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Animal-friendly methods for rodent behavioral testing in neuroscience research

Edited by
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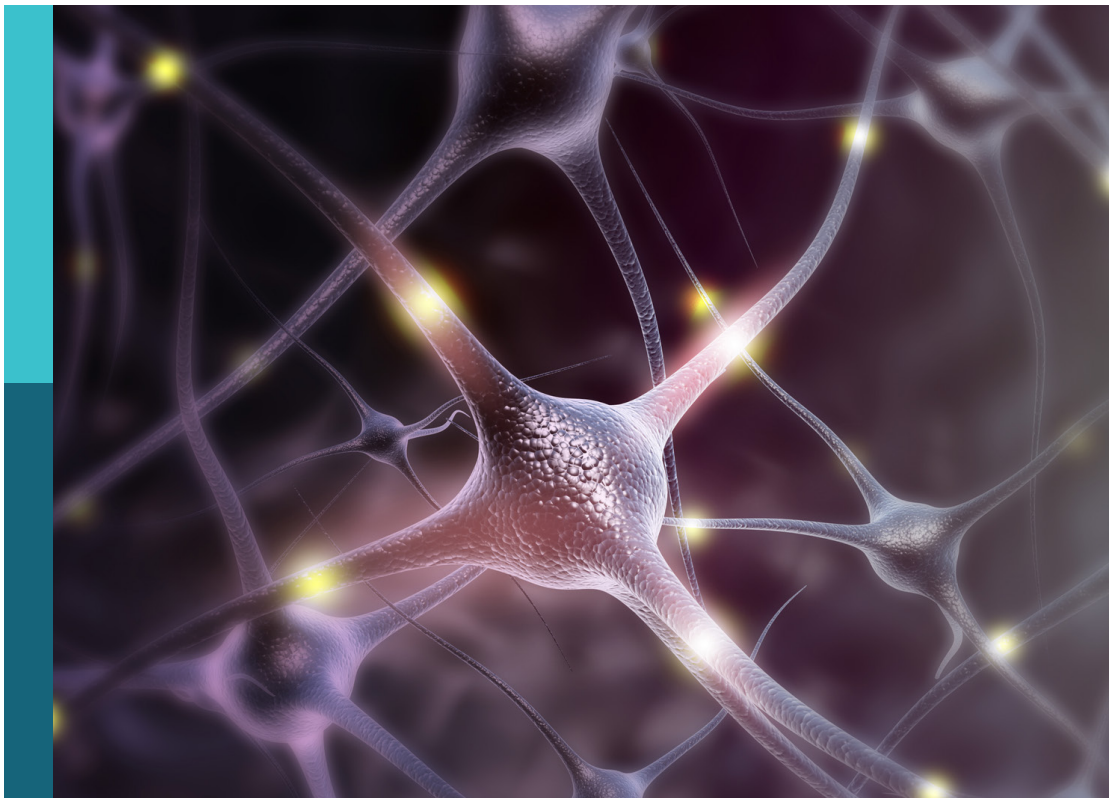

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Animal-friendly methods for rodent behavioral testing in neuroscience research

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Editorial: Animal-friendly methods for rodent behavioral testing in neuroscience research

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Editorial on the Research Topic
Animal-friendly methods for rodent behavioral testing in neuroscience research

Background

Rodents have been employed in modern scientific research from the 17th century. However, in the early period of experimental science, scientists often had scarce attention for animal welfare. For instance, in 1659 Irish-English chemist Robert Boyle (1627–1691) performed suffocation tests on mice placed in extremely rarefied air and measured the time the animals took to die without air to breathe (Boyle, 1660). On the other hand, Italian biologist Francesco Redi (1626–1697) performed terminal starvation tests on both domestic mice and field mice to discover how much time they could survive without food, finding that both species were dead within 3 days (Redi, 1684). In the following century, rodents were employed mainly in lethal experiments, for example the toxicological studies of the Italian naturalist Felice Fontana (1730–1805), director of the Museum of Natural History of Florence from 1775, who used guinea pigs to assess the effects of inflammable air (Fontana, 1779), curare venom (Fontana, 1780, 1781, 1787), cherry-laurel poison (Fontana, 1781, 1787) and viper venom (Fontana, 1781, 1787). While toxicological studies were driven by a practical utility, other studies of that time, by inflicting pointless suffering, appear as merely cruel.

Compared to other non-human animals such as dogs and cats, it has been relatively more difficult for humans to empathize and sympathize with rodents such as mice and rats, probably also because they have often been viewed as pest animals infesting urban environments or damaging orchards, agricultural cultivations and cereal deposits (Stenseth et al., 2003), and because they were perceived as "lower" animals. Indeed, the idea that rodents are cognitively inferior animals could be one of the reasons for which the welfare of laboratory rodents has often been overlooked, especially in the past centuries of scientific research. Importantly, the cognitive limitedness of rodents has been challenged by the neuroscientific and psychological investigations of the past few decades, which have revealed increasingly complex cognitive, emotional and social skills for these animals

(Langford et al., 2006; Miller, 2006; Rutte and Taborsky, 2007, 2008; Viana et al., 2010; Ben-Ami Bartal et al., 2011; Dolivo et al., 2016; Zentall, 2016; Schweinfurth and Taborsky, 2018a,b; Sivaselvachandran et al., 2018; Ueno et al., 2018; Mogil, 2019; Reinhold et al., 2019; Templer, 2019; Cox and Reichel, 2020; Venniro and Golden, 2020; Joo et al., 2021; Kim et al., 2021; Rutishauser, 2021; Hernandez-Lallement et al., 2022; Engelhardt and Taborsky, 2023; Keysers and Gazzola, 2023; Misiołek et al., 2023; Yu et al., 2024).

However, it is important to underline that the criterion for the right for animal welfare should not be the cognitive level of a species, but rather its ability to feel. In the words of the British philosopher Jeremy Bentham (1748–1832), one of the first to criticize specist prejudices and the adoption of intelligence as criterion to decide whether a given species deserves welfare concerns: "The French have already discovered that the blackness of skin is no reason why a human being should be abandoned without redress to the caprice of a tormentor. It may come one day to be recognized, that the number of legs, the villosity of the skin, or the termination of the os sacrum, are reasons equally insufficient for abandoning a sensitive being to the same fate. What else is it that should trace the insuperable line? Is it the faculty of reason, or perhaps, the faculty for discourse? [...] the question is not, Can they reason? nor, Can they talk? but, Can they suffer?" (Bentham, 1789). Actually, as argued by philosopher Sahar Akhtar in *The Oxford Handbook on Ethics and Animals*, for non-human animals pain may be even worse than for humans, as non-human animals lack the possibility to rationalize about the causes of their pain, or to imagine a future in which the pain has ceased (Akhtar, 2011). As eloquently expressed by the American bioethicist Bernard E. Rollin (1943–2021): "If they are in pain, their whole universe is pain; there is no horizon; they are their pain" (Rollin, 1999).

Behavioral studies on rodents kept in a controlled environment (i.e., not in nature) began to be carried out much later than physiological studies. Indeed, behavioral studies on captive rodents were first performed in 1822 (Moss, 1836) and became more common from the 1870s (Jillson, 1871; Lockwood, 1871; Tenney, 1872; Perkins, 1873; King, 1883; Stephens, 1887; Davis, 1889). Nevertheless, these first behavioral studies were merely observational. Behavioral testing of rodents started instead in the 1890s (Stewart, 1894, 1898; Lombard, 1895; Mills, 1895, 1898; Kline, 1899a,b; Small, 1899).

Rodent behavioral testing has since then been employed in an increasingly growing number of studies to investigate brain functions, and has become a gold-standard method in modern neuroscience. With the study of behavior, came also a greater attention for the welfare of laboratory rodents, both because their cognitive abilities were better understood and because of the awareness that affecting the welfare of the animals could impact the scientific results of behavioral studies. As noted by Small, one of the pioneers of rodent behavioral testing: "the experiments must conform to the psycho-biological character of an animal if sane results are to be obtained" (Small, 1901). Nevertheless, the first behavioral methods developed for laboratory rodents often still had a great margin of improvement for optimization of animal welfare.

Indeed, the vast majority of rodent behavioral tests designed up to the 1950s was based on punishments and rewards. Unfortunately, both these approaches can lead to a certain degree of animal pain or suffering. Punishments required the employment of painful stimuli, typically electric shocks. Tests as passive avoidance and fear conditioning can be performed using only a single brief shock, but other tests, as active avoidance, can require tens or even hundreds of shocks, which make them an extreme challenge for the psychological welfare of the animals. On the other hand, tests based on rewards, which apparently may seem more ethical, actually still induce suffering in the animals, as food rewards are almost always associated with a food restriction protocol, in order to motivate the animals to seek food. In this case, the rodents are starved for days before starting the test and kept under food restriction for the whole duration of the test. For the radial maze, for example, animals will suffer hunger for 2 weeks (3–4 days of pre-training phase and 10 days of training). Actually, the distress during the testing session is only a minimal part compared to the stress lived outside of the testing session, which is prolonged and continuous. Analogously, liquid rewards commonly rely on a previous water restriction protocol, in order to use thirst as motivation for reward-seeking.

Animal stress is not only an ethical issue *per se*, but is also an important factor that puts at risk the reliability and reproducibility of scientific results. From the 1960s, many tests have been designed that do not employ punishments or rewards, being based on spontaneous behaviors of the rodents. For instance, in Boissier and Simon's 16-hole-board (Boissier and Simon, 1962) or File and Wardill's 4-hole-board (File and Wardill, 1975; d'Isa et al., 2021a), mice are induced to look inside the holes of a board simply by their natural curiosity. In Ennaceur and Delacour's object recognition test mice are exposed to objects, which are spontaneously explored on the basis of their novelty (Ennaceur and Delacour, 1988; d'Isa et al., 2014). In the object location test, the displacement of an object is used to create a source of novelty and hence induce higher levels of exploration (Ennaceur et al., 1997). Maze examples are the spontaneous alternation T-maze (d'Isa et al., 2021b) and the continuous alternation Y-maze (Gerlai, 1998). The attention of the biomedical community for animal welfare sharply increased over the course of the past 40 years. To provide a metric, a search in the PubMed biomedical archive shows that the number of new scientific articles mentioning the phrase "animal welfare" remained constantly under 50 for each decade from the 1940s (when the first article with "animal welfare" was published) to the 1970s, but underwent an explosion in the 1980s, reaching more than 1000 hits (Figure 1A). From the 1980s, the annual number of new articles progressively increased up to present, indicating an escalating growth, and got to more than 1600 in 2023 alone (Figure 1B).

In May 2022, with the present Research Topic, we launched a call to encourage works on animal-friendly behavioral testing methods for rodents. The call was received with enthusiasm by the scientific community. Indeed, the article collection that we are glad to present here comprises 20 contributions by 70 authors from countries across the world, ranging from Norway to Mexico and from California to Japan. Several different approaches have been explored, from automated home-cage monitoring to robotic rats, and from seminatural environments to freely-accessible mazes directly connected to the home-cage. We will hereon briefly describe the Research Topic contributions.

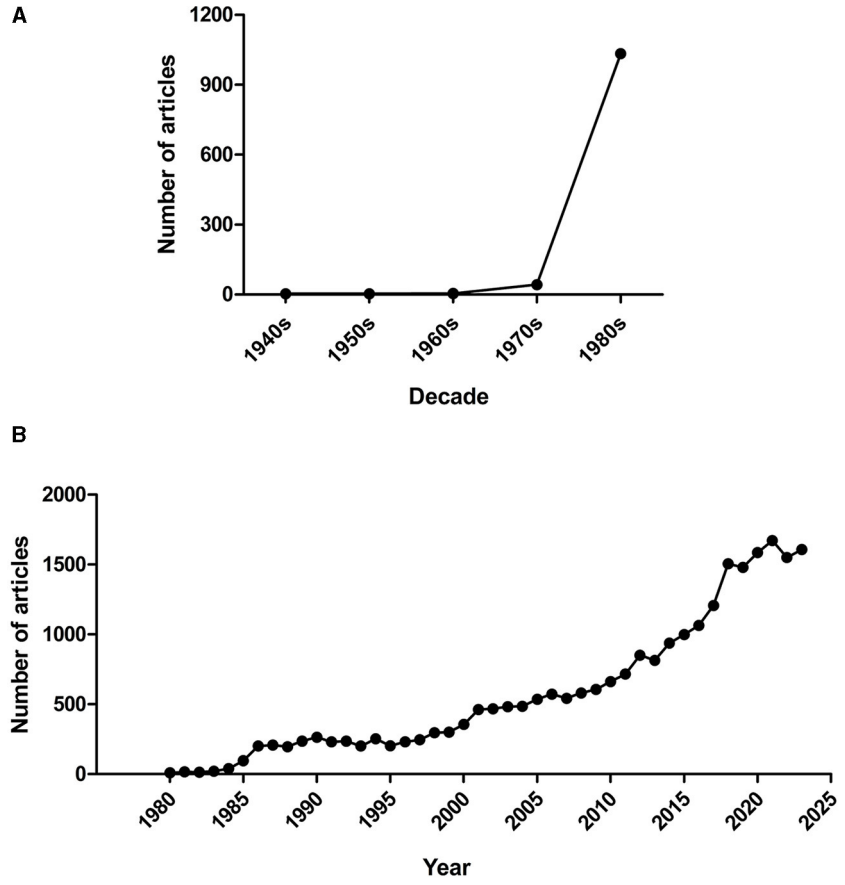

FIGURE 1
Number of publications mentioning "animal welfare". **(A)** Number of articles, listed in the PubMed database, mentioning the phrase "animal welfare", per decade (from the 1940s, when the first article employed the phrase, to the 1980s). **(B)** Number of articles, listed in the PubMed database, mentioning the phrase "animal welfare", per year (from 1980 to 2023).

General concepts

Two articles of the Research Topic deal with general concepts related to animal-friendly testing. In the first, d'Isa and Gerlai underline the importance of the employment of knowledge of the species-specific peculiarities of a given species to design behavioral tests that produce valid results (not random one-time responses to an artificial situation) and that are based on animal-friendly motivators. This combined maximization of ethological validity and animal welfare is a key feature of what the authors defined as ethological neuroscience. Additionally, the authors present a rating scale for behavioral tests, which is based on their impact on animal welfare and features 12 levels, from A (animal-friendly) to L (lethal). It is the hope of the authors that in future an increasing number of A-level behavioral tests will be designed.

Comparative psychologist Charles I. Abramson has investigated, over the course of almost half a century, the behavior of more than 40 species. In the present Research Topic, Abramson explains the importance of comparative psychology for neuroscientists. Indeed, according to Abramson, the comparative approach can be a valuable *forma mentis* for the study of behavior. For instance, the analysis of the behavioral differences of closely related species, or even strains, can help to elucidate, by subtraction, the genetic and neural underpinnings of behavior, and it can allow to identify more easily species-specific peculiarities that can be useful for the design of species-tailored behavioral tests that optimize animal welfare.

In the following paragraphs we will describe the contributions dealing with specific behavioral tests, subdivided into two categories: closed-session and open-session behavioral tests. In the first, the subject animals are brought to a specific testing environment different from their living environment, they are tested at a specific hour of the day chosen by the experimenter, the duration is a fixed short period (generally between 1 and 60 min) and the animals are returned to their living environment only at the conclusion of the testing session. On the other hand, in open-session behavioral tests, the animals: (1) remain in their living environment; (2) are given the opportunity to approach freely a series of interactive testing elements; (3) can choose the moment of the day when they want to start behavioral testing and for how long to engage in the testing; (4) undergo open-session testing, meaning that they can stop and resume behavioral testing in any moment, alternating testing with their regular living activities, such as feeding or sleeping.

Closed-session animal-friendly behavioral tests

Among closed-session tests, a good example of animal-friendly behavioral testing is the paced mating test, standardized by Mary Erskine (1946–2007) in the 1980s (Erskine, 1985, 1987; Erskine et al., 1989) and made more widely popular by Raúl Paredes and collaborators between the late 1990s and the early 2000s (Paredes and Alonso, 1997; Paredes and Vazquez, 1999; Martinez and Paredes, 2001; Paredes and Martinez, 2001). In the present Research Topic, Ventura-Aquino and Paredes describe the usefulness of this test to investigate behavioral, neuroendocrine and neuroplastic changes in female rats and mice following sexual experience. In traditional non-paced mating tests, the females are exposed to a sexually active male and cannot escape from its approaches. In such a situation, sexual activity may lose its rewarding value and become stressful for the females. In contrast, in nature, female rats and mice have the possibility to accept or reject sexual approaches from the male, a possibility which prompts male courtship efforts and is at the basis of biological evolution through sexual selection. Paced mating reproduces in laboratory the same possibility, enabling the females to choose if, when and for how long engage in sexual activities with the males. Such protocol, on the one hand, increases the ethological validity of the behavioral test and, on the other hand, it increases the animal welfare of the experimental subjects.

Comparative psychologist Shigeru Watanabe, who has been professor at Keio University in Tokyo for over 40 years, contributed to the Research Topic with two works [Watanabe (a, b)]. In the first, he proposes the possibility to employ mirror-based tests which use the mirror as an animal-friendly reward not requiring previous food or water deprivation [Watanabe (a)]. In the second, Watanabe reviews the use of a non-invasive and contactless technique, infrared thermography, to evaluate social judgements in mice and highlights the potential of this non-invasive tool for the animal-friendly study of cognition and emotion in rodents [Watanabe (b)]. This approach has currently been employed in various rodents, including laboratory mice (Watanabe, 2015), laboratory rats (Wongsaengchan et al., 2023) and wild mice (Delacoux and Guenther, 2023), as well as in many non-rodent species (Mota-Rojas et al., 2021).

Behavioral neuroscientist Sergio Pellis, who has been investigating play behavior for over 45 years, and colleagues propose the rough-and-tumble play of juvenile rats as a natural behavior offering a unique window to study the processes of the social brain (Pellis et al.). Indeed, the rough-and-tumble play is a playful confrontation, highly pleasurable for the participants, in which competition for physical dominance is moderated by cooperation, including self-limitation and turn taking, which leads to a voluntary exchange of the dominant and submissive roles. This complex play behavior is particularly suitable to test social decision-making in rats.

In social interaction tests featuring encounters between unfamiliar adult rodents, a subject animal is exposed to an unfamiliar stimulus animal, and the behavior of the subject animal is scored. However, such encounters have the problem that, especially with males, fights may often occur, with the possibility of pain and injuries for the animals involved. In specific paradigms as the resident-intruder test, the risk of fight-related injuries is even higher (Koolhaas et al., 2013). Different solutions can be imagined to solve this issue. Harda et al. performed a partitioned social interaction test, in which the two animals were separated by a transparent perforated barrier that allowed the mice to see and smell, but not touch, each other, as well as a second test with the stimulus mouse placed inside a protective wire-mesh cup in an open-field arena. Through these tests, the authors showed that C57BL/6N mice have a sub-strain specific resistance to ketamine-induced social behavior deficits. Robotics engineer Siddall proposes a solution that additionally allows physical interaction: the employment of robotic animals as stimulus animals. In particular, Siddall reports the characteristics of 13 models of robotic rats that have been developed over the course of the past 20 years, and describes which features the robotic rats of the future should possess to be employed effectively in behavioral research. The use of robotic rats would not only make social interaction tests safe, but it would also, since the behavior of the robotic rats is programmable or remotely controllable by the experimenters, allow an unprecedented control over the experimental design.

The pup retrieval test is currently the leading procedure to assess maternal behavior in rodents. Winters et al. present an automated version of the test that, for the first time, allows synchronous video-recording of maternal behavior and audio-recording of pup vocalizations, which allows to assess bidirectionally the dam-pup dyadic interaction. This new test, named BAMBI (Bidirectional Automated Mother-pup Behavioral Interaction), is performed in the home-cage and employs artificial intelligence for computer vision allowing body part tracking and pose estimation, as well as for automated audio-recognition of pup ultrasonic calls.

Finally, Nunes, who has been studying squirrels for over 35 years, argues how animal-friendly rodent behavioral tests can be used also in the field. In particular, Nunes describes animal-friendly behavioral tests that can be performed *in situ* on free-living ground squirrels, including tests for motor coordination, the caution-boldness continuum, docility and problem-solving.

Open-session animal-friendly behavioral tests

The best example of open-session animal-friendly behavioral testing are the automated home-cage monitoring systems (Mingrone et al., 2020; Voikar and Gaburro, 2020; Grieco et al., 2021; Kahnau et al., 2023), which avoid potentially stressful animal handling, do not require removal of the animals from their home-cage for testing in unfamiliar and hence potentially anxiogenic environments, permit the animals to be tested in a social context together with their mates and respect the circadian rhythms of the tested subjects. The most widely known of these systems is IntelliCage, conceived by Hans-Peter Lipp and collaborators in the early 2000s at the University of Zurich (Galsworthy et al., 2005; Lipp, 2005; Lipp et al., 2005). In this smart cage, the interactions of mice or rats with specific elements (visits to the corners, nose-pokes to nose-holes and licks of the bottle-nipples)

are recorded automatically and continuously, allowing to design different experimental protocols for the evaluation of motor and cognitive functions. In our Research Topic, Lipp, who has been studying animal behavior for 50 years, traces with colleagues the history of the development and the future perspectives of this animal- and user-friendly automated behavioral testing system (Lipp et al.). The authors also present an evolution of IntelliCage: greater-sized chambers endowed with the same interactive elements of the smart home-cages. These IntelliCage-like environments, which could be named IntelliChambers, have already been tested with marmosets and could be particularly useful for the behavioral testing of rodents that require abundant space to move along three dimensions, such as squirrels and chinchillas.

Four experimental works of our Research Topic employed IntelliCage. Many standard learning protocols in IntelliCage use controlled water access as the motivational driver. However, this may lead to water restriction in slow learners. Bramati et al. present a new IntelliCage learning protocol in which mice have permanent access to plain water but can additionally be rewarded with saccharin-sweetened water if during the task they perform a correct choice. Through this appetitively motivated learning protocol, the authors showed that environmental enrichment enhances hippocampus-dependent spatial learning in female mice (Bramati et al.). Nevertheless, while this purely appetitive motivator was effective for simple tasks, an excessive number of mice lost interest in the sweet reward when challenged with more difficult hippocampus-dependent tasks. To solve this issue, Ma et al. compared, in female mice, the purely appetitive task (correct choice: saccharin; wrong choice: water) with other two new tasks, in which the second option (water) was devalued by (a) the addition of bitter-tasting quinine, or (b) increasing the number of work (nose-pokes) required to obtain it. Compared to the previous protocol (saccharin vs. water), these two novel combined incentive-disincentive protocols showed a strong improvement of both task engagement and task performance. Nigri et al. tested the Bramati protocol in male mice, finding that for the males the performance levels dropped even more rapidly than for the females when switching from simple to complex learning tasks, suggesting a higher motivational cost for the males. New protocols optimizing the performance of males are yet to be tested, but a suggestion could come from the combined incentive-disincentive protocols conceived by Ma et al.. Finally, Wu et al. employed a water-motivated IntelliCage protocol in which access to water could be denied only for a maximum of 2.5 h, in order to avoid dehydration and psychological stress derived from thirst. Through this protocol, the authors found that stimulation or inhibition of $GABA_B$ receptors in the insula of epileptic rats led to, respectively, reduced or increased memory, in both spatial and non-spatial operant tasks.

Another home-cage behavioral monitoring system is the Home Cage Analyser (HCA; Bains et al., 2016). Here, Bains et al. present a new method for HCA based on a computer vision algorithm capable of measuring climbing on the wire lid of the home-cage. Home-cage monitoring of climbing behavior allowed early detection (at 8 weeks) of motor impairment in the N171-82Q mutant mouse, a widely employed model of Huntington's disease, suggesting an interesting new behavioral marker for this neurological disease. Additionally, in healthy mice, a sex effect was found, with females spending more time climbing than males.

Julius Emmrich's team at the German Center for the Protection of Laboratory Animals has recently developed a new refined version of the radial maze which is fully automated, handling-free, voluntary and does not require food or water deprivation (Mei et al., 2020). In this test, the maze is connected through a tube to the home-cage, and the mice can freely decide when to explore the maze and perform the spatial memory testing. In the present Research Topic, the same team perfected the method and directly compared the refined radial maze with the classical radial maze (Kohler et al.). Both tests showed significant learning in healthy mice and detected spatial impairments in lipopolysaccharide (LPS)-injected mice.

Hernández-Arteaga and Ågmo describe the benefits of employing seminatural environments for rodent behavioral testing. These settings, which reproduce in the laboratory an environment similar to the natural one, are particularly appropriate for the study of sexual behavior. Indeed, in seminatural environments, as in nature, males and females equally control the sexual interactions (Bergheim et al., 2015). As in closed-session paced mating, in seminatural environments male sexual approaches are escapable by females. Moreover, females perform proceptive behaviors that incite male copulation and that can be considered as an index of female sexual motivation. Importantly, Bergheim and colleagues found that, in a seminatural environment, the almost totality of copulatory acts (96%) were performed within 5 s from a female proceptive behavior, indicating a high level of sexual motivation in the females. Additionally, sexual interactions were initiated by females as frequently as by males. Overall, seminatural environments not only are research tools more suitable for the animal welfare of the female subjects, but additionally they represent a more realistic and ethologically valid model of bidirectional socio-sexual interactions between males and females.

Finally, Parsons et al. outline the advantages of the free exploratory paradigm (FEP), which can be used both in the laboratory (as in Kohler et al.) and in the wild (Parsons et al., 2023). Indeed, by placing free-access test chambers in natural environments, rodent behavior can be assessed without handling, relying on spontaneous activity, avoiding the need of keeping animals in captivity and in a context with a higher ecological validity. Moreover, non-conventional species of rodents, such as field mice, can be studied and heterozygosity-enriched groups could be employed.

Conclusions

The present Research Topic includes numerous different approaches for animal-friendly behavioral testing. In future, hopefully, each of these approaches will be further developed and new approaches will be found. However, the most interesting frontier of the evolution of animal-friendly behavioral testing could be, rather than the amelioration of a single approach,

the combination of different approaches. For instance, robotic rats could be placed in seminatural environments for rat-robot social interactions. IntelliChambers could host seminatural environments, as well as robotic rats. And so on. Since several new approaches and technologies have become available, scientists will be free to use all their creativity and ingenuity to combine these options at best and design optimal paradigms for animal-friendly behavioral testing.

Author contributions

RdI: Conceptualization, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. SF: Conceptualization, Writing – review & editing. RB: Conceptualization, Writing – review & editing.

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Conflict of interest

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

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Designing animal-friendly behavioral tests for neuroscience research: The importance of an ethological approach

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Behavioral tests have three key elements: (1) a motivating factor (what motivates the animal in the test); (2) an observable behavior (which behaviors we may expect the animal exhibits in response to the test); (3) a measurable outcome (a quantifiable variable associated with the behavioral response).

For example, in the rodent step-through passive avoidance test (see d'Isa et al., 2014 for a brief history of the test), the animal is released into a strongly illuminated chamber connected to a dark zone. Being naturally photophobic and preferring dark areas, mice and rats will rapidly move from the well-illuminated zone into the dark zone, a behavior that in the wild is useful to avoid being seen by predators. When the animal enters the dark zone, it receives an electric shock. After the initial exposure to this apparatus (the training), the animal is released a second time in the same apparatus for a memory test, but this time without the shock deliverer being active. If the animal remembers receiving the shock, the dark zone should now be perceived as dangerous and hence avoided. In this test, the motivation is fear, the observable behavior is avoidance of a dangerous (dark) zone, and the quantifiable outcome is the latency to enter the dangerous zone, which thus serves as an index of memory. The longer is the latency, the stronger is the memory of the past electric shock exposure.

Behavioral testing of rodents in a laboratory setting started in the 1890's with the studies of Thomas Wesley Mills (1847–1915) from McGill University (Montreal, Canada) and of Linus Ward Kline (1866–1961) and Willard Stanton Small (1870–1943), both from Clark University (Worcester, Massachusetts, USA) (Mills, 1895, 1898; Kline, 1899a,b; Small, 1899, 1900, 1901). These studies were preceded by ethological, purely observational, studies on rodent behavior in the wild, e.g., Mills's studies on squirrel behavior (Mills, 1888, 1890, 1893), but it was only in the period 1895–1900 that behavioral tests for studying rodent behavior and psychology in a laboratory setting started to be designed. Mills also observed the behavior of two squirrels he captured and kept for few months, reporting, for example, how ethologically relevant behaviors could be observed, like nest-building and food storing, and how one of them learned to eat from his hand and enjoyed running on a running wheel that was installed in its home-cage (Mills, 1888). Although this was a first step for the study of rodent behavior in a

controlled environment, the report with the findings was only anecdotal and appeared inserted within a paper on the behavior of squirrels in the wild. Two important elements of scientific testing were absent: systematic observations (i.e., observations at pre-set time-points according to a specific rationale) and choice of one or more quantitatively measurable behavioral outcomes as variables of interest. A systematic and quantitative study of rodent behavior in laboratory had yet to come.

Mills was the first, in the mid 1890's, to introduce for rodents the ontogenetic diary method, which consisted of observing and describing step by step the different developmental stages of a species, starting from the day of birth (Mills, 1895). Applying this method, he studied systematically the physical and psychological development of guinea pigs through a daily monitoring in a laboratory setting. In addition to purely observational studies of development, he also used some basic behavioral tests, e.g., reflex tests or taste reactivity tests, in which only qualitative responses were recorded (Mills, 1895, 1898). On the other hand, Kline and Small introduced behavioral tests aimed at specifically evaluating cognition. In 1898, a Clark University colleague of Kline and Small, Colin Campbell Stewart (1873–1944), published a study on the effects of alcohol, barometric pressure and type of diet on rat daily voluntary wheel running activity, as assessed by a revolving drum connected to automatic counters recording the total number of revolutions (Stewart, 1898). Stewart was the first to perform a quantitative rodent test of motor activity. The experiments of Stewart, from the Biology Faculty, inspired Kline, from the Psychology Faculty, the idea to choose rats as animal models for his research on learning processes (Kline, 1928; Miles, 1930). Kline designed a rat problem box (Kline, 1899a), while Small, who worked in the same laboratory as Kline, was the first to use a maze in the history of behavioral neuroscience (Small, 1901). In Kline's test, the task was finding the entrance of a box and retrieving the food contained inside it. Time to retrieve the food was recorded over multiple trials to assess learning. In Small's test, the task was finding food placed in the central zone of a complex maze inspired by the design of Hampton Court Maze, the well-known hedge labyrinth in England. Time to find the food and number of errors (entering a blind alley) were recorded over multiple trials. The ones performed by Kline and Small were the first quantitative rodent cognitive tests in the history of behavioral neuroscience. Regarding the motivating factor, both Kline and Small employed hungry rats in their cognitive tests, and food deprivation became a widely employed protocol in the subsequent studies using appetitive (reward-based) learning tasks.

However, since the dawn of behavioral neuroscience, researchers have been aware of the fact that hunger is only one possible motivator. Small himself wrote: "I trust the reader will not "jump" to the conclusion that no other motive would be workable. Hunger is merely the most fundamental" (Small, 1900). Noticing that well-fed rats still retrieved food during the task, he added: "Their performance of the task without the incitement of hunger can hardly be accounted for except upon the basis of a hoarding instinct almost as imperative as hunger" (Small, 1900). Nevertheless, these specifications seem to have been scarcely considered by the following researchers. Indeed, most behavioral tests designed up to the 1950's were based on rewards or punishments and commonly employed food deprivation or electric shocks, respectively.

Another classical avoidance task is, for example, the shuttle-box active avoidance, first conceived by Lucien Warner in the early 1930's (Warner, 1932), where instead of having to stay in the illuminated zone to avoid the shock, i.e., instead of not moving and being passive, the appropriate response is to actively move over to an opposite zone to avoid the shock when a stimulus (a tone or a light) announces its imminent release. In this task, the experimenter may have to employ a large number of shocks (even hundreds) over several days of training before animals reach high rates of shock avoidance and cognitively impaired animals could still show low rates of shock avoidance even at the end of the training (Montag-Sallaz and Montag, 2003; Cain, 2019). Painful stimulation, along with being ethically undesirable when dealing with any sentient organism, also leads to methodological complications for the experimenters. Indeed, pain generates stress, which is a major confounding factor in animal research. Still today, classical avoidance tests employ electric shocks, while many other traditional tests, although not utilizing painful stimuli, feature highly stressful conditions including starvation, water deprivation or pharmacologically induced sickness to motivate the animals to perform a task. However, an increasingly accepted view is that absence of pain and reduction of stress during behavioral testing are fundamental for both animal welfare and reproducibility of experimental results, and unless pain or stress is the main focus of the study, these conditions should be avoided as much as possible. In order to respond to such ethical and methodological concerns, several animal-friendly tests have been designed. However, since not all behavioral domains of investigation currently have such animal-friendly options, the development of new animal-friendly tests is an important goal for modern behavioral neuroscience.

How to design an animal-friendly behavioral test

Compared to shuttle-box active avoidance, step-through passive avoidance, introduced in the 1960's (Kopp et al., 1966; Jarvik and Kopp, 1967), is considerably less stressful and in the test session no shock delivery is actually present. Nevertheless, the training session still features a brief painful stimulus. In an ideal animal-friendly behavioral test, the motivating factor should not be painful or stressful. Furthermore, the observable behavior should be natural (i.e., an ethologically

relevant species-specific motor or postural pattern). Finally, the outcome variable associated with this behavioral response should be practical to measure in a laboratory setting through a method that is safe for the animals (e.g., direct observation, videorecording, videotracking, audiorecording of ultrasonic vocalizations, photocell actimetry, weight sensors, infrared thermometry and other non-invasive methods). These are the three main characteristics that a behavioral test should have to be qualified as animal-friendly. Let us first focus on the motivational aspect of behavioral tests, the first of the key components mentioned above.

Ethologists have been stressing the argument that motivating factors are species-specific (Gerlai and Clayton, 1999a; Gerlai, 2021). A stimulus that is appetitive (rewarding) or aversive (punishing) for one species, may be neutral, or may have the opposite reinforcing value to another. Even among closely related species, the rewarding value of a stimulus may be remarkably different. Among felids, for instance, tigers like to bath in water, while lions do not. Hence a swimming pool may be a reward for the former, but not for the latter species, as found, for example, by Allison Hedgecoth who provided a water pool to a lioness and a tigress living together in the same environment in the Noah's Ark Animal Sanctuary of Locust Grove, Georgia, United States (Harries et al., 2020). The main issue, however, in the behavioral neuroscience literature is that systematic analysis of what motivates animals used in laboratory settings is often lacking, or that ethology research often does not intersect with biomedical studies.

Considering, for example, laboratory rodents, the house mouse (*Mus musculus*) and the common rat (*Rattus norvegicus*) are the two most widely used species in biomedical research. Their employment is almost universal in translational research studying mechanisms of central nervous system disorders. Most studies that require aversive stimuli with rodents use electric shocks. But electric shocks are rather unnatural stimuli. However, almost no one considers what consequences may result from the unnatural aspect of this stimulus. It is just assumed that pain is pain, and that electric shock-induced pain is relevant and strongly motivating. Most scientists do not even consider what complication this electricity passively running through the body of the animal, including its brain, may cause with respect to neuronal activity: such electric currents may alter synaptic function and numerous underlying molecular mechanisms. Similarly, studies that employ appetitive stimuli, almost always use food that the experimenter picks out based on tradition, habit or just personal preference. Comparative analyses of what food types, food quantities, food textures and food sizes are most preferred by rats or mice are often not considered, or have not even been conducted. Briefly, as animals are, through evolutionary processes, adapted to their natural environment, they possess species-specific characteristics that represent genetic predispositions, instincts in colloquial terms, that determine, or at least heavily influence what they like, what they dislike, and how they respond to these stimuli. Taking these species-specific features into account is thus a must in animal-friendly experimental designing (Gerlai and Clayton, 1999a,b). In rodents, a typical animal-friendly motivating factor is neophilia (attraction for novelty), which drives, for instance, object exploration behavior in the object recognition test (d'Isa et al., 2014), head-dipping in the hole-board test (d'Isa et al., 2021a) and arm alternation in the spontaneous alternation T-maze (d'Isa et al., 2021b). A similar example is the continuous spontaneous alternation test using a T-maze, which utilizes novel place preference to study short-term spatial memory in rodents (Gerlai, 1998).

Regarding the second key element mentioned above, the observable behavior in an animal-friendly test should be naturally displayed by the animal (e.g., should be part of the ethogram). Preferably, it should be a spontaneous behavior, an instinctive response that requires no pre-training, during which typically punishments and rewards are used by the experimenter to lead to a target behavior. Punishments are commonly painful stimuli (as electric shocks), while rewards, as food or liquids, are often associated with food-deprivation or water-deprivation, in order to use hunger or thirst as motivating factors. Lack of the need for pre-training makes the test more animal-friendly because it avoids punishments and deprivations, and is also time-saving for the experimenter. It is, however, also possible to use conditioned behaviors in an animal-friendly way, if certain conditions are respected. In particular, rewards should not be associated with a previous aversive state. Chow and colleagues, for instance, designed a reward-based cognitive test for gray squirrels in which no food-deprivation or water-deprivation was employed (Chow et al., 2017). The motivation of the rodents was ensured simply by using food rewards (hazelnuts) that were different from their daily diet (seeds, fresh fruit and vegetables), i.e., novelty alone was sufficient to motivate the animals. Novelty-seeking and exploratory drive (i.e., the motivation to learn about new places and/or new inanimate or animate components of the environment) are almost universal among animal species, and certainly have been shown for laboratory rodents (Gerlai et al., 1990; Crusio, 2001). In fact, stabilizing natural selection has been inferred for exploratory behaviors from fish to mammals, as it leads to an optimal level of activity ensuring the ability of the animal to find resources, including food, water and mates, as well as escape routes leading away from predators (Gerlai et al., 1990; Crusio, 2001). The use of novelty as a motivator may not be appropriate in some research contexts and, for certain studies, aversive stimulation may be required. However, even in such cases, painful punishments could be and should be substituted with non-painful aversive alternatives, for example, air-puffs. Indeed, air-puffs have been efficiently employed to elicit robust conditioned place avoidance negating the need for using any painful stimuli (d'Isa et al., 2011). Even for studies specifically focused on fear reactions, alternatives to painful stimulation

are available. Odor of predators (e.g., fox's urine, or an extract from it) has been efficiently used to induce avoidance reactions and fear without previous painful stimulation (Blanchard et al., 2003).

The main steps for designing an animal-friendly test can be summarized as follows: (a) prepare a list of behaviors typical of the species (the ethogram), along with what stimuli may induce these behaviors, i.e., the motivating forces; (b) exclude behaviors induced by pain, physical suffering or psychological stress; (c) from the remaining, choose a behavior that can be studied through an apparatus that can be used in a laboratory setting; (d) choose which outcomes could be measured, safely for the animals, in the most efficient and precise way in order to provide quantitative experimental data. Let us examine an experimental example of how these steps may be accomplished.

A typical behavior of rodents is food hoarding, that is, collecting and hiding food as supply storage for times of food scarcity. This behavior can be observed in more than 180 rodents (Zhang et al., 2022). This is an adaptive behavior that is observable both in nature and in the laboratory setting. It is an instinctive behavior that does not require pre-training. Two main strategies are adopted by food hoarding rodents. Scatter hoarders, as gray squirrels, hide food in many dispersed small hoards. On the other hand, larder hoarders, as hamsters, store food in one large hoard, named the larder. A classification of the hoarding strategies of 183 rodents is provided by Zhang and colleagues (Zhang et al., 2022). These hoarding behaviors may be utilized by the experimenter to devise behavioral tests of motivation (during the food accumulation phase) or of spatial memory (during the subsequent phase of food retrieval from the spatially separated hoards). For motivation tests, easier to study in larder hoarding rodents, the measurable outcome could be the total weight of the seeds or pellets collected and stored in a fixed amount of time. For spatial memory tests, which would be best studied with scatter hoarding rodents, the recorded outcome could be the number of errors in finding the hoarding sites containing the previously stored food. Alternatively, spatial memory could be studied also in larder hoarders if, during the accumulation phase, the sources of food are multiple. Number of errors (returning to an already depleted food site) would serve as memory index. An apparatus for the testing of food hoarding behavior in a laboratory setting has been realized, for example, by Robert Deacon at Oxford University (Deacon, 2006).

An ethological approach may be useful to devise animal-friendly behavioral tests for two reasons. On the one hand, it may help researchers to choose among the elements of the ethogram a behavior that does not require painful or stressful motivating factors. On the other hand, among a taxonomical family of species (for example rodents), it may help researchers to select the most suitable species for a certain test. Let us return to the example we mentioned above. Laboratory mice are larder hoarders, just like hamsters, but their propensity to hoard is relatively low under baseline conditions. In order to avoid food-deprivation, long testing sessions may be required to obtain replicable results, including, e.g., overnight testing sessions (Deacon, 2006). Hamsters, on the other hand, have a high propensity to hoard (Vander Wall, 1990; Harris, 2017). Up to 90 kg of food have been found in hamster burrows (Nowak and Paradiso, 1983). Among food hoarders, they display a specific behavior known as cheek pouching, that is accumulating food in cheek pouches, specialized pockets that allow food transportation. Instead of eating the food items, hamsters keep the food items in their mouth to carry them to a safe place for storage (the larder). Importantly, hamsters easily show this behavior even when they are not hungry, with a latency to hoard within 2 min (Montoya and Gutiérrez, 2016). This peculiarity of hamsters makes them particularly suitable as animal models for scientists who want to design an animal-friendly reward-based memory test that does not require any previous starvation.

Another rodent, the chinchilla (*Chinchilla lanigera*), displays a peculiar behavior known as sand-bathing: when presented with a box full of sand, it will readily start rolling in the box, rotating along its longitudinal axis, to rub its fur in the sand (Stern and Merari, 1969). This natural and spontaneous behavior can be easily elicited in a laboratory setting and sand could be used as an animal-friendly reward in instrumental learning tests without the need of any previous deprivation condition (Redman, 1974).

Eastern woodrats (*Neotoma floridana*), also known as pack rats, have a special attraction for shiny objects, which they readily approach, pick up and bring to their nest, where they collect them (Bradley et al., 2022). This natural tendency of woodrats could be used in behavioral tests, employing small metal objects, as stripes or balls of aluminum foil, as motivators (Kaufman and Kaufman, 1984).

Of course, the issue is that quite often neurobiological, genetic, or other methods may not be as readily available, or as sophisticated, for such species as hamsters and chinchillas as for the favorites of biomedical research, mice and rats. How can we solve this conundrum? Firstly, we could improve biotechnological methods for the so called "alternative" species. Secondly, we could improve our understanding of the ethology, the natural species-specific behavioral characteristics, of the preferred model organisms, e.g., of mice and rats. Certainly, advances in both of these areas have been made during the past few decades. Regarding the first area, numerous novel techniques may now be equally useable with mice and hamsters (and many other species). The CRISPR/Cas technology is a clear example (Kampmann, 2020). Concerning the second area, there have been research efforts adopting ethological approaches in mouse neurobehavioral genetics, as for instance testing mouse mutants in the wild (Dell'Omo et al., 2000; Vyssotski et al., 2002) or in laboratory environments more closely resembling a natural habitat, like Eco-HAB (Puścian et al., 2016; Winiarski et al., 2022). Anders Ågmo's research group at University of Tromsø recreated a seminatural environment in the laboratory for the evaluation of rat behavior (Chu and Ågmo, 2014),

a method that has been employed in several subsequent studies (Chu et al., 2015; Houwing et al., 2019; Le Moëne et al., 2020; Heinla et al., 2021). In the testing sessions, which may last days, rat behavior is continuously video-recorded and subsequently scored off-line by the researchers. Notably, another important ethological approach of the new century is testing the animals not in a setting designated uniquely for testing sessions, but rather in the permanent housing environment in which they commonly live (Mingrone et al., 2020; Voikar and Gaburro, 2020). In nature, most rodents build burrows (or occupy pre-existing holes or burrows) to use them as homes (long-term inhabiting spaces), in which they return to sleep, store food, seek shelter from the elements, keep warm, hide from predators, give birth, raise the pups and share a social life with conspecifics. Rodents develop a strong bond with their home and show a territorial behavior toward it, actively defending it from possible invaders. In the laboratory, if an unfamiliar conspecific is placed in the home-cage of mice or rats, the intruder will rapidly be attacked by the resident animal (Koolhaas et al., 2013; Ruzza et al., 2015). The home-cage is the place where laboratory rodents feel safest and where they are more likely to display spontaneous natural behaviors. Thus, the idea of testing in the home-cage has been gaining considerable attention in the past few years, and several home-cage automated multi-variable recording systems have been developed, e.g., the IntelliCage (Galsworthy et al., 2005; Kiryk et al., 2020; Iman et al., 2021), PhenoMaster (Urbach et al., 2008; König et al., 2020), Actual-HCA (Bains et al., 2016; Mitchell et al., 2020) and SmartKage (Ho et al., 2022). Automated home-cage testing systems have several advantages: (a) they allow behavioral phenotyping without human interference and without the consequent handling-related stress; (b) the animals are not tested in an external apparatus but in their familiar and well-known housing environment, which eliminates confounds arising from anxiety; (c) data collection is not restricted to a specific moment of the day, but can be performed continuously, 24 h a day, 7 days a week, allowing a more precise and realistic assessment of behavior; d) long longitudinal studies (lasting weeks, months or years), or even life-long studies, can be performed on the same animals with a continuous behavioral assessment, which is particularly relevant for developmental neuroscience and aging neuroscience; (e) interactive elements (e.g., levers, nose-poking ports, motorized doors and running wheels) may be installed in these home-cages, allowing not only detailed motor assessment, but also complex cognitive testing; (f) animals are tested in a natural social context while living together with other conspecifics, thus providing motor and cognitive measurements with a higher ethological validity and allowing additionally to monitor and analyze complex social interactions. Some of these automated home-cage testing systems are modular, allowing the connection of multiple cages to create a more complex envinronment. For instance, IntelliCage can be connected to two social boxes containing different social stimuli (Mitjans et al., 2017), while in ColonyRack mice can freely roam across 70 cages, arranged in a two-sided rack with fivs columns and seven rows, in which the cages are connected both horizontally and vertically (Zocher et al., 2020; Kempermann et al., 2022). The most recent innovation within this automated behavioral testing approach is connecting home-cages to mazes (Mei et al., 2020; Kohler et al., 2022), granting the experimental subjects free access to the novel test environment. This allows the animals to decide voluntarily when and for how long they explore the maze, similarly to what would happen in nature when rodents decide to leave their burrow for external exploratory excursions.

We believe that bringing closer the fields of ethology and neurobehavioral genetics or behavioral neuroscience will be the solution and will lead to cross-fertilization of these fields. Similarly to how the application of neuroscience-related knowledge to ethology led to the birth of neuroethology (i.e., the study of the neural basis of natural behaviors), the reverse could lead to an ethologically based neuroscience, or ethological neuroscience, which can be defined as the employment of knowledge of the natural behavior of animals in the wild to develop animal models of behavior and behavioral tests for neuroscience research. This ethologically based neuroscience can lead to animal-friendly testing approaches that will not only be more oriented toward the welfare of the animals involved, but also will provide more reliable and more replicable results for the experimenters.

Concluding remarks: Reproducibility, replicability and refinement

Reproducibility is when we obtain the same results repeatedly by using identical methods (Kafkafi et al., 2018; Gerlai, 2019), whereas replicability is when we reach similar conclusions by adopting different methodologies (Kafkafi et al., 2018; Gerlai, 2019). Minimizing stress of the tested animals is a value in itself from an ethical point of view. However, since stress is a confounding factor that increases variability of experimental outcomes, minimizing stress is also fundamental to achieve methodologically sound scientific research. Why does research that ignores species-specific features lead to increased variability? Why is stress a confounding factor that reduces reproducibility? These are intriguing questions that would deserve specific research. The answer may lay in the fact that stress causes activation of the hypothalamic–pituitary–adrenal (HPA) axis, which in turn alters physiological processes regulating cognition and behavior (Moreira et al., 2016). HPA reactivity depends on genetic, epigenetic and environmental

TABLE 1 Rating of the impact of behavioral tests on animal welfare.

Welfare rating	Features	Behavioral tests	References
A	• Spontaneous behaviors • Conditioning only through rewards not associated with previous aversive situations or through non-painful disincentives • No food deprivation • No water deprivation • No forced water immersion • No painful stimuli • No distressful conditions	Novel object recognition	d'Isa et al., 2014
		Object location test	Murai et al., 2007
		Object exploration test	Steinbach et al., 2016
		Spontaneous alternation T-maze	d'Isa et al., 2021b
		Continuous alternation Y-maze	Detrait et al., 2010
		Continuous alternation T-maze	Gerlai, 1998
		Spontaneous 8-arm radial maze (no food deprivation, unbaited)	Haga, 1995
		Spontaneous 6-arm radial maze (no food deprivation, unbaited)	Alessandri et al., 1994; Opitz et al., 1997
		Spontaneous Dashiell hexagonal maze (no food deprivation, unbaited)	Giménez-Llort et al., 2007
		Free access rewarded 8-arm radial maze (no food deprivation, baited)	Mei et al., 2020; Kohler et al., 2022
		Hole-board test	d'Isa et al., 2021a
		Locomotor activity test	Visigalli et al., 2010
		Open-field test (in dim light)	McReynolds et al., 1967; Võikar and Stanford, 2023
		Emergence test	Paré et al., 2001
		Sociability test in the three-chambered apparatus	Gu et al., 2022
		Social vs. object preference test in the three-chambered apparatus	Lammert et al., 2018
		Social novelty preference test in the three-chambered apparatus	Kaidanovich-Beilin et al., 2011
		Social recognition test	Jacobs et al., 2016
		Opposite-sex partner preference test in the satellite cages apparatus	Linnenbrink and von Merten, 2017
		Mate choice test in the three-chambered apparatus	Nomoto et al., 2018; Guarraci and Frohardt, 2019
		Paced mating sexual behavior test	Zipse et al., 2000; Nedergaard et al., 2004
		Paced mating-induced conditioned place preference	Paredes and Alonso, 1997; Camacho et al., 2009
		Two-bottle taste preference test	Gaillard and Stratford, 2016; Strekalova, 2023
		Saccharin consumption test	Inostroza et al., 2012
		Voluntary wheel running	Goh and Ladiges, 2015
		Successive alleys test	Deacon, 2013a
		Nest-building test	Neely et al., 2019; Dorninger et al., 2020
		Burrowing test	Deacon, 2009
		Food hoarding test (without food deprivation)	Deacon, 2006
		Marble burying test	Angoa-Pérez et al., 2013; Witkin and Smith, 2023
		CatWalk gait analysis	Crowley et al., 2018; Pitzer et al., 2021
		IntelliCage automated home-cage testing	Vannoni et al., 2014; Kiryk et al., 2020
		SmartKage automated home-cage testing	Ho et al., 2022

(Continued)

TABLE 1 (Continued)

Welfare rating	Features	Behavioral tests	References
		Eco-HAB automated home-cage testing	Puścian et al., 2016; Winiarski et al., 2022
		PhenoMaster automated home-cage testing	Robinson et al., 2013; König et al., 2020
		Actual-HCA automated home-cage testing	Bains et al., 2018; Mitchell et al., 2020
B	• Low psychological stress • No food deprivation • No water deprivation • No forced water immersion • No painful stimuli	Barnes maze	Rosenfeld and Ferguson, 2014
		Open-field test (in bright light)	Seibenhener and Wooten, 2015
		Light-dark transition test	Takao and Miyakawa, 2006
		Elevated plus maze	Walf and Frye, 2007
		Rotarod	Papale et al., 2017
		Pole test	Zhu et al., 2017
		Beam walking test	Luong et al., 2011
		Pup retrieval test	Lee et al., 2021; Winters et al., 2022
		Pup-rewarded auditory learning test	Besosa et al., 2020
C	• Moderate psychological stress • No food deprivation • No water deprivation • No painful stimuli	Morris water maze	d'Isa et al., 2011
		Grip strength test	Mandillo et al., 2008
		Inverted screen test	Deacon, 2013b
		Prepulse inhibition test	Valsamis and Schmid, 2011; Ioannidou et al., 2018
		Visual threat flight test	Huang et al., 2020; Yang et al., 2020
D	• Brief food deprivation (up to 18 h) • Brief water deprivation (up to 9 h) • No painful stimuli	Socially transmitted food preference test	Wrenn et al., 2003; Plucinska et al., 2012
		Social vs. food preference test	Reppucci and Veenema, 2020
		Hyponeophagia test	Deacon, 2011
		Water-rewarded social cooperation test	Feng et al., 2021; Shin and Ko, 2021
E	• High psychological stress • No food deprivation • No water deprivation • No painful stimuli	Forced swimming test	Castagné et al., 2011
		Tail suspension test	Can et al., 2012
		Predator odor test	Otsuka, 2017
		Counter-current swimming test	Matsumoto et al., 1996; Mizunoya et al., 2002
F	• Brief painful stimulation (≤2 s)	Passive avoidance test	Papale et al., 2017
		Single-shock fear conditioning	Poulos et al., 2016
		Shock-probe defensive burying test	Fucich and Morilak, 2018
G	• Repeated (2–5 events) or extended (>2 and ≤ 10 total s) painful stimulation	Multiple-shock fear conditioning	Shoji et al., 2014; Müller and Fendt, 2023
		Multiple-shock passive avoidance	Takahashi et al., 2018
		Tail-flick test	Chidiac et al., 2021
		Hot plate test	Lee et al., 2018
H	• Prolonged food restriction (from 18 h to weeks) • Prolonged water restriction (from 9 h to weeks)	8-arm radial maze	Crusio and Schwegler, 2005
		Cross maze	Pittenger et al., 2006

(Continued)

TABLE 1 (Continued)

Welfare rating	Features	Behavioral tests	References
		Food-rewarded 5-choice serial reaction time task	Asinof and Paine, 2014
		Novelty-suppressed feeding test	Fukumoto and Chaki, 2015
		Water-rewarded 5-choice serial reaction time task	Birtalan et al., 2020
		Water-rewarded auditory decision making test	Jaramillo and Zador, 2014
		Water-rewarded labyrinth	Rosenberg et al., 2021
I	• Sickness induction	Lithium chloride-induced conditioned taste aversion	Lavi et al., 2018
		Lithium cloride-induced conditioned place aversion	Frisch et al., 1995
J	• Repeated (>5 events) or extended (>10 total s) painful stimulation	Shuttle-box active avoidance	Montag-Sallaz and Montag, 2003
		Learned helplessness test	Vollmayr and Henn, 2001; Silveira and Joca, 2023
		Formalin test	Teng and Abbott, 1998
K	• Potential physical injury (mild to severe)	Resident-intruder aggression test	Koolhaas et al., 2013
		Resident-intruder violence test	Haller et al., 2006; Koolhaas et al., 2013
L	• Lethal tests	Terminal sleep deprivation	Everson et al., 1989
		Drowning test	Richter, 1957

A suggested rating scale for behavioral tests in order of severity (from A to L) of the impact on the wellbeing and welfare of laboratory rodents is presented. For each rating class, features are described and a list of example behavioral tests is provided. The scale comprises twelve welfare classes, from A (no negative impact on animal welfare) to L (most severe negative impact). Important notes:

1. Test impact on animal welfare is species-specific. The suggested rating is specific for crepuscular/nocturnal rodents (as mice, rats and hamsters). For different species of rodents, the ratings may differ. For instance, for the degu (*Octodon degus*), a diurnal rodent species in which adults show strong preference for the lit compartment in the light-dark transition test (Popović et al., 2009), the Barnes maze would have a rating of A instead of B.

2. This classification of behavioral tests is based on the normal responses expected from wild-type or untreated/unmanipulated control rodents. However, as test-associated stress always derives from the interaction between the test and the experimental manipulations (e.g., mutations induced or drug treatment employed), even behavioral tests that are minimally stressful for untreated wild-types may lead to high distress in manipulated animals. Thus, the experimenter must always consider not only what wild-type control animals will do in the test, but also closely monitor how the mutant/treated animals respond in pilot studies, and revise the experimental protocol or choose alternative tests accordingly.

3. This scale is ordinal, but not linear. We are not assuming equal distances between classes.

4. Due to the degree of suffering inflicted on the animals, the tests reported in category J are considered unethical according to current ethical standards, and nowadays would not be approved by the institutional animal care and use committees (IACUCs), nor would receive legal authorization, in most countries performing scientific research.

5. The rating scale is only a suggested scale, a non-comprehensive working document that is meant to be debated, updated and expanded by the rodent research community.

factors (Holmes et al., 2005), which makes it more difficult to predict than instinctive responses. Let us make some overarching theoretical points. Most animal research includes human handling. Human handling is extremely difficult to standardize (Crabbe et al., 1999). Even if handling was perfectly standardized, stress reactivity of the animals would not. Animals experiencing more stress due to the experimental procedures will be more responsive to human handling, which then will lead to elevated error variation in the behavioral test. In order to maximize experiment reproducibility, the best option is to minimize handling-related stress (Gouveia and Hurst, 2017). Considering, for instance, mice, although tail picking is the most commonly employed method of handling (Ueno et al., 2020), this method features tail lifting, tail suspension and swinging the animal over a void, which are highly stressful for the mice. Indeed, tail lifting, compared with alternative handling methods that do not require tail lifting, increases anxiety in the open-field test (Gouveia and Hurst, 2019) and elevated plus maze (Hurst and West, 2010), and it has been shown to reduce exploratory activity (Gouveia and Hurst, 2017), to increase aversion for the human handler in voluntary interaction test (Hurst and West, 2010) and to impair responsiveness to sucrose reward, indicating a reduction of reward's hedonic value (Clarkson et al., 2018). Several animal-friendly approaches are now available to avoid the negative impact of human handling on mice: (a) adopting non-aversive manual handling techniques, as open-hand retrieval through the cupping method (Hurst and West, 2010; Gouveia and Hurst, 2017, 2019; d'Isa et al., 2021b; Davies et al., 2022); (b) employing a tool to handle the mice, as a plastic handling tunnel (Hurst and West, 2010; Gouveia and Hurst, 2013, 2017, 2019; Sensini et al., 2020; Davies et al., 2022); (c) using automated home-cage testing systems in which behavioral outcomes are recorded without physical interaction with the human experimenter (Kiryk et al., 2020; König et al., 2020; Mitchell et al., 2020; Ho et al., 2022; Kohler et al., 2022; Winiarski et al., 2022).

Furthermore, not knowing the species-specific characteristics of the studied organism, for example, applying

inappropriate motivators, forcing the animal to exhibit behavioral responses it would not normally perform, and measuring the behavior under artificial conditions that do not have much to do with the natural environment in which the animal evolved, all can elevate random error, simply because the individuals tested this way may have to find unique solutions to the problems, considerably increasing individual differences in the study (Gerlai and Clayton, 1999a,b). To put it in the words of the aforementioned pioneer of experimental behavioral research Willlard Stanton Small, "the experiments must conform to the psycho-biological character of an animal if sane results are to be obtained" (Small, 1901).

Animal-friendly tests utilizing species-specific features of the studied organism may not be always available or applicable, but, when they are, they should be employed as a first option, in order to maximize both animal welfare and repeatability of experimental results. When fully animal-friendly tests are not available, then the least stressful available test should be employed. In Table 1 we present a rating scale for behavioral tests based on their impact on animal welfare. This rating is not meant to be final, but rather a starting point to stimulate reflection and discussion on the differential stress impact of behavioral tests. We hope that in future an increasing number of studies will employ tests of class A (animal-friendly) and B (minimally stressful) and that, in accordance with a progressive refinement principle, new animal-friendly tests will be designed to substitute the more stressful alternatives.

Author contributions

Rd'I and RG: conceptualization, writing, revision, and final approval of the manuscript. Both authors provided funding. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

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Why the study of comparative psychology is important to neuroscientists

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The purpose of this contribution is threefold. First, is to acquaint neuroscientists with the area of psychology known as comparative psychology. Comparative psychology is the oldest of the organized social sciences with the term appearing as early as 1808. Many of the myriad issues of experimental design routinely faced by comparative psychologists are directly applicable to neuroscience. These issues include consistent definitions of psychological phenomena, the use of Morgan's canon to reduce unbridled anthropomorphism, and observation oriented modeling as a new statistical procedure to increase replication. Second, is a discussion of early comparative methods that may be of value to contemporary neuroscientists. Third, how the comparative approach can help the neuroscientist limit unfounded generalizations across species and develop more animal-friendly behavioral testing options tailored for the species or strain of interest. The articles closes with some recommendations on how comparative psychologists and neuroscientists can work more closely together.

KEYWORDS

comparative psychology, Morgan's canon, systematic variation, neuroscience, definition

Introduction

I would like to thank Dr. Raffaele d'Isa for inviting me to share my opinion on the value of comparative psychology for neuroscientists. Comparative psychology (CP) is the application of the comparative method to problems in psychology (Abramson, 2018). CP is the oldest of the organized psychologies and arguable one of the first social sciences where researchers learned to make scientifically valid comparisons.

The issues of experimental design faced by comparative psychologists in its 215 year history are directly applicable to neuroscience. These issues include the importance of consistent definitions, the use of systematic variation as a control procedure, and an appreciation of Morgan's canon to reduce unbridled anthropomorphism. I have discussed the importance of comparative psychology in several previous publications (e.g., Abramson, 1994, 1997, 2013, 2015, 2018; Abramson and Wells, 2018; Abramson and Levin, 2021).

Brief history

The phrase "comparative psychology" appeared as early as 1808 in German (vergleichende psychologie) used by the physiological anthropologist Liebsch (1808), 1812 in Latin (psychologia comparata) used by the physician Hoffbauer (1812) and 1827 in Italian (psicologia comparata) used by the philosopher Poli (1827). In his book chapter *Of the science of comparative psychology. Origin, principles, critique, truthfulness and useful application of comparative psychology*, Poli (1827) defines CP as "the science that studies and analyzes the instincts, the functions and the habits of beasts in relation to the analogous human faculties, with the aim to explain better the phenomena of thought and feeling in man.[1]" In 1836 the phrase was first used in French (psychologie comparée) by the physician Lélut (1836). Describing the field of observation of CP, Lélut mentions CP of animal species, of human races, of human ages and of mental pathologies. In English, the phrase was used in 1841 by psychiatrist and ethnologist Prichard (1841) and in 1858 by zoologist Weinland (1858). In 1864 Flourens (1864) published the first book with the phrase as its title: *Comparative Psychology* [*Psychologie comparée*, in the original French version].[2] In 1876 Spencer (1876) published "*The comparative psychology of man*." In 1880 Ludwig Büchner wrote, in his *"Mind in Animals,"* that "Comparative anatomy, i.e., the study of bodies, which we have long followed, must necessarily have beside it comparative psychology, the study of minds" (Büchner, 1880), and in 1882 George Romanes used the term "comparative psychology" in his "*Animal Intelligence*" (Romanes, 1882). Of particular interest, Alfred Binet, who developed intelligence tests that eventually became known as the Stanford-Binet Intelligence Quotient (IQ) test, published a book in 1889 on the "psychic life of micro-organisms" where he highlights the benefits of comparative psychology (Binet, 1889; Abramson and McCarthy, 2022).

The first CP society appeared in 1885 in Montreal, Canada: the Association for the Study of Comparative Psychology (Mills, 1887; Murray, 1990). In contrast, the American Psychological Association (APA) was founded in 1892 and the Society for Neuroscience was established in 1969—respectively, 7 and 84 years after the first CP Society.

Comparative psychology has always been identified with neuroscience. One only has to look at any CP textbook to appreciate that all contain at least one chapter related to the "physiology of behavior." Moreover, in 1947 the APA created the *Journal of Comparative and Physiological Psychology*. This collaboration between comparative psychology and neuroscience was recognized until 1982, when the journal was split into the *Journal of Comparative Psychology* and *Behavioral Neuroscience*.

I will comment on several issues that I believe will be useful for neuroscientists. These are: inconsistent definitions, the use of systematic variation as a control procedure, the value of Morgan's canon to limit anthropomorphizing, and the advantage of using observation orientated modeling to analyze data. I will also mention some early techniques that may be useful for contemporary neuroscience research and close with some recommendations.

Inconsistent definitions

Neuroscience studies often deal with psychological and behavioral concepts. Unfortunately, many neuroscientists, especially those coming from a molecular background, overlook providing definitions for these concepts or take existing definitions for granted. This attitude is problematic and can lead to errors in both experimental design and data interpretation. Sometimes there is not a clear concept behind the term employed, so that the term is vague. In other cases, definitions for those concepts exist in the literature, but they are many and varied, so it is actually not possible to know which definition the authors of the study embrace.

Comparative psychology, on the other hand, being a branch of psychology, connects animal research with psychological theorizing. Hence, it could provide neuroscientists with the required theoretical support and help them develop objective definitions for psychological and behavioral concepts. When neuroscience studies use psychological concepts, clear definitions should always be provided, or at least, references to the scientific literature clarifying the theoretical background adopted by the authors.

The use of inconsistent definitions reduces the ability to replicate research and creates a situation where data obtained by neuroscientists may well rest upon an ever changing foundation of weak behavioral knowledge. If we are not more careful, psychology-related sciences and social science in general could become a discredit field much as Richard Feynman stated in a BBC interview in 1981 (Tavares, 2014).

One of the best examples of inconsistent definitions can be found in the study of learning. Neuroscientists may be surprised to discover that there are no consistent definitions of classical conditioning and operant conditioning (Abramson and Wells, 2018). Moreover, there are no consistent definitions of, for example, learning (Kimble, 1961; Bullock and Quarton, 1966), behavior (Levitis et al., 2009; Cvrčková et al., 2016), tool use (Crain et al., 2013), intelligence and personality (Sternberg, 1984; Sternberg and Detterman, 1986; Schlinger, 2003; Legg and Hutter, 2007). All of these areas are of interest to neuroscientists. How can a neuroscientist study a behavioral phenomenon when the definitions of that phenomenon is consistently shifting? The answer is you cannot.

1 Title and quotation translated from the original Italian by Dr. Raffaele d'Isa.

2 Flourens had previously used the phrase comparative psychology in 1861 in his book *De la raison du génie et de la folie*, in which he dedicated to the topic a whole chapter entitled *De la psychologie comparée et du sense intime* (Flourens, 1861).

In regards to intelligence, the intelligence of plants has become a popular area of neuroscience research (e.g., Abramson and Chicas-Mosier, 2016; Abramson and Calvo, 2018). How much faith can a neuroscientist have that they are investigating the "neuroscience of intelligence" (or learning, or tool use, or behavior, or personality) if there are no consistent definitions of what intelligence is? The answer is you cannot.

One of the most egregious examples is the definition of cognition. Frankly, I am not sure that anyone actually knows what "cognition" is. The founding editor of the journal *Cognition* was once asked to define it. The response was "Whatever I like" (Amsel, 1989). In one study, 12 leading cognitive textbooks were examined and 12 different definitions found (Abramson, 2013). How can a neuroscientist rationally study "cognition" if the term is so ambiguous?

Another issue is whether male/female differences among non-human animals should be referred to as gender differences or sex differences. I recently had the opportunity to review a paper on the exploratory behavior of male and female woodlice where the authors referred to sex differences as "gender differences." While I found the notion of gender differences in woodlice, or any non-human animal problematic, it nicely illustrates how psychological concepts developed for humans (such as personality) are seeping into the natural science community to the determent of the science. A definition of what distinguishes gender from biological sex, and a comparative analysis directed toward understanding if, and in which non-human animals gender can be present, would be most welcome.

Systematic variation

In addition to definitional problems, the neuroscientist should be aware of what is known in the CP literature as "systematic variation." Systematic variation is a control procedure where the experimenter "systematically varies" possible explanations before reaching a conclusion (Abramson, 1994). Systematic variation is a reminder to neuroscientists that alternative explanations must be evaluated before inferring that, for example, a species, subspecies, strain or sex difference actually exists.

Consider, for instance, a human study in which females outperform males on an intelligence test. Setting aside problems with the definition of intelligence, neuroscientists not familiar with comparative research methods might conclude that "females are more intelligent than males." While this may be correct, it cannot be concluded before possible explanations are "systematically varied." Males may not be motivated to complete the task. Therefore, motivation will have to be systematically varied and if the differences among males and females persist, then motivation is ruled out. Once motivation is ruled out, the researcher may direct attention to the properties of the intelligence test. Perhaps the test itself contains some inherent methodological bias favoring females. If, using a methodologically different test assessing the same type of intelligence, females still outperform males, then the researcher may be confident that a difference between the sexes exists for this particular task. While the above example focuses on humans, the logic of systematic variation is exactly the same when considering experimental designs with non-human animals.

Morgan's canon

Systematic variation is a control method that limits unsupported generalizations related to comparative research. Another comparative strategy useful for neuroscientists is known as Morgan's canon. Morgan's canon is an epistemological position that encourages researchers to limit their speculations when making comparisons (Karin-D'Arcu, 2005).

The original statement of the canon appeared in Conwy Lloyd Morgan's *Introduction to comparative psychology* (Morgan, 1894). As the original statement was often misunderstood, he clarified the canon in a later publication (Morgan, 1903). As Morgan states (1903, page 59):

"In no case is an animal activity to be interpreted in terms of higher psychological processes, if it can be fairly interpreted in terms of processes which stand lower in the scale of psychological evolution and development. To this, however, it should be added, lest the range of the principle be misunderstood, that the canon by no means excludes the interpretation of a particular activity in terms of the higher processes, if we already have independent evidence of the occurrence of these higher processes in the animal under observation."

The canon contains several important principles for neuroscience research. First, researchers must not assume a higher level of processing if a lower level can satisfactory account for the data. Secondly, one must view with caution the tendency to anthropomorphize human explanations of behavioral phenomena to non-human animals. Third, a researcher must not overlook the possibility that a more reasonable and fundamental explanation of a non-human animal's behavior may be appropriate also when observing the same behavior in humans.

Statistical analysis—Observation oriented modeling

A difficult challenge facing neuroscience researchers is what statistics to use. I suggest neuroscientists consider Observation Oriented Modeling (OOM) (Grice, 2011; Grice et al., 2012). OOM is a collection of methods requiring researchers to hypothesize an expected pattern of results and then determine how many individuals or entities match that predicted pattern (Grice, 2021). OOM has been used in a number of investigations

including social reinforcement delays (Craig et al., 2012), timing (Craig et al., 2014, 2015), and taste aversion learning (Varnon et al., 2018). The program is easy to use and well supported. While I know of no specific case where OOM has been used in neuroscience research, I believe it is worth looking into.

Importance of resisting reductionism

Natural sciences place an emphasis on reductionism. This is easily seen in fields such as chemistry, genetics, molecular biology and indeed neuroscience where their traditions favor experimental designs that focus on internal validity and reducing variability due to external factors (i.e., the factors different from the experimental factor or factors of interest). This type of variability is defined as "noise" and considered a possible source of uncontrolled error in the experiment. Internal validity evaluates if the experimental design, conduct and data analysis answer the experimental questions of a study without bias, whereas external validity refers to the extent to which the experimental finding can be generalized to a different contexts (Andrade, 2018). Research in CP has consistently shown that while internal validity is important, it should not be at the expense of external validity (Steckler and McLeroy, 2008).

While the reduction of noise in experimental design is important, the neuroscientist should remember that human and non-human animals live in a world of noise. Mice, and other rodent models so favored in behavioral neuroscience, live in a world of constantly changing environmental conditions including temperature and humidity fluctuations, and exposure to stressors such as pesticides and pollutants, all of which influences development across the life span. There is a real danger that the reductionist models do not represent external validity as non-human animal models often are studied, maintained, and created in temperature-controlled, humidity-controlled and specific pathogen free (SPF) environments with little contact with outside environmental influences—i.e., noise. In my opinion, one method to ensure external validity is to incorporate systematic variation into the experimental designs used by neuroscientists. At the very least, there should be some recognition by neuroscientists that the reductionist models may not represent the entire picture, could be misleading, and could represent a disciplinary standard detrimental to the quality of the science.

Comparative methods to investigate rodent behavior

I suggest neuroscientists examine some of the early to mid-20th century research methods developed by comparative psychologists. Many of these methods are no longer in use and, in my view, just waiting to be rediscovered and adapted for contemporary research. Of particular interest to neuroscientists is that they were designed specifically to investigate human phenomena in non-human animals from a comparative perspective.

One of the most interesting is the work of Walter Samuel Hunter on the delayed reaction in animals and children (Hunter, 1913). The monograph describes a learning task where the subject must delay its response before a reward is obtained. This task has been used to compare the performance of children, rats, dogs, and raccoons. Many other tasks can be found in Norman Leslie Munn's *Handbook of psychological research on the rat* (Munn, 1950). There is literally page after page of fascinating material including experimental designs related to what is considered "cognitive" such as reasoning and the use of logic. Another excellent source is the three volume set on comparative psychology by Carl John Warden, Thomas Nichols Jenkins, and Lucien Haynes Warner (Warden et al., 1935, 1936, 1940). Once again, a fascinating array of methods and experimental paradigms are presented.

Why are these techniques not generally known? I believe it is the lack of interest in history generally, and of the history of psychology in particular. Professors of neuroscience probably do not realize that before the introduction of simple mazes and runways, comparative psychologists of the first decades of 20th century confronted their organisms with an array of sophisticated problems. These problems include multiple unit mazes, elevated mazes, temporal mazes, jumping stands, and a variety of situations in which the organism must escape an enclosure by solving a puzzle (Munn, 1950). Many of these techniques were designed to explore what are now considered "cognitive" processes. However, and this is often overlooked, processes such as learning and insight were then studied and interpreted within a behaviorist but not a cognitivist framework (Abramson, 2018; Abramson and Levin, 2021).

Importantly, I would like to note that research performed by comparative psychologists during its golden age used a variety of organisms. As time progressed, the range of organisms became restricted to mostly rats and pigeons, as did the type of apparatuses used—a situation similar to what is facing the contemporary neuroscientist, which employs mainly mice and rats.

Such a situation should serve as a warning to neuroscientists that it is dangerous to rely on a single or even a few species to base conclusions on. For example, there are 38 species of mouse and they differ in many respects related to sensory abilities, natural history and behavior. Nevertheless, in neuroscience *Mus musculus* is generally considered the standard mouse. Generalizing findings from a single species to an entire genus is fraught with difficulty and wrong generalizations can easily be made. Analogously, the mouse strain C57BL/6 is often

considered the standard strain, leading to a widespread bias in the choice of the experimental subjects (Zilkha et al., 2016).

Discussion

Problems with definitions, anthropomorphism, and difficulties with replication, are all problems addressed by CP. Arguably, the most important contribution that CP can give to neuroscience is the "comparative" approach itself.

Neuroscientists often perform experiments on a single model and may believe that their discoveries are universally valid. The results of a memory study of mice, for example, are considered to be valid for "memory" in general, not for "mouse memory." Many neuroscience investigations have a strong translational goal and what is found in a model organism is implicitly considered to be related to what happens in humans. Taking this relationship for granted is very dangerous. A major reason why treatments that are found to work in model non-human animals often do not work when applied to humans, is because species-specific differences are present and not appreciated until it is too late.

Comparative psychology, on the other hand, emphasizes that each species has its own specific natural history, behavioral tendencies, learning practices and neural processes. Thus, a model developed with one species should be tested also with closely related species within a family or even closely related strains within a same species. Only in this way can generalizations among models be safely made. When differences are found, since the genetic and neural organization between the experimental subjects is so similar, it would be much easier, by subtraction, to identify the genetic and neural substrates of the observed difference between the species or the strains. Such a comparative approach would be very useful in neuroscience research to identify, by contrast, the neurobiological underpinnings of behavior.

Practically speaking, behavioral neuroscientists should try to assess "cognitive" and behavioral function in multiple species. Since rodents are the most popular model organism in neuroscience, the same task could be tested, for example, in mouse, rat, hamster, and gerbil. If, more specifically, mice are used, then experiments should not be limited to the use of one single strain, but the discovery should be reconfirmed (or disproved) by testing at least three or four different strains. Sex differences should also be taken into account. Too often in neuroscience, and generally in biomedical sciences, experiments are performed on only one sex (generally male) and results have been generalized as universally valid. Experiments on females could lead to completely different results. Indeed, a more frequent inclusion of females in neuroscience studies would avoid inappropriate generalizations deriving from a sex bias (Prendergast et al., 2014; Zilkha et al., 2016).

A comparative approach would require a higher number of experimental subjects. Nonetheless, it would help to ameliorate the reproducibility crisis that biological sciences are currently facing. In the end, obtaining solid results could actually lead to a reduction in the total number of experimental animals used, since a lower number of independent studies would be required to reconfirm the results. Furthermore, even if multiple sexes, strains or species are not used in the same study, the important point would be at least to adopt the comparative approach as a *forma mentis*, to avoid inaccurate generalizations. If only one of many options can be employed in a study, for instance a single sex or a single strain, a rationale for that choice should be provided, based on general knowledge of biological processes or on previous experimental data.

Comparative psychology could be helpful in avoiding failure of behavioral experiments and useless employment of animals. For instance, a recent good example of adoption of a comparative approach is a study by König et al. (2020) in which voluntary physical activity and energy expenditure were measured in both sexes of 30 strains of mice, recording the parameters in both the light phase (photophase) and dark phase (scotophase) of the day. Interestingly, the study found that not all strains, and within some strains not both sexes, had light-dark cycles. If an experiment on circadian rhythms of physical activity has to be performed, choice of a strain with no light-dark variation would lead to failure of the experiment. A comparative knowledge of the different strain and sex characteristics can lead to the choice of the most suitable model for the target behavior, reducing the number of failed experiments and hence the total number of animals needed to obtain a valid result.

Comparative psychology can also help neuroscientists develop more animal-friendly behavioral testing options tailored for the species or strain of interest. For instance, in a recent CP study, the palatability of over 30 types of food was assessed in rats and significant differences among rat strains were found regarding food type preference (Dews et al., 2022). Such comparative data may help neuroscientists choose the best food reward in appetitively motivated learning tests, optimizing training and avoiding the necessity of food deprivation to motivate the animals.

A consideration of CP will also encourage the behavioral neuroscientist to have at least a working knowledge of their model's natural and evolutionary history. Where does their model organism live? Does it invade diverse environments or is it restricted to a narrow niche? Does it eat meat, plants, or both? Does the model organism live alone or in groups? Only with such information (i.e., noise), and acting upon it, can the behavioral neuroscientist ensure that their models make contact with the natural environment.

Finally, I would like to offer some recommendations. First, behavioral neuroscientists should acquaint themselves with CP. As I have endeavored to show in this opinion article, CP has much to recommend it for behavioral neuroscience in

terms of both research design and general overall strategy. Second, I would strongly encourage behavioral neuroscientists to collaborate with comparative psychologists in the design and interpretation of their experiments.

For readers interested in obtaining source material about comparative psychology, see Abramson (2018). This article contains information related to review articles, textbooks, history, and recommended papers. It was part of a special issue on comparative psychology appearing in the *International Journal of Comparative Psychology*. The remaining 12 articles in that special issue focus on methodology, applied aspects, and teaching, respectively (Abramson and Hill, 2018). In addition, there is a companion issue solely dedicated to the teaching of CP (Abramson, 2020). The 12 articles in that special issue describes over 50 inquiry-based activities. Both special issues can be downloaded free of charge.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Being friendly: paced mating for the study of physiological, behavioral, and neuroplastic changes induced by sexual behavior in females

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Paced mating in rats is an experimental condition that allows the evaluation of sexual behavior in a way that closely resembles what occurs in seminatural and natural conditions enabling the female to control the rate of the sexual interaction. In conventional non-paced mating tests, females cannot escape from male approaches, which may lead to an unrewarding overstimulation. Paced mating is an alternative laboratory procedure that improves animal welfare and has a higher ethological relevance. The use of this procedure contributed to the identification of physiological and behavioral factors that favor reproduction. Paced mating includes motivational and behavioral components differentiating quantitative and qualitative characteristics that are critical for the induction of the rewarding properties of mating. These positive consequences ensure that the behavior will be repeated, favoring the species' survival. Sexual reward is an immediate consequence of paced mating, mediated mainly by the endogenous opioid system. Paced mating also induces long-lasting neuroplastic changes, including gene expression, synthesis of proteins, and neurogenesis in sex-relevant brain areas. The interest in paced mating is growing since the complexity of its elements and consequences at different levels in a laboratory setting resembles what occurs in natural conditions. In this review, we analyze the classic studies and recent publications demonstrating the advantages of using paced mating to evaluate different aspects of sexual behavior in females.

KEYWORDS

paced mating, positive affective state (reward), motivation, opioids, rats

1. Introduction

Scientific studies about sexual behavior started in the late 18th century, with outstanding growth in the post-Second World War period. These early rodent studies mainly focused on behavioral elements and the rewarding properties of mating in males (Agmo, 2007a). At that time, mating tests were usually conducted in a standard cage where the male controlled the rate of sexual interactions (non-pacing, NP). When tested under that condition, female sexual behavior remained relatively stable until they showed rejection and avoidance behaviors, questioning whether sexual interaction was rewarding for females. Early experimental reports employing operant tasks demonstrated that sexually receptive females showed high motivation

to access a male. Females were trained to lever press to have access to a male with whom to mate (Figure 1A). The return latencies to the lever were inversely related to the amount of stimulation the females received. In particular, latencies were shorter after a mount alone than after an intromission and both post-mount latencies and post-intromission latencies were shorter than after an ejaculation, which generally takes place after several intromissions (Bermant, 1961; Bermant and Westbrook, 1966). The authors concluded that females could work on spacing the stimulation they received, making the sexual contacts positively reinforcing (Bermant and Westbrook, 1966). Reinforcement refers to the increase in the probability that a response will be repeated, while reward refers to the ability to elicit an approach behavior to an incentive. In the case of mating, the incentive a male or female induces approach behavior in the appropriate hormonal conditions see Paredes (2009, 2014) for a discussion.

Other studies failed to demonstrate that sexual behavior could be reinforcing for female rats. For example, when female rats were trained in a straight runway to interact with different stimulus animals, females in estrous ran faster than anestrous females to interact with sexually active males. However, the estrous females ran equally fast to interact with sexually active or passive males. The interpretation of these results was that mating was not reinforcing for female rats and that social interaction was the main reward (Bolles et al., 1968). There were clear differences in the methods used in the two studies. With the lever press, the females can indeed pace the sexual interaction depending on the type of stimulation they receive. In the alley running, females received sexual stimulation every 10 min. Therefore, they were not able to pace the sexual interaction. Moreover, since they could run to a sexually or passive male, social contact could confound the interpretation of the results. Aside from operant tasks, other methods such as crossing an electrified grid, partner preference, and conditioned place preference (CPP) have clearly demonstrated that sexual interactions under appropriate conditions are appetitive to female rats see Paredes and Vazquez (1999) for a review.

It was not until the development of a standardized method to allow the female to control the sexual interaction (paced mating; PM) that this type of question could be addressed systematically. One of the key advantages of PM, developed by Mary Erskine (1946–2007) in the 1980s (Erskine, 1985, 1987, 1989), is that it partially resembles what occurs in the wild. This methodology allows researchers to evaluate different aspects of female sexual behavior under laboratory conditions. Since the 1980s, the number of studies employing PM has significantly grown, making this method a valuable tool to increase our understanding of the motivational and rewarding properties of mating in females. The consequences of PM at the reproductive and neuroplastic levels have also been explored, undoubtedly opening a new field of study on sexual behavior in females, a topic almost ignored previously.

In the following sections, we will describe the behavioral elements of female sexual behavior in the rat to continue with a general description of PM. We will then briefly review studies demonstrating that PM induces a reward state mediated by opioids. We will also describe the long-term plastic changes in neurogenesis induced by PM. We will explore how PM is employed to evaluate pharmacological strategies and their consequences on female sexual behavior. Finally, we will briefly mention how PM can be combined with magnetic

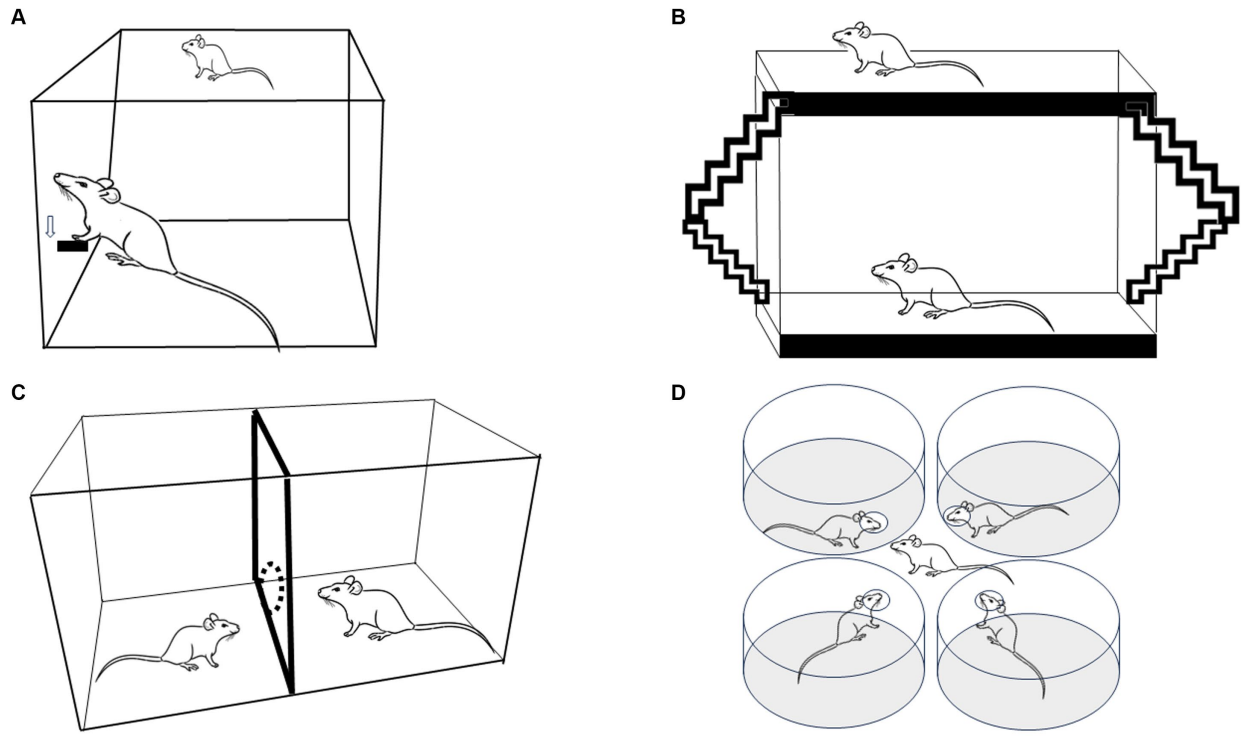

FIGURE 1
Four experimental strategies used to evaluate paced mating in female rats. **(A)** Lever press to obtain the presence of a sexually active male. **(B)** The bilevel chamber in which the females use the ramps to move up and down, changing the level in the arena. **(C)** The two-compartment arena in which females can pace the sexual interaction crossing through a hole that communicates both compartments. **(D)** Multiple partner preference choice.

resonance imaging to study the neural circuits controlling sexual behavior. Most of the reports were conducted in ovariectomized (ovx) and hormonal-primed rats. Otherwise, corresponding specifications will be noted.

2. Sexual behavior in the female rat

Observations in the laboratory, as well as in seminatural conditions, of female sexual behavior in rats identified that they display periodic solicitations, also called proceptive, appetitive, or paracopulatory behaviors, that influence the rate of mating by triggering mounts from the male (Erskine, 1989). Proceptive behaviors in the female rat include ear wiggling, hopping, darting around the male, and approaching and withdrawing movements. Proceptive behaviors have been considered as a motivational index and fluctuate along mating tests, i.e., they decay as sexual stimulation is extended but increase with the presentation of a novel sexual partner (Ventura-Aquino and Fernández-Guasti, 2013). The consummatory element, the lordosis posture, consists of the spinal dorsiflexion and elevation of the rump when the male mounts the female. This position enables the male to insert the penis into the female's vagina (vaginal intromission) and reflects the level of sexual receptivity of the female. Usually, receptivity is measured by calculating the lordosis quotient (LQ), which is the percentage of mounts that trigger the lordosis posture in the female. Additionally, there is a four-level scale of lordosis rating (0: absent to 3: exaggerated) depending on its degree (lordosis intensity, LI) (Hardy and DeBold, 1972). Usually, lordosis indicates the sexual responsiveness of the female, and it stays stable as long as the receptivity period lasts (Agmo, 2007b; Blaustein, 2009; Ventura-Aquino and Fernández-Guasti, 2013).

3. PM components

PM is a method very easy to set up since it does not require sophisticated equipment; it is inexpensive, and it is very reliable. The protocol requires only an observation cage (which can be made up of Plexiglass), females in the appropriate hormonal condition and a sexually trained male to pace the sexual interaction. Paced mating has been tested in cycling females in proestrus and Ovx hormone-primed subjects showing similar behavioral patterns with some differences depending on the hormonal scheme (Brandling-Bennett et al., 1999; Zipse et al., 2000). Most PM studies are conducted in an arena made of clear Plexiglass (40 × 60 × 40 cm), divided in two by a removable partition with one hole at the bottom, 4–7 cm in diameter, as described initially by Erskine (1985, 1989) and Figure 1C. In this way, the female can go back and forth between both compartments, whereas the male, which is usually bigger, cannot go through the hole when pursuing the female. When the male is about the same size as the female, he can be trained to stay in his compartment by gently tapping him on the nose (Erskine, 1985, 1989). Some studies have used a partition with two, three, or four holes to allow the female to move from one compartment to the other (Erskine and Hanrahan, 1997; Rossler et al., 2006; Coria-Avila et al., 2008; Snoeren et al., 2011). It has been reported that females who pace in a four hole mating cage show a shorter interintromission interval (Yang and Clemens, 1996) and a higher number of hops and darts (Ismail et al., 2008, 2009). In our experience (Camacho et al., 2009a), the sexual behavior parameters are the same if the females pace the sexual interaction through one or three holes. Moreover, both with one hole or three hole partitions, a positive affective reward state is induced, suggesting that pacing the sexual interaction through one or more holes has the same consequences on female sexual behavior as revealed by a subsequent conditioned preference for the mating chamber (Camacho et al., 2009a). Males appear to be more sensitive to the context in which copulation occurs, showing more behavioral differences if they mate in 1 or 4 hole pacing chambers (Ismail et al., 2008, 2009).

Different parameters can be obtained from paced mating tests. For instance, it is possible to calculate the percentage of exits (%E) after receiving mounts (%EM), intromissions (%EI), or ejaculations (%EE), but also the time that the female takes to return to the male side, defined as return latencies after mounts (MRL), intromissions (IRL) or ejaculations (ERL). The %E represents the female's ability to discern the stimulation received and correlates with the stimulation intensity, i.e., %EM is lower than %EI, and both %EM and %EI are lower than %EE." On the other hand, latencies to return are considered indicators of the female motivation to resume mating, i.e., shorter latencies reflect higher motivation and vice versa (Erskine, 1992; Coopersmith et al., 1996).

In traditional mating tests, rejection and aggressive behaviors towards the male can be observed when testing is extended and the females have received repeated stimulation or when the estrous period is finishing, reflecting a lowering of sexual motivation in females. Prolonged vaginal penetration increases the frequency of rejection behaviors and reduces the intensity and probability of subsequent lordosis (Bermant and Westbrook, 1966; Hardy and DeBold, 1972). Moreover, lordosis is inhibited after a brief period of intensive mounting by the male rat (Hardy and DeBold, 1972). These studies indicate that sexual interaction in the female rat has appetitive and aversive components. The aversive properties of mating are highly reduced when females pace their sexual contacts (Erskine, 1989; Paredes and Alonso, 1997; Paredes, 2009).

3.1. Other methods in which females control the rate of sexual stimulation

The original method described by Erskine, allowing the female to control the sexual stimulation received, significantly contributed to the understanding of the behavioral and physiological advantages of paced mating. Other groups modified the method, always allowing the female to control the sexual stimulation, to analyze different components of female sexual motivation. The modified methods are the bilevel chambers and the multiple partner preference/choice test.

In the bilevel chambers (51 × 70 × 15 cm boxes made of Plexiglas), the female can pace the sexual interaction by forcing the males to chase them while they run between levels, as shown in Figure 1B, Mendelson and Gorzalka (1987), Pfaus et al. (2000), and Pfaus et al. (1999). Moreover, animals can use ramps to move from one level to the other. The narrow chamber keeps the animals in a side-ways position, which is optimal for viewers. Measures registered in this method are anticipatory level changing before the introduction of the male (considered as sexual motivation parameter), latency and frequency of proceptive behaviors, lordosis quotient, lordosis intensity, and the number of rejections (Pfaus et al., 1999).

The multiple partner preference/choice test is another model in which females control the stimulation they receive during sexual interaction. The arena comprises four cylinders, each with a hole facing the central zone. Each cylinder contains a tethered sexually experienced male stimulus animal that can display sexual behavior but cannot leave the cylinder (Figure 1D). In this way, the female can choose to interact with any of the four males (Ferreira-Nuno et al., 2005). As occurs in paced mating tests, the percentage of exits after intromission or ejaculations is higher than the percentage of exits after mounts. The females spent a significantly longer time with a preferred male (Ferreira-Nuno et al., 2005), demonstrating again that females can discriminate and select sexual stimulation.

It should be clear by now that the ability of females to control or pace the sexual interaction can be observed using different methodologies including bar pressing (Bermant, 1961; Peirce and Nuttall, 1961; Bermant and Westbrook, 1966), mating with tethered males (Edwards and Pfeifle, 1983), bilevel chambers (Mendelson and Gorzalka, 1987; Pfaus et al., 1999, 2000), multiple partner preference/choice arena (Ferreira-Nuno et al., 2005) and paced mating with one hole (Erskine, 1989; Paredes and Alonso, 1997) or four holes (Yang and Clemens, 1996; Yang and Clements, 2000). Fewer rejections behaviors are observed using these methodologies, indicating reduced aversive stimulation. One important characteristic of these different methods used in laboratory conditions is that the females display behavioral patterns similar to those observed in seminatural or natural conditions (Barnett, 1975; Mcclintock and Adler, 1978; Mcclintock and Anisko, 1982), including solicitations, hopping and darting. The possibility to study the sexual interactions in natural or seminatural environments allows a more natural context and a fine-tuned analysis of this motivated behavior but PM remains the best option when rodents are tested in a laboratory condition.

Early classical studies demonstrated that the copulatory pattern of male and female rats in the wild and seminatural conditions is promiscuous. Estrous is synchronized among females, and mating occurs in groups with several males and females (Barnett, 1975; Robitaille and Bovet, 1976; Mcclintock and Adler, 1978; Mcclintock and Anisko, 1982). In group mating, males and females repeatedly change partners. For the female, there is no order sequence of stimulation they received. Several intromissions do not necessarily precede an ejaculation. A female can start mating with a male who has intromitted several times with other females and can receive an ejaculation without previous intromissions. In group mating, males and females experience the same amount of copulation (Mcclintock et al., 1982). Recent studies by Chu and Agmo (2015), Chu et al. (2017), and Hernandez-Arteaga and Agmo (2023) have evaluated sexual behavior in seminatural conditions. The arena consists of an open area with tunnels and burrows where 3 males and 4 female rats are housed together for several days. When tested under these conditions, females prefer a specific male, receiving more intromissions and ejaculations from this male than from other males (Chu and Agmo, 2015; Chu et al., 2017). It is clear that in group mating in seminatural or natural conditions, both sexes control the rate of sexual interaction, receiving a sufficient amount of stimulation from one or several members of the opposite sex, which makes sex rewarding and sexual behavior repeated in the future see Martinez and Paredes (2001) and Paredes (2009, 2014) for a discussion.

3.2. PM in mice

Although most pacing studies have been performed in rats, few studies have evaluated PM in mice following a similar methodology. Since size differences between male and female mice are not as evident as that observed in rats, in one study, the authors used a Plexiglass barrier (10 cm tall) to divide the male from the female side. The male was tethered and could not leave his side of the cage, while the female mice could jump the barrier to be with the male. Female sexual behavior was compared when they mated in the PM and NP paradigms. The authors found that, like female rats, female mice can pace the sexual interaction. They took longer to return to the male side after an ejaculation than after a mount or an intromission (Johansen et al., 2008).

In another study, the authors used a similar design as that used in rats with a Plexiglass partition with four holes at the bottom of sufficient size to allow the female mice but not the male to move from one side to the other. The authors used significantly smaller females, around 25 g, than males, around 45 g (Farmer et al., 2014). One important point that needs to be considered when evaluating PM in female mice is that proceptive behaviors are not so evident as in rats. Acceptances, defined as the percentage of approaches by the male that terminate in mounts or intromissions, are used as a measure of sexual receptivity in female mice (Johansen et al., 2008). Unfortunately, the behavioral, physiological, and neuroplastic changes induced by PM in mice have not been studied as much as in rats. This clearly represents an opportunity for future studies.

Another important contribution of the paced mating method is that it allows a clear dissociation of the appetitive components of female sexual behavior, leading to an understanding of the rewarding aspects of this behavior. Moreover, what we have learned about paced mating and sexual reward has also contributed to our general understanding of reward and conditioning. In the following section, we will describe the rewarding aspects of paced mating.

4. PM, reward, and neuroplasticity

4.1. PM and reward

A relevant contribution of PM studies is the demonstration of a reward state after mating in females, evaluated by the conditioned place preference (CPP) paradigm (Paredes and Alonso, 1997; Camacho et al., 2009a). CPP is conducted in an arena divided into three compartments, two with distinctive and contrasting characteristics and a neutral one in the middle. The CPP evaluates approach behavior towards environments associated with a previous reinforcing event (food, drug, sex). In the pre-test, the preferred compartment is determined on the basis of the times spent in each of the two lateral compartments. Later, the animal is placed in the preferred compartment without any reinforcing stimulus. On alternate days, the female mates and immediately thereafter, is placed in the non-preferred (reinforced) compartment. In this way, the state induced by mating is associated with the non-preferred compartment. After three non-reinforced and three reinforced sessions, the preference is tested again. The change of the original preference is widely accepted as an objective way to determine the induction of a positive conditioned affective reward state. In the case

of mating, the preference change by PM is similar to that induced by a dose of morphine (1 mg/kg) in both sexes (Camacho et al., 2009b; Arzate et al., 2011). Robust evidence indicates that PM is rewarding in both sexes. That is, males and females, need to control the rate of sexual interactions to find sex rewarding. More specifically, females need to mate in a pacing chamber, and males need to control the access to the female (Martinez and Paredes, 2001). However, some studies have described sexual reward in NP conditions. For example, Oldenburger et al. (1992) evaluated CPP in females after six conditioning sessions where mating occurred in the non-preferred compartment alternated with sessions where females stayed alone in the other compartment in counterbalance sessions (Oldenburger et al., 1992). Only a weak effect on conditioning was observed. The authors compared the time spent in the compartments in 5-min epochs, finding statistical differences only in the last 5-min period (Oldenburger et al., 1992). When mating occurs in the conditioning cage, the females can associate both the appetitive and aversive components of mating, reducing the effect of conditioning.

A study by Meerts and Clark (2007) reported that females develop CPP in NP conditions. Two experiments were performed. In experiment 1, females could pace or not the sexual interaction until they received 15 intromissions, including ejaculations. Both groups developed CPP. In experiment 2, one group of females received 15 paced intromissions with the same male, and another group received the same number of intromissions with different males. In this case, females developed CPP only when a single male provided the stimulation, but not when the male was replaced after the first ejaculation with a second one (Meerts and Clark, 2007). In a follow up study, they found that artificial vaginal cervical stimulation (VCS) induces CPP (Meerts and Clark, 2009). In fact, the importance of timing the stimulation to induce sexual reward in females was demonstrated by Jenkins and Becker (2003a). They lengthened the sexual stimulation in NP by retiring the male after each intromission to mimic the interintromission interval observed in PM conditions (Jenkins and Becker, 2003a). Females developed CPP in PM conditions when the male was removed to mimic the female preferred interval (Jenkins and Becker, 2003a). One possible explanation for the different results between our studies and those by Meerts and Clark is that the males used in their studies ejaculated after around 6 intromissions, and our males required around 10–12 intromissions to ejaculate. On average, their females received about 2 ejaculations, because their females received 15 intromissions before they were placed in the conditioning cage (Meerts and Clark, 2007). The postejaculatory intervals, at least two for each female, could reduce the aversive components of NP, enhancing the rewarding effects. Another possibility is that Long-Evans rats (used in the studies by Meerts and Clark) are more sensitive to the appetitive effects of mating than the Wistar rats used in our studies. In fact, when female Wistar rats mate in PM conditions with the same male until receiving 15 intromissions, a clear CPP is observed. However, no CPP is produced if the female mates in NP conditions with the same male (Camacho et al., 2009a).

Another critical aspect of PM is the amount of stimulation required to induce the reward state. In females, at least 10 intromissions (with or without ejaculation) are needed (Paredes and Vazquez, 1999; Martinez and Paredes, 2001; Camacho et al., 2009b). When PM is extended to around 25 intromissions, CPP is still present independently of the number of ejaculations received (Arzate et al., 2011). For males, 15 intromissions or ejaculation are required to induce CPP.

4.2. Reward state induced by PM is mediated by opioids

The reward state induced by PM is prevented by the systemic administration of naloxone, an opioid receptor blocker, in males (16 mg/kg; Agmo and Berenfeld, 1990; Agmo and Gomez, 1993) and females (4 mg/kg; Paredes and Martinez, 2001). Similarly, when naloxone is infused directly into the medial preoptic area (MPOA), the ventromedial hypothalamus (VMH) and the amygdala (AMG) of females, CPP is also blocked suggesting a central role of opioids in sexual reward (Garcia-Horsman et al., 2008).

We also evaluated if sexual behavior could induce a reward state of the same intensity as a morphine injection. One group of females was allowed to pace the sexual interaction before being placed in the non-preferred compartment. In alternate sessions, they received a morphine injection before being placed in the preferred compartment. A second group received the reversed treatment. Only the females placed in the originally non-preferred compartment after paced mating developed CPP, suggesting that paced mating induces a reward state of higher intensity than a morphine injection of 1 mg/kg. In the same study, we also demonstrated that females that pace the sexual interaction for 1 h continue mating and develop CPP. No CPP was observed in the females that mated for 1 h without pacing the sexual interaction (Arzate et al., 2011), further demonstrating the biological relevance of the female's ability to space the coital stimulation received during mating.

4.3. PM and neuroplasticity

We have also demonstrated that PM induces permanent neuroplastic changes in Ovx females hormonally primed with estradiol benzoate (EB) and progesterone (P). For example, a single PM session is enough to promote newborn cells in the granular layer of the accessory olfactory bulb (AOB), evaluated 15 days later. This effect was blocked by administering naloxone (4 mg/kg/i.p.), suggesting that opioids have an essential role in neurogenesis induced by PM (Santoyo-Zedillo et al., 2017). Subsequent studies showed that after four sessions of PM, one session per week, females showed more newborn cells integrated into the granular and the mitral layers of the AOB when they paced the sexual interaction compared to females that mated without pacing and to a control group. Moreover, after 10 PM sessions, one per week, the number of cells in the glomerular layer of the AOB and the granular layer of the MOB was higher compared to control and NP groups at day 45 (Alvarado-Martínez and Paredes, 2018; Portillo et al., 2020). These studies clearly indicate that PM induces long-term plastic changes that could explain this mating condition's behavioral and physiological changes (Bedos et al., 2018). To date, the functional implications and relevance of PM induced neurogenesis are still a matter of study.

5. Sensory pathways important for PM

Whenever a behavior induces a reward state, it is more likely to be repeated in the future, and in the case of mating, this eventually

impacts the species' survival. In this regard, genitosensory stimulation under PM is qualitatively and quantitatively different from NP. For example, PM intromissions are usually longer (616 ± 30 vs. 527 ± 30 msec) than in NP conditions, suggesting that PM intromissions are a more intense stimulus than NP intromissions (Erskine et al., 1989). Additionally, steroids hormones, i.e., EB and P increase the responsiveness to stimulation by enlarging the field of the pudendal and pelvic nerves, which corresponds to the cutaneous areas of the flanks, perineum, clitoral sheet, and the caudal reproductive and urinary systems, including vagina, cervix, and bladder (Komisaruk et al., 1972). Thus, the female's capability to discern the type and intensity of sexual stimulation received highly depends on the neural input. For example, a study evaluated PM in ovariectomized females 14 days after transection of pudendal (Pu), pelvic (Pe), or pudendal + pelvic (PuPe) nerves. Females were treated with EB for 7 days or with EB + P for 14 days. After treatment with EB, all groups (Pu, Pe, and PuPe) showed decreased pacing behavior compared to sham controls. When EB + P was administered, only the Pe and PuPe groups showed a reduction in pacing behavior (Erskine, 1992). These results suggest that P reduces the effects of autonomic nerve transection, increasing the threshold of the VCS favoring the return to the male side with a shorter latency. They also indicate that the afferent inputs from the vagina, cervix, and the surrounding skin via the pelvic and pudendal nerves to the spinal cord are relevant for the display of the pacing pattern in association with ovarian hormones, especially in combination with the well documented antinociceptive effect of P (Meyerson, 1967; Gilman and Hitt, 1978; Kim et al., 2012; Hornung et al., 2020). This combination of sensory stimulation and ovarian hormones allows the female to discern the stimulation they receive during mating and contributes to the physiological and behavioral consequences induced by PM.

6. Neuroendocrine responses induced by PM

6.1. Prolactin

The VCS received under PM is critical to trigger neuroendocrine responses. For example, in PM conditions, intromissions induce a twice-daily prolactin surge activating the luteal function and abbreviate the receptivity period favoring pregnancy. In NP mating conditions, more intromissions are required to generate the same physiological changes (Erskine and Kornberg, 1992). The prolactin release is correlated with the induction of pregnancy or pseudopregnancy independently of the mating condition. However, PM is more efficient in inducing this response because noradrenergic neurons convey genitosensory inputs to mating-responsive forebrain areas such as the medial amygdala that projects to the VMH where prolactin is released (Erskine and Hanrahan, 1997; Northrop et al., 2010). Additionally, the role of PM favoring reproduction is shown in intact females that mate under PM conditions which have bigger litters than females mated under NP conditions (Coopersmith and Erskine, 1994).

6.2. Progesterone and oxytocin

Mating under both conditions induces similar acute increases in progesterone (P) and 5 alpha-Androstane-3 alpha, 17 beta-diol (3 alpha-Diol), suggesting that PM and NP cause similar levels of stress (Frye et al., 1996). When PM was tested in combination with ovarian hormones, no effect on basal anxiety was found for sexual history, while an anxiolytic effect was found for progesterone (Arnold et al., 2019). Nevertheless, in the same study it was found that paced mating reduced anxiety after an acute stressor, suggesting that PM provides an increased resilience to stress (Arnold et al., 2019). When females mate in traditional mating chambers, an increase in anxiety-related behaviors is observed, without behavioral changes in the PM group (Nyuyki et al., 2011). In the study by Nyuyki and colleagues, anxiety-related behaviors were measured in the elevated plus maze and the black-white box tests after a 30-min mating test. Hormonal priming induced anxiolytic effects, compared to non primed females, when the females mated in PM. On the other hand, mating under NP abolished this anxiolytic effect of hormonal priming. Additionally, primed rats that underwent NP showed higher anxiety-related behaviors than primed rats that experienced PM. The same authors also showed that oxytocin (OT) is released in the paraventricular nucleus of the hypothalamus in the PM group but not in the NP condition. The administration of an OT antagonist partially prevented the anxiolytic effects of PM. These results indicate that PM triggers activation in the OT system that enhances anxiolysis by sex steroids that might facilitate the establishment of sexual reward, reducing the aversive components of mating through this protective-stress effect.

6.3. Dopamine

Dopamine (DA) is another neurotransmitter released during PM conditions, as evaluated by *in vivo* microdialysis. The authors placed a cannula in the *nucleus accumbens* (NAc) and monitored minute-by-minute DA levels during mating tests. They only found a peak of extracellular DA before the first intromission in the PM group. The authors proposed that DA is released in response to cues associated with predicting a rewarding state induced by PM compared with NP (Jenkins and Becker, 2003b). However, there is evidence that DA does not participate in the rewarding properties of sexual behavior. For example, DA antagonists do not block the reward state induced by sexual behavior in males (Agmo and Berenfeld, 1990) or females (Garcia Horsman and Paredes, 2004). In fact, it has been suggested that DA induces generalized behavioral arousal (Alcaro et al., 2007). Moreover, a series of studies evaluating the role of DA in reward by Berridge and Robinson have shown the DA does not mediate hedonic pleasure of reinforces, "DA systems appear necessary for wanting incentives but not for liking them" (Berridge and Robinson, 1998). Whatever the neuromodulator involved, it is clear, as demonstrated by several groups, that sexual behavior induces a reward state. Although opioids are the most likely candidates, DA and OT could also participate directly or indirectly through an interaction with the opioid system in the reward state induced by sexual behavior in males and females observed in rats (Miller and Baum, 1987; Agmo and Berenfeld, 1990; Hughes et al., 1990; Mehrara and Baum, 1990; Oldenburger et al., 1992; Paredes and Martinez, 2001; Kippin and van der Kooy, 2003; Harding and McGinnis, 2004; Meerts and Clark, 2007; Paredes, 2014), mice (Kudwa et al., 2005) and hamsters (Meisel and Joppa, 1994; Bell et al., 2010).

6.4. Gene expression

In addition to the endocrine responses, mating also modifies gene expression differentially according to mating conditions. A report evaluating the immediate early gene expression by FOS immunoreactivity (FOS-IR) 1 h after receiving 5 or 15 intromissions under NP and PM in rats found increased FOS-IR in brain areas relevant for reproduction, such as the MPOA, the VMH, and the bed nucleus of the stria terminalis (BNST) in both NP and PM groups compared with controls and females which received mounts only and those who stay in their home cage. In contrast, the posterodorsal medial nucleus of the amygdala (MePD) showed increased FOS-IR only in the PM group proportionally with the number of intromissions. The authors proposed that the MePD is a region that receives inputs from other areas and serves as a center to modulate behavioral and neuroendocrine responses induced by mating (Erskine and Hanrahan, 1997).

7. Behavioral pharmacology of paced mating

As mentioned, PM mating is a feasible way to evaluate neurobiological mechanisms of sexual motivation and reward in females. For this reason, its use is extended in preclinical trials to explore its validity in human conditions, mainly regarding sexual dysfunctions. In the following section, we will describe how paced mating has been used to evaluate different compounds and doses in combination with other methods as a valuable tool to study different aspects of sexual behavior and motivation. The growing interest in new pharmacological agents for treating female sexual dysfunctions, mainly associated with the motivational components, makes the PM method ideal for dissecting drug effects, especially those affecting mood and anxiety.

7.1. Psychotropic drugs and PM

A study evaluated sexual and anxiety-like behaviors after weekly administration (4 in total) of ketamine (10 mg/kg/i.p.) in PM conditions. Females in the ketamine group spent more time in the male's compartment. They showed a reduced percentage of exits after a mount and shorter latencies to return to the male side after an intromission compared with controls. However, the effects were attenuated by sexual experience (Guarraci et al., 2020). In addition, ketamine did not affect anxiety-like behavior in the elevated plus maze test. The authors proposed that ketamine elevated the pain threshold in females. This effect could diminish the aversive components during mating. They also evaluated if previous sexual experience could influence the effect of a single dose of fluoxetine or ketamine in female rats in PM. The fluoxetine group spent less time in the male's compartment and showed longer return latencies after ejaculations, whereas ketamine did not modify sexual behavior (Marshall et al., 2020).

Serotonin elicits bimodal effects on sexual behavior in females, depending upon the receptor subtype involved. For example, 5-HT_{1A} and 5-Ht_{1B} agonists inhibit proceptivity and receptivity in Ovx hormonally primed female rats, whereas 5-HT_2 and 5-HT_3 stimulation facilitate lordosis behavior and its antagonism provokes the opposite (Snoeren, 2019). However, most studies evaluating the role of different neurotransmitters in female sexual behavior have been done in NP conditions. As aforementioned, the mating condition is crucial since the effects could be other if the female controls or not the sexual interaction. For example, a study on the effects of the chronic treatment of paroxetine (10 or 20 mg/kg/p.o. 56 days) in Ovx hormonally sub-primed and fully primed females tested under PM showed no changes in any sexual behavior parameters for four 30-min sexual behavior tests (once a week). After day 21 of treatment, females also received weekly doses of the 5-HT_{1A}/5-HT_7 receptor agonist 8-Hydroxy-2-(dipropylamino) tetralin hydrobromide (8-OH-DPAT, 0.1 or 0.3 mg/kg/i.p.), alone and in combination with the selective 5-HT_{1A} antagonist WAY-100635 (0.3 mg/kg/s.c.). Sexual behavior was tested 30 min after those treatments to evaluate the possible 5-HT_{1A} desensitization by chronic paroxetine. The 8-OH-DPAT agonist reduced, in a dose dependent manner, proceptive behaviors in sub-primed and fully primed groups treated with vehicle and showed a right-shift dose–response curve in females treated with paroxetine, indicating receptor desensitization, whereas cotreatment with WAY100635 counteracted these inhibitory effects. The results indicate that chronic paroxetine treatment does not modify sexual behavior in females in PM, even after 5-HT1A desensitization (Snoeren et al., 2011).

7.2. Psychomotor active drugs and PM

The repeated administration of some psychoactive drugs facilitates the rewarding effects of mating (indicative of cross-sensitization). Studies showed conflicting results in female rats in PM tests. For example, a single dose of d-amphetamine (1.0 mg/kg, i.p.) increased the percentage of exits following mounts and intromissions. When rats received *d*-amphetamine chronically (1.0 mg/kg, i.p. daily for 3 weeks) and were tested 1 week after the final injection, they showed shorter latencies to return after mounts than controls (Afonso et al., 2009). Another report showed that females displayed more proceptive behaviors in a bilevel chamber test 21 days after the last of three doses of *d*-amphetamine (1 mg/kg, i.p. every other day). Results suggest that there is a cross-sensitization by *d*-amphetamine and sexual behavior in females after a chronic treatment that is not explained by increased locomotor activity since those effects are presented after a washout period. However, when *d*-amphetamine is infused in the NAc (40 µg/0.5 µL), there is a lack of effect in PM. In contrast, infusion into the MPOA (10 µg/0.5 µL) showed similar effects to those presented after an acute administration. After an MPOA lesion, i.e., females spend less time in the male's compartment and leave the male side more frequently after receiving a mount without affecting lordosis (Guarraci and Bolton, 2014). The authors proposed that d-amphetamine infused directly into the MPOA causes excessive dopaminergic activity and the inhibition of sexual behavior.

The effects of Methamphetamine (MA) on PM have also been evaluated since its use increases sexual activities, including those associated with high risks, especially in women. Ovariectomized and hormonally primed rats received three doses of MA (5 mg/kg/i.p./day). Four hours after the last MA injection, rats were tested in PM tests that lasted 25 min to avoid locomotor effects. Females in the MA group showed shorter MRL, ERL, and reduced %E after intromissions.

Females also displayed more proceptive behaviors and reduced rejection components. Moreover, their LQ and LI were higher compared with controls. Additionally, MA increased spinophilin protein expression (a dendritic spine density marker) in the medial AMG, suggesting neuroplastic changes in synaptic transmission. However, it is unclear if the plastic change is induced by sexual behavior itself because NP was not evaluated (Holder and Mong, 2010). To assess the possible cross-sensitization by MA, they evaluated PM in female rats 21 or 6 days after the last injection in two schemes of chronic MA (1 mg/kg/every other day for a total of 3 days, or 1 mg/kg/daily/for 12 consecutive days). Methamphetamine did not modify sexual behavior when tested after 6 or 21 days of abstinence. Results indicate that MA failed to induce a cross-sensitization with sexual behavior (Thibodeau et al., 2013).

Methamphetamine might enhance sexual motivation in females depending on its interaction with the excitatory dopamine receptor subtype 1 (DR1) and progestins in the MePD, a brain site where multisensory sexually-relevant stimuli and generalized arousal increase the incentive value for a sexual partner (Rudzinskas et al., 2019). It is proposed that MA induces DA release in the MePD that activates DR1. This activation favors the estrogen receptors (ER) translocation to the nucleus, increasing the progesterone receptors (PR) transcription in a ligand-independent manner. In this way, MA increases P sensitivity by up-regulating PR to facilitate sexual motivation even in subthreshold doses of P (Rudzinskas et al., 2019). The facilitatory role of P and its metabolites in establishing sexual reward induced by PM in females was previously reported after their i.v. administration (Gonzalez-Flores et al., 2004).

Another psychomotor active drug tested in PM is caffeine. A single moderate dose of this substance (15 mg/kg/s.c.) reduced the return latency after ejaculation and increased motor activity. In a partner preference test, females in the caffeine group also visited more times the male than the female, although the time spent in the male's compartment was similar to the control group. The results indicate that caffeine induces a general activation that might secondarily stimulate approach behavior to a sexual partner (Guarraci and Benson, 2005).

7.3. Prosexual drugs and PM

Paced mating has been used to evaluate drugs with potential effects on sexual activity in preclinical studies. For example, the phosphodiesterase type-5 (PDE-5) inhibitor, zaprinast, which increases genital blood flow in women, was tested in rats (Clark et al., 2009). They received one of three different doses (1.5, 3, and 6 mg/kg/i.p.) 20 min before testing. Zaprinast increased contact-return latency after ejaculation in a dose–response fashion without modifying receptivity. The authors proposed that since zaprinast enhances blood supply, it also increases vaginal sensitivity, which explains the increase in contact return latencies.

Another drug tested is PT-141, a melanocortin receptor agonist, to evaluate its effects on female sexual behavior. Different groups of females received doses of 50, 100, and 200 μg/kg/ml/s.c. of PT-140 5 min before PM tests (30 min duration) in unilevel or believer chambers. Females in the 100 and 200 μg/kg groups showed increased proceptive behaviors in both types of tests without affecting lordosis or pacing measurements. The authors concluded that PT-141 enhances solicitation in females, which indicates that the melanocortin central system is relevant for sexual motivation (Pfaus et al., 2004).

Unfortunately, no pharmacological studies have directly compared a particular drug's effects in both PM and NPM. This is important for future studies in which the pharmacological effects of a drug want to be evaluated on sexual behavior, considering the physiological and behavioral differences induced by PM and NPM.

8. PM under different animal models of human diseases

Some studies have used PM as a model to study sexual dysfunctions in women since it is possible to dissociate motivational and consummatory aspects after pharmacological treatments.

8.1. Pacing and nociceptive conditions

Vulvar pain is an underdiagnosed condition that affects around 10% of women and disrupts sexual function, but also causes high levels of distress and interpersonal difficulties (Farmer et al., 2014; Schlaeger et al., 2019; Chisari et al., 2021; Torres-Cueco and Nohales-Alfonso, 2021). For these reasons, different methods, including PM, have been used to study its physiopathological mechanisms. Farmer et al. (2014) evaluated if pain reduces sexual motivation in female mice using an arena divided into two compartments with four holes at the bottom to pace the sexual interaction. They used a model of inflammatory pain induced by injecting (1) zymosan A (0.5 mg/mL/10 μL, s.c.) into the genital area (center-posterior vulva or center-dorsal penile shaft) or in the hind paw, or (2) 2% λ-carrageenan (s.c. dissolved in 10 μL of saline) into the right cheek or the ventral tail. In that way, they evaluated different combinations of inflammatory pain inducers and areas. Four hours after injecting one of the compounds and confirming the induction of the nociceptive response, female sexual behavior was observed for 1 h under PM conditions. Females received fewer mounts in all groups with inflammatory pain, spent less time in the male's compartment, and displayed fewer proceptive behaviors, compared with pain-free controls, without affecting receptivity. These effects were reversed with the treatment of an anti-inflammatory (pregabalin) or a prosexual drug (apomorphine or melanotan II; Farmer et al., 2014). On the other hand, male sexual behavior was unaffected in all pain induced groups. These results indicate that there are sex differences in the incentive value of mating towards an aversive context, i.e., pain. In females, sexual incentive motivation is inhibited, whereas, in males, pain is overcome by mating.

In another report regarding pain-related conditions and PM behavior, females were implanted with an autologous endometriotic tissue in the intestine of two experimental groups of female rats. One group was Ovx and hormonally primed. The second group was maintained on natural proestrus, and both were tested 50 days after the implant under PM conditions. There were no differences in the percentage of exits or in the return latencies after mounts, intromissions, or ejaculations. However, the amount of endometriotic tissue implanted was positively correlated with the contact return latency following ejaculation. The authors concluded that endometriotic implants slightly modify the sensitivity to vigorous sexual stimulation (Clark et al., 2011). However, that study did not

corroborate the presence of vaginal hypersensitivity to confirm that subjects felt pain.

8.2. Pacing and hyperglycemic conditions

The administration of streptozotocin (STZ), a toxin that induces the death of beta cells in the pancreas, depletes insulin production, that causes hyperglycemia. When STZ is administered neonatally, insulin depletion is partial, with mild glucose elevation in the blood. If STZ is administered in adult rats, the deficit is even higher, inducing severe hyperglycemia. A study evaluated sexual behavior in rats using both STZ models under PM and NP conditions. The results showed a reduction of LQ in females with severe hyperglycemia tested in NP conditions, whereas, in the mild hyperglycemia group, there were no changes in the LQ or LI in NP and PM groups (Hernandez-Munive et al., 2019). However, pacing behavior was disrupted in the PM group since females did not exit the male compartment after receiving a mating stimulus. A possible explanation for this result is a sensory disruption induced by the persistent hyperglycemic state that leads to decreased neuronal activity in the lumbosacral dorsal horn in female rats treated with streptozotocin (Nakagawa et al., 2020). The number of aggressions (boxing, bites, and lateral postures) was higher in the NP condition, than in the PM condition. The authors proposed that the aversiveness of the NP condition favored rejection behaviors towards the male. The exogenous supplementation of insulin prevented aggressive behavior in females. The results suggest that the hyperglycemic state induces changes in vascularity and morphology of the vaginal tissue (Kim et al., 2006), neuropathy (Tripathi et al., 2016) and increases anxiety-like behaviors (Aksu et al., 2012). These factors together disrupt the execution of PM, increasing the aversive components of mating (Hernandez-Munive et al., 2021).

Most of the early studies done to evaluate the effects of different neurotransmitters or pharmacological compounds upon sexual behavior were done in traditional mating chambers where the male controls the sexual interaction. From the above described studies is clear that different compounds and doses modify sexual behavior depending if they were tested under PM or NP conditions. By using PM, the authors can reduce the aversive components of mating and increase the appetitive aspects of this behavior, dissociating the motivational aspects of mating. These conditions resemble what occurs in natural and seminatural conditions, and it is a better model to approach the study of sexual dysfunctions (Table 1).

9. Magnetic resonance imaging studies and PM

In recent years, imaging studies have been growing since this technique is non-invasive and can be used in longitudinal protocols to evaluate anatomical and functional changes over time. Manganese-enhanced magnetic resonance imaging (MEMRI) enables the evaluation of neural activity since manganese is an analog of calcium which enters and accumulates in neurons with their depolarization. Additionally, since manganese's paramagnetic properties enhance contrast in T1 weighted images, its accumulation is related to neuronal activation. Using MEMRI, our group evaluated the activity of several brain areas immediately after 1, 5, and 10 PM sessions (one test per week) in Ovx and hormonally primed female rats. The results showed no differences after the first PM test in all the groups. After the 5th PM test, the signal intensity increased in areas related to the sociosexual behavior circuit, such as the OB, the bed nucleus of the stria terminalis (BNST), the AMG, the MPOA, and the VMH. This increase continued at session 10. On the other hand, areas associated with the reward circuit (the NAc, the striatum, the hippocampus, and the VTA) showed no changes until session 10th. This was the first longitudinal study demonstrating that the sociosexual circuit is activated before and with a higher intensity than the reward circuit, and this activation is modified by sexual experience (Aguilar-Moreno et al., 2022). Further studies will evaluate if the motivational and consummatory aspects of sexual behavior activate different brain circuits.

10. PM and animal welfare

Increasing attention is being devoted by researchers to the issue of animal welfare among laboratory rodents and of refinement of behavioral procedures (Jirkof et al., 2019; Voikar and Gaburro, 2020; O'Malley et al., 2022; d'Isa and Gerlai, 2023). From the evidence described above it is clear that PM improves animal welfare compared to NP. As already described, PM favors reproduction by inducing a higher release of luteinizing hormone and prolactin (Erskine and Kornberg, 1992), increases the probability of the females getting pregnant, and those pregnant females sire more pups than females in the NP condition (Erskine, 1985, 1989). Additionally, while NP increases anxiety-related behaviors, PM does not (Nyuyki et al., 2011). Importantly, PM reduces anxiety after an acute stressor, suggesting that PM confers resilience to stress (Arnold et al., 2019). Moreover, PM induces a reduction in rejection and aggressive behaviors compared to NP (Erskine, 1989). Furthermore, females can pace the sexual interaction in a 1 h test or longer without an increase in aggressive and rejection behaviors, as is usually observed in NP tests (Arzate et al., 2013; Ventura-Aquino and Fernández-Guasti, 2013). Another essential advantage of PM is that, under this condition, sexual behavior is rewarding in male and female rodents (Martinez and Paredes, 2001). This reward state is mediated by opioids in males and females (Paredes, 2014). Together, these findings indicate that PM improves animal welfare compared to NP, representing a valuable refinement of rodent sexual behavior models.

11. Conclusion

The development of the friendly PM methodology was crucial to improve our understanding of the physiological and behavioral consequences associated with the possibility of controlling the rate of sexual stimulation. It also demonstrated that females could discriminate the intensity of the stimulation they received, either a mount and intromission or an ejaculation. It has also contributed to the study of the motivational and rewarding properties of mating in females. It can also be used to study long term plastic changes, neurogenesis induced by PM. The interest in PM is growing since the complexity of its elements and consequences at different levels makes this paradigm a valuable tool for studying physiological, behavioral, motivational, rewarding, and plastic changes in a laboratory setting resembling natural conditions. Moreover, PM is also employed to

TABLE 1 Differences between paced mating (PM) and non-paced mating (NP) in different parameters of female rats.

Parameter	Comparison	Interpretation	References
Litter size	More pups in PM than NP	PM favors reproduction	Coopersmith and Erskine (1994)
Prolactin surges induced by mating	Higher in PM than NP	PM induces prolactin release favoring reproduction	Erskine and Kornberg (1992)
Rejection behaviors	Higher in NP than in PM	Paced mating is less aversive	Erskine (1989)
CPP: reward state induced by PM	Consistently induced by PM under different conditions	PM facilitates sexual reward even when mating is extended	Paredes and Alonso (1997) and Arzate et al. (2011)
CPP: reward state induced by NP	Weak effect CPP only when males ejaculated after few intromissions	CPP not consistently induced Males ejaculating after few intromissions have not been observed by other groups	Oldenburger et al. (1992) and Meerts and Clark (2007)
Anxiety	PM does not increase basal anxiety, while NP increases anxiety-related behaviors PM reduces post-stress anxiety Steroid-primed rats exposed to PM show lower anxiety than steroid-primed rats exposed to NP	NP is anxiogenic, while PM is not PM confers resilience to stress PM, but not NP, allows progesterone-mediated anxiolysis	Arnold et al. (2019) and Nyuyki et al. (2011)
FOS immunoreactivity	Increase FOS-IR in the MPOA, the VMH and the BNST in NP and PM. Increased FOS-IR in the MePD only in PM	MePD modulates qualitative as well as quantitative aspects of PM	Erskine and Hanrahan (1997)
Neurogenesis Rostral Migratory stream Accessory olfactory bulbs Main olfactory bulbs	PM and NP induce more cells More neurogenesis 15 and 45 days after PM More neurogenesis 15 and 45 days after repeated PM	PM induces long-term neuroplastic changes mediated by the endogenous opioid system. PM and NP induce long-term neuroplastic changes when the stimulation is repeated.	Alvarado-Martínez and Paredes (2018), Bedos et al. (2018), and Portillo et al. (2020)

CPP, conditioned place preference; MPOA, medial preoptic area; VMH, ventromedial hypothalamus; BNST, bed nucleus of the stria terminalis; MePD, posterodorsal medial amygdala.

evaluate new pharmacological strategies to treat female sexual dysfunctions that could impact sexual health. However, we must cautiously extrapolate the results obtained in PM studies to the human clinical field.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

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

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Are mirrors aversive or rewarding for mice? Insights from the mirror preference test

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1. Introduction

Visual stimulation *via* mirror images has been examined among various animals, including fish, birds, rodents, monkeys, and great apes. Most of these species demonstrate social or aggressive behavior toward mirrors. Although mice have poor vision and predominantly use olfaction to gather information from the surrounding environment, visual stimulation through mirrors appears to be effective also in this species. Research investigating the effects of mirror exposure in mice found that the presence of mirrors has similar effects to the presence of cage mates. Restraint in a small holder induces hyperthermia (stress-induce hyperthermia: SIH) in mice but a restrained mouse surrounded by similarly restrained cage mates shows less SIH (Watanabe, 2015). A restrained mouse surrounded by mirrors instead of the cage mates also shows reduced SIH, suggesting that the images reflected by the mirrors are a substitute for conspecifics (Watanabe, 2016). However, there is mixed evidence on the effect of mirrors on mice. In a study on chronic mirror-image stimulation, Fuss et al. (2013) found that a mirror placed for 5 weeks in the cage of single-housed mice had no effect on anxiety and depression-like behaviors. Nevertheless, the presence of the mirror increased exploratory behavior, enhancing both rearing in the novel cage exploration test and head-dipping in the hole-board test. Conversely, pharmacological studies have used mirrors to induce anxiety in mice.

This suggests that mirrors have contrasting effects on mice. In the present article, we will examine mirror-based rodent behavioral tests and compare their individual characteristics to understand the effect of mirrors on mice. Moreover, we will describe under which conditions mirrors could be used as rewards. Indeed, mirror-based behavioral tests would be particularly useful for behavioral neuroscience research. Since the mirror reward does not require previous starvation or water deprivation, it would be an animal-friendly alternative to classical appetitively motivated learning tests employing food or water as reward. Refinement of current behavioral tests is important both to maximize animal welfare and to reduce stress-associated variability, hence improving reproducibility of scientific results (d'Isa and Gerlai, 2023).

2. Mirror chamber test to examine anxiety in mice

2.1. Design of a mirror chamber test

Various methods have been employed to measure anxiety in rodents, including the elevated plus maze, the light-dark box test, the conflict test and the social defeat test (see Belzung and Griebel, 2001; Parle et al., 2010). As a method alternative to the elevated plus-maze test, Toubas et al. (1990) invented the mirror chamber test, which consisted of a mirrored cube, open on one side, that was placed in a square Plexiglas box (Figure 1A). This cube (30 cm × 30 cm × 30 cm) was constructed from five pieces of mirrored glass (three side panes, a top pane, and a floor

pane) with a single open side. The mirror chamber was placed in a container box (40 cm × 40 cm × 30.5 cm). A sixth mirror was placed on the container wall and positioned such that it faced the single open side of the mirrored chamber. The container thus formed a 5 cm corridor surrounding the mirrored chamber. Mice (Balb/c) were placed in the corner of the corridor and allowed to move around the container for 30 min. Latency to enter the mirrored chamber was used as an index of anxiety. The authors injected the mice with different doses of diazepam, a known anxiolytic, and found that the latency decreased in a dose-dependent manner. This evidence indicates that the mirror chamber test is an effective tool in pharmacological studies. The authors claimed that the method was simple, non-punishing, rapid, and quantitative.

2.2. Behavioral indexes of anxiety

Alongside latency measures of anxiety, other methods included measuring the number of entries into the mirrored chamber and the total time spent in the mirrored chamber (Reddy and Kulkarni, 1997; Paterson et al., 2010). Toubas et al. (1990) measured the number of animals that exhibited a latency longer than 200s. Kliethermes et al. (2003) employed a modified mirror chamber without a ceiling, placed mice inside the chamber as start position, and used voluntary re-entry time (defined as the total time spent in the mirror chamber minus the initial latency to exit the chamber). These studies reported agreement between conventional latency measurements and alternative indices.

2.3. Control conditions

Various control conditions have been employed in mirror studies. For example, Toubas et al. (1990) used an inverted mirror chamber, where the corridors had mirrors (instead of the chamber) and found a significant difference in mean latencies between entering the mirror chamber (1039s) and the inverted mirror chamber (14s). The short latency in entering the inverted mirror chamber may reflect an avoidance of mirrored corridors. Similar findings were obtained by Lamberty (1998), who compared the latency between three chambers: a mirror chamber, a white chamber, and a gray chamber. Balb/c and DBA mice avoided these three chambers, while C57BL/6 mice spent a longer time in the gray chamber than in the white and mirror chambers. The author suggested that brightness may explain the avoidance of mirror and white chambers. In this case, the avoidance of mirrors would not derive from aversion toward the images of the mirror, but rather from aversion toward the strong light reflected by the mirror, related to mice's natural photophobia.

3. Mirror preference test

In a two-choice test of mirror preference (Sherwin, 2004), C57BL/6 mice were placed in an apparatus comprising two cages connected by a tunnel. They occupied the mirror cage 47.6% of the time demonstrating no significant differences between preferences for mirror and non-mirror conditions. Similarly, in a study of mirror preference, Fuss et al. (2013) observed for 5 min how much time C57BL/6 mice spent in the chambers. Consistent with Sherwin's (2004) findings, mice did not demonstrate any preference or aversion to the mirrors. More recent studies innovated the mirror preference test by using two successive tests rather than simultaneous choices of preference. For example, Ueno et al. (2020) used an apparatus that was divided into two areas (Figure 1B). After overnight exposure to a mirror in their home cages, the researchers measured time spent by C57BL/6 mice in the central empty area and in board areas for 20 min. Subsequently, the mirror was placed in the board area and the time spent in each area was measured again, thus examining mirror preference using two successive tests. The mice spent approximately the same amount of time in areas with and without the mirror, demonstrating a consistent lack of preference for, or avoidance of, mirrors.

Using a similar two-choice place-preference experiment design, Yakura et al. (2018) tested rats' preferences for mirrors. They utilized an elaborate apparatus with an empty chamber on one end and a mirror, a video-recorded image of a rat, or a still image of a rat at the opposite end (Yakura et al., 2018). The rats spent significantly more time in the mirror chamber and video-recorded image chamber than in their respective empty chambers. Analogously, Watanabe (2016) used a two-choice place preference apparatus (Figure 1C) to test how much time C57BL/6 mice spent in the mirror compartment vs. the compartment with an opaque screen, when they were observed for 10 min. Notably, 17 of the 24 mice demonstrated significant preferences for the mirror, contradicting the findings of Sherwin (2004) and Ueno et al. (2020).

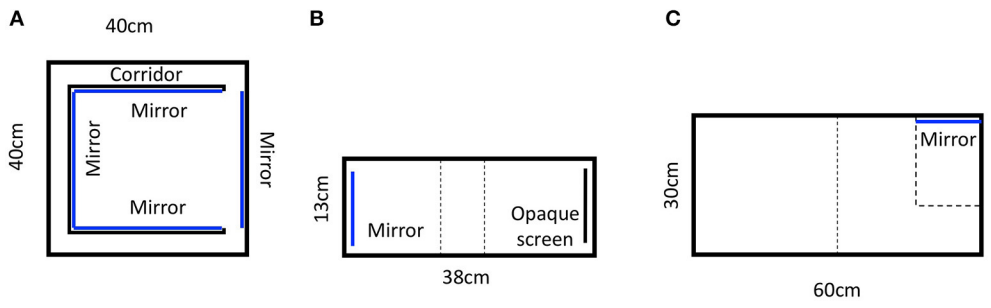

FIGURE 1
Apparatus of mirror experiments. **(A)** Toubas et al. (1990), **(B)** Watanabe (2016), **(C)** Ueno et al. (2020). Blue lines represent mirrors.

There are several procedural differences between these studies and Watanabe's (2016) study. Firstly, Sherwin (2004) repeatedly measured preferences every 10 min for 24 h, while Ueno et al. (2020) compared mirror and non-mirror preferences by using successive tests. Secondly, the illumination used by Watanabe (2016) was relatively low (11.0 lux). Unfortunately, the luminance of the apparatus in other experiments is not known. However, mice are naturally photophobic (i.e., they are averse to bright light) and this aversion may have masked a possible mirror preference. Another confound in the studies by Sherwin (2004) and Ueno et al. (2020) is that mirrors tend to appear much brighter than control objects in well-lit environments due to their reflective nature. This could have elicited an aversion to the mirrors, masking the mice's preference for them.

4. Mirrors vs. live animals

Watanabe (2016) also demonstrated that mice preferred mirrors over unfamiliar live mice, but not over familiar live mice (cage mates). Their aversion to unfamiliar mice, and similar preferences for mirrors and cage mates contrasts with the findings of Ueno et al. (2020). In this simultaneous presentation test with a mirror and a stranger (enclosed in a transparent cage), mice showed a preference for the stranger, demonstrating that they were able to discriminate between unfamiliar conspecifics and mirrors. It is important to note that in this experiment, the stranger mice were placed in transparent cages endowed with holes that were 1 cm in diameter. This allowed visual, tactile and olfactory stimulation. Nose contact and sniffing may have driven the preference for chambers with unfamiliar conspecifics. Since olfaction is a primary sense in mice, olfactory curiosity may be a stronger motivator than visual curiosity. This behavior has been observed in another study that employed a perforated partition to separate an unfamiliar conspecific from subject mice. The mice displayed approach behavior and spent longer time in its proximity (Harda et al., 2022). Conversely, Watanabe (2016) separated the subject mice *via* a transparent partition without holes, which excluded the possibility of proximal investigation through nose contact and sniffing of the unfamiliar conspecific. By removing the possibility of physical contact and confounds arising from olfaction, and focusing on visual stimulation, the method of Watanabe (2016) allowed to evaluate more accurately the ability of the subject mice to discriminate between the visual appearance of an unfamiliar conspecific and the visual image reflected in the mirror. Thus, this paradigm provides a less biased comparison between the effects of mirrors and unfamiliar conspecific exposure.

5. What is measured in the mirror chamber test?

In the mirror chamber test, mice avoided the mirror chamber, suggesting that the presence of the mirror had an anxiogenic effect. However, it is important to carefully assess and distinguish the causes of this anxiety. For instance, there is evidence that mirror placement could be crucial. Watanabe (2016) placed mirrors only on the side walls of the chamber, reflecting natural and realistic images of conspecifics, and this had an anti-stress effect on mice. In contrast, in the mirror chamber test mirrors were placed on the side wall, the floor and the ceiling. Mirrors placed in these positions reflect unusual and unnatural images of conspecifics, which may induce anxiety in mice. Another critical confounding factor in the mirror chamber test is the narrow corridor around the mirror chamber. Mice display a tendency known as thigmotaxis, or wall-hugging, which is a preference for narrow spaces. They have an aversion to open spaces and prefer to stay close to lateral barriers. The behavior of mice in the mirror chamber test is the summation of two factors: first, the effect of being exposed for the first time to unnatural images of conspecifics; second, aversion for open spaces and consequent thigmotaxis. Moreover, as the measurement of mirror preference (or aversion) is sensitive to procedural details, such as behavioral adaptation to the mirror, time of testing, and lighting conditions, these methodological details should be standardized for pharmacological testing. In addition to the procedural differences, difference in strain of experimental subjects also affects the results.

Importantly, under unbiased conditions, where these confounds are controlled by employing equally sized and shaped chambers, natural reflected images and low luminosity, mirrors appear to be rewarding for mice and have a positive effect on their affective state.

6. Mirrors as rewards

The reward value of a stimulus (i.e., its effectiveness as a conditioning stimulus) can be evaluated through a two-choice preference test. Research demonstrated that bright lights and unnatural reflected images are anxiogenic factors that can produce bias in the preference test and hence should be avoided. However, under unbiased conditions (natural reflected images and low luminosity), mirrors appear to be rewarding for mice, that show a clear preference for mirrors over opaque control objects (Watanabe, 2016). Additionally, mirrors showed an anti-stress effect on mice, as revealed by non-invasive infrared thermography assessment of stress-induced hyperthermia (Watanabe, 2016). Considering these results, it is possible that mirrors might be effective reinforcers in conditioning paradigms. Conditioning can be of two types. Respondent (classical or Pavlovian) conditioning features the establishment of an association between two stimuli (conditioned and unconditioned stimuli), whereas operant (instrumental or Skinnerian) conditioning features a contingency of three events, stimulus (discriminative stimulus), behavior (operant) and a reinforcer (d'Isa et al., 2011). Respondent conditioning can be assessed through the conditioned place preference (CPP) test (Tzschentke, 2007) and the conditioned place aversion (CPA) test (Schechter and Meechan, 1994). CPP and CPA have been used mostly in pharmacological studies but aversive state without drug injection has also been employed, for example, water-flood induced CPA in mice (Goltseker and Barak, 2018). To test the potential of mirrors as a reward in respondent conditioning, mice could be trained in a CPP apparatus with two chambers: on one side, a chamber with a vertically-striped wall facing a wall with attached a flat opaque object; on the other side, a chamber with a horizontally-striped wall facing a wall with attached a flat mirrored object (equal in size to the opaque object). The experimental subject is allowed to stay in one chamber on day

one and in the other chamber on day two. After repeating this conditioning procedure, the subject will undergo a test. In the test session, the two objects should be removed, along with the partition separating the two chambers, and mice will be allowed to move freely across the chambers. Preference between the vertically striped and horizontally striped chambers will provide an index of the rewarding value of the mirror. Indeed, non-pharmacological place conditioning procedures are particularly useful, since the drugs commonly used in the ordinal pharmacological CPP can interfere with memory and learning (Goltseker and Barak, 2018).

On the other hand, the reinforcing value of a mirror in operant conditioning can be assessed by measuring, for example, lever pressing to access a mirror. The strength of its reinforcing value should be measured by a progressive ratio schedule in which the number of responses required to receive reinforcement is gradually increased until the subject stops responding.

Mirror-based tests could become a new class of animal-friendly learning tests. Indeed, mirrors could be employed as animal-friendly reinforcers to study learning and memory processes. Investigation of mirror-induced conditioning could lead to the development of new behavioral tests that do not require punishment or prior stressful conditions, such as food or water deprivation.

Author contributions

SW conceived and wrote the manuscript, created the figures, and acquired funding.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Infrared thermography for non-invasive measurement of social inequality aversion in rodents and potential usefulness for future animal-friendly studies

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Infrared thermography is a method that detects thermal radiation energy and can measure the body surface temperature of animals from a distance. While rectal temperature has traditionally been used to measure animals' core temperature, thermal imaging can avoid the stress and potential rise of body temperature deriving from handling of the animals. Additionally, being non-invasive and contactless, thermal imaging allows free movement of the animals. The validity of this technique as a psychophysiological method has been proven in a series of stress-induced hyperthermia (SIH) studies of mice under social inequality conditions. Restraint in a holder elicits SIH in mice. A restrained mouse surrounded by freely moving cage mates displays increased SIH suggesting that social inequality enhances the stress. Social inequality can be examined also in unrestrained mice, in particular through unequal distribution of food. In this protocol, a food-deprived mouse is given a small piece of cheese, while its cage mate is given a large piece of cheese. This inequity causes SIH, suggesting social inequality aversion in mice. Thus, social inequality in different situations similarly increased SIH. Importantly, in future studies infrared thermography could also be used to evaluate emotional arousal states different from stress (for example to assess reactivity to rewards or in social and sexual preference tests). Moreover, the technique could be used to investigate also cognitive arousal induced by novelty. Indeed, infrared thermography could be a particularly useful tool for animal-friendly studies of cognition and emotion in rodents.

KEYWORDS

infrared thermography, stress-induced hyperthermia, inequality aversion, advantageous inequality, disadvantageous inequality

1. The mechanism of infrared thermography

Infrared radiation is emitted naturally from any object with a temperature higher than zero. The relationship between the infrared radiation and the surface temperature of an object is expressed by the Stephen–Boltzmann formula, using an ideal object called a black body. The radiation ratio of a black body is equal to 1.0; however, when infrared

thermography is performed on an animal, the radiation ratio of its body surface is estimated by matching the temperature recorded by an infrared thermometer to that obtained by a contact thermometer. Importantly, infrared thermography is non-invasive and contactless, allowing the animals to move freely without any disturbance during temperature measurement, which makes this method completely animal-friendly and particularly suitable for behavioral research. In the case of rodents, the temperature of the interscapular region is commonly used for measurement purposes. To obtain accurate measurements, it is best to remove the hair of the animals by shaving or use nude mice, as, although hair does not produce heat, it can maintain it (Fiebig et al., 2018). Since radiation travels in straight lines, measuring the intensity of thermal radiation using an acute angle reduces the radiation received. Therefore, although continuous long-term thermographic recording is feasible and can be applied to freely moving wild animals (Vinne et al., 2020), this angle dependency may result in data variability.

2. Analysis of social inequality aversion by stress-induced hyperthermia

Stress causes several autonomic responses, including changes in the heart rate, blood pressure, and respiration rate. Stress also increases body temperature, a phenomenon known as stress-induced hyperthermia (SIH; Bouwknecht et al., 2007). A variety of stressors induce hyperthermia, including being in a novel cage (Houtepen et al., 2011), physical restraint (Thornhill et al., 1979; Van der Heyden et al., 1997; Van Eijl et al., 2006), social threat (Keeney et al., 2001; Pardon et al., 2004), and fear conditioning (Marks et al., 2009). The rectal temperature has traditionally been used to measure the core temperature of animals (e.g., Van der Heyden et al., 1997); however, infrared thermography has been employed as a non-invasive alternative (Conley and Hutson, 2007; Hishimura and Itoh, 2009; Houtepen et al., 2011). Compared to rectal temperature measurement, thermal imaging can avoid the stress and the consequent potential rise of body temperature deriving from handling of the animals. Although stress induction is, by definition, not stress-free, it is important to employ stress-free methods for the experimental measurements. On the one hand, this represents a refinement of the experimental procedure, increasing animal welfare. On the other hand, when studying stress processes it is particularly useful to choose a measurement method that does not interfere with the variable of interest.

According to Nakamura (2015), the central mechanisms of SIH are as follows. Psychological stress activates two groups of neurons in the dorsomedial hypothalamus (DMH): the dorsal DMH neurons and the ventral DMH neurons. Dorsal DMH neurons send glutamatergic input to sympathetic premotor neurons in the rostral medullary raphe to drive brown adipose tissue (BAT) thermogenesis, whereas ventral DMH neurons send direct input to the paraventricular hypothalamus to activate the hypothalamus-pituitary-adrenal axis, releasing stress hormones. BAT is a specialized organ for rapid heat production. In mice, it is found mainly in the interscapular region, an area highly innervated by the sympathetic nerves (Robinson et al., 2016).

Body temperature commonly shows individual variations and is easily affected by environmental changes such as being in a novel experimental setting. To account for this, instead of using the absolute temperature as a dependent variable, the difference between the temperature at baseline and the temperature in the experimental condition can be used. Additionally, it is important to make sure that animals are well adapted.

3. Social inequality aversion in rodents

Humans seek to punish unfair behavior of others (Fehr and Gachter, 2002), indicating strong inequality aversion in this species. Inequality aversion has also been observed in primates (Brosnan and de Waal, 2014) and, to a certain extent, in dogs (Range et al., 2008, 2012). However, there have been many challenges in identifying social inequality aversion in other species (Oberliessen and Kalenscher, 2019) and there still are contradictory discussions on the subject. An important topic of discussion is the method used to measure aversion. Since aversion leads to the behavioral avoidance of its source and induces physiological stress, it can be measured both behaviorally and physiologically. The author has employed infrared thermography to measure aversion as an autonomic response in mice and obtained consistent results, which will be reviewed in the following paragraphs.

3.1. Social inequality in restraint stress

Placing animals in cylindrical holders induces restraint stress. Mice that were restrained in holders alone in the presence of freely moving cage mates (the social inequality condition) have been shown to exhibit a greater degree of SIH (Watanabe, 2015) compared to those that were restrained in the presence of other equally restrained cage mates (the social equality condition). The outcome of the first condition is indicative of social inequality aversion (**Figure 1A**), whereas the outcome of the second condition is indicative of social buffering.

Stress has a memory-enhancing effect on aversive experiences (Hashimoto and Watanabe, 2005; Miracle et al., 2006; Roozendaal et al., 2009), and Watanabe (2011) investigated this effect in mice under three social conditions. The experience of restraint stress enhanced the aversive memory of a floor that delivered an electric shock (the single stress condition). The aversive memory enhancement was reduced in mice that were restrained in the presence of similarly restrained cage mates and increased in the presence of freely moving cage mates. Corticosterone levels, which are a common biomarker of stress, were highest after restraint stress was applied with freely moving cage mates, and lowest after restraint stress was applied with restrained cage mates. These results are consistent with those of the aforementioned thermography experiment, which demonstrated that social inequality enhances SIH (Watanabe, 2015). This stress enhancement could be explained by the possibility of predation. Indeed, the social equality condition has a dilution effect against predation, whereas a restrained animal among freely moving conspecifics could easily be a target of predation.

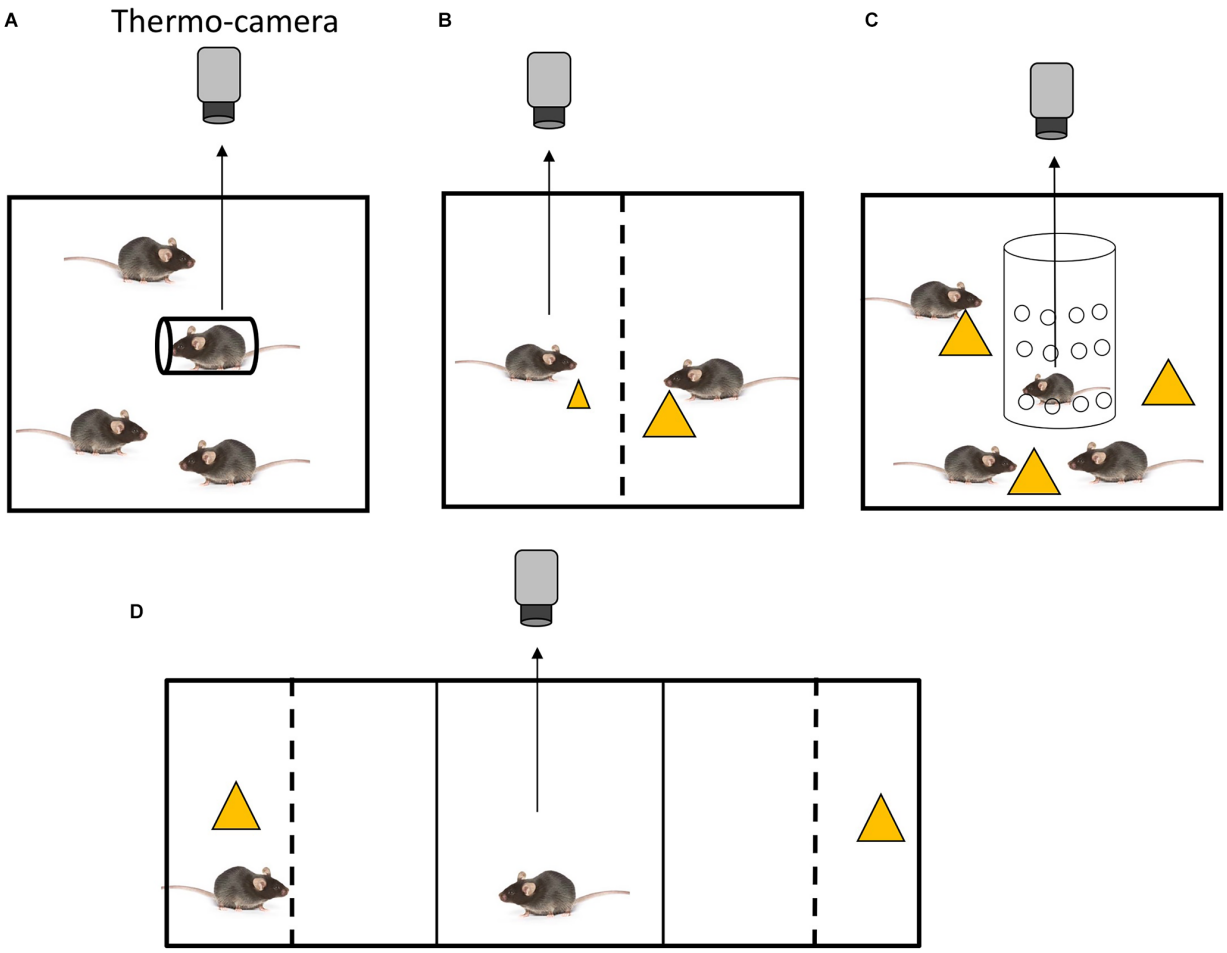

FIGURE 1
Experimental settings for the assessment of social inequality aversion in mice. **(A)** Setting to test inequality aversion to restraint stress (Watanabe, 2015). A mouse holder for blood sampling (diameter, 3 cm; length, 10 cm) was used to restrain the mouse. All animals were placed in cages (20 × 15 × 13 cm). **(B)** Setting to test inequality aversion in food distribution (Watanabe, 2017). An arena made of transparent acrylic plastic was employed. A central partition, a transparent acrylic plastic barrier with small holes (diameter: 0.5 cm; distance between holes: 0.3 cm), divided the arena into two equally sized chambers. Each chamber measured 19 × 11 × 20 cm. One mouse received a small piece of cheese, while the other one received a large piece. **(C)** Setting to test disadvantageous inequality aversion in food distribution (Watanabe, 2019). The experimental chamber was a 20 × 25 × 20 cm acrylic box. A transparent cylindrical tube (diameter, 10 cm; height, 20 cm) made of acrylic and featuring several holes (diameter of 0.5 cm with a distance of 0.3 cm between each hole) was placed vertically in the center of the experimental chamber. The test mouse was then placed in the cylinder. Yellow triangles indicate pieces of cheese. **(D)** Setting for the simultaneous recording of behavioral preference and body temperature (Watanabe, 2017). The apparatus used for the behavioral tests was a conventional conditioned place preference apparatus (MED ENV3015) with three compartments: two lateral compartments (16 × 13 × 12 cm) and a central compartment (6 × 13 × 12 cm). The central compartment was connected to the two lateral compartments through guillotine doors. A box made of gray acrylic plate was placed in each lateral compartment, so that the external appearance of the lateral compartments was identical. In each box, a transparent acrylic partition was placed 5 cm from the end wall to create a separated stimulus area. In this setting preference between a cage mate eating cheese (left) and a piece of cheese without cage mate (right) was tested. The subject mouse was placed in the center.

Social inequality is divided into two types: disadvantageous and advantageous. For humans, both types of inequality have aversive properties (Fehr and Schmidt, 1999). A restrained mouse surrounded by freely moving cage mates experiences disadvantageous inequality, whereas a free mouse surrounded by restrained cage mates experiences advantageous inequality. The body temperature of a freely moving mouse in the presence of restrained cage mates has been previously investigated, but no clear increase in temperature has been observed. Therefore, the existence of aversion to advantageous inequality in mice is questionable (Watanabe, 2019). A preference test between a compartment with a freely moving cage mate and that with a restrained mate did not show signs of avoidance of the condition with the restrained mate (Watanabe, 2012), but using a conditioned place conditioning protocol, the freely moving mice showed place aversion for the chamber where the mate was restrained (Watanabe, 2012), indicating that a certain degree of advantageous inequality aversion could be present in mice.

3.2. Social inequality in food delivery

Restraint stress is a well-established method for inducing stress in rodents, but it implies a high level of physical restriction. Alternative stress induction protocols allow analysis of social inequality aversion in freely moving mice. For instance, the aversive

property of inequitable food delivery has been demonstrated in several non-human primates (Yamamoto and Takimoto, 2012; Brosnan and de Waal, 2014; see Bräuer et al., 2009, and Sheskin et al., 2014 for contradictory discussion). The body temperature of mice has been examined under social equality and inequality conditions of food delivery in a test chamber with two compartments, each containing a mouse (Watanabe, 2017). In the equality condition, the same amount of food (cheese) was provided to two similarly food-deprived mice. However, in the inequality condition, different amounts of food were provided to the two food-deprived mice (**Figure 1B**). An increase in body temperature was observed in mice that were given a small piece of cheese while their cage mate received a large piece. Thus, this finding indicates that social inequality in food delivery leads to SIH. Interestingly, when one mouse was given laboratory chew, which is considered to be less preferable, and the other was given cheese, which is considered to be more preferable, the former did not exhibit SIH. Hence, mice appear to be sensitive to quantitative, but not qualitative, inequality. On the contrary, capuchin monkeys showed inequality aversion when they received a cucumber (non-preferred food) while their counterparts received grapes (preferred food; Brosnan and de Waal, 2014). However, because the relative value of these foods might differ between mice and capuchin monkeys, it is premature to conclude that qualitative inequality aversion does not occur in mice.

Analogously to the restraint experiment, when a previously food-deprived mouse is tested in a situation in which it does not receive food and it is surrounded by cage mates that are consuming food (disadvantageous inequality condition), it has been shown to exhibit SIH (Watanabe, 2019; **Figure 1C**). When a mouse receives food while being surrounded by food-deprived cage mates that do not have access to food (advantageous inequality condition), an increase in body temperature has been observed, although it was not significantly higher than that found in the equality condition. This increase could be explained by the fact that the eating mouse was confined in a smaller area of the arena. The freely moving cage mates could not physically access the eating mouse, which was confined in an area delimited by an acrylic cylinder, but they could gather around the acrylic cylinder wherein the subject was eating cheese. This behavior of the freely moving cage mates might have caused in the eating mouse a low stress deriving from the risk of food pilferage. This behavior of the freely moving cage mates might have caused in the eating mouse a low stress deriving from confinement, which could explain the small increase (not significant) in body temperature. In summary, disadvantageous inequality aversion was observed in mice in the presence of both unpleasant and pleasant stimuli (restraint and food, respectively), while advantageous inequality aversion was not. Notably, pre-feeding of the test mouse has been shown to attenuate SIH under disadvantageous conditions (Watanabe, 2019). Therefore, the sight of cage mates that are eating does not seem to have an aversive effect on pre-fed mice with limited access to food. These findings indicate that, when a mouse is exposed to conspecifics that are eating while its access to food is limited, it may perceive the situation as a potential depletion of food, which leads to an aversive effect. However, pre-feeding the mouse reduces this aversive effect.

Additionally, Oberliessen et al. (2016) examined inequality aversion in rats using a T-maze choice paradigm, which showed a preference for equal food delivery compared to unequal delivery. Thus, the rats involved in this study also showed inequality aversion.

4. Contradiction between behavioral preference and autonomic response

Thorndike defined satisfaction as "that animal does not nothing to do avoid, often doing something which maintains or renews it, and the annoying state as that animal does nothing to preserve, often doing something which puts an end to it" (Thorndike, 1911, page 2). Behavioral aversion to social inequality is measured as the time spent in two compartments (Watanabe, 2017). In an experiment performed using a two-choice apparatus, the test mice were able to observe the content of two compartments, one containing a cage mate eating cheese and one containing cheese alone (**Figure 1D**). Due to the presence of the partition, the subjects could not physically access the cage mate or cheese. Test mice spent more time in a compartment with a cheese-eating cage mate than in a compartment that included either cheese alone or a cage mate alone. This finding suggests that disadvantageous inequality may have a "satisfactory effect" in the sense of Thorndike's definition.

In a second experiment performed in the same apparatus, behavioral preferences and body temperatures were simultaneously recorded. As in the previous experiment, mice spent more time in the unequal condition compartment (observing a cheese-eating cage mate) and an increase in body temperature was also recorded. Thus, social inequality induced both an aversive autonomic response (SIH) and an approaching behavior, potentially indicating satisfaction. The sight of mice engaged in eating behavior has informative value regarding the availability of food resources for the non-eating mice, leading to interest and approach behavior despite the fact that the sight itself induces stress.

Observing conspecifics in pain constitutes another type of inequality paradigm. Approach behavior towards conspecifics in pain may have several explanations. Watanabe (2012) reported that mice spent more time in the compartment where there was a cage mate injected with a small amount of low-dose formalin, an irritant compound, in its paw formalin compared to the time spent in the compartment with an intact cage mate. Langford et al. (2010) also reported that mice placed in the presence of a cage mate confined in a container spent more time with the cage mate if it was in pain than if it was without pain. Thus, the injured cage mate had a satisfactory effect on the subjects according to Thorndike's definition. However, approaching a cage mate may also indicate a kind of rescue behavior, information-seeking behavior about possible danger, or even, although less likely, schadenfreude (a rewarding effect deriving from the observation of the conspecific in pain). Approaching an injured conspecific may also be dangerous because of possible infection. In fact, after mice are primed with cadaverine, a compound with the odor of decomposed animal tissues, they have been shown to avoid conspecifics that exhibit sickness behavior (Renault et al., 2008). Approach behavior could also be a response to the ultrasonic vocalizations of the mouse in pain (Ko et al., 2005). In addition, subordinate mice were shown to

approach dominant mates in pain more than non-dominant mates without pain, whereas dominant mice did not approach subordinate mates in pain (Watanabe, 2012). Hence, social rank order affects approaching behavior to conspecifics in pain. Interestingly, human schadenfreude is also sensitive to social rankings (Feather, 2008).

Although mice in the aforementioned study were shown to approach the formalin-injected cage mate, a conditioned place preference test with formalin-injected mates found conditioned aversion to the compartment associated with the formalin-injected mate (Watanabe, 2012). Therefore, the choice preference and conditioned place preference tests yielded contradictory results. A possible explanation could be that in the conditioned place preference, the compartment previously associated with an injured cage mate no longer held informative value (hence losing the possibility to attract the interest of the test mice), while the memory of the aversive value of the event would still be present, leading to conditioned place aversion. However, further studies are required to clarify this possibility. Combining the recording of physiological indices with behavioral testing provides a new window for studying social inequality aversion, and thermography is a promising tool for such studies.

5. Thermography in other animals

Infrared thermography has been used to study emotional responses in a wide variety of animals, including macaques, chimpanzees, marmosets, dogs, cats, rabbits, pigs, horses, cattle. and sheep (Travain and Valsecchi, 2021). The advantages of thermography, namely the simultaneous recording of multiple individual animals without disturbing their behavior, make it a valuable new tool for animal research. Indeed, a promising area of research is the recording of wild animals. For instance, Heintz et al. (2019) measured the temperatures of wild chimpanzees in Budongo Forest, Uganda, when exposed to vocalizations of conspecifics. Another promising area of research is animal welfare of farm animals (Mota-Rojas et al., 2021), as demonstrated by a study conducted by Cannas et al. (2018) who used this method to measure freely moving sheep. As previously mentioned, although infrared thermography measurements are angle-sensitive, the post-hoc selection of recorded data enables to provide reliable results.

6. New directions: potential of infrared thermography as an animal-friendly method to study rodent cognition and emotion

Psychological arousal can be divided into cognitive arousal (associated with cognitive processing and attention) and affective arousal (associated with emotional experience). We have shown, in the previous paragraphs, that infrared imaging can detect changes in the level of psychological stress, which is a type of affective arousal.

Although the DMH-BAT system is a thermoregulation system driven by stress, alternative mechanisms, as increase of heart beat and blood pressure, can also lead to increase of body temperature, and infrared thermography could hence be used to evaluate also psychological arousal states different from stress in future studies. Mice are neophilic, i.e., they are naturally attracted by novelty. Cognitive arousal can be elicited in mice by presentation of a novel stimuli vs. a familiar stimulus. Affective arousal, on the other hand, can be elicited by delivery of a reward or other emotionally salient stimuli.

It would be interesting to test infrared thermography in cognitive and affective essays unrelated to stress induction and verify if this technique is able to detect significant thermal changes during interaction with the test stimulus in comparison to interaction with the control stimulus. Several behavioral tests would be suitable for this type of comparison. Regarding cognitive arousal, thermal imaging could be used to measure thermal changes during exploration of novel vs. familiar objects in the novel object recognition test (d'Isa et al., 2014) or during exploration of novel vs. familiar environments in the hole-board test (d'Isa et al., 2021). Concerning affective arousal, infrared thermography could be employed in tests for reactivity to rewards, in the social preference test (Lammert et al., 2018; Gu et al., 2022), in the sexual preference test (Linnenbrink and von Merten, 2017; Nomoto et al., 2018; Guarraci and Frohardt, 2019) or even in social communication studies, for example to evaluate reactivity to conspecific ultrasonic vocalizations in the two-choice vocalization playback test (Asaba et al., 2015).

Researchers have recently highlighted the importance of employing animal-friendly tests in behavioral neuroscience, underlining how these tests would improve both animal welfare and validity of scientific results (Voikar and Gaburro, 2020; d'Isa and Gerlai, 2023). Indeed, infrared thermography could be a very useful tool for animal-friendly studies of cognition and emotion in rodents, which makes it a particularly promising method for behavioral neuroscience.

Author contributions

SW conceived, wrote, and revised the manuscript and created the figures.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The rough-and-tumble play of rats as a natural behavior suitable for studying the social brain

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Play fighting, the most commonly reported form of social play, involves competition to gain an advantage (Aldis, 1975), but there are two features that make it different from serious fighting. First, it is highly pleasurable and associated with a positive affective state (Vanderschuren et al., 2016). Second, the competition is moderated by cooperation, ensuring that the interactions have a degree of reciprocity or turn taking between partners (Palagi et al., 2016a). That is, the player with the advantage may voluntarily relinquish it, thus allowing a role reversal to occur, with the original defender gaining the opportunity to become the attacker (Pellis and Pellis, 2017). Thus, although actions performed during play fighting can be accurately described as involving attack, defense, and counterattack, the context of their use should not be confused with that of aggression. For this reason, we will refer to this kind of play as rough-and-tumble play (RTP), to highlight the cooperative aspect of these interactions. Nonetheless, competition during RTP can create ambiguity as to whether a partner may be taking unfair advantage of the situation, with effective communication being important to avoid the risk of escalating to aggression or to partners being ostracized if they play too roughly (Palagi et al., 2016b). Creating and resolving ambiguity, which requires balancing competition and cooperation, also provides a vehicle by which juveniles and adolescents can train socio-cognitive skills (Pellis and Pellis, 2009, 2017).

Laboratory rats have been an important model species with which to study the neurobiology of RTP (Siviy and Panksepp, 2011; Siviy, 2016; Vanderschuren et al., 2016). As shown in Figure 1, RTP in rats involves competition to gain access to the partner's nape of the neck, which is nuzzled with the snout if contacted (Pellis and Pellis, 1987; Siviy and Panksepp, 2011). A variety of tactics are used to attack and defend the nape, including launching counterattacks following a successful defense (Himmler et al., 2013; Pellis et al., 2022). Rats are a particularly good model species for studying RTP, as this behavior not only differs from serious fighting in the ways described above, but also because serious fighting involves attacking other body targets, namely the flanks and rump, which are bitten if contacted (Blanchard et al., 1977; Pellis and Pellis, 1987). Consequently, it can be readily discerned when a playful encounter escalates to serious fighting, as the aggressor switches from attacking the nape to biting the partner's posterior (Stark and Pellis, 2020).

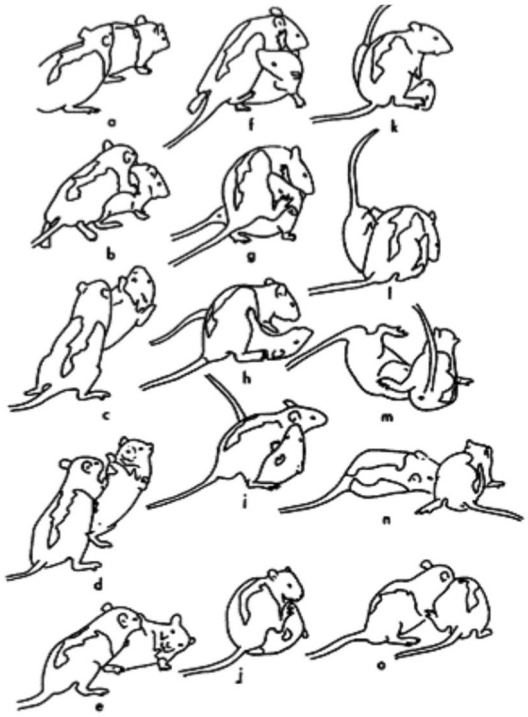

FIGURE 1
A sequence of play fighting is shown for a pair of juvenile rats. The rat on the left approaches the partner **(a)**, and reaches toward its nape from the rear **(b)**, but before contact can be made, the partner rotates around its longitudinal axis **(c)** to face its attacker **(d)**. By moving forward, the attacker pushes the defender onto its side **(e)**. The defender then rolls over onto its back as the attacker continues to reach for its nape **(f–h)**. Once in the supine position, the defender launches an attack on its partner's nape **(i)**, but fails due to its partner's use of its hind foot **(j,k)**. Eventually, the rat on top **(l)** is pushed off by the supine animal **(m)**, which then regains its footing **(n)**. The original defender then lunges toward its partner's nape **(o)**. (Reprinted from Pellis and Pellis, 1987, with permission).

In laboratory rats, RTP begins to emerge in the third week after birth, peaks in occurrence between the fourth and fifth week and then declines with the onset of puberty, but continues into adulthood, albeit at a lower level (Thor and Holloway, 1984; Pellis and Pellis, 1990, 1997). While the motivation to play can be modified by early experiences mostly derived from the mother (Parent and Meaney, 2008; Van Hasselt et al., 2012), once play begins at around 17 days of age, it takes about 10 days for the repertoire of tactics used during play to mature fully and this maturation appears to be little influenced by rearing experiences (Himmler et al., 2015).

Neither the age-typical changes in the frequency of RTP nor the availability of the full behavioral repertoire used during RTP depend on the cortex, but cortical systems, especially those of the prefrontal cortex, are critical to allow rats to modulate aspects of their playful responses, depending on their partner's actions and their identity (Pellis and Pellis, 2016). Even though both sexes and all strains of rats thus far studied share the same basic play repertoire, there are subtle differences that can be informative. For example, under some rearing and testing conditions, males initiate more nape attacks (Thor and Holloway, 1984), and in early adulthood are more likely to use defensive tactics that makes their RTP seem rougher (Pellis, 2002). Similarly, there are differences across strains in the relative frequency of nape attacks and in the use of defensive tactics that lead to evading contact or promoting close-quarter wrestling (Himmler et al., 2016). These differences have provided valuable tools for using sex and strain differences to identify the roles of specific neural systems and neural networks in regulating particular aspects of play (Siviy, 2020; VanRyzin et al., 2020).

Given that RTP is naturally occurring, no experimental training is needed to teach the animals to play. Moreover, as rats have a degree of sophistication in their RTP that is comparable to that of many primates and other social mammals (Pellis and Pellis, 2017), they are an ideal laboratory species to study not only play, but also, by extension, aspects of the social brain that make play possible. For example, the development of the medial prefrontal cortex (mPFC) and associated socio-cognitive skills is influenced by the experience of RTP with peers in the juvenile period (about 28–40 days post birth; Bell et al., 2010; Baarendse et al., 2013; Schneider et al., 2016), and the mPFC has a crucial role in coordinating actions with partners as juveniles and as adults in both playful and non-playful social interactions (Bell et al., 2009; Van Kerkhof et al., 2013; Himmler et al., 2014; Stark and Pellis, 2020). That is, social play is a valuable window into the social brain. Two recent developments illustrate the opportunities provided by the study of play in rats.

Individual differences and the drivers of play

Even within members of the same sex and same strain, not all individuals play to the same degree—some rats consistently play more than others (Lampe et al., 2017; Lesscher et al., 2021). Such individual differences provide an opportunity to refine the search for the neural mechanisms that regulate play. For instance, attack and defense during RTP tend to involve independent mechanisms (Himmler et al., 2016). Within a strain, high players not only initiate more nape attacks, but also preferentially use a different suite of the rat-typical defensive tactics than do low players (Pellis et al., 2022). As the use of ultrasonic vocalizations (USV) can be critical for communication during play, facilitating role reversals and avoiding escalation to aggression (Kisko et al., 2017; Burke et al., 2020), rats with different styles of RTP may need to modify their use of such calls (Pellis et al., 2022) to play together effectively, making RTP in rats a useful window into subtle social communication processes.

There is strong evidence that the difference in launching nape attacks is linked to differences in mesolimbic dopamine activity (Vanderschuren et al., 2016; Siviy, 2020). That mesolimbic dopamine activity does not just affect the launching of nape attacks, but also the motivation to engage in play, has been shown by operant conditioning methods, in which dopamine manipulations affect how hard rats will work for the reward of access to a playmate (Achterberg et al., 2016). While opioid systems have been implicated in the rewards derived from engaging in play (Vanderschuren et al., 2016; Achterberg et al., 2019), exactly which neural circuits are associated with what types of playful actions and styles of play remains to be investigated. In addition, how cortical and subcortical neural circuits that are known to be involved in regulating affective and aversive USV (Brudzynski, 2013) are modulated to accommodate different styles of play remains to be determined. However, attempts to explain the neural mechanisms associated with individual differences in RTP have started to emerge in the literature (e.g., Reppucci et al., 2020).

Group dynamics and partner choice

Initial studies of play in rats involved observing them in their home enclosures with the whole litter present to assess the occurrence of RTP (Baeninger, 1967; Meaney and Stewart, 1981; Pellis and Pellis, 1983). Such a collective paradigm, although naturalistic, makes testing the effects of specific treatments on play nearly impossible. Consequently, a dyadic paradigm was developed—rats are tested in pairs in an enclosure to which they have been habituated following a period of social isolation to increase their motivation to engage in play (Panksepp and Beatty, 1980; Panksepp, 1981). This dyadic paradigm has now become the most widely used experimental paradigm for testing RTP in rats (Pellis et al., 2022). Variations on the theme include the length of pre-test social isolation and whether experimental rats are tested with a same condition partner, an untreated partner, or both. In addition, rats can be partnered either with a familiar rat or a stranger. As the rats in the dyadic paradigm are tested for a fixed duration (5–20 min being most common), the effects on both the overall amount of play, as measured by the number of nape attacks launched, and the style of play, as measured by the frequency of use of the different defensive tactics, can be compared between experimental and control pairs. This level of control is especially important for pharmacological manipulations, as the animals need to be tested when the drug reaches its peak effects on the brain (e.g., Field and Pellis, 1994; Achterberg and Vanderschuren, 2020). However, the downside to the level of control achieved by the dyadic paradigm is that the rats lose the ability to choose their play partner, as it is the experimenter who selects the partner.

Not all partners are equally attractive as play mates (Holloway and Suter, 2004; Pellis et al., 2006). When rats are tested in groups, in which multiple partners are available, play is not distributed evenly. Rather, some partners are favored over others, and this is not simply a by-product of which of the animals are in closest proximity—rats will leave the company of animals in one part of the enclosure and travel to the other side to initiate play with a particular animal (Pellis et al., 2022). That is, some potential play partners are preferred over others. Experimental manipulations may alter the ability of rats to discriminate between partners (Pellis et al., 2006), and this may go undetected in the standard, dyadic play paradigm, in which interaction with only one partner is possible, potentially leading to the false conclusion that the experimental manipulation has no effect as the amount of play is the same as that of the control. Thus, multi-animal paradigms are needed to offset the disadvantages that have come with the advantages gained from the dyadic paradigm. Indeed, more sophisticated methods are becoming available for continuous recording and scoring of behavior in a home cage (Greico et al., 2021). A combination of dyadic testing, enabling more control in how a particular rat deals with a particular partner, and home cage, group testing, allowing the animal to exercise control over with whom to play and where, will capture the advantages of both approaches, and so assess a deeper analysis of the effects of treatments on social behavior.

Combining naturalistic observations with experimental manipulations

As indicated by the two examples above, so-called outliers in naturally occurring behavior like RTP can be highly informative about underlying biological processes, rather than, as is typically the case in many contrived experiments, being viewed as noise that interferes with the interpretation of results and future replication. However, while outliers can focus our attention on phenomena that may be missed when comparing group means, some degree of experimental control over the behavior may be needed to identify what is most relevant to the animals. That is, naturalistic observations and experimental manipulations can be used together in an iterative manner. An example will illustrate this synergy.

In a group setting, rats preferentially play with particular partners (Pellis et al., 2022), but what makes one partner more attractive than another? As indicated above, some rats are more playful than others and also have differing styles of play. Therefore, one possibility is that rats seek out partners with congruent styles of play. If this is so, this could be tested in a dyadic setting by matching congruent and incongruent pairs. However, this is still contaminated by the fact that the rat has no choice but to play with the partner available—and in such a context, both partners may need to make compromises in how they play. Another approach is to allow the subject to play with rats having different play styles, and then give the subject a choice in an operant conditioning paradigm. If play style is important, then the rat should be willing to work harder to

access the partner with their preferred style. Similarly, other features of potential partners could be tested.

Studying RTP can be a valuable tool both for basic research on communication and other processes that are involved in regulating social behavior (Palagi et al., 2016b), and for translational research on neurodevelopmental disorders (Burke et al., 2017). Indeed, identifying that turn taking, as shown by the occurrence of role reversals, is a key feature of RTP that promotes the development of socio-cognitive skills (Pellis et al., 2019; Stark et al., 2021), has been important for engineering therapeutic play contexts that similarly promote the development of those skills in children (e.g., Diamond et al., 2007; Nijhoff et al., 2018).

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Conflict of interest

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C57BL/6N mice show a sub-strain specific resistance to the psychotomimetic effects of ketamine

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Repeated administration of subanesthetic doses of ketamine is a model of psychosis-like state in rodents. In mice, this treatment produces a range of behavioral deficits, including impairment in social interactions and locomotion. To date, these phenotypes were described primarily in the Swiss and C3H/HeHsd mouse strains. A few studies investigated ketamine-induced behaviors in the C57BL/6J strain, but to our knowledge the C57BL/6N strain was not investigated thus far. This is surprising, as both C57BL/6 sub-strains are widely used in behavioral and neuropsychopharmacological research, and are *de facto* standards for characterization of drug effects. The goal of this study was to determine if C57BL/6N mice are vulnerable to develop social deficits after 5 days withdrawal from sub-chronic ketamine treatment (5 days, 30 mg/kg, i.p.), an experimental schedule shown before to cause deficits in social interactions in C57BL/6J mice. Our results show that sub-chronic administration of ketamine that was reported to cause psychotic-like behavior in C57BL/6J mice does not induce appreciable behavioral alterations in C57BL/6N mice. Thus, we show that the effects of sub-chronic ketamine treatment in mice are sub-strain specific.

KEYWORDS

ketamine, schizophrenia, social behavior, locomotion, C57BL/6 mice

Introduction

Repeated administration of subanesthetic doses of ketamine is a widely used model of psychosis-like state in rodents (Neill et al., 2010; Frohlich and Van Horn, 2014). Psychotomimetic effects of ketamine are attributed to the antagonism of N-methyl-D-aspartate (NMDA) receptors (Nowacka and Borczyk, 2019). In humans, acute administration of ketamine induces psychosis-like symptoms, including hallucinations and impaired cognitive abilities (Krystal et al., 1994). In mice, sub-chronic (5–24 days) administration of subanesthetic (5–100 mg/kg) doses of ketamine produces deficits in

social interactions (Vasconcelos et al., 2015; da Silva Araújo et al., 2017; Onaolapo et al., 2017; Ogundele and Lee, 2018; Sultana and Lee, 2020), pre-pulse inhibition (Monte et al., 2013; Vasconcelos et al., 2015; da Silva Araújo et al., 2017), auditory processing (Maxwell et al., 2006; Amann et al., 2009), and cognition (Featherstone et al., 2012). This treatment also induces despair-like behavior (Chatterjee et al., 2011, 2012; Choudhury et al., 2016) and locomotor hyperactivity (Monte et al., 2013; Vasconcelos et al., 2015; Choudhury et al., 2016; da Silva Araújo et al., 2017; Onaolapo et al., 2017) (though hypoactivity was also reported; Sultana and Lee, 2020). Ketamine effects have been studied at different time points after cessation of drug treatment (up to 6 months). Some of the studies reported altered behaviors over extended periods of time (Chatterjee et al., 2011; Featherstone et al., 2012). To date, these effects were described mainly in the Swiss mouse strain (Chatterjee et al., 2012; Monte et al., 2013; Vasconcelos et al., 2015; Choudhury et al., 2016; da Silva Araújo et al., 2017; Onaolapo et al., 2017; Ben-Azu et al., 2018) and C3H/HeHsd mice (Maxwell et al., 2006; Featherstone et al., 2012). Relatively few studies employed C57BL/6 mice: the C57BL/6J strain (Amann et al., 2009; Sultana and Lee, 2020), C57BL/6Hsd (Maxwell et al., 2006), and in some cases the substrain was not specified (Choudhury et al., 2016; Ogundele and Lee, 2018).

The goal of this study was to determine if C57BL/6N mice are vulnerable to develop social deficits after 5 days withdrawal from sub-chronic ketamine treatment. To induce behavioral changes, we used a ketamine administration scheme shown before to cause deficits in social interactions in C57BL/6J mice (Sultana and Lee, 2020). Furthermore, we wanted to determine if a selective kappa opioid receptor antagonist, norbinaltorphimine, could affect behaviors induced by sub-chronic ketamine administration. This part of the study was prompted by the observation that nonselective kappa opioid receptor ligands (e.g., naltrexone and buprenorphine) have immediate antipsychotic effects in schizophrenic patients, and by the recent hypothesis that kappa opioid antagonists could be effective in the treatment of schizophrenia (Clark and Abi-Dargham, 2019).

Materials and methods

Animals

Male C57BL/6N mice from Charles River Laboratories (Germany) aged 12 weeks at the start of the procedures were used as subject animals (later referred to as "actors"). Male C57BL/6J mice aged 7 weeks from the colony at the Maj Institute of Pharmacology Polish Academy of Sciences were used as stimulus animals (later referred to as "partners"). Actors arrived at the Maj Institute of Pharmacology 2 weeks prior to the experiment. Mice were handled by the experimenter for 4 days before the experiment. Both actors and partners were group-housed (three to four animals per cage) in a normal dark-light cycle (dark 6 p.m.–6 a.m.), in cages containing gnawing blocks and nesting material, with food and water *ad libitum*. Drug injections and behavioral testing were performed during the light phase, as was done in the previous study that reported social deficits in C57BL/6J mice after withdrawal from sub-chronic ketamine injections (Sultana and Lee, 2020). The tests started 1 h into the light phase or later, and were completed no later than 1 h before the onset of the dark phase. Prior studies that investigated the impact of the testing phase on the results of social interaction tests show that behavioral scores obtained in the dark and light phases are similar (Yang et al., 2008). All procedures were performed in accordance with the European Union (directive no. 2010/63/UE) and Polish regulations concerning animal research. Experimental plan was pre-approved by the 2nd Local Ethics Committee in Krakow, Poland (permit number 306/2020). No animals were excluded from the study due to health issues.

Drugs and treatment schedule

Experimental schedule was taken from (Ogundele and Lee, 2018; Sultana and Lee, 2020). Mice were randomly allocated to the three treatment groups listed in **Table 1** (n = 8 for each group, i.e., 24 animals in total). Ketamine (ketamine hydrochloride, Biowet, Poland) was administered once a day for 5 days (i.p., 30 mg/kg, 5 μl/g). Five days after the last injection social interactions with a novel conspecific were assessed in the partition test (**Figure 1A**). Four hours before the test, animals received norbinaltorphimine (norbinaltorphimine dihydrochloride, 0347, Tocris, UK) injection (i.p., dissolved in saline, 10 mg/kg, 5 μl/g). Three days after the partition test, social interaction with an unfamiliar conspecific placed under an enclosure was measured in the open field. Norbinaltorphimine was reported to act for up to 2 weeks (Lalanne et al., 2017), thus a single injection is sufficient to achieve persistent effects during both behavioral tests, performed 3 days apart.

Partition test: Interaction with a novel conspecific behind the wall

The cage design and test were inspired by Kudryavtseva (2003) and Langford et al. (2010). One day before the test, partner animals were habituated to partner's compartment for 6 min. The test consisted of three phases: habituation, sociability test (trial 1) and social memory test (trial 2), 5 min each (**Figure 1B**). The testing cage (length: 50 cm; width: 12 cm; height: 24.5 cm) was divided into different compartments through transparent plastic walls. In the habituation phase, the cage was divided into two compartments by one plastic wall: a

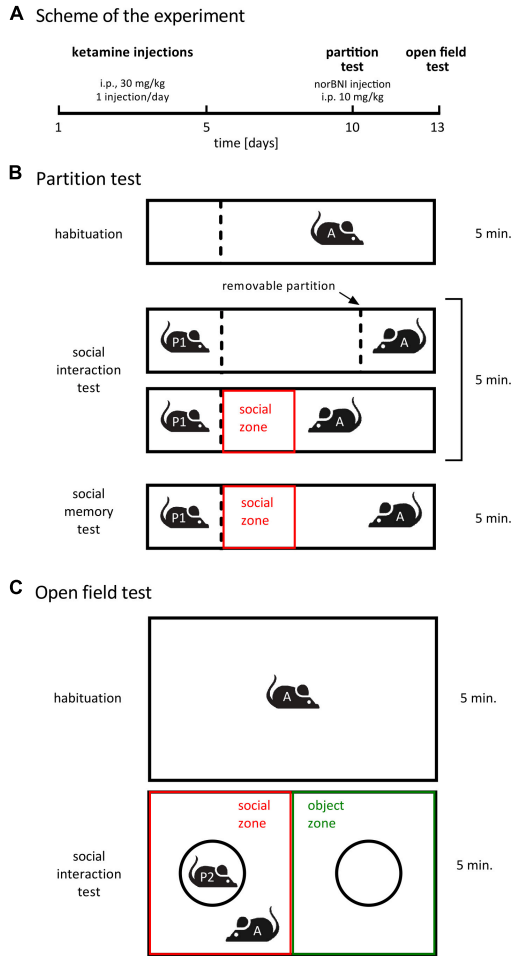

FIGURE 1
Treatment schedule and behavioral paradigms. **(A)** A summary of the treatment schedule. norBNI indicates norbinaltorphimine. **(B)** A scheme of the partition test. The dotted lines represent a transparent perforated Plexiglas wall. The red box shows the area that was designated as social interaction zone during video analysis. "A" stands for "actor" (subject animal), "P" stands for "partner". **(C)** A summary of the open field test. The circles represent wire cups. The red and green rectangles represent, respectively, the social zone and the object zone during video analysis.

smaller compartment (1/4 of the cage) on the left, which was designated to be the partner's compartment during the second phase, and a larger compartment (3/4 of the cage), in which the actor was released. The wall dividing the compartments was transparent and perforated, so that during the test phase animals could see and smell, but not touch each other. During habituation, the actor was able to move freely in the larger compartment, but was not able to enter partner's compartment. After habituation, the actor animal was moved to a transport cage for a short time. During this time, a second plastic wall was introduced to the cage, separating the small compartment (1/4 of the cage) on the right, opposite to the partner's compartment,

TABLE 1 Treatment groups.

Group name	Weight[g] shown as mean (SEM)	Phase 1: Psychotic-like symptoms induction	Phase 2: Psychotic-like symptoms rescue
Sal sal	25.58 (0.35)	Saline	Saline
Ket sal	25.38 (0.21)	Ketamine i.p., 30 mg/kg, 5 μl/g	Saline
Ket norBNI	25.46 (0.48)	Ketamine i.p., 30 mg/kg, 5 μl/g	Norbinaltorphimine, i.p., 10 mg/kg, 5 μl/g

from the rest of the cage. This second small compartment is referred to as the actor's compartment. Finally, in the test phase, a partner mouse (unfamiliar adult C57BL/6J male) was placed in the partner's compartment. Next, the actor was immediately placed in the actor's compartment and the transparent wall separating the actor from the rest of the cage was lifted, while the transparent wall separating the partner's compartment from the rest of the cage was kept in its place. Latency to the first approach to the partner's compartment was measured. After the first trial and 30 min of inter-trial interval, a second 5 min trial with the same partner animal was performed. The aim of the second trial was to measure social memory (Donegan et al., 2020). Reduction of time spent investigating a partner animal on the second vs. first encounter is regarded as an indicator of social recognition. We compared time spent in social zone (the 12.5 cm × 12 cm rectangular zone close to the partner's compartment) between the first and second trial of the partition test. No difference in the time spent in social zone during the first compared to second trial was observed in any of the groups, i.e., the test did not show any indication of social memory. Thus, the results of the second trial are not shown.

Open field test: Interaction with a novel confined conspecific

Open field test was performed as previously published (Ogundele and Lee, 2018; Sultana and Lee, 2020). The test constituted of two phases: habituation and social interaction test, 5 min each (**Figure 1C**). For habituation, the tested mouse was introduced into an empty open field arena open field arena (length: 55 cm; width: 37.5 cm; height: 20.5 cm). Next, two wire cups (9 cm diameter, B-0197, Bionovo, Poland) were placed in the cage: one covering an unfamiliar conspecific (adult C57BL/6J male) and one empty (as a novel object).

Data analysis

Locomotor activity and time spent in social zone during partition test, and time spent in social and object zones during

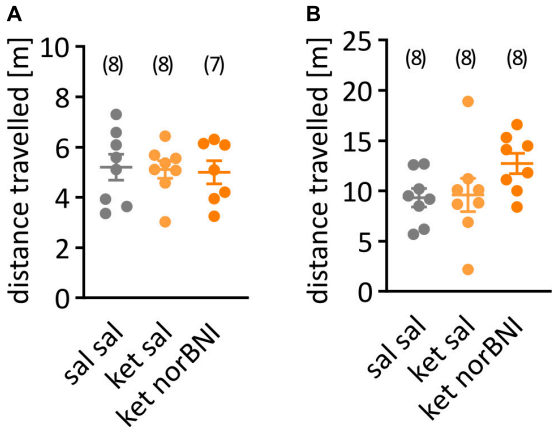

FIGURE 2
Locomotor activity during the habituation to the partition and open field tests. **(A)** Distance traveled during the habituation to the partition apparatus. **(B)** Activity during habituation to the open field. The group that received only saline is labeled "Sal sal", ketamine followed by saline "ket sal", and ketamine followed by norbinaltorphimine "ket norBNI". Each dot represents an individual mouse. The bar and whiskers correspond to mean and SEM respectively. Group sizes are indicated above the graph.

open field test were measured automatically with EthoVision 15 software (Noldus, The Netherlands). In the partition test, the social zone was defined as a 12 ×12.5 cm rectangular adjacent to the partner's compartment (Figure 1B). In the open field test social and object zones were defined as half of the cage containing a cup with a partner or an empty cup, respectively (Figure 1C). Zones were defined digitally, no physical barriers between the zones were present in the experimental cages. Time to the first approach to the partner's zone in partition test and time spent investigating the cups in the open field test were scored manually using the BORIS software package (Friard and Gamba, 2016) by observers blind to the treatment.

Statistical analysis was performed using GraphPad Prism 9. Before group comparisons, the data were scanned for outliers using the Grubbs' test. When an outlier result was identified, it was removed from the specific comparison, but left in all other comparisons. Outlier measurements were identified in the following variables: distance moved in the partition test (Figure 2A, ket norBNI group), time to first approach in the partition test (Figure 3A, sal sal and ket norBNI groups), time spent investigating social cup in the open field test (Figure 4D, ket sal group), time spent investigating empty cup in the open field test (Figure 4E, all groups), percent of time spent investigating social cup (Figure 4F, sal sal group). Departure from normality was tested using the Shapiro-Wilk test. In cases where significant departure from normality was detected, results were analyzed using nonparametric tests: Kruskal-Wallis followed *post-hoc* with the Dunn test for multiple group comparisons, or alternatively Wilcoxon test for within-subject comparison (social memory). In cases where no departure from normality was detected, data were analyzed with one-way ANOVA followed *post-hoc* with Tukey's HSD. Alpha level was set to 0.05. One mouse from the "ket norBNI" group escaped the cage during the habituation phase in the partition test, and was excluded from the analysis of locomotor activity during partition test.

Results

First, we tested whether sub-chronic ketamine administration affected locomotor activity in C57BL/6N mice, as it does in Swiss or C57BL/6J mice. Locomotor activity was

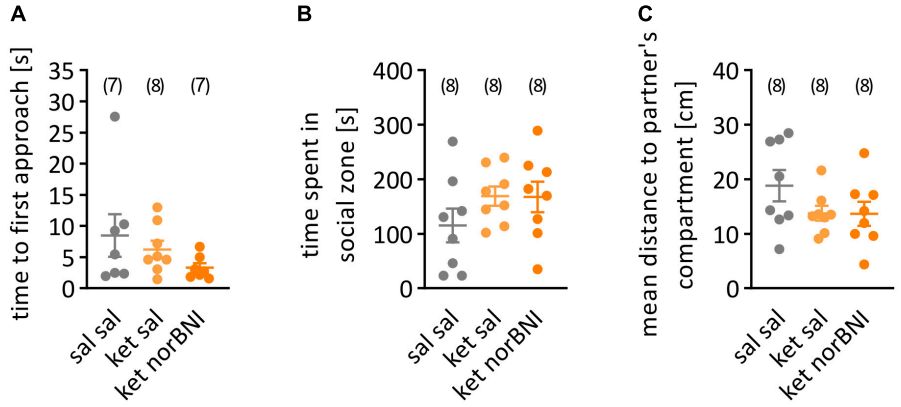

FIGURE 3
Social interaction in partition test. **(A)** time to first approach, **(B)** time spent in social zone, **(C)** mean distance to partner's compartment. "Sal sal" corresponds to treatment with saline throughout the procedure, "ket sal" represents subchronic ketamine treatment and then saline injection before the test, and "ket norBNI" is ketamine treatment followed by single injection of norbinaltorphimine. The lines represent the mean and SEM. The numbers above the graphs indicate group sizes.

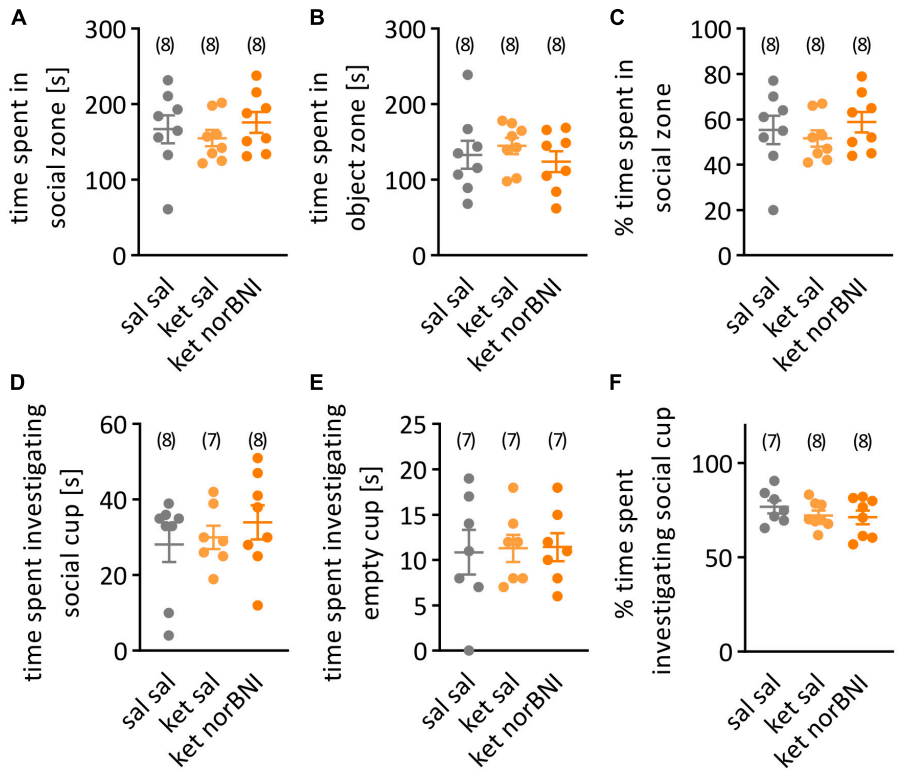

FIGURE 4
Social interaction in the open field. **(A)** Time spent in in the half of the cage with the partner mouse under the wire cup. **(B)** Time spent in the empty-cup zone. **(C)** Percent time spent in the social zone. **(D)** Time spent investigating the wire cup containing a partner mouse. **(E)** Time spent investigating empty cup. **(F)** Percent time spent investigating social cup. Groups are labeled as follows: "Sal sal" is the control group with only saline injections, "ket sal" corresponds to ketamine treatment and then saline, and "ket norBNI" is ketamine followed by norbinaltorphimine. Results are shown as mean and SEM. The numbers above the graphs indicate group sizes.

studied 5 and 8 days after the last ketamine injection. Ketamine treatment, alone or combined with norbinaltorphimine injection, did not affect distance traveled in the partition test [**Figure 2A**, $F_{(2,20)} = 0.046$, $p = 0.955$] or in the open field [**Figure 2B**, $F_{(2,21)} = 0.41$, $p = 0.669$].

In order to determine if sub-chronic ketamine administration causes impairment in social interactions in C57BL/6N mice, similar to the C57BL/6J strain (Sultana and Lee, 2020), the partition test was performed. Three behavioral parameters were assessed: time to first approach to partner's compartment, time spent in the social zone, and mean distance to partner's compartments. No statistically significant effects of ketamine or ketamine plus nolbinaltorphimine treatment were observed in the time to first approach (**Figure 3A**, $H = 2.75$, $p = 0.262$), the time spent in the social zone of the apparatus [**Figure 3B**, $F_{(2,21)} = 1.38$, $p = 0.273$] or the mean distance from the partner's compartment [**Figure 3C**, $F_{(2,21)} = 1.77$, $p = 0.195$].

Finally, in the open field test, time spent in social and object zones as well as time spent sniffing both cups (empty and containing a conspecific) were analyzed. Again, none of the parameters significantly differed between the groups [**Figures 4A,B**, for both time spent in social (A) and object (B) zones, $F_{(2,21)} = 0.5$, $p = 0.616$, **Figure 4C**, Percent time spent in social zone, $F_{(2,21)} = 0.52$, $p = 0.599$, **Figure 4D**, Time spent investigating social cup, $F_{(2,20)} = 0.52$, $p = 0.6024$, **Figure 4E**, Time spent investigating object cup, $F_{(2,18)} = 0.25$, $p = 0.976$, **Figure 4F**, Percent time spent investigating social cup, $F_{(2,20)} = 0.79$, $p = 0.465$].

Discussion

Our results show that withdrawal from sub-chronic ketamine administration in the dose that caused psychotic-like symptoms in C57BL/6J mice (Sultana and Lee, 2020) did not cause such symptoms in C57BL/6N animals. No differences were observed in locomotor activity in a novel environment or social interaction with a same-sex conspecific. The same regimen of ketamine administration was shown to reduce locomotor activity in C57BL/6J mice (Sultana and Lee, 2020). Conversely, similar ketamine administration schedules were repeatedly shown to induce hyperactivity in Swiss mice (Monte et al., 2013; Vasconcelos et al., 2015; Choudhury et al., 2016;

da Silva Araújo et al., 2017; Onaolapo et al., 2017) and C57BL/6 mice, with sub-strain not specified (Choudhury et al., 2016).

A possible explanation of the lack of significant behavioral alterations in ketamine-treated animals are sub-strain differences. The C57BL/6N and C57BL/6J sub-strains emerged from the two colonies that were separated in 1951. These sub-strains differ genetically as a result of accumulated spontaneous mutations (Simon et al., 2013), which probably underlie differences in baseline measures in blood pressure, metabolism and behavior (Bryant et al., 2008; Simon et al., 2013; Åhlgren and Voikar, 2019). Several reports indicate that C57BL/6J mice show more interest toward novel mouse in social approach test (Åhlgren and Voikar, 2019), enhanced social interaction (Matsuo et al., 2010; Pinheiro et al., 2016), and express a decreased amount of anxiety-like behavior in the open field (Matsuo et al., 2010; Simon et al., 2013; Åhlgren and Voikar, 2019) in comparison to C57BL/6N mice. Average percentage of time spent in the social zone in the open field test in the C57BL/6N control group (**Figure 4C**, 55%) is lower than in the 6J strain where it was reported to be approximately 70% (Ogundele and Lee, 2018; Sultana and Lee, 2020). Treatment with ketamine (30 mg/kg) was reported to decrease this to 50% (Ogundele and Lee, 2018) or 35% (Sultana and Lee, 2020). Therefore, we don't interpret the observed lack of difference in social interaction after sub-chronic ketamine treatment in our research as an indication of a floor effect.

Furthermore, the literature indicates that differences in behavior can potentially exist not only between strains and sub-strains themselves, but also arise from the environmental factors such as laboratory environment or derivation from different vendors (Åhlgren and Voikar, 2019). The results presented here demonstrate, that the minor differences between sub-strains and standardized environments are sufficient to dramatically affect the behavioral phenotype. Importantly, we observed no ketamine-induced impairments, thus the putative differences appear to offer resilience to the psychotomimetic effects.

Additionally, a second factor that may contribute to the lack of appreciable ketamine effects in this study could be the dose of ketamine used or the treatment length. We applied the same ketamine treatment scheme (5 days, 30 mg/kg per day) that was described before to induce wide-range of psychotic-like behavioral alterations in C57BL/6J mice (Ogundele and Lee, 2018; Sultana and Lee, 2020). However, earlier studies on Swiss mice usually used either higher doses of ketamine (Chatterjee et al., 2011; Choudhury et al., 2016) or longer treatment schedules (Featherstone et al., 2012; Monte et al., 2013; Ben-Azu et al., 2018). In one study that included both Swiss and C57BL/6 subjects, authors noted that C57BL/6 showed strong ketamine-induced impairments at doses that had no apparent effects in Swiss mice (Choudhury et al., 2016). In this study Swiss mice were given 100 mg/kg per day and C57BL/6 mice 70 mg/kg (Choudhury et al., 2016). It is possible that administration of 60 mg/kg ketamine for 5 days would induce expected behavioral deficits in C57BL/6N mice too. Alternatively, longer treatment with lower ketamine dose would be necessary. Chatterjee et al. (2011) mention that in Swiss mice the enhancement of immobility in forced swim test was observed after 10, but not after 5, days of ketamine administration (however, the data obtained after 5 days are not shown). We cannot exclude the possibility that social impairment in C57BL/6N mice would emerge after a longer ketamine treatment.

It should be also noted that in this study ketamine treatment was administered in adult C57BL/6N animals, while in the previous report where the same treatment regimen affected social behavior of C57BL/6J mice, animals received ketamine in the late adolescent period (post-natal days 45–60) (Sultana and Lee, 2020). Speculatively, the effects of ketamine treatment could stem from disrupted late brain development, and thus the age of treatment could be critical for the phenotype to develop. While such possibility cannot be entirely excluded, it appears unlikely. In mice, behavior of late adolescent (i.e., sexually mature) males is, in most respects, similar to the behavior of adults (Bell, 2018; Reiber et al., 2022). Moreover, sensitive periods for the development of social behavior, i.e., periods when environmental disturbances influence later behavior most, were described to occur much earlier in the mouse ontogeny (Dyer and Southwick, 1974; Makinodan et al., 2012; Bicks et al., 2020).

Finally, the apparent difference in the response of the C57BL/6N and C57BL/6J sub-strains to ketamine treatment resembles their different sensitivity to addictive substances. The C57BL/6N shows smaller increase in motor activity after acute or repeated treatment with psychostimulants (Kumar et al., 2013), weaker response to nicotine-induced phenotypes (Akinola et al., 2019) and also differences in sensitivity to the effects of alcohol (Crabbe, 1983; Seemiller et al., 2022). The extent of the differences in drug sensitivity is surprising, considering the very small differences in genotype (Simon et al., 2013), nevertheless, it cautions that no assumption of equivalence in drug-induced phenotypes between the two sub-strains should be made.

Conclusion

In summary, we have shown that sub-chronic administration of high ketamine dose in a scheme that causes psychotic-like behavior in C57BL/6J mice does not induce negative schizophrenia-like symptoms in C57BL/6N mice. We conclude that there are sub-strain differences in the behavioral reaction to sub-chronic ketamine administration in mice.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/ and https://doi.org/10.6084/m9.figshare.15022764.v1.

Ethics statement

This animal study was reviewed and approved by the 2nd Local Ethics Committee, Krakow, Poland.

Author contributions

ZH: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review and editing, visualization, project administration, and supervision. KM: investigation and writing—original draft. MK and MC: formal analysis. JRP: conceptualization, methodology, writing—review and editing, project administration, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Ethorobotic rats for rodent behavioral research: design considerations

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The development of robots as tools for biological research, sometimes termed "biorobotics", has grown rapidly in recent years, fueled by the proliferation of miniaturized computation and advanced manufacturing techniques. Much of this work is focused on the use of robots as biomechanical models for natural systems. But, increasingly, biomimetic robots are being employed to interact directly with animals, as component parts of ethology studies in the field and behavioral neuroscience studies in the laboratory. While it has been possible to mechanize and automate animal behavior experiments for decades, only recently has there been the prospect of creating at-scale robotic animals containing the sensing, autonomy and actuation necessary for complex, life-like interaction. This not only opens up new avenues of enquiry, but also provides important ways to improve animal welfare, both by reducing or replacing the use of animal subjects, and by minimizing animal distress (if robots are used judiciously). This article will discuss the current state of the art in robotic lab rats, providing perspective on where research could be directed to enable the safe and effective use of biorobotic animals.

KEYWORDS

ethorobotics, laboratory rodents, biorobotics, biohybrid interaction, robotic rats, ethology, bioinspired robotics

1 Introduction

Rodents (*Rodentia*) are remarkable animals, which have successfully occupied almost every habitat on earth, and account for almost 50% of all observed mammal species. Among the rodents, the *Muridae* family are ubiquitous in the modern world, due to their commensal relationship with humans. Domesticated rats (*Rattus norvegicus forma domestica*) and mice (*Mus musculus domesticus*) have become some of the most important model animals in modern biology. In the US, as many as 100 million rodents are used in experiments in a given year (Carbone, 2021). Many of these experiments involve social behavior tests featuring multiple rats interacting with one another, which can be used (for example) to gauge the psychoactive effects of pharmaceuticals, or to phenotype animal models of neurological disorders. Unfortunately, some of these social tests face ethical issues deriving from the risk of physical harm to the animal subjects. These issues are particularly relevant in the tests featuring encounters among unfamiliar individuals. For instance, one of the most common tests of aggressivity is the resident-intruder test (Koolhaas et al., 2013; Ruzza et al., 2015), in which a stimulus rat or mouse (the intruder) is placed in the home-cage of another rat or mouse (the resident). Since rats and mice are highly territorial, the intrusion of a new rat or mouse elicits aggressive reactions aimed at territorial defense. While the variable of interest is the resident animal's reaction to the intrusion, the following fight may lead to injuries and even death of the experimental animals, raising important issues regarding animal welfare. The recent d'Isa-Gerlai rating scale for the impact on behavioral tests on animal

welfare, which features 12 levels from A (animal-friendly) to L (lethal), rates the resident-intruder test K due to the risks of physical harm (d'Isa and Gerlai, 2023). The employment of robotic rodents as intruders could completely eliminate injuries deriving from fight, representing a notable refinement of aggression tests in behavioral neuroscience. Moreover, social experiments with animals are complex and face ethical issues with reproducibility. If sufficiently "life-like" robotic rodents can be developed, an opportunity is presented to simultaneously improve the repeatability with which social interaction can be tested (since the behaviors of the robotic intruder are programmable by the experimenters) and animal welfare, by reducing both the number of rodents required for a given experimental cohort and the incidence of injury to experimental subjects from violent interaction with conspecifics. But before such robot-rat interactions (Figure 1A) can enter into wide use, significant challenges remain in replicating the suite of behaviors necessary for a scientifically useful interaction.

Biorobotics is a subdiscipline of robotics which seeks to which develops biomimetic or bio-inspired robots, which may be used not only as technological applications to help society and/or the environment, but also as scientific tools for biological research, for instance through the use of synthetic abstractions of biological systems (Tamborini and Datteri, 2022). Fueled by the miniaturization of computation and sensing, and the proliferation of advanced manufacturing, the increasing sophistication with which robots can be built offers ways to test biological hypotheses in a highly controlled fashion, while also reducing the need for intrusive animal experimentation. These bio-inspired robots can be used to elucidate biomechanical principles in support of animal studies (Siddall et al., 2021), or simply to provide an accessible way of engaging a wider audience in educational projects (Siddall et al., 2023). Indeed, the growing autonomy with which robots can be imbued now offers a chance to use biorobotics in more sophisticated ways, and to test interactive effects.

The development of robots capable of interacting with live animals for behavioral studies (often called "ethorobotics") has been employed in a number of ways (Romano et al., 2018), most often through animatronic replication of body language and visual navigation cues. This is a fast growing area of research with a 5-fold growth in the past decade (Figure 1B). Rodents have a long history as model animals, and consequently robotic rats have the most research attention in robot-animal interaction studies. However, the range of animal robot interactions is rapidly growing, and recent studies have shown the influencing of collective behavior with robotic cues, e.g., in schooling fish or swarming bees (Romano et al., 2018).

Ethorobotics may provide useful tools for behavioral research. Indeed, automated mechanical systems have long been used in rodent behavioral experiments, and mobile robots have been used to manipulate the social behavior of a variety of animals, including insects (Gribović et al., 2017), fish (Polverino et al., 2013), and birds (Gribovskiy et al., 2015), not to mention the vast amount of research devoted to human-robot interaction. But it has only recently become possible to endow robots with more complex interactive behavior. Recent work has closed the loop between animal and robot in zebrafish (Bonnet et al., 2018; Khalil et al., 2019) and the techniques for animal-interactive robot control are developing rapidly (Landgraf et al., 2021), though the field is still nascent. Collectively, the work on ethorobotics to date has shown the ways in which robots can lead to more sophisticated and controllable animal experiments, and there is a clear benefit to directing more robotics work toward rodents, as they are the most widely studied animal models.

While both rats and mice are widely used as test subjects in behavioral biology, this paper will focus primarily on rats, for four principal reasons. Firstly, rats are larger than mice (200–400 g vs. 20–30 g), a size difference which is enough to make robotic rat development possible with off the shelf electronics and actuation, whereas the miniaturization needed for mouse-scale robots would currently require a drastically greater development effort. Secondly, rats are employed both as subject animals in rat studies and as stimulus animals in mouse studies, for instance in the predator threat test for mice (Blanchard et al., 1998, 2003). Thirdly, rats are more expensive to breed and maintain than mice, so their substitution would have a greater economical impact on laboratories. Lastly, mouse and rat colonies must be kept separated. The availability of robotic rats would allow laboratories endowed only of a mouse facility to perform mouse-rat interaction experiments without opening a rat facility.

2 State of the art in biohybrid rodent studies

To date, around a dozen different prototype rat robots have been presented in the literature, many of which have been tested interacting with live rats in multiple follow-up studies. Table 1 collects body dimensions, movement speeds and estimated power consumption (based on battery provision) for various robotic rodents. Table 1 is limited to rodent-mimicking hardware designed with animal interaction in mind—several other works exist exploring rodent interaction with off the shelf robots (e.g., Del Angel Ortiz et al., 2016), using rat-biomimicry to develop novel hardware (e.g., Pearson et al., 2007), implementing rat-like behaviors to test neuromechanical hypotheses *in-silico* (e.g., Fend et al., 2004), or testing virtual robots (Merel et al., 2019). While many robots take the approach of attempting to replicate as directly as possible the kinematics of rat movement, currently the robots most widely tested in animal interactions use simplified geometry and rely on wheels (Shi et al., 2011, 2012, 2015; Wiles et al., 2012; Heath et al., 2018; Yamada et al., 2021). This allows them to move at similar speeds to rats over engineered/flat surfaces (∼1 m/s), but comes with the cost of being unable to move over more complicated terrain, or to replicate rat body postures (e.g., rearing Yuanzhong et al., 2022). Most of the robots in Table 1 use off the shelf servomotors which are liable to produce ultrasonic noise, and of the robots in Table 1, only "PiRat" has been specifically designed to minimize motor whine in rat auditory frequencies. Quadrupedal robots (Laschi et al., 2006; Ishii et al., 2009a,b; Lucas et al., 2019; Shi et al., 2022) thus far have not been able to attain biological movement speeds. Rats move with a lower center of gravity, with more bent limbs (Figure 2B) and different hindlimb kinematics compared to the cursorial animals widely studied for quadrupedal locomotion (e.g., dogs), and more research is needed to adapt the compliance and force control needed for efficient legged locomotion. Soft robotics techniques are present in existing rat

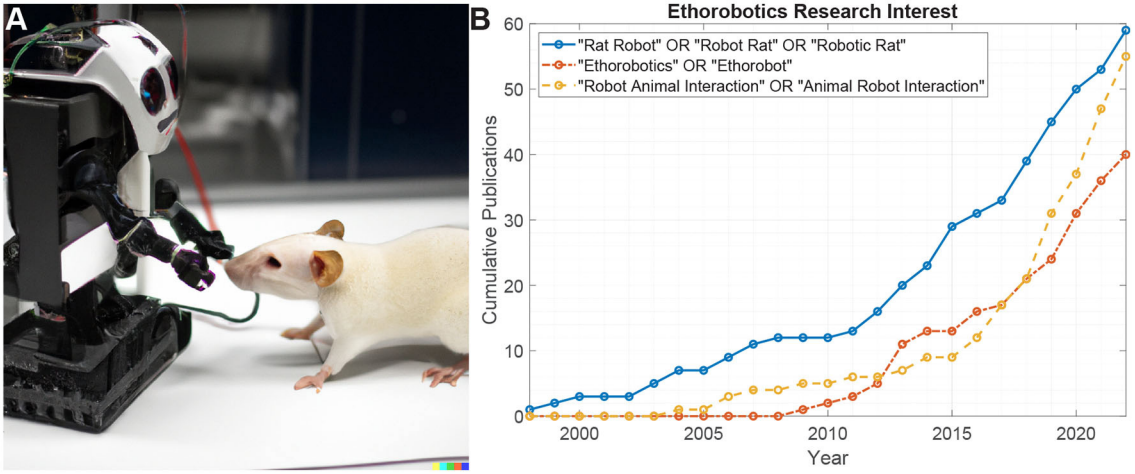

FIGURE 1
(A) AI-generated concept sketch of a laboratory rat interacting with a robot. **(B)** Web of science citation analysis for different search terms related to robotic rodents and ethorobotics. Robotic rats are some of the longest pursued "ethorobots", and the most widely studied in animal-robot interaction research.

TABLE 1 Comparison of the designs of previously developed robotic rats, with a column indicating which robots have been tested interacting with a live rodent.

Robot	Mass (*g*)	Speed (*m/s*)	Size (*mm*)	DOF	Locomotion type?	Power (*W*)	Interaction tested?	References
Psikharpax	–	0.3	500	∼10	Wheels	–		Meyer et al., 2005
Rat-Robot	340	–	146	13	Legs	∼10	✓	Patanè et al., 2007
WR-1	1150	0.03	270	15	Legs	–	✓	Ishii et al., 2009a
WR-2	850	0.02	240	15	Legs	16		Ishii et al., 2009b
WR-4	850	1	270	10	Wheels	–	✓	Shi et al., 2011
WR-3	1,000	1	240	14	Wheels	–	✓	Shi et al., 2012
iRat	600	0.5	180	2	Wheels	9	✓	Wiles et al., 2012
WR-5	700	1	240	13	Wheels	15	✓	Shi et al., 2015
PiRat	240	1.1	123	2	Wheels	–	✓	Heath et al., 2018
NeRmo	275	0.3	117	13	Legs	18		Lucas et al., 2019
WR-7	–	0.24	230	3	Wheels	–		Yamada et al., 2021
Soft Rat	–	–	240	6	None	–		Yuanzhong et al., 2022
SQuRo	220	0.2	136	12	Legs	12		Shi et al., 2022

Robotic legged locomotion cannot yet produce life-like movement speeds, and so many robots employ wheels.

robots (Lucas et al., 2019; Shi et al., 2022; Yuanzhong et al., 2022), but of the legged robots in Table 1, only one (Lucas et al., 2019) has a design giving close attention to limb compliance. Given the vast amount of data and analysis of rat running, and the availability of simulated neuromechanical models (Merel et al., 2019), there is ample material available for further robotic development.

As well as allowing more life-like and efficient motion for legged robots, the use of elastic elements limits force and offers intrinsic safety. However, social interaction does not necessarily require absolute biomechanical mimicry. The "iRat" platform (Wiles et al., 2012) has already been used in a cooperative interaction and has been proven effective in inducing a positive response in live rodents (Quinn et al., 2018), while an undisguised legged robot was used to elicit faster learning in a Skinner's box experiment (Patanè et al., 2007). Among the robots that have been presented, the only instance of a pro-social response was found in Quinn et al. (2018), when rats showed a preference for helping a trapped robot that was made to move in a social fashion over a randomly moved robot. However, it cannot be established that the robot was treated as a conspecific by the rats, or whether another motivation was present [rats actively work to access enrichments (National Research Council, 2008), for example]. More effort is needed to establish the extent to which an inanimate object can be recognized socially by

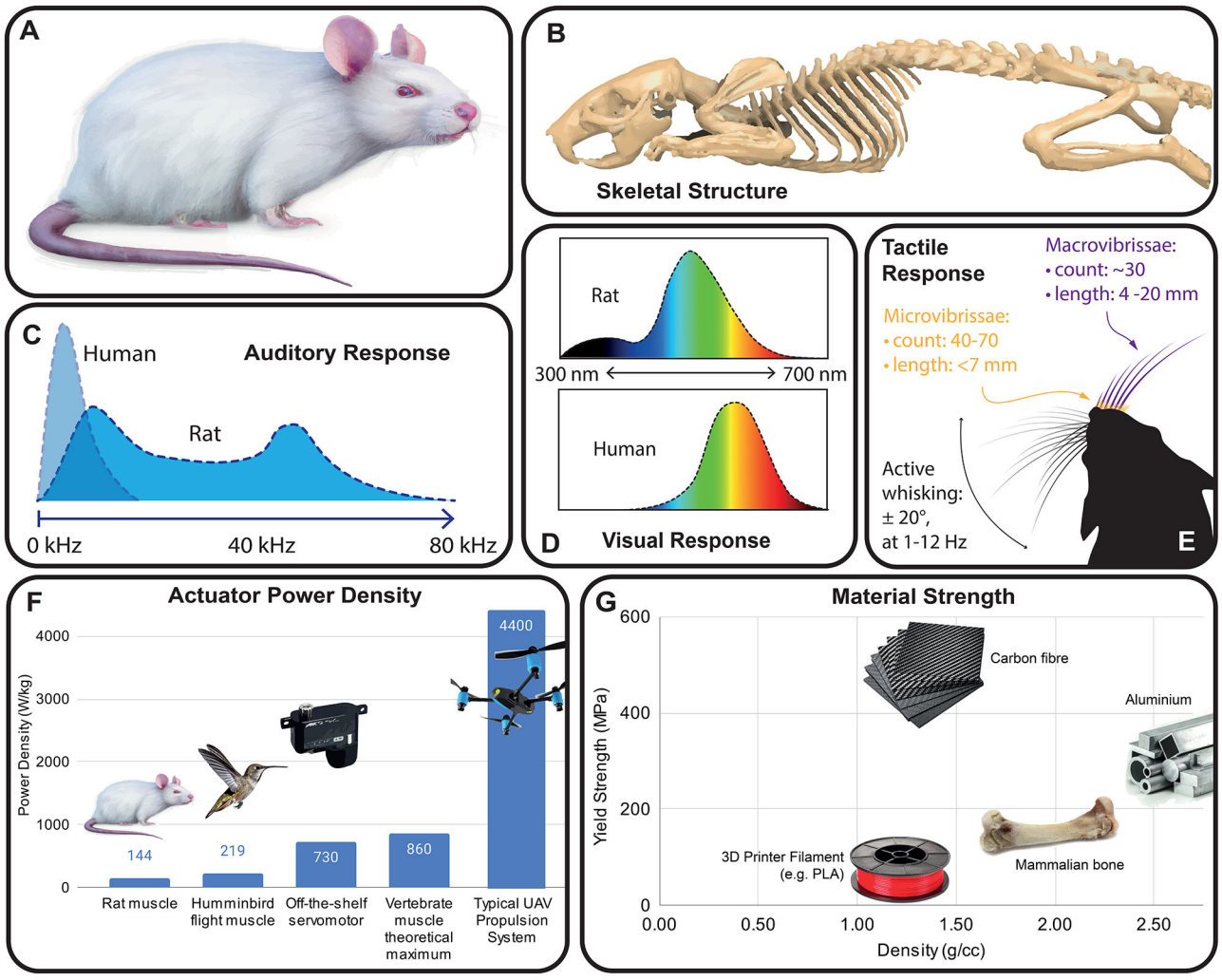

FIGURE 2
Rat anatomy and salient sensory features. **(A)** Sketch of a widely used albino rat strain (e.g., Wistar, Sprague Dawley). **(B)** Rat skeletal anatomy [CT scan data from Doney et al. (2013)]. **(C)** Audio response of a typical rat compared to human hearing [data from Kelly and Masterton (2005)]. **(D)** Normalized spectral sensitivity of rats and humans, showing effect of rat UV opsins [data from De Farias Rocha et al. (2016)]. **(E)** Vibrissae overview [data from Brecht et al. (1997)], showing macro and micro-vibrissae, and active whisking kinematics. **(F)** Mass-specific power of animal muscles vs. off the shelf electric motors [muscle data taken from Pennycuick and Rezende (1984), Martin et al. (2000), Reiser et al. (2013)]. **(G)** Typical strength vs. density for common robotics materials vs. animal bone.

a rat, and hence understand whether disguising of robots is effective/necessary.

3 The sensory environment of a laboratory rat

Rats navigate their environment in a way which is alien to humans (Burn, 2008), and as a consequence is not intuitive to robot designers (Figure 2). While many laboratory rats appear markedly different from their wild relatives (Figure 2A), even domesticated species have been shown to retain many of the behaviors of their "wild" suite when allowed to express them (Berdoy, 2002). Rats are nocturnal, with elongated bodies adapted for stealth and access to confined spaces (Figure 2B). They are short-sighted and possess limited color vision (Figure 2D), but have highly developed olfaction, gustation and hearing (Figure 2C) as well as a sophisticated suite of tactile senses, including vibrissal perception (Figure 2E). These senses have different sensorial ranges, and here we will go through them in approximate order of the sensorial range.

Of the suite of primary rodent senses, perhaps the most difficult to control and interpret in a laboratory environment are olfaction and gustation, which unfortunately also provide rats with some of their strongest high-level navigation cues. Rodents sniff at a frequency of up to 12 Hz (Spencer et al., 2021) (representing a significant data rate) and smell directly affects social response [notably for interaction experiments, smell affected cooperation in Gerber et al. (2020)]. The miniaturization of "e-nose" sensors, and the growing ease with which artificial intelligence can be applied to their data implies that better biomimetic smell classification in robots is well within reach of current technology. Indeed, it

has been possible to classify certain rodent odors with electronic noses for decades (Montag et al., 2001). However, the ability of rodents to localize and track scents is far more complex, involving the exploitation of airflow differences between nostrils and active "casting" of the nose to trace the source of olfactory cues, and rat-like chemotaxis is currently considerably more challenging for an artificial system. Importantly, olfaction could not be required in the robotic rat if it used not as subject animal, but rather as stimulus animal for living rats. Nevertheless, emission of scent could be fundamental for appropriate social responses from living rats.

After scent or lack thereof, the immediately obvious problem with the use of an electromechanical rat is its audio signature. Rats' large hearing range (up to 100 kHz, Figure 2C) implies that the whine of electric motors is much more audible. Off the shelf servomotors typically used in robotics (and in many of the robots in Table 1) use control pulse frequencies of around 9 kHz, leading to a "whine" at the same frequency. While many more sophisticated drivers use frequencies of up to 40 kHz (making the motor whine inaudible to humans), most motor drivers would need to be operated at their maximum viable frequency to be fully inaudible to rats. Additionally, the lower frequency noise from gearboxes and other mechanisms should be considered.

Just as the differing auditory response of rats has implications for robot design, so does the broadened rat visual response, in particular the presence of UV sensitive opsins (Figure 2D). A differing spectral response means that color as perceived by humans is not a reliable basis for mimicry. Rat fur is known to fluoresce under UV light (Tumlison et al., 2021) and has even been suggested to provide rudimentary sensation of infrared radiation (Baker, 2021). Fur maintenance is clearly important to rats, who spend 50% of waking time grooming (Lambert, 2011).

Finally, rats' sense of their environment is strongly tactile. The upper lips of rats contain around 100 vibrissae/whiskers (Figure 2E), whose follicles are dense with nerve endings and which remain constantly in motion as the animal moves (Brecht et al., 1997). Whisking of rat vibrissae at up to 12 Hz gives the animals constant information about positions and textures of the substrates they move across, and their tactile response is sufficiently sensitive to accurately measure airflow (Yu et al., 2016). Interestingly, Tony Prescott's laboratory at the University of Sheffield (United Kingdom) has designed a biomimetic whisking scratchbot capable of tactile sensing through active movement of the vibrissae, on the model of the rat whisker sensory system (Pearson et al., 2007, 2010; Prescott et al., 2009; Fox et al., 2012). While providing a robotic rat with complex tactile abilities would not be necessary if the robotic rat is employed as stimulus animal, the presence of whiskers and of whisker movements could be particularly important to induce adequate social reactions in living rats.

4 Design considerations for robotic rats

From a coarse engineering perspective, the power provision necessary to replicate rat locomotion is within the power and energy density limits of current electrical actuators and batteries. Maximal exertion observed in respirometry tests of running rats is roughly 3 kcal over 30 min, or 7 W of average output power (Paes et al., 2016). This could be delivered with 2A of current from a single lithium battery, with a 50 gram battery being sufficient for an hour of high-intensity exercise in a hypothetical rat-like robot. This is significantly less power than is typically used in robotic rats (mean robot power in Table 1 = 13.4 W) despite their lower movement speeds, highlighting the gap in locomotion efficiency that currently exists between animal and robot. Yet, mammalian muscle power output reaches around 200 Watts per kilogram muscle mass, which is below the peak performance of high-end hobbyist servo motors (~700 W/kg), and well below high performance brushless motors (Figure 2A). Similarly, engineered materials can readily match or exceed the specific strength of animal bone (Figure 2B), although it should be noted that bone has a flexibility which can only be matched by composites maintaining a strength similar to animal bone.

This is not to trivialize the challenge of replicating rat locomotion; power provision is not the metric for success in biohybrid robot design—e.g., the compliance, distributed sensing and evolutionary tuning of muscoskeletal systems have profound effects on locomotion ability (Spröwitz et al., 2013). This simply means that many observed behaviors in rats are potentially mechanically replicable without requiring novel miniaturization of available technology. Size is important; larger rats will attempt to dominate smaller rats in social encounters, and so social interaction requires that robots be miniaturiseable to a sufficient extent that they do not intimidate (without sacrificing mobility) and it is encouraging that current actuation is sufficient to achieve this goal.

To date, a strong focus of rat robot design has been on kinematic similarity to the animal. This is useful to replicate natural movement and interaction behaviors—rat interaction is often physical, and has distinct "choreographies" (Lambert, 2011) that life-like interactors will need. However, far less attention has been given to assimilating robots to the full suite of rodent senses, in order to enhance the probability that they will induce the intended behaviors in living rats. Humans have a profound visual bias, and more attention could be given to mimicking other aspects of robotic rat's "appearance", including audio, scent and tactile similarity. Additionally, the robot rat could be endowed with senses itself and this could make its behavior even more natural. For instance, the first phase of most social interactions involves exploration of the perimeter of the interaction space by both the resident and the newcomer rat. Fortunately, basic wall-following is one of the easier behaviors to replicate in a robot with contact switches or proximity sensors (this a common feature of robotic vacuum cleaners, for example).

One advantage that any rat robot has is the ability to use external sensors to augment its perception. Automated behavior tracking has been in use in laboratory ethology for over 20 years (Isik and Unal, 2023), and has rapidly improved with the advent of deep-learning (Mathis et al., 2018; Nilsson et al., 2020). External gas sensors, microphones and cameras can all be used to choreograph robot behavior within an enclosure and augment computational power, so there is limited need to compact processing power into a mobile robot beyond convenience/transport. In addition, many behaviors could be remotely-controlled directly by the experimenters, which could decide in real time the most appropriate reaction for the robotic rat. Consequently, rather than providing the rat robot with a complex

embedded sensorial system, the primary considerations should be locomotion and appearance.

Regarding movement, rodents commonly live in cages endowed with a bedding of soft sawdust. Existing robotic rats typically can only move on smooth, flat surfaces and are incapable of locomotion on sawdust. While some social tests can be performed outside the home-cage, other tests (such as the resident-intruder test) must be performed in the home-cage of the resident animal. This issue could be easily solved by placing a rubber mat or a transparent plexiglass sheet on the sawdust of the home-cage before the test, which would give the robotic rat the possibility to walk.

Rat robots may also need to reflect rat behavior in order to provoke a natural response. The series of rat robots developed at Waseda University (Table 1) devote considerable design effort to accurately mimick the posture and rearing behaviors of natural rats, and elicited statistically significant response behaviors in live rats. However, many rat posture cues are much more subtle than rearing: ear wiggling, whisker protraction, eye tightening and mouth opening are all present during social interaction (Ebbesen and Froemke, 2021). Unlike the primary muscles used for locomotion, replicating facial muscles weighing fractions of a gram represents a technological challenge. Mimicking facial expressions at scale would likely require the use of novel actuation (e.g., shape memory alloys or other smart actuators).

Outside of technical considerations, several features of modern robotics research practice could be ethically problematic when transposed into biological research. Firstly, it is not typical for robotics research articles to require full reproducibility of the prototypes of the authors. Full sharing of the code, design files, and manufacturing instructions needed to reproduce robotics work is uncommon in published articles - the reporting standard is the minimum technical detail needed for comprehension, not for full replication. Secondly, design for decontamination is not typically required of research robots, and widespread use of robots in rodent studies will either require design for disposability or levels of ingress protection normally reserved for medical robots, which would allow the use of detergents and alcoholic solutions to clean and disinfect the rat robots.

Finally, the prototyping-led, Edisonian design approaches common in robotics research do not lend themselves to judicious use of animal subjects, nor the minimum level of scientific quality needed to justify the use of living creatures. Establishing new robotic experiment paradigms will require extensive animal testing with a sufficient number of subjects. Existing ethical guidelines establish general best practice, but have little to no information on the use of animatronics (Van Sluyters and Obernier, 2003). However, if animatronic robots will be used to interact with living animals, important safety criteria need to be considered and should become part of the robot design requirements. For example, since rodents often explore new items by mouthing them, it is important that no elements on the surface of the robot can be bitten off or ingested by the animals. Indeed, robot prototypes should be used with animals only after safety considerations have been implemented in their design.

5 Conclusion

While there are vastly more avenues of enquiry into robot-rat interaction than can be collected into a perspective article, surveying the literature suggests some key topics that need research attention before robots can be effectively deployed as tools for behavioral research:

- The extent to which it is possible to elicit social behaviors in a rat with an inanimate object has not been established. Research into rodents and other taxa has demonstrated the importance of biomimetic appearance and movement (Landgraf et al., 2021), but controlled tests of the relative importance of appearance, sound, scent, and posture are needed to establish clearer design requirements for robots. The "helping behavior" paradigm (Bartal et al., 2011), in which a "trapped" robotic rat induces rescue behavior in a living rat, has already been employed with robots (Quinn et al., 2018) and provides a safer initial way to test rat responses without requiring direct animal-robot contact (a "trapped" robot also reduces locomotion performance requirements).
- Appropriate manipulation of olfaction is a consistent challenge in rodent studies, and is particularly acute in robot interactions. To date, robot interaction studies have used neutral scent marks to distinguish robots (Quinn et al., 2018), but given the importance of scent to social interaction, effort should be put into testing the integration of scents which have a more reactive effect on the subject animals.
- The use of compliant/soft elements in robotic rats should be increased. Soft structures improve the intrinsic safety of interacting robots, as well as providing locomotion benefits. To date only minor elastic elements have been employed in robotic rats.
- Finally, to create repeatable, reproducible experiments, robot autonomy is required. Using human operated robots will be sufficient to make progress in the areas listed above, but behaviors will ultimately need to be fully automated, so that generalizable experiments can be run by different researchers across institutions. Automated chasing of an individual by a robot is already possible (Heath et al., 2018), but a fuller suite of robot postures/responses requires subtler timing and perception, almost certainly requiring advanced machine learning for visual classification of animal behaviors, which is an already an active topic of research (Nilsson et al., 2020), but not yet integrated into robot development.

Current rodent behavioral tests of social behavior (such as interaction with unfamiliar subjects or the resident-intruder test) may lead to fight and consequently to pain and injury of the animals. The employment of robotic rats in social interaction tests, especially the tests of aggressivity (as the resident-intruder test), can avoid the risks for the health of the subjects and would notably improve animal welfare. In the resident-intruder test, if a robotic rat is used as stimulus rat (the intruder), wounds and deaths deriving from fight could be completely avoided and the aggressive responses of the subject rat (the resident) could be measured in total safety.

This would be an enormous refinement of aggressivity tests in behavioral neuroscience. Moreover, several characteristics of the robot stimulus animal (such as the posture and the vocalizations) could be controlled by the experimenters, to better understand the effects of single behavioral variables on the social behavior of the subjects. In addition to improving both the ethical and experimental quality of animal testing, the fact that rats are already widely used as models for psychological disorders implies that there are new avenues of inquiry that could be opened up by more controllable interaction studies. Rats derive real and measurable health benefits from social interaction (Hermes et al., 2009), and robotic rats may provide a more controllable means of ameliorating stress in isolated captive animals. Furthermore, the insights gained in robot-rat interaction may also find application in conservation breeding programs, particularly for endangered species, with robotic predators allowing appropriate fear-conditioning of individuals before release into the wild. Indeed, technological conservation tools have recently been defined as "the next generation of engineering-biology collaborations" (Schulz et al., 2023).

In this article the many practical advantages that could be brought by hypothetical rat robots have been outlined, but the question of what type of knowledge robotic rat experiments would yield deserves further attention. The level of sophistication at which a robotic animal moves from being a particularly complex but still fundamentally mechanical experiment to being a viable simulation of animal-animal interaction is not clear at this stage. Even if a robot was shown to convincingly replicate social interaction, it would still be necessary to determine what behavioral features may have been lost because of the abstractions and simplifications inherent in any synthetic copy of a living animal.

Finally, it should also be noted that robotic rat research could lead to a cross-fertilization between biology and robotics. Biology could benefit from the applications deriving from robotics, and robotics could benefit from biological knowledge to develop bioinspired prototypes. At a basic engineering level, rats are remarkably adaptable animals, with abilities to traverse terrain that would benefit many robotics applications such as inspection and non-invasive ecological monitoring. Such a well-studied animal as the rat should be a target for biomimetic roboticists even without the significant potential direct benefits to biology research. In order to achieve such cross-fertilization, a cross talk between robotics engineers and biological scientists should be started. This could be done by increasing the participation of biological scientists in robotics congresses such as Living Machines (Hunt et al., 2022) and the long-running From Animals to Animats (Cañamero et al., 2022), and of robotics engineers in biological congresses such as the ones of the Society for Neuroscience (SfN) and of the Federation of European Neuroscience Societies (FENS). Robotics engineers should try to write in biological journals and biological scientists should try to write in robotics journals. Collaborations between robotics labs and biological labs should be promoted. Funding agencies could launch funding offers for such collaborative projects. Indeed, the cross-fertilization between robotics and biology could be one of the most fruitful of the next decade.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

RS: Conceptualization, Investigation, Visualization, Writing – original draft, Writing -review & editing.

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BAMBI: A new method for automated assessment of bidirectional early-life interaction between maternal behavior and pup vocalization in mouse dam-pup dyads

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Vital early-life dyadic interaction in mice requires a pup to signal its needs adequately, and a dam to recognize and respond to the pup's cues accurately and timely. Previous research might have missed important biological and/or environmental elements of this complex bidirectional interaction, because it often focused on one dyadic member only. In laboratory rodents, the Pup Retrieval Test (PRT) is the leading procedure to assess pup-directed maternal care. The present study describes BAMBI (Bidirectional Automated Mother-pup Behavioral Interaction test), a novel automated PRT methodology based on synchronous video recording of maternal behavior and audio recording of pup vocalizations, which allows to assess bidirectional dam-pup dyadic interaction. We were able to estimate pup retrieval and pup vocalization parameters accurately in 156 pups from 29 dams on postnatal days (PND) 5, 7, 9, 11, and 13. Moreover, we showed an association between number of emitted USVs and retrieval success, indicating dyadic interdependency and bidirectionality. BAMBI is a promising new automated home-cage behavioral method that can be applied to both basic and preclinical studies investigating complex phenotypes related to early-life social development.

KEYWORDS

early-life, communication, mouse, behavior, pup retrieval, ultrasonic vocalizations, automation

Introduction

Neonatal mouse pups depend on their dam for nutrition, thermoregulation, and protection (Nowak et al., 2000). They produce acoustic signals to communicate their vital needs, and particularly ultrasonic vocalizations (USVs) are essential to evoke maternal care behaviors, such as retrieval in pups that have dangerously strayed from the nest (Wöhr et al., 2010; Bornstein et al., 2017). Early-life USVs can be used to study the genetic and neural basis of early-life communication and to assess early-life communicative defects and their impact on social development (Hahn and Lavooy, 2005; Scattoni et al., 2009; Reynolds et al., 2017; Potasiewicz et al., 2019). Moreover, early-life maternal care has been shown to affect the pup's physical and functional development in a very broad sense (Caldji et al., 2000; Meaney et al., 2000; Braungart-Rieker et al., 2001; Shin et al., 2008; Curley and Champagne, 2015).

Establishing effective bidirectional communication does not only require that the pup is able to signal distress effectively, but also that the dam is able to perceive, process and respond accurately and timely to these cues (Shin et al., 2008; Bornstein et al., 2017). The separation-induced USV test has been used to assess the quantity and quality of pup USVs after separation from its dam and litter (Hahn and Lavooy, 2005), but it is essentially a unidirectional behavioral assay that focusses on the pup. On the other hand, assays such as the pup retrieval test (PRT) or USV playback tests center on maternal behaviors, such as search and retrieval (Sewell, 1970; Smotherman et al., 1974; Ehret and Haack, 1982; Ehret, 1992, 2005). Some studies implemented both unidirectional procedures separately, but assessed statistical association afterward (Wöhr and Schwarting, 2008; Bowers et al., 2013; Abuaish et al., 2020). Combining both procedures in one behavioral assay has several advantages. First, the behavioral readouts can be sampled in a single assay, which reduces workload, and microenvironmental variability, originating from differences in animal transportation and handling, for example (Sukoff Rizzo and Silverman, 2016; Ey et al., 2020). Second, communication and social competence can be investigated as a bidirectional process in the same animals (Vogel et al., 2019). Third, the complex interaction between deficits in dam and pup can be investigated (Kelly et al., 2000). The latter is particularly important in rodent models of disorders with early-life communication deficits, such as autism or fetal alcohol syndrome (Kelly et al., 2000; Bosque Ortiz et al., 2022). Therefore, we present BAMBI (Bidirectional Automated Mother-pup Behavioral Interaction test), a combined, automated approach to assess early-life communicative bidirectionality in laboratory mice. The automated PRT, as described in Winters et al. (2022), was expanded with simultaneous recording and automated detection of pup USVs.

Abbreviations: COLAB, Google COLAB; DAS, deep audio segmenter; DLC, deep lab cut; FPS, frames per second; GD, gestational day; HR, hazard ratio; LMT, live mouse tracker; PND, postnatal day; PRT, pup retrieval test; px, pixel; SimBA, simple behavioral analysis; USV, ultrasonic vocalization; USVs10 s, number of USVs recorded during the first 10 s of the test.

Materials and methods

Animal housing and breeding

C57BL/6J mice from Janvier Labs (Le Genest-Saint-Isle, France) and the KU Leuven Animal Facility (Leuven, Belgium) were time-specifically bred and kept at a 12/12-h light-dark cycle (lights on at 7 a.m.), with *ad libitum* water and food in conditioned rooms (22°C, humidity 30%). The morning after mating was considered as gestational day (GD) 0.5. On GD0.5, dams were housed individually for the remainder of the pregnancy and pregnancies were confirmed between GD7.5 and GD10.5 by recording weight evolution based on Heyne et al. (2015). All experimental procedures were approved by the Animal Ethics Committee of KU Leuven (P028/2018), in accordance with European Community Council Directive 86/609/EEC, the ARRIVE guidelines and the ILAR Guide to the Care and Use of Experimental Animals.

Experimental groups

In compliance with the reduction principle, mice for the present methodological work were obtained from an independently designed pharmacological study, in which pregnant dams were injected with valproic acid sodium salt (VPA) in order to generate pups representing a neurodevelopmental model of autism. Pups were pharmacologically treated to attempt a rescue of the behavioral impairment. More specifically, pregnant dams (N = 44) received a single subcutaneous injection with 60 mg/ml VPA (Sigma Aldrich, Taufkirchen, Germany) dissolved in saline solution on GD12.5. The day of birth was considered as PND0. To standardize nest composition, nests were culled to six pups on PND0 and every nest needed to have at least four pups with both sexes present. These restrictions resulted in 29 dams with viable progeny and a total of 156 pups for further testing. Pups were subcutaneously injected daily from P1-7 with a low (0.5 mg/kg) or a high (2 mg/kg) dose of THIQ (N-[(1R)-1-[(4-Chlorophenyl)methyl]-2-[4-cyclohexyl-4-(1H-1,2,4-trazol-1-ylmethyl)-1-piperidinyl]-2-oxoethyl]-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide; Bio-techne, Abingdon, UK) or a PBS-DMSO control vehicle [doses adapted from Mastinu et al. (2018)]. THIQ was dissolved in PBS and 3.5% DMSO. In total, nine litters (48 pups) were injected with low THIQ dose, 10 l (50 pups) with a high THIQ dose and 10 l (58 pups) with the PBS-DMSO control vehicle. The pharmacological effects are not the focus of the present study and will be described in a separate study. In the current study the aim is to present a proof of principle demonstration of the feasibility and validity of a new automated method for behavioral testing of early life mother-pup bidirectional interactions. Since pharmacological effects were not relevant for the present study, we employed a general linear model (GLM) in which drug effect was set as fixed factor, in order to correct for drug effects (see Calculation of parameters and statistics), and the three drug groups (low-dose THIQ, high-dose THIQ and vehicle) were pooled into one group.

FIGURE 1
Image of the BAMBI testing setup. BAMBI test was performed in the home-cage and included a cup on PND7-13 to prevent the pups from crawling back into the nest. An ultrasound microphone was placed approximately 5 cm above the test pup's corner in order to minimize interference from USVs emitted by the pups in the nest (in the opposite corner). A video camera was mounted above the home-cage.

For the present study, animals were divided into the following groups: Dams (n = 29) and pups (n = 156). For the pup sex effect analysis, animals were divided into three groups: dams (n = 29), male pups (n = 72) and female pups (n = 84). For the subsequent analyses investigating the general behavioral interactions between mother and pups, we corrected pup sex effects through a GLM model in which pup sex was set as fixed factor and we pooled male and female pups together. Mice were tested at five time-points: pup postnatal day (PND) 5, 7, 9, 11, and 13.

Pup retrieval test (PRT) protocol

The PRT was performed as described previously by Winters et al. (2022). Briefly, the test is performed in the home-cage which is placed inside a Styrofoam box (**Figure 1**; 370 mm × 300 mm × 330 mm) to create a visually isolated environment. A single pup was removed from the nest and placed in a clean, glass cup pre-heated to 35°C using a heating pad. A trial was started by a beep when the dam was on the nesting site. Hereafter, the pup was placed in the furthest corner from the nest. Trials had a maximum duration of 100 s after the beep, and when the pup was not retrieved within this time, it was returned to the nest by the experimenter. On PND7-13, since pups had developed more mature motor skills, they were kept from crawling back into the nest by placing them in a cup (**Figure 1**; 90 mm diameter and 55 mm height) as described by Esposito et al. (2019). Per dam, the PRT was repeated six times on PND5, and due to practical limitations four times per dam on PND7-13. For all test ages, pup sex was counterbalanced per dam and pups were not marked during this test to avoid odor interference. Pups thus could not be identified, meaning that a pup might have been tested more than once. Maternal trial sequence was defined as the order of trials within a dam on a specific testing day. During each trial, PRT performance was scored by the experimenter performing the test (two experimenters in total) for latency to retrieval (s) and retrieval success (0 = not retrieved; 1 = retrieved).

Ultrasonic vocalization recording and pre-processing

Pup USVs were recorded using an ultrasound microphone (Dodotronic Ultramic UM250K, Rome, Italy) connected to a personal computer equipped with Avisoft SASLab Lite software (Avisoft, Bioacoustics, Berlin, Germany). The microphone was placed approximately 5 cm above the pups' corner or cup to minimize interference by USVs emitted by the pups in the nest. USVs were recorded for 100 s, with a sampling rate of 250 kHz and 16 bits. Audacity® open-source software (Version 3.1.3)[1] was used to remove DC (direct current) offset and a equalization (EQ) curve filter was used to remove all signal below 40 kHz (**Figure 2**).

Synchronization of USV and behavioral pup retrieval recording

A Foscam C2 IP-camera (EUport, Wageningen) was mounted over the home-cage to record maternal behavior. Per dam, one video was recorded including six PRT trials on PND5 or four PRT trials on PND7-13. A PRT trial started after the dam was back on the nest, and a beep, manually played on a smartphone, introduced the placement of the pup in the furthest corner of the home-cage. Synchronization of behavioral and audio data was done by identifying the beep using frame-precision Shotcut software (version 22.10.25, Meltytech, LLC).[2] This means video or audio signals can be split precisely per individual frame. Here, videos were recorded using a frame rate of 30 frames per second (fps) and videos were split before the first frame in which the beep was audible. The end of the video was defined 100 s after the first frame with an audible beep. Similarly, the audio recordings were recorded in Avisoft with a sampling rate of 250 kHz, whereas Shotcut was used to remove signal before the beep using a frame rate of 25 fps. Again, the start of the recording was defined as the sampling point before the first fragment in which the beep was audible. For USV recordings, the end was not defined as Avisoft automatically ended sampling after 100 s.

1 https://www.audacityteam.org/
2 www.shotcut.org

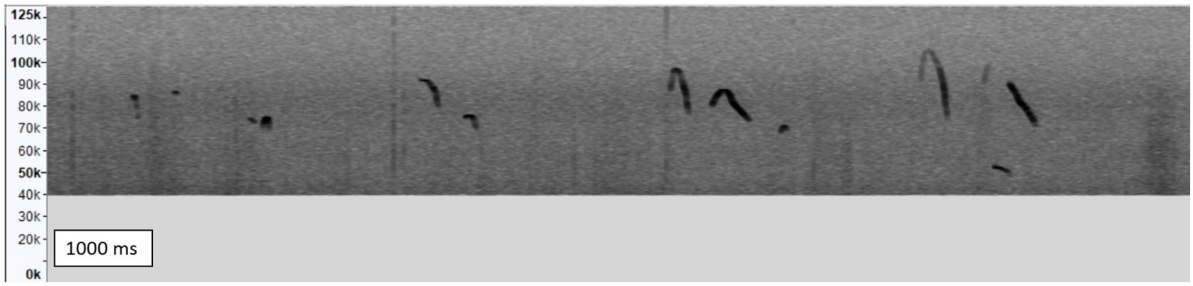

FIGURE 2
Exemplary spectrogram of ultrasonic vocalizations emitted by pups. Frequency (Hz) is shown on the y-axis over a fixed time of 1,000 ms on the x-axis.

Automated detection of pup USVs using deep audio segmenter

Ultrasonic vocalization detection was performed using a custom-build model with Deep Audio Segmenter (DAS; 29). DAS 0.26.7 was installed in an Anaconda environment with Python 3.9.13 and training was performed using the DAS notebook on Google Colaboratory [COLAB; (Steinfath et al., 2021)]. Thirty-two audio recordings were pseudo-randomly selected across two different experiments at five ages (postnatal day 5, 7, 9, 11, and 13) and both sexes. Across all recordings, 2,189 pup vocalizations were manually annotated as segments which is equivalent to 67.7 s of pup USVs. Per audio recording, 80% of the annotated USVs were assigned to the training dataset, 10% to the testing dataset and 10% to the validation dataset. A pup USV network was trained in Google COLAB and structural training parameters were chosen based on Steinfath et al. (2021) and can be found in **Supplementary File 1**. DAS automatically stops training as the validation loss of the model has not improved in 20 epochs (Steinfath et al., 2021). The pup vocalization model did not improve after 44 epochs and performance of this detection model was assessed using precision, recall, F1 scores and overall accuracy. Precision is the percentage of "true cases" per "detected cases." Recall on the other hand is the percentage of "true cases" per "manually annotated cases." The F1 score is the harmonic mean of precision and recall.

Data were post-processed for quality control using a custom-build R script to resolve inaccuracies. In the videos, the time was recorded between the first detectable beep segment and the first frame where the hand of the researcher was completely out of the setup after placing the pup in it. All detected USVs were removed from the recording during this time interval.

Expanded body part tracking

The resulting dataset included 212 frames and was used to re-train the original network from Winters et al. (2022). DeepLabCut 2.2b8 [DLC; (Mathis et al., 2018)] was installed in an Anaconda environment with Python 3.7.7 on a laptop equipped with an Intel Core i5-8350U CPU and 8 GM RAM and Windows 10 64-bit operating system. Training, evaluation and analysis of the expanded model was performed using DLC in Google COLAB.[3]

Learning strategy and performance evaluation of the PRT pose estimation model

The PRT dam–pup tracking model developed by Winters et al. (2022) was trained to track only PND5 pups in a home-cage without a cup. As C57BL/6J pup body shape changes significantly between PND5 and PND13, and the use of a cup is a significant context change that elicits different maternal poses, the model needed to learn these changes. A two-phase hybrid learning strategy was used similar to Gorssen et al. (2022). In the first phase, fourteen extra single trail video recordings were selected because of their variability in pup age and/or modulated home-cage environment. Fifteen frames per video were extracted using k-means clustering in DLC and labeled manually. Additionally, using the original model 10 extra outlier frames per video were extracted using the DLC "jump" algorithm. Labels in these outlier frames were manually refined and frames were only annotated if both dam and pup were visible. The resulting dataset included 212 frames and the original PRT model was retrained with a 95:5 train/test ratio using the same features as Winters et al. (2022). The model was trained for 47 000 iterations and had a mean pixel error over all body parts of 4.29 px for the training dataset and 14.35 px for the test dataset.

In the second training phase, all original labeled data was combined with the data from the first training phase. The output model from the first training phase was then re-trained using the entire dataset with a 95:5 train/test ratio. After 18 000 iterations, the model had a mean pixel error over all body parts of 6.34 px for the training dataset and 10.11 px for the test dataset. Applying a p cut-off ($p = 0.10$) improved mean pixel error on the training dataset to 5.54 px (or 1.96 mm), and 8.82 px (or 3.13 mm) for the test dataset. Average pixels per millimeter did differ between the original dataset and the data used to extend the dataset. Distance calculation, performed in Simple Behavioral Analysis [SimBA; (Nilsson et al., 2020)] as described by Winters et al. (2022), showed an average of

3 https://colab.research.google.com/

2.27 (*SD* = 0.3) px/mm in the original dataset, and an average of 2.87 (*SD* = 0.16) px/mm for the newly annotated data.

A custom-build R script was used to post-process the data (quality control) and to estimate retrieval time. First, a time correction was applied to ensure tracking started at the precise moment the pup was placed in the nest. Hereafter, the rolling median (90 frames) of the distance of pup to the nest was calculated to correct for inaccurate tracking in the first seconds of the PRT. The first frame where the rolling median >85 mm was determined. If this was not the first frame, the distance of pup to nest for all previous frames was set to 85 mm, as pups started at least >85 mm from the nest at the start of PRT. Frames with a mean pup tracking probability over all body parts <0.01 were discarded, as these estimates were considered unreliable. Next, a smoothing algorithm was used to approximate the distance of a pup to the nest using the *stat_smooth* function in R (loess method) with a smoothing factor of 0.25. Observed values deviating more than 15 mm from the smoothing estimate were set to missing. After these quality control steps, retrieval time was estimated as the first frame a pup entered the nest.

Calculation of parameters and statistics

A custom-build R-script (RStudio, Inc., Boston, MA) was used to allow direct comparison between parameters of video and audio analysis. Mean USV duration was calculated as the mean duration of all USVs emitted by the same pup within one trial. USV rate before retrieval was calculated as shown below:

$$USV\ rate\ (\frac{USVs}{s}) = \frac{Number\ USVs\ before\ retrieval}{Retrieval\ time\ (s)}$$

Statistical analyses were performed using the GLM package in R for (binomial) regression models and survival package in R for survival analysis *via* multivariate Cox regression for the trait retrieval success. All models were corrected for USV rate or average USV duration (covariate), sex (fixed effect), day of testing (fixed effect), maternal trial (covariate), and experimental condition (fixed effect).

Results

Performance evaluation of DAS audio detection

The USV detection algorithm accomplished an overall accuracy of 99.7%. Noise was predicted with a precision of 99.8 %, a recall of 99.9%, and a F1 score of 99.9%. Pup USVs were predicted with a precision of 94.3%, a recall of 90.2%, and a F1 score of 92.2%.

Validation of retrieval parameters

To validate the performance of the automated PRT, automatically estimated retrieval times were compared with manual recordings. Retrieval success was estimated with an accuracy of 90.4% (95% *CI* = 87.9–92.5), a sensitivity of 81.0% and specificity of 94.4%. The confusion matrix (Table 1) showed inconsistencies in the prediction of retrieval success of 65 of 670 (9.7%) data entries. After visual inspection, 8 files (Manual: pup not retrieved; Automated: pup retrieved) involved pups walking themselves back into the nest; for 11 files the automated retrieval estimation was more accurate than manual scores; and for 46 files manual scores were more accurate than automated estimations due to tracking errors.

TABLE 1 Confusion matrix raw data.

	Manual not retrieved	Manual retrieved
Automated not retrieved	162	27
Automated retrieved	38	451

For estimated retrieval time, Pearson correlations between manual recordings and automated analysis were high (r = 0.86). However, estimates using video analysis were on average 2.4 (*SD* = 17.8) seconds faster than manual recordings. Within test day (PND5-13), Pearson correlations ranged between r = 0.80 and r = 0.92 (PND5: r = 0.80; PND7: r = 0.80; PND9: r = 0.92; PND11: r = 0.92; PND13: r = 0.86). To establish translatability for the current methodology, manual and automated recordings with a difference larger than 30 s were flagged based on the distribution of differences (Figure 3A). A total of 54 records were flagged of which 31 previously inspected retrieval inconsistencies and the remaining 23 records were visually inspected (Figure 3B). To ensure methodological correctness, 41 automated pup retrieval time estimations were corrected to their manual estimation. Also, pups that walked themselves into the nest were removed from the dataset as bidirectional behavior might be affected. The final dataset is visualized in Figure 3C and the confusion matrix is shown in Table 2. This corrected dataset had an accuracy of 95.1% (95% *CI* = 93.2–96.6), sensitivity of 89.6% and specificity of 97.28%.

Correlations between USV parameters

Correlational analysis showed that the total number of USVs emitted before retrieval was correlated with the USV rate before retrieval (r = 0.84; p < 0.001), mean USV duration (r = 0.44; p < 0.001) and first USV event (r = −0.30; p < 0.001). The same pattern was observed for separate test days (Supplementary Figure 2).

Repeatability of traits over test days

Repeatability of traits was assessed by looking at the Pearson correlation matrix within a trait over time for the mean value of pups with the same sex within dams (Supplementary Figure 3). For maternal retrieval time, repeatability was generally moderate to high for consecutive test days, significant and consistently positive (r = 0.32–0.63; p < 0.05–0.001). The correlations suggest that dams who retrieve their pups faster on PND7 generally also will do so on the other days of testing. Pearson correlations between

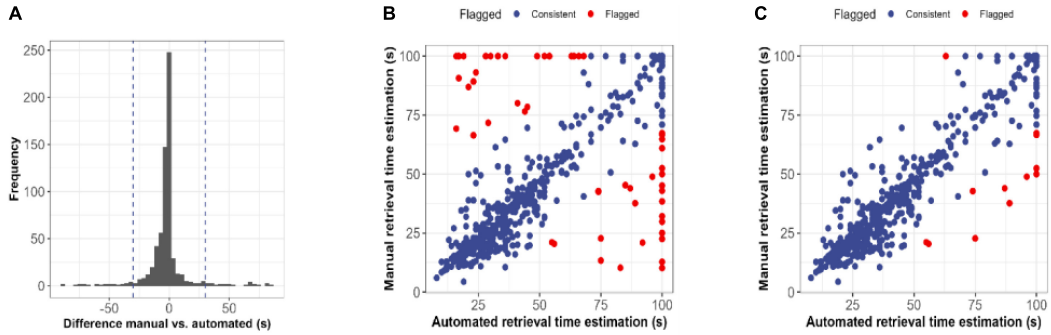

FIGURE 3
Post-processing quality control of retrieval time estimations. **(A)** Histogram representing the difference in seconds between manual and automated estimations of the retrieval time. Automated estimations of retrieval time were on average 2.4 s faster than manually registered estimations. **(B)** Scatterplot displaying the relationship between raw manual and automated estimations. Differences smaller than 30 s were accepted and shown in blue, whereas differences larger than 30 s were flagged for visual inspection. **(C)** Scatterplot displaying the relationship between corrected manual and automated estimations. After visual inspection of the flagged estimates of figure, the final estimate was either accepted (red) or corrected to the manual estimation.

PND5 and the other days of testing were the lowest which might be due to the fact that this was the only day in which the cup paradigm was not used.

Repeatabilities for USV rate and mean USV duration were similarly assessed. Correlations were less pronounced, although most correlations were positive (Supplementary Figures 3, 4). Particularly PND7 gave moderate correlations with the other test days for USV rate (r = 0.34–0.50; p < 0.05–0.001) and for mean USV duration (r = 0.39–0.59; p < 0.01–0.001) although not with PND13 data (r = 0.12). For latency to first USV emission, no clear pattern was observed although most correlations were positive (Supplementary Figure 5).

Analysis of pup sex effect

No significant differences between pup sexes were found for USV rate before retrieval (p = 0.81), indicating that the number of USVs, proportioned to the retrieval time, was comparable between pup sexes. However, USVs emitted by male pups had a significantly shorter duration compared to the USVs emitted by females (p < 0.001). Nevertheless, this did not seem to affect maternal behavior. No significant effect of pup sex on maternal retrieval was observed (p = 0.07).

Analysis of bidirectionality

Correlational analysis of PND5-13 data combined (Supplementary Figure 6), indicated a positive association between pup retrieval time and the amount of USVs the pup emitted (r = 0.54; p < 0.001), suggesting that pups that vocalized more were retrieved later. Hereafter, we looked at USV emission rate (number of USVs/retrieval time) and the number of USVs recorded during the first 10 s of the test ($USVs_{10\ sec}$), as most pups were retrieved after 10 s (5 pups <10 s). This was done to correct for the fact that pups that are retrieved slower, also have more time to emit USVs. However, retrieval time was still positively correlated with USV emission rate (r = 0.24; p < 0.001). Interestingly, a significantly positive correlation was also found between retrieval time and $USVs_{10\ sec}$ (r = 0.23; p < 0.001).

Hereafter, we performed correlational analyses for each day separately, to exclude the use of the cup and/or age as cofounding variables for these results (Supplementary Figure 6). For total number of USVs emitted before retrieval, moderate, positive correlations were found with retrieval time for all testing days (r = 0.45–0.61; p < 0.001). This suggests that pups with a higher amount of vocalizations were generally retrieved later. Next, a correction for retrieval time was made by either looking at USV rate or $USVs_{10\ sec}$. Here, a significant positive correlation was only found on PND7-9 (r = 0.31–0.33; p < 0.01) for USV rate and on PND7 and PND13 (r = 0.21–0.29; p < 0.05) for $USVs_{10\ sec}$. It should be noted that non-retrieved pups were assigned a retrieval time of 100 s, which might bias correlations.

The previous results query whether there is a difference in the number of vocalizations emitted by pups that are retrieved and those not retrieved. Binomial regression analysis of PND5-13 data combined, showed that the USV rate was a significant predictor of retrieval success (HR = 0.58; p < 0.001), which was also indicated by the boxplot (Figure 4B). The hazard ratio (HR) of 0.58 indicates that a USV rate increase of 1 USV/s reduces the probability of being retrieved by 42%. Hereafter, analyses were performed for each day separately, to exclude the use of the cup and/or age as cofounding variables for these results. Figure 5 shows that median USV rate was higher in non-retrieved pups than in retrieved pups, although this difference was small on PND5 and PND13. Binomial regression analyses confirmed these results with negative estimated HR's on each test day (HR = 0.46–0.86) with only significant effects found

TABLE 2 Confusion matrix corrected data.

	Manual not retrieved	Manual retrieved
Automated not retrieved	172	13
Automated retrieved	20	465

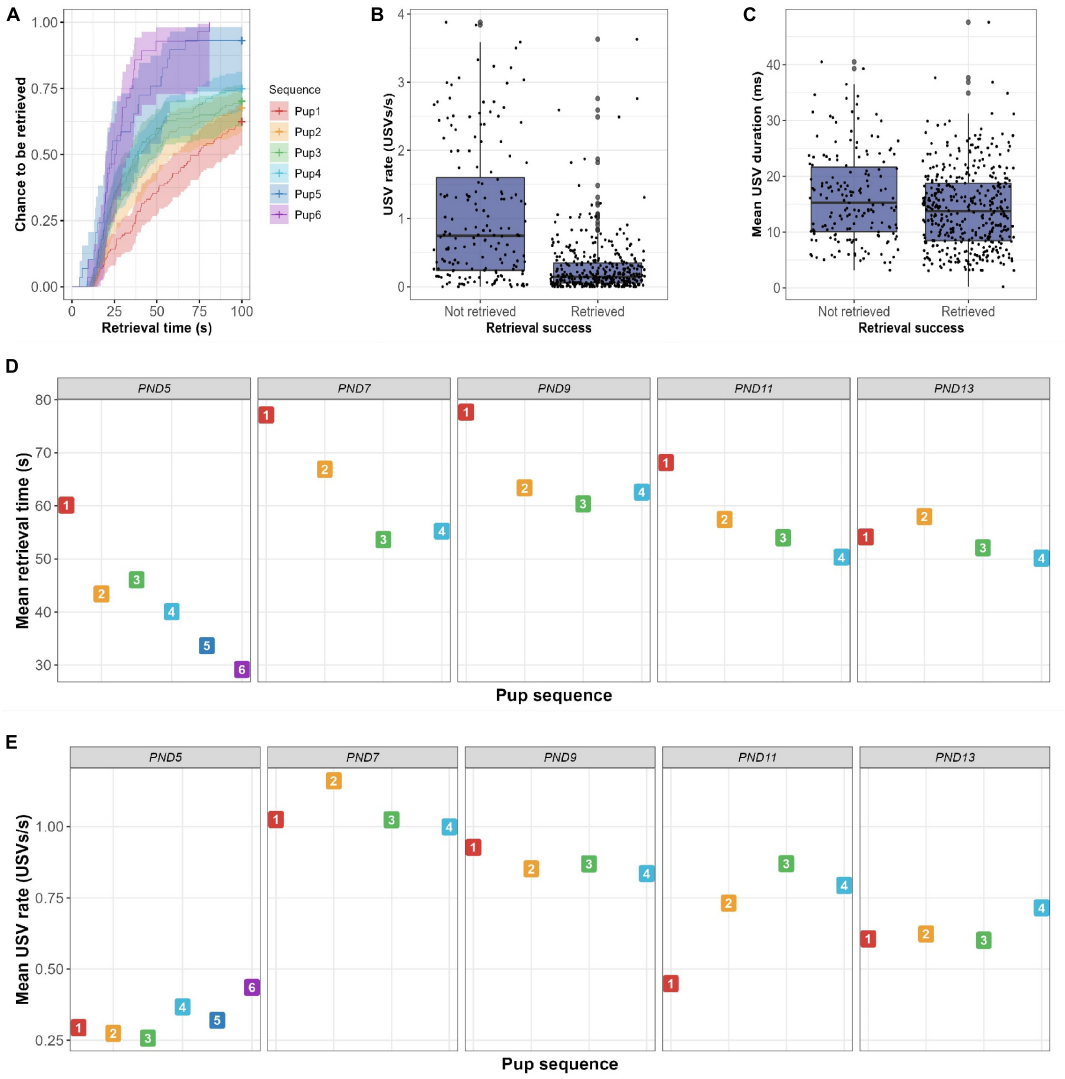

FIGURE 4
Results of bidirectionality analysis. **(A)** Survival plot representing the estimated probability to be retrieved over time in the PRT per maternal trial. Both retrieval time and chance to be retrieved increased as the maternal trial number increased, suggesting a maternal learning effect. **(B)** Boxplot showing the number of USVs emitted per second when pups are either not retrieved or retrieved. Pups with a higher USV rate had a higher probability not to be retrieved ($p < 0.001$). **(C)** Boxplot showing the mean duration of USVs per pup when pups are either not retrieved or retrieved. Pups with a higher mean USV duration had an increased chance not to be retrieved ($p < 0.001$). **(D)** Mean plot showing the mean retrieval time per maternal trial per day. Although retrieval time decreases significantly for trials within days ($p < 0.001$), the learning effect was not significant between days ($p = 0.22$). **(E)** Mean plot showing the mean USV rate per maternal trial sequence per day. USV rate was not affected by repeated trials ($p = 0.59$), whereas test day significantly did ($p = 0.02$).

on PND7, PND9, and PND11. The range of HR between 0.46 and 0.86 over separate test days indicates that a USV rate increase of 1 USV/s reduces the probability of being retrieved by 14–54%.

Furthermore, we wanted to see whether this could be explained by a few poorly retrieving dams (i.e., dams retrieving on fewer than 50% of the trials), such dams were removed from the dataset ($n = 7$ dams). However, the effect of USV rate on retrieval success was still significant after removing poorly retrieving dams ($p < 0.001$). As shown in **Supplementary Table 1**, some pups ($n = 67$) did not vocalize before retrieval, although 64 of these pups were still retrieved by their dams. Of these 64 trials, 48% occurred on PND5, 20% on PND11, and 19% on PND13. Retrieval without pup vocalization is more common in pups with repeated maternal measurements, i.e., with a later position in maternal trial sequence within a litter (**Figure 4A**). Moreover, the sequence of maternal trial was found to influence retrieval success significantly (**Figure 4A**; $P < 0.001$).

The significant effect of maternal trial suggests a learning effect, and as such, provides another possible explanation for the faster retrieval in pups that have a lower vocalization rate. That is, exposing a dam to multiple trials might affect her retrieval behavior and/or might affect pup vocalization rate. However, as shown in **Figure 4E**, USV rate was not significantly affected by maternal trial ($p = 0.59$), although test day did ($p = 0.02$). Over all days, a maternal learning effect was found to be statistically significant (**Figure 4A**; $HR = 1.19$; $p < 0.001$). The HR indicates that an increase in maternal trial by one increases the probability of pup retrieval by 19%. As shown in **Figure 4D**, this maternal learning effect was manifest within repeated trials on the same day ($p < 0.001$), but did not translate between days ($p = 0.22$).

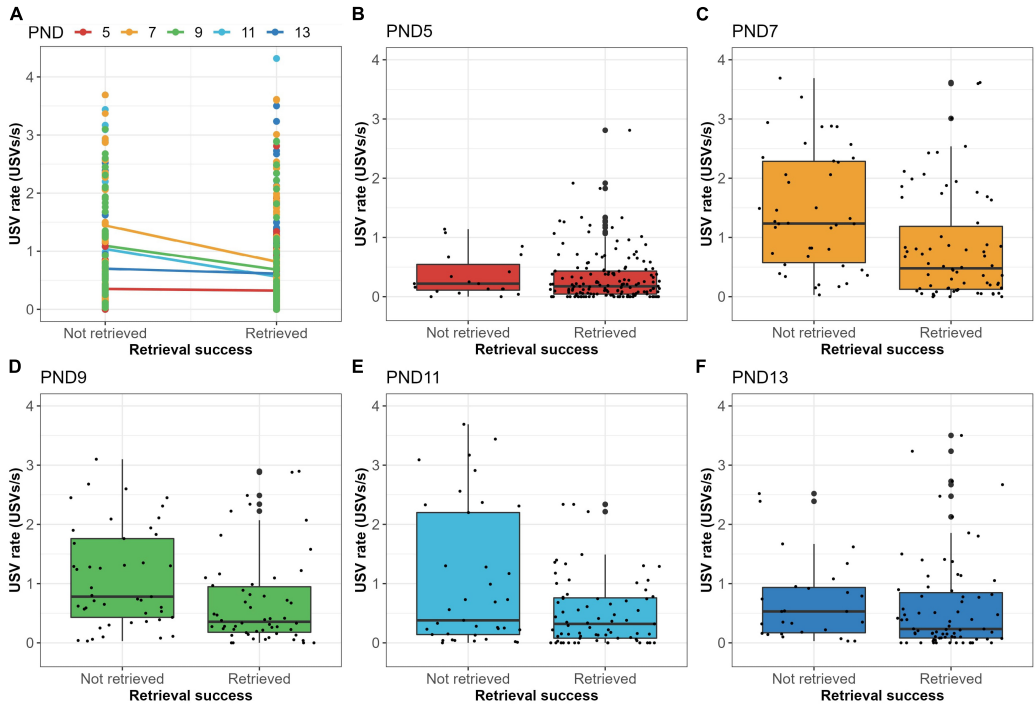

FIGURE 5
USV rate vs. retrieval success for each test day separately. **(A)** Plot with linear regression of USV rate vs. retrieval success scored as a binary variable for each test day separately. For all test days (PND5-13), USV rate was higher in non-retrieved pups than in retrieved pups, although regression estimates were close to 0 (horizontal regression line) for PND5 and PND13. **(B)** Boxplot showing the USV rate (USVs per second) for pups which are either retrieved or not retrieved on PND5. **(C)** Boxplot showing the USV rate (USVs per second) for pups which are either retrieved or not retrieved on PND7. **(D)** Boxplot showing the USV rate (USVs per second) for pups which are either retrieved or not retrieved on PND9. **(E)** Boxplot showing the USV rate (USVs per second) for pups which are either retrieved or not retrieved on PND11. **(F)** Boxplot showing the USV rate (USVs per second) for pups which are either retrieved or not retrieved on PND13.

Lastly, the average duration of pup vocalizations was positively correlated with retrieval time ($r = 0.14$; $p < 0.001$), which was most pronounced on PND7-11 (**Supplementary Figure 7**). Pups emitting USVs with a longer average duration had a lower probability of being retrieved (**Figure 4C**). The estimated effect in a binomial model was −0.053 ($p < 0.001$) which corresponds with a decreased hazard by a factor of 5% for one extra millisecond of USV.

Discussion

Bidirectional dam-pup dyad interactions are critical for pup survival. However, most studies investigated dyadic members and behaviors unilaterally (Wöhr and Schwarting, 2008; Abuaish et al., 2020). In the current study, we describe BAMBI (Bidirectional Automated Mother-pup Behavioral Interaction test) to assess bidirectional dam-pup interaction in laboratory mice. This approach combines the automated PRT described by Winters et al. (2022) with synchronous ultrasonic audio recording and subsequent automated USV detection. At first, we demonstrated the transferability of the previously established dam-pup model to a novel experiment with different traits. Further, a model was developed to detect simultaneously recorded pup USVs with high accuracy. Lastly, we applied this methodology on PRT data sampled on PND5, 7, 9, 11, and 13. Indeed, through synchronous video recording of maternal behavior and audio recording of pup vocalizations, BAMBI allowed to test bidirectional early-life mother-pup interactions in an unprecedented way.

We were able to expand the publicly available model (Winters et al., 2022), and optimized its performance for PRT data with different subject and environmental traits such as the inclusion of a cup. We used a hybrid learning strategy to increase variability relatively fast while minimizing bias. This hybrid learning strategy combined manual annotation of k-means selected frames and refinement of outlier frames selected by the DLC "jump" algorithm. In our first attempts, these newly annotated data were added to the annotated dataset of Winters et al. (2022) and retrained. However, pose estimation performance on videos with novel traits was insufficient (data not shown). We hypothesized this might be due to representation bias whereas the original dataset with robust PRT poses on PND5 outweighed the novel dataset with higher pose variability (Krishnan et al., 2021). Therefore, we used a two-step learning approach similar to Gorssen et al. (2022). In a first step, the original model was retrained only with the newly annotated data, whereas in a second step, all annotated data were used to ensure the algorithm performed well on both the original and new data. The automated retrieval estimate can be seen as proof-of-concept and had a high accuracy of 90.4% over all test days. For future research, two remarks on this learning approach should be kept in mind. First, the train and test error after the second retraining step should be interpreted and reported with caution. That is, all data has been

used in previous training phases and thus the test data might not be completely new anymore. Second, we found a difference in the average pixels per millimeter when comparing the original dataset and the dataset of the current study. Again, this indicates that the retraining pixel errors should be interpreted with caution.

Further, we were able to develop a model to detect ultrasonic vocalizations in the PRT accurately and automatically using DAS (Steinfath et al., 2021). Despite the wide range of available automated detection options, we chose to work with DAS based on a few selection criteria. First, both the toolbox and its basis software (i.e., Python) are completely open source. Second, the system is versatile which is necessary as this PRT assay intends to investigate early-life communicative deficits, and thus, the emitted vocalizations might not be as expected (Scattoni et al., 2008; Bowers et al., 2013; Ey et al., 2013; Shahrier and Wada, 2018). The system therefore should be easily adaptable and relatively flexible. Third, the system should be able to handle background noise as the PRT is performed in freely moving animals, which are interacting with their environment. As argued in the work of Ey et al. (2020), most available automated systems cannot (yet) handle background noise. However, the main limitation of DAS is that the output is limited to the temporal parameters start and end time of the vocalization. Although this was not a problem for the current study, it is a restriction when investigating communicative deficits. Additional spectrographic output parameters should be an integral part of communicative assessment to fully understand eventual deficits.

An obstacle in the current study was the synchronization of video and audio recordings. Both recording data were sampled using different software and could be synchronized by introduction of a beep at the start of the trial. Although we were able to precisely retrace this beep with frame accuracy, this required an intensive step of data processing. To find its way to standard operational practices, an integrated recording system would significantly reduce human involvement and workload. An exemplary integrated recording system was described in Ey et al. (2020). In this work, behavioral monitoring was done using the Live Mouse Tracker [LMT, (de Chaumont et al., 2019)] system in which synchronized USV sequences were recorded using the Avisoft UltraSoundGate Recording system's trigger function. The Avisoft burst recording yield an advantage when working with long-term recordings (Ey et al., 2020). However, in the PRT paradigm a maximum time of 100 s is defined and, as previously mentioned, intends to investigate abnormalities in early-life communicative behaviors. The use of burst recordings should be used with caution as it could miss deviant vocalizations and thus could lead to loss of data which cannot be corrected afterward. Other options exist as most Avisoft Ultra Sound Gates have the possibility to connect a TTL cable, which can be used to start ultrasound recording together with another software, e.g., video recording.

Lastly, we demonstrated the effectiveness of our combined methodology by applying it on PRT data sampled on PND5, 7, 9, 11, and 13. It is important to add a note regarding the selection of the study subjects. In compliance with the reduction principle, mice of the present study were obtained from an independently designed pharmacological study. As a consequence, in the absence of controls for experimental disease models, subjects were exposed to VPA and pharmacological treatment, possibly affecting their behavior. Importantly, the aim of the present work was not to investigate pharmacological effects, but rather to present a proof of principle demonstration of the feasibility and validity of a new automated method for behavioral testing of early life mother-pup bidirectional interactions. Nevertheless, in order to address the issue of not being pharmacologically naive, statistical analyses performed in the current study employed a correction for pharmacological treatments as a confounding variable, by using a GLM model in which drug effect was set as a fixed effect, which allowed to pool the different drug groups into a single group (see Experimental groups). Therefore, the general relationships between pup vocalizations and maternal retrieval found in our study can be considered relevant for future research.

We found an association between maternal retrieval success and pup calling behavior. Counterintuitively, we found that pups that were retrieved had a lower call rate during maternal separation than non-retrieved pups (**Figure 4B**), which was most pronounced on PND7-13. This effect was not caused by certain poorly retrieving mothers, nor testing day. Previous research (D'Amato et al., 2005; Wöhr and Schwarting, 2008) reported a negative relationship between maternal caregiving behaviors and separation-induced pup calling. These studies found that high levels of maternal caregiving behavior in the first days of life lead to reduced numbers of USV later in life, probably because of reduced anxiety. In the same line, maternal carrying has been shown to have soothing effects on pup physiology including cardiac deceleration, immobility response and a reduction of emitted USVs, whereas the absence of this calming response has been reported to hinder maternal retrieval efficacy (Yoshida et al., 2013). Altogether, these findings seemingly go against a robust set of evidence from playback literature which show that pup USVs elicit retrieval behavior (Sewell, 1970; Smotherman et al., 1974; Ehret and Haack, 1982; Ehret, 1992, 2005). Our hypothesis is that USVs do elicit retrieval behavior, but is dependent on a great number of factors (Wöhr et al., 2008) and an excessive amount of USV vocalizations might negatively influence maternal retrieval efficacy. This negative relationship might be due to a miscommunication in the mother-pup dyad. However, further research is necessary to test this hypothesis.

Studies that used maternal retrieval and separation-induced vocalizations separately suggested that these factors might be related. The present simultaneous registrations further confirm and detail this relationship. For example, we found that vocalizations during the first 10 s actually predicted retrieval success, notwithstanding corrections for age and maternal trial sequence. Still, this should not be taken as evidence that pup behavior tunes maternal behavior, as behavioral testing only started on PND5. In our results, we found a peak in USV rate at PND7-9 (**Figure 4D**), which corresponds with previous findings in literature (Sungur et al., 2016). However, future research might consider earlier time points as communicative fitness might already be affected before PND5 in either quality and/or quantity of vocalizations.

Further, we show that dams subjected to repeated retrieval trials show a significant learning curve within the same test day, although this does not translate to an inter-day effect (**Figure 4D**). Between PND7 and 9 this might be explained by the introduction of a cup in the home-cage. However, translation is still limited on the other four days that the cup is present. Research has shown that experience improves pup retrieval success (Stolzenberg et al., 2012; Dunlap et al., 2020). Mice tend to use a spatial memory-based strategy when engaged repetitively in pup search and retrieval (Dunlap et al., 2020). Therefore, an overall decrease in retrieval

time was to be expected as pups were always placed in the same corner. Additionally, Dunlap et al. (2020) report that retrieval behavior further improves by sensory learning of associated cues. The beep at the start of the trial in the current experiment could have predicted the presence of an separated pup in the home-cage. Our findings seem to contradict the findings of Dunlap et al. (2020) although the number of retrieval repetitions is significantly higher than in our PRT procedure, and the test environment might play a role in the valence of pup stimuli (Stolzenberg et al., 2012). For the interpretation of USVs, this means that the functional relevance of USV emission is particularly high at the beginning. After repeated testing, USV emission seems to be less and less relevant, as evidenced by the fact that retrieval behavior even occurred in the absence of USV emission probably due to maternal learning. However, this maternal learning curve could also be used as a behavioral read-out.

In the present study, we adapted our previous automated home-cage PRT (Winters et al., 2022) and we combined video recording of maternal behavior with synchronous audio recording of pup vocalizations in order to assess bidirectional dam-pup dyadic interaction. Our methodology expands the automated pup retrieval test with automated detection of pups' ultrasonic vocalizations. Moreover, we validated our results and showed that the number and rate of ultrasonic vocalizations are associated with retrieval success. BAMBI is a promising new automated home-cage behavioral method that can be applied to both basic and preclinical studies on early-life social development.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**. All models used for this study are publicly available at: doi: 10.17605/OSF.IO/VEJ4H. Further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the Animal Ethics Committee of KU Leuven (P028/2018).

Author contributions

CW and RD'H designed the experimental strategy. CW optimized experimental procedures, labeled the data, and wrote the manuscript with input from WG, MW, and RD'H. CW and WG conceptualized and wrote the code. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2023.1139254/full#supplementary-material

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Animal-friendly behavioral testing in field studies: examples from ground squirrels

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Field studies of behavior provide insight into the expression of behavior in its natural ecological context and can serve as an important complement to behavioral studies conducted in the lab under controlled conditions. In addition to naturalistic observations, behavioral testing can be an important component of field studies of behavior. This mini review evaluates a sample of behavioral testing methods in field studies to identify ways in which behavioral testing can be animal-friendly and generate ethologically relevant data. Specific examples, primarily from studies of ground squirrels, are presented to illustrate ways in which principles of animal-friendly behavioral testing can be applied to and guide testing methods. Tests conducted with animals in their natural habitat and that elicit naturally occurring behavioral responses can minimize stress and disturbance for animals, as well as disruption of the larger ecosystem, and can have high ethological validity. When animals are trapped or handled as part of a study, behavioral testing can be incorporated into handling procedures to reduce overall disturbance. When behavior is evaluated in a testing arena, the arena can be designed to resemble natural conditions to increase the ethological relevance of the test. Efforts to minimize time spent in testing arenas can also reduce disturbance to animals. Adapting a behavioral test to a species or habitat conditions can facilitate reduced disruption to subjects and increased ethological relevance of the test.

KEYWORDS

animal-friendly, animal welfare, behavioral testing, field study, ground squirrel, rodent

Introduction

Behavioral testing typically involves exposing an animal to a specific situation to assess a behavioral variable, and is an important component of neuroscience which can help elucidate elements of behavior under standardized conditions (Hernández-Arteaga and Ågmo, 2023). Laboratory studies are amenable to experimentally manipulating variables and conducting behavioral tests in controlled settings, and are important in establishing causal relationships between neural systems and expression of behavior. Field studies of behavior are less controlled, but allow for evaluation of behavior under naturalistic conditions in the context of the behavioral ecology of animals, and can serve as an important complement to laboratory studies (Nunes and Monroy Montemayor, 2023). In some cases, field studies provide information about behavior through basic observation of animals. For example,

observation can provide information about motor skills associated with behavior and social interactions among individuals, as well as about how they vary among groups of individuals and change during development or across the lifespan (Meyer and Weber, 1996; Rho et al., 2007; Blumstein et al., 2013; Lee and Moss, 2014; Palagi, 2018; Gallo et al., 2021; Nolfo et al., 2021). Behavioral testing in a naturalistic field setting can reinforce observations, and in some cases provide a more feasible alternative to observation. For example, behavioral testing can be useful in the study of nocturnal or secretive animals whose behavior is difficult to directly observe, or in studies of rare events such as the threat of predation that might occur infrequently during regular observations (Tinbergen, 1948; Holekamp, 1986; Brehm et al., 2020). In developmental studies, behavioral testing can allow for finer-scale evaluation of behavior at specific time points or evaluation of behavioral changes across developmental periods. Moreover, behavioral testing can allow for data to be collected under uniform conditions, thereby controlling for possible variations in animals' social or physical environments (Nunes and Monroy Montemayor, 2023).

Recently, d'Isa and Gerlai (2023) proposed guidelines for behavioral testing in lab settings that focus on the well-being of animals and relevance of the testing to the question being evaluated. They noted that minimizing stress during tests contributes to the ethical treatment of subjects, and also reduces possible confounding effects of stress on the outcome of tests. They further suggested that minimizing subjects' contact with human handlers and designing tests that reflect the expression of behavior in naturally occurring contexts increase the reliability and replicability of tests, making results of tests more generalizable to settings beyond the lab. The guidelines proposed by d'Isa and Gerlai (2023) for animal-friendly behavioral testing in lab studies are also applicable to field studies. However, minimizing disruption to subject animals and the wider ecosystem are additional considerations in field studies. Trapping and handling methods, habitat features including anthropogenic alterations to the environment, and in some cases the presence of humans can generate physiological stress responses and influence behavior in free-living animals (Calsi and Bentley, 2009; Johnstone et al., 2012; Boonstra, 2013; Yardimci et al., 2013; Balestri et al., 2014; Huber et al., 2017; Boyle et al., 2021; Fardell et al., 2021). Benefits of field studies include evaluation of behavior in the context in which it naturally occurs and under which it evolved; however, behavioral testing that causes a high degree of disturbance to animals or their habitat can alter this context and negate the value of studying behavior in the field (Buchanan et al., 2012; Sikes et al., 2016).

Here I evaluate behavioral testing in field studies of free-living animals. Rodents are commonly used as model systems in lab and field studies of behavior. Ground squirrels in particular are amenable to behavioral studies in the field because they are diurnal, have relatively short life cycles (making developmental or longitudinal studies tractable), have relatively small home areas, typically occur at moderate to high population density within their habitats, and are fairly easy to handle (Wolff and Sherman, 2007). I assess behavioral testing methods in the context of their friendliness and ethological relevance to subject animals and provide some specific examples, primarily from studies of ground squirrels. I focus on basic tenets of animal-friendly testing including (1) minimizing stress to subject animals, (2) reducing disturbances to subject animals and their habitat, (3) creating standardized conditions for tests, and (4) developing tests germane to the ethology and behavioral ecology of animals. The goal here is to illustrate basic ways that these principles can be applied to and guide behavioral testing of free-living animals.

Motor skill and development

Field studies of motor development have helped elucidate various features of behavior, including development of anti-predator behavior, benefits of juvenile play, the timing of natal dispersal, and energetic costs of behavioral development (Nunes et al., 2004; Berghänel et al., 2015; Carter et al., 2019; Gallo et al., 2021). Development of motor and executive areas of the brain extends into the juvenile period in a wide range of animals (Watson et al., 2006; Stiles and Jernigan, 2010; White and Sillitoe, 2013; Sakai and Sugiyama, 2018), and field studies of motor development can help to identify possible periods of motor and behavioral development in the brain of species not commonly studied in the lab (Carter et al., 2019). In studies of larger animals or animals with relatively long periods of juvenile development, evaluation of motor function and motor development typically involves longitudinal observation or videotaping of motor skills displayed during regular activity, to monitor performance of behavior and improvement in motor skill and coordination over time (Berghänel et al., 2015; Carter et al., 2019; Gallo et al., 2021).

Behavioral testing to evaluate motor skill might not provide the same ecological context as naturalistic observations, but can allow for assessment of motor skill on a finer scale and with greater standardization of conditions than basic observations. For example, Nunes et al. (2004) evaluated development of motor skill in juvenile Belding's ground squirrels (*Urocitellus beldingi*) with tests that required increasingly skilled behavior to progress through the task (**Figure 1**). Squirrels in this species have a relatively short period of juvenile development and because of their small size can be collected and handled with relatively simple and quick procedures, helping to minimize overall disruption to the animals. Testing that involved progression through different skill levels revealed emergence of new skills and increased motor proficiency at different points of development, and controlled for the possibility that the testing procedure itself provided practice and promoted development of specific skills. To mitigate disruptions associated with testing, tests were conducted in squirrels' home areas, which avoided transporting squirrels. Squirrels were tested immediately after being collected and were released immediately after tests were completed, to minimize the time they were removed from their home environment.

Alarm calls

Many species across a range of taxa use alarm calls to communicate information about predators or other potential threats (Slobodchikoff, 2010; Gill and Bierema, 2013; Townsend and Manser, 2013). Within a species, animals can vary alarm vocalizations to encode specific information such as the degree or imminence of danger posed by a predator or potential threat (Zuberbühler et al., 1997; Zuberbühler, 2000; Murphy et al., 2013;

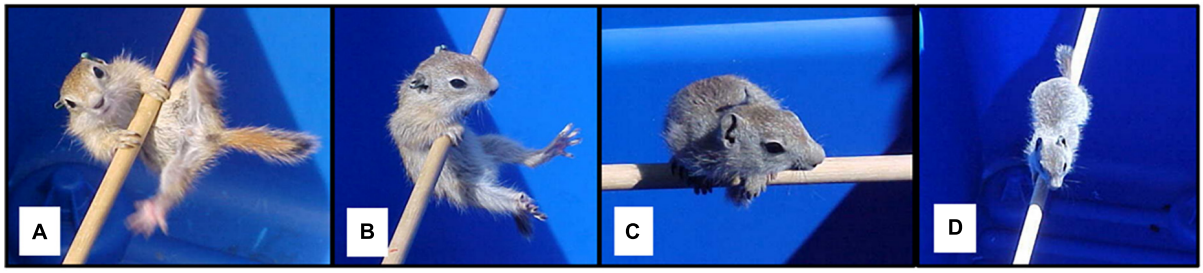

FIGURE 1
Motor skill test for Belding's ground squirrels. Squirrels were placed on a cylindrical wooden rod **(A)**, and their responses were observed. Squirrels could immediately fall, hang on rod **(B)**, climb onto the rod and perch with the body perpendicular to the rod **(C)**, balance on the rod with body perpendicular to the rod **(D)**, walk along the rod, or jump from the rod to the edge of the arena. Squirrels were given scores based on the final outcome of the test, with scores increasing with the difficulty of skills needed to achieve an outcome. Tests were terminated when the squirrel fell off the rod, jumped to the rim of the arena, or after 1 min, whichever came first. Adapted from Nunes et al. (2004).

Coye et al., 2015; Carlson et al., 2017). Because alarm calls communicate information about possible danger, they can elicit specific vigilant or antipredator behavioral responses, as well as physiological responses, in conspecifics who hear the calls (Mateo, 2010; Silvestri et al., 2019; McRae, 2020; Lawson et al., 2021). Evaluation of alarm calls during trapping procedures can provide information about the health status of yellow-bellied marmots (*Marmota flaviventer*; Nash et al., 2020). Moreover, playing recordings of alarm vocalizations can serve as a minimally disruptive testing method for evaluating various elements of vigilant or antipredator behavior. Recordings present stimuli that animals encounter during regular activity, and evoke responses germane to the behavioral ecology of animals. For example, playback of alarm calls have been an important component of behavioral testing in studies assessing variation among individuals in antipredator behavior, the influence of social relationships on perceptions of threat and safety, and responsiveness to communication and signaling from different species or different populations of the same species (Aschemeier and Maher, 2011; Lea and Blumstein, 2011; Makenbeach et al., 2013; Blumstein et al., 2017; Lengagne et al., 2020).

Studies of ground squirrels involving playback of alarm calls have also evaluated the trade-off between body condition and vigilance. Arenz and Leger (2000) supplemented some juvenile thirteen lined ground squirrels (*Ictidomys tridecenlineatus*) with high energy food to manipulate body mass and body condition. They observed the vigilant and foraging behavior of juveniles, and found that unsupplemented juveniles foraged more and displayed less vigilant behavior than did supplemented juveniles. Bachman (1993) similarly manipulated body condition of adult and yearling female Belding's ground squirrels by supplementing some squirrels with high energy food. She later set up behavioral testing stations with high energy food, and played recordings of alarm calls to assess vigilant responses when squirrels came to feed. Unprovisioned squirrels expressed less vigilant behavior and were more likely to continue feeding when alarm calls were played. These two studies took different approaches to evaluate similar research questions, but their approaches acted synergistically to increase the reliability of the finding that animals may reduce vigilance in favor of foraging when they have smaller energy reserves. Behavioral testing provided evaluation of behavior under relatively uniform conditions, whereas naturalistic observations demonstrated a tradeoff between vigilance and foraging in the daily activity of individuals.

Temperament

Expression of behavior varies among individuals, and behavioral traits of individuals that show consistency over time and across situations are generally referred to as temperament. Elements of temperament comprise behaviors that vary along continua. For example, the caution-boldness continuum includes responses to risks or threats, the avoidance-exploration continuum includes responses to novel objects or situations, and the docility continuum includes the degree to which responses in a situation are passive vs. active (Sih et al., 2004; Réale et al., 2007, 2010; Herde and Eccard, 2013; Petelle et al., 2013). Evaluation of temperament has a range of important applications to the study of human mental health, neural correlates of behavior, physiological responses to stress, the welfare of captive animals, social behavior and social interaction, antipredator behavior, space use, dispersal, behavioral development, and an array of ecological variables (Carere et al., 2001; Dingemanse et al., 2004; Both et al., 2005; Boon et al., 2008; Clary et al., 2014; Vetter et al., 2016; Rasmussen and Belk, 2017; Hecht et al., 2021; MacGregor et al., 2021; Pomerantz and Capitanio, 2021; Wauters et al., 2021; Skinner et al., 2022; Luciano et al., 2023; Nunes and Monroy Montemayor, 2023). Here I discuss behavioral testing methods related to assessing elements of temperament, and provide examples of methods used to evaluate development of temperament along the caution-boldness and docility continua in Belding's ground squirrels.

Flight-initiation distance tests (henceforth flight tests) gauge the distance at which an individual flees from an approaching human and are commonly used to evaluate temperament along the caution-boldness continuum (Ydenberg and Dill, 1986; Blumstein, 2003; Runyan and Blumstein, 2004). Flight is an antipredator response, and flight tests are considered to provide a measure of caution or boldness in response to a threat (Cooper, 2009; Petelle et al., 2013). Flight tests elicit a response among subjects, but do not require trapping or handling, minimizing stress to subject animals and disturbance to the local habitat. Flight tests have been

an integral component of a range of studies addressing diverse research questions related to energetic influences on behavior, behavioral strategies in reproduction, behavioral adaptations to local environmental conditions, species distributions based on interactions between behavior and habitat, and behavioral responses to climate change (Shuai et al., 2019, 2022; Pereira et al., 2020; Satterfeld and Johnson, 2020; Stamoulis et al., 2020; Díaz et al., 2021; Hamao et al., 2021; Ventura et al., 2021; Mikula et al., 2023).

The ethological relevance of flight tests can vary. Some species do not distinguish between human intruders and natural predators, and flight distances during tests do not differ when individuals are approached by a human compared to a predator (e.g., Asunsolo-Rivera et al., 2023). However, other species have nuanced responses to threats and discriminate between different levels of threat or different types of predators, and flight distances in response to human intruders can differ from those in response to actual predators (Allan et al., 2021; Morelli et al., 2022). Thus, in studies specifically evaluating antipredator behavior, rather than temperament in general, behavioral observations of responses to predators would increase the reliability of results obtained from flight tests.

Prior interactions with people and levels of local human activity can influence the outcomes of flight tests. During repeated trials over a short time period, test subjects can become habituated to human intruders and flee at shorter approach distances (Petelle et al., 2013). Similarly, in areas with high human population density, animals can become acclimated to people and flee at shorter distances during flight tests (Ekanayake et al., 2022). In some cases, influences of human activity on results of flight tests can be applied to understanding human-wildlife coexistence and can provide insights into behavioral responses to environmental changes caused by anthropogenic activity (Pettit et al., 2021; Mikula et al., 2023).

Because flight tests do not involve trapping or handling animals and mimic disturbances individuals might encounter during regular activity, they can be useful in evaluating behavioral development without introducing variables that could potentially influence developmental processes. Shehan et al. (2023) developed a flight test to assess a possible association between play behavior and the development of cautious responses in juvenile Belding's ground squirrels (**Figure 2**). They evaluated distances at which juvenile squirrels first noticed and then fled from a human intruder, with greater distances reflecting greater caution. They observed that caution increased as juveniles got older and increases were positively correlated with rates of social play, raising the possibility that play behavior may have a role in development of cautious responses in young squirrels.

Ramos et al. (2023) noted that individual responses to trapping or handling can provide information about temperament and suggested that disturbances to animals can be reduced by incorporating assessment of temperament into regular data collection procedures that involve trapping and handling. Evaluation of docility in particular is amenable to being integrated into handling methods. For example, Kannan et al. (2022) used passive vs. active responses of captive goats (*Caprus hircus*) while being weighed as a measure of excitability. Petelle et al. (2013) used passive vs. active responses of free-living yellow-bellied marmots while in traps as a measure of docility. Underhill et al. (2021) evaluated docility in free-living mice (*Peromyscus leucopus* and *P. maniculatus*) and DeRango et al. (2019, 2021) evaluated docility in free-living Galápagos sea lions (*Zalophus wollebaeki*) as the degree to which individuals struggled while being handled. Measurements of docility during handling and trapping have limits in that they do not directly reflect behaviors expressed during regular activity in animals' natural habitat. However, they are generally considered to represent tendencies toward reactive or proactive behaviors not related to threats or novelty, and have been important in studies of behavioral and physiological stress responses, behavioral plasticity, behavioral development, stability of individual behavior across the lifespan, and the degree to which behavioral traits can predict other features of behavior (Réale et al., 2000, 2009; Petelle et al., 2013, 2015, 2017; DeRango et al., 2019, 2021; Underhill et al., 2021; Kannan et al., 2022).

Hurst-Hopf et al. (2023) evaluated the relationship between play behavior and the development of temperament along the docility continuum in Belding's ground squirrels. Docility tests were incorporated into handling procedures, and consisted of holding juvenile squirrels and videotaping their responses for 30 s (**Figure 3**). Responses shifted to being less passive and more active as juveniles got older. This shift was correlated with rates of social play, raising the possibility that play behavior may refine development of temperament in young squirrels. Responses during docility tests were not directly generalizable to specific behaviors within the behavioral repertoires of squirrels, but contributed to formulation of a developmental hypothesis suggesting that as juvenile squirrels venture farther from the natal burrow, behavioral responses become more proactive to facilitate gathering of information about the social and physical environment, while cautious responses increase to reduce vulnerability to predation (Nunes and Monroy Montemayor, 2023).

Remote monitoring

Technologies that allow for monitoring animals remotely without the presence of people can reduce disruption to animals and their habitats and eliminate confounding effects that may be associated with human observers nearby (Trathan and Emmerson, 2014). Radio-frequency identification (RFID) systems have important applications for remote monitoring in behavioral testing in free-living rodents as well as a range of other vertebrates (Dell'Omo et al., 1998; Ousterhout and Semlitsch, 2014; Fetherman et al., 2017; Hughes et al., 2021; Stryjek et al., 2021; Harrison and Kelly, 2022). In RFID systems, a small passive-integrated transponder (PIT) tag is implanted subcutaneously using a minimally-invasive procedure. The PIT tag facilitates lifetime identification of an individual without external tagging or marking. Antennas can be set up to read PIT tags and record the presence or movement of animals at burrow entrances or nesting sites, natural foraging patches, experimental feeding stations, or established runways regularly used by animals (Dell'Omo et al., 1998). Remote monitoring with RIFD technology can have important applications in a range of studies of free-living rodents including evaluation

of exploratory behavior, risk perception and aversion, structure of social grouping, environmental effects on social affiliation and activity patterns, and effects of social connection on disease transmission and immune system responses (Perony et al., 2012; Schuett et al., 2012; Scheibler et al., 2013, 2014; Halliday et al., 2014; König et al., 2015; Lopes et al., 2016, 2020; Bleicher et al., 2018; He et al., 2019; Evans et al., 2021).

Heuristic approaches to design new animal-friendly behavioral tests

Finding ways to adapt behavioral tests to a specific research question, species, or habitat conditions can increase the ethological and ecological relevance of a study and reduce disruption to subjects. For example, Drayton and Santos (2017) evaluated the

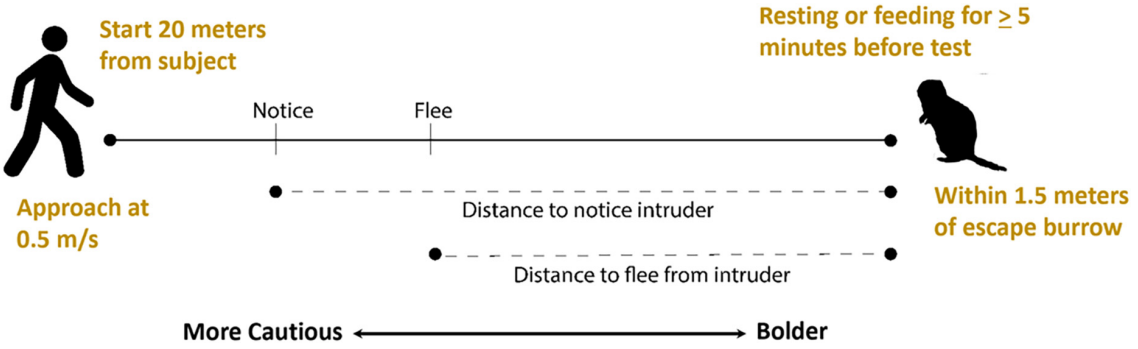

FIGURE 2
Flight initiation distance (flight) tests for Belding's ground squirrels. A human intruder identifies a subject who has been feeding or resting continuously for at least 5 min, starts at a set distance from the squirrel, walks at a constant rate toward the squirrel, and marks the distances at which the squirrel notices and flees from the intruder with greater distances reflecting greater caution.

FIGURE 3
Docility tests for Belding's ground squirrels. Squirrels are held and their responses are recorded for 30 s. Responses such as remaining still **(A)** are scored as passive, and responses such as biting the handler's glove **(B)** or struggling to escape **(C)** are recorded as active. Docility scores are calculated as the number of seconds during tests that juveniles are passive. Adapted from Hurst-Hopf et al. (2023).

FIGURE 4
Problem-solving test for Belding's ground squirrels. A squirrel is placed in a testing arena, and the amount of time needed for the squirrel to escape is recorded **(A)**. Methods of escape include using objects from the squirrel's natural environment such as branches **(B)** and rocks **(C)**.

degree to which non-human animals are aware of what other individuals know. They worked with a population of rhesus macaques (*Macaca mulatta*) on the island of Cayo Santiago in Puerto Rico where macaques are accustomed to the presence of humans. They set up a testing station with behavioral tests that involved macaques following the gaze of a human, and conducted tests when macaques entered the testing area on their own. Drayton and Santos (2017) considered specific features of the population from which subjects were drawn, taking advantage of the macaques' freedom to roam across the island and familiarity with humans to design an animal-friendly behavioral test that did not involve handling macaques or interfering with their regular activity. Moreover, they made use of a behavioral response (gaze-following) present in the animals' natural behavioral repertoire. Macaques followed the gaze of a human observing an object, and the macaques' gaze-following varied with how familiar the human was with the object, suggesting that macaques are cognizant of what other individuals know.

Marks et al. (2017) evaluated the relationship between play behavior in juvenile Belding's ground squirrels and development of the ability to navigate novel situations. They designed a behavioral test that involved placing a juvenile squirrel in an unfamiliar testing arena and recording the amount of time the squirrel needed to escape from the arena. Although the test was conducted in an arena rather than the squirrels' natural habitat, attempts were made to have the arena mimic the natural habitat by equipping it with objects that squirrels encounter in their habitat during regular activity, such as branches and rocks, that could be used as an aid to escape from the arena (**Figure 4**). Tests were terminated after 1 min if squirrels had not escaped by then, to minimize disturbance to squirrels. In addition to minimizing disturbance, limiting the amount of time subjects spend in a testing arena and the number of times they are placed in the arena reduce the likelihood that they will become familiar with the arena or acclimated to testing procedures, which could affect the outcomes of tests conducted in the arena in the future (Ozawa et al., 2011). The time that juvenile squirrels took to escape from the testing arena was found to be associated with their play behavior, suggesting that play might help prepare young animals to navigate unfamiliar situations.

Conclusion

Naturalistic observations and behavioral testing can importantly complement each other in field studies. Observations place results in the context of animals' behavioral ecology, and behavioral testing allows for evaluation of behavior under standardized conditions. Animal-friendly tests that are minimally disruptive not only benefit the welfare of animals but also generate ethologically relevant results. Animal-friendly tests can use a variety of approaches to increase their ethological and ecological relevance to the research question or animals being studied. Tests conducted with subjects in their natural habitat ideally involve eliciting behaviors expressed by the animals during regular activity. When animals are trapped or handled in a study, behavioral tests can be designed to evaluate responses to handling, thereby maximizing data collection during handling and eliminating the need for separate testing. When behavior is evaluated in a testing arena, arranging the arena to resemble natural conditions can support the ethological relevance of the test, and minimizing time spent in the arena can reduce disruption to subjects. Taking into account the behavior and ecology of a species when designing or adapting a behavioral test for free-living animals can help to maximize the overall relevance of the test.

Author contributions

SN conceived, wrote, and edited this mini review.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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IntelliCage: the development and perspectives of a mouse- and user-friendly automated behavioral test system

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IntelliCage for mice is a rodent home-cage equipped with four corner structures harboring symmetrical double panels for operant conditioning at each of the two sides, either by reward (access to water) or by aversion (non-painful stimuli: air-puffs, LED lights). Corner visits, nose-pokes and actual licks at bottle-nipples are recorded individually using subcutaneously implanted transponders for RFID identification of up to 16 adult mice housed in the same home-cage. This allows for recording individual in-cage activity of mice and applying reward/punishment operant conditioning schemes in corners using workflows designed on a versatile graphic user interface. IntelliCage development had four roots: (i) dissatisfaction with standard approaches for analyzing mouse behavior, including standardization and reproducibility issues, (ii) response to handling and housing animal welfare issues, (iii) the increasing number of mouse models had produced a high work burden on classic manual behavioral phenotyping of single mice. and (iv), studies of transponder-chipped mice in outdoor settings revealed clear genetic behavioral differences in mouse models corresponding to those observed by classic testing in the laboratory. The latter observations were important for the development of home-cage testing in social groups, because they contradicted the traditional belief that animals must be tested under social isolation to prevent disturbance by other group members. The use of IntelliCages reduced indeed the amount of classic testing remarkably, while its flexibility was proved in a wide range of applications worldwide including transcontinental parallel testing. Essentially, two lines of testing emerged: sophisticated analysis of spontaneous behavior in the IntelliCage for screening of new genetic models, and hypothesis testing in many fields of behavioral neuroscience. Upcoming developments of the IntelliCage aim at improved stimulus presentation in the learning corners and videotracking of social interactions within the IntelliCage. Its main advantages are (i) that mice live in social context and are not stressfully handled for experiments, (ii) that studies are not restricted in time and can run in absence of humans, (iii) that it increases reproducibility of behavioral phenotyping worldwide, and (iv) that the industrial standardization of the cage permits retrospective data analysis with new statistical tools even after many years.

KEYWORDS

home-cage testing, animal welfare, automated behavioral analysis, standardization, ethology and ecology, comparative and evolutionary neuroscience, marmoset *(Callithrix jacchus)*, reproducibility

1 Introduction

IntelliCage® is a home-cage system with four operant conditioning boxes integrated into the corners of the housing cage and has been marketed since 2003. The design of the IntelliCage was developed by neurobehavioral scientists experienced in mouse testing since 1978, which then was turned into an industrialized product by NewBehavior AG (Zürich). The need for such a system was rooted in four initially independent threads.

1.1 Dissatisfaction with standard phenotyping approaches

Firstly, there was a growing dissatisfaction, or more poetically, a disenchantment, with the interpretation of classic mouse behavioral tests, as discussed in detail by Lipp and Wolfer (2022). This was not based on lack of publications. On the contrary, in the early 1990s, the group of Hans-Peter Lipp and David Wolfer at the University of Zürich (Switzerland) ran one of the few behavioral laboratories specialized in testing mice, which resulted in many cooperative projects that were published in high-ranking journals. However, conceptually, the field disintegrated rapidly. One reason was the uncritical adaptation of tests designed originally for rats and then transferred to mouse neuroscience and behavioral genetics. By and large, a mouse test battery included a mix of operant and fear conditioning tasks with various maze procedures reflecting different cognitive theories. Yet, the tests employed were presented in a piece-meal fashion depending on whether they fitted a specific interpretation. In extreme cases, behavioral outcomes after genetic manipulations were considered by molecular biologists merely as an icing on the cake, not infrequently accompanied by withholding behavioral data questioning the hypothesis, or by not citing contradictory publications. Unfortunately, no one wondered how mice behaved and learned naturally and how this might fit with the laboratory data. At least in rats, the work of the Blanchards in Hawaii permitted interpreting various rat behaviors in ethological terms (Blanchard and Blanchard, 1988), while early approaches of assessing the behavior and interactions of electronically identified mice in interconnected mouse cages were never followed up (Ely et al., 1972, 1976). Thus, interpreting mouse behavior became largely a theory of how mice ought to behave, categorizing movements of mice as proxies for hypothetical brain processes.

For example, a series of studies had focused on behavioral differences associated with a minor variation of the hippocampal mossy fiber system, the extent of the infrapyramidal mossy fiber (IIP-MF) distribution. Earlier studies had shown that genetic and epigenetic variations of this trait were correlated with behavioral test scores as observed after hippocampal lesions (Lipp et al., 1989; Schöpke et al., 1991; Bernasconi-Guastalla et al., 1994; Hausheer-Zarmakupi et al., 1996), yet other studies showed that the IIP-MF were also correlated with strength of handedness (Gruber et al., 1991; Hausheer-Zarmakupi et al., 1996) and intermale aggression (Guillot et al., 1994; Sluyter et al., 1994). The latter findings did not fit well with theories perceiving the hippocampus as a substrate for spatial memory and processing but were instead compatible with earlier theories postulating a generally inhibitory role of the hippocampus for behavior. Because of such ambiguity, the hippocampal community apparently lost interest and, for more than 25 years, the role of the IIP-MF in behavioral control remained mainly obscure and overlooked. Interestingly, the relation between mossy fibers and behavior has recently been investigated through IntelliCage (Bramati et al., 2023).

Similarly to the case of mossy fibers, our behavioral studies of knockout mice missing the prion protein PrP (Büeler et al., 1992) did not reveal any significant behavioral changes, in accordance with other functional studies (Weissmann, 2004; Castle and Gill, 2017). Given that we had used only a few classic tests, it was not clear whether the removal of the PrP gene had hidden negative side-effects preventing the use of the knockout technique as a method protecting animals from prionic infections, as shown much later for cattle (Richt et al., 2007).

Since much information about the ecological validity of behavioral data obtained in the laboratory was missing, a NATO conference was launched to discuss studying brain and behavior in semi-naturalistic environments (Alleva et al., 1995; Lipp and Wolfer, 1995; Nadel, 1995). Eventually, the Lipp/Wolfer group research group decided to set-up outdoor pens in Russia (Lipp and Wolfer, 2013), realized with the support of behavior geneticist Inga I. Poletaeva and bear researcher Valentin S. Pazhetnov. The first goal was to monitor natural selection as a tool to estimate the functional importance of missing genes or hippocampal mossy fiber variations. Later, they used the same pens to study learning processes of feralized mice outdoors (Dell'Omo et al., 2000; Lewejohann et al., 2004), which reinforced their intention to develop a test system more compatible with real world conditions. After all, house mice (*Mus musculus*) show amazing problem-solving abilities enabling them to adapt even to urban environments (Lipp and Wolfer, 2013; Vrbanec et al., 2021).

To be fair, the actual situation has changed by the rediscovery that the key to understanding mouse behavior in standard phenotyping and translational research is to study how mice act in social contexts and naturalistic environments (Smith, 2023), combined with analyzing their variable problem-solving strategies (Le et al., 2023). Most recently, the importance of an "ethological neuroscience" based on ethologically relevant behavioral tests has been emphasized by behavioral neuroscientist Raffaele d'Isa and neuroethologist Robert Gerlai (d'Isa and Gerlai, 2023). This interest in natural behavior of animals is now transferred to studies in humans, boosted by an NIH budget of 25 million USD to develop outdoor tracking of human daily activities (Smith, 2023).

1.2 Animal-unfriendly testing

The second reason to develop a more realistic yet animal-friendly test system was animal welfare. The field of behavioral testing of genetically modified mice emerged around 1990, facing the need to adapt test systems for mice that had been developed and used predominantly in rats. Among these tests, two did not fit mice's evolutionary behavioral framework (the collection of instinctual behaviors) preparing them to cope with daily routines, namely the water maze (Morris et al., 1982) and shock-induced fear conditioning (Fanselow, 1994). Nonetheless, just these two rather stressing tests became standard procedures for assessing memory and learning of mice. Another main problem was the aversion of mice to being handled by humans, especially by males (Sorge et al., 2014; Georgiou

et al., 2022), and their slow responding to various handling-habituation procedures. Finally, a large body of observations has shown that routine procedures such as transport to the test facility can increase plasma corticosterone levels up to 24 h after transport (Drozdowicz et al., 1990), while handling itself has mostly unpredictable effects on behavioral measures influenced by anxiety (Bailey et al., 2006; Deacon, 2006; Drude et al., 2011; Heredia et al., 2012; Lopez-Salesansky et al., 2016; Do et al., 2020; McCarson, 2020; Sensini et al., 2020; Marcotte et al., 2021; Hogue et al., 2022). Thus, minimizing handling of experimental mice would seem a useful strategy to apply in any kind of behavioral test (Wahlsten et al., 2003; Bailey et al., 2006).

1.3 Standardization and reproducibility problems

The field also realized soon that the results of behavioral studies could often not be replicated by other laboratories (Crabbe et al., 1999; Crabbe and Wahlsten, 2003) or, worse, failed replication in the own one, specifically for fear-related tests such as the elevated plus maze (Wahlsten et al., 2006). The simplest solution to deal with this problem was to avoid replication of experiments, as there was no foreseeable benefit in doing this, and to call for more stringent standardization, preferably by having others adopting one's own methods. However, as most laboratories had developed their own protocols, and dimensions of apparatus differed with manufacturers, procedural standardization in the field faced resistance and strongly delayed acceptance of newly invented tests or protocols, specifically in the pharmaceutical industry with huge proprietary behavioral databases for drug testing. Thus, *procedural standardization* met a stalemate, only mitigated by the growing awareness for careful description of behavioral studies (du Sert et al., 2020). On the other hand, the progressive growth of veterinary control and services pushed toward *environmental standardization* in animal housing, resulting in strict control of illumination, temperature, and humidity, as well as minimized contact with humans and germs, thus constantly reducing environmental stimulation. It was obvious to most observers that housing single mice in cages containing only sawdust bedding represented a maximally impoverished environment, but even keeping mice in social groups was opposed considerably by reviewers of papers till a study could show that variation of group-house mice in behavioral test situations was not exceeding the statistical variation of individually housed animals (Wolfer et al., 2004).

1.4 Too much work with standard behavioral phenotyping

The fourth and final reason to envision a new test system was very simple and practical. The number of mice used for behavioral research had exploded. From 1940 to 1989, a PubMed search for "mice" and "behavior" found some 1,800 papers, mostly referring to behavior genetics, drug testing and neuroscience, but only one paper reporting behavioral analysis of transgenic mice (Finger et al., 1988). From 1990 to 2023 the number of papers referring to behavioral testing of genetically modified mice alone rose to 28,000. Because of its previous activities, the Lipp/Wolfer laboratory was one of the earliest to have a comprehensive mouse test battery for collaborative efforts, but it was facing soon personnel and space limits, despite streamlining behavioral phenotyping by automated recording and data analysis. On average, the time to complete a standard manual phenotypic testing of 30–40 mice (including recording of spontaneous activity, water maze, radial maze, avoidance learning, and data analysis) took 3–6 person/months. Given the constraints of academic teaching, expanding the size and staff of the labor was not a satisfactory solution. Therefore, from 1998 to 2001, the Lipp/Wolfer laboratory intensified its efforts to develop a home-cage-based behavioral testing system that could be user- and animal-friendly by harboring mouse groups, permit efficient and automated high-throughput analysis of mouse behavior, and fulfill long-lasting standardization criteria at the procedural level. The goal was achieved in 2002 (Figure 1) when the system was first presented at the Society for Neuroscience Meeting and in journals (Bohannon, 2002; Gerlai, 2002). The IntelliCage was then marketed from 2003 to 2008 by the spin-off company NewBehavior (Zürich, Switzerland) and afterwards by TSE-Systems International (Berlin, Germany).

2 Review body

The following sections will:

- Briefly describe the outdoor studies which provided important input to the design of the IntelliCage
- Discuss the IntelliCage's design features and provide a comprehensive description of the most recent IntelliCage system, currently lacking in the literature
- Review early validation studies from 2003 to 2007
- Present selected papers illustrating some principal uses of the IntelliCage and review the relations between water maze and IntelliCage
- Sketch the degree of acceptance of the system and present some past and upcoming research lines, including a discussion of its inherent limitations
- Describe extensions of the IntelliCage with other home-based analysis systems
- Outline the adaptation of the IntelliCage to larger species and the potential incorporation of new features
- Indicate the present and future state of high-level data analysis in the IntelliCage.

The final conclusions will summarize the insights that the IntelliCage system has brought to the field, chiefly from a conceptual point of view. Our review intends to complement rather than replace an earlier review of the IntelliCage system based on publications till 2018 (Kiryk et al., 2020), which includes discussion of several fundamental studies not analyzed here.

2.1 Three proof-of-principle outdoor studies

While discussing the potential advantages and costs of the resource-consuming project that would have later lead to the development of IntelliCage, it was clear that such a system would

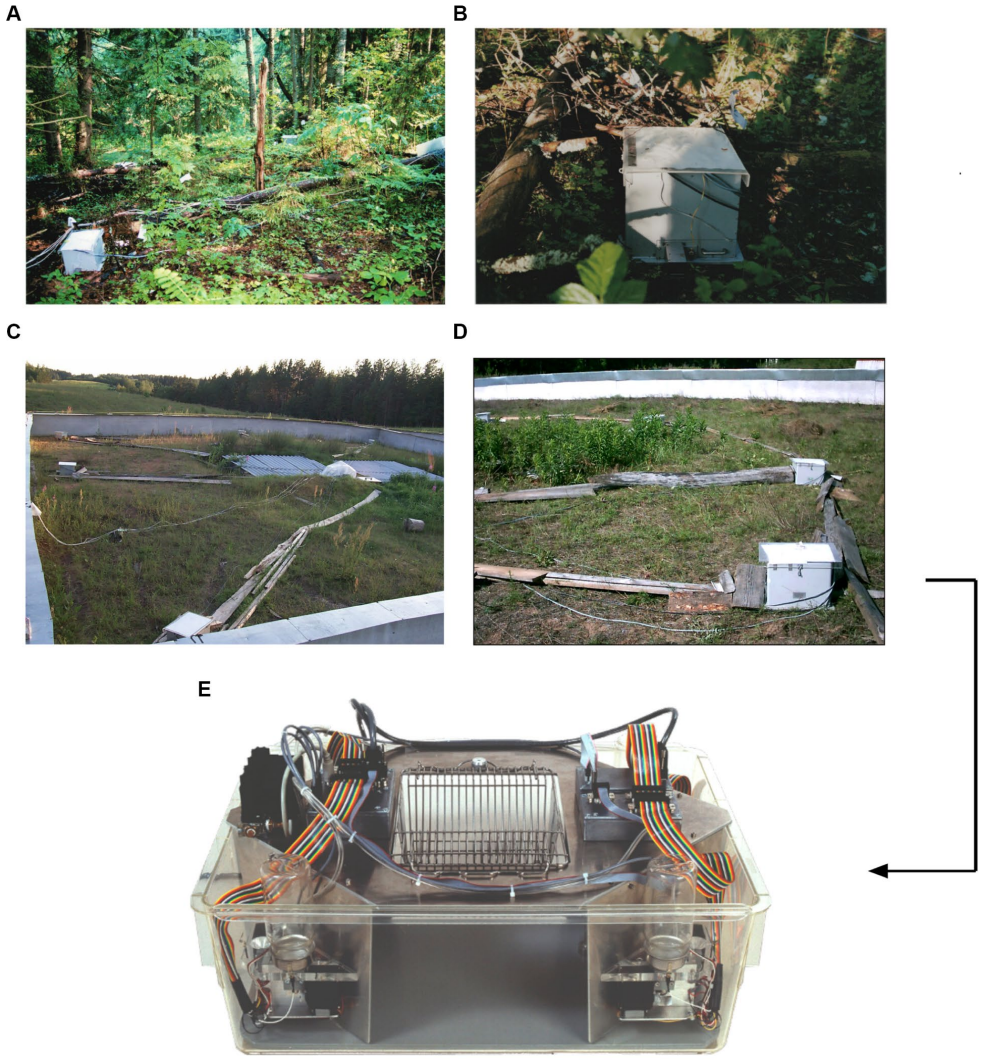

FIGURE 1
From outdoor feeder boxes in Russia to a tool in the laboratory. The conceptual origin of IntelliCage were feeder boxes placed in the forest or in outdoor pens for recording and controlling the patrolling of wild and feralized mice (Lipp and Wolfer, 2013). **(A)** Set-up of feeder boxes to study natural learning in wild mice. **(B)** Closer view of a feeder box in the forest. Experiments in the forest failed because feeder boxes were partially destroyed by roaming bear cubs smelling the mouse food. **(C)** Outdoor pen (20 × 20 m) in the Russian field station Chisti Les containing eight feeder boxes and a central computer controlling the boxes. **(D)** Closer view of an automated feeder box recording entries of mice tagged with transponder chips. Food was only delivered upon a new visit. **(E)** First prototype of an IntelliCage operating on MS-DOS, constructed by Alexei Vyssotski and Giacomo Dell'Omo. **(A,B)** Courtesy of Patricia D'Adamo.

be met with skepticism. The foreseeable main objection would be the belief that behavior of mice must be studied by separating them, because their social interaction would be a confound factor and make the results unreliable. The origin of this idea is not documented. Likely, it reflects a tendency to standardize behavioral testing thoroughly by excluding any external distractions, possibly also the Western culture habit of separating students for exams. Hence, it was necessary to show that mouse behavior as observed in the laboratory can also be assessed reliably under uncontrollable environmental conditions and in social contexts. The condition for this approach was the identification of individual animals by means of radiofrequency identification (RFID), made possible in the mid 1990s by the availability of implantable glass transponders. The technique was refined in two studies (Dell'Omo et al., 1998, 2000). Mice lived for prolonged periods in subterranean shelters from which they could roam and visit feeder sites at varying places. The feeders were either of simple types (just a circular antenna around some mouse food) or more complex ones that could deliver (or withhold) a food upon entry of a transponder-tagged mouse. This allowed simple spatial learning and assessment of patrolling patterns by replacing the feeders. The Lipp/Wolfer group also realized that access to feeder boxes must be strictly restricted to single individuals, because they were expecting that a mouse would visit a feeder, be identified inside, and be rewarded with a small portion of grains. Yet, the mice surprisingly outsmarted the researchers by visiting the boxes in small groups and sharing the food portion (Dell'Omo et al., 2000). Lipp and collaborators conducted three outdoor studies.

In a first, only partially published, study transgenic mice ectopically expressing the neural cell adhesion molecule L1 in astrocytes (Kadmon et al., 1990) were investigated in the laboratory

for water maze learning (Lipp and Wolfer, 1998). Overall, the differences were subtle but hinted to a superior flexibility of the transgenic mice after platform reversal. A batch of mice of either sex (49 transgenics and 22 wildtypes) was then transferred to Russia and released in an outdoor pen for studying survival (Vyssotski et al., 2000). A spatial learning study was then performed by placing food at variable distances from the central shelter. For 18 days, mice were fed in the shelters, then food was exclusively placed in the most distant locations, followed by some changes in placements. The first replacements showed that the transgenics appeared faster at the new sites ($p < 0.05$), thus confirming the conclusions of the water maze study (see Supplementary Figure S1).

A second study used a similar approach (Lewejohann et al., 2004). The mice had been genetically modified by eliminating a non-messenger RNA coined BC1 (Skryabin et al., 2003). BC1 RNA is a small non-messenger RNA common in dendritic microdomains of neurons in rodents and is probably an evolutionary novelty in a rodent ancestor dating back 110 million years ago. Thus, it was hypothesized that the mice should have intact evolutionary old mechanisms governing escape and spatial learning, but that mutants lacking the molecule might show deficits in exploratory behavior requiring a more finely tuning of simple spatial and escape behaviors. Different lines were tested in three laboratories, and one line was also transferred to Russia for studying long-term survival and outdoor learning abilities. The laboratory tests showed unimpaired spatial learning in the mutants, while tests aimed at assessing exploratory behavior revealed deficits in the BC1-KO mice. When tested in the Russian outdoor pen according to similar schemes as the L1 transgenics, the mice deficient in BC1 appeared significantly later at newly placed food sites, confirming the results from the three laboratories.

The third study focused on a mouse model in which the receptor trkB for the brain derived neurotrophin factor (BNDF) had been eliminated postnatally, resulting in mice in which the loss of trkB was restricted to the forebrain (Minichiello et al., 1999). These mice underwent standard behavioral tests in the Lipp/Wolfer laboratory, showing no differences in passive avoidance, no memory impairment in contextual freezing, and only minor impairment on the radial maze, while improvement in two-way avoidance learning hinted at hippocampal deficits (Jarrard, 1980). In the water maze, however, the homozygous mutants were unable to learn the task due to strong thigmotaxis (wall hugging) that even persisted when the escape platform was visually marked (Figure 2A), while the wildtype and heterozygous mice could not be separated statistically. Mice were also investigated for changes in long-term potentiation in hippocampal slices. Here, all genotypes were statistically different from each other (Figure 2B), suggesting that the presence of one functional allele for trkB was mitigating the LTP impairment. A batch of mice was then transferred to Russia for outdoor testing in a radial maze equivalent, in which eight boxes were grouped around two central shelters (Figure 2C). Transponder-tagged mice of all genotypes were tested over 21 days for development of correct box visits, just one visit per box/day being allowed. Because of the potential memory problem of the homozygous mutants as evidenced in the water maze, every third day food was placed inside the shelter to prevent starvation. This was not a complete reversal because the outside boxes were still active (Vyssotski et al., 2002a). All mice learned the task, but on the days with free food inside, wildtype mice quickly abandoned outside patrolling and ate the food inside, whereas the homozygous mutants just continued their usual patrolling. Intriguingly, the heterozygous animals were significantly different from both wildtype and homozygous mutants, corresponding to the earlier LTP data.

These three studies showed that genetically dependent behavioral differences observed by single mouse testing in the laboratory were replicated in outdoor studies. While the differences in the L1 and BC1 study were not dramatic, they were in the same direction. One would have expected that a weak phenotype would disappear under largely uncontrollable outdoor conditions, but this was not the case. Moreover, the outdoor testing of the trkB mutants showed much more precise results as the intermediate scores of the trkB heterozygous mice corresponded exactly to their intermediate position in LTP scores. This was unlikely a chance event. The main lesson was clear: *patrolling of feeder boxes or conditioned patrolling over 20–40 days without human interference gave the same results and came to the same conclusions as many weeks of daily single mouse testing in conventional test batteries in the laboratory*. Another lesson was that the main behavioral factor distinguishing the various genotypes in pens were *problems in spatial reversal learning and switching strategies*. Overall, the pen data reflected the real cognitive problems of mice, namely finding nutrients in a familiar territory under daily changing conditions, yet without facing shock grids or inescapable ponds, and this justified the development of test systems emulating the daily world of mice in natural conditions.

2.2 IntelliCage: design features for a home-cage system housing mice in a socially enriched environment

Before presenting the IntelliCage in detail, we consider here the design features derived from practical experience in the laboratory and outdoors. The outdoor studies implied that the system: (a) needed to run without human supervision for 2–3 weeks with minimal handling; (b) should present retreat opportunities allowing some separation of non-sociable mice; (c) should have at least four sites for patrolling; (d) should provide access to reward sites at which mice could be identified individually; (e) should present a simple set of sensory stimuli guiding patrolling and choices at a given location.

However, the conditions found in laboratories or mouse facilities required restrictions or changes. First, the system ought to be easily stored and cleaned. Therefore, we chose a commercially available rat cage (model 2000 of Tecniplast, Buguggiate) and equipped the IntelliCage with four red mouse houses of the same company, allowing to separate non-sociable mice during rest. Other additional equipment required (Makrolon cages, water bottles and nipples) was available from standard laboratory providers. To minimize disturbance of mice and facilitate cleaning, the plate holding all apparatus could be lifted and placed on a cage with new bedding but could be decomposed easily for maintenance. The corners to be visited needed to be controlled individually, so most of electronic circuitry was placed inside them, remaining connected to a main controller located on a plate closing the top opening of the cage. A tubular RFID antenna with an inner diameter of 30 mm limited access to a corner for a single mouse. The antenna tube was placed at a height of 58 mm which was easily accessible for climbing into it, while the corner was free from bedding material. In contrast to the outdoor pen, we decided to use liquid as reward, because this allowed for quantifiable delivery of water solutions to identified subjects for controlled periods of

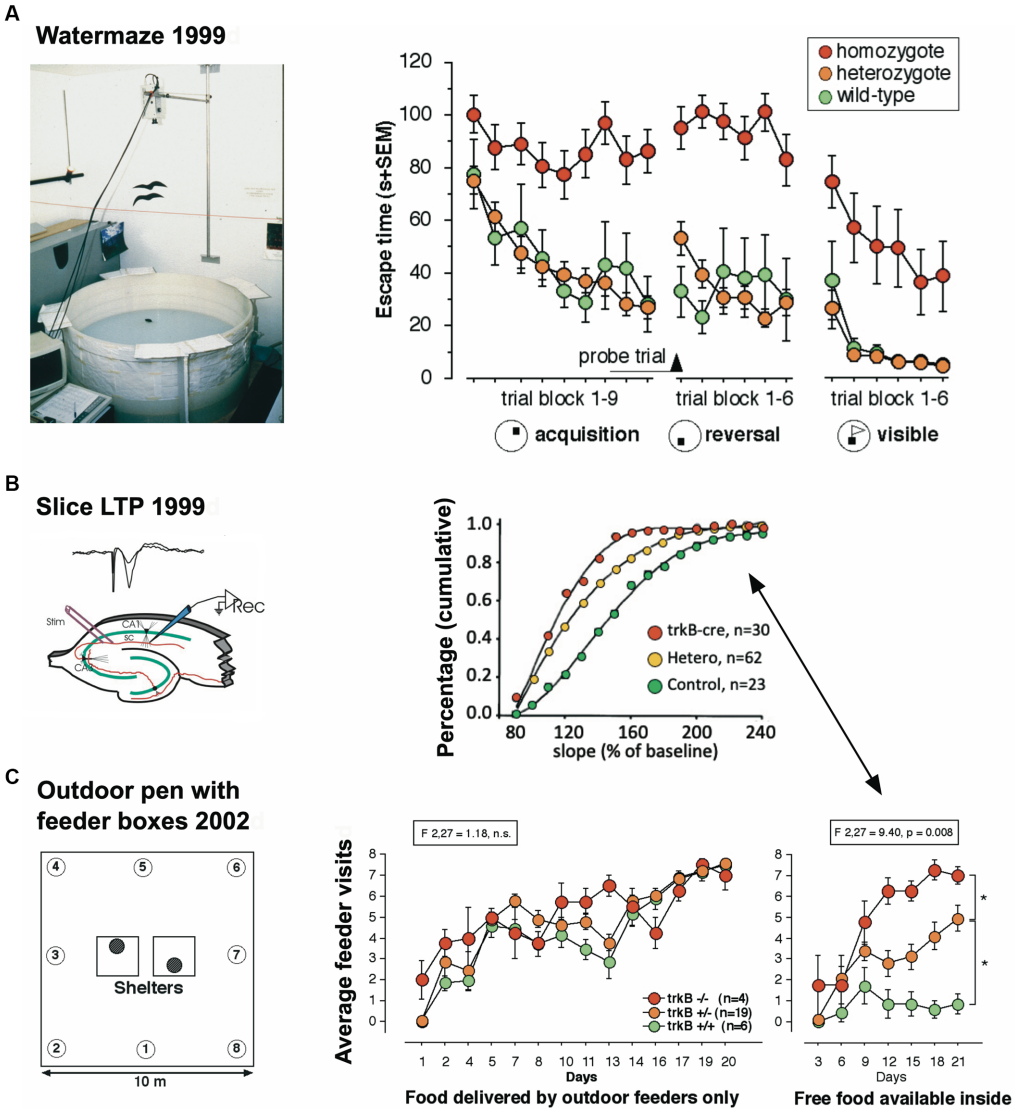

FIGURE 2
An eye-opening study comparing the spatial learning of trkB mutant mice in the lab with the behavior in a semi-naturalistic situation **(A)** trkB mutant mice were tested for water maze learning and showed a severe impairment, mostly visible in the homozygous mutants, while the heterozygous mice behaved like the controls. Modified after Minichiello et al. (1999). **(B)** In the same study, hippocampal slices had shown intermediate LTP values for the heterozygous animals. Modified after Minichiello et al. (1999). **(C)** Outdoor patrolling behavior of the same trkB mutant line in the Russian field station Chisti Les over 21 days. The mice had to patrol 8 boxes to obtain maximal food reward. Every third day, patrolling the loaded boxes was not necessary as food was placed inside the shelter, offering an opportunity for a one-day place reversal learning. Notably, the homozygous mutants ignored this opportunity, which was instead regularly exploited by the wildtype controls. Intriguingly, the heterozygous mutants felt in-between the groups, as would have been expected from the LTP data. Modified after Vyssotski et al. (2002a).

time. Delivery of food reward cannot be controlled that way as pellets are carried around and can be eaten by cagemates. Since most small-scaled dry mazes offering reward face problems with partial reinforcing (the mice do not care to move on after a wrong choice), we also added an air-puff system delivering a moderate, non-painful, punishment depending on adjusting the valves for pressurized air available in most laboratories. Such air-puffs can also serve to expel mice taking corners for sleeping places. In terms of controllable sensory stimuli, we decided to present them only in form of simple visual LED patterns or differently tasting liquids. This required the placement of two bottles per corner, each one freely available or, depending on the experimental protocol, potentially only accessible by nose-poking.

2.3 System description

2.3.1 Hardware

Figure 3A shows the most recent industrialized version of the IntelliCage that was developed from the prototype shown in Figure 1. Each corner contains a motherboard running a firmware that sends the signals from the sensors (RFID, temperature, light barrier, lickometer) to the main controller board on top of the cover plate and sends input from the main controller to the actors (LED, door sliders and valves delivering air-puffs). The hardware settings allow for conditioning of mice by sensing the activity of individuals and acting by applying the

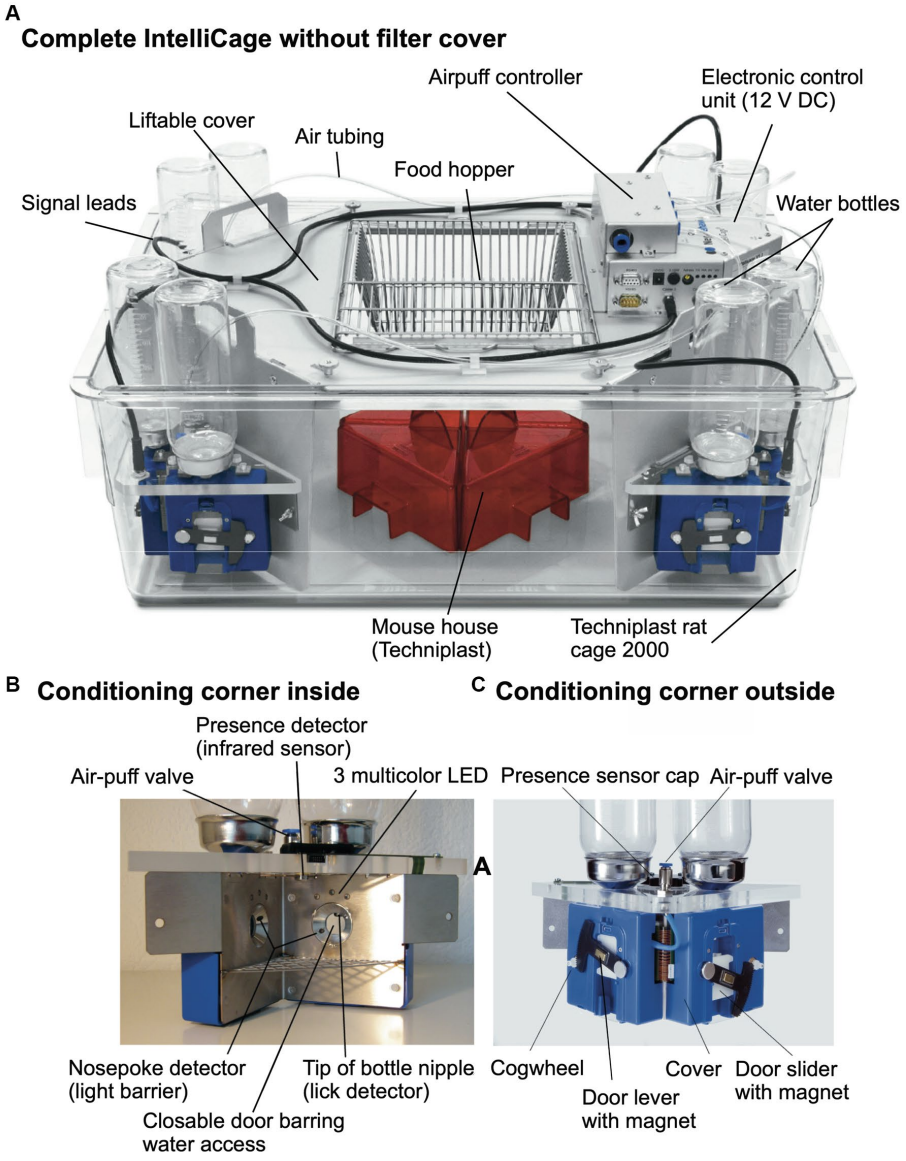

FIGURE 3
Modern IntelliCage since 2006. **(A)** Complete view of the system integrated into a commercially available polycarbonate rat cage (20.5 cm high × 58 × 40 cm at top, 55 × 37.5 cm at bottom, Tecniplast 2000, Buguggiate, Italy). The entire cover plate with the corners can be lifted for cleaning or exchanging the cage body. The electronic control unit integrates light and temperature sensors. It connects with up to 8 IntelliCages running the same or different programs. **(A)** Combination of 4 standard Tecniplast mouse-houses permits preferential huddling of mice. **(B)** Inside view of the conditioning corner faced by the mouse when advancing through the ring antenna. Walls, nose-poke-holes and grids are made from stainless steel. **(C)** Outside view of the conditioning corner. The sliding doors are moved by means of a cogwheel-operated mechanism preventing squeezing of the mouse nose. Part of the operating circuitry is integrated in the blue plastic container.

appropriate responses. The RFID antenna and the temperature sensor together identify the *presence* of a mouse in the corner, *nose-poking* is recorded by breaking an infrared light beam crossing the opening to the bottles and *licking* activity can be registered when the mouse uses muzzle or tongue to touch the drinking nipples of the water bottle (Figure 3B). In response, *door opening/closing* can be initiated via the door slider, *LED lightening can be induced* and some *air-puff* can be given (Figure 3C). LED and door control can be exercised independently on the two sides per corner, hence allowing for left–right, as well as gustatory, discrimination conditioning. These constant input/output options ensure replicability and standardization over time and testing with other species in different environments. More information about hardware and some of its peculiarities are found in Supplementary Figure S2 (Dos and don'ts in the IntelliCage).

2.3.2 Software

The unique feature of the IntelliCage system is its flexible software architecture that has remained largely unchanged for 20 years. Its central

piece is the *Designer* application that sets the response of the system to mouse behavior by uploading a file generated with a proprietary graphical user interface (GUI). Here, units representing actors, sensors, and other instrumental operators can be logically connected using drag-and-drop functions. Many named designs can be constructed and stored by the user, and their activation sequence can be interconnected logically or by temporal schedules. Figure 4 shows examples for the two classes of protocols typically run in the IntelliCage, patrolling and local operant conditioning. For spatial learning, one or several mice have access to water in a defined corner only (Figure 4A). An example of data obtained in this way is shown by the results of a study using serial reversal learning behavior of MHB-Cre:DTA mice lacking medial habenular cells (Kobayashi et al., 2013). These mice showed an impaired ability of spatial reversal learning (Figure 4C), combined with other behavioral deficits, specifically higher impulsivity as also shown in the IntelliCage. To assess impulsivity and processes depending on inhibitory control, the protocols are more complex, as shown for a discount-delay procedure that measures how well mice can solve a conflict between easy access to plain water and the need to wait a defined time for obtaining a sucrose reward (Figure 4B). Interestingly, this procedure is able to identify strain differences (Figure 4D). These two programming examples demonstrate the ability of the IntelliCage to test simultaneously behaviors related to patrolling and to analyze locally the ability of problem-solving. Further graphic examples of such control files can be found under[1].

The ***Controller application*** responds to the three inputs (visits, nose-pokes and licks) according to an experimental file assembled by the designer program (see above). Opening doors is seen as rewarding, closing or not opening doors as negative punishments, air-puffs as

1 http://www.xbehavior.com/packages/intellicage

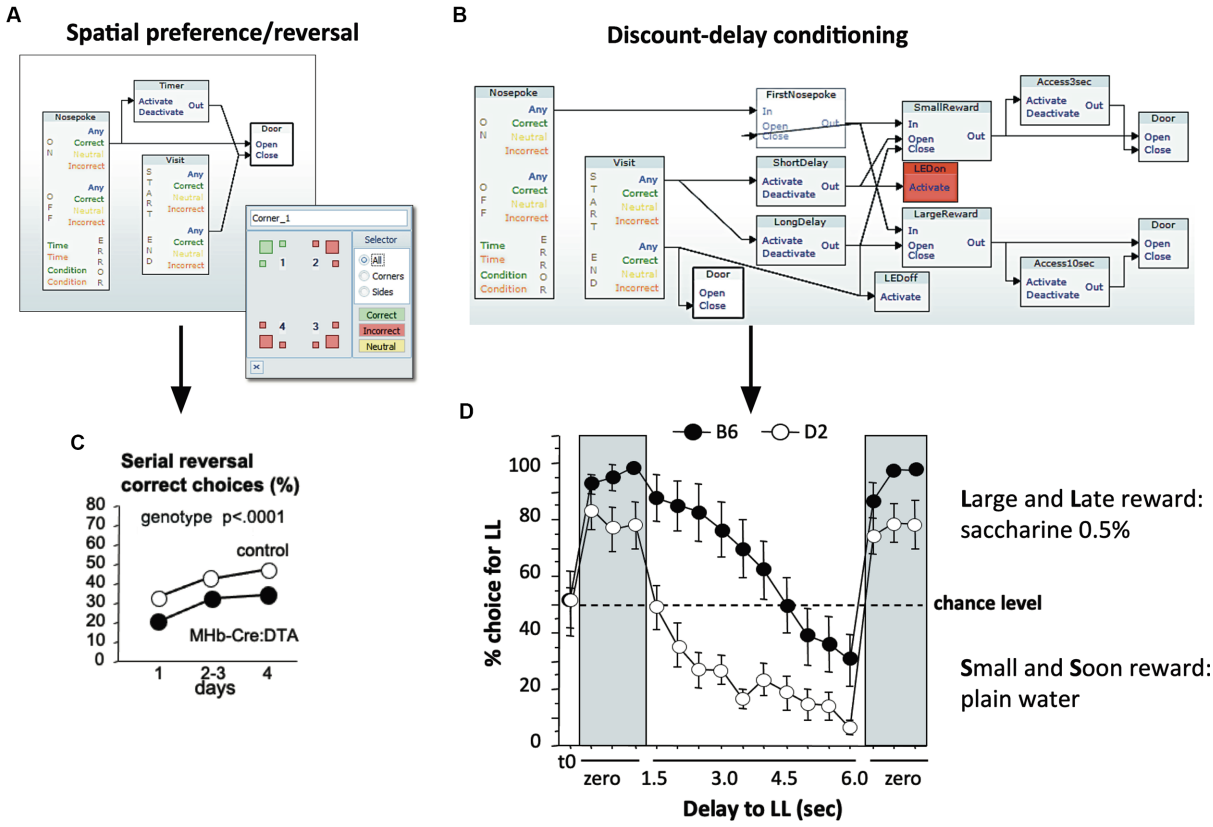

FIGURE 4
Designing simple and complex tasks in the designer program with a graphic user interface (GUI). **(A)** Graphic design for spatial learning. This requires a simple sequence: a specific corner is assigned to one or several animals. Upon identification of an assigned individual, a timer is activated and the door leading to the drinking nipple opens. The door closes after a defined period or after the mouse has left the corner. **(B)** Graphic design for discount delay-conditioning. This procedure measures how well mice can solve a conflict between easy access to plain water and the need to wait a defined time for obtaining a sucrose/saccharine reward. Upon entering a corner, the mouse is identified, two timers are activated according to the learning progress of the mouse, and an LED signal is activated to mark the beginning of the procedure. After having made a nose-poke choice towards one of the bottles, the system will deny access to the sweetened bottle if the nose-poke is too early. The recording of the animal's actions indicates its ability to inhibit learned local movements, yet also a sense for time at short-term scales. **(C)** Data example of simple spatial programming: MHB-Cre:DTA mice carrying a mutation causing postnatal ablation of medial habenular cells are impaired in their ability of spatial reversal learning, however combined with other behavioral deficits (Kobayashi et al., 2013). **(D)** Strain comparison using discount-delay conditioning. C57BL/6 and DBA/2 mice typically differ in their ability of controlling behavior under conflicting situations (Wolfer et al., 2012). Saccharine preference was established rapidly in both strains when there was no imposed delay. Upon increasing waiting times, DBA/2 mice quickly switched to drink plain water, while C57BL/6 mice maintained a preference for saccharine, also with increasing waiting times, but eventually switched to the plain water solution. Presenting immediate reward re-established the saccharine preference in both strains. Example set up by Elisabetta Vannoni.

positive punishment, and LED light configuration were supposed to potentially convey information for instrumental conditioning, or can be used to deter animals. The Controller also presents to the user real-time information in the control panel (Figure 5A). The data are constantly assembled and analyzed using simple statistics showing the progress of the experiment, either for a single mouse or as group average. For example, checking the frequency of corner visits permits determining most or least preferred corners for a given mouse (Figure 5B). The controller can also present ongoing cumulative learning curves that show whether the scores of two experimental groups (such as hippocampally lesioned mice and their controls) coincide or diverge (Figure 5C). The behavior of individual mice can also be singled out. For example, plotting individually the saccharine preference (which can be obtained by presenting pairs of bottles containing either plain water or saccharine) rapidly identifies mice with strong preference, ambivalence, or even initial avoidance of the sweet taste (Figure 5D). Yet, the final collective scores indicated a weak yet significant preference for the entire sample. Other screens show

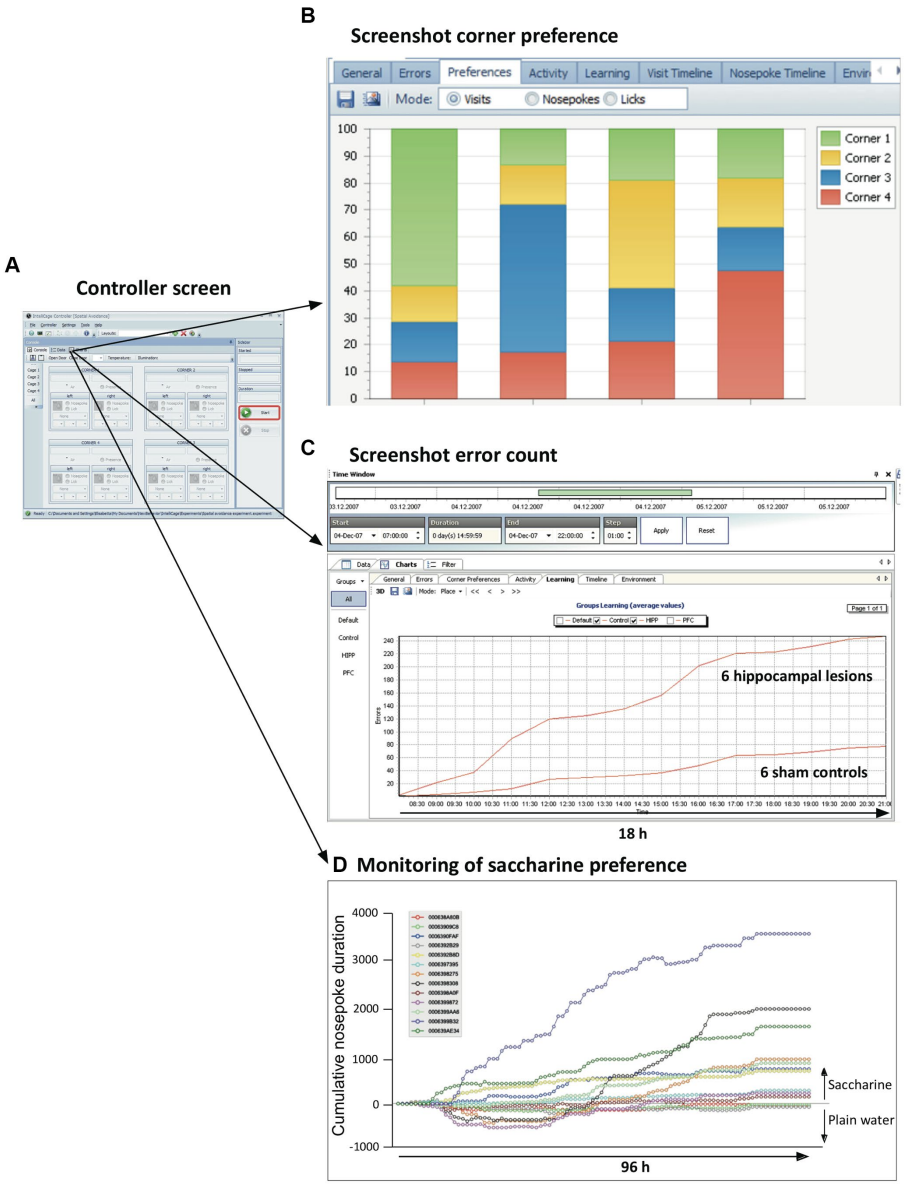

FIGURE 5
Ongoing information provided on-screen by the controller. **(A)** The default controller screen just shows the activity state of sensors and actors. Yet, the menu provides numerous opportunities to call the actual state of the data in both alphanumerical and graphic form. The graphs can be selected for single animals, subgroups, or all mice in the cage. **(B)** Quick monitoring of corner preferences by individual mice. **(C)** Continuous monitoring of behaviors considered as errors or success permits to recognize developing trends resulting from treatments. The screen shows the mean cumulative error rate in reversal learning as observed in a group of mice with hippocampal lesions. **(D)** Individual learning or preference curves can also be plotted, e.g., for saccharine preference. Note that the final mean score of the animals in the cage is around 900, because some of the mice ignored or even avoided saccharine. Also note that every experiment can be graphically replayed (from archive files), for individuals or for treatment groups, by using selectable time windows from seconds to weeks, thus recognizing the development of odd behavior patterns of treatment groups or strangely behaving animals.

actograms, separately for visits, nose-pokes and licks, and the time course of temperature and illumination. All graphs can be interactively shortened or expanded to inspect different phases of mouse activity during the experiment, from very short time windows to day-long plots. The registered data are stored continuously on the PC as text files and turned into zipped files (archives) when the experiment is stopped by exiting the Controller run. The archive files themselves cannot be manipulated, following the recommendation of good laboratory practice (GLP).

Post-experiment visualization of observations and some basic statistical views are provided by the *Analyzer application*. This can read as many archive files as intended, *and replay the entire experiment and its* var*ious stages*, while the user selects the parameters for creating the tabular outputs. These may include subsets of animals, selected time windows, or types of responses such as licks, corner visits and outputs. The tables can then be transferred to a variety of statistical programs or databases such as Excel but must be further analyzed by the user. The entire palette of graphs that were produced by the controller during the experiment can also be obtained in Analyzer.

For in-depth data analyses, however, one would turn to a *pre-assembled software* that allows for detailed data preparation and sophisticated statistical analyses even for users inexperienced in applying statistical software themselves. To our knowledge, there are three software packages facilitating such data analysis, two based on Python (Dzik et al., 2018; Ruffini et al., 2021) and one using R (Voikar et al., 2018). All of them can extract and rearrange data from IntelliCage archive files, but for Python applications, the statistical analysis is left to the user's skills, for example Esmaeili et al. (2022). On the other hand, FlowR (XBehavior, Bänk, Switzerland) is based on a graphic GUI combining R-protocols (Figure 6A), that has been developed by the same persons having implemented the Programmer and the Designer application for the IntelliCage, being thus familiar with the architecture of the data as well as with the behavioral meaning of the protocol files. It also includes pre-assembled advanced statistics (Figures 6B,C), so that persons inexperienced in statistics can just import the archive files for getting the statistics with a few clicks[2]. Moreover, it has been used in a variety of IntelliCage studies (Fischer et al., 2017; Hardt et al., 2017; Ajonijebu et al., 2018; Vogel et al., 2020; Simmons et al., 2021a; Tran et al., 2021; Hahnefeld et al., 2022; Stephan et al., 2022; Vasić et al., 2022; Hühne-Landgraf et al., 2023).

2.4 Early validation studies

Novel systems need time to be accepted by peers or reviewers. In a phase from 2000 to 2004, the earlier versions of the IntelliCage system were tested using various mouse models. This could not be done by systematic studies, but the Lipp/Wolfer laboratory had access to a variety of mouse models that were sent for testing or were leftovers from other studies. From these mice, samples could be used for proof-of-principle studies showing the potential results with graphs to be presented at conferences and meetings. However, some of these earlier studies provided interesting insights as shown in Figure 7.

As one of the advantages of the IntelliCage was the opportunity of testing non-domesticated rodents (since handling during behavioral assessment is not required), two systems were shipped to Russia for studying wild mice from the local populations around the field station and were employed successfully in comparing bank voles (*Clethrionomys glareolus*) against wood mice (*Apodemus sylvaticus*), resulting in a first peer-reviewed IntelliCage paper (Galsworthy et al., 2005). To this end, the IntelliCage had to be placed in a rather primitive and largely uncontrollable environment, namely a log cabin serving as animal house for field studies. Because behavioral test systems are usually run under visual and acoustic isolation in special boxes, there were some concerns whether the observed species differences might not simply reflect uncontrollable events such visitors and outdoor noises. To check this objection, one of the students there, the late Nada Ben Abdallah, had obtained a batch of irradiated Russian mice for a pilot study checking different radiation intensities and their effects on spontaneous activity over a short period. The IntelliCages were placed in the same environment (Figure 7A). The data showed systematic differences that remained without scientific value as it was impossible to verify posthumously the details of the treatments. Yet they showed again that the IntelliCages were able to recognize systematic group effects in partially noisy and uncontrollable environments. Of note, however, is that IntelliCages were used later to reveal irradiation-induced behavioral changes (Barlind et al., 2010; Karlsson et al., 2011; Huo et al., 2012; Roughton et al., 2012; Ben Abdallah et al., 2013; Kalm et al., 2013, 2016; Osman et al., 2014; Kato et al., 2018; Sato et al., 2018).

IntelliCages proved their sensitivity in detecting subtle behavioral changes in DBA/2 mice whose grand-grand-fathers had received postnatal thyroxine injection, supposed to trigger transgenerational changes in brain and behavior (Vyssotski et al., 2000; Vyssotski, 2011). Because these mice had undergone different behavioral standard tests before and could not be used for further studies, they were placed in summer 2003 for a curiosity-check in IntelliCages placed on a table in a histology lab for 20 days. The simple task only required the mice to consume water in a specific corner, by punishing with air-puffs visits to other places (a task which is normally learned quickly by mice). However, these mice showed a persistent error rate that was also audible because of regular hissing of the air-blowers. The error rates even rose after 10 days, and were, for this period, significantly higher in the offspring of the ancestors treated with thyroxine (Figure 7B, see also Lipp, 2005). Because the summer 2003 was exceptionally hot and the laboratories were not climatized, we suspect that the mice sought some cooling and that the air-puffs could have become rewarding, which would explain the persistent error rates. However, at present the cause of the behavioral group difference detected by the IntelliCages remains obscure. IntelliCages used later also discovered epigenetic or paternally transmitted behavioral changes (Gapp et al., 2014; Ajonijebu et al., 2018), proving the sensitivity of the system.

During a collaborative project, the Lipp/Wolfer laboratory tested the effects of lacking CREB (cAMP responsive element binding protein) on mouse behavior (Balschun et al., 2003) and received from the same laboratory that generated the mutants a set of older mice that were carrying a double mutation (CREB/CREM) for preliminary testing. Likewise, some mice and their controls with a CreLox-deletion of the mineralocorticoid receptor (MCR) were also available from a

2 See http://www.xbehavior.com/packages/intelliCage/.

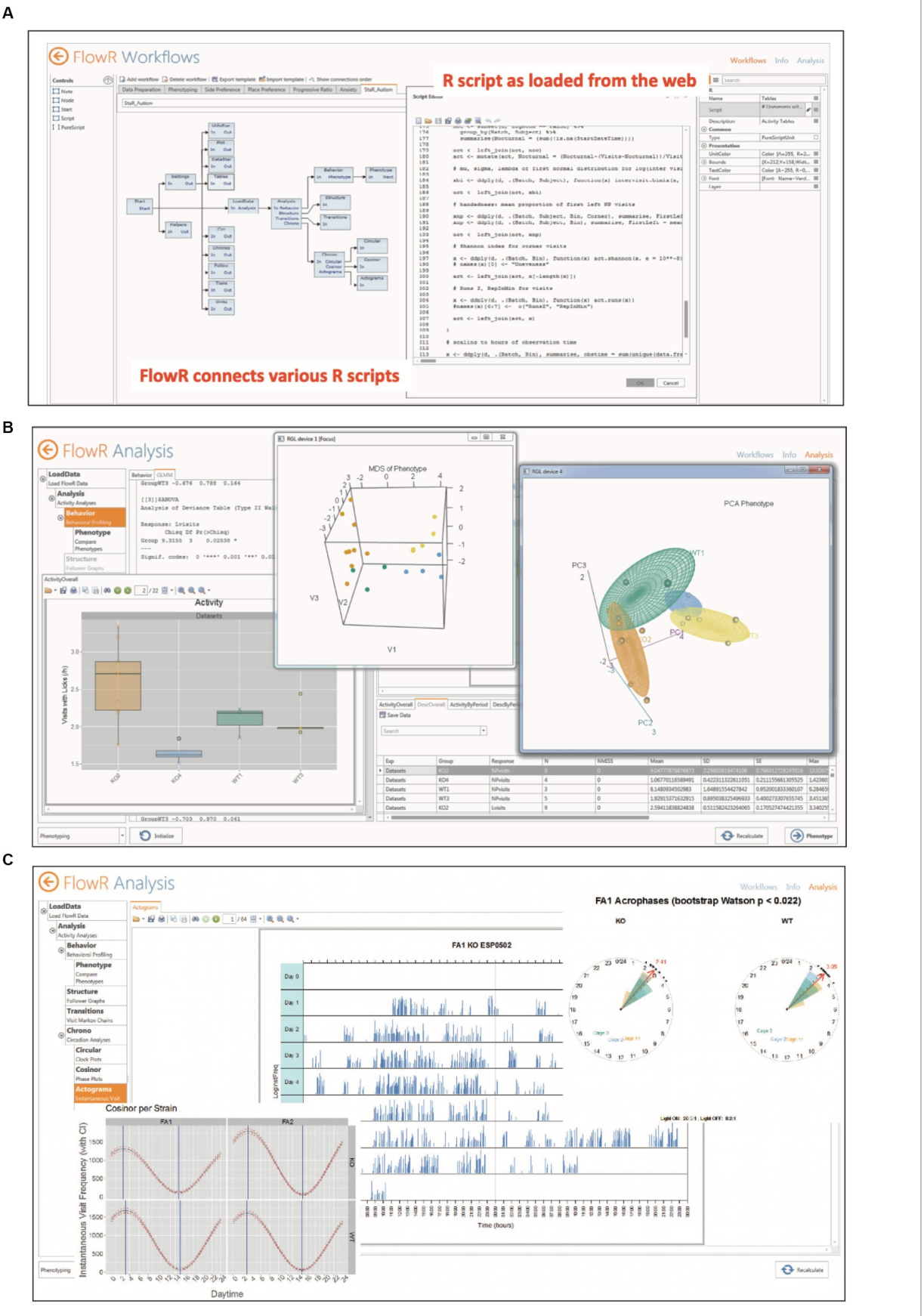

FIGURE 6

(Continued)

FIGURE 6 (Continued)
Automated statistical analysis of IntelliCage data by FlowR. **(A)** Graphic interface for creating a workflow connecting various R scripts for simple or complex statistics. The program reads in archive files from IntelliCage experiments, leaving the original data intact. **(B)** The extracted data are read-in and analyzed by pre-assembled R-routines including publication-ready graphic displays and statistical analysis in PDF format. Shown here are simple bar graphs, and 3D multidimensional scaling and principal component analysis. The analysis requires a minimum of computer experience and knowledge in R or other statistics programs. **(C)** Chronometric analysis including simple activity plots, cosinor analysis and vector rose plots of acrophases for rapid comparison of groups. Picture provided by courtesy of XBehavior.

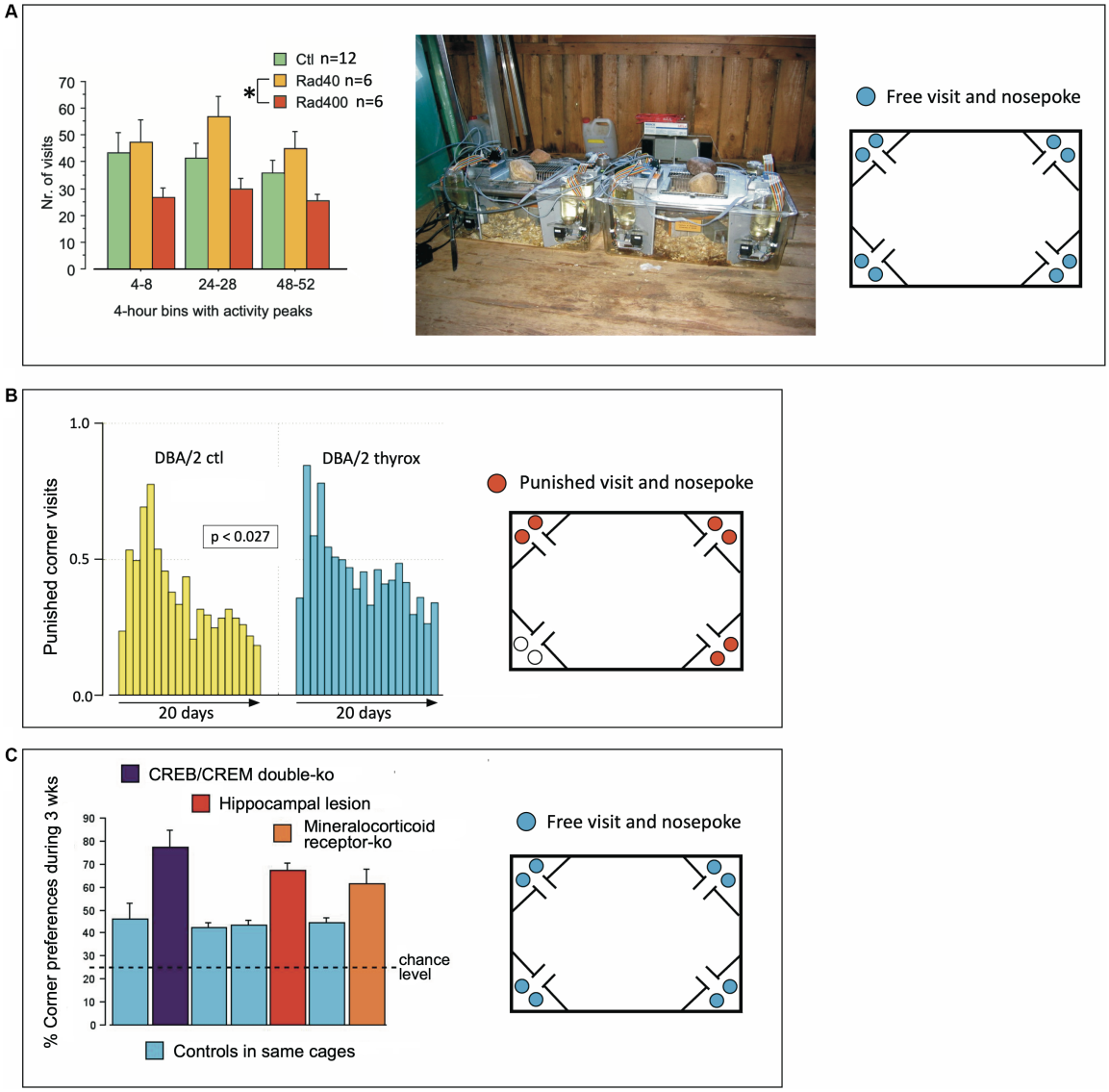

FIGURE 7
Early validation studies of IntelliCages in 2003 and 2004. **(A)** Estimating robustness of expected differences in a largely uncontrollable environment in a Russian field station. Two old-type IntelliCage were used to run a pilot study with cranially irradiated mice, but the information provided by the radiologists was lost due to the untimely death of Nada Ben Abdallah who was running the study. However, an IntelliCage data archive file could be recovered (by Pascal Zinn) and permitted to run a data analysis using the stored information only. There were clearly some differences between treatment groups that cannot be interpreted, however. On the other hand, the data demonstrate that IntelliCages can reveal significant behavioral differences between treatment groups even in noisy environments. The same cages were also used in that year to study differences between wild voles and mice (Galsworthy et al., 2005). **(B)** IntelliCages revealing extremely subtle transgenerational effects. Two IntelliCages housed 20 female DBA mice, 9 controls and 11 animals whose grand-grand-fathers (3 generations ago) had received postnatal thyroxine injections that changed brain and body features that were transmitted paternally (yet variably) over 3 generations of dam-raised DBA/2 mice. For details see Vyssotski et al. (2002b) and Vyssotski (2011). The observed behavior was how frequently the mice were visiting corners where they received air-puffs, which was rarely observed in other studies. The cages were situated in a non-climatized laboratory. Given the heat of summer 2003, we suspect that some mice were actively seeking the air-blows, which in this context provided a rewarding cooling. Modified after Lipp (2005) and Lipp et al. (2005). **(C)** Pooled presentation of non-systematic IntelliCage tests with knockout mice provided by collaborators and not being used in conventional tests, including a few mice with hippocampal lesions available for pilot studies. CREB/CREM double mutants and mice with knockout of the mineralocorticoid receptor were provided by Peter Gass and Thomas Lemberger in Heidelberg. Data were presented repeatedly by Lipp (2006) and Wolfer et al. (2012).

collaborating laboratory (Berger et al., 2006), and the Lipp/Wolfer laboratory had some mice with hippocampal lesions and their controls from its own studies (Voikar et al., 2010). Because of different treatment history and age of the mice, they were tested only for adaptation behavior over 4 weeks. The common feature characterizing the mice with various malfunctions of the brain was clearly a high degree of corner preference (Figure 7C), while the control mice included in four cages showed practically equal results despite of their different backgrounds. Repetitive visits of the same corner were later found in more detailed analysis of mice with hippocampal lesions (Voikar et al., 2018) and appear to be a simple yet reliable sign of substantial cerebral malfunction in rodents. Normal mice show a preference for one or two corners, and patrol the others occasionally, so that abnormally high corner preference during the adaptation period can easily be detected on screen (Figure 5B).

2.5 Influential studies promoting the use of IntelliCages

Here we present and discuss some papers that were important for the acceptance and understanding of the IntelliCage system.

2.5.1 Differential activation of neurons in the mouse amygdala according to motivation and learning task

One of the first studies was conducted by Ewelina Knapska at the Nencki Institute of Experimental Biology in Warsaw (Poland) to analyze whether the central amygdala (CEA) in rodents (a connective bottleneck and a chief output structure to subcortical structures) was specifically involved in signaling rewarded learning, against a prevailing concept perceiving the amygdala as processing aversive and fear-related signals (Knapska et al., 2006) (Figure 8A). Learning-dependent activation of neurons in the amygdala nuclei was visualized by the c-Fos technique. The hypothesis predicted that the CEA would be selectively activated during rewarded, yet not during fear-related learning. Handling stress had to be avoided, and the experiment needed to be completed fast. Therefore, the IntelliCage system was chosen. Technically, the approach was demanding because learning-dependent c-Fos activation can only be observed during a short time window of 1–2 h, which required that the mice had to learn rapidly a spatial preference or avoidance task, and that the controls were also consuming water or sucrose solutions without learning. Thus, mice were divided in two groups and assigned to an IntelliCage for preference or for avoidance learning, respectively. During an adaptation period, access to liquids was restricted and only allowed for 3 h, which caused high corner visit activity necessary to establish a rapid place preference learning. During this period, the individual corner preferences of the mice were also established. For the c-Fos test, all bottles in the reward test cage contained a sucrose solution and half of the mice in the IntelliCage could consume sucrose wherever they wanted. However, for the other half, access to sucrose was only permitted in their least preferred corner, which required a rapid place learning against their earlier spatial preference. In the IntelliCage assigned to avoidance learning, half of the mice could consume plain water wherever they wanted, yet the other half received air-puffs when visiting their preferred corner, enforcing avoidance of this location. The results obtained with this very elegant design of balancing motivations and learning requirements showed then that c-Fos activation of neurons in the CEA occurred chiefly after having learned a spatially defined sucrose preference, but not in the mice that consumed reward everywhere. Conversely, avoidance-dependent spatial learning did not entail c-Fos activation, nor was it increased in the controls showing consummatory drinking of plain water only. This study showed that IntelliCages could be used successfully in tackling complex neurobehavioral questions.

2.5.2 Subtle re-arrangements of cues in the IntelliCage reveal impairments in mice generated as model for intellectual disability

Mutations of the gene *Arhgef6* in humans are known for causing X-linked intellectual disability (Figure 8B). The constitutive knockout mouse model of this syndrome underwent a series of behavioral tests including IntelliCage tests (Ramakers et al., 2012). Water maze learning did show modest differences, but not the radial maze. In a place learning test in the IntelliCage, mutants were more active, but learned the simple task as rapidly as the wildtypes. However, the task was then complicated inasmuch the mice not only had to learn the position of a rewarded corner, but also whether the left or the right bottle in a corner was providing water. This subtle change in task complexity was also associated with increased locomotor activity of the knockout mice, implying poor adaptation to a situational change. IntelliCages have also been employed in other mouse models of intellectual disability or autism (Viosca et al., 2009; Puścian et al., 2014; Fischer et al., 2017; Mitjans et al., 2017; Jensen et al., 2019; Syding et al., 2022).

2.5.3 Assessing short-term flexibility and rule learning in 3 rodent species

Behavioral flexibility denotes the ability of animals and humans to adapt their ongoing behavior when facing environmental changes (Figure 8C). It does not only include a cognitive component but also various parallel adaptations of motor and motivational systems, which ultimately result in a decision whether an ongoing motor activity is maintained or changed (Lipp and Wolfer, 2022). Because of such multi-level processing, it is unsurprising that impairment of many brain systems leads to gross or subtle impairment of behavioral flexibility, which is not easily analyzed. Especially, water maze data offer only limited statistical clues for interpretation (Lipp and Wolfer, 1998; Wolfer et al., 2004). On the other hand, the IntelliCage system provides opportunities for analyzing even subtle changes in behavioral flexibility. The initial task was devised by Endo et al. (2011, 2012) and included learning a shuttling routine between diagonally opposite corners. After several sessions (usually days), the positions of the active corners are switched, and the mice must relearn the new positions. This procedure provides two measures. After a new reversal, the error rates are high but decline rapidly, showing the ability of the mouse to adapt its behavior within a limited time, a classic *reversal task*. The second measure is the comparison of initial error rates after the reversal that gradually decline after every reversal, thus providing a rare measure for *rule learning*. As the original protocol is time-consuming, new versions of the test are based on a self-paced reversal, usually after a mouse has reached a criterion of at least 30% correct responses. This less tedious (automated) procedure allows for testing of older animals and different species hard to study in common behavioral

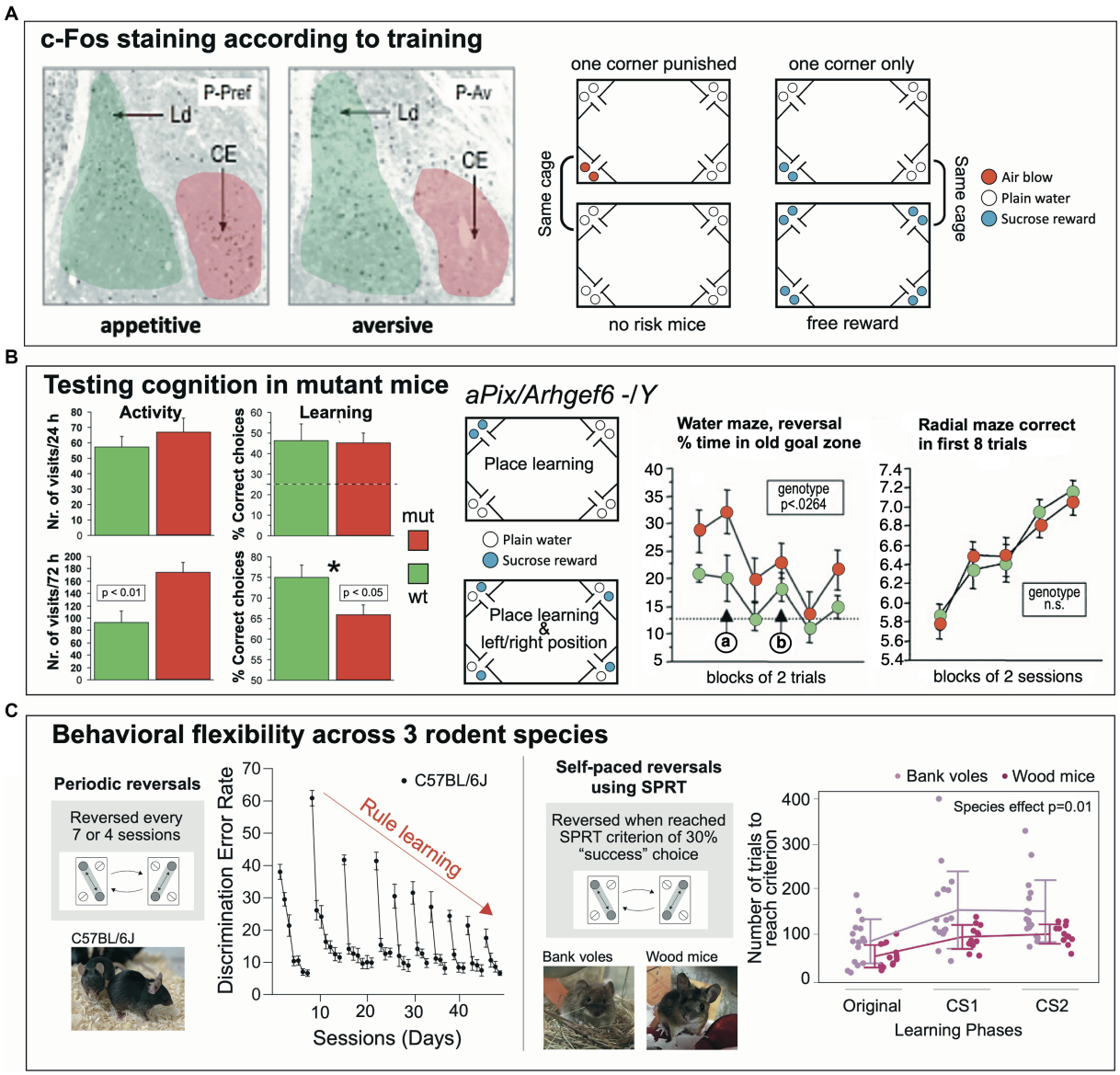

FIGURE 8
Influential studies giving rise to different directions of IntelliCage use. **(A)** The central amygdala (CE) shows activation of neurons as indicated by c-Fos-Expression when mice in an IntelliCage had to learn to visit a corner to obtain sweet reward. Their companions in the same cage, that had free access to sucrose solution in all corners, did not show activation of the central amygdala, indicating that the c-Fos activation was not due to a gustatory sensation. In a second cage, one group of mice had access to plain water in all corners, while their companions received air-puffs after having visited their preferred corner (as identified during the adaptation period). This study showed that the IntelliCage can provide unique testing procedures for dissecting the involvement of neuronal structures in motivationally different tasks during similar learning requirements tasks. Figure redrawn after Knapska et al. (2006); see also there for methods. CEm, central nucleus (amygdalae) medial part; Ld, lateral nucleus (amygdalae) dorsal part; P-Av, place avoidance task; P-Pref, rewarded place preference learning. **(B)** Mutations of the gene Arhgef6 in humans are known for causing intellectual disability. The corresponding mouse model underwent a series of behavioral tests including IntelliCage tests. In a simple place learning test, mutants were more active but learned the simple task as the wildtypes. Complicating the task by introducing left/right differences in the corners was associated with increased activity of the mutants, associated with higher error rates. Water maze learning showed modest differences, but the radial maze did not. Figure redrawn from Ramakers et al. (2012). **(C)** Two paradigms of behavioral flexibility based on learning a switching routine for obtaining water. The initial task was devised by Endo et al. (2011) and included learning a shuttling routine between diagonally opposite corners. After several sessions (usually days), the position of the active corners are switched and the mice must relearn the new positions, thus providing a measure for *spatial reversal learning*. The error rates after a new reversal are initially high, but gradually decline after every reversal, providing a measure for *rule learning*. As this protocol is time consuming, new versions were developed by one of us (Toshihiro Endo), based on a self-paced reversal (SPRT), usually after a mouse has reached a criterion between 30% correct responses. This less tedious (automated) procedure is particularly suitable for older animals and different wild species hard to test in common behavioral laboratories due to handling difficulties. For example, wood mice (*Apodemus sylvaticus*) learn this procedure easily as compared to bank voles (*Clethrionomys glareolus*). The power of this IntelliCage approach is that higher cognitive abilities of rodents can be assessed subtly and without stress. Graphs were modified after Endo et al. (2011) and Jörimann et al. (2023).

laboratories. Wood mice learn this procedure rather easily as compared to bank voles, and this behavioral difference is associated with the size of their medial habenular nuclei (Jörimann et al., 2023). The power of this IntelliCage approach is that higher cognitive abilities of rodents can be assessed subtly and without stress. Since observed behavioral flexibility in patrolling probably depends on

many brain systems, the IntelliCage allows for additional tests not depending on locomotion, for example by assessing the degree of impulsivity by a reaction time task in which animals must withhold a response for some time. Such a procedure identified higher impulsivity (or less patience) in the bank voles. Of note is that a similar coherence between behavioral flexibility measures and the impulsivity test (the reaction time task) was observed when analyzing the medial habenular system in mHB:DTA transgenic mice (Kobayashi et al., 2013).

2.5.4 Induction of social stress and its assessment

By its design, the IntelliCage system aims at minimizing stress of mice and appears to be less useful for studies involving stress. Nonetheless, there have been several studies specifically focusing on stress (Branchi et al., 2010, 2013a,b; Kulesskaya et al., 2014; Bergamini et al., 2016; Milior et al., 2016; Mohammadi et al., 2017; Akbergenov et al., 2018; Serchov et al., 2020; Picard et al., 2021; Poggini et al., 2021; Li et al., 2023b; Nagaeva et al., 2023).

Most of them used IntelliCage as a tool for efficiently estimating sweet preference against plain water to obtain a measure of anhedonia following various exposures to external stress, while others used a variety of protocols documenting impairment of various forms of learning thought to be affected by stress. Among these, the study of Gapp et al. (2014) is of particular interest, as the stressing treatments were applied to the fathers, while an (unexpected) behavioral improvement was found in the offspring. Others used the IntelliCage itself to deliver subtle forms of social stress. For example, Branchi et al. (2010, 2013a,b) produced social stress in male mice by daily mixing the populations of two IntelliCages and could show that communal nesting in childhood mitigated the reduction in sucrose preference as observed in stressed yet normally raised mice. Likewise, mixing two strains of inbred mice (female C57BL/6 and DBA/2) increased, somewhat surprisingly, stress markers and anhedonia as measured by saccharine preference in C57BL/6 (Kulesskaya et al., 2014). One may note, however, that many strain differences in learning paradigms persisted even under induced social unrest, most likely because the prolonged observation times in the IntelliCage cancel short-term effects of social interactions.

2.6 Acceptance by the field and coverage of topics

Some 20 years after its first presentation, the IntelliCage system has now been accepted widely, as the term is used even without reference to the trademark name (Plum et al., 2023). Likewise, its ability to produce equal experimental outcomes in different locations has been repeatedly verified (Lipp et al., 2005; Krackow et al., 2010; Codita et al., 2012; Kobayashi et al., 2013). After a period with low publication volume, the number of papers having used IntelliCage technology rose to 295 on October 15 2023 and is likely to reach 300 soon (Figure 9A). A focus of development has been the Nencki Institute of Experimental Biology in Warsaw, having summarized the work with IntelliCages up to 2018 (Kiryk et al., 2020). We have updated the main table of their paper focusing on behavioral protocols in Supplementary Table S1, and provide here primarily clinical classifications (Table 1; Figure 9B). A complete list of IntelliCage papers is provided in Supplementary References (for details see legend of Figure 9A).

Most studies using IntelliCages refer to basic physiology and neurodegeneration, chiefly by comparing specific genetically modified mouse models. A more diverse cluster of studies is the development of behavioral methods in the IntelliCage, often employed in translational psychiatry. The predominant topic in addiction studies is, unsurprisingly, alcohol abuse, because cage-mates can be exposed to different concentrations while monitoring the behavioral consequences directly. Mouse models with immune defects and developmental disorders have also been productively used, while other topics are less represented. The IntelliCage is also mentioned increasingly in patent applications (not listed here), indicating its usefulness as an unbiased behavioral system providing data from a standardized set-up everywhere in the world. Interestingly, true high-throughput phenotyping studies were infrequent, conducted chiefly by the industry (Oakeshott et al., 2012; Balci et al., 2013; Alexandrov et al., 2015), but laboratories performing longtime follow-up studies profited from the reduced iterative workload in phenotyping (e.g., Codita et al., 2010; Radwanska and Kaczmarek, 2012; Plank et al., 2016; Masuda et al., 2018; Iman et al., 2021b). Taken together, the diversity of scientific fields which have profited from the use of IntelliCage underscores the versatility of the system. We present a selection of papers according to clinical criteria in Table 1 and draw attention to useful reviews and discussions of the system using other criteria (Kiryk et al., 2020; Iman et al., 2021a; Varholick et al., 2021).

We are unaware of substantial criticism of the IntelliCage system, as home-cage-based testing systems are mostly well perceived by behavioral science and the public. However, a noteworthy perspective article by Crabbe and Morris (2004) about conflicting concepts underlying high-throughput testing questioned the need for automation and speed in animal testing, calling instead for heuristic reflections before action, according to a "festina lente" principle (acting too fast retards progress).

Given the high number of divergent IntelliCage papers and topics, we will refrain from discussing them further and we will focus on selected studies showing interesting directions. Readers interested in how the IntelliCage compares to the increasing number of home-cage-based testing systems can find tabulated comparisons (Kiryk et al., 2020; Mingrone et al., 2020; Voikar and Gaburro, 2020; Iman et al., 2021a; Coulibaly, 2022; Kahnau et al., 2023b).

2.7 Research trends in past and future

2.7.1 Analyzing spontaneous activity – a simple but effective tool

Spontaneous locomotor activity in the home cage is a sensitive tool for assessing various pathologies. For example, simple movement sensors over the cages of single-housed mice permitted to distinguish and monitor the impact of various prion strains on spontaneous locomotor behavior after inoculation in mouse brains (Dell'Omo et al., 2002). Given that IntelliCages always require an adaptation period before conducting any study, Vannoni et al. (2014) compared 1,552 mice from 32 mouse models for their spontaneous behavior during a one-week adaptation period. The only variables assessed were visits, nose-pokes and licks. The data were then analyzed by factor analysis, that identified 11 factors

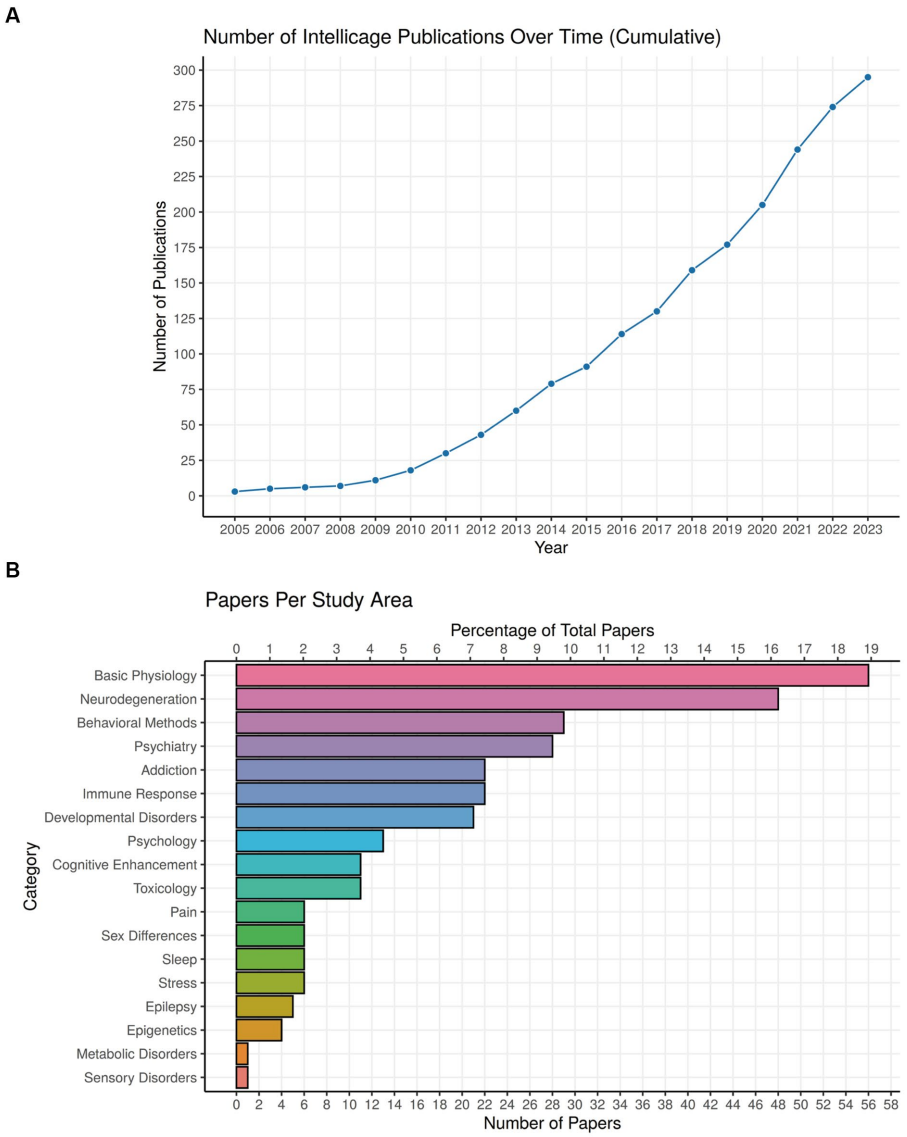

FIGURE 9
Use of IntelliCage systems in behavioral research. **(A)** Cumulative plot of papers dealing practically with or describing IntelliCages since 2005. The year 2023 includes publications at 15 October 2023, including some reviews and discussion papers. Searching criteria in Google Scholar (screening the entire paper) were: presence of the keyword "IntelliCage" together with (i) Primary journal articles that use "IntelliCage" as part of the methodology, (ii) Review papers/textbook chapters only if they focus on rodent behavior, (iii) Preprints (bioRxiv), (iv) Articles in languages other than English. Anything else is not included, for example conference abstracts, theses, articles that only mention "IntelliCage" without actual use or specific focus on it, etc. A complete list of papers, ordered alphabetically or chronologically, can be found in Supplementary References. **(B)** Proportions of IntelliCage papers classified according to scientific fields. For a description of the main disease classifications, see Table 1.

accounting for 83% of the variance, which could be grouped into four clusters. One accounted for corner and side preferences (27%), a second for parameters describing activity during corner visits such as nose-pokes and visit duration (21%), a third one for drinking activities such as lick number and frequency (20%) and a fourth one related to ultradian activity variations. Because the study included large samples of inbred strains, it could prove high stability of strain comparisons over time, while the inclusion of many hippocampally lesioned mice showed that these mice could be easily recognized by the IntelliCage and that their abnormal behavioral profiles often coincided with mutations suspected to carry hippocampal deficits. These results might appear boring to specialists in mouse phenotyping but would justify a close look at the first week in any IntelliCage study using an adaptation period. Some mouse models with obvious hypo- or hyperactivity may account in part for these results, but recent video-computer analysis of mouse spontaneous behavior in a simple circular arena for a short timespan has recognized subtle non-reinforced fluctuations in activity that hint at a periodic self-regulation by striatal and dopaminergic systems (Markowitz et al., 2018, 2023). It would seem possible that the IntelliCage system is picking up such small motor idiosyncrasies of the mice simply by sampling their activity for a long time. Obviously, such data sets would profit from re-analysis by artificial intelligence.

TABLE 1 Publications between 2005 and October 2023 grouped according to main clinical fields.

Addiction (non-alcohol)	Skupio et al., 2017; Ajonijebu et al., 2018; Iman et al., 2021a,b
Aging	Mechan et al., 2009; Berry et al., 2012; Albuquerque et al., 2013; Too et al., 2016a; Fischer et al., 2020; Oizumi et al., 2020; Barranco et al., 2022; Li et al., 2023b
Alcohol abuse disorder	Radwanska and Kaczmarek, 2012; Parkitna et al., 2013; Smutek et al., 2014; Holgate et al., 2017; Mijakowska et al., 2017; Stefaniuk et al., 2017; Beroun et al., 2018; Koskela et al., 2018, 2021a,b; Iman et al., 2021b; Stefaniuk et al., 2021; Hühne et al., 2022; Pagano et al., 2022; Caly et al., 2023; Frycz et al., 2023; Nalberczak-Skóra et al., 2023; Stefaniuk et al., 2023
Anxiety	Safi et al., 2006; Jensen et al., 2016; Sano et al., 2016; Fischer et al., 2017; Raab et al., 2018; Sasaki et al., 2023
Brain lesional or ischemic damage	Voikar et al., 2010; Vannoni et al., 2014; Voikar et al., 2018; Dzirkale et al., 2023
Chemical exposure	Onishchenko et al., 2007; Zhu et al., 2010; Endo et al., 2012; Ogi et al., 2013, 2015; Aung et al., 2016; Sano et al., 2016; Xia et al., 2016; Yang et al., 2017; Kimura et al., 2020; Wen et al., 2021; Sasaki et al., 2023; Zhu et al., 2023
Developmental disorders	Ramakers et al., 2012; Puścian et al., 2014; Fischer et al., 2017; Mitjans et al., 2017; Fröhlich et al., 2019; Jensen et al., 2019; Garrett et al., 2020; Horigane et al., 2020; Morello et al., 2020; Balan et al., 2021; Meng et al., 2021; Puścian and Knapska, 2022; Puścian et al., 2022; Syding et al., 2022; Viosca et al., 2009; Xiao et al., 2022
Immune system	Too et al., 2014a,b,c, 2016a,b; Cathomas et al., 2015a; Pan et al., 2019; Arinrad et al., 2021; Markova and Knyazheva, 2021; Wilke et al., 2021; Markova et al., 2022; Plum et al., 2023
Irradiation	Jaholkowski et al., 2009; Barlind et al., 2010; Huo et al., 2012; Roughton et al., 2012; Ben Abdallah et al., 2013; Kalm et al., 2013; Osman et al., 2014; Kalm et al., 2016
Mood disorders	Branchi et al., 2010, 2013a,b; Cathomas et al., 2015a,b; Alboni et al., 2016; Bergamini et al., 2016; Jastrzębska et al., 2016; Milior et al., 2016; Mohammadi et al., 2017; Kato et al., 2018; Marwari and Dawe, 2018; Poggini et al., 2019; Serchov et al., 2020; Serykh et al., 2020; Markova and Knyazheva, 2021; Poggini et al., 2021; Sun et al., 2021; Volkmann et al., 2021; Markova et al., 2022; Yamamoto et al., 2023
Neuro-degeneration	Kiryk et al., 2008; Rudenko et al., 2009; Codita et al., 2010; Kiryk et al., 2011; Oakeshott et al., 2011; Sekiguchi et al., 2011; Weyer et al., 2011; Oakeshott et al., 2012; Balci et al., 2013; Gumucio et al., 2013; Oakeshott et al., 2012; Ryan et al., 2013; Menalled et al., 2014; Stribl et al., 2014; Urbach et al., 2014; Alexandrov et al., 2015; Benraiss et al., 2016; Koss et al., 2016; Masuda et al., 2016; Simmons et al., 2016; Masuda et al., 2018; Rudenko et al., 2019; Mehr et al., 2020; Nieraad et al., 2020; Cisbani et al., 2021; Mifflin et al., 2021; Tikhonova et al., 2021; Winslow et al., 2021; Yesiltepe et al., 2022
Schizophrenia	Peltola et al., 2015; Nakamura et al., 2021; Mätlik et al., 2022; Stephan et al., 2022
Seizures and epilepsy	Orock et al., 2018; Liu et al., 2020; Wu et al., 2023
Traumatic brain injury	Muthuraju et al., 2012, 2013; Vogel et al., 2020; Lopez-Caperuchipi et al., 2021; Simmons et al., 2021a,b; Hahnefeld et al., 2022

2.7.2 Embedding IntelliCages in phenotyping batteries: IntelliCage versus water maze

Increasingly more laboratories are now integrating IntelliCages in their standard test batteries, offering themselves or to collaborators refined behavioral analysis of mouse models. However, it took time to convince the field that the IntelliCage system was able to produce data that were fitting the results from other classic tests. Given the omnipresence of the standard Morris water maze test (MWM) in most phenotyping laboratories, reports having tested mice in parallel in both IntelliCages and other apparatus often included the water maze, which, being capable of detecting spatial impairments, is often taken as a proxy for hippocampal malfunction. In analogy, it was (and is) frequently assumed that deficiencies in spatial learning within the IntelliCage would represent an animal-friendly alternative to the rather stressful water maze procedure, which requires forced swimming. Therefore, we present a short overview of 25 identified studies having reported similar or dissimilar treatment effects in water maze and IntelliCage and we try to define the common denominator in both tasks.

Only five out of 25 studies reported discordant results. Male mice but not females exposed prenatally to methylmercury showed several behavioral deficits in the IntelliCage, yet not in the water maze (Onishchenko et al., 2007). Notably, the adult male cohorts were formed at the age of 4 weeks (see also male–female differences below). Viosca et al. (2009) investigated the behavior of a mouse model of the human Costello syndrome and found moderate impairment in the MWM. However, the IntelliCage was apparently only used to document the apparent hypo-locomotion of the mutant mice. Voikar et al. (2018) reported that LRRTM1-deficient mice (lacking a gene for a specific type of neural cell adhesion molecule) showed several behavioral peculiarities including an aversion to enter narrow tubes. This was associated with normal MWM learning but retarded acquisition of IntelliCage tests, most likely reflecting some form of claustrophobia. Koss et al. (2016) studied mutant Tau knock-in mice for progressive changes in cognitive development. They showed no differences in the MWM (except for swim speed in older mice) but reduced behavioral flexibility in the IntelliCage as indicated by impaired rewarded place reversal learning. Wilke et al. (2021) observed behavioral differences in mouse models of encephalitis aggravated by injection of diphtheria-toxin ablating pyramidal neurons (DTA). Mice after DTA induction showed hyperactivity and deficits in the water maze but, surprisingly, no significant treatment effects in the IntelliCage using various tasks.

Six studies were done in the context of simple screening for potential cognitive problems in mouse models without making specific functional predictions and showed no or rather subtle

differences in the two behavioral paradigms (Kulesskaya et al., 2014; Netrakanti et al., 2015; Peltola et al., 2015; Roccaro-Waldmeyer et al., 2018; Festa et al., 2019; Arinrad et al., 2023). Lack of treatment effects then either reflect insensitivity of both MWM and IntelliCage in revealing deficits, or true absence of effects. In two cases, however, parallel testing was based on clear hypotheses. For example, Jaholkowski et al. (2009) tested cognitive versus sensory deficits in CyclinD2 mutant mice lacking adult neurogenesis and found no impairment in the MWM and IntelliCage, yet deficits in olfactory tasks. Likewise, d'Isa et al. (2011) clarified a long-standing controversy about the role of the RasGRF1 protein in different knockout models, showing no spatial memory differences between mutants and wildtypes in both the water maze and IntelliCage protocols based on corner avoidance, while clear differences between mutants and wildtypes in contextual fear conditioning pointed at different roles of RasGRF1 in specific memory tasks.

A predicted similar loss of function in both assays, mostly in combination with a variety of other behavioral tests, was reported in five studies. Kiryk et al. (2011) analyzed the behavior of transgenic mice with a mutation of the human amyloid precursor protein (APP. V717I) at different ages. A deficit in spatial learning in both tasks was observed in all three age groups. However, the APP mice learned much better when co-housed with the wild-type littermates than when housed only with other APP mutants, suggesting a form of social learning that appeared to be modulated by different circadian activity of the transgenics. Lan et al. (2011) compared mice having undergone postnatal hypoxia and found deficits in punished reversal learning of males in the IntelliCage while the parallel deficits in the MWM approached significance only. A comprehensive behavioral phenotyping of the Ts65Dn mouse model of Down syndrome showed deficits in both tasks (Faizi et al., 2011). In the IntelliCage, these researchers used rewarded and punished learning for 4 days, removed the animals for 72 h and checked, as probe trial, corner preference and avoidance, the latter showing deficits in the mutant mice. Ryan et al. (2013) studied PLP1 triple knock-in Alzheimer mice at various age stages and reported deficits in both paradigms but noted that the IntelliCage was more sensitive in revealing impairments. Synaptic electrophysiology and hippocampus dependent behavior in mice lacking the cAMP-guanine nucleotide exchange factor II (cAMP-GEFII) were studied by Lee et al. (2015) who found impairment in long-term depression in hippocampal slices and moderate deficits in reversal learning paradigms in the MWM and IntelliCage.

Four papers in rats showed parallel loss of function. A study analyzing rats lacking the G protein-coupled estrogen receptor (GPER) reported that both female and male rats were slow to learn the MWM and showed modest impairment in place and reversal learning in the IntelliCage (Zheng et al., 2020). Cao et al. (2021) tested rats kept isolated after weaning in the MWM, IntelliCage and an own type of video-controlled dry maze, and claimed equal impairment, however without providing in-depth analysis. Li et al. (2023a) investigated the effect of juvenile isolation stress in 6 weeks old male and female rats, using ill-defined MWM tests and more extensive IntelliCage procedures. The authors claim deficits in the water maze and impairments in IntelliCage which include a reduced number of visits and nose-pokes in a punished left–right discrimination task and in the reversal test. A further paper analyzed postoperative cognitive dysfunction after splenectomy in aged rats, finding that operated animals were handicapped in both tasks after operation and that the pre-operatively administered drug Maresin appeared to mitigate such impairments (Li et al., 2023b).

Three studies reported parallel gain of function in both the MWM and IntelliCage. Konopka et al. (2010) induced a gene deletion (Dicer1) in the forebrain of adult mice that impaired, for a defined period, the transcription of non-coding messenger RNAs thought to be important for modification or stability of synapses. Somewhat surprisingly, the treated mice showed superior MWM learning including probe trial scores, and in the IntelliCage better sucrose-rewarded place learning. Schroeder et al. (2021) fed aged mice with the nutritional additive spermidine and found subtly improved spatial learning in the MWM and a trend also in the IntelliCage. Interestingly, the spermidine-fed mice were also better in a serial reaction time task permitting nose-pokes only during a visually signaled time window. Barth et al. (2023) tested mice deficient for the growth factor-like protein 7 (EGFL7), showing upregulated adult hippocampal neurogenesis. Both tests, learning and probe trial in the water maze and learning/reversal learning of corner preferences or avoidance in the IntelliCage, were slightly improved in the knockout mice.

What conclusions can be drawn from these studies? Clearly, gain of function in both tests is the most compelling argument that a common cerebral factor or process is underlying parallel behavioral changes in the MWM and IntelliCage. Probably this brain process relates to behavioral flexibility and not to a special form of memory. In the MWM mice must suppress inappropriate search strategies even when the position of the hidden platform is known (Lipp and Wolfer, 1998). Probe trials do demonstrate that mice have developed a spatial memory, but the usual scores only show how insistently they search over the old platform position (Wolfer et al., 1998), while impaired spatial reversal learning in the water maze is the most distinct behavioral sign after chronic hippocampal lesions (Lipp and Wolfer, 2022). Likewise, in the IntelliCage, learning the spatial position of the corners is rather fast, both by reward or by punishment, and treatment-dependent effects become visible mostly after positional changes, that is spatial reversal learning. Thus, in both tasks mice must adapt their movements to changing situations and the tests are excellent detectors for a variety of changes in brain structures of which the hippocampus is only one of many. Whether the type of spatial memory in the two tasks is equivalent is unknown. In the IntelliCage, its presence can be tested by removing and re-introducing the mice after some time. However, care must be taken to distinguish between punishing a visit from punishing a visit with nose-poke, as the former includes spatial memory and the latter combines memory for place with a special movement in that place (Voikar et al., 2010). Finally, from a practical point one should note that the motivational levels in the water maze are usually constant, while the IntelliCage permits to increase motivation for rewarded place learning by sweetening water or strengthening air-puffs. On the other hand, locomotor hyperactivity induced by treatments can confound IntelliCage testing but is less important in the water maze. To our knowledge, we are unaware of a study that, after having assessed specifically individual mouse behavior in the MWM and IntelliCage, analyzed intercorrelations between the two tests. In most of the cited studies, the two apparatus are part of a test battery, which is likely to complicate statistical analysis.

2.7.3 Increasingly sophisticated protocols

The laboratory of Hannelore Ehrenreich in Göttingen (Germany) focused on developing sophisticated IntelliCage

protocols to identify higher-order cognitive functions in mice (Mitjans et al., 2017; Pan et al., 2019; Arinrad et al., 2021), following several years of validation and protocol evaluation (Dere et al., 2018). Besides the usual assessment of various forms of patrolling, they developed a so-called "mental-time-travel protocol" (MTT). After adaptation to nose-poking for water, access time was limited to 2 h, and each mouse had to face one corner delivering air-puffs whose position changed in a predictable sequence over 4 days in a training cycle. The pattern was then repeated for a second round of 4 days and the preference to each corner on each day of the second round was used to assess MTT abilities. Each corner per day was considered either currently (=0 days after punishment), recently (=1 day after punishment), intermediately (=2 days after punishment) or longer ago punished (=3 days after punishment), and the data obtained were used to calculate a curve (percent corner preferences versus days after punishment) whose steepness (expressed as trendline) reflects the quality of the MTT thought to represent memory for traveled time and place.

2.7.4 Immunology and gut-brain axis

Immunology and brain-gut interactions are a topic rapidly gaining relevance. Mice can be immunized by injection of ovalbumin (egg white) and will subsequently avoid drinking sweetened water if this contains ovalbumin (Cara et al., 1994). This simple paradigm was implemented in IntelliCages to study the role of mast cells in developing antigen-avoidance behavior (Plum et al., 2023). Plum and colleagues immunized, by intraperitoneal injection of ovalbumin, wildtype and knockout mice lacking mast cells, and placed them in IntelliCages to test them together. In each corner, one bottle contained a mix of ovalbumin and sucrose, the other plain water, but left/right positions were counterbalanced. Over 12 days, non-immunized mice from either control group developed a strong preference for the bottles containing sucrose and ovalbumin, while immunized mice with intact mast cells began to avoid the sweetened antigen-containing bottles increasingly. However, the KO-mice without mast cells maintained the sucrose preference, providing compelling evidence that mast cells were part of a signaling pathway for immunoglobulin E (IgE)-mediated allergies, transmitting antigen signals from the gut to the brain – amazing results and a top paper obtained with the help of a simple IntelliCage test.

2.7.5 Testing mice with human genes

Ongoing studies by IntelliCage users are usually not communicated, but we anticipate some interesting results from Svante Pääbo's laboratory in Okinawa (Japan), where transgenic mice carrying gene variants that are specific to modern humans and to Neanderthals are tested for their potential to change behavior. The outcome will be certainly of interest to a wide audience.

2.8 Inherent limitations and problems of the IntelliCage

Before discussing upcoming developments of the conventional mouse IntelliCage, some of its inherent limitations and problems should be addressed. Minor technical problems are dealt with in the Supplementary Figure S2 (Dos and don'ts in the IntelliCage).

2.8.1 Testing females, males or both?

In most IntelliCage papers, only female mice were tested. One reason is that cages housing males over prolonged periods become smelly rather quickly. However, the main concern is intermale aggression and fights possibly interfering with behavioral testing. A systematic review by Varholick et al. (2021) analyzing traditional behavioral phenotyping and intermale aggression found little evidence for differences between dominant and subordinate male mice, which would justify the testing of group-housed males in IntelliCages. Assessing aggressive yet non-violent behavior between males (usually black C57BL/6 mice) in an IntelliCage would require expensive constant video-monitoring of individually recognized mice, as attempts by simple surveillance cameras did not identify aggressors (Mifflin et al., 2021). A possibly simpler solution might be to determine sleeping places and latrine areas (Makowska et al., 2019) by means of RFID tracking, as early studies in multi-cage systems have shown that subordinate and scarred male mice were forced to sleep in latrine areas (Ely et al., 1972), while recording movements in individually ventilated cages (IVC) cages housing 4–5 mice showed that male mice usually avoid the latrine area (Ulfhake et al., 2022). For more information about tracking, see the Section 2.10.2 "Tracking of mice in the IntelliCage" below.

Because IntelliCage studies testing both female and male mice using similar protocols are infrequent and not easily found by literature searches, they shall be discussed briefly. Clearly, they do not provide a coherent picture. There is a single study without genetic and treatment differences, showing that male mice develop a stronger preference for alcohol (Smutek et al., 2014). A developmental toxicity study assessing the neurotoxicity of the neonicotinoid acetamiprid did not reveal treatment effects on behavioral flexibility in the IntelliCage for both sexes (Sano et al., 2016), while exposing mice to early bisphenol A led to opposite treatment effects in corner visit patterns for adult males and females (Ogi et al., 2013). Saccharine preference of control mice in the IntelliCage was equal in both sexes (Morello et al., 2020).

More pronounced learning deficits in female mice as compared to males was observed in aged (16 months) CH3 mice that were exposed prenatally to arsenic (Aung et al., 2016), and irradiation of young mice caused a greater impairment of initial place learning in adult females than in males, in agreement with clinical observations (Roughton et al., 2012). In mice with a mutation of the AMBRA gene (thought to be linked to female autism), mutant females, but not males, lost preferences for sex pheromones as evidenced by connecting IntelliCages to boxes containing different scents (Mitjans et al., 2017).

On the other hand, comparative IntelliCage testing often found increased resiliency in females toward treatment effects or mutations. Onishchenko et al. (2007) found behavioral differences after developmental exposure to methylmercury only in males and not in females. Mice having undergone sub-lethal hypoxia after birth showed, at the age of 6 weeks, moderately impaired spatial reversal learning in males but not in females, while both sexes showed persistent incorrect nose-poking in corners delivering air-puffs, yet less pronouncedly in females (Lan et al., 2011). Berry et al. (2012) investigated aged (21–24 months) male and female $P66^{Shc-/-}$ mice, known for longevity, in the IntelliCage. They found higher initial exploration in mutant mice, yet more pronounced in females, while later testing for spatial learning revealed no genotype effects but better acquisition by the females (Figure 10A), surprisingly no more

differences during reversal learning (Figure 10B). In an early life stress model, adult male mice showed treatment effects by being more subordinate than females in a water access competition test (Benner et al., 2014). Finally, Mifflin et al. (2021) compared 12-month-old male and female APP/PS1 and non-transgenic mice, and found that females performed better in a variety of IntelliCage tasks except for impulsivity tests. Taken together, there is no doubt that IntelliCages studies can recognize sex differences in a variety of tasks.

The studies above provide some hints that the basic motor activity of male mice is a main factor generating sex differences, but to better understand these differences, we must await for the results of an ongoing study comparing systematically male and female C57BL/6 mice in the IntelliCage. A crucial factor to stabilize social interactions in the IntelliCage is the procedure preparing both male and female mice for testing. The probably most important factor is the time span allowed for social adaptation within an IntelliCage-sized cage before recording and testing. We provide a detailed description in the Supplementary Figure S2 "Dos and don'ts in the IntelliCage."

2.8.2 Comparability between laboratories

Specifically designed studies have shown similar experimental outcomes in different laboratories, as stated before. However, it should not be inferred that the absolute values of activities in the IntelliCage are equal, but rather that the relative differences between treated mice or strains were similar in different places. This is exemplified in a large study (Krackow et al., 2010) in which laboratories in Stockholm, Hamburg, Zürich and Rome tested synchronously a total of 288 mice of the strains C57BL/6, DBA/2, and their F1 hybrids, raised and shipped by the same supplier. The statistical split-plot design assessed a variety of measures for differences between laboratories, strains and lab-by-strain interactions (see also in Krackow et al., 2010). For example, the grand average of the nocturnal activity scores of these mice for spontaneous nocturnal corner visits showed DBA/2 mice

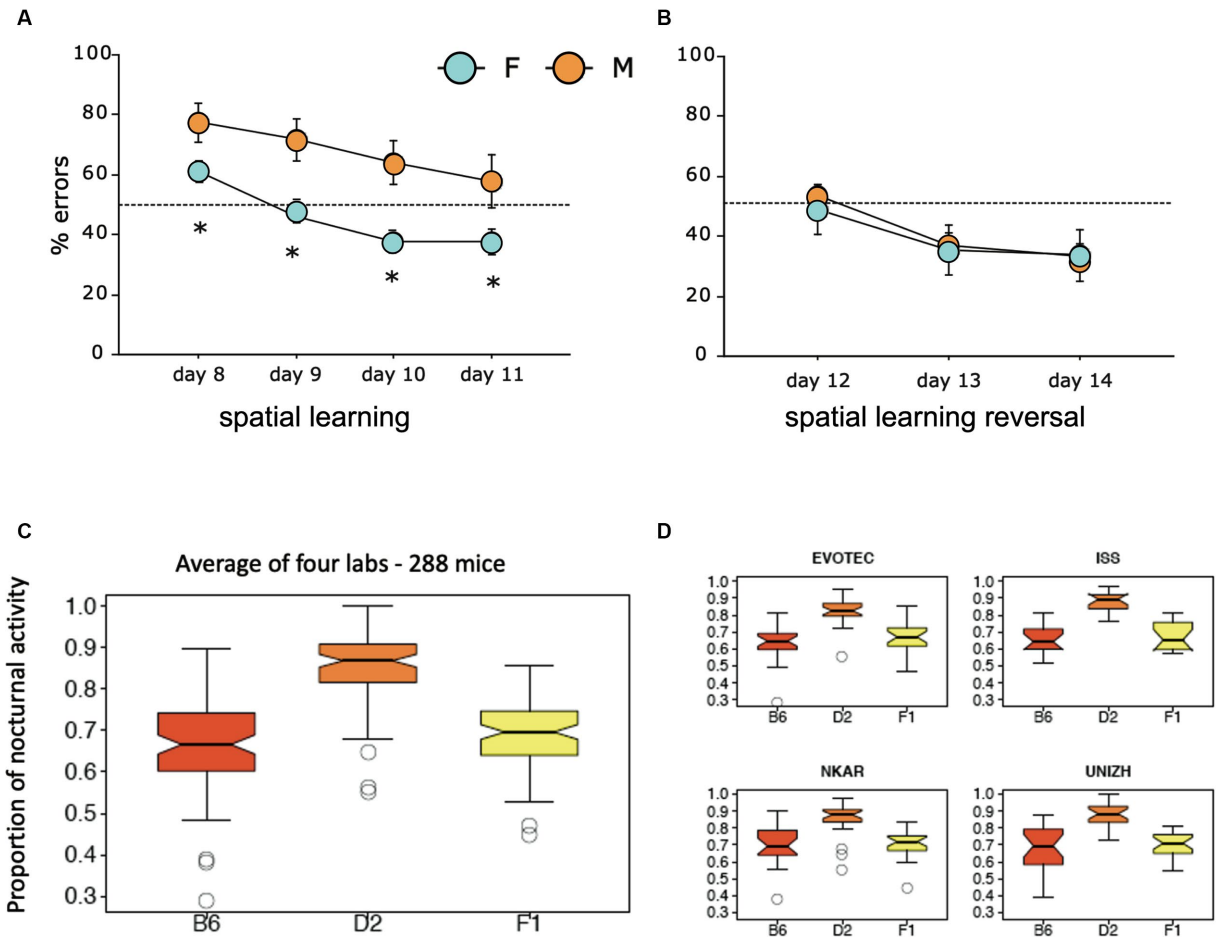

FIGURE 10
Male–female differences and inter-laboratory comparisons. **(A)** Significantly less errors in spatial learning of aged females ($n = 15$) as compared to males ($n = 14$), $*p < 0.05$, means and S.E.M. After 7 days of habituation to the IntelliCage environment with free access to all corners for drinking, water delivery occurred for 4 days only in the corner opposite to the one preferred during the adaptation period. **(B)** Reversal phase: during days 12–14, all mice underwent a 3-day spatial learning reversal where the only corner available for drinking was the preferred one during the adaptation phase (Berry et al., 2012). **(C)** Grand average of 288 mice of different strains (C57BL/6, DBA/2, F1 B6xD2) as observed for nocturnal activity in four different laboratories across Europe, indicating a highly significant strain effect (Krackow et al., 2010). **(D)** Laboratory-specific results show clear differences among the single strains at Karolinska Institutet (NKAR) in Stockholm, Evotec in Hamburg, Istituto Superiore di Sanità (ISS) in Rome; and University of Zürich (UNIZH), but the laboratory-specific comparisons revealed in all cases comparable strain differences. For details of the statistical design, see Krackow et al. (2010).

being clearly much more active than both other strains (Figure 10C), while absolute activity scores of strains could vary depending on place, but the strain order was the same (Figure 10D). Some years later, a transatlantic multi-lab study expecting to find equal motor activities of C57BL/6 mouse groups kept under stringent isolation in IVC conditions reported the same result: mice in three different labs showed significantly different behavioral activity level despite all efforts to environmental standardization and shielding (Pernold et al., 2019).

We consider each group of mice within an IntelliCage to establish an idiosyncratic social setting which ought to be taken into account in comparison of overt behaviors between treatments or genotypes, even in the case of inbred mice that can show minor yet stable differences in neuroanatomy and behavior. For this reason, when possible, housing of mice in IntelliCage should feature mixed groups (experimental and control mice being housed together in the same IntelliCage). However, conditioning tasks are carried out individually by each mouse within the corners, quite independently of social status and interactions. Hence, we consider conditioning success amenable for taking individual mice as independent data points in analyses. If similar statistically significant differences between treatment groups can be observed in two or more IntelliCages at the same location, we expect them to be robust even in case of possible environmental differences between cages. Should there be unusual differences between single cages (e.g., Kiryk et al., 2011), digital graphical replay of critical experiments may identify specific environmental conditions or activity patterns within cages (see also the legend of Figure 5).

2.9 Combining IntelliCages with add-ons

Supported by FP6 grants of the European Union, the projects Intellimaze and Noveltune were launched for expanding the home-cage concept by developing add-ons that can be connected to IntelliCages. The primary goal was to implement behavioral tests that were difficult to conduct in standard IntelliCages. Figure 11A shows a layout of the realized add-ons. The center piece is an IntelliCage whose software (designer, controller and analyzer) can also control communication with add-ons (Figure 11A). Mice can reach a so-called *social box* through tubes permitting RFID identification of mice passing inside, as well as their direction, and in this *social box* mice can find other mice or deposited scents or pheromones (Dere et al., 2018; Pan et al., 2019). Two social boxes permit to establish preference for socially relevant smells, analogously to the more complex RFID-based ECO-HAB system, which permits circulation of mice without a central home-cage (Puścian et al., 2016). If performance in a certain device should not be disturbed by partners, an *animal gate* can regulate access by blocking and opening the passage (Figure 11B). The animal gate also contains an inbuilt scale for monitoring the weight of mice, an opportunity useful to observe the consumption of different diets in attached cages, or to assess the impact of experimental manipulations or social stress. Because IntelliCage was not suited for presenting auditory cues, the consortium developed an *audiobox* which uses an IntelliCage corner placed at some distance in a sound-attenuated box and permits the use of IntelliCage software for self-paced auditory conditioning, resulting in publications that appeared even in particularly high-ranking journals (de Hoz and Nelken, 2014; Atlan et al., 2018; de Hoz et al., 2018; Chen et al., 2019; Chen and de Hoz, 2023). One may note that Kahnau et al. (2023a,b) used a reverse approach by keeping the mice in a home-cage connected to an empty IntelliCage from where the mice had temporary access to solitary auditory conditioning as controlled by an animal gate. Most of the add-ons are available from TSE-Systems, while another add-on is a floor plate made of RFID antennae described later under "Tracking of mice on the floor of IntelliCage" (Section 2.10.2).

2.10 Current and future modifications of IntelliCage

2.10.1 Minor modifications

There have been several smaller or larger modifications that can be technically integrated in the IntelliCage system. Their usefulness depends on whether the intended use of an IntelliCage is testing mouse models under identical conditions, in which case modifications may jeopardize long-term comparability and reproducibility, or even patent applications. But when the goal is to tackle specific behavioral problems in an efficient and animal-friendly manner, modifications can be useful.

For experimenters wanting to avoid cage patrolling and social interaction between animals, it is possible to use a transparent *cage divider* that leaves 4 compartments with one corner, which is basically a two-faced operant conditioning box. It is slightly less

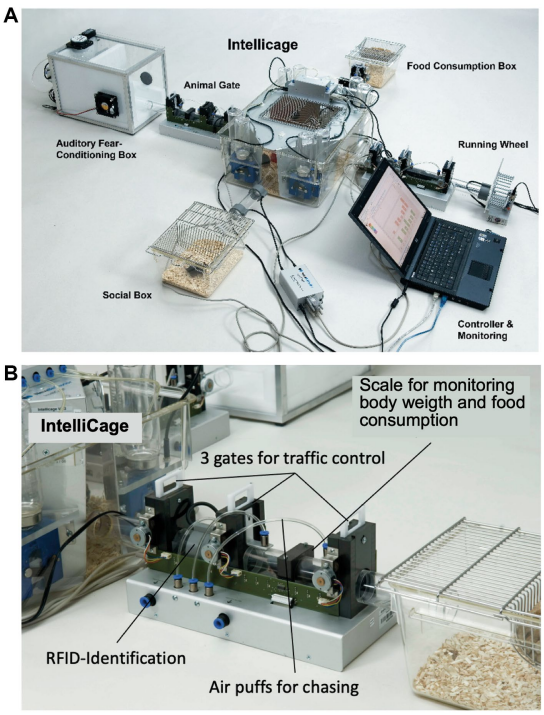

FIGURE 11
IntelliCage add-ons that can be controlled by the IntelliCage system. **(A)** Overview of all add-ons constructed under the FP6 programs "Intellimaze" and "Noveltune." Picture provided by the University of Zürich. **(B)** Animal gate permitting or denying access to different test systems. The gate contains three doors regulating to-and-from traffic to external devices, supported by air-puffs driving away dawdling mice. The first compartment contains the RFID reader. For a figure showing an outside home cage connected to the IntelliCage for auditory testing, see Kahnau et al. (2023a,b). The add-on most successfully used up-to-now is the audiobox.

animal-friendly because mice lack full contact with conspecifics, but mice are nonetheless not totally isolated as in individual home-cage testing systems, as they are able to see the other mice beyond the transparent dividers. Such dividers can also be useful when testing incompatible males.

The standard tubular RFID antenna poses a problem when studying *obese mice*. As observed in a variety of mouse models (Lutz and Woods, 2012), many obese mice will be unable to squeeze through the standard tube. Occasionally, small ramps may help to facilitate corner entry, but increasing obesity needs RFID readers with enlarged diameter as offered by TSE-Systems. Nevertheless, these larger RFID readers present some limitations when housing control and obese mice together, because two control mice may enter the compartment. This may lead to undesirable interactions during conditioning. A workaround solution is to mount two large RFID readers and keep two normal ones. This entails some limitations in the precise assessment of cage patrolling, but many intra-corner protocols will work.

Another modification under development is the modification of the signaling LED by a *touchscreen panel*. Presently, the LEDs include three programmable light sources, one on each side of the corner. Integrating a small touchscreen would permit to emulate the old LED arrangement yet could also present more complex visual schemes for discrimination learning. LCD (or LED) screens allow the presentation of simple as well as complex visual stimuli and have been extensively used in touchscreen and virtual reality experiments with rodents (Lopatina et al., 2020; Palmer et al., 2021). Reward consumption or refusal would still be possible as before by nose-poking because most of the touchscreen tasks developed benefit much more from the flexibility of the stimulus presentation than touching the correct position on a screen as a response element (Sullivan et al., 2021).

2.10.2 Tracking of mice across the floor of IntelliCage

An often-requested add-on is the ability to follow the trajectories of the mice across the cage floor, although it has been shown that the locomotor activity of mice can be measured indirectly using the number of visits and by calculating which mouse follows corner visits of a cagemate. Typical findings such as circadian activity, habituation or age-dependent decline can be observed using this indirect measurement of locomotor activity (Codita et al., 2010; Krackow et al., 2010). Data generated with the PhenoCube, a modulated IntelliCage combined with a video tracking system, indicated a good correlation between general activity and corner visits in two Huntington's disease models (Oakeshott et al., 2012; Balci et al., 2013).

On the other hand, there is a broad interest to monitor and understand the social interactions, hierarchy, and general home-cage activity in the center part of the IntelliCage. A relatively simple solution is to place RFID-antennae under the IntelliCage so that the positions of various mice can be recorded (for figures see Klein et al., 2022). Such systems are available commercially, e.g., from TSE-Systems as Trafficage, which has an intentionally low spatial resolution[3], or from Phenovance, which offers a higher resolution by using 5 × 5 cm RFID antenna tiles fitting under an IntelliCage (Figure 12). Transponders will be available that record both the floor activity and the corner visits of mice in an IntelliCage. This will permit to evaluate the individual distances between mice in the IntelliCage, as well as the speed and the trajectory of movements, yet it will miss fine interactions between animals. Nevertheless, the amount of data being analyzed can still be managed by ordinary laptop computers, making such extensions affordable. In our view, this new system could be most useful to estimate the amount of activity in an IntelliCage generated by social interactions.

However, to assess a detailed interaction between two or more mice, the only solution are video systems tracking head, tail and body direction of one or several mice. Earlier attempts to check the animals by video were limited to mounting small cameras observing the interior of the cage but being unsuitable for quantitative tracking (Mifflin et al., 2021; Winslow et al., 2021). The analysis of automated movement tracking in mice has made much progress in the last years (Peleh et al., 2019) and video tracking of a single mouse can resolve extremely fine movement variants of spontaneous behavior (Markowitz et al., 2018, 2023). However, the challenges of video tracking multiple mice without visible tags both in dark and under light in a cage where mice have places in which they can hide is still enormous. Typically, a combination of RFID antenna tiles and multiple cameras is necessary to recalibrate individual tracking (de Chaumont et al., 2019). Nonetheless, once achieved an effective motion tracking system, there will be newly developed software packages dealing with the immense data sets recorded, chiefly based on artificial intelligence (AI) including machine learning, such as DeepLabCut which enables body point estimation and tracking of individual animals housed singly or in a group (Mathis et al., 2018; Nath et al., 2019). There is an increasing number of AI-based technologies available to track and further analyse fine body movements according to trained classifiers for mouse behavior which are objective and independent of human definitions (Nilsson et al., 2020; Dunn et al., 2021; Fong et al., 2023; Sakamoto et al., 2023). Thus, one may expect further support by video techniques for the fine-grained analysis of mouse behavior in an IntelliCage, but the amount of data analysis associated with it (the

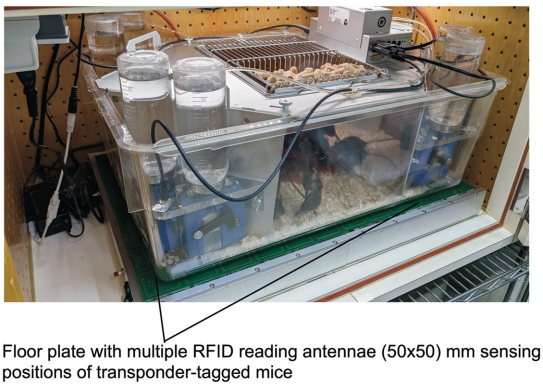

FIGURE 12
RFID floor plate (Phenovance) fitting exactly under the IntelliCage, comprising 50 × 50 mm antennas capable of recording proximity and trajectories of moving mice even when multiple mice are on the same antenna. Although the RFID floor plate requires a different type of transponder from that used in IntelliCage, these two types of transponders do not interfere with each other. Picture courtesy by Phenovance.

3 http:/www.tse-systems.com/service/trafficage

so-called data footprint) will require careful consideration of specific experimental questions to be asked.

2.10.3 Olfactory testing?

An interesting extension would be the possibility to train mice for olfactory discrimination in the IntelliCage. Volatile chemicals characteristic for the smell of food, of a predator or of a receptive female in the environment provide ethologically important information to many animals and are particularly important for mice and other rodents. The recent coronavirus (COVID-19) pandemic with the loss of smell as a typical symptom, and olfactory deficiencies in early stages of Alzheimer's disease raised awareness of this sensory modality in humans. Olfactory assessment might be of special interest to many groups phenotyping Alzheimer mouse (AD) models, as olfactory dysfunction is considered as a pre-cognitive biomarker of AD. Furthermore, olfactory dysfunction is associated with several animal models of neurodevelopmental disorders, such as autism spectrum disorders, which have been intensively characterized in the IntelliCage (Puścian et al., 2014) and other devices (Lyons-Warren et al., 2021).

Currently, our understanding of how animals learn to use this information in the home-cage is limited, mainly due to the relatively challenging experimental settings required (Reinert et al., 2019; Reinert and Fukunaga, 2022). Presently, olfactory stimuli can be placed on top of the corner compartments of an IntelliCage to check olfactory novelty, but finer assessment is not possible. A solution without much modification would be to use animal gates to connect the IntelliCage to testing arenas analysing olfactory signals by ethological approaches such as the ECO-HAB system (Puścian et al., 2016), or to more complex test batteries as described by Zhang et al. (2022). Caglayan et al. (2021) used an RFID based animal sorter to link a home-cage with an external olfactory operant conditioning tool. Similarly, the AutonoMouse system (Erskine et al., 2019; Reinert et al., 2019; Ackels et al., 2021) allows RFID-based olfactory phenotyping of group-housed mice within the same apparatus.

As the IntelliCage is equipped with air-blowers, the available tubing and valve system might be modified for determining olfactory acuity just in front of the nose-poke holes, and by adding a ventilating system preventing spread of the olfactory traces in the IntelliCage. The concept is not new and has been presented before in a self-constructed arrangement (Kudryavitskaya et al., 2020). Thus, providing a defined air source in the IntelliCage corner combined with an olfactometer might allow to use the IntelliCage for olfactory phenotyping. Several behavioral tests in the categories of odor recognition, odor discrimination, and even episodic and emotional memory have been described in mice (Zhang et al., 2022) and could be transferred to the IntelliCage.

2.11 Adapting the IntelliCage to larger species

2.11.1 Rat IntelliCage

The first prototypes of rat IntelliCages were produced in 2006 but needed some adaptation to rat behavior to make the systems run satisfactorily. The first adaptation was size, because rats need more space than offered by commercial rat cages (Figure 13A). The second adaptation included larger corners and larger tubular RFID-antennas (Figure 13B). However, the IntelliCage software could be used to run this system without modifications, because the inputs (presence, nose-poke, licks) as well as the outputs (opening/moving doors, activating LED and delivering air-puffs) were the same, and so the adaptation work was chiefly mechanical and permitted rapid use of the rat systems. Nonetheless, it took some years until the first rat paper appeared (Urbach et al., 2014), but then others followed, 13 over the past 5 years, proving that the mouse IntelliCage system could be adapted successfully to larger rodent species (Yang et al., 2017; Oliveros et al., 2018; Pelsőczi et al., 2020; Zheng et al., 2020; Cao et al., 2021; Esmaeili et al., 2022; Pham et al., 2022; Shishelova et al., 2022; Xiao et al., 2022; Li et al., 2023a,b; Pupikina and Sitnikova, 2023; Wu et al., 2023).

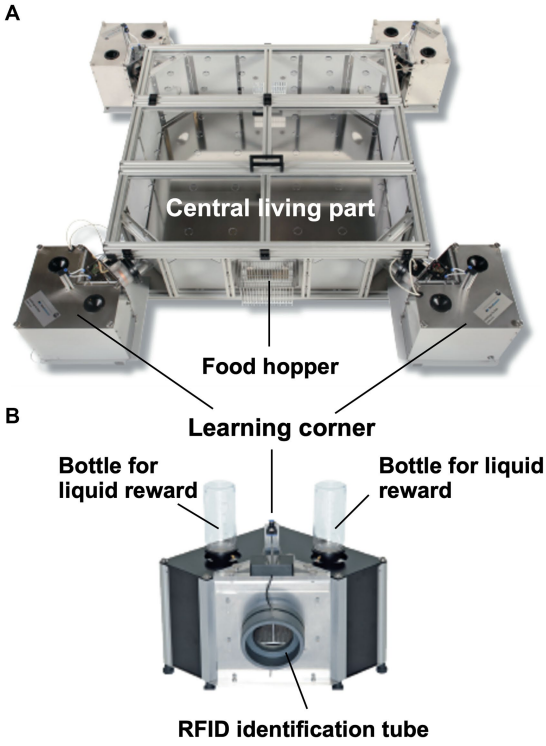

FIGURE 13
IntelliCage for rats. **(A)** Larger housing space required adaption of the IntelliCage system, and **(B)** adaptation of the corner system. The software of the mouse IntelliCage runs also with the rat system. Picture provided by TSE-Systems International.

2.11.2 Marmoset IntelliCage

Marmosets (*Callithrix jacchus*) have become increasingly popular in behavioral neuroscience, partly because they have an easily recognizable behavioral repertoire (Lipp and Hunsperger, 1978), partly because the Japanese government has launched a massive initiative with the goal of implementing marmosets as a primate alternative to rodents, including transgenic models (Okano et al., 2016). This has resulted in many institutions keeping marmosets in Japan, but the actual legal standards in animal welfare would preclude publications of their data in most Western journals. Thus, it appeared attractive to implement a large and animal-friendly home-cage system for these small primates. Because marmosets have a long tongue, they can easily reach the nipple of drinking bottles through openings in a rat IntelliCage corner (Figure 14A). At the Yamasue Laboratory of Hamamatsu University (Japan), to emulate an IntelliCage-like environment, corners were placed in a room housing several marmosets (Figures 14B,C).

The marmosets adapted quickly to the test situation (Figures 14D,E). Experimental protocols were assembled by the normal designer application and the controller used the same interface and analytics online

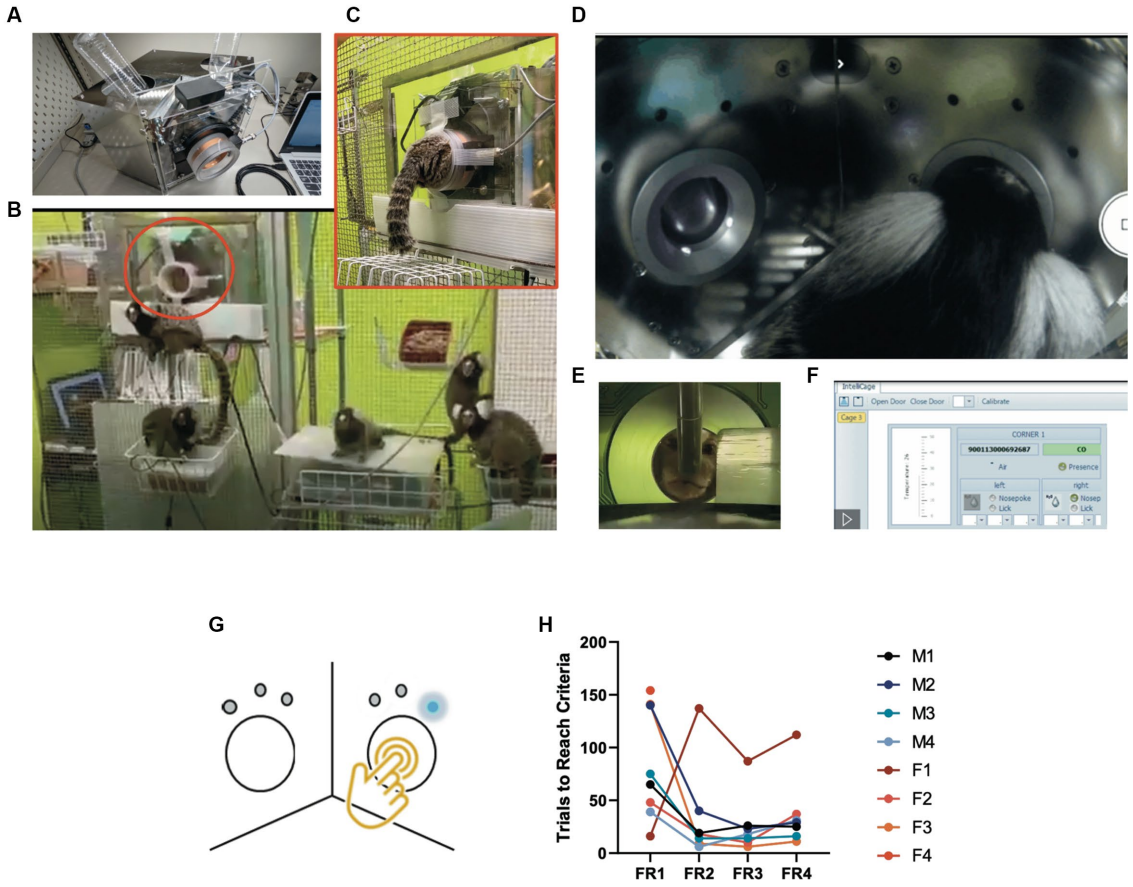

FIGURE 14
IntelliCage system for marmosets (*Callithrix jacchus*) as constructed by Seico Benner and Toshihiro Endo. **(A)** Modified rat corner. **(B)** Animal-friendly marmoset housing at the Yamasue Laboratory, Department of Psychiatry, Hamamatsu University School of Medicine (Hamamatsu, Japan). The position of a rat corner is marked by a red circle. Other corners can be placed everywhere, provided cabling and backside of the corner are protected. Mounting at the wall is the easiest solution. **(C)** Marmoset entering the tubular RFID identifier, which blocks access to others while one subject is working inside. **(D)** Inside view of the conditioning corner corresponding to the arrangement in Figure 4G. **(E)** Outside view of a corner, with the operating gate blocking access to the nipples. **(F)** Screenshot of the standard IntelliCage controller during training of monkeys showing activated signals for presence and nose-poke. **(G)** Set-up of a simple fixed-ratio (FR) discrimination learning task during which the monkey has to nose-poke or to touch the light barrier in front of the closed barrier several times to open the barrier. The position of the rewarded site is signaled by LEDs. The programming was done with the designer program. **(H)** Proof-of-principle pilot study demonstrating differential learning by 8 marmosets under fixed ratios (FR1–FR4, the latter indicating that 4 pokes/touches are required for gate opening). Data published by Benner (2022). Videos showing the various actions are provided in Supplementary Videos S1, S2.

(Figure 14F and Supplementary Videos S1, S2). Simple fixed ratio protocols for nose-poking were easily learned by most monkeys, even though marmoset groups often contain strong-willed individuals that do not like to be conditioned. However, these individual personality differences could make them even better models for translational research in neuroscience and neuropsychiatry (Figures 14G,H). The only restriction for set-ups of IntelliCage-like systems in large rooms is that a computer running the normal IntelliCage controller program can only handle 4 corners per location. Thus, for placing more than 4 test corners in a room, the IntelliCage system needs a program variant, IntelliCage StaR (available from Neurospex GmbH and XBehavior GmbH, Bänk, Switzerland) that avoids this restriction.

2.12 Classifying home-cage behavioral phenotypes by machine learning algorithms

Behavioral phenotypes are usually quantified by an arbitrary array of activity measures per subject during voluntary behavioral expression, e.g., Voikar et al. (2018), as well as by focusing on endpoints defined by the experimenter as responses to a sequence of conditioning tasks, e.g., Fischer et al. (2017), Voikar et al. (2018), and Volkmann et al. (2021). Many outcomes of conventional behavioral studies have proven to be highly inconsistent between experiments, which has often been attributed to strain, animal keeping, handling, lab maintenance, staff attitude, or environmental differences (Crabbe and Wahlsten, 2003; Wahlsten et al., 2003; Codita et al., 2012). Experimental reproducibility also suffers from the absence of an agreed-upon robust method to categorize and summarize behavioral phenotypes. Approaches in IntelliCage range from attempting to extract the most concise number of measures in order to reflect individual spatiotemporal activity patterns (Krackow et al., 2010) to calculating arbitrarily many (linear) combinations of measured variables allowing the algorithm to pick up any classifying patterns that might be hidden in the data (van Dijk et al., 2016, 2019).

In many areas of behavioral phenotyping research, the ultimate goal is to translate some intervention effect into changes of the subjects' condition, e.g., assess whether a drug applied to knockout

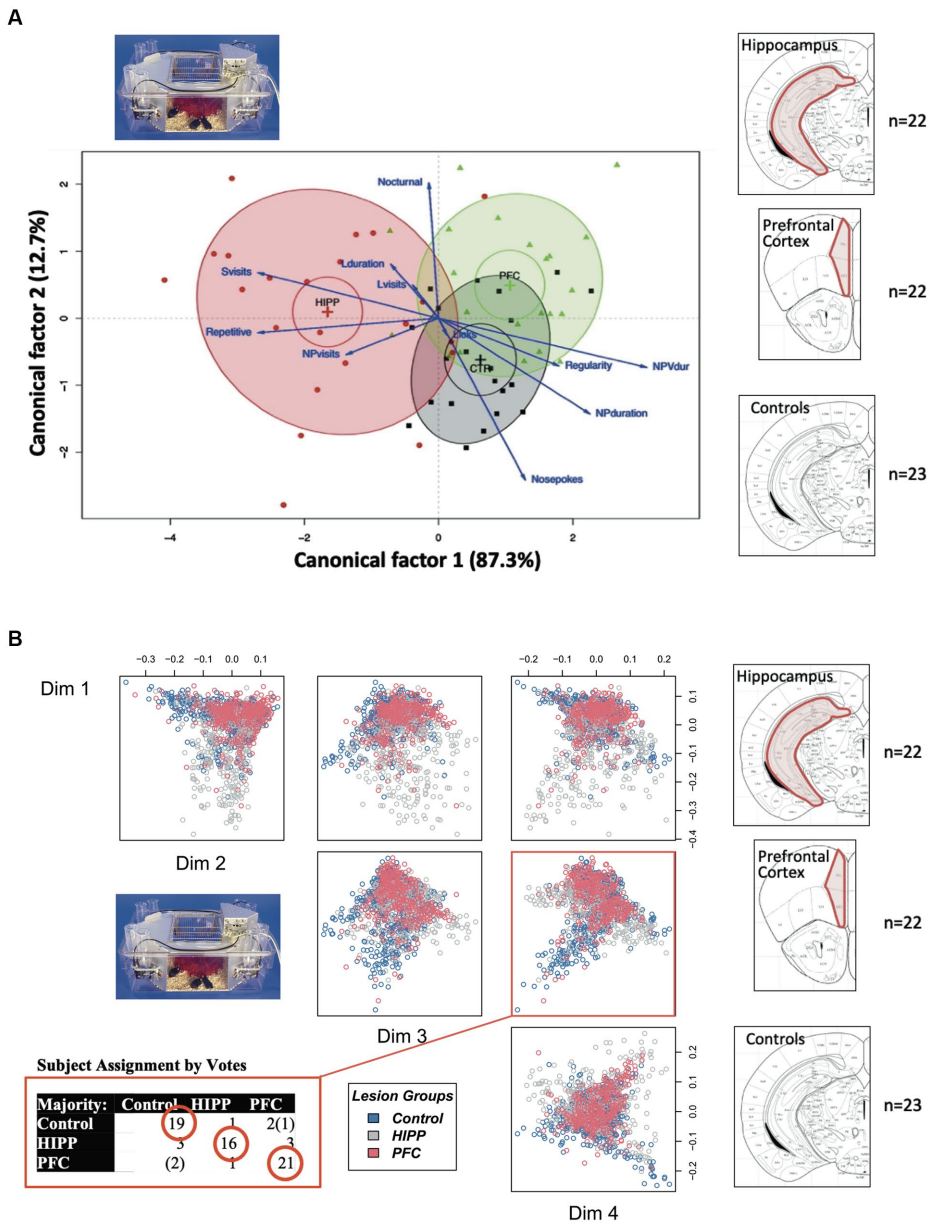

FIGURE 15
Comparison of conventional (linear) multivariate statistics and machine learning. **(A)** Standard canonical discrimination analysis of mouse learning in the IntelliCage showing decent separation of hippocampally lesioned mice (HIPP) from controls in the same cage, but considerable overlap between controls and prefrontal lesions (PFC). Data from Voikar et al. (2018). **(B)** Random forest analysis: same data analyzed by the random forest algorithm, which eventually could separate the three groups much better. Data presented by Krackow and Lipp (2023).

mice showing a "depression-like" phenotype restores "normal" behavior. To this end, the actual nature of the underlying behavioral variables (and their interactions) is not of relevance, but rather their operational value, i.e., whether a subject changes its phenotype in an expected or intended direction. Traditionally, some kind of "biomarker" serves as a proxy to indicate intervention effectiveness in translational research, e.g., Branchi et al. (2010). Hence, trained classifiers that could predict a subject's phenotype might significantly short-cut such translational research on effectiveness of interventions: instead of first searching for a reliable "biomarker," one might only have to check if the behavioral phenotype returns to the "normal" class after intervention. This is in fact the rationale of the approach for drug testing as used by PsychoGenics Inc. (Paramus, New Jersey) in their phenotyping tools SmartCube, NeuroCube and PhenoCube, the latter being a modified IntelliCage (Alexandrov et al., 2015).

To explore the potential of learning algorithms like convolutional neural networks (CNN) for extracting patterns that could be used to predict classes and, hence, in the future, to evaluate interference effects, a random forest model was trained to separate behavioral phenotypes of control and two lesioned groups of C57BL/6J female mice using data from Voikar et al. (2018). Trained classifiers would have the advantage. Over classical models like canonical discriminant analysis. of not ultimately assume linearity (and monotonicity), yet also allowing for predictive classification of unknown samples.

Figure 15 compares the outcome of a canonical discriminant analysis (which ultimately represents a linear multivariate variance model) with

a typical classification procedure used in machine learning (random forest algorithm). Both methods classify subjects, according to their behavioral responses during non-conditioning periods within the long-term experimental sequence, into type-of-lesion groups (hippocampal, prefrontal and controls). Canonical discriminant analysis could not clearly differentiate between control and prefrontally lesioned mice, despite of the expected impairment in the latter (Figure 15A). The preliminary random forest evaluation given in Figure 15B appeared to separate the three groups of mice in the test sample quite well, possibly due to the basically non-linear approach. This is a most promising finding for the feasibility and profitability of AI and machine learning in behavioral phenotyping approaches in the translational realm. Clearly, the standardized way of stimulus presentation and response assessment in IntelliCage during the last 20 years system offers a unique opportunity for comparative retrospective analyses of data sets by means of AI.

3 Conclusion

- Among the many methods for behavioral testing of mice, the IntelliCage is certainly one of the most animal-friendly systems, as (1) it reduces interference with human handling, (2) it avoids testing in possibly anxiogenic environments external to the home-cage, (3) it provides social housing enriched by cognitive testing, and (4) it permits a wide range of patrolling-based and classical operant conditioning protocols (including gustatory and visual signal discrimination) without the employment of painful motivators such as electrical shocks. Even recording simple spontaneous activity is of high heuristic value.
- IntelliCage is also user-friendly, because it reduces the workload of experimenters associated with standard testing of mice, facilitates data analysis, and allows more time for reflection and planning, while caretakers profit from easy handling and cleaning.
- The mouse IntelliCage has a proven record for long-term sustainable standardization and reproducibility for behavioral testing, drug discovery, translational research, toxicology, and neuroscience, as reflected in its increasing use for patent application. Because of its simple design, the same hardware, software, and even similar protocols can be used for comparative testing of rodents and small primates. Thus, it has set and can set comparative standards for the future.
- IntelliCage systems can be expanded and modified for extracting more behavioral information if required, but at the cost of losing standardization and reproducibility.
- Within the field of behavioral testing, the IntelliCage is just one, yet efficient, tool and its output data may require to be checked by manual experiments.
- Despite IntelliCage's friendliness for users, these should carefully consider the possibilities, but also the limitations, in using this tool. High throughput behavioral phenotyping without clear concepts may entail misleading results – the *festina lente* concept should prevail whenever applicable.
- IntelliCage output can be classified according to different views from ethology, experimental animal psychology and even artificial intelligence. Biased by ethological thinking, we believe that the IntelliCage is emulating a small world in which little cohorts of mice can move, explore, and solve problems by adjusting their ongoing activity to tasks presented by a computer, thus demonstrating flexibility at various levels of their brains, that were adapted evolutionarily to small worlds as well. Therefore, we expect or hope that the IntelliCage can help deciphering the enigma of behavioral flexibility and adaptability that mice share with humans.

Author contributions

H-PL: Conceptualization, Data curation, Project administration, Visualization, Writing – original draft, Resources, Writing – review & editing. SK: Software, Visualization, Writing – review & editing, Writing – original draft. ET: Data curation, Visualization, Writing – review & editing. SB: Conceptualization, Methodology, Visualization, Writing – review & editing. TE: Conceptualization, Methodology, Visualization, Writing – review & editing. HR: Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

H-PL was representative of the consulting company Neurospex GmbH and of the software company XBehavior GmbH in Bänk (Switzerland), HR was employed by the TSE-Systems International GmbH in Berlin (Germany) and TE was representative of the Phenovance LLC in Chiba (Japan).

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2023.1270538/full#supplementary-material

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Environmental enrichment improves hippocampus-dependent spatial learning in female C57BL/6 mice in novel IntelliCage sweet reward-based behavioral tests

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The IntelliCage is an automated home-cage system that allows researchers to investigate the spontaneous behavior and learning abilities of group-housed mice. The IntelliCage enables us to increase the standardization and reproducibility of behavioral outcomes by the omission of experimenter–mouse interactions. Although the IntelliCage provides a less stressful environment for animals, standard IntelliCage protocols use controlled water access as the motivational driver for learning. To overcome possible water restrictions in slow learners, we developed a series of novel protocols based on appetitive learning, in which mice had permanent access to plain water but were additionally rewarded with sweetened water upon solving the task. C57BL/6NCrl female mice were used to assess the efficacy of these sweet reward-based protocols in a series of learning tasks. Compared to control mice tested with standard protocols, mice motivated with a sweet reward did equal to or better in operant performance and place learning tasks. Learning of temporal rules was slower than that in controls. When faced with a combined temporal x spatial working memory task, sweet-rewarded mice learned little and chose plain water. In a second set of experiments, the impact of environmental enrichment on appetitive learning was tested. Mice kept under enriched environment (EE) or standard housing (SH) conditions prior to the IntelliCage experiments performed similarly in the sweet-rewarded place learning task. EE mice performed better in the hippocampus-dependent spatial working memory task. The improved performance of EE mice in the hippocampus-dependent spatial working memory task might be explained by the observed larger volume of their mossy fibers. Our results confirm that environmental enrichment increases complex spatial learning abilities and leads to long-lasting morphological changes in the hippocampus. Furthermore, simple standard IntelliCage protocols could easily be adapted to sweet rewards, which improve animal welfare by removing the possibility of water restriction. However, complex behavioral tasks motivated by sweet reward-based learning need further adjustments to reach the same efficacy as standard protocols.

KEYWORDS

IntelliCage, reward, motivation, environmental enrichment, hippocampus, plasticity, working memory, animal welfare

1. Introduction

Under normal and diseased conditions, behavioral phenotyping allows for the objective and quantitative investigation of complex cognitive processes such as spatial learning and memory, emotionality, and exploratory drive. Classical behavioral tests to investigate such processes were well-established, such as the Morris Water Maze task (Morris, 1981; Vorhees and Williams, 2006) or the open field and elevated mazes (Walsh and Cummins, 1976; Pellow et al., 1985; Shepherd et al., 1994; Stanford, 2007). Despite their effectiveness, these and other classical tests have some limitations. First, the necessary human intervention in classical behavioral testing was a source of stress for the animals, and this affected both animal welfare and the quality of the collected data (Balcombe et al., 2004; Deacon, 2006; Spruijt et al., 2014; d'Isa and Gerlai, 2023). Even simple handling increased the corticosterone levels in rats (Armario et al., 1986). Second, different laboratory environments may lead to differences in behavioral outcomes, even if experimental and/or environmental conditions were strictly standardized (Crabbe et al., 1999; Jaric et al., 2022). To overcome these limitations, new automated phenotyping systems based on video, infrared, radiofrequency identification (RFID) or sensor plates have been developed and used for measurements in home cages (see Voikar and Gaburro, 2020 for a comprehensive overview). These home-cage systems limit the animal–human interaction and concomitantly provide a standardized environment, thereby increasing reliability and eventual reproducibility in future experiments (Krackow et al., 2010; Endo et al., 2011; Spruijt et al., 2014; Kiryk et al., 2020; Grieco et al., 2021). While most of these home-cage systems are designed to test single animals, one exception is the IntelliCage, where up to 16 mice can be tested together (Galsworthy et al., 2005). The IntelliCage (NewBehavior AG and TSE-systems) is an RFID transponder-based, fully automated, and programmable apparatus to study cognitive abilities in group-housed mice over long periods of time (Masuda et al., 2018). Basically, the IntelliCage is a large home cage with four computer-controlled operant chambers fitted into the cage corners. A chamber can be visited by one mouse at a time, while the presence and identity of the animal are registered. Inside the chamber, two water bottles are hidden behind doors. The mouse can access water as a reward by nose poking the door. Whether or not a given door opens after a nose poke depends on the specific task. Once experimental mice are placed in the IntelliCage, they remain in this IntelliCage testing environment with their social group, and all experimental procedures, either on a group or individual level, are managed remotely. Animal activity, measured by visits to chambers, and performance within the chamber were monitored for each animal 24 h a day. This is in stark contrast to classical behavioral tests, where the behavioral responses of animals are usually assessed in novel environments over short periods of time. Many studies pointed out the relevance of the higher sensitivity of IntelliCage testing in detecting exploratory behaviors, circadian rhythm, and learning abilities ranging from simple place learning to complex delay-discounting tasks in wild-type and mutant mice (see Kiryk et al., 2020; Iman et al., 2021 for reviews). Even though the benefits of the IntelliCage test environment are obvious (minimal human intervention, social housing, and a stimulating yet familiar environment), animals with severe learning impairments might become water-restricted, requiring constant monitoring and, if necessary, their removal from the experiment. In line with the three Rs principles (*Reduction*, *Refinement*, and *Replacement*), we, therefore, sought to overcome the potential consequences of water restriction by creating and testing learning protocols based on a sweetened reward, exploiting the known saccharin preference of C57BL/6 mice (Bachmanov et al., 2001). In these sweet reward-based learning protocols, water was always accessible, while sweet rewards could only be collected if animals made a correct choice. In the first set of experiments, we tested the efficacy and power of the sweet reward-based learning protocols compared to standard protocols, where access to water depended on solving the task correctly. We hypothesized that a sweet reward would be sufficient for several IntelliCage learning tasks but that high cognitive challenges may offset the reward and decrease learning as animals turn to free-access plain water. If so, we wanted to define this turning point and use this information to design modified sweet reward-based protocols.

In the second set of experiments, we explored the effect of environmental enrichment in early adulthood on performance in the sweet reward-based learning paradigms in the IntelliCage. Positive effects of environmental enrichment on animal welfare were well-documented (Bayne, 2018), among other beneficial effects ranging from improvements in spatial learning and memory tests (Frick et al., 2003; Kulesskaya et al., 2011; Hendershott et al., 2016), decreased reward-seeking behaviors (van der Harst et al., 2003; Wood et al., 2006), and neuronal modifications in the hippocampus (Duffy et al., 2001; Hirase and Shinohara, 2014). In this study, female C57BL/6NCrl mice were housed either under standard housing (SH) or enriched environment (EE) conditions and tested afterward on sweet reward-based learning tasks in the IntelliCage.

The sweet-rewarded learning protocols included tests for operant performance, temporal learning, impulsivity, place preference learning, spatial sequence learning (chaining), and reversal learning. After behavioral testing, alterations in brain morphology due to housing conditions were examined by volumetric analysis of the hippocampal fields.

2. Materials and methods

2.1. Animals

Eight-week-old female C57BL/6NCrl mice ($N = 56$) were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were group-housed under an inverted light–dark cycle (light on from 20:00 p.m. to 08:00 a.m.). After 1 week of adaptation, mice were injected with a radiofrequency identification (RFID) transponder (Planet ID® GmbH, Germany) under isoflurane inhalation anesthesia (5% isoflurane, 0.7 l/min oxygen). At the age of 10 weeks, mice were randomly assigned to the experimental groups as follows: the Reward-Control experiment ($N = 24$) and the Reward-Housing experiment ($N = 32$). Mice remained in an inverted light–dark cycle for the entire experimental phase.

All experimental procedures (introduction into experimental setups, changes in experimental setups, or remote changes in IntelliCage protocols) were performed at ∼09:00 a.m., which is 1 h into the animal's dark phase. All animal experiments were conducted under permit No. ZH041/18,29918 of the Canton Zurich Veterinary Office.

2.2. Reward-Control experiment

Mice were randomly assigned to the control ($N = 12$) or sweet reward ($N = 12$) group. Each group was tested separately in an IntelliCage equipped with four red shelters, food *ad libitum*, and the operant chambers providing access to water bottles. An extension cage (T3) was permanently connected to each IntelliCage, so mice could be confined either to the IntelliCage or the extension cage while cleaning the other cage. All water bottles in the IntelliCage of the control group contained plain water. In the sweet reward group, each corner of the IntelliCage contained one bottle of plain water and one bottle of sweetened water (saccharin solution: 0.5% saccharin Sigma Aldrich in water). In the sweet reward group, the bottles containing plain water were accessible at any visit in every corner for the entire duration of the experiment, while the bottles with sweetened water were accessible only after a correct response.

Mice were given 3 days of habituation in the IntelliCage (free adaptation, FA), where all doors were open and all bottles were accessible without limitations. In the nose poke adaptation (NPA) phase for operant performance, doors in front of the bottles were closed by default and could be opened for 3 s by a nose poke to the door. In the control group, mice had to perform a nose poke at any door to get access to water. In the sweet reward group, the doors hiding water opened at the beginning of a visit for 3 s without a nose poke, while the doors hiding sweetened water only opened when a nose poke was performed.

In the drinking session adaptation (DSA) phase for time learning, mice could only receive a reward during 4 x 1 h sessions, which were evenly distributed over 24 h. For the control group, water was only accessible during those sessions. Outside the drinking sessions, all doors remained closed. For the sweet reward group, doors hiding saccharin opened after a nose poke during the drinking sessions, while the water doors opened at any visit without a nose poke, regardless of the time of the day.

In the chaining acquisition (CA) task for time x spatial working memory, mice could receive a reward in the corner adjacent to the most recently visited corner in which at least one nose poke had been made. Half of the mice in each IntelliCage had to rotate in a clockwise direction, the other half were assigned to an anti-clockwise direction. As in the task before, the reward (water for the control group, saccharin for the sweet reward group) could only be received upon nose poke in the correct corner during the drinking session. For the sweet reward group, plain water remained available at any time in any corner.

Finally, a recovery phase (with conditions equal to the NPA phase) was followed by a simple place preference (PP) task for place learning, in which each animal could receive water (control group) or saccharin (sweet reward group) in one out of four corners only. Corner assignments were balanced within groups. Again, for the sweet reward group, plain water was accessible in all four corners.

2.3. Reward-Housing experiment

At the age of 10 weeks, mice were randomly assigned into environmentally enriched (EE, $N = 16$) or standard-housed (SH, $N = 16$) groups (Figure 1). The setup for the EE mice (eight animals per group) consisted of four tube-connected cages (two T2 and two T3), all provided with water, food, mouse shelters, and nesting material (Figure 1A). A large running wheel was fit into one of the T3 cages. To simulate a dynamically changing environment, eight objects of different shapes, colors, and materials were added once a week to the cages, and one object was relocated. SH mice were housed for 8 weeks in groups of eight animals in normal cages (T3) with water and food pellets *ad libitum*. Cages contained two polycarbonate mouse shelters (ZOONLAB® GmbH) and nesting material (Figure 1A).

After 8 weeks of housing either under EE or SH conditions, mice were again randomized into two groups of 16 animals each (Figure 1B) and tested in two IntelliCage (Figure 1B). In the IntelliCage, test setup and environmental conditions were the same for both groups. For both SH and EE mice, sweet reward protocols were used, that is, free access to water at any time and sweetened water rewards only if mice performed correctly.

The IntelliCage experiments started with the free adaptation (FA) and nose poke adaptation (NPA) phases, followed by the reaction time task (RTT, Kobayashi et al., 2013; Jörimann et al., 2023) to assess impulsivity. After the first nose poke initiated the trial, mice had to withhold the second nose poke for a random delay (between 0.5 and 2.5 s) before a sweet reward was given. Premature nose pokes on the sweet reward door during the delay were punished by trial abortion, and the mouse had to leave the chamber before having the possibility to start a new trial. Prior to this test, two pre-training phases (T0 and T1) were run in which premature nose pokes did not have consequences. The RTT task was followed by an NPA recovery phase. Mice were then trained for the place preference (PP) task for place learning, followed by the spatial working memory chaining acquisition (CA) and chaining reversal (CR) task. In CR, mice had to visit corners in the opposite direction than during CA. The chaining tasks in the Reward-Housing experiment did not include a time component as in the Reward-Control experiment.

2.4. Histology

Animals of the Reward-Housing experiment were deeply anesthetized (pentobarbital 50 mg/kg body weight) and perfused transcardiacally with ice-cold phosphate-buffered saline (PBS), followed by sulfide solution and, lastly, with 4% paraformaldehyde (PFA) with 15% picric acid. Brains were removed and post-fixed in PFA + picric acid at 4°C overnight. Left hemispheres were embedded with a 2-hydroxyethylmethacrylate (HEMA)-based polymerizate (Technovit 7100, Kulzer GmbH, Wehrheim, Germany) according to the manufacturer's instructions. Embedded

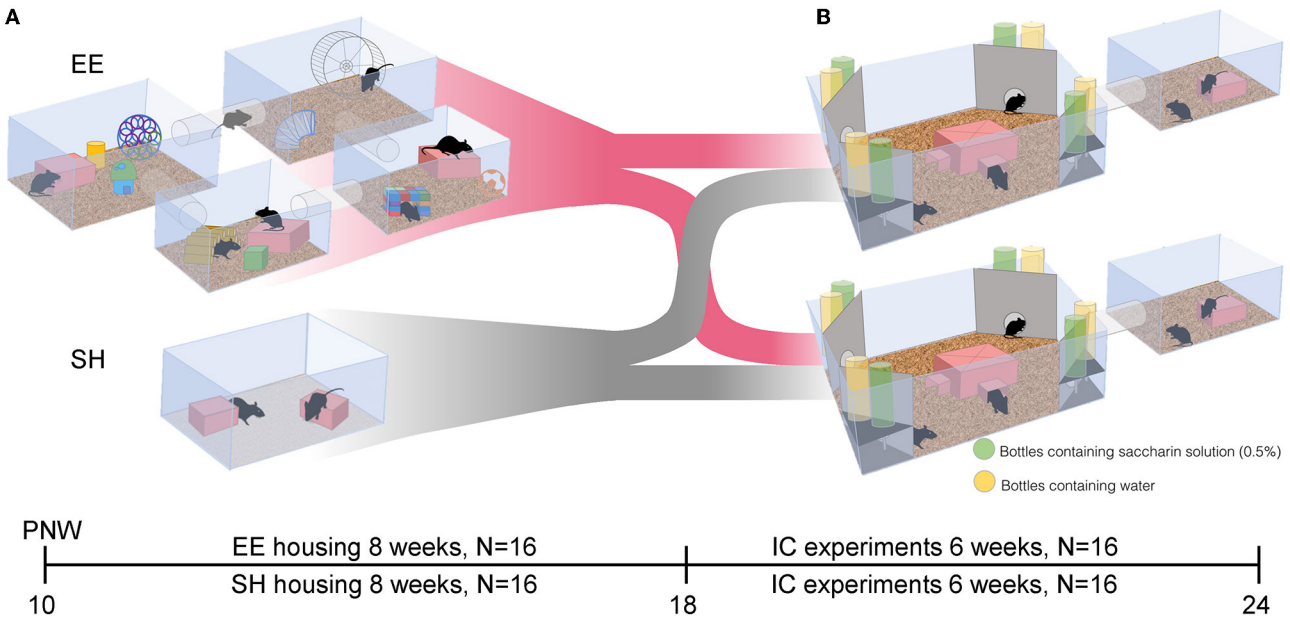

FIGURE 1
Experimental setup of the Reward-Housing experiment. **(A)** At post-natal week (PNW) 10, female C57BL/6NCrl mice were assigned either to the enriched environment (EE) or the standard housing (SH) condition. In the EE condition, mice were stimulated by a running wheel and the addition and relocation of objects in a system of four tube-connected cages over 8 weeks. At PNW 18, mice from the EE and SH conditions were randomized and transferred into IntelliCage for behavioral testing. **(B)** Behavioral testing was conducted in an IntelliCage (IC, large cage). Corners of the IntelliCage hold experimental chambers where mice had access to free water (yellow bottles) or a sweetened reward (green bottles, 0.5% saccharin in water). Food *ad libitum* was provided in the IntelliCage only, while bedding and mouse shelters were provided in the IntelliCage and the extension cage (tube-connected smaller cage).

tissue was cut into coronal sections of 20 µm thickness with a rotational microtome (Microm HM325). Every 10th section was collected in a 24-well plate filled with distilled water, mounted in the correct anatomical order on slides, and dried.

For Timm staining, slides were incubated into a developer solution containing gummi arabicum (1:1 in distilled H_2O), citrate buffer (citric acid and tri-sodium citrate), hydroquinone solution, and $AgNO_3$ 34% at 37°C for 40 min. Slides were rinsed in tap water and incubated for 1 min in 1% sodium thiosulfate. After two more washes in distilled water, sections were counterstained by Giemsa solution diluted 1:5 in KH_2PO_4 for 15 min at room temperature, dehydrated, and embedded.

2.5. Stereological volume analysis of the hippocampus

The volumes of the hippocampal regions were estimated with a design-based stereological method, the Cavalieri estimator (Slomianka, 2021) on the Timm and Giemsa stained sections. Every 10th section containing the hippocampal formation from its rostral to the caudal extent was analyzed using a Zeiss Axio Imager.M2 microscope (magnification 2.5x and 10x) with the Stereo Investigator software 10 (MBF Bioscience, Williston, Vermont USA). Prior to analysis, animal identity was coded. For all regions, a point grid of 100 µm on x- and y-axes was generated and overlaid on each section containing the hippocampus. Seven hippocampal regions were analyzed: granule cell layer of the dentate gyrus (GC); the molecular layer of the dentate gyrus (MOL-DG); hilus of the dentate gyrus (HIL); CA1 including stratum pyramidale, stratum radiatum, oriens, and lacunosum-moleculare; CA3 including stratum pyramidale, stratum radiatum, oriens, and lacunosum-moleculare; subiculum (SUB) and suprapyramidal and infrapyramidal mossy fibers (SI-MF). On average, 15.3 sections (SD = 1.4) in each animal were analyzed.

2.6. Statistical analysis

Behavioral data of the Reward-Control and Reward-Housing experiments were exported with the IntelliCage Analyzer software and processed in R (version 4.2.0) for statistical and graphical analyses. Packages used were dplyr, reshape2, lme4, nlme, emmeans, and ggplot2. For statistical analysis, behavioral parameters of each experimental phase were calculated in three time periods: performance on the 1st day, the last day, and aggregated days in between. Repeated ANOVA was used to analyze the main effects of groups and days in each learning phase, including interactions. If the main effects were significant, Tukey's *post-hoc* testing was applied. One-way ANOVA was used to test for group differences in hippocampal volumetric data, followed by Benjamini–Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995) to adjust *p*-values across all hippocampal regions. The correlation between hippocampal volumetric data and the behavioral parameter was tested with Pearson's correlation. The Shapiro–Wilk normality test was run on

behavioral and hippocampal data, and the Box-Cox transformation was applied if necessary. In graphs, untransformed data with mean, SEM, and individual data points in the background are shown.

In the Reward-Housing experiment, two EE mice had to be excluded from the experiment due to elephant teeth (overgrowth of incisors due to the misalignment of mandibular and maxillary teeth) and low drinking (below 100 licks per day) in IntelliCage, respectively. In addition, behavioral data from the 2nd day of RTT (day 16) were excluded from the analysis due to technical problems. Two exclusions of selected data are mentioned in the result section; otherwise, no data were excluded.

3. Results

3.1. Reward-Control experiment

Visualization of the consumption of saccharin vs. water over the entire experimental phase provides a general survey of animal performance and task complexity (Figure 2). Overall, appetitively motivated mice consumed more liquid (saccharin plus water) than controls (water only) over the entire experimental phase [$F_{\text{group (1,22)}} = 26.9$, $p < 0.0001$, Figure 2G]. Mice in the sweet reward group showed a strong preference for saccharin consumption in phases when tasks were simple (days 1–8, days 24–33, Figures 2A, H). However, saccharin consumption dropped dramatically in the phase of challenging tasks [$F_{\text{phase (2, 22)}} = 157.2$, $p < 0.0001$, Figure 2H]. There was no evidence for a difference between the control and sweet reward groups for general activity, assessed as the mean number of corner visits per day [$F_{\text{group (1, 22)}} = 0.2$, $p = 0.7$]; however, there was evidence for a phase effect [$F_{\text{phase (2, 44)}} = 44.8$, $p < 0.0001$]; *post-hoc* analysis revealed an increase in activity ($p = 0.001$) in the phase of challenging tasks (days 9–23) in control mice (Figure 2I). To check for novelty responses, we analyzed visit activity as a response to environmental or rule changes, that is, during the 1st day of the IntelliCage experiment, the 1st day of the DSA protocol, and the 1st day of the PP protocol. A significant group difference could only be established for the 1st day of DSA ($p = 0.006$, Figure 2J) where control mice increased their visits; otherwise, groups responded similarly to novelty.

Simple learning was analyzed during NPA and PP. The sweet rewards improved operant performance in the NPA phase (Figure 2B) in the sweet reward group compared to controls [$F_{\text{group (1, 22)}} = 10.16$, $p < 0.01$]. Data suggested that sweet rewards prevented a drop in performance on the last day of NPA in the reward group (Figure 2B). In the simple place preference task (PP, Figure 2E), both the sweet reward and control groups learned to visit the rewarded corner equally well and correct corner visits increased over time [$F_{\text{day (2, 46)}} = 211.8$, $p < 0.0001$]. *Post-hoc* comparison for each time point revealed that the performance gain was achieved after the 1st day in both groups ($p < 0.0001$, Figure 2E).

In the complex learning tasks, the sweet reward group preferred free water over saccharin; nevertheless, the analysis of performance revealed some improvements. Time learning in the drinking adaptation phase (DSA, Figure 2C) was analyzed by the percentage of visits during drinking sessions. Effect size was different for groups [$F_{\text{group (1,22)}} = 19.5$, $p < 0.001$], time points [$F_{\text{day (2, 44)}} = 53.9$, $p < 0.0001$], and interaction [$F_{\text{groupxday (1, 44)}} = 9.8$, $p < 0.001$]. *Post-hoc* analysis suggested that controls performed better than the sweet reward group after the 1st day ($p < 0.001$); however, the sweet reward group improved over time ($p < 0.001$). In the chaining acquisition task (CA, Figure 2D), which is a combined time x spatial working memory task, the sweet reward group performed markedly worse than the control group [$F_{\text{group (1, 22)}} = 855.1$, $p < 0.0001$]. *Post-hoc* testing revealed that controls improved after the 1st day ($p < 0.0001$), while in the sweet reward group, there was no evidence of an improvement over time.

In the sweet reward group, consumption of free available water during the three NPA phases was low (on average between 1 and 11%). Importantly, saccharin consumption during the three phases of NPA did not decline significantly over time [$F_{\text{phase (2, 46)}} = 2.05$, $p = 0.14$, Figure 2F], suggesting that the attractiveness of the sweetened reward did not change over time. Note that two mice of the sweet reward group lost preference for saccharin over time (outliers in Figure 2F) and increased plain water consumption.

3.2. Reward-Housing experiment

In this experiment, standard and enriched environment-housed mice had access to saccharin as a sweet reward for correct performance, while plain water was always available. A graphical overview of liquid consumption over the entire experimental period indicates which tasks were difficult for the mice to solve (Figure 3A). The main focus of the analysis was on group differences due to housing conditions, e.g., between the enriched environment (EE) and standard-housed (SH) groups. Over the entire experimental phase, there was no indication of a different preference for saccharin over water between housing groups [$F_{\text{group (1,28)}} = 0.4$, $p = 0.5$], as shown in the selected analysis of FA, PP, and CA/CR phases (Figure 3G). As observed in the Reward-Control experiment before, saccharin consumption dropped dramatically in the challenging RTT and CA/CR phases (Figures 3A, B, G). Overall activity (visits per day) was indistinguishable between groups [$F_{\text{group (1, 28)}} = 1.9$, $p = 0.2$], confirmed by the analysis of selected experimental phases (Figure 3H).

Furthermore, there was no evidence for a group difference in novelty responses, analyzed by visit activity as a response to environmental or rule changes, that is the 1st day of Intellicage testing and the 1st days of RTT, PP, and CA (Figure 3I). Novelty exploration, assessed at the start of the experiment as the latency to visit the IntelliCage corners for the first time, revealed faster exploration in the EE group [$F_{\text{group (1, 27)}} = 31.0$, $p < 0.0001$], and *post-hoc* analysis indicated that the latency to visit the second, third, and fourth corners was shorter in EE-housed mice (Figure 3J). In this analysis, one SH mouse was an extreme outlier (latency by more than two SD higher than the mean) and was excluded from the analysis.

Analysis of learning was performed for the RTT, PP, and chaining tasks. In the RTT task assessing impulsivity (Figure 3B1), both groups scored equally high in premature poke repetition on the 1st day and learned to withhold repetitive poking on the saccharin door in the following days [$F_{\text{day (2, 56)}} = 76.3$, $p < 0.0001$]. Liquid consumption, however, indicated that mice mainly switched

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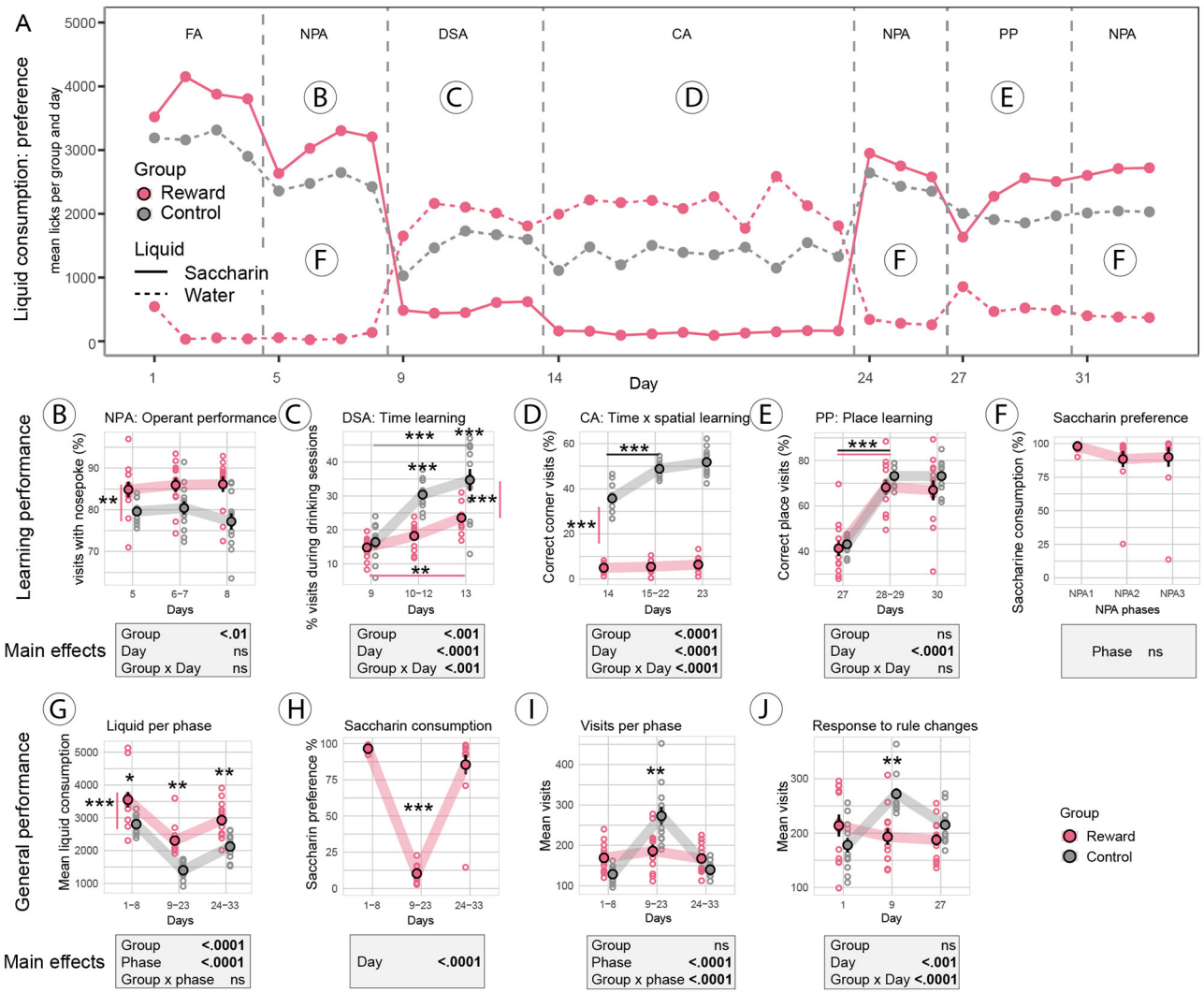

FIGURE 2
Reward-Control experiment. **(A)** Liquid consumption over the entire experimental phase visualizes tasks where the sweet reward motivated mice engaged in the tasks by drinking saccharin (solid red line high), in comparison to tasks where sweet reward consumption dropped dramatically below free water consumption (dashed red line high). Mice in the control group could only access plain water (dashed gray line). **(B)** Operant performance in the nose poke adaptation (NPA) task was improved in the sweet reward group ($p < 0.01$) compared to controls without a drop in performance over days. **(C)** In the drinking session adaptation (DSA) task, access to water (controls) or saccharin (sweet reward group) was restricted to 4x1 h a day. Mice in the sweet reward group made less visits during drinking sessions ($p < 0.001$) but improved over days ($p < 0.001$). **(D)** In the chaining acquisition (CA) task, rewards were only available during drinking sessions, and rewarded corners rotated after every visit. Mice in the sweet reward group made fewer correct visits ($p < 0.0001$) and could also not improve over days, while control animals showed rapid learning. **(E)** In the place preference (PP) task, rewards were available in only one corner and corner assignments were randomized between mice. Sweet reward and control groups performed equally well; learning was significantly improved after the 1st day of testing ($p < 0.0001$). **(F)** Saccharin consumption in the sweet reward group remained stable in the three NPA phases. Note that two mice (outliers in NPA2 and NPA3) increasingly preferred free water over saccharin. Under standard protocols, these two mice would most likely have to be excluded from the experiment. **(G)** Total consumption of liquid (saccharin plus water) increased in the sweet reward group throughout the experiment ($p < 0.0001$). **(H)** Saccharin consumption dropped dramatically during the DSA and CA tests in the sweet-rewarded group. **(I)** Mean number of visits was higher in controls than in sweet-rewarded mice during the challenging DSA and CA tasks (days 9–23, $p = 0.001$). **(J)** Novelty response, assessed as the number of visits during days of rule change, was higher in controls when a challenging task was introduced (day 9, start of DSA task, $p = 0.006$). Mice in the sweet reward group did not increase activity due to rule changes. ***: p-value < 0.001; **: p-value < 0.01; *: p-value < 0.05.

to water consumption (see Figure 3A). Saccharin consumption remained low, and most of the sweet rewards (62%) were received after the shortest delay of 0.5 s (data not shown). However, mice also found a workaround in this test. Detailed analysis suggested that mice increasingly consumed both water and saccharin during the same visit [$F_{\text{day (2, 56)}} = 7.4$, $p = 0.001$] by initiating the trial with a first (correct) nose poke to the saccharin door, then switching to free water consumption, and, once the delay was over and the sweet reward door opened, also consuming saccharin water (Figure 3B2).

In the PP task (Figure 3C), both groups improved over time [$F_{\text{day (2, 56)}} = 111.0$, $p < 0.0001$], without evidence for a group

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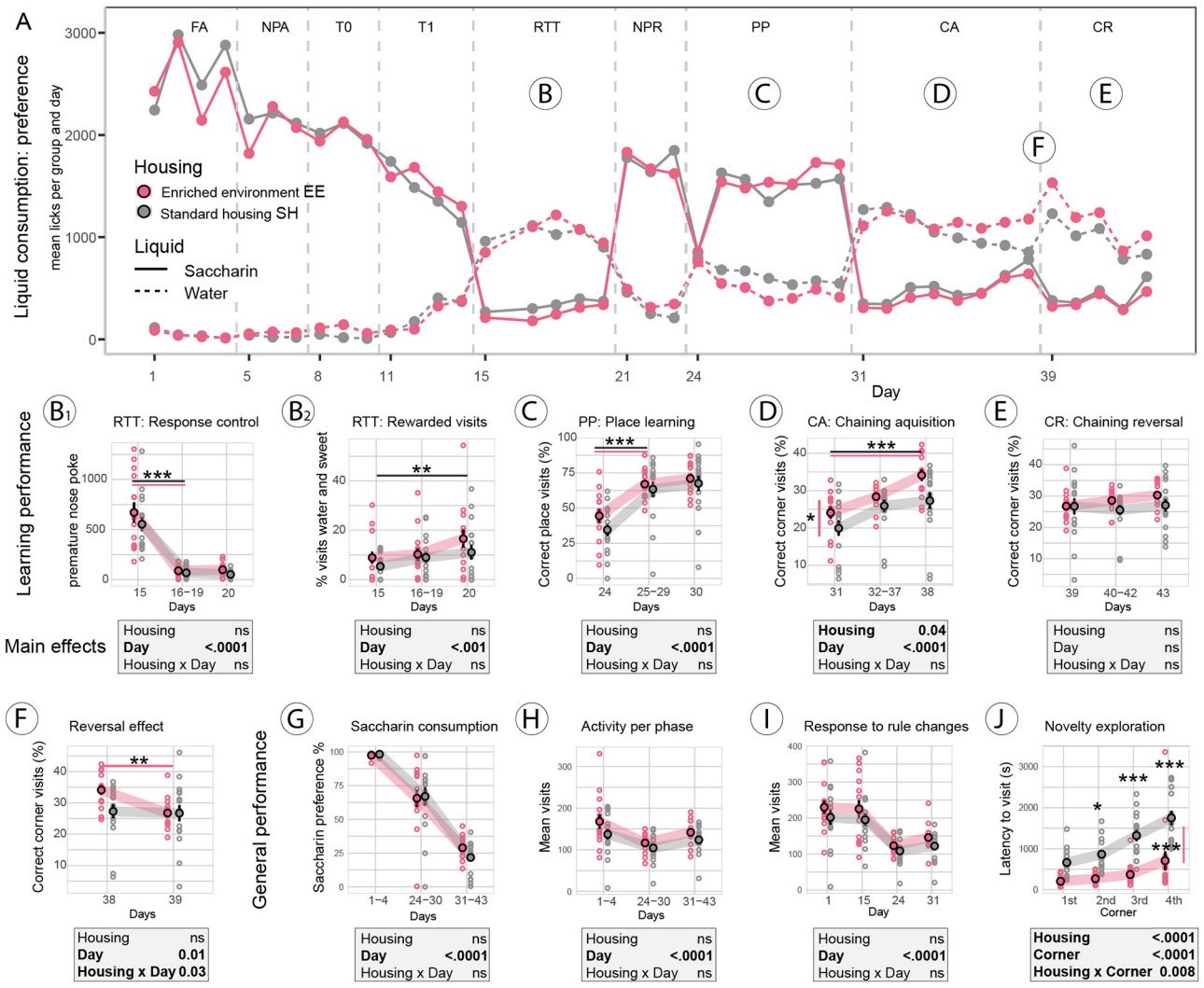

FIGURE 3
Reward-Housing experiment. **(A)** Overview of saccharin (solid line) vs. water (dashed line) consumption in the enriched environment (EE, red) and standard-housed (SH, gray) groups. Saccharin consumption dropped below water consumption in complex learning tasks. **(B)** In the reaction time task (RTT) assessing impulsivity, **(B2)** mice of both groups learned equally well to withhold premature nose pokes after the 1st day of testing ($p < 0.0001$). **(B2)** Detailed analysis of visits revealed that the percentage of visits in which mice consumed both water and saccharin increased over time (day effect $p = 0.001$), indicating that mice learned a workaround in this task. **(C)** In the place preference (PP) task, no group difference was found; all mice learned to visit the correct corner after the 1st day ($p < 0.0001$). **(D)** Correct corner visits were improved in EE mice ($p = 0.04$) in the chaining acquisition (CA) task; over days, both EE ($p < 0.0001$) and SH ($p < 0.001$) improved. **(E)** In the chaining reversal (CR) task, none of the groups could improve. **(F)** In the analysis of the reversal effect, comparing the last day of CA to the 1st day of CR, we found a group x day interaction ($p = 0.03$), the effect was due to the EE mice performing worse after the rule change ($p = 0.008$), while correct corner visits in SH mice were indifferent to the rule change. **(G)** General assessment of sweet reward consumption revealed that saccharin preference did not differ between groups. **(H)** Visit activity during easy or complex phases did not differ between groups. **(I)** Visit activity in response to the rule change did not differ between groups. **(J)** However, novelty exploration, measured as the latency to visit all four corners of the IntelliCage for the first time at the beginning of the experiments, showed faster exploration in the EE group ($p < 0.0001$). ***: p-value < 0.001; **: p-value < 0.01; *: p-value < 0.05.

difference. The chaining acquisition (CA) task in this experiment was designed as a spatial working memory task without a time component, that is, correct responses required visiting corners consecutively in a clockwise or anti-clockwise fashion without restriction to specific time windows. Mice improved with correct corner visits over time [$F_{\text{day (2, 56)}} = 27.5$, $p < 0.0001$, Figure 3D], and EE animals performed overall better in this task [$F_{\text{group (1, 28)}} = 4.6$, $p = 0.04$]. In the chaining reversal (CR) task (Figure 3E), the direction of the rewarded corners for each animal was reversed. The recovery of correct performance for the group or time during the CR task was not significant. To investigate the reversal effect in response to the spatial rule change, performance during the last day of CA and the 1st day of CR was compared (Figure 3F). Correct corner visits declined [$F_{\text{day (1, 28)}} = 6.7$, $p = 0.01$], and *post-hoc* comparison indicated that the decline was due to the drop in performance of EE mice ($p < 0.01$), indicating that SH mice did not show a reversal effect as performance remained on chance level (~25% of correct corner visits), both during the last day of CA and the 1st day of CR. After the rule change, the performance of EE mice was on a chance level too.

3.3. Larger mossy fibers correlate with the reversal effect in the spatial sequence task

Hippocampal fields (Figure 4A) were analyzed using the Cavalieri method. The precision of the volumetric estimations was tested by calculating the coefficient of error (CE) with a smoothness constant of m = 0 (Gundersen and Jensen, 1987; Slomianka and West, 2005). CE was low and varied between 0.03 and 0.13 (Table 1). The ratio CE over the relative group variance was smaller than 0.5 (range 0.1–0.3), indicating that measurement precision did not limit our ability to detect volumetric changes between groups (Slomianka, 2021). There was no evidence for differences between SH and EE mice in the volume of the dentate gyrus granule cell layer, dentate gyrus molecular layer, hilus, CA1, CA3, and subiculum. However, we found a housing effect in the volume of the terminal field of the mossy fibers (Figures 4B, C and Table 1).

The suprapyramidal and infrapyramidal mossy fibers (SI-MF) were larger in the EE group [$F_{group(1, 28)} = 9.6$, $p = 0.005$, adjusted for multiple comparisons $p = 0.036$, Table 1]. We tested SI-MF volume against the reversal effect in the CA to the CR task (see Figure 3F). The reversal effect was expressed as the percentage of correct corner visits on the last day of the CA task (day 38) divided by the same parameter on the 1st day of CR (day 39).

Hence, reversal effects larger than 1 indicated learning of the previous rule and a drop in performance after the rule change. Data indicated a significant within-group correlation of SI-MF volume with the reversal effect ($t = 2.1$, df = 28, $p = 0.04$, Figure 4D), suggesting that mice with larger SI-MF showed an increased reversal effect.

4. Discussion

Automated home-cage systems provide powerful tools for the reproducible and standardized assessments of spontaneous behavior and cognitive abilities in laboratory rodents (Spruijt et al., 2014; Voikar and Gaburro, 2020; Grieco et al., 2021). Of the currently commercially available systems, the IntelliCage is the only home-cage system for high-throughput screening of the behavioral performance of group-housed mice. We tested the benefits and limitations of sweet reward-based tests in the IntelliCage while avoiding water restriction, thus improving animal welfare in this automated behavioral phenotyping system. The predilection of C57BL/6 mice for saccharin over a wide range of concentrations (0.1–20.5 g/l, Bachmanov et al., 2001) was exploited in the present study as a sweet reward-based driver for learning. Saccharin was preferred over sucrose because of the metabolic effects implied by the prolonged consumption of the latter on body weight and enzymatic activity (Black et al., 1998). Compared to controls, which could gain only water as a reward, mice with sweet rewards were more eager to engage in the tasks, as they showed increased liquid consumption over the entire period of testing, without a significant drop in saccharin preference over time.

4.1. Efficiency of sweet reward-based learning in the IntelliCage

The sweet reward-based protocols can easily be applied in tests for explorative behaviors and circadian rhythm, since the behaviors observed during FA and NPA were consistent with those achieved with standard protocols. Furthermore, our findings indicated that, in operant performance, place learning and, to some extent, time learning, sweet reward protocols did not compromise learning efficiency while improving animal welfare. However, the chaining task, combining time learning with spatial working memory learning, posed too much of a challenge for the sweet reward group. These mice switched to plain water consumption and did not engage in learning the task. We used this finding to redesign

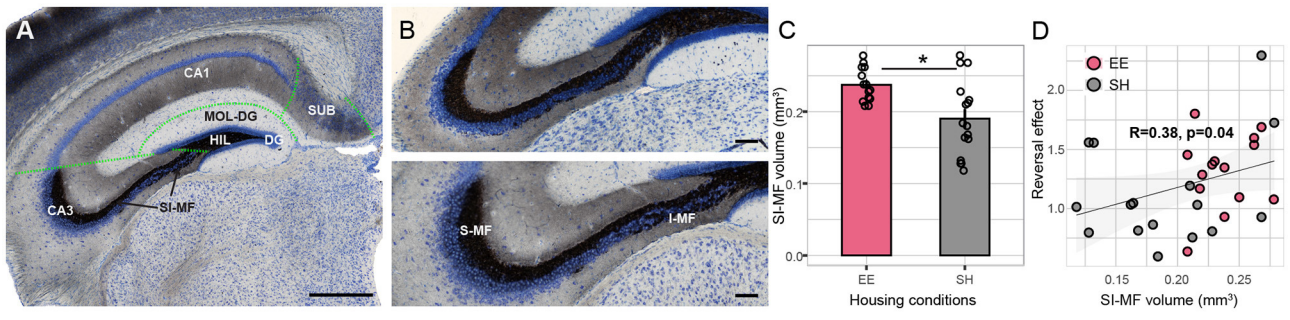

FIGURE 4
Mossy fiber volume correlates with housing conditions. **(A)** Representative Timm stained, coronal section of the hippocampal formation, counterstained with Giemsa. Volumetric analysis was performed for the dentate gyrus granule (DG) cell layer, dentate gyrus molecular layer (MOL-DG), and hilus (HIL). In the CA3 and CA1 regions, pyramidal layers and associated layers of stratum oriens, radiatum, and lacunosum-moleculare were pooled for the volumetric analysis. Lastly, subiculum (SUB) and supra-infrapyramidal mossy fibers (SI-MFs) were measured. Green dashed lines indicate the boundaries of hippocampal fields. **(B)** Enlarged view of the suprapyramidal (S-MF) and infrapyramidal (I-MF) mossy fibers in the dorsal region of the hippocampus of SH (upper panel) and EE (lower panel) mice. **(C)** Total volume of SI-MF was larger in EE mice compared to SH mice ($p = 0.036$); on average, 15 sections per animal were analyzed, spanning the entire rostral to the caudal axis of the hippocampus. **(D)** A significant positive within-group correlation between SI-MF size and reversal effect after the rule change was found ($p = 0.04$); reversal effect was calculated as the percentage of correct corner visits on the last day of the CA task divided by the same parameter on the 1st day of CR. Reversal effects larger than 1 indicate learning of the previous spatial sequence rule and a drop in performance after the rule change. EE, Enriched environment; SH, standard housing; Scale bar A = 500 mm, B = 100 mm. *: p-value < 0.05.

TABLE 1 Cavalieri estimations of hippocampal volumes and group statistics.

	Estimated volume (mm^3)				Group statistics			Estimate precision
Hippocampal fields	**EE mean**	**EE SD**	**SH mean**	**SH SD**	***p*-value**	***F***	***p*-adjusted (BH)**	**CE (m = 0)**
Dentage gyrus granule cell layer	0.30	0.02	0.29	0.03	0.97	0.00	0.971	0.06
Dentage gyrus molecular layer	1.18	0.13	1.16	0.11	0.70	0.15	0.971	0.03
Hilus	0.18	0.03	0.16	0.03	0.24	1.43	0.969	0.13
CA3	1.64	0.19	1.65	0.14	0.85	0.03	0.971	0.05
CA1	2.64	0.26	2.65	0.25	0.97	0.00	0.971	0.03
Subiculum	1.42	0.14	1.41	0.16	0.89	0.02	0.971	0.04
Mossy fiber supra/infrapyramidal (SI-MF)	0.24	0.02	0.19	0.05	**0.005**	9.56	**0.036**	0.08
Hippocampus total	7.59	0.69	7.52	0.59	0.76	0.09	0.971	-

Unilateral volumetric measurements.
EE, enriched environment-housed group; SH, standard-housed group; SD, standard deviation; BH, Benjamini–Hochberg correction for multiple comparisons; CE, coefficient of error; m, smoothness factor.
Significant group comparisons are indicated in bold.

the chaining protocol for the Reward-Housing study, where the time component was removed from the spatial working memory task. Even though performance at the end of this version of the chaining task was below controls, both SH and EE mice learned the task by significantly improving over time. The reaction time task (RTT, Kobayashi et al., 2013) assesses impulsivity and motor response control. Mice receiving sweet rewards learned to withhold premature nose pokes on the saccharin door. However, data indicated that successful inhibition of nose pokes did not increase sweet reward consumption; rather, mice switched mainly to plain water or found a workaround by switching to water consumption to pass the delay time. As observed in our study, mice are not prone to waiting. A decline in the willingness to wait for a sweetened reward was already apparent in the training phases preceding the RTT test, where the rewarding stimulus lost attractiveness even before premature nose pokes had negative consequences. The RTT task in the IntelliCage is a powerful test to detect impulsivity in mice (Kobayashi et al., 2013; Masuda et al., 2016; van Dijk et al., 2016). However, the test is quite challenging for the animals (Jörimann et al., 2023). Modifications to the current sweet reward-based RTT protocol would be highly desirable. Alterations could be achieved by making either saccharin more attractive and water less appealing or by preventing double takes of water and a sweet reward during the same visit.

4.2. Environmental enrichment improves complex spatial learning

Differences in behavior between the EE- and SH-housed mice were observed for the learning complex spatial rules and a reversal effect after the rule change, while both experimental groups displayed the same ability in simple place learning. Improvement in spatial learning and retention after environmental enrichments, usually assessed in the Morris water maze (MWM), is well-documented (Kempermann et al., 1998; Wolfer et al., 2004; Leggio et al., 2005; Bennett et al., 2006; Nithianantharajah and Hannan, 2006; Hüttenrauch et al., 2016, to name but a few). Hippocampal lesion experiments have shown that both MWM and various forms of spatial sequence learning in the IntelliCage are hippocampus-dependent learning tasks (D'Hooge and De Deyn, 2001; Voikar et al., 2018). Spatial learning and memory processes in the hidden platform version of the MWM can be based on different strategies using extramaze cues, proximal cues, or praxis (learning a sequence of movements, Janus, 2004). Spatial sequence learning tasks in the IC, such as the chaining task or the patrolling task (Onishchenko et al., 2007; Albuquerque et al., 2013), might depend less on extramaze cues as the IntelliCage is smaller and relatively enclosed. In addition, mice will be predominantly active during the dark phase when local cues might be more relevant. Moreover, correct performance in spatial sequence tasks in the IntelliCage depends on spatial working memory, as correct corner visits are predictable based on the location of the previous correct visit. Spatial sequence learning tasks in the IntelliCage are more similar to the 8-arm radial maze used to assess spatial working memory (Reinstein et al., 1983). In this study, mice in the EE group performed better than SH mice in the acquisition phase of the spatial working memory-dependent chaining task and showed a stronger reversal effect after the rule change in the chaining reversal phase. In contrast to the chaining task, EE had no effect on simple place preference learning, both groups were equally successful in learning this task. Place recognition, necessary to solve the place preference task in the IntelliCage, is hippocampus-independent, as hippocampal lesion experiments have shown before (Voikar et al., 2018). Stimulation with a dynamically changing environment prior to the IntelliCage experiments had no impact on place recognition abilities. EE conditions might affect higher spatial skills and more complex aspects of spatial memory, leading to the formation of more intricate cognitive maps necessary to learn adaptive spatial rules, as in the chaining task.

4.3. Enlarged suprapyramidal and infrapyramidal mossy fibers after environmental enrichment

Providing a stimulating environment that fits species-specific needs improves the wellbeing of laboratory rodents (summarized by Smith and Corrow, 2005; Neville et al., 2023). It has been shown that the benefits of EE for wild-type rodents and animal

models of brain disorders are multilevel, encompassing visual, motor, cognitive, and somatosensory systems (for a review, see Nithianantharajah and Hannan, 2006). The expression of genes related to synaptic function and cellular plasticity is altered in the cortex and hippocampus of mice reared under enriched conditions (Rampon et al., 2000a; Hüttenrauch et al., 2016). Morphological changes in the hippocampus after EE include increased synapse density in the CA1 region (Rampon et al., 2000b) and a larger cell size of pyramidal neurons in CA1 with longer dendrites in the CA1 and dentate gyrus (Faherty et al., 2003). EE in C57BL/6 mice over 11 months increases the number of dentate gyrus granule cells and leads to a volumetric increase of the cell layers of the dentate gyrus and CA1 (Hüttenrauch et al., 2016). Furthermore, EE promotes adult neurogenesis of granule cells in laboratory rodents (Kempermann et al., 1997), and axonal growth of the newly born neurons preferentially contributes to the infrapyramidal mossy fiber field, leading to a net increase of infrapyramidal mossy fibers after enriched conditions (Römer et al., 2011). In the present study, EE for 8 weeks prior to the IntelliCage behavioral experiments did not lead to a volumetric change in the cell layer of the dentate gyrus. However, we found a persisting volumetric increase of the suprapyramidal and infrapyramidal terminal fields of the mossy fibers in EE mice, while none of the other hippocampal fields showed volumetric changes in the EE mice compared to SH mice. Our finding of enlarged mossy fiber terminal fields due to EE is supported by evidence both for the suprapyramidal and infrapyramidal regions. EE increases the number, size, and complexity of local terminal arborization complexes of mossy fibers, as well as synapse number and dendritic spine length in the suprapyramidal mossy fiber field (Galimberti et al., 2006; Gogolla et al., 2009). Even though we did not separately assess suprapyramidal vs. intrapyramidal and infrapyramidal mossy fibers, it is intriguing to note that larger intrapyramidal and infrapyramidal mossy fibers have been associated with more efficient navigation strategies in the MWM and radial maze (Crusio et al., 1987; Pleskacheva et al., 2000), as well as increased retention in the MWM (Schöpke et al., 1991), suggesting that larger mossy fibers stabilize ongoing behavior and facilitate the processing and use of complex spatial information (Crusio, 2001). This corresponds well with our observation of better performance in the spatial working memory task and increased reversal effects in EE mice. Moreover, within-group covariance analysis revealed a significant positive association between the reversal effect and suprapyramidal and infrapyramidal mossy fiber sizes.

4.4. Explorative behavior and reward-seeking behavior in appetitively motivated learning tasks

Based on previous studies underlining the positive influence of environmental enrichment on explorative behavior by mitigating anxiety-like behaviors (Chapillon et al., 1999; Moreno-Jiménez et al., 2019), we expected to observe increased explorative behavior in EE mice in the IntelliCage. Enrichment did, indeed, lead to shorter latency in exploring the IntelliCage at the beginning of the experiments. However, exploratory behavior in response to rule changes during the following experimental phases was not significantly higher in the EE group, possibly due to an environmental habituation effect. Alternatively, the IntelliCage itself can be considered a form of environmental enrichment (see Figure 1), as it provides both social interactions as well as increased physical activity in a complex environment. The continuous IntelliCage enrichment could have compensated for the previous housing conditions for SH mice. However, the IntelliCage enrichment did not mask the improved spatial working memory performance of mice exposed to the EE condition. Thus, IntelliCage experiments are still a suitable tool to study the effects of previous EE on cognitive performance. Appetitively motivated learning depends on the equal and continuous attractiveness of the reward for both experimental groups. A large body of evidence shows that seeking behavior declines under EE conditions, in particular concerning substances of abuse (Stairs and Bardo, 2009; Olsen, 2011). Our findings in female C57BL/6 mice indicated that preference for sweet rewards was not different between the EE and SH groups, which is in agreement with previous reports of equal sucrose preference in male C57BL/6 mice under EE or social housing conditions, while ethanol preference was reduced in EE mice (Holgate et al., 2017).

In summary, we showed that phenotyping mice in the IntelliCage can be improved further in terms of animal welfare by introducing sweetened water as a reward, while always providing the option to drink plain water, avoiding water deprivation in slow learners. A sweet reward as a motivational driver in the IntelliCage is sufficient to induce robust operant performance and simple place learning. Significant spatial sequence learning and time learning can be achieved with sweet reward-based learning, although the extent is smaller than in standard protocols. Sweet reward-based motivation is not sufficient to induce complex spatial sequence × time learning or successful performance in the impulsivity task. In the present study, only female mice were tested. Previously, female or male mice have been tested using sweet rewards for preference or place learning in the IntelliCage (Kiryk et al., 2020). When both sexes have been investigated, no sex difference in sweet reward preference has been reported (Morello et al., 2020). This is in contrast to conventional saccharin consumption studies, where male C57BL/6 mice showed higher intake compared to female mice (control animals in Di Segni et al., 2019). A formal test of sex-dependent performance in sweet reward-based learning tasks in the IntelliCage is currently missing and could be the subject of future research. Finally, additional studies testing refined IntelliCage protocols might improve the effectiveness of sweet reward-based learning on complex tasks.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Canton Zurich Veterinary Office, Switzerland. The study was

conducted in accordance with the local legislation and institutional requirements.

Author contributions

GB: Investigation, Formal analysis, Writing—original draft. PS: Investigation, Writing—original draft. MN: Methodology. DPW: Resources, Formal analysis, Supervision, Writing—review and editing. IA: Conceptualization, Formal analysis, Supervision, Writing—review and editing.

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Conflict of interest

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

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Refinement of IntelliCage protocols for complex cognitive tasks through replacement of drinking restrictions by incentive-disincentive paradigms

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The IntelliCage allows automated testing of cognitive abilities of mice in a social home cage environment without handling by human experimenters. Restricted water access in combination with protocols in which only correct responses give access to water is a reliable learning motivator for hippocampus-dependent tasks assessing spatial memory and executive function. However, water restriction may negatively impact on animal welfare, especially in poor learners. To better comply with the 3R principles, we previously tested protocols in which water was freely available but additional access to sweetened water could be obtained by learning a task rule. While this purely appetitive motivation worked for simple tasks, too many mice lost interest in the sweet reward during more difficult hippocampus-dependent tasks. In the present study, we tested a battery of increasingly difficult spatial tasks in which water was still available without learning the task rule, but rendered less attractive either by adding bitter tasting quinine or by increasing the amount of work to obtain it. As in previous protocols, learning of the task rule provided access to water sweetened with saccharin. The two approaches of dual motivation were tested in two cohorts of female C57BL/6 N mice. Compared to purely appetitive motivation, both novel protocols strongly improved task engagement and increased task performance. Importantly, neither of the added disincentives had an adverse impact on liquid consumption, health status or body weight of the animals. Our results show that it is possible to refine test protocols in the IntelliCage so that they challenge cognitive functions without restricting access to water.

KEYWORDS

home cage monitoring, incentive learning, disincentive learning, appetitive learning, aversive learning, animal welfare, 3R principles, C57BL/6 N mice

Introduction

Traditionally, behavioral analysis of laboratory animals is performed using batteries of tests that are conducted in a specific experimental setup. To name but a few, these include simple conditioning chambers (Heron and Skinner, 1939), the nesting (Deacon, 2006a) and burrowing (Deacon, 2006b) tests to assess the intactness of complex instinctive behaviors or the open field

test to quantify emotionality and spontaneous activity (Hall, 1934). An especially broad range of experiments has been developed to explore different facets of memory (see Ghafarimoghadam et al., 2022, for a comprehensive review), including the Morris water maze (Morris et al., 1982; D'Hooge and De Deyn, 2001) and Barnes maze tasks (Barnes, 1979) as tests of spatial learning capability.

While these tests remain crucial to phenotyping new animal models of neurological diseases, they share a number of drawbacks: Animals are exposed to an unfamiliar and thus stressful environment, they need to be separated from their cage mates and the necessity for human intervention introduces undesirable variability across laboratories and experimenters (Crabbe et al., 1999; Chesler et al., 2002; Wahlsten et al., 2003).

To address this issue, systems that automatically monitor animals in their home cage environment have been developed, using various mechanisms such as infrared beams, video tracking and operant task machines inside the cage (Voikar and Gaburro, 2020).

The IntelliCage system (New Behavior AG, TSE systems, see Figure 1A for a photograph of the apparatus) remains the most flexible of these concepts (Kiryk et al., 2020; Lipp et al., 2023) and offers the advantage of social housing. It uses transponder-based radio-frequency identification and four learning corners that each contain two operant conditioning walls with a motorized door regulating access to the nipple of a drinking bottle. Although the inputs required from the animals are simple ("visits" to the learning corners are measured by presence of body heat and transponder signal, "nose pokes" on the operant doors are recorded through infrared beams and "licks" at the bottle nipples are registered by contact sensors), adjustments to the spatial and temporal sequence of correct doors and various complications such as light indicators, unpleasant air-puffs or olfactory cues allow for an extremely broad range of possible experiments to study many aspects of rodent behavior.

A disadvantage of the IntelliCage system is the fact that in most learning tasks, drinking is provided only if tasks are correctly completed, which means that thirst is the primary motivational driver. Drinking sessions are usually limited to 2–4 intervals of 1–2 h a day to improve learning. Under these circumstances, animals that learn poorly or insist on wrong choice patterns are at risk for dehydration. Finding a way to replace drinking restrictions is an ethically desirable goal, as it reduces suffering imposed on laboratory animals through refinement of the experimental process in accordance with the last element of the 3-R principles "replace, reduce, refine" (Russell, 1960).

Concerns regarding the impact of liquid deprivation on animal welfare are increasing. In Switzerland, water deprivation of mice is only considered mild if it lasts for less than 12 h, deprivation periods of 12–24 h are classified as moderate constraint (severity grade 2 according the Swiss Federal Food Safety and Veterinary Office FSVO; FSVO, 2018). In the recently proposed d'Isa-Gerlai rating scale for the impact of behavioral tests on animal welfare, on a 12-level scale ranging from A (animal-friendly) to L (lethal), water deprivation with duration > 9 h has been rated H (d'Isa and Gerlai, 2023).

One way to replace thirst as the primary learning incentive is to make plain water constantly available, but reward correct task completion with a liquid of more attractive taste. C57BL/6 mice are known to prefer water containing nutritive sugars as well as non-nutritive sweeteners (Sclafani et al., 2010). In a previous study (Bramati et al., 2023), we showed that appetitive learning based solely on preference for sweetened water can work sufficiently well in simple learning tasks, but is insufficient for reliably providing the stronger motivation needed to learn more complex tasks.

For this reason, we sought a way to improve motivation while still constantly providing drinking water to all animals. To do so, we decided to add a disincentive to the constantly available water. In this study, we compare two ways to achieve this: the addition of bitter tasting quinine, which has been shown to be disliked by mice (Masamoto et al., 2020; Kahnau et al., 2023) and the introduction of a "gambling" mechanism that denied access to plain water in 75% of responses for plain water, which increases the amount of effort needed to obtain the same volume of water. Our aim was to expand the range of tasks that can be successfully employed in a reward learning paradigm and explore the limits of our new approach. To this end, we tested our methods in a series of increasingly difficult place-learning tasks and compared them to purely appetitive motivation.

Materials and methods

All experimental procedures were approved by the Cantonal Veterinary Office of Zurich (License No. 060/2021).

Animals

A total of 69 female C57BL/6 N mice (Charles River Laboratories, Germany) were used in the study. Their age was 2 months on arrival in our facility and they were housed in a 12:12-h reversed light–dark cycle (lights off 08:00–20:00). Radio frequency identification transponders were implanted subcutaneously into the neck region under inhalation anesthesia with isoflurane. Recovery from the implantation procedure and stability of transponder placement was observed during a 7-day period. Mice were introduced to IntelliCages at the age of 3 months, which is when experiments started.

Animals were randomly assigned to two experimental and two control groups. Control groups were subjected to a series of appetitive-learning tasks that included saccharin alone as incentive, while experiment groups were exposed to the combined incentive-disincentive paradigm, with either quinine or decreased reward probability as the disincentive.

Mice that scored fewer than 100 licks daily (which indicates a clear drop from the usually observed baseline of approximately 1,000 licks a day) were temporarily transferred to a separate cage, where they were provided with an *ad libitum* supply of drinking water. If sufficient drinking did not resume after a few days, they were excluded from the study and placed back into normal housing. In total, 3 animals were temporarily transferred to separate cage and returned, whereas 13 were permanently excluded (6 from neutral groups and 7 from disincentive groups).

Nomenclature

The four learning corners are numbered in a clockwise manner, starting from the upper left (Figure 1A). Each learning corner has two operant doors. The operant doors on the long edges of the cage are termed "Task side" doors (where Saccharin could be obtained for correct responses), whereas the ones located on the short edges are

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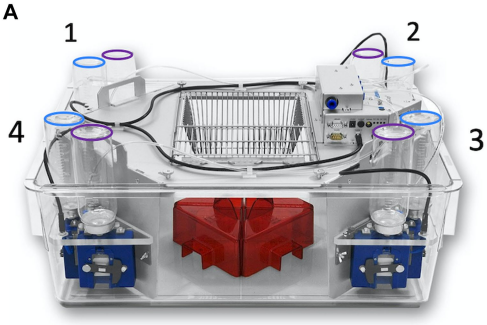
A



B

Groups	Task side ↓ ↑ cont	Task side ↓ ↑ cond*	Joker side ← → cont	Joker side ← → cond**
Contr. 1+2 (n=17+18)	Sacch. 0.5%	Nose-poke	Plain water	Visit (100%)
Quinine (n=17)	Sacch. 0.5%	Nose-poke	Quinine 0.3 mM	Visit (100%)
Lotto (n=17)	Sacch. 0.5%	Nose-poke	Plain water	Visit (25%)

*only in correct corner, **in any corner

C

Phase	Change trigger	Change pattern	Reversal	Example sequence
Free adaptation (FA)	-	-	-	None - all doors always open
Nose-poke adapt. (NPA I-VIII)*	-	-	-	None - all corners always correct
Place preference (PP Acq + Rev)	-	-	Shift to diagonal	1 *3*
Serial reversal (SR)	Day	Pseudo-random shift	-	1 3 2 4 1 3 2
Place time task (PT Acq + Rev)	Time (mid-phase)	Shift to adjacent (two used)	Same with remaining corners	1 2 1 2 1 2 1 *3 4 3 4 3 4 3*
Diag. sequencing (DS Acq + Rev)	Correct response	Shift to diagonal (two used)	Same with remaining corners	1 3 1 3 1 3 1 *2 4 2 4 2 4 2*
Chaining (C Acq + Rev)	Task response	Shift to adjacent (all used)	Inversed direction	1 2 3 4 1 2 3 *4 3 2 1 4 3 2*

*Disincentives (quinine or reduced probability of door opening) were introduced in NPA II, incentive (saccharin) in NPA III

D

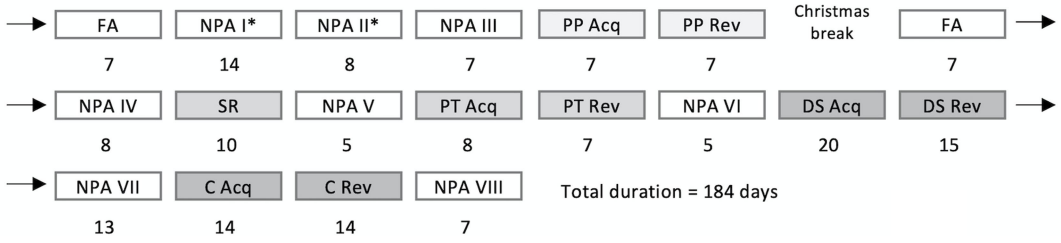


FIGURE 1
Overview of study design. **(A)** Photograph of an IntelliCage: Red rodent house in the middle, learning corners with drinking bottles on the side. Purple = task side bottles, blue = joker side bottles. Note: The added extension cage is not shown here. **(B)** Programming and content of drinking bottles. Task sides opened for 3 s after a nose poke, whereas joker sides opened for 3 s on every corner visit. Joker sides were made unattractive using either bitter tasting quinine solution or decreased opening probability in experiment groups. Task sides were made more attractive with saccharin solution for all groups. Task side bottles were only available in correct corners, whereas joker side bottles were accessible in all four corners. **(C)** Phases.

(Continued)

FIGURE 1 (Continued)
Shading reflects difficulty. Reversal sequences in red italic numbers. **(D)** Timeline. Shading again reflects difficulty. *Note that modifications were gradually introduced in NPA I-III. NPA I, No disincentive or incentive; NPA II, Only disincentive; NPA III-VIII, Both disincentive and incentive (saccharin).

called "Joker side" doors. The initial stage of each learning task is called "acquisition stage," and the following phase with inversed conditions is termed "reversal stage." For the sake of brevity, groups are named after the joker side doors (control groups were termed "neutral" and incentive/disincentive experiment groups were called "disincentive"). "Lotto" and "Quinine" were chosen as short names for, respectively, the experiment with the gambling condition and the experiment with the bitter solution.

IntelliCages

A total of 8 IntelliCages were used, with 8 to 9 animals per cage. They were placed inside a standard T2000 cage (610 × 435 × 215 mm) connected to a Type III extension cage of dimensions 425 × 266 × 155 mm to provide more living space. Food was provided *ad libitum* on a hopper on top of the cage. Saccharin solution was exchanged every 2–3 days to ensure uniform quality, quinine and plain water bottles were replaced every 4–5 days.

Incentives and disincentives

In our study, we chose the artificial sweetener saccharin as a sweet reward, because it prevented confounding effects such as weight gain or changing body composition. We provided the animals with a 0.5% saccharin sodium salt hydrate solution (Sigma-Aldrich S1002), because we found in a previous experiment (unpublished data) that this concentration—while repulsively oversweet to the human taste—is the most attractive for mice (see also Cathomas et al., 2015). As disincentive, we used a bitter solution containing 0.3 mM quinine monohydrochloride dihydrate (Acros Organics A0420352). The approach with decreased reward probability allowed co-housing of the two groups using the same drinking bottles (4 mixed IntelliCages) and the Quinine mice were separated in 2 neutral and 2 disincentive cages.

Protocols

Free adaptation (FA; see Figures 1B–D for details and durations of all phases): First, animals were habituated to the IntelliCage environment in a free adaptation phase of 7 days, during which all doors were always open.

Nose poke adaptation (NPA): Animals were introduced to the concepts of the learning tasks in these phases. During NPA I, joker side doors opened for 3 s at the beginning of every visit to a learning corner. Task side doors, on the other hand, could be opened once per visit for 3 s using a nose poke. In NPA II, the disincentive condition was introduced for the experimental groups: for the quinine group, joker side bottles were replaced with quinine solution and in the lotto group, joker side doors now immediately closed after a nose poke 75% of the time. In the next phase, NPA III, all task side bottles were replaced with sweet saccharin solution, completing the habituation to the experimental setup. NPA IV-VIII used the same protocol as NPA III and were interposed between learning tasks to reset task performance to pre-task levels.

Place preference (PA): For each animal, one corner was set to be "correct," allowing the usage of the task side, whereas the task side door remained permanently closed in all other corners. The correct corner remained constant for the entire acquisition stage and was shifted diagonally for the reversal stage. Conditions for joker sides remained the same as in NPA III. To avoid bias due to previous spontaneous preference, the most and the least preferred corners of NPA III were never assigned as correct. To avoid cage effects, correct corners were balanced as well as feasible within each cage.

Serial reversal (SR): The correct corner changed every 24 h in this phase. Intentionally, a changing pattern too complex for animals to learn (shift to diagonal, then shift to long-side adjacent and so forth) was chosen to provide a pseudo-random pattern for the mice. This task did not have a reversal stage.

Place time task (PT): The correct corner now moved back and forth between two adjacent corners for the entire acquisition stage, changing position in the middle of each phase of the light–dark cycle (02:00 and 14:00). In the reversal stage, the other two corners were used.

Diagonal sequencing (DS): The correct corner changed diagonally after every correct task response, increasing task difficulty compared to fixed time-dependent rules. For the reversal stage, the remaining two corners were used.

Chaining (C): Correct corners now changed after every task response (correct or incorrect), and now included all four corners in a clockwise or counterclockwise sequence. Direction was individually assigned to prevent imitation learning. In the reversal stage, direction was reversed.

Apart from the NPA interludes, tasks followed immediately after each other and task duration was modulated dynamically based on continuous observation of the learning curve.

Detailed temporal analysis of door movement

In order to improve our understanding of the animals' experience during our experiments and to define hits and hit-rates, we analyzed the exact pattern of door opening and closing by comparing raw data output from the IntelliCage with video recordings produced in a test setup without animals. We found that door opening started 0.17 s (see Supplementary Figure 2A for all timepoints) after the trigger (visit or nose poke) and the first licks were registered 0.4 s post-trigger. Doors were fully opened after 1.4 s. Closing of the door started with a latency of 0.13 s and the last licks occurred 0.7 s after the closing trigger (nose poke, automatic 3 s timer or premature end of visit). The doors were fully closed 1.37 s after the trigger and motor movement stopped shortly thereafter (1.4 s post-trigger). We observed that the first licks were registered at a time where the door position does not yet allow actual drinking—it is probable that mice prematurely started licking

the metal of the bottle cap in anticipation. The last licks were registered at a door position that seemed reasonable as the last possible moment for drinking, which is why we estimated that the first moment of effective drinking occurred at this door position as well (0.9 s after opening trigger). Motor activity lasted slightly longer than the process of door movement (1.5 s).

In conclusion, with a timer setting of 3 s reward presentation (the time window allowing licks to be recorded) lasted for 3.3 s (t + 0.4 s until t + 3.7 s) and the presumed drinking window had a duration of 2.8 s (t + 0.9 s until t + 3.7 s). During analysis, hits were defined as nose pokes overlapping with reward presentation at task doors (task hits) or joker doors (joker hits). Since joker doors were programmed to accept visit onset as opening trigger, reward presentation started sooner and, sometimes, the door was already open when the first nose poke was made. This resulted in slightly reduced hit durations (Supplementary Figure 2B) and markedly reduced latencies between hitting nose pokes and licks at joker doors (Supplementary Figure 2C).

Parameters

As the IntelliCage system's output files only report basic variables, post-processing steps were applied to obtain composite variables such as task responses (visits to a corner with at least one nose poke on the task door), joker responses (visits with at least one nose poke on the joker door) or hits (stratified into joker and task hits).

As a measure of door preference and of motivation to engage in task leaning, we calculated the task response ratio R:

$$R = \frac{2 + 2 * Task\ responses}{2 + Joker\ responses + Task\ responses}$$

This value tends to 0 after many responses exclusively on the joker side and to 2 after a large number of responses exclusively on the task side. A value of 1 indicates the absence of a door preference.

As a measure of learning and task performance, we also calculated the false rate, which was defined as the percentage of task responses in incorrect corners. In the absence of a learning effect, this value is expected to be around 75%, with a significant reduction indicating successful learning of the task rule.

Statistical analysis

Statistical analysis was performed using R software (version 4.3, used with packages ggplot2, plyr, nlme, moments, lmtest and psych). To evaluate the effect of the two methods of dual motivation, a linear model was used with two between subject factors: group (control: neutral = saccharin alone as incentive, experiment: disincentive = combined incentive-disincentive) and experiment (Quinine = quinine as disincentive, Lotto = plain water with access denied with 75% probability). The full model was set as y ~ (group*experiment*time) + Error (name), with "name" corresponding to animal ID. In the analyses shown in Supplementary Figures 2B,C, door was used instead of name. A within subject factor time was added to the model in order to explore learning effects and their dependence on group and experiment factors. Significant interactions were explored by splitting the model. Significant effects of time were further explored using partial models. Variables with strongly skewed distributions or strong correlations between variances and group means were subjected to Box-Cox transformation before statistical analysis. The significance threshold was set at 0.05. The false discovery rate (FDR) control procedure of Hochberg was applied to groups of conceptually related variables within single tests to correct significance thresholds for multiple comparisons. Similarly, FDR correction was applied during post-hoc testing. Partial ω^2 served as measure of effect size. Comparisons of group means against chance values were performed using one-sample *t*-tests.

Results

Corner preference strongly established for all groups in nose poke adaptation phases

During NPA I, a subtle but significant preference to nose poke at joker doors emerged (Figure 2A). This was likely explained by the fact that the opening of these doors was triggered by the beginning of the corner visit, giving access to water more rapidly (Supplementary Figure 2C). One prospective quinine cage showed a spontaneous preference for task doors, creating a general preference in the disincentive group of the quinine experiment. This had already been observed during free adaptation (data not shown). After the introduction of the disincentive in NPA II, preference shifted to the task door in both experimental groups (Figure 2B). The addition of saccharin to task side bottles during NPA III further increased the preference for this side in the disincentive groups, and the control groups developed a preference for the sweet liquid as well, although a small group difference persisted (Figure 2C).

Comparable false rate, but enhanced task response ratio in place preference task

In the acquisition stage, we found a strong decrease in the percentage of false corner choices compared to baseline, where the correct corner was not yet noticeably different. In this phase, neither disincentive group showed a false corner choice rate that was significantly better than the controls. However, a group x time bin interaction effect revealed a somewhat steeper learning curve for the disincentive groups (Figure 3A). Task response ratio was significantly higher in disincentive groups, as well (Figure 4A). While graphs suggested a somewhat stronger effect in the Lotto group, this remained below the threshold of significance.

During the reversal stage, false rates dropped sharply and significantly, but did not fully reach the levels of the acquisition phase. The patterns of the acquisition phase were replicated, with no significant difference in false rates, but significant effects on improvement rate and task response ratio (Figures 3B, 4B).

Improved learning in serial reversal task

In this task, we saw a significantly reduced false rate in both groups compared to baseline, with better performance in disincentive

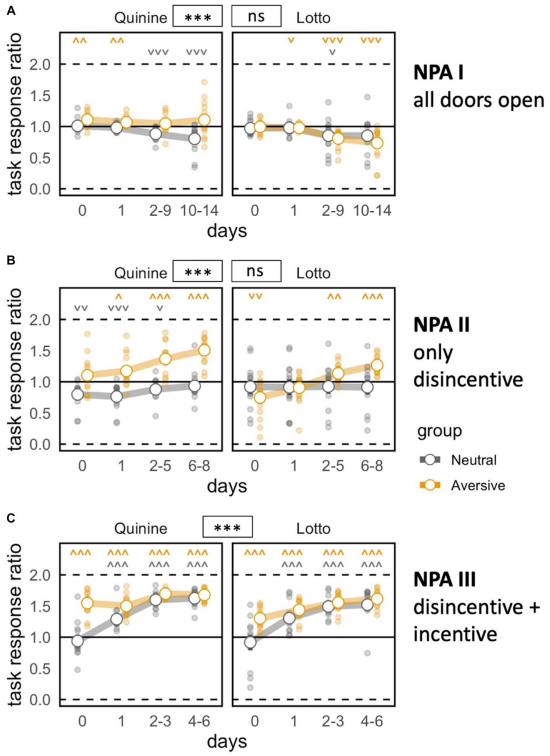


FIGURE 2
Task motivation during nose poke adaptation phases. Zero indicates total preference for joker door, 1 means no door preference, 2 means total preference for task door. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate group effects. ˅ ^, ˅˅ ^^, ˅˅˅ ^^^ stand for significant deviation from chance level according to one-sample *t*-tests. NPA, nose poke adaptation phase. Day 0 indicates last day of previous phase (pre-task baseline). **(A)** Mice develop a preference for more easily accessible joker doors except in the prospective disincentive group of Quinine experiment [time bin: $F(3,144) = 19.22$, $p < 0.0001$ $\omega^2 = 0.16$, group: $F(1,48) = 7.132$, $p = 0.0103$ $\omega^2 = 0.11$, group × time bin: bin $F(3,144) = 0.6729$ ns, group × experiment: $F(1,48) = 33.44$, $p < 0.0001$ $\omega^2 = 0.39$. group × experiment × time bin: $F(3,144) = 12.36$, $p < 0.0001$ $\omega^2 = 0.11$]. **(B)** Preference shifts to task doors upon introduction of disincentive. Experiment effect persists, probably because of pre-existing spontaneous preference [time bin: $F(3,147) = 41.56$, $p < 0.0001$, $\omega^2 = 0.20$, group: $F(1,49) = 28.43$, $p < 0.0001$, $\omega^2 = 0.35$., group × time bin: $F(3,147) = 20.61$, $p < 0.0001$, $\omega^2 = 0.11$, group × experiment: $F(1,49) = 18.99$, $p < 0.0001$, $\omega^2 = 0.26$, group × experiment × time bin: $F(3,147) = 4.812$, $p = 0.0032$, $\omega^2 = 0.02$]. **(C)** Control groups catch up in terms of task response ratio after incentive is introduced [time bin: $F(3,147) = 232.3$, $p < 0.0001$, $\omega^2 = 0.55$, group $F(1,49) = 24.45$, $p < 0.0001$, $\omega^2 = 0.31$, group × time bin: $F(3,147) = 26.73$, $p < 0.0001$, $\omega^2 = 0.12$, group × experiment $F(1,49) = 0.1323$ ns. Group × experiment × time bin: $F(3,147) = 12.95$, $p < 0.0001$, $\omega^2 = 0.06$].

groups, but no evidence for a difference between the two disincentive groups (Figure 3C). False rates after baseline reached a plateau and did not further improve across days, which indicates that the mice, as expected, did not understand the corner change pattern and learned each target position as a new task. Task response ratio fell significantly across time bins. However, disincentive groups still showed a significantly higher task response ratio than controls (Figure 4C).

When trials within days (where the same corner remained correct) were grouped into block bins (corresponding to deciles of the trial number within that time period), false rates steeply declined for all groups, with disincentive groups again displaying more robust learning (Figure 3D). Block bin analysis also showed that overall task response ratio steadily and significantly, but slowly increased within days, with higher levels in disincentive groups (Figure 4D).

Intact learning in place time task with stronger performance of disincentive groups

False rates dropped significantly from chance levels at baseline. The fact that false rates continued to fall after the implementation of the task rule across time bins showcases the animals' ability to understand the simpler back-and-forth change pattern employed here (Figure 3E). While disincentive groups performed better, a potential trend toward stronger learning in the Quinine group compared to the Lotto group remained not significant.

Task response ratio fell significantly after the introduction of the task rule, but remained stronger in the disincentive groups (Figure 4E).

During reversal, the pattern of the acquisition phase was mostly replicated, with significant effects of time bin and disincentive group and a non-significant trend toward stronger performance in the Quinine group compared to the Lotto group (Figures 3F, 4F).

Diagonal sequencing with only disincentive groups retaining above-chance task response ratio

False rates dropped significantly from chance levels at baseline and we again saw a steady decline in false rate, which was expected in a task with a constant (or rather, constantly changing) task rule. However, false rates did not reach the levels seen in the previous phases, mirroring the increased difficulty of the task. Groups showed a similar pattern as in the previous phase, with better performance of disincentive groups (Figure 3G).

Task response ratio fell significantly after the introduction of the task rule, but remained stronger in the disincentive groups. It should be noted that task response ratio only remained above chance for disincentive groups (Figure 4G).

In the reversal stage, false rates still dropped, but not as strongly as in the acquisition phase. While all groups started clearly above chance levels (because baseline was recorded under the acquisition task rule), only disincentive groups were able to reach levels below chance (Figure 3H). Task response ratio was also lower than in the acquisition phase. While disincentive groups never fell below chance levels, controls were always below chance, meaning they preferred to reliably receive plain water at joker doors (Figure 4H).

Disincentive groups with preserved learning performance into chaining task

False rates in the acquisition phase were overall higher than in the diagonal sequencing task, reflecting the fact that difficulty increased yet again. Here, the false rates of disincentive groups were no longer lower than in controls, but there was a significant interaction between group and time bin, indicating a steeper learning curve in disincentive

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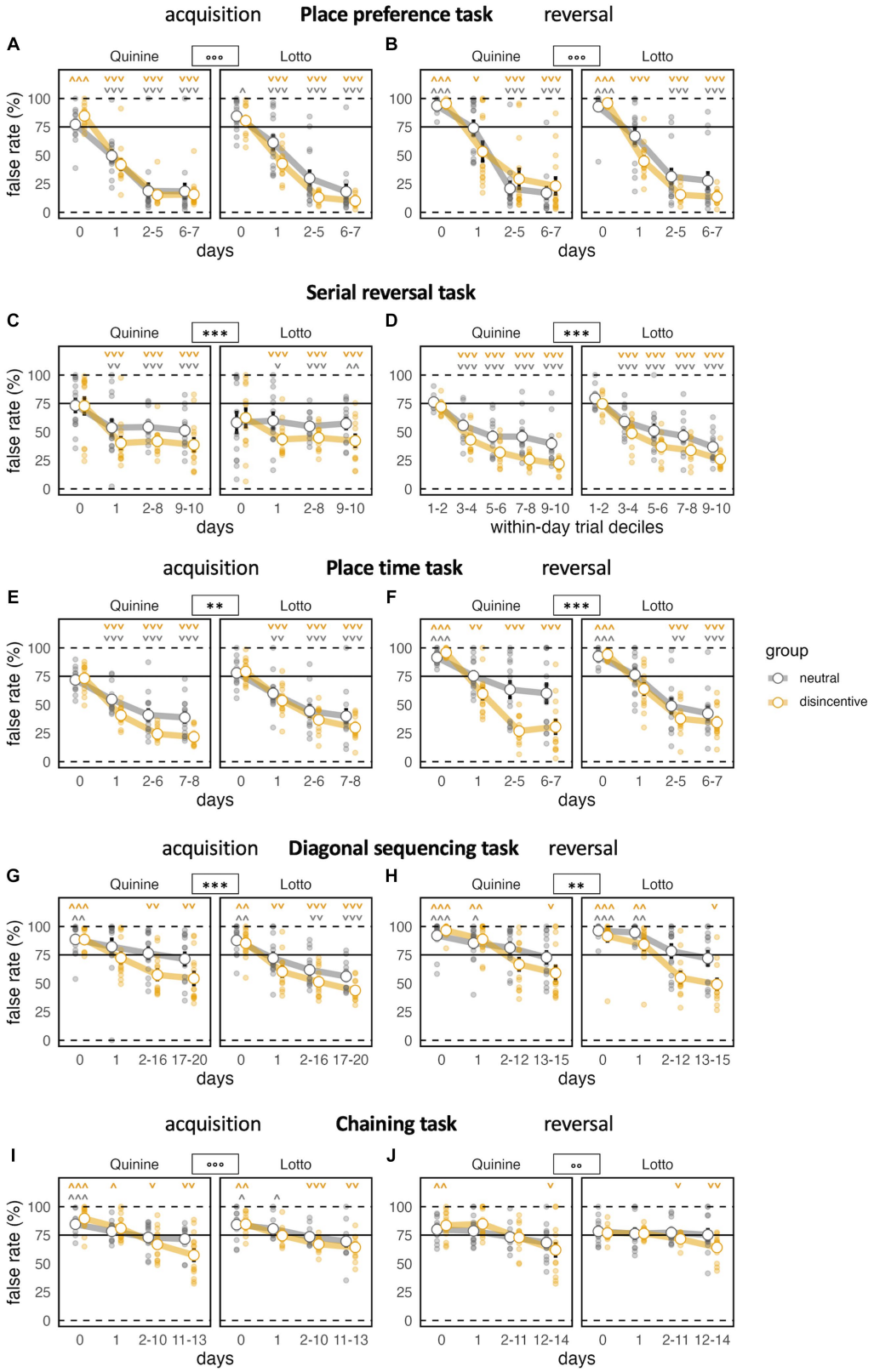

FIGURE 3
Task performance across learning phases. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate group effects, ° indicate group × time bin interaction (only shown when group effect not significant). ˅^, ˅˅^^, ˅˅˅^^^ stand for significant deviation from chance level according to one-sample *t*-tests. Day 0 indicates last day of previous NPA for acquisition **(A,C,E,G,I)**, last day of acquisition phase for reversal **(B,D,F,H,J)**. **(A)** All groups learn well, the disincentive group of the Lotto experiment slightly faster [time bin: $F(3,144) = 521.0$, $p < 0.0001$, $\omega^2 = 0.76$, group: $F(1,48) = 3.459$, $p = 0.0690$, $\omega^2 = 0.05$, group × time bin: $F(3,144) = 5.852$, $p = 0.0008$, $\omega^2 = 0.03$, group × experiment $F(1,48) = 3.236$, $p = 0.0783$, $\omega^2 = 0.04$, group × experiment × time bin: $F(3,144) = 0.8052$ ns].

(Continued)

FIGURE 3 (Continued)

(B) Again, all groups learn well, the disincentive group of the Lotto experiment slightly faster [time bin: $F(3,141) = 280.9$, $p < 0.0001$, $\omega^2 = 0.72$, group: $F(1,47) = 2.787$ ns, group × time bin $F(3,141) = 6.192$, $p = 0.0006$, $\omega^2 = 0.05$, group × experiment: $F(1,47) = 2.276$ ns, group × experiment × time bin: $F(3,141) = 2.465$, $p = 0.0649$, $\omega^2 = 0.01$]. **(C)** Learning is intact, but not progressive after first day in serial reversal (SR) task. Disincentive groups perform better, irrespective of experiment [time bin: $F(3,138) = 10.57$, $p < 0.0001$, $\omega^2 = 0.14$, group: $F(1,46) = 12.76$, $p = 0.0008$, $\omega^2 = 0.20$, group × time bin: $F(3,138) = 1.833$ ns, group × experiment: $F(1,46) = 0.1020$ ns, group × experiment × time bin: $F(3,138) = 0.2525$ ns]. **(D)** Progressive performance in serial reversal task when analyzed by block bins, which correspond to deciles of numbers of trials within a day/task. Disincentive groups perform better and learn faster, irrespective of experiment [block bin: $F(1,220) = 663.8$, $p < 0.0001$ $\omega^2 = 0.61$, group: $F(1,46) = 27.14$, $p < 0.0001$, $\omega^2 = 0.35$, group × block bin: $F(1,220) = 9.778$, $p = 0.0020$, $\omega^2 = 0.02$, group × experiment: $F(1,46) = 0.3550$ ns, group × experiment × block bin: $F(1,220) = 0.6738$ ns]. **(E)** Good performance in all groups, disincentive groups learn faster and perform better [time bin: $F(3,138) = 323.2$, $p < 0.0001$, $\omega^2 = 0.67$, group: $F(1,46) = 9.302$, $p = 0.0038$, $\omega^2 = 0.15$, group × time bin: time bin $F(3,138) = 9.999$, $p < 0.0001$, $\omega^2 = 0.05$, group × experiment: $F(1,46) = 1.521$ ns, group × experiment × time bin: $F(3,138) = 0.1349$ ns]. **(F)** All groups learn the reversal stage with modest performance of controls in the Quinine experiment. Again, disincentive groups learn faster and perform better [time bin: $F(3,138) = 153.4$, $p < 0.0001$, $\omega^2 = 0.61$, group: $F(1,46) = 18.40$, $p < 0.0001$, $\omega^2 = 0.27$, group × time bin: $F(3,138) = 10.51$, $p < 0.0001$, $\omega^2 = 0.09$, group × experiment: $F(1,46) = 1.011$ ns, group × experiment × time bin: $F(3,138) = 1.941$ ns]. **(G)** All groups learn, starting off above chance level. Disincentive groups learn faster and perform better. False rate of controls in the Lotto experiment does not fall significantly below chance level [time bin: $F(3,138) = 80.43$, $p < 0.0001$, $\omega^2 = 0.40$, group: $F(1,46) = 10.44$, $p = 0.0023$, $\omega^2 = 0.16$, group × time bin: $F(3,138) = 4.488$, $p = 0.0049$, $\omega^2 = 0.03$, group × experiment: $F(1,46) = 0.0192$ ns, group × experiment × time bin: $F(3,138) = 0.1339$ ns]. **(H)** All groups improve, starting clearly above chance level. Disincentive groups learn faster and perform better. False rate of controls in both experiments fails to fall significantly below chance level [time bin: $F(3,138) = 91.22$, $p < 0.0001$, $\omega^2 = 0.42$, group: $F(1,46) = 7.935$, $p = 0.0071$, $\omega^2 = 0.13$, group × time bin: $F(3,138) = 8.974$, $p < 0.0001$, $\omega^2 = 0.06$, group × experiment: $F(1,46) = 1.628$ ns, group × experiment × time bin: $F(3,138) = 0.0904$ ns]. **(I)** Starting above chance level, all groups still improve, but more slowly than in previous tasks. Disincentive groups learn faster while control groups fail to improve significantly below chance level [time bin: $F(3,132) = 80.11$, $p < 0.0001$, $\omega^2 = 0.39$, group: $F(1,44) = 2.541$ ns, group × time bin: $F(3,132) = 7.810$, $p < 0.0001$, $\omega^2 = 0.05$. group × experiment: $F(1,44) = 1.086$ ns group × experiment × time bin: $F(3,132) = 3.4$ 28, $p = 0.0191$, $\omega^2 = 0.02$]. **(J)** Learning is overall slower than during acquisition. Disincentive groups learn slightly faster and unlike controls reach a final false rate significantly below chance [time bin: $F(3,132) = 25.33$, $p < 0.0001$, $\omega^2 = 0.19$, group: $F(1,44) = 0.5358$ ns, group × time bin: $F(3,132) = 5.160$, $p = 0.0021$, $\omega^2 = 0.04$, group × experiment: $F(1,44) = 0.6728$ ns, group × experiment × time bin: $F(3,132) = 0.2386$ ns].

groups. In this phase, there was also a significant interaction between time bin, group and disincentive type, with a steeper learning curve in the Quinine experiment (Figure 3I). Task response ratio decreased over time, more strongly in control groups. Controls fell below chance levels (Figure 4I).

During chaining reversal, false rates overall were the highest recorded in any phase, but the decline across time bins was still significant. Again, false rate in disincentive groups was no longer reduced, but their learning curves were significantly steeper. Task response ratios were also low throughout this phase, but significant changes were noticeable for time bins and group (Figures 3J, 4J).

Lick numbers and task motivation decreased across phases, but were increased in disincentive groups

When comparing the number of total licks per day across phases, we found that values decreased over the course of the study both for nose poke adaptation and learning phases. After an initial drop, numbers stabilized at a level of approximately 1,000 licks per day. There was no evidence for decreased lick numbers in disincentive groups compared to controls (Supplementary Figures 1B,D).

Task response ratio also dropped markedly across phases in control as well as in disincentive groups. In learning tasks (Supplementary Figure 1C), this can be explained by increasing task difficulty, but the decrease in the nose poke adaptation phases (Supplementary Figure 1A) also shows an overall loss of motivation. Task response ratio remained significantly enhanced for disincentive groups during NPA as well as learning phases.

Discussion

Our findings confirm and expand the results of previous studies that examined reward learning in IntelliCage (Bramati et al., 2023). The combination of incentive and disincentive resulted in an overall stronger motivation to learn, which is reflected by consistently higher task response ratio and results in better performance and/or higher learning rate, as well as the preservation of the learning effect even into more difficult hippocampus-dependent learning tasks.

However, when comparing our study to IntelliCage experiments based on drinking restrictions, it appears that our approach still elicited a somewhat weaker learning response in difficult learning tasks: For instance, animals in previous studies (Kobayashi et al., 2013; Akbergenov et al., 2018; Mätlik et al., 2018; van Dijk et al., 2019) were all able to deliver false rates of 30–40% on average in the most difficult task used in this study (chaining), which exceeds our results of around 60% in this phase. On the other hand, a recently published study using captured wild rodents (Jörimann et al., 2023) found chaining phase false rates that were roughly comparable to ours.

Future studies using our protocols would not necessarily need to include the most difficult tasks used here. Depending on the research question, simpler memory tasks such as diagonal sequencing or the place time task may already suffice. However, it is important to always include a very simple task such as place preference (PP) to check the intactness of basal sensorimotor functions, as it is usually done in classical Morris Water Maze testing by adding a much easier cue-based version as control (Vorhees and Williams, 2006).

Even though task motivation as measured by task response rates was consistently improved by the use of our dual-motivation protocols, these protocols could not prevent a decline of task engagement with increasing task difficulty. Task engagement and also total liquid consumption measured by lick number deteriorated over the course of our study even when examining the interposed nose poke adaptation phases. This suggests that a certain habituation effect was present. Animals might have lost their initial fascination with the sweet taste stimuli and increasingly limited their efforts to the minimum required to prevent dehydration. Possible ways to address this could include a quicker progression to more difficult learning tasks or the replacement of saccharin solution with plain water during nose

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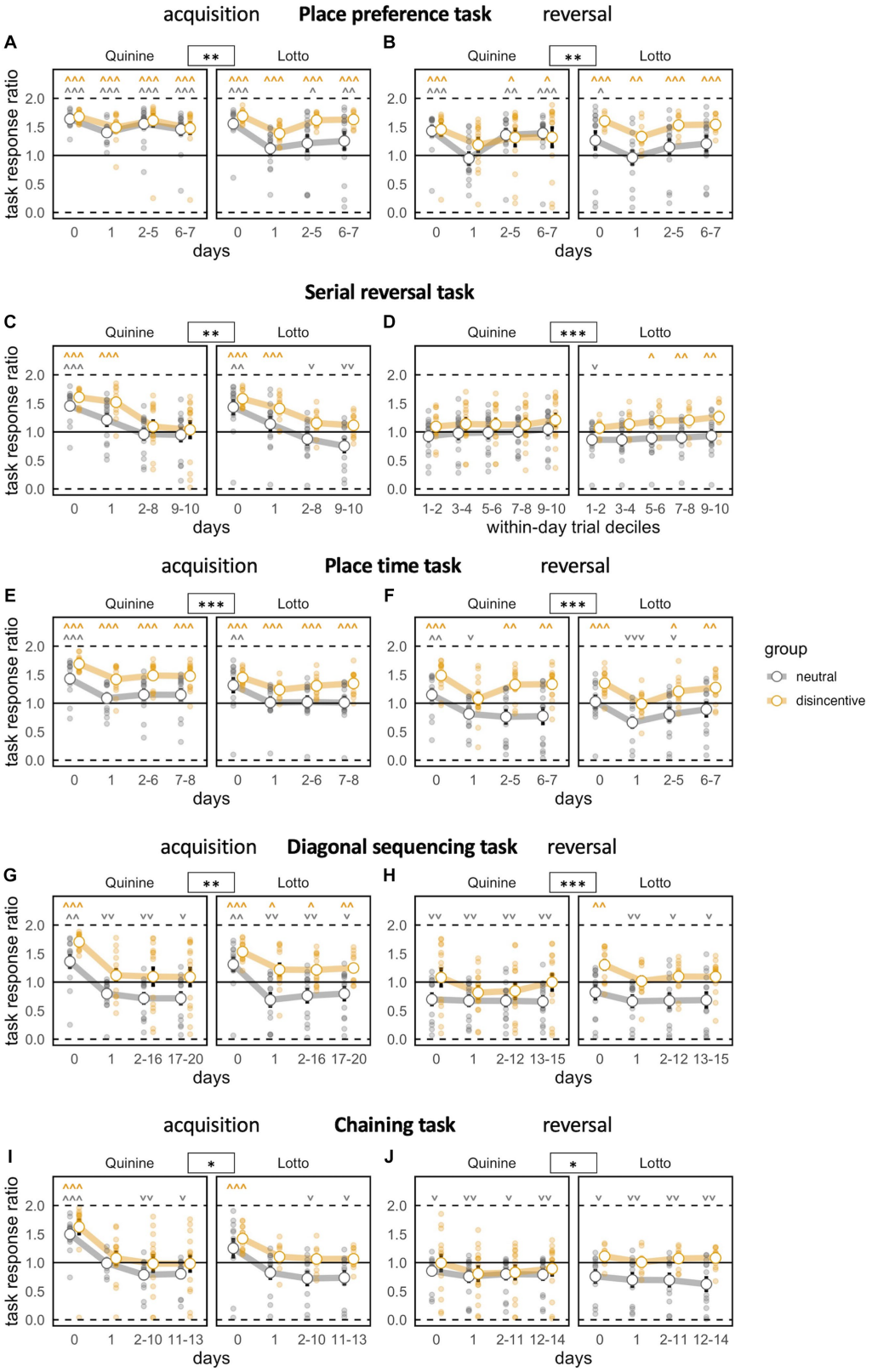

FIGURE 4
Task motivation across learning phases. Zero indicates total preference for joker door, 1 means no door preference, 2 means total preference for task door. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate group effects. ˅^, ˅˅ ^^, ˅˅ ^^^ stand for significant deviation from chance level according to one-sample t-tests. Day 0 indicates last day of previous NPA for acquisition **(A,C,E,G,I)**, last day of acquisition phase for reversal **(B,D,F,H,J)**. **(A)** Preference to respond at task doors decreases transiently as task begins. Controls, especially of the Lotto experiment, show weaker preference to respond at task doors [time bin: $F(3,144) = 45.21$, $p < 0.0001$, $\omega^2 = 0.25$, group: $F(1,48) = 9.446$, $p = 0.0035$, $\omega^2 = 0.14$, group × time bin $F(3,144) = 0.7165$ ns, group ×

(Continued)

FIGURE 4 (Continued)

experiment: $F(1,48) = 3.566$, $p = 0.0650$, $\omega^2 = 0.05$, group × experiment × time bin: $F(3,144) = 0.7024$ ns]. **(B)** Preference to respond at task doors decreases transiently as target corner changes. Controls, especially of the Lotto experiment, show weaker preference to respond at task doors [time bin: $F(3,141) = 39.05$, $p < 0.0001$, $\omega^2 = 0.16$, group: $F(1,47) = 5.789$, $p = 0.0201$, $\omega^2 = 0.09$, group × time bin: $F(3,141) = 0.4827$ ns, group × experiment: $F(1,48) = 3.566$, $p = 0.0650$, $\omega^2 = 0.05$, group × experiment: × time bin: $F(3,141) = 1.548$ ns]. **(C)** Task response ratio decreases persistently, to levels slightly above chance in disincentive groups, to levels slightly below chance in controls [time bin: $F(3,138) = 82.57$, $p < 0.0001$, $\omega^2 = 0.43$, group: $F(1,46) = 11.46$, $p = 0.0015$, $\omega^2 = 0.18$, group × time bin: $F(3,138) = 1.069$ ns, group × experiment: $F(1,46) = 0.0008$ ns, group × experiment × time bin: $F(3,138) = 0.5364$ ns]. **(D)** Progressive increase in motivation in serial reversal task when analyzed by block bins, which correspond to deciles of numbers of trials within a day/task. Only the disincentive group of the Lotto experiment develops a significant preference to respond at task doors [block bin: $F(1,220) = 102.8$, $p < 0.0001$, $\omega^2 = 0.03$, group: $F(1,46) = 7.317$, $p = 0.0095$, $\omega^2 = 0.12$, group × block bin: $F(1,220) = 8.082$, $p = 0.0049$, $\omega^2 = 0.00$, group × experiment: $F(1,46) = 0.1098$ ns, group × experiment × block bin: $F(1,220) = 5.600$ $p = 0.0188$ $\omega^2 = 0.00$]. **(E)** Task response ratio decreases persistently as task begins. Only disincentive groups maintain a significant preference to respond at task doors [time bin: $F(3,138) = 63.50$, $p < 0.0001$, $\omega^2 = 0.25$, group: $F(1,46) = 25.28$, $p < 0.0001$, $\omega^2 = 0.34$, group × time bin: $F(3,138) = 1.685$ ns, group × experiment: $F(1,46) = 1.439$ ns, group × experiment × time bin: $F(3,138) = 0.9273$ ns]. **(F)** Task response ratio drops further as the rule is reversed, followed by partial recovery. While disincentive groups reestablish preferential responding at task doors, controls transiently prefer to respond at joker doors [time bin: $F(3,138) = 34.97$, $p < 0.0001$, $\omega^2 = 0.19$, group: $F(1,46) = 32.07$, $p < 0.0001$, $\omega^2 = 0.39$, group × time bin: $F(3,138) = 2.420$, $p = 0.0688$, $\omega^2 = 0.01$, group × experiment: $F(1,46) = 0.4442$ ns, group × experiment × time bin: $F(3,138) = 0.1100$ ns]. **(G)** Task response ratio markedly and persistently decreases as task begins. Controls prefer to respond at joker doors throughout the task, while controls slightly favor responding at task doors [time bin: $F(3,138) = 72.26$, $p < 0.0001$, $\omega^2 = 0.31$, group: $F(1,46) = 21.02$, $p < 0.0001$, $\omega^2 = 0.29$, group × time bin: $F(3,138) = 1.134$ ns, group × experiment: $F(1,46) = 1.108$ ns, group × experiment × time bin: $F(3,138) = 0.9775$ ns]. **(H)** Task response ratio decreases further as the task rule is reversed. Controls consistently prefer to respond at joker doors, while controls respond near chance level [time bin: $F(3,138) = 6.490$, $p = 0.0004$, $\omega^2 = 0.03$, group: $F(1,46) = 12.68$, $p = 0.0009$, $\omega^2 = 0.20$, group × time bin: $F(3,138) = 1.786$ ns, group × experiment: $F(1,46) = 1.585$ ns, group × experiment × time bin: $F(3,138) = 1.023$ ns]. **(I)** Task response ratio strongly decreases as task begins, without recovery. Controls prefer to respond at joker doors, while controls respond near chance level [time bin: $F(3,132) = 102.7$, $p < 0.0001$, $\omega^2 = 0.33$, group: $F(1,44) = 5.754$, $p = 0.0208$, $\omega^2 = 0.09$, group × time bin $F(3,132) = 1.133$ ns, group × experiment: $F(1,44) = 0.2809$ ns, group × experiment × time bin: $F(3,132) = 0.3633$ ns]. **(J)** Motivation further decreases and shows a similar pattern as in acquisition [time bin: $F(3,132) = 9.649$, $p < 0.0001$, $\omega^2 = 0.02$, group: $F(1,44) = 6.945$, $p = 0.0116$, $\omega^2 = 0.11$, group × time bin: $F(3,132) = 2.637$, $p = 0.0523$, $\omega^2 = 0.00$, group × experiment: $F(1,44) = 0.2784$ ns, group × experiment × time bin: $F(3,132) = 1.153$ ns].

poke adaptation interludes. The latter would provide a sensation of novelty once learning resumes and could condition the mice to strictly associate sweet rewards with task completion.

A potential limitation of our study lies in the fact that we exclusively tested female animals. In a previous experiment (Nigri et al. submitted to the same special issue of Frontiers in Behavioral Neuroscience), we observed that females show a more robust motivation to learn reward-learning tasks (see also Chen et al., 2021, for sex differences in reward learning) and thus, it remains to be shown whether our protocol sufficiently motivates males. If learning is unsatisfactory in this case, the paradigm could be further escalated by combining the two disincentives, simultaneously decreasing the chance for access to joker doors and replacing their bottle content with quinine solution.

Aside from potentially replacing traditional thirst-based IntelliCage learning protocols, adapted versions of our protocols could also be used to investigate specific effects of genetic modifications or pharmacological compounds on reward learning as compared to thirst-driven learning. For instance, many neurodegenerative diseases have specific impacts on the sensitivity to reward (see Perry and Kramer, 2015, for a review on the topic). Combined protocols could help to describe deficient behavioral phenotypes more specifically. Perhaps, some mouse models might also show impairments in reward learning experiments that would have remained masked under the binary task rules of conventional protocols.

We found no consistent differences in efficacy between the two disincentives. However, both have some advantages and disadvantages. The Lotto approach allows co-housing of both groups, which eliminates the possibility of cage effects. During the nose poke adaptation phases, the emergence of such a cage effect in of the quinine IntelliCages complicated analysis to a certain degree. On the other hand, using quinine as the disincentive limits the paradigm to taste preference alone, which facilitates interpretation on a neurological level. Meanwhile, the Lotto group was exposed to a combination of an attractive taste stimulus and a disincentive, which could best be described as an unattractive low reward probability/low reward value gambling task (Pittaras et al., 2020). This complicates the underlying cognitive mechanisms and, consequently, the interpretation of findings from such an experiment series.

In conclusion, we demonstrated that a combined approach of positive and negative drivers can be used to provide motivation even for complex learning tasks, which stands in contrast to the more rapidly waning effect of positive/appetitive motivation alone. Importantly, the introduction of the disincentives did not lead to a reduction in the number of licks and thus did not expose animals to the risk of dehydration. The protocols we described can be used to replace conventional spatial learning tasks that rely on drinking restrictions, improving animal welfare. However, our study also highlights the limitations of this approach. Even with the improved paradigm presented here, our results suggest a somewhat weaker learning performance than seen in conventional approaches. Because of this, more research in this direction is needed to further exploit the vast possibilities of modified IntelliCage protocols in the service of animal welfare.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Cantonal Veterinary Office of Zurich (License No. 060/2021). The study was conducted

in accordance with the local legislation and institutional requirements.

Author contributions

DW: design and concept of the study and statistical analysis. XM, BS, and IA: mouse behavioral phenotyping. DW, AS, and IA: local support and coordination with planning, protocols, equipment and animal orders. XM, BS, AS, IA, MN, GB, and DW: discussing the data. AS: writing the manuscript and preparing the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DW was involved in the development of the IntelliCage system.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2023.1232546/full#supplementary-material

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Appetitively motivated tasks in the IntelliCage reveal a higher motivational cost of spatial learning in male than female mice

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The IntelliCage (IC) permits the assessment of the behavior and learning abilities of mice in a social home cage context. To overcome water deprivation as an aversive driver of learning, we developed protocols in which spatial learning is motivated appetitively by the preference of mice for sweetened over plain water. While plain water is available at all times, only correct task responses give access to sweetened water rewards. Under these conditions, C57BL/6J mice successfully mastered a corner preference task with the reversal and also learned a more difficult time-place task with reversal. However, the rate of responding to sweetened water decreased strongly with increasing task difficulty, indicating that learning challenges and reduced success in obtaining rewards decreased the motivation of the animals to seek sweetened water. While C57BL/6J mice of both sexes showed similar initial taste preferences and learned similarly well in simple learning tasks, the rate of responding to sweetened water and performance dropped more rapidly in male than in female mice in response to increasing learning challenges. Taken together, our data indicate that male mice can have a disadvantage relative to females in mastering difficult, appetitively motivated learning tasks, likely due to sex differences in value-based decision-making.

KEYWORDS

IntelliCage automated system, appetitive learning, 3R refinement, C57BL/6J mice, sex differences, animal welfare

Introduction

The behavioral characterization of wild-type and genetically modified mouse strains has become a powerful tool for investigating the molecular basis of normal brain functions (van der Staay et al., 2009; Voikar, 2020) and dysfunctions (Moore et al., 2021; Brilkova et al., 2022; Shcherbakov et al., 2022). In this context, most of the experimental studies conducted with rodents have traditionally used male subjects, very rarely offering adequate comparisons between males and females. This is likely associated with the assumption that females might display a larger variability due to the estrous cycle (Prendergast et al., 2014; Shansky, 2019). However, differences between the sexes have been documented at every level of neuroscience, from single neurons in cell culture to systems-level processes measured by neuroimaging (Andreano and Cahill, 2009; Arnold, 2010; Clayton and Collins, 2014; Meyer et al., 2017). The

claim that these neurobiological sex differences extend to the behavioral level has typically been more controversial. Given that free-living male *Mus musculus* have larger territories and venture farther away than females (Pocock et al., 2004, 2005), one would predict sex differences in spatial learning and exploratory behavior in laboratory tests. However, while literature reports indicate large and reliable male advantages for rats in radial-maze and water-maze protocols (Jonasson, 2005), experimental findings have remained contradictory in laboratory mice (Frick et al., 1999; Võikar et al., 2001; Hendershott et al., 2016). For example, evidence suggests differential performance by male and female mice in spatial navigation tasks (Kundey et al., 2019) and object recognition tasks (Frick and Gresack, 2003). In line with these observations, experimental studies reported poorer performance in the water maze combined with increased serum corticosterone levels in females (Beiko et al., 2004). In contrast, the equivalent performance of female and male C57BL/6J mice in the open field and water-maze task have been reported in previous studies (Fritz et al., 2017). Sex differences can also emerge in decision-making where an animal is given a choice between an option that provides a smaller but guaranteed gain and an option that provides a larger gain but also could provide a loss. In humans, it is well-established that men tend to be more risk-seeking than women in a wide domain of decision-making (Fornwagner et al., 2022), gambling (Raylu and Oei, 2002; van den Bos et al., 2013a), and financial risk-taking (Dwyer et al., 2002; Eckel and Grossman, 2002; Charness and Gneezy, 2012). In contrast, studies on non-human animals, including common laboratory mice, have been limited in their conclusions.

Along with the widespread use of the behavioral phenotyping approach, a large variety of rodent behavioral tests has been established to evaluate various forms of cognitive functions (Morris, 1981; Pellow et al., 1985; Vorhees and Williams, 2006; Hånell and Marklund, 2014). Despite their efficacy, classical tests still must cope with a few limitations. In fact, traditional behavioral tests typically involve social isolation, sensory deprivation, exposure to unfamiliar apparatus with very short observation time, and repeated handling by humans. The resulting stress responses introduce artifacts and reduce test reliability (Crabbe et al., 1999; Chesler et al., 2002; Deacon, 2006; Endo et al., 2011; Voikar and Gaburro, 2020). In addition, an anxiety-inducing experimenter effect is always present (Nigri et al., 2022). These shortcomings have, therefore, created an urgent need to develop new, more efficient approaches to behavioral phenotyping of mice. Therefore, a number of computer-assisted technologies for automatically capturing rodent behavior in the home cage over long periods of time have been developed (Gerlai, 2002; Spruijt and Devisser, 2006; Goulding et al., 2008; Endo et al., 2011; Kahnau et al., 2023). Among them, the IntelliCage (IC) is a unique approach because the system is specifically designed for the cognitive assessment of group-housed mice. Advantages of such automated testing in the home cage compared to manual assessments include continuous monitoring, observation in a familiar environment, and examination of combinations of behaviors rather than single behaviors (Richter, 2020; d'Isa and Gerlai, 2023). Moreover, experimental paradigms and protocols can be freely programmed and executed with this system, thus allowing maximum flexibility in the experimental design. The automated generation and collection of data by standardized procedures allow for high data comparability and reproducibility among different laboratories. Additionally, the apparatus also minimizes the need for the experimenter's handling, thus reducing the artifacts that interfere with the activities of the mice.

Even though the IntelliCage system offers the mentioned advantages, thirst remains the driver of learning and only correct responses grant access to drinking water in typical IntelliCage learning tasks. Thus, poor learning or insisting on wrong response patterns may result in water deprivation, which negatively impacts animal welfare. To refine the approach in accordance with the 3R principles (replace, reduce, and refine), we designed IntelliCage learning tasks in which successful learning gives access to a sweet reward while plain water is constantly available. In a previous study, we were able to show that this purely appetitive motivation is sufficient to drive the learning of female mice in simple IntelliCage tasks but fails in more complex hippocampus-dependent tasks (Bramati et al., 2023). This was achieved by exploiting the known preference for saccharin of C57BL/6J mice (Bachmanov et al., 2001). In the present study, we sought to determine whether using access to a saccharin reward as a sole and purely appetitive learning incentive could also be used to motivate male mice to learn simple IntelliCage tasks and whether they would lose interest in learning at a similar turning point as female mice if task difficulty is increased.

In the above-mentioned previous study (Bramati et al., 2023), the mice had the option to first respond to saccharin and switch to plain water during the same visit as a backup after not being rewarded with saccharin in an incorrect corner. The second aim of the present study was to test whether this option of double choices could have contributed to their rapid decline in performance as learning tasks became more difficult. To this end, we introduced a modified protocol enforcing an exclusive choice of either plain water or sweet water reward during each visit and compared it with the standard protocol used in the previous study. C57BL/6J mice, the most commonly used inbred strain in behavioral genetics, were deliberately chosen in both studies. For many behavioral domains, they are considered to display a medium-level phenotype (Crawley et al., 1997), which allows a feasible detection of upward and downward behavioral changes at the baseline and in response to various manipulations (Stiedl et al., 1999; Cabib et al., 2000; Võikar et al., 2005).

Materials and methods

Animals and environment

All the animal experiments were carried out at the Institute of Anatomy, University of Zurich, in accordance with the European legislation (Directive 2010/63/EU) and have been approved by the veterinary office of the Canton of Zurich (License number 060/2021).

Male and female C57BL/6J mice were bred at the Institute of Anatomy housing facility. Animals ($N = 29$, $F = 16$, $M = 13$) were weaned at 21 days and kept in the same-sex groups in standard Type III cages (temperature 21.9 ± 0.3°C and relative humidity 60.2 ± 9.6%) under a 12/12 inverted light-dark cycle (light on 20:00–08:00) for an adaptation period. A maximum of two pups per sex per litter were group-housed to avoid litter effects. Food and water were provided *ad libitum*. The radio frequency identification (RFID) transponders (Planet ID GmbH, Essen, Germany), (Zeldovich, 2016) were injected subcutaneously in the dorso-cervical region under isoflurane inhalation anesthesia 1 week before the behavioral testing. At the age of 8 months, C57BL/6J mice were randomly assigned to two experimental groups, the inclusive choice ($N = 15$, $M = 7$, $F = 8$) and the exclusive choice ($N = 14$, $M = 6$, $F = 8$), and introduced to the

IntelliCage apparatus. While the IntelliCage 1 accommodated 5 male mice (inclusive choice = 3, exclusive choice = 2), the IntelliCage 2 accommodated 8 males (inclusive choice = 4, exclusive choice = 4). The IntelliCages 3 and 4 accommodated 8 female mice each (inclusive choice = 4, exclusive choice = 4). In line with the 3Rs principles, we adopted recommendations to prevent aggression between the group-housed male mice, aiming to avoid fighting episodes and improve animal welfare. To facilitate species-specific behaviors reducing the prevalence of aggression (Van Loo et al., 2001), we provided environmental enrichment by increasing cage complexity. In particular, we provided transparent tubes (diameter: 4 cm; length: 15 cm) connecting each IntelliCage with a freely accessible extension cage (Figure 1A) (425 × 266 × 155 mm). As spot cleaning when needed, rather than a weekly full cage change, is associated with a lower prevalence of aggression (Lidster et al., 2019), we cleaned either the IntelliCage or the extension cage per time every 10 days, also retaining some clean and dry nesting material and transferring them during cage changes. Additionally, we consistently monitored the animals by behavioral observations (fighting, chasing, mounting, and submissive behavior) and physical evidence (tail wounds, rump and back wounds/hair loss, and urogenital wounds). Adopting the above-mentioned recommendations lets us avoid fighting episodes that could have interfered with the acquisition of behavioral data.

Behavioral procedures

The IntelliCage system

Behavioral testing was conducted in the IntelliCage system (TSE Systems, Bad Homburg, Germany), which is a fully automated cage system designed for the assessment of cognitive abilities in group-housed small rodents (Lipp, 2005; Kiryk et al., 2020; Lipp et al., 2024). The apparatus (Figure 1A) consists of a polycarbonate cage (20.5 cm high, 58 × 40 cm top, 55 × 37.5 cm bottom, Techniplast, 2000P, Buguggiate, Italy) equipped with four triangular operant test chambers (15 × 15 × 21 cm) fitted into each corner. Each chamber contains two drinking bottles, accessible via two round openings that can be opened and closed with motorized doors. Mice that access a chamber are identified by a circular RFID antenna at its entrance, and the duration of their visit is determined by both the antenna reading and a temperature sensor that detects the presence of the animal inside the corner. During a visit, the number and duration of individual nosepokes at each door are recorded using infrared (IR)-beam sensors. Licking episodes at each bottle are monitored using lickometers. Additionally, an extension cage was connected to each IntelliCage via a tube, and behavioral experiments started simultaneously for all animals by opening the connecting tubes. The system has individual controllers, and they are connected to a central PC running the software that permits the design and control of experiments remotely and the analysis of the recorded data (IntelliCage Plus, TSE Systems, Bad Homburg, Germany).

Design of the novel appetitively motivated protocols in the IntelliCage

To promote appetitive learning by exploiting the strong preference of C57BL/6J mice for saccharin over plain water (Bachmanov et al., 2001), we developed novel protocols based on the possibility of choosing between saccharin and plain water (Figure 1B). For each corner, one side provided a bottle of plain water (joker side), while the other side had a bottle of sweetened water containing 0.5% saccharin (task side). The joker door opened automatically for 3 seconds at the beginning of any visit during every protocol, while the task door opened for 3 seconds only in response to a nosepoke in a correct corner. Thus, while water was available for free at the joker sides, the mice had to acquire and follow the rules of the respective learning task to obtain sweet rewards at the task sides. To avoid spontaneous bias to respond at task or joker side, the sweetened water bottles were placed on the left side in two corners and on the right side in the two other corners. Mice were assigned to two experimental groups: the *exclusive choice group* ($M = 6$, $F = 8$) and the *inclusive choice control group* ($M = 7$, $F = 8$). A first poke at the joker side prevented the opening of the task door during the same visit in the *exclusive choice group*. Similarly, a first poke at the task side immediately triggered the closing of the joker door, thereby shortening the availability of water. Instead, the task and joker doors operate independently, allowing successful responses on both sides during the same visit in the *inclusive choice control group*. The *exclusive choice* protocol was designed to test whether not having the possibility to choose both saccharin and water as a backup would increase the motivation of the mice to learn to access saccharin.

Adaptation phases

Free adaptation: (FA 10, 8 days): Animals were first habituated for 10 days in the IntelliCage environment in a free adaptation stage with all doors open and free access to plain water at any time. During the following 8 days, doors remained constantly open, and each corner provided both a bottle of plain water and a bottle containing 0.5% saccharin solution. This let the mice learn where the water and saccharin were available.

Nosepoke adaptation (NPA, 8 days): All doors were closed by default. The doors hiding plain water opened at the beginning of any visit for 3 seconds. The doors hiding the 0.5% saccharin solution could be opened with a nosepoke once per visit, with time to drink limited to 3 seconds.

Learning tasks

Corner preference acquisition (PPRA1, 7 days; PPRA2, 9 days) and reversal learning (PPRR, 6 days): for acquisition training, each mouse was assigned to one correct corner based on its corner preference during NPA (either the second- or third-favorite corner was assigned with a balanced distribution). All doors were closed by default. The doors hiding plain water opened at the beginning of every visit in every corner, while the doors hiding saccharin opened for 3 seconds once per visit only in response to a nosepoke in the correct corner. After cleaning the cages, acquisition training was continued without changing the correct corners (PPRA2). The correct corner was moved to the opposite corner for each mouse in the reversal phase, with conditions for the joker sides remaining the same.

Place time acquisition (PPTA, 11 days) and reversal learning (PPTR, 7 days): as for corner preference acquisition, each mouse is assigned to an initial correct corner with the other corners being incorrect. But the correct corner changed position every 12 hours, moving to the right at 14:00 every day and back to the original position at 02:00. Correct and incorrect corners operated in the same way as during the corner preference task. In the reversal phase, mice had access to the saccharin solution in the corners diagonally opposite the ones assigned in the acquisition stage.

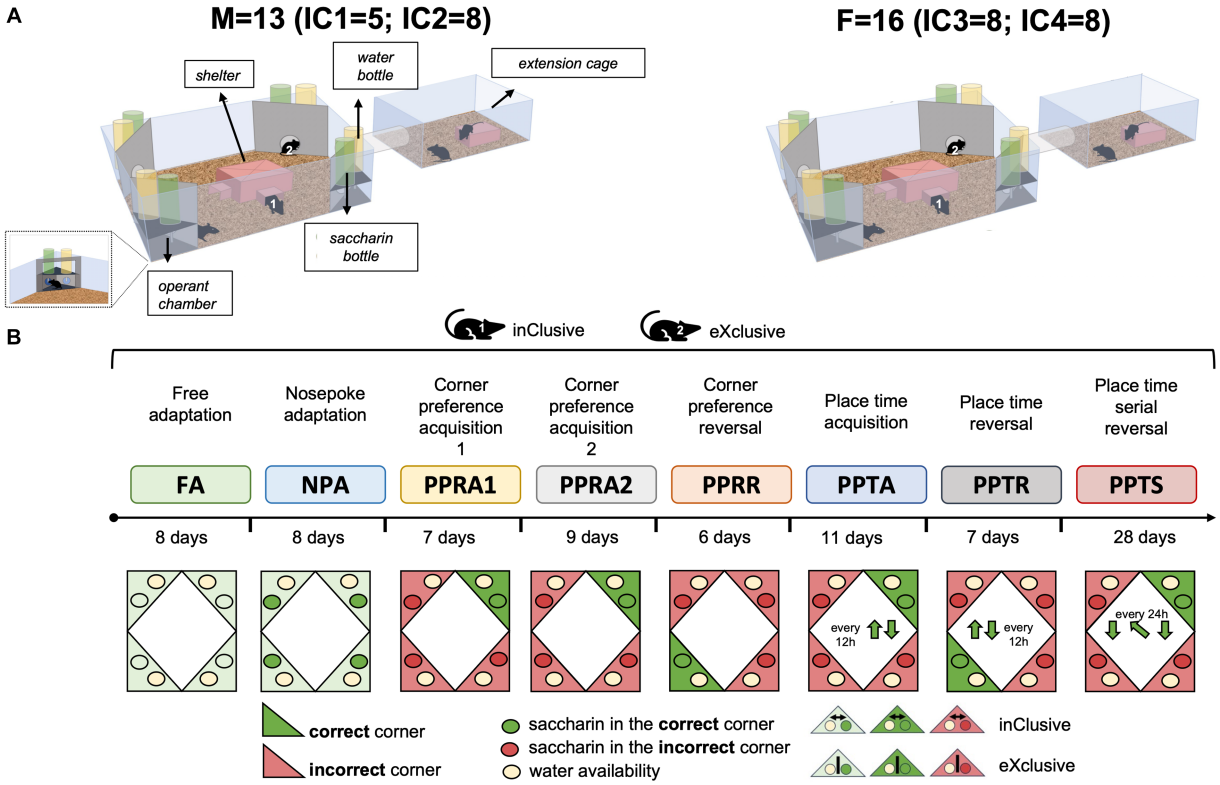

FIGURE 1
Apparatus and task paradigm. **(A)** Overview of the IntelliCage apparatus. C57BL/6J males ($N = 13$) and females ($N = 16$), assigned to two experimental groups, the exclusive choice group ($F = 8$, $M = 6$) and inclusive choice control group ($F = 8$, $M = 7$), were group-housed in the IntelliCage system (IC1, IC2, IC3, and IC4). While the IntelliCage 1 accommodated 5 male mice (inclusive choice = 3, exclusive choice = 2), the IntelliCage 2 accommodated 8 males (inclusive choice = 4, exclusive choice = 4). The IntelliCages 3 and 4 accommodated 8 female mice each (inclusive choice = 4, exclusive choice = 4). Their behavioral response, corner visits, nosepokes, and licks were monitored in a fully automated manner during the experimental tasks. **(B)** Diagram of behavioral test sequence based on appetitive learning.

Place time serial reversal (PPTS, 28 days): The protocol consisted of seven alternations between place time acquisition and reversal, each lasting 4 days, starting and ending with an acquisition.

Experimental parameters

Post-processing steps were applied to obtain composite variables from the IntelliCage system's output file (Ma et al., 2023). They include task responses defined as visits to a corner with at least one nosepoke on the task door, and joker responses defined as visits with at least one nosepoke on the joker door and hits stratified into joker and task hits. In this context, we calculated the task response ratio R, as indicated below.

$$R = \frac{2 + 2^{*}\text{Task responses}}{2 + \text{Joker responses} + \text{Task responses}}$$

This value tends to 0 after many responses exclusively on the joker side and to 2 after a large number of responses exclusively on the task side. A value of 1 indicates the absence of a door preference. We calculated the false rate, which is defined as the percentage of task responses in incorrect corners as a measure of learning and task performance. In the absence of a learning effect, this value is expected to be around 75%, with a significant reduction indicating successful learning of the task rule.

Statistical analysis

Behavioral data were extracted with the IntelliCage Analyzer software and further processed using Excel. The statistical analysis was conducted using a linear model with sex (male and female) and choice group (inclusive and exclusive) as between-subject factors. Within-subject factors were added as needed to explore the dependence of behavior on time or corner side. Significant interactions were explored by splitting the model. Significant effects of time were further explored using partial models. Variables with strongly skewed distributions or strong correlations between variances and group means were subjected to Box–Cox transformation before statistical analysis, as indicated in figure legends. The significance threshold was set at 0.05. The false discovery rate (FDR) control procedure of Hochberg was applied to groups of conceptually related variables within single tests to correct significance thresholds for multiple comparisons. Similarly, FDR correction was applied during post-hoc testing. One-sample t-tests were used to compare values against chance levels. The statistical analyses and graphs were obtained using R version 4.3.0,

complemented with the package ggplot2. In line graphs, untransformed data are plotted as mean + SEM with individual data points in the background.

Results

Male mice showed a stronger preference for responding exclusively at the saccharin sides during the free adaptation stage

With the free adaptation stage, we aimed to let the mice explore the new environment, learning where the water and saccharin were available. During the pre-task baseline, when all bottles contained water, there was no spontaneous bias to respond at task or joker sides (responses = visits with at least one nosepoke). Overall, mice switched to preferential responding at task sides instantly upon introducing saccharin, males more strongly than females (Figure 2A). They overall preferred to respond exclusively at the saccharin side, while visits with a response to the water side were below the chance level (Figure 2B). In this context, male mice more strongly avoided responding at both sides than female mice and showed a stronger preference for responding exclusively at the task side (Figure 2B). In line with these observations, the drinking preference overall changed rapidly upon the introduction of saccharin. The mice eventually almost exclusively consumed saccharin without evidence of a sex effect (Figure 2C). In accordance, the lick frequency increased strongly and instantly in response to the introduction of saccharin at the task sides without evidence of a sex effect (Supplementary Figure S1).

The exclusive choice group responded more exclusively for saccharin during the nosepoke adaptation stage, confirming the functioning of the learning protocols

Following the free adaptation stage, door operation was activated at the task and joker sides during the nosepoke adaptation stage. Overall, the percentage of responses with nosepokes overlapping with the accessibility of saccharin bottles, defined as task hits, dropped when door operation was activated and recovered rapidly to about 93% as mice adapted to the movement of doors (Figure 3A). Moreover, they dropped more strongly in the exclusive group and remained lower throughout the stage, reflecting unsuccessful attempts to drink saccharin after a first response at the joker side (Figure 3A). On the other hand, the percentage of responses with nosepokes overlapping with the accessibility of water bottles, defined as joker hits, overall dropped to 57% when the door operation was activated without evidence of recovery (Figure 3B). This happened since mice could not control the door with nosepokes and came too late when they poked first at the saccharin door and then at the water door. While the exclusive choice group and the control inclusive choice group showed a similar initial drop in the joker hit rate, the control group learned to switch faster to the joker side after a first response to the task side, as indicated by the diverging curves (Figure 3B). Confirming how the designed protocols worked as intended, exclusive hits (either task or joker) were more frequent in the exclusive choice group, with dual hits almost never occurring (Figure 3C). In line with this observation, the exclusive choice group more strongly avoided responding to both sides than the inclusive choice control group, showing a stronger preference for responding exclusively to the task side. Therefore, the exclusive choice group was more exclusive in its choice to respond to saccharin as intended with the designed protocols (Figure 3D). During the nosepoke adaptation stage, we also observed some sex effects, which were in line with our observations made during the free adaptation stage. While the hit rate of males dropped less strongly at task sides (Supplementary Figure S2A) when the new protocol was introduced, it showed a stronger transient drop than in females at joker sides (Supplementary Figure S2B). Sexes were similar with respect to the overall distribution of hit rates across sides during nosepoke adaptation (Supplementary Figure S2C), but as during free adaptation, males continued to respond more exclusively than females at task sides (Supplementary Figure S2D).

The presence of saccharin motivates mice to learn the corner preference acquisition and reversal tasks

The learning performance of C57BL/6J male and female mice was first addressed in the corner preference acquisition and reversal tasks. During corner preference acquisition 1 (PPRA1), place errors were slightly above chance during the pre-task baseline when all corners were still rewarded with saccharin and decreased robustly below chance, indicating the mice successfully learned the place rule (Figure 4A). While there was no evidence for an overall sex effect on performance, error numbers decreased somewhat more slowly in males than females (Figure 4A). The response task ratio decreased strongly at the beginning of the learning task and continued to decrease during the task, reaching near indifference at the end of training (Figure 4B). Corner preference acquisition 2 (PPRA2) continued with the same target corner as corner preference acquisition 1 (PPRA1) after cage cleaning. To note, there was no statistical evidence for an effect of cage change on place error rate, indicating that cage cleaning was not interfering with their performance (Figure 4C). In addition, there was no evidence of a sex effect on the overall learning performance (Figure 4C). In line with this observation, the response task ratio remained near indifference without evidence of a change over time. In addition, no evidence of a sex effect on the response task ratio was observed (Figure 4D). Looking at the reversal stage, place error rates decreased robustly and reached levels below chance, indicating that the mice learned the new rule. There was no evidence for a sex effect on learning performance (Figure 5A). To note, the mice overall shifted toward responding preferentially at the water sides, as suggested by the decreased response task ratio in the corner preference reversal stage (Figure 5B).

Mice learn the place time task, with males performing more poorly and preferentially responding at water sides compared to females

The learning performance of C57BL/6J male and female mice was then evaluated in the place time acquisition and reversal tasks. Overall, place errors decreased robustly, indicating that the mice learned the

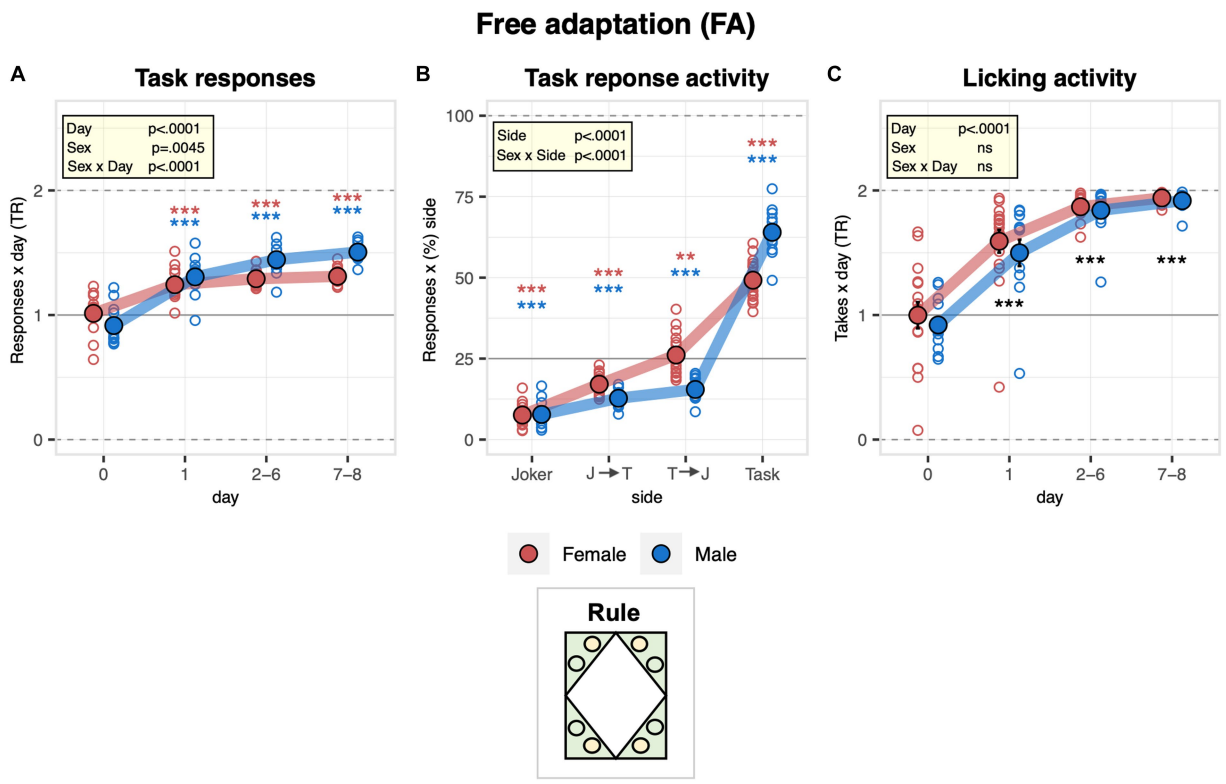

FIGURE 2
Activity of male and female mice during the free adaptation stage in the IntelliCage. During the free adaptation stage (8 days), saccharin was introduced to task sides for the first time while doors always remained open. During day 0, that indicates the last day of the previous stage (pre-task baseline, all bottles still contained plain water). One-sample *t*-tests against chance (solid horizontal line): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ referring to the comparison of pooled groups against chance. One-sample *t*-test results are shown for groups separately in red and blue when a significant interaction with sex is present. **(A)** Response task ratio plotted as a function of day. Ratio defined as (2 + 2 × Task)/(2 + Joker + Task), chance = 1, range = 0–2. Responses defined as visits with poke(s) at task and joker sides, respectively (ANOVA: day $F_{3,72} = 103.7$ $p < 0.0001$ $\omega^2 = 0.70$, sex × day $F_{3,72} = 12.28$ $p < 0.0001$ $\omega^2 = 0.21$, Box–Cox λ 1.50). Response task ratio increased more strongly in male mice and stabilized at a level of about 1.4. **(B)** Percentage of responses plotted as a function of side sequence during the visit (joker side only, joker side followed by task side, task side followed by joker side, task side only) and averaged across the entire stage. All except joker side only is counted as a task response, all but task side only is considered a joker response (ANOVA: taste $F_{3,72} = 288.6$ $p < 0.0001$ $\omega^2 = 0.92$, sex × taste $F_{3,72} = 14.23$ $p < 0.0001$ $\omega^2 = 0.33$, Box–Cox λ 0.500). Overall, mice preferred to respond exclusively for saccharin while visits with a response plain water were below the chance level. In addition, males more strongly avoided responding to both sides than females and showed a stronger preference for responding exclusively to the task side. **(C)** Drinking task ratio plotted as a function of the day (ANOVA: day $F_{3,72} = 192.2$ $p < 0.0001$ $\omega^2 = 0.79$, Box–Cox λ 5.00). The ratio increased rapidly upon the introduction of saccharin to reach levels close to 2, indicating that the mice overall almost exclusively consumed saccharin.

place time acquisition rule, reaching a plateau on the second experimental day (Figure 6A). From the second day onward, males made more place errors (Figure 6A). Looking at the place time reversal stage, mice also learned the new place rule, as indicated by the robust decrease in the place error rates during the task (Figure 6B). In contrast, there was no improvement across repeated goal changes in the place time serial reversal task, indicating that the mice could not learn to adapt more efficiently to the changing pairs of target corners (Figure 6C). However, there was a robust decrease in place error rate within each task as mice adapted to the new corner pair (Figure 6D). During the place time task, choices to respond at water or saccharin doors showed a striking sex difference. Male mice switched to preferential responding for water at task onset, and their responding at task sides further decreased as the task progressed. By contrast, females still responded preferentially for saccharin at baseline and did not develop a preference for responding at water sides throughout the task (Figure 6E). Throughout the reversal stage, males preferred to respond to water. Females also shifted toward preferential responses for water, but clearly fewer than males (Figure 6F). In line with these observations, the preference of male mice to respond to water remained clearly stronger than that of females during the place time serial reversal stage (Figure 6G).

Preference to respond for saccharin is generally lost with the introduction of the first task

To examine the behavior of mice across the different experimental protocols, we analyzed their overall task activity throughout nosepoke adaptation stages before, between, and after learning tasks. At the transition from free to nosepoke adaptation, lick numbers dropped by about 50% and remained stable thereafter, with only very small decreases after learning stages without evidence for a consistent effect of sex on lick numbers (Supplementary Figure S3A). Moreover, no evidence for a choice group effect on the overall lick number was

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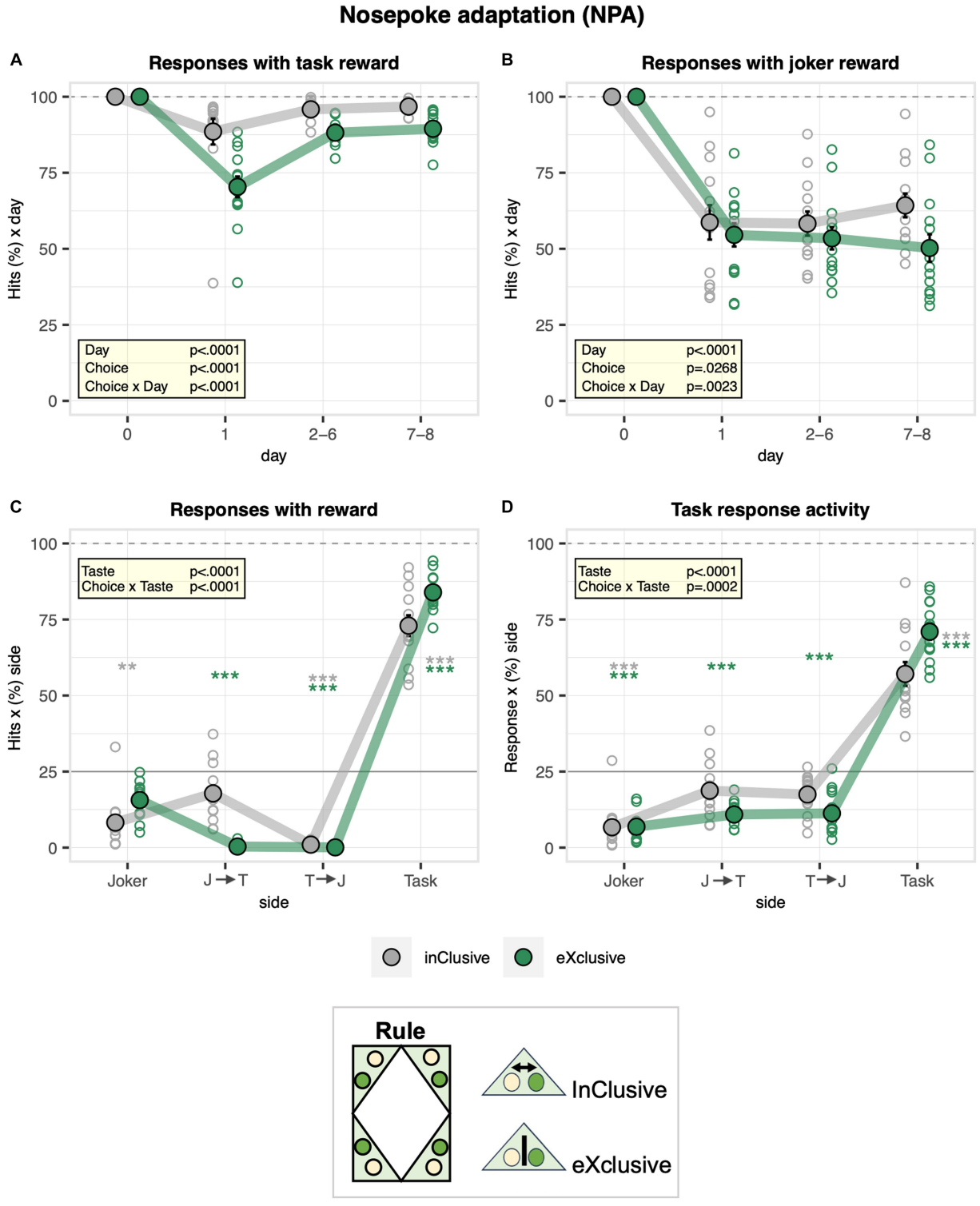

FIGURE 3
Activity of the inclusive and exclusive experimental groups during the nosepoke adaptation stage. One-sample *t*-tests are shown for groups separately in gray and green when a significant interaction with choice is present: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$. **(A)** Percentage of nosepokes overlapping with accessibility of saccharin bottles plotted as a function of the day (ANOVA: day $F_{3,69} = 143.9$ $p < 0.0001$ $\omega^2 = 0.77$, choice x day $F_{3,69} = 17.46$ $p < 0.0001$ $\omega^2 = 0.28$, Box–Cox λ 4.00). Overall, the percentage of responses with nosepokes overlapping with the accessibility of saccharin bottles dropped when door operation was activated. It dropped more strongly in the exclusive experimental group. **(B)** Percentage of responses with nosepokes overlapping with the accessibility of water bottles plotted as a function of the day (ANOVA: day $F_{3,69} = 57.08$ $p < 0.0001$ $\omega^2 = 0.58$, choice x day $F_{3,69} = 5.320$ $p = 0.0023$ $\omega^2 = 0.10$, Box–Cox λ 0.500). Overall, the percentage of nosepokes overlapping with the accessibility of water bottles dropped to 57% when the door operation was activated, without evidence of recovery. The inclusive, experimental group learned to switch faster to the joker side after a first response to the task side. **(C)** Percentage of responses with nosepokes overlapping with accessibility of water and saccharin reward plotted as function of task sequence during the visit (joker side only, joker side followed by task side, task side followed by joker side, and task side only) and averaged across the entire stage (ANOVA: choice x taste $F_{3,69} = 48.30$ $p < 0.0001$ $\omega^2 = 0.64$, Box–Cox λ 0.500). Exclusive nosepokes overlapping with accessibility

(Continued)

FIGURE 3 (Continued)

of both water and saccharin were more frequent in the exclusive choice group with dual hits almost never occurring. **(D)** Percentage of responses plotted as function of side sequence during the visit (joker side only, joker side followed by task side, task side followed by joker side, task side only) and averaged across the entire stage (ANOVA: choice × taste $F_{3,69} = 7.418$ $p = 0.0002$ $\omega^2 = 0.20$, Box–Cox λ 0.500). The exclusive choice group was more exclusive in its choice to respond for saccharin as intended with the designed protocols.

observed (Supplementary Figure S3B). Looking at the response numbers, they overall increased strongly at the transition from free to nosepoke adaptation to decrease again after the learning stages without statistical evidence for a sex effect (Supplementary Figure S3C). They were also similar in the two experimental groups (Supplementary Figure S3D). Looking specifically at the preference to respond to saccharin, it increased throughout the pre-learning stages but dropped after the learning stages without evidence for recovery during nosepoke adaptation interludes (Figure 7A). Moreover, it was slightly higher in males during the pre-learning stages but dropped more strongly after learning than in females (Figure 7A). In line with this observation, a stronger decrease in the preference for drinking saccharin after the learning stages was observed in males compared to females (Figure 7B). These data indicate how the motivation of males to respond to saccharin did not fully recover when saccharin became available in all corners again after spatial learning tasks. Looking at the two experimental groups, the preference to respond to saccharin was slightly higher in the exclusive choice group during pre-learning nosepoke adaptation, but the effect was lost after the learning stages (Figure 7C). No evidence for a choice group effect on the preference for drinking saccharin was detected (Figure 7D).

No evidence for better learning performance in the exclusive choice group

The learning performance of the two experimental groups was addressed in the corner preference acquisition/reversal tasks and place time acquisition/reversal/serial reversal tasks. There was no statistical evidence for improved performance of the exclusive choice group during corner preference acquisition 1 (Supplementary Figure S4A) or corner preference acquisition 2 (Supplementary Figure S4B). In line with this observation, no statistical evidence for an enhancing effect of the exclusive choice protocol on the preference to respond for saccharin was observed in corner preference 1 (Supplementary Figure S4C) or corner preference 2 (Supplementary Figure S4D) acquisition. During the reversal stage, the two experimental groups were similar in terms of both learning performance ($F_{1,23} = 0.0632$ ns, Supplementary Figure S4E) and preference to respond to saccharin (Supplementary Figure S4F). Moreover, there was no evidence for improved performance or learning rate in the exclusive choice group during the place time acquisition (Supplementary Figure S5A), reversal (Supplementary Figure S5B), and serial reversal (Supplementary Figures S5C, D) tasks. In line with this observation, the two experimental groups showed a similar preference to respond to saccharin in the acquisition (Supplementary Figure S5E), reversal (Supplementary Figure S5F), and serial reversal (Supplementary Figure S5G) stages.

Discussion

In the present study, we compared task engagement and learning performance of male and female C57BL/6J mice in the IntelliCage in a set of increasingly difficult appetitively motivated spatial learning tasks. In all tasks, successful learning gave access to a sweet reward, while plain water was freely available to prevent water deprivation in poor learners and to create a purely appetitive incentive for learning. In line with a previous study (Bramati et al., 2023), our results confirm that this purely appetitive incentive is sufficient to drive learning in simple but not in more demanding IntelliCage tasks. In addition, we observed that male mice, despite being attracted more strongly by the sweet reward when it was available for free, were less successful than females in engaging in learning to obtain access to sweet reward and performed more poorly in demanding IntelliCage tasks. Finally, we found that a modification of the protocol enforcing an exclusive choice of either plain water or sweet water reward failed to improve performance in female and male mice, even though it prevented the use of plain water as backup during incorrect responses.

Given the well-documented sex differences in both physiology and behavior, it is mandatory that both female and male subjects are tested to capture sex-dependent aspects of disease mechanisms and when mouse models are used for modeling a human population (Shansky, 2018). Thus, to be valid, cognitive tests must be applicable to subjects of both sexes. Given that the IntelliCage system is generally suitable for testing mice of both sexes (Kiryk et al., 2020; Lipp et al., 2024), we deemed it necessary to assess task performance in our appetitively motivated protocols for IntelliCage not only in female but also in male mice. The attractiveness of saccharin, the sweet reward used in our study, to male C57BL/6 mice is well-documented (Bachmanov et al., 2001). As expected, there was no evidence of a sex difference in the almost exclusive choice to drink saccharin solution when both saccharin solution and plain water were freely available during the adaptation stages of our experiment. Because saccharin consumption depends not only on motivation but also on learning success, we used response preference, which also includes nosepokes without licking, as a measure of the motivation to engage in learning tasks. The baseline preference of males for responding at saccharin sides during baseline conditions was even slightly stronger than in female mice. This confirmed that saccharin as a sweet reward was sufficiently attractive to mice of both sexes.

However, when we evaluated learning performance in the set of learning tasks, we observed that the performance of males deteriorated even more rapidly with increasing task difficulty than that of female mice. As already detailed in the introduction section, there is little evidence for a genuine disadvantage of male mice relative to females in learning spatial tasks, and it has been shown previously that male mice of various strains learn challenging spatial tasks well in IntelliCage if they are motivated by water deprivation (Endo et al.,

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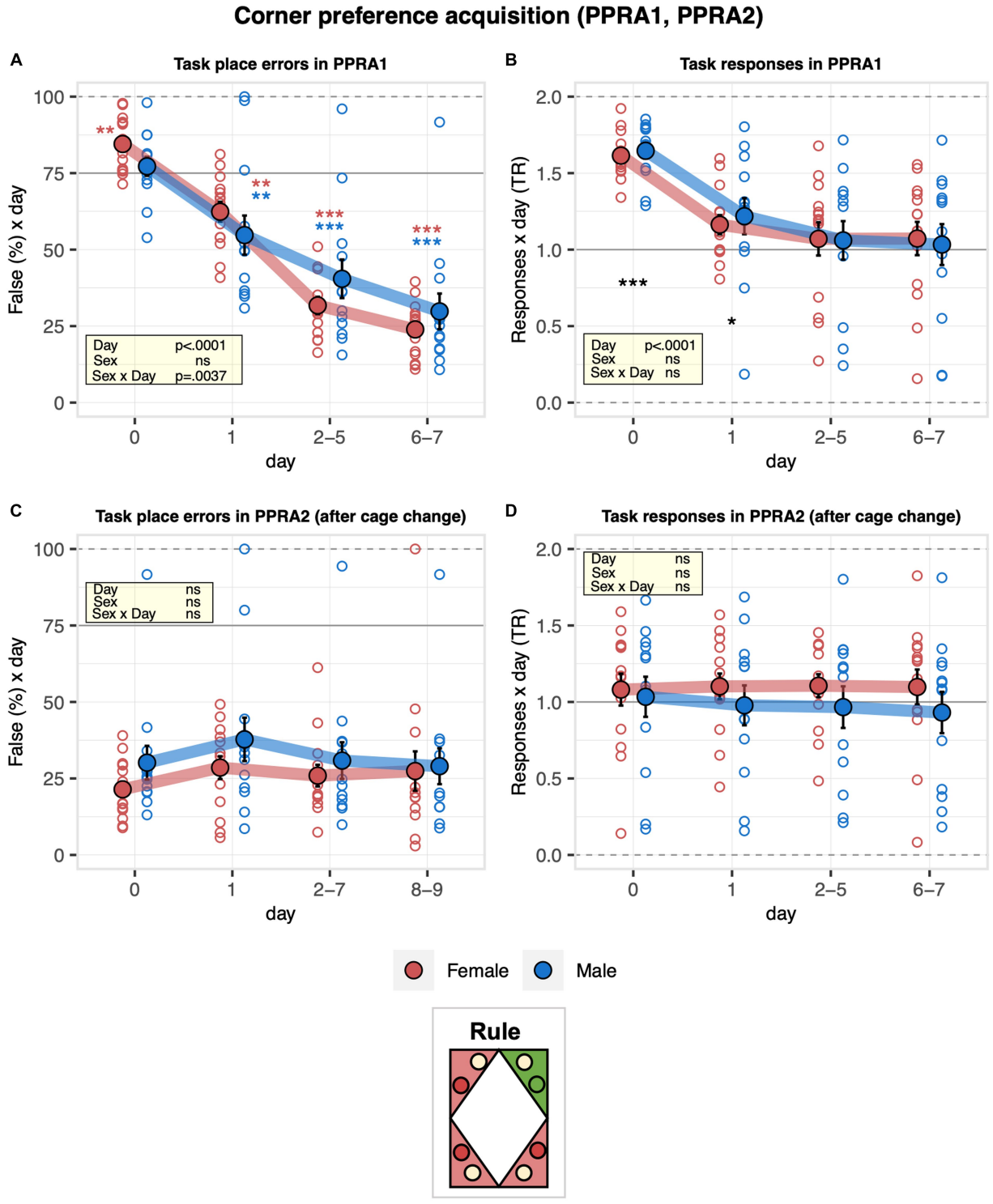

FIGURE 4
Learning performance of males and females in the corner preference acquisition stage. During corner preference acquisition 1 (PPRA1, 7 days), water was available at joker sides in all corners, but saccharin could only be obtained at the task side of a single target corner, which remained the same throughout the task. Corner preference acquisition 2 (PPRA2, 9 days) continued with the same target corner as in corner preference acquisition 1 after cleaning the cages. Percentage of place errors corresponds to task responses to incorrect corners plotted as a function of the day, with Day 0 corresponding to the last 2 days of nosepoke adaptation with saccharin still available in all corners. Response task ratio was plotted as a function of the day. Ratio defined as (2 + 2 × Task)/(2 + Joker + Task), chance = 1, range = 0–2. One-sample *t*-tests against chance (solid horizontal line): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ referring to the comparison of pooled groups against chance. One-sample *t*-test results are shown for groups separately in red and blue when a significant interaction with sex is present. **(A)** Percentage of place errors during PPRA1 (ANOVA: day $F_{3,69} = 157.6$ $p < 0.0001$ $\omega^2 = 0.66$, sex × day $F_{3,69} = 4.925$ $p = 0.0037$ $\omega^2 = 0.05$). Place errors were slightly above chance during pre-task baseline and decreased robustly below chance indicating the mice successfully learned the place rule. Error numbers decreased somewhat more slowly in males than females. **(B)** Response task ratio during PPRA1 (ANOVA: day $F_{3,69} = 68.12$ $p < 0.0001$ $\omega^2 = 0.41$, Box–Cox λ 3.00). Response task ratio decreased strongly at the beginning of the

(Continued)

FIGURE 4 (Continued)

learning task and continued to decrease during the task, reaching near indifference at the end of training. **(C)** Percentage of place errors during PPRA2 (ANOVA: day $F_{3,69} = 1.585$ ns, sex $F_{1,23} = 1.254$ ns, Box–Cox λ 0.500). There was no statistical evidence for an effect of cage cleaning on place error rate. In addition, there was no evidence of a sex effect on the overall learning performance. **(D)** Response task ratio during PPRA2 (ANOVA: day $F_{3,69} = 0.1422$ ns, sex $F_{1,23} = 0.4463$ ns, Box–Cox λ 2.00). Response task ratio remained near indifference without evidence of a change over time and without evidence of a sex effect.

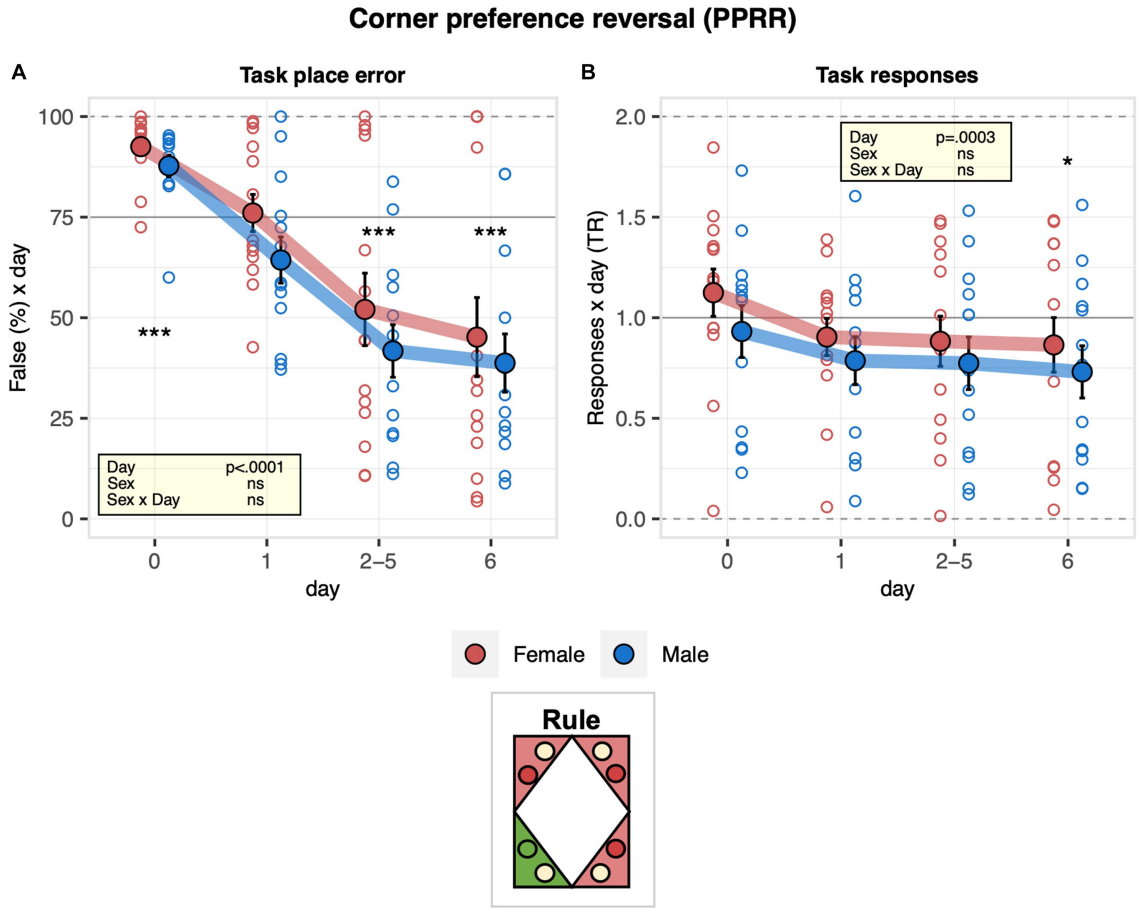

FIGURE 5
Learning performance of males and females in the corner preference reversal stage. During corner preference reversal (PPRR, 6 days), the target corner was opposite to the one used in corner preference acquisition 1 and 2 (PPRA1, PPRA2). One-sample *t*-tests against chance (solid horizontal line): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ referring to the comparison of pooled groups against chance. **(A)** Percentage of place errors corresponding to task responses to incorrect corners plotted as a function of the day with day 0 corresponding to the last 2 days of corner preference acquisition 2 (ANOVA: day $F_{3,69} = 50.62$ $p < 0.0001$ $\omega^2 = 0.40$ sex $F_{1,23} = 1.151$ ns). Place error rates decreased robustly and reached a level below chances, indicating how the mice learned the new rule without evidence for a sex effect on learning performance. **(B)** Response task ratio plotted as a function of the day, with day 0 corresponding to the last 2 days of corner preference acquisition 2. Ratio defined as (2 + 2 × Task)/(2 + Joker + Task), chance = 1, range = 0–2 (ANOVA: day $F_{3,69} = 7.027$ $p = 0.0003$ $\omega^2 = 0.04$). Mice overall started to respond preferentially at the water sides.

2011). But evidence is emerging that sex differences play a role in value-based decision-making (van den Bos et al., 2013b; Orsini and Setlow, 2017; Shansky, 2018; Grissom and Reyes, 2019; Chen et al., 2021; Cox et al., 2023). This is relevant because the spatial IntelliCage protocols evaluated in the present study rely fully on appetitive motivation, thereby eliminating the need to secure sufficient liquid intake in the interest of body homeostasis as a powerful driver of learning. As a consequence of this design and unlike in conventional IntelliCage tasks, value-based decision-making becomes the main driver of learning and adapting behavior to the changing location of reward. Particularly relevant to our specific setting are observations that motivation to engage in a task is modulated by action value more strongly in female than in male mice (Cox et al., 2023) and that male mice can be more prone than females to adhere to exploratory choice patterns in value-based decision-making tasks (Chen et al., 2021). Engaging in an exploratory response pattern across corners in our appetitively motivated spatial IntelliCage tasks reduces the success rate of responding for saccharin, and this may potentiate the impact of such sex differences on task motivation and learning performance. This interpretation is supported by our observation that task performance and preference to respond to saccharin decreased in parallel. The fact that male mice perform worse in some of the

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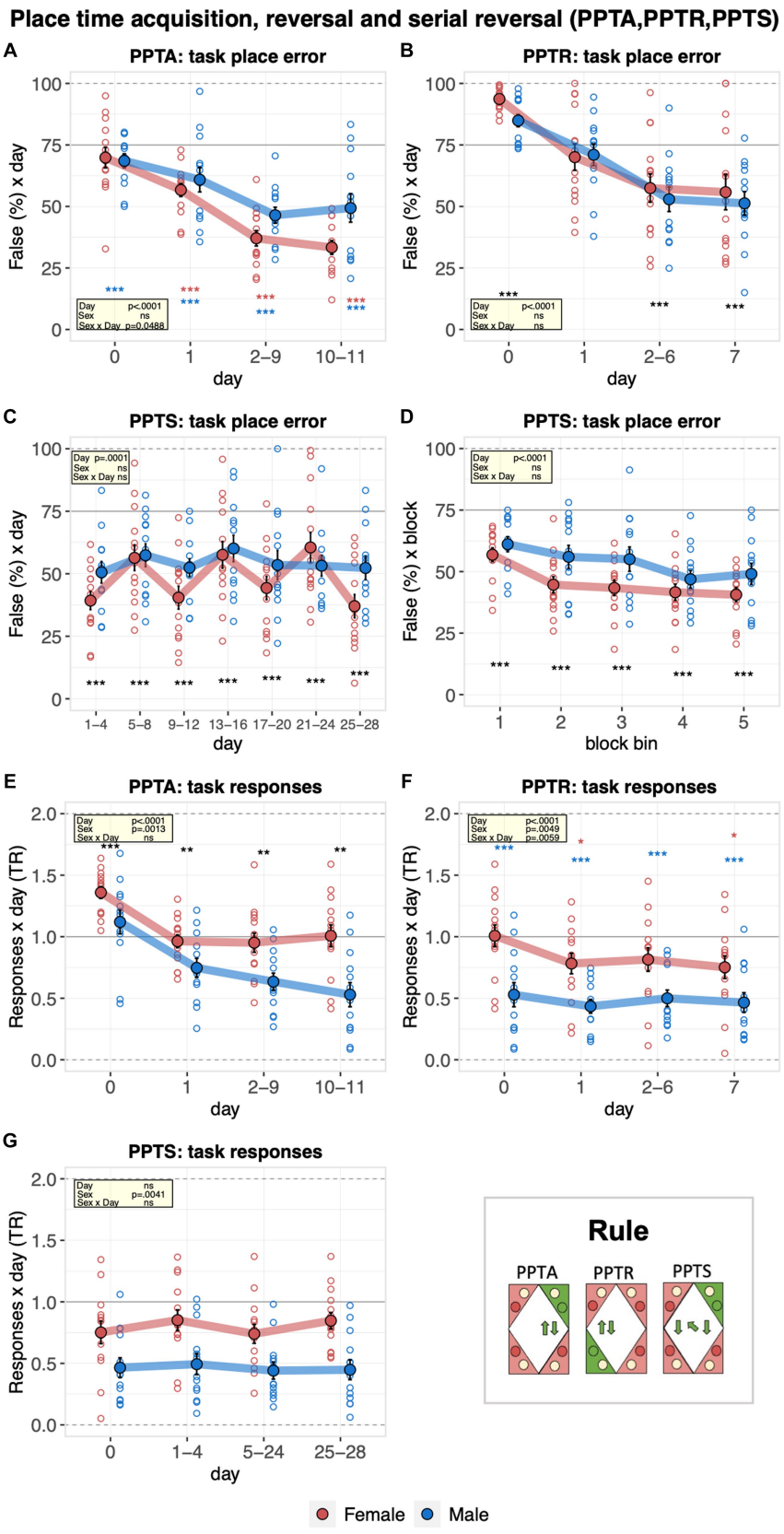

FIGURE 6
Learning performance of males and females in the place time acquisition, reversal, and serial reversal stages. During the place time acquisition (PPTA, 11 days), the target corner moved to the right at 14:00 every day and back to the original position at 02:00. During the place time reversal (PPTR, 7 days), a new corner pair, with target corner opposite to the one used in time place acquisition and again moved to the right at 14:00 every day and back to the original position at 02:00, was defined. During the place time serial reversal (PPTS, 28 days), 7 alternations between place time acquisition

(Continued)

FIGURE 6 (Continued)

and reversal, each lasting for 4 days, starting and ending with the acquisition, were defined. One-sample *t*-tests against chance (solid horizontal line): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ referring to the comparison of pooled groups against chance. One-sample *t*-test results are shown for groups separately in red and blue when a significant interaction with sex is present. **(A)** Percentage of place errors during PPTA corresponding to task responses to incorrect corners plotted as a function of the day with day 0 corresponding to the last 2 days of nosepoke adaptation III with saccharin available in all corners (ANOVA: day $F_{3,69} = 38.24$ $p < 0.0001$ $\omega^2 = 0.44$, sex × day $F_{3,69} = 2.758$ $p = 0.0488$ $\omega^2 = 0.04$). Overall, place errors decreased robustly indicating how the mice learned the place time acquisition rule. Males made significantly more place errors from the second day onward. **(B)** Percentage of place errors during PPTR corresponding to task responses to incorrect corners plotted as a function of the day with day 0 corresponding to the last 2 days of place time acquisition (ANOVA: day $F_{3,69} = 37.33$ $p < 0.0001$ $\omega^2 = 0.42$). Overall, mice learned the place acquisition rule. **(C)** Percentage of place errors during PPTS corresponding to task responses to incorrect corners plotted as a function of the day (7 × 4 days, average of each alternation, ANOVA, sex × day $F_{6,138} = 1.930$ $p = 0.0801$ $\omega^2 = 0.02$). There was no general learning of the place time serial reversal task. **(D)** Percentage of place errors during PPTS corresponding to task responses to incorrect corners plotted as a function of block bin (first, second, third, fourth, and fifth 20% responses in each alternation, averaged across alternations, ANOVA: block bin $F_{1,104} = 55.03$ $p < 0.0001$ $\omega^2 = 0.12$). A robust decrease of place error rate within each task was observed. **(E)** Response task ratio during PPTA plotted as a function of the day, with day 0 corresponding to the last 2 days of nosepoke adaptation III with saccharin available in all corners. Ratio defined as (2 + 2 × Task)/(2 + Joker + Task), chance = 1, range = 0–2 (ANOVA: sex $F_{1,23} = 13.40$ $p = 0.0013$ $\omega^2 = 0.33$). Response task ratio of male mice dropped at task onset, continued to decrease during the task and reached levels clearly indicating preferential responding at water sides. **(F)** Response task ratio during PPTR plotted as a function of the day with day 0 corresponding to the last 2 days of place time acquisition (ANOVA: sex $F_{1,23} = 9.705$ $p = 0.0049$ $\omega^2 = 0.26$; sex × time bin $F_{3,69} = 4.531$ $p = 0.0059$ $\omega^2 = 0.01$). Response task ratio of male mice remained at very low levels throughout the task, indicating persistent preferential responding at water sides compared to female mice. **(G)** Response task ratio plotted as a function of the day (7 × 4 days, average of each alternation). (ANOVA: sex $F_{1,23} = 10.14$ $p = 0.0041$ $\omega^2 = 0.27$). Response task ratio of male mice remained consistently below that of females.

protocols presented here is a limitation that needs to be addressed by improving the protocols. On the other hand, the ability of these protocols to pick up sex differences in decision-making that are not evident in aversively motivated conventional or IntelliCage tasks also indicates that they may also be more suitable to detect alterations of decision-making which may be relevant phenotypic changes in mouse models of brain disease (Perry and Kramer, 2015).

We speculated that having the option to first respond for saccharin and to switch to plain water as a backup after not being rewarded with saccharin in an incorrect corner could lower the cost of incorrect responses and reduce the motivation to learn the task rule. Therefore, we tested a modification of the protocol enforcing an exclusive choice of either plain water or sweet reward during every visit, thereby preventing using plain water as backup during incorrect responses. Our results provide no evidence for a consistent beneficial effect of this modification on task performance. Most likely, this is due to the fact that the mice spontaneously tended to make exclusive responses either at the saccharin or plain water side. Even when allowed and rewarded, double responses for both saccharin solution and plain water were infrequent already during baseline conditions (Figures 3C,D). When challenged by increasingly difficult learning tasks, the mice reduced response for saccharin completely and independently of the protocol and did not adopt a double responding strategy. Obviously, the perceived value of plain water as a backup was too small for the animals to have a significant negative impact on learning motivation.

We deliberately chose saccharin as a sweet reward instead of sucrose because of the metabolic effects that may be induced by the prolonged consumption of a caloric reward on body weight and enzymatic activity (Black and Moyer, 1998). The strain dependence of the preference for saccharin in mice (Bachmanov et al., 2001) is a potential further limitation of the protocols proposed in the present study. In addition, experimental manipulations in mouse models of neurodegenerative disease may alter reward processing at a basic level and thereby compromise the attractiveness of saccharin as a reward (Perry and Kramer, 2015). Therefore, the baseline preference to respond and consume saccharin solution will need to be checked carefully during the adaptation stages in any study using these protocols. Saccharin may need to be replaced by another tastant or by sucrose—or in some cases, one may even need to revert to protocols that use water deprivation as a negative incentive for learning.

In conclusion, IntelliCage protocols which are based on sweet rewards and prevent water deprivation in poor learners by providing continuous access to water, permit to optimize animal welfare and refine the assessment of learning in mouse models following the 3R principles (replace, reduce, refine). However, the validity of such learning tasks still needs to be improved. Learning engagement also needs to be secured in more demanding learning tasks by modifying sweet reward-based protocols in ways that provide a stronger incentive for learning in female mice and even more so in male mice that are less willing to engage in learning for a sweet reward. This could, for example, be achieved by attaching a price tag to the constantly available water to make it less attractive and to create a double incentive for learning. Indeed, we have recently found that introducing a disincentive component either by adding bitter-tasting quinine to the freely available water or by reducing the probability of water delivery at joker sides indeed improves motivation and performance of female mice in challenging spatial tasks in IntelliCage (Ma et al., 2023). However, whether such an approach could also motivate male mice to learn difficult tasks remains to be shown.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All the animal experiments were carried out at the Institute of Anatomy, University of Zürich in accordance with the European legislation (Directive 2010/63/EU) and having been approved by the veterinary office of the Canton of Zürich (License number 060/2021). The study was conducted in accordance with the local legislation and institutional requirements.

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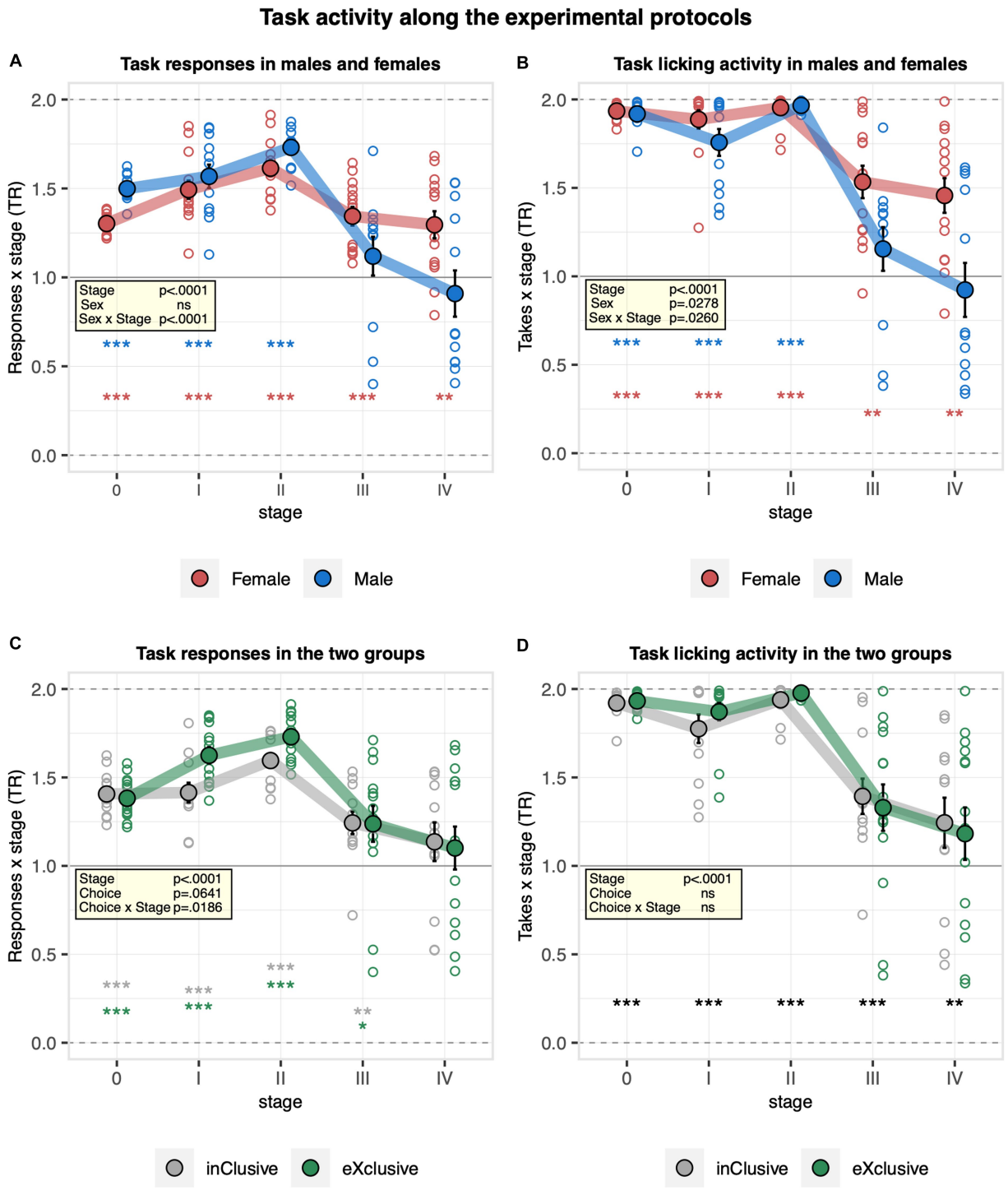

FIGURE 7
Activity of the mice at the task sides during nosepoke adaptation stages before, between, and after learning tasks. Response task ratio was plotted as a function of stage, each corresponding to the last 2 days of a phase (0 free adaptation saccharin/water, I first nosepoke adaptation, II continued pre-learning nosepoke adaptation, III nosepoke adaptation after corner preference training, and IV final nosepoke adaptation after time place training). Ratio defined as (2 + 2 × Task)/(2 + Joker + Task), chance = 1, range = 0–2. The licking task ratio was calculated based on takes = responses with drinking at task and joker sides, respectively. One-sample *t*-tests against chance (solid horizontal line): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ referring to the comparison of pooled groups against chance. One-sample *t*-test results are shown for groups separately when a significant interaction with either sex or choice is present. **(A)** Response task ratio in males and females (ANOVA: phase $F_{4,88} = 33.38$ $p < 0.0001$ $\omega^2 = 0.47$, sex × phase $F_{4,88} = 7.275$ $p < 0.0001$ $\omega^2 = 0.15$, Box–Cox λ 3.50). The response task ratio increased throughout the pre-learning stages but dropped after the learning stages, without evidence for recovery during nosepoke adaptation interludes. It was slightly higher in males during the pre-learning stages but dropped more strongly after learning than in females. **(B)** Licking task ratio in males and females (ANOVA: sex × phase $F_{4,88} = 2.909$ $p = 0.0260$ $\omega^2 = 0.05$, Box–Cox λ 5.00). Males showed a stronger decrease in the drinking task ratio after the learning stages. **(C)** Response task ratio in the inclusive and exclusive groups (ANOVA: choice × phase $F_{4,88} = 3.130$ $p = 0.0186$ $\omega^2 = 0.06$, Box–Cox λ 3.50). The response task ratio was higher in the exclusive choice group during the pre-learning stages, but the effect was lost after the learning stages. **(D)** Drinking task ratio in the inclusive and exclusive groups (choice $F_{1,22} = 0.2115$ ns, Box–Cox λ 5.00). No evidence for a choice group effect on the drinking task ratio was observed.

Author contributions

MN: Investigation, Writing – original draft, Writing Review & Editing, Visualization. GB: Investigation, Writing – original draft. ACS: Writing – original draft. DW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing Review & Editing, Visualization.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2024.1270159/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Licking frequency of male and female mice during the free adaptation stage in the IntelliCage. Average licks per hour plotted as function of day with Day 0 corresponding to the last day of the previous stage (pre-task baseline, when all bottles still contained plain water) (ANOVA: day F3,72=21.34 p <.0001 ω^2=.18, sex F1,24=1.236 ns). Lick frequency increased instantly in response to the introduction of saccharin at the task sides. There was no evidence for a sex effect on lick frequency, suggesting that male and female mice consumed similar amounts of liquid.

SUPPLEMENTARY FIGURE S2
Activity of male and female mice during the nosepoke adaptation stage. One-sample t-test results shown for groups separately in red and blue when a significant interaction with sex is present: ***p<.001 **p<.01 *p<.05. **A)** Percentage of nosepokes overlapping with accessibility of saccharin bottles plotted as function of day (ANOVA: day F3,69=143.9 p<.0001 ω^2=.77, sex F1,23=6.040 p=.0219 ω^2=.17, sex x day F3,69=2.571 ns, sex x choice F1,23=1.802 ns, Box-Cox λ 4.00). Percentage of nosepokes overlapping with accessibility of saccharin bottles dropped more strongly in females but sexes became indistinguishable by the end of the stage. **B)** Percentage of responses with nosepokes overlapping with accessibility of water bottles plotted as function of day (ANOVA: day F3,69=57.08 p<.0001 ω^2=.58, sex F1,23=.3964 ns, sex x day F3,69=3.550 p=.0188 ω^2=.06, sex x choice F1,23=1.110 ns, Box-Cox λ .500). Percentage of responses with nosepokes overlapping with accessibility of water bottles dropped more strongly in males but soon recovered to levels slightly higher than females. **C)** Percentage of responses with nosepokes overlapping with accessibility of water and saccharin reward plotted as function of task sequence during the visit (joker side only, joker side followed by task side, task side followed by joker side, task side only) and averaged across the entire stage (ANOVA: sex x taste F3,69=2.546 ns, sex x choice x taste F3,69=2.560 ns, Box-Cox λ .500). **D)** Percentage of responses plotted as function of side sequence during the visit (joker side only, joker side followed by task side, task side followed by joker side, task side only) and averaged across the entire stage (ANOVA: sex x taste F3,69=7.355 p=.0002 ω^2=.20, sex x choice x taste F3,69=.9204 ns, Box-Cox λ .500). Males more strongly avoided to respond at both sides than females and showed a stronger preference for responding exclusively at the task side.

SUPPLEMENTARY FIGURE S3
Activity of the mice during nosepoke adaptation stages before, between and after learning tasks. **A, B)** Average licks per day plotted as function of phase each corresponding to the last 2 days of a stage (0 free adaptation saccharin / water, I first nosepoke adaptation, II continued pre-learning nosepoke adaptation, III nosepoke adaptation after corner preference training, IV final nosepoke adaptation after time place training; ANOVA: phase F4,88=46.46 p<.0001 ω^2=.47, Box-Cox λ .500). At the transition from free to nosepoke adaptation, lick numbers dropped by about 50% and remained stable thereafter with only very small decreases after learning stages, without evidence for a consistent effect of sex on lick numbers. No evidence for a choice group effect on the overall lick number was observed. **C, D)** Average responses per hour plotted as function of phase (ANOVA: phase F4,88=60.69 p<.0001 ω^2=.50, Box-Cox λ .500). Overall, responses increased strongly at the transition from free to nosepoke adaptation to decrease again after the learning stages without statistical evidence for a sex effect. Responses were also similar in the two experimental groups.

SUPPLEMENTARY FIGURE S4
Learning performance of the inclusive and exclusive groups in the corner preference acquisition and reversal stages. During corner preference acquisition 1 (PPRA1, 7 days), water was available at joker sides in all corners, but saccharin could only be obtained at the task side of a single target corner which remained the same throughout the task. Corner preference acquisition 2 (PPRA2, 9 days) continued with same target corner as in corner preference acquisition 1 after cleaning the cages. During corner preference reversal (PPRR, 6 days) the target corner was defined as opposite to the one used in corner preference acquisition 1 and 2. Ratio defined as (b + 2 x Task) / Proofs_Legends (b + Joker + Task), b=2, chance = 1, range = 0-2. One-sample t-tests against chance (solid horizontal line): ***p<.001 **p<.01 *p<.05 referring to the comparison of pooled groups against chance. **A)** Percentage of place errors in PPRA1 corresponding to task responses to incorrect corners plotted as function of day with Day 0 corresponding to the last 2 days of joker adaptation with saccharin still available in all corners (ANOVA: choice F1,23=.0326 ns). **B)** Percentage of place errors in PPRA2 corresponding to task responses to incorrect corners plotted as function of day with Day 0 corresponding to the last 2 days of corner preference acquisition 1 (ANOVA: choice F1,23=.1233 ns, Box-Cox λ .500). **C)** Response task ratio plotted as function of day in PPRA1 with Day 0 corresponding to the last 2 days of joker adaptation with saccharin still available in all corners. (ANOVA: choice F1,23=2.277 ns, Box-Cox λ 3.00). **D)** Response task ratio plotted as function of day in PPRA2 with Day 0 corresponding to the last 2 days of corner preference acquisition 1 (ANOVA: choice F1,23=.4011 ns, Box-Cox λ 2.00). **E)** Percentage of place errors during PPRR corresponding to task responses to incorrect corners plotted as function of day with Day 0 corresponding to the last 2 days of corner preference acquisition 2 (ANOVA: choice F1,23=.0632 ns). **F)** Response task ratio plotted as function of day in PPRR with Day 0 corresponding to the last 2 days of corner preference acquisition 2 (ANOVA: choice F1,23=.2247 ns).

SUPPLEMENTARY FIGURE S5
Learning performance of the inclusive and exclusive groups in the place time acquisition, reversal and serial reversal stages. During place time acquisition (PPTA, 11 days), the target corner moved to the right at 14:00 every day and back to the original position at 02:00. During place time reversal (PPTR, 7 days), a new corner pair was defined with target corner opposite to the one used in time place acquisition and again moving to the right at 14:00 every

day and back to the original position at 02:00. During place time serial reversal (PPTS, 28 days), 7 alternations between place time acquisition and reversal each lasting for 4 days, starting and ending with acquisition, were defined. One-sample t-tests against chance (solid horizontal line): ***p<.001 **p<.01 *p<.05 referring to the comparison of pooled groups against chance. **A)** Percentage of place errors during PPTA corresponding to task responses to incorrect corners plotted as function of day with Day 0 corresponding to the last 2 days of joker adaptation III with saccharin available in all corners (ANOVA: choice F1,23=2.463 ns). **B)** Percentage of place errors during PPTR corresponding to task responses to incorrect corners plotted as function of day with Day 0 corresponding to the last 2 days of time place acquisition (ANOVA: choice F1,23=.7869 ns). **C)** Percentage of place errors corresponding to task responses to incorrect corners plotted as function of day (7x4 days, average of each alternation) during PPTS (ANOVA: choice F1,23=2.106 ns). **D)** Percentage of place errors corresponding to task responses to incorrect corners plotted as function of block bin (first, second, third, fourth and fifth 20% responses in each alternation, averaged across alternations) during PPTS (ANOVA: choice F1,23=1.516 ns). **E)** Response task ratio plotted as function of day during PPTA with Day 0 corresponding to the last 2 days of joker adaptation III with saccharin available in all corners. Ratio defined as (2 + 2 x Task) / (2 + Joker + Task), chance = 1, range = 0-2 (ANOVA: choice F1,23=1.626 ns). **F)** Response task ratio plotted as function of day during PPTR with Day 0 corresponding to the last 2 days of time place acquisition. (ANOVA: choice F1,23=1.482 ns). **G)** Response task ratio plotted as function of time bin (7x4 days, average of each alternation) during PPTS. (ANOVA: choice F1,23=1.550 ns).

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Modulation of $GABA_B$ receptors in the insula bidirectionally affects associative memory of epilectic rats in both spatial and non-spatial operant tasks

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Background: Stimulation of gamma-aminobutyric acid (GABA) activity through GABA receptor agonists is the basic mechanism of many anticonvulsant drugs. Nevertheless, many of these GABergic drugs have adverse cognitive effects. We previously found that GABAB receptors ($GABA_B$Rs) in the insula regulate operant associative memory in healthy rats. The present study aimed at investigating the effects of $GABA_B$R modulation in the insula on operant associative memory in epileptic rats, along with the underlying mechanisms.

Methods: The lithium-pilocarpine model of temporal lobe epilepsy (TLE) was established in male Sprague–Dawley rats. A 22-gauge stainless-steel guide cannula was surgically implanted into the granular insula cortex of the epileptic rats. Baclofen (125 ng/μl, 1 μl), CGP35348 (12.5 μg/μl, 1 μl), or saline (1 μl) was slowly infused through the guide cannula. The Intellicage automated behavioral testing system was used to evaluate operant associative memory of the epileptic rats, including non-spatial operant tasks (basic nosepoke learning and skilled nosepoke learning) and spatial operant tasks (chamber position learning). The expression of the $GABA_B$R subunits GB1 and GB2 in the insula was examined by immunofluorescence and Western blotting.

Results: The Intellicage tests demonstrated that baclofen significantly impaired basic nosepoke learning, skilled nosepoke learning and chamber position learning of the epileptic rats, while CGP35348 boosted these functions. Immunofluorescence staining revealed that GB1 and GB2 were expressed in the insula of the epileptic rats, and Western blotting analysis showed that baclofen enhanced while CGP35348 inhibited the expression of these subunits.

Conclusion: $GABA_BRs$ in the insula bidirectionally regulate both spatial and non-spatial operant associative memory of epileptic rats. Effects of $GABA_BRs$ on cognition should be taken into account when evaluating new possible treatments for people with epilepsy.

KEYWORDS

$GABA_BR$, insula, Intellicage, epilepsy, operant associative memory

Introduction

The gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and a major player in the pathogenesis of epilepsy (Treiman, 2001; Sperk et al., 2004). When the balance between the inhibitory tone and neuronal excitation is perturbed, epileptic seizures may arise. GABA acts through two classes of receptors: $GABA_ARs$ (ligand-gated ion channels) and $GABA_BRs$ (G-protein coupled receptors). Several studies have reported the critical role of $GABA_ARs$ in epileptogenesis (Chuang and Reddy, 2018; Maljevic et al., 2019), and a number of anticonvulsants, such as phenobarbital, valproic acid, benzodiazepines, and topiramate, act through $GABA_ARs$, potentiating the inhibitory effects of GABA (Jankovic et al., 2021). However, these $GABA_AR$ modulators can have adverse effects on the cognitive functions of patients. For example, topiramate is known to cause treatment-emergent adverse events on cognition in epileptic patients (Mula, 2012). These issues with $GABA_AR$-targeting anticonvulsants have prompted the search for new GABA modulators with an improved therapeutic profile, including allosteric $GABA_AR$ agonists (Zeman et al., 2016; Jankovic et al., 2021) or selective $GABA_BR$ modulators (Avoli and Levesque, 2021).

Emerging evidence supports the involvement of $GABA_BRs$ in epileptogenesis. In humans, a $GABA_B$ receptor ($GABA_BR$) polymorphism (G1465A) has been associated with a high risk of temporal lobe epilepsy (TLE) and disease severity in TLE patients (Gambardella et al., 2003). Moreover, $GABA_BR$ expression and efficacy were downregulated in human TLE cortical tissues (Teichgraber et al., 2009). In a kindling-induced rat model of epilepsy, it has been shown that stimulation of GABAergic neurotransmission through $GABA_BR$ agonist baclofen (BLF) had an anti-convulsant effect, while inhibition of GABAergic activity through $GABA_BR$ antagonist CGP35348 had a pro-convulsant effect in developing rats of 12 and 25 days (Mares and Slamberová, 2006). Furthermore, BLF showed an anti-convulsant effect in pentylenetetrazol-induced model of epilepsy in developing rats of 7, 12, 18, and 25 days (Kubová et al., 1996). In adult rats, BLF reduced pentylenetetrazol-induced seizures (Chen et al., 2004) and electroshock-induced seizures (Hyder Pottoo et al., 2022). Similarly, BLF reduced seizures in a mouse pentylenetetrazole kindling model of epilepsy (De Sarro et al., 2000).

Stimulation of GABAergic activity through GABA receptor agonists is the basic mechanism of many anticonvulsant drugs and represents a useful therapeutic strategy for people with epilepsy (Treiman, 2001; Jankovic et al., 2021).

Nevertheless, GABAergic neurotransmission also has an important role in memory processes, with, generally, agonists of GABA receptors impairing cognitive function and antagonists potentiating it (Makkar et al., 2010; Kasten and Boehm, 2015; Heaney and Kinney, 2016). Hence, treatment with the agonist BLF, especially if chronic, could lead to cognitive impairment. Indeed, in healthy rats, it has been observed that BLF impaired spatial memory (Nakagawa et al., 1995; Nakagawa and Takashima, 1997; Arolfo et al., 1998; Deng et al., 2009; Kumar et al., 2017). Additionally, our group found that BLF impaired several operant learning tasks (Wu et al., 2017).

The aim of the present study is to understand if BLF may lead to cognitive impairments also in epileptic rats. To achieve this goal, we tested the effect of BLF on the memory of lithium chloride (LiCl)-pilocarpine-induced epileptic rats, a model of temporal lobe epilepsy. In order to comprehend if the memory function of epilectic rats is bidirectionally regulated by GABAergic neurotransmission, and have a specular confirmation of the association between GABAergic neurotransmission and memory function, we also tested the cognitive performance of rats treated with the GABA receptor antagonist CGP35348 (in a non-convulsive dosage).

TLE is the most common form of focal epilepsy (Vinti et al., 2021). The insula, also known as the "hidden fifth lobe," is a part of the cerebral cortex positioned deep within the lateral fissure. The insular lobe has a relevant role in TLE, with epileptic activity often invading it from the temporal cortex and in some cases even originating in it (Isnard et al., 2000; Blauwblomme et al., 2013; Barba et al., 2017). Regarding its functions, the insula has long been associated with taste memory (Yiannakas and Rosenblum, 2017) and has been recently linked to non-gustatory learning, in particular object recognition memory formation (Martin et al., 2012, 2021; Korczyn et al., 2013; Bermudez-Rattoni, 2014; Titiz et al., 2014). Additionally, pharmacological inhibition of insula in mice impaired associative memory,

Abbreviations: GABA, gamma-aminobutyric acid.

disrupting conditioned responses to reward-associated cues, in particular cue-triggered reward approach (Kusumoto-Yoshida et al., 2015). Associative memory can be studied in animal models through two different conditioning paradigms. While classical (Pavlovian) conditioning features the formation of an association between two stimuli (an S-S association), operant conditioning features an association between a stimulus and a behavioral response (an S-R association) (d'Isa et al., 2011). Our group previously found that the insula is involved in operant associative learning of conditioned nosepoking via $GABA_BRs$ (Wu et al., 2017). In particular, we observed that intra-insula infusion of the $GABA_BR$ agonist baclofen impaired position learning, punitive learning, and punitive reversal learning in normal rats, while the antagonist CGP35348 enhanced these learning abilities (Wu et al., 2017), indicating that proper functioning of $GABA_BRs$ in the insula is critical for maintaining operant associative memory. However, what effect the modulation of $GABA_BRs$ in the insula of epileptic rats could have on memory function is yet to be investigated.

In the present study, we used the Intellicage system to assess the effects of intra-insula infusion of baclofen or CGP35348 on operant associative memory functions in LiCl-pilocarpine-induced epileptic rats. Also, the underlying insular $GABA_BR$ expression levels were analyzed. Our current findings shed new light on the cognitive effects of GABAergic drugs in epilepsy, indicating a memory-impairing effect for $GABA_BR$ agonist baclofen and a cognitive enhancing effect for $GABA_BR$ antagonist CGP35348.

Materials and methods

Reagents

Primary antibodies against $GABA_BR$ subunits GB_1 and GB_2 were purchased from Abcam (UK). The selective $GABA_BR$ agonist baclofen and antagonist CGP35348 were obtained from Sigma–Aldrich (USA).

Animals

Male Sprague–Dawley rats (6–8-week-old, 250–300 g)(10 rats/group) were obtained from the Animal Center of Ningxia Medical University (Yinchuan, Ningxia, China). Rats were kept under a 12:12 h light/dark cycle (lights on at 8 a.m.) with free access to food and water. Each rat was housed in a separate cage in order to avoid damage to the cannula implants and harm to the rats. All animal studies were conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China on October 31, 1988. All animal protocols were approved by the Ethics Committee of the Animal Center of Ningxia Medical University.

TLE model

A lithium-pilocarpine TLE model was established as described previously (Andre et al., 2001). Briefly, lithium chloride (LiCl) in saline (127 mg/kg) was injected into the rat abdominal cavity. After 18 h, hyoscyamine sulfate [1 mg/kg, intraperitoneally (i.p.)] was administered to mitigate the peripheral effects of pilocarpine, and 30 min later, pilocarpine (30 mg/kg, i.p.) was injected to induce status epilepticus (SE). If the rat did not exhibit behavioral seizures ($\geq$class 4 on the scale of Racine) within 30 min of pilocarpine injection, an additional dose (10 mg/kg, i.p.) was administered every 30 min until clinical signs were observed. The total amount of pilocarpine administered in each rat did not exceed 60 mg/kg, while diazepam (10 mg/kg, i.p.) was administered to stop the seizure 1 h after the onset of SE or after the rat exhibited dehydration symptoms. During the induction session, each rat was scored for epileptic signs according to Racine's scale. Successful induction was defined as induction of epileptic seizures $\geq$class 4 of the Racine scale. With the described protocol, we obtained a success rate of over 70%. For the present work, a pool of over 75 rats underwent induction. Among the rats showing successful induction, 50 rats were randomly selected as experimental animals. No rat died during induction nor during the whole period of experimental testing.

Surgery

After 1 week of acclimatization, the epileptic rats were randomly divided into five groups (10 rats/group): control, sham, saline (NaCl), baclofen (BLF), and CGP35348 (CGP). The rats in the sham, NaCl, BLF, and CGP groups underwent surgical implantation of a bilateral cannula aimed at the granular insular cortex according to a standardized protocol (Balderas et al., 2015). Briefly, the animals were anesthetized with 10% chloral hydrate (4 ml/kg, i.p.) and mounted on a stereotaxic frame. A 22-gauge stainless-steel guide cannula was inserted into the granular insula cortex according to coordinates from the Paxinos and Watson brain atlas (mm from bregma: AP = + 1.2; ML = $\pm$ 5.5; mm from skull surface: DV = −6.5) (Paxinos and Watson, 1986). The cannula was anchored to the skull using stainless steel screws and acrylic cement.

Bilateral intra-insula microinjection

The animals were allowed to recover for 14 days after the cannula implantation surgery. In order to evaluate the

effects on memory function, the rats received slow bilateral intra-insula microinjection (1 μl/0.5 min) as follows: sham, no treatment; NaCl, NaCl at 0.3 nmol/μl; BLF, baclofen at 125 ng/μl (St Onge and Floresco, 2010); CGP, CGP35348 at 12.5 μg/μl (Ataie et al., 2013). The injection needle (50 G) was left in place for 1 min post-injection to prevent backflow. Infusions were administered on each day of behavioral testing. After the infusion was complete, the rats were allowed to rest for 30 min before behavioral testing.

Signal transponder implantation

Signal transponders were implanted above the scapula using the injector system of the Intellicage device. The implantation was carried out under 10% chloral hydrate (4 ml/kg, i.p.) 14 days after the surgery and 24 h prior to introduction into the Intellicage. The transponders were used to follow the movement of the rats during behavioral testing (Urbach et al., 2014). Visits of each rat to each corner of the Intellicage were detected through radio-frequency identification (RFID) antennas installed in the cage.

Intellicage

The Intellicage (TSE Systems GmbH, Germany) is a new automated group-testing system that allows standardized rodent behavioral phenotyping in a social context and without interaction with the experimenter during the test. It features four operant conditioning chambers positioned in each corner of the cage. Each chamber is equipped with a transponder-reader antenna that registers the number of visits by the rat. Inside each chamber there are two doors (the left and the right door), each giving access to a water-drinking bottle. Each water bottle is separately gated by one door, and door opening is controlled by an infrared beam-break sensor that detects correct nosepoking (inserting the nose in a small hole near the door). The number of chamber entries and nosepokes are automatically recorded and processed with the Intellicage software. Total dimensions of the rat Intellicage are: 118 cm x 118 cm x 46 cm (rat system, center cage + corner + water bottles). Currently, the Intellicage system is one of the most advanced commercial apparatuses for automated rodent behavioral testing (Kiryk et al., 2020; Iman et al., 2021), and its first application in behavioral research on insular functions was published by our research group (Wu et al., 2017).

Behavioral test

The rats were transferred to the Intellicage 2 weeks post-surgery. The behavioral information was collected from 9:00 to 12:00 a.m. Each rat was tested in daily sessions of 30 min. Rats were tested in groups of 10, randomized by experimental group (2 for each of the 5 experimental groups). Each day, 5 sessions were performed, with 10 rats in each session (50 total rats tested each day). The Intellicage was cleaned with ethanol 70% at the end of each session. Each rat was tested in one single session per day. The rats were removed from the Intellicage at the end of each daily testing session and maintained in their home-cage with free access to food and water until the next testing session. Water bottles were removed from the home-cages from 7:00 to 9:00 a.m. of each testing day. Although absence of water for 2 h does not cause an actual physiological water deprivation in rats, the disappearance of the familiar water bottles from the home-cage shortly before the test was aimed at promoting research of alternative sources of water in the Intellicage.

Intellicage testing experimental design (**Figure 1**):

1. Free exploration: 8 days in the Intellicage with free access to all water bottles (all doors open). The number of chamber visits was recorded to evaluate exploratory behavior of the animals. This phase also served as familiarization with the Intellicage environment and with the other rats. The subsequent learning tasks (nosepoke learning, chamber position learning and door position learning) were performed only after these 8-days of contextual and social familiarization.
2. Basic nosepoke learning: 8 days in the Intellicage with access to water bottles granted by one correct nosepoke. In each chamber, doors were closed and could be opened only by nosepoking. Number of nosepokes was recorded.
3. Behavioral extinction: 1 day in the Intellicage with free access to all water bottles (all doors open) to extinguish the previous learning.
4. Skilled nosepoke learning: 8 days in the Intellicage with access to water bottles granted by five correct nosepokes. In each chamber, doors were closed and could be opened only by nosepoking. Number of nosepokes was recorded.
5. Behavioral extinction: Same as in step 3.
6. Chamber position learning 1: A chamber is located in each of the four corners of the Intellicage. The least visited chamber identified from the skilled nosepoke learning test was designated as "correct," and the three remaining chambers were designated as "incorrect." In order to avoid any possible spatial bias, each rat was tested in all four possible spatial configurations ("correct" corner in North-West, North-East, South-East or South-West). Four consecutive 2-day tests were performed. The "correct" chamber was rotated 90°clockwise every 2 days. The rats had access to all chambers, but drinking water was allowed only in the "correct" chamber. In each chamber, the left door was open, while the right door was closed. Rats could obtain water only through the left bottle of the "correct" chamber (the left bottles of the three "incorrect" chambers

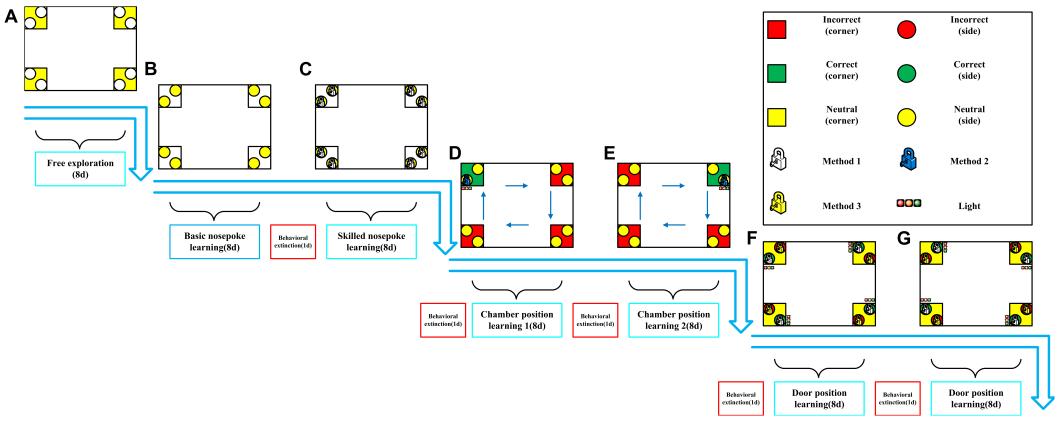

FIGURE 1
Intellicage behavioral testing experimental design. **(A)** Free exploration. **(B)** Basic nosepoke learning. **(C)** Skilled nosepoke learning. **(D)** Chamber position learning 1. **(E)** Chamber position learning 2. **(F)** Door position learning 1. **(G)** Door position learning 2.

were empty). Three multi-color LEDs over the left door of the "correct" chamber served as visual cues for the correct site.

The number of visits to each chamber was recorded. The experiment was completed in 8 days. Number of visits and percentage of visits to the "correct" chamber were used to evaluate chamber position learning.

1. Behavioral extinction: Same as in step 3.
2. Chamber position learning 2: The testing conditions were similar to those in chamber position learning 1, except that the rats could access the water bottle only through the right door of the "correct" chamber. In each chamber, right doors were open and left doors were closed. Three multi-color LEDs over the right door of the "correct" chamber served as visual cues for the correct site.
3. Behavioral extinction: Same as in step 3.
4. Door position learning 1: The left side of each chamber was designated as the "correct" side and the right side as the "incorrect" side. The door of the "correct" side would open after five nosepokes and stay open for 10 s, and door at the "incorrect" side would also open after five nosepokes but stay open for only 3 s. Three multi-color LEDs over the left doors served as visual cues for the correct sites. Water drinking was allowed at both sites in all chambers. Number of nosepokes to each door was recorded. The experiment was completed in 8 days.
5. Behavioral extinction: Same as in step 3.
6. Door position learning 2: The testing conditions were the same as those in door position learning 1, except that the right side of each chamber was designated as the "correct" side and the left side as the "incorrect" side. Three multi-color LEDs over the right doors served as visual cues for the correct sites.

Tissue sample collection

After completing all behavioral tests in Intellicage, on the day after the rats were euthanized under 10% chloral hydrate. Left and right insula tissues were collected for immunofluorescence staining and Western blot analysis.

Immunofluorescence staining

The insular tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in optimal cutting temperature (OCT) compound, and sectioned. GB1 and GB2 were detected by immunofluorescence staining. Briefly, after antigen retrieval in citric acid buffer, the sections were blocked in serum and incubated with anti-GB1 (1:300) or anti-GB2 (1:500) antibody at 4°C overnight. After washing in phosphate-buffered saline (PBS), the samples were incubated at room temperature with a FITC-labeled secondary antibody for 1 h. The unbound antibody was removed with PBS washes. After blocking with an anti-quencher, the samples were analyzed by immunofluorescence imaging. The cell nuclei were counterstained with DAPI. The imaging data were processed with the ImageJ 1.48 analysis system.

Western blotting

The insula tissues were placed on ice and lysed in lysis buffer. The total protein content was determined using the

BCA method. The proteins were separated by SDS/PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk for 1 h, the membranes were probed with anti-GB1 (1:300) or anti-GB2 (1:500) antibody at 4°C overnight and incubated with an IRDye 800CW dye-labeled secondary antibody (1:5,000). The immunoreactive bands were detected on an Odyssey infrared laser imaging system and quantified by gray intensity analysis. The protein levels were normalized to those of GADPH.

Statistical analysis

All data are presented as the mean ± standard error of mean (SEM) and analyzed using the SPSS 21.0 software. Overall behavioral responses (total responses throughout all days of testing) and Western blotting results were analyzed using one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) *post hoc* test in case of significant effect of experimental group. Daily behavioral responses were analyzed through a two way ANOVA for repeated measures, followed by Fisher's LSD *post hoc* test in case of significant effect of experimental group. For percentages of correct visits, chance level analysis was performed by comparing the percentages of each experimental group against chance level (25%) through a one-sample *t*-test. In all analyses, a p-value < 0.05 was considered statistically significant.

Results

Intra-insula baclofen impaired while CGP35348 boosted the operant associative memory of TLE rats

Rats underwent a series of behavioral protocols in the Intellicage as described in **Figure 1**: a free exploration test, two non-spatial operant tasks (basic nosepoke learning and skilled nosepoke learning), two spatial operant tasks (chamber position learning 1 and chamber position learning 2) and finally two tasks in which both nosepoking and spatial discrimination were required (door position learning 1 and door position learning 2). Overall behavioral responses totalized over the course of all days of testing are showed in **Figure 2**.

During the 8-day free exploration test, in which all doors were open, the five groups of epileptic rats showed similar exploratory capacities under unrestricted conditions, as evaluated by the number of visits to the water bottle chambers ($F(4,45) = 2.006$, $p = 0.110$; **Figure 2A**). However, during the basic nosepoke learning test, when the rats had to learn to perform a correct response (a nosepoke) to open a door to access the water bottle, a significant effect of experimental group was found ($F(4,45) = 14.708$, $p < 0.0001$; **Figure 2B**). The BLF rats nosepoked significantly less ($p = 0.0003$), while the CGP rats nosepoked significantly more ($p = 0.0007$) than the NaCl rats, indicating that baclofen decreased while CGP35348 increased the basic nosepoke learning ability of epileptic rats. Similarly, a significant effect of experimental group was found also in skilled nosepoke learning ($F(4,45) = 17.919$, $p < 0.0001$; **Figure 2C**), in which rats had to nosepoke five times to access the water bottles. BLF rats exhibited a lower ($p = 0.003$) while the CGP rats displayed a higher ($p < 0.0001$) number of nosepokes than the NaCl rats.

Subsequenly, we tested the rats in two spatial tasks: chamber position learning 1 and chamber position learning 2 (**Figures 2D, E**), in which a bottle with water was placed in one of the four corner chambers (the "correct chamber"), while the other three corner chambers contained empty bottles (the "incorrect" chambers). Rats had to learn the position of the bottle with water and simply visit the "correct" chamber (no nosepoking required) to drink. Effect of experimental group was significant for both spatial tasks (chamber position learning 1: $F(4,45) = 43.741$, $p < 0.0001$; chamber position learning 2: $F(4,45) = 79.111$, $p < 0.0001$). In both spatial tasks, baclofen impaired the performance, while CGP35348 improved it. In comparison with the NaCl group, BLF rats showed a significant decrease in the total number of correct visits (chamber position learning 1: $p < 0.0001$; chamber position learning 2: $p < 0.0001$), while CGP rats displayed a significant increase (chamber position learning 1: $p < 0.0001$; chamber position learning 2: $p = 0.007$).

Additionally, in order to perform a chance level analysis, we proportioned the number of visits in the correct corner to the total number of visits (number correct visits + number of incorrect visits), obtaining the percentage of visits to the correct corner (**Figures 2F, G**). Since, in the spatial tasks, one corner was correct and 3 corners were incorrect, for the percentage of correct visits the chance level performance was 25%. Compared against chance level, all groups apart from the BLF rats showed significant learning, in both chamber position learning 1 (control: $p = 0.0008$; sham: $p < 0.0001$; NaCl: $p < 0.0001$; BLF: $p = 0.564$; CGP: $p < 0.0001$) and chamber position learning 2 (control: $p = 0.0008$; sham: $p < 0.0001$; NaCl: $p < 0.0001$; BLF: $p = 0.074$; CGP: $p < 0.0001$). Moreover, in comparison with percentages of NaCl rats, percentages of BLF were significantly lower in chamber position learning 1 ($p = 0.0002$) and chamber position learning 2 ($p < 0.0001$), while percentages of CGP were significantly higher in chamber position learning 1 ($p = 0.004$).

Regarding door position learning, the experiments were not valid as the control groups developed a significant preference for the wrong side. Hence it was not possible to use this test to evaluate the memory of the experimental groups. Since the experiments are invalid, we are not showing the results. Probably the experimental protocol did not work because the difference between the value of the reward of the two sides (3 s

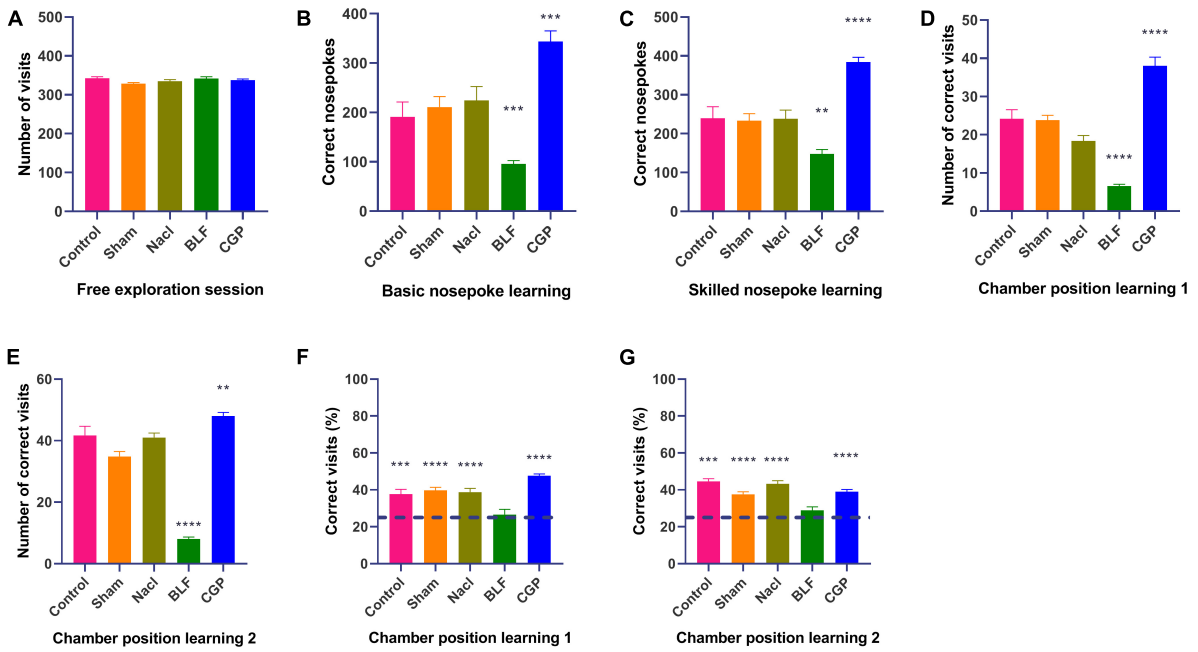

FIGURE 2
Intellicage behavioral testing: overall results (sum of all 8 days of testing). **(A)** Number of visits in free exploration. **(B)** Number of nosepokes in basic nosepoke learning. **(C)** Number of nosepokes in skilled nosepoke learning. **(D)** Number of correct visits in chamber position learning 1. **(E)** Number of correct visits in chamber position learning 2. **(F)** Percentage of correct visits in chamber position learning 1. **(G)** Percentage of correct visits in chamber position learning 2. $N = 10$ rats/group. The asterisks indicate significance against the NaCl group in **(A–E)** or against chance level (25%) in **(F,G)** $^{**}p = 0.01$; $^{***}p = 0.001$; $^{****}p = 0.0001$.

of water vs 10 s of water) was insufficient to induce a preference for the correct side in the rats of the control groups. We further comment on this issue in the Discussion.

Additionally, we performed a single day analysis in order to investigate in more detail differences between experimental groups. For the free exploration paradigm (Figure 3A), two-way ANOVA for repeated measures confirmed no significant effect of experimental group ($F(4,45) = 2.006$; $p = 0.110$).

For basic nosepoke learning (Figure 3B), a significant effect of experimental group was found ($F(4,45) = 14.708$; $p < 0.0001$). Compared with NaCl, BLF rats showed a significantly lower performance on day 1 ($p = 0.0009$), day 2 ($p = 0.025$), day 3 ($p = 0.005$), day 5 ($p < 0.0001$), day 6 ($p = 0.003$), day 7 ($p = 0.002$) and day 8 ($p = 0.003$), whereas CGP rats showed a significantly higher performance on day 1 ($p = 0.004$), day 2 ($p = 0.013$), day 3 ($p = 0.10$), day 4 ($p = 0.008$), day 5 ($p = 0.006$), day 7 ($p = 0.003$) and day 8 ($p < 0.0001$). Performance of CGP rats was significantly higher than the one of BLF rats on day 1 ($p < 0.0001$), day 2 ($p < 0.0001$), day 3 ($p < 0.0001$), day 4 ($p < 0.0001$), day 5 ($p < 0.0001$), day 6 ($p < 0.0001$), day 7 ($p < 0.0001$) and day 8 ($p < 0.0001$).

For skilled nosepoke learning (Figure 3C), a significant effect of experimental group was found ($F(4,45) = 17.919$; $p < 0.0001$). Compared with NaCl, BLF rats showed a significantly lower performance on day 2 ($p = 0.026$), day 3 ($p = 0.001$), day 4 ($p = 0.047$), day 5 ($p = 0.005$), day 6 ($p = 0.027$), day 7 ($p = 0.035$) and day 8 ($p = 0.022$), whereas CGP rats showed a significantly higher performance on day 1 ($p < 0.0001$), day 2 ($p = 0.0006$), day 3 ($p < 0.0001$), day 4 ($p = 0.001$), day 5 ($p < 0.0001$) day 6 ($p = 0.024$) and day 7 ($p < 0.0001$). Performance of CGP rats was significantly higher than the one of BLF rats on day 1 ($p < 0.0001$), day 2 ($p < 0.0001$), day 3 ($p < 0.0001$), day 4 ($p < 0.0001$), day 5 ($p < 0.0001$), day 6 ($p < 0.0001$), day 7 ($p < 0.0001$) and day 8 ($p = 0.0001$).

For the spatial tasks, four 2-day tests were performed, one for each spatial configuration (correct corner in North-West, North-East, South-East or South-West), in order to avoid any possible spatial bias. The average performance for all four spatial configurations was considered for analysis. For chamber position learning 1 (Figure 3D), a significant effect of experimental group was found ($F(4,45) = 13.505$; $p < 0.0001$). Compared with NaCl, BLF rats exhibited a significantly reduced performance on day 1 ($p = 0.005$) and day 2 ($p = 0.004$), while CGP rats showed a significantly increased performance on day 1 ($p = 0.013$). Performance of CGP rats was significantly higher than the one of BLF rats on day 1 ($p < 0.0001$) and day 2 ($p < 0.0001$). For chamber position learning 2 (Figure 3E), a significant effect of experimental group was found ($F(4,45) = 8.887$; $p < 0.0001$). Compared with NaCl,

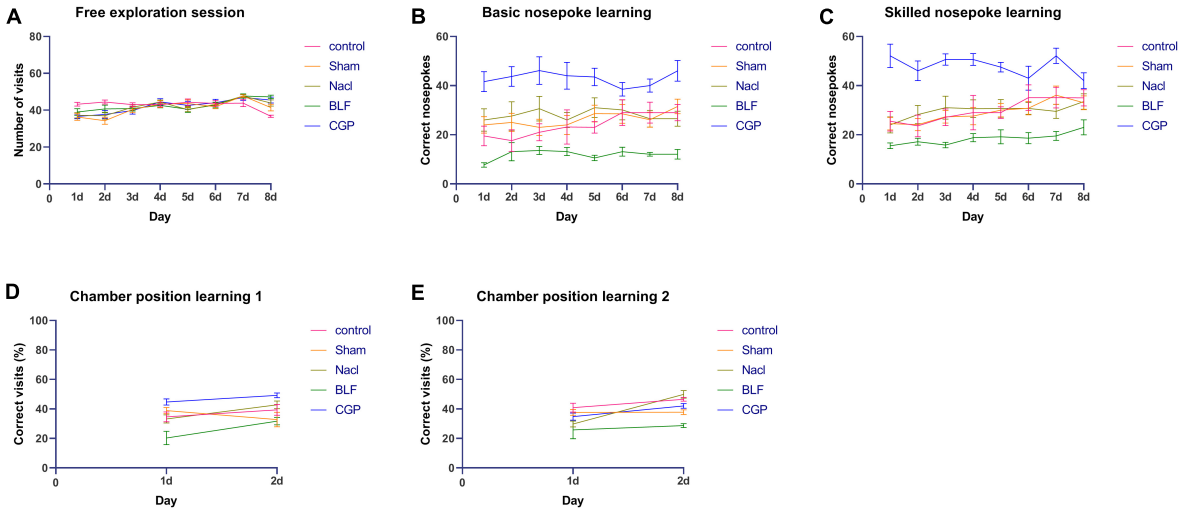

FIGURE 3
Intellicage behavioral testing: daily results. **(A)** Number of visits in free exploration. **(B)** Number of nosepokes in basic nosepoke learning. **(C)** Number of nosepokes in skilled nosepoke learning. **(D)** Percentage of correct visits in chamber position learning 1. **(E)** Percentage of correct visits in chamber position learning 2. *N* = 10 rats/group. *N* = 10 rats/group.

on day 2, the performance of BLF rats was significantly reduced ($p < 0.0001$), while the performance of CGP rats was significantly augmented ($p = 0.003$). Performance of CGP rats was significantly higher than the one of BLF rats on day 2 ($p < 0.0001$).

Collectively, these results demonstrated that bilateral intra-insula infusion of baclofen impaired associative memory of TLE rats, while the infusion of CGP35348 boosted this function.

$GABA_BR$ was expressed in the insula of TLC rats

In a previous study (Wu et al., 2017), we detected GB1 and GB2, the two subunits of $GABA_BR$, in the insula of normal Sprague-Dawley rats. In the present study, the immunofluorescence results revealed positive GB1 and GB2 staining in the insula tissues of the epileptic Sprague-Dawley rats (Figure 4), indicating that $GABA_BR$ was expressed in the insula of these rats.

Baclofen increased while CGP35348 decreased insular $GABA_BR$ expression in TLC rats

In a previous study (Wu et al., 2017), we found that baclofen increased while CGP35348 decreased GB1 and GB2 expression in the insula of normal Sprague-Dawley rats. In the present study, the expression of GB1 and GB2 was evaluated in the insula of epileptic Sprague-Dawley rats by Western blot analysis (Figure 5). A significant effect of experimental group was found for both GB1 expression ($F(4,45) = 46.034$, $p < 0.0001$) and GB2 expression ($F(4,45) = 31.841$, $p < 0.0001$). Compared to NaCl-treated rats, the baclofen-treated rats showed higher insular expression of GB1 ($p < 0.0001$) and GB2 ($p < 0.0001$), while the CGP35348-treated rats exhibited lower insular expression of GB1 ($p < 0.0001$) and GB2 ($p < 0.0001$). These findings indicate that, similarly to the effects observed in normal rats, baclofen induced while CGP35348 inhibited $GABA_BR$ expression in the insula of epileptic rats.

Discussion

Many antiepileptic drugs have adverse cognitive effects, which can significantly impact the quality of life of people with epilepsy (Hermann et al., 2010). Thus, understanding the neural network involved in epilepsy treatment-associated cognitive dysfunction is critical for improved disease management. In this study, we investigated the effects of $GABA_BR$ modulation in the insula on the operant associative memory functions of epileptic rats. The memory functions were evaluated using the Intellicage system, one of the most advanced automated devices for rodent behavioral testing. The current experiments demonstrated that bilateral intra-insula infusion of the $GABA_BR$ agonist baclofen impaired operant associative memory of epileptic rats, while the infusion of the GABABR antagonist CGP35348 boosted this function. Next, we confirmed $GABA_BR$ expression in the insula by immunofluorescence staining. We also found that baclofen induced while CGP35348 inhibited $GABA_BR$ expression in the

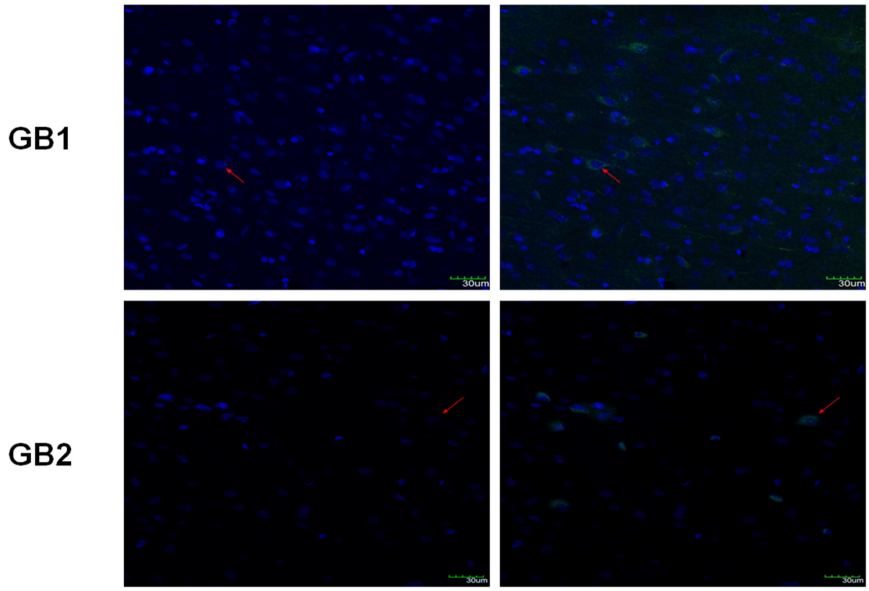

FIGURE 4
Immunofluorescence staining of insula tissues for GB1 and GB2 expression. The red arrows indicate cells showing positive staining for both GB1 and DAPI or GB2 and DAPI. Scale bars: 30 μm.

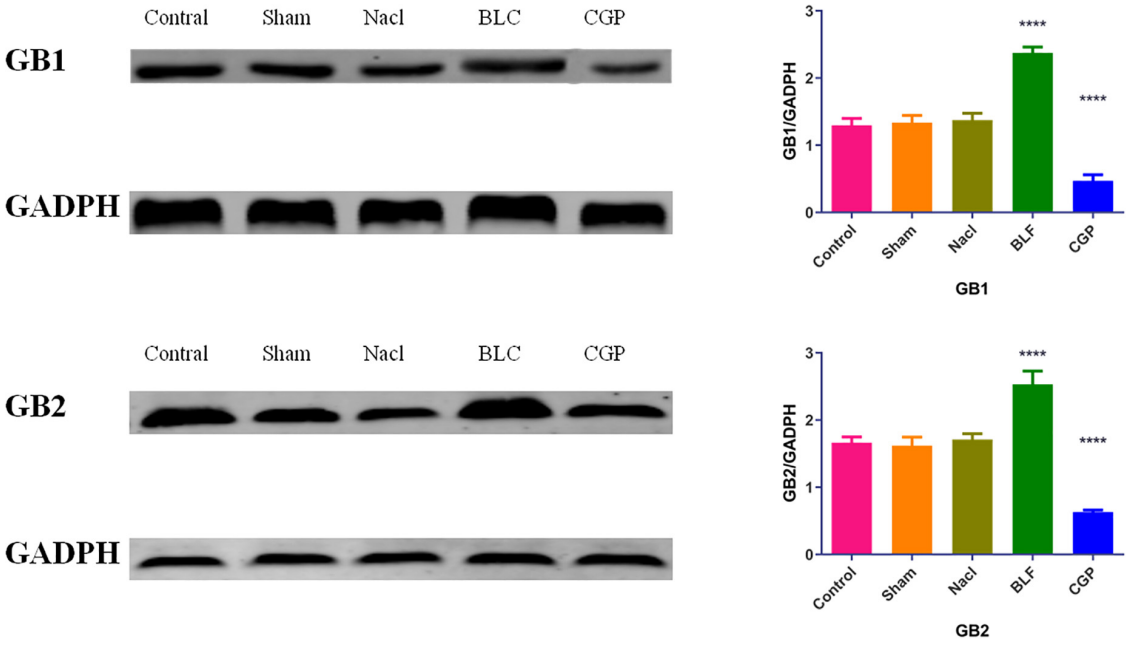

FIGURE 5
Western blot analysis of GB1 and GB2 levels in insula tissues. *N* = 10/group. The asterisks indicate significance against the NaCl group: ****$p < 0.0001$.

insula, further supporting the theory that the effects of baclofen and CGP35348 were mediated through $GABA_BR$ modulation in the insula.

Our current findings indicate that $GABA_BR$ modulation in the insula has a strong effect on associative memory of TLE rats, which should be taken into account when considering $GABA_BR$ modulators for potential epilepsy treatment.

In Intellicage experiments, two non-spatial tasks (basic nosepoke learning and skilled nosepoke learning) and two spatial tasks (chamber learning 1 and chamber learning 2) were

found altered by modulation of gabaergic neurotransmission in insula. In door position learning, we were unable to obtain valid results as the control groups developed a significant preference for the wrong side, so it was not possible to use this test to evaluate the drug-treated rats. The reason for which we could not obtain valid results with this experimental protocol could be that the difference between the rewarding values of the rewards provided in the two sides (3 s of water vs 10 s of water) was insufficient to induce a preference for the correct side in the rats of the control groups. In future experiments, rather than providing as reward water in both sides, the difference between reward values of the two sides could be augmented by dispensing in one side water and in the other side a saccharin solution, which rats naturally prefer over water.

Diving deeper into cognitive processes, why does modulation of insular gabaergic neurotransmission bidirectionally alter operant learning? In which specific psychological process is insula involved? Four hypotheses can be made to explain the changes in operant learning: a) alteration of place recognition; b) alteration of cue recognition; c) alteration of the stimulus-response association; d) alteration of the rewarding value of water. All four cases may lead to an alteration of operant conditioning. The last hypothesis (d) can be discarded, since in the free exploration paradigm, in which no nosepokes were required to access the water bottles, visits to the bottles were comparable across groups. This indicates that there were no differences in locomotor activity, motivation to explore and reward value of water. The other three hypotheses remain valid possibilities. Additionally, it should be considered that multiple alterations could be present together. Both spatial operant tasks (chamber position learning 1 and 2) were altered. In these tasks, the correct sites for the behavioral response were determined by the spatial position and by the presence of spatially-specific visual cues (the three multicolor LEDs). Nevertheless, the fact that alterations were present also in the non-spatial operant tasks (basic nosepoke learning and skilled nosepoke learning), in which nosepoking (inserting the nose in a hole) is required to access water but regardless of the spatial position (any chamber and any door lead to the reward), suggests that an alteration of spatial memory alone cannot be responsible for the observed behavioral phenotype and that also an alteration of the basic ability to form stimulus-response links is present. In operant conditioning a behavioral response (as nosepoking or approach) is linked to a stimulus (as a luminous visual cue or a specific place). Insula could be involved in behavioral reactivity to the stimulus. Indeed, a previous study on mice found that insula inhibition impaired cue-reactivity (Kusumoto-Yoshida et al., 2015). Future experiments, employing specifically designed behavioral protocols, could help to dissect the role of insula in modulating these single components of operant learning.

The majority of the studies on the effects of baclofen and CGP35348 have focused on the hippocampus as the central node of memory regulation (Arolfo et al., 1998; Deng et al., 2009; Gillani et al., 2014; Lee et al., 2016). Our previous study, for the first time, showed that $GABA_BRs$ in the insula are involved in memory regulation (Wu et al., 2017). The present study showed that $GABA_BRs$ in the insula are also involved in the regulation of operant associative memory in epileptic rats. These findings shed light on the site and mechanisms of memory regulation and spur the development of novel treatments for patients with cognitive impairment. Importantly, in the present work we did not employ healthy rats. We focused on epilectic rats because our main aim was to understand if positive and negative modulation of insular gabaergic neurotransmission can lead to, respectively, reduced and increased memory also in epileptic rats, similarly to what we had previously found in healthy rats (Wu et al., 2017). In future experiments, it will be useful to test together six groups of rats: healthy-sham, healthy-BLF, healthy-CGP, epileptic-sham, epileptic-BLF and epileptic-CGP. The comparison between the two sham groups (receiving no drugs) will indicate if a cognitive impairment is present in epileptic rats for these tasks. On the other hand, the other groups will show if the drug-induced increase and decrease of cognitive function is of comparable size between healthy and epileptic rats. Next, we plan to investigate the role of the insula in the regulation of cognitive behavior, the underlying molecular mechanisms, and its interactions with the other brain regions of the memory network. Also, the Intellicage system would be utilized in future studies because, unlike many conventional behavioral tests that require a high degree of animal handling and interaction with the experimenter, the automated Intellicage system provides an environment that closely resembles a natural social context with minimal human interference.

In summary, we found that $GABA_BRs$ in the insula bidirectionally regulate the operant associative memory of epileptic rats. Cognitive impairment induced by stimulation of $GABA_BRs$ and cognitive enhancement induced by inhibition of $GABA_BRs$ should be taken into account when evaluating new possible treatments for people with epilepsy.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by Rats were provided from the Animal Center of Ningxia Medical

University (China) and all experiments were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China on October 31, 1988, No. (2016-124). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

NW contributed to experimental designing, planning of the experiments, performing the experiments, data analysis, and writing the manuscript. TS contributed to experimental designing, planning of the experiments, and manuscript writing. XW took part in experimental designing, planning of the experiments, data analysis, and writing the manuscript. HC contributed to experimental designing and planning of the experiments. ZZ contributed to experimental designing, planning of the experiments, data analysis, data visualization, manuscript writing, manuscript revision, and funding acquisition. All authors read and approved the final manuscript.

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Conflict of interest

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

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Longitudinal home-cage automated assessment of climbing behavior shows sexual dimorphism and aging-related decrease in C57BL/6J healthy mice and allows early detection of motor impairment in the N171-82Q mouse model of Huntington's disease

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Monitoring the activity of mice within their home cage is proving to be a powerful tool for revealing subtle and early-onset phenotypes in mouse models. Video-tracking, in particular, lends itself to automated machine-learning technologies that have the potential to improve the manual annotations carried out by humans. This type of recording and analysis is particularly powerful in objective phenotyping, monitoring behaviors with no experimenter intervention. Automated home-cage testing allows the recording of non-evoked voluntary behaviors, which do not require any contact with the animal or exposure to specialist equipment. By avoiding stress deriving from handling, this approach, on the one hand, increases the welfare of experimental animals and, on the other hand, increases the reliability of results excluding confounding effects of stress on behavior. In this study, we show that the monitoring of climbing on the wire cage lid of a standard individually ventilated cage (IVC) yields reproducible data reflecting complex phenotypes of individual mouse inbred strains and of a widely used model of neurodegeneration, the N171-82Q mouse model of Huntington's disease (HD). Measurements in the home-cage environment allowed for the collection of comprehensive motor activity data, which revealed sexual dimorphism, daily biphasic changes, and aging-related decrease in healthy C57BL/6J mice. Furthermore, home-cage recording of climbing allowed early

detection of motor impairment in the N171-82Q HD mouse model. Integrating cage-floor activity with cage-lid activity (climbing) has the potential to greatly enhance the characterization of mouse strains, detecting early and subtle signs of disease and increasing reproducibility in preclinical studies.

KEYWORDS

automated, neurodegeneration, motor function, reproducible, welfare, Huntington's disease (HD), digital biomarkers

1. Introduction

Advances in the field of genetics mean that mouse models are increasingly sophisticated, and more closely than ever before the model human disease (Mingrone et al., 2020). For diseases affecting neuromuscular systems, there is also an increasing range of assays and tests for mice that measure parameters such as coordination and muscle strength (Mandillo et al., 2014). Disorders of the central nervous system are often accompanied by deficits in motor function, and affect a number of aspects of movement, from locomotion and balance to finer tasks such as reaching and grasping (Tucci et al., 2007; Preisig et al., 2016). Climbing on the cage top and locomotor activity on the cage-floor have been found to be important indicators of motor function and form the natural activity routine of mouse motor behavior (Nevison et al., 1999; Borbélyová et al., 2019).

Currently, a number of tests, including grip strength and gait analysis (Tucci et al., 2007; Preisig et al., 2016), are used to study the progression of degenerative diseases. These are limited to measuring aspects of motor function, which can also be dependent on external factors, such as experimenter expertise, the timing of the test, testing conditions, and the motivation of the test subject (Balzani et al., 2018; Baran et al., 2022). In addition, a number of these tests are known to be affected by subtle factors, such as the order of testing and the amount of handling before testing, and crucially, repeated testing in progressive conditions may itself alter the results of subsequent tests (McIlwain et al., 2001; Paylor et al., 2006; Mingrone et al., 2020). Therefore, issues of reproducibility and consistency have to be overcome as researchers strive for greater translatability in preclinical research. This is particularly true for pharmacological investigations that require chronic administration of substances, especially as the short periods of time and potential external confounders affect the integrity and completeness of the result. The probability of clinical success for substances tested in such studies is therefore reduced (Kaffman et al., 2019).

Investigating perturbations in the home-cage activity of undisturbed mice over extended periods can greatly enrich standard out-of-cage phenotyping and provide novel insights into subtle and progressive conditions at early time points. A number of systems have been developed to investigate motor activity over extended periods of time in single-housed as well as group-housed mice. However, measuring both cage-lid climbing and cage-floor movement simultaneously in group-housed mice remained a technical challenge (Bains et al., 2018).

Over the past few years, there has been a concerted effort toward overcoming these challenges by housing the mice in testing chambers for extended periods of time and automatically measuring non-evoked activity (Bains et al., 2018). Voluntary wheel running has proved to be a robust indicator of motor-function deficits from an early stage, as it measures a number of motor parameters over several weeks (Lana-Elola et al., 2021). However, concerns such as single housing and the detrimental effect of exercise on certain genetically altered mouse strains that serve as models for diseases such as Huntington's disease (HD; Corrochano et al., 2018), including the model used in the current study, in which wheel running may itself affect the phenotype expression, remain an issue. In addition, the subtler indicators of changes in motor function, such as activity around anticipation to light phase change, are not identified through wheel-running activity (Bains et al., 2016).

Despite some early promise shown by gene-targeting therapies, there is currently no disease-modifying treatment for HD, and therapy is focused on the management of symptoms and improving quality of life (Kim et al., 2021; Kwon, 2021). Progressive loss of neurons is a characteristic feature of this neurodegenerative condition. HD is caused by an unstable expansion within the trinucleotide poly(CAG) tract in exon 1 of the huntingtin gene, located on the short arm of Chromosome 4 (Menalled et al., 2012; Corrochano et al., 2014; Cepeda and Tong, 2018), and is characterized by progressive motor deficits such as loss of coordination, tremors, and hypokinesis (Schilling et al., 1999). The hallmark histopathology of HD is cell death in the striatum and cerebral cortex, which results in miscommunication between the basal ganglia and the cerebral cortex. This manifests as uncontrolled, involuntary movements (chorea), cognitive deficits, and psychiatric symptoms (Cepeda and Tong, 2018). The condition is a progressive, ultimately fatal, neurodegenerative disorder.

This study used the B6-TgN(HD82Gln)81Dbo/H, also known as the N171-82Q, model of HD, first published in 1999, in which damage to the basal ganglia structures causes a hyperkinetic disorder (chorea) in combination with a loss of voluntary movements (bradykinesia and rigidity). These phenotypes become evident at around 10.5 weeks of age and manifest as abnormal gait and other behavioral and physiological abnormalities, such as lower grip strength, disturbed limb dynamics, and rigidity of the trunk, as well as a tendency toward a lower body weight (Schilling et al., 1999; Ferrante, 2009; Preisig et al., 2016). Automated analysis of gait in the lateral and ventral plane has proved to be very useful in the early detection of the subtle changes in limb movement that

recapitulate the hyperkinetic phenotype observed in this model, which is detected as early as 10.5 weeks of age (Preisig et al., 2016).

Voluntary locomotion in mouse disease models is highly clinically relevant because it provides an insight into the physiology of the condition, as well as the behavioral motivation of the individual, and is a fundamental readout of the phenotype used in the diagnosis in human patients (Karl Kieburtz et al., 1996; Reilmann et al., 2014). A method that measures the ways in which animals move in non-provoked situations could potentially be a powerful tool for detecting early, and complex, temporal phenotypes.

Advanced image analysis, which can highlight changes in the animal's gait in both the lateral and the ventral plane, has thus far proven to be the most sophisticated way of extracting subtle motor phenotypes earlier than 13 weeks of age in the model used in our study (Preisig et al., 2016).

In this study, we demonstrate a new tool for the automated analysis of motor activity, which encompasses climbing as well as locomotion on the cage-floor, in undisturbed mice over multiple light: dark cycles. Through its application to the N171-82Q model of HD, we have uncovered a robust complex phenotypic profile for disease progression, including early features of motor dysfunction that are fundamental in developing reproducible digital biomarkers for therapeutic testing, especially when targeting the prodromal stages of HD.

In meeting these challenges, we developed an algorithm to automatically annotate climbing behavior from high-definition video captured from a side-on view of the home-cage. The resulting automated climbing behavior annotations provide an important additional parameter set that further enriches the activity profile captured by the Home Cage Analyser system (HCA; Actual Analytics Ltd., UK; Bains et al., 2016). Our study shows that it is possible to measure two robust indicators of activity simultaneously (cage-floor activity and cage-lid activity) in group-housed mice from the inbred strain C57BL/6J. Using this technology, we have also demonstrated the advantages of using a more comprehensive recording of motor activity to reveal early signs of degeneration in a genetically altered mouse model of HD (N171-82Q; Schilling et al., 1999).

2. Materials and methods

2.1. Animals and husbandry

All mice used in the study were bred in the Mary Lyon Centre at MRC Harwell and were housed in individually ventilated cages (IVCs; Tecniplast BlueLine 1284) in groups of three mice per cage on Eco-pure spruce chips grade 6 bedding (Datesand, UK), with shredded article shaving nesting material and small cardboard play tunnels for enrichment. The mice were kept under controlled light (light 07:00–19:00; dark 19:00–07:00), temperature (22°C ± 2°C), and humidity (55% ± 10%) conditions. They had free access to water (25 p.p.m. chlorine) and were fed *ad libitum* on a commercial diet [SDS Rat and Mouse No.3 Breeding diet (RM3)]. All procedures and animal studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986, UK, Amendment Regulations 2012 (SI 4 2012/3039).

For the first study, 18 male and 18 female mice, in six cages of three mice each, from the inbred strain C57BL/6J were recorded at three time points: 13–14 weeks, 30–31 weeks, and 52–53 weeks of age. For the second study, mice from the mutant strain B6-TgN (HD82Gln) 81Dbo/H (HD), were recorded at three time points: 8 weeks, 13 weeks, and 15–16 weeks of age. Twenty-seven hemizygous (Hemi) males carrying the HD transgene, along with 24 male wild type (WT) littermate controls, and 33 hemizygous females carrying the HD transgene, along with 24 female WT mice, were housed in same-genotype groups of three mice per cage. Using the cage as the experimental unit, a sample number of six was calculated to be the most appropriate sample size based on data from previous studies (**Supplementary Dataset 1**). Therefore, the first study of C57BL/6J mice, had $n = 6$ for both sexes. Additional animals were included in the HD study to compensate for the high attrition rate experienced with this model. Therefore, 9–11 cages of hemizygous mice were included in the study to avoid under-powering the later time points (**Supplementary Table 1**, **Supplementary Dataset 2**). Data from all cages were included in the analysis and appropriate statistical methods (described below) were used to account for the differences in the group sizes. Mice were housed with colony-mates born within the same week in cages containing animals of the same genotype.

Three days prior to recording sessions, the animals were transferred to clean home cages with fresh bedding, nesting material, and a cardboard rodent tunnel as enrichment material, in line with the standard husbandry procedures for IVC cages. The cages were then placed in an IVC rack in the experimental room for the animals to acclimatize. For each recording, the cages were randomly assigned to an HCA rig. On the first day of recording, each cage was placed onto the ventilation system, within the rig, as would occur during a normal husbandry procedure.

Animal welfare checks were carried out visually twice daily. At the end of the recording period, the home cages were removed from the HCA rigs and returned to their original positions on the IVC racks.

2.2. Microchipping and data collection

Radio frequency identification microchips were injected subcutaneously into the lower left or right quadrant of the abdomen of each mouse at 12 weeks of age for the C57BL/6J study and 7 weeks of age for the B6-TgN(HD82Gln)81Dbo/H study. These microchips were contained in standard ISO-biocompatible glass capsules (11.5 × 2 mm; PeddyMark Ltd., UK). The procedure was performed on sedated mice (Isoflo; Abbott, UK) after topical application of local anesthetic cream on the injection site prior to the procedure (EMLA Cream 5%; AstraZeneca, UK) as described in Hobson et al. (2020). The animals were allowed to recover from the microchip procedure for at least 1 week before being placed in the HCA rigs for data collection. The procedure for data collection has been described previously in Bains et al. (2016) and Hobson et al. (2020), briefly, microchipped animals were placed in the HCA rigs for 72 h of continuous recording for every age group. At the end of the 72-h recording period for that age, the animals in their IVCs were returned to the standard IVC rack, until the animals reached the next age

group to be recorded, where the procedure was repeated for another 72 h.

2.3. Measurement of climbing and validation

Mice were housed in an individually ventilated cage which has a metal wire lid that allows climbing. Mouse activity was videorecorded through a high definition, infra-red camera of the Home Cage Analyser system (HCA; Actual Analytics Ltd., UK), and videos were subsequently analyzed offline through a video-recognition algorithm. Through this method, climbing behavior is measured on a frame-by-frame basis by numerically characterizing the pattern of motion occurring within a pre-defined region around the cage lid and quantifying its similarity to a set of key reference examples of climbing and non-climbing behavior (selected programmatically from a large set of human "training" annotations) to yield a classification decision. More specifically, the local trinary pattern representation proposed by Yeffet and Wolf (2009), is used to characterize motion within a 690 × 385 pixel region adjacent to the cage lid as a 16,384-dimensional vector; this particular representation was shown in Burgos-Artizzu et al. (2012) to provide an effective, yet computationally efficient, means of distinguishing between different mouse behaviors. Local trinary pattern vectors were extracted for every video frame across more than 7 h of human-annotated video footage—encompassing over 130 separate bouts of climbing—and were used to train a linear support vector machine classifier (SVM; Fan et al., 2008) to distinguish between climbing and non-climbing instances. To leverage the correlation between consecutive video frames, a temporal voting window was applied, such that the final classification of a given video frame represented the consensus over a wider time period spanning the frame in question (the underlying logic is to reduce spurious "single frame" detections, while conversely preventing erroneous "splitting" of longer bouts of climbing on the basis of a single misclassified frame.) A leave-one-out cross-validation procedure was applied over the available "training" set of 15 discrete 30-min video segments, in order to identify: (i) the SVM regularization parameter values; and (ii) the temporal aggregation parameters that—on average—yielded the best generalization performance. The final classifier, generated from the full set of available training data using the parameters revealed by the preceding cross-validation process, was then tested on a further 2.5 h of—unseen—annotated test videos. These videos were scored by three separate annotators, whose consensus was derived using a "majority voting" rule, to yield a single "gold standard" annotation for each video: comparison to this gold standard annotation yielded 86.6% frame-by-frame accuracy (where 76.9% of climbing frames and 89.2% of non-climbing frames were correctly classified, with climbing behavior accounting for approximately 21% of the test data). Considering this test data in terms of 5-min time bins, automatically annotated climbing time correlates well with human annotated climbing time, as confirmed by Spearman's rank coefficient ($\rho = 0.862$; $n = 30$; $p < 0.000000001$).

It is however worth noting that there are other ways to combine the human annotations. For example, during training, a frame was treated as climbing if any one annotator deemed it so (the purpose of this was to ensure that no example of climbing was missed). If the test annotations are combined in this way, a greater proportion of the data is considered to be climbing (approximately 30%), and the accuracy scores of the automated climbing annotations change accordingly (85.6% frame-by-frame accuracy with 65.9% of climbing frames, and 94.3% of non-climbing frames correctly classified). Correlation over 5 min time bins is lower, albeit broadly similar ($\rho = 0.836$; $n = 30$; $p < 0.00000001$).

2.4. Data analysis

2.4.1. Linear mixed-effects modelling

To account for dependence between data recorded over separate days from the same cage (repeated measures) and to avoid pseudo-replication, statistical analyses were conducted using linear mixed-effects modelling. To adjust for parameters with non-normal distributions, data were box-cox transformed prior to analysis. Any subsequent modelling satisfied assumptions of normally distributed residuals.

We constructed a linear mixed-effects model of either distance travelled or time spent climbing (continuous variables) as a function of the effect of sex, age of caged mice, and genotype (all categorical fixed effects). Cage ID was modelled as the random effect intercept with day of recording as the random effect slope. This structure allows for cages to vary randomly in their baseline distance moved or time spent climbing value, and for this relationship to vary randomly according to the day of recording. It will account for time-dependent and cage-specific fluctuations in activity over the 3 days of recording.

$$Mean\ activity \sim Age{:}Genotype{:}Sex + (day\ of\ recording|CageID)$$

This model was compared to other model iterations with different combinations of sex, age, and genotype with or without the interaction term and random effects structure. An ANOVA test was run to determine the statistical significance of the interaction between Age: Genotype: Sex and to inform model selection. Random effects and fixed effects found not to have a statistically significant contribution to model fit were eliminated. Models were fit using R's "lmer" function.

2.4.2. Time-frames of interest

The time-frames of interest in the current study were defined as the 30 min directly preceding lights being turned on (06:30–07:00) and 30 min directly preceding lights being turned off (18:30–19:00). This definition was consistent between both parameters of interest: distance travelled (mm) and time spent climbing (seconds). When analyzing activity during the time-frames of interest, we first summed data per time bin (6 min) for the mouse within a cage for distance travelled and cumulative climbing in the cage per time bin (6 min) for time spent climbing. We then calculated the average activity per cage across the 5 time bins.

One of the early indicators in clinical presentation of a number of neurodegenerative conditions is a perturbed circadian rhythm

(Carter et al., 2021). We have previously shown that C57BL/6J mice show peak activity in the dark phase, and that mouse activity varies around a change in light phases in a strain-specific manner (Bains et al., 2016). In the current study, these changes were particularly relevant, as mouse models of neurodegenerative diseases, including HD, have known sleep disturbances, that manifest as changes to the time of onset and/or offset of the active periods (Werdann and Zhang, 2020). Sleep onset latency towards the end of the active period is a particularly sensitive measure (Morton et al., 2005), therefore the first 30 min and the last 30 min of the active period were chosen as the time frames of interest for further investigation.

2.4.3. *Post-hoc* analysis

We conducted pairwise *post-hoc* comparison tests by computing the estimated marginal means (least-squares means) for factor combinations and correcting for multiple comparisons using the Benjamini–Hochberg method to decrease the false discovery rate. This process was run using R's "emmeans" function, which returned adjusted p values. These values were used to indicate the statistical significance of the genotype effect at various levels of factor combination.

While the algorithm for automated behavior annotation is proprietary, the analysis is openly available as a part of this manuscript; please see "Data availability statement" in this manuscript. The datasets for the experiments in this manuscript are also openly available on request.

All climbing data were converted from a number of frames to time spent climbing in seconds prior to analysis and visualization, as the authors believe that to be a more intuitive parameter. The number of frames is converted to time in seconds as follows:

$$\text{Time Spent Climbing in Seconds} = (\text{Number of frames} * 40)/1{,}000$$

Each individual frame is 40 ms long.

3. Results

3.1. Multiday recording in mouse home cage shows sexual dimorphism in C57BL/6J mice and reveals age-related decrease in activity

The data from the females of inbred strain C57BL/6J show significantly higher cage-floor activity, described as distance travelled in mm (**Figure 1G**) in the dark phase, as compared to males at 3 months of age ($n = 6$, $p < 0.0001$). This difference in cage-floor activity during the dark phase persists as the mice age, as seen in the later time points of 7 months ($p = 0.0001$) and 12 months ($p < 0.01$; **Figure 1G**, $n = 6$). The activity shows a clear circadian rhythm (**Figure 1**). Individual comparisons between each age group, per sex, also reveal statistically significant differences in the dark phase for both females and males at 12 months of age as compared to 3 months and 7 months of age (**Supplementary Figure 1**, **Supplementary Dataset 2**).

3.2. Automated climbing annotation in mouse home cage records complex sexual dimorphism in C57BL/6J mice and reveals age-related decrease in activity

The data from the females of inbred strain C57BL/6J show significantly higher cage-lid climbing, described as time spent climbing in seconds (**Figure 2**), as compared to males at 3 months of age (**Figure 2G**, $n = 6$, $p < 0.0001$), this difference in cage-lid climbing during the dark phase persists as the mice age as seen at the later time points of 7 months (**Figure 2G**, $n = 6$, $p < 0.0001$) and 12 months (**Figure 2G**, $n = 6$, $p < 0.0001$). The activity shows a clear circadian rhythm (**Figure 2**). Individual comparisons between each age group per sex reveal statistically significant differences in the dark phase for females only, at 12 months of age as compared to 3 months and 7 months of age (**Supplementary Figure 2**, **Supplementary Dataset 2**).

3.3. Early detection of activity phenotype in mouse model of Huntington's disease

The data from the HD model recapitulate the clear circadian rhythm and sex differences seen in the inbred strain, in which females were significantly more active than males in both measures of activity (cage-floor activity, as seen in **Figures 3A–C** and **Figures 4A–C** and cage-lid climbing activity, as seen in **Figures 3F–H** and **Figures 4F–H**). The total activity over the dark and light phases of hemizygous (Hemi) HD mice compared to the WT HD mice was not statistically different from each other for both sexes.

There is, however, a specific time-of-day-dependent deficit in activity seen in Hemi HD mice as compared to WT HD mice, at the transition between dark and light phases for females, not seen in the transition between light and dark phases (18:30–19:00; **Figure 3D**). This difference was apparent as early as 8 weeks when the animals showed no overt signs of the disease onset (**Figure 3E**, $n = 8$ WT/11 Hemi, $p < 0.01$). The decrease in cage-floor activity at this time became even more apparent at 13 weeks of age (**Figure 3E**, $n = 7$ WT/7 Hemi, $p < 0.0001$). By 15–16 weeks of age the mice showed clear signs of disease and differences between the two genotypes were distinct (**Figure 3E**, $n = 7$ WT/3 Hemi, $p < 0.0001$).

This decrease in activity at the end of the dark phase (06:30–07:00) is also seen in male Hemi HD mice as compared to WT HD mice, allowing for the phenotype to be detected as early as 8 weeks of age (**Figure 4E**, $n = 8$ WT/9 Hemi, $p < 0.05$), persisting into the next time point of 13 weeks of age (**Figure 4E**, $n = 8$ WT/6 Hemi, $p < 0.05$), and becoming obvious at 15–16 weeks of age (**Figure 4E**, $n = 8$ WT/5 Hemi, $p < 0.001$). Once again this decrease in activity is not seen in the transition between light and dark phases (18:30–19:00; **Figure 4D**).

This specific time-of-day-dependent deficit in cage-floor activity is mirrored in cage-lid climbing, described as time spent climbing, where female Hemi HD mice show a significant decrease in time spent climbing compared to female WT HD mice, at the transition between dark and light phases (06:30–07:00), not

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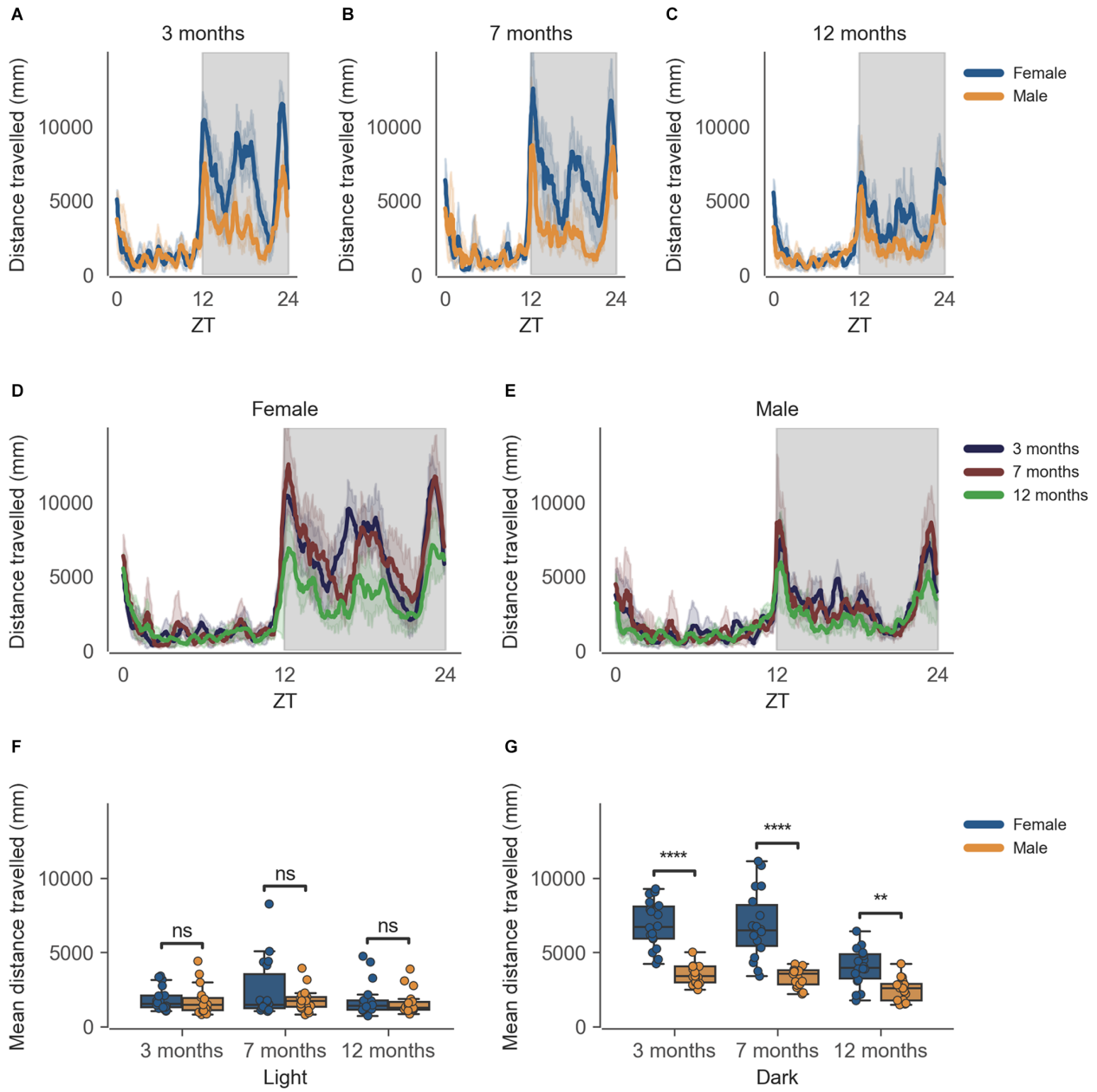

FIGURE 1
Distance travelled by mice during the dark phase varies according to sex and across age during passive home-cage monitoring. **(A)** Distance travelled (mm) over zeitgeber time in female and male cages of 3-month-old mice during the recording session, binned into 6-min time bins and averaged over a 24-h period. The line represents the mean distance over time across cages of a sex group; the shaded error band represents a 95% confidence interval. Data from individual mice within a cage were summed to produce one time series per cage. The gray shaded areas on the plot represent darkness. **(B,C)** Same as **(A)** but for 7-month-old and 12-month-old mice, respectively. **(D)** Distance travelled over zeitgeber time in female cages of mice of all ages. The line represents the mean distance over time across cages of a sex and age group; the error-shaded area represents 95% confidence interval. **(E)** Same as **(D)** but for male cages. **(F)** Boxplot of mean distance travelled during light phase with cages split by age and by sex. Distance travelled within the light phase was averaged across time per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(G)** Same as **(D)** but for the dark phase. ns $p > 0.05$, $^{**}p < 0.05$, $^{****}p < 0.0001$.

seen in the transition between light and dark phases (18:30–19:00; Figure 3I). Once again, this difference was apparent as early as 8 weeks when the animals showed no overt signs of the disease onset (Figure 3J, $n = 8$ WT/11 Hemi, $p < 0.0001$). As seen with cage-floor activity, the decrease in cage-lid climbing at this time became even more apparent at 13 weeks of age (Figure 3J, $n = 7$ WT/7 Hemi, $p < 0.0001$) and by 15–16 weeks of age the mice showed clear signs of disease and differences between the two genotypes were distinct (Figure 3J, $n = 7$ WT/3 Hemi, $p < 0.0001$).

As with cage-floor activity, time spent climbing also follows the same pattern in male Hemi HD mice as

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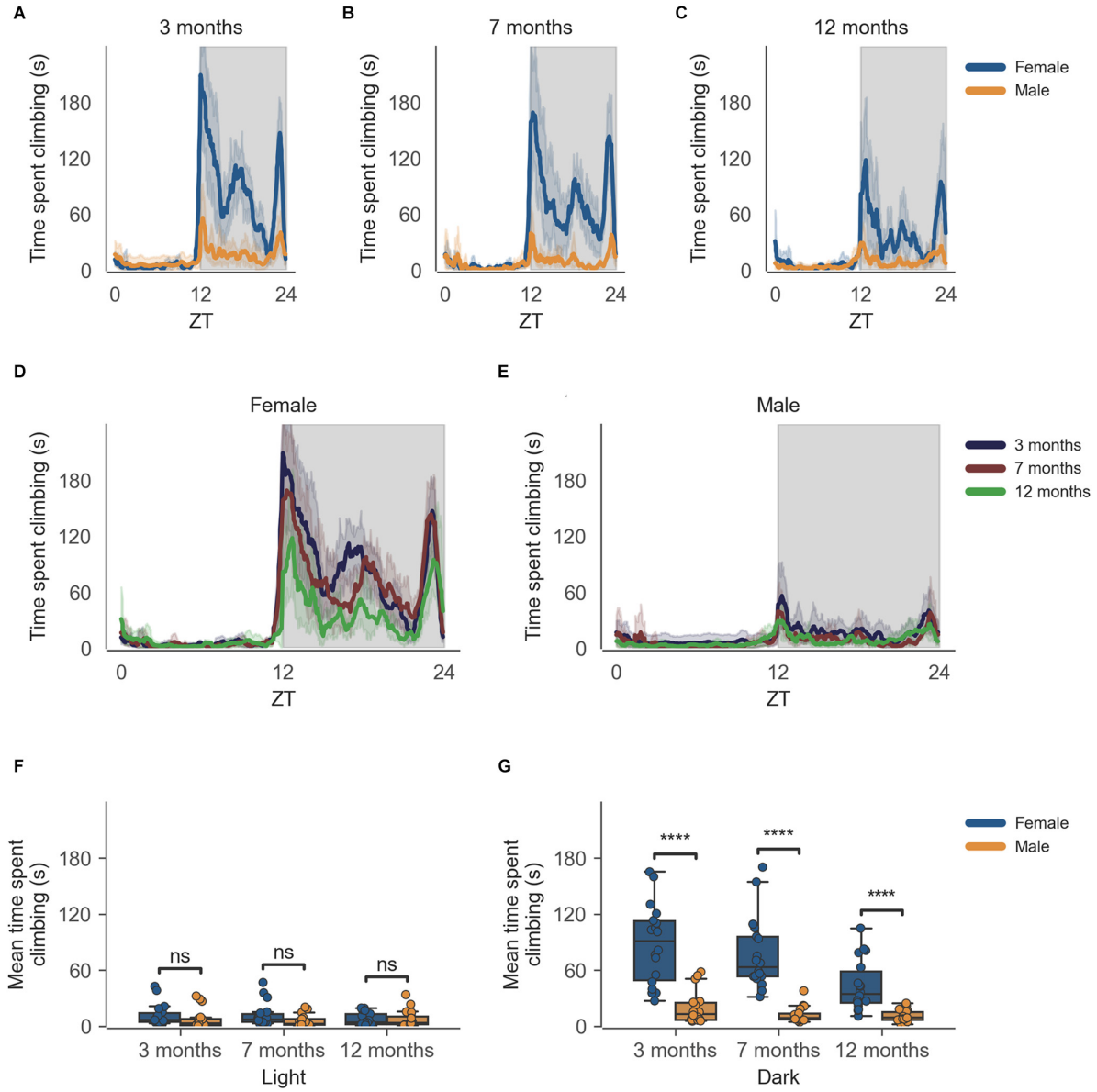

FIGURE 2
Time spent climbing by mice during dark phase varies according to sex and across age during passive home-cage monitoring. **(A)** Time spent climbing over zeitgeber time in female and male cages of 3-month-old mice during recording session, binned into 6-min time bins and averaged over a 24-h period. Line represents mean time spent climbing over time across cages of a sex group; the shaded error band represents a 95% confidence interval. Data from individual mice within a cage were summed to produce one time series per cage. The gray shaded areas on the plot represent darkness. **(B,C)** Same as **(A)** but for 7-month-old and 12-month-old mice, respectively. **(D)** The time spent climbing over zeitgeber time in female cages of mice of all ages. The line represents the mean distance over time across cages of a sex and age group; the error-shaded area represents a 95% confidence interval. **(E)** Same as **(D)** but for male cages. **(F)** Boxplot of mean time spent climbing moved during light phase with cages split by age and by sex. Time spent climbing within the light phase was averaged across time per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(G)** Same as **(D)** but for the dark phase. ns $p > 0.05$, ****$p < 0.0001$.

compared to male WT HD mice, where a statistically significant difference in genotypes is observed at 8 weeks of age (Figure 4J, $n = 8$ WT/9 Hemi, $p < 0.05$), persisting to the next time point of 13 weeks of age (Figure 4J, $n = 8$ WT/6 Hemi, $p < 0.001$) and becoming obvious at 15–16 weeks of age (Figure 4J, $n = 8$ WT/5 Hemi, $p = 0.0001$). Again this decrease in activity is not seen in the transition between light and dark phases (18:30–19:00; Figure 4I).

4. Discussion

Classically, with a few exceptions, all behavior testing is carried out during the light phase, in which resting animals are removed from their home cage and placed in a novel environment away from their cage mates (Bains et al., 2018). Such out-of-cage tests are known to be influenced by factors such as ambient noise, lighting and odors, and handling methods, resulting in stress and anxiety-like responses. Home-cage monitoring, by contrast, is free

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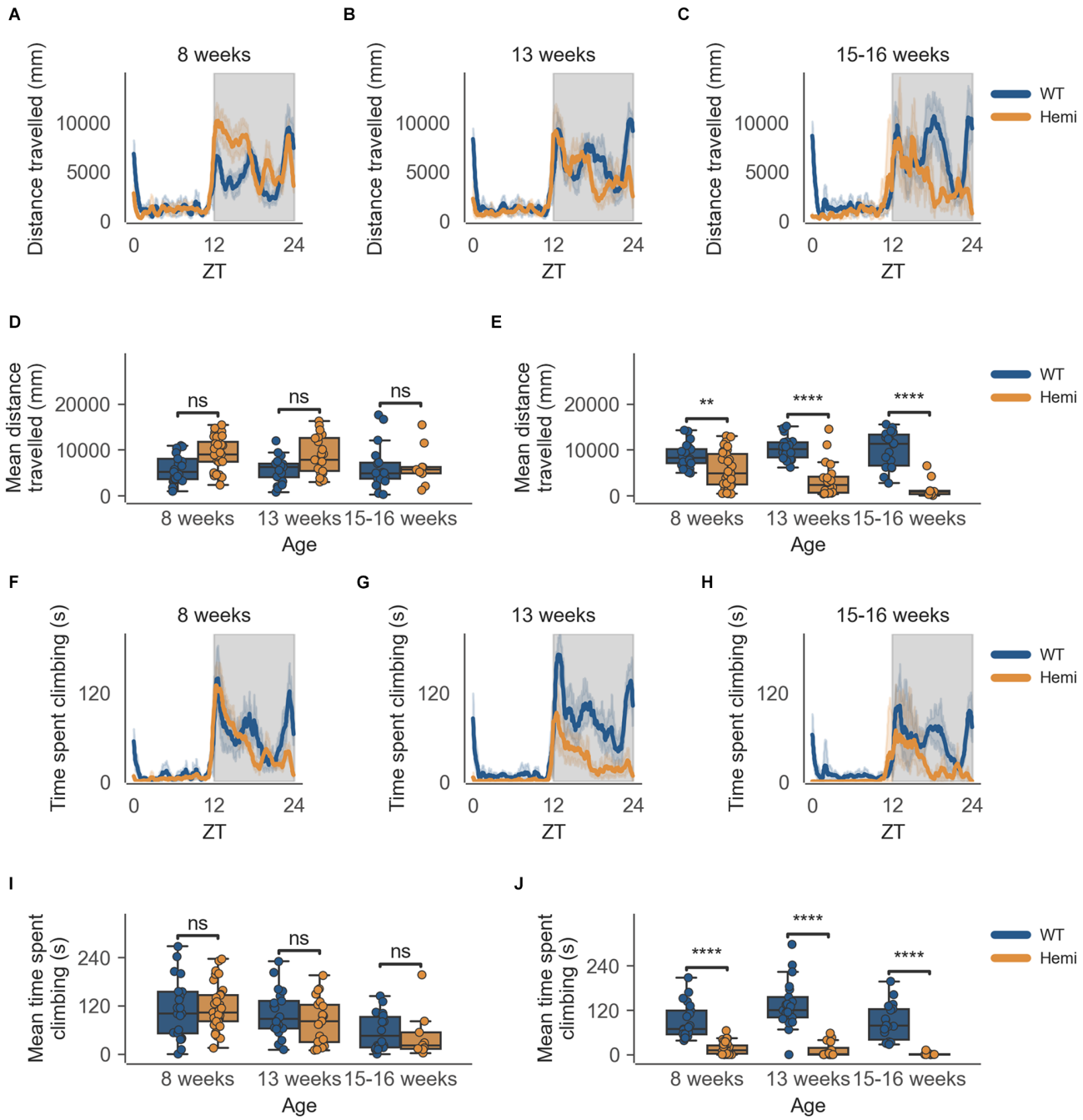

FIGURE 3
Distance travelled and climbing activity varies according to genotype in female mice and across age at the conclusion of the dark phase, but not at beginning of the dark phase. **(A)** Distance travelled over zeitgeber time in female-only cages of 8-week-old mice, split according to genotype, during the recording session, binned into 6-min time bins and averaged over a 24-h period. The line represents the mean distance over time across cages of a genotype group, the shaded error band represents a 95% confidence interval. Data from individual mice within a cage was summed to produce one time-series per cage. The gray shaded areas on the plot represent darkness. **(B,C)** Same as **(A)** but for 13-week-old and 15–16-week-old mice, respectively. **(D)** Boxplot of mean distance travelled during first 30 min of darkness within female-only cages split by age and genotype. Distance travelled was averaged across the first 30 min of darkness per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(E)** Same as **(D)** but for last 30 min of darkness. **(F)** Time spent climbing over zeitgeber time in female-only cages of 8-week-old mice, split according to genotype, during the recording session, binned into 6-min time bins and averaged over a 24-h period. The line represents the mean time spent climbing over time across cages of a genotype group, the shaded error band represents a 95% confidence interval. Data from individual mice within a cage was summed to produce one time-series per cage. The gray shaded areas on the plot represent darkness. **(G,H)** Same as **(F)** but for 13-week-old and 15–16-week-old mice, respectively. **(I)** Boxplot of mean time spent climbing during first 30 min of darkness within female-only cages split by age and genotype. Time spent climbing was averaged across the first 30 min of darkness per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(J)** Same as **(I)** but for last 30 min of darkness. ns $p > 0.05$, **$p < 0.01$, ****$p < 0.0001$.

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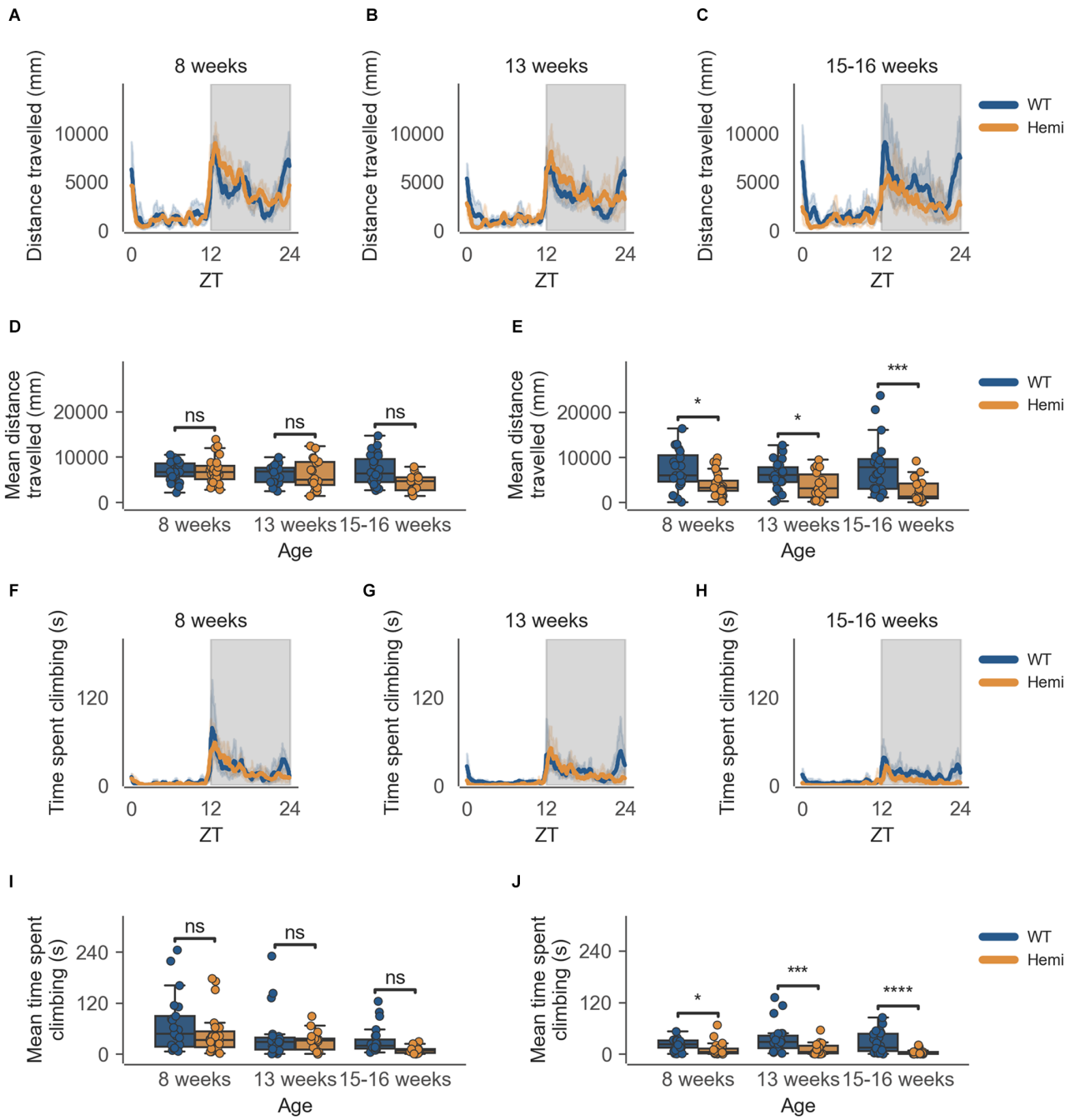

FIGURE 4
Distance travelled and climbing activity varies according to genotype in male mice and across age at conclusion of dark phase, but not at beginning of dark phase. **(A)** Distance travelled over zeitgeber time in male-only cages of 8-week-old mice, split according to genotype, during the recording session, binned into 6-min time bins and averaged over a 24-h period. Line represents mean distance over time across cages of a genotype group, the shaded error band represents a 95% confidence interval. Data from individual mice within a cage was summed to produce one time-series per cage. The gray shaded areas on the plot represent darkness. **(B,C)** Same as **(A)** but for 13-week-old and 15–16-week-old mice, respectively. **(D)** Boxplot of mean distance travelled during first 30 min of darkness within male-only cages split by age and genotype. Distance travelled was averaged across the first 30 min of darkness per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(E)** Same as **(D)** but for last 30 min of darkness. **(F)** Time spent climbing over zeitgeber time in male-only cages of 8-week-old mice, split according to genotype, during the recording session, binned into 6-min time bins and averaged over a 24-h period. The line represents the mean time spent climbing over time across cages of a genotype group, the shaded error band represents a 95% confidence interval. Data from individual mice within a cage was summed to produce one time-series per cage. The gray shaded areas on the plot represent darkness. **(G,H)** Same as **(F)** but for 13-week-old and 15–16-week-old mice, respectively. **(I)** Boxplot of mean time spent climbing during first 30 min of darkness within male-only cages split by age and genotype. Time spent climbing was averaged across the first 30 min of darkness per cage and per day. Three data points per cage (3 days of recording) were modelled using a linear mixed-effects model to account for repeated-measures and least-squares means estimated to return adjusted p values of levels of factor combinations. **(J)** Same as **(I)** but for last 30 min of darkness. ns $p > 0.05$, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

of such influences as these behavioral confounds are removed. Therefore, the welfare burden on the animals is much lower. Avoidance of stressors deriving from out-of-cage testing, on the one hand, leads to a more animal-friendly behavioral testing and, on the other hand, increases the validity and reproducibility of results (Voikar and Gaburro, 2020; d'Isa and Gerlai, 2023). Two further advantages of home-cage testing are the possibility to test the animals in a social environment, while they are group-housed, and the possibility to record behaviors outside of the normal observation hours, allowing for continual monitoring of progressive phenotypes over both light and dark cycles and not just at specific time points. Phenotyping through tests lasting a few minutes performed during the conventional working hours of researchers is an approach that risks missing critical milestones for disease emergence and/or progression. This is particularly true for aged mice, as it has been shown that spontaneous locomotor activity declines with age (Nakamura et al., 2011; Yanai and Endo, 2021) and snapshots of these milestones may not be enough to reveal complex phenotypes that change with time of day, or are particularly exaggerated at certain times of the day in relation to the light: dark cycle, as shown in this study.

The advantage of observing the mice undisturbed within their home cage over multiple light:dark cycles is that, in addition to the observed phenotype, it is also possible to disentangle the temporal appearance of such phenotypic traits by focusing the analysis on specific time-frames of interest. The data from C57BL/6J show that there is a clear sexual dimorphism in the overall activity of the animals and that both males and females show peak activity in the dark phase. These data, therefore, point to a clear circadian influence. This method of analysis allows one to also investigate the influence of ultradian parameters on measures such as cage-floor activity and cage-lid climbing. The importance of understanding these circadian and ultradian effects is highlighted in the HD study, in which the males show very low baseline activity in both mutant and WT strains. We have already shown that the HCA system is capable of detecting statistically significant changes in activity around the light phase changes between various background strains (Bains et al., 2016). Here we extend this concept to draw out clinically relevant, subtle, and early phenotypic changes by focusing on specific times of interest such as the first 30 and last 30 min of the dark phase.

Through the current study, we showcase a recently developed automated behavior annotation tool for climbing behavior in standard IVCs under group-housed conditions. We have previously shown the capabilities of the system in investigating cage-floor activity in group-housed mice in their home cages (Bains et al., 2016). Here, we show that measuring climbing is part of the standard motor behavior repertoire of mice and can greatly enhance the existing dataset to investigate motor phenotypes in much greater detail and with minimal experimenter intervention.

Sexual dimorphism in climbing behavior has been reported previously in singly tested C57BL/6Ntac mice, using the LABORAS system, in which the main aim of the study was to investigate the difference in response to novelty between the sexes. Furthermore, parameters were only measured for 10 min (Borbélyová et al., 2019). More recently, a study on the effect of age, sex, and strain on cage-lid climbing in single-housed mice has also reported sexual dimorphism, as well as strain and age differences, in single-housed mice, over 24 h, peaking in the dark phase (Zhang et al., 2021). The data from the inbred strain C57BL/6J, in the current study show significantly high cage-lid climbing as well as cage-floor activity in females as compared to males at all three age time points, with most of the activity observed in the dark phase. To the best of our knowledge, this is the first system of its kind that can detect cage-level climbing activity in group-housed mice within their home cage for extended periods of time, without the need for removal into specialized and/or novel caging.

Serious motor and cognitive deficits that are the hallmark of HD are often preceded, by decades, by more subtle changes in circadian rhythms and motor function (Wang et al., 2018; Wiatr et al., 2018). Therefore, an approach that screens for the chronic and progressive nature of such conditions is more clinically relevant than one that screens for acute signs of motor deficits that manifest at a much later stage of the disease. One such approach is to focus on behaviors that are elective and not essential to survival, such as grooming, playing, or climbing, as they reflect the animal's emotional or motivational state, which would be ethologically more relevant for a preclinical model (Zhang et al., 2021). Therefore, a perturbation in such behaviors could reflect a suboptimal health state, especially in conditions that are chronic and progressive rather than acute.

The onset of HD is often insidious and progressive and the phenotypes are biphasic; at early stages of the condition involuntary functions are affected and in the later stages the directly controlled, voluntary functions begin to fade. This means that motor phenotypes are often expressed as hyperkinesia in the early stages and akinesia in the later stage (Kim et al., 2021). Therefore, it becomes imperative to investigate such conditions longitudinally and for extended periods of time as the "snapshot in time" investigations, such as those that investigate motor activity in an open field, may not be representative of a clinically relevant disease profile.

The HD data in the current study show a significant increase in signal in both cage-floor and cage-lid climbing activities around the transition between the light-to-dark and dark-to-light phases. There is ample evidence to show that spontaneous cage-lid climbing is mediated through the dopaminergic system and therefore depends on the motivation and arousal state of the mouse (Costall et al., 1982; Palmiter, 2008; Brooks and Dunnett, 2009). As mice are active during the dark phase, the arousal states coincide with the transition periods between the light and dark phases of the circadian cycle; we have already shown that the most active periods as seen from cage-floor activity, are around these transition times (Bains et al., 2016). The current study shows that this is also true for cage-lid climbing. In addition, despite the decrease in total activity with age, this increase in cage-lid climbing and cage-floor activity around dark-to-light phase transition persists. This aspect is of particular interest in those models in which the genetic manipulation modelling the disease would result in a greater decrease in activity with age, as compared to wild-type counterparts. However, as the activity in the wild-type mice also decreases with age, any decrease in activity due to the genotype is therefore hard to discern in conventional testing paradigms.

The importance of this finding is highlighted in the set of experiments carried out using the mouse model of HD, N171-82Q. These data recapitulate the sex differences seen in the C57BL/6J strain experiments, in which females were significantly more active

than males in both measures of activity, with this difference persisting across all time points. However, of note is a specific time-dependent decrease, compared with WT mice, in cage-lid climbing activity at the transition between dark and light phases, which was apparent as early as 8 weeks, when the animals showed no overt signs of the disease onset. The decrease in cage-floor activity at this time was also observed at 8 weeks of age; however, the decrease in cage-floor activity became even more apparent at 13 weeks of age. By 15–16 weeks of age the mice showed clear signs of disease and differences between the two genotypes were distinct. This last finding is of particular interest as clinical case studies, as well as mouse models of HD, are known to present with sleep disturbances, one of the hallmarks of the condition (Pallier et al., 2007). Whilst the mechanism is not fully understood, there is some evidence that this change may be attributed to increased pathology either in the brain region controlling circadian rhythms—the suprachiasmatic nucleus—or in a pathway further downstream (Pallier et al., 2007).

This study recapitulates the aspect of the disease in which the offset of activity in HD mice is observed sooner than that in their WT counterparts, for both cage-floor activity, as well as cage-lid climbing. Indeed, a 2005 study comparing the circadian activity patterns of human patients with a different mouse model of Huntington's disease (R6/2), reported a similar pattern of decline in activity towards the end of the active phase with disease progression (Morton et al., 2005). In human patients, this manifests as spending longer time in bed. However, in the absence of a complete circadian screen, which would be outside the scope of this study, it would not be over-anthropomorphizing to say that HD mice begin their rest period earlier than their wild-type counterparts and remain at rest for longer, from the earliest stages of the disease.

In progressive degenerative conditions, neuronal dysfunction occurs before any overt signs of the condition become apparent in the behavior. As neurons are unable to regenerate, most therapies under development focus on neuroprotection, with the aim of slowing the progression of the disease and, where possible, delaying the onset (Jin et al., 2014; Kumar et al., 2015). This necessitates the development of models in which the therapeutic window aims to target the pre-manifestation period, in order to minimize neuronal loss. Therefore, any models that can identify the earliest manifestation of the mutation are invaluable in the investigation of the disease progression and identification of early biomarkers (Levine et al., 2004).

It is important to remember, however, that animal behavior is complex, and that external factors, such as the effects of diet and exercise, can have an impact on disease progression (Dutta et al., 2021). The model used in the current study, N171-82Q, is known to have a more variable phenotype than that of the more severe R6/2 model, even though the motor phenotype and weight loss generally becomes evident at 11 weeks of age (Ferrante, 2009). Such disease models are generally complex in their development, and part of the required improvement in animal research is the development of tools with the ability to capture this complexity both in terms of different phenotypes measured and the timings of their appearance. The factors driving spontaneous cage-lid climbing are not fully understood, but it is clear that this activity is affected by a decline in welfare (Zhang et al., 2021). Thus, we can say that investigating the total non-evoked motor function repertoire of animals in progressive and degenerative conditions is the first step toward early phenotype recognition and this approach can be extended to other mutant models showing complex phenotypes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

Animal studies described here were subject to the guidance issued by the Medical Research Council in Responsibility in the Use of Animals for Medical Research (July 1993), were dependent on an institutional Animal Welfare and Ethical Review Body evaluation and were carried out in compliance with the Animals (Scientific Procedures) Act 1986, UK, Amendment Regulations 2012 (SI 42012/3039).

Author contributions

RB was responsible for the experimental design, experimental procedure, data collection and manuscript preparation. HF carried out bioinformatics and statistical analysis. RS was responsible for the system design, including automated climbing algorithm. JA contributed to the study design and system design. MS contributed to the study design. PN contributed to the study design, carried out circadian and activity data analysis, and prepared the manuscript. SW contributed to the study design, including animal procedures, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

RS and JA were employed by and were shareholders in Actual Analytics Ltd at the time the research was performed and therefore

declare a competing financial interest. Actual HCA is commercially available from Actual Analytics Ltd.

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

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Supplementary material

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Assessing spatial learning and memory in mice: Classic radial maze versus a new animal-friendly automated radial maze allowing free access and not requiring food deprivation

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The radial arm maze (RAM) is a common behavioral test to quantify spatial learning and memory in rodents. Prior attempts to refine the standard experimental setup have been insufficient. Previously, we demonstrated the feasibility of a fully automated, voluntary, and stress-free eight-arm RAM not requiring food or water deprivation. Here, we compared this newly developed refined RAM to a classic manual experimental setup using 24 female 10–12 weeks old C57BL/6J mice. We used a lipopolysaccharide (LPS)-induced model of systemic inflammation to examine long-term cognitive impairment for up to 13 weeks following LPS injection. Both mazes demonstrated robust spatial learning performance during the working memory paradigm. The refined RAM detected spatial learning and memory deficits among LPS-treated mice in the working memory paradigm, whereas the classic RAM detected spatial learning and memory deficits only in the combined working/reference memory paradigm. In addition, the refined RAM allowed for quantification of

an animal's overall exploratory behavior and day/night activity pattern. While our study highlights important aspects of refinement of the new setup, our comparison of methods suggests that both RAMs have their respective merits depending on experimental requirements.

KEYWORDS

memory, spatial learning, behavioral test, radial arm maze (RAM), maze, automation, LPS (lipopolysaccharide)

Introduction

Replacement, Reduction, and Refinement constitute the 3R principles which have guided animal behavior research since its introduction in 1959 (Russell and Burch, 1959). Although considerable progress has been made in the past decades toward achieving these principles (Fenwick et al., 2009; Bayne et al., 2015; Lewis, 2019; Lee et al., 2020), reducing an animal's pain, suffering, and distress while ensuring scientific validity of results remains a constant challenge.

Mazes are commonly used for behavioral tests to assess spatial learning and memory in rodents. Among a variety of different types, the eight-arm radial arm maze (RAM) is one of the most frequently used methods. It was introduced by Olton and Samuelson (1976) and has since been used to test the cognitive performance of mice in various disease models including Alzheimer's disease (Choi et al., 2018), posttraumatic stress disorder (El Hage et al., 2006), depression (Yadav et al., 2013), and sepsis (Semmler et al., 2007; Weberpals et al., 2009; Anderson et al., 2015).

Traditionally, testing in the classic RAM is performed manually and requires food and/or water deprivation. For animals being tested in the classic RAM, manual handling and food and/or water deprivation may result in a substantial degree of stress. The classic RAM setup thereby also introduces a variety of possible confounders. The close olfactory, visual, auditory, and tactile interactions between experimenter and animal may result in anxiety and handling stress among experimental animals, which, in turn, may endanger the reproducibility of an experiment (Hurst and West, 2010; Gouveia and Hurst, 2017; Gulinello et al., 2019). Other confounding effects are caused by water and/or food deprivation which are commonly used to motivate foraging (Vorhees and Williams, 2014). In addition, testing animals in the RAM can be quite time-consuming for the experimenter as animals cannot be tested simultaneously.

To address these shortcomings of the classic RAM, we recently demonstrated the feasibility of a fully automated, voluntary, and handling-free refined version of the RAM allowing free access and not requiring food or water deprivation (Mei et al., 2020). There have been various other attempts to refine the classic RAM setup including automated detection of an animal's location or pellet intake using cameras, photoelectric or pressure sensors as well as automation of some mechanical parts of the RAM but none were handling-free, allowed free access and did not require food or water deprivation (Peele and Baron, 1988; Miyakawa et al., 2001; Dubreuil et al., 2003; Brillaud et al., 2005; Risher et al., 2013).

With this study, we aimed to compare the classic manual setup with the refined automated version of the RAM. We used an established mouse model of lipopolysaccharide (LPS)-induced systemic inflammation (Barichello et al., 2019; Savi et al., 2021), which has been shown to elicit long-term memory impairment in rodents in a classic RAM setup (Semmler et al., 2007; Weberpals et al., 2009), to induce long-term cognitive deficits. By reflecting on the respective merits of the two methods, we contribute to the refinement of future RAM experiments supporting the application of the third of the three 3R principles.

Materials and methods

Ethical statement

All experimental procedures were reviewed and approved by the State Office for Health and Social Affairs [Landesamt für Gesundheit und Soziales (LaGeSo), Berlin, Germany], Berlin (G290/15) and were carried out in accordance with the German animal protection law and local welfare guidelines at the German Federal Institute for Risk Assessment [Bundesinstitut für Risikobewertung (BfR), Berlin, Germany]. Reporting of the study complies with the ARRIVE 2.0 guideline (Percie du Sert et al., 2020).

Animals, housing, husbandry, and setting

We used female C57BL/6J mice, that were 10–12 weeks old at the beginning of the study, obtained from Charles River Laboratories, Sulzfeld, Germany at the age of 6–8 weeks. Animals were kept under specific-pathogen-free conditions according to FELASA recommendations. Housing conditions were as follows: Room temperature 23 ± 1°C, humidity

60 ± 5%, inverse 12:12 h light:dark cycle [lights on: 20:00, lights off: 8:00)]. Animals were group-housed in type III polycarbonate cages (1290D Euro standard Type III, Techniplast, Italy) equipped with environmental enrichment tools (red transparent plastic nest box and nesting material), with *ad libitum* access to food (autoclaved pellets; Lasvendi, LASQCdiets TM ROD16-H) and water. All persons entering the laboratory rooms wore single-use coveralls (Microgard 1,500, Ansell Microgard, Kingston Upon Hull, UK), gloves and surgical masks to reduce potential olfactory confounding effects. We cleaned the arms of each maze daily to remove droppings. Upon completion of each experimental group, we cleaned and disinfected the RAMs thoroughly using warm water, soap, and an alcohol-based disinfectant. All procedures and experiments were performed in the same facility as where animals were housed.

Apparatus

The classic RAM was made from polycarbonate and consisted of eight equally spaced, rectangular arms (length: 30 cm, width: 5 cm, and height: 20 cm) that were open at the top and which extended from a central octagonal platform (13 cm across) (**Figure 1B**). At the end of each arm, there was a small cavity in which a pellet was placed. The experimenter manually placed the animal onto the central platform of the maze using a containment box to minimize handling stress. Likewise, upon completion of an experimental session, animals were removed from the classic maze using a containment box. We scheduled experiments in the classic RAM at the same time each day during the active (lights off) phase.

We described the refined RAM previously (Mei et al., 2020). In short, it consisted of eight transparent tubes radiating outwards from an octagonal holding platform (**Figures 1A,E**). An automated pellet dispenser was located at the end of each arm (**Figure 1D**) which dispensed a pellet once an animal entered a correct arm. The maze was connected to a standard home cage *via* an animal sorter device (**Figure 1C**). Animals could freely access the refined RAM from their home cage at any time of the day. Only one home cage (containing six animals with a random combination of LPS-treated and control animals) was connected to the refined RAM at any given time. An RFID reader and photoelectric sensors allowed to determine the location of an animal within the refined RAM. The animal sorter device ensured that only one animal could enter the maze at a given time.

For both mazes, illuminated visual cues including different objects like a candleholder and picture frames presenting various geometrical patterns were placed next to the maze as distal cues. The central platforms contained tactile cues constituting local cues for additional tactile orientation. We used sucrose enriched pellets (Purified Rodent Tablets 5TUL) from Test Diet, Richmond, USA.

Transponder implantation

To allow for animal identification, radio-frequency identification (RFID) transponders were implanted subcutaneously in all mice at least 2 weeks prior to the beginning of the experiments. Glass-covered, biocompatible RFID transponders (dimensions: 2.1 mm × 12 mm; model: passive 125 kHz glass transponder; EURO I.D. Identifikationssysteme GmbH & Co., KG, Frechen, Germany) with individual identification numbers were sterilized and loaded in an applicator device. We implanted the transponders subcutaneously in the nuchal region. Anesthesia was induced with 3% isoflurane delivered in 100% oxygen for 45 s before the implantation procedure. For analgesia animals received meloxicam (1 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) subcutaneously once during the procedure. Following implantation, mice were observed for up to 48 h for signs of complications. Presence and functionality of the RFID transponder was checked before the start of the experiment.

Experimental design

This was a randomized, blinded method-comparison study. We conducted an *a priori* sample size calculation based on findings from previous classic RAM studies assessing long-term cognitive deficits following LPS-injection using G*Power (Faul et al., 2007). The study was designed with 80% power to detect a relative 25% difference in combined working/reference memory performance. *A priori* power analysis using a repeated measures ANOVA with Tukey's *post-hoc* test under the following assumptions $\alpha = 0.05$, $\beta = 0.2$ and based on mean and SD obtained from preliminary experiments determined the number of required experimental units at 12 animals per group.

First, animals were randomly assigned to one of two treatment groups (LPS-treated or control group). The number of animals per group after randomization was 13 in the LPS-treated group and 11 in the control group. Second, animals were randomly assigned to one of two experimental groups because we used a cross-over design (for an illustration of the experimental design, see **Figure 1F**). The first experimental group began in the refined RAM and was subsequently tested in the classic RAM. The second experimental group began in the classic RAM and was subsequently tested in the refined RAM. We performed this cross-over testing twice with two groups of 12 animals in sequence: The first group of 12 animals was randomly divided in two subgroups of six animals. After both subgroups had finished the experiments in both mazes, the second group of 12 animals followed in the same manner. The six animals of one subgroup remained together in one cage for the entire time of the experiment without contact to animals from other cages. Due to technical reasons, the duration between injection and start of experiments varied from 5 to 10 weeks

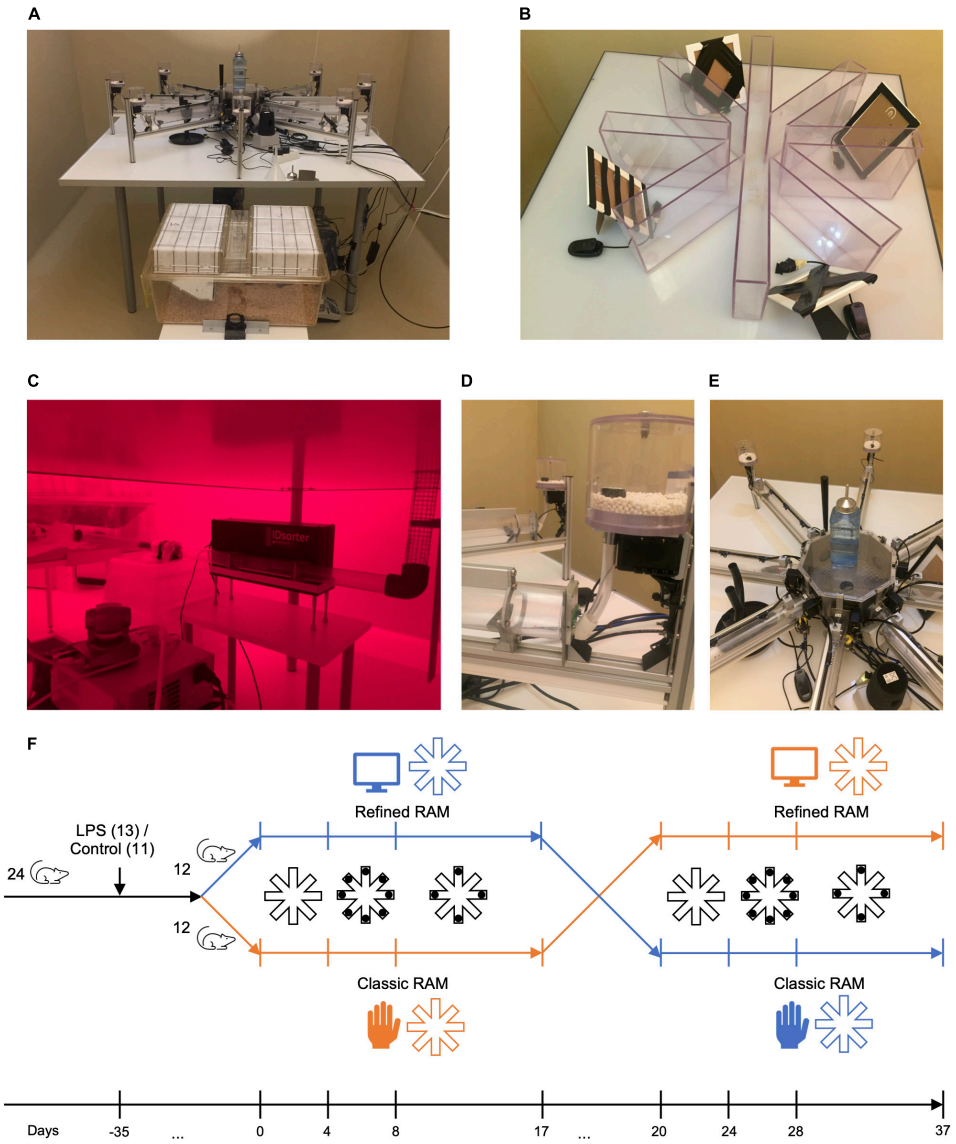

FIGURE 1
Setup of the refined and classic radial arm mazes (RAM) and timeline. **(A)** Refined RAM connected to the animals' home cage; **(B)** Setup of the classic RAM including extra-maze visual cues; **(C)** Radio-frequency identification-based animal sorter device and climbing wire connecting the home cage to the refined RAM (pictured under red light conditions during the animal's active phase); **(D)** Automated sucrose enriched pellet dispenser; **(E)** Central platform of the refined RAM including extra-maze visual cues; **(E)** Setup of the classic RAM showing extra-maze visual cues; **(F)** Timeline of the experiments in the refined and the classic RAM. We used a cross-over design; one group of animals was tested in the refined RAM first and continued in the classic RAM after a washout phase of 3 days and *vice versa*. Experimental phases included habituation (refined RAM: habituation cage, classic RAM: maze, with pellets distributed in the home cage/maze), working memory paradigm (eight arms baited), and combined working/reference memory paradigm (four arms baited). RAM, radial arm maze.

for the working memory paradigm and 7–13 weeks for the combined working/reference memory paradigm for both mazes.

Methods to prevent bias

Animals were randomized to treatment groups, experimental groups, and to rewarding arm pattern in the combined working/reference memory paradigm using the Research Randomizer tool.[1] The researcher conducting the experiments was blinded regarding treatment group assignment until the end of data analysis.

1 www.randomizer.org

Experimental procedures

Treatments

To induce a systemic inflammatory response, animals were treated with LPS, a cell wall component of Gram-negative bacteria. LPS (from *Salmonella enterica* serotype, Lot # 056M4115V, Sigma-Aldrich St. Louis, MO, USA) at a dose of 1.5 mg/kg or physiological phosphate-buffered saline solution were administered intraperitoneally on two consecutive days at the beginning of the active (i.e., light-off) phase at 8:00 with a volume of 10 μl/g. After injection, animals were monitored closely using a sickness score adapted from the murine sepsis score. These procedures were performed as previously described (Mei et al., 2018, 2019).

Food deprivation

For the classic RAM, animals were food deprived for 8 h before testing. Animals were weighed twice daily. First, before the food was removed and second, before the beginning of the testing session. If weight loss exceeded 15% compared to baseline weight, food deprivation would have been stopped until baseline weight had been regained. Recorded weight loss never exceeded 5%. We did not food deprive animals for the refined RAM.

Habituation phase

We habituated animals to the experimental setup for 3–5 days. For the classic RAM, we placed sucrose enriched pellets all over the maze and animals were allowed to move freely within the maze for up to 30 min per day. For the refined RAM, we used a habituation cage followed by exploration of the refined RAM as described previously (Mei et al., 2020).

Working memory paradigm

The actual experiment consisted of a working memory paradigm and a combined working/reference memory paradigm. During the working memory paradigm, a sucrose enriched pellet was placed by the end of each of the eight arms of the maze (manually in the classic RAM; the refined RAM dispensed a pellet when an animal visited a correct arm for the first time during a session). The working memory paradigm lasted for 4 days. We assessed spatial working memory performance during the working memory paradigm by considering an animal's reentry into a previously visited arm as a working memory error. We present correct choices (i.e., first entries to reward-baited arms during one experimental session) as three different ratios (see the section "Behavioral parameters").

Combined working/reference memory paradigm

During the combined working/reference memory paradigm, a sucrose enriched pellet was placed by the end of each of the four randomly selected arms of the maze. The configuration of the four randomly selected arms remained the same for every individual mouse throughout the combined working/reference memory paradigm. The combined working/reference memory paradigm lasted for 9 days and began immediately after the end of the working memory paradigm in the refined RAM and on the day following the end of the working memory paradigm in the classic RAM. We considered an animal's reentry into a previously visited baited arm as spatial working memory error. In addition, we considered a (re-)entry into an unbaited arm as spatial reference memory error.

Sessions

In the classic RAM, animals were tested once per day during working memory paradigm and combined working/reference memory paradigm in the classic RAM. Animals could voluntarily enter the refined RAM for up to ten times per day during both phases. Start and end of sessions were defined as follows. For the classic RAM, a session started when the mouse was released in the central platform of the maze, while for the refined RAM a session started when an animal voluntarily entered the maze. Sessions were terminated upon task completion (visiting all eight arms in the working memory paradigm and visiting all four baited arms in the combined working/reference memory paradigm) or when 10 min elapsed. At the end of a session (when task was completed or when the time-out limit was reached), in the classic RAM mice were retrieved by the experimenter and returned to their home-cage, while in the refined RAM all arms closed apart from the one containing the mouse. When the mouse left the last arm and returned to the central platform, the last visited arm closed, too, leaving available only the path leading back to the home-cage.

Behavioral parameters

Our outcomes were working memory errors and reference memory errors. In addition, we report the time animals needed to complete one session (i.e., entering all baited arms) and three different ratios: the ratio of correct entries to the sum of all entries [calculation: correct entries to reward-baited arms divided by the sum of all arm entries; i.e., correct entries ratio (all arms visited)], the ratio of correct entries within the first four arm visits [calculation: correct entries to reward-baited arms within the first four arms visits of a session divided by the number of reward-baited arms (eight in the working memory paradigm, four in the combined working/reference memory paradigm), i.e., correct entries ratio (first four arms visited)], and the ratio of correct entries within the first eight arm visits [calculation: correct entries to reward-baited arms within the first eight arms visits of a session divided by the number of reward-baited arms, i.e., correct entries ratio (first eight

arms visited)] (Wenk, 2004). This approach, normalizing the number of correct entries by the total number of baited arms, avoided underscoring the performance of mice in the eight arm ratio in the relative comparison with the four arm ratio. This approach guarantees that each addition of a correct entry is correctly scored as an increase in the ratio. In the refined RAM, since multiple daily sessions were performed by mice, the values of the behavioral parameters were averaged across daily sessions in order to obtain a single daily value. To ensure spatial learning, we excluded the data generated by mice which had two or fewer maze entries during working memory paradigm or three or fewer maze entries during combined working/reference memory paradigms, respectively.

Data analysis and statistical methods

All data values are shown in mean ± standard deviation (SD) unless indicated otherwise. All experiments in the classic RAM were video recorded and analyzed manually. A custom-made software controlled the refined RAM and recorded experimental data.

Statistical analysis was performed using SPSS (Version 26.0). We analyzed data using linear mixed models. We used random intercept models that account for the clustering of measures within individuals. The measures of the behavioral outcomes served as dependent variable; treatment (LPS/control), maze type (refined/classic RAM), experimental order of maze type (first refined, then classic, or first classic, then refined) and interactions of treatment*time, maze type*time, treatment*maze type, and maze type*experimental order of maze type as factors; and time (days) as covariate. Deviation from normal distribution was checked with histograms and we log-transformed the data before analysis if they were not sufficiently normally distributed. We report model-based marginal means and group differences with 95% confidence intervals (CIs) as well as within-group differences. A two-sided significance level of $\alpha = 0.05$ was used.

Results

Thirteen animals were randomized to the LPS-treatment group; eleven animals were randomized to the control group. One animal had to be killed following LPS-injection because it exceeded the pre-defined sickness severity cut-off score.

Refined radial arm maze

Figures 2A–C and **Supplementary Figures 2A,B** show model-derived adjusted means for each treatment group as well as adjusted treatment effects (group differences) on the first and last days of the working memory paradigm (eight arms baited). The corresponding descriptive statistics are displayed in **Figures 3A–F** and **Supplementary Figures 3A–D**. Spatial learning performance is summarized in **Supplementary Table 1**.

During the working memory paradigm, we observed treatment group differences on the last day of the paradigm for working memory errors [treatment effect: 0.53 (log-transformed), 95% CI: 0.05–1.01, $P = 0.032$; **Figure 2A**], correct entries ratio (all arms visited) (treatment effect: −0.12, 95% CI: −0.23–0.00, $P = 0.042$; **Figure 2B**), and session duration [treatment effect: 0.29 (log-transformed), 95% CI: 0.04–0.54, $P = 0.022$; **Figure 2C**], which indicates spatial learning and memory deficits among LPS-treated animals. There was no relevant treatment group difference on the first day of the paradigm neither for session duration, working memory errors nor for correct entries ratios. Working memory errors and session duration decreased over time among control animals [working memory errors: difference day 1 day 4: 0.63 (log-transformed), 95% CI: 0.06–1.19, $P = 0.030$; session duration: difference day 1 day 4: 0.44 (log-transformed), 95% CI: 0.14–0.74, $P = 0.004$; **Supplementary Table 1**], indicating spatial learning performance. There was no effect of time on working memory errors among LPS-treated animals.

Figures 2D–G and **Supplementary Figures 2C,D** show model-derived adjusted means for each treatment group as well as adjusted treatment effects (group differences) on the first and the last days of the combined working/reference memory paradigm (four arms baited). During the combined working/reference memory paradigm, we found no relevant group differences. Reference memory errors (LPS-treated animals: difference day 1 day 9: 1.97, 95% CI: 1.09–2.85, $P < 0.001$; control animals: difference day 1 day 9: 1.22, 95% CI: 0.34–2.10, $P = 0.007$), correct entries ratio (first four arms visited) (LPS-treated animals: difference day 1 day 9: −0.24; 95% CI: −0.34–0.14, $P < 0.001$; control animals: difference day 1 day 9: −0.14, 95% CI: −0.24–0.04, $P = 0.006$), correct entries ratio (all arms visited) (LPS-treated animals: difference day 1 day 9: −0.19; 95% CI: −0.27–0.11, $P < 0.001$; control animals: difference day 1 day 9: −0.14, 95% CI: −0.22–0.07, $P < 0.001$), and session duration (LPS-treated animals: difference day 1 day 9: 0.33 (log-transformed), 95% CI: 0.13–0.53, $P = 0.001$; control animals: difference day 1 day 9: 0.21 (log-transformed), 95% CI: 0.01–0.41, $P = 0.040$) improved over time in both treatment groups; correct entries ratio (first eight arms visited) improved among LPS-treated animals (difference day 1 day 9: −0.07; 95% CI: −0.13–0.01, $P = 0.019$) (**Supplementary Table 1**).

Taken together, these results indicate a subtle deficit in spatial learning and memory among LPS-treated mice compared to control animals. To account for a potential washout of the treatment effect due to multiple daily sessions, we analyzed the first four sessions of the working memory paradigm and the first nine sessions of the combined

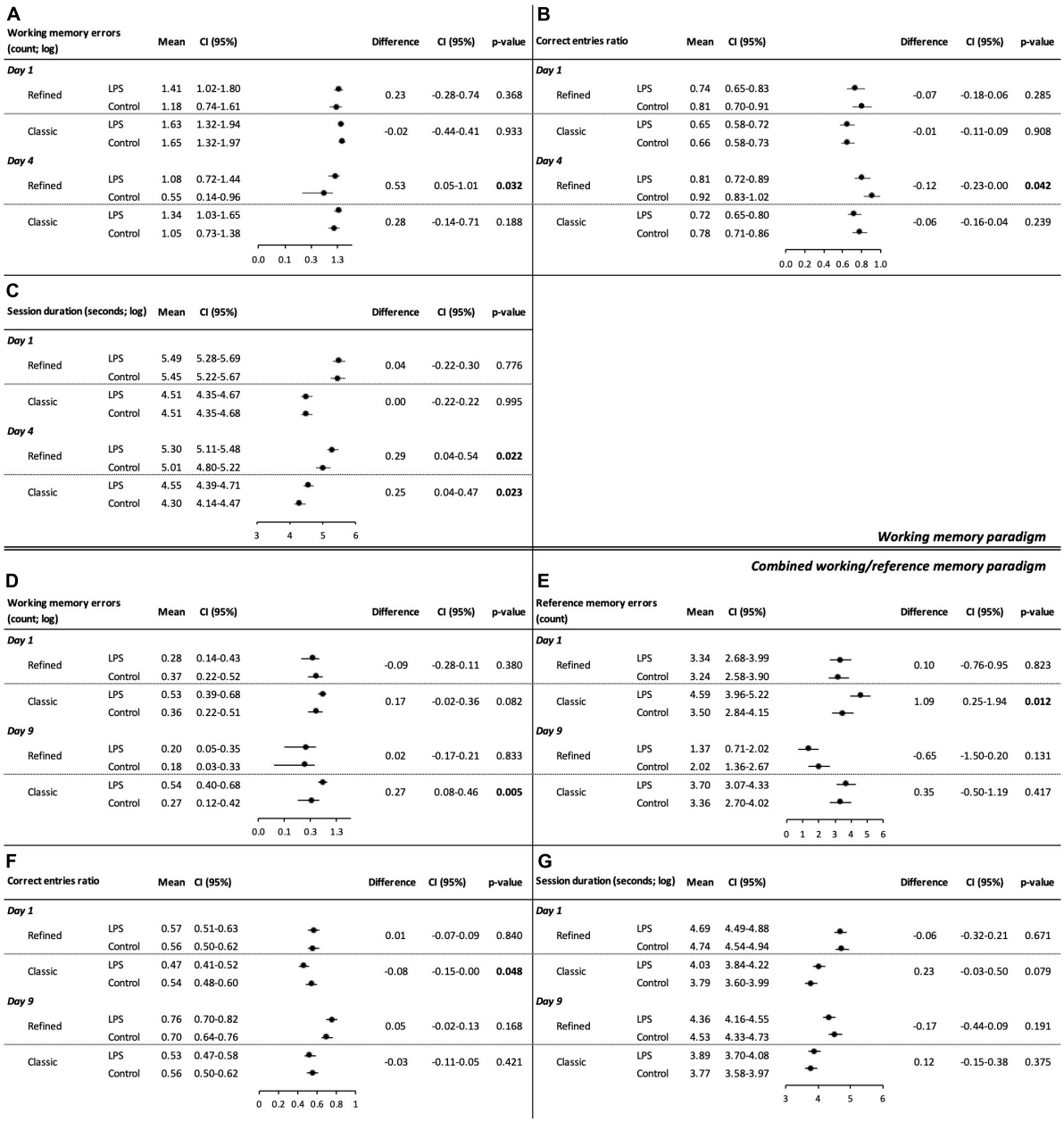

FIGURE 2
Cognitive performance in the refined and classic radial arm mazes during the working memory paradigm **(A–C)** and the combined working/reference memory paradigm **(D–G)**. Separate linear mixed model analyses were conducted. Model-derived estimated marginal means and group differences for **(A)** working memory errors (log-transformed), **(B)** correct entries ratio (all arms visited), and **(C)** session duration (log-transformed) on the first (1 day) and last day (4 day) of the working memory paradigm are shown. Model-derived estimated marginal means and group differences for the combined working/reference memory paradigm on the first (1 day) and last day (9 day) of the paradigm: **(D)** working memory errors (log-transformed), **(E)** reference memory errors, **(F)** correct entries ratio (all arms visited), and **(G)** session duration (log-transformed).

working/reference memory paradigm, separately. We did not observe a significant difference between treatment groups during the first sessions of a paradigm in the refined RAM (**Supplementary Figure 1**).

Classic radial arm maze

During the working memory paradigm, there was a significant treatment group difference for session duration on

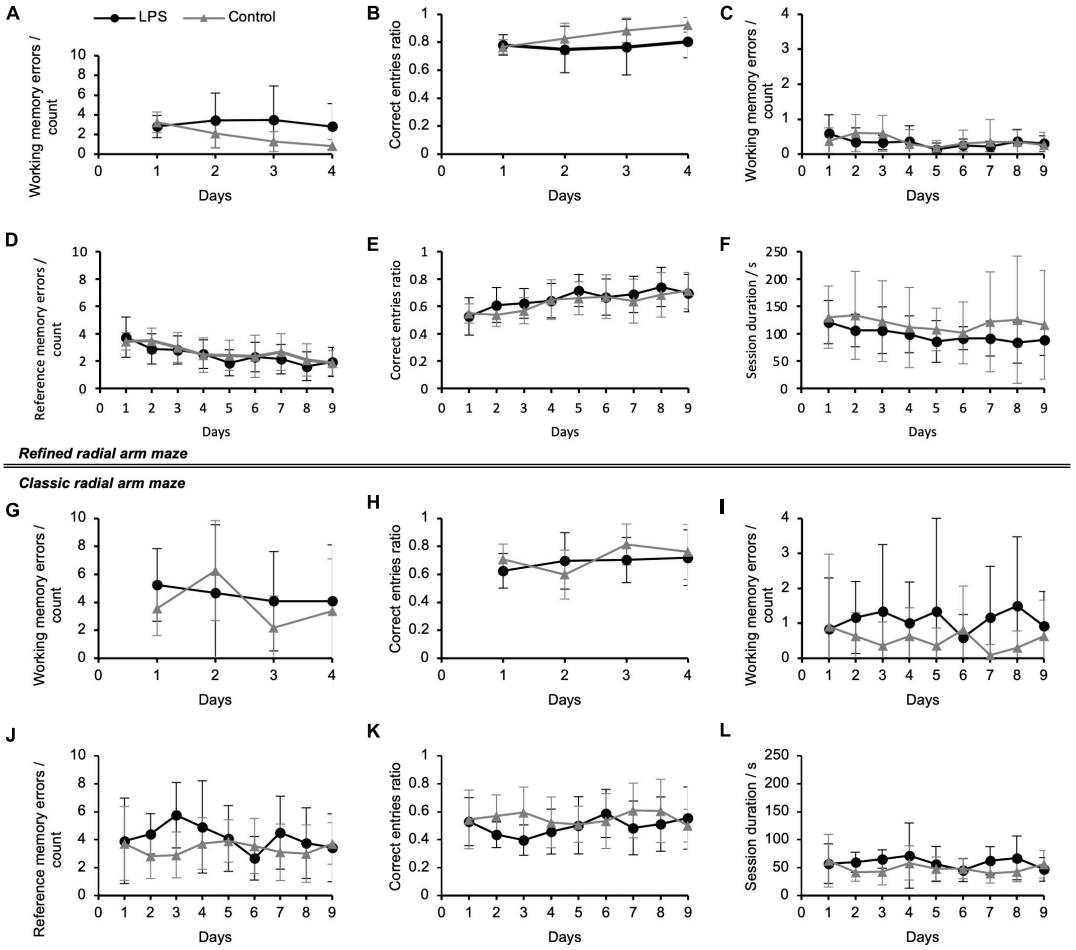

FIGURE 3
Spatial working and reference memory performance of mice following lipopolysaccharide (LPS)-injection in the refined radial arm maze (RAM) during working memory paradigm **(A,B)** and combined working/reference memory paradigm **(C–F)** and in the classic RAM during working memory paradigm **(G,H)** and combined working/reference memory paradigm **(I–L)**. Refined RAM: **(A)** Average number of working memory errors per animal per day (re-entries into an arm which had already been visited during a session) during the working memory paradigm; **(B)** Correct entries ratio (all arms visited) during the working memory paradigm; the ratio expresses the fact that the animals reached the maximum number of correct entries in most of the sessions (also in panel **E**); **(C)** Average number of working memory errors per session during the combined working/reference memory paradigm; **(D)** Average number of spatial reference memory errors (i.e., number of entries and re-entries to unbaited arms per session) per animal per day; **(E)** Correct entries ratio (all arms visited) during the combined working/reference memory paradigm; **(F)** Average session duration from entering the refined RAM until all baited arms had been visited and the animal exited the RAM per animal per day; maximum session duration was: 10 min. Working memory paradigm: $N = 9$ (LPS-treated group), $N = 6$ (control group); combined working/reference memory paradigm: $N = 11$ (LPS-treated group), $N = 11$ (control group). Classic RAM: **(G)** Working memory errors and **(H)** correct entries ratio (all arms visited) during the working memory paradigm; **(I)** working memory errors, **(J)** reference memory errors, **(K)** correct entries ratio (all arms visited) and **(L)** session duration during the combined working/reference memory paradigm. $N = 12$ (LPS-treated group), $N = 11$ (control group). Data are presented as mean (±SD). Half of the individuals tested with the refined RAM had previously been trained on the classic RAM and vice versa. RAM, radial arm maze.

the last day of the paradigm [treatment effect: 0.25 (log-transformed), 95% CI: 0.04–0.47, $P = 0.023$; Figure 2C]. We did not find group differences neither for working memory errors nor for the correct entries ratios (corresponding descriptive statistics are summarized in Figures 3G–L and Supplementary Figures 3E–H). Working memory errors [difference day 1 day 4: 0.59 (log-transformed), 95% CI: 0.14–1.04, $P = 0.010$] decreased and correct entries ratio (first eight arms visited) (difference day 1 day 4: −0.10, 95% CI: −0.19–0.01, $P = 0.026$) and correct entries ratio (all arms visited) increased (difference day 1 day 4: −0.13, 95% CI: −0.23–0.02, $P = 0.021$) among control animals (Supplementary Table 1). There was no effect of time on working memory errors among LPS-treated animals.

During the combined working/reference memory paradigm, we observed a treatment effect for working memory errors on the last day of the paradigm [treatment effect: 0.27 (log-transformed), 95% CI: 0.08–0.46, $P = 0.005$; Figure 2D] and for reference memory errors (treatment effect: 1.09, 95% CI:

0.25–1.94, P = 0.012; **Figure 2E**), correct entries ratio (first four arms visited) (treatment effect: −0.09, 95% CI: −0.18–0.00, P = 0.047, **Supplementary Figure 2C**), correct entries ratio (first eight arms visited) (treatment effect: −0.06, 95% CI: −0.12–0.00, P = 0.040, **Supplementary Figure 2D**), and correct entries ratio (all arms visited) (treatment effect: −0.08, 95% CI: −0.15–0.00, P = 0.048; **Figure 2F**), on the first day of the paradigm, respectively. These results indicate spatial learning and memory deficits among LPS-treated animals. There was a trend toward poorer cognitive performance among LPS-treated animals for working memory errors and session duration on the first day of the paradigm, albeit not statistically significant [working memory treatment effect: 0.17 (log-transformed), 95% CI: −0.02–0.36, P = 0.082, **Figure 2D**; session duration treatment effect: 0.23 (log-transformed), 95% CI: −0.03–0.50, P = 0.079, **Figure 2G**]. Apart from a decrease of reference memory errors (difference day 1 day 9: 0.89, 95% CI: 0.03–1.75, P = 0.042) and an increase of the correct entries ratio among LPS-treated animals (first four arms visited) (difference day 1 day 9: −0.10, 95% CI: −0.19–0.00, P = 0.048), neither LPS-treated nor control animals showed a significant change in performance over time, indicating overall poor spatial learning performance (**Supplementary Table 1**).

Taken together, these results indicate that LPS-treated animals had subtle cognitive deficits which could be detected both in the refined and classic RAM.

Activity in the refined radial arm maze

In addition to cognitive performance, continuous data acquisition in the refined RAM allowed us to quantify an animal's day/night activity pattern and exploratory behavior. During the animals' active phase (i.e., lights off), both groups entered the maze more frequently than during the inactive phase (i.e., lights on), representing a physiological day/night activity pattern (Refinetti, 2004; Arakawa et al., 2007; Pioli et al., 2014; Saré et al., 2021; **Figure 4C**). Latency to first entry to the maze as an indicator of exploratory behavior was 2.65 (±3.38) days for the control and 0.92 (±1.18) days for the LPS-treated groups, respectively, indicating within- and between-groups variations whereas the between-groups difference was not significant (**Figure 4D**). The average number of maze entries per day remained largely unchanged during the duration of the experiment for both groups (**Figures 4A,B**).

Data exclusion

Due to a low number of daily maze entries to the refined RAM, we excluded eight animals (three from LPS-treated group; five from control group) during the working memory paradigm and one animal from the LPS-treated group during the combined working/reference memory paradigm. We excluded two of 322 maze visits (0.6%; 1 day for one animal from the LPS-treated groups; 1 day for one animal from control group) to the classic maze due to corrupted video files. We excluded 30 of 402 maze visits to the refined RAM (7.4%; 11/402 = 2.7% from LPS-treated group; 19/402 = 4.7% from control group) during the working memory paradigm and 121 of 1,881 maze visits (6.4%; 66/1,881 = 3.5% from LPS-treated group; 55/1,881 = 2.9% from control group) during the combined working/reference memory paradigm due to errors of the control software of the refined RAM.

Discussion

The aim of our study was to compare the advantages and limitations of a classic manual eight-arm radial maze with those of a fully automated refined equivalent. A particular strength of our study was that the same animals were used in both apparatuses, which allowed a direct, intra-individual comparison.

During the working memory paradigm, LPS-treated animals demonstrated a worse cognitive performance in the refined RAM but not in the classic RAM. During the combined working/reference memory paradigm, LPS-treated animals performed worse in the classic RAM but not in the refined RAM. Overall, LPS-induced cognitive deficits were subtle. In addition to cognitive performance, which both mazes readily detected, continuous data acquisition in the refined RAM allowed quantification of an animal's exploratory behavior and day/night activity pattern.

Previous studies in mice found mixed effects of LPS-induced systemic inflammation on cognitive performance in the RAM. In one study, working and reference memory deficits persisted for up to 2 months following 5 mg/kg LPS injection (Weberpals et al., 2009), whereas another study found no effect of 5 mg/kg LPS injection on working memory performance one month after injection (Anderson et al., 2015). In the present study, we used a relatively low LPS dosage (2 × 1.5 mg/kg), which may explain the subtle treatment effect. In addition, the interval between the injections and the beginning of the working memory paradigm was comparatively long (up to 13 weeks) for some animals due to unexpected technical challenges of the refined RAM. A washout of the treatment effect during multiple daily sessions might have further reduced discrimination power in the refined RAM. However, we did not observe a significant difference between treatment groups even during the first sessions of a paradigm in the refined RAM. Future studies should consider limiting the number of daily sessions or the time period during which animals can access the refined RAM to avoid potential washout effects. Using different experimental setups such as object recognition and open field test, others have shown that

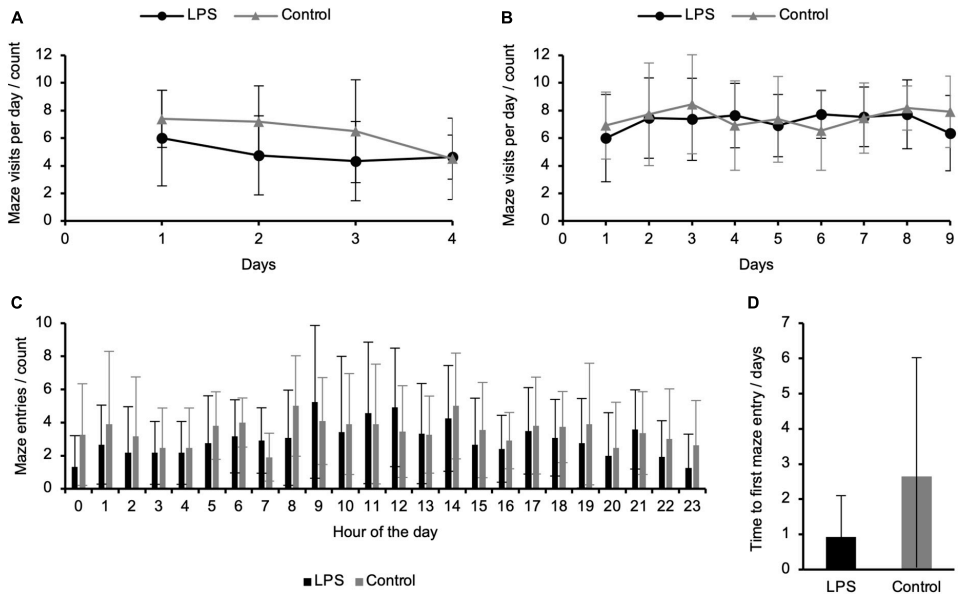

FIGURE 4
Exploratory activity and day/night activity of control and lipopolysaccharide (LPS)-treated animals in the refined radial arm maze (RAM). **(A)** Average number of individual entries to the RAM per day during the working memory paradigm and **(B)** combined working/reference memory paradigm remained largely unchanged; **(C)** Average sessions per hour of LPS-treated and control animals across all days of the combined working/reference memory paradigm (active phase, lights off: 8.00–20.00; inactive phase, lights on: 20.00–8.00) showed a physiological increase of locomotor activity during the active phase; **(D)** Average latency to first entry to the RAM during the working memory paradigm did not reveal a significant difference between the LPS-treated and the control group. Data are presented as mean (±SD). Working memory paradigm: *N* = 9 (LPS-treated group), *N* = 6 (control group); combined working/reference memory paradigm: *N* = 11 (LPS-treated group), *N* = 11 (control group).

TABLE 1 Advantages and limitations of the two versions of the radial arm maze.

	Refined radial arm maze	Classic radial arm maze
Overall animal stress level during experiment	Low	Standard
Food restriction	Not required	Required
Required preparation	Transponder implantation	Transponder implantation
Interaction of animal and experimenter	Low to none	High
Daily effort for experimenter	Low (few minutes)	High (hours; depending on number of animals and sessions)
Effort in case of damage/error	High	Low
Effort to analyze data	Low	Low
Measurement of exploratory and day/night activity pattern	Possible	None
Food reward smell masking	Yes	No
Data exclusion	High	Low
Equipment-associated costs	High	Standard

cognitive function following sepsis improves over time, which supports our findings (Tuon et al., 2008; Comim et al., 2011).

Latency to first entry to the refined RAM appeared to be shorter among LPS-injected animals, albeit not significantly. Previous studies showed decreased exploratory activity following systemic inflammation in rodents (Haba et al., 2012; Anderson et al., 2015; Ye et al., 2019). However, these studies measured exploratory behavior right after LPS-induced systemic inflammation, i.e., during acute sickness behavior. Future studies should assess the potential long-term effects of

LPS on exploratory behavior once animals have recovered from acute sickness.

Apart from sensitivity and variations of measurement, other characteristics must be considered for a comprehensive method comparison. In terms of animal welfare, the refined RAM had the important advantage of not requiring food or water deprivation; such deprivation is standard procedure to increase an animal's reward seeking behavior in the classic RAM. Whereas there have been successful previous attempts to refrain from food deprivation in six-arm and eight-arm radial mazes, none of these mazes allowed free access (Fitzgerald et al., 1988; Haga, 1995; Opitz et al., 1997). In addition, experiments in the refined RAM lasted for up to 3 weeks without manual handling by an experimenter whereas animals required daily handling in the classic RAM. In addition to handling itself, other potentially confounding factors including an experimenter's level of experience or sex could not affect animal performance in the refined RAM. By lowering an animal's stress level during cognitive testing, the refined RAM may thus reduce between- and within-subject variations, which, in turn, may improve characterization of cognitive performance and reproducibility of experimental studies. Further studies are needed to quantify the effect of the refined RAM on stress levels.

Another conspicuous difference between the classic and the refined experimental procedure was the time required to set up and run the experiments. While animals in the classic maze required daily handling by an experienced experimenter lasting up to 15 min per animal per day, the refined maze only required a 5 min, basic daily inspection by an animal technician. Thus, while the experimenter spent drastically less time on conducting experiments in the refined RAM, the overall duration of the experiment was around 8 days longer in the refined compared to the classic RAM. This was because animals could voluntarily enter the refined RAM at a time of their choosing, which necessitated to prolong experiments until animals were sufficiently trained. Experimental time could possibly be reduced if experimental pellet feeders provided whole diet pellets, and overall food availability would largely be through these pellet feeders as done in another study using the same automation technology (Caglayan et al., 2021). Regarding the smell of the pellets, the refined RAM holds the advantage of masking the smell when an animal is on the central platform since a pellet is only dispensed at the moment a mouse enters a baited arm. Additionally, in the refined RAM, only one cage at a time is connected to the maze. This should be taken into account when planning experimental designs in which the age of the animals and/or the timing from the treatment is of importance.

The refined RAM as a custom-made device is expensive to purchase and maintain. In the future, however, commercialization of the refined RAM could reduce costs. Finally, the necessity to exclude data was comparatively high in the refined RAM. This was due to unexpected hardware and software errors during all stages of the experiment. Future improvement will likely address and solve these issues causing the data exclusion rate to decrease over time. **Table 1** summarizes the respective characteristics of the two methods.

Limitations of our study include the relatively small sample size which, however, was in the range of other studies using the LPS-model to assess long-term cognitive deficits in a radial arm maze. In addition, we used only one disease model. It would be of interest to compare data from additional disease models in the future to further evaluate sensitivity also in terms of group differences. Since the animals in our study were female, it remains to be seen how male mice perform in the refined RAM. Another weakness is the relatively high data exclusion rate. We took a rigorous approach by excluding software output with only small errors. However, minimizing data exclusion remains both a challenge and a goal for future experiments in this setting. This could include prolonging the initial habituation phase in the refined RAM until all animals regularly enter the maze *ad libitum*. Lastly, our study design allowed for up to 10 daily sessions in the refined RAM versus one daily session in the classic RAM. Future studies should carefully consider limiting the number of daily sessions or the time period during which the maze can be entered.

In conclusion, this is the first study to compare a classic manual eight-arm RAM to a fully automated refined setup. While both mazes proved to be solid testing tools, the refined RAM delivered more sensitive and comprehensive data whereas the classic RAM required less data exclusion. The refined RAM therefore represents a valid new method with promising potential in terms of more differentiated data acquisition in a stress-free, voluntary environment for the animal and with only little effort needed by the researcher. In time-sensitive experimental settings which do not allow for flexibility in adjusting the schedule, however, the classic RAM might still be the preferable version. Despite some obvious disadvantages and limitations, the refined RAM constituted a refinement over the classic RAM procedure as it did not require food deprivation or manual handling, thus improving animal welfare. Future studies should demonstrate this in other disease models and further optimize this approach to refine spatial memory tests.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the State Office for Health and Social Affairs (Landesamt für Gesundheit und Soziales, LaGeSo), Berlin (G290/15).

Author contributions

JK, JE, ME, SB, and YW designed the study. JK performed the behavioral experiments and analysis. JK, JM, and JE interpreted the data. JK and JE prepared the figures and wrote the manuscript. All authors reviewed the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.1013624/full#supplementary-material

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Seminatural environments for rodent behavioral testing: a representative design improving animal welfare and enhancing replicability

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The low replicability of scientific studies has become an important issue. One possible cause is low representativeness of the experimental design employed. Already in the 1950's, Egon Brunswick pointed out that experimental setups ideally should be based on a random sample of stimuli from the subjects' natural environment or at least include basic features of that environment. Only experimental designs satisfying this criterion, representative designs in Brunswikian terminology, can produce results generalizable beyond the procedure used and to situations outside the laboratory. Such external validity is crucial in preclinical drug studies, for example, and should be important for replicability in general. Popular experimental setups in rodent research on non-human animals, like the tail suspension test or the Geller-Seifter procedure, do not correspond to contexts likely to be encountered in the animals' habitat. Consequently, results obtained in this kind of procedures can be generalized neither to other procedures nor to contexts outside the laboratory. Furthermore, many traditional procedures are incompatible with current notions of animal welfare. An approximation to the natural social and physical context can be provided in the laboratory, in the form of a seminatural environment. In addition to satisfy the basic demands for a representative design, such environments offer a far higher level of animal welfare than the typical small cages. This perspective article will briefly discuss the basic principles of the generalizability of experimental results, the virtues of representative designs and the coincidence of enhanced scientific quality and animal welfare provided by this kind of design.

KEYWORDS

replicability, external validity, animal welfare, seminatural environments, generalizability

Introduction

The use of non-human animals for modeling human behavior, disease, and other conditions is based on the premise that human situations can be recreated in these animals. However, most animal models focus on a particular aspect of the situation to be modeled, without holistically considering the processes that occur neither in humans nor in the species used for modeling the human condition. However, the behavioral patterns displayed vary depending on the characteristics of the environment in a

species-specific way (Fierro Toscano and Andrade, 2016). Ignoring the subtle interaction between environment and organism when studying behavior may have costly consequences.

The aims of the present article are to briefly introduce what has been labeled the replicability and generalizability crisis as well as the low predictive value of many animal studies. Then we will propose that a different kind of design, representative design, could enhance replicability and generalizability of animal models of human conditions, thereby improving the predictive value. It will also be mentioned that considerations of animal welfare were not given any fundamental importance in many of the established animal tests. We will argue that a representative design, in the form of a seminatural environment, much improves animal welfare.

Before entering into the specific subjects of the present contribution we need to define some basic concepts, including the distinction between animal models and animal tests. Some have defined "tests as behaviors that can be evaluated, whereas an animal model is an animal that has been manipulated as to score higher in these tests" (Söderlund and Lindskog, 2018, p. 669). Others use "model" as synonym to "test," i.e., a specific procedure aimed to predict the effects of a manipulation, for example the administration of a drug, in humans suffering from disease or dysfunction (Cryan et al., 2005; Planchez et al., 2019). In the article, we use behavioral test when referring to the exposure of an organism to a specific situation in order to assess a behavioral variable of interest and behavioral model when a behavior pattern of an organism is considered as representative of the behavior of another, generally more complex, organism.

The reliability of scientific studies

Replicability, repeatability, or reproducibility refer to the likelihood of obtaining similar results with a new dataset in a procedure identical or similar to the procedure used in the original study (Kenett and Shmueli, 2015; Patil et al., 2019). The low replicability of scientific studies has been of concern for many years. It has been suggested that more than half of the claims made in scientific publications are false (Ioannidis, 2005). Low replicability has been reported for the neurosciences (Button et al., 2013) as well as the medical (Prinz et al., 2011) and social sciences (Camerer et al., 2018), including psychology. In fact, several failed intents to replicate landmark studies in psychology (Open Science Collaboration, 2015; Wagenmakers et al., 2016) originated a phenomenon labeled "the replicability crisis."

The remedies for low reproducibility is thought to be enhanced scientific rigor, meaning that, for example, statistical methods should be strengthened, the analysis plan should be prepublished, collaboration across labs should be stimulated, data should be made openly available, and detailed experimental procedures should be reported (Munafo et al., 2017; Stevens, 2017; Ganley et al., 2022; Lu and Daugherty, 2022).

The predictive value of studies in non-human animals

If we are testing drugs in non-human animals with the purpose to predict clinical effects in humans, we are not only facing a replicability problem but also questions concerning the validity of the test. This becomes especially evident if the test is intended to represent a human psychopathology such as depression, anxiety or schizophrenia, or one of the sexual dysfunctions. Since these conditions have no equivalent in non-human animals, suppositions must be made concerning the correspondence between the behavior expressed in the animal test and the alterations observed in human psychopathology. These suppositions are often questionable. Indeed, whether popular rodent tests of anxiety, like the elevated plus maze, the open field or the dark/light transition test really represent the human anxiety condition (Ennaceur, 2014; Ennaceur and Chazot, 2016) or if they have any predictive validity or not (e.g., Rosso et al., 2022) are subjects of endless debates. The same is the case for other animal tests designed to be representative of human mental disease (Commons et al., 2017; Białoń and Wąsik, 2022). Thus, as soon as animal behavior is used as a model for human psychopathology, besides the problems of replicability, we have the quandary of the validity of the animal model itself. To these difficulties we have to add the uncertainty of generalizations from one species to another.

In the last sentence of the preceding paragraph, generalization means the extent to which the behavioral effects of an experimental manipulation, such as drug treatment, obtained in one species also would occur in other species. This is different from the use of the term generalization in statistics. There, it refers to whether the effects found in a random sample are applicable to the population from which the sample was drawn. There are many vicissitudes even in this kind of generalization, and a generalizability crisis in inferential statistics is presently of considerable concern (Yarkoni, 2022). A third kind of generalization refers to the applicability of results obtained under strictly controlled laboratory conditions to situations outside of the laboratory.

The generalization of effects observed in one species to another species combined with generalizations from the experimental conditions used in the preclinical studies to effects in the clinic is apparently not particularly successful. About 90% of all clinical drug trials fail, even though they are based on the best available animal data (Sun et al., 2022). The success rate is particularly low for CNS active drugs (6.3% vs. 13.3% for non-CNS drugs; Gribkoff and Kaczmarek, 2017). The dismal predictive validity of the preclinical studies made most established pharmaceutical companies in Europe, Japan, and the US to shut down their CNS research facilities many years ago (Abbott, 2011).

The problems of replicability within a species and the poor generalizability of effects from one species to another combined with the uncertainty concerning the validity of the animal model may seem unsurmountable. Over the years, many solutions have been offered (e.g., Meyerson and Lindström, 1973; Olivier et al., 1990; Peters et al., 2015; Kafkafi et al., 2018; Storey et al., 2021), but their success has been limited or non-existent since none of these problems has been eliminated. However, the recent proposal (Voelkl et al., 2020, 2021) that systematic incorporation of confounding factors, leading to "controlled heterogenization" would improve external validity and reproducibility is interesting. The complicated statistical procedures and large samples required for this approach may reduce its feasibility, though. Nevertheless, data suggest that heterogenization indeed improves replicability and generalizability, at least in animal models of ischemic stroke (Usui et al., 2021).

It is possible that an entirely different kind of experimental design, involving holistic considerations about the processes that occur both in humans and in the species used for modeling the human condition, might improve generalizability within a species as well as applicability to context outside of the laboratory. Indirectly, it might even improve interspecies generalizations, and perhaps enhance the validity of the animal models.

Animal welfare

Besides the many problems outlined above, studies in animals have been criticized because of concerns for animal welfare (e.g., Brown and Winnicker, 2015; d'Isa and Gerlai, 2023). These concerns are not necessarily related to worries about scientific reliability, but they acquire additional weight when it is pointed out that a substantial part of the scientific effort is wasted because of lack of reliability and clinical relevance. It has been claimed that about 28 billion US$ are spent on irreproducible research every year in the United States alone (Freedman et al., 2015). Provided that some studies require that animals are subjected to varying levels of discomfort, it can be argued that the discomfort inflicted on them is pointless since the data obtained may be both unreliable and without clinical relevance, despite claims to the contrary (see Stanford, 2020, for an excellent discussion). The social standing of science would be much improved if we could develop experimental setups assuring some degree of welfare for the subjects and a high degree of replicability and generalizability, including to the clinic.

The problems with standard behavioral tests

In standard behavioral tests for laboratory rodents, animals are housed in home-cages and their behavior is evaluated in specific test sessions, performed outside the home-cage, which last generally between a few minutes and 1 h. There has been a long tradition to design such experimental procedures so that the animals' behavioral repertoire becomes as limited as possible. For example, when studying learning, be it in a T-maze, in a Skinner box, or on a radial maze, the researcher tries to eliminate all stimuli that are considered irrelevant, thereby avoiding distractions that might perturb the animals' performance. Odors are normally eliminated from the setup, unnecessary visual stimuli likewise, and sounds can either be reduced as much as possible or masked by a white noise. The response options are also limited to what is considered of interest, like running in the aseptic runway of the maze and turning either to the left or the right, or pressing the manipulandum, or walking back and forth on the arms of the radial maze. In the case we study sexual behavior, a heterosexual couple is enclosed in a barren arena where they can choose between sleeping, fighting, or copulating. In the Porsolt test, the options are to try the impossible escape or give up and drown. In sum, the setup is arranged in such way that there are no distracting stimuli and few response options. This experimental ideal was brilliantly exposed by American psychologist Kenneth Spence (1907-1967) in his classic 1956 book (Spence, 1956).

The approach described in the preceding paragraph is excellent for hypothesis testing, and is often labeled systematic design (Brunswik, 1947). Since the experimental subjects' behavioral repertoire has been limited to the behaviors of interest and since irrelevant stimuli have been eliminated, at least as far as possible, the systematic design is a powerful tool to test specific hypothesis.

The notion of representative design

A key notion in experimental design is that the experimental subjects should be a random sample of the population. If the experimental groups were not composed according to this notion, all the statistical tests now being an integral part of any scientific endeavor would be meaningless, because they are all based on the assumption of a random sample. The results obtained in the sample can be generalized to the population from which the sample was drawn only if the sample was random. It is common to talk of a representative sample, when special care has been taken in the sampling procedure.

In addition to the requirement of a random sample of subjects, it has been suggested that the experimental design should include random samples of potentially relevant variables or of procedures appropriate for evaluating the research question (Petrinovich, 1989; Dhami et al., 2004; Araujo et al., 2007; Scholz, 2017). Such a design would be labeled "representative design." According to the Brunswikian notions, it would not be sufficient to include additional subject variables such as sex, age, degree of deprivation, etc. Variations of context (procedure) are an indispensable part of a representative design.

The term was originally proposed by the psychologist Egon Brunswik (1903 – 1955). Although forgotten by many young psychologists, Brunswik was quite influential in the 1950's and for several years thereafter. He was of Hungarian origin, educated in Vienna, where he got his Ph.D. in psychology in 1927. In 1937, he moved to Berkeley where he remained until his death in 1955. During his time in Vienna, Brunswik occasionally participated in the Vienna circle, a group of neopositivist philosophers animated by German philosopher and physicist Moritz Schlick (1882 – 1936) and including, among others, Austrian mathematician and logician Kurt Friedrich Gödel (1906 – 1978), Austrian philosopher and sociologist Otto Neurath (1882 – 1954) and German philosopher Rudolf Carnap (1891 – 1970). The emphasis on the logical foundations of knowledge and theory construction typical of the Vienna circle are basic to Brunswik's ideas (Leary, 1987). Brunswik believed that humans and animals live in environments that are chaotic and constantly changing. Certain stimuli in the environment are reliable predictors of important events and are considered ecologically valid in brunswikian terms. Most stimuli have no predictive value and can be safely ignored. Brunswik's famous double lens model (Brunswik, 1955) provides an illustration of how ecologically valid stimuli function. In order to determine the ecological validity of a stimulus, the stimulus needs to be evaluated in a representative design in the sense described in the preceding paragraph.

Even though the concept of representative design originated in studies of perception, it can be applied to any field of behavioral inquiry. If we are interested in finding out if a drug

has antidepressant properties in preclinical tests, for example, we have many test procedures to choose from. A recent review of the most used current animal tests of major depression listed more than 20 (Planchez et al., 2019). Actually, the total number of tests supposed to represent depression is far larger than that. Thus, it can be maintained that there is a population of tests for studying major depression. According to the notion of representative design, we should draw a random sample from that population, and then use all the sampled tests in our experiment. Such a random sample of test procedures would assure that our results can be generalized to the entire population of test procedures usable for testing antidepressant drugs.

In practice, a representative design as described here is cumbersome and extremely costly. It has been suggested that an acceptable approximation could be to introduce crucial elements of the subject's natural habitat in the experimental setup (Petrinovich, 1980).

In humans, rather than introducing elements from the habitat into the laboratory, experiments can be performed outside the laboratory. In fact, there are many recent examples of research performed in people's natural environment (Sliwinski et al., 2018; Richmond and Burnett, 2022). We will not further discuss the application of representative design to studies in humans, but we find it important to mention that it is quite feasible. Instead, we will focus on designs suitable for experiments in rodents.

There are fields of inquiry that would not benefit from the use of representative designs. Many physiological processes, like water reabsorption in the loop of Henle, or the release of thyroid stimulating hormone in response to cold, can be adequately studied without any representative design. In fact, such designs are relevant particularly in behavioral studies. Nevertheless, even in behavioral experiments, they may not always be needed. The molecular mechanisms involved in estradiol's facilitation of lordosis may be perfectly understood by using extremely simple designs, and the results are generalizable to all contexts in which lordosis is displayed. They are also perfectly replicable (Pfaff, 2017). Whether they can be generalized from rats to women is an entirely different question, particularly since lordosis is not a basic part of sexual behavior in women.

It is mainly when complex behavioral phenomena are the subject of study that representative designs become crucial. This is also the case when hypotheses about the adaptive value or biological functions of behavior are to be made.

Seminatural environments as representative design

If the aim of an experiment is to determine the effects of a drug or of a manipulation of the brain on behavior, then procedures like the Porsolt test are entirely unsuitable. In experiments with this wide purpose, we need to employ a design allowing the experimental subjects to express as much as possible of their behavioral repertoire. Then we could even see whether the drug or manipulation has any unexpected or novel effects, i.e., we would make discovery research (Foletti and Fais, 2019). Preferably, this should be done in an environment offering a rich variation of stimuli acting on several sensory modalities. Ideally, a complete ethogram should be established, and the observation time should be long enough to record several occurrences of relevant behavior patterns. Modern computational techniques have reached a stage in which complex behavior patterns can be automatically identified and described in excruciating detail, even when several animals are observed simultaneously (Egnor and Branson, 2016; Kennedy, 2022; Lauer et al., 2022). This amazing progress makes the kind of studies mentioned above feasible without excessive investment of labor.

A way to combine the requirements of a representative design and discovery research is to create a complex test environment allowing the subjects to express as much as possible of their natural behavioral repertoire. This becomes possible when the basic features of the natural habitat are preserved in the experimental procedure. There are several examples of experimental setups in animal research that satisfy these demands (e.g., McClintock, 1981; Blanchard et al., 1995, 2001; Ragnauth et al., 2005; Weissbrod et al., 2013). The Ragnauth et al. (2005) environment, employed at the Rockefeller University, is illustrated in **Figure 1A**. In rats, the essential features are the presence of several conspecifics, the availability of something similar to a burrow, and a reasonably large physical space. Studies of wild rats have systematically shown that several individuals share a burrow, that they are sociable and that sexual interactions involve several individuals (Barnett, 1958a; Calhoun, 1962; Robitaille and Bovet, 1976; Schweinfurth, 2020). In agreement with this, at the University of Tromsø we built a two-dimensional copy of a rat burrow (**Figure 1B**), based on data from Calhoun (1962) and on the seminatural environment described by McClintock and Adler (1978). The burrow was connected to a large open field. Lighting was so arranged that the burrow was kept in constant darkness for the rats, but illuminated with infrared light (850 nm) for the video cameras. The open field had a day beginning and ending with a 30 min period of increasing and decreasing light intensity, respectively, simulating sunrise and dusk. During the night, the light intensity was about 10 lx at floor level, not much different from the light provided by a full moon. Experiments lasted 8 days, and groups of 4 female and 3 male rats were always used. The sex ratio is close to what is found among adult rats in nature. Since the environment include the basic features of the natural habitat, we consider it appropriate to call it seminatural. Detailed descriptions of this environment can be found elsewhere (Chu and Ågmo, 2014, 2015b).

It is important to note that careful studies have revealed that laboratory rats share most behavioral characteristics with wild rats (Boice, 1977, 1981; Flannelly and Lore, 1977; Price, 1980), the main exception being that wild rats are far more neophobic than laboratory rats (Barnett, 1958b). However, there are also data showing that wild rats captured in an urban environment are not more neophobic than laboratory rats (Koizumi et al., 2021). Thus, we maintain that the seminatural environment is as valid for laboratory rats as it would be for wild rats, and that observations in this environment can be generalized to the natural habitat.

Descriptions of sociosexual interactions in this environment have revealed a considerable number of features that had not been detected in standard tests of sexual behavior, performed in heterosexual couples in a small observation arena. Among these are the sudden transition from non-receptivity to full receptivity

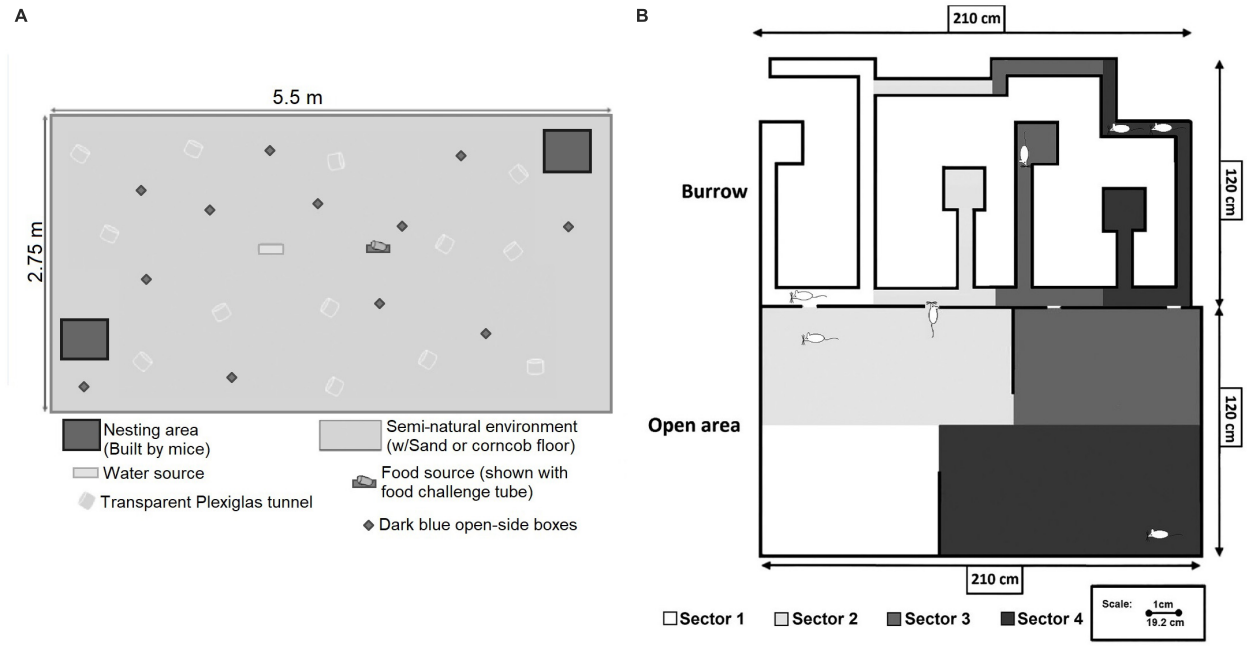

FIGURE 1
(A) The seminatural environment used in studies in mice in the Pfaff laboratory at the Rockefeller University. It consisted of a large space with food and water sources, as well as materials for nest building. For a detailed description, see Ragnauth et al. (2005) from which the figure is reproduced with permission from Wiley. **(B)** The seminatural environment used in the Ågmo laboratory at the University of Tromsø. It consisted of complex burrow system and a large open area. For analysis of localization of behavior, the environment was divided into four sectors. Reprinted from Le Moëne and Ågmo (2018) with permission from Elsevier. Further details can be found in that paper as well as in Chu and Ågmo (2014).

at the beginning of behavioral estrus (Chu and Ågmo, 2015a; Le Moëne et al., 2020a). In the standard observation environment, in which the female has no escape from a sexually active male, the transition is gradual. Another feature not evident in the standard environment is that males and females equally control the sexual interactions (Bergheim et al., 2015). Indeed, seminatural environments provide the female with ample opportunities to control sociosexual interactions, at difference to most standard environments in which the male appears to dominate (Chu and Ågmo, 2014, 2023). The fact of providing the females with these opportunities, reproducing the situation occurring in nature, makes seminatural environments a more realistic model of biredirectional socio-sexual interactions between males and females. In addition, it also makes seminatural environments research tools more suitable for the welfare of the female subjects.

In the studies mentioned in the preceding paragraph, as well as in many others, the purpose was to understand the dynamics of rat sexual behavior, without the slightest intention to generalize the results to other species. What we pretended, though, was to be able to generalize our findings to rat behavior outside the laboratory. Valid generalizations to the natural habitat make it possible to present fruitful analyses of the adaptive value of behavior patterns, rather than the sterile speculations based on data from standard procedures lacking external validity.

Seminatural environments are useful not only for detailed descriptions of animal behavior, but they can also be used in experiments. Early examples were the introduction of a predator (a cat) in the open area of the visible burrow system in studies of defensive behavior (Blanchard and Blanchard, 1989; Blanchard et al., 1991). More recent examples are studies on the role of the estrogen receptors α and β in several hypothalamic nuclei in female sociosexual interactions (Snoeren et al., 2015). In our laboratory, we have also introduced different kinds of events in the environment. Among what we believe to be emotionally positive events are the odor of lavender, the sudden availability of chocolate pellets or the sound of a sonata by Mozart. Emotionally negative events can also be used, for example a strong white noise or fox odor (Le Moëne and Ågmo, 2018; Le Moëne et al., 2020b). The behavioral consequences of these events can then be described in untreated rats, in rats where hormone receptors have been manipulated (Le Moëne et al., 2019), in rats treated with anxiogenic or anxiolytic drugs (Le Moëne and Ågmo, 2019), or whatever treatment found of interest. The use of the seminatural environment, i.e., a representative design or a design with external validity, should make it legitimate to generalize the findings to rat behavior in all kinds of situations inside and outside the laboratory. However, while intraspecies generalizations of the results can be made, it would be very risky to maintain that we can generalize to other species. Furthermore, in the studies mentioned above, there was no intention to model human pathologies, and no speculations as to clinical relevance of the results were made. Nevertheless, it has been suggested that procedures based on spontaneous behaviors being part of the natural repertoire are needed for developing valid models of human disorders (Puscian and Knapska, 2022).

Variable environments, for example seminatural environment, are more representative of real biological systems and therefore

have greater predictive validity and replicability. However, one of the main challenges that a researcher faces when using variable environments in animal models is precisely the need for better standardization of research protocols and understanding the inherent variability in biological systems, which could reduce the sensitivity of the experimental assessment (Voelkl et al., 2020).

Employing seminatural environments to model human behavioral and psychiatric disorders

So far, no attempt has been made to use a seminatural environment for describing the behavior of any animal model of human disease. However, in principle this could be extremely helpful. For example, studies of rats prenatally treated with valproic acid, a model of autism (Nicolini and Fahnestock, 2018), in seminatural environments could provide a much richer behavioral characterization than any of the procedures currently used. Such a characterization could be important for a better understanding of the behavioral alterations in autism and provide an opportunity for evaluating treatments. Any of the many transgenic rat strains, supposedly modeling pathologies such as schizophrenia (Uzuneser et al., 2019), Parkinson's disease (Paldino et al., 2022) or depression (Matthes et al., 2019) could also be studied, just to mention a few examples. There is no doubt that such studies could shed new light on many of the behavioral alterations hitherto poorly understood. This, in turn, may open doors to the neurobiological bases of these alterations.

Animal welfare in seminatural environments

Quantifications of animal welfare is a tricky issue (Le Moëne and Ågmo, 2017). However, there is consensus concerning the basic importance for animal welfare of having the opportunity to express a substantial proportion of the natural behavioral repertoire (Miller et al., 2020). In fact, compared to standard laboratory tests, seminatural environments offer a high degree of welfare to the animals (Makowska and Weary, 2016). It appears that such environments satisfy most of the recently proposed criteria for animal-friendly tests (d'Isa and Gerlai, 2023). The subjects are allowed to interact with conspecifics while having the possibility to avoid or escape from social contact. Moreover, the subjects are provided with a relatively large and complex space to move in, which gives them the possibility to express a substantial part of their behavioral repertoire. The environmental disturbances introduced in some experiments could be considered part of rats' natural habitat. While walking around in the garbage dump, rats will be exposed to odors of all kinds, including urine and feces from the cats and dogs in the neighborhood, they can find highly palatable as well as uneatable food, and suddenly be victims of loud noises. All these events, and many more, may occur in rapid succession during any nocturnal walk outside the burrow. They might be aversive, but it is known that rats' emotional responses to aversive events are attenuated when conspecifics are present (Kiyokawa and Hennessy, 2018; Denomme and Mason, 2022), as is the case in the seminatural environment. Moreover, all aversive stimuli mentioned here are of short duration, and it is known that rats resume their normal activities within less than 5 min after the end of an aversive event, like strong white noise (Le Moëne et al., 2020b). The fact that all events occur in a well-known, safe environment probably contributes to this. Thus, we propose that seminatural environments, in addition to providing a higher number of stimuli positively modulating the affective state of rats, also provide an enrichment buffer which enhances the rats' resilience to stress and to possible aversive stimuli.

TABLE 1 Advantages and disadvantages of seminatural environments.

Advantages	Disadvantages
The data can be generalized to rats' behavior outside the laboratory, including the natural habitat.	There are no established standards for the design of seminatural environments. Heterogenous conditions might reduce the sensitivity of the experimental assessment.
It is possible to describe, in excruciating detail, the interactions among several animals observed simultaneously.	At present it is unclear whether these environments are helpful for modeling human psychopathologies.
Allow researchers to observe a higher number of fine behavioral features than possible in standard tests.	Demanding in lab space and time investment required for performing experiments as well as for data collection and analysis.
In addition to observational studies, they can be used in naturalistic experimental conditions, for example for evaluating emotion-inducing events occurring in the environment.	Not suitable for high throughput studies.
They offer a higher degree of welfare to animals because they satisfy most of the criteria for animal-friendly tests.	It could be necessary to complement the results obtained with data from additional procedures when modeling human psychopathologies.
They may reduce the number of animals required for appropriate power because of the huge number of data points obtained from each animal.	

Conclusion

Seminatural environments not only satisfy requirements for a representative design, thereby assuring external validity and improved replicability, but also enhance animal welfare. The drawback of this kind of environment is the low throughput. Drug screening, for example, would be entirely impracticable in such environments. On the other hand, seminatural environments can be helpful for testing animal models of human psychopathologies and they have recently been proposed as a paradigm that could revolutionize translational psychiatry (Shemesh and Chen, 2023). We have summarized the advantages and disadvantages of seminatural environments in **Table 1**.

The use of seminatural environments has remained at a rather low, stable, level for several decades. However, the enormous progress in automated analyses of videorecorded behaviors, even in group living animals, have made studies in seminatural environments easier to implement and consequently more attractive. Indeed, seminatural environments are a promising tool for both neuroscientific and psychiatric translational research.

Author contributions

Both authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of interest

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

Publisher's note

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Making a case for the free exploratory paradigm: animal welfare-friendly assays that enhance heterozygosity and ecological validity

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"Rodents are not test tubes with whiskers"
– author unknown

Introduction

Rodents, laboratory rats and mice, have been used as models in experimental research for almost two centuries (Keeler, 1947; Bolles and Woods, 1964; Nishioka, 1995; Guénet and Bonhomme, 2003). During this time, it has been assumed that rodent suffering was a necessary part of the tremendous scientific advancement, and thus the means justified the ends. After centuries of unregulated research, animal welfare committees were instituted in Europe, America and Australasia to limit animal suffering (Steneck, 1997; Curzer et al., 2016). While licenses to conduct research on animals are often burdensome to obtain (Curzer et al., 2016), there has been strong variance across nations in expectations for the license, the review process and compliance (Varga, 2013). Institutions in some nations, for instance, are not financially-equipped to perform random onsite inspections, or hire veterinarians to assess or enforce conditions of the license. Those critical of the process, such as Rollin (2002) have invoked the idiom *"the fox guarding the hen-house"* to describe the seemingly voluntary nature of compliance for researchers in these circumstances. Regardless, some common assays that can cause needless or unjustified suffering are still used (Mason et al., 2004; Carbone, 2019), and some licenses that are appropriately established are not followed closely-enough (Jerusalem Post, 2023). Meanwhile, science is more broadly communicated than ever, and the general public and media are becoming more aware of this suffering, particularly as we learn more about the animals themselves. For instance, rodents were historically viewed as vermin or pests. Yet it is now widely recognized that rodents are sentient (Bartal et al., 2011, 2014; Mogil, 2012; Mason, 2021), and like any animal, they deserve an expansion of our "compassion footprint" (Bekoff, 2010; Cochrane, 2013; Dunayer, 2013).

Amidst challenges to the current system (Varga, 2013) mounting data on animal suffering (Buckland and Nattrass, 2020; Webb et al., 2020), and calls from animal rights groups (McMahon et al., 2012) recommending the replacement of laboratory animals altogether (Gruber and Hartung, 2004; Langley et al., 2007; Robinson et al., 2019), there is a clear need to take additional steps to limit suffering. One such approach is to develop alternative assays. Fortunately, there are available assays which promote more positive affective states for rodents (Jirkof et al., 2019), while minimizing the number of animals bred into captivity and/or euthanized. Here we argue the value of one of many such approaches, the free exploratory paradigm (FEP; Griebel et al., 1993), which is a paradigm that allows animals to freely enter and exit a test apparatus. We suggest that the FEP can improve rodent welfare in both laboratory and field assays. We then discuss how the FEP can be utilized to improve the quality of data from some of these experiments.

As a team composed of field ecologists, ethologists, physiologists and neuroscientists, we study rodents in the field and laboratory. Our experiences with rodents do not align with historical attitudes and opinions. Rodents have traditionally had a reputation, particularly in some nations, as animals that "deserved to die" (Buckland and Nattrass, 2020). This poor perception of rodents was mostly worldwide, but it was epitomized by a survey of 200 households in Cape Town, South Africa. Almost one-fifth of participants answered they were "happy" for rodents to suffer before death, and only one third cared whether rodent control was humane (Buckland and Nattrass, 2020). In the centuries that unregulated rodent research took place, attitudes toward rodents used in scientific research could have reflected social attitudes. Common tests that have historically been known to cause suffering included moderate deprivation and reward studies, forced swim tests and forced copulation assays. In the latter case, sexually-receptive females are first paired with a male and later substituted with non-receptive females. These non-consensual copulatory assays were "justified" by the authors as a means to better understand (human) male sexual violence. Despite the unquestioned importance of laboratory animals to scientific progress over 200 years, suffering has become institutionalized. Not only is suffering bad for welfare, but stress within the laboratory causes data- distortion and reduces the justification of such studies (Bailey, 2018). Fortunately, we are now far enough along in advancements and technology, that we can raise the standard of justification for a few historic assays that have limited usefulness.

For instance, all members of our international team have experience in field research. Several of our team members have been approached by laboratory researchers who wish to expand their studies to the field. The reasons for the transition are varied, yet one experience stands out. When MHP inquired about the forced swim test to a research team who recruited him to help, they explained that the assays were used to train future research students and for the benefit of any theoretical knowledge that was gained by using it. The principal researcher, who worked at a major research institution, had not considered whether there were tangible outcomes to medicine or society. However, some of these researchers-in-training would likely carry the same assays forward when they train their own students. One can see how this attitude, if embodied elsewhere, could become a cyclical process that perpetrates suffering. This occurs when students become desensitized to rodents' suffering (Balcombe, 2000), develop "compassion fatigue" (LaFollette et al., 2020), or falsely assume suffering is justifiable, because "vermin" are not thought of as having "feelings, emotions and/or memories."

Research over the past 15 years, however, has shown laboratory rodents experience a wide range of feelings, emotions, regret and intelligence—being far more sentient than previously thought (Webb et al., 2020; Crump, 2022; Webster, 2022). While all animals, sentient or not, deserve our compassion (Bekoff, 2010), society has historically given more rights to animals thought to express memories, intelligence or sentience (Cochrane, 2013; Dunayer, 2013). For instance, we now know rats and mice show a high degree of empathy (Crawley, 2004; Bartal et al., 2011, 2014; Cox and Reichel, 2020) and remorse (Steiner and Redish, 2014). Rats are smart (Davis, 1996), have exceptional memories and can assess time (Kononowicz et al., 2022). Among rats driving robotic cars, those living in enriched environments had more robust driving skills (Crawford et al., 2020). All 17 rats of the latter study assayed had a higher concentration of dehydroepiandrosterone while driving, indicating they were experiencing the reward of learning a new skill (Crawford et al., 2020). The media, so important in steering social expectations, also widely-reported rats' ability to play, be tickled, and express joy through ultra-high frequency vocalizations (Mällo et al., 2007; Hammond et al., 2019; Burke et al., 2022). In a highly-cited, and attitude-shifting paper, these "chirps" were shown to be analogous to laughter (Panksepp and Burgdorf, 2000). Finally, in a finding that went "viral" on social media, researchers found that rats move to the beat ("danced") of an eclectic range of popular music from Mozart to Michael Jackson (Ito et al., 2022). This public knowledge is helping shift attitudes, which is in turn compel animal rights advocates and researchers to explore alternatives. While a common concern of researchers is that their research outcomes could be compromised by welfare-friendly designs, we will argue that, in some situations, the FEP may actually improve data quality and research outcomes.

Ironically, the researchers who we referred to earlier, contacted us, not to improve the welfare of the animals, but to increase the value of their own research. The principal complaint was that *"laboratory animals were a product of indolence and lacked genetic variability."* To eliminate variation, which might permit smaller effects to be detected, laboratory studies often used genetically-homogenous strains. Not only have laboratory animals been deprived of heterozygosity, the processes of domestication has modified the behavior and physiology of these animals. Such studies make it difficult to have broad conclusions. To eliminate further variation, laboratory studies also test animals in standardized environmental conditions that often do not reflect their natural environments in which they evolved. Thus, traditional tests purposefully remove interfering contextual variables (Rader, 1997, 2004; Würbel, 2000; Wolff, 2003; Voelkl et al., 2020). Yet these environmental variables intentionally removed from standardized assays may be essential for understanding treatment of some illnesses (Nesse, 1994; Mobbs and Kim, 2015; Oppenheim, 2019).

For instance, much neuroscience research focuses on fear or anxiety. These are natural states (Blumstein, 2020) that are elicited

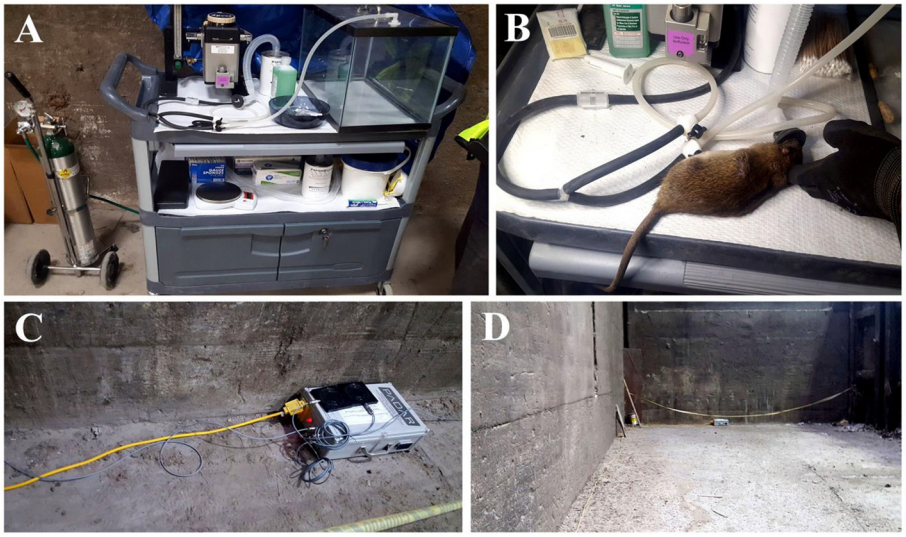

FIGURE 1
Field FEP comprised of free ranging animals with continuous surveillance using passive microchip readers and video recorders in an urban environment. **(A)** Mobile outdoor laboratory; **(B)** gas anesthesia system for RFID implantation; **(C)** antennas placed within "rat runways"; **(D)** natural (non-modified) landscape within an urban warehouse setting.

by cues of threats and may be modified in the absence of shelter, the presence of conspecifics, or when visibility changes (Orrock and Danielson, 2009; Parsons and Blumstein, 2010). The lack of these sorts of natural contexts in research assays, as well as a lack of genetic variation, has led to a perceived crises in some sub-disciplines when laboratory outcomes do not relate to practice (Manjili, 2013; Drucker, 2016; Fendt et al., 2020; Stryjek et al., 2021a). Furthermore, we understand that non-welfare-friendly designs may create uninterpretable data. This occurs when data is compromised after being collected from stressed animals (e.g., data distortion; Bailey, 2018). Yet the FEP, as we describe below, could improve research outcomes to address each of these crises.

The FEP can be a more welfare-friendly approach when used in the laboratory (Stryjek et al., 2012; Kohl et al., 2018; Mei et al., 2020; Kohler et al., 2022) or the field (Stryjek et al., 2018; Bedoya-Pérez et al., 2021; Parsons et al., 2023). This type of assay is similar in some respects to home cage testing (Grieco et al., 2021), where animals are tested in the place they live in order to minimize the stresses of transport and handling. It also allows animals to choose if and when they visit an experimental test. They are neither deprived nor punished beforehand, and they choose whether to remain or leave a test arena. There are many examples of FEP and we will give only generalized examples of how they might operate.

For instance, an FEP test may involve experimental chambers whereby animals are attracted by food, shelter or conspecifics, and assayed under video surveillance or direct observation (Bedoya-Pérez et al., 2021; Parsons et al., 2023). In some circumstances, even more realistic assays may be constructed using the natural landscape such as common "rat runways" instead of chambers (Figure 1; Parsons et al., 2019). While we recognize these approaches are not sufficient for all research questions, we will highlight the benefits, a few types of hypotheses that may be addressed, and potential advantages over traditional tests.

Field

In addition to being welfare-friendly, FEP in the field offers other advantages. Free-living rodents are assumed to be genetically variable and possess their full faculties. This is in contrast to lab animals, which are inbred, have smaller brains, adrenal glands, and different sized brain structures including the basolateral complex of the amygdala, main olfactory bulb, and accessory olfactory bulb (Koizumi et al., 2018), among others. The differences are exacerbated by albinism which is frequent among laboratory rodents and causes impairment in various senses (e.g., Lockard, 1968; Sachs, 1996; Prusky et al., 2002). Additionally, such studies require limited (or no) handing, an unnatural stressor (Sensini et al., 2020) that can influence outcomes. This could be especially relevant to researchers that prefer to pre-identify subjects prior to testing. Additionally, studying animals in their natural environment allows more accurate study of environmental contexts that are missing in standardized laboratory trials. Context is essential because decision rules are often context-specific (e.g., Pinho et al., 2019; Heissenberger et al., 2020). Contexts, such as the availability of conspecifics, competitors, shelter and predators are expected to modify a variety of behaviors of interest. This can be especially important in fear and anxiety studies (Orrock et al., 2004; Orrock and Danielson, 2009; Parsons et al., 2018). Indeed, laboratory and field trial outcomes in olfactory-based research often differ (Apfelbach et al., 2005), and many of these differences can be explained by variable contexts (Parsons et al., 2018; Fendt et al., 2020; Stryjek et al., 2021b). The most parsimonious approach to improve welfare outcomes and increase experimental contexts may be to move laboratory-style chambers into the field (Figure 2; Modlinska and Stryjek, 2016; Stryjek et al., 2018; Bedoya-Pérez et al., 2021; Parsons et al., 2023). A wide range of possible experimental topics are discussed in Stryjek et al. (2021a)

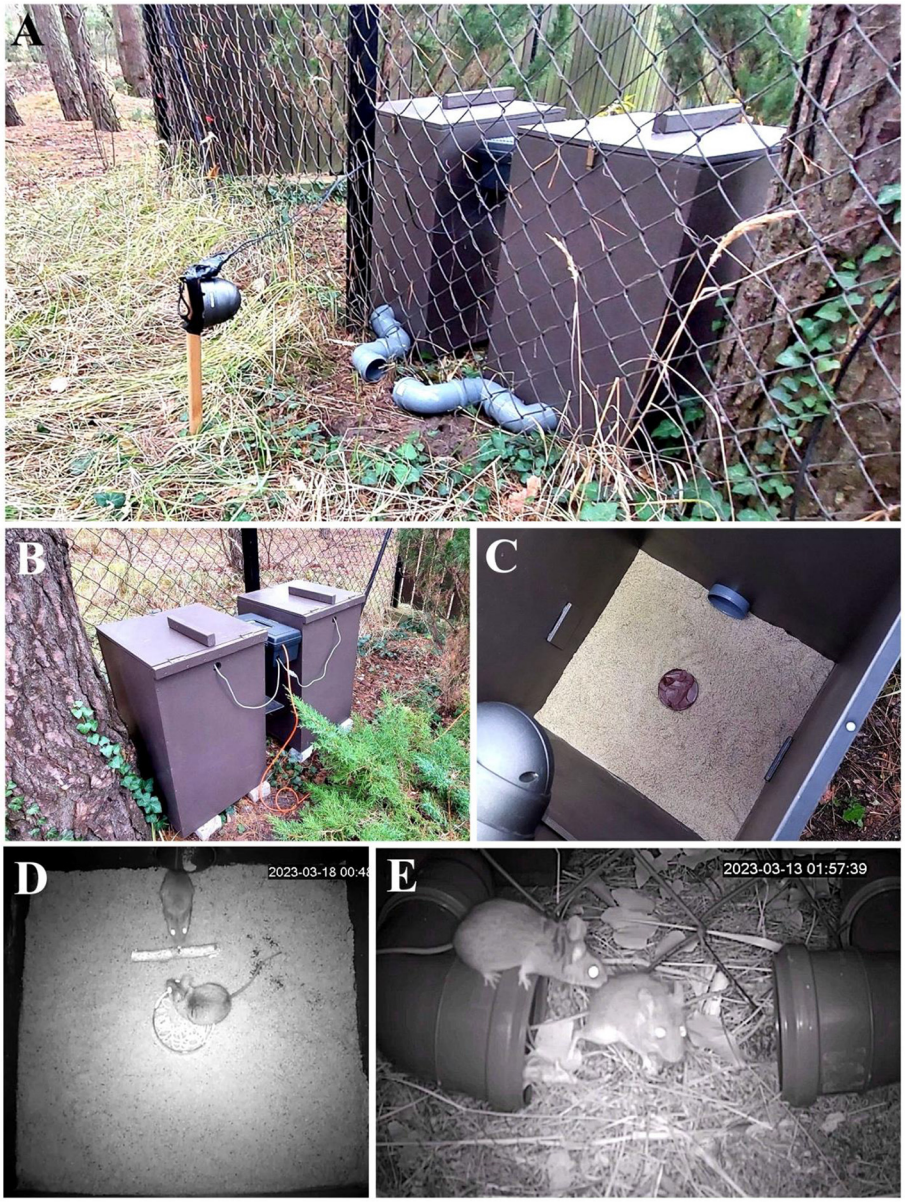

FIGURE 2
Field FEP in natural conditions demonstrating an assay that eliminates all animal handling. Laboratory-style boxes used for a study on free-ranging rodents (Apodemus mice; *A. agrarius* and *A. flavicollis*). **(A, B)** Two wooden chambers deployed near mice's habitat. **(C)** The inside of the experimental chamber with floor covered with sand layer and chocolate cream as a bait. **(D)** Video-still showing yellow-necked mice (*A. flavicollis*) during social interaction inside one of the chambers. **(E)** Video still showing yellow-necked mice during social interaction near the entrance pipes.

and these include studies of novelty, cognition, problem-solving, sensory acuity, behavioral responses to stress (stress resilience), and social behavior.

Millions of rodents are bred and sold to research laboratories each year. By testing wild animals in the wild, fewer rodents have to be bred and there is no need to kill free-living animals. Testing animals in their natural environment may help address the "crisis" of translational medicine as reported by Oppenheim (2019), where he argued that findings from the laboratory are not reliable predictors of clinical outcomes. Finally, studying animals in the wild may be a pathway to identify promising new model system that could be brought back into the laboratory, where they could be more systematically studied using a FEP.

Laboratory

FEP assays when conducted in the laboratory are also welfare-friendly, while the advantages for improving research outcomes are not as straight forward as those in the field. Wild animal studies can be important model systems and by bringing them into laboratory trials they can increase genetic variability and help

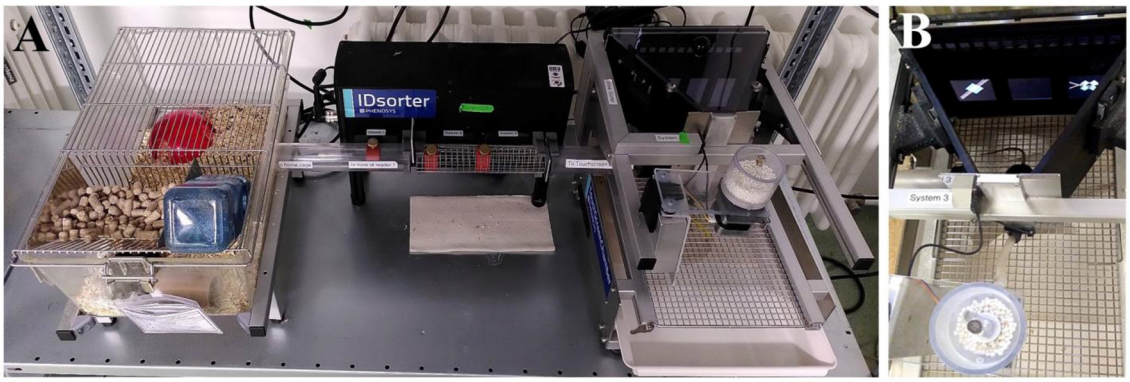

FIGURE 3
(A) Homecage (left), sorter system (middle), and touchscreen box (right). **(B)** Touchscreen box with pictures of a compound discrimination task on the screen (part of the attentional set shifting task measuring cognitive flexibility).

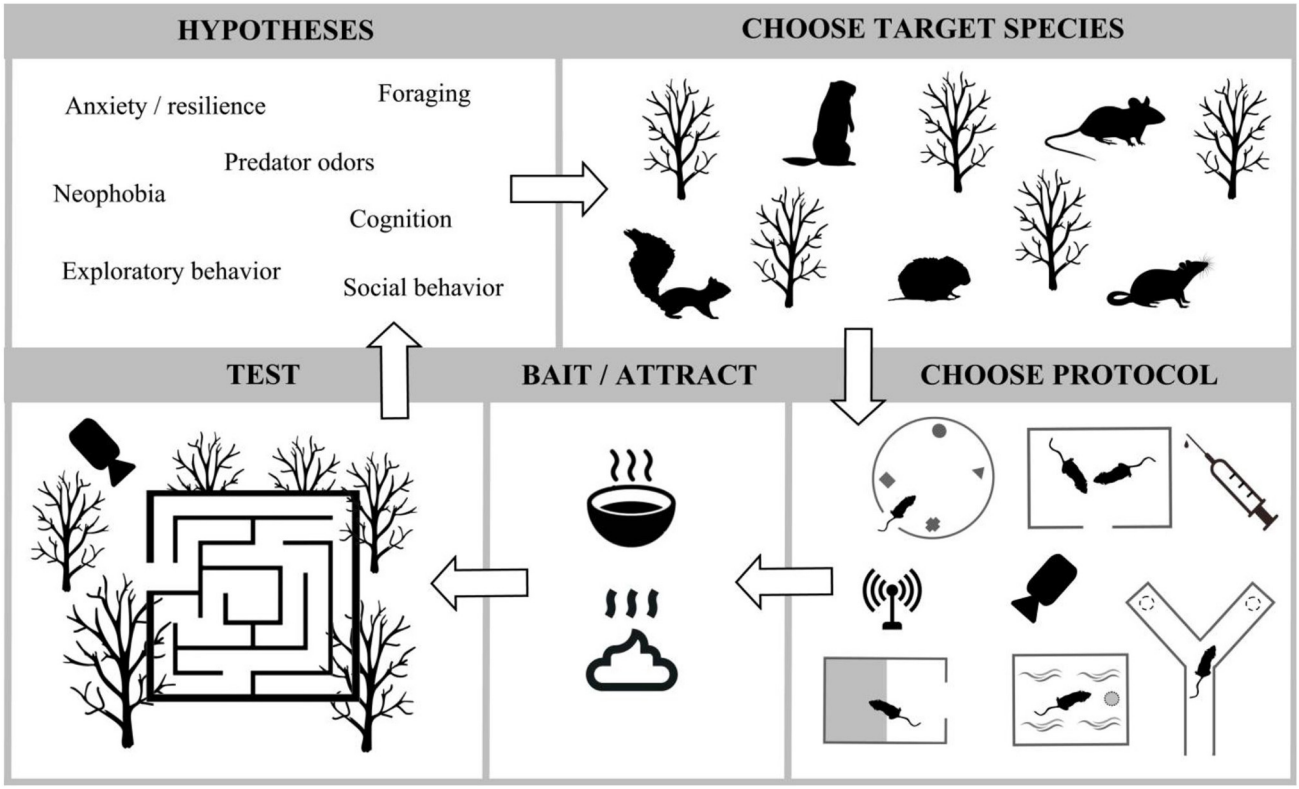

FIGURE 4
Infographic relating a few generalized types of hypotheses that can be addressed in a range of FEP assays in either the laboratory or the field. As more people work within this paradigm, more types of research questions will be addressed.

us better understand natural behavioral variation (Stryjek et al., 2013; Dolivo and Taborsky, 2015; Kiyokawa et al., 2017; Koizumi et al., 2018; Schneeberger et al., 2020). However, animals are still captive. Additionally, animals in traditional paradigms are forced to explore/take part in the study, which elevates stress and distorts the result and can even eliminate behaviors under study. Yet, research in recent decades explored how the FEP could be applied in the lab. Laboratory FEP setups usually consist of a home cage and a testing device, e.g., operant walls (Kiryk et al., 2020), a touch screen box (Rivalan et al., 2017) or mazes such as the radial arm maze (Mei et al., 2020; Kohler et al., 2022). Figure 3 shows an example where the two are connected by a so-called sorter that recognizes the RFID-chipped mouse, ensures that only one animal enters the test device alone, and then starts individual tests in the test device via a connected computer. Mice or rats explore such a setup without food deprivation once they have access to it, quickly

become accustomed to the set up, and learn within a few days that they are rewarded (e.g., with sucrose solution or pellets) for solving tasks in the test device. Such a FEP setup can theoretically run 24/7, and the animals visit the test device during both their active and passive phases, making more visits during the active period but surprisingly performing very similar in both phases. Compared to the classical procedure, where animals are manually placed in the boxes for training, the complete training procedure in such an application is several times faster. These studies show that the FEP is useful in field and laboratory conditions.

Conclusions

There is increasing desire from researchers (Jirkof et al., 2019; Buckland and Nattrass, 2020; d'Isa and Gerlai, 2023), animal welfare advocates, and the public to shift our attitudes about rodents used in experimental research. Indeed, a contingent of researchers and advocates are calling for the replacement of laboratory animals altogether (Gruber and Hartung, 2004; Langley et al., 2007). This has all been happening while traditional rodent research has been under scrutiny (Oppenheim, 2019; Voelkl et al., 2020, 2021) because laboratory animals lack genetic diversity, and because experimental laboratory situations are not similar enough to the "real world" to justify suffering if studies produce questionable results (Manjili, 2013; Drucker, 2016; Matusz et al., 2019; Oppenheim, 2019; Fendt et al., 2020). We recognize however, that laboratory animals are our only means for success in some areas of biomedicine. So, our position is not so strong that we recommend replacing animals altogether, and we recognize that not all research questions can be adequately addressed by the FEP. They do however offer, for some researchers, an intermediate, transitional step, whereby study protocols are explicitly designed to optimize animal welfare and to produce interpretable findings. Ultimately, the FEP, whether the designs we have highlighted, or new designs built to address new questions, can dramatically improve the welfare of rodents, while, when used in the wild, can reduce the number of animals bred and euthanized.

The most important misconception we have addressed relates to a common concern about adapting new practices relates to the false assumption that research outcomes will be compromised. FEP in the field may improve research outcomes because they incorporate genetic diversity, minimize animal handling, and take place in a natural environment where many contexts can be isolated or understood in concert with one another. This would satisfy animal welfare concerns and at the same time, address issues about translatability of findings (Drucker, 2016; Oppenheim, 2019). Laboratory FEP may be designed to improve outcomes and welfare in three ways: (1) by increasing heterozygosity when wild animals are brought into controlled settings and allowed to freely enter the designed apparatus; (2) when naturalistic contexts such as availability of conspecifics and shelter are incorporated into laboratory FEP settings; and (3) by minimizing animal handling, we decrease animal stress which is known to cause data distortion (Bailey, 2018). In short, improved welfare also increases data quality. While these types of assays have great potential to improve welfare and for more translatable outcomes, we would be remiss if we did not acknowledge potential shortfalls to be considered during the design and implementation. First, when used in the field where predators are nearby, we recommend deployment of video cameras to look for potential negative impacts on the subjects. In the laboratory, and when using wild-caught animals, care should be taken while catching them and acclimating them; some species may be unsuitable for captive living (Stryjek, 2010; Stryjek et al., 2021b). We hope in the next 10 years, that many variations of the FEP are created to continue addressing our most pressing research questions (for an exhaustive list see Stryjek et al., 2021a, and also Figure 4) in neuroscience, ethology and clinical medicine.

Author contributions

This invited opinion came about through extended discussions between MP, RS, MF, YK, PB, and DB. MP, RS, MF, YK, PB, and DB wrote and edited the draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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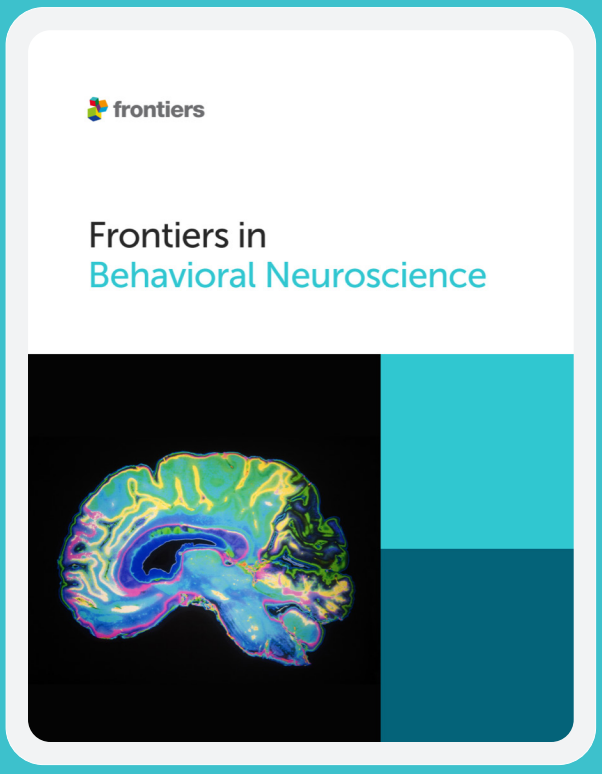

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