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Pharmacological validation of individual animal locomotion, temperature and behavioural analysis in group-housed rats using a novel automated home cage analysis system: a comparison with the modified Irwin test

Karen Tse^{a,*}, Rowland Sillito^b, Amy Keerie^{a,Φ}, Rachel Collier^{c,Ψ}, Claire Grant^d, Natasha A Karp^e, Cathy Vickers^f, Kathryn Chapman^{f,Υ}, J Douglas Armstrong^{b,g}, William S Redfern^a

^a Safety and ADME Translational Sciences, Drug Safety and Metabolism, IMED Biotech Unit, AstraZeneca, Babraham Research Campus, Cambridge, CB22 3AT, UK

^b Actual Analytics Ltd, Appleton Tower, 10 Crichton Street, Edinburgh, EH8 9LE., UK

^c Laboratory Animal Sciences, Drug Safety and Metabolism, IMED Biotech Unit, AstraZeneca, Babraham Research Campus, Cambridge, CB22 3AT, UK

^d Safety and ADME Translational Sciences, Drug Safety and Metabolism, IMED Biotech Unit, AstraZeneca, Alderley Park, Cheshire SK10 4TG, UK

^e Quantitative Biology, Discovery Science, IMED Biotech Unit, AstraZeneca, Cambridge Science Park, Cambridge CB4 0WG, UK

^f NC3Rs, Gibbs Building, 215 Euston Road, London NW1 2BE, UK

^g School of Informatics, University of Edinburgh, Appleton Tower, 10 Crichton Street, Edinburgh EH8 9LE, UK

*Corresponding author: AstraZeneca, Fleming Building, B623 Babraham Research Campus, Babraham, Cambridgeshire, CB22 3AT, Email: karen.tse@astrazeneca.com; Tel: +44 (0) 1625 234 578

Present addresses:

^Φ Sheffield Institute of Translational Neuroscience (SITraN), University of Sheffield, 385a Glossop Road, Sheffield S10 2HQ, UK

^Ψ DMPK, Charles River, The Robinson Building, Chesterford Research Park, Saffron Walden CB10 1XL, UK

^Υ The Milner Therapeutics Institute, University of Cambridge, Cambridge CB2 1QN, UK

Abstract

Background: The ActualHCA™ system continuously monitors the activity, temperature and behavior of group-housed rats without invasive surgery. The system was validated to detect the contrasting effects of sedative and stimulant test agents (chlorpromazine, clonidine and amphetamine), and compared with the modified Irwin test (mIT) with rectal temperature measurements.

Methods: Six male Han Wistar rats per group were used to assess each test agent and vehicle controls in separate ActualHCA™ recordings and mIT. The mIT was undertaken at 15, 30 mins, 1, 2, 4 and 24h post-dose. ActualHCA™ recorded continuously for 24h post-dose under 3 experimental conditions: dosed during light phase, dark phase, and light phase with a scheduled cage change at the time of peak effects determined by mIT.

Results: ActualHCA™ detected an increase stimulated activity from the cage change at 1-2h post-dose which was obliterated by chlorpromazine and clonidine. Amphetamine increased activity up to 4h post-dose in all conditions. Temperature from ActualHCA™ was affected by all test agents in all conditions. The mIT showed effects on all 3 test agents up to 4h post-dose, with maximal effects at 1-2h post-dose. The maximal effects on temperature from ActualHCA™ differed from mIT. Delayed effects on activity were detected by ActualHCA™, but not on mIT.

Conclusions: Continuous monitoring has the advantage of capturing effects over time that may be missed with manual tests using pre-determined time points. This automated behavioural system does not replace the need for conventional methods but could be implemented simultaneously to improve our understanding of behavioural pharmacology.

Keywords:

Behaviour; Continuous monitoring; Group-housed; Home cage; Irwin; Locomotion; Methods; Sedative; Stimulant; Temperature.

Abbreviations:

ActualHCA™ Actual Home Cage Analyzer

AUC	Area under the curve
CNS	Central nervous system
mIT	Modified Irwin test
RFID	Radiofrequency identification
%MPS	Percentage of maximum possible score

1. Introduction

Neurobehavioural assessments of drugs in rodents provide insights into pharmacological effects on the central nervous system (CNS) and are often conducted using manual measurements at pre-selected time points. Automated methods of monitoring locomotor activity and behavior in laboratory rats have been emerging that can continuously assess the responses to test agents (Alexandrov et al., 2015; Dunne et al., 2007; Van de Weerd et al., 2001). Automated systems have advantages over conventional manual observations that are often susceptible to observer bias, are of shorter duration and performed during the light phase only (Alexandrov et al., 2015; Dunne et al., 2007; Van de Weerd et al., 2001). However, both these and more conventional locomotor activity systems require the use of single-housing (Redfern et al., 2017). In contrast, modern laboratory practices have moved away from single-housing rats as it affects their behavior and welfare (Balcombe, 2006). Moreover, automated methods such as continuous temperature monitoring requires invasive surgical implantation of radiotelemetry transmitters (Ansah et al., 1996; Bishop et al., 2001; Deveney et al., 1998; Harkin et al., 2002; Ossenkopp et al., 1994). A recent approach that mitigates these concerns is the ActualHCA™ system (Actual Analytics, UK), which was developed as part of the “Rodent Big Brother project” funded by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), UK, to continuously monitor the activity and temperature of individual rats simultaneously when group-housed in their home cage environment on a cage rack, without the need for invasive surgery (Redfern et al., 2017).

The present study was conducted to validate the ActualHCA™ system by assessing its ability to detect changes in activity, temperature and behaviours in response to stimulant and sedative test agents, and to compare the results to the conventional manual approach: the modified Irwin test. The Irwin test is a comprehensive, systematic qualitative observational assessment that was introduced to evaluate the neurobehavioural effects of drugs on mice (Irwin, 1968). It has since been modified for use in rats and is currently used in safety pharmacology studies recommended by the International Conference on Harmonisation (ICH) S7A guideline for assessing

the effects of new chemical entities, to help protect clinical trials participants and patients from potential adverse effects (["International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use \(ICH\)," ; Redfern & Wakefield, 2006](#)). It is also used as a general observational test on rodents for assessing the neurobehavioural effects in disease models (Blokland et al., 1995; Hunter et al., 2000; Roux et al., 2004).

Pharmacological validation of the system was conducted using well-characterised sedatives (chlorpromazine and clonidine), and a stimulant (amphetamine). Chlorpromazine was originally introduced as a neuroleptic agent in humans, and causes reduced activity, ataxia and lowered body temperature in rats (Mattsson et al., 1996; Moscardo et al., 2007). Clonidine is a centrally-acting α_2 -adrenoreceptor agonist, which results in reduced activity and lowered body temperature in rodents (Drew et al., 1979; Drew et al., 1977; Ewart et al., 2013; Moscardo et al., 2007; Van der Laan & De Groot, 1988). Amphetamine is a CNS stimulant first synthesised in 1927 for the treatment of narcolepsy and mild depression (Heal et al., 2013). Since then it has been useful for the treatment of attention deficit hyperactivity disorder (Heal et al., 2013). Amphetamine works predominantly by increasing and sustaining the level of extracellular dopamine (Calipari & Ferris, 2013), and causes increase in locomotion, rearing and temperature in rats (Fog, 1970; Mattsson et al., 1996; Moscardo et al., 2007).

The objective of this study was to explore how the capability of this new technology compares to that of the conventional modified Irwin test as a means in detecting functional effects of test agents with known pharmacological effects on the CNS (Table 1). We also propose how the ActualHCA™ system could be integrated into the modified Irwin test to augment it.

2. Materials and methods

2.1. Drugs

Chlorpromazine hydrochloride was purchased from Sigma Aldrich, UK and was formulated with sterile water. The formulated chlorpromazine was stored in the dark at 4°C. Clonidine hydrochloride was purchased from Sigma Aldrich, UK and was formulated with sterile water. The formulated clonidine was stored in the dark at room temperature. D-Amphetamine sulfate salt was purchased from Tocris, UK and was formulated with sterile water. The formulated amphetamine was stored in the dark at room temperature.

2.2. Ethical statement

The sample size of $n=6$ per treatment group was selected to have sufficient power to detect the effects reported on the modified Irwin test (Ewart et al., 2013). The Irwin test is designed to identify rare event symptoms as such we infrequently observe these within the control data and therefore seeing multiple symptoms within the treatment is a significant effect. This design also gives a good sensitivity on subcutaneous temperature measured using ActualHCA™, which was achievable with $n=6$ because of the low variability of the data. Two tailed Student's T-test for temperature gives a power = 0.9 to detect a 1°C change with a variability of 0.48°C (Lenth, 2009). The activity measure using ActualHCA™ has lower sensitivity when time points are considered in isolation due to high variation, this means the screen can detect median to large-sized activity effects that are sustained over multiple time points. A total of 72 rats were used for the work described in this paper. All procedures were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 and associated guidelines, approved by institutional ethical review committees (Alderley Park Animal Welfare and Ethical Review Board; Babraham Institute Animal Welfare and Ethical Review Board) and conducted under the authority of the Project Licences (40/3368 and 70/8307, respectively). All animal facilities have been approved by the United Kingdom Home Office Licensing Authority and meet all current regulations and standards of the United Kingdom.

This manuscript has been prepared to meet the ARRIVE reporting guidelines (Kilkenny et al., 2010).

2.3. Animals

Twelve male Han Wistar (CrI:W1 (Han)) rats were used for the assessment of each drug and assessment methodology: with 6 animals in the control and 6 in the treatment group. Rats (weight range 200-275g; age range 7-13 weeks at the start of data acquisition) were purchased from Charles River Laboratories, Margate, UK with vendor-supplied health reports indicated that the rats were free of known viral, bacterial and parasitic pathogens. All rats were allocated to cages in 3s by body weight to balance the distribution of body weights between cages after arrival. Rats remained housed in their social group throughout the duration of the study. All rats were habituated for 7 days to the semi-barrier facility with temperature and relative humidity of +19°C to 23°C and 40% to 70%, respectively, prior to the start of any procedures. Welfare of each rat was assessed throughout by daily monitoring of appearance, behavior and cage environment. All rats had free access to food (RM1E, IRR 0.25 pelleted diet, Special Diet Services, UK) and water from the site drinking water supply. Standard cages (Tecniplast model number 2000P, Italy) measured approximately 61.2 x 43.5 cm of floor space, and individually-ventilated cages (Tecniplast model number 1500IU, Italy) measured approximately 59 x 38 cm of floor space. Both types of cages had 1-1.5 cm layer of 4 mm³ bedding material consisted of aspen wooden chips (Eco-Pure™ Aspen Chips 4), soft nesting material, ENVIRO-dri sizzle nest and Aspen chew blocks (Datesand, UK).

Naïve rats were used for the Irwin experiments prior to the start of dosing. Rats tested with the ActualHCA™ system were implanted in-house with a temperature-sensitive passive radiofrequency identification (RFID) transponder (BioTherm13, Biomark, USA) subcutaneously in the ventral abdominal midline at least 7 days prior to the start of dosing. For the Irwin experiments, the holding and experimental room light cycle were in the darkness from 18:00h to 06:00h, and lit from 06:00h to 18:00h. For the ActualHCA™ experiments, the holding and experimental room light cycle in the facility were in darkness from 19:00h to 07:00h, and lit from 07:00h to 19:00h.

For the Irwin experiments, each cage contained a cardboard tunnel as environmental enrichment. For the ActualHCA™ experiments, each cage contained a red translucent play tunnel in order for the camera to see through the tunnel, which appears clear under infrared light (Redfern et al., 2017). For each experiment, six rats were used per treatment group and a vehicle counterpart (sterile water) was used as control per test agent. A single oral gavage dose of 30 mg/kg chlorpromazine, or 0.3 mg/kg clonidine, or 10 mg/kg amphetamine, or their vehicle counterparts (10 mL/kg) was administered to the rats. The dose levels for chlorpromazine, clonidine and amphetamine were selected based on information derived from the literature, which showed extensive sedative and stimulant effects in neurobehavioural assessments (Ewart et al., 2013; Strang, 2005).

2.4. Modified Irwin tests

On the day of the experiments, rats were weighed and administered the appropriate drug treatment, and observed at 15, 30 minutes, 1, 2, 4 and 24 hours post-dose in sequential order based on the order of dosing. The observer was not blind to treatment regimes to prevent dosing errors. Blinding was not practically possible within our animal facility because the treatment groups are currently used as a point of identification on the cage cards, and there was only one operator. This operator, who undertakes Irwin assessments routinely, has no investment in the outcome of the experiment and did not analyse the data. Furthermore, the effects of the drugs were pronounced and readily identifiable. The Irwin parameters (Ewart et al., 2013; Irwin, 1968) were assessed systematically, starting with the home cage observation followed by the handling observation. The incidence of rats exhibiting symptoms was recorded. The observed symptoms for each rat were scored from 1 to 3 based on severity of the symptom, where '1' indicates mild, '2' indicates moderate and '3' indicates pronounced (see Supplementary Table S1). The observed symptoms for each treatment group at each observed time point were then presented as the dose group mean of the percentage of the maximal possible score (%MPS) [score/max score*100]. Rectal temperature was measured at 1, 4 and 24h post-dose for chlorpromazine and amphetamine; and at 1h post-dose for clonidine, using a

thermometer with a flexible temperature-sensitive probe (Physitemp thermalert model TH-8) inserted approximately 2 cm past the anal sphincter.

2.5. ActualHCA™ monitoring: microchip implantation

The transponders were purchased in pre-sterilised and pre-loaded single-use needle form. All rats were anaesthetised with isoflurane (induced at 4.5%, maintained at 3-4% with 2L oxygen) prior to the implantation. The ventral abdominal region was shaved and swabbed using antiseptic pre-surgical scrub. A small (<5mm) transverse incision was made through the skin using sterile scissors, to allow insertion of sterile blunt scissors to create a subcutaneous pocket using blunt dissection. A pre-sterilised trocar needle (pre-loaded with the transponder) was used to introduce the transponder using the plunger, in a rostrocaudal orientation. The wound was then sealed using topical tissue adhesive (GLUture®, Abbott Laboratories, UK). The rat remained under anaesthesia until the adhesive had dried. Rats were left to recover from 3-10 days post-implantation to ensure the transponder did not fall out before experimentation. Of 36 rats implanted in this study, the RFID transponder extruded externally from the implantation site in 1 rat, and was replaced. There were no other welfare issues associated with the implantation.

2.6. ActualHCA™ monitoring

The system can record temperature and horizontal (ambulatory) activity via the implanted RFID transponder, and the behavior from the side-on high definition infrared video camera (Redfern et al., 2017). For each test agent, 2 days' baseline data were collected prior to agent administration and data collection continued for 24 hours post-administration. An additional 3 days were allowed between treatments to ensure wash-out of the test agents (Table 2). For each test agent, a concurrent randomised design was used with 6 animals treated with vehicle and 6 animals treated with test agent. Through the continuous monitoring, all rats were assessed in 3 different experimental conditions: 'light phase' - dosed during the light phase (at approximately 09:00h); 'dark phase' - dosed during the dark phase (at approximately 20:00h); 'cage change' - dosed during the light phase followed by a cage change at the maximal effect of the drug determined from the Irwin test (chlorpromazine: 2h post-dose; clonidine: 1h post-dose; amphetamine: 2h post-

dose). Two cages (each containing 3 rats) were randomly allocated per treatment group based on the pre-dose mean ambulatory activity per cage (expressed as the number of transitions for that cage) measured over 24 h period, in order to balance the baseline activity levels for the vehicle and the drug-treated groups as much as possible. All rats were weighed on the day of dosing and dosed with the appropriate drug treatment.

Raw data of movement and subcutaneous temperature for individual rats were recorded via the transponder. Vertical activity was determined using the video footage and processed using ActualHCA™. Vertical activity is defined as any movement above a vertical threshold, set at 8 cm above the cage floor at the far wall of the cage (Redfern et al., 2017). Raw activity, temperature data and the video files were filtered to remove any artefactual noise (Redfern et al., 2017) and binned into 30-minute bins using ActualHCA™ (version 2.2.4.). Manual 'Irwin-style' behavioural assessment using the video footage recorded during the 'light phase' was performed *post hoc* at the same pre-determined time points as the conventional modified Irwin test that was conducted live in the laboratory in a separate group of animals. The 'light phase' was chosen for dosing regimen as this approach was most comparable to the conventional Irwin experiment. Additional delayed time points for the manual 'Irwin-style' behavioural assessments were also performed using the ActualHCA™ video, based on the activity profile detected by ActualHCA™. All home cage observed elements of the modified Irwin test were manually assessed using the video (see Supplementary Table S1), and only effects with abnormal findings were reported. The purpose was to confirm the delayed effects detected from the RFID transponder with behavioural assessment. The additional time points for chlorpromazine were at 01:00h, 02:00h and 03:00h (16, 17 and 18 hours post-dose, respectively); clonidine at 21:00h and 23:30h (12 and 14.5 hours post-dose); and amphetamine at 17:00h, 21:00h and 23:00h (8, 12 and 14 hours post-dose, respectively). The manual video assessment took place using the first 5 minutes of the 30-minute-binned video footage.

2.7. Statistical analysis

The data analysis treated each animal as the experimental unit. Effects in the Irwin test were considered significant if half or more of the rats in a given treatment group exhibited the symptom (e.g. $\geq n=3$ out of 6)(Ewart et al., 2013). This is appropriate for these symptoms because they are rare symptoms in the control data. In effect, the operator is comparing the treated group to the baseline historic data and the local controls are contributing to this data. For example, a power greater than 0.854 is obtained with a one sample test of proportions when the abnormality rate of interest is greater than 50% and the baseline abnormality rate is less than 7.5% (Lenth, 2009). Body temperature was analysed using a two-tailed student's t-test adjusted to control the false discovery rate to 5% for datasets for a variable and drug using the Benjamini and Hochberg methodology (Benjamini & Hochberg, 1995). For filtered activity, temperature, and vertical activity data, statistically significant differences were identified by comparing drug-treated groups with their time-matched vehicle counterparts using a two-tailed student's t-test. The multiple testing problem was managed by adjusting the p values to control the false discovery rate to 5% in each dataset which comprised of all the tests for a variable and drug using the Benjamini and Hochberg methodology (Benjamini & Hochberg, 1995). Area under the curve (AUC) was calculated for activity, temperature and vertical activity of all animals using the composite trapezoid rule for 0-24h post-dose. The AUC for drug-treated groups was then compared with their vehicle counterparts using a two-tailed student's t-test. P-values were adjusted to control the false discovery rate to 5% using the Benjamini and Hochberg methodology and those of less than 0.05 was considered significant (Benjamini & Hochberg, 1995).

2.8. Data availability

Data are freely available at the Zenodo repository
(<https://doi.org/10.5281/zenodo.1012739>)

3. Results

3.1. Modified Irwin test

In our study, the effects of chlorpromazine, clonidine and amphetamine were consistent with their known pharmacology (Ewart et al., 2013; Fog, 1970; Holman et al., 1971; Mattsson et al., 1996; Moscardo et al., 2007). The duration of effects for all 3 drugs were observed to be from 15 mins to 4 hours post-dose (Table 3). For chlorpromazine, the majority of and most pronounced behavioural effects were observed at 2 hours post-dose, with 7 significant findings and spontaneous activity decreased reaching up to 100% severity score (Table 3), and the mean maximum decrease in body temperature of 1.3°C occurred at 1 hour post-dose (Table 4). For clonidine, the majority of and most pronounced effects were observed at 1 hour post-dose, with 6 significant findings and spontaneous activity decreased reaching up to 83% severity score (Table 3), where the mean maximum decrease in body temperature of 2.1°C also occurred (Table 5). For amphetamine, the majority of and most pronounced effects were observed at 2 hours post-dose, with 5 significant findings and touch response increased reaching up to 83% severity score (Table 3), and the mean maximum increase in body temperature of 1°C occurred at 4 hours post-dose (Table 6).

3.2. Effects on activity detected by ActualHCA™

3.2.1. Chlorpromazine

The activity profile of chlorpromazine differed between the 3 experimental conditions. When dosed during the light phase, chlorpromazine caused a statistically significant reduction in ambulatory and vertical activities immediately post-dose compared to its time-matched vehicle group ($P < 0.05$; Figure 1A and B). However, a delayed reduction in ambulatory activity was more prominent at 01:00h and 02:00h overnight (16 and 17 hours post-dose, respectively)(Figure 1A and B). The AUC_{0-24h} overview measure highlighted statistically significant reduction in ambulatory activity ($P = 0.000047$) and in time spent in vertical activity ($P = 0.00014$) when chlorpromazine

was dosed during the light phase. When dosed in the dark phase, chlorpromazine caused a statistically significant delayed reduction in ambulatory and vertical activities at 10:00h the following morning (14 hours post-dose)(Figure 1C and D). The AUC_{0-24h} overview measure highlighted significant reduction in ambulatory activity ($P=0.013$) and in time spent in vertical activity ($P=0.012$) when chlorpromazine was dosed during the dark phase. When dosed during the light phase with a scheduled cage change at 2 hours post-dose, chlorpromazine caused a statistically significant reduction in stimulated ambulatory activity from 2 to 2.5 hours post-dose and in vertical activity at 1 and 2 hours post-dose (Figure 1E and F). The AUC_{0-24h} overview measure highlighted a statistically significant reduction in ambulatory activity ($P=0.00075$) but no significant effects on time spent in vertical activity ($P=0.60$) when chlorpromazine was dosed in the light phase with a scheduled cage change. In conclusion, the transient stimulation of activity caused by a cage change in the vehicle animals was prevented by chlorpromazine treatment, which is in agreement with the effects of chlorpromazine causing reduced activity (Moscardo et al., 2007).

3.2.2. Clonidine

The activity profile of clonidine also differed between the 3 experimental conditions. When dosed in the light phase, clonidine caused a statistically significant delayed increase in ambulatory activity at 06:00h and 07:00h the next day (21 and 22 hours post-dose) and delayed increase in vertical activity from 04:00h to 07:30h the next day (19 to 22.5 hours post-dose)($P<0.05$; Figure 2A and B). The AUC_{0-24h} measure highlighted no significant reduction in ambulatory activity ($P=0.97$), but the reduction was statistically significant on time spent in vertical activity ($P=0.003$) when clonidine was dosed during the light phase. This effect in the light phase was inconsistent with the pharmacology of clonidine from the literature as clonidine was shown to reduce activity in rats (Drew et al., 1979; Drew et al., 1977; Ewart et al., 2013). When dosed in the dark phase, clonidine caused no changes in ambulatory activity up to 24 hours post-dose (Figure 2C). However, clonidine caused a statistically significant reduction in time spent in vertical activity from 02:30h to 06:30h overnight (6.5 to 10.5 hours post-dose, respectively)($P<0.05$; Figure 2D). The AUC_{0-24h} measure highlighted no

significant reduction in ambulatory activity ($P=0.13$) but the reduction was statistically significant on time spent in vertical activity ($P=0.0017$) when clonidine was dosed in the dark phase. When dosed in the light phase with a scheduled cage change at 1 hour post-dose, clonidine caused a significant reduction in stimulated ambulatory activity from 1 to 1.5 hours post-dose, a delayed significant reduction in activity at 22:30h (13.5 hours post-dose) and a significant delayed increase in activity at 07:00h (22 hours post-dose) hours post-dose ($P<0.05$; Figure 2E). Clonidine also caused a significant reduction in stimulated time spent in vertical activity from immediately post-dose to 1.5 hours post-dose ($P<0.05$; Figure 2F). The AUC_{0-24h} measure highlighted significant reduction in ambulatory activity ($P=0.040$) and in time spent in vertical activity ($P=0.0016$) when clonidine was dosed during the light phase with a scheduled cage change. In summary, the transient stimulation of activity caused by a cage change in the vehicle animals was obliterated by clonidine treatment, which was in agreement with the effects of clonidine causing reduced activity in rats (Drew et al., 1979; Drew et al., 1977; Ewart et al., 2013).

3.2.3. Amphetamine

The effects of amphetamine in all 3 experimental conditions were consistent with its pharmacology from the literature (Fog, 1970; Mattsson et al., 1996; Moscardo et al., 2007). When dosed in the light phase, amphetamine caused a significant increase in ambulatory and vertical activities from immediately post-dose to 6.5 hours post-dose ($P<0.05$; Figure 3A and B). The AUC_{0-24h} measure highlighted a significant increase in ambulatory activity ($P=0.012$) and in time spent in vertical activity ($P=0.0015$) when amphetamine was dosed during the light phase. When dosed in the dark phase at 20:00h, amphetamine caused a significant increase in ambulatory and vertical activities from 0.5 to 8 hours post-dose ($P<0.05$; Figure 3C and D). The AUC_{0-24h} measure highlighted a significant increase in ambulatory activity ($P=0.0078$) but effects were not significant on time spent in vertical activity ($P=0.065$) when amphetamine was dosed in the dark phase. When dosed in the light phase with a scheduled cage change at 2 hours post-dose, amphetamine caused significantly increased stimulated ambulatory and vertical activities from 0.5 to 6.5 hours post-dose ($P<0.05$; Figure 3E and F). The AUC_{0-24h} measure highlighted significant increase in ambulatory activity ($P=0.00092$) and in time spent in vertical activity

($P=0.00069$) when amphetamine was dosed during the light phase with a scheduled cage change.

3.3. Effects on subcutaneous temperature detected by ActualHCA™

3.3.1. Chlorpromazine

The effects of chlorpromazine on subcutaneous temperature were consistent in all 3 experimental conditions and in concordance with its known pharmacology (Mattsson et al., 1996; Moscardo et al., 2007). The maximum decrease in temperature of 1.5°C when dosed during the light phase occurred 3.5 hours post-dose (Figure 4A), when dosed during the dark phase, a maximum decrease of 1.4°C occurred at 3 hours post-dose (Figure 4B), and when dosed during the light phase with scheduled cage change, a maximum decrease of 1.7°C occurred 2.5 hours post-dose (Figure 4C). The $\text{AUC}_{0-24\text{h}}$ measure highlighted statistically significant effects when dosed in the light phase ($P=0.016$), however, effects were not statistically significant when dosed in the dark phase ($P=0.14$) or dosed in the light phase with a scheduled cage change ($P=0.16$).

3.3.2. Clonidine

The effects of clonidine on subcutaneous temperature were consistent in all 3 experimental conditions and in concordance with its known pharmacology (Drew et al., 1979; Drew et al., 1977; Ewart et al., 2013; Moscardo et al., 2007). The maximum decrease in body temperature of 1.5°C when dosed during the light phase occurred at 2.5 hours post-dose (Figure 4D), when dosed during the dark phase, a maximum decrease of 2.7°C occurred at 4.5 hours post-dose (Figure 4E), and when dosed during the light phase with scheduled cage change, a maximum decrease of 2.9°C occurred at 1.5 hours post-dose (Figure 4F). The $\text{AUC}_{0-24\text{h}}$ measure was not statistically significant when dosed in the light phase ($P=0.46$), however, effects were statistically significant both when dosed in the dark phase ($P=0.0043$) and when dosed in the light phase with a scheduled cage change ($P=0.0017$).

3.3.3. Amphetamine

The effects of amphetamine on subcutaneous temperature were consistent in all 3 experimental conditions and in concordance with its known pharmacology (Fog, 1970; Mattsson et al., 1996; Moscardo et al., 2007). The maximum increase in temperature of 1.5°C when dosed during the light phase occurred at 2 hour post-dose (Figure 4G), when dosed during the dark phase, a maximum increase of 1.8°C occurred at 2 hours post-dose (Figure 4H), and when dosed during the light phase with a scheduled cage change, a maximum increase of 1.9°C occurred at 1.5 hours post-dose (Figure 4I). However, the AUC_{0-24h} measure was not statistically significant when dosed in the light phase (P=0.99), dosed in the dark phase (P=0.48) nor when dosed in the light phase with a scheduled cage change (P=0.10).

3.4. Manual behavioural assessment from ActualHCA™ video vs. conventional modified Irwin test

To validate the manual behavioural assessment collected from ActualHCA™ videos, an independent experiment undertaking the conventional home cage observation component of the modified Irwin test data was performed on control and treated animals. The effects of chlorpromazine and clonidine on decreased spontaneous activity were consistent between both methods. The effects of amphetamine on increased spontaneous activity, rearing and sniffing were also consistent between both methods (Tables 3, 4 and 5). An effect of chlorpromazine on abnormal gait was observed from the video footage, but not from the live test (Table 4). An effect of chlorpromazine on piloerection was observed in the live test, but not from the video footage (Table 4). Piloerection caused by amphetamine in the live test were observed on several time points, which were also more pronounced than what was seen with chlorpromazine. However, significant piloerection from the video footage was only observed at one time point, and was seen as less pronounced (Table 6). Since piloerection as a phenotype does not affect all animals exposed to chlorpromazine then the difference between the two studies could arise from a sampling effect or it could be due to level of resolution of the ActualHCA™ video.

3.5. Delayed behavioural findings from the ActualHCA™ monitoring

Three additional time points of 'Irwin-style' *post hoc* assessment for each test agent were performed using the video footage captured with ActualHCA™ during the 'light phase' dosing protocol. These time points were selected after evaluating the test agents on the 24h post-dose activity profile detected from the RFID transponder. At these additional time points, chlorpromazine was observed to cause significantly decreased spontaneous activity at 16 and 17 hours post-dose, in the video (Table 7), where the automated ambulatory activity was significant decreased compared to vehicle (Figure 1). Clonidine was observed to cause significantly increased spontaneous activity at 14.5 and 21 hours post-dose, in the video (Table 7), where the automated ambulatory activity showed decreasing trends at 12 and 14.5 hours post-dose, and significant increased activity at 21 hours post-dose (Figure 2). Amphetamine significantly decreased spontaneous activity at 12 and 14 hours post-dose, in the video (Table 7), where the automated ambulatory activity was significant decreased at 8, 12 and 14 hours post-dose (Figure 3).

4. Discussion

4.1. The added value of the ActualHCA™ system

The ActualHCA™ system improves automated detection compared to other systems through the ability to monitor group housed animals without the need for specialised housing or invasive surgery. The objective of this study was to validate the ActualHCA™ system pharmacologically with test agents and to compare the results with a manual qualitative observational assessment (modified Irwin test) that is widely used for assessing the neurobehavioural effects of new chemical entities. The pharmacological validation study showed that the ActualHCA™ system was able to detect effects on ambulatory and vertical activities, and subcutaneous temperature for the test agents chlorpromazine, clonidine and amphetamine. The observed findings were consistent with the known pharmacology of these test agents in rats (Fog, 1970; Heal et al., 2013; Mattsson et al., 1996; Moscardo et al., 2007; Van der Laan & De Groot, 1988). Their effects on activity and temperature, as detected using the modified Irwin test and ActualHCA™ were in agreement, despite using different cohorts of animals in independent experiments. These results show that the automated monitoring of the ActualHCA™ system can be used as an alternative method for assessing locomotor activity and temperature effects of new chemical entities.

Compared to an automated continuous monitoring method, a disadvantage of the conventional modified Irwin test is the use of pre-determined time points which can miss the maximum effect. For example, the effect of chlorpromazine on subcutaneous temperature using ActualHCA™ was consistent in all 3 experimental conditions, with maximum effects observed within 2.5 to 3.5 hours post-dose. However, from the live modified Irwin test, where body temperature measurements were set at 1, 4 and 24 hours post-dose, the maximum effect on temperature was observed at 1 hour post-dose. A further benefit of automated continuous monitoring is the ability to detect delayed effects. Any functional effects that do not directly correlate with the pharmacokinetics of a test agent (e.g. delayed effects) can be missed when using manual pre-determined time points. In 2 of the 3 drugs studied,

delayed effects were observed. For example with chlorpromazine, an effect on ambulatory activity was observed at 16 and 17 hours post-dose, whereas, based on the neurobehavioural profile of chlorpromazine using conventional manual observations, the effects on behavior would have been expected to have subsided by 8 hours post-dose (Moscardo et al., 2007). Any compensatory or rebound effects following the initial response to test agents may also be missed with pre-determined time points for manual observation based on the pharmacokinetic profile. This was demonstrated with clonidine and amphetamine, where delayed reverse effects of their pharmacology were observed. The rebound effect of clonidine has previously been demonstrated following 3 daily doses on locomotor activity (Van der Laan & De Groot, 1988).

Most routine behavioural observations and measurements in rodents are conducted during the light phase for practical reasons. However, as rats are nocturnal, sedative effects may not be detected during the light phase. A scheduled cage change was included within the experiment to introduce a novel environment which would transiently increase exploratory activity. The rationale for doing this was that this might provide a brief window to detect sedative effects during the light phase. This strategy was successful, as demonstrated with 2 sedative agents used in this study. The ActualHCA™ system can acquire data during the dark phase where animals are naturally active. however, dosing prior to a transient stimulation has benefits. This is analogous to a conventional assessment of locomotor activity in a novel arena, except that rats are group-housed with their cagemates. The data from the scheduled cage change will be comparable with other studies, which are frequently performed during the light phase. Furthermore, drug metabolism has been shown to differ depending on the time of day (Gachon & Firsov, 2011; Radzialowski & Bousquet, 1968), therefore dosing during the dark phase can potentially alter the pharmacokinetic profile of the test agent.

Another advantage of continuous measurements of body temperature is the reduction of stress leading to a more representative measure. Body temperature measurements for the modified Irwin test require handling the animals and inserting

a rectal probe at each pre-determined time point. This method limits the number of body temperature measurements performed each day. The body temperature of animals also increases due to the stress of the handling procedure (Balcombe et al., 2004; Clement et al., 1989; Dilsaver et al., 1992; Eikelboom, 1986; Moe & Bakken, 1997). Although, the ActualHCA™ measures subcutaneous temperature rather than core temperature, the rectal temperature used at the modified Irwin test is also not equivalent to core temperature (Dilsaver et al., 1992). Mean rectal temperature in the rat ranges from 30-38°C with higher temperatures detected the further the probe pasts the anal sphincter (Lomax, 1966). Mean core temperature from the rat ranges from 36.5 to 38°C, depending on the time of day (Gordon et al., 2002). Subcutaneous temperature measured using the ActualHCA™ in the rat ranges from 35.1 to 37.2°C, with a mean subcutaneous temperature of 36.0°C ± 0.05 (mean ± 95% confidence interval) during the light phase, and 36.2°C ± 0.07 (mean ± 95% confidence interval) during the dark phase (Redfern et al., 2017). Rectal temperature can reach the range of core temperature when the probe reaches beyond 4 cm past the anal sphincter (Lomax, 1966). However, the temperature probe for modified Irwin test was inserted 2 cm past the anal sphincter, therefore the temperature measured would be lower than core temperature. To measure core temperature continuously would require invasive surgical implantation of an intraperitoneal telemetry device (Gordon et al., 2002).

Maximum benefit from the system can be obtained by combining the continuous home cage monitoring with the modified Irwin test. The video footage enables *post hoc* assessments of the observational 'non-interactive' elements of the modified Irwin test. The elements that require handling e.g. traction response, touch response, or those that require close observations e.g. pupil size cannot be assessed. The *post hoc* assessment can be implemented wherever the user feels is most appropriate, for example at the compound's T_{max} (see Supplementary Figure S1). The use of an automated behavioural system does not directly replace the need for conventional approaches, but can work in synergy to provide non-subjective, additional measurements that are not achievable through manual observations, including measurements and observations during the dark phase in undisturbed animals. Therefore, the ActualHCA™ system could be combined both with *post hoc* Irwin

home cage observations on demand and a planned conventional modified Irwin test at the Tmax. From our observations on the benefits of a cage change to present a transient novel environment, a conventional modified Irwin test could be combined with this event in the light phase as our data clearly demonstrated that this would reveal the effects of sedative agents without obscuring the effect of a stimulant.

Obviously the technology has numerous other potential applications besides integration into the modified Irwin test. Various authors have encouraged the inclusion of behavioural endpoints in rodent repeat-dose toxicology studies over the last three decades (Evans, 1989; Luft & Bode, 2002; Redfern, 2015; Redfern et al., 2013; Zbinden, 1984). Deployment of ActualHCA™ in early repeat-dose toxicology studies in rats would provide valuable additional data on adverse effects of new molecular entities on activity, behavior and temperature, as we have demonstrated with these three reference drugs after a single dose. Such effects can increase, diminish or remain the same on repeated dosing (Redfern et al., 2013). Another obvious application in the field of CNS safety assessment is in the detection of drug dependence. Typically, these studies involve repeat dosing of rodents daily for (say) 3 weeks, followed by cessation of dosing. The animals are then monitored using a combination of manual and automated approaches to look for signs of physiological and behavioural withdrawal phenomena (Anon, 2006, 2017; Balster, 1991; Porsolt et al., 2002; Swedberg, 2013). Depending on the pharmacological class, these withdrawal syndromes commonly involve changes in horizontal and vertical activity, and temperature (Ohmura et al., 2011). Furthermore, ActualHCA™ has potential applications across the entire spectrum of behavioural neuroscience and drug discovery: activity/behavioural phenotyping of different rat strains and transgenic animals (e.g. (McDermott & Kelly, 2008)); circadian biology (e.g., (De La Iglesia et al., 2008)); fever studies (e.g. (de Melo Soares et al., 2017)); neurological and other disease models (e.g. (Percie du Sert et al., 2017)). In the field of animal husbandry and welfare it would provide the opportunity for stringent, objective, undisturbed evaluation of preference for different bedding, caging and environmental enrichment etc. (Balcombe, 2006). It would also enable early identification of individual animals at risk in repeat-dose toxicity studies, disease models, etc., as they would be

expected to exhibit hypoactivity and hypothermia (Gordon, 1991; Percie du Sert et al., 2017).

4.2. Considering clinical translation

The purpose of an Irwin test prior to clinical trials (Phase I) is to protect healthy volunteers and patients from harmful effects of candidate drugs on the nervous system. A recent analysis of the predictive value of the Irwin test was undertaken on 141 candidate drugs that had proceeded to be tested in Phase I single ascending dose studies (Mead et al., 2016). This translational analysis was limited to common adverse effects, and found that these were poorly predicted from the Irwin parameters, included amongst these were somnolence and fatigue. A possible impediment to translation is the observation that there are inter-laboratory differences in outcomes from the Irwin test (Ewart et al., 2013). These can arise from strain, age or batch differences between rats, inter-observer differences in interpretation/assessment/scoring of behaviours, or from operational differences affecting baseline activity of the rats (Moscardo et al., 2007). Operational differences include the level of prior handling of the animals, lighting levels and background noise, the time allowed from moving animal racks to the area where the Irwin test will be conducted before commencing the procedures, the time from removal of a cage from the rack to commencement of the observations – and even the time from opening the cage lid to the observations (Ewart et al., 2013). Automated detection of effects on activity (which are plausibly associated with somnolence or fatigue), and extension of manual Irwin observations beyond the conventional working day and into the animal's active dark phase, could contribute to improving the translation between preclinical assessment of CNS effects in rodents and CNS safety outcomes in Phase I. Using ActualHCA™, this could be achieved without additional 'observer resources'.

4.3. Summary

This study has demonstrated that ActualHCA™ can detect pharmacological changes in activity, temperature and behavior from two sedatives and a stimulant. Compared

with the modified Irwin test, ActualHCA™ can consistently detect changes as seen in the modified Irwin test, albeit those limited to ‘non-interactive’ elements of this test. Furthermore, ActualHCA™ can provide advantages from the longer duration of continuous automated measurements which can highlight additional behavioural effects of the test agents to those achievable using conventional approaches. Using automated home cage behavioural monitoring of group-housed rats can help improve our understanding of the pharmacological effects of test agents on the CNS.

Conflict of interest statement

The authors RS and JDA are employed by or were shareholders in Actual Analytics Ltd at the time the research was performed and therefore declare a competing financial interest. ActualHCA™ is commercially available from Actual Analytics Ltd. For the authors associated with AstraZeneca and NC3Rs, there are no development or marketed products to declare.

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Figures and Tables for “Pharmacological validation of individual animal locomotion, temperature and behavioural analysis in group-housed rats using a novel automated home cage analysis system: a comparison with the modified Irwin test”

Test agent	Animal cohort	ActualHCA™ system	Animal cohort	Modified Irwin test
Chlorpromazine	1	Dosed and assessed on 3 different experimental conditions: 'Light phase', 'dark phase' & 'cage change'	4	Dosed and assessed conventionally
Clonidine	2	Dosed and assessed on 3 different experimental conditions: 'Light phase', 'dark phase' & 'cage change'	5	Dosed and assessed conventionally
Amphetamine	3	Dosed and assessed on 3 different experimental conditions: 'Light phase', 'dark phase' & 'cage change'	6	Dosed and assessed conventionally

Table 1. A study design table, illustrating the different experimental objectives, tested by ActualHCA™ and the conventional modified Irwin test

A table illustrating the study design and the cohort of animals used for each experiment and each test agent. The objective was to compare the capability of ActualHCA™ vs. the conventional modified Irwin test, in detecting functional effects of test agents with known pharmacological effects on the CNS in group-housed rats.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week -3			Arrival	Habituate	Habituate	Habituate	Habituate
Week -2	Habituate	Habituate	Transponder implantation:	Recovery	Recovery	Recovery	Recovery
Week -1*	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery
Week 1	Pre-dose (Day -2)	Pre-dose randomisation (Day -1)	Day 1 dosing – condition 1 (light phase)	Day 2	Washout	Washout	Washout
Week 2	Pre-dose (Day -2)	Pre-dose Randomisation (Day -1) dosing after lights off (dark phase)	Day 1 condition 2 (dark phase)	Day 2	Washout	Washout	Washout
Week 3	Pre-dose (Day -2)	Pre-dose Randomisation (Day -1)	Day 1 dosing – condition 3 (T_{max} cage change)	Day 2	Washout	Washout	Washout

Table 2. A study timing table, illustrating the timelines of the different experimental conditions tested using ActualHCA™ per test agent. A table illustrating the timelines from animals arrival to radiofrequency identification (RFID) transponder implantation, and dosing of the test agent for each experimental condition. *Additional week for recovery was only required when the RFID transponder extruded externally from the implantation site and required replacement.

Group	Symptoms observed	15 min	30 min	1h	2h	4h	24h
Vehicle for chlorpromazine	No symptoms	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Chlorpromazine (30 mg/kg orally)	Spontaneous activity decreased	17% (3)	56% (6)	78% (6)	100% (6)	56% (5)	0% (0)
	Piloerection	0% (0)	0% (0)	17% (2)	33% (6)	39% (5)	0% (0)
	Touch response decreased	0% (0)	17% (2)	72% (6)	50% (4)	83% (6)	0% (0)
	Body tonus decreased	0% (0)	50% (5)	50% (5)	22% (3)	33% (5)	0% (0)
	Traction response decreased	0% (0)	6% (1)	28% (3)	6% (1)	11% (1)	6% (1)
	Sedation	0% (0)	11% (1)	56% (6)	56% (6)	56% (6)	0% (0)
	Pinna reflex decreased	0% (0)	11% (1)	11% (1)	33% (3)	11% (1)	11% (1)
	Ptosis	0% (0)	17% (2)	56% (6)	50% (5)	44% (6)	0% (0)
	Abnormal urination (increased)	0% (0)	0% (0)	0% (0)	0% (0)	22% (4)	0% (0)
Vehicle for clonidine	Traction response decreased	22% (2)	22% (2)	44% (4)	39% (4)	33% (3)	11% (1)
Clonidine (0.3 mg/kg orally)	Spontaneous activity decreased	50% (6)	61% (6)	83% (6)	83% (6)	67% (6)	0% (0)
	Flat body posture	22% (1)	33% (3)	33% (3)	11% (1)	0% (0)	0% (0)
	Piloerection	17% (2)	17% (3)	28% (3)	56% (5)	22% (3)	0% (0)
	Touch response decreased	0% (0)	39% (4)	6% (1)	11% (1)	22% (3)	0% (0)
	Traction response decreased	44% (3)	22% (2)	28% (3)	50% (4)	44% (3)	6% (1)
	Body tonus decreased	17% (2)	17% (3)	6% (1)	6% (1)	11% (1)	0% (0)

	Ptosis	0% (0)	22% (1)	28% (3)	33% (3)	44% (4)	0% (0)
	Absence of blink reflex	67% (6)	56% (5)	56% (5)	11% (1)	33% (3)	0% (0)
	Abnormal respiration	67% (6)	67% (6)	56% (5)	67% (6)	61% (6)	0% (0)
Vehicle for amphetamine	No symptoms	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Amphetamine (10 mg/kg orally)	Spontaneous activity increased	50% (6)	83% (6)	67% (6)	67% (6)	67% (6)	0% (0)
	Rearing increased	17% (3)	33% (3)	67% (6)	67% (6)	67% (6)	0% (0)
	Sniffing increased	17% (3)	17% (3)	17% (3)	17% (3)	50% (6)	0% (0)
	Piloerection	33% (3)	50% (6)	33% (3)	50% (6)	22% (4)	0% (0)
	Touch response increased	0% (0)	50% (6)	72% (6)	83% (6)	44% (4)	0% (0)
	Traction response decreased	22% (3)	17% (1)	0% (0)	17% (1)	6% (1)	6% (1)
	Pinna reflex decreased	11% (1)	0% (0)	44% (4)	22% (2)	33% (3)	0% (0)

Table 3: The neurobehavioural effects from the modified Irwin test for chlorpromazine, clonidine and amphetamine exposure in rats.

The table shows the percentage of maximum possible score (%MPS) and the number of animals with the observation in brackets for symptoms where 1 or more animals after oral administration of the respective compounds (n=6 per group per compound) exhibit the symptom. No effect is scored as 0. Data in **bold** are considered to be a significant effect in the modified Irwin test observation, whereby they have been observed in animals in the test agent groups with $\geq n=3$ of the same symptom observed in their respective vehicle groups. The observed symptoms for each rat were scored from 1 to 3 based on severity of the symptom. The observed symptoms for each treatment group at each observed time point were then presented as the dose group mean of the %MPS [score/max score*100]. The clonidine data presented were also published in Ewart et al. 2013.

Observed time point	Abnormal gait	Abnormal respiration	Pilo-erection	Spontaneous activity decreased	Temperature: Mean difference (95%CI)
<u>Modified Irwin Test data</u>					<u>Rectal probe</u>
15 min	0% (0)	0% (0)	0% (0)	17% (3)	
30 min	0% (0)	0% (0)	0% (0)	56% (6)	
1h	0% (0)	0% (0)	17% (2)	78% (6)	-1.3°C (-1.04 to -1.53)
2h	0% (0)	0% (0)	33% (6)	100% (6)	
4h	0% (0)	0% (0)	39% (5)	56% (5)	-1.0°C (-0.43 to -1.54)
24h	0% (0)	0% (0)	0% (0)	0% (0)	+0.7°C (0.23 to 1.17)
<u>Home Cage observation from ActualHCA™</u>					<u>RFID transponder</u>
15 min	11% (2)	0% (0)	0% (0)	0% (0)	
30 min	28% (5)	6% (1)	0% (0)	56% (5)	-0.7°C (-0.15 to -1.20)
1h	22% (4)	0% (0)	0% (0)	67% (6)	-0.6°C (-0.001 to -1.23)
2h	0% (0)	6% (1)	0% (0)	67% (6)	-0.6°C (-0.17 to -1.38)
4h	0% (0)	0% (0)	0% (0)	56% (5)	-1.1°C (-0.34 to -1.92)
24h	0% (0)	0% (0)	0% (0)	0% (0)	+0.3°C (-0.07 to 0.70)

Table 4: Comparison of the parameters observed in the home cage using both the modified Irwin test and from ActualHCA™, following the administration of chlorpromazine or vehicle in rats.

The observed behavioural effects for each rat were scored from 1 to 3 based on severity of the symptom (n=6/group). No effect is scored as 0. The observed behavioural effects for each method at each observed timepoint are presented as the dose group mean of the percentage of maximum possible severity score [score/max score*100], and the number of rats exhibited the behavioural effect in brackets. Behavioural effects are considered significant and highlighted in **bold**, in the modified Irwin test or ActualHCA™, whereby they have been observed in the animals in the test agent groups with $\geq n=3$ with the same symptoms observed in their respective vehicle groups. None of the symptoms listed were observed with the vehicle groups. The effect of the drug on temperature is presented as the mean difference between vehicle and chlorpromazine groups, with the 95% confidence interval (CI) in brackets. Effects on temperature were analysed using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. Blank cells indicate the assessment was not performed at that timepoint, therefore data are not available.

Observed time point	Abnormal gait	Spontaneous activity decreased	Temperature: Mean difference (95%CI)
Modified Irwin Test data			Rectal probe
15 min	0% (0)	50% (6)	
30 min	0% (0)	61% (6)	
1h	0% (0)	83% (6)	-2.1°C (-1.44 to -2.82)
2h	0% (0)	83% (6)	
4h	0% (0)	67% (6)	
24h	0% (0)	0% (0)	
Home Cage observation from ActualHCA™			RFID transponder
15 min	11% (2)	44% (6)	
30 min	0% (0)	67% (6)	-0.7°C (-0.08 to -1.30)
1h	0% (0)	50% (6)	-0.9°C (-0.21 to -1.68)
2h	0% (0)	0% (0)	-1.0°C (-0.53 to -1.49)
4h	0% (0)	50% (6)	-0.5°C (-1.07 to 0.11)
24h	0% (0)	0% (0)	-0.1°C (-0.73 to 0.60)

Table 5: Comparison of the parameters observed in the home cage using both the modified Irwin test and from ActualHCA™, following the administration of clonidine or vehicle in rats.

The observed behavioural effects for each rat were scored from 1 to 3 based on severity of the symptom (n=6/group). No effect is scored as 0. The observed behavioural effects for each method at each observed timepoint are presented as the dose group mean of the percentage of maximum possible severity score [score/max score*100], and the number of rats exhibited the behavioural effect in brackets. Behavioural effects are considered significant and highlighted in **bold**, in the modified Irwin test or ActualHCA™, whereby they have been observed in the animals in the test agent groups with $\geq n=3$ with the same symptoms observed in their respective vehicle groups. None of the symptoms listed were observed with the vehicle groups. The effect on temperature is presented as the mean difference between vehicle and chlorpromazine groups, with the 95% confidence interval (CI) in brackets. Effects on temperature were analysed using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. Blank cells indicate the assessment was not performed at that timepoint, therefore data are not available.

Observed time point	Pilo-erection	Rearing increased	Sniffing increased	Spontaneous activity increased	Temperature: Mean difference (95%CI)
Modified Irwin Test data					Rectal probe
15 min	33% (3)	17% (3)	17% (3)	50% (6)	
30 min	50% (6)	33% (3)	17% (3)	83% (6)	
1h	33% (3)	67% (6)	17% (3)	67% (6)	+0.7°C (0.23 to 1.17)
2h	50% (6)	67% (6)	17% (3)	67% (6)	
4h	22% (4)	67% (6)	50% (6)	67% (6)	+1.0°C (0.59 to 1.34)
24h	0% (0)	0% (0)	0% (0)	0% (0)	-0.3°C (-0.67 to 0.19)
Home Cage observation from RBB video footage					RFID transponder
15 min	0% (0)	33% (6)	67% (6)	67% (6)	
30 min	17% (3)	33% (6)	67% (6)	67% (6)	+0.9°C (0.26 to 1.59)
1h	6% (1)	22% (4)	67% (6)	67% (6)	+1.4°C (0.74 to 2.10)
2h	0% (0)	17% (3)	28% (5)	67% (6)	+1.4°C (0.39 to 2.40)
4h	0% (0)	11% (2)	28% (5)	61% (6)	+0.7°C (-0.36 to 1.74)
24h	0% (0)	0% (0)	0% (0)	0% (0)	+0.1°C (-0.59 to 0.81)

Table 6: Comparison of the parameters observed in the home cage using both the modified Irwin test and from ActualHCA™, following the administration of amphetamine or vehicle in rats.

The observed behavioural effects for each rat were scored from 1 to 3 based on severity of the symptom (n=6/group). No effect is scored as 0. The observed behavioural effects for each method at each observed timepoint are presented as the dose group mean of the percentage of maximum possible severity score [score/max score*100], and the number of rats exhibited the behavioural effect in brackets. Behavioural effects are considered significant and highlighted in **bold**, in the modified Irwin test or ActualHCA™, whereby they have been observed in the animals in the test agent groups with $\geq n=3$ with the same symptoms observed in their respective vehicle groups. None of the symptoms listed were observed with the vehicle groups. The effect of the drug on temperature is presented as the mean difference between vehicle and chlorpromazine groups, with the 95% confidence interval (CI) in brackets. Effects on temperature were analysed using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. Blank cells indicate the assessment was not performed at that timepoint, therefore data are not available.

Figure 1. The effects of chlorpromazine compared with vehicle, on activity in rats.

Male Han Wistar rats ($n=6/\text{group}$) were dosed orally with chlorpromazine at 30 mg/kg, or vehicle (10 mL/kg). Ambulatory activity, expressed as the number of transitions (left panel), and in time spent rearing, expressed in seconds (right panel) were recorded for 24h from immediately post-dose using ActualHCA™. A) + B); dosing occurred during the light phase at approximately 09:00h. C) + D); dosing occurred during the dark phase at approximately 20:00h. E) + F); dosing occurred during the light phase at approximately 09:00h, with a scheduled cage-change at 11:00h. At each time point the treatment data were compared to vehicle using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 2. The effects of clonidine compared with vehicle, on activity in rats.

Male Han Wistar rats (n=6/group) were dosed orally with clonidine at 0.3 mg/kg, or vehicle (10 mL/kg). Ambulatory activity, expressed as the number of transitions (left panel), and in time spent rearing, expressed in seconds (right panel) were recorded for 24h from immediately post-dose using ActualHCA™. A) + B); dosing occurred during the light phase at approximately 09:00h. C) + D); dosing occurred during the dark phase at approximately 20:00h. E) + F); dosing occurred during the light phase at approximately 09:00h, with a scheduled cage-change at 10:00h. At each time point the treatment data were compared to vehicle using a student's t-test with multiple testing adjusted to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. *P<0.05; **P<0.01.

Figure 3. The effects of amphetamine compared with vehicle, on activity in rats.

Male Han Wistar rats ($n=6/\text{group}$) were dosed orally with amphetamine at 10 mg/kg, or vehicle (10 mL/kg). Ambulatory activity, expressed as the number of transitions (left panel), and in time spent rearing, expressed in seconds (right panel) were recorded for 24h from immediately post-dose using ActualHCA™. A) + B); dosing occurred during the light phase at approximately 09:00h. C) + D); dosing occurred during the dark phase at approximately 20:00h. E) + F); dosing occurred during the light phase at approximately 09:00h, with a scheduled cage-change at 11:00h. At each timepoint the treatment data were compared to vehicle using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 4. The effects of chlorpromazine, clonidine and amphetamine compared with vehicle, on temperature in rats.

Male Han Wistar rats ($n=6/\text{group}$) were dosed orally with chlorpromazine at 30 mg/kg (A, B, C), clonidine at 0.3 mg/kg (D, E, F), amphetamine at 10 mg/kg (G, H, I), or vehicle (10 mL/kg) and the subcutaneous temperature monitored. The left panel (A, D, G): dosing during the light phase at approximately 09:00h. The middle panel (B, E, H): dosing during the dark phase at approximately 20:00h. The right panel (C, F, I): dosing during the light phase at approximately 09:00h, with a scheduled cage-change at 10:00h for clonidine, and 11:00h for chlorpromazine and amphetamine. At each timepoint the treatment data were compared to vehicle using a student's t-test with multiple testing adjustment to control the false discovery rate to 5% within each datasets for a variable and drug using the Benjamini and Hochberg methodology.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Chlorpromazine	Timepoints (post-dose)		
	16h	17h	18h
Spontaneous activity decreased	56% (5)	28% (3)	6% (1)
Spontaneous activity increased	0% (0)	0% (0)	0% (0)
Clonidine	Timepoints (post-dose)		
	12h	14.5h	21h
Spontaneous activity decreased	0% (0)	0% (0)	0% (0)
Spontaneous activity increased	0% (0)	17% (3)	50% (6)
Amphetamine	Timepoints (post-dose)		
	8h	12h	14h
Spontaneous activity decreased	11% (2)	17% (3)	22% (3)
Spontaneous activity increased	0% (0)	0% (0)	0% (0)

Table 7. Results from the additional modified Irwin test conducted from ActualHCA™ video at 3 delayed time points for chlorpromazine, clonidine and amphetamine in rats.

The observed behavioural effects for each rat were scored from 1 to 3 based on severity of the symptom (n=6/group). No effect is scored as 0. The observed behavioural effects for each method at each observed timepoint are presented as the dose group mean of the percentage of maximum possible severity score [score/max score*100], and the number of rats exhibited the behavioural effect in brackets. Behavioural effects are considered significant and highlighted in **bold**, whereby they have been observed in the animals in the test agent groups with $\geq n=3$ with the same symptoms observed in their respective vehicle groups. None of the symptoms listed were observed with the vehicle groups.

5. References

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