Nakamura & Ukita, 1967). For the first 8-min after intravenous injection heroin concentrations in both arterial and venous circulation remain higher than that of all other active metabolites, including 6-MAM (Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a; Rook et al., 2006) [but see (Kosel et al., 2008)]. In the rat, after intravenous administration, heroin plasma concentrations peak immediately both in the blood (T_{max} = 0.0-min in the venous circulation) and in the brain (T_{max} = 1.5-2 min in the striatal extracellular fluid). It is then metabolized by esterases ($t_{1/2b}$ = 3-min in the venous circulation; $t_{1/2b}$ = 1-min in the striatal extracellular fluid) and becomes undetectable within 10-30 min (Gottas et al., 2013).

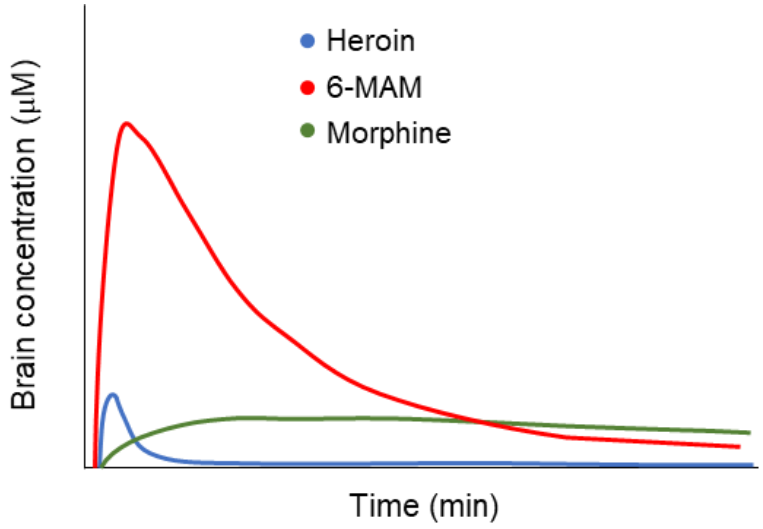

Figure 8. *Concentrations of heroin (blue), 6-MAM (red), and morphine (green) in the striatal extracellular fluid of rats, after an intravenous injection of 1.3 µmol (≅4 mg/kg) of heroin. Adapted from Gottås et al. (Gottas et al., 2013).*

Pharmacokinetics of 6-MAM

Following intravenous administration of heroin, the peak concentration of 6-MAM occurs at approximately the same time as heroin, both in the venous and arterial circulation (**Figure 9**). The C_{max} is similar to that of heroin in the arterial circulation but considerably lower in the venous circulation (Girardin et al., 2003; Rentsch, Kullak-Ublick, Reichel, Meier & Fattinger, 2001; Rook et al., 2006). As detailed in the previous section, plasma concentrations of 6-MAM remain lower than that of heroin for the first 8-min after intravenous injection [but see (Kosel et al., 2008)]. The $t_{1/2b}$ of 6-MAM is longer than that of heroin, although estimates vary greatly from study to study (3-52 min), and can be detected in the plasma for hours, at a time when heroin has already disappeared (Girardin et al., 2003; Rentsch, Kullak-Ublick, Reichel, Meier & Fattinger, 2001; Rook et al., 2006). In contrast, intravenous injection of heroin in the rat results in peak plasma and striatal concentrations of 6-MAM much higher than those of heroin, with a T_{max} of 2-min in the venous blood and 8-min in the striatum (Gottas et al., 2013). This is likely due to inter-species differences in esterase activity (Bahar, Ohura, Ogihara & Imai, 2012). Given its high lipophilicity, 6-MAM passively diffuses across the blood-brain barrier (Gulaboski et al., 2007). It was proposed, based on data from subcutaneous injection of heroin in mice, that the rapid increase in 6-MAM brain

concentration is mainly due to the deacetylation of heroin in the blood, before it enters into the brain (Boix, Andersen & Mørland, 2013). Notably, the striatal C_{max} of 6-MAM after heroin administration in the rat is about 50% higher than after equimolar doses of 6-MAM (Gottås, Boix, Øiestad, Vindenes & Mørland, 2014), indicating that, at least in the rat, a significant fraction of brain 6-MAM results from the local deacetylation of heroin.

Pharmacokinetics of morphine

The second hydrolytic step in the metabolism of heroin mostly depends on liver carboxylesterase-2, which deacetylates 6-MAM to morphine (Kamendulis, Brzezinski, Pindel, Bosron, Dean & therapeutics, 1996). Following heroin intravenous administration in humans, morphine plasma levels rise quickly with a T_{max} ranging between 4 and 8-min (**Figure 9**) (Gyr et al., 2000; Rook et al., 2006). The T_{max} after intranasal or intramuscular injection of heroin is considerably longer, ranging between 10 and 90-min (Cone, Holicky, Grant, Darwin & Goldberger, 1993; Girardin et al., 2003; Skoppl, Ganssmann, Cone & Aderjan, 1997). Plasma levels decline at a much slower pace than for heroin or 6-MAM, with a $t_{1/2b}$ of about 3-4 h (Gyr et al., 2000; Rook et al., 2006). The T_{max} and $t_{1/2b}$ after morphine administration have similar values (Hasselström & Säwe, 1993). In the rat, after intravenous heroin administration, morphine concentrations peak at 10-12.6 min in the blood and at 24-min in the striatum (Gottås, Boix, Øiestad, Vindenes & Mørland, 2014) and then decline very slowly. Morphine metabolism mostly depends on its glucuronidation in the liver. Morphine glucuronidation yields M6G and morphine-3-glucuronide (M3G). Following heroin intravenous administration and inhalation in humans, the ratio of M6G/M3G formation is about 1:6-8 (Rook et al., 2006). In contrast, in the rat, morphine glucuronidation yields, under normal conditions, almost exclusively M3G (Milne, Nation & Somogyi, 1996). However, repeated exposure to opioids can dramatically alter morphine glucuronidation both in humans and rats. Antonilli et al. (2003) found higher concentrations of M6G and lower concentrations of M3G in people with heroin use disorder relative to heroin-naïve patients receiving morphine for pain control.

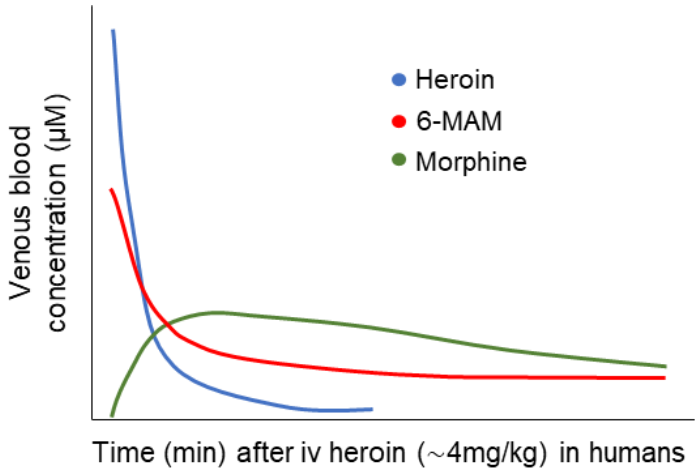

Figure 9. *Pharmacokinetics profile of heroin by intravenous administration and its main metabolites. Time course of venous concentrations of heroin (blue line), 6-MAM (red line), and morphine (green line), after an intravenous injection of heroin (≅4 mg/kg) in humans. Adapted from Milella et al. (2023).*

3.3.2 Pharmacodynamics of heroin and its metabolites

The opioid system and opioid receptors

The pharmacology of the endogenous opioid system is highly intricate (Che & Roth, 2023; Le Merrer, Becker, Befort & Kieffer, 2009) and here the main focus will be on the mu-opioid receptors (MOR) (Pasternak & Pan, 2013) since heroin and its metabolites all are MOR agonists (Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983). All opioid receptors, including MOR, are Gi/o-protein-coupled receptors, whose canonical transduction cascade depends on the action of the αi subunit (with inhibition of adenylyl cyclase and reduced synthesis of cAMP) and of the βγ subunits [resulting in reduced conductance of voltage-gated Ca^{2+} channels and the G protein-coupled inwardly rectifying potassium (GIRK) channels] (Pasternak & Pan, 2013; Williams et al., 2013). These effects ultimately result in the hyperpolarization of the cell and a reduction in neuronal excitability. However, this common perspective was challenged over the years on multiple fronts. Indeed, it was shown that MORs are coupled with alternative transduction mechanisms [e.g., β-arrestin-2 and protein kinase C, (PKC)], as a function of the ligand ('biased agonism') (Conibear & Kelly, 2019). Both β-arrestin-2 and PKC were implicated in the development of tolerance (defined as the need to increase the dose to produce the same effect) after chronic exposure to MOR agonists (Williams et al., 2013). In addition, a variety of omo- and hetero-dimers (involving other

types of opioid receptors and non-opioid G protein-coupled receptors) were identified, each with distinctive transduction pathways (Gomes, Jordan, Gupta, Trapaidze, Nagy & Devi, 2000; Gupta, Décaillot & Devi, 2006).

MORs are encoded by a single structural gene (OPRM1), but there is evidence for alternatively spliced variants of coding exons of the mRNA resulting in polymorphisms. Pasternak and colleagues have long hypothesized that the complexity of splicing (which far exceeds that of receptor subtypes identified using pharmacological tools) might account for the qualitative and quantitative differences in the effects of MOR agonists and the incomplete cross-tolerance among them (Pan, Xu, Xu, Rossi, Matulonis & Pasternak, 2009; Pasternak & Pan, 2013; Schuller et al., 1999).

Endogenously MORs are activated by both enkephalins and β-endorphins. Functionally, MOR activation induces analgesia and reward by exerting its influence across various sites in the central and peripheral nervous system. Acute activation of MORs leads to analgesia, increased locomotor activity, respiratory depression, pruritus, constipation, and immunosuppression (Ninković & Roy, 2013; Pattinson, 2008; Volkow & McLellan, 2016).

Pharmacodynamics of heroin

Heroin is typically considered a prodrug that acts via its conversion to 6-MAM and then to morphine. This is because in vitro opioid-binding studies suggested that heroin acts through its metabolites since it has a very low affinity for the MOR in brain homogenates and its affinity for MOR is much lower than that of morphine and 6-MAM (Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983; Way, Young & Kemp, 1965). By contrast, heroin efficacy, assessed by G-protein activation in GTPγS binding assays, is higher than that of morphine and M6G, and at least comparable to that of 6-MAM (Selley, Cao, Sexton, Schwegel, Martin & Childers, 2001). Accordingly, in CXBK mice, characterized by reduced sensitivity to morphine and by partial deficiency in mu-opioid receptor (MOR) expression, as well as in antisense probes studies targeting exon-1 of the MOR, the analgesic effect of heroin and M6G is retained, while morphine analgesia is suppressed (Schuller et al., 1999).

Pharmacodynamics of 6-MAM

As noted above, 6-MAM has a greater affinity than heroin at MOR (Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983) but the same transduction efficacy, higher than that of downstream metabolites (i.e., morphine) (Selley, Cao, Sexton, Schwegel, Martin & Childers, 2001).

Pharmacodynamics of morphine

Morphine has a slightly higher affinity for the MOR (Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983) but lower efficacy in activating the G-protein cascade relative to 6-MAM or heroin (Selley, Cao, Sexton, Schwegel, Martin & Childers, 2001). The heroin-morphine analgesic potency ratio in humans was estimated to be between 2:1 and 4:1 when administered subcutaneously, intravenously or intramuscularly (Kaiko, Wallenstein, Rogers, Grabinski & Houde, 1981; Robinson, Rowbotham & Smith, 1991) and 1.5:1 when given orally (Twycross, 1977). Comparative studies generally agree that heroin analgesia has faster onset but shorter duration, than morphine analgesia (Twycross, 1977). In addition, heroin has a more favorable profile, in terms of side-effects (e.g., nausea, respiratory depression, dysphoria) than morphine (Haemmig & Tschacher, 2001; Seevers, Pfeiffer & Therapeutics, 1936).

3.3.3. Distinct roles of heroin and its metabolites in heroin effects

As mentioned earlier, heroin has conventionally been regarded as merely a prodrug (see section ‘Pharmacodynamics of heroin’), exerting its effects through its metabolites (Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983; Way, Young & Kemp, 1965). The prevailing assumption is that the effects of heroin exclusively depend on the effects mediated by its metabolites. Moreover, the literature commonly asserts that these effects are primarily attributed to morphine, with limited attention given to the contribution of the other metabolites, such as 6-MAM (Andersen, Ripel, Boix, Normann & Mørland, 2009; Umans & Inturrisi, 1981; Umans & Inturrisi, 1982). Recent emerging evidence indicates that the sequela of heroin effects (e.g. subjective experience, withdrawal effects, etc.) following intravenous injection, are dictated by both heroin and its metabolites. Their dynamic interplay collectively contributes to the overall effects and the progression of heroin addiction.

Humans typically report that heroin 'rush' is particularly robust after intravenous injections (Dorn & South, 1987; Faupel, 1991). The 'rush' of comprises sensations of warmth and pleasure, followed by an extended period of sedation or 'tranquil-high' (Chessick, 1960; Dorn & South, 1987; Faupel, 1991; Kosel et al., 2008; Seecof & Tennant, 1986).

After administration, heroin peaks in the blood at approximately 30-s, coinciding with the 'rush' that some users report (Chessick, 1960; Seecof & Tennant, 1986). In the meanwhile, heroin is still distributed into the brain and other peripheral compartments, and plasma esterases hydrolyze heroin to 6-MAM. Indeed, in humans heroin remains by far the prevailing opioid in plasma for about 8-min (Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a) (**Figure 9**). In rats, in contrast, the opposite occurs, with 6-MAM being the main opioid in blood and brain for about 20-min (Gottas et al., 2013) (**Figure 8**). This suggests that heroin, or the combination of heroin and 6-MAM mediates the rapid 'rush' given by heroin. The rapid 'rush' is typically followed by a relatively extended period known as the 'tranquil-high' or 'nodding,' lasting up to 270-min (**Figure 10**) (Comer, Collins, MacArthur & Fischman, 1999). This period is characterized by sedation and the absence of heroin craving, since immediately following heroin administration craving drops for at least 60-min (Gerber et al., 2012; Walter et al., 2013). Since at this point, the main metabolites of heroin present are 6-MAM and morphine, probably the combination of 6-MAM and morphine mediates the 'tranquil-high' sensation.

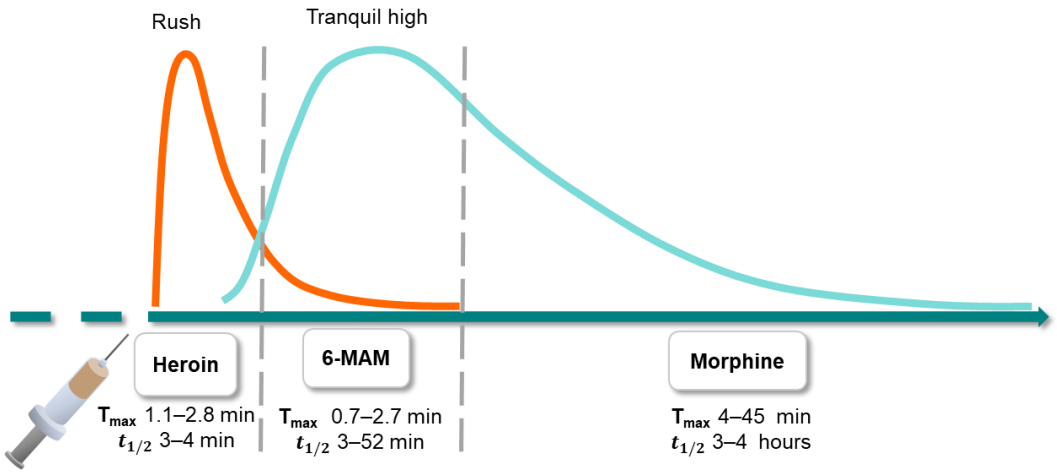

Figure 10. *The hypothetical role of heroin and its metabolites in the sequela of heroin effects after intravenous injection.*

However, Inturrisi et al. (1983) showed that, in in-vitro examination in crude membrane preparations from rat brains, heroin has a lower affinity for the MOR than that of 6-MAM and this suggests that even in the presence of higher concentrations of heroin, MOR would preferentially bind 6-MAM. However, the translation of these findings to humans remains uncertain due to specific binding site limitations in crude membranes and potential species-specific differences. Thus, to the extent that during the first minutes after intravenous injection, heroin, and 6-MAM coexist in the blood and the brain, there is no reason to dismiss the role of heroin itself in producing the heroin 'rush'.

One of the approaches to distinguish the roles of heroin and 6-MAM is to inhibit cholinesterase activity and block the deacetylation of heroin to 6-MAM. Peripheral administration of the cholinesterase inhibitor tri-ortho-tolylphosphate (which does not cross the blood-brain barrier) increases the analgesic potency of heroin (but not that of 6-MAM or morphine) in the mouse (Gianutsos et al., 1986). However, the effects of this manipulation on heroin reward in both animals and humans remain unexplored. Another approach is comparing the brain concentration profiles of heroin and 6-MAM with the time-course of early neurobiological effects in experimental animals. However, potential species-specific differences persist, and conducting such studies in humans is currently impractical.

An alternative approach to exploring the distinct roles of heroin and 6-MAM involves using antibodies that selectively target these compounds. Traditionally, studies have focused on developing antibodies against heroin metabolites. For instance, Avvisati et al. (2019) treated rats with a monoclonal antibody (mAb) targeting 6-MAM. In a self-administration study, they demonstrated that the mAb decreased the re-acquisition of 6-MAM self-administration, but not that of heroin (Avvisati et al., 2019). More recently, Lee et al. (2022) developed a mAb selectively targeting heroin. Through antinociception, pharmacokinetics, and overdose assays, they showed that the mAb against heroin effectively mitigates heroin's psychoactive and lethal effects. While these findings await extension to other behavioral assays, this study suggests that heroin itself may hold pharmacological effects.

After heroin injection, morphine concentrations surpass those of heroin and 6-MAM at about 10-min after intravenous administration of heroin. Morphine binds to MOR with lower affinity than 6-MAM

(Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983) and has lower potency than 6-MAM and heroin (Selley, Cao, Sexton, Schwegel, Martin & Childers, 2001). Nonetheless, morphine has intrinsic rewarding effects and contributes to the overall subjective and behavioral aftereffects of heroin injections. Indeed, the temporal profiles of morphine concentrations do not align with the heroin 'rush' (Chessick, 1960; Seecof & Tennant, 1986), but it overlaps with the subsequent, prolonged feelings of contentment or 'tranquil-high' appreciated by some users (Dorn & South, 1987; Faupel, 1991).

Collectively, these observations underscore that, despite the extensive history of heroin misuse, our understanding of the pharmacology of heroin is still limited. Further studies are needed to understand the dynamics of heroin pharmacology and disentangling the contributions of heroin and its metabolites in the neurobehavioral effects of heroin.

3.4. Summary

In **Chapter 2** I reviewed the most common way in which individuals instrumentalize cocaine and heroin use. In **Chapter 3**, I focused on the pharmacokinetics and pharmacodynamics profile of cocaine and heroin. Below I will contrast cocaine and heroin, outlining how their distinct pharmacokinetics and pharmacodynamics profiles account (at least in part) for their distinct instrumentalization. These observations are relevant for the refinement of animal models of drug addiction[5].

Both cocaine and heroin, when administered intravenously, produce an immediate sensation of euphoria or 'rush' (Seecof & Tennant, 1986). However, the sensation differs qualitatively between the two drugs (Seecof & Tennant, 1986). Cocaine 'rush' is characterized by excitement and increased energy, but also anxiety and agitation, lasting approximately 30-40 min before individuals experience a strong craving

[5] The discussion is focused on intravenous administration since the next Chapter will be centered on animal models of intravenous drug self-administration.

for the drug (Jaffe, Cascella, Kumor & Sherer, 1989; Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978; Resnick, Kestenbaum & Schwartz, 1977). On the other hand, heroin 'rush' is described as an intense and immediate sensation of pleasure, warmth, and relaxation, lasting approximately 2-3 min, followed by a 'tranquil-high' or 'nodding' phase that can last up to 270-min. During this period, there is sedation and an absence of heroin craving due to a reduction in craving for at least 60-min following heroin administration (Gerber et al., 2012; Walter et al., 2013).

In combining this information with the pharmacokinetics profiles of these two drugs, the patterns of drug use among cocaine and heroin users can now be more comprehensively understood. Cocaine users typically administer small doses of cocaine repeatedly every 10-30 min throughout a binge episode (Gawin, 1991; Gawin & Kleber, 1985; Siegel, 1977; Siegel, 1984; Siegel, 1985), use days long breaks between episodes. This pattern is well explained by the drug's 'short' half-life [16-87 min, (Cone, 1995)], as well as the phenomenon of cocaine-induced craving which drives individuals to self-administer cocaine whenever its effects diminish (Jaffe, Cascella, Kumor & Sherer, 1989; Javaid, Fischman, Schuster, Dekirmenjian & Davis, 1978). In contrast, experienced heroin users tend to maintain a more chronic state of intoxication over months or years, typically consuming heroin in 2-4 injected doses per day (Darke, 2011; Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Ross, McCurdy, Kilonzo, Williams, Leshabari & hygiene, 2008). The longer inter-dose intervals in heroin compared to cocaine users is no doubt influenced by the sustained effects of heroin metabolites (Andersen, Ripel, Boix, Normann & Mørland, 2009; Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983; Milella, D'Ottavio, De Pirro, Barra, Caprioli & Badiani, 2023; Rook et al., 2006) and the resultant, sustained reduction in craving following heroin administration (Gerber et al., 2012; Walter et al., 2013).

These considerations do not directly support the application of the highly standardized self-administration procedures [originally conceived and developed for cocaine, (refer to **Chapter 4**)] featuring pre-selected experimenter-imposed unit-doses spaced by timeouts. While these strategies reliably maintain drug self-administration, they create a 'unique case' of drug use behavior that disregards the unique pharmacokinetics and pharmacodynamics profile of every drug (Morgan, Liu, Oleson & Roberts, 2009; Roberts, Gabriele & Zimmer, 2013; Roberts & Zimmer, 2020).

In the forthcoming chapter, I will undertake an examination of the animal models developed over the years highlighting some of their putative weaknesses when applied indiscriminately to drugs with very different pharmacological profiles.

4. Historical excursus of preclinical models of drug addiction

4.1. Introduction

In the previous chapters, I described the putative interplay occurring between the pharmacokinetic and pharmacodynamic properties of heroin and cocaine, in relation to their instrumentalization. In this chapter I discuss animal models of drug self-administration along with the more recent incorporation of drug-vs-nondrug rewards in discrete-choice procedures.

I will first provide a historical overview of preclinical drug self-administration procedures. This will include a description of the various steps that followed the implementation of drug self-administration in laboratory animals. Subsequently, I will provide a comprehensive description of the most used self-administration procedures proposed so far. This includes an examination of the long-access procedure (Ahmed & Koob, 1998), the DSM-IV-based procedure (Deroche-Gamonet, Belin & Piazza, 2004), and the intermittent-access procedure (Zimmer, Oleson & Roberts, 2012). Finally, I will discuss the implementation of drug-vs-nondrug reward discrete choice procedures in self-administration studies.

It is important to highlight that self-administration is just one of the various animal models used by addiction neuroscientists. Over the years, several models, including psychomotor sensitization and conditioned place preference, were proposed. In these models, the animal receives a drug without any contingency, enabling the assessment of drug delivery independent from requiring a voluntary drug taking response. While these models contributed to understanding the neurobiological mechanisms of drugs, a comprehensive description of these preclinical models exceeds the scope of this dissertation. Interested readers are directed to the literature for more in-depth details (Kalivas & Stewart, 1991; Robinson & Becker, 1986; Robinson & Berridge, 1993a; Stewart & Badiani, 1993b; Tzschentke, 2007).

Glossary 2. Behavioral assessment of drug-related behaviors in laboratory animals.

Progressive ratio: reinforcement schedule where the number of responses required to obtain a reward increases progressively after each reward delivery.

Operant extinction: reduction of the frequency of a behavior that was previously reinforced by withholding the reinforcement.

Escalation of drug taking: gradual and progressive rise in drug consumption over time, .

Drug-seeking: Typically refers to non-reinforced lever presses during tests for relapse or reinstatement in different animal models. These tests are done under extinction conditions.

Reinstatement of drug seeking: resumption of drug-seeking behavior after extinction of the drug-reinforced responding, triggered by exposure to drug, drug cues, drug context, or stress.

Drug-induced reinstatement: resumption of drug-seeking behavior after extinction triggered by a non-contingent exposure to the self-administered drug or other drugs.

Cue-induced reinstatement: Typically refers to resumption of drug-seeking behavior after extinction triggered by contingent exposure to discrete drug cues that function as conditioned reinforcers during the reinstatement test.

Stress-induced reinstatement: resumption of drug-seeking behavior after extinction triggered by exposure to a stressor, such as intermittent foot shock.

Incubation of drug craving: increase of drug-seeking behavior, triggered by drug-related cues, during prolonged periods of abstinence.

Behavioral economics: it measures intake as a function of price to determine how resistant baseline "free" or "ideal" drug intake is to increasing prices (i.e., how strong is the "demand" for the drug), this is done by giving rodents less drug (across a descending series of doses) each time they respond on an FR1 reinforcement schedule.

4.2. History of operant drug self-administration in rodents

In an earlier period, the common opinion was that addiction is a phenomenon exclusively human (Lindesmith, 1938; Lindesmith, 1946; Sanchis-Segura & Spanagel, 2006; Spragg, 1940), a hypothesis that was reemphasized in recent times, when the validity of preclinical models of drug addiction was questioned (Field & Kersbergen, 2020). Supporting this assertion, Alfred R. Lindesmith, a key theorist of one of the major sociological theories of addiction, argued that addiction is a complex condition that depends on language and causal inter-relationships between subjective symptoms to the social, economic, and environmental context (Lindesmith, 1938; Lindesmith, 1946) and therefore not amenable to modeling in non-human animals (Lindesmith, 1938; Lindesmith, 1946).

Contrary to this dogmatical viewpoint, a pivotal study conducted by Spragg, in 1940, showed that chimpanzees would 'work intentionally' for morphine (Spragg, 1940). In that study, chimpanzees, made dependent on morphine (through non-contingent drug administration until withdrawal symptoms were observed), would engage in a series of responses to obtain morphine injections. Similarly, years later, Headlee et al. (1955) in 1955 and Weeks (1962) in 1962 demonstrated that rats, previously made dependent on codeine or morphine, learn to engage in a series of behaviors (head turning or lever pressing) which results in the delivery of an intraperitoneal or intravenous injection of a morphine. These studies marked the beginning of modern self-administration studies in rodents.

However, it is noteworthy that the preclinical models of addiction proposed in those studies were based on the common belief that addiction depends on physical dependence (Lindesmith, 1938), indeed laboratory animals were always pre-treated with the drug before the self-administration. In those times, it was considered that only drugs that can induce physical dependence and overt withdrawal symptoms including cramps, nausea, and vomiting like benzodiazepines, alcohol, and opiates, but not psychostimulants, might induce addiction (Jellinek, 1960; Lindesmith, 1938; Solomon & Corbit, 1974). In this framework, addictive drugs are considered drugs that act as negative reinforcers, increasing the behavior of drug use because of their ability to eliminate the unpleasant experience of withdrawal. Despite evidence in humans that supports the presence of specific withdrawal patterns (that differ from those of other addictive drugs) during abstinence from cocaine or amphetamine (Gawin, 1991; Kramer,

Fischman & Littlefield, 1967), the ‘physical dependence theory’ falls short of explaining; (1) the fact that individuals voluntarily consume addictive drugs because of their pleasurable effects (McAuliffe & Gordon, 1974) or (2) why drug use gets established in initially nondependent subjects (Chein, Gerard, Lee & Rosenfeld, 1964; Zinberg & Jacobson, 1976).

Largely based on these considerations, preclinical researchers implemented psychostimulant self-administration in their labs (Thompson, 1968). The first evidence of animals self-administering psychostimulant drugs came from a study conducted by Deneau et al. (1969). In this study, they illustrated that naïve monkeys voluntarily self-administered cocaine and d-amphetamine. Furthermore, they also demonstrated that monkeys spontaneously learned and maintained self-administration of morphine, codeine, pentobarbital, and ethanol, without prior conditioning and without displaying evident withdrawal symptoms. Similarly, Kumar et al. (1968) demonstrated that naïve rats also acquire self-administration of morphine and maintain this behavior in the absence of physical dependence.

This evidence laid the foundation for the interpretation of drug addiction within the framework of Skinnerian theory and operant conditioning principles.

Initially, the adoption of drug self-administration in laboratory animals encountered many obstacles. The initial studies reported a high number of deaths due to several factors that are summarized below. First, animals were trained to self-administer drugs with continuous unrestricted access, 23 hours a day, in a fixed ratio 1 (FR1) schedule of reinforcement (Bozarth & Wise, 1985). Second, drug doses delivered at each lever pressing (unit-doses) were arbitrarily and empirically selected to: (1) sustain self-administration behavior in animals (Deneau, Yanagita & Seevers, 1969) and (2) to assess toxicity associated with self-administration of high doses of the drug (considered an important component of their abuse potential) (Collins, Weeks, Cooper, Good & Russell, 1984; Deneau, Yanagita & Seevers, 1969; Johanson, Balster & Bonese, 1976). Those pre-selected high unit-doses of a drug were reported to disrupt lever pressing and self-administration behaviors, resulting in erratic patterns of drug taking with very few injections per day and long periods of abstinence. This was particularly evident with psychostimulants (Balster & Schuster, 1973b).

These limitations initially prevented the use of these procedures to investigate the effects of drug pretreatments, schedule changes, or lesions over an extended period of several weeks (Roberts, Brebner, Vincler & Lynch, 2002). Notably, a widespread sense of dissatisfaction stemmed also from the fact that the patterns of psychostimulant self-administration were extremely irregular and did not align with those observed with non-drug rewards or other addictive drugs like alcohol, opioids, and barbiturates (Johanson, Balster & Bonese, 1976; Kelleher, 1975).

To mitigate these challenges, researchers modified the drug reinforcement schedules in the following ways: (1) to reduce animal mortality, the daily access to the drug was limited to 2-6 hours (Balster & Schuster, 1973a; Wilson, Hitomi & Schuster, 1971); (2) to achieve a steadier pattern of consumption and decrease behavioral toxicity symptoms, they opted for lower drug doses (Balster & Schuster, 1973b; Kelleher, 1975; Wilson, Hitomi & Schuster, 1971). Additionally, to improve the likelihood of lever-pressing behavior, strategies such as increasing the number of responses needed for a reward or implementing a timeout between successive injections were adopted (Goldberg, 1973).

In conclusion, these stratagems, in conjunction with standardized training procedures, led to the current behavioral assays for the neuropharmacological investigation of drug addiction (Roberts, Morgan & Liu, 2007).

I would like to stress in this context that the selection of dose, dosing frequency, and route of administration was driven more by practical considerations than by behavioral or pharmacological evaluations (Sim-Selley, Selley, Vogt, Childers & Martin, 2000). It is not rare to read in papers sentences concerning the rationale behind the reduction of the drug dose to boost lever pressing (Corre et al., 2018; Fuchs & See, 2002) or as a stratagem to increase the lever pressing during reinstatement tests (see **Glossary 2**) (Shen, Scofield, Boger, Hensley & Kalivas, 2014). Furthermore, many articles improperly report in the materials and methods section the implementation of a timeout between drug injections as a preventive measure against overdoses (Conrad et al., 2008; He, Wang, Li, Wang, Freyberg & Dong, 2023; Mameli et al., 2009; Rocha et al., 1998).

4.3. ‘More is better’: upward shift in dose-response function in the long-access, but not short-access procedure

The realization that laboratory animals could voluntarily self-administer most addictive drugs represented a significant step forward in the addiction field. Until the 1990s, many preclinical addiction studies were based on the premise that voluntary drug consumption alone could lead to addiction in laboratory animals. Notably, the studies mentioned above did not provide valid behavioral evidence of addiction-like behavior in drug-exposed rats, which, led to the refinement of the previously adopted behavioral procedures (Ahmed, 2010; Ahmed, 2012).

A starting point was to improve the face validity and eventually predictive validity of the animal models by incorporating in them some of the critical features of human drug addiction. Among the various self-administration procedures proposed, one of the most popular and widespread across laboratories was developed in 1998 by Serge Ahmed and George Koob, commonly known as ‘long-access’ procedure (Ahmed & Koob, 1998). This procedure represented the first attempt to improve animal models of addiction, aiming at observing addiction-like behaviors in rats, beyond simple drug consumption.

The investigation of Ahmed and Koob (1998) began with the analysis of the ‘short-access’ procedure, featured by limited access to the drug (up to 2 hours). A practical consideration for the duration of the training sessions was that 2 hours were deemed adequate to assess the effects of different manipulations on self-administration behavior (Bozarth, 1987). Under these conditions, rats showed very stable levels of drug intake over many weeks (Goldberg & Stolerman, 1986). However, as outlined in the preceding chapter and remarked by Ahmed and Koob in their seminal study, a crucial aspect of human drug addiction is the escalation of drug use (Ahmed & Koob, 1998; Siegel, 1977; Siegel, 1984) an aspect that was not captured in procedures where drug access was limited to a few hours per day. To address this issue, Ahmed and Koob (1998) investigated whether providing rats with extended access to cocaine, a procedure termed 'long-access', would result in an escalation of drug intake. They compared the progression of cocaine intake in rats trained to self-administer the drug 1 hour/day (short- access group) or 6 h/d (long-access group). Over the testing days, the long-access group exhibited escalated drug intake (primarily observed during the first hour) compared to the short-access rats (Ahmed & Koob, 1998).

Notably, the authors also reported a shift upward in the cocaine dose-response function, indicating an increase in the 'hedonic set point' (Ahmed, Kenny, Koob & Markou, 2002; Ahmed & Koob, 1998). Later, Ahmed et al. (2000) extended these findings to heroin self-administration. They observed that, like cocaine, the long-access procedure, but not the short-access, induced escalated drug intake (Ahmed, Walker & Koob, 2000).

Further studies strengthened the case that the long-access procedure, comparatively to the short-access, was a better model to study human drug addiction. Below, I will detail the key findings that support this concept:

1) Long-access procedure was associated with a markedly increased effort to acquire cocaine (Christensen, Silberberg, Hursh, Roma & Riley, 2008; Paterson & Markou, 2003) and heroin (Lenoir & Ahmed, 2008) when subjected to a progressive ratio reinforcement schedule (refer to **Glossary 2**).

2) Long-access procedure was associated with the development of punishment-resistant drug taking and seeking (APA, 2013; Lüscher, Robbins & Everitt, 2020; Wolffgramm & Heyne, 1995). For example, Ahmed (Ahmed, 2011) investigated the impact of punishment on cocaine self-administration in rats trained under long-access and short-access conditions. Although both groups of rats decreased their cocaine consumption when subjected to an immediate electric shock following each infusion, only the short-access group sustained this decrease after the removal of the shock. These results are supported by additional research indicating that the long-access procedure can lead to compulsive behavior (Pelloux, Everitt & Dickinson, 2007; Vanderschuren & Everitt, 2004). It is important to note that evaluating compulsive opioid use is complicated by its pain-relieving properties, which can occlude the assessment of persistence despite adverse consequences, such as electric shock. This complexity contributes to the scarcity of literature in this area.

3) Long-access procedures in the context of heroin self-administration demonstrated greater resistance to extinguishing heroin-seeking compared to the short-access (Ahmed, Walker & Koob, 2000; Zhou et al., 2009). Notably, these observations have not been replicated with cocaine (Mantsch, Baker, Francis, Katz, Hoks & Serge, 2008; Mantsch, Yuferov, Mathieu-Kia, Ho & Kreek, 2004; Sorge & Stewart, 2005). Rats trained under long-access conditions to cocaine typically exhibited suppressed responding during the initial phases of abstinence compared to rats with limited drug access, but an increased

reinstatement of drug-seeking after prolonged withdrawal (3 weeks) (Ferrario, Gorny, Crombag, Li, Kolb & Robinson, 2005; Grimm, Hope, Wise & Shaham, 2001; Sorge & Stewart, 2005).

In summary, the evidence indicates that the long-access procedure, as opposed to the short-access procedure, more reliably induces addiction-like behaviors in rodents. From these studies, it was posited that under these conditions, the amount of drug consumed is a critical factor in the development of addiction-like behaviors.

As previously argued, a pivotal aspect defining individuals using drugs is the patterns of drug taking. In this respect, short-access and long-access procedures employ fixed doses and restricted access time to drugs, leading to the regularization of patterns of drug taking (Ahmed & Koob, 1998; Ahmed, Walker & Koob, 2000; Morgan, Liu, Oleson & Roberts, 2009; Roberts, Brebner, Vincler & Lynch, 2002). The typical pattern described is characterized by two distinct moments. The initial period of the self-administration session, typically the first 20-min of drug access (Ahmed, Walker & Koob, 2000), is characterized by a 'loading' phase featured by a high rate of infusions (also known as 'bursts' of infusions), which likely reflects the animal attempt to increase the brain-drug concentration above a 'satiety threshold' (Ahmed & Koob, 1998; Ahmed, Walker & Koob, 2000; Tsibulsky & Norman, 1999). After this loading phase, single infusions spaced apart are typically observed. This pattern of responding is often referred to as the 'maintenance' phase, during which animals titrate brain-drug concentrations to a steady state (Tsibulsky & Norman, 1999; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012).

Our understanding of heroin self-administration is limited within the broader context of patterns of drug taking, largely because most of the literature focuses on cocaine.

4.4. Interindividual differences in the prolonged access DSM-IV-based model

As outlined in the preceding chapter, epidemiological studies noted that a relatively small proportion of individuals exposed to addictive drugs develop addiction (Anthony & Helzer, 2002). Typically, these individuals transit from recreational drug use to irregular and excessive drug intake, resulting in tolerance, physical dependence, and addiction. Building on the previous background and because of the growing realization that mere drug self-administration is necessary, but not sufficient, for inducing and identifying

an addiction-like profile or phenotype in laboratory animals, in 2004, Deroche-Gamonet and colleagues (2004) introduced a multi-symptomatic rodent model of cocaine addiction that modeled some of the DSM-IV criteria.

In this procedure, rats were trained for three-months to self-administer cocaine in three daily 40-min sessions separated by 15-min OFF periods. Across the training, three behaviors, based on the DSM-IV criteria, were assessed: (1) persistent drug seeking during periods of drug unavailability (responding during the 15-min OFF periods); (2) high motivation for self-administering cocaine (progressive ratio responding), and 3) willingness to take the drug despite adverse consequences (foot shock punishment) (see **Glossary 2**). Based on the performance of these three measures, an 'addiction' score was assigned to rats. Approximately 20% of the rats met all three 'addiction' criteria, displaying heightened vulnerability to relapse. Notably, this procedure was used only with psychostimulant drugs [cocaine (Deroche-Gamonet, Belin & Piazza, 2004) and methamphetamine (Venniro et al., 2018)] and has never been extended to opioid drugs.

To my knowledge, a singular study using the DSM-IV-based procedure stands out for examining the patterns of cocaine use in rats, specifically comparing those deemed ‘3crit’ (characterized by a high addiction severity score) versus ‘0crit (characterized by a low addiction severity score). (Belin, Balado, Piazza & Deroche-Gamonet, 2009). The authors revealed that short inter-infusion intervals, commonly referred to as burst-like intake, served as a predictive factor for the subsequent severity of cocaine use following prolonged self-administration training. Similarly, Martin-Garcia et al. (2014) used the DSM-IV-based procedure and reported that the ‘high-frequency’ group (characterized by shorter inter-infusion intervals) demonstrated a heightened susceptibility to cocaine-induced reinstatement compared to rats in the ‘low-frequency’ group (characterized by longer inter-infusion intervals).

In summary, the DSM-IV-based procedure was applied exclusively to psychostimulants and has not been broadly adapted for other drugs like heroin. With this narrow focus it is quite surprising that, later, it was proposed a multistep general theory of addiction. It is important to highlight the bias of this ‘general theory’, which is largely derived from data on cocaine or amphetamine, as evidenced by the frequent mentions of cocaine compared to heroin (81 vs 2 respectively) (Badiani, 2014).

4.5. ‘Less is more’: intermittent spikes in drug brain levels increase motivation for drug

The seminal studies by Ahmed et al. (1998) and Deroche-Gamonet et al. (2004) consistently supported the notion that both the duration and quantity of drug exposure play crucial roles in inducing addictive-like behaviors. This contributed to disseminating the prevailing assumption that the outcome is predominantly determined by the level of drug exposure (Ahmed, 2012; Allain, Minogianis, Roberts & Samaha, 2015; Jonkman, Pelloux & Everitt, 2012).

Extending beyond the mere considerations of the duration and quantity of drug exposure, as previously described in **Chapter 2**, human studies revealed that the pattern of drug taking is a critical indicator of the severity of drug consumption and addiction.

In this context, Roberts et al. (2002) initiated a comprehensive investigation into the patterns of cocaine-taking with the goal of closely mimicking the patterns of cocaine-taking observed in humans. These patterns involve irregular episodes of binge cocaine self-administration followed by periods of abstinence (Gawin, 1991). Building on historical studies revealing drug toxicity after cocaine bingeing (Bozarth & Wise, 1985; Deneau, Yanagita & Seevers, 1969; Johanson, Balster & Bonese, 1976) and the work of Miczek’s Lab (Mutschler, Covington & Miczek, 2001; Mutschler & Miczek, 1998; Tornatzky & Miczek, 2000), their goal was to establish a procedure featuring the highest daily cocaine intake as possible but avoiding the large loss of experimental sample due to drug toxicity, commonly observed in those studies (Roberts, Brebner, Vincler & Lynch, 2002).

Their approach involved providing rats with extended access periods while limiting the hourly consumption of cocaine. This was achieved by restricting the opportunities for self-administering cocaine to a defined number of discrete trials per hour. For example, they allowed rats to self-administer cocaine during ‘binge’ periods, lasting from 24 to 72 hours, during which access to cocaine was provided on discrete trials (i.e., 5 trials per hour, 24 hours per day). Using these schedules of drug self-administration, they reported that access conditions, along with the patterns of drug taking, played a critical role in modulating motivation for drugs, as assessed in the progressive ratio schedule of reinforcement (see **Glossary 2**). In a subsequent study, Zimmer et al. (2011) used the hold-down procedure (where holding the lever down turned the syringe pump on and subsequently releasing the lever turned the pump off),

originally introduced by Morgan et al. (2009). The experimental design consisted of repeated ('intermittent' and hence the name of the procedure) access periods (5-min each) followed by forced OFF periods of varying durations (10–25 min). Through this approach, they manipulated brain-cocaine concentrations within a session and demonstrated that longer OFF periods (i.e., 25-min) resulted in lower brain concentrations.

Capitalizing on these studies and with the aim of replicating the intermittent binge pattern of cocaine use observed in humans, Zimmer et al. (2012) finally proposed a procedure commonly referred to as intermittent-access procedure. Here, during the 6-hour daily sessions, cocaine was available in 12 epochs of 5-min – referred to as binge periods – separated by 25-min OFF periods during which the drug was not available – referred to as drug OFF periods. Under these conditions, rats showed a pattern of cocaine self-administration characterized by closely spaced infusions ('bursts'). This pattern of drug self-administration resulted in spiking, rather than steady cocaine brain concentrations (observed under long-access conditions, **Figure 11**). In their study, Zimmer et al. (2012) also conducted a comparison of the intermittent-access, short-access, and long-access procedures. They observed that the total intake in the intermittent-access group was similar in magnitude to the short-access group but substantially lower relative to the long-access group. Despite those remarkable differences in cocaine intake, when assessing the impact of the intermittent-access procedure on the motivation to take cocaine (in a behavioral economic procedure, see **Glossary 2**) they found that the intermittent-access group was associated with a greater motivation for cocaine relative to the long-access group. This result was later reproduced by James et al. (2019).

Subsequent studies investigated other drug-related behaviors in rats trained to self-administer cocaine under short-access, long-access, and intermittent-access conditions. For instance, Nicolas et al. (Nicolas et al., 2019) observed that incubation of craving was higher after intermittent-access than after long-access to cocaine. James et al. (2019) showed that lever pressing on the first day of extinction training, and both cue- and drug-induced reinstatement, following extinction training, were higher after intermittent-access relative to short-access and long-access conditions.

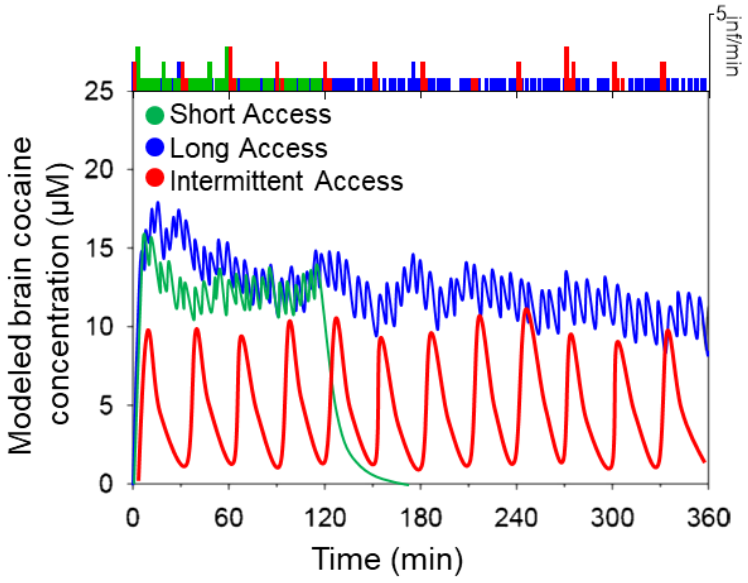

Figure 11. *The pattern of drug taking and estimated cocaine brain concentrations for representative animals tested during three distinct self-administration procedures: short-access (green line), long-access (blue line), and intermittent-access (red line). Adapted from Allain et al. (2015).*

As far as the interindividual differences are concerned, Garcia et al. (2020), by contrasting the long-access and intermittent-access procedures to cocaine observed interindividual differences only in the latter group. Specifically, 40% of rats in the intermittent-access group exhibited escalated drug intake, while 60% maintained stable intake levels across sessions. In addition, the subpopulation of rats that escalated their cocaine intake displayed a higher degree of locomotor sensitization, and higher levels of cue-induced reinstatement (see **Glossary 2**).

A key insight from these studies is that contrary to earlier assertions (Ahmed, 1998; Deroche-Gamonet, 2004), it is the temporal pattern of cocaine self-administration, rather than the total amount consumed, that significantly affects the motivation to seek and use cocaine. This revision emphasizes the importance of the timing and frequency of drug use on motivational behaviors.

As already stated in the introduction, in addiction neuroscience, most drug self-administration procedures were developed for cocaine and subsequently translated to other addictive drugs without pharmacokinetics and pharmacodynamics considerations. Not surprisingly in 2020 O'Neal et al. (2020)

used the intermittent-access procedure with heroin. In their study, the authors demonstrated that intermittent- access effectively maintained heroin self-administration and led to the manifestation of interindividual differences in various addiction-like behaviors. Rats deemed as 'high-risk', compared to 'low-risk', exhibited higher heroin self-administration, higher seeking during drug-unavailable OFF periods, elevated motivation for heroin (as revealed by the progressive ratio), increased responding throughout extinction training, and stronger cue-induced reinstatement.

The sole study available that directly compares the intermittent-access procedure with the more commonly used long-access and short-access procedures was conducted with fentanyl. Fragale et al. (2021) showed that intermittent-access to fentanyl, when compared to rats trained under long-access or short-access conditions, resulted in a more pronounced escalation of fentanyl intake, heightened motivation for fentanyl on a behavioral economics task, sustained drug seeking during abstinence and extinction, and increased cue-induced reinstatement of extinguished fentanyl seeking (see **Glossary 2**).

As noted previously, drug pharmacokinetics and pharmacodynamics influence the patterns of drug taking, and cocaine, fentanyl, and heroin differ significantly in these factors. In my discussion, I will contend that it is imprudent to directly transfer procedures such as long-access and intermittent-access, which were primarily developed for cocaine, to other addictive drugs without considering the pharmacokinetics/pharmacodynamics of the specific drug and considering how individuals with addiction may instrumentalize the drug.

4.6. Optimizing drug self-administration procedures by introduction of non-drug alternatives: evolution of choice procedures and contemporary applications

In a natural setting, individuals with drug addiction persist in 'chasing the high' or pain relief given by the drug at the expense of more adaptive alternative socially valued reinforcers (APA, 2013; Banks & Negus, 2017; Bornstein & Pickard, 2020; Heilig, Epstein, Nader & Shaham, 2016; Heyman, 2009; Hogarth, 2020; Volkow, Baler & Goldstein, 2011). This progressive neglect of alternative rewards typically results in a significant decline in the individual's socio-economic status (e.g., job loss, marital dissolution,

legal repercussions, etc.). Under normal circumstances, these adverse consequences should be sufficient to motivate and sustain abstinence from drug use (Faupel, 1991; Siegel, 1977). By contrast, individuals with addiction persist in seeking and using drugs, indicating a compromised ability to make rational, and voluntary choices toward adaptive alternative socially valued reinforcers (Bechara, 2005; Heyman, 2009; Redish, 2004). Correspondingly, in laboratory human studies, employing choice procedures between drug and non-drug rewards, it was demonstrated that individuals who use opioids place a lower value on money (an alternative non-drug socially valued reward) compared to non-drug users (Madden, Petry, Badger & Bickel, 1997). Based on this evidence, addiction diagnosis adopts a behavior-centric approach, suggesting that these disorders stem from an imbalanced allocation of behaviors between the substance being abused and other non-drug reinforcers. This concept of misallocation is also reflected in the DSM criteria for addiction (APA, 2013; Banks & Negus, 2017; Wesson, Smith & Monograph, 1985).

Several preclinical studies examined drug self-administration and related neurobehavioral alterations in a context where other options were available. Studies have also proposed that the limited availability of alternative options could clarify why nearly all laboratory animals learn to self-administer drugs and eventually relapse to drug-seeking (Ahmed, 2010; Ahmed, 2012; Ahmed, Badiani, Miczek & Müller, 2020). Based on that, it was suggested that a main challenge in preclinical addiction research lies in distinguishing which laboratory animals are genuinely addicted to the drug and which ones simply use it as an activity due to the absence of better alternatives (Ahmed, 2010; Herrnstein, 1970).

Investigations aimed at mimicking the characteristic maladaptive choice of addiction in laboratory animals were carried out in some of the earliest animal studies on drug addiction and stemmed from much older insights (Spragg, 1940; Tatum & Seevers, 1929).

In 1940 Spragg (Spragg, 1940) found that chimpanzees who were physically dependent on morphine and had withdrawal symptoms opted for morphine instead of fruit. Subsequently, several studies employing choice procedures between food and cocaine or heroin in non-human primates provided compelling evidence supporting the influential role of alternative reinforcers in drug self-

administration (Aigner & Balster, 1978; Elsmore, Fletcher, Conrad & Sodetz, 1980; Griffiths, Wurster & Brady, 1975; Nader & Woolverton, 1991; Woolverton & Balster, 1979; Wurster, Griffiths, Findley & Brady, 1977). Carroll and her colleagues later extended these results to rats (Carroll & Lac, 1993; Carroll, Lac & Nygaard, 1989). They demonstrated that providing rats with concurrent access to sweet water reduced the likelihood of rats acquiring cocaine self-administration and maintained cocaine self-administration after acquisition (Carroll & Lac, 1993; Carroll, Lac & Nygaard, 1989).

Building on this evidence, the laboratory directed by Ahmed expanded the generality and validity of the choice procedure across a broad spectrum of factors and conditions (Cantin et al., 2010; Lenoir & Ahmed, 2008; Lenoir, Cantin, Vanhille, Serre & Ahmed, 2013; Lenoir, Serre, Cantin & Ahmed, 2007). Indeed, in their first study, investigating the choice between cocaine and sweet water, almost all rats (90%) preferred the non-drug reward (Lenoir, Serre, Cantin & Ahmed, 2007). Certainly, these findings were surprising, prompting Ahmed and colleagues to thoroughly examine all aspects that might influence the preference for sweet water over the drug. In the case of cocaine, their investigations revealed that the preference for sweet water persisted despite various manipulations, including higher cocaine intake, an escalation of cocaine dose in the choice procedure, and even when the rats were under the effects of cocaine (Lenoir, Serre, Cantin & Ahmed, 2007).

Several subsequent studies have built upon and expanded these investigations to include other addictive drugs, such as methamphetamine, heroin, fentanyl, and alcohol. In brief, certain studies employing discrete choice procedures involving drugs like methamphetamine, heroin, and fentanyl, and palatable food pellets, indicated that nearly all the rats consistently preferred food over drugs, even after extended daily exposure to relatively high doses of drugs (Caprioli, Zeric, Thorndike & Venniro, 2015; Reiner et al., 2020; Venniro, Zhang, Shaham & Caprioli, 2017). Notably, this phenomenon was termed choice-based voluntary abstinence (Caprioli et al., 2015; Venniro et al., 2017; Venniro, Zhang, Shaham & Caprioli, 2017).

Other studies have instead observed interindividual differences in drug preference using discrete choice procedures. For instance, Lenoir et al. (2008) observed that, after extended access exposure to

heroin, approximately 51% of rats exhibited a preference for heroin over sweet water. Augier et al. (2018) discovered that 15% of rats chose alcohol over sweet water. Heinsbroek et al. (2021) reported an average population preference of 50% for heroin versus palatable food pellets in rats. Finally, Padovan-Hernandez et al. (2022) reported that around 23.5% of rats persisted in displaying a preference for cocaine over sucrose pellets.

Of note, the discrepancies observed between studies presented above were mainly attributed to the fact that drug preference is extremely sensitive to several aspects. For instance, manipulations that modify the relative cost (i.e., response requirement) (Nader & Woolverton, 1990), the magnitude of the reward (food or drugs) (Cantin et al., 2010; Nader & Woolverton, 1990; Panlilio, Secci, Schindler & Bradberry, 2017), pre-choice drug exposure, and reward availability (Tunstall & Kearns, 2014), as well as the delay of the reward (food or drugs) (Canchy, Girardeau, Durand, Vouillac-Mendoza & Ahmed, 2021; Panlilio, Secci, Schindler & Bradberry, 2017; Secci, Factor, Schindler & Panlilio, 2016; Woolverton & Anderson, 2006).

Overall, these discoveries fueled the concept that providing alternative non-drug rewards to laboratory animals in drug self-administration procedures could have diminished drug taking (if not entirely suppressed), leaving only a small proportion of rats sustaining drug consumption. Thus, investigators suggested that choice procedures could be used as a screening for addiction ‘vulnerable’ versus addiction ‘resilient’ rats, an idea supported by several reports (Ahmed, 2010; Ahmed, 2018; Lenoir, Cantin, Vanhille, Serre & Ahmed, 2013; Lenoir, Serre, Cantin & Ahmed, 2007). Building on this body of evidence, Augier et al. (2018) used a choice procedure that revealed approximately 15% of rats preferring alcohol to sweet water. This study identified a molecular mechanism underpinning the preference for alcohol over an alternative reward, shedding light on the development of addiction in vulnerable individuals Similarly, Heinsbroek et al. (2021) identified a neural circuit involved in opioid preference that limits the selection of heroin when food is presented as an alternative reward. These studies represent initial steps in exploring the neural correlates of interindividual differences in drug versus natural reward preferences.

From a refinement perspective, however, while the food versus drug choice procedure may be a valid method for identifying vulnerable animals, studies revealed an additional translational gap between preclinical animal models and the human condition (Heilig, Epstein, Nader & Shaham, 2016). In natural settings, numerous alternatives to drug use exist, and food is not the sole reward that competes with and is effective in reducing drug use. Among these highly rewarding alternatives, social interactions play a significant role in diminishing the inclination toward drug consumption (Azrin, Acierno, Kogan, Donohue, Besalel & McMahon, 1996). Notably, one of the most effective behavioral treatments for drug addiction, the community reinforcement approach, promotes abstinence by providing voluntary social interactions with social reinforcers, such as support groups and positive work environments (Hunt & Azrin, 1973; Silverman, DeFulio & Sigurdsson, 2012). In a similar vein, earlier preclinical investigations reported that social interaction can influence drug taking. The passive exposure of a rat engaged in self-administration to an abstinent partner exhibits a protective effect, reducing cocaine intake in the self-administering rat (Giorla et al., 2022; Robinson, Fronk, Zhang, Tonidandel & Smith, 2017; Robinson, Lacy, Strickland, Magee & Smith, 2016; Smith, 2012; Strickland & Smith, 2014).

Based on this evidence, with the aim of narrowing the translational gap in choice procedures, Venniro et al. (2018) introduced an animal model that incorporates a choice procedure where rats are allowed to voluntarily choose between interacting with a social partner or taking addictive drugs. They used this procedure to investigate the impact of rewarding social interaction on drug self-administration, in rats that underwent procedures designed to mimic crucial aspects of human addiction [e.g. escalation of drug intake (Ahmed & Koob, 1998)]. They illustrated that voluntary social interaction prevented drug taking in rats. Rats exhibited a strong preference for social interaction over methamphetamine and heroin, abruptly ceasing to self-administer drugs when social rewards were provided. An interesting aspect was that this effect was independent of the amount of exposure to methamphetamine and the severity of addiction, established based on the three-criteria DSM-IV-based model previously described (Deroche-Gamonet, Belin & Piazza, 2004). Subsequent studies further illustrated that the protective effect of social interaction on drug self-administration extends to other drugs, such as heroin and cocaine (Venniro, Panlilio, Epstein & Shaham, 2021; Venniro, Russell, Zhang & Shaham, 2019).

However, as previously anticipated drug choice procedures are sensitive to experimenter-imposed contingencies. In their initial study, Venniro et al. (2018) illustrated that drug preference over social could be elicited by introducing a delay between social-lever press and social reward or by punishing social-lever presses with foot shock. However, the increased drug preference, suggesting potential susceptibility to drug addiction, did not correlate with any addiction measures from established models (e.g., intense drug consumption or seeking behavior and resilience to punishment in drug self-administration). In a subsequent study, by Shaham's Lab, it was shown that rats shift their preference for remifentanil over social interaction in a dose-dependent manner, i.e. when high doses of remifentanil are provided rats prefer drug over social interaction (Chow et al., 2022). Similarly, Marcus et al. (2022) corroborated these findings with cocaine, showing that at higher doses of cocaine, rats preferred the drug over social interaction. Finally, St. Onge et al. (2022) observed that the fentanyl versus social interaction choice procedure was sensitive to environmental manipulations such as the dose of the drug provided and the response requirement for the drug. Specifically, as the unit-dose or response requirement for fentanyl increased, the preference for fentanyl progressively rose, reaching almost exclusive fentanyl choice with the largest dose of fentanyl or the highest response requirement.

In the studies discussed above, the experimental manipulations applied were designed to induce a shift toward the drug reward. Across these manipulations, all tested laboratory animals consistently shifted their preference toward the drug reward, with no observed interindividual differences. Notably, the study conducted by Venniro et al. (2021) demonstrated considerable interindividual variability after applying experimental manipulations. In this study, the preference for social access was diminished by delaying both rewards or social reward alone, or by increasing response requirements for social reward. However, as of now, genuine evidence supporting the existence of interindividual differences in social versus drug choice is lacking.

4.7. Conclusions

In this chapter, I offered an overview of the historical evolution and status of animal models of drug addiction. This section will outline the inconsistencies among the primary drug self-administration

procedures in addiction research, as well as the disparities between these procedures and the findings from human studies. This overview will serve as a foundation for the experimental design of the present dissertation.

The predominant observation is that the refinement of the intravenous self-administration procedures largely revolved around human cocaine addiction-based considerations. The same effort was not applied to other addictive drugs. This observation holds significance due to the vast diversity observed in drug taking behaviors among individuals, which is predominantly influenced by the distinct characteristics inherent to the drug being consumed.

Another notable observation is that, while all the various preclinical addiction models proposed strived to replicate specific human behaviors in rats, all these models were grounded on the same 'reductionistic' approach. Self-administration procedures were developed with the primary objective of achieving relatively high and regular patterns of drug taking across various addictive drugs, individuals, and self-administration sessions (Goldberg, 1973; Goldberg & Stolerman, 1986; Kelleher, 1975). To obtain the regular patterns of drug taking described, self-administration procedures were based on 'discrete dimension strategies' (Morgan, Liu, Oleson & Roberts, 2009). These strategies entail experimenter-imposed unit-doses of drugs, interspersed by timeouts (Kelleher, 1975), pre-selected to maintain a steady and high rate of response, aligning more closely with the response rate observed with food reinforcers, which are elective reinforcers commonly used within the Skinnerian framework (Goldberg, 1973; Yokel, 1987). Despite that pre-selected experimenter-imposed unit-doses, spaced by timeouts, can reliably maintain drug self-administration, the implementation of these strategies creates a 'unique case' of drug taking behavior (Morgan, Liu, Oleson & Roberts, 2009; Roberts, Gabriele & Zimmer, 2013; Roberts & Zimmer, 2020). This strategy prevents the laboratory animal from self-selecting the appropriate dose-time relationship of drug administration. Furthermore, it is worth noting that this logic percolated in the choice procedures, which traditionally utilize an experimenter-imposed fixed unit-dose of drug against an alternative reward and disregarding the evidence that at the beginning of the sessions, rats typically display 'drug loading' behavior, even if spaced apart by the imposition of timeouts.

These observations represent a weakness of animal models of drug addiction for two main reasons.

Firstly, they challenge the fundamental principle of the dose-effect relationship and its role in shaping drug effects and consumption patterns. Typically, the pharmacological effects of drugs are closely tied to the administered dose. However, many studies utilize small doses on the ascending limb (e.g. 0.25 mg/kg/inf of cocaine), despite reports indicating a preference for larger doses (e.g., 1.5 mg/kg/inf of cocaine) in laboratory animals (Desai, Tron Esqueda & Norman, 2023; Pickens, Thompson & therapeutics, 1968). Of note, Roberts and Zimmer argued that single unit-doses are insufficient to achieve the 'preferred drug brain levels' (Roberts & Zimmer, 2020). Similarly, in human laboratory drug self-administration studies, it was shown that individuals with heroin addiction effectively adjust their operant work to obtain the desired amount of heroin (Mello & Mendelson, 1987), but they prefer to wait several hours for taking large doses of heroin, instead of taking a smaller dose every few hours (Meyer & Mirin, 1979).

Secondly, regular patterns of drug intake are rarely observed in individuals with drug addiction. Consequently, this raises concerns regarding the face validity and appropriateness of replicating the heterogeneity observed in drug-related behaviors among drug users. These limitations highlight the need for refinement and adaptation of animal models to better capture the complexities of human drug addiction.

5. Increased heroin intake and relapse vulnerability in intermittent-access relative to long-access[6] self-administration: Sex differences in rats[7]

5.1. Abstract

Background. Studies using intermittent-access drug self-administration procedures show increased motivation to take and seek cocaine and fentanyl, relative to long-access. In this study, I examined the effects of intermittent-access and long-access self-administration on heroin intake, patterns of self-administration, and cue-induced heroin-seeking, after forced or voluntary abstinence, in male and female rats. I also estimate brain levels of heroin and its active metabolites.

Methods. Rats were trained to self-administer a palatable solution and then heroin (0.075 mg/kg per inf) either continuously (6 h/d; 10 days) or intermittently (6 h/d; 5-min access every 30-min; 10 days). Brain levels of heroin and its metabolites were estimated using a pharmacokinetic software. Next, heroin-seeking was assessed after 1 or 21 abstinence days. Between tests, rats underwent either forced or voluntary abstinence. The estrous cycle was measured using a vaginal smear test.

Results. Intermittent-access exacerbated heroin self-administration and was characterized by a burst-like intake, yielding higher brain peak concentrations of heroin and 6-MAM concentrations. Moreover, intermittent-access increased cue-induced heroin-seeking during early, but not late abstinence. Heroin-seeking was higher in females after intermittent-access, but not long-access, and this effect was independent of the estrous cycle.

Discussion. Intermittent-access to heroin in rats resembles critical features of heroin use disorder: a pattern of drug taking characterized by repeated large doses of heroin and higher relapse vulnerability

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[6] In the original publication, the term 'continuous' was used to describe the long-access procedure. However, in this dissertation, the term 'continuous' refers to a procedure that differs from the long-access.

[7] This Chapter is extracted from D'Ottavio G, Reverte I, Ragozzino D, Meringolo M, Milella MS, Boix F*, et al.* (2023). Increased heroin intake and relapse vulnerability in intermittent relative to continuous self-administration: Sex differences in rats. Br J Pharmacol 180**:** 910-926. https://doi.org/10.1111/bph.15791

during early abstinence. This has significant implications for refining animal models of substance use disorder and for a better understanding of the neuroadaptations responsible for it.

5.2. Specific aims of the project

The goal of the experiment was to compare the effect of two different drug self-administration procedures, long-access, and intermittent-access on: (1) drug taking and related patterns of drug taking; (2) estimated brain concentrations of heroin and its active metabolites; (3) incubation of heroin craving, after forced or voluntary abstinence; (4) sex differences and the role of the ovarian hormones on craving. The experiment consisted of three phases: self-administration training, an abstinence period (either forced or voluntary), and relapse tests. After the relapse tests, the estrous cycle was measured in the female rats.

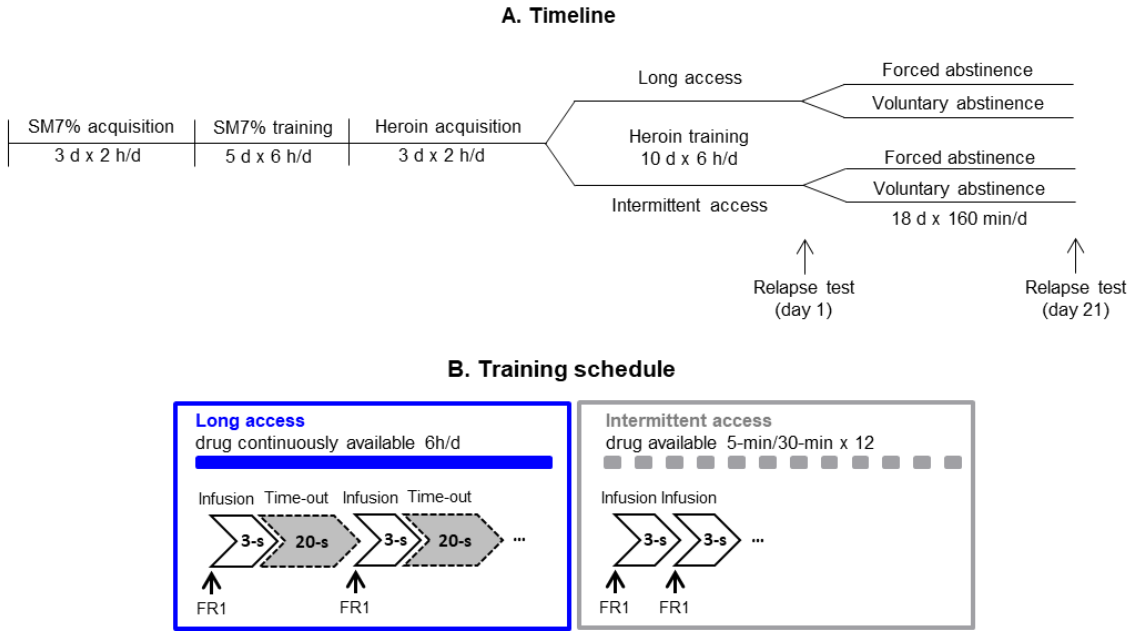

Figure 12. *(**A**) Timeline of the experiment and (**B**) Training schedule.*

5.3. Materials and Methods

Subjects. A total of 124 male and female Sprague-Dawley rats (Charles River, Lecco) were used. Rats were 5-6 weeks old at the beginning of the experiment (150-175 g male, 125-150 g female). The rats were pair-housed prior to the surgery and then housed individually after surgery. Rats were maintained on a reversed 12-h light/dark cycle (lights off at 6 AM) with free access to standard laboratory chow and water throughout the entire experiment. All the procedures followed the guidelines of national law (DL 26/2014) on the use of animals for research based on the European Communities Council Directive (2010/63/UE) and were approved by the ethics committee of the Italian Ministry of Health and by the local Ethical Committee of the Santa Lucia Foundation. 12 rats (5 male, 7 female) were excluded due to catheter problems or sickness. 10 female rats after the self-administration training were used for another study.

Drug. Heroin hydrochloride (diamorphine) (S.a.l.a.r.s., Como, Italy) was dissolved in sterile saline (0.9% NaCl). For the self-administration training, a unit-dose of 0.075 mg/kg/infusion was chosen based on a previous study (O'Neal, Nooney, Thien & Ferguson, 2020).

Self-administration apparatus. Rats were trained in self-administration chambers located inside sound-attenuating cubicles, fitted with an electric fan, and controlled by a custom-made system. Each chamber was equipped with a stainless-steel grid floor, and two operant panels were placed on the left and right walls. The left panel of the chamber was equipped with a house light that signaled the insertion and subsequent availability of the heroin-paired active (retractable) lever. Responses on this lever activated the infusion pump and the discrete white-light cue located above the lever and heroin was delivered through a modified cannula (Plastics One; Roanoke, VA, USA) connected to a liquid swivel (Instech; Plymouth Meeting, PA, USA) via polyethylene-50 tubing that was protected by a metal spring. In addition, the left wall was equipped with an inactive (stationary) lever, responses on this lever had no consequence (i.e., non-reinforced). The right panel was equipped with the palatable solution-paired active (retractable) lever. Responses on this lever activated the infusion pump and the three-light cue located

above the lever. The 1-ml palatable solution was delivered to a receptacle located near the solution-paired lever, connected with silicon tubing to a syringe that contained the palatable solution.

Experiment 1. A comparison between long-access and intermittent-access to heroin

Palatable solution self-administration. The training procedure was similar to the one described in previous studies (Caprioli et al., 2015; Caprioli, Zeric, Thorndike & Venniro, 2015; Venniro et al., 2017). However, the palatable food pellets (TestDiet, Catalogue #1811155) were substituted with a palatable solution since the cages used were not equipped with pellet dispensers. The palatable solution contained a concentration of 7% sucrose and 7% maltodextrin (SM7%), matching the amount of carbohydrates contained in the palatable food pellets previously used. Rats (n=53 male, n=71 female) were first trained to self-administer SM7% 2 h/d for 3 days (acquisition phase; maximum rewards = 20) and then for 6 h/d for 5 days (training phase; maximum rewards = 55). The training sessions started with the illumination of the house light and the insertion of the SM7% solution-paired lever (that remained inserted throughout the session); responses on this lever resulted in the delivery of 1 ml of the SM7% solution (15-s), paired with the illumination of the three-light cue (20-s).

Intravenous surgery. Rats underwent intravenous catheterization after palatable solution self-administration. Rats were anesthetized with isoflurane (5% induction, 2–3% maintenance) and injected with Carprofen (2 mg/kg, subcutaneous injection; Zoetis Italia srl), immediately after the surgery and for the following 5 days to relieve pain and decrease inflammation. The silastic catheter was inserted into the jugular vein as previously described (Caprioli, Zeric, Thorndike & Venniro, 2015). The distal end of the catheter was placed into the right jugular vein and was attached to the proximal end of a modified 22-gauge cannula placed on the back in the mid-scapular region. The catheters were flushed daily with a 0.2 ml sterile saline solution containing gentamicin (4.25 mg/ml; Fatro S.p.A.) to prevent occlusion during the recovery, training, and abstinence phases. Rats were allowed to recover for a minimum of 5-7 days. The catheter patency was tested daily with the sterile saline and gentamicin solution and if during the training the catheter failed the test, the rats underwent intravenous catheterization of the left jugular vein, with the same procedure for the right, or were eliminated from the study.

Heroin self-administration. Heroin self-administration training was divided into two phases: acquisition and training. In the acquisition phase, rats were trained to self-administer heroin (0.1 ml/3-s; 0.075 mg/kg/infusion) 2 h/d for 3 days (maximum infusions = 20) on a fixed ratio 1 (FR1), 20-s timeout reinforcement schedule. The sessions started with the insertion of the two levers (active and inactive) and the illumination of the house light. Responses on the active lever (FR1) were reinforced by unit-doses of heroin, paired with the cue light, followed by a 20-s timeout during which lever pressing was not reinforced and the cue light was on. Subsequently, for the training phase, rats were divided into two groups (matched in terms of their total heroin intake during acquisition, **Statistical Table 1**): one group was trained using the long-access procedure (n=26 male, n=37 female) and the other using the intermittent-access procedure (n=27 male, n=34 female). In the long-access condition, the rats had long-access to heroin 6 h/d on FR1, 20-s timeout reinforcement schedule. The sessions started with the insertion of the two levers and the illumination of the house light. Responses on the active lever were reinforced by unit-doses of heroin, paired with the cue light, followed by a 20-s timeout during which lever pressing was not reinforced and the cue light was on. In the intermittent-access condition, the rats had access to heroin for a total of 60 min during each 6-hour daily session, comprised of 12, 5-min ON periods (drug available) and 12, 25-min OFF periods (drug unavailable and levers retracted). During ON periods, heroin was available according to an FR1 reinforcement schedule with no timeout imposed (except for the time of the infusion) as a result of lever pressing. The length of the OFF periods was set based on a previous study (O'Neal, Nooney, Thien & Ferguson, 2020) and corresponded to the time needed for blood- and brain-levels of heroin and 6-MAM to dissipate (Gottas et al., 2013). Each 5-min ON period started with the insertion of the two levers and illumination of the house light and ended with the retraction of the levers and shutdown of the house light. Responses on the active lever were reinforced by unit-doses of heroin, paired with a cue light (3-s), followed by no timeout. The rats were trained in long-access or intermittent-access conditions for 10 days (training phase; maximum infusions = 90/day) and after every three consecutive drug self-administration sessions, the preference between palatable solution and heroin was assessed using the following discrete-choice test procedure.

Discrete-choice procedure. The discrete choice test sessions were conducted using the same parameters (dose of heroin and dose of SM7% solution per reward and stimuli associated with the two active retractable levers) used during the training phase. Rats were allowed to choose between the heroin-paired and the SM7% solution-paired levers in a discrete choice procedure. Each 160-min choice session was divided into 20 discrete trials that were separated by 6-min. Briefly, each trial began with the presentation of the house light followed 10-s later by the insertion of both the SM7% solution-paired and heroin-paired levers. Rats then could select one of the two levers. The operant response requirement for the lever's selection was set to two consecutive responses (FR2) to avoid accidental choices (Vandaele, Cantin, Serre, Vouillac-Mendoza & Ahmed, 2016). If the rats responded within 2 mins, they received the reward corresponding with the selected lever. Reward delivery was signaled by the heroin-associated or SM7% solution-associated cue (20-s), the retraction of both levers and the house light turned off. If the rats failed to respond on either active lever within 2 mins, both levers were retracted, and the house light was turned off with no reward delivery.

Estimated brain levels of heroin and its metabolites. The theoretical brain levels of heroin and its active metabolites, 6-MAM and morphine were estimated in representative rats using the FitMultiMicroExtravascular model for multiple administered doses, in the software program Kinetica v.5.1 (Thermo Fisher Scientific Inc). The input for the calculation was the timing of single unit-doses during the 10th self-administration session and the absolute dose amount (μmol) of heroin received per infusion, after correcting the dose to the weight of each animal. The estimations were based on the kinetic parameters acquired from the fitting of the brain extracellular fluid concentrations in a study by Gottås et al. (2013) (**Table 1**). In this study, the authors assessed levels of heroin and its metabolites, in blood and brain extracellular fluid, in male Sprague-Dawley rats, after a single passive intravenous heroin administration (1.3 mg; for a detailed account of the pharmacokinetics modeling refer to **Chapter 4)**.

One methodological consideration in the present study is that brain concentrations were estimated from intravenous heroin self-administration data based on parameters derived from an acute intravenous administration in male rats (Gottas et al., 2013). While the present pharmacokinetics findings should therefore be interpreted with caution, it is unlikely that this aspect would significantly alter the estimated

brain levels. This is because previous clinical studies based on intravenous, oral, and subcutaneous administration of opioids (oxycodone, codeine, and morphine) did not report significant changes in acute vs chronic opioid metabolism (Kimbrough et al., 2020; Zernig et al., 2007). Finally, brain levels of heroin and its metabolites were estimated only in male rats, since two studies revealed sex differences in opioid pharmacokinetics after intraperitoneal or oral administration (Chan, Edwards, Wyse & Smith, 2008; Djurendic-Brenesel, Mimica-Dukic, Pilija & Tasic, 2010). Notably, in the present study, no sex differences in patterns of heroin-taking were observed, including differences in total intake, frequency of intake, and inter-infusion intervals. This suggests that the pharmacokinetic determinants of the frequency and pattern of intravenous heroin self-administration across sexes are similar.

The following differential equations were used by Kinetica v.5.1 (Thermo Fisher Scientific Inc) to calculate the parameters to fit the brain extracellular fluid (ECF) concentrations of heroin, 6-MAM, and morphine, where Co refers to the concentration at time t; A, B, and C are coefficients that describe the exponential functions; α, β, and γ are exponents that describe the shape of the measured concentration curve for each phase; Ka is the absorption rate; and tl refers to t-lag at time t.

- One compartment:

$$Co = A\,\frac{Ka}{Ka-\propto}(e^{-\propto tl} - e^{-ka\,tl})$$

- Two compartments:

$$Co = A\,\frac{Ka}{Ka-\propto}(e^{-\propto tl} - e^{-ka\,tl}) + B\,\frac{Ka/}{Ka-\beta}\,(e^{(-\beta\,tl)} - e^{(-ka\,tl)})$$

- Three compartments:

$$Co = A\,\frac{Ka}{Ka-\propto}(e^{-\propto tl} - e^{-ka\,tl}) + B\,\frac{Ka}{Ka-\beta}\left(e^{(-\beta\,tl)} - e^{(-ka\,tl)}\right) + C\,\frac{Ka}{Ka-\gamma}\,(e^{(-\gamma\,tl)} - e^{(-ka\,tl)}$$

From these “macro” constants, the pharmacokinetics parameters (**Table 1**) are calculated as follows:

- One compartment:

$$Kel = \alpha$$

- Two compartments:

$$Co = A + B$$

$$K_{21} = \frac{A * \beta + B * \alpha}{Co}$$

$$K_{12} = \alpha + \beta - (K_{21} + Kel)$$

$$Kel = \frac{\alpha * \beta}{K_{21}}$$

- Three compartments:

$$Co = A + B + C$$

$$a = \alpha + \beta + \gamma$$

$$b = \frac{C * \alpha + B * \alpha + A * \gamma + B * \gamma + A * \beta + C * \beta}{-Co}$$

$$c = \frac{C * \alpha * \beta + B * \alpha * \gamma + A * \beta * \gamma}{Co}$$

$$K_{31} = \frac{-b - \sqrt{(b^2 - 4c)}}{2}$$

$$K_{21} = -b - K_{31}$$

$$Kel = \frac{\alpha * \beta * \gamma}{K_{21} - K_{31}}$$

$$K_{12} = \frac{(\beta * \gamma + \alpha * \beta + \alpha * \gamma - K_{21} * a - Kel * K_{31} + K_{21}^2)}{(K_{31} - K_{21})}$$

$$K_{13} = a - (Kel + K_{12} + K_{21} + K_{31})$$

These same equations are used during simulation to calculate the theoretical concentrations when the parameters are known. For the administration of multiple doses, the software uses the superposition principle by independently computing the concentrations for each dose administered and afterward adding the calculated concentrations for each time point of the session.

Parameter	**Ka**	**lag**	**Volume**	**Kel**	**K12**	**K21**	**K13**	**K31**
Unit	min^{-1}	min	L	min^{-1}	min^{-1}	min^{-1}	min^{-1}	min^{-1}
Heroin[1]	0.749072	0.757626	0.401859	2.18171				
6-MAM[2]	1.00001	1.52153	0.417121	0.0695501	0.00802414	0.032289		
Morphine[3]	0.050732	1.22008	0.873335	0.0418547	0.114092	0.115256	0.0182552	0.012605

Table 1. *Pharmacokinetics parameters used in Gottås et al. (2013) to fit the concentrations of heroin, 6-monoacetylmorphine (6-MAM), and morphine in the brain extracellular fluid after intravenous administration of 3 µmol (1.3 mg) heroin in the rat (Boix, Andersen & Mørland, 2013). These same parameters were applied to the FitMultiMicroExtravascular model of the software program Kinetica v.5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) to simulate the brain concentrations taking into account the times of the single unit-doses during the 10th self-administration training session.*
[1] One compartment extravascular model
[2] Two compartments extravascular model
[3] Three compartments extravascular model
Ka: Absorption rate constant from the injection site. Lag: Time taken to appear in the brain following administration. Kel: Elimination rate constant from the brain. Volume: Volume of distribution. KXY: Transfer rate constant between compartments X and Y (1 = measured compartment)

Abstinence period group assignment. After the relapse test on Abstinence day 1, long-access and intermittent-access rats were randomly assigned to the forced or voluntary abstinence condition.

Voluntary abstinence group. Rats (long-access n=9 male / n=13 female, intermittent-access n=12 male / n=14 female) were allowed to choose between heroin (one infusion) and palatable solution (one delivery) during 20 discrete-choice trials for 18 days (Caprioli et al., 2015).

Forced abstinence group. The rats were brought (long-access n=12 male / n=14 female, intermittent-access n=15 male / n=13 female) to their home cages and handled twice a week.

Relapse test. Rats were tested for heroin-seeking under extinction conditions on Abstinence day 1 and 21 (after forced or voluntary abstinence). The duration of the test sessions was 30-min to minimize the carryover effect of extinction learning on Abstinence day 1, which may decrease drug-seeking on Abstinence day 21 (Caprioli et al., 2015). The sessions began with the illumination of the house-light, followed 10-s later by the insertion of the heroin-paired lever; the house-light remained on for the duration of the session. Lever presses during the tests resulted in the contingent presentation of the light cue, previously paired with unit-doses of heroin, but no heroin infusion was delivered.

Estrous cycle. In female rats (n=48) the estrous cycle was monitored daily (for 5 days) before the relapse test and immediately after the relapse test on Abstinence day 1 and Abstinence day 21 by a vaginal cytological test (Nicolas et al., 2019). A cotton tip, moistened with saline, was rolled into the vaginal orifice to collect the vaginal smear (Goldman, Murr & Cooper, 2007). Then the cotton tip was rolled onto a microscope slide and analyzed within 5-min, using an Olympus BX51 microscope (20x magnification). Samples were classified into two macro-phases: estrus and non-estrus [as previously described (Nicolas et al., 2019)]. The estrus phase is characterized by a prevalence of cornified cells. The non-estrus phase includes: (1) proestrus with a prevalence of nucleated epithelial cells; (2) metestrus with the same proportion of leucocytes, nucleated epithelial cells, and cornified cells; (3) diestrus with a prevalence of leukocytes.

Statistical analysis.

The statistical analysis was undertaken only for studies where each group size was at least n=5. The group sizes are the number of independent observations, and the statistical analyses were performed using these independent observations. The group size for female rats was much larger than the group size for male rats since female rats, after the relapse tests, were further divided into two groups based on their estrous cycle phase. Data were analyzed with the statistical program SPSS (Version 25, GLM procedure; SPSS) or GraphPad Prism (Version 8.0.1; GraphPad Prism). I included outliers in the data analysis and presentation. The level of probability (p), for determining group differences, was set at $p<0.05$. I followed significant main effects and interactions ($p<0.05$) with post-hoc tests (Fisher's PLSD)

which were conducted only if the F values in the analyses achieved the appropriate level of statistical significance and the statistical measures of homogeneity of variance were not significant. For the palatable solution self-administration, the data were analyzed separately for rewards using the between-subject factor of Sex (male, female) and the within-subject factor of Session. For heroin self-administration, data were analyzed separately for unit-doses using the between-subject factors of Sex and Access (long, intermittent) and the within-subjects factor of Session. For the discrete choice tests and voluntary abstinence, I normalized the indifference level between palatable food pellets and heroin (preference score) at 0 using the following formula: [1 - (% drug choices/50%)] (Lenoir, Serre, Cantin & Ahmed, 2007) and analyzed the data using the between-subject factors of Sex and Access and the within-subjects factor Session. For the long-access group, the average of the inter-infusion intervals in the 10th self-administration session was analyzed using the between-subject factor of Sex. For the intermittent-access group, the average infusion during the 5-min ON periods in the 10th self-administration session was analyzed using the between-subject factor of Sex. For the cumulative infusions in the 10^{th} self-administration session, the cumulative 1-min infusions were analyzed using a multifactorial analysis and the between-subject factor of Sex. For the min x min infusions, in the intermittent-access group, the average of unit-doses earned during each min was analyzed using the between-subject factor of Sex and the within-subject factor of Session. For the relapse tests, the active-lever presses were analyzed using the between-subjects factors of Sex, Access, and Abstinence condition (forced, voluntary) and the within-subjects factor of Abstinence day (1, 21) and I included the inactive lever presses as a covariate. For the estrous cycle in the relapse test Abstinence day 1, the active lever presses on Abstinence day 1 were analyzed using the between-subjects factors of Access and Cycle phase (estrus, non-estrus). In all analyses of the relapse tests, the number of inactive-lever presses was used as a covariate to statistically control for the effect of the abstinence period on non-specific (training independent) lever presses during testing.

5.4. Results

The multifactorial ANOVAs yielded multiple main and interaction effects, thus only significant effects, which are critical for data interpretation, were reported in this section (see **Statistical Table 1** for a complete reporting of the statistical analyses and their exact p-values).

Experiment 1. A comparison between long-access and intermittent-access to heroin

Sucrose+Maltodextin 7% (SM7%) self-administration. During acquisition, male and female rats increased their SM7% intake and lever pressing over sessions (**Figure 13A-B, left**). Female rats self-administered significantly more ml/kg of SM7% relative to their body weight compared to male rats (**Figure 13A, left**). During training, female rats had a significantly higher SM7% intake relative to male rats (**Figure 13A, right**).

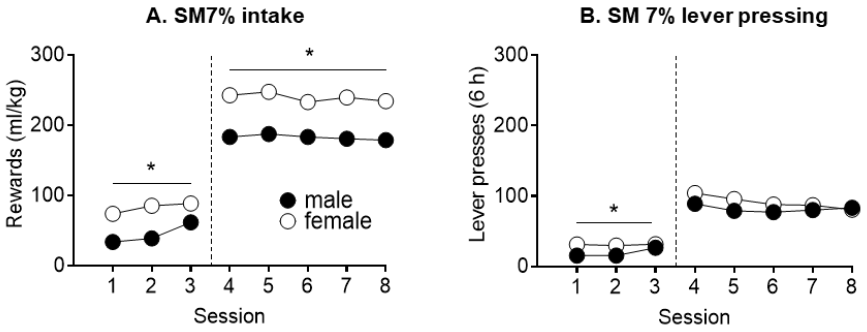

Figure 13. *SM7% self-administration. (**A**) Timeline of the experiment. (**B**) SM7% self-administration. Intake (left): mean±SEM number of SM7% rewards earned (1 ml infusion) during sessions. Lever pressing (right): mean±SEM number of lever presses during sessions. *Different from males, p<0.001 (males n=53 / females n=71).*

Heroin self-administration. During acquisition, no sex differences were observed in the number and frequency of infusions (infusions/min) and active lever presses (**Figure 14A, B, C**). During training, male and female rats increased their heroin intake over time in both access conditions, but the total heroin and frequency of intake were significantly higher in the intermittent-access relative to the long-access condition (**Figure 14A, B**). Notably, the intermittent-access group, both male and female, earned most of the infusions during the first min of the 5-min ON periods (**Figure 15B**). Additionally, a progressive increase in heroin intake during the first min was evident across sessions (**Figure 15B**). Furthermore, the

pattern of drug taking in either the intermittent-access or the long-access conditions did not differ between sexes (**Figure 14A, 14A**), and the estrous cycle did not influence heroin self-administration regardless of the access conditions (**Figure 16**). Notably, the lack of sex differences in our study agrees with those from earlier studies, demonstrating similar heroin self-administration in male and female rats and mice over a range of unit-doses (Mayberry et al., 2022; Stewart, Woodside & Shaham, 1996; Towers, Tunstall, McCracken, Vendruscolo & Koob, 2019; Venniro, Russell, Zhang & Shaham, 2019).

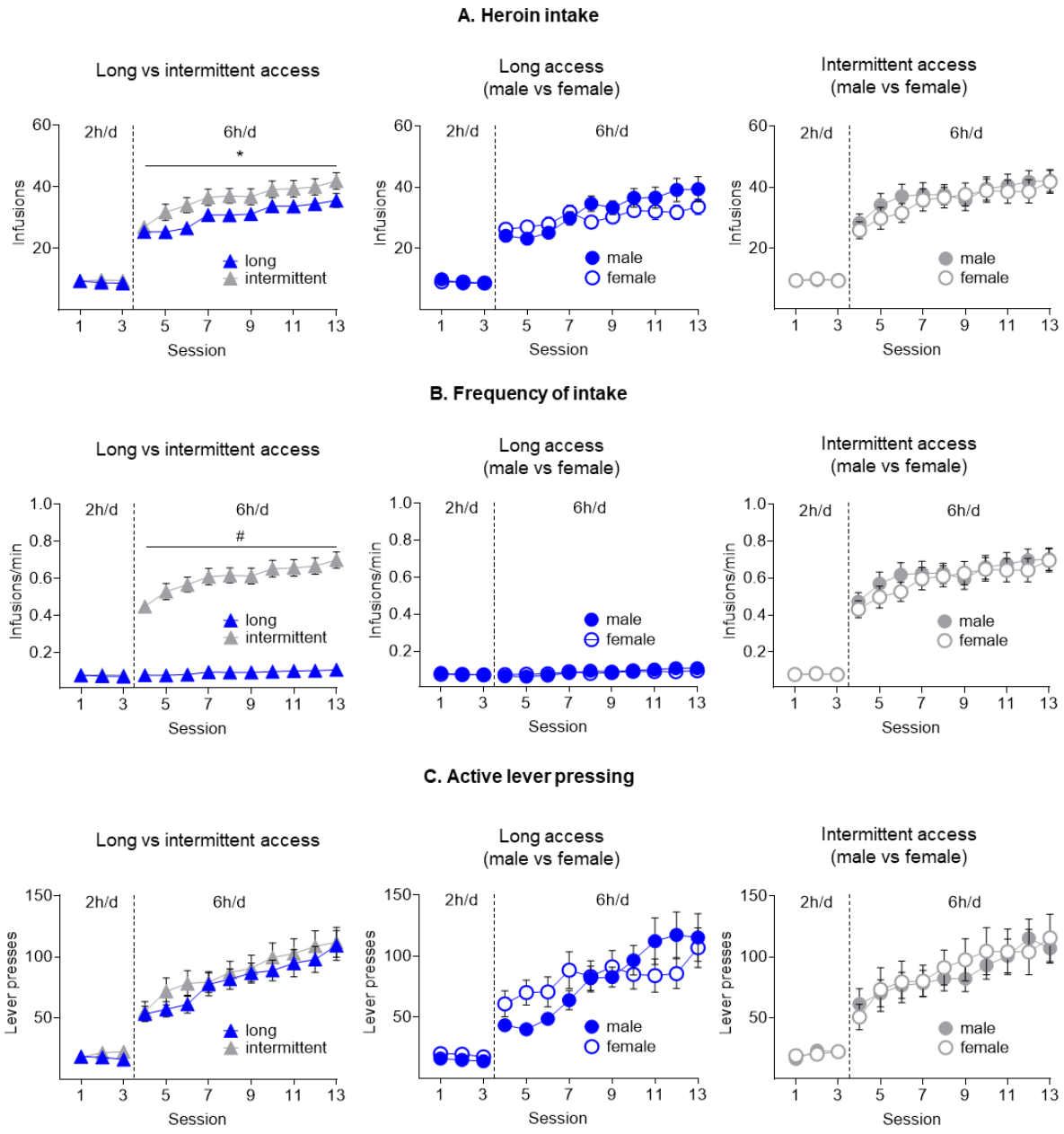

Figure 14. *Long-access and intermittent-access heroin self-administration in male and female rats. (**A**) Heroin intake. Mean±SEM number of heroin infusions (0.075 mg/kg/infusion) per session. * Different from long-access condition p<0.05.(**B**) Frequency of intake. Mean±SEM number of heroin infusions per min of access per session. # Different from long-access condition p<0.05. (**C**) Active-lever pressing. Mean±SEM active lever presses per session. (long-access: males n=26 / females n=37; intermittent-access males n=27 / females n=34).*

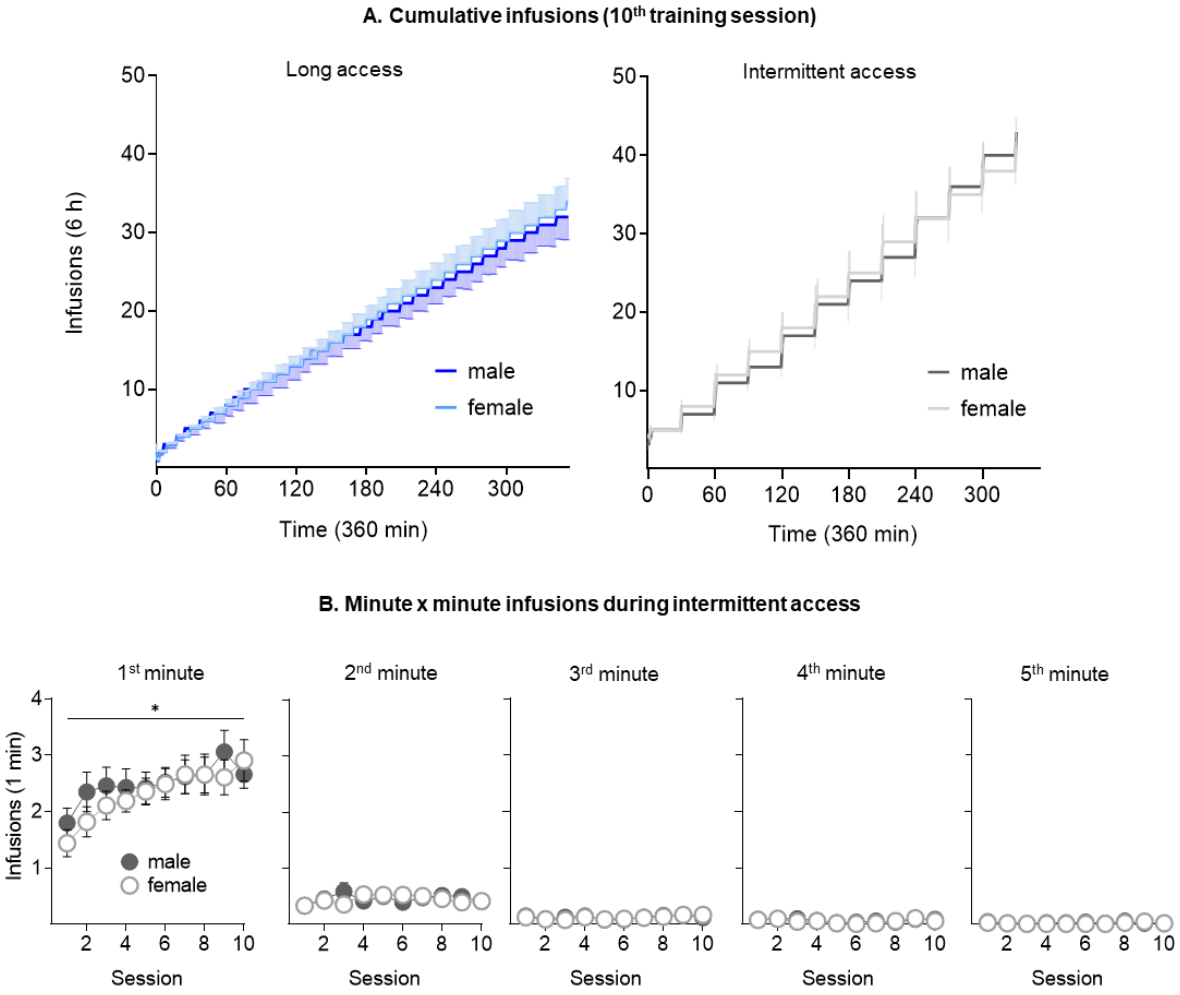

Figure 15. *Temporal pattern of infusions. (**A**) Cumulative infusions (10th training session). Cumulative infusions earned during the 10th self-administration session in male and female rats trained under either long-access (left) or intermittent-access (right). (**B**) Min x min infusions during each 5-min ON intermittent-access. Mean±SEM average of heroin infusions earned during each min of the sessions. *Intake increases throughout sessions, p<0.05. (long-access: males n=26 / females n=37; intermittent-access: males n=27 / females n=34).*

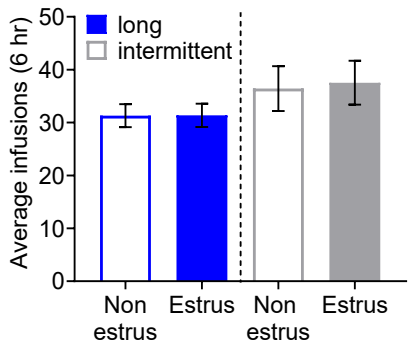

Figure 16. *Effect of estrous cycle on long-access or intermittent-access heroin self-administration in female rats. Heroin intake: estrous cycle. Mean±SEM of the average of infusions earned during non-estrus and estrus phase in the last five days of heroin self-administration (n=48).*

Estimated brain concentrations of heroin, 6-MAM, and morphine. A kinetic simulation model carried out on data from the last session of self-administration (10th training session; **Figure 17**), showed sharp peaks of brain heroin levels with fast rises and falls to zero, consistent with the short half-life of heroin (~0.9-min). These peaks coincide with the infusion of heroin and were higher in rats in the intermittent-access relative to the long-access condition. The short half-life of brain heroin (Gottas et al., 2013) implies little accumulation in the long-access condition. During the 20-s timeout immediately following infusion, the low concentrations of heroin that reach the brain are already being metabolized to 6-MAM by carboxylesterases.(Andersen, Ripel, Boix, Normann & Mørland, 2009; Gottås, Boix, Øiestad, Vindenes & Mørland, 2014). 6-MAM levels rise fast (T_{max} ~4.3-min) but decline much slower, due to its longer half-life ($t_{1/2}$ ~23.3-min vs. ~0.9-min) (Gottas et al., 2013). In both the long-access and intermittent-access conditions, 6-MAM reached ~5 fold higher concentrations than heroin). However, in the intermittent-access condition 6-MAM peaks were up to 4-fold higher than in the long-access condition. In both access conditions, morphine increased throughout the session.

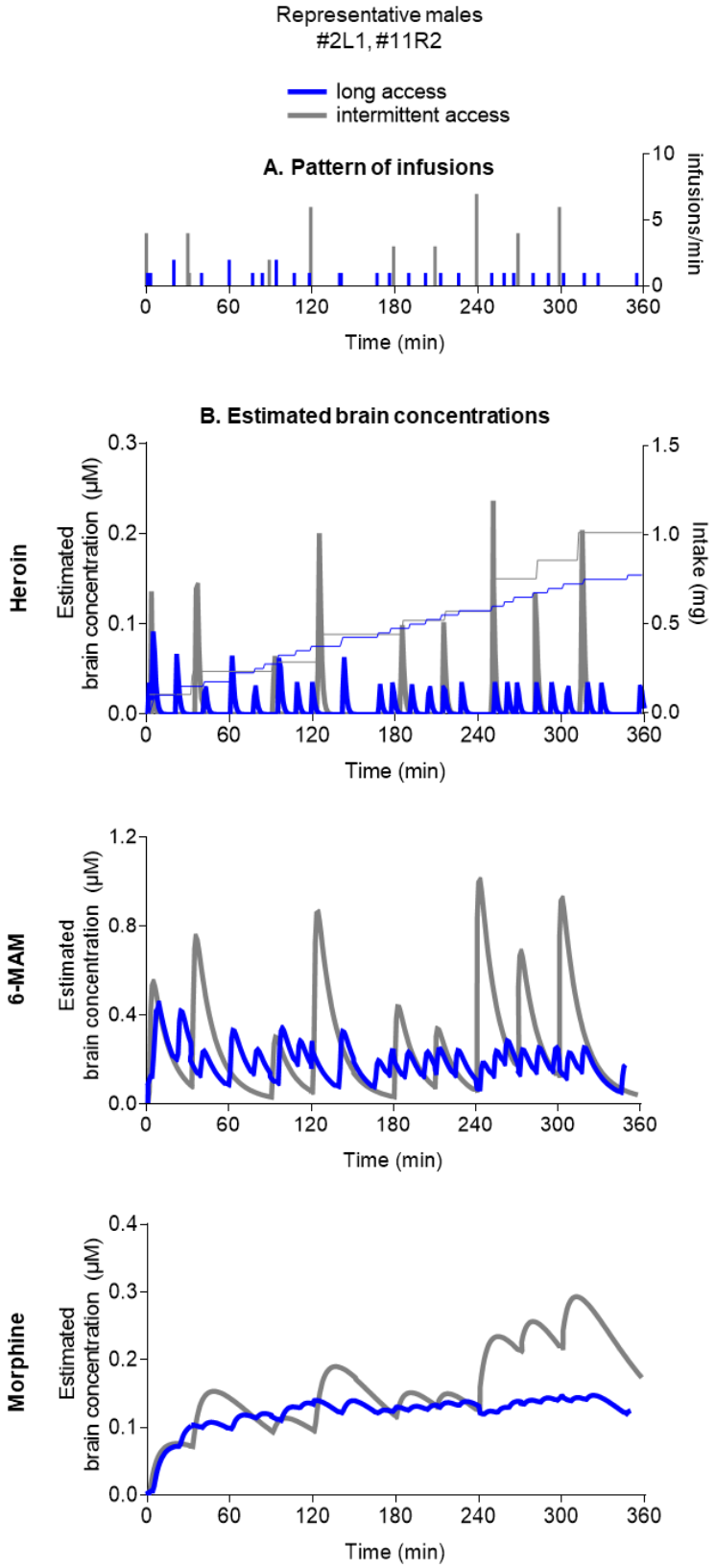
Representative males
#2L1, #11R2
long access
intermittent access
A. Pattern of infusions
infusions/min
Time (min)
B. Estimated brain concentrations
Heroin
Estimated brain concentration (μM)
Intake (mg)
Time (min)
6-MAM
Estimated brain concentration (μM)
Time (min)
Morphine
Estimated brain concentration (μM)
Time (min)

Figure 17. *Cumulative heroin intake and estimated brain concentrations of heroin and its active metabolites in representative male rats trained under either long-access or intermittent-access to heroin. (A) Pattern of infusions. (B) Estimated brain concentrations. Estimated brain concentrations of heroin, 6-MAM, and morphine (left axis) and cumulative intake (right axis) throughout the 10th heroin self-administration training session of representative rats (infusions: long-access = 31; intermittent-access = 39). Note the scales are adapted to the levels of each compound.*

Voluntary abstinence. During the voluntary abstinence, rats from both access conditions displayed a strong preference for SM7% relative to heroin and increased choices for SM7% across sessions (**Figure 18**).

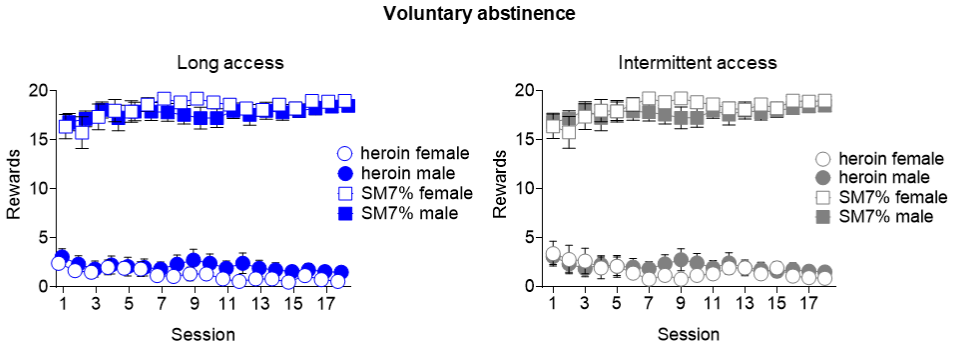

Figure 18. *Voluntary abstinence. Mean±SEM of SM7% reward and heroin infusions earned during the 18 discrete-choice sessions in male and female rats trained under either long-access (left) or intermittent-access (right). (long-access males n=9 / females n=13, intermittent-access males n=12 / females n=14).*

Incubation of heroin craving. Incubation of heroin craving was observed in the long-access but not in the intermittent-access condition after forced abstinence, with higher lever pressing on Abstinence day 21 than on Abstinence day 1. In particular, rats in the intermittent-access condition did not show an 'incubated' profile because of the higher heroin-seeking on Abstinence day 1, which was similar to Abstinence day 21 (**Figure 19A, left and center**). The time course of heroin-seeking on Abstinence day 1 was significantly higher in the intermittent-access relative to the long-access condition (**Figure 19B, left**). No differences between access conditions were observed on Abstinence day 21 (**Figure 19C, left**). In the long-access condition, voluntary abstinence prevented the incubation of heroin craving (**Figure 19A, center**), as previously reported (Venniro, Zhang, Shaham & Caprioli, 2017). In the intermittent-access condition, lever responding on both Abstinence day 1 and 21 was higher in female rats than in male rats; this effect was independent of the abstinence condition (**Figure 19A, right**).

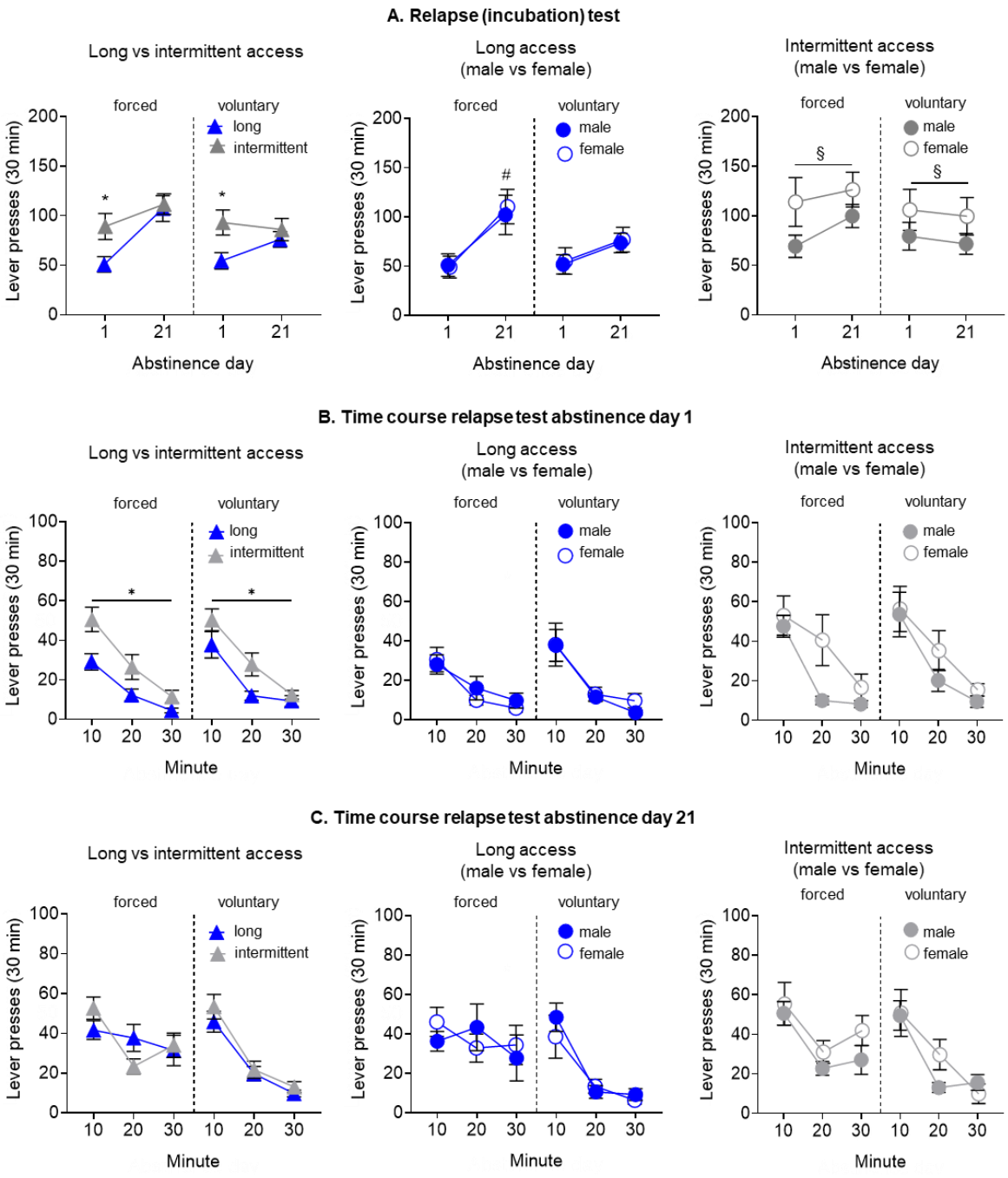

Figure 19. *Relapse tests. (**A**) Relapse (incubation) test. Each left side of the graphs shows the data from the forced abstinence condition, while the right side from the voluntary abstinence condition. Data are mean±SEM of lever presses on the active lever during the 30-min extinction test on Abstinence day 1 and on Abstinence day 21. * Different from long-access, p<0.05. # Different from Abstinence day 1, p<0.05. § Different from males, p<0.05. (**B**) Time course relapse test Abstinence day 1. Data are mean±SEM of lever presses at each 10-min of the test session on Abstinence day 1. * Different from long-access. (**C**) Time course relapse test Abstinence day 21. Data are mean±SEM of lever presses at each 10-min of the test session on Abstinence day 21. (voluntary abstinence: long-access males n=9/females n=13, intermittent-access males n=12 / females n=14; forced abstinence: long-access males n=12 / females n=14, intermittent-access males n=15 / females n=13).*

Incubation of heroin craving and the estrous cycle. I observed no significant effect of the estrous cycle on the incubation of heroin craving (**Figure 20**). Female rats, in both access conditions, displayed a similar lever pressing during the estrus and non-estrus phase during the relapse tests.

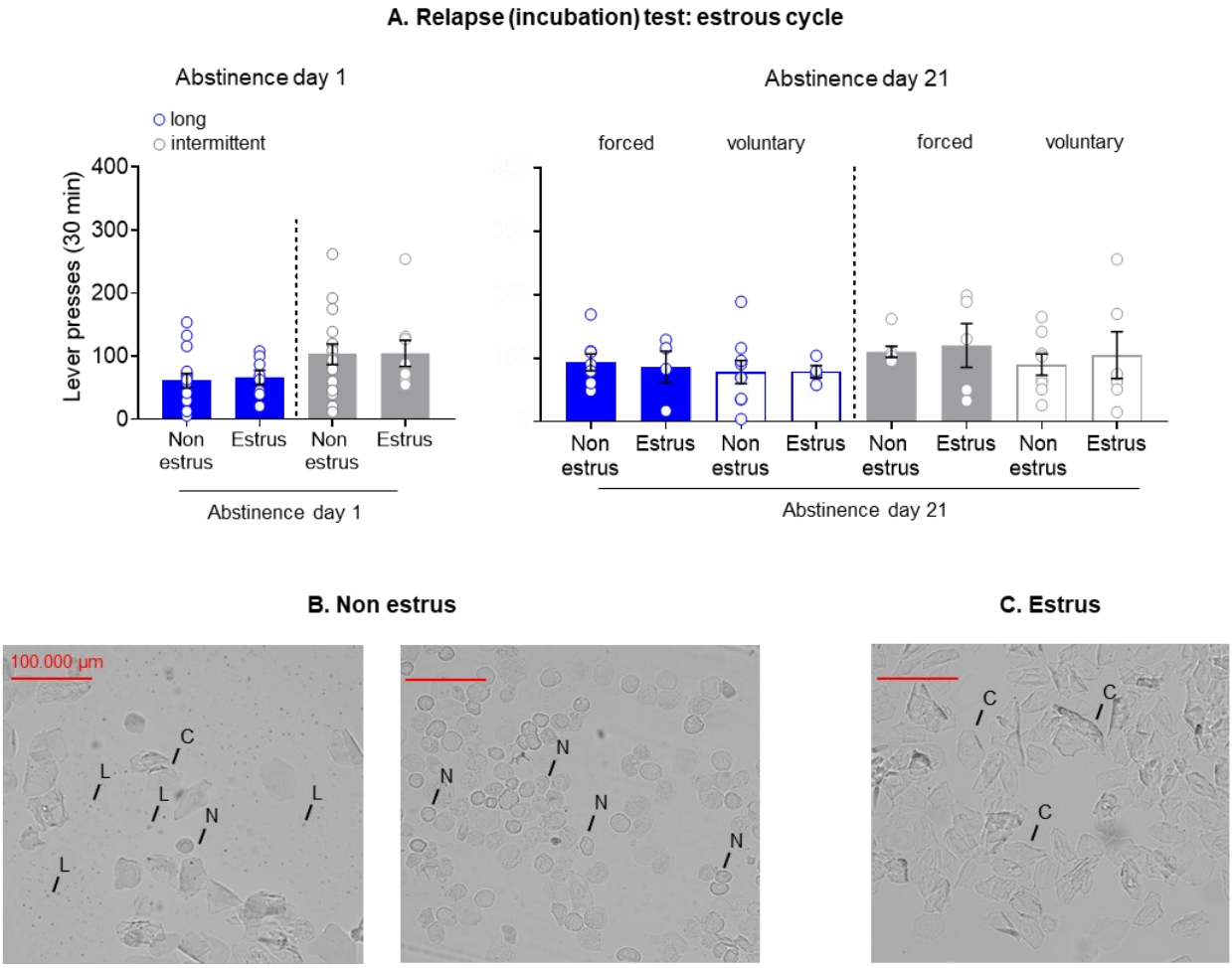

Figure 20. *Effect of the estrous cycle in incubation of craving after long-access or intermittent-access heroin self-administration in female rats. (**A**) Relapse (incubation) test: estrous cycle. Mean±SEM of lever presses on the active lever during the extinction sessions on Abstinence day 1 (left) and 21 (right). (**B**) Non estrus. Representative wet, unstained vaginal smears of the non-estrus phase of the rat estrous cycle. Metestrus (left): same proportion of leucocytes (L), nucleated epithelial cells (N), and cornified cells (C). Proestrus (right): prevalence of nucleated epithelial cells. (**C**) Estrus. Representative wet, unstained vaginal smears of the estrus phase of the rat estrous cycle: prevalence of cornified cells. (Abstinence day 1: long-access non-estrus n=14 / estrus n=9; intermittent-access non-estrus n=16 / estrus n=9. Abstinence day 21 voluntary abstinence: long-access non-estrus n=9 / estrus n=4; intermittent-access non-estrus n=8/estrus n=6. Abstinence day 21 forced abstinence: long-access non-estrus n=8 / estrus n=4; intermittent-access non-estrus n=5 / estrus n=4).*

5.5. Discussion

There are three main findings in this study. First, the overall heroin intake and estimated peaks in brain levels of heroin and 6-MAM were much higher during intermittent-access relative to the long-access conditions. Second, intermittent-access to heroin was followed by higher heroin-seeking during early abstinence (Abstinence day 1) that remained stable over time (Abstinence day 21). This phenomenon was more pronounced in female rats. Third, the estrous cycle was not associated with the magnitude of relapse to heroin-seeking, regardless of training conditions.

5.5.1. Heroin intake and estimated brain concentrations of heroin and its active metabolites during intermittent-access and long-access self-administration

The main unexpected finding was that despite the much shorter drug access, heroin intake was significantly higher in the intermittent-access than in the long-access condition (**Figure 14A**). This higher intake was accompanied by patterns of drug taking characterized by closely spaced infusions (bursts) mainly concentrated in the first minute of each 5-min access period (**Figure 15A**). In contrast, the long-access condition (**Figure 15B**) featured a more regular pattern of intake, with single infusions spaced apart, and with peaks and troughs in 6-MAM levels maintained within a tighter range (**Figure 15A**). In the following paragraphs, we discuss two distinct, but not mutually exclusive explanations, accounting for these divergent results.

The *first* interpretation of this data is based on the kinetics of heroin metabolism. Following intravenous administration, heroin is very quickly metabolized into 6-MAM and then into morphine (Gottas et al., 2013; Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983; Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a; Way, Kemp, Young & Grassetti, 1960), both pharmacologically active compounds (Andersen, Ripel, Boix, Normann & Mørland, 2009; Kvello, Andersen, Boix, Morland & Bogen, 2020; Umans & Inturrisi, 1982).

The divergent results across access conditions might therefore be due to the long timeout imposed after each injection (20-s in our study; but can vary up to 40-s in other studies) present in the long-access but not in the intermittent-access condition (see Materials and Methods).

Heroin has a very short terminal half-life [~0.9-min (~54-s) in the rat brain (Gottas et al., 2013)]. Therefore, in the long-access condition, during the 20-s timeout, carboxylesterases are already metabolizing heroin to 6-MAM (Andersen, Ripel, Boix, Normann & Mørland, 2009; Gottås, Boix, Øiestad, Vindenes & Mørland, 2014), a phenomenon that counteracts heroin accumulation in the brain. This is exemplified by the low heroin peaks observed in the long-access relative to the intermittent-access condition (**Figure 17B**). On the other hand, the lack of this timeout period in the intermittent-access condition allowed rats to infuse heroin in a burst-like pattern. This pattern of drug taking led to a significant heroin and 6-MAM accumulation in the brain (**Figure 17B**). In other words, the rats trained under long-access conditions couldn't reach the high peaks observed in the intermittent-access condition, no matter the rate of lever pressing.

The metabolite 6-MAM is thought to contribute to the rapid onset of heroin effects (Andersen, Ripel, Boix, Normann & Mørland, 2009; Gottås, Boix, Øiestad, Vindenes & Mørland, 2014; Gottas et al., 2013; Perekopskiy & Kiyatkin, 2019; Solis, Cameron-Burr, Shaham & Kiyatkin, 2017) and it is known to be intrinsically rewarding (Avvisati et al., 2019; Hubner & Kornetsky, 1992; Kvello, Andersen, Boix, Morland & Bogen, 2020). The bursts-like heroin pattern occurring in the intermittent-access condition also caused an accumulation of 6-MAM, as exemplified by the higher 6-MAM peaks relative to the long-access condition (**Figure 17B**). Such high levels might have induced intoxicating or sedative effects (Andersen, Ripel, Boix, Normann & Mørland, 2009; Gottås, Boix, Øiestad, Vindenes & Mørland, 2014; Gottas et al., 2013), with rats returning to self-administer another burst of heroin infusions as soon as the 6-MAM concentrations significantly drop. In contrast, in the long-access condition, rats were more likely to self-administer heroin with inter-infusion intervals of about 10-15 min, a pause compatible with the time course of 6-MAM concentrations after intravenous heroin administration (Gottas et al., 2013). In the long-access condition the levels of 6-MAM fluctuated around a steady state.

According to the highlighted hypothesis, rats in the intermittent-access condition were more likely to experience the rewarding and intoxicating consequences of high levels of heroin self-administration. Conversely, rats in the long-access condition, due to the timeout, did not reach the same elevated brain levels of heroin. It is therefore plausible that the timeout imposed in the long-access procedure could have occluded the acquisition of heroin self-administration. Indeed, the behavioral pattern observed

reflects more accurately the pharmacokinetics profile of 6-MAM. An external observation in support of this hypothesis comes from an intracranial self-stimulation (ICSS) study. Black and colleagues (1985) demonstrated that even minor delays can occlude the acquisition of ICSS.

To further corroborate this hypothesis, drug brain peak concentrations of heroin, 6-MAM, and morphine were simulated in two rats self-administering the same number of unit-doses of heroin, but one with no timeout and the other with 20-s timeout between unit-doses (**Figure 21**). The simulation revealed that brain peak concentrations of heroin were halved in the timeout condition compared to the no-timeout condition, and the peak of 6-MAM was delayed in the timeout condition relative to the no-timeout condition. To conclude, timeout hinders reaching high brain heroin levels, impeding heroin self-administration acquisition, and encouraging rats to self-administer heroin to maximize the rewarding effect of its metabolite 6-MAM.

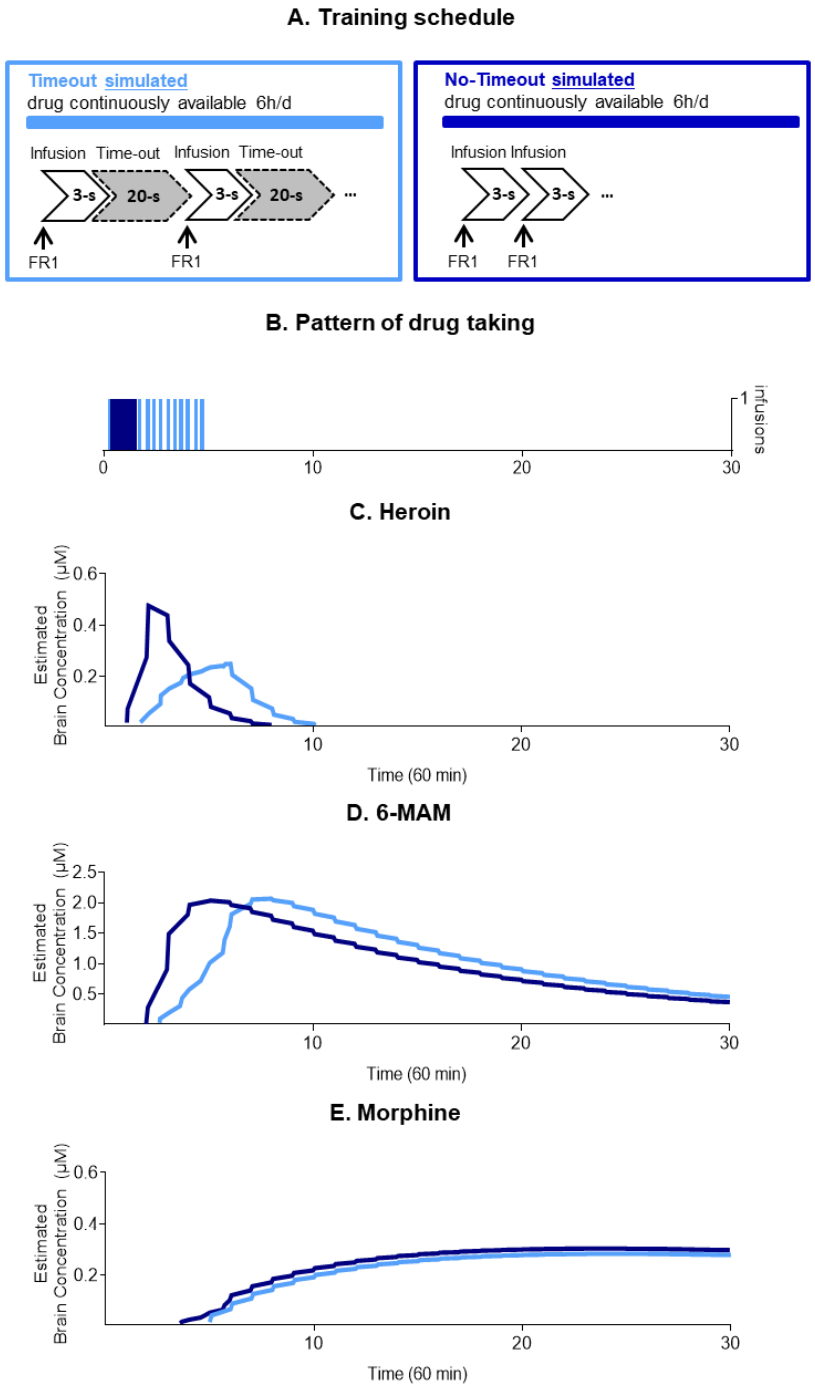

Figure 21. *Estimated brain concentrations of heroin and its active metabolites in representative rats self-administering heroin without timeout and a simulated rat self-administering heroin with timeout. (**A**) Training schedule. (**B**) Pattern of drug taking. (**C**) Estimated brain concentrations of heroin. (**D**) Estimated brain concentrations of 6-monoacthylmorphine (6-MAM). (**E**) Estimated brain concentrations of morphine.*

Notably, in both access conditions, brain concentrations of morphine increased throughout the session and decreased slightly between injections. Indeed, the intervals between unit-doses were 10-15 min, which is shorter than the half-life of morphine (~50-min) (Gottas et al., 2013) (see **Figure 15A**, **16B**). This finding suggests that morphine does not contribute significantly to the differences observed between heroin access conditions or the temporal dynamics during self-administration, a phenomenon that most of the historical literature would not have predicted. Indeed, morphine is still widely believed to be the main, if not the only, metabolite responsible for the effects of heroin (Oldendorf, Hyman, Braun & Oldendorf, 1972).

Although the metabolic breakdown of heroin is well known, the pharmacological activity of its active metabolites and their association with the acute and chronic effects are still surprisingly overlooked and will require further research.

The *second* explanation resides in the learning determinants of drug taking. Previous studies, in rats trained under long-access conditions, suggested that animals self-administer drugs to achieve a specific drug 'satiety threshold' [also known as 'compulsion zone' (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006)] and learn to titrate drug levels around this threshold (self-administering drug injections whenever drug levels in the body drop below the threshold) (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006; Panlilio, Katz, Pickens & Schindler, 2003; Tsibulsky & Norman, 1999). Thus, rats in the intermittent-access condition could have learned to self-administer several injections to maintain drug levels in the body above the drug satiety threshold during the 25-min OFF period. While this is a plausible explanation that warrants further studies, it should be noted that studies directly comparing long-access and intermittent-access to cocaine showed that drug intake was significantly lower in the intermittent-access relative to long-access condition (Algallal, Allain, Ndiaye & Samaha, 2020; James, Stopper, Zimmer, Koll, Bowrey & Aston-Jones, 2019; Nicolas et al., 2019; Zimmer, Oleson & Roberts, 2012), a finding directly in opposition to present results. Notably, a similar dissociation in drug intake between psychostimulants and opioids was previously reported by Panlilio et al. (2003) in rats trained to self-administer cocaine or remifentanil, a short-acting opioid with a half-life of 0.7-min (Haidar, Moreton, Liang, Hoke, Muir & Eddington, 1997).

In agreement with the preclinical literature on heroin self-administration (Bossert et al., 2021; Mayberry et al., 2022; Stewart, Woodside & Shaham, 1996; Venniro, Zhang, Shaham & Caprioli, 2017), sex differences in the total intake were not observed (**Figure 14A**) or the pattern of heroin self-administration in either the long-access or intermittent-access conditions (**Figure 15A-B**). These results match the clinical evidence reporting no sex differences in the amount of heroin consumed (Gjersing & Bretteville-Jensen, 2014; Kennedy, Epstein, Phillips & Preston, 2013), the number of injections during drug taking periods (Ross, McCurdy, Kilonzo, Williams, Leshabari & hygiene, 2008) or in the degree of enjoyment of use (Kennedy, Epstein, Phillips & Preston, 2013). However, these data were at odds, with an earlier study reporting higher heroin intake in females compared to male rats (Lynch & Carroll, 1999). This discrepancy, as previously discussed (Venniro, Zhang, Shaham & Caprioli, 2017), is likely the result of the significantly lower unit-dose (0.015 mg/kg), a fifth of the unit-dose used in the present study.

5.5.2. Heroin relapse

A second relevant finding in the present study is that, similar to a recent finding with oxycodone (Samson, Xu, Kortagere & España, 2022), rats in the intermittent-access condition after a forced abstinence did not show ‘incubated’ drug craving, a phenomenon that was consistently observed after forced abstinence from long-access to heroin (Fanous, Goldart, Theberge, Bossert, Shaham & Hope, 2012; Shalev, Morales, Hope, Yap & Shaham, 2001; Theberge et al., 2012; Venniro, Zhang, Shaham & Caprioli, 2017), and both long-access and intermittent-access to cocaine (Gueye, Allain & Samaha, 2019; Nicolas et al., 2019). The lack of ‘incubated’ heroin craving during abstinence following the intermittent-access condition is likely due to the already-high level of heroin-seeking observed on abstinence Day 1 (**Figure 19A**). This behavioral pattern strikingly resembles the high levels of craving during the initial transition to abstinence typically observed in treatment-refractory active users being prescribed heroin maintenance (Blanken, Hendriks, Koeter, van Ree & van den Brink, 2012), in heroin users seeking treatment at different time points during abstinence (Wang et al., 2012), in abstinent users on methadone substitution therapy (Arafa, Enaba, Baz, Gomaa, Ragab & Tarek, 2024; Blanken, Hendriks, Koeter, van Ree & van den Brink, 2012), and in users who completed therapy (Childress, McLellan & O'Brien, 1986).

From a mechanistic perspective, it is known that several factors, including sex, context, and schedule of drug reinforcement affect the development and time course of sensitization of the dopamine system (Lefevre et al., 2020; Robinson & Berridge, 1993b; Stewart & Badiani, 1993a; Vanderschuren, Tjon, Nestby, Mulder, Schoffelmeer & De Vries, 1997). Indeed, several studies have shown that the high motivation for drug and drug-associated cues observed after an intermittent-access to cocaine is mediated by a sensitized dopamine response, which is not present in rats trained under long-access conditions (Calipari, Ferris, Zimmer, Roberts & Jones, 2013; Kawa, Valenta, Kennedy & Robinson, 2019). This sensitized dopamine response resembles the enhanced dopamine response to drug and drug cues observed in clinical imaging studies (Jasinska, Stein, Kaiser, Naumer & Yalachkov, 2014; Samaha, Khoo, Ferrario & Robinson, 2021). Based on the rationale provided above it could be speculated that the repeated bursts of high heroin concentrations produced by the intermittent- access could induce an extremely rapid sensitization of relevant neural substrates, resulting in an intense cue-induced craving since the very early phases of abstinence. An alternative mechanistic explanation can be found in the reward allostatic hypothesis of substance use disorder (Koob & Le Moal, 2001). In the intermittent-access condition, the repeated abstinence periods (lasting 25-min), that divide the drug taking periods of heroin (12 epochs), would repeatedly trigger compensatory mechanisms, downregulating the ‘reward system’ and determining a persistent high heroin craving (Koob, 2020). Further preclinical and clinical studies will be required to fill this gap in the literature.

Another observation was that in the intermittent-access condition, drug-seeking was significantly higher in female rats relative to males (**Figure 19A**). This is in agreement with clinical studies showing shorter abstinence periods and higher reactivity to heroin-associated cues in women, relative to men (Petry & Bickel, 2000; Yu et al., 2007). A ‘telescoping effect’ in the transition to substance use disorder and a high relapse rate in women was suggested (Brady & Randall, 1999) to be driven by ovarian hormones (Becker & Chartoff, 2019). With psychostimulants, vulnerability to relapse is increased during the follicular/estrus phase (Nicolas et al., 2019). Nevertheless, evidence for the role of ovarian hormones in opioid-seeking has not been established (Knouse & Briand, 2021). Thus, the role of the estrous cycle in the incubation of heroin craving was explored, after a prolonged forced or voluntary abstinence. Our results indicated that the estrous cycle does not influence cue-induced heroin-seeking, with a similar

intensity of heroin-seeking behavior displayed during the estrus and non-estrus phases of the cycle (**Figure 20**). This is consistent with recent studies demonstrating that show that estrous cycle does not impact heroin self-administration nor incubation of craving (Mayberry et al., 2022), and at a causal level treatment with estradiol or progesterone does not influence cue-induced reinstatement of heroin-seeking (Vazquez, Frazier, Reichel & Peters, 2020). Furthermore, as recently reviewed by Nicolas et al. (2022), preclinical studies demonstrated that sex differences exist in the reinstatement and incubation of cocaine seeking, but not in the reinstatement or incubation of methamphetamine or opioid seeking. The distinct neurobiological mechanisms involved in relapse to opioids and psychostimulants may help account for these results, with different classes of drugs interacting differently with ovarian hormones (Badiani, Belin, Epstein, Calu & Shaham, 2011).

Finally, after both long-access and intermittent-access to heroin, the food choice-based voluntary abstinence completely suppressed heroin taking, regardless of the training conditions (**Figure 18**). In addition, in confirmation of previous findings (Venniro, Zhang, Shaham & Caprioli, 2017), a lack of sex differences in voluntary abstinence was observed (**Figure 18**). These results are consistent with those of Reiner et al. (2020), showing no sex differences in voluntary abstinence from fentanyl, and are also consistent with the lack of sex differences in the efficacy of contingency management in promoting abstinence in humans (Epstein, Schmittner, Umbricht, Schroeder, Moolchan & Preston, 2009).

5.6. Statistical table 1

Figure	Data	Primary statistic	Factor name	*p*-value	F-value
Figure 13A	SM7% intake (acquisition phase)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	**p=0.001*** **p=0.001*** **p=0.001***	$F_{2,240}$=52.272 $F_{1,120}$=54.315 $F_{2,240}$=7.782
Figure 13A	SM7% intake (training phase)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	p=0.646 **p=0.001*** p=0.375	$F_{4,480}$=0.623 $F_{1,120}$=91.739 $F_{4,480}$=1.039
Figure 13B	SM7% lever pressing (acquisition)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	**p=0.001*** **p=0.001*** **p=0.004***	$F_{2,120}$=8.477 $F_{1,120}$=20.317 $F_{2,120}$=5.873
Figure 13B	SM7% lever pressing (training phase)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	**p=0.001*** p=0.099 **p=0.049***	$F_{4,120}$=6.975 $F_{1,120}$=2.768 $F_{4,120}$=2.725
Figure 14A	Heroin intake (acquisition phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	p=0.479 p=0.996 p=0.315 p=0.406 p=0.181 p=0.664	$F_{2,236}$=0.739 $F_{2,118}$=0.000 $F_{2,118}$=1.017 $F_{2,236}$=0.904 $F_{2,236}$=1.724 $F_{2,236}$=0.410
not shown	Total heroin intake (acquisition phase)	Two-way ANOVA	Access (between) Sex (between) Access X Sex	p=0.315 p=0.996 p=0.862	$F_{3,120}$=1.017 $F_{3,120}$=0.000 $F_{3,120}$=0.030
Figure 14A	Heroin intake (training phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	**p=0.001*** p=0.550 **p=0.049*** p=0.229 p=0.262 p=0.058	$F_{9,1080}$=36.061 $F_{9,120}$=0.359 $F_{9,120}$=3.960 $F_{9,1080}$=1.462 $F_{9,1080}$=1.273 $F_{9,1080}$=3.673
Figure 14B	Heroin frequency of intake (acquisition phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	p=0.436 p=0.996 p=0.315 p=0.344 p=0.181 p=0.594	$F_{2,240}$=0.739 $F_{1,120}$=0.000 $F_{1,120}$=1.017 $F_{2,240}$=0.904 $F_{2,240}$=1.724 $F_{2,240}$=0.410
Figure 14B	Heroin frequency of intake (training phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	**p=0.001*** p=0.737 **p=0.001*** p=0.350 **p=0.001*** p=0.099	$F_{9,1080}$=30.322 $F_{1,120}$=0.113 $F_{1,120}$=156.573 $F_{9,1080}$=1.108 $F_{9,1080}$=17.035 $F_{9,1080}$=2.018

Figure 14C	Heroin active lever pressing (acquisition phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	p=0.375 p=0.372 p=0.074 p=0.645 **p=0.041*** p=0.412	$F_{2,236}$=0.851 $F_{1,118}$=0.992 $F_{1,118}$=3.257 $F_{2,236}$=0.439 $F_{2,236}$=4.262 $F_{2,236}$=0.889
Figure 14C	Heroin active lever pressing (training phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	**p=0.001*** p=0.911 p=0.485 **p=0.008*** p=0.594 **p=0.030***	$F_{9,1035}$=24.656 $F_{1,115}$=0.013 $F_{1,115}$=0.490 $F_{9,1035}$=2.453 $F_{9,1035}$=0.675 $F_{9,1035}$=2.705
Heroin inactive	lever pressing (acquisition phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	p=0.689 p=0.218 p=0.732 p=0.954 p=0.613 p=0.852	$F_{2,236}$=0.372 $F_{1,118}$=1.532 $F_{1,118}$=0.118 $F_{2,236}$=0.048 $F_{2,236}$=0.490 $F_{2,236}$=0.161
Heroin inactive	lever pressing (training phase)	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	**p=0.001*** p=0.412 p=0.466 p=0.717 p=0.480 p=0.877	$F_{9,1062}$=4.971 $F_{1,118}$=0.679 $F_{1,118}$=0.535 $F_{9,1062}$=0.692 $F_{9,1062}$=0.950 $F_{9,1062}$=0.498
Figure 15A	Cumulative infusions (10^{th} session) (intermittent-access)	Two-way RM ANOVA	Long-access Sex (between) Intermittent-access Sex (between)	 p=0.085 p=0.067	 $F_{1,63}$=1.875 $F_{1,60}$= 1.736
Figure 15B	Infusions 1^{st} min (intermittent-access)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	**p=0.001*** p=0.661 p=0.215	$F_{9,59}$=13.256 $F_{1,59}$=0.194 $F_{9,59}$=1.571
Figure 15B	Infusions 2^{nd} min (intermittent-access)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	p=0.307 p=0.936 p=0.290	$F_{9,59}$=1.062 $F_{1,59}$=0.007 $F_{9,59}$=1.141
Figure 15B	Infusions 3^{rd} min (intermittent-access)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	p=0.223 p=0.905 p=0.557	$F_{9,59}$=1.151 $F_{1,59}$=0.014 $F_{9,59}$=0.349
Figure 15B	Infusions 4^{th} min (intermittent-access)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	p=0.131 p=0.439 p=0.511	$F_{9,59}$=2.347 $F_{1,59}$=0.607 $F_{9,59}$=0.437
Figure 15B	Infusions 5^{th} min (intermittent-access)	Two-way RM ANOVA	Session (within) Sex (between) Session X Sex interaction	p=0.410 p=0.426 p=0.711	$F_{9,59}$=1.005 $F_{1,59}$=0.643 $F_{9,59}$=0.698

Figure 16	Heroin intake: estrous cycle (5 days)		Long-access Intermittent-access	p=0.985 p=0.853	t=0.0187 t=0.1857
not shown	Discrete-choice tests Preference score	Three-way RM ANOVA	Session (within) Sex (between) Access (Between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	p=0.798 p=0.120 p=0.386 p=0.191 p=0.123 p=0.860	$F_{1,55}$=0.066 $F_{1,55}$=2.488 $F_{1,55}$=0.822 $F_{1,55}$=0.013 $F_{1,55}$=2.452 $F_{1,55}$=0.031
Figure 18	Voluntary abstinence Preference score	Three-way RM ANOVA	Session (within) Sex (between) Access (between) Session X Sex interaction Session X Access interaction Session X Sex X Access interaction	**p=0.001*** p=0.695 p=0.054 p=0.515 p=0.953 p=0.818	$F_{17,765}$=4.010 $F_{1,45}$=0.156 $F_{1,45}$=3.630 $F_{17,765}$=0.949 $F_{17,765}$=0.502 $F_{17,765}$=0.686
Figure 19A (left)	Relapse (incubation) test	Four-way RM ANOVA	Abstinence day (within) Access (between) Sex (between) Abstinence condition (between) Abstinence day X Sex interaction Abstinence day X Access interaction Abstinence day X Abstinence condition interaction Abstinence day X Sex X Access interaction Abstinence day X Sex X Abstinence condition interaction Abstinence day X Access X Abstinence condition interaction Abstinence day X Sex X Access X Abstinence condition interaction	**p=0.001*** **p=0.017*** p=0.081 p=0.095 p=0.991 **p=0.009*** **p=0.004*** p=0.462 p=0.606 p=0.631 p=0.714	$F_{1,94}$=14.699 $F_{1,94}$=5.895 $F_{1,94}$=3.105 $F_{1,94}$=2.841 $F_{1,94}$=0.003 $F_{1,94}$=7.239 $F_{1,94}$=8.826 $F_{1,94}$=0.544 $F_{1,94}$=0.126 $F_{1,94}$=0.232 $F_{1,94}$=0.136
Figure 19A (left)	Relapse (incubation) test (Abstinence day 1)	Three-way ANOVA	Sex (between) Access (between) Abstinence condition (between)	p=0.131 **p=0.001*** p=0.941	$F_{1,94}$=2.325 $F_{1,94}$=12.056 $F_{1,94}$=0.006
Figure 19A (center)	Relapse (incubation) test (long-access)	Three-way RM ANOVA	Abstinence day (within) Sex (between) Abstinence condition (between) Abstinence day X Sex interaction Abstinence day X Abstinence condition interaction Abstinence day X Sex X Abstinence condition interaction	**p=0.001*** p=0.702 p=0.183 p=0.569 **p=0.011*** p=0.992	$F_{1,44}$=24.872 $F_{1,44}$=0.149 $F_{1,44}$=1.829 $F_{1,44}$=0.329 $F_{1,44}$=6.985 $F_{1,44}$=0.0001
Figure 19A (right)	Relapse (incubation) test (intermittent-access)	Three-way RM ANOVA	Abstinence day (within) Sex (between)	p=0.447 **p=0.046*** p=0.291	$F_{1,50}$=0.586 $F_{1,50}$=4.245 $F_{1,50}$=1.138

			Abstinence condition (between)	p=0.626	$F_{1,50}$=0.240
			Abstinence day X Sex interaction	p=0.099	$F_{1,50}$=2.826
			Abstinence day X Abstinence condition interaction	p=0.628	$F_{1,50}$=0.238
			Abstinence day X Sex X Abstinence condition interaction		
Figure 19B	Time course relapse test (Abstinence day 1)	Four-way RM ANOVA	Min (within)	**p=0.001***	$F_{2,94}$=74.953
			Sex (between)	p=0.141	$F_{2,94}$=2.209
			Access (between)	**p=0.001***	$F_{2,94}$=12.416
			Abstinence condition (between)	p=0.082	$F_{2,94}$=0.001
			Sex X Access interaction	p=0.082	$F_{2,94}$=2.535
			Sex X Abstinence condition interaction	p=0.854	$F_{2,94}$=0.131
			Access X Abstinence condition interaction	p=0.176	$F_{2,94}$=1.779
			Sex X Access X Abstinence condition interaction	p=0.401	$F_{2,94}$=0.918
Figure 19C	Time course relapse test (Abstinence day 21)	Four-way RM ANOVA	Min (within)	**p=0.001***	$F_{2,94}$=34.607
			Sex (between)	p=0.193	$F_{2,94}$=1.719
			Access (between)	p=0.550	$F_{2,94}$=0.550
			Abstinence condition (between)	**p=0.009***	$F_{2,94}$=7.167
			Sex X Access interaction	p=0.682	$F_{2,94}$=0.383
			Sex X Abstinence condition interaction	p=0.063	$F_{2,94}$=2.809
			Access X Abstinence condition interaction	p=0.374	$F_{2,94}$=0.988
			Sex X Access X Abstinence condition interaction	p=0.395	$F_{2,94}$=0.933
Figure 20	Relapse (incubation) test: estrous cycle (Abstinence day 1)	Two-way ANOVA	Access (between)	**p=0.013***	$F_{1,44}$=5.874
			Cycle (between)	p=0.837	$F_{1,44}$=0.043
			Access X Cycle interaction	p=0.903	$F_{1,44}$=0.015

6. The impact of timeout and unit-dose on heroin and cocaine patterns of drug taking, drug-seeking, and sociability

6.1. Abstract

Background. During their drug-use history, cocaine and heroin users gain mastery and control over their drug consumption. Indeed, they self-regulate the dosage, route, speed, and frequency of administration as a function of the expected effects (e.g., avoiding withdrawal, experiencing euphoria, etc.). Counterintuitively, most preclinical self-administration and choice procedures use discrete dimension strategies, featured by the presence of experimenter-imposed timeout periods between consecutive drug injections of unit-doses (discrete) or its absence (continuous), which prevent the experimental animal from self-selecting the appropriate dose-time relationship of administration. Here, discrete to continuous dimension strategies (i.e., self-selected doses without timeout) on drug-related behaviors were compared.

Methods. Patterns of drug taking, and estimated drug-brain levels (pharmacokinetics profiling) were analyzed in rats trained under distinct self-administration training conditions, characterized by discrete or continuous dimensions strategies. Additionally, the motivation to take and seek drugs was assessed across training conditions and in the context of drug-vs-social choice procedures.

Results. The patterns of drug taking and related pharmacokinetics profiling were profoundly different across both training conditions and the drug under examination. Notably, overdoses were not observed in rats trained without a timeout, contrary to what the literature would have anticipated. Rather, the lack of timeout was associated with stronger motivation to take and seek drugs. Finally, heroin, but not cocaine, continuous-access self-administration induced social withdrawal in rats in drug-vs-social choice procedures.

Conclusions. Here, evidence supporting the use of continuous strategies over discrete ones in self-administration and choice procedures was presented. These approaches better reflect the nuances of human drug-related behaviors and likely engage better the relevant underlying neurobiological mechanisms.

6.2. Specific aims of the project

In this series of experiments, the goal was to contrast the impact of timeout and unit-doses strategies on drug self-administration and drug-vs-social choice procedures.

Drug self-administration procedure. Two experiments were carried out to investigate the impact of timeout and unit-doses[8] on heroin and cocaine self-administration, using both within-subject and between-subject designs. An FR1 schedule of reinforcement with short injection times (3-s) and no timeouts between injections [strongly comparable to the hold-down drug self-administration procedure introduced by Morgan et al. (2009)] was used. In experiment 2, a within-subject design was used to probe whether the timeout affects heroin and cocaine self-administration. The same rats were trained to self-administer drugs for 18 days with or without experimenter-imposed timeout between injections using a two-lever drug self-administration procedure and (1) patterns of drug taking; (2) drug-seeking under extinction conditions, (3) preference (in a discrete-choice procedure), and (4) motivation [(in a recently developed progressive ratio procedure (Roberts & Zimmer, 2020)] were compared on the two access conditions. In experiment 3, the same research question was analyzed using a between-subject design. Three groups of rats were trained to self-administer heroin or cocaine for 15 days under three different self-administration training schedules: (1) long-access (drugs continuously available 6-h/d, FR1 20-s timeout), (2) intermittent-access (drugs intermittently available 6-h/d divided in 5-min access every 30-min, FR1 no-timeout), or (3) continuous-access (drugs continuously available 6-h/d, FR1 no-timeout) schedule. Then, rats from the three training schedules were compared on: (1) overt behavior during self-administration (Alcantara et al., 2011; Seip, Reed, Ho & Kreek, 2012), (2) patterns of drug taking, and

[8] In the original investigation, Morgan D, Liu Y, Oleson EB, & Roberts DC (2009). Cocaine self-administration on a hold-down schedule of reinforcement in rats. Psychopharmacology (Berl) 201**:** 601-609. used a continuous dimension strategy, known as hold-down procedure. This approach assumes that doses of the drug delivered (unit-doses) should not be imposed by the experimenter, but rather selected by the rats themselves. To this aim, rats were trained to depress a lever for specific durations, directly correlating with the magnitude of the cocaine delivered. Due to limitations of the system used for this investigation, a procedure was implemented where each lever press corresponds to an infusion, and no timeouts follow the injections, i.e. FR1 no-timeout. Notably, Morgan et al. Ibid. demonstrated that a fixed ratio 1 (FR1) schedule without timeouts yields comparable behavior to the hold-down procedure, at least with cocaine.

resultant drug brain levels during the last self-administration session, and (3) drug seeking under extinction conditions.

Drug-vs-social discrete choice procedure. In experiment 4, it was tested whether the possibility to self-select the preferred dose in the preferred time in the drug-vs-social choice would affect the preference between drug and social interaction. Rats were trained to get access to a social partner and then heroin or cocaine (drugs continuously available 6-h/d, FR1 no-timeout) and tested for drug seeking. Then rats' preference for drug or social interaction was tested in two different choice conditions: (1) in the first condition, the rats were tested in the typical choice procedure where they were allowed to choose between 1 unit-dose of social interaction (1-min access) or drug (heroin dose 0.075 mg/kg/inf or cocaine 0.5 mg/kg/inf; (2) in the second condition, the rats were tested in a choice procedure where they were allowed to choose between 5-min access to the social partner or to the drug (a time sufficient to self-administer the preferred dose in the preferred time).

Below the experimental details will be outlined.

6.3. Materials and Methods

Subjects. A total of 228 male Sprague-Dawley rats (Charles River, Lecco) were used for the study. At the beginning of the experiments, the rats were 5-6 weeks old and weighed between 150-175 g. Before the surgery, the rats were pair-housed, and after the surgery, they were housed individually. For the social choice experiments, the rats were divided before the social self-administration training. Throughout the entire experiment, the rats were maintained on a reversed 12-hour light/dark cycle (lights off at 6 AM) with free access to standard laboratory chow and water. All the procedures followed the guidelines of national law (DL 26/2014) on the use of animals for research based on the European Communities Council Directive (2010/63/UE) and received approval from the ethics committee of the Italian Ministry of Health and the local Ethical Committee of the Santa Lucia Foundation. 19 rats were excluded due to catheter problems or sickness.

Drug. The heroin hydrochloride used in the study was donated from the Drug Supply Program of the National Institute on Drug Abuse (NIDA) and dissolved in sterile saline (0.9% NaCl). For drug self-administration training, unit-doses of 0.075 mg/kg/infusion for heroin and 0.5 mg/kg/infusion for cocaine were chosen based on previous studies (Gueye, Allain & Samaha, 2019; O'Neal, Nooney, Thien & Ferguson, 2020).

Intravenous surgery. To perform the intravenous surgery, the rats were anaesthetized with isoflurane (5% induction, 2–3% maintenance) and administered Carprofen (2 mg/kg, subcutaneous injection; Demas Italia Srl) immediately after the surgery and for the following 5 days to relieve pain and decrease inflammation. A silastic catheter was inserted into the jugular vein as previously described (Caprioli et al., 2015). The rats' catheters were flushed daily with a 0.2-ml sterile saline solution containing gentamicin (4.25 mg/ml; Fatro S.p.A.) to prevent occlusion during the recovery, training, and abstinence phases. After a minimum of 5-7 days of recovery, the catheter patency was tested daily with sterile saline and gentamicin solution. If the catheter failed the test during the training, intravenous catheterization of the left jugular vein was performed with the same procedure as for the right, or the rats were eliminated from the study.

Self-administration apparatus. The rats were trained in self-administration chambers placed inside sound-attenuating cubicles, which were equipped with an electric fan and controlled by a custom-made system. The operant chambers had different components based on the specific experiment. The drug was delivered through a modified cannula (Plastics One; Roanoke, VA, USA) connected to a liquid swivel (Instech; Plymouth Meeting, PA, USA) via polyethylene-50 tubing that was protected by a metal spring.

Estimated brain levels of cocaine and heroin and its metabolites. To estimate the estimated brain levels of heroin and its active metabolites (6-MAM and morphine), and cocaine, the software program Kinetica v.5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used with its FitMultiMicroExtravascular model for multiple administered doses. The estimations were based on the times of the single infusions during the last session of drug self-administration. The mean brain concentrations in **Table 2** were extracted from Pan et al. (1991) and the parameters of the best-fitted

model were used to estimate the theoretical brain levels from the infusion times. For heroin and its metabolites, the estimated pharmacokinetics parameters (absorption, elimination, intercompartmental distribution rate constants, time lag, volume of distribution) best fitting the mean brain extracellular fluid data for each opioid in the study by Gottås et al. (2013) were used (see **Table 1, Chapter 5**). The brain concentrations were estimated from the absolute dose (μmol) received per infusion (adjusted to the weight of each animal) and were calculated in 0.5-min intervals for 8 hours, starting at the beginning of the session and up to 2 hours after its end. However, in the graphs, only the brain levels for the 6 hours of the session are presented.

Parameter	**Ka**	**lag**	**Volume**	**Kel**	**K12**	**K21**	**K13**	**K31**
Unit	min^{-1}	min	L	min^{-1}	min^{-1}	min^{-1}	min^{-1}	min^{-1}
Cocaine[1]	0.586706	n/a	0.122182	0.090295	0.067599	0.047336	n/a	n/a

Table 2. *Pharmacokinetics parameters extracted from Pan et al. (1991) used to fit the concentrations of cocaine in the brain extracellular fluid after intravenous administration of 7.5 mg/kg cocaine in the rat. These same parameters were applied to the FitMultiMicroExtravascular model of the software program Kinetica v.5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) to simulate the brain concentrations taking into account the times of the single unit-doses during the last self-administration training session.*

[1] Extravascular two compartments model without lag
Ka: Absorption rate constant from injection site. Kel: Elimination rate constant from brain. Volume: Volume of distribution. KXY: Transfer rate constant between compartment X and Y (1 = measured compartment).

Experiment 2. The role of timeout in heroin and cocaine self-administration: a within-subject study

Self-administration chambers. Chambers were equipped with a stainless-steel grid floor, and two operant panels were placed on the left and right walls. The left panel of the chamber was equipped with a house light and one active (retractable) lever. Responses on this lever activated the infusion pump and the discrete white-light cue located above the lever. The right panel was equipped with one active (retractable) lever. Responses on this lever activated the infusion pump and the three-light cue located above the lever. Left or right levers and relative cues were assigned randomly, to timeout or no-timeout conditions (see below).

Drug self-administration. Drug self-administration training was divided into two phases: acquisition and training. In the acquisition phase, rats were trained to self-administer drug 2-h/d for 4d (acquisition phase; maximum infusions = 20/day). In the training phase, rats were trained 3-h/d for 14d (training phase; maximum infusions = 90/day). During both acquisition and training phase, rats were trained to self-administer drug on two different levers: one lever associated with a timeout condition (that reflects the long-access condition) and one lever associated with a no-timeout condition (that reflects the continuous-access condition). The timeout or no-timeout levers were randomly presented on alternate days. The sessions started with the insertion of one of the two levers (timeout OR no-timeout) and the illumination of the house light. Responses on the lever associated with the timeout condition (FR1) were reinforced by unit-doses of drug, paired with the cue light (3-s), followed by a 20-s timeout (cue light on), during which lever pressing was not reinforced and the cue light was on. Responses on the lever associated with the no-timeout condition (FR1) were reinforced by unit-doses of drug, paired with the cue light (3-s).

Seeking test. Rats were tested for drug-seeking on both levers (timeout and no-timeout) under extinction conditions the days after the last choice session. Rats were tested on the two levers three days apart. The test days were counterbalanced to avoid any confounding factors (abstinence days, lever-paired cues, etc.). The duration of the test sessions was 30-min to avoid a carryover effect on the second test. The sessions began with the illumination of the house-light, followed 10-s later by the insertion of the drug-paired lever; the house-light remained on for the duration of the session. Lever presses during the tests resulted in the contingent presentation of the light cue, previously paired with unit-doses of drug, but no drug infusion was delivered. After the last cue-induced seeking test, rats were divided (matched for their total drug intake during the self-administration training, **Statistical Table 2**) into two groups. One group of rats was tested in a discrete choice procedure and the other group of rats was tested in a multi-day progressive ratio procedure.

Discrete-choice procedure. A subgroup of rats was tested for preference between timeout and no-timeout conditions in a discrete choice procedure where the rats were allowed to choose between the timeout-paired and no-timeout-paired levers. The discrete choice sessions lasted for 6-h and were conducted using the same parameters (dose of drug and stimuli associated with the two levers) selected

for the training phase. Each 6-h discrete choice session was divided into 6 trials separated by 55-min. The length of the inter-trial intervals was set based on the time needed for morphine to dissipate (Gottas et al., 2013). Each trial began with the flashing of the house light for 30-s (a discriminative stimulus that signaled the choice session), followed by the fixed lighting of the house light and the insertion of both the timeout-paired and not-timeout-paired levers. Rats then had to select one of the two levers. The operant response requirement for the lever's selection was set to 5 consecutive responses (FR5) to avoid accidental choices. If the rats responded within 3-min, they had access to the selected lever for a total of 5-min. If the rats failed to respond on either active lever within 3-min, both levers were retracted, and the house light was turned off with no reward delivery. Rats were tested using the discrete choice precedure for a total of 7 days.

Multi-day progressive ratio. A subgroup of rats was tested in a progressive ratio procedure, on both the timeout and the no-timeout levers, on alternate days in a random sequence. The progressive ratio procedure was identical to the training procedure except that access to the drugs was dependent upon an increasing number of lever presses (ratio value). The number of lever presses (ratio value) required was incremented within sessions and between days through the following progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, etc. (Richardson & Roberts, 1996; Roberts & Bennett, 1993). Based on a recent study by Roberts and Zimmer (Roberts & Zimmer, 2020), the completion of the ratio requirement provided access to 3-min to the drug under FR1 schedule. Sessions were 3-h in length, after which time the levers were retracted. No restrictions were placed on the time required to complete the seeking ratio and no timeout periods were imposed after the 3-min access period. Each subsequent daily session started 1-step back to the ratio value reached on the day before. The rats were excluded if they did not exceed the final ratio value reached on the day before, corresponding to the breakpoint.

Experiment 3. The role of timeout in drug self-administration: comparison between long-access, intermittent-access, or continuous-access to heroin and cocaine

Self-administration chambers. Chambers were equipped with a stainless-steel grid floor and one operant panel placed on the left wall. The panel of the chamber was equipped with a house light and the

drug-paired active (retractable) lever. Responses on this lever activated the infusion pump and the discrete white-light cue located above the lever. In addition, the left panel was equipped with an inactive (stationary) lever that had no reinforced consequences.

Mechanical Nociceptive von Frey Test. Mechanical sensitivity was tested with the von Frey Test only in rats that would then be trained for heroin, but not cocaine self-administration, because it was shown that mechanical sensitivity does not change after cocaine self-administration (Edwards et al., 2012). Rats were tested before the drug self-administration (7 days after the intravenous surgery) and after every three consecutive drug self-administration sessions (in the morning, 12 hours into withdrawal) (Edwards et al., 2012; Kallupi et al., 2020). The rats were put in boxes on an elevated metal mesh floor and allowed 10 min for habituation before examination. The plantar surface of each hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.04–2.0 g, 2Biological Instruments, Besozzo, Varese, Italy), presented perpendicular to the plantar surface (7–8 s for each hair). The 50% paw withdrawal threshold (PWT) was determined using Dixon's up-down method (Chaplan, Bach, Pogrel, Chung & Yaksh, 1994; Maftei et al., 2014).

Drug self-administration. Drug self-administration training was divided into two phases: acquisition and training. In the acquisition phase, rats were trained to self-administer drug 2-h/d for 3d (acquisition phase; maximum infusions = 20/day). After the acquisition, rats were divided (matched for their total drug intake during acquisition, **Statistical Table 2**) into three groups that underwent three different drug self-administration training: long-access, intermittent-access, and continuous-access to drug. In the training phase, rats were trained 6-h/d for 12 days (training phase; maximum infusions: heroin = 90/d; cocaine = 150/d).

(1) Long-access training: drug continuously available 6-h/d; fixed ratio 1 (FR1) with 20-s timeout. Sessions started with the insertion of the two levers (active and inactive) and the illumination of the house light. Responses on the active lever (FR1) were reinforced by unit-doses of drug paired with the cue light (3-s), followed by a 20-s timeout during which lever pressing was not reinforced and the cue light was on.

(2) Intermittent-access training: drug available in 12 epochs of 5-mins (ON periods) separated by 25-mins (OFF periods), FR1 no-timeout. Each 5-min ON period started with the insertion of the two levers

and illumination of the house light and ended with the retraction of the levers and shutdown of the house light. Responses on the active lever were reinforced by unit-doses of drug, paired with a cue light (3-s), followed by no experimenter-imposed timeout.

(3) Continuous-access training: drug continuously available 6-h/d; FR1 no-timeout. Sessions started with the insertion of the two levers (active and inactive) and the illumination of the house light. Responses on the active lever (FR1) were reinforced by unit-doses of drug, paired with a cue light (3-s), followed by no experimenter-imposed timeout.

Behavioral repertoire assessment. Rats' behavior was recorded during the first hour of the last drug self-administration training session. Sessions were recorded via a digital camera, videos were analyzed offline, and data were manually scored using Behavioral Observation Research Interactive Software [BORIS; (Friard & Gamba, 2016)]. The number of bouts was noted as well as the length of each bout. The following behaviors were scored: grooming (corporal grooming and plucking/pulling at fur or digits), chewing, walking, stupor/immobility, hand/foot licking, and sniffing (Alcantara et al., 2011; Seip, Reed, Ho & Kreek, 2012).

Seeking test. Rats were tested for drug-seeking under extinction conditions on abstinence days 1 and 21. The duration of the test sessions was 30-min to minimize the carryover effect of extinction learning on day 1, which may decrease drug-seeking on day 21 (Caprioli et al., 2015). The sessions began with the illumination of the house-light, followed 10-s later by the insertion of the drug-paired lever; the house-light remained on for the duration of the session. Lever presses during the tests resulted in the contingent presentation of the light cue, previously paired with unit-doses of drug, but no drug infusion was delivered. After the relapse test on day 1, the rats were brought to their home cages and handled twice a week during abstinence.

Experiment 4. Drug versus social interaction choice

Self-administration chambers. Chambers were equipped with a stainless-steel grid floor, and two operant panels were placed on the left and right walls. The left panel was equipped with a house light and the drug-paired active (retractable) lever. Responses on this lever activated the infusion pump and the

discrete white-light cue located above the lever. The right panel was equipped with the social partner-paired active (retractable) lever. Responses on this lever activated the three-light cue located above the lever and determined the opening of the guillotine-style sliding door.

Social self-administration. The training procedure was similar to the one described in previous studies (Venniro et al., 2018). Rats were trained to press a lever to have access to a social partner 2-h/d for 6d. The resident rats were housed with their social partner (cage mate) until 7 days prior to social interaction self-administration, and each resident rat lever pressed for its previously paired partner. The training sessions started with the illumination of the house light and the insertion of the social partner-paired lever (that remained inserted throughout the session); responses on this lever resulted in access to the social partner (1-min), paired with the illumination of the three-light cue (1-min).

Drug self-administration. After the social self-administration, rats were trained to self-administer the drug 6-h/d for 15 days under continuous-access conditions. Drug self-administration training sessions started with the insertion of the lever and the illumination of the house light. Responses on the active lever (FR1) were reinforced by infusion of a unit-dose of drug (3-s), paired with the cue light (3-s). Rats were randomly assigned to the two choice conditions. After every three consecutive drug self-administration sessions, rats were tested for drug versus social interaction preference in a discrete choice procedure (see below).

Seeking test. Rats were tested for drug-seeking under extinction conditions on abstinence day 1. The duration of the test sessions was 30-min. The sessions began with the illumination of the house-light, followed 10-s later by the insertion of the drug-paired lever; the house-light remained on for the duration of the session. Lever presses during the tests resulted in the contingent presentation of the light cue, previously paired with unit-doses of drug, but no drug infusion was delivered.

Discrete trial choice. After the self-administration training, rats were divided (matched for their total drug intake during the training, **Statistical Table 2**) into two groups which were tested in two different choice procedures. The choice procedure was conducted using the same parameters (dose of drug and time of access to the social partner per reward and stimuli associated with the two active retractable

levers) selected for the training phase. Rats were allowed to choose between the drug-paired and the social partner-paired levers. Rats were tested for a total of 7 days.

(1) Discrete choice '1 vs 1 unit-dose'. Choice sessions lasted for 120-min. Each 120-min choice session was divided into 15 discrete trials that were separated by 8-min. Each trial began with the presentation of the house light followed 10-s later by the insertion of both the social partner-paired and drug-paired levers. Rats then had to select one of two levers. The operant response requirement for the lever's selection was set to two consecutive responses (FR2) to avoid accidental choices. If the rats responded within 3-min, they received the reward corresponding with the selected lever. Reward delivery was signaled by the social partner-paired cue (1-min) or drug-paired cue (20-s), the retraction of both levers and the turning off of the house light. If the rat failed to respond on either active lever within 3-min, both levers retracted, and the house light was turned off with no reward delivery (Venniro et al., 2018).

(2) Discrete choice '5 vs 5 min access'. Choice sessions lasted for 6-h. Each 6-h choice session was divided into 6 discrete trials that were separated by 55-min. Each trial began with the flashing of the house light for 30-s (a discriminative stimulus that signaled the choice session), followed by the illumination of the house light and the insertion of both the social partner-paired and drug-paired levers. Rats then had to select one of the two levers. The operant response requirement for the lever's selection was set to 5 consecutive responses (FR5) to avoid accidental choices. If the rats responded within 3-min, they had access to the selected lever for a total of 5-min under FR1 schedule. The duration of access to the social partner was matched with the time allocated to drug access. Notably, Chow et al. (2022) demonstrated that the time of access to the social partner does not impact progressive ratio responding. If the rats failed to respond on either active lever within 3-min, both levers were retracted, and the house light was turned off with no reward delivery.

Discrete choice with incremental access time to drug or social partner. A subgroup of rats was tested for 4 days in a choice procedure with incremental access time to drug (in the case of cocaine) or social partner (in the case of heroin). To allow rats to evaluate each option separately before expressing their choice, each choice session was preceded by 4 sampling trials spaced by 55-min. During the sampling, each trial began with the flashing of the house light for 30-s (discriminative stimulus that signaled the session), followed by the fixed illumination of the house light and the insertion of only one of the two

levers (drug-paired, social-paired) in the following order: drug – social – drug – social. The order of lever presentation (i.e., whether drug- or social-paired lever was presented first) was counter-balanced across rats. During the sampling, rats were required to complete each trial to advance (Chow & Beckmann, 2021). During the choice (4 trials), each trial began with the flashing of the house light for 30-s (discriminative stimulus that signaled the session), followed by the illumination of the house light and the insertion of both the social partner-paired and drug-paired levers. During sampling and choice, the response requirement was set to 5 (FR5) consecutive responses. Rats trained to self-administer heroin were tested for preference between drug and social interaction in a choice procedure where the access time to the social partner was randomly increased across testing days from 1-min to 15-min and the access time to heroin was maintained constant to 1-min (see **Figure 34**). On the contrary, rats trained to self-administer cocaine were tested for preference between drug and social interaction in a choice procedure where the access time to cocaine was randomly increased across testing days from 1-min to 15-min and the access time to the social partner was maintained constant to 1-min (see **Figure 34**).

Discrete-choice trial choice on late abstinence. On day 60 from the beginning of the experiment, after all the behavioral procedures, rats were tested for discrete choice after prolonged abstinence from drug. This choice test was preceded by 4 sampling trials, as described above, to allow rats to evaluate each option separately before expressing their choice. After the sampling, the rats were allowed to choose between the social partner-paired and drug-paired levers. During the choice (4 trials), each trial began with the flashing of the house light for 30-s (discriminative stimulus that signaled the session), followed by the illumination of the house light and the insertion of both the social partner-paired and drug-paired levers.

Statistical analysis

Experiment 2. For the training phase, total drug intake and total lever presses for infusions/number of lever presses were analyzed using the within-subjects factor of Session. Drug intake and lever presses during self-administration training were analyzed using a GLMM as a function of Access (timeout, no-timeout) condition. In the GLMM, the Access condition was used as a fixed effect, while Sessions and Rat were used as random effects in a crossed design. For the relapse test, active lever presses during the

cue-induced seeking tests were analyzed using the Wilcoxon matched-pairs signed-rank test with Access condition (timeout, no-timeout) as within-subjects factor; Cues (white light or three-light cue) and Abstinence Day (1,3) were included as covariates. Relative to the choice procedure, the preference (preference score) in the choice tests was calculated by normalizing the indifference level between timeout and no-timeout choices at 0 using the following formula: [1 - (% timeout lever choices/50%)] (Lenoir, Serre, Cantin & Ahmed, 2007). Preference score across sessions was analyzed using the within-subjects factor of Session. Finally, for the progressive ratio test, the final ratio was completed, and the total number of lever presses were analyzed using the Access condition (timeout, no-timeout) as within-subjects factor.

Experiment 3. For the training phase, drug intake and lever pressing data for infusions/number of lever presses were analyzed using the between-subject factor of Access condition (long-access, intermittent-access, and continuous-access) and the within-subjects factor of Session. Regarding the pattern of drug self-administration and pharmacokinetic data, estimated based on behavioral data collected during the last session of drug self-administration, total drug intake of the last day of self-administration, number of infusions per peak, mean drug brain peak concentrations (calculated from valley to maxima), mean drug brain concentration and mean drug brain peak concentrations slope, were analyzed using the between-subject factor of Access condition. Concerning the behavioral observations, collected during the last self-administration session, each behavior (Stupor, Walking, Chewing, Grooming, Sniffing, Hand licking) was analyzed using the between-subject factor of Access condition. Regarding the relapse test on abstinence day 1, the number of active-lever presses was analyzed using the between-subject factor of Access condition. Drug-seeking between Abstinence day 1 was compared by analyzing the number of active lever presses using the between-subject factor of Access condition; the inactive lever was included as a covariate. Then, withdrawal signs (hyperalgesia, body weight) were analyzed using the between-subject factor of Access (long-access, intermittent-access, and continuous-access) and the within-subjects factor of Time (baseline, test days). Finally, cluster analysis was conducted to investigate whether the presence or absence of timeout would lead to distinct subpopulations regardless of the training conditions (Brittany et al., 2024; Garcia-Rivas et al., 2024; Navarrete et al., 2024; Venniro, Panlilio, Epstein & Shaham, 2021). The hierarchical TwoStep Cluster analysis procedure (SPSS TwoStep

Cluster Component) was used. This method assumes independence among variables in the cluster model and does not use a target field. To determine which number of clusters was the best, each of the cluster solutions was compared using the Akaike Information Criterion (AIC) (Vrieze, 2012). For clustering, two features were used: (1) mean peak concentrations reached on the final session of self-administration, (2) drug seeking on Abstinence day 1. These two measures were selected for several reasons. Total drug intake was disregarded, particularly with cocaine, as reductions were influenced by experimental constraints. Total brain concentrations weren't used due to their direct relation with drug intake and peak brain concentrations. Finally, drug-seeking behavior on Abstinence day 21 of abstinence showed comparability across all training conditions.

Experiment 4. For the training phase, drug intake and lever presses for infusions/number of lever presses were analyzed using the between-subject factor of Choice condition (1 vs 1, 5 vs 5) and the within-subjects factor of Session. Relative to the choice procedure, the preference (preference score) in the choice tests was calculated by normalizing the indifference level between social and drug choices at 0 using the following formula: [1 - (% social choices/50%)]. Then, the preference score across sessions was analyzed using the between-subject factor of Choice condition (1 vs 1, 5 vs 5) and the within-subjects factor of Session. Relative to the relapse test, the number of active-lever presses was analyzed using the between-subjects factor of Choice condition (1 vs 1, 5 vs 5). Later, cluster analysis was conducted as described above, based on previously used measures (mean peak concentrations and drug seeking). After clustering, it was examined whether these two clusters could predict the classification of rats into resilient versus vulnerable groups. The mean preference scores over the last three choice sessions, during periods of stable preference, were analyzed utilizing the between-subject factor of Cluster (vulnerable, resilient). Then z-score was calculated for five features: (1) mean intake during the entire drug self-administration training, (2) mean drug brain concentrations, (3) mean brain peak concentrations, (4) drug seeking, (5) choice during the last three days of testing and I analyzed the z-scores utilizing the between-subject factor of Cluster.

6.4. Results

Data were analyzed with the statistical program SPSS (SPSS, Version 25), GraphPad Prism (Version 8.0.1), or R. Before any analysis, all data were evaluated for normality. Outliers were included in the data analysis and presentation. The level of probability (p), for determining group differences, was set at $p<0.05$. Significant main effects and interactions ($p<0.05$) were followed with post-hoc tests (Fisher's PLSD OR Dunn) which were conducted only if the F values in the analyses achieved the appropriate level of statistical significance and the statistical measures of homogeneity of variance were not significant. In all the experiments data for heroin and cocaine were analyzed separately (see **Statistical Table 2** for a complete reporting of the statistical analyses and their exact p-values).

Experiment 2. The role of timeout in drug self-administration: a within-subject study

Drug self-administration and drug-related behaviors. The rats increased their heroin and cocaine intake over time in both access conditions (timeout and no-timeout) (**Figure 22D left, 22F left**). Of note, the total heroin, but not cocaine intake was significantly higher in the no-timeout, relative to the timeout condition (**Figure 22D right, 22F right**). By contrast, lever presses for heroin but not cocaine was significantly higher on the timeout, relative to the no-timeout condition (**Figure 22E, 22G**). Under extinction conditions, the rats exhibited stronger seeking for heroin and cocaine no-timeout, as indicated by the higher number of lever presses on the no-timeout, relative to the timeout lever (**Figure 23D, 23G**). Rats expressed a strong preference for the no-timeout condition for both drugs during the choice tests (**Figure 23E, 23H**). Finally, in the progressive ratio test, the rats displayed higher breaking points and cumulative number of lever presses for the no-timeout, relative to the timeout condition, indicating a stronger motivation for heroin and cocaine self-administration without timeout (**Figure 23F, 23I**).

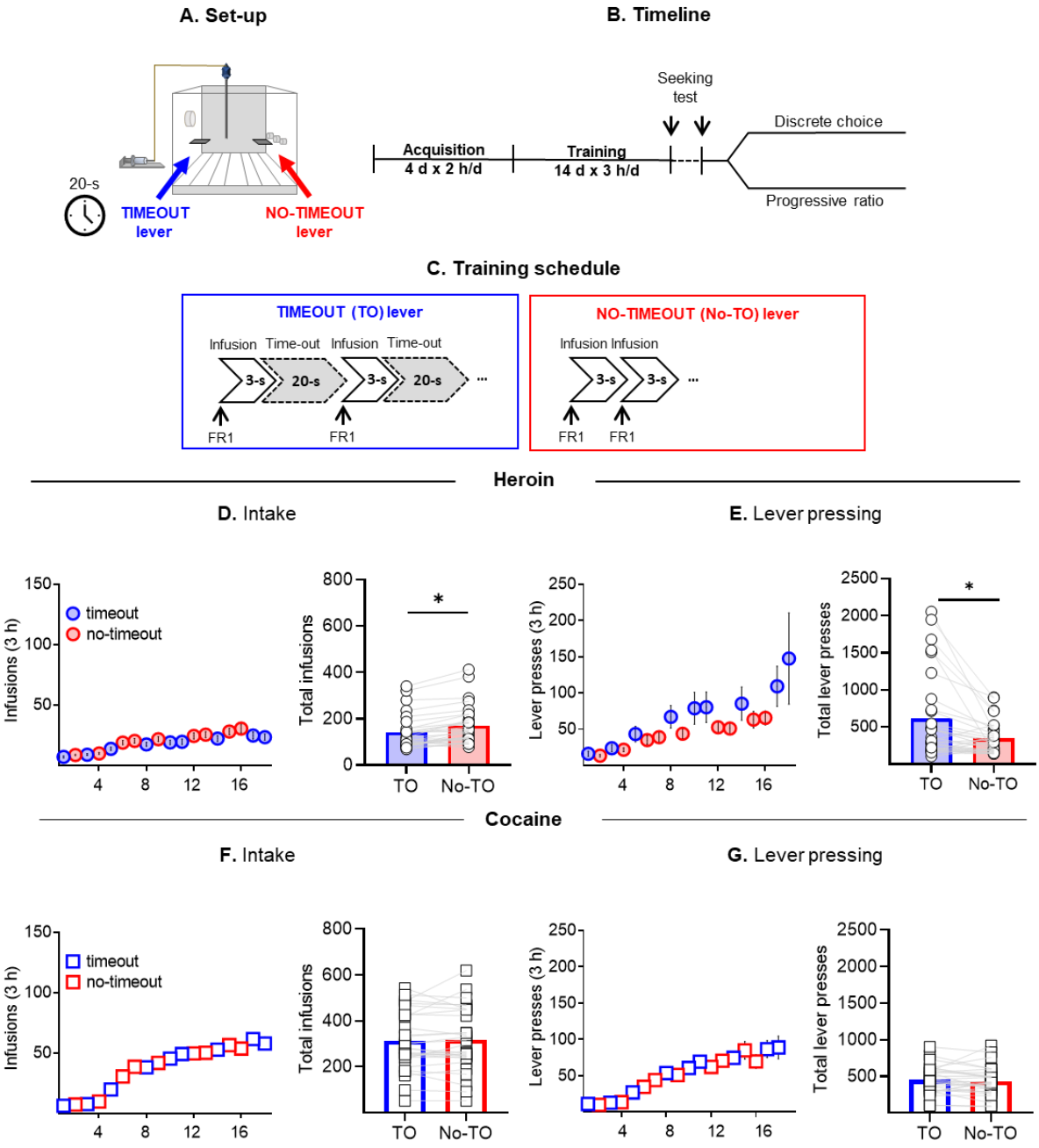

Figure 22. *The impact of timeout on heroin and cocaine self-administration: comparison between timeout and no-timeout conditions: a within-subject design. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and F**) Drug intake. Mean ± SEM number of drug infusions per session (left) and individual data of number of total drug infusions (right). (**E and G**) Lever pressing. Mean ± SEM number of lever presses per session (left) and individual data of number of total lever presses (right). *Different from timeout condition, $p < 0.05$ (heroin n = 29; cocaine n = 28).*

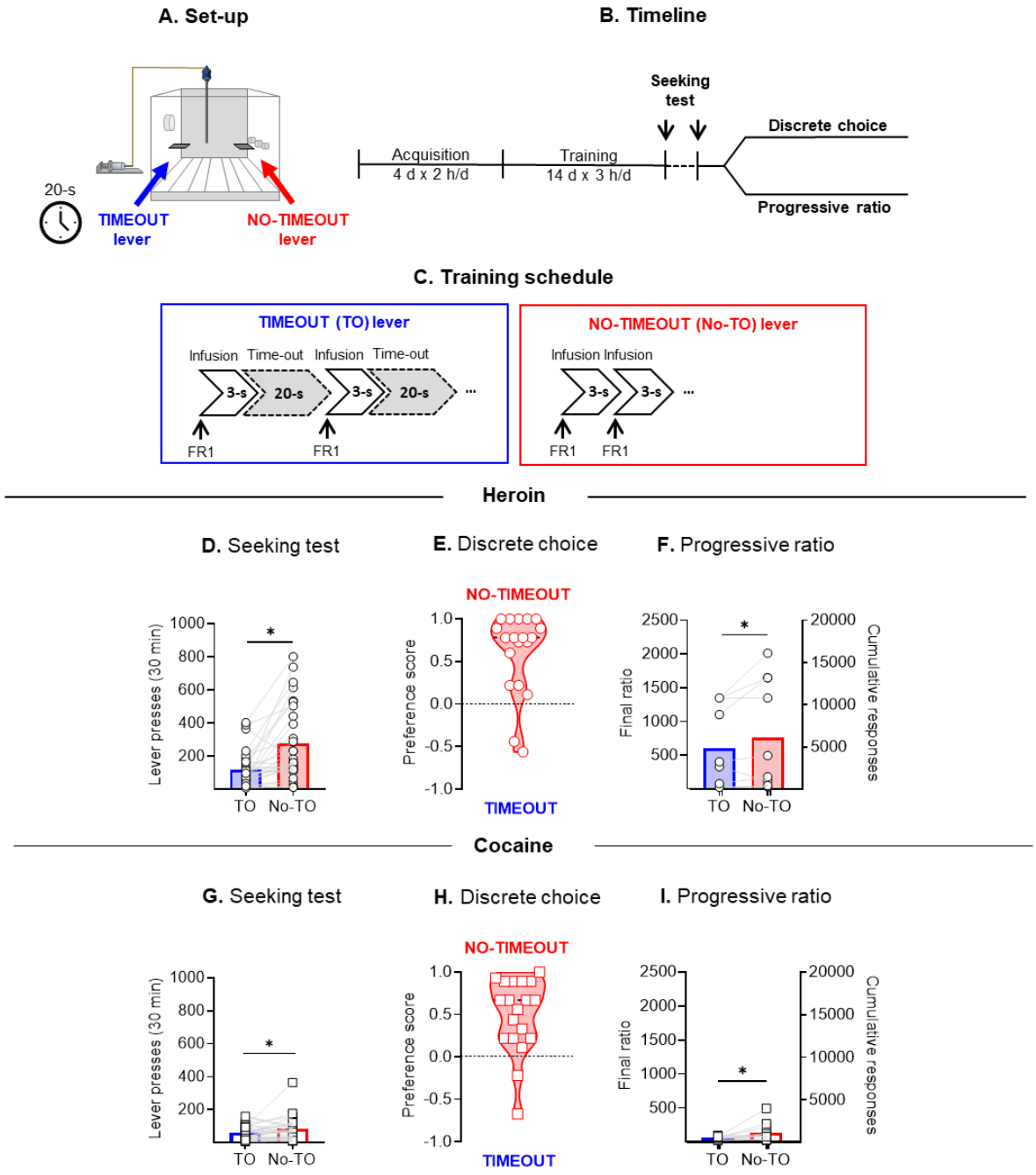

Figure 23. *The impact of timeout on motivation to seek and take heroin and cocaine: comparison between timeout and no-timeout conditions in a within-subject design. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and G**) Seeking test. Individual data of number of lever presses on the active lever during the 30-min extinction tests. (**E and H**) Discrete choice. Individual data of average preference score during the discrete choice sessions. (**F and I**) Progressive ratio. Individual data of final ratio completed and cumulative number of lever presses during the multi-day progressive ratio test. *Different from timeout condition, $p < 0.05$ (heroin n = 29; cocaine n = 28).*

Experiment 3. The role of timeout in drug self-administration: comparison between long-access, intermittent-access, or continuous-access to heroin and cocaine

Drug intake. Rats reliably acquired drug self-administration and increased their drug intake and lever pressing during the acquisition training (**Figure 24D, 24G**). After acquisition the rats were divided into three different groups matched for their drug intake (**Statistical Table 2**). During drug self-administration training, in all access conditions, the rats increased their drug intake and lever pressing over time. However, strong differences between drugs and access conditions were observed. Total heroin intake was significantly higher in the intermittent- and continuous-access conditions, relative to the long-access condition (**Figure 24E**). Notably, differences in drug intake were not accompanied by changes in the development of hyperalgesia, but long-access rats exhibited reduced heroin-induced body weight loss compared to intermittent and continuous-access rats (see below **Figure 29E, 29F**). On the contrary, total cocaine intake was lower in the intermittent-access condition, relative to long- and continuous-access conditions. However, total cocaine intake was higher in the continuous- relative to the long-access condition (**Figure 24E, 24H**). Of note, the quantitative differences in drug intake were associated with qualitative differences in the pattern of drug taking (**Figure 25D, 26D, 26E**).

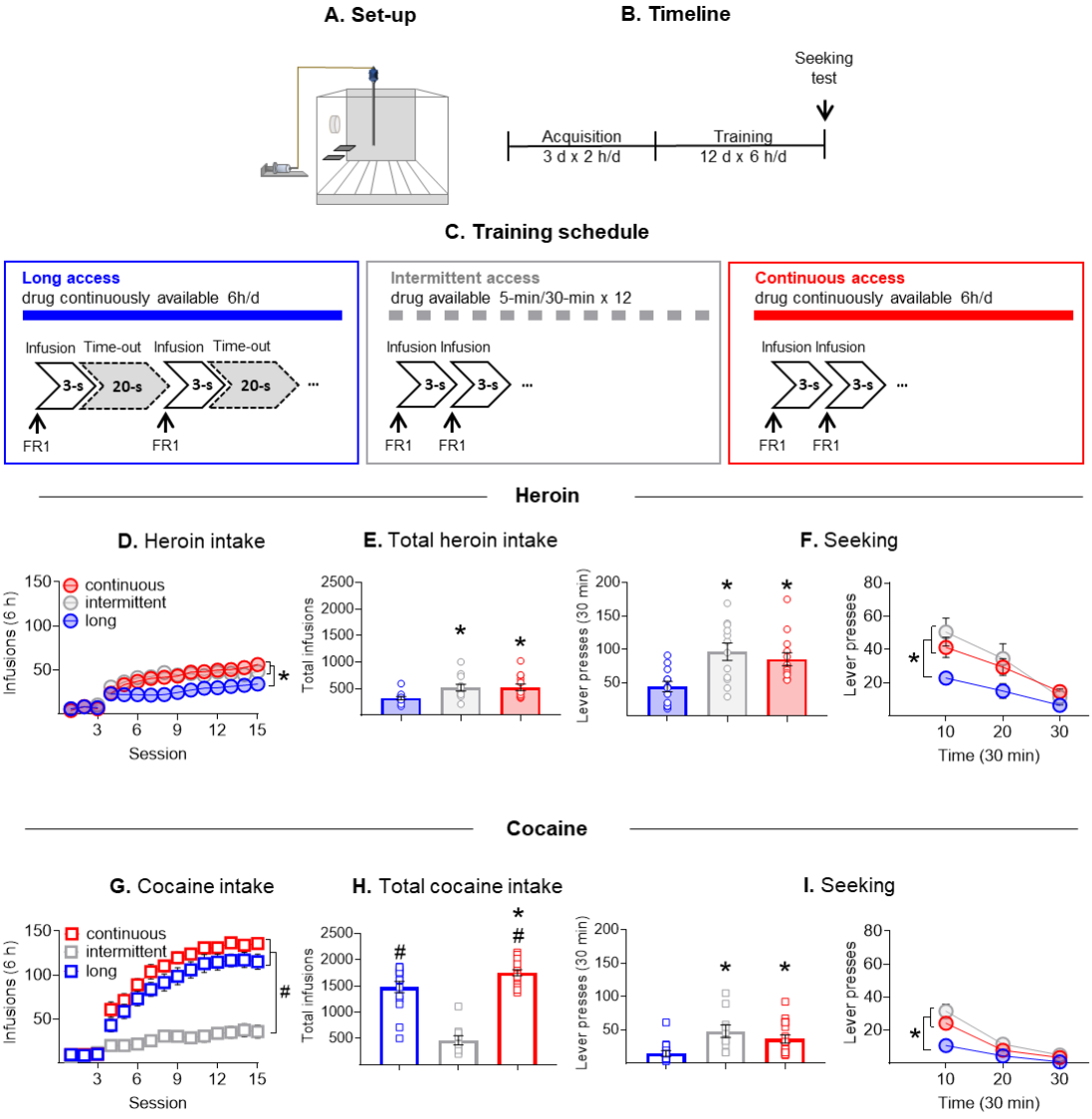

Figure 24. *The impact of timeout in heroin and cocaine self-administration and seeking: a comparison between long-access, intermittent-access, or continuous-access to drug in a between-subject design. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and G**) Drug intake. Mean ± SEM number of drug infusions per session. (**E and H**) Total heroin intake. Individual data of number of total drug infusions. (**F and I**) Seeking test on Abstinence day 1. Individual data of number of lever presses on the active lever during the 30-min extinction tests (left) and time course of lever presses extinction on the active lever during the 30-min extinction tests (right). Data are mean ± SEM of lever presses at each 10 min of the test session on day 1. * Different from long-access, $p < 0.05$; # Different from intermittent-access, $p < 0.05$ (heroin n = 37; cocaine n = 41).*

Patterns of drug taking and estimated brain levels of drug. Regarding heroin, rats trained under long-access conditions displayed a pattern of drug taking characterized by few infusions (typically maximum 2 consecutive unit-doses, **Figure 26E**) spaced by inter-infusion intervals of more than 10-min (**Figure 26D**). On the contrary, rats trained under intermittent- and continuous-access conditions self-administered heroin in 'bursts' of infusion (Belin, Balado, Piazza & Deroche-Gamonet, 2009): they took several infusions in a row, typically spaced by very short inter-infusions intervals (about seconds) (more than 2 consecutive injections, **Figure 26D, 26E**). Burst episodes, in the intermittent-access procedure, were separated by the programmed 25-min OFF, on the contrary rats in the continuous-access condition self-imposed 'abstinence' periods of several minutes (typically more than 20-min) between 'burst' episodes (**Figure 26D**). Notably, consistently with results in Experiment 1 in **Chapter 5**, 'burst' episodes were accompanied by fast-rising high brain peak concentrations of heroin and 6-MAM that were significantly higher in intermittent- and continuous, relative to long-access conditions (**Figure 27E**, **27F**, **28E**). Notably, morphine brain levels were significantly higher in continuous, relative to long- and intermittent-access conditions (**Figure 28F**). In the case of cocaine, no differences in the pattern of intake were observed between the long-access and continuous-access conditions (**Figure 25F**, **25G**). Patterns of cocaine-taking in long-access and continuous-access conditions were characterized by a 'loading' phase, featured by a high rate of infusions, followed by a 'maintenance' phase, during which infusion became more spaced, as observed elsewhere (Panlilio, Katz, Pickens & Schindler, 2003; Zimmer, Dobrin & Roberts, 2011). These patterns were accompanied by comparable brain cocaine peak concentrations and brain cocaine concentrations (**Figure 27H**, **27I**). While, in the intermittent-access condition the pattern of intake was characterized by 'burst' episodes separated by the programmed 25-min OFF (**Figure 26F**, **26G**). Notably, this pattern was accompanied by brain cocaine peak concentrations and brain cocaine concentrations that were lower than rats trained under long-access and continuous-access conditions (**Figure 27H**, **27I**).

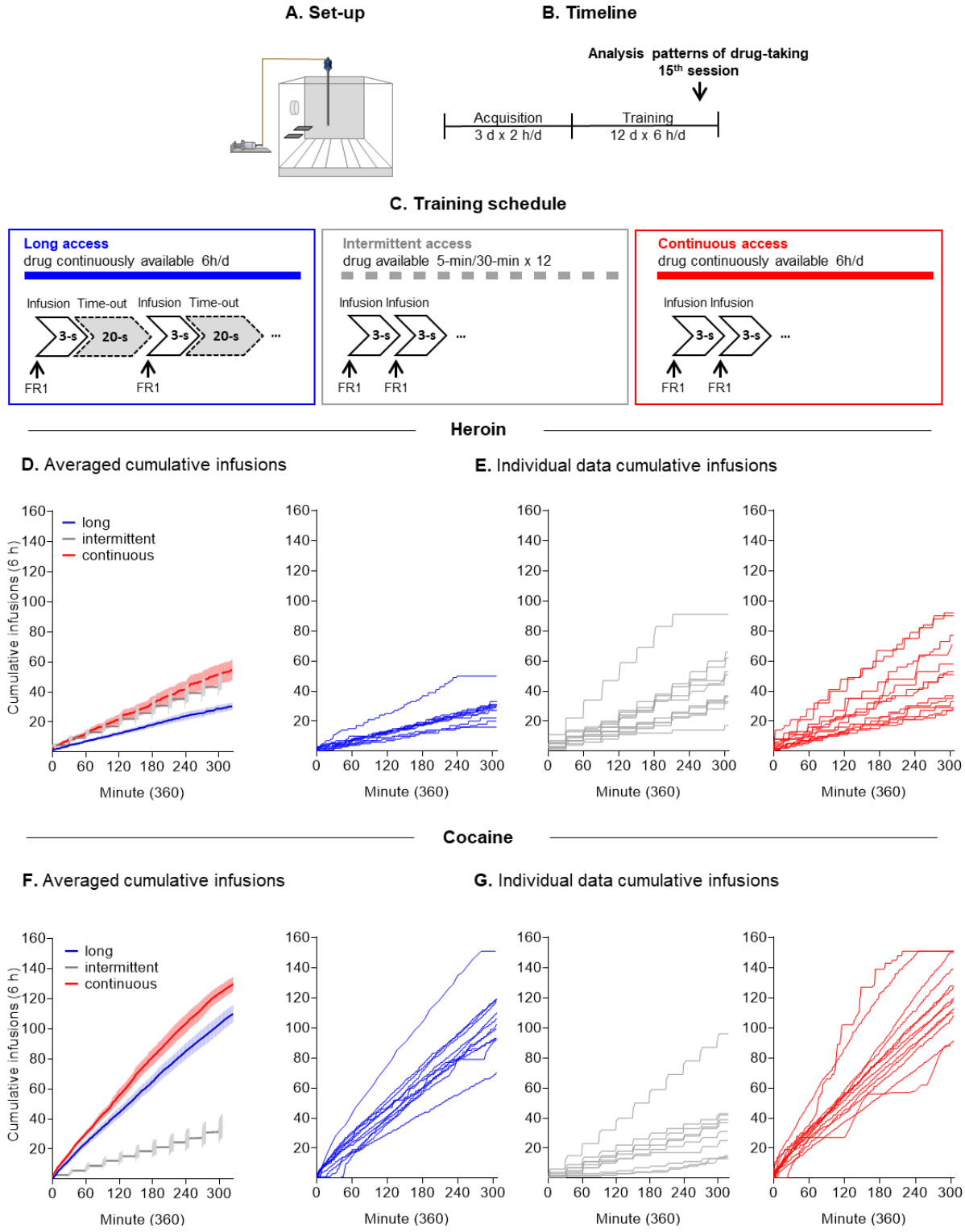

Figure 25. *The impact of timeout in heroin and cocaine self-administration: patterns of drug taking on the last drug self-administration session. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. Mean (**D and F**) and individual (**E and G**) cumulative infusions in the last self-administration session. (heroin: n = 37; cocaine: n = 41).*

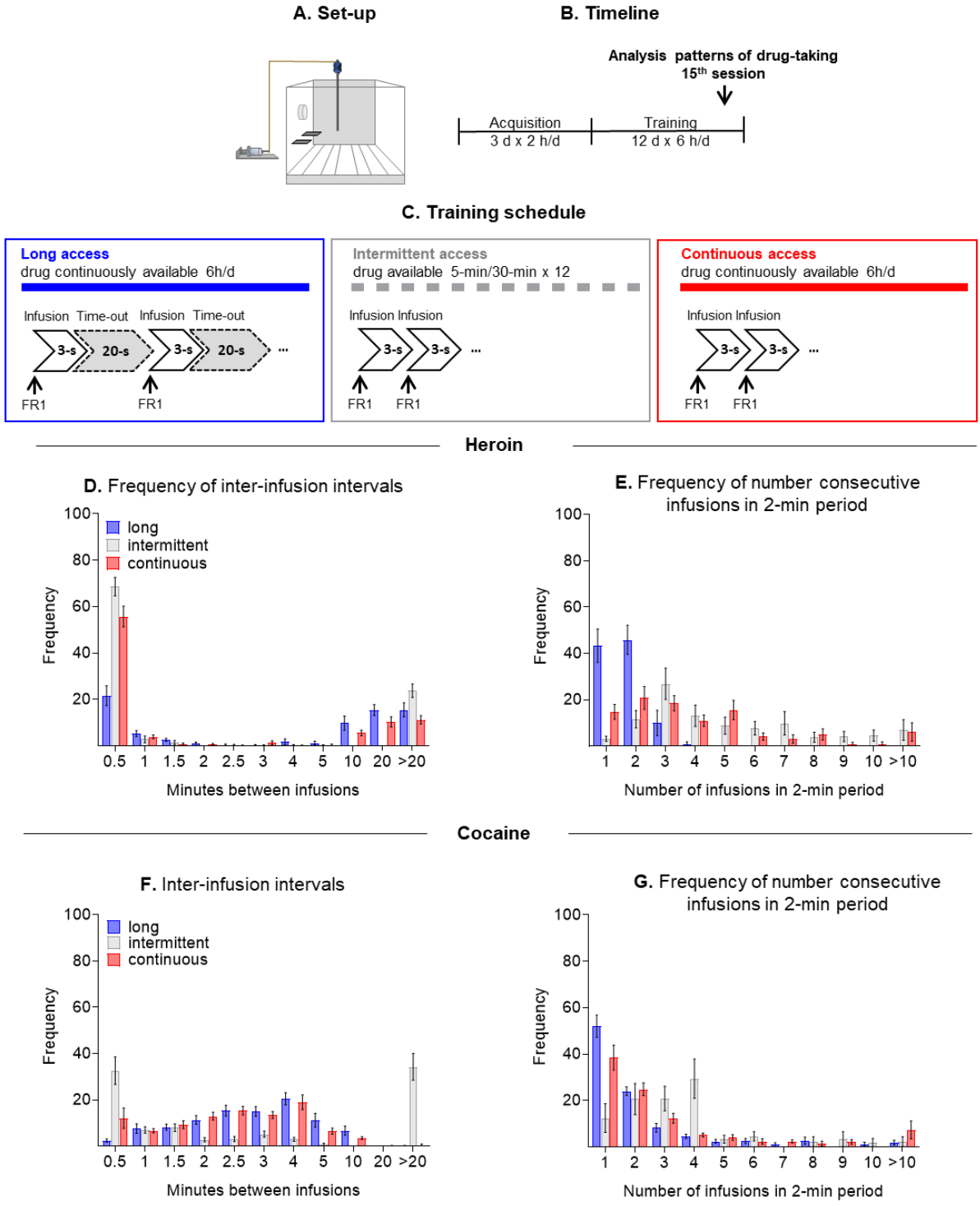

Figure 26. *The impact of timeout in heroin and cocaine self-administration: frequencies of mean inter-infusion intervals and mean number of consecutive infusions per 2-min periods in long-access, intermittent-access, or continuous-access to drug. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and F**) Frequency of inter-infusion intervals. (**E and G**) Frequency of number of consecutive infusions per 2-min periods. (heroin: n=37; cocaine: n=41).*

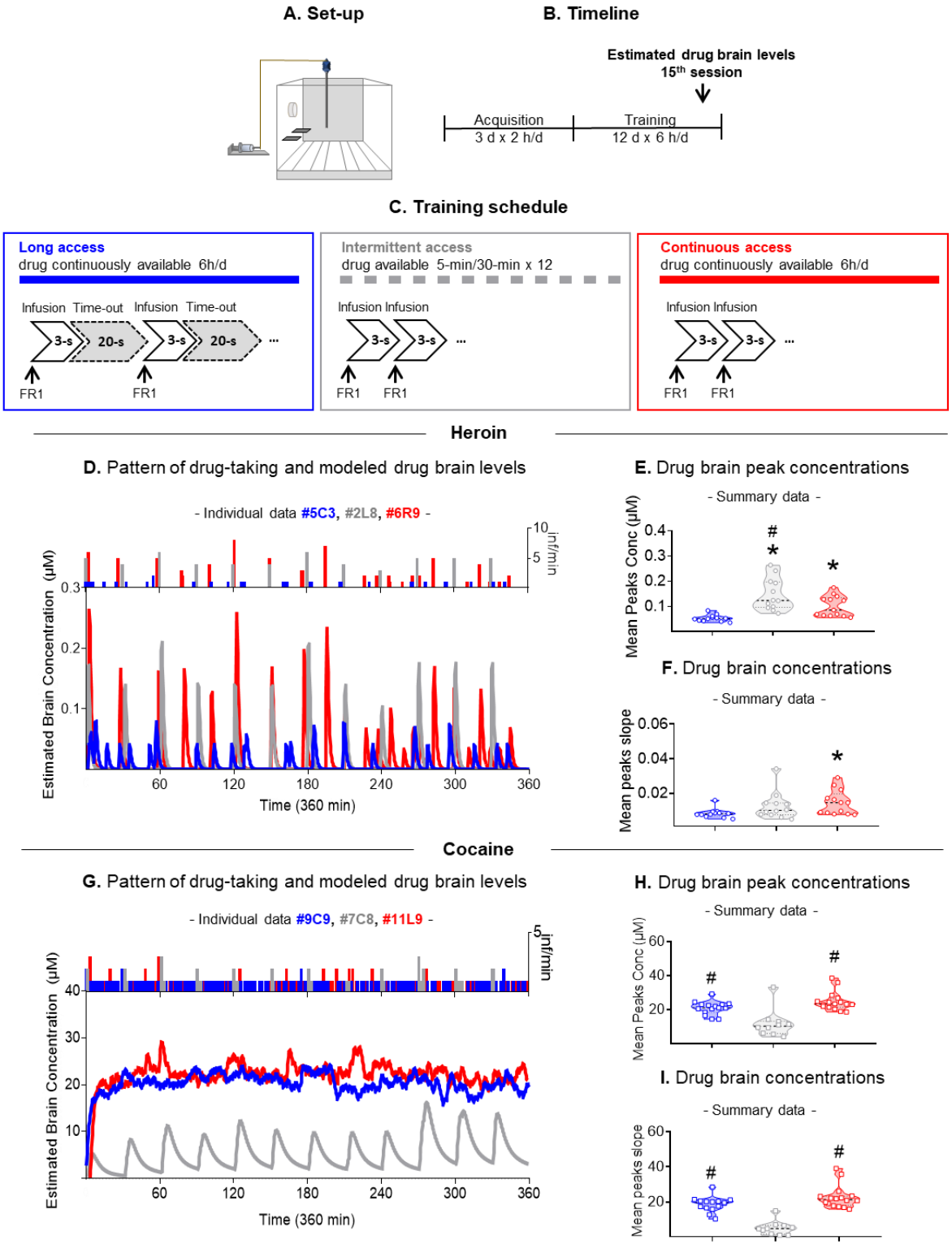
A. Set-up
B. Timeline
Estimated drug brain levels
15th session
Acquisition
3 d x 2 h/d
Training
12 d x 6 h/d
C. Training schedule
Long access
drug continuously available 6h/d
Infusion Time-out Infusion Time-out
3-s 20-s 3-s 20-s ...
FR1 FR1
Intermittent access
drug available 5-min/30-min x 12
Infusion Infusion
3-s 3-s ...
FR1 FR1
Continuous access
drug continuously available 6h/d
Infusion Infusion
3-s 3-s ...
FR1 FR1
Heroin
D. Pattern of drug-taking and modeled drug brain levels
- Individual data #5C3, #2L8, #6R9 -
Estimated Brain Concentration (µM)
inf/min
Time (360 min)
E. Drug brain peak concentrations
- Summary data -
Mean Peaks Conc (µM)
F. Drug brain concentrations
- Summary data -
Mean peaks slope
Cocaine
G. Pattern of drug-taking and modeled drug brain levels
- Individual data #9C9, #7C8, #11L9 -
Estimated Brain Concentration (µM)
inf/min
Time (360 min)
H. Drug brain peak concentrations
- Summary data -
Mean Peaks Conc (µM)
I. Drug brain concentrations
- Summary data -
Mean peaks slope

Figure 27. *Patterns of drug intake and pharmacokinetic modelling of brain concentrations of heroin and cocaine in representative rats trained under long-access, intermittent-access or continuous-access to drug in the last session of drug self-administration training. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and G**) Pattern of infusions and estimated drug brain levels in representative rats (heroin infusions: long-access = 30, intermittent-access = 55, continuous-access = 86; cocaine infusions: long-access = 137, intermittent-access = 44, continuous-access = 146) (**E and H**) Estimated drug brain peak concentrations. Individual data of mean concentration of drug brain peaks. (**F and I**) Estimated drug brain concentrations Individual data of mean drug brain concentrations. Note the scales are adapted to the levels of each compound. * Different from long-access, $p < 0.05$; † Different from long-access and continuous-access, $p < 0.05$; # Different from intermittent-access, $p < 0.05$. (heroin: $n = 37$; cocaine: $n = 41$).*

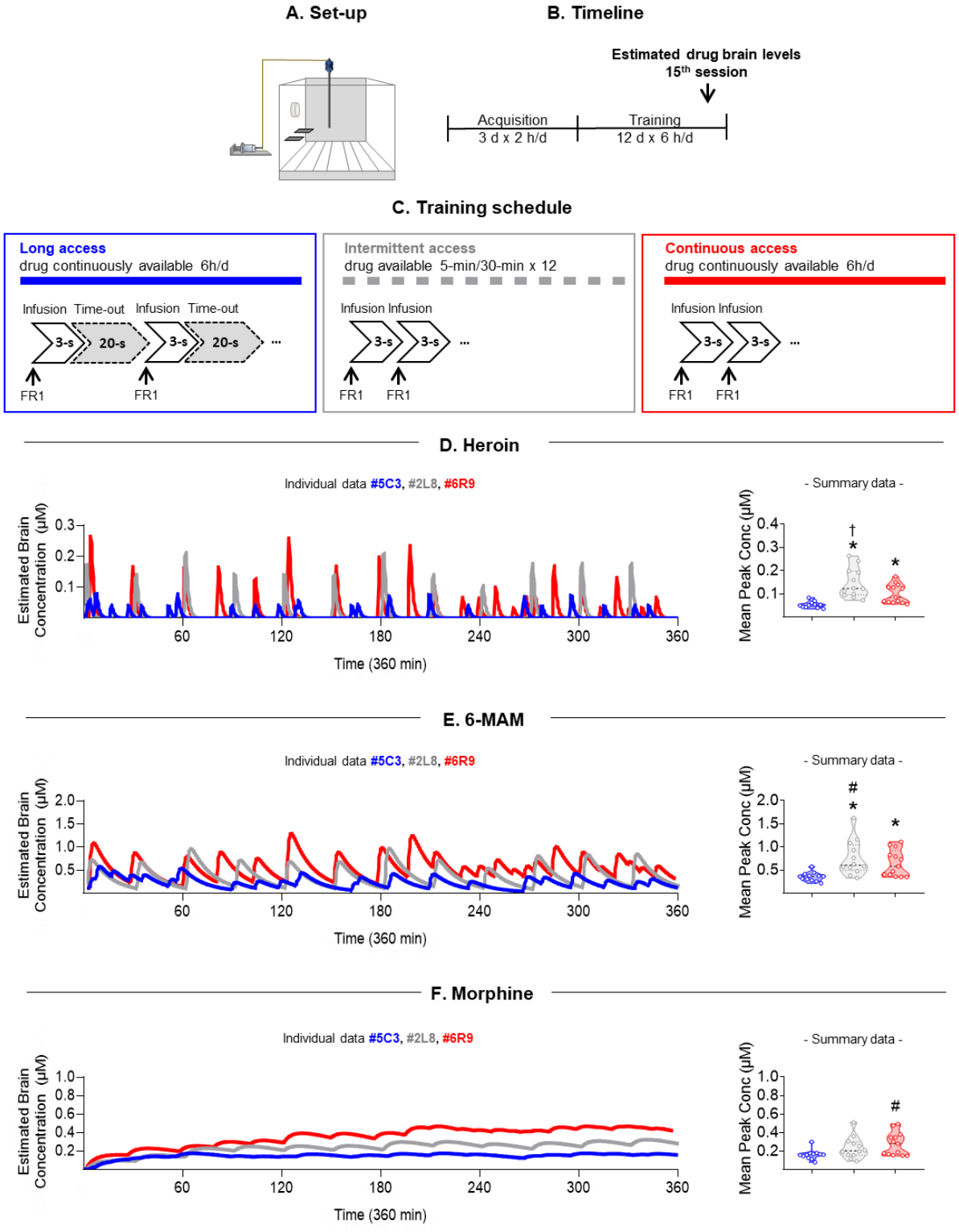
A. Set-up
B. Timeline
Estimated drug brain levels
15th session
Acquisition
3 d x 2 h/d
Training
12 d x 6 h/d
C. Training schedule
Long access
drug continuously available 6h/d
Infusion Time-out Infusion Time-out
3-s
20-s
FR1
Intermittent access
drug available 5-min/30-min x 12
Infusion Infusion
Continuous access
drug continuously available 6h/d
D. Heroin
Individual data #5C3, #2L8, #6R9
- Summary data -
Estimated Brain Concentration (µM)
Mean Peak Conc (µM)
Time (360 min)
E. 6-MAM
F. Morphine

Figure 28. *Estimated brain concentrations of heroin and its active metabolites in representative rats trained under long-access, intermittent-access or continuous-access to heroin in the last session of drug self-administration training. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. For each line: estimated drug brain concentrations (left) of (**D**) heroin, (**E**) 6-MAM and (**F**) morphine in representative rats (infusions: long-access = 30, intermittent-access = 55, continuous-access = 86); individual data of mean drug brain peak concentrations (right) of (**D**) heroin, (**E**) 6-MAM and (**F**) morphine. Note the scales are adapted to the levels of each compound. * Different from long-access, p < 0.05; † Different from long-access and continuous-access, p < 0.05; # Different from long-access and intermittent-access, p < 0.05. (n=37).*

Drug-seeking. Overall, the rats displayed stronger seeking for heroin and cocaine self-administration without timeout, as indicated by the higher number of lever presses in the intermittent- and continuous-access conditions (where the timeout is not imposed) relative to the long-access condition (see above **Figure 24F, 24I**). Incubation of drug craving was observed in the long-access but not in intermittent and continuous-access conditions after forced abstinence, with higher lever pressing on abstinence day 21 than on day 1 (**Figure 29D, 29G**). Rats in the intermittent and continuous-access conditions did not show an ‘incubated’ profile because of the higher drug-seeking on Abstinence day 1, which was similar to day 21 (**Figure 29D, 29G**).

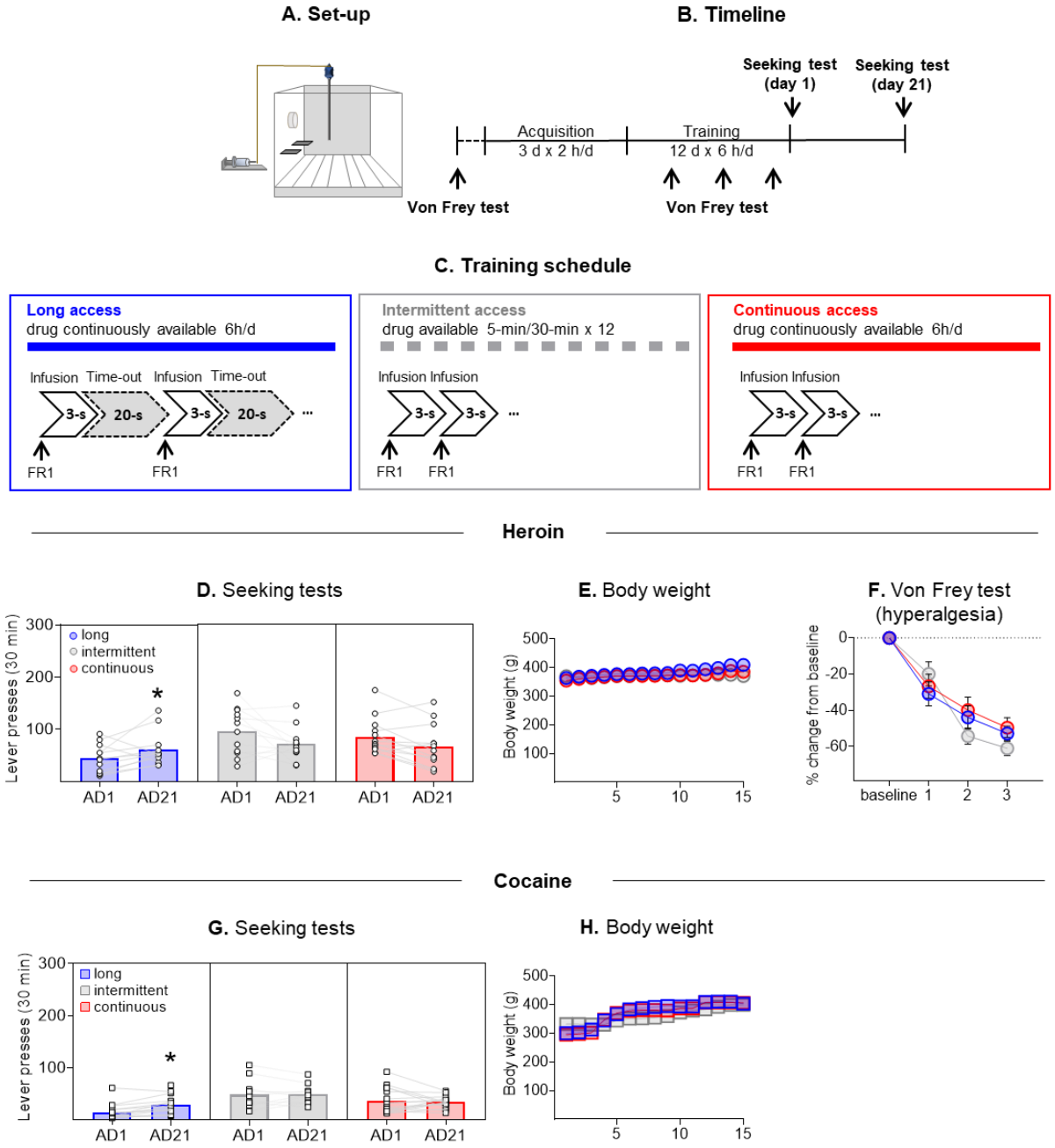

Figure 29. *The impact of timeout in heroin and cocaine self-administration: craving and withdrawal signs after long-access, intermittent-access, or continuous-access to drug. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Training schedule. (**D and G**) Seeking test on Abstinence day 1 and 21. Individual data of number of lever presses on the active lever during the 30-min extinction tests. (**E and H**) Body weight. Mean ± SEM of body weight per session. (**F**) Von Frey test (hyperalgesia). Mean ± SEM of % change from baseline in paw withdrawal per session. *Different from day 1, p < 0.05 (heroin: n=37; cocaine: n=41).*

Behavioral repertoire. In rats trained to self-administer heroin, the overall behavioral patterns during the initial hour of their final self-administration session were comparable across conditions, except for differences noted in stupor and locomotion. Specifically, Stupor was higher in intermittent and continuous-access conditions relative to long-access conditions (**Figure 30D**). By contrast, walking was higher in long-access conditions, relative to intermittent and continuous-access conditions (**Figure 30L**). In rats trained to self-administer cocaine, locomotory activity (walking) was higher in intermittent, relative to long-access and continuous-access conditions (**Figure 30J**).

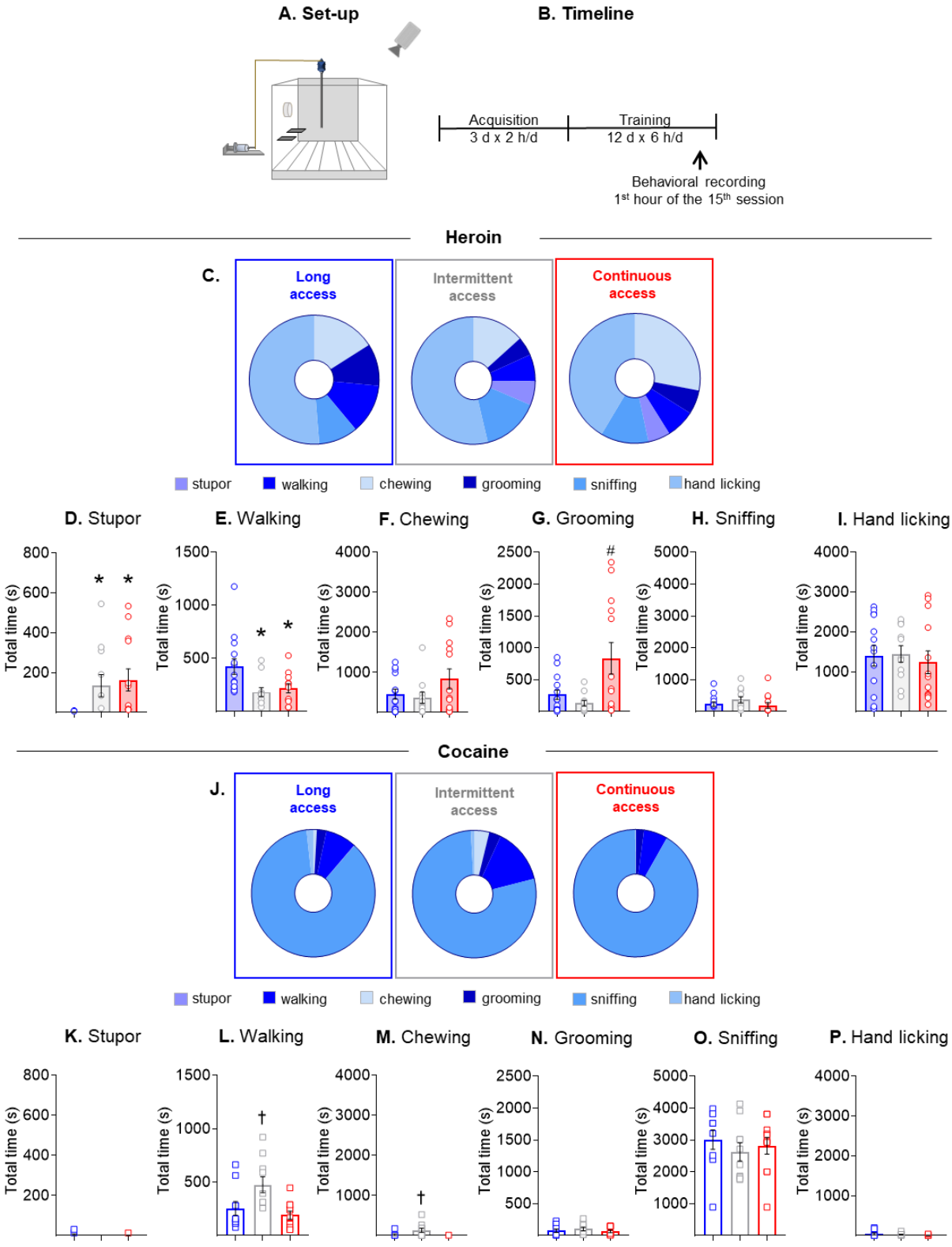
A. Set-up
B. Timeline
Acquisition
3 d x 2 h/d
Training
12 d x 6 h/d
Behavioral recording
1st hour of the 15th session
Heroin
C.
Long access
Intermittent access
Continuous access
stupor
walking
chewing
grooming
sniffing
hand licking
D. Stupor
E. Walking
F. Chewing
G. Grooming
H. Sniffing
I. Hand licking
Total time (s)
Cocaine
J.
K. Stupor
L. Walking
M. Chewing
N. Grooming
O. Sniffing
P. Hand licking

Figure 30. *Behavioral repertoire assessment in the first hour of the last session of drug self-administration training. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C and J**) Pie charts illustrating the average time allocated to each behavior. (**D-I and K-P**) Individual data of total time (s) of observed (**C and I**) stupor, (**D and J**) walking, (**E and K**) chewing, (**F and L**) grooming, (**G and M**) sniffing, (**H and N**) hand licking. * Different from long-access, $p < 0.05$; # Different from long-access and intermittent-access, $p < 0.05$; † Different from long-access and continuous-access, $p < 0.05$. (heroin: n=31; cocaine: n=31).*

Influence of timeout on drug-seeking and brain peak concentrations: insights from cluster analysis.

The three distinct self-administration procedures resulted in behavioral outcomes that exhibited similarities in certain aspects and disparities in others. Cluster analysis was conducted on the behavior of all tested individuals, agnostic to the training conditions of individuals, to explore whether distinct behaviorally-defined subpopulations of rats would be identified, and further, to determine if putative subpopulations would map onto the presence or absence of timeout during training. For both heroin and cocaine, the cluster analysis revealed that the most pertinent number of clusters was two. Regarding heroin, the two clusters (Cluster A n=21; Cluster B n=16) were predicted almost equally by the Seeking (predictor importance = 1.00) and Mean Brain Peak concentrations (predictor importance = 0.95), suggesting that both measures could discriminate the two subpopulations (**Figure 31 top**). Accordingly, disparities in the two clusters exhibited consistent alignment, with rats from Cluster B exhibiting higher brain heroin peaks and higher heroin seeking relative to rats from Cluster A (**Figure 32F, 32G**). Cluster A was comprised of n=12 long-access (100%), n=4 intermittent-access (33.3%), and n=5 continuous-access rats (38.5%); Cluster B was comprised of: n=0 long-access (0%), n=8 intermittent-access (66.6%), and n=8 continuous-access (61.5%). Concerning cocaine, the most discriminating feature between the two clusters (Cluster A n=25; Cluster B n=16) was the Seeking (predictor importance = 1.00), relative to Mean Brain Peak concentrations (predictor importance = 0.20) (**Figure 31 bottom**). This was apparent as the two clusters displayed contrasting trends in the two measures, with rats from Cluster A exhibiting similar brain cocaine peaks (**Figure 32I**) but lower cocaine seeking relative to rats from Cluster B (**Figure 32L**). Cluster A was comprised of n=14 long-access (93.3%), n=1intermittent-access (10%), n=10 continuous-access (62.5%); Cluster B was comprised of: n=1 long-access (6.6%), n=9 intermittent-access (90%), and n=6 continuous-access (37.5%).

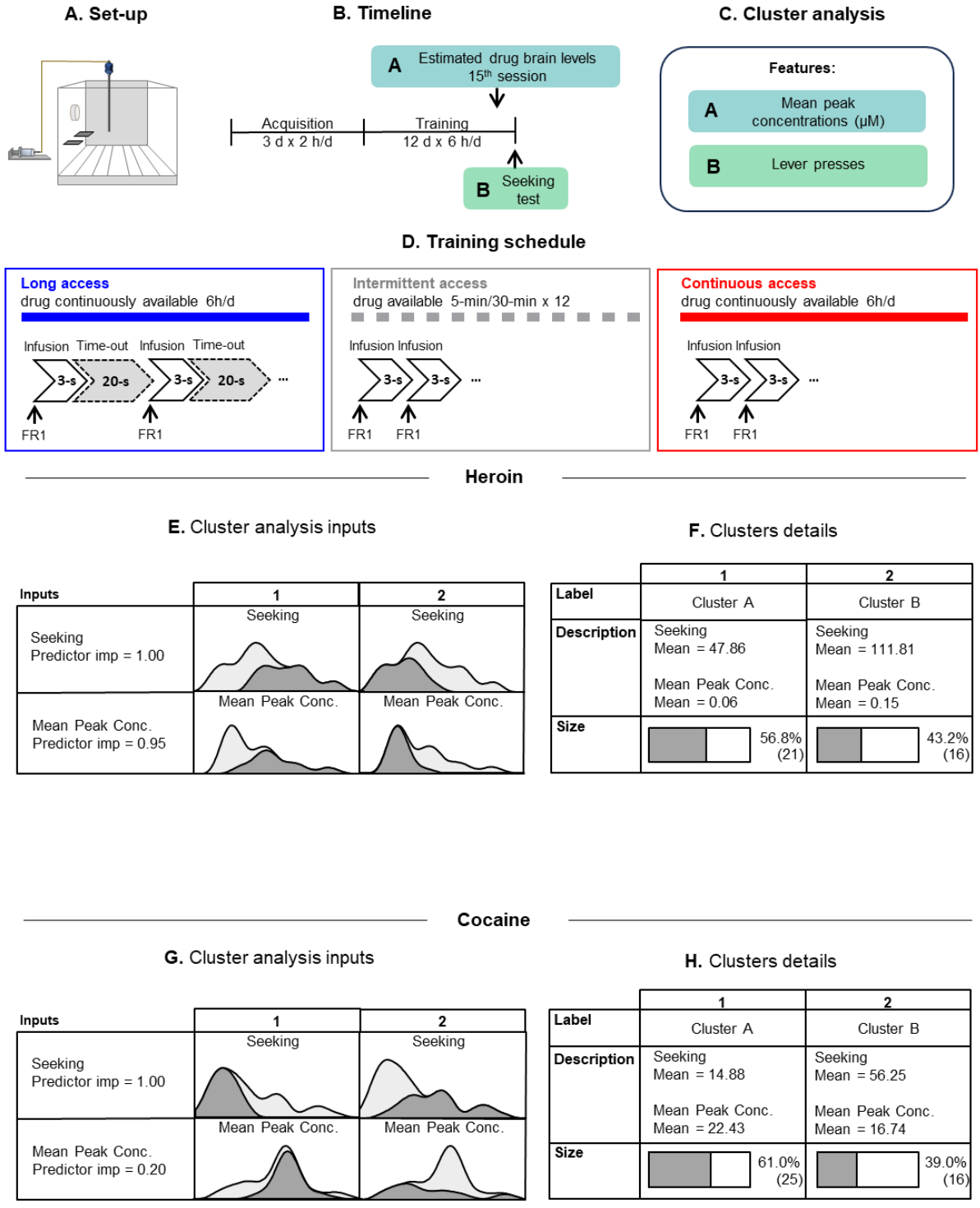

Figure 31. *Cluster analysis of rats trained under long-access, intermittent-access or continuous-access to heroin and cocaine. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Features used for cluster analysis. (**D**) Training schedules. (**E and G**) Cluster analysis inputs. Features predictor importance and related populations distribution of drug seeking and drug brain peak concentrations. (**F and H**) Clusters details. Details regarding the average drug-seeking, brain peak concentrations, and the sizes of Cluster A and B.*

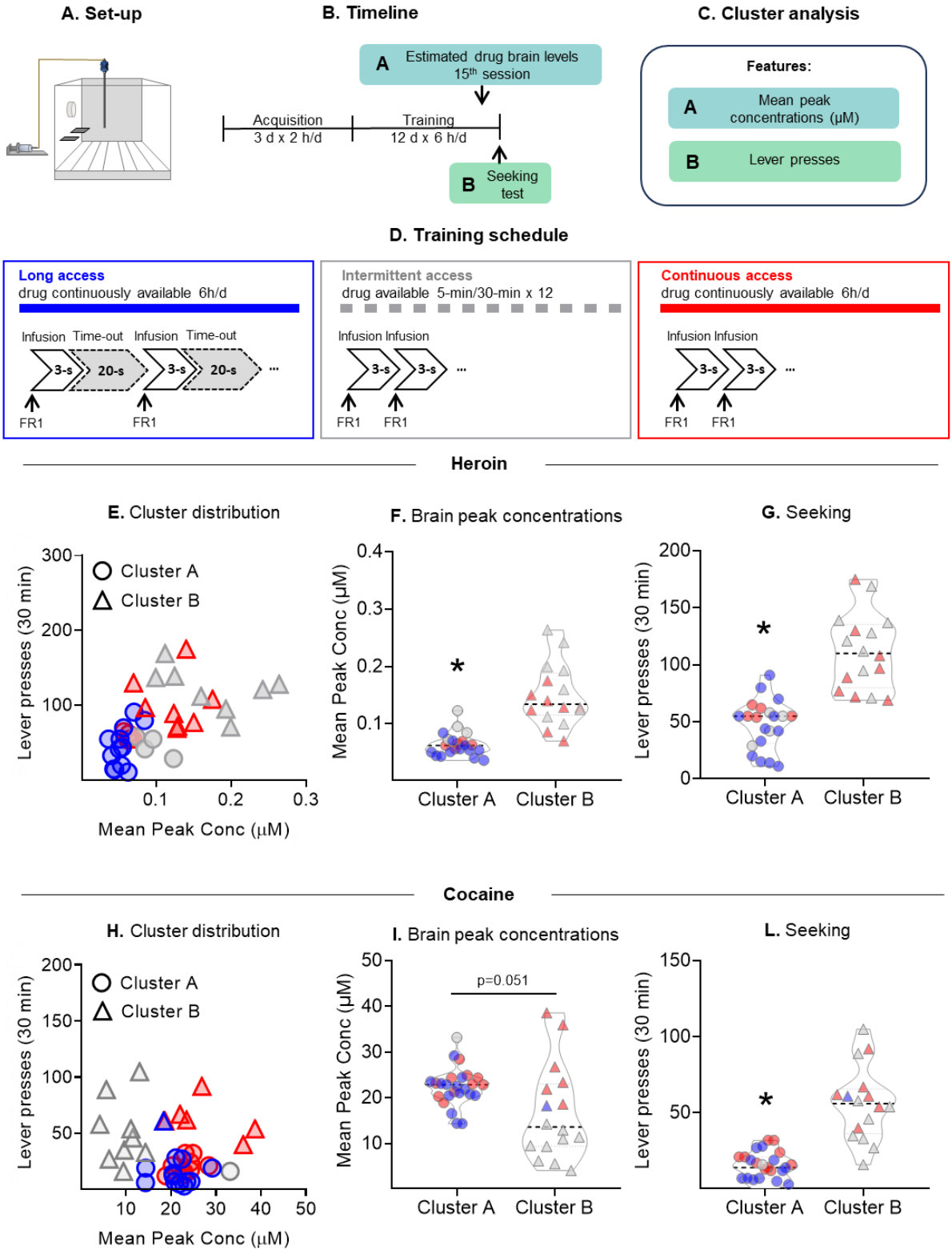
A. Set-up
B. Timeline
C. Cluster analysis
A Estimated drug brain levels 15th session
Acquisition 3 d x 2 h/d
Training 12 d x 6 h/d
B Seeking test
Features:
A Mean peak concentrations (μM)
B Lever presses
D. Training schedule
Long access
drug continuously available 6h/d
Infusion Time-out Infusion Time-out
3-s 20-s 3-s 20-s
FR1 FR1
Intermittent access
drug available 5-min/30-min x 12
Infusion Infusion
3-s 3-s
FR1 FR1
Continuous access
drug continuously available 6h/d
Infusion Infusion
3-s 3-s
FR1 FR1
Heroin
E. Cluster distribution
F. Brain peak concentrations
G. Seeking
Cluster A
Cluster B
Lever presses (30 min)
Mean Peak Conc (μM)
Cocaine
H. Cluster distribution
I. Brain peak concentrations
L. Seeking
p=0.051

Figure 32. *Cluster analysis of rats trained under long-access, intermittent-access or continuous-access to heroin and cocaine. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Features used for cluster analysis. (**D**) Training schedules (**E and H**) Cluster distribution. Distribution of individuals across the two factors used for cluster analysis. (**F and I**) Brain peak concentrations. Individual data of mean concentration of drug brain peaks in rats from Cluster A and B. (**G and L**) Seeking. Individual data of drug seeking in rats from Cluster A and B. Note the scales are different for each drug. * Different from Cluster B, $p < 0.05$. (heroin: $n = 37$; cocaine: $n = 41$).*

Experiment 4. Choice between drugs and social interaction

Discrete choice between drugs and social interaction in two different choice procedures. During the self-administration training, the rats increased their number of social and drug rewards earned over time (**Figure 33D, 33G**). After the drug self-administration, the rats were divided into two groups, matched for drug intake (**Statistical Table 2**): one group could choose between 1 unit-dose of drug or 1-min of access to the social partner (1 unit-dose drug vs 1-min access to social peer), as has been previously used in drug vs. social mutually-exclusive choice procedures. To allow the drug reward alternative to more closely approximate the social reward alternative in terms of offering a continuous reinforcement opportunity during a fixed availability period, the other group was offered a choice between 5-min of access to drug self-administration on a continuous schedule or 5-min access to the social partner (5-min drug access vs 5 min social access). In this preparation, the rat could select either the social or drug alternative, and then have 5-min to engage with that reinforcer in a self-selected ideal manner. Groups of heroin and cocaine-trained rats that were tested in the choice procedure '1 unit-dose drug vs. 1-min social', displayed a strong preference for social interaction over drug rewards (**Figure 33F, 33I**), as previously described (Venniro, Panlilio, Epstein & Shaham, 2021; Venniro, Russell, Zhang & Shaham, 2019). Regarding the choice procedure where the rats could self-select the preferred drug dose in the preferred time, '5-min drug access vs 5 min social access', heroin and cocaine-trained rats displayed opposite drug vs. social preferences . Cocaine rats preferred social interaction over cocaine, even if they could self-administer high doses of drug (**Figure 33I**), and their preference did not change when the time of access to the drug was increased (**Figure 34F**). Contrarily, heroin-trained rats, displayed a range of choice behaviors, with a subpopulation of heroin-trained rats preferring heroin to social interaction (**Figure 33F**). In addition, their preference for drug increased when the time of access to the social partner was increased (**Figure 34D**).

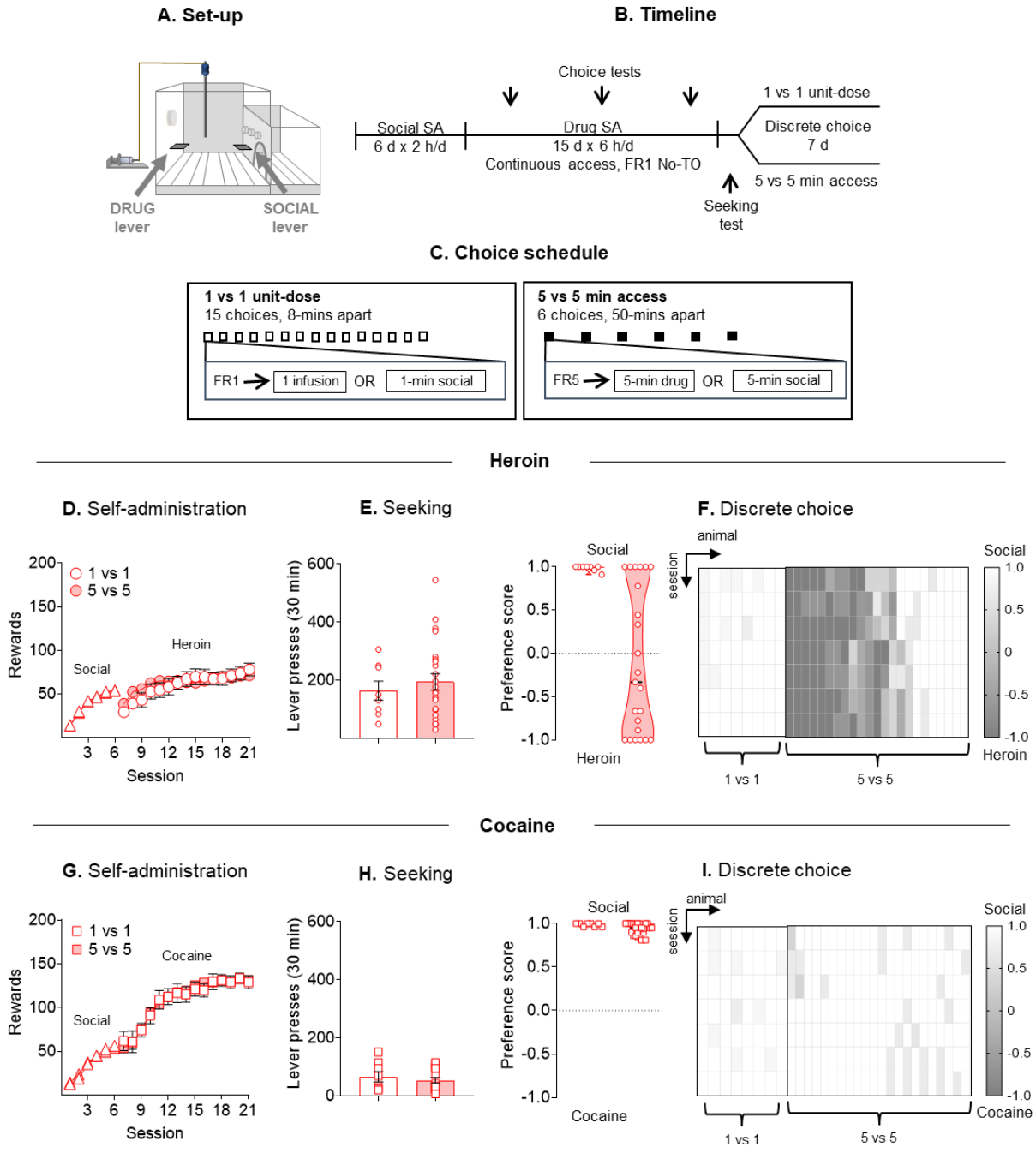

Figure 33. *Discrete choice between drugs and social interaction in two different choice procedures. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Choice schedule. (**D and G**) Social and drug self-administration. Mean ± SEM number of social and drug rewards per session. (**E and H**) Seeking test. Individual data of number of lever presses on the active lever during the 30-min extinction tests. (**F and I**) Discrete choice. Individual data of mean preference score (left). Heatmap of individual social preference scores (right) across the 11 discrete choice sessions for rats tested in the two different choice procedures. White indicates a preference for social interaction (score = 1) and dark grey indicates a preference for drug (score = -1). (heroin: '1 vs 1 choice' n = 8, '5 vs 5 choice' n = 23; cocaine: '1 vs 1 choice' n = 8, '5 vs 5 choice' n = 25).*

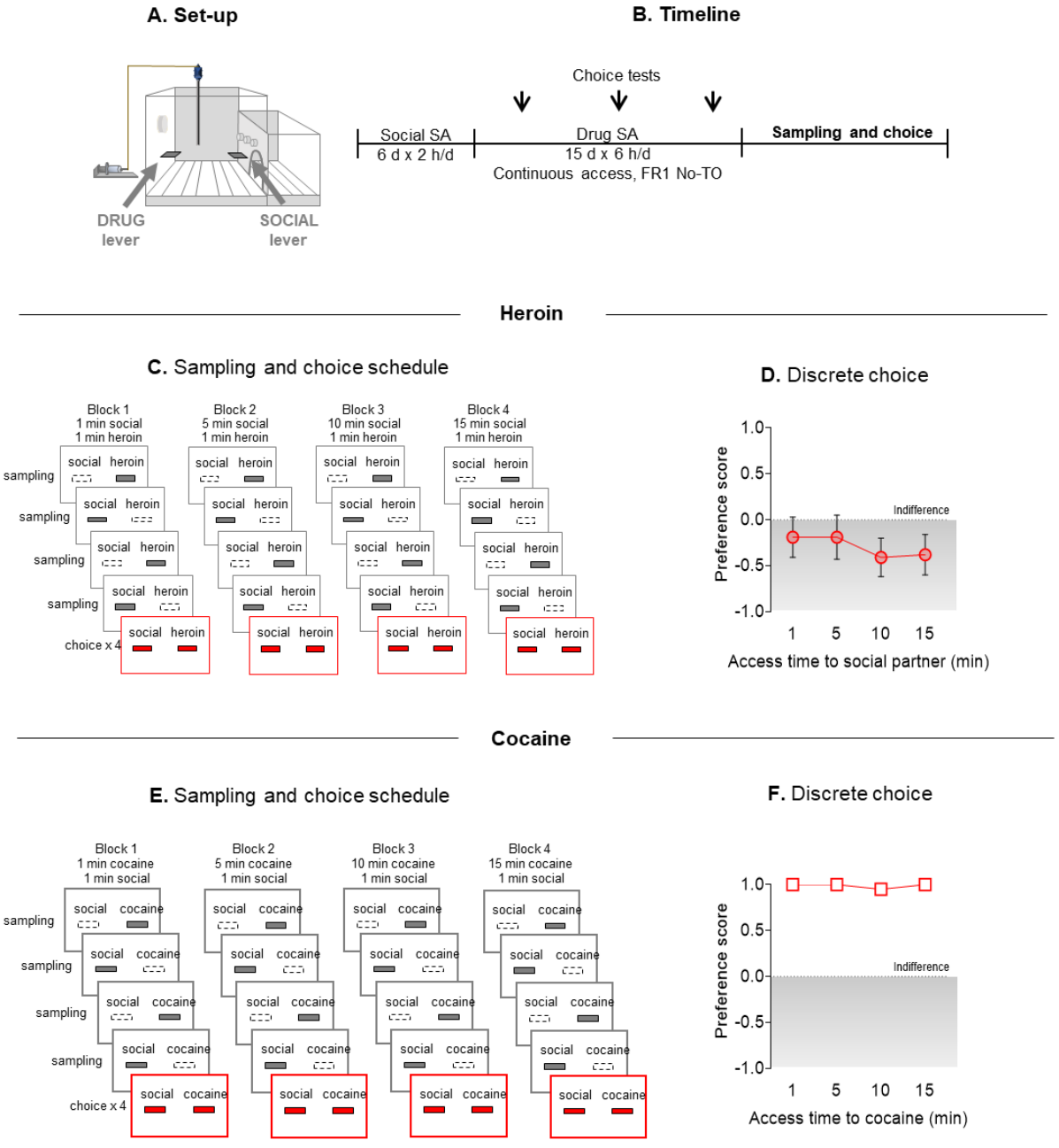

Figure 34. *Effects of procedural manipulations on drug/social preference: sampling and discrete choice. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C and E**) Sampling and choice schedule. (**D**) Discrete choice. Heroin vs social interaction with incremental time of access to the social partner. Mean ± SEM preference score across all the access time conditions (1=social, -1=heroin). (**F**) Discrete choice. Cocaine vs social interaction with incremental time of access to cocaine (1=social, -1=cocaine). Mean ± SEM preference score across all the access time conditions. (heroin: n = 15; cocaine: n = 10).*

Interindividual differences: insights from cluster analysis. The choice procedure ‘5 vs 5’ resulted in notable interindividual variances in drug preference among heroin-trained rats, but not those trained with cocaine. To ascertain whether these differences in heroin preference were predictable, cluster analysis was conducted on measures of drug self-administration and seeking. The analysis was extended to include cocaine, as, despite their strong inclination towards social interaction, there were occasional instances where cocaine-trained rats opted for the drug. Whether these sporadic choices could be anticipated through the analysis was sought. The cluster analysis indicated that the optimal number of clusters for both drugs was two. In the case of heroin, the most distinguishing factor between the two clusters, labeled as resilient (n=10) and vulnerable (n=13), was the Mean Brain Peak concentrations, with a predictor importance of 1.00, compared to the Seeking behavior which had a predictor importance of 0.65 (**Figure 35 top**). Rats from these separate clusters showed differences in severity scores, with rats from the vulnerable group displaying higher scores on the severity index (**Figure 36E**). This implies that variances in drug taking and -seeking behaviors may serve as predictors of drug preference and susceptibility to addiction. Additionally, there were differences in drug preference, with the vulnerable group displaying a greater preference for the drug compared to the resilient group (**Figure 36F**). Regarding cocaine, the primary distinguishing factor between the two clusters, denoted as resilient (n=13) and vulnerable (n=9), was predominantly the Seeking behavior, with a predictor importance of 1.00, while the predictor importance for Mean Brain Peak concentrations was 0.09 (**Figure 35 bottom**). Rats from the two clusters displayed discrepancies in severity, with those from the vulnerable group showing higher scores on the severity index (**Figure 36H**). However, unlike heroin, the two clusters did not exhibit disparities in drug preference during the choice procedure (**Figure 36I**).

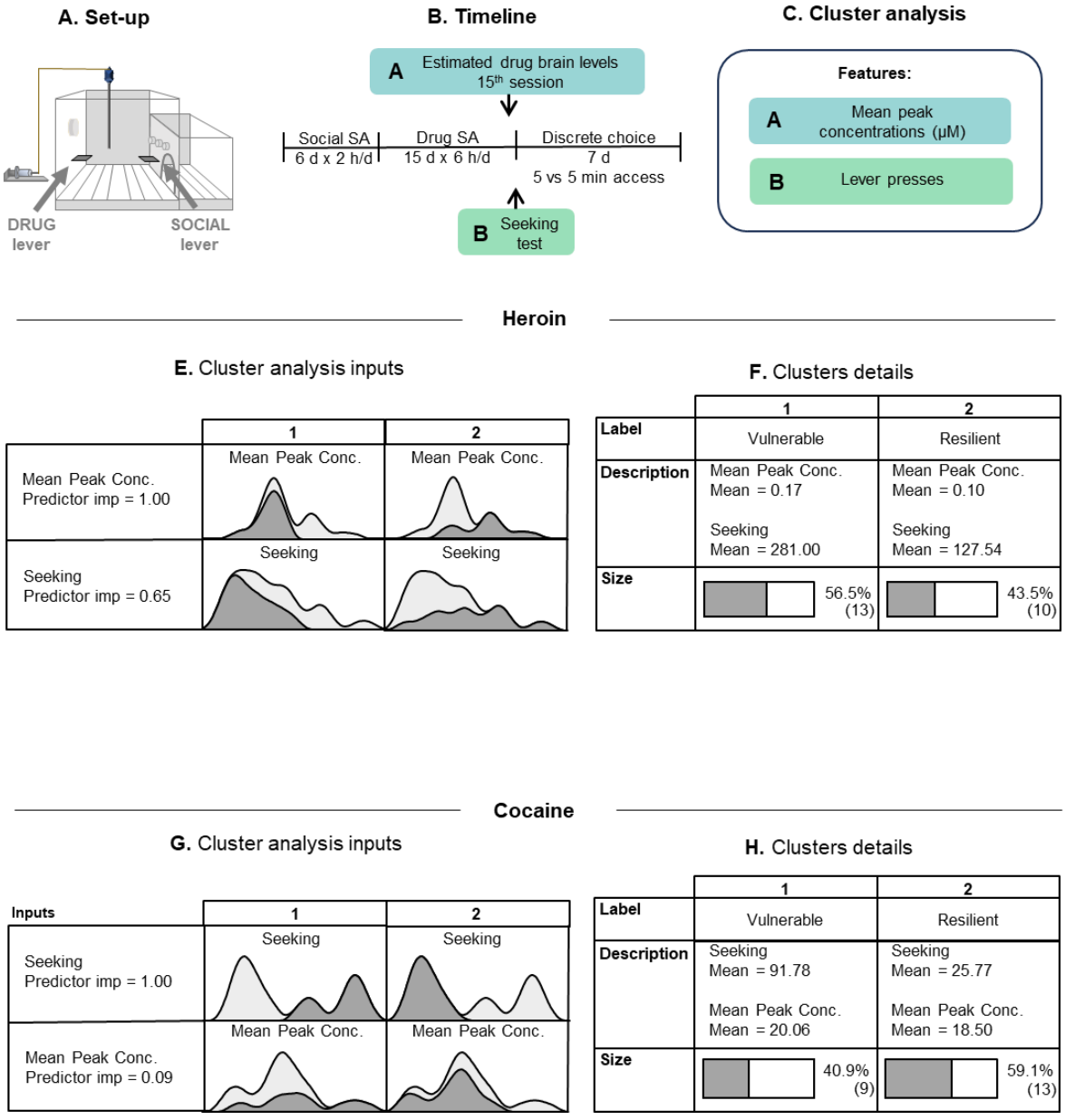

Figure 35. *Cluster analysis of rats tested on the '5 vs 5 min' choice procedure. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Features used for cluster analysis. (**D**) Training schedules. (**E and G**) Cluster analysis inputs. Features predictor importance and related populations distribution of drug seeking and drug brain peak concentrations. (**F and H**) Clusters details. Details regarding the average drug-seeking, brain peak concentrations, and the sizes of vulnerable and resilient clusters.*

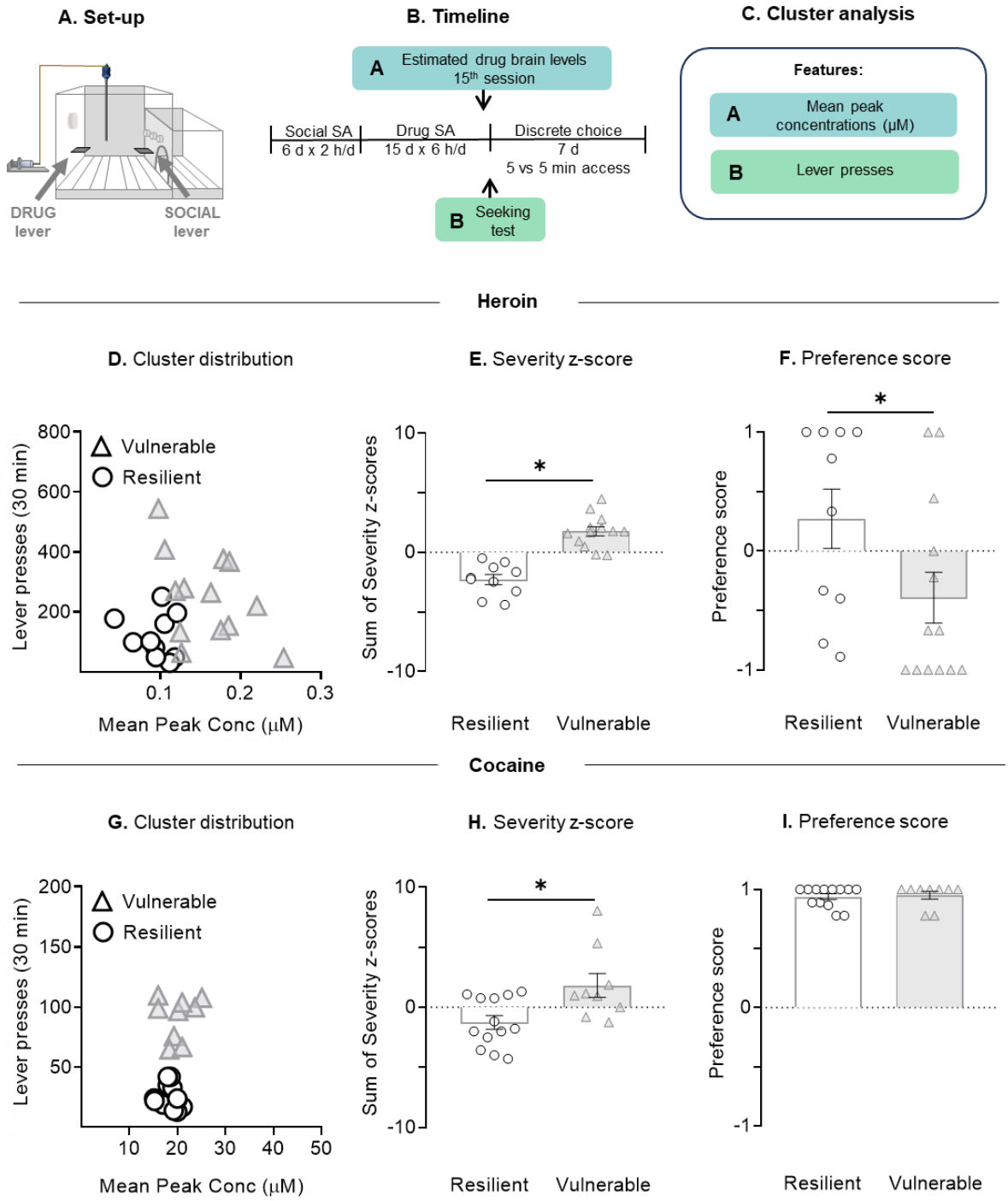

Figure 36. *Cluster analysis of rats tested on the '5 vs 5 min' choice procedure. (**A**) Experimental set-up. (**B**) Experimental timeline. (**C**) Features used for cluster analysis. (**D**) Training schedules. (**D and G**) Cluster distribution. Distribution of individuals across the two factors used for cluster analysis. (**E and H**) Individual data of severity score in resilient and vulnerable rats. (**F and I**) Individual data of mean social preference scores during the last 3 sessions of social choice in in resilient and vulnerable rats. Preference for social interaction score = 1), preference for drug (score = -1). * Different from resilient, $p < 0.05$. (heroin: n = 23; cocaine: n = 22).*

6.6. Discussion

In this chapter, I conducted three experiments to probe the effects of timeout and unit-doses on drug self-administration performance and choice procedures.

The first experiment, employing the within-subject design, revealed that the absence of timeout between drug unit-doses significantly increased the motivation to take and seek heroin and cocaine, with rats showing a strong preference for drug self-administration without a timeout.

The second experiment used a between-subject design to compare three conditions: (1) long-access (drugs continuously available 6-h/d, FR1, 20-s timeout), 2) intermittent-access (drugs intermittently available 6-h/d divided in 5-min access every 30-minin, FR1, no-timeout), or 3) continuous-access (drugs continuously available 6-h/d, FR1, no-timeout) access schedule. The absence of timeout had minimal impact on cocaine-taking patterns and resultant drug brain concentration both in the continuous-access and long-access groups. To the contrary, as previously reported, cocaine-taking patterns were influenced by the intermittent-access procedure, because of the experimenter imposed 25-min OFF periods. Notably, the absence of timeout heightened drug-seeking behavior, particularly during early abstinence. In contrast, heroin-taking patterns were predominantly affected by the presence of timeout between unit-doses. The absence of timeout, irrespective of the training conditions (intermittent-access and continuous-access), promoted burst-like patterns of drug taking behavior, characterized by self-administration of multiple unit-doses of heroin in quick succession. This behavior resulted in intermittently high brain peaks of heroin concentrations and subsequent intoxicating effects in a subgroup of rats. The dissociation between heroin and cocaine was attributed to their distinct pharmacokinetics and pharmacodynamics profiles.

Finally, in the third experiment, two choice procedures were contrasted: (1) choosing between 1 unit-dose of social interaction (1-min access) or of the drug; (2) choosing between 5-min access to the social partner or to the drug (a time sufficient load up the preferred dose in the preferred time). Results indicated again a dissociation: all cocaine-trained rats displayed a strong preference for the social peer regardless of the choice procedure adopted. In contrast, more than 50% of heroin-trained rats preferred heroin, but

only if allowed to choose the preferred dose in the preferred time. The resultant social withdrawal was predicted (via a cluster analysis) by peak concentrations of heroin in the brain and drug-seeking.

6.6.1. The role of the experimenter-imposed timeout on cocaine and heroin taking and seeking: a within-subject design approach

In **Chapter 5**, I compared long-access and intermittent-access to heroin. The principal discovery was that the intermittent-access group, despite having significantly shorter periods of drug access as determined by the experimenter, exhibited a notably higher heroin intake compared to the long-access group. This outcome stands in stark contrast to existing literature on intermittent-access with cocaine (Kawa, Bentzley & Robinson, 2016; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012). The interpretations of this counterintuitive finding were attributed to two factors: (1) the timeout imposed between unit-doses (present in the long-access but absent in the intermittent-access procedure) might have counteracted heroin brain accumulation (leading to a reduced heroin intake) and 2) the intermittency in drug access might have promoted a compensatory drug taking to overcome periods of unavailability (25 minutes OFF periods). This compensatory behavior may be driven by the need to maintain drug levels in the brain above the threshold for drug satiety (Desai, Tron Esqueda & Norman, 2023; Panlilio, Katz, Pickens & Schindler, 2003; Tsibulsky & Norman, 1999), or to mitigate withdrawal/stressful effects induced by periods of unavailability[9].

Based on these premises, the main goal of the experiments designed in this chapter, was to dissect the relative contribution of (1) timeout between unit-doses (experimentally imposed in the long-access procedure) from (2) the 25 min OFF periods (experimentally imposed in the intermittent-access procedure), on the patterns of drug taking and drug seeking. Because the unexpected heroin findings contradicted previous findings in the context of cocaine self-administration, (Allain, Minogianis, Roberts & Samaha, 2015; Kawa, Allain, Robinson & Samaha, 2019; Kawa, Bentzley & Robinson, 2016; Samaha,

[9] This interpretation was proposed by one of the reviewers during the first submission of the paper.

Khoo, Ferrario & Robinson, 2021; Zimmer, Oleson & Roberts, 2012), I decided to compare heroin to cocaine self-administration, both differing in their pharmacokinetics and pharmacodynamics profiles.

Through the use of a within-subject design, the *first* main finding of the current study indicates that the lack of timeout following drug infusion significantly influenced the motivation to take and seek drugs, leading to increased drug motivation in both heroin and cocaine self-administration experiments. Similarly, in drug-seeking tests, under extinction conditions, rats pressed more on levers associated with no-timeout vs timeout drug self-administration (**Figure 22D, 22G**). In a discrete choice procedure where rats could select between timeout and no-timeout drug self-administration levers, the rats exhibited a strong preference for drug self-administration without timeout between unit-doses (**Figure 22H, 22H**). Finally, in a progressive ratio procedure, rats worked harder for the lever associated with no-timeout (**Figure 22F, 22I**).

A potential interpretation of these findings is that the imposition of timeout between unit-doses slows down the accumulation of drug in the brain and consequently may limit rats' ability to choose the preferred dose in the preferred time (Morgan, Liu, Oleson & Roberts, 2009; Roberts & Zimmer, 2020). Prior studies demonstrated that the speed of cocaine injection significantly influences drug-related behaviors in rats (Samaha & Robinson, 2005). Faster delivery of cocaine to the brain (tested dose ranging from 5-s to 90-s), led to higher cocaine intake and cocaine-seeking (Wakabayashi, Weiss, Pickup & Robinson, 2010) stronger psychomotor sensitization (Samaha, Li & Robinson, 2002), and motivation to take cocaine (Minogianis, Lévesque & Samaha, 2013). It is worth mentioning that no prior preclinical studies have reported a systematic investigation of the effects of the speed of drug injection in heroin or opioid self-administration. However, the current findings are consistent with data on cocaine and suggest a significant influence of the speed of heroin delivery/accumulation on drug self-administration and related behaviors.

This led to the *second* main finding of the present study: timeout had a stronger impact on heroin but not cocaine self-administration. This distinction was evident in several measures. Firstly, the absence of timeouts led to increased heroin intake, whereas it did not affect cocaine intake (**Figure 22D, 22F**).

Secondly, heroin-trained rats showed heightened lever pressing when subjected to training conditions involving timeouts between heroin unit-doses, a response not observed with cocaine (**Figure 22E, 22G**). Thirdly, lever pressing during the drug seeking (relapse) tests and final ratio in progressive ratio tests were notably higher in heroin-trained rats than in cocaine-trained rats (**Figure 23**).

A caveat of these findings resides in (1) the utilization of a short access training (3-h per session) and 2) carryover confounding effect of the training conditions (the within-subject design; where the same rat was trained on two levers -one paired with a timeout and the other had no such consequence). We therefore decided to corroborate and generalize these findings by employing extended access procedures (6-h per session) and a between-subject design.

6.6.2. The role of the experimenter-imposed timeout on cocaine and heroin taking and seeking: a between-subject design approach

To better understand the differential impact of timeout on heroin and cocaine self-administration, distinct groups of rats underwent training using different self-administration schedules. A comparison was made between long-access and continuous-access, which differed only in the presence of a timeout between unit-doses. Additionally, a group trained under intermittent-access was included to gain a better understanding of the separate roles played by (1) the timeout between unit-doses and 2) the 25-min OFF period imposed between access periods (**Figure 23C**). Drug brain levels were estimated and compared across the three different procedures.

The between-subject design study corroborated the findings of the within-subject study, indicating that the absence of a timeout led to heightened motivation to seek both heroin and cocaine. However, distinct effects of the presence or absence of a timeout were observed on drug intake, patterns of drug taking, and resultant drug brain levels, highlighting a significant influence of timeouts on patterns of heroin taking. As such, the findings for cocaine and heroin will be separately discussed below, followed by an integrative summary at the end of the section.

The impact of timeout on cocaine taking and seeking

The long-access and continuous-access procedures (**Figure 24G, 24H**) resulted in a higher overall cocaine intake relative to the intermittent-access, as previously reported (Kawa, Bentzley & Robinson, 2016; Zimmer, Oleson & Roberts, 2012). However, cocaine seeking during early abstinence was remarkably similar between rats trained under intermittent-access and continuous-access conditions, supporting a critical role for the absence of time out in the motivation to seek the drug. Notably, the reduction in cocaine intake in intermittent-access rats was mainly attributable to the experimenter-imposed limit to drug access, as also confirmed by the cluster analysis. This constraint led to a cocaine pattern featured by a self-administration of multiple consecutive unit-doses during ON periods of access (**Figure 25D, 26G**) taken in less than 1-min (**Figure 25D, 26F**) and fluctuating brain levels of cocaine (**Figure 27G**), which increased during periods of drug availability and decreased to near-zero levels during OFF periods. This resulted in total brain levels of cocaine and brain peak concentrations that were lower relative to rats trained under long-access and continuous-access conditions (**Figure 27H, 27I**), as previously reported (Samaha, Khoo, Ferrario & Robinson, 2021; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012).

In the long-access and continuous-access conditions, rats consistently displayed the typical pattern of cocaine self-administration characterized by a ‘loading’ phase (at the beginning of the session), featured by infusions of several unit-doses at a high rate, followed by a 'maintenance phase' of single unit-doses spaced apart (Ahmed & Koob, 1998; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012). This pattern was associated with comparable maximum brain peak concentrations and total brain concentrations of cocaine (**Figure 27G, 27H, 27I**). These results are consistent with the observations by Ahmed and Koob (Ahmed & Koob, 1999) showing that the reduction of timeout from 20-s to 4-s did not affect rats’ patterns of drug taking and total intake. Furthermore, the brain levels of cocaine achieved with or without timeout are consistent with previous findings indicating that different speeds of drug delivery led to similar peaks of cocaine in the brain (Minogianis et al., 2019). Finally, as outlined in **Chapter 5**, it is proposed that one of the key factors sustaining drug taking behavior in rats is their learning to self-administer drugs to achieve a certain 'satiety threshold' of the drug, also known as the 'compulsion zone.'

They then regulate their drug levels to remain around this threshold, administering further doses whenever their drug levels fall below it (Desai, Tron Esqueda & Norman, 2023; Panlilio, Katz, Pickens & Schindler, 2003; Tsibulsky & Norman, 1999). The results of the present study provide additional support for the existence of a 'compulsion zone' for cocaine (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006; Panlilio, Katz, Pickens & Schindler, 2003; Suto & Wise, 2011; Tsibulsky & Norman, 1999). This phenomenon suggests a nuanced regulatory mechanism governing cocaine intake, wherein rats adjust their drug taking behavior in response to fluctuations in drug levels within the brain, after reaching a preferred cocaine brain level threshold (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006; Panlilio, Katz, Pickens & Schindler, 2003; Tsibulsky & Norman, 1999). Other studies suggested that this behavior is also related to the maintenance of dopamine levels in the striatum (Gerber & Wise, 1989; Suto & Wise, 2011), suggesting that cocaine self-administration is the result of a complex interplay between pharmacokinetics, drug reinforcement, and neural mechanisms.

Notably, the high drug seeking during early abstinence marked the observation of incubation of drug craving in training conditions where the timeout was not imposed (Grimm, Hope, Wise & Shaham, 2001) (**Figure 29G**). This observation mirrors findings in the within-subject study and studies on the rate of drug delivery indicating that the rapid delivery of cocaine heightens motivation to seek cocaine (Minogianis, Lévesque & Samaha, 2013; Samaha & Robinson, 2005; Wakabayashi, Weiss, Pickup & Robinson, 2010). However, the absence of incubation may have been influenced by the within-subject design (early and late abstinence test) and the significantly short test duration. Therefore, future studies should explore these aspects by examining rats using a between-subject design and longer test durations. Of relevance, long-access rats display the lowest drug-seeking (**Figure 24H**).

The impact of timeout on heroin taking and seeking

The lack of timeout in the intermittent-access and continuous-access procedure, resulted in a remarkably similar intake that was significantly higher relative to the long-access procedure. Most of the intermittent-access and continuous-access rats displayed patterns characterized by bursts-like events,

featured by 3-5 unit-doses injected in less than 2-min[10] (**Figure 26D, 26E**). These patterns resulted in fast-rising high brain peaks of heroin (**Figure 27E**). It should be noted that the continuous and long-access procedures differ only in terms of the imposition of a 20-s timeout following drug delivery. Despite this, those burst-like patterns were never observed in rats trained under long-access conditions. (**Figure 26D, 26E, 27D, 27E**). Regarding brain levels of heroin metabolites, the burst-like patterns observed in intermittent-access and continuous-access conditions led to higher brain peak concentrations of both 6-MAM, and morphine compared to the long-access condition (**Figure 28E, 28F**).

In addition to these aspects, the analysis of the behavioral repertoire of rats trained under intermittent-access and continuous-access conditions was remarkably different from the long-access condition (**Figure 30**). The most noteworthy observation was the manifestation of 'behavioral stupor' (Seip-Cammack, Reed, Zhang, Ho & Kreek, 2013), often referred to as opioid-induced catalepsy (De Ryck, Schallert & Teitelbaum, 1980; De Ryck & Teitelbaum, 1984; Turski, Havemann & Kuschinsky, 1982) observed consistently and exclusively in rats trained under intermittent-access and continuous-access conditions (**Figure 5**). This cataleptic behavior and reduced locomotor activity in intermittent-access and continuous-access conditions was mainly observed following burst-like events, thus it could have been induced by the higher doses of heroin administered. Of note, studies investigating opioid-induced catalepsy demonstrated that low doses of morphine only produce an excitatory effect, while high doses lead to initial depression followed by a delayed excitatory effect (Babbini & Davis, 1972). However, due to technical constraints inherent in the recording system used in this study, a systematic behavioral analysis of rats was not conducted concerning the temporal dynamics of drug injections. Additionally, the effects of heroin and its metabolites were not systematically matched and evaluated concerning observed behaviors. Therefore, further investigations are warranted to study this aspect and elucidate the intricate relationship between drug administration dynamics and resultant behaviors in more detail.

[10] A 2-minute interval was selected to account for consecutive injections, particularly in the long-access condition where a timeout between injections was imposed.

To summarize, the intermittent-access and continuous-access conditions differed significantly from the long-access conditions. Moreover, the observed behavioral variability among rats subjected to intermittent-access and continuous-access suggests the existence of interindividual differences. This inference is consistent with a previous study utilizing intermittent-access (O'Neal, Nooney, Thien & Ferguson, 2020). To gain further insight into this phenomenon, a cluster analysis was conducted using two primary features: 1) mean peak concentrations reached during the final self-administration session, and 2) drug seeking on Abstinence day 1. This analysis revealed the existence of two distinct clusters, named Cluster A and Cluster B. These clusters differed in both measures, with rats in Cluster B exhibiting higher mean peak concentrations and greater drug-seeking behavior compared to those in Cluster A (**Figure 32**). Importantly, all rats from the long-access group were categorized into Cluster A, indicating a distinct behavioral profile. In contrast, rats trained under intermittent-access and continuous-access conditions were distributed across both clusters. This suggests that a subset of rats trained under intermittent-access (33.3%) and continuous-access (38.5%) conditions displayed behavior resembling that of rats trained under long-access conditions (**Figure 31E, 31F, 31G**).

Finally, differences between these two conditions emerged, necessitating further discussion. Indeed, the rate of increase in brain drug levels was higher in rats under intermittent-access compared to continuous-access (**Statistical Table 2**). This could be attributed to the experimental design, which forced rats in the intermittent-access condition to self-administer the drug rapidly due to time constraints (5-min ON of access to heroin followed by 25-min OFF period). Additionally, brain levels of heroin and 6-MAM were higher in rats under intermittent-access relative to continuous-access (**Figure 28D, 28E**). This difference may be explained by the occasional single drug unit-doses in rats under continuous-access, as their infusions were not restricted during the session by experimenter-imposed limits on drug access (i.e. 25-min OFF).

Another relevant aspect stemming from the studies outlined above is that the lack of timeout increased drug-seeking under extinction conditions during initial periods of abstinence, with rats trained under long-access conditions (with timeout between unit-doses) showing the lowest drug-seeking (**Figure**

23F). This aspect recapitulates previous findings from the within-subject study (discussed above) and mirrors previously described cocaine findings.

Together these heroin findings suggest the rejection of one of the hypotheses driving the current study. It was initially proposed that periods of drug unavailability, as imposed in the intermittent-access procedure, might trigger compensatory behavior aimed at maximizing drug intake during available periods to alleviate withdrawal or stress induced by the unavailability periods. However, contrary results lend further credence to the notion that the absence of a timeout slows down heroin delivery to the brain. This slowdown prevents its accumulation and potentially hinders rats from reaching preferred drug brain concentrations and associated effects. For a more in-depth discussion on this topic, readers are referred to the discussion provided in **Chapter 2**.

Insights from pharmacokinetics on the impact of timeout on cocaine and heroin taking

One of the main findings of the present series of experiments was the distinct impact of the absence of timeout over cocaine and heroin-taking patterns. Specifically, heroin self-administration under continuous-access conditions resulted in fast-rising high brain peaks of heroin relative to the long-access procedures (differing only for 20-s experimenter-imposed timeout). This difference was not observed with cocaine. Below, I will offer a tentative explanation based on the pharmacokinetics differences of these two drugs.

Heroin has a shorter half-life than cocaine (Gottas et al., 2013) (~0.9-min; a tenth of cocaine, ~10-min) thus the experiment-imposed timeout [20-s in our study; but can vary up to 40-s (Corre et al., 2018)] likely counteract the accumulation of heroin in the brain. In doing so it would lessen the rat's ability to achieve the preferred dose in the preferred time (and associated effects). In contrast, in virtue of the half-life of cocaine (~10-min) (Pan, Menacherry & Justice, 1991) the timeout likely exerts only a negligible impact on its brain accumulation. Accordingly, Minogianis et al. (2019) showed that different speeds of drug delivery led to similar peaks of cocaine in the brain.

In addition to this, the metabolism of cocaine and heroin leads to the formation of unique metabolites featured by distinct pharmacokinetics and pharmacodynamics profiles relative to their parent compounds.

Heroin in particular yields several pharmacologically active metabolites known to significantly contribute to the acute reinforcing and physiological effects of heroin (Andersen, Ripel, Boix, Normann & Mørland, 2009; Inturrisi, Max, Foley, Schultz, Shin & Houde, 1984; Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983) (**Chapter 3**). Because of the absence of time out, the concentrations of 6-MAM and morphine were significantly higher relative to the training conditions featured by time out (long-access). The absence of timeout training condition has therefore altered the dose-effect relationship of the metabolites (relative to the no timeout condition). In the present study, the pharmacokinetics modeling combined with the observational assessment of rats (data not shown) suggests a multistage course of effects likely driven by 6-MAM and morphine. The elevated levels of 6-MAM resulting from the very rapid increase in heroin concentrations may impact performance due to the sedative effects associated with 6-MAM. Notably, this interpretation supports previous reports suggesting that implementing a timeout after drug infusions increases lever pressing by reducing the direct pharmacological effects of drugs on behavioral performance (Kelleher, 1975). Due to the scarce understanding of the impact of norcocaine on behavior, it is premature to speculate on this matter.

The differences highlighted in the patterns of drug taking and other drug-related behaviors are significant. As emphasized in the introduction, the traditional approach to developing animal models of drug addiction revolved around creating a standardized, one-size-fits-all self-administration procedure characterized by a uniform and highly regular drug taking pattern across all addictive drugs (Roberts, Morgan & Liu, 2007). However, the findings presented here challenge this viewpoint and suggest that a uniform approach to studying drug addiction may not be applicable across all drugs. Therefore, further research is warranted to understand deeper into these differences and to develop a more nuanced understanding of the complexities involved in drug addiction. This could potentially pave the way for the development of customized self-administration procedures for each drug, ultimately leading to the development of more effective treatment strategies tailored to the specific drugs.

Key point: timeout strategies disrupt rats' ability to self-administer the preferred drug-dose in the preferred time

One significant finding from this series of experiments was the notable influence of the timeout period on drug loading. This effect was particularly evident in drugs with (1) very short half-lives and (2) a metabolism leading to the production of numerous active metabolites, such as heroin.

Results showed that the timeout strongly influenced the pattern of heroin-taking. However, more remarkably, distinct patterns of heroin-taking also led to *qualitatively* different behavior. Specifically, the stupor behavior was never observed in rats trained under long-access conditions (with timeout) (**Figure 30D**). Indicating that the dose-time relationship does play a role in this behavioral outcome, highly sought after. By contrast, certain behaviors such as hand licking to self-injury and chewing, commonly observed in rats exposed to heroin (Lenoir & Ahmed, 2007; Seip, Reed, Ho & Kreek, 2012), remained unchanged (**Figure 30D**, **30I**). These differences are unexpected considering that morphine brain concentrations were comparable at least between rats trained in the long-access and intermittent-access procedures (**Figure 28F**).

This phenomenon is supported by various evidence. Pharmacokinetic and pharmacodynamic studies consistently demonstrated a non-linear dose-effect relationship when the same total dose is administered in divided amounts at different intervals (Holford & Sheiner, 1981; Wagner, 1968). This effect of timing between doses is strictly linked to the metabolism of the drug in the body: the faster the metabolism the stronger the impact of timing on the drug effects. For example, remifentanil, a fast-acting opioid agonist with a very short half-life [8-10 min, (Bürkle, Dunbar & Van Aken, 1996)], must be infused at a constant rate at specific doses to achieve a sufficient analgesic effect (Bürkle, Dunbar & Van Aken, 1996) while avoiding tolerance side-effects (Vinik & Kissin, 1998). Suggesting that the dosing-timing relationship can differentially influence different effects. In a different context, using morphine, which has a significantly longer half-life [3–4 h (Gyr et al., 2000; Rook et al., 2006)] relative to remifentanil, Marsch et al. (2001) showed that administering the same dose of morphine at different infusion rates resulted in heterogeneous effects. Specifically, certain effects of morphine did not differ from placebo, while others

did. This suggests that the dose-time relationship can differently impact the multitude of effects elicited by drugs.

Based on this evidence, the spectrum of effects from repeated unit doses, intersperse with timeouts, may qualitatively differ from those arising from self-selected dosing in the preferred time. Indeed, drugs like heroin and cocaine produce a wide range of effects, characterized by diverse dose-effect relationships. Moreover, the progression of these effects is a nonlinear function of the interaction between the parent drug and its metabolites, each with distinct pharmacokinetic and pharmacodynamic profiling. In essence, the cumulative experience induced by spaced unit doses may not equate to a fraction of the experience produced by multiple doses consumed in rapid succession. Accordingly, clinical studies have shown that some heroin users tend to opt for consuming large doses of heroin to experience an intense 'rush', waiting for longer intervals between doses, rather than taking smaller doses at shorter intervals (McAuliffe & Gordon, 1974; Mirin & Meyer, 1979).

Building upon these insights, I transitioned my investigation to drug-vs-social choice procedures, to understand how these parameters might have influenced earlier reports that compared the rewarding potential of drugs to non-drug reinforcement. These procedures offer a means to replicate one of the hallmark criteria of drug addiction: social withdrawal triggered by heightened motivation for drugs. Within these procedures, unit-dose strategies are commonly used to evaluate the relative preference between drug consumption and social interaction in rats. I therefore designed an experiment to investigate the impact of unit-doses in drug versus social choice procedures, and below, I will discuss the results.

6.6.3. Unit dose strategies occlude heroin preference but not cocaine in drug-vs-social choice

Unit-doses of drugs commonly used in self-administration studies which maintain high levels of ongoing behavior may be suboptimal rewards when offered as a single infusion drug reward (Morgan, Liu, Oleson & Roberts, 2009; Roberts & Zimmer, 2020) in the context of discrete choice procedures. This is because a single unit-dose (e.g. (Papastrat et al., 2023; Venniro, Panlilio, Epstein & Shaham, 2021; Venniro et al., 2020; Venniro, Russell, Zhang & Shaham, 2019; Venniro et al., 2018), even though on the descending limb of the dose-effect curve, might not be rewarding enough relative to an alternative

incentive (e.g. social interaction). It should be noted that the naturally occurring behavior of rats at the beginning of the training sessions is 'drug-loading' phase featured by a high rate of infusions (also known as 'bursts' of infusions), which likely reflects the animal attempt to increase the brain-drug concentration above a 'satiety threshold'.

To investigate the robustness of this concern, I used a revised mutually exclusive choice procedure. In this experiment, rats were initially trained under no-timeout conditions, enabling them to learn self-administration of drugs based on their preferred dose-time relationship. Subsequently, rats were given the option to choose between a fixed period of access to drug or social interaction during which they could engage with selected alternative as they self-selected to. I compared two choice procedures: one that employs unit-dose strategies for rewards and one that allows them to self-select their preferred drug dose in each trial (rats had access to the drug for 5-min, a period sufficient for them to load up on high doses of the drug) (**Figure 12C**).

The main finding that emerged from this experiment was a dissociation between cocaine and heroin (**Figure 32F, 32I**). In particular: 1) in the context of cocaine, an inflexible social preference regardless of the choice procedure was observed; 2) in the context of heroin, marked interindividual differences were observed, but only when rats were allowed to self-administer their preferred drug dose-time relationship over social interaction. Indeed, a subgroup of rats (50%) chose heroin over social interaction, exhibiting voluntary social withdrawal in favor of drug consumption (**Figure 32F**). Specifically, the choice procedure '5 vs 5' revealed significant interindividual differences in drug preference among heroin-trained rats (but not cocaine-trained rats).

Following the same logic described above, I conducted cluster analysis, using two primary features: (1) mean peak concentrations reached during the last self-administration session and (2) drug seeking on Abstinence day 1. Two distinct clusters were revealed, deemed as 'resilient' and 'vulnerable' (**Figure 35**). Rats from these separate clusters showed differences in severity scores, with rats from the vulnerable group displaying higher scores on the severity index (**Figure 36E, 36H**). However, only in heroin-trained rats, the degree of severity was associated with a significant preference for heroin over social interaction (**Figure 36F, 36I**). This revealed once again a dissociation between heroin and cocaine and suggests that

(1) drug brain levels and seeking could serve as predictors of the severity of heroin addiction in rats, and (2) the severity of cocaine addiction in rats is not linked to social withdrawal, at least under my experimental conditions, this screening strategy.

Below I will provide a putative explanation underlying why in the context of heroin choice, but not cocaine choice, we observed interindividual differences.

The analysis of the pattern of cocaine self-administration suggests that under continuous-access conditions, rats exhibited a sustained self-administration of unit-doses of cocaine, leading to brain cocaine levels above a specified threshold, also referred to as the 'compulsion zone' (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006; Tsibulsky & Norman, 1999). Accordingly, in a recent study, Roberts and Zimmer (Roberts & Zimmer, 2020) showed that rats remain motivated to self-administer cocaine if the dosage provided can restore drug levels to a desired zone. The choice procedure used here prevented rats from sustaining this pattern of drug taking even when rats were given access to cocaine for 5-min every 60-min. Therefore, it is plausible that the experimenter-imposed intermittency in the choice procedure diminished the motivation for cocaine, as the rats were unable to attain and sustain specific drug brain levels. In other words, this procedure occluded rats from entering a cycle of compulsive cocaine consumption or steady intoxication (Jaffe, Cascella, Kumor & Sherer, 1989; Norman & Tsibulsky, 2006). In support of this interpretation, previous studies using self-administration procedures with concurrent access to a social partner, albeit not in a choice context, showed that the opportunity to engage in social interaction simply decreased, but did not abolish cocaine intake (Giorla et al., 2022; Smith, 2012). Future studies should investigate whether social preference can be displaced by providing rats with a choice from a social partner and cocaine access enabling them to enter the 'compulsion zone'. In support of this, Vandaele et al. (2016) showed that in a scenario where rats were given the possibility to choose between cocaine and saccharin, under the influence of drug effects, they predominantly opted for cocaine over saccharin only when the effects of cocaine took place.

The observations in the present study are in contrast with a recent report by Marcus et al. (2022) revealing dose-dependent cocaine preference over social interaction in a discrete choice setting. However, there are significant procedural disparities between this prior study and the current

investigation. The most significant distinction is that rats were separated by a perforated metal barrier, whereas in the present study, full-contact social interaction was used. In other words, in Marcus et al. (2022) study, cocaine preference over social was likely elicited because of a social devaluation. Of note, recent research in mice underscored the significance of physical contact for social reward, suggesting that animals require physical interaction to perceive the presence of others and fulfill their social needs (Liu et al., 2023; Wu et al., 2021). This fundamental difference in social interaction protocols may be a key factor contributing to the variations observed between the present study.

To the contrary, our analysis of the pattern of heroin self-administration in the absence of timeout revealed a different attitude by a subgroup of rats (relative to cocaine), featured by burst-taking episodes followed by long periods of inactivity. This temporal pattern is remarkably similar to our choice procedure featured by experimenter-imposed intermittency (5-min access every 60-min). Notably, the cluster analysis revealed that those rats self-administering heroin to attain higher brain peak concentrations of heroin invariably preferred heroin over social interaction (**Figure 15D, 15E**).

A *second* aspect, potentially contributing to the distinct responses to heroin and cocaine may be that chronic exposure to heroin, particularly in susceptible individuals, could have led to social withdrawal in rats, an effect not observed with cocaine. Previous studies in rats investigating the effects of addictive drugs on social interaction revealed that acute injections of cocaine reduce social interactions (Achterberg, Trezza, Siviy, Schrama, Schoffelmeer & Vanderschuren, 2014; Thiel, Okun & Neisewander, 2008). In contrast, conflicting findings were reported on acute morphine and social interactions in rats: some indicate an increase (Panksepp, Jalowiec, DeEskinazi & Bishop, 1985; Trezza & Vanderschuren, 2008) and others a decrease (Panksepp, Najam & Soares, 1979) in social interaction. To my knowledge, no study has extensively investigated the effects of chronic exposure to opioid or psychostimulant drugs on sociability in rats. One single study investigated social behavior in rats with a history of heroin self-administration, uncovering deficits in such behavior (Tomek, Stegmann & Olive, 2019). However, such research has not yet been expanded to include rats exposed to cocaine.

Evidence is also available from studies using mice as experimental animals. In general, researchers found that opioid drugs, but not psychostimulants, induce social deficits in mice (Becker, Kieffer & Le

Merrer, 2017; Goeldner et al., 2011; Lutz et al., 2014; Piccin & Contarino, 2020; Pomrenze et al., 2022; Valentinova et al., 2019). The findings from these studies support the dissociation observed in our studies between opioids and psychostimulants (Becker, Kieffer & Le Merrer, 2017), mirroring the results obtained with the choice procedure.

Interindividual differences in heroin self-administration, heroin seeking, and social withdrawal

In this section, I will provide further considerations on the interindividual differences observed in the heroin group.

As previously mentioned, the cluster analysis on brain peak concentrations of heroin and heroin-seeking unveiled interindividual differences, with rats classifiable into two distinct clusters, mostly predicted by the mean brain peak concentrations of heroin (**Figure 34 top**). We deemed the two clusters respectively as 'Vulnerable' and 'Resilient' (**Figure 35D**). Assigning a z-score to five measures (such as total drug intake, mean drug brain concentrations, mean brain peak concentrations, drug seeking and preference score during the last three days of testing), vulnerable rats exhibited higher severity scores (**Figure 35E**). Overall, these rats displayed a significantly higher preference for heroin when given a choice between heroin and social interaction (**Figure 35F**). These aspects together indicate that social withdrawal was predicted by the emergence of a burst-like pattern of heroin self-administration, featured by high brain peak concentrations of heroin. Further studies should explore whether this pattern (leading to intoxicating effects) causally contributed to the social deficit per se.

These findings shed new light on the significance of patterns of drug taking, by expanding upon prior evidence on cocaine, which suggested that the pattern of drug taking could serve as an early predictor of addiction severity (Belin, Balado, Piazza & Deroche-Gamonet, 2009). This study revealed that such patterns were associated with higher scores in measures commonly associated with addiction, including drug intake, and seeking behaviors. In addition, contrary to previous investigations (Venniro et al., 2018) examining whether rats with high severity scores would show heightened susceptibility to preferring methamphetamine over social rewards, the present findings indicate that heroin preference was predicted by two measures: the development of a burst-like pattern of heroin self-administration and elevated drug seeking during early stages of abstinence (**Figure 35D, 35E, 35F**).

Future studies should corroborate the present findings by including a comprehensive characterization of other related and orthogonal measures of addiction-like behavior (e.g. responding despite adverse consequences, behavioral economic demand curve analysis, etc.).

6.7. Statistical table 2

Figure	Data	Primary statistic	Post-hoc test	Comparison	p-value	F/t/r/z statistic
Figure 22D	Heroin training Intake	Generalized Linear Mixed-Effects Model			p=0.07	
Figure 22D	Heroin training Total intake	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.001***	z=13.500
Figure 22E	Heroin training Lever presses	Generalized Linear Mixed-Effects Model			**p = 0.021***	
Figure 22F	Cocaine training Intake	Generalized Linear Mixed-Effects Model			p=0.75	
Figure 22F	Cocaine training Total intake	Two-tailed, paired t-test		Timeout vs No-Timeout		
Figure 22G	Cocaine training Lever presses	Generalized Linear Mixed-Effects Model			p=0.85	
Figure 23D	Heroin seeking test	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.001***	z=-4.011
not shown	Heroin training Intake Access assignment (choice or PR test)	One-way RM ANOVA		Access x Session interaction	p=0.344	$F_{13,351}$=1.129
				Main effect of Access	**p=0.001***	$F_{1,27}$=0.014
				Main effect of Session	p=0.908	$F_{13,351}$=12.832
not shown	Preference score	One-way RM ANOVA		Main effect of Session	**p=0.001***	$F_{8,144}$=4.685
Figure 23E	Preference score	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.001***	T_{18}=-4.693212
Figure 23F	Progressive ratio (Final ratio)	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.012***	z=-2.524
Figure 23F	Progressive ratio (Cumulative lever presses)	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.047***	z=1.988
Figure 23G	Cocaine seeking test	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.025***	z=2.244
not shown	Cocaine training Intake Access assignment (choice or PR test)	One-way RM ANOVA		Access x Time interaction	p=0.510	$F_{13,338}$=0.801
				Main effect of Access	**p=0.001***	$F_{13,338}$=27.088
				Main effect of Session	p=0.908	$F_{1,26}$=0.585
not shown	Preference score	1-way RM ANOVA		Main effect of Time	**p=0.001***	$F_{8,144}$=4.685
Figure 23H	Preference score	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.010***	T_{16}=-2.928311
Figure 23I	Progressive ratio (Final ratio)	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.041***	z=-2.047
Figure 23I	Progressive ratio (Cumulative lever presses)	Two-tailed, paired t-test		Timeout vs No-Timeout	**p=0.047***	z=1.988
Figure 24D	Heroin acquisition Intake	Two-way RM ANOVA		Access x Session interaction	p=0.088	$F_{4,68}$=2.612
				Main effect of Access	p=0.174	$F_{2,34}$=1.843
				Main effect of Session	**p=0.001***	$F_{2,68}$=15.975
			PLSD	Long vs Intermittent	p=0.137	
			PLSD	Long vs Continuous	p=0.822	

			PLSD	Intermittent vs Continuous	p=0.084	
not shown	Heroin acquisition Lever pressing	Two-way RM ANOVA		Access x Session interaction	p=0.131	$F_{4,68}$=1.967
				Main effect of Access	p=0.066	$F_{2,34}$=2.955
				Main effect of Session	**p=0.001***	$F_{2,68}$=8.427520
			PLSD	Long vs Intermittent	p=0.452070	
			PLSD	Long vs Continuous	p=0.119196	
			PLSD	Intermittent vs Continuous	p=0.023374	
Figure 24D	Heroin training Intake	Two-way RM ANOVA		Access x Session interaction	**p=0.002***	$F_{22,374}$=2.159322
				Main effect of Access	**p=0.001***	$F_{2,34}$=4.573282
				Main effect of Session	**p=0.001***	$F_{11,374}$=17.061538
			PLSD	Long vs Intermittent	p=0.014970	
			PLSD	Long vs Continuous	p=0.011052	
			PLSD	Intermittent vs Continuous	p=0.941453	
not shown	Heroin training Lever pressing	Two-way RM ANOVA		Access x Session interaction	p=0.551659	$F_{22,374}$=0.932557
				Main effect of Access	p=0.352560	$F_{2,34}$=1.075164
				Main effect of Session	**p=0.001***	$F_{22,374}$=9.885
			PLSD	Long vs Intermittent	p=0.168837	
			PLSD	Long vs Continuous	p=0.722435	
			PLSD	Intermittent vs Continuous	p=0.289704	
24F	Heroin seeking test AD1	One-way ANOVA		Main effect of Access	**p=0.003***	$F_{2,34}$=6.904106
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.008***	
			PLSD	Intermittent vs Continuous	p=0.450	
Figure 24F	Heroin seeking test AD1 (Time course)	Two-way RM ANOVA		Access x Time interaction	p=0.380	$F_{4,68}$=1.066165
				Main effect of Access	**p=0.003***	$F_{2,34}$=6.904106
				Main effect of Time	**p=0.001***	$F_{2,68}$=18.424894
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.008***	
			PLSD	Intermittent vs Continuous	p=0.450	
Figure 24G	Cocaine acquisition Intake	Two-way RM ANOVA		Access x Session interaction	p=0.298	$F_{4,76}$=1.248610
				Main effect of Access	p=0.989	$F_{2,38}$=0.010858
				Main effect of Session	**p=0.044***	$F_{2,76}$=3.244245

			PLSD	Long vs Intermittent	p=0.968043	
			PLSD	Long vs Continuous	p=0.885801	
			PLSD	Intermittent vs Continuous	p=0.930287	
not shown	Cocaine acquisition Lever pressing	Two-way RM ANOVA		Access x Session interaction	p=0.746	$F_{4,76}$=0.486377
				Main effect of Access	p=0.494	$F_{2,38}$=0.719322
				Main effect of Session	p=0.971	$F_{2,76}$=0.029248
			PLSD	Long vs Intermittent	p=0.429579	
			PLSD	Long vs Continuous	p=0.253236	
			PLSD	Intermittent vs Continuous	p=0.822640	
Figure 24H	Cocaine training Intake	Two-way RM ANOVA		Access x Session interaction	**p=0.001***	$F_{22,418}$=4.706878
				Main effect of Access	**p=0.001***	$F_{2,38}$=47.056141
				Main effect of Session	**p=0.001***	$F_{11,418}$=47.606853
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.026***	
			PLSD	Intermittent vs Continuous	**p=0.001***	
not shown	Cocaine training Lever pressing	Two-way RM ANOVA		Access x Session interaction	**p=0.004***	$F_{22,418}$=2.057190
				Main effect of Access	**p=0.001***	$F_{2,38}$=35.004707
				Main effect of Session	**p=0.001***	$F_{11,418}$=23.446173
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	p=0.248799	
			PLSD	Intermittent vs Continuous	**p=0.001***	
Figure 24I	Cocaine seeking test AD1	One-way ANOVA		Main effect of Access	**p=0.001***	$H_{2,37}$=13.06
			PLSD	Long vs Intermittent	**p=0.003***	
			PLSD	Long vs Continuous	**p=0.015***	
			PLSD	Intermittent vs Continuous	p=0.211759	
Figure 24I	Cocaine seeking test AD1 (Time course)	Two-way RM ANOVA		Access x Time interaction	**p=0.002***	$F_{2,76}$=4.778906
				Main effect of Access	**p=0.003***	$F_{2,38}$=6.705851
				Main effect of Time	**p=0.001***	$F_{2,76}$=71.675902
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.018***	
			PLSD	Intermittent vs Continuous	p=0.176919	
not shown	Heroin intake last self-	One-way ANOVA		Main effect of Access	**p=0.012***	$F_{2,34}$=5.102462

	administration session		PLSD	Long vs Intermittent	p=0.071686	
			PLSD	Long vs Continuous	**p=0.003***	
			PLSD	Intermittent vs Continuous	p=0.206866	
not shown	Mean heroin infusions per 2-min period	One-way ANOVA		Main effect of Access	**p=0.001***	$F_{2,34}$=11.432818
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.013***	
			PLSD	Intermittent vs Continuous	**p=0.031***	
Figure 27E	Mean brain concentration of heroin per peak	One-way ANOVA		Main effect of Access	**p=0.001***	$H_{3,37}$=21.63
			Dunn	Long vs Intermittent	**p=0.001***	
			Dunn	Long vs Continuous	**p=0.005***	
			Dunn	Intermittent vs Continuous	p=0.425	
not shown	Mean peaks slope	One-way ANOVA		Main effect of Access	**p=0.001***	$F_{2,34}$=12.829708
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.014***	
			PLSD	Intermittent vs Continuous	**p=0.014***	
Figure 27F	Mean brain concentration of heroin	One-way ANOVA		Main effect of Access	**p=0.029***	$H_{2,34}$=7.076
			Dunn	Long vs Intermittent	p=0.1219	
			Dunn	Long vs Continuous	**p=0.008***	
			Dunn	Intermittent vs Continuous	p=0.2833	
not shown	Cocaine intake last self-administration session	One-way ANOVA		Main effect of Access	**p=0.001***	$F_{2,38}$=65.662886
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	p=0.052	
			PLSD	Intermittent vs Continuous	**p=0.001***	
not shown	Mean cocaine infusions per 2-min period	One-way ANOVA		Main effect of Access	**p=0.011***	$F_{2,38}$=5.037645
			PLSD	Long vs Intermittent	**p=0.003***	
			PLSD	Long vs Continuous	p=0.241857	
			PLSD	Intermittent vs Continuous	**p=0.038***	
Figure 27H	Mean brain concentration of cocaine per peak	One-way ANOVA		Main effect of Access	**p=0.001***	$H_{3,38}$=17.16
			Dunn	Long vs Intermittent	**p=0.021***	
			Dunn	Long vs Continuous	p=0.3361	
			Dunn	Intermittent vs Continuous	**p=0.001***	
not shown	Mean peaks slope	One-way ANOVA		Main effect of Access	**p=0.001***	$F_{2,38}$=7.833116
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	p=0.491967	

			PLSD	Intermittent vs Continuous	**p=0.003***	
Figure 27I	Mean brain concentration of cocaine	One-way ANOVA		Main effect of Access	**p=0.001***	$H_{2,38}$=39.0636
			Dunn	Long vs Intermittent	**p=0.001***	
			Dunn	Long vs Continuous	p=0.4000	
			Dunn	Intermittent vs Continuous	**p=0.001***	
Figure 28E	Mean brain concentration of 6-MAM per peak	One-way ANOVA		Main effect of Access	**p=0.004***	$F_{2,34}$=6.576353
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.014***	
			PLSD	Intermittent vs Continuous	p=0.341533	
Figure 28F	Mean brain concentration of morphine per peak	One-way ANOVA		Main effect of Access	**p=0.027***	$F_{2,34}$=4.027738
			PLSD	Long vs Intermittent	p=0.086302	
			PLSD	Long vs Continuous	**p=0.008***	
			PLSD	Intermittent vs Continuous	p=0.319473	
Figure 29G	Heroin seeking test AD21	One-way ANOVA		Main effect of Access	p=0.4306	$H_{2,37}$=9.885
			PLSD	Long vs Intermittent	p=0.6291	
			PLSD	Long vs Continuous	p=0.999	
			PLSD	Intermittent vs Continuous	p=0.999	
Figure 29G	Heroin seeking test AD1-AD21 (incubation)	Two-way RM ANOVA		Main effect of Access	**p=0.002***	$F_{2,38}$=3.568233
				Main effect of Time	p=0.154903	$F_{2,38}$=2.106263
				Access x Time interaction	**p=0.038***	$F_{2,38}$=3.568233
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.042***	
			PLSD	Intermittent vs Continuous	p=0.054	
Figure 29E	Heroin body weight	Two-way RM ANOVA		Main effect of Access	p=0.050833	$F_{2,34}$=3.256211
				Main effect of Session	**p=0.001***	$F_{14,476}$=11.640652
				Access x Session interaction	p=0.111988	$F_{28,476}$=1.978551
			PLSD	Long vs Intermittent	**p=0.041***	
			PLSD	Long vs Continuous	**p=0.028***	
			PLSD	Intermittent vs Continuous	p=0.902470	
Figure 29F	Heroin Von Frey test (hyperalgesia)	Two-way RM ANOVA		Main effect of Access	p=0.721673	$F_{2,35}$=0.329241
				Main effect of Time	**p=0.001***	$F_{3,105}$=64.784474
				Access x Time interaction	p=0.653393	$F_{6,105}$=0.695964
			PLSD	Long vs Intermittent	p=0.467960	

			PLSD	Long vs Continuous	p=0.954505	
			PLSD	Intermittent vs Continuous	p=0.502545	
Figure 29G	Cocaine seeking test AD21	One-way ANOVA		Main effect of Access	**p=0.009***	$F_{2,38}$=5.379362
			PLSD	Long vs Intermittent	**p=0.003***	
			PLSD	Long vs Continuous	p=0.439134	
			PLSD	Intermittent vs Continuous	**p=0.015***	
Figure 29G	Cocaine seeking test AD1-AD21 (incubation)	Two-way RM ANOVA		Main effect of Access	**p=0.002***	$F_{1,38}$=7.370770
				Main effect of Time	p=0.154903	$F_{2,38}$=2.106263
				Access x Time interaction	**p=0.038***	$F_{2,38}$=3.568233
			PLSD	Long vs Intermittent	**p=0.001***	
			PLSD	Long vs Continuous	**p=0.042***	
			PLSD	Intermittent vs Continuous	p=0.054	
Figure 29H	Cocaine body weight	Two-way RM ANOVA		Main effect of Access	p=0.497680	$F_{2,28}$=0.715480
				Main effect of Session	**p=0.001***	$F_{14,392}$=177.701531
				Access x Session interaction	**p=0.001***	$F_{28,392}$=8.894700
			PLSD	Long vs Intermittent	p=0.670820	
			PLSD	Long vs Continuous	p=0.245480	
			PLSD	Intermittent vs Continuous	p=0.465851	
Figure 30D	Stupor	One-way ANOVA		Main effect of Access	**p=0.022***	$F_{2,35}$=4.272452
			PLSD	Long vs Intermittent	**p=0.038***	
			PLSD	Long vs Continuous	**p=0.010***	
			PLSD	Intermittent vs Continuous	**p=0.650**	
Figure 30E	Walking	One-way ANOVA		Main effect of Access	**p=0.008***	$F_{2,35}$=5.561852
			PLSD	Long vs Intermittent	**p=0.005***	
			PLSD	Long vs Continuous	**p=0.010***	
			PLSD	Intermittent vs Continuous	p=0.711706	
Figure 30F	Chewing	One-way ANOVA		Main effect of Access	p=0.168282	$F_{2,35}$=1.876017
			PLSD	Long vs Intermittent	p=0.723563	
			PLSD	Long vs Continuous	p=0.135962	
			PLSD	Intermittent vs Continuous	p=0.082847	
Figure 30G	Grooming	One-way ANOVA		Main effect of Access	**p=0.007***	$F_{2,35}$=5.706851
			PLSD	Long vs Intermittent	p=0.549023	
			PLSD	Long vs Continuous	**p=0.011***	

			PLSD	Intermittent vs Continuous	**p=0.003***	
Figure 30H	Sniffing	One-way ANOVA		Main effect of Access	p=0.350569	$F_{2,35}$=1.080226
			PLSD	Long vs Intermittent	p=0.277315	
			PLSD	Long vs Continuous	p=0.725470	
			PLSD	Intermittent vs Continuous	p=0.164989	
Figure 30I	Hand licking	One-way ANOVA		Main effect of Access	p=0.837110	$F_{2,35}$=0.178706
			PLSD	Long vs Intermittent	p=0.893258	
			PLSD	Long vs Continuous	p=0.652065	
			PLSD	Intermittent vs Continuous	p=0.578697	
Figure 30K	Stupor	One-way ANOVA		Main effect of Access	p=0.125447	$F_{2,28}$=1.336917
			PLSD	Long vs Intermittent	p=0.251169	
			PLSD	Long vs Continuous	p=0.659784	
			PLSD	Intermittent vs Continuous	p=0.578697	
Figure 30L	Walking	One-way ANOVA		Main effect of Access	**p=0.005***	$F_{2,28}$=6.316419
			PLSD	Long vs Intermittent	**p=0.015***	
			PLSD	Long vs Continuous	p=0.452044	
			PLSD	Intermittent vs Continuous	**p=0.002***	
Figure 30M	Chewing	One-way ANOVA		Main effect of Access	**p=0.011***	$F_{2,28}$=5.375668
			PLSD	Long vs Intermittent	**p=0.016***	
			PLSD	Long vs Continuous	p=0.676351	
			PLSD	Intermittent vs Continuous	**p=0.005***	
Figure 30N	Grooming	One-way ANOVA		Main effect of Access	p=0.525433	$F_{2,28}$=0.658553
			PLSD	Long vs Intermittent	p=0.436744	
			PLSD	Long vs Continuous	p=0.757179	
			PLSD	Intermittent vs Continuous	p=0.272315	
Figure 30O	Sniffing	One-way ANOVA		Main effect of Access	p=0.639362	$F_{2,28}$=0.454506
			PLSD	Long vs Intermittent	p=0.348536	
			PLSD	Long vs Continuous	p=0.628988	
			PLSD	Intermittent vs Continuous	p=0.629811	
Figure 30P	Hand licking	One-way ANOVA		Main effect of Access	p=0.117956	$F_{2,28}$=2.323206
			PLSD	Long vs Intermittent	p=0.244506	
			PLSD	Long vs Continuous	p=0.051472	
			PLSD	Intermittent vs Continuous	p=0.326871	
Figure 32F	Brain peak concentrations	Two-tailed, unpaired t-test		Main effect of Cluster	**p=0.001***	T_{35}=6.763

Figure 32G	Seeking	Two-tailed, unpaired t-test		Main effect of Cluster	**p=0.001***	T_{35}=7.709
Figure 32I	Brain peak concentrations	Two-tailed, unpaired t-test	Welch's correction	Main effect of Cluster	p= 0.051	T_{18}=2.093
Figure 32L	Seeking	Two-tailed, unpaired t-test	Welch's correction	Main effect of Cluster	**p=0.001***	T_{18}=6.541
Figure 33D	Social self-administration	Two-way RM ANOVA		Choice x Session interaction	p=0.898	$F_{5,155}$=0.028257
				Main effect of Choice	p=0.867598	$F_{1,31}$=0.028257
				Main effect of Session	**p=0.001***	$F_{5,155}$=81.815445
Figure 33D	Heroin self-administration	Two-way RM ANOVA		Access x Session interaction	p=0.109025	$F_{14,434}$=2.041447
				Main effect of Choice	p=0.748158	$F_{1,31}$=0.104938
				Main effect of Session	**p=0.001***	$F_{14,434}$=20.532306
Figure 33E	Heroin seeking	Two-tailed, unpaired t-test		1:1 vs 5:5 Choice	p=0.563	T_{31}=0.584
Figure 33F	Preference score	Two-way RM ANOVA		Choice x Session interaction	p=0.226284	$F_{6,186}$=1.376076
				Main effect of Choice	**p=0.002***	$F_{1,31=}$11.250930
				Main effect of Session	p=0.990098	$F_{6,186=}$0.143614
Figure 33F	Preference score	Two-tailed, unpaired t-test		1:1 vs 5:5 Choice	**p=0.001***	$T_{24.229=}$5.971613
Figure 33F	Preference score (Choice 1 vs 1)	Two-tailed, paired t-test		Social vs Heroin	**p=0.001***	$T_{7=}$-81.992683
Figure 33F	Preference score (Choice 5 vs 5)	Two-tailed, paired t-test		Social vs Heroin	p=0.802304	$T_{24=}$0.253156
Figure 33G	Social self-administration	Two-way RM ANOVA		Choice x Session interaction	p=0.509672	$F_{5,155}$=0.859805
				Main effect of Choice	p=0.898299	$F_{1,31}$=0.016606
				Main effect of Session	**p=0.001***	$F_{5,155}$=105.119213
Figure 33G	Cocaine self-administration	Two-way RM ANOVA		Access x Session interaction	p=0.994890	$F_{14,434}$=0.289621
				Main effect of Choice	p=0.937921	$F_{1,31}$=0.006165
				Main effect of Session	**p=0.001***	$F_{14,434}$=50.126369
Figure 33H	Cocaine seeking	Two-tailed, unpaired t-test		1:1 vs 5:5 Choice	p=0.248795	T_{31}=-1.175370
Figure 33I	Preference score	Two-way RM ANOVA		Choice x Session interaction	p=0.961218	$F_{10,310}$=0.363913
				Main effect of Choice	p=0.628799	$F_{1,31=}$0.238401
				Main effect of Session	**p=0.001***	$F_{10,310=}$5.022495
Figure 33I	Preference score	Two-tailed, unpaired t-test		1:1 vs 5:5 Choice	p=0.226799	$F_{30.704=}$-1.233365
Figure 33I	Preference score (Choice 1 vs 1)	Two-tailed, paired t-test		Social vs Cocaine	**p=0.001***	T_{7}=-63.561781
Figure 33I	Preference score (Choice 5 vs 5)	Two-tailed, paired t-test		Social vs Cocaine	**p=0.001***	T_{24}=-72.883616
Figure 34D	Preference score	One-way RM ANOVA			**p=0.046***	$F_{3,45=}$3.617772
Figure 34F	Preference score	One-way RM ANOVA			p=0.406	$F_{3,30=}$1.00000

Figure 36E	Sum Severity z-score	Two-tailed, unpaired t-test		Resilient vs Vulnerable	**p=0.001***	$T_{21=}2.944807$
Figure 36F	Preference score	Two-tailed, unpaired t-test		Resilient vs Vulnerable	**p=0.043***	
Figure 36H	Sum Severity z-score	Two-tailed, unpaired t-test		Resilient vs Vulnerable	**p=0.009***	$T_{20=}2.857$
Figure 36I	Preference score	Two-tailed, unpaired t-test		Resilient vs Vulnerable	p=0.6478	

7. General discussion

7.1. Synopsis

The overarching objective of the present dissertation was to refine existing preclinical models of drug addiction, aiming to capture the intricate nuances underlying instrumentalization of self-administered drugs using rodent models. In pursuit of this objective, by adopting a comparative approach, in **Chapter 2** I conducted an extensive analysis of patterns of drug taking adopted by individuals using cocaine and heroin, integrating insights from the pharmacokinetic and pharmacodynamic characteristics of the two drugs under investigation (**Chapter 3**).

The integration of this data revealed a convergence between the pharmacokinetic and pharmacodynamic profiles and the respective drug taking behaviors observed for each substance, yet a divergence is evident between cocaine and heroin.

Based on these considerations, in **Chapter 4** I conducted a critical examination of available animal models of drug addiction used in addiction research without strict pharmacokinetic and pharmacodynamic considerations on the drug under investigation. Drawing on insights from previous investigations (Roberts, Brebner, Vincler & Lynch, 2002; Roberts & Zimmer, 2020), the most apparent weakness was linked to the perpetuation of a misconception from early drug self-administration procedures: the implementation of discrete dimension strategies (Morgan, Liu, Oleson & Roberts, 2009). These strategies consider the implementation of fixed unit-doses and timeouts, to establish a standardized, ‘one-size-fits-all approach’ characterized by consistent patterns of drug taking across all types of addictive drugs, prioritizing a convergent rather than divergent approach (Badiani, Belin, Epstein, Calu & Shaham, 2011). However, this approach has two main concerns: (1) it does not consider the pharmacokinetics and pharmacodynamics determinants of patterns of drug taking, and consequently (2) it fails to capture the intricate nuances of patterns of drug taking observed in humans using different drugs.

Building upon this evidence, by adopting a comparative approach, I carried out a series of experiments (described in **Chapters 5** and **6**) investigating the impact of timeouts and unit-doses in

cocaine and heroin self-administration and drug-vs-social choice procedures. Results demonstrated that employing discrete dimension strategies in self-administration and choice procedures decreases the drive to seek and take drugs in rats. However, more remarkable, it occludes the observations of distinct patterns of drug taking between heroin and cocaine and interindividual differences in social withdrawal in favor of heroin use.

These findings were separately discussed in the preceding chapters, examining their similarities and differences to other preclinical studies on drug self-administration and choice procedures, as well as interpreting their significance in relationship with pharmacokinetics and pharmacodynamics variables. Reflecting the primary objective of this dissertation, which is to improve animal models of drug addiction, the subsequent sections will be dedicated to analyzing the collected data in a broader context. This analysis will aim to evaluate the relevance of these findings in the context of clinical observations and to offer insights that may guide future investigative efforts in this field.

7.2. Patterns of drug use: bridging the gap between rat and human behavior

The comparative approach used throughout the dissertation underscored a critical distinction in the patterns of drug taking adopted by individuals with cocaine and heroin addiction, indicating that the 'one-size-fits-all approach' to drug self-administration procedures could be detrimental. Collectively, the results obtained from the experiments presented above highlighted that the implementation of unit dose strategies primarily influenced the patterns of drug taking for heroin compared to cocaine, mainly due to their different pharmacokinetics and pharmacodynamics profiles.

Below, I will discuss these results in relation to the patterns of drug taking exhibited by individuals using heroin and cocaine. The primary aim is to provide insight into the most effective procedures to employ when investigating cocaine or heroin addiction. These insights will be discussed separately for each drug.

Unraveling the dynamics of patterns of binge cocaine-taking: insights from clinical and preclinical studies

The most common pattern of drug taking observed among individuals using cocaine, regardless of the development of addiction or career stage (Siegel, 1977), is characterized by cycles of long periods of drug binging interspersed by abstinence periods. During binges cocaine taking is highly regular: cocaine is taken repeatedly, every 10-30 min, resulting in frequent use episodes (Gawin, 1991; Gawin & Kleber, 1985; Siegel, 1977; Siegel, 1984; Siegel, 1985; Van Beek, Dwyer & Malcolm, 2001). This regular pattern of cocaine-taking can be observed in rats provided with extended access to the drug (6-h/d), but not in rats provided with intermittent-access. Notably, this pattern can be observed independently of the presence or absence of timeout between unit-doses (**Figure 25E, 25G, 27G**), suggesting that the implementation of timeout has only a limited impact on patterns of drug taking (see **Chapter 6** for further discussion).

The pattern of cocaine-taking adopted during a binge begins with an initial phase of ‘controlled’ cocaine use, oriented to experiencing the effects of cocaine (Gawin, 1991). As the binge progresses, cocaine-taking transitions to a phase characterized by uncontrolled and 'compulsive' behavior (Gawin, 1991; Gawin & Kleber, 1986; Ward, Haney, Fischman & Foltin, 1997). In this phase, individuals report being unable to abstain when cocaine is available, prompting them to preselect amounts of cocaine before initiating a binge since they anticipated being unable to cease usage before exhausting their supply (Gawin & Kleber, 1986).

A comparable 'compulsive' cocaine-taking in rats was observed by integrating data on the dynamics of patterns of drug taking and drug brain levels. In self-administration procedures where cocaine is continuously available, rats consistently display a pattern characterized by an initial period of loading, with burst-like cocaine-taking reaching a specific threshold of drug body levels, followed by a maintenance phase (Ahmed & Koob, 1998; Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012). During the maintenance phase rats enter a 'compulsion zone' [also referred to as ‘satiety threshold’ (Gerber & Wise, 1989; Suto & Wise, 2011)] during which they repeatedly self-administer cocaine at extremely

regular intervals, maintaining drug brain levels above the threshold (Desai, Tron Esqueda & Norman, 2023; Norman & Tsibulsky, 2006; Tsibulsky & Norman, 1999). Notably, the architecture of the intermittent-access procedure prevents rats from entering a 'compulsion zone' (Zimmer, Dobrin & Roberts, 2011; Zimmer, Oleson & Roberts, 2012) while allowing repeated experimenter-imposed cycles of 'loading phases', not very common in individuals with cocaine addiction.

Despite direct evidence of a clear distinction between the loading and maintenance phase in humans has not been found (Angarita et al., 2010) and drug levels throughout a drug self-administration session has yet to be investigated, the perspective of 'compulsive' and uncontrolled cocaine-taking during a binge is supported by several factors. The subjective and physiological effects of cocaine typically increase concurrently with the first few injections of cocaine (Foltin & Fischman, 1998), but following initial doses, the effects of cocaine do not exhibit further increases with subsequent doses, indicating the development of acute tolerance to these effects during a binge phase (Foltin & Fischman, 1998). As a result, during the late phases of a binge, subjects typically report being unable to reach the level of euphoria achieved with the first doses taken, regardless of the size of dosage increases (Gawin & Kleber, 1986). Therefore, following the initial doses of cocaine, the pattern of drug taking becomes increasingly compulsive and is no longer driven by the desire to experience cocaine effects. Individuals report being unable to wait for the next dose (Ward, Haney, Fischman & Foltin, 1997). Importantly, this uncontrolled cocaine-taking behavior is elucidated by the phenomenon wherein cocaine itself induces a craving for additional doses (Jaffe, Cascella, Kumor & Sherer, 1989), a craving that often reemerges shortly after each dose of cocaine (Van Dyke, Barash, Jatlow & Byck, 1976).

Together these observations suggest that cocaine-taking during binges, in both humans and rats, might be 'compulsive' and sustained by the direct effects of cocaine, including drug-induced craving, rather than by the desire to achieve subjective effects of the drug (Gawin, 1991; Gawin & Kleber, 1985; Siegel, 1977; Siegel, 1984; Siegel, 1985). This observation is further supported by the fact that in the midst of a binge phase individuals typically report ceasing taking cocaine due to exhaustion and the onset of adverse drug effects, such as anxiety, agitation, and paranoia (Foltin & Fischman, 1998; Gawin, 1991; Reinarman, Murphy & Waldorf, 1994; Van Beek, Dwyer & Malcolm, 2001) and not because they are

satiated by the drug effects (Van Beek, Dwyer & Malcolm, 2001). Finally, together these aspects can explain the death due to cocaine intoxication observed in the very initial studies characterized by unlimited access to cocaine (Bozarth & Wise, 1985; Deneau, Yanagita & Seevers, 1969), potentially driven by a compulsion that makes it difficult to stop cocaine-taking.

Based on the evidence provided above, it can be inferred that the most suitable self-administration procedure for mimicking patterns of cocaine-taking in rats involves providing continuous-access to the drug. This procedure allows for the observation of an initial 'goal-directed-loading' phase followed by a 'compulsive' phase of cocaine intake, as indicated by the highly regular pattern of drug taking during the maintenance phase and the maintenance of consistent cocaine brain levels.

The intermittent-access procedure, which occludes sustained brain levels of cocaine over time, may not be the ideal model for mimicking human patterns of cocaine-taking. Additionally, procedures where the drug is continuously available also mirror another significant aspect of cocaine addiction: the 'crash' and reduced motivation for cocaine observed immediately after the last binge (Gawin, 1991; Gawin & Kleber, 1986). This is apparent in rats exhibiting low motivation to take cocaine following long-access, as opposed to high motivation for cocaine following intermittent-access to cocaine, in progressive ratio procedures (Algallal, Allain, Ndiaye & Samaha, 2020; Minogianis, Lévesque & Samaha, 2013; Zimmer, Oleson & Roberts, 2012).

Even from a treatment perspective, Gawin (1991) has advocated that a significant objective in cocaine addiction treatment is the cessation of the cycle involving self-administration of repeated doses of cocaine during a binge phase. However, few studies directly examined this phenomenon and the effects of repeated self-administration of cocaine in a binge pattern [e.g., (Hatsukami, Thompson, Pentel, Flygare, Carroll & Psychopharmacology, 1994)]. Future studies should investigate which are the mechanisms maintaining compulsive cocaine-taking during binges (Mutschler, Covington & Miczek, 2001; Mutschler & Miczek, 1998; Tornatzky & Miczek, 2000).

Interindividual differences in patterns of heroin-taking: parallels between human behavior and rat models in the absence of timeout

Among heroin users, interindividual differences in patterns of heroin-taking were reported (Zinberg, Harding, Stelmack & Marblestone, 1978; Zinberg & Jacobson, 1976). Accordingly, an empirical categorization of heroin users was proposed based on the severity of patterns of heroin-taking, including frequency and quantity, as well as the level of physical dependence (Johnson, 1984; Zinberg, Harding, Stelmack & Marblestone, 1978). Individuals diagnosed with heroin addiction typically display the most severe patterns of heroin-taking, characterized by few use episodes per day, during which they consume large doses of heroin, leading to intense euphoria, followed by several hours of abstinence, mostly characterized by sedation (Alksne, Lieberman & Brill, 1967; Darke, 2011; Haasen, Verthein, Degkwitz, Berger, Krausz & Naber, 2007; Ross, McCurdy, Kilonzo, Williams, Leshabari & hygiene, 2008).

The current study revealed that this spectrum of interindividual differences in heroin-taking patterns, spanning from low to severe heroin taking-patterns, could be reproduced in rats under training conditions without timeout between unit doses, leading to elevated brain levels of heroin, followed by periods of brief abstinence (**Figure 26, 27, 32, 36**), closely resembling those observed in humans. These patterns were consistently absent when the timeout was experimentally imposed (Ahmed, Walker & Koob, 2000; D'Ottavio et al., 2023), a fact that found a solid explanation in the pharmacokinetics and pharmacodynamics profile of heroin featured by a very short half-life and a complex breakdown leading to a plethora of active metabolites. Additionally, according to human literature, rats displaying the most severe patterns of drug taking, featured by high brain peak concentrations of heroin, were those exhibiting higher scores in severity of addiction-like behaviors, such as high drug-seeking during early phases of abstinence and social withdrawal in drug-versus-social choice procedures (**Figure 36**).

The striking similarity between the patterns of drug taking observed in rats and humans supports the significance of the refinement proposed for heroin self-administration procedures. Moreover, these considerations lay the groundwork for a multitude of investigations. Below, I will outline the potential implications of these findings.

This improved animal model can help us study why some individuals are more likely to become addicted to heroin than others. By examining how the patterns of drug taking adopted by vulnerable rats account for high scores in addiction-like behaviors, it could be learned what causes a severe addiction leading to social isolation. Conversely, looking at what prevents addiction in other rats, could provide valuable insights to improve the effective management and treatment of heroin use in therapeutic contexts. As advanced by clinical studies embracing harm-reduction strategies, understanding the patterns of 'controlled' heroin use would help in designing treatment strategies aimed at mitigating drug-related harm (Harding, 1988; Harding, Zinberg, Stelmack & Barry, 1980) such as heroin-assisted substitution therapies (Harding, 1988; Harding, Zinberg, Stelmack & Barry, 1980).

These aspects gain even greater relevance considering the recent viewpoint proposed by the Food and Drug Administration (Administration, 2020), which suggests that patterns of drug taking serve as strong indicators of opioid addiction treatment efficacy. Indeed, a main question for future studies is to evaluate the predictive validity of the animal model proposed here (Epstein, Preston, Stewart & Shaham, 2006; Venniro, Banks, Heilig, Epstein & Shaham, 2020), by examining the effectiveness of approved medications for heroin addiction, such as methadone, in mitigating intoxicating effects and decreasing bursts of intake episodes (Dole, Nyswander & Kreek, 1966). This investigation could provide several insights into treatment strategies for opioid addiction, also considering that there are significant interindividual differences in response to methadone maintenance (Belding, McLellan, Zanis & Incmikoski, 1998; Casati, Piontek & Pfeiffer-Gerschel, 2014; Faggiano, Vigna-Taglianti, Versino, Lemma, Drugs & Reviews, 1996).

In addition to these aspects, the similarity in heroin-related behaviors between rats and humans could serve as a foundation for the investigation of the complex dynamics of heroin pharmacology and untangle the contributions of both heroin and its metabolites to the neurobehavioral effects of the parent compound (Andersen, Ripel, Boix, Normann & Mørland, 2009; Inturrisi, Max, Foley, Schultz, Shin & Houde, 1984; Inturrisi, Schultz, Shin, Umans, Angel & Simon, 1983; Jenkins, Keenan, Henningfield & Cone, 1994; Milella, D'Ottavio, De Pirro, Barra, Caprioli & Badiani, 2023; Rook, Huitema, van den Brink, van Ree & Beijnen, 2006a; Rook et al., 2006). Indeed, it remains an open question in the heroin literature,

necessitating further investigation to enhance our understanding of the neurobiological mechanisms underlying heroin addiction (Milella, D'Ottavio, De Pirro, Barra, Caprioli & Badiani, 2023).

In conclusion, with further validation, this procedure shows promising potential to enhance our understanding of the neurobiological mechanisms underlying heroin addiction and improve current strategies for managing heroin addiction.

7.3. Social withdrawal induced by heroin, but not cocaine, exposure in rats: a discrepancy with clinical findings?

The studies outlined above revealed that the use of discrete dimensions strategies, such as unit-doses, in the social-versus-drug choice procedure, masked social withdrawal in a subset of rats exposed to heroin but not cocaine. From a preclinical standpoint, these findings are significant due to the challenge of detecting this phenomenon in conventional social-versus-drug choice experiments, where nearly all rats exhibit a preference for social rewards over drug consumption (Venniro, Panlilio, Epstein & Shaham, 2021; Venniro et al., 2020; Venniro, Russell, Zhang & Shaham, 2019; Venniro et al., 2018). Nonetheless, this outcome starkly contrasts with the clinical domain, highlighting the need for a comprehensive investigation of this phenomenon to clarify the distinctions between human and animal behaviors.

Note: the core of the introduction of the present dissertation focused on patterns of drug taking and the role of pharmacokinetics and pharmacodynamics in explaining these patterns, useful for gaining insights on the refining of self-administration and choice procedures in animal models. For this reason, the social context and the various social factors implicated in addiction were not addressed. Thus, in the following section, maintaining a comparative approach, before getting deep into the interpretation of the findings described above, I will provide a brief overview of the social behaviors, and personality characteristics of individuals with heroin or cocaine addiction.

In the clinical realm, social withdrawal is commonly observed in individuals with both heroin and cocaine addiction. Individuals with addiction persist in pursuing the euphoric effects induced by drugs, disregarding more socially adaptive activities (APA, 2013; Babor, Meyer, Mirin, McNamee & Davies,

1976; Banks & Negus, 2017; Bornstein & Pickard, 2020; Heilig, Epstein, Nader & Shaham, 2016; Heyman, 2009; Hogarth, 2020; Volkow, Baler & Goldstein, 2011). Accordingly, the social repercussions of drug addiction (i.e. social disconnection, loss of significant relationships, loss of employment, etc.) are often cited as a motivating factor for seeking treatment (Marlatt, Tucker, Donovan & Vuchinich, 1997; Rounsaville, Spitzer & Williams, 1986). Additionally, studies consistently reported that individuals with cocaine and heroin addiction commonly exhibit a more extensive impairment in social skills, including decreased empathy, impairments in social cognition, and smaller social network sizes) (Carlyle, Rowley, Stevens, Karl & Morgan, 2020; Ferrari, Smeraldi, Bottero & Politi, 2014; Kroll, Nikolic, Bieri, Soyka, Baumgartner & Quednow, 2018; Kroll et al., 2019; Kroll et al., 2018; McDonald, Darke, Kaye & Torok, 2013; Preller et al., 2014; Tobler et al., 2016; Tomei, Besson, Reber, Rougemont-Bücking & Grivel, 2017). Therefore, social impairments are identified as criteria for making a formal diagnosis of addiction in the DSM-5 (APA, 2013).

Clinical studies indicate that although social withdrawal is frequently observed in addiction, the underlying reasons may differ between individuals addicted to heroin versus cocaine. Anecdotal evidence suggests that cocaine users are typically more socially functional, extroverted, and well-integrated compared to heroin users (Gerra, Bertacca, Zaimovic, Pirani, Branchi & Ferri, 2008). In support of this perspective, clinical studies reported that individuals with heroin addiction are typically considered socially inept (Kurtines, Hogan & Weiss, 1975) and, compared to cocaine users, exhibit greater social inhibition, higher levels of social deviance, and are more frequently diagnosed with antisocial personality disorder (Conway, Swendsen, Rounsaville & Merikangas, 2002; Fieldman, Woolfolk & Allen, 1995; Flynn et al., 1995). Similar observations were reported by Gerra et al. (2008), who found that individuals predominantly using heroin (i.e. heroin as the drug of choice) display higher social introversion compared to those predominantly using cocaine. In addition, individuals predominantly using cocaine demonstrate higher direct aggressiveness and paranoia (Gerra, Bertacca, Zaimovic, Pirani, Branchi & Ferri, 2008; Hopwood, Baker, Morey & Differences, 2008), the latter is typically absent in individuals with heroin addiction (Hopwood, Baker, Morey & Differences, 2008; Maremmani, Rovai, Rugani, Bacciardi, Dell'Osso & Maremmani, 2014; Nevid, Gordon, Barris, Sperber & Haggerty, 2019). Notably, paranoia is a critical adverse effect of cocaine addiction (Brady, Lydiard, Malcolm & Ballenger, 1991; Gambill & Kornetsky,

1976) and may exacerbate social withdrawal in individuals with cocaine addiction (Rice-Licare & Delaney-McLoughlin, 2014). Accordingly, while cocaine is often sought after for its perceived ability to enhance social functioning (Müller & Schumann, 2011; Siegel, 1977; Siegel, 2005), individuals commonly find themselves experiencing decreased sociability during the latter stages of a cocaine binge, as negative effects such as paranoia and anxiety begin to manifest (Foltin & Fischman, 1998).

It is important to note that several studies revealed the impact of personality traits on determining the preference for one drug over another, therefore personality characteristics may contribute to an individual's vulnerability to drug addiction and the inclination to the specific ‘drug of choice’ (Conway, Kane, Ball, Poling, Rounsaville & dependence, 2003; Conway, Swendsen, Rounsaville, Merikangas & dependence, 2002; Gerra, Bertacca, Zaimovic, Pirani, Branchi & Ferri, 2008; Hopwood, Baker, Morey & Differences, 2008; O'Connor, Berry, Morrison & Brown, 1995).

In addition to these personality-related aspects, studies exploring social dimensions (such as social cognition, empathy, etc.) in individuals with cocaine addiction consistently reported several comorbid conditions to cocaine addiction, such as alcohol or other substance abuse, ADHD, and major depression (Kroll et al., 2018; Preller et al., 2014): all conditions linked to social impairment and withdrawal (Åkerlind, Hörnquist & medicine, 1992; Kennedy, Foy, Sherazi, McDonough & McKeon, 2007; Wehmeier, Schacht & Barkley, 2010). Therefore, the presence of other co-occurring disorders raises uncertainty about whether the observed social deficits are solely attributed to cocaine addiction. Hukla et al. (2014) in a comparative study of individuals classified as recreational cocaine users or individuals with cocaine addiction indicates that social-decision-making related deficits were evident in both groups, suggesting that social deficits are a predisposing factor for cocaine addiction rather than being a consequence of cocaine addiction. Analogous comparative studies have not yet been conducted on individuals with heroin addiction.

In summary, the literature reviewed above indicates significant differences in social functioning among individuals with heroin and cocaine addiction. Furthermore, it suggests that the nature of social impairments in addiction remains ambiguous, with uncertainty regarding whether these impairments are

pre-existing conditions predisposing individuals to drug addiction or are induced by drug use itself (Verdejo-Garcia, 2014).

Although this evidence may support the notion previously suggested that animal models might not fully replicate the complex dynamics of human drug addiction (Field & Kersbergen, 2020), preclinical studies offer an exclusive opportunity to control and dissect the variables known to contribute to social impairments in addiction. Of note, preclinical studies suggest that social withdrawal observed in people with opioid addiction may primarily be attributed to the direct effects of opioids (Goeldner et al., 2011; Pellissier, Gandia, Laboute, Becker & Le Merrer, 2018; Piccin & Contarino, 2020; Piccin, Courtand & Contarino, 2022; Pomrenze et al., 2022; Valentinova et al., 2019), since mice exposed to morphine but not cocaine display reduced social interaction (Becker, Kieffer & Le Merrer, 2017). This evidence combined with differences observed in individuals using cocaine and heroin corroborates the distinctions between cocaine and heroin in inducing social withdrawal observed in the present study.

Additional clinical studies with larger sample sizes, longitudinal designs, and comprehensive assessments are necessary to elucidate the relationship between social deficits, drug use, and comorbid psychiatric conditions. Conducting such investigations can offer insights into the fundamental mechanisms and causal pathways leading to social withdrawal. This knowledge can enhance the development of more effective prevention and intervention strategies for individuals with addiction. Additionally, it can provide valuable guidance for preclinical studies aiming to replicate human social withdrawal observed in animal models. Such research endeavors are crucial for unraveling the neurobiological underpinnings of this condition.

7.4. Concluding remarks

In conclusion, the findings discussed above highlight several critical points.

Firstly, using discrete dimension strategies, featured by unit-doses and timeouts, in animal models of drug self-administration and choice presents limitations, as these approaches restrict the observation of

‘naturalistic’ patterns of drug taking, shaped by the pharmacokinetics and pharmacodynamics profiles of the drug under investigation.

This leads to the second point; the pharmacological properties of the drugs being studied should be considered when using self-administration procedures to study the neurobiology of drug addiction. Indeed, as described above different drugs may necessitate different self-administration procedures, tailored to the specific pharmacological characteristics of the drug under investigation.

This is linked to a third point, comparative studies are essential to recognize distinctions at both behavioral and neurobiological levels among different drugs (Badiani, Belin, Epstein, Calu & Shaham, 2011). Convergent theories and perspectives on addiction to different drugs may not comprehensively capture their unique characteristics. Recognizing differences among drugs and their instrumentalization can guide more tailored interventions, leading to more effective treatment approaches specific to each addictive drug.

Fourthly, the relationship between social withdrawal, drug use, and comorbid psychiatric conditions is still unclear and both clinical and preclinical studies yielded mixed results. Thus, additional studies are needed to understand fundamental mechanisms and causal pathways leading to social withdrawal in individuals with addiction.

In summary, a comprehensive understanding of all these factors is vital for advancing our understating of addiction and developing more effective interventions to address this complex condition.

7. References

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