Received: 28 October 2024 | Revised: 7 February 2025 | Accepted: 18 February 2025

DOI: 10.1111/bph.70023

RESEARCH ARTICLE

Behavioural and pharmacokinetic analysis of heroin and cocaine self-administration: Effects of timeout on self-administration and choice in male rats

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Funding information
We acknowledge financial support under the National Recovery and Resilience Plan (NRRP), Mission 4, Component 2, Investment 1.1, Call for tender No. 1409 published on 14.9.2022 by the Italian Ministry of University and Research (MUR), funded by the European Union – NextGenerationEU – grant No. P202274WPN (to DC, FF, RC). The research was also supported by the Sapienza University of Rome Fondi di Ateneo AR122181634AA6CD (to GD),

Background and Purpose: Heroin and cocaine users tailor their dosage, frequency and administration route to maximise the drugs' effects or prevent withdrawal symptoms. Counterintuitively, preclinical self-administration and choice experiments employ, almost invariably and regardless of the pharmacokinetic properties of the drug under examination, fixed unit-doses and timeouts (after unit-doses) largely resulting in uniform drug-taking patterns. This uniformity contrasts with the large variability observed in humans, which serves as critical indicator of addiction severity and treatment success. Here, by combining behavioural and pharmacokinetics assessments, we revealed that drug self-administration procedures without timeouts may overcome this limitation.

Abbreviations: FR, Fixed Ratio; TO, timeout; No-TO, no-timeout.

RM11916B0E316F23 (to DC) and a grant from the 'Enrico ed Enrica Sovena Foundation', which funded the fellowship of Mr. Soami F. Zenoni.

Experimental Approach: We analysed heroin- and cocaine-taking patterns and seeking and estimated drug-brain levels in the presence or absence of timeout under different training conditions.

Key Results: Removing timeouts had a profound effect on heroin-taking patterns and seeking, promoting the emergence of burst-like intake, yielding higher brain peak concentrations of heroin. In contrast, the removal of timeout had marginal impact on cocaine-taking patterns and seeking.

Conclusion and Implications: The removal of timeout during self-administration revealed distinct cocaine and heroin patterns, with the latter closely resembling human heroin use patterns.

KEYWORDS

addiction, animal models, opioids, pharmacokinetics, psychostimulants, self-administration

1 | INTRODUCTION

Cocaine and **heroin (diamorphine)** addictions persist as global health concerns, prompting ongoing research for novel treatments (Kampman, 2019; Kreek et al., 2019). Preclinical research mainly contributes investigating the neurobiological effects of drug exposure by employing a plethora of animal models, with self-administration along with its variants as the 'gold standard' procedure (Ahmed, 2010, 2012; Roberts et al., 2007; Venniro et al., 2020). Over time, drug-self-administration procedures underwent a process of refinement to reflect, as accurately as possible, key aspects of human addiction. Accordingly, various models were proposed [including long access (Ahmed & Koob, 1998), intermittent drug-taking (Zimmer et al., 2012), choice between drug and non-drug rewards (Venniro, Panlilio, et al., 2021) and the Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV)-based individual differences model (Deroche-Gamonet et al., 2004)] leading to significant advancements in the field (Venniro et al., 2020). However, the extensive preclinical data amassed thus far has not resulted in 'forward translation' during the last two decades (Ahmed, 2010; Field & Kersbergen, 2020; Venniro et al., 2020).

The present study contributes to existing literature by analysing the impact of the experimenter-imposed constraint, the timeout in between infusions. This factor directly affects the animals ability to titrate the optimal drug loading speed. This study examines this across two widely misused substances, cocaine and heroin, which have distinct pharmacokinetic and pharmacodynamic profiles.

In real-world scenarios, humans display the ability to 'instrumentalise' the intrinsic drug properties to achieve desired effects, responding to internal and external factors (e.g. drug availability, tolerance or sensitisation to drug effects or side effects of excessive drug consumption) (Alksne et al., 1967; Busto & Sellers, 1986; Gawin & Kleber, 1986; McAuliffe & Gordon, 1974; Müller & Schumann, 2011; Siegel, 1977, 1985). Heroin and cocaine users display markedly distinct drug instrumentalization. For example, they adopt distinct frequencies, dosages and routes of administration (Darke, 2011; Gawin, 1991; Gawin & Kleber, 1985; Haasen et al., 2007; Ross et al., 2008; Siegel, 1977, 1984, 1985; Zinberg, 1984). These differences typically reflect the pharmacokinetic and pharmacodynamic properties of the drugs (Busto & Sellers, 1986). Notably, a relative common feature of cocaine and heroin users is transitioning from slower to faster routes of administration, with the aim of intensifying drug effects (Gossop et al., 1992; Gossop et al., 2004; Siegel, 1985; Strang et al., 1992; Strang et al., 1998). This is particularly evident in a

What is already known?

- Human patterns of cocaine and heroin-use differ and are influenced by their distinct pharmacokinetic profiles.
- Preclinical self-administration experiments feature standardised cocaine and heroin-taking patterns, possibly shaped by imposed constraints (timeouts/unit-doses).

What does this study add?

- Timeouts profoundly affect heroin-taking patterns, but not cocaine, because of their distinct pharmacokinetic profiles.
- Timeouts reduce heroin and cocaine taking and seeking.

What is the clinical significance

- We propose a self-administration procedure that more closely mimic real-world drug use patterns.
- Continuous refinement of addiction animal models expands knowledge and supports the development of novel medications.

subset of individuals, often considered vulnerable, featuring excessive drug instrumentalization [referred to as over-instrumentalization (Müller & Schumann, 2011)] leading to the development of severe and harmful patterns of use, culminating in addiction (Anthony & Helzer, 2002; Koob et al., 2015; Siegel, 1977; Zinberg, 1984; Zinberg et al., 1978).

Despite clear evidence of differences in how various addictive drugs are used and 'instrumentalized' in humans, preclinical addiction research traditionally relied on a generalised 'across-drugs-convergent' modelling approach. This strategy arguably relies on the evidence that drugs are positive reinforcers (Goldberg, 1973; Thompson, 1968; Wise & Bozarth, 1987; Robert A. Yokel, 1987). In this framework, experimenters designed drug self-administration procedures with the goal of achieving regular drug-taking patterns, comparable to natural rewards (Goldberg, 1973; R. A. Yokel & Pickens, 1973). Regular patterns can be maintained by using 'over-constrained' self-administration procedures characterised by implementation of discrete (as opposed to continuous) dimension strategies (Morgan et al., 2009; Roberts et al., 2013; Roberts & Zimmer, 2020), involving experimenter-imposed unit-doses of drug interspersed by timeouts. These modeling strategies significantly enriched our mechanistic understanding of addiction. However, their intrinsic design favours a regular pattern of drug-self administration limiting our ability to address questions related to the differences in the patterns of drug-taking and drug seeking across different drugs, likely reflecting the instrumentalization of the interoceptive properties of the drug under scrutiny. Indeed, as discussed elsewhere (Morgan et al., 2009), these strategies might create a 'unique case' of drug-taking behaviour that: (1) disregards drug metabolism, which often leads to the formation of active drug metabolites, contributing to the trajectory of the behavioural effects (Andersen et al., 2009; Busto & Sellers, 1986; Wang et al., 2001); (2) prevents an optimal drug loading speed titration (Morgan et al., 2009; Roberts & Zimmer, 2020); (3) masks differences in patterns of drug-taking between different addictive drugs and (4), masks interindividual differences in the pattern of self-administration (Roberts & Zimmer, 2020).

Here we argue that, by employing self-administration procedures devoid of temporal constraint (and therefore promoting an optimal drug loading speed titration) (Morgan et al., 2009) we facilitate the emergence of spontaneous drug use patterns, which are a direct reflection of the unique interaction between the pharmacological properties of the drug, the set and setting (Roberts et al., 2007; Zinberg, 1984).

To address this issue, we first explored the impact of timeouts, typically imposed after unit-doses, on cocaine and heroin taking and seeking. Next, we compared three self-administration procedures and analysed their pharmacokinetics correlates (Allain et al., 2015; Busto & Sellers, 1986; D'Ottavio et al., 2023): (i) 6-h continuous-access with 20-s timeout (Ahmed & Koob, 1998), (ii) 6-h continuous-access without 20-s timeout and (iii) 6-h intermittent-access without timeout (Zimmer et al., 2011; Zimmer et al., 2012) to investigate the impact of timeouts and drug access time on patterns of drug taking and drug seeking, as well as their impact on drug brain levels.

Overall, our study represents a step forward in refining animal models of addiction to be achieved by relaxing the constraints in self-administration experiments to reveal bona fide individual differences in animal models of addiction.

2 | METHODS

Procedures followed the guidelines of the Italian national law (DL 26/2014) on the use of animals for research based on the European Communities Council Directive (2010/63/UE) and approved by the ethics committee of the Italian Ministry of Health and by local Ethical Committee of the Italian Ministry of Health and the local Ethical Committee of the Santa Lucia Foundation. In all the experiments, the investigators were not blind to the training conditions. Further, all animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.1 | Subjects

A total of 143 male Sprague–Dawley rats (CD [IGS] code 001, Charles River, Lecco, IT; RRID:RGD_734476) were used for the study. At the beginning of the experiments, the rats were 5–6 weeks old and weighed between 150 and 175 g. The rats were pair-housed (1291H cage, Tecniplast S.p.A., Varese, IT) before the surgery and individually afterwards. Throughout the entire experiment, the 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. Eleven rats were excluded because of catheter problems or sickness.

We used only male rats because exploring these differences was not the primary aim of the current study. Our hypotheses were primarily informed by behavioural and pharmacokinetic analyses of drug self-administration conducted in a previous study (D'Ottavio et al., 2023). In this context, we emphasize that no studies examined potential sex differences in the pharmacokinetics of heroin and cocaine metabolism following intravenous administration of these drugs. Future research should explore potential sex differences in greater detail (Docherty et al., 2019).

2.2 | Drugs

All drugs were dissolved in sterile saline (0.9% NaCl; B. Braun Group). For drug self-administration training, we used unit-doses of 0.075 mg kg^{-1} per infusion for heroin hydrochloride (diamorphine) and 0.5 mg kg^{-1} per infusion for cocaine hydrochloride. We selected these doses based on previous studies that indicate they support reliable drug self-administration under both continuous (Airavaara et al., 2011; Wee et al., 2007) and intermittent-access (Gueye et al., 2019; O'Neal et al., 2020) schedules, as well as promote a high

rate of responding under progressive ratio schedules (Roberts & Bennett, 1993; Shaham & Stewart, 1994). More importantly, these doses fall on the descending limb of the drug dose–response curve (Martin et al., 1996; Piazza et al., 2000; Pickens et al., 1968), suggesting they are *high* enough to sustain response while being *low* enough to allow rats to quickly reach the 'preferred concentration.' As we will see in the results section, this aspect will have a profound impact on the experimental groups where the timeout constrain is removed. Notably, higher doses would have constrained the titration capability of rats during the self-administration and as a consequence reduced their ability to adjust the number of drug infusions to their preferred levels.

2.3 | Intravenous surgery

We anaesthetised rats with **isoflurane** (5% induction, 2–3% maintenance; Demas S.r.l., Rome, IT) and injected them with **carprofen** (2 mg kg^{-1}, subcutaneous injection; Zoetis Italia S.r.l., Rome, IT), immediately after the surgery and for the following 5 days to relieve pain and decrease inflammation. We inserted a silastic catheter into the jugular vein as previously described (Caprioli et al., 2015). During the anaesthsia, where body temperature was monitored and maintained using a heathing blanket, we placed the distal end of the catheter into the right jugular vein and attached the proximal end of a modified 22-gauge cannula placed on the back in the mid-scapular region. We allowed rats to recover for a minimum of 5–7 days. We flushed the catheters daily with a 0.2 ml sterile saline solution containing **gentamicin** (4.25 mg ml^{-1}; Fatro S.p.A., Bologna, IT) to test the catheter patency and prevent occlusion during the recovery, training and abstinence phases. If during the training phase the catheter failed the patency test, we catheterised the left jugular vein using the same procedure, or we eliminated the rat from the study.

2.4 | Self-administration apparatus

We trained rats in self-administration chambers placed inside sound-attenuating cubicles controlled by a custom-made system (Caprioli et al., 2007; Celentano et al., 2009). 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.

2.5 | Estimated brain levels of cocaine, heroin and its metabolites

We chose to estimate drug brain levels using pharmacokinetic parameters calculated from previous *in vivo* studies. This is because, assessing brain levels of heroin, its metabolites and cocaine is challenging with currently available techniques, such as microdialysis. The need for repetitive sampling interferes with animal behaviour. In addition, these methods lack the sensitivity needed to detect the low brain concentrations resulting from the doses used in this study. Based on these considerations, brain levels of heroin and its active metabolites [6-monoacetylmorphine (6-MAM) and **morphine**], along with cocaine were estimated using Kinetica v.5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with its FitMultiMicroExtravascular model for multiple administered doses. Heroin, its metabolites and cocaine brain levels were estimated based on the times and the absolute dose (μmol) received per infusion (adjusted to the weight of each animal) during the last self-administration session, and on the pharmacokinetics parameters (absorption, elimination, intercompartmental distribution rate constants, time lag, volume of distribution) best fitting the mean brain extracellular fluid data in *in vivo* studies. Brain concentrations were calculated in 0.5 min intervals for 6 h. Pharmacokinetics parameters for heroin and its metabolites were obtained from the study by Gottas et al. (2013) (see Table S1), while pharmacokinetics parameters for cocaine were obtained from the study by Pan et al. (1991) (see Table S2).

2.6 | Experiment 1. The role of timeout in heroin and cocaine self-administration: a within-subject study

2.6.1 | 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 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 succeeding discussion).

2.6.2 | 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 drugs 2 h day^{-1} for 4 days (acquisition phase; maximum infusions = 20 per day). In the training phase, rats were trained 3 h d^{-1} for 14 days (training phase; maximum infusions = 90 per day). During both acquisition and training phase, we trained rats to self-administer drugs on two different levers: one lever associated with timeout and one lever associated with no-timeout. 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 [Fixed Ratio 1 (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. The cue light remained on for a total of 23 s. 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).

2.6.3 | Drug seeking

We tested rats for drug seeking under extinction conditions on both levers (timeout and no-timeout) in the days following the last choice session. We tested rats on the two levers 3 days apart. The test days were balanced 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, we divided the rats (matched for their total drug intake during the self-administration training, Table S3) into two groups. We tested one group of rats in a discrete choice procedure and the other group of rats was tested in a multi-day progressive ratio procedure.

2.6.4 | Discrete choice

We tested a subgroup of rats 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 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 six trials separated by 55 min. The length of the inter-trial intervals was set based on a pilot study (data not shown) and corresponded to the time needed for morphine to begin dissipating (Gottas et al., 2013). Each trial began with the flashing of the house light for 30 s (a discriminative stimulus that signalled 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 levers selection was set to five 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. We tested rats starting from the day following the final drug self-administration session, continuing for a duration of 7 days.

2.6.5 | Multi-day progressive ratio

We tested a subgroup of rats in a recently developed progressive ratio procedure (Roberts & Zimmer, 2020) 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, etc. (Richardson & Roberts, 1996; Roberts & Bennett, 1993). The completion of the ratio requirement provided access to 3 min to the drug under FR1 or FR1 20 s timeout 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 one step back to the ratio value reached on the day before. The test ended for the rats if they did not exceed the final ratio value achieved the previous day, which corresponded to the breakpoint.

2.7 | Experiment 2. The role of timeout in drug self-administration: comparison between continuous-access timeout, intermittent-access, or continuous-access no-timeout to heroin and cocaine

2.7.1 | 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.

2.7.2 | Mechanical nociceptive von Frey test

We only tested mechanical sensitivity with the von Frey test in rats that would then be trained for heroin, because mechanical sensitivity does not change after cocaine self-administration (Edwards et al., 2012). We tested rats before (7 days after the intravenous surgery) and after every three consecutive drug self-administration training sessions (in the morning, 18 h into withdrawal) (Edwards et al., 2012; Kallupi et al., 2020). We put rats in boxes on an elevated metal mesh floor and allowed 10 min for habituation before examination. We stimulated the plantar surface of each hind paw 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). We determined

the 50% paw withdrawal threshold (PWT) using Dixon's up-down method (Chaplan et al., 1994; Maftei et al., 2014).

2.7.3 | 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 day^{-1} for 3 days (acquisition phase; maximum infusions = 20 per day). After the acquisition, we divided rats (matched for their total drug intake during acquisition, Table S3) into three groups that underwent three different drug self-administration training: continuous-access timeout, intermittent-access and continuous-access no-timeout to drug. In the training phase we trained rats 6 h day^{-1} for 12 days (training phase; maximum infusions: heroin = 90 per day; cocaine = 150 per day).

1. Continuous-access timeout: drug continuously available 6 h d^{-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. The cue light remained on for a total of 23 s.
2. Intermittent-access: drug available in 12 epochs of 5 min (ON periods) every 25 min (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 timeout.
3. Continuous-access no-timeout: -drug continuously available 6 h day^{-1}; 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 timeout.

2.7.4 | Behavioural assessment

We recorded rats' behaviour during the first hour of the last drug self-administration training session. Sessions were recorded via a digital camera, videos were analysed offline and data were manually scored using Behavioural Observation Research Interactive Software [BORIS; (Friard & Gamba, 2016); RRID:SCR_021509]. The videos were analysed by an experimenter that was blind to the training conditions. We scored the length of each bout of the following behaviours: grooming (corporal grooming and plucking/pulling at fur or digits), chewing (chewing grid floor or lever), walking, stupor/immobility, hand/foot licking and sniffing (Alcantara et al., 2011; Seip et al., 2012).

2.7.5 | Seeking test

We tested rats for drug seeking under extinction conditions on abstinence days 1 and 21. Duration of the test sessions was 30 min to minimise 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 seeking test on day 1, we brought the rats to their home cages and handled them twice a week during abstinence.

2.8 | Statistical analysis

All the data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). Data were analysed with the statistical programmes SPSS (SPSS, Version 25; RRID:SCR_002865), GraphPad Prism (Version 8.0.1; RRID:SCR_002798), XLSTAT (RRID:SCR_016299) or R (RRID:SCR_001905). Before any analysis, all data were evaluated for normality. We used either parametric or non-parametric tests based on the results. 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 analysed separately (see Table S3 for a complete reporting of the statistical analyses and their exact P-values).

2.8.1 | Experiment 1

For the training phase, we analysed total drug intake and total lever presses for infusions/number of lever presses using the within-subjects factor of Session. We analysed drug intake and lever presses during self-administration training using a General Linear Mixed Models (GLMM) as a function of Access (timeout, no-timeout) condition. In the General Linear Mixed Models, we used the Access condition as a fixed effect, while we used Sessions and Rat as random effects in a crossed design. For the seeking test, we analysed active lever presses during the cue-induced seeking tests using the Wilcoxon matched-pairs signed-rank test with Access condition (timeout, no-timeout) as within-subjects factor; we included Cues (white light, three-light cue) and Abstinence Day (1,3) as covariates. Relative to the choice procedure, we calculated the preference (preference score) in the choice tests by

normalising the indifference level between timeout and no-timeout choices at 0 using the following formula:- [1 – (% timeout lever choices/50%)] (Lenoir et al., 2007). We analysed the preference score across sessions using the within-subjects factor of Session. Finally, for the progressive ratio test, we analysed the total number of lever presses using the Access condition (timeout, no-timeout) as within-subjects factor.

2.8.2 | Experiment 2

For the training phase, we analysed drug intake and lever pressing data for infusions/number of lever presses using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout) and the within-subjects factor of Session. Regarding the pattern of drug self-administration and pharmacokinetics data, estimated based on behavioural data collected during the last session of drug self-administration, we analysed total drug intake of the last session of self-administration, number of bursts-like events (defined as ≥3 infusions in less than 5 min, similar to what previously described by Belin et al. (2009), 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, using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout). Concerning the behavioural observations, collected during the last self-administration session, we analysed each behaviour (stupor, walking, chewing, grooming, sniffing and hand licking) using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout). Regarding the seeking test on abstinence day 1, we analysed the number of active-lever presses using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout). We compared drug seeking on Abstinence Day 1 and 21 by analysing the number of active lever presses using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout) and the within-subjects factor of Abstinence Day (1, 21); the inactive lever was included as a covariate. Then, we analysed withdrawal signs (hyperalgesia and body weight) using the between-subject factor of Access (continuous-access timeout, intermittent-access and continuous-access no-timeout) and the within-subjects factor of Time (baseline and test days).

2.9 | Materials

The drugs used in the study were donated from the Drug Supply Program (Research Triangle Institute, NC277092194, USA) of the National Institute on Drug Abuse (NIDA, Baltimore, MD, USA). Details of other materials and suppliers are provided in the specific sections.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023).

3 | RESULTS

3.1 | Experiment 1. The role of timeout in drug self-administration: a within-subject study

The rats increased their heroin and cocaine intake over time in both access conditions (timeout and no-timeout) (Figure S1D left, S1F left). Heroin intake was higher without timeout (Figure S1D right), while lever presses for heroin increased with timeout (Figure S1E right). Notably, this effect was not observed with cocaine (Figure S1F right, S1G right). The rats exhibited stronger seeking for heroin and cocaine no-timeout (Figure 1d, g), strong preference for the no-timeout condition for both drugs during the choice tests (Figure 1e, h) and higher final ratios and cumulative number of lever presses for the no-timeout, relative to the timeout condition (Figure 1f, i).

3.2 | Experiment 2. The role of timeout in heroin and cocaine self-administration: comparison between continuous-access timeout, intermittent-access or continuous-access no-timeout

3.2.1 | Drug intake

Across all access conditions (intermittent, continuous timeout and continuous no-timeout), drug intake and lever pressing increased over time (Figure 2d, g). However, heroin intake was markedly higher in both intermittent- and continuous-access no-timeout, relative to the timeout condition (Figure 2d, e). Interestingly, these differences in intake did not affect hyperalgesia development, although rats in the timeout condition experienced less heroin-related weight loss than those in other conditions (Figure S2E, S2F). Conversely, cocaine intake was lower in intermittent-access compared to continuous-access (both timeout and no-timeout) conditions. However, total cocaine intake was higher in the continuous-access no-timeout relative to the continuous-access timeout condition (Figure 2g, h).

3.2.2 | Drug seeking

Overall, the rats displayed stronger seeking for heroin and cocaine self-administration without timeout (Figure 2f, i). We observed incubation of drug craving exclusively under timeout conditions, with increased

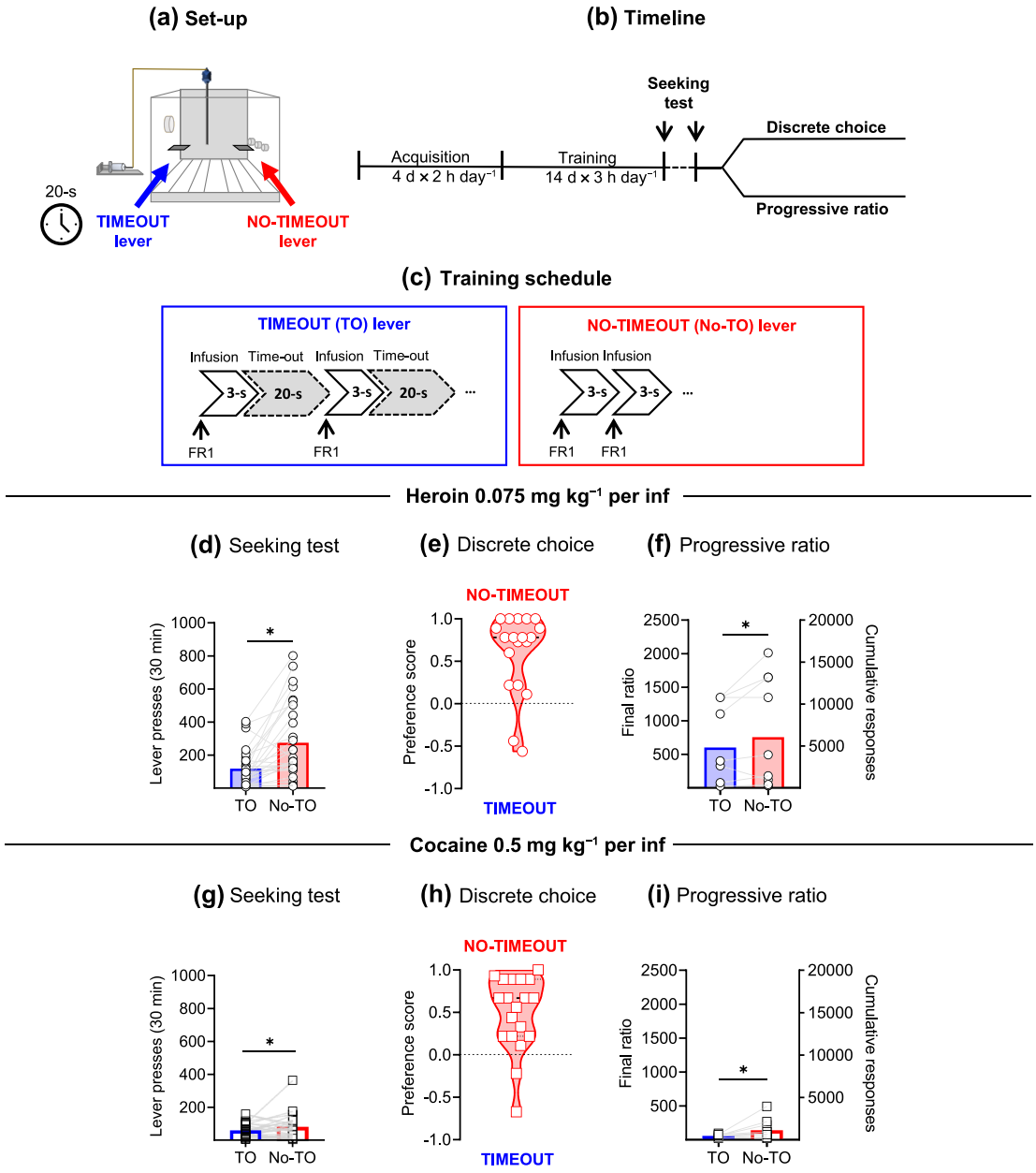

FIGURE 1 The impact of timeout on motivation to seek and take heroin and cocaine: comparison between timeout and no-timeout conditions in a within-subject design. (a) Experimental set-up. (b) Experimental timeline. (c) Training schedule. (d and g) Seeking test. Individual data of number of lever presses 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 $n = 28$).

lever pressing on Abstinence Day 21 compared to Day 1 (Figure S2D, S2G).

3.2.3 | Drug-taking patterns and estimated brain levels of drug

The different training conditions (Figure 2c) were associated with qualitative differences in the pattern of drug-taking (Figures S3, S4), with strong differences between heroin and cocaine. Regarding heroin, rats trained under continuous-access timeout conditions displayed a pattern of drug-taking characterised by few infusions (typically maximum two consecutive unit-doses) spaced by inter-infusion intervals of more than 10 min (Figure S4E). While without timeout (both intermittent and continuous-access), rats consumed heroin in rapid, consecutive 'bursts' (Belin et al., 2009): several infusions in a row, spaced by very short inter-infusions intervals (more than two consecutive injections in less than 5 min, Figure S4E, S4F). As in our

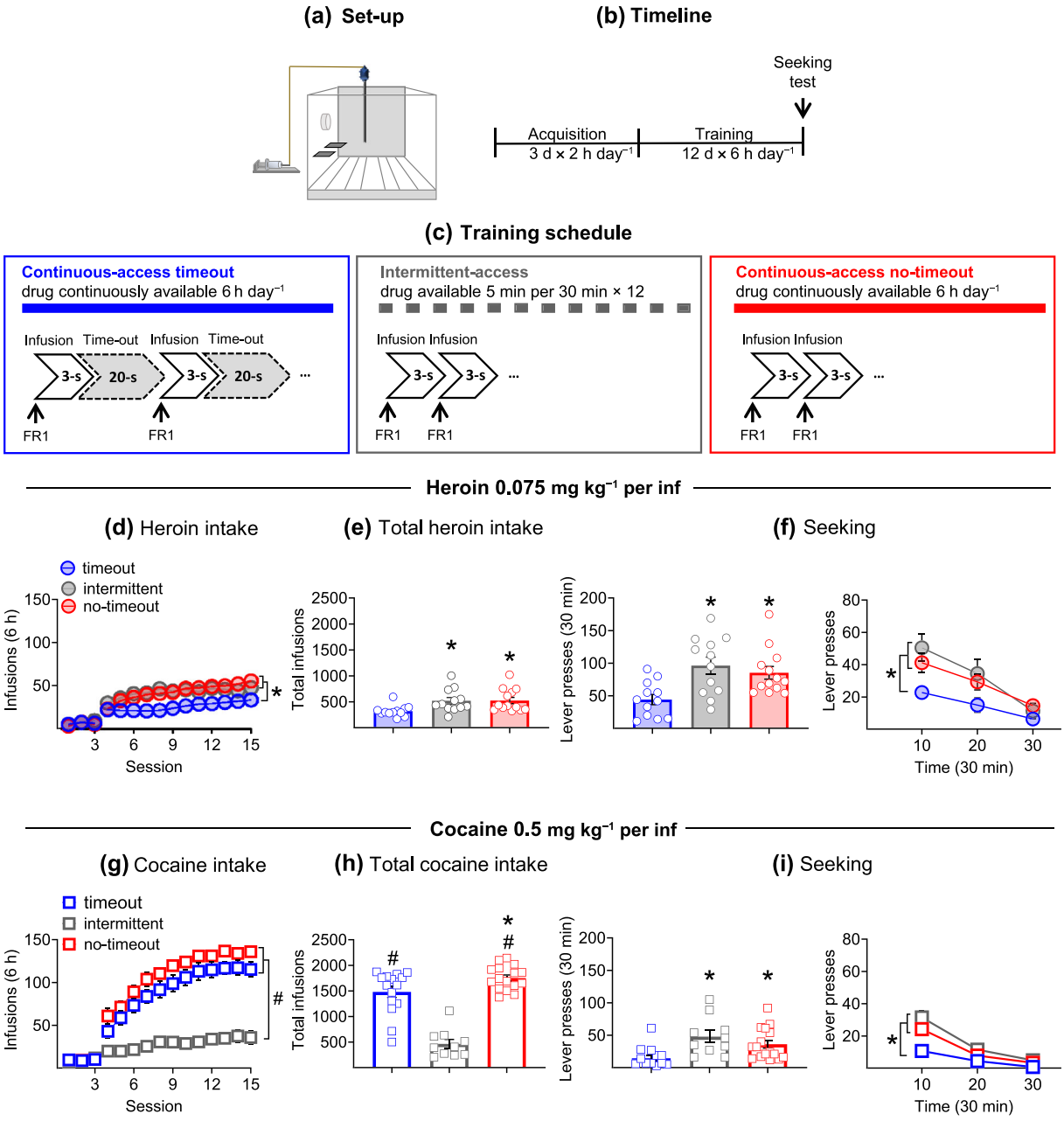

FIGURE 2 The impact of timeout in heroin and cocaine self-administration and seeking: a comparison between continuous-access timeout, intermittent-access or continuous-access no-timeout to drug in a between-subject design. (a) Experimental set-up. (b) Experimental timeline. (c) Training schedule. (d and g) Drug intake. Mean ± SEM number of drug infusions per session. (e and h) Total drug 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 continuous-access timeout, $P < 0.05$; # different from intermittent-access, $P < 0.05$ (heroin $n = 37$; cocaine $n = 41$).

previous study (D'Ottavio et al., 2023), 'burst' episodes were accompanied by fast-rising high brain peak concentrations of heroin and 6-MAM that were significantly higher in intermittent- and continuous-access no-timeout, relative to timeout conditions (Figure S5D, S5E). Morphine brain levels, though, were significantly higher only in continuous-access no-timeout, relative to continuous-access timeout and intermittent-access conditions (Figure S5F). Regarding cocaine, in both continuous-access timeout and no-timeout conditions, patterns of cocaine followed a 'loading'—where several unit-doses were self-administered at high rate in succession—then 'maintenance' pattern—characterised by self-administration of fewer unit-doses every few minutes—leading to similar brain cocaine levels (Figure 3g, h, i), whereas 'burst' events were more frequent without timeout (Figure S4G, S4H). In intermittent-access, 'burst' episodes were

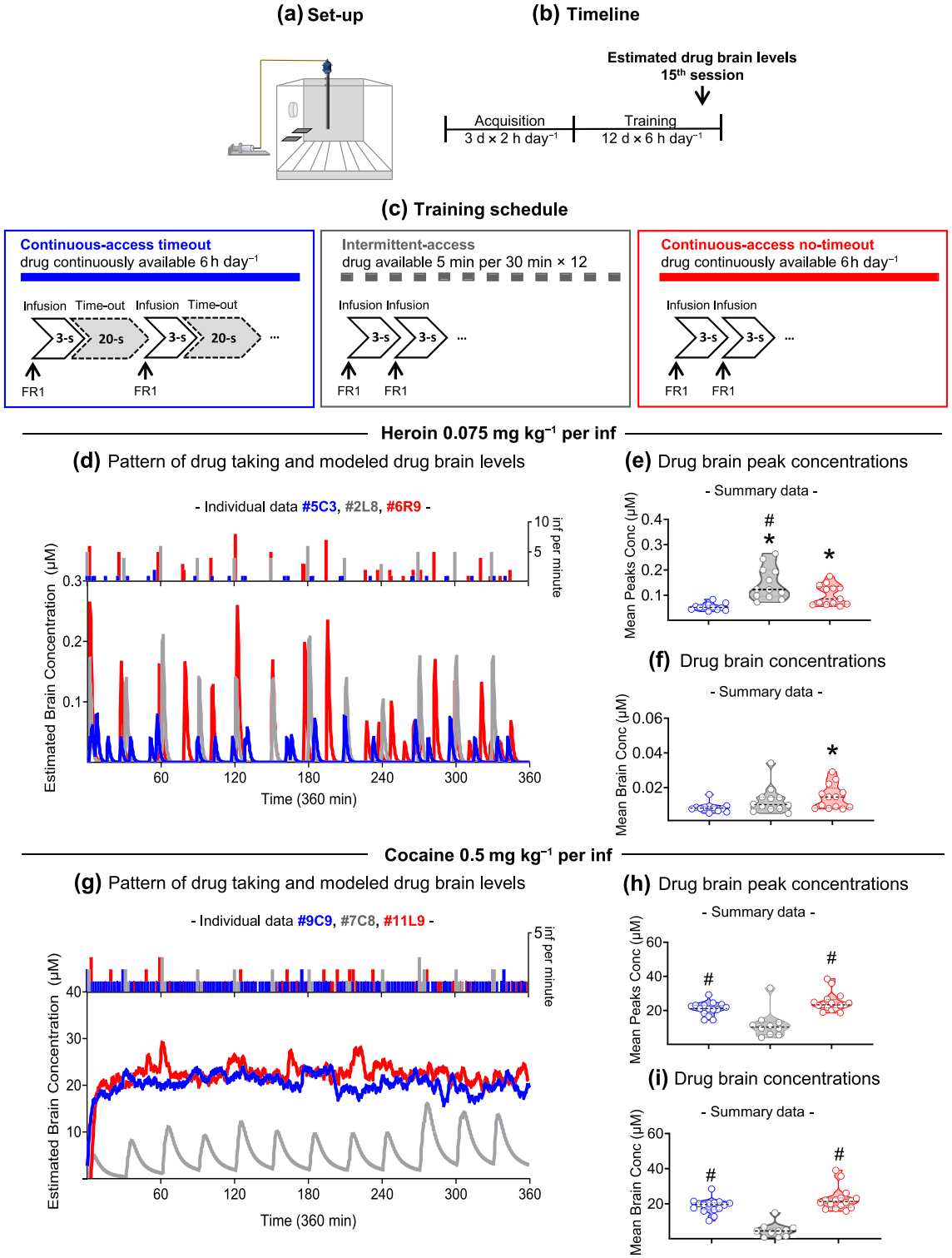

FIGURE 3 Patterns of drug intake and pharmacokinetic modelling of brain concentrations of heroin and cocaine in representative rats trained under continuous-access timeout, intermittent-access or continuous-access no-timeout to drug in the last session of drug self-administration training. (a) Experimental set-up. (b) Experimental timeline. (c) Training schedule. (d and g) Pattern of drug-taking and estimated drug brain levels in representative rats (heroin infusions: continuous-access timeout = 30, intermittent-access = 55, continuous-access no-timeout = 86; cocaine infusions: continuous-access timeout = 137, intermittent-access = 44, continuous-access no-timeout = 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 continuous-access timeout, $P < 0.05$; # different from intermittent-access, $P < 0.05$. (heroin: $n = 37$; cocaine: $n = 41$).

separated by programmed time OFF (25 min) (Figure 3g). Notably, this pattern was accompanied by brain cocaine peak concentrations and brain cocaine concentrations that were lower than in rats trained under continuous-access timeout and no-timeout conditions (Figure 3h, i).

3.2.4 | Behavioural repertoire

We observed that during the first hour of the last self-administration session, heroin-trained rats showed similar behaviours across conditions. However, stupor was higher in intermittent and continuous no-timeout conditions, compared to continuous-access timeout (Figure S7D), but walking was higher in continuous-access timeout conditions (Figure S7E). In cocaine-trained rats, walking was higher in intermittent conditions than in continuous-access timeout or no-timeout conditions (Figure S7K).

4 | DISCUSSION

We explored the effects of discrete versus continuous dimension strategies on drug self-administration and choice. Through a comparative analysis of cocaine and heroin taking and seeking in different self-administration procedures, we found that absence of timeouts between unit-doses: (1) increases rats' drive to seek and take drugs and (2) promotes individual differences in drug-taking patterns for heroin (but not cocaine) resembling human heroin-taking patterns.

4.1 | Optimising drug self-administration models in addiction research: an analysis of the effect of experimenter-imposed timeouts

Originally, timeouts were implemented in drug self-administration procedures to generate regular drug-taking patterns (Goldberg, 1973). Over time, the rationale for their implementation shifted to overdose prevention. By and large, this remains the main reason for their systematic implementation, as referred to in materials and methods sections of preclinical addiction studies using self-administration procedures (i.e. Conrad et al., 2008; He et al., 2023; Mameli et al., 2009; Rocha et al., 1998). Here, we demonstrated that removing the timeout, at the standard training unit-doses used in self-administration studies, did not result in overdoses in either cocaine or heroin self-administration. Instead, the absence of timeouts led to relevant individual differences in drug-taking patterns, with significant differences between cocaine and heroin. Below we will discuss, initially separately for cocaine and heroin, these differences in the context of the drug-taking modalities often observed in humans.

4.1.1 | Cocaine

Cocaine addiction in humans is often characterised by patterns of repeated 'binges', which involve cycles of prolonged and continuous drug-taking (lasting several hours and can last for days in more severe instances) with periods of abstinence and resulting in the so called 'crash' (Gawin, 1991; Gawin & Kleber, 1985, 1986; Siegel, 1977, 1984, 1985; Van Beek et al., 2001). During binges, cocaine is consumed in a regular pattern, with repeated use occurring every 10–30 min. This redosing interval aligns with cocaine's pharmacokinetics, including its time to maximum blood concentration and half-life (Javaid et al., 1978). Indeed, human cocaine self-administration studies that simulate binge-like drug-taking in laboratory settings showed that, during binges, plasma cocaine levels either stabilise at steady-state concentrations (Fischman et al., 1976; Hatsukami et al., 1994) or increase with consecutive doses (A. Ward et al., 1997).

Considering these aspects of human drug-taking behaviour and the canonical definition of binge-like drug taking in clinical literature, the continuous-access procedure (also known as long-access), as originally proposed by Ahmed and Koob (Ahmed & Koob, 1998), serves as an effective model for replicating this pattern. This holds true even when a timeout is removed (continuous-access no-timeout), as it does not significantly disrupt the cocaine-taking pattern and associated brain cocaine levels. One significant difference, though, between the continuous-access timeout and our continuous-access no-timeout, is that it results in an overall higher motivation (Figure 1), likely because of an increased frequency of bursts events (Figure S4) (Belin et al., 2009).

More recently, preclinical researchers proposed an alternative model to the classical continuous-access procedures discussed earlier: the intermittent-access drug self-administration (Allain et al., 2015; Gueye et al., 2019; Kawa et al., 2019; Zimmer et al., 2012). This procedure was proposed as a more accurate model to mimic the binge-like patterns of human cocaine use and it is featured by an experimenter imposed intermittent-access to cocaine (every 25 min). This strategy arguably results in intermittent spikes in brain and body levels of cocaine, with concentrations decreasing after each taking event (Zimmer et al., 2011). However, this pattern does not often occur in humans with cocaine addiction that is characterised by a binge and compulsive-like use, as shown by clinical observations and laboratory cocaine self-administration studies (Fischman et al., 1976; Gawin, 1991; Gawin & Kleber, 1985, 1986; Hatsukami et al., 1994; Siegel, 1977, 1984, 1985; Van Beek et al., 2001; Ward et al., 1997). Despite the paucity of human studies investigating patterns of cocaine use (which are largely needed), we believe that intermittent-access more likely models the early stages of cocaine use or recreational-like use in humans (Siegel, 1984, 1985) and should therefore be used to study this phase.

4.1.2 | Heroin

Regarding heroin, patients typically report taking large amounts (burst-like events) in a few daily episodes, which leads to intense euphoria (Alksne et al., 1967; Darke, 2011; Haasen et al., 2007; McAuliffe & Gordon, 1974; Ross et al., 2008). These patterns of heroin taking in our study are largely recapitulated under no-timeout conditions (intermittent-access and the continuous-access no-timeout). In other words, the absence of timeout promotes burst-like heroin self-administration followed by periods of inactivity. Notably, as previously reported for intermittent-access to heroin (O'Neal et al., 2020), our continuous-access resulted in pronounced individual differences, with a subpopulation of rats that exhibit very high heroin intake, large and frequent bursts and elevated heroin brain levels, as well as intense drug seeking. Future research should investigate the biological mechanisms and correlates that underpin these individual differences.

What might be the reason behind the striking heroin pattern differences among the timeout and no-timeout access training conditions?

In our previous study (D'Ottavio et al., 2023), we speculated that timeout-related effects are linked to the drug's metabolism: the faster the metabolism the stronger the impact of timeout on drug's effects. Timeout between unit-doses prevents rats from self-administering drug at high frequency and, considering heroin's brief half-life in the rat brain [∼0.9 min, (Gottas et al., 2013)], this may hinder brain heroin accumulation, preventing rats from reaching fast-delivered high brain heroin concentrations, which would affect the behavioural outcomes (Andersen et al., 2009; Avvisati et al., 2019; D'Ottavio et al., 2023; Gottas et al., 2013; Gottås et al., 2014; Hubner & Kornetsky, 1992; Kvello et al., 2020; Perekopskiy & Kiyatkin, 2019; Solis et al., 2017). Given the longer half-lives of 6-MAM and morphine, the timeout would have a lesser impact on their accumulation in the brain. To support this hypothesis and corroborate our data, we simulated brain concentrations of heroin, 6-MAM and morphine under self-administration conditions with and without timeout. The simulated brain concentrations of heroin were halved and about 3 min delayed, maximal brain concentrations of 6-MAM were about 2 min delayed, while morphine brain concentrations were only minimally affected during timeout compared to no-timeout conditions (Figure S6), changes which would influence the experience of rush and intoxication. Further studies are needed to test our simulation *in vivo*, for example by using engineered drug sensors (Muthusamy et al., 2024), and to assess whether the imposition of a timeout influences the self-administration of heroin metabolites, particularly 6-MAM and morphine. Overall, this supports the hypothesis that the differences observed in rats trained with or without timeout are more likely influenced by the effect of the timeout on brain accumulation of heroin and 6-MAM, rather than on the brain accumulation of morphine. Notably, historical literature posits that heroin primarily functions as a delivery mechanism for its metabolites (Inturrisi et al., 1983; Oldendorf et al., 1972; Way et al., 1960). However, our findings suggest a more intricate interaction, highlighting the roles of both heroin itself and its metabolites in influencing behavioural outcomes (D'Ottavio et al., 2023; Milella et al., 2023).

4.1.3 | Cocaine versus heroin

As previously mentioned, the experimenter-imposed timeout between infusions profoundly affected heroin-taking pattern but not the cocaine-taking pattern. From a pharmacokinetic perspective, this discrepancy might be because of the unique pharmacokinetic properties of each drug. Cocaine has longer half-life in the rat brain—ranging from 8 to 11 min (Pan et al., 1991)—than heroin (≅90 s) (Gottas et al., 2013). This suggests that timeout have a minimal effect on brain cocaine accumulation. Accordingly, Minogianis et al. (2019) demonstrated that varying the speed of cocaine delivery did not affect cocaine's brain levels. Of note, similar studies have not yet been conducted on heroin and further research is needed to explore how variations in delivery speed might influence heroin brain levels. Such studies would help to clarify the potential effects of delivery methods and dosing regimens on heroin accumulation and distribution in the brain, as well as their effects on behaviour.

Taken together, our study reinforces the notion that pharmacokinetic profiles of drugs are crucial in determining behavioural response to drugs (Muthusamy et al., 2024) and patterns of drug-taking and drug seeking (Busto & Sellers, 1986).

4.2 | Implications for the development of refined animal models in addiction and mechanistic studies

From a refinement perspective, the significance of our data is amplified in light of the Food and Drug Administration's recent emphasis on drug use patterns as critical indicators of addiction severity and treatment success for substance use disorders (Amin-Esmaeili et al., 2024; Food and Drug Administration, 2020; Panlilio et al., 2020), highlighting the necessity for animal models that accurately reflect human drug consumption to advance neurobiological research and treatment. Classical neuroscience research consistently demonstrated that training and experience significantly influence brain plasticity, with different experiences inducing a range of dissociable alterations in the brain, including changes in spine density, synapse formation and metabolic activity (Kolb & Whishaw, 1998; Rosenzweig & Bennett, 1996; Zatorre et al., 2012). Additionally, prior studies showed that different patterns of non-contingent opioid exposure (continuous vs. intermittent) lead to different synaptic plasticity profiles (increased vs. decreased neuronal activity) (Lefevre et al., 2023) and that different cocaine self-administration patterns (continuous vs. intermittent) have distinct effects on dopaminergic signalling (Calipari et al., 2013). Based on this body of evidence, we hypothesise that the distinct training conditions used in our study, which result in divergent self-administration patterns, likely induce different forms of brain plasticity, potentially explaining the divergent addiction-like behaviours (Belin et al., 2009; Kasanetz et al., 2010). However, no studies systematically explored the neuroplastic changes produced by distinct heroin and cocaine self-administration patterns and their link to addiction-like behaviours. Future research is needed to investigate how these patterns cause lasting brain changes that drive addiction.

4.3 | The relationship between timeout, drug-taking patterns and unit-doses

A main observation in our study is that rats did not limit themselves to the self-administration of single and spaced drug unit-doses. Despite considerable variability, when trained without timeout, rats displayed the tendency to self-administer multiple unit-doses in bursts. These findings challenge the idea that the experimenter-imposed unit-dose per se correspond to the drug's 'ideal' rewarding effect. Accordingly, Roberts and Zimmer (2020), using a novel progressive ratio procedure, demonstrated that rats self-administering cocaine remain motivated to lever-press only if the dose of drug provided allow them to reach and maintain preferred drug brain levels. Notably, a single unit-dose of cocaine is not sufficient to reach these levels. We replicated these findings with cocaine (Roberts & Zimmer, 2020) and generalised them to heroin, with rats reaching an unusually high final ratio—over 2000—this result is unprecedented compared to previous studies using fixed unit-doses of heroin and cocaine. Overall, when rats were given access to their preferred drug dose, they self-administered multiple unit-doses of the drug and achieved high final ratios never documented in studies using progressive ratio schedules with single unit-doses (Richardson & Roberts, 1996; Roberts & Bennett, 1993; Roberts & Zimmer, 2020; S. J. Ward et al., 2005). In our study, rats displayed higher motivation when their preferred/'ideal' dose was delivered faster, as observed by higher lever-pressing on the no-timeout lever. Accordingly, previous studies showed that rapid cocaine delivery significantly enhances motivation to take and seek the drug, as well as increases progressive ratio performance, drug-seeking behaviour and relapse rates (Bouayad-Gervais et al., 2014; Martín-García et al., 2014; Minogianis et al., 2013; Samaha et al., 2002; Samaha & Robinson, 2005; Wakabayashi et al., 2010). While the specific influence of heroin delivery speed has not been fully explored, our data indicate that a faster heroin delivery intensifies heroin taking and seeking.

In addition to this, we found that increased motivation for drugs, induced by no-timeout training, resulted in strong drug seeking from the early days of abstinence, remaining stable over time. This finding is unexpected, considering prior evidence suggesting that drug seeking intensified over time (a phenomenon termed incubation of craving) (Grimm et al., 2001). This phenomenon has been consistently reported for various addictive drugs and natural rewards (Chow et al., 2025; Venniro, Reverte, et al., 2021). Both our lab and others noted a lack of incubation of craving following intermittent access to opioids or alcohol, with high seeking during early abstinence (Abstinence Day 1) driving this absence (D'Ottavio et al., 2023; Gage et al., 2023; Samson et al., 2022). Here, we observed a similar effect under no-timeout conditions, with high seeking during early abstinence, regardless of the drug access schedule or amount consumed. Here we propose two explanations for this phenomenon. First, the within-subject design used may have caused carryover effects from high extinction on Abstinence Day 1 to extinction testing on Day 21. Further studies with a between-subject design are therefore needed to confirm whether the lack of incubation persists. Second, environmental cues associated with drug availability are key relapse-promoting factors, while cues signalling drug unavailability may suppress drug-seeking behaviour in rats, acting as 'conditioned inhibitors' (Rescorla, 1969) or 'negative occasion setters' (Schmajuk et al., 1998; Weiss et al., 2001). Previous studies showed that drug omission cues can suppress major relapse-promoting factors like drug-predictive cues, stress and drug exposure (Kearns et al., 2005; Laque et al., 2019). Given this, it is possible that the timeout (signalled by a cue light) acted as a cue for drug unavailability, thereby reducing drug-seeking behaviour during early abstinence. Future studies should further explore this aspect.

Overall, our results suggest that the absence of timeout more strongly impacts heroin than cocaine taking and seeking. Indeed, heroin-trained rats showed higher intake and seeking, when trained without timeout. Additionally, to our knowledge, this is the first study to describe patterns of heroin taking characterised by burst-like events, while the cocaine-taking patterns observed here were documented in several prior studies (e.g. Ahmed & Koob, 1998; Zimmer et al., 2011). However, one methodological limitation remains in this regard. In the present study, we used a single dose of heroin and cocaine, leaving it uncertain whether higher doses would produce similar results. Future studies should explore this phenomenon in greater depth to determine how varying doses may influence the observed effects.

Beyond this consideration, in this context, it is important to note that, historically, the choice of doses and their timing in self-administration studies were often about convenience and standardisation (Bozarth, 1987; Martin et al., 1996; Piazza et al., 2000; Pickens et al., 1968; Sim-Selley et al., 2000). For example, studies justify dose reductions during self-administration training as a way to increase lever-pressing during self-administration and reinstatement tests (Corre et al., 2018; Fuchs & See, 2002; Shen et al., 2014). In addition, previous research investigated behavioural allocation between drugs and non-drug rewards in choice procedures, mostly used unit-dose strategies (Lenoir & Ahmed, 2008; Vandaele et al., 2016; Venniro et al., 2017; Venniro et al., 2018; Venniro et al., 2019; Venniro, Panlilio, et al., 2021). Nevertheless, our study challenges this viewpoint and suggests that the rewarding effects of drugs are closely linked to the administration of preferred doses at preferred time (Roberts & Zimmer, 2020). Thus, future studies should explore whether the provision of access to the preferred dose in the preferred timing might affect drug preference in choice procedures.

4.4 | Concluding remarks

Our results highlight the need to recognise the distinct pharmacokinetic properties of each drug, questioning the use of standardised drug self-administration protocols. We, therefore, suggest a reevaluation of current methodologies used in preclinical drug self-administration models, such as the use of unit-doses and timeout, advocating for approaches devoid of experimenter-imposed constraint.

AUTHOR CONTRIBUTIONS

G. D'Ottavio: Conceptualisation; methodology; formal analysis; investigation; data curationl writing—original draft; visualisation; funding acquisition. **S. Pezza**: Investigation. **J. Modoni**: Formal analysis. **I. Reverte**: Formal analysis. **C. Marchetti**: Investigation. **S. F. Zenoni**: Investigation. **S. De Pirro**: Writing—review and editing. **D. Maftei**: Investigation. **R. Lattanzi**: Writing—review and editing. **G. Esposito**: Writing—review and editing. **D. Ragozzino**: Writing—review and editing. **E. Merlo**: Writing—review and editing. **M. Venniro** and **R. Ciccocioppo**: Writing—review and editing; funding acquisition. **F. Fumagalli**: Writing—review and editing; funding acquisition. **M. S. Milella**: Writing—original draft. **A. Badiani**: Writing—review and editing. **F. Boix**: Formal analysis; data curation; writing—review and editing. **D. Caprioli**: Conceptualisation; project administration; resources; writing—original draft; supervision; funding acquisition. All authors reviewed and approved the final version of the manuscript prior to submission.

ACKNOWLEDGEMENTS

We thank Dr. Yavin Shaham for his comments on an earlier version of this manuscript, Miss Alana Sullivan for proofreading the manuscript, and Drs. Andrea Termine and Carlo Fabrizio for their assistance with data analysis. A previous version of this manuscript was published as a preprint on bioRxiv: https://www.biorxiv.org/content/10.1101/2024.07.08.602268v1.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflicts of interest (financial or otherwise) related to the content of the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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How to cite this article: D'Ottavio, G., Pezza, S., Modoni, J., Reverte, I., Marchetti, C., Zenoni, S. F., De Pirro, S., Maftei, D., Lattanzi, R., Esposito, G., Ragozzino, D., Merlo, E., Venniro, M., Ciccocioppo, R., Fumagalli, F., Milella, M. S., Badiani, A., Boix, F., & Caprioli, D. (2025). Behavioural and pharmacokinetic analysis of heroin and cocaine self-administration: Effects of timeout on self-administration and choice in male rats. *British Journal of Pharmacology*, *182*(13), 2968–2985. https://doi.org/10.1111/bph.70023