Development and Validation of a Social Home Cage System for Rat Cognitive Testing: The Serotonin Case

Armen Andreasyan

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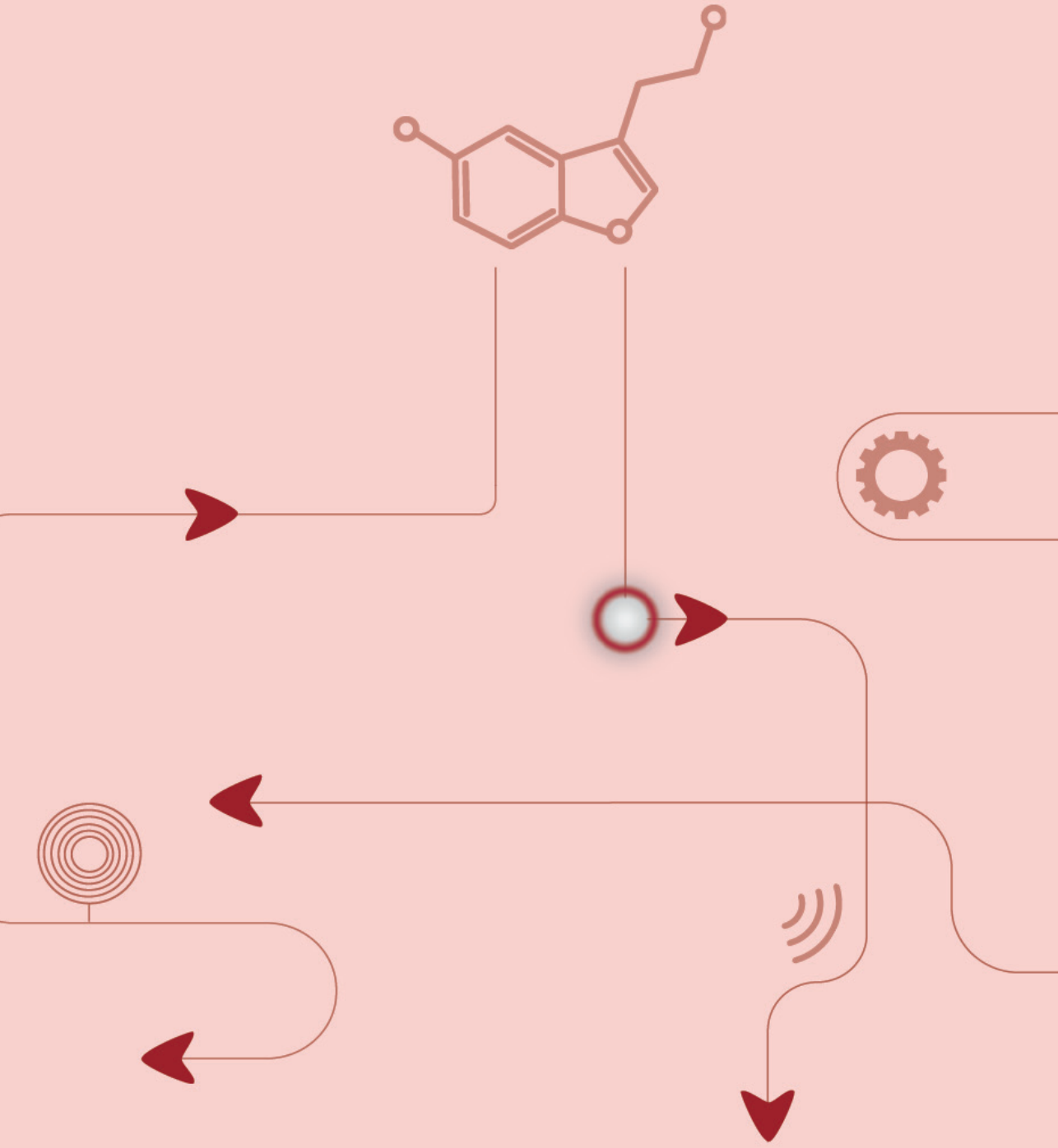

Chapter 1

Introduction

Global Burden of Neurological and Psychiatric Disorders

When it comes to understanding the societal impact of mental health conditions, economic markers serve as powerful indicators, revealing the vast scale of their consequences and the urgency for effective prevention and intervention strategies. Neurological and psychiatric disorders together impose an enormous global burden on health and society. Recent estimates indicate that in 2021 over 3 billion people worldwide - more than one-third of the global population - were living with a neurological condition ("Over 1 in 3 People Affected by Neurological Conditions, the Leading Cause of Illness and Disability Worldwide", 2024). Neurological disorders have become the leading cause of disability globally, with the total disability-adjusted life years (DALYs - a public health metric combining years of life lost due to premature death and years lived with disability) from these conditions rising by ~18% since 1990 (GBD 2021 Nervous System Disorders Collaborators 2024). In parallel, mental health disorders are highly prevalent: as of 2019 approximately 1 in 8 people (around 970 million worldwide) were living with a mental disorder, most commonly anxiety or depression ("Mental Disorders", 2025). The COVID-19 pandemic further exacerbated this, with global rates of anxiety and depression spiking by over 25% in 2020 ("Mental Disorders", 2025). Beyond the human toll, the economic costs are staggering - a Lancet Commission projected that mental health conditions could cost the world up to $16 trillion in lost productivity and health expenses between 2010 and 2030 if we fail to act (Patel et al. 2018). These sobering statistics underscore an urgent need for improved strategies in brain health research and intervention on a global scale.

Executive Function and the Case for Early Intervention

Amid this crisis, **executive functions** (EF) have emerged as a critical focus for understanding and improving mental health diagnosis and outcomes. EFs are the top-down cognitive abilities - including inhibitory control, working memory, and cognitive flexibility - that enable regulation of thoughts, emotions, and actions in daily life (Diamond 2013; Esmaili et al. 2023). Notably, executive dysfunction is a common thread across many psychiatric disorders, making EF a transdiagnostic target for research (Esmaili et al. 2023). Deficits in EF are observed in conditions ranging from ADHD to depression and schizophrenia, and such deficits can predict poorer clinical outcomes and impair an individual's capacity to benefit from treatments. Crucially, EF skills develop rapidly in childhood and adolescence, a period when the brain is highly plastic. Early-life stress and adverse childhood experiences (ACEs) can disrupt the development of executive and self-regulation skills, which in turn elevates the risk for learning difficulties, mental health problems, and even criminality

in adulthood (Wexler and Alexander 2020). Conversely, strengthening EF in young people is increasingly seen as a powerful preventive approach. School-based programs that train attention, self-control, and other EF skills have shown lasting benefits, protecting at-risk children from developing later mental health issues. In short, early intervention targeting executive function and self-regulation may foster resilience and alter life-long trajectories, offering a promising avenue to ameliorate the burden of psychiatric disorders before they fully manifest.

Preclinical Cognitive Testings: Rodent Models and Limitations of Traditional Behavioral Tests

To unravel the biological mechanisms behind cognition and mental illness - and to develop early interventions - researchers rely heavily on rodent models (Homberg, Wöhr, and Alenina 2017). Rodents (especially rats) provide tractable systems to probe genetic, molecular, and circuit-level factors influencing brain and behavior. However, traditional rodent behavioral testing paradigms come with significant limitations. Conventional cognitive tests (e.g. maze navigation, operant conditioning in Skinner boxes) often require removing animals from their home cage, extensive handling, and testing them in isolated apparatus under tightly controlled conditions. Such procedures can induce stress and anxiety, which confound performance measures and reduce translational relevance (Berger et al. 2018; Towers et al. 2019). Moreover, to ensure experimental control, animals are typically tested one at a time in solitary enclosures, and the time of testing may vary within the experimental subjects per day and across different studies. Such factors raise concerns about the ecological validity of findings from traditional methods. In response, there is growing recognition that next-generation behavioral setups must account for animals' welfare and naturalistic behaviors. For instance, a study (Bueno-Guerra 2021) reveals that 71.4% of researchers reported a lack of consensus in defining relevant concepts such as cognition. Furthermore, 56.1% of the apparatuses used do not address species-specific ecological needs, for example, testing elephants with generic cognitive tasks instead of problems they typically face in the wild, and there's a need for collaboration with engineers to address current technological requirements. Recent critiques have highlighted the need for paradigms that minimize human interference and better mimic ethological conditions (Bailey 2018). In essence, while rodent models remain indispensable, improving how we conduct behavioral assays is crucial for both ethical reasons and the quality of obtained data.

Advances in Rodent Cognitive Testing

Technological innovations are now driving a transformation in rodent cognitive testing, aiming to address these limitations. One major advance has been the adoption of touchscreen-based cognitive testing platforms for rodents. While touchscreens are not inherently part of a rodent's natural environment, they can be implemented in ways that reflect species-typical behaviors. For example, tasks can be designed around natural actions such as nose-poking, foraging-like exploration, or approaching novel stimuli, and integrated into home-cage setups. This approach preserves the advantages of automated, standardized testing while aligning with rodents' ecological and behavioral tendencies. Inspired by the CANTAB touchscreen battery used in human neuropsychological testing, Bussey and colleagues developed analogous tasks for rats and mice that animals solve by touching stimuli on a screen for rewards (Bussey et al. 1994). This approach enables a diverse range of cognitive functions to be assessed in a standardized and automated manner. Unlike aversive maze tasks, touchscreen protocols typically use appetitive rewards and mild food motivation, which reduces stress and anxiety during testing (Horner et al. 2013). The computer-controlled paradigm also ensures consistent stimulus presentation and data recording, improving reproducibility and throughput. Indeed, the touchscreen operant platform has been successfully used to probe learning, memory, attention, and executive function in rodent models of Alzheimer's, schizophrenia, and other disorders in tasks closely paralleling human tests (Romberg et al. 2013). Nonetheless, some ecologically relevant tasks may deliberately depart from direct human-test parallels in order to prioritize species-specific validity and better capture natural behavioral repertoires. However, even these advanced systems usually involve training animals in dedicated testing chambers separate from the home cage, often one animal at a time. Thus, they only partially address social housing, as animals are still removed from their group and tested individually in unfamiliar environments, which can influence behavior and reduce ecological validity. Recognizing this gap, researchers have begun integrating cognitive testing into more ethologically relevant, home-cage based setups. For example, fully automated home-cage testing systems now allow rodents to perform cognitive tasks voluntarily, without human presence, and often during their normal active periods. In a recent study, (Huang et al. 2023) demonstrated that rats trained on a *fully-automated, experimenter-free* touchscreen system (with the animals self-initiating trials) learned cognitive tasks much faster and could complete many more trials per day compared to traditional methods - all while drastically reducing experimenter involvement (Huang et al. 2023). This highlights the promise of merging high-tech operant

platforms with self-pacing conditions: when rodents can engage with tasks on their own schedule and without the anxiety of human handling, their true cognitive capacities can be measured more efficiently and authentically. Still, implementing such systems in group-housed scenarios presents challenges (e.g. tracking individual animals, preventing competition at the apparatus), and until recently, few solutions existed to allow *socially housed* rodents to participate in cognitive tests freely. It is also important to highlight that the commercially available solutions mentioned are great, but they introduce a lack of flexibility for future development. Our lab's experience shows that rapid modifications are often crucial to address the very specific goals of certain studies. Since we focus on various genetically modified rat models, it is essential for us to develop an in-house solution that can be adapted to different settings and integrated into larger systems.

To summarize, we found that despite advances, existing rodent behavioral assays maintain critical limitations:

1. **Social Isolation**: Conventional paradigms isolate animals in test boxes, disrupting natural social dynamics.
2. **Handling Stress**: Frequent human interaction for transport and setup elevates stress hormones and skews performance.
3. **Fixed Testing Windows**: Experimenter-determined schedules often conflict with rodents' nocturnal activity cycles.
4. **Lack of Complex Cognitive Focus in Home Cage Systems**: Most automated home-cage platforms track locomotion or simple behaviors, such as rearing or grooming, but do not support structured cognitive tasks.

Proposed Solutions To address these gaps, we designed a system that:

1. **Maintains Group Housing:** Allows continuous social interactions in a shared central cage, preserving normal hierarchy and affiliative behaviors.
2. **Eliminates Routine Handling:** Automates animal entry and exit to testing chambers using gated tunnels and Radio-Frequency Identification (RFID), minimizing human-animal contact to cage cleaning only.
3. **Enables Self-Paced Testing:** Touchscreens are available eight hours per day (as a result of optional choice, otherwise unlimited availability is possible), permitting rodents to initiate tasks on their own motivational schedule.
4. **Integrates Complex Cognitive Protocols:** Integrates complex cognitive protocols by implementing visual discrimination, reversal learning,

and working memory tasks directly within the home-cage connected chambers as well as monitors and records socializing tendencies.

The Social RatPad: Socially Inclusive Testing System

Building on these solutions, we developed in this thesis the Social RatPad system - an automated, socially inclusive touchscreen testing platform. First, we took it into account guideline requirements (table 1). These documents outline the minimal standards for social housing, environmental enrichment, and cage area per animal, which must be addressed to ensure both ethical compliance and high-quality scientific outcomes. Table 1 summarizes the key requirements extracted from the Guide for the Care and Use of Laboratory Animals and the Directive 2010/63/EU of the European Parliament and of the Council, providing the foundation for the system's development.

Then we addressed the specific core requirements outlined earlier. Its core features include:

- **Dual Home-Cage-Chamber Configuration**: A central group cage connects via two tunnels to separate touchscreen chambers, enabling one-animal-at-a-time entry without manual sorting.
- **RFID-Based Identification & Gating**: Each rat carries an implantable RFID tag. Turnstile gates and RFID readers control chamber access and log every entry, ensuring individual attribution of performance data.
- **Capacitive Touchscreen & Reward Detection**: High-sensitivity capacitive screens detect deliberate touches while sensors confirm reward retrieval, reducing false inputs and equipment contamination.
- **Continuous, Voluntary Engagement**: The system runs standard touchscreen tasks (habituation, must-touch, visual discrimination, reversal, TUNL) in a self-serve mode for eight-hour periods, allowing rapid learning curves and high trial counts.
- **Real-Time Monitoring & Logging**: Automated software captures timestamps, trial metrics, delivering high-fidelity datasets without experimenter interference.
- **Long-Term Autonomous Operation:** Designed for extended, low-maintenance use, the system minimizes the need for daily manual cleaning or calibration. Unlike similar setups, which require frequent maintenance due to dust-sensitive components, the Social RatPad's closed design, capacitive sensors, and simplified reward mechanisms ensure stable performance even in long-duration experiments.

Table1. Comprehensive summary of housing, enrichment, and area requirements for laboratory animals based on established guidelines

Requirement Category	Guideline (Document)	Requirements& Details
Social Housing	Guide for the Care and Use of Laboratory Animals, 8th edition	- Social animals should be housed in stable pairs or groups of compatible individuals unless required alone for experimental reasons (Page 51).- Structural adjustments may be needed for social housing to prevent dominance (Page 51).- Appropriate social interactions among conspecifics are crucial for normal development and well-being (Page 64).
	Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010	- Social animals, except naturally solitary ones, should be socially housed in stable groups (Page L 276/56).- Single housing should be for the minimum period necessary (Page L 276/56).- Introduction or re-introduction of animals should be monitored to avoid social problems (Page L 276/56).- Solitary housing for sociable strains (short-term: mild severity; prolonged isolation: moderate severity) (Page L 276/58).
Environmental Enrichment	Guide for the Care and Use of Laboratory Animals, 8th edition	- Enrichment should provide sensory, motor stimulation, and cognitive challenges according to species-specific characteristics (Page 52-53).- Consideration of animals' natural behavior during evaluation of suitable housing (Page 63).
	Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010	- Space should be sufficiently complex to allow expression of a wide range of normal behavior, including cognitive activities (Page L 276/56).- Enrichment techniques should extend available activities, including cognitive ones, adapted to species' needs (Page L 276/56).
Area Size	Guide for the Care and Use of Laboratory Animals, 8th edition	- For rats over 500g: Floor area per animal should be ≥451.5 cm^2 (Page 59).
	Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010	- For rats over 600g: Floor area per animal should be ≥1500 cm^2.

Building on these requirements and design considerations, the Social RatPad was developed as a fully automated system that enables rodents to engage in cognitive tasks within their natural social environment. A key innovation of this platform is that tasks are no longer bound to fixed schedules or experimenter presence but instead can be initiated and performed voluntarily by the animals themselves. The cognitive tasks run continuously in a self-serve mode, allowing the rats to dictate their own training schedule - they can initiate trials at will, and complete multiple sessions per day, without any experimenter present. This represents a union of high-precision digital monitoring with the naturalistic element of voluntary participation in a social setting. By dramatically reducing human intervention and allowing rodents to train at their own volition, the Social RatPad aims to reduce stress and improve animal welfare while also leading to data with greater translational relevance. In sum, this innovative platform provides an opportunity to assess executive functions and other cognitive behaviors in rats under far more ethologically valid conditions - a crucial step toward bridging the gap between laboratory research and real-world neurobehavioral functioning.

Serotonergic Modulation of Cognition: Insights from Genetic Models

Alongside advances in behavioral testing, progress in understanding the neuro-chemical underpinnings of cognition has highlighted the serotonin (5-HT) system as a key player. Serotonin and serotonergic pathways are believed to play a crucial role in origin and process of neurological and psychiatric disorders. Serotonergic drugs are widely used within various disorders including depression, schizophrenia and obsessive-compulsive disorders (Homberg 2012). Serotonin is associated with cognitive and behavioral processes, particularly, memory (Seyedabadi et al. 2014), learning (Al-Zahrani et al. 1996), decision-making (Homberg 2012), cognitive flexibility (Evers et al. 2007), executive functions (Sargin et al. 2019), consciousness flows (Müller 2022), emotions (Meneses and Liy-Salmeron 2012), sleep patterns and circadian rhythm (Bacqué-Cazenave et al. 2020), movement (Bacqué-Cazenave et al. 2020), etc.

In the realm of cognition, accumulating evidence indicates that 5-HT profoundly affects executive functions and behavioral flexibility. For example, boosting 5-HT signaling (whether through selective serotonin reuptake inhibitors (SSRIs) or via genetic disruption of the serotonin transporter) can facilitate cognitive flexibility, improving performance in tasks requiring adaptation to changing rules (Homberg 2012). Conversely, low serotonin

states have been linked to impairments in memory, increased perseveration, and impulsivity (Cools, Roberts, and Robbins 2008). To achieve more specific insights, researchers have turned to genetic models in rodents that inherently alter 5-HT levels or signaling. Two such models used in the present work are rats with constitutive knockouts of the serotonin transporter (SERT, also known as 5-HTT) and of tryptophan hydroxylase 2 (TPH2), the brain-specific enzyme for 5-HT synthesis.

SERT knockout rats have abnormally high extracellular serotonin from early development onward (due to lacking the transporter that normally recycles 5-HT). Notably, SERT knockout (KO) rats exhibit behavioral phenotypes analogous to human anxiety and mood disorders - they show increased depression and anxiety-like behaviors, as well as excessive impulsivity and altered reward sensitivity (Homberg et al. 2007). The rats display increased anxiety-like behavior in adulthood, as shown by reduced exploration of open arms in the elevated plus maze and heightened passive coping responses in the forced swim test, suggesting greater stress reactivity and altered emotional regulation (Olivier J et al. 2010). Consistent with these traits, serotonin transporter deficiency in rats has been shown to improve inhibitory control but not behavioural flexibility (Homberg et al. 2007). These animals have been proposed as a model of developmental 5-HT excess and its neuropsychiatric consequences (Homberg 2012). Recent studies also reveal subtle cognitive differences in serotonin-transporter knockout (SERT KO) rats. For instance, information processing and learning strategies appear to be shifted: one report using a touchscreen visual discrimination/reversal task found that SERT KO rats responded to stimuli more quickly than wildtypes in initial phases of testing, but were less able to adjust their behavior when task contingencies changed in later stages, suggesting a deficit in stimulus generalization (Guo et al. 2021). On the other hand, some findings hint that life-long elevated serotonin might enhance certain aspects of flexibility; (Nonkes 2012) observed improved reversal learning in SERT KO rats, hypothesizing that high 5-HT activates cognitive control circuits. Overall, the SERT KO model underscores serotonin's complex, bidirectional influence on behavior: While adult SSRI treatment alters serotonin signaling transiently, lifelong 5-HTT deletion produces neurodevelopmental adaptations that differ markedly. SERT KO rats typically show increased anxiety and enhanced behavioral flexibility, indicating distinct, developmentally rooted effects rather than parallels to SSRI-induced anxiolytic and antidepressive effects. Importantly, all cognitive assessments in SERT KO rats have so far been carried out in adulthood, leaving a knowledge

gap regarding how cognition develops during earlier life stages. The Social RatPad now enables, for the first time, testing cognitive performance in adolescence under socially enriched and automated conditions, offering new opportunities to track developmental trajectories.

In contrast to serotonin-transporter knockout (SERT-KO) rats, tryptophan-hydroxylase-2 knockout (TPH2-KO) rats completely lack the ability to synthesize serotonin in the brain, yielding an endogenous model of serotonin deficiency from gestation through adulthood. Studying these rats offers a window into how serotonin absence affects neurodevelopment and behavior. Strikingly, TPH2 KO rats display an inverse anxiety profile to SERT KOs: they are less anxious in adulthood than normal rats in exploratory tests, despite having no serotonin (Meng et al. 2022). At the same time, they show abnormally high levels of aggression and impaired social communication, indicating that serotonin is crucial for normal social behavior and impulse control (Meng et al. 2022). These pronounced behavioral changes appear to be accompanied by downstream neurobiological alterations, including diminished sensitivity of 5-HT_1A serotonin receptors (Peeters et al. 2019); despite the near absence of serotonin, 5-HT_1A receptors remain functional and respond to exogenous agonists like NLX-112, with the requirement for higher doses indicating long-term adaptations that shape aggression regulation and likely involve other neurotransmitter systems such as GABA.

Behaviorally, the cognitive profile of TPH2 KO rats is still being elucidated (e.g. we lack data on emotional, cognitive and behavioural profile of the model in juvenile and adolescence stages). Recent work has shown that constitutive serotonin depletion leads to pronounced alterations in social behaviors, elevated stress responses, and disorganized social hierarchies, despite no deficits in standard cognitive tests (Alonso et al. 2023). Moreover, inducible, partial serotonin depletion has been linked to impaired decision-making and reduced social recognition, with certain individuals displaying heightened vulnerability to these effects (Alonso et al. 2024). Also, the initial data indicate potential impairments in tasks with an emotional or social component (Angoa-Pérez et al. 2014).

Outline of the Thesis

This thesis builds upon the themes outlined above to explore how early-life changes and novel technologies can advance our understanding of executive function and its neurochemical modulators. We address the global need

for better translational models by leveraging the Social RatPad system - enabling cognitive testing in a more natural, socially inclusive context - and by examining rats with perturbed serotonin levels (TPH2 KO model) to probe how this neurotransmitter influences cognitive development. By integrating these approaches, we aim to investigate how altered serotonin signaling affects executive function, laying the groundwork for future studies in this system and ultimately guiding earlier, more precise interventions for neuropsychiatric disorders.

By early-life changes, we refer to manipulations or monitoring of serotonin levels and related neurodevelopmental processes during the prenatal, juvenile, and adolescent periods. These stages are critical for the maturation of neural circuits underlying executive function, emotion regulation, and social behavior, and disruptions in serotonin signaling during these windows can have long-lasting effects on brain architecture and behavioral outcomes. For example, genetic models such as tryptophan-hydroxylase-2 knockout (TPH2-KO) rats and serotonin-transporter knockout (SERT-KO) rats allow the study of lifelong or developmentally induced serotonin deficiencies, providing insight into how altered serotonergic activity shapes cognitive trajectories from adolescence into adulthood. The Social RatPad system enables cognitive assessment during these formative stages, addressing a gap in earlier research that primarily focused on adult animals.

This thesis aims to develop, validate, and apply an automated, socially inclusive touchscreen testing system for rats (the Social RatPad system) to enable cognitive assessment in a group-housed environment. By integrating novel hardware and software components, the system addresses existing limitations in rodent cognitive testing, such as isolation or limited testing schedules. It further explores how brain serotonin fluctuations - using transgenic rat models - affect the development of executive functions and social behaviors from adolescence into adulthood (a test that becomes possible with the novel testing environment).

The thesis is organized as follows:

Chapter 2: Development and Validation of the Social RatPad system. We address the technological gap in existing systems by creating a platform that allows continuous, stress-free cognitive testing in a social homecage environment and describe the developmental and validation steps of the system.

Chapter 3: Automating the Social RatPad. Building on the previous chapter, we enhance the system by integrating additional automated components such as sensor-driven tubes, automated sorting mechanisms, and access gates. This refinement aims to reduce human intervention and maintain a stable and naturalistic setting for cognitive assessment, also allowing true social housing without human interference.

Chapter 4: Behavioral and Cognitive Development During Adolescence in TPH2 KO rats - We use the automated Social RatPad system to study behavioral and cognitive development in adolescent transgenic rat models with genetically altered serotonin levels. By comparing data from TPH2 KO rats with wild-type controls, we provide new insights into the role of serotonin in cognitive and behavioral maturation.

Chapter 5: Conclusion and Future Directions - This chapter summarizes the key findings, discusses their implications for neuroscience research and therapeutic strategies, and suggests directions for future research.

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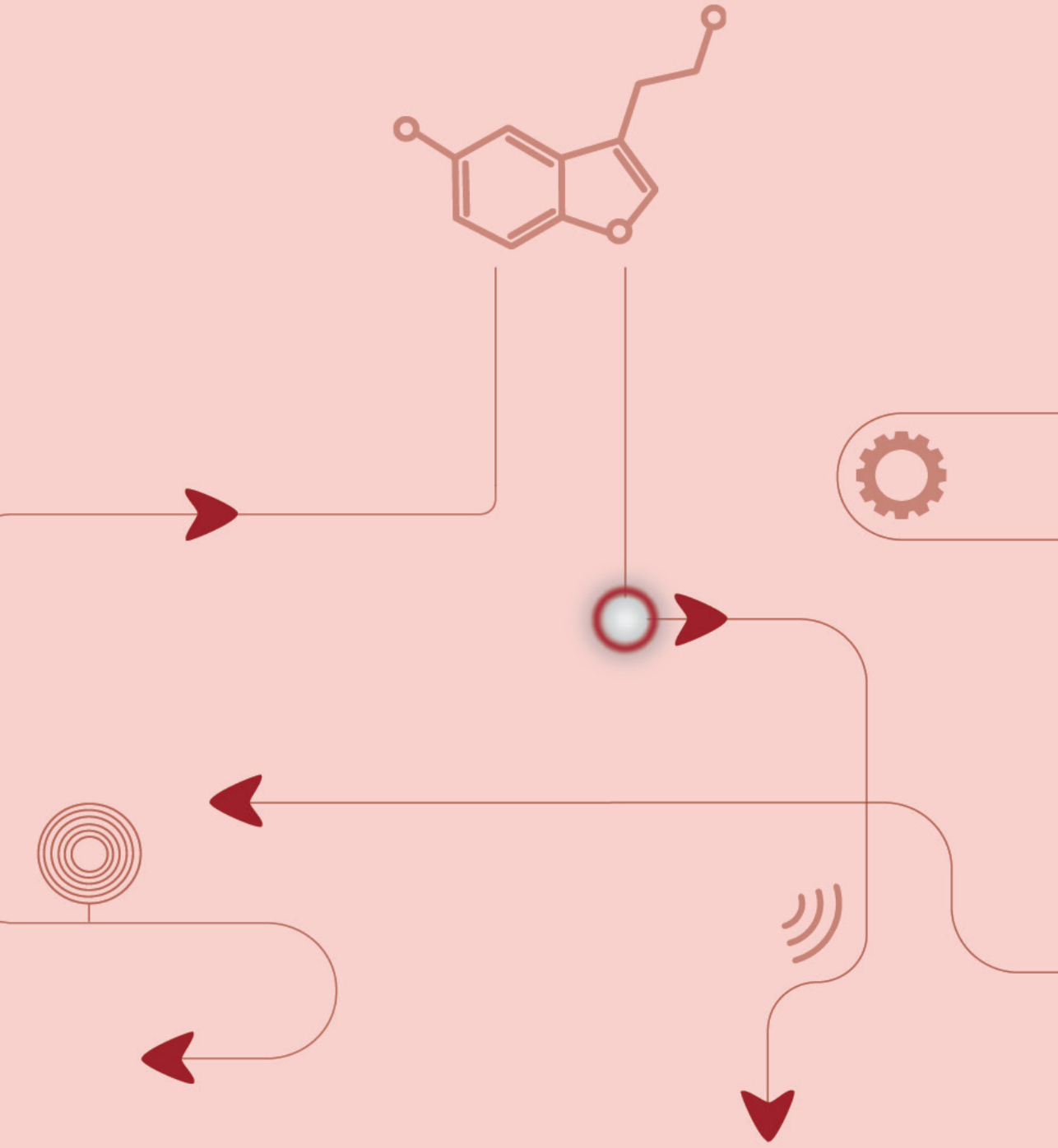

Chapter 2

Development and Validation of the Social RatPad: An Automated Touchscreen-Based Cognitive Assessment Tool for Rodents

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Abstract

Cognitive assessment in rodents is essential for understanding the neural substrates of learning, memory, and decision-making, and for translating preclinical findings into treatments for human neuropsychiatric and neurodegenerative disorders. Accurate, low-stress cognitive assessment in rodents demands tools that combine translational validity with animal welfare. Here, we describe the development and validation of the Social RatPad, an automated, touchscreen-based home-cage system that enables self-paced training and testing in socially housed animals. Across three developmental stages, we iteratively refined both hardware and software: Prototype 1 contrasted infrared versus capacitive screens in modified operant boxes; Prototype 2 implemented a standalone 8" capacitive touchscreen for working-memory (TUNL) tasks; and the Social RatPad integrated two capacitive RatPads with a shared central cage linked by tunnels, allowing animals to voluntarily enter and exit testing chambers without handling.

In Prototype 1, Long-Evans rats reached a visual-discrimination criterion (70% correct) significantly faster on the capacitive RatPad than in the standard infrared system. Prototype 2 demonstrated that capacitive screens support complex working-memory assessment: learning curves, delay-dependent performance declines, and sensitivity to spatial separation in the TUNL task were statistically indistinguishable from those in commercial infrared chambers. Finally, Social RatPad-housed Wistar rats reliably acquired Must-Touch, Visual Discrimination, and Reversal Learning tasks - meeting performance criteria within anticipated session ranges - while remaining in a familiar, group-housing context.

These findings establish that capacitive touchscreen technology, when embedded in an home-cage environment, can match or exceed traditional systems in training efficiency, working-memory demand sensitivity, and ecological validity.

Introduction

Assessment of cognitive processes in animals has a long history. Various methods have been incorporated to understand some species-specific cognitive abilities, aiming to compare analogous skills in humans (Bräuer et al. 2020). These methods range from mere observations (Tinbergen 1974) to attempts to create assessing tools resembling the natural environment of the animals (Alonso et al. 2023). Interestingly, one famous historical example that already combined both the mere observation and recreation of the natural environment throughout the testing was highlighted in an experiment conducted by Charles Darwin (Korb and Salewski 2011), who demonstrated that earthworms are capable of visual discrimination.

Throughout the development of cognitive assessment tools, psychologists were initially the primary interested group in certain assessing devices. Researchers, while developing paradigms regarding cognitive processes, used these tools to test various proposed hypotheses, such as operant learning. There was a need for standardized approaches to decrease variability. That is where B.F. Skinner introduced the famous chamber arrangement (Skinner 1966) to give the researchers control over environmental conditions while recording the animals' cognitive performance.

Afterward, to meet some scientific goals (assessing various cognitive processes and skills, such as spatial learning, recognition, discrimination, memory functions, and executive functions), the introduction of mazes (Morris Water Maze (Morris 1984), T-maze (Olton and Samuelson 1976), Radial Arm Maze (Olton and Samuelson 1976) and puzzles happened. To some degree, those tools were effective. However, a lot of points taking place during the experiments led to controversial views on the effectiveness of such methods. Particularly, on the opposing side to these tools, motivational and welfare aspects (Berger et al. 2018), high stress due to handling (Towers et al. 2019) and other concerns were highlighted.

With the advancement of technologies, human cognitive assessment shifted from questionnaires to touchscreens. Cognitive batteries to assess various cognitive processes were introduced. Among them was the CANTAB (Roque et al. 2011). Those technological and paradigmatic developments required a new view in cognitive assessment methods and consequently the tools also in animal science. The CANTAB battery, for example, after being utilized with

humans, was also adapted for monkeys and rodents (Chudasama and Robbins 2003) shortly after.

Touchscreen-based evaluation of rodents' cognitive capabilities has its roots in the attempts to adapt the CANTAB for rats (Bussey et al. 1994). However, it became more standardized after the Bussey-Saksida chamber (Bussey and Saksida 2007) was introduced. The authors also highlighted some possible ways of arranging the testing procedures within a touchscreen-based chamber. Those paradigms became, we may say, classical. It has been demonstrated that those tasks are effective in assessing the animals' capabilities in Pavlovian conditioning, visual discrimination, adaptive learning, motivation, inhibition, etc. Touch-screen based systems allowed increased translational values (Romberg et al. 2013), more complex tasks (Talpos et al. 2010), advancing overall cognitive research (Cook et al. 2004).

Besides the scientific need for the advancements of the assessing tools, the well-being of the animals became strongly highlighted over time, and various requirements were arranged to keep the animals out of stress. As an example, while within the Skinner box, the need to control environmental conditions for the researchers was highlighted, the new requirements for animal well-being demanded control over the environment from the animal's side, leading to a suite of limitations that undermine both animal welfare and data validity:

Traditional single-animal testing approaches face several limitations. First, animals are often removed from group housing and tested once at a time, leading to social isolation that disrupts natural hierarchies and buffering. Secondly, frequent transport and manual placement into testing chambers can induce handling stress, elevating corticosterone levels and anxiety, thereby confounding performance outcomes. Third, experimenter-determined fixed testing schedules frequently clash with rodents' nocturnal activity peaks, resulting in suboptimal engagement and variable motivation. In addition, sterile test boxes lack species-typical enrichment, leading to environmental impoverishment and reduced ecological validity of observed behaviors. Furthermore, single-animal assays are characterized by low throughput and high labor intensity, as they demand continuous experimenter oversight, slowing data collection and increasing workload. Finally, repeated light exposure and handling at inconsistent times can cause circadian disruption, alter sleep-wake cycles and further biasing cognitive readouts.

A home-cage system could overcome these challenges by preserving social housing, eliminating routine handling, and allowing animals to self-initiate trials on their natural circadian schedule within a richly enriched environment. This approach not only reduces stress and improves welfare, but also yields higher-throughput, more consistent, and ecologically valid measures of cognition.

Observations and discussions within the field of neuroscience also highlighted the need for home-cage assessment of cognitive processes. Various attempts to create better environmental conditions and automated testing protocols took place. In examples where such environments were created for monkeys, crucial points were highlighted: Animals have autonomy (to choose the time points for testing), opportunities to socialize, and chances to engage in playful experiences (Berger et al. 2018; Fizet et al. 2017; Fagot and Bonté 2010). However, few attempts to address those points within the testing with rodents happened. Therefore, there is a growing need to adapt these principles to rodent research, creating environments that not only enhance ecological validity but also allow for greater autonomy, socialization, and naturalistic experiences, thereby enabling a more comprehensive understanding of cognitive processes in these animals.

In this study we aimed to develop an automated touchscreen homecage system. Homecage systems may address several crucial limitations of currently available cognitive assessment systems. These limitations primarily concern enrichments that enable choice (a crucial aspect of cognitive testing) and housing. Achieving higher translational value requires providing rodents with more complex tasks beyond simple choice tests between two visually - similar stimuli. Standard lever-based systems are ineffective for this purpose, as similar levers cannot target complex attention, perception, and discrimination modules simultaneously. In contrast, devices used in human studies, such as touchscreens, provide the opportunity to test rodents with different types of stimuli illustrated with a variety of interactive options. Touchscreens offer the potential for developing more complex protocols while being easily adjustable for the researchers.

The second crucial aspect we addressed is housing. Separated housing and testing chambers necessitate frequent relocations, which expose animals to stress. This stress stems from sudden, involuntary changes in location and handling, forcing animals out of their social housing to perform tasks.

Consequently, animals may become distracted during tasks due to stress from sudden relocation and isolation from their housed companions.

The development of the system went through three stages: two prototypes of a standalone RatPad and the introduction of Social RatPad. The two prototypes consisted of capacitive touchscreens, reward modules, a food and water dispenser, and were different in some parameters, like the touchscreen size. To address the lack of social housing we connected two standalone Ratpads connected to a shared cage, resulting in Social Ratpad. In this version the animals were free to travel to testing chambers and back to the central cage.

Methodology

Animals

Cognitive testing in RatPad prototype 1 compared with standalone touchscreen operant box

RatPad: Young Long Evans rats (Rattus norvegicus) of both sexes, N=6, aged 21 days, were housed in Eurostandard type IV cages upon arrival. During the experimental phase, each rat was individually housed in Eurostandard type III cages for a maximum of two months. The rats were subjected to a reversed day-night cycle, exposed to red light during the day and white light at night, to promote activity during behavioral tests. They underwent food restriction, being allowed to consume only 90% of their preferred food, measured individually based on their consumption during weekends when they received a predetermined amount of feed. The rats were fed pellets, with a second prototype pellet dispenser that was developed to improve reliability and minimize pellet grinding. The housing room was maintained at a constant temperature (21 ± 1 °C) and humidity (40–60%), a 12:12 h reversed light/dark cycle was applied, with lights on from 07:00 to 19:00, and the cages contained sawdust bedding. Randomization was applied in group allocation and test order. All experimental procedures carried out were approved by the Central Committee Animal Experiments (Centrale Commissie Dierproeven, CCD) and conducted according to the Dutch Animal Experiments Act.

Standalone system: A separate group of Long Evans rats (N = 16) was tested in a conventional standalone touchscreen operant box (Campden Instruments). The animals were of comparable age (postnatal day 27 at testing onset)

and housed under the same environmental conditions as the RatPad group, including sawdust bedding, reversed 12:12 h light/dark cycle (lights on from 07:00 to 19:00), and temperature- and humidity-controlled rooms (21 ± 1 °C, 40–60% humidity). Food restriction and reward type (sucrose solution) were identical to those used in the RatPad condition.

Cognitive testing in RatPad prototype 2 compared with standalone touchscreen operant box

RatPad: Long Evans rats (3 males, 3 females) obtained from Janvier at postnatal day (PND)21 was utilized to pilot test the Trial-Unique Non-Matching to Location (TUNL) task on a 10-inch capacitive touchscreen, aimed at assessing working memory. The experiment began on postnatal day 21 (PND21). The rats were housed under a reversed day-night cycle to promote activity during behavioral tests, the rats were socially housed in groups of three, receiving ad libitum feeding except for 3-4 hours before cognitive testing sessions, during which food was withheld to motivate performance. While randomization was applied in group allocation and test order, blinding was deemed unnecessary for this pilot study. The housing room was maintained at a constant temperature (21 ± 1 °C) and humidity (40–60%), a 12:12 h reversed light/dark cycle was applied, with lights on from 07:00 to 19:00, and the cages contained sawdust bedding.

Standalone operant box: Long Evans rats (7 males, 8 females) obtained from Janvier were utilized at PND21. The rats were housed initially in group settings for acclimatization but were individually housed in type III cages during experimental phases. Food restriction was applied, and the day-night cycle was reversed to test the animals during their active phase. In the study, food restrictions were implemented to motivate the animals for the cognitive task, with a maximum food allowance of 90% of their daily consumption. This restriction began five days after the animals arrived and was carefully monitored to ensure adequate nutrition while maintaining motivation for the task. They received an excess of food during the weekend. The housing room was maintained at a constant temperature (21 ± 1 °C) and humidity (40–60%), a 12:12 h reversed light/dark cycle was applied, with lights on from 07:00 to 19:00, and the cages contained sawdust bedding.

Cognitive testing in Social RatPad

Wistar male rats (N=8), obtained from Charles River, were utilized to pilot the Social RatPad system. The rats arrived at the facility on PND 21. They

were housed in the Social RatPad. Food and water were provided ad libitum in the central cage. The housing room was maintained at a constant temperature (21 ± 1 °C) and humidity (40–60%), a 12:12 h normal light/dark cycle was applied, with lights on from 07:00 to 19:00, and the cages contained sawdust bedding.

Equipment

RatPad Prototype 1

To enable the comparison of cognitive assessment results in relation to touchscreen types, we assembled a specialized testing setup. We adapted MedAssociates operant chambers, originally equipped with an infrared touchscreen, by installing a capacitive touchscreen (iPad 2) in one of the chambers. This allowed direct comparison between the traditional infrared touchscreen and the newly added capacitive touchscreen. The system was controlled by TBES (Touch-screen Based Evaluation System; (Wolf et al. 2014), allowing precise measurement of cognitive task performance across both touchscreen types (figure 1).

RatPad prototype 2

RatPad prototype 2 (designed and produced by Metris B.V.) consists of of a 8-inch capacitive touchscreen (8.0-inch IPS capacitive touchscreen display with a resolution of 1920 x 1200 pixels and 24-bit color depth, featuring a MediaTek MT8163A 64-bit Processor, ARM Mali-T720 MP2 GPU, 2 GB RAM, and 32 GB internal storage).

To enhance RatPad's functionality and precision, we utilized an 8-inch capacitive touchscreen, which offers a higher resolution (1920 x 1200 pixels) and improved processing power. This change was made to improve touch sensitivity and response accuracy while also creating a more compact, efficient setup. Alongside this, we implemented a mask with designated response areas (10 x 10 cm each) to further reduce unintentional touches, a common issue in previous versions. The upgraded PC-controlled RatPad software now allows precise task control, providing a streamlined and accurate testing environment. These developments aim to improve the accuracy, usability, and reliability of the cognitive tasks conducted within the RatPad.

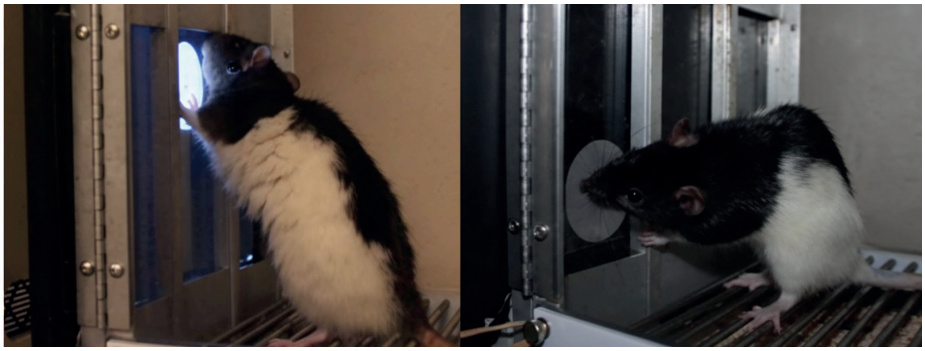

Figure 1. Rat performing the Must-Touch task in a modified Med Associates chamber equipped with an iPad and TBES software.

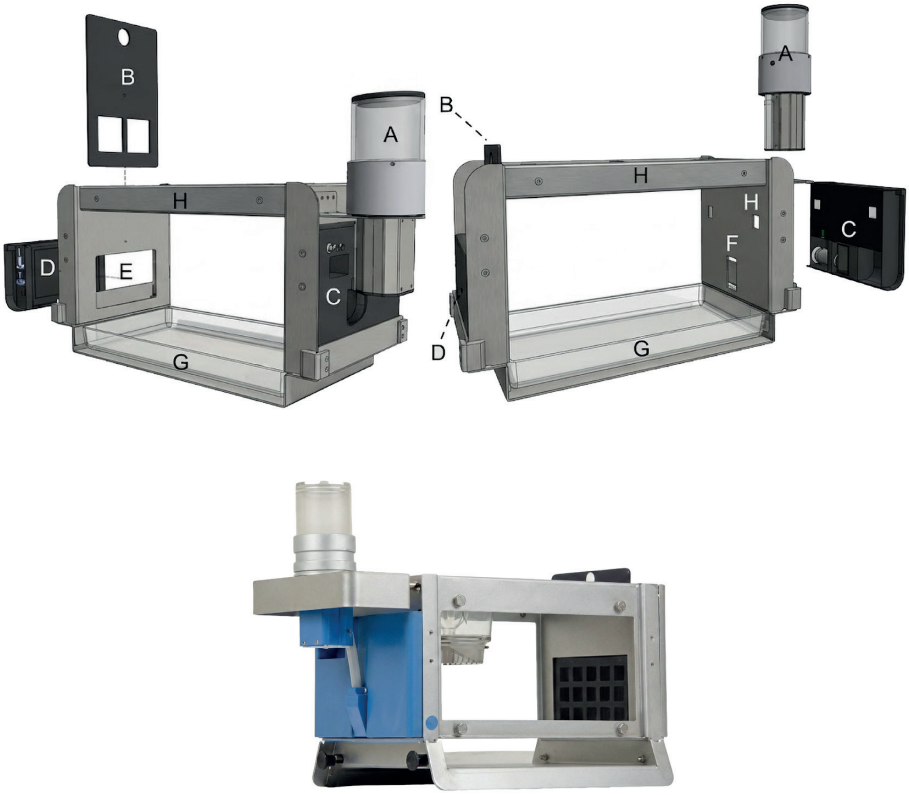

Figure 2. RatPad operant cage. (A) Pellet dispenser; (B) Mask; (C) Electrical component with power point; (D) Touchscreen. (E) Response Area. (F) Reward receptacle. (G) Bottom. (H) Punish light. On the right is the 4th prototype.

Social Ratpad

The Social RatPad system consisted of two standalone RatPads (Size is identical to - Type III Eurostandard) connected to a central cage (Type IV Eurostandard cage). The RatPad systems were linked to the main living area by tubes (dimensions: 60 mm inner diameter, 70 mm outer diameter, and length of 30mm, made of plexiglass). Special connectors, designed by Metris B.V., were used to connect the tubes to the cages. These connectors could be removed during cleaning sessions. The software was rearranged, allowing initiation of pre-defined sessions throughout the testing long cycles. The data collection was automated. To prevent data mixing, a separator was placed in the middle of the central cage. This divider was transparent and equipped with holes to allow for vocalization and scent communication between the animals while they were separated. The separator was removed daily for 2-3 hours to allow the rats to socialize. A red retreat tunnel was provided for each of the two animals to use as shelter. Tasks were conducted approximately 5 times daily at predefined time points, typically starting at 18:00 and repeating every 3 hours. A normal day-night cycle was maintained throughout the experiment.

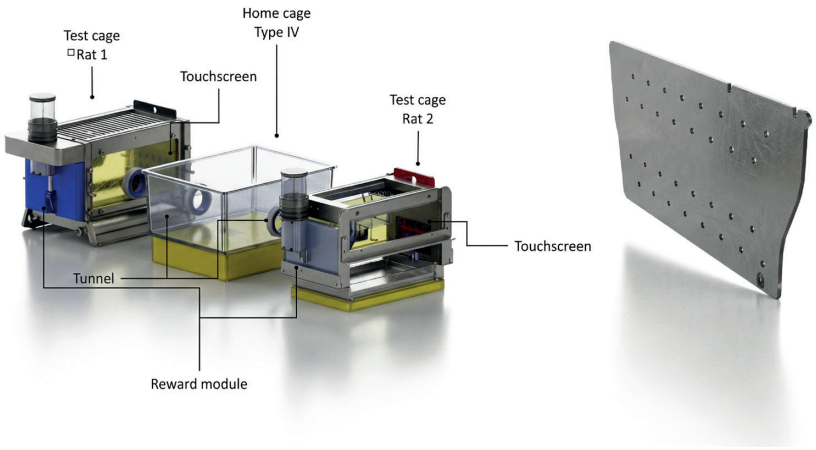

Figure 3. Social RatPad: removable divider installed to create separate living and testing areas (right side shown).

Standalone touchscreen boxes:

Training for the infrared touchscreen setup was performed using six to eight operant conditioning chambers from Campden Instruments Ltd. (USA) (as shown in Figure 1). Each trapezoidal chamber had dimensions of 30 cm

in height, 25 cm in width at the screen, 13 cm in width at the magazine, and 33 cm in length. The chambers were housed within larger sound-attenuating cubicles that measured 61 cm in height, 53.2 cm in width, and 54 cm in length. The operant boxes contained a white house light (28 V, 100 mA), magazine unit, pellet dispenser, and infrared screen (22.5 cm x 30 cm). The touchscreen was covered with a mask that had two 10 cm x 10 cm response areas. Within the infrared setup, the white circle stimulus was 8 cm in diameter, and the other stimuli were 4.5 cm x 4.5 cm. The touchscreen tasks were controlled by a PC with ABET II software from Campden and Lafayette Instruments Ltd. (USA).

Tasks

RatPad Prototype 1 and Social RatPad

To test RatPad prototype 1 and Social RatPad, we used a reversal learning task. The animals were trained for this task along three main training stages, as follows: After habituation, during which the animals were rewarded only for poking the screen, they underwent the Must Touch task where only poking the circle was rewarded. Subsequently, during the visual discrimination task, two different stimuli were presented on the screen, one considered correct and the other incorrect. Once they learned the task, they proceeded to the reversal learning stage. The progressive stages illustrated in Figure 4 include:

Stage 1: *Habituation (a)* involved acclimatization of the rat to the environment. Following an inter-trial interval (ITI) of 10 seconds, the trial began, and a reward was delivered without requiring any interaction from the subject. The trial ended immediately after the reward delivery.

Stage 2: *Must Touch (b)* introduced a simple stimulus on the touchscreen for 30 seconds during each trial. The subject had to touch the screen to receive a reward (S+); if the subject failed to respond or interact with an incorrect area (S-), no reward was provided.

Stage 3: *Visual Discrimination (c*) required the subject to distinguish between two presented stimuli: a correct (S+) and an incorrect (S-) option. The trial started after a 10-second ITI, and the stimuli is displayed for 30 seconds. Touching S+ resulted in reward delivery, while touching S- resulted in no reward. If the subject failed to respond, the trial was marked as an omission.

Stage 4: *Reversal Learning (d)* built on visual discrimination but reversed the reward contingencies for the stimuli. The previously correct stimulus (S+) now became incorrect (S-), and vice versa. As in the previous stages, S+ led to reward delivery, S- resulted in no reward, and a lack of response led to an omission.

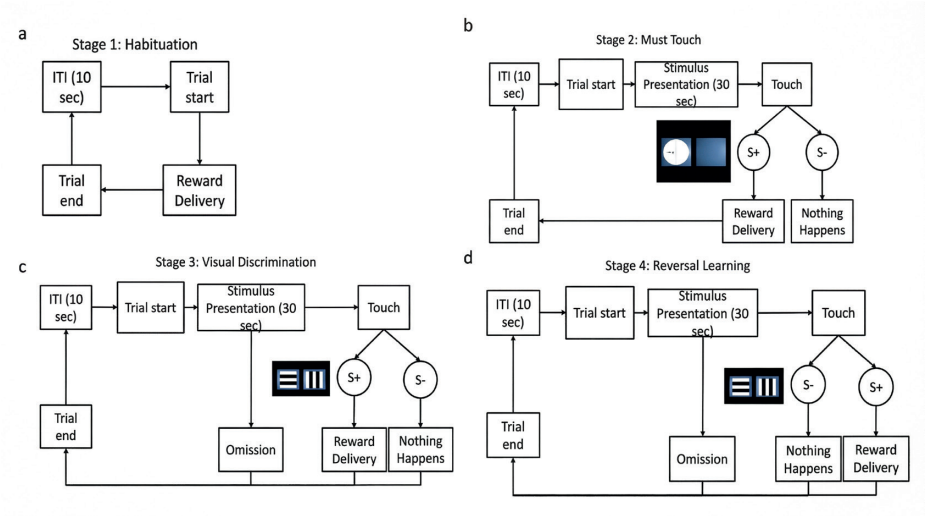

Figure 4. Demonstration of key stages and their parameters in the workflow for the following tasks: (A) Habituation; (B) Must Touch; (C) Visual Discrimination; and (D) Reversal Learning.

In the case of Social RatPad the rats had predefined sub-sessions (5 sub-sessions during each training night from 8 PM to 8 AM, lasting 30 minutes each). During these trials, rats had fewer overall trials because they could miss the sub-sessions while being engaged in other activities or sleeping during those 30 minutes. For the Must-Touch, Visual Discrimination, and Reversal Learning tasks in the Social RatPad, we calculated the number of sessions each rat required to meet the performance criterion (80% correct responses: total correct trials ÷ [correct + incorrect trials] x 100%.

RatPad prototype 2

The experiment spanned five days a week over approximately three months, with each testing session lasting one hour. The rats were tested multiple times throughout the day, each session lasting around 1 hour. The rats were trained to perform a delayed trial-unique non-matching to location (TUNL) task in either the Standalone RatPad or Campden set-up.

All behavioral tests were performed during the dark phase of the day, from 9:00 AM to 5:00 PM, and each animal underwent Must-Touch, Visual Discrimination, and Reversal Learning tasks, with a maximum of two tests per day, until the set acquisition criteria were achieved. Before starting the TUNL task, the animals received pre-training that included AutoShaping, must touch A-C, and punishment for incorrect behavior (figure 4). To motivate the animals, standard grain pellets were used as a reward in combination with food restriction.

The training procedures were carried out in accordance with the instructions provided in the manual by Campden Instruments Ltd for the delayed trial-unique non-matching to location (TUNL) task, and they were further optimized based on relevant literature, such as the study by (Talpos et al. 2010). During the task, the subject is presented with a sample stimulus at a specific location on the touchscreen. When poked, after a short delay, multiple stimulus was displayed, including the original location and new ones. The rodent was required to select a stimulus at a location different from the sample (non-matching location) to receive a reward.

After TUNL acquisition, all animals were tested with a longer time manipulation between sample and choice phase for 4 trials, with 2 regular TUNL trials in between. The increased time was pre-set to 6 seconds between phases. This allowed us to explore how increased delays would affect performance in animals after acquisition.

TUNL B and C were an additional set of manipulations after acquisition. TUNL B increased distance so the two stimuli in the choice phase were diagonal and thus harder to differentiate. TUNL C had both stimuli of the choice phase adjacent to each other, thus relying more heavily on pattern separation than the standard TUNL or TUNL B. The horizontal configuration mirrored the initial TUNL training, whereas the adjacent and diagonal configurations were novel to the animals. (Figure 5.)

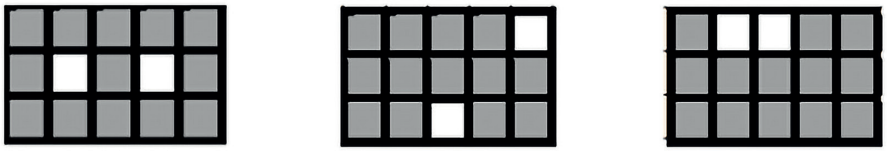

Figure 5. Example of distance differences between sample and choice stimuli in TUNL Tasks: (Left) TUNL, (Middle) TUNL B, and (Right) TUNL C. TUNL had stimuli one square apart in a horizontal line. TUNL B had stimuli placed diagonally. TUNL C had stimuli adjacent to each other.

Data Analysis

This study employed a variety of statistical tests to assess differences in learning and memory performance across groups, tasks, and systems. All analyses were conducted using RStudio 2023.12.1 Build 402 (Ocean Storm Release) for Windows (© 2009-2024 Posit Software, PBC), with figures generated to illustrate the results of these comparisons. Significance was set at $P < 0.05$.

Comparison of Days to Criterion Achievement

To determine if there was a significant difference in the number of days required to reach the learning criterion (70% correct responses; total correct trials ÷ [correct + incorrect trials] x 100%) during the first and second development stages in the RatPad and Campden systems, we conducted a Student's t-test.

Visual discrimination and reversal learning

For progression through various training stages (AutoShaping, Must Touch A, Must Touch B, Must Touch C, and Punish Incorrect tasks) for TUNL task, descriptive statistics (mean ± standard deviation) were calculated to determine the average number of sessions required by each sex within both systems (RatPad and Campden). The same was completed for the Social RatPad. No further inferential statistics (e.g., Student's t-tests) were conducted because this part of the study was exploratory, involved a limited number of animals, and primarily aimed to demonstrate the technical feasibility and face validity of the new system rather than to test group-level hypotheses.

TUNL Task

To examine learning progression in the TUNL task over 15 sessions, we tracked the percentage of correct responses for both the RatPad and Campden groups. Student's t-tests were conducted to assess differences in performance between groups, with particular focus on early and mid-sessions ($p < 0.05$) to detect significant learning differences. To compare the number of sessions required to reach the 70% of daily correct trials criterion between the RatPad

and Campden groups we conducted a Welch Two Sample T-test. This test was chosen to account for potential differences in variance between groups.

Performance under short and long delay conditions was analyzed using a paired t-test. To analyze how variations in spatial distance affected task performance, we combined and averaged correct response percentages from TUNL B and TUNL C tasks and grouped them into three distance clusters (closest, intermediate, furthest). A two-way ANOVA was performed to evaluate the main effects of the system (RatPad vs. Campden) and distance cluster on the percentage of correct responses, as well as the interaction effect between these factors.

Social RatPad Task Performance Analysis

Descriptive statistics (mean ± standard deviation of daily correct trial percentages, together with plotted individual and group learning curves) were used to summarize task performance across rats, with figures illustrating daily performance progress in each task. No comparative statistical tests were conducted for the Social RatPad due to the lack of a similar control group, but observed trends in learning rates were documented. For reference, the criterion used in the learning analyses was defined as reaching 70% of daily correct trials (see Figure 12).

Results

RatPad prototype 1 Compared to Campden

First, we compared visual discrimination performance using infrared versus capacitive touchscreens. It took an average of 13 days for the 'RatPad' rats and 18 days for the 'Campden' rats to achieve the criterion of 70% correct.

A Student's t-test revealed a significant difference between the two groups ($t = -2.73$, $p = 0.020$), indicating that the rats in the RatPad group achieved the criteria in significantly fewer days compared to those in the Campden group.

The two female rats engaged in RatPad testing reached criterion on the seventh and ninth days, respectively. The two male rats achieved criterion on the sixteenth and twentieth days. These results were obtained with correction trials, where the rats were provided with the opportunity to correct an incorrect choice.

RatPad Prototype 2 performance compared with Campden Chamber performance

In this study, we first evaluated the progression of male and female rats through various training stages before the TUNL task, focusing on five key stages: AutoShaping, Must Touch A, Must Touch B, Must Touch C, and Punish Incorrect tasks.

As shown in Figure 7, the average number of sessions required to complete each training stage varied by system and sex. For AutoShaping, RatPad males required 4.00 ± 1.00 sessions, RatPad females 5.33 ± 2.08, Campden males 3.25 ± 1.98, and Campden females 2.88 ± 1.13 sessions. During Must Touch A, the averages were higher in the RatPad groups (7.33 ± 0.58 for males, 8.33 ± 1.53 for females) than in the Campden groups (3.25 ± 1.49 for males, 3.25 ± 1.75 for females). For Must Touch B, the session counts were similar for RatPad males and females (both 2.33 ± 0.58), while Campden males averaged 4.38 ± 2.33 and females 3.38 ± 1.19. In Must Touch C, RatPad males averaged 3.00 ± 1.00 and females 2.33 ± 0.58, compared to 4.13 ± 2.23 for Campden males and 4.00 ± 1.20 for females. Finally, in the Punish Incorrect stage, the RatPad groups required substantially more sessions (15.00 ± 1.73 for males, 14.33 ± 3.79 for females) than the Campden groups (8.13 ± 3.31 for males, 6.63 ± 2.56 for females). These results illustrate consistent differences in training progression across systems and sexes, with RatPad animals generally requiring more sessions in certain stages.

Next, we examined how rats performed in the TUNL (Trial-Unique Nonmatching-to-Location) task across sessions, focusing on their ability to learn and adapt to increasingly demanding spatial memory conditions. The TUNL task specifically measures spatial working memory by requiring animals to remember and select a correct location on a touchscreen after a brief delay. Difficulty increases with smaller distances between the sample and choice stimuli, which makes the discrimination harder. We measured learning progression over 15 sessions by tracking the percentage of correct responses under these varying spatial separations (Figure 8). The analysis included two experimental systems, RatPad and Campden, further divided by sex. All groups demonstrated learning progression over the sessions, indicated by increasing or stable correct response rates. Because the dataset for session-by-session learning progression was unbalanced and exploratory, we limited that analysis to descriptive statistics. However, for subsequent comparisons where each animal contributed complete data (e.g., performance at criterion, delay, and distance effects), inferential tests were applied as appropriate.

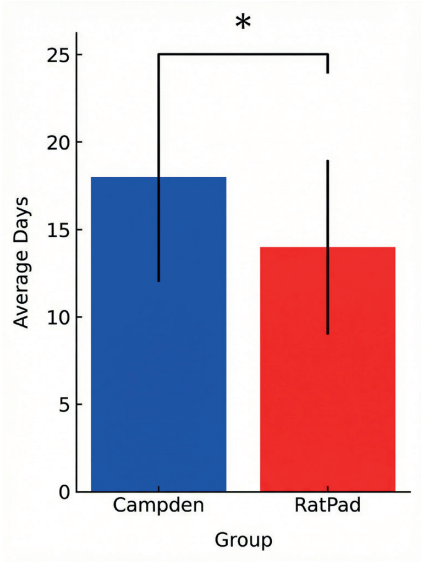

Figure 6. Comparison of average days to reach learning criterion between RatPad and Campden groups. Male and female rats are combined. Bars represent mean days required to reach the criterion, with error bars indicating SEM. Rats trained in the RatPad system reached the criterion faster than those in the Campden setup ($p < 0.05$, Student's t-test). * $p < 0.05$.

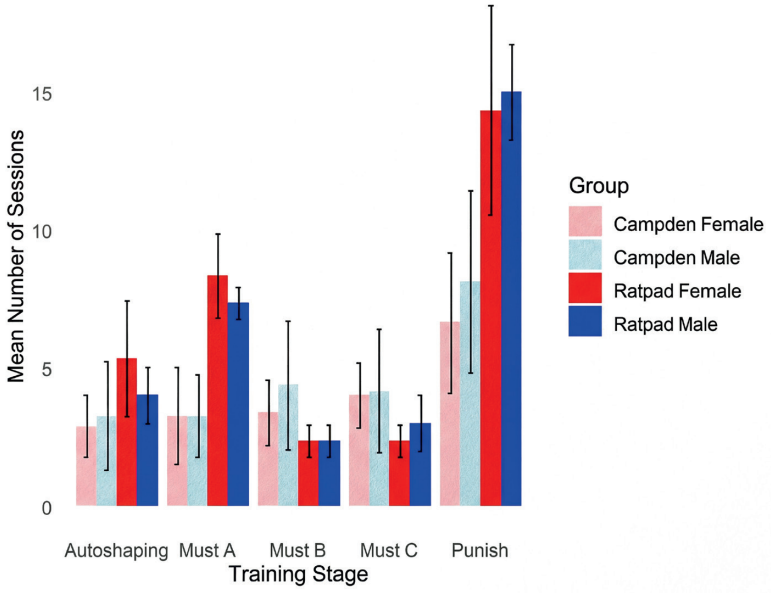

Figure 7. Mean number of sessions required for male and female rats to complete each training stage prior to the TUNL task across two experimental cohorts (RatPad and Campden). Stages include AutoShaping, Must Touch Variants (A, B, C), and Punish Incorrect. Bars represent group means with error bars indicating standard deviation.

For the RatPad system, male rats showed an increase in correct responses from session 1 (34%) to session 15 (54%), while female rats maintained relatively stable performance from 44% to 49%. In contrast, Campden system rats demonstrated clearer upward trends: males improved from 59% to 71%, and females from 56% to 65% over the same sessions (Figure 8). Visual inspection of the learning curves suggested differences among the four groups - RatPad Male, RatPad Female, Campden Male, and Campden Female - were most evident during early (sessions 1-5) and mid-training sessions (sessions 6-10), where performance trajectories separated more clearly before converging somewhat in later sessions. However, formal statistical testing across all 15 sessions was not conducted. The apparent differences varied by session and group and this part of the study involved a limited sample size, making it better suited for descriptive rather than inferential analysis. The aim was to identify general learning trajectories and feasibility patterns rather than to test specific group-level hypotheses.

These results indicate that all groups were capable of improving or maintaining stable performance over training, but that the rate and pattern of learning progression differed depending on system and sex. This highlights the importance of considering system-specific design features and sex differences when interpreting learning performance in the TUNL task.

To determine whether overall accuracy differed between the two touchscreen systems once animals had learned the task, we extracted, for every rat, the first TUNLA session in which it reached the acquisition criterion of % correct. We then calculated the mean percentage-correct criterion for the six RatPad animals (71.38%) and the sixteen Campden animals (72.04 %; Figure 9, "Performance at 70 % criterion"). These two independent means were compared using a student t-test, and no statistically significant difference between the groups was found ($t = -0.63$, $df = 18.29$, $p = 0.535$). The 95% confidence interval for the difference in means ranged from -2.86 to 1.54. These findings suggest that, although the rats in the RatPad group took more sessions to reach the learning criterion, their performance, once learned, was comparable to that of the rats in the Campden group.

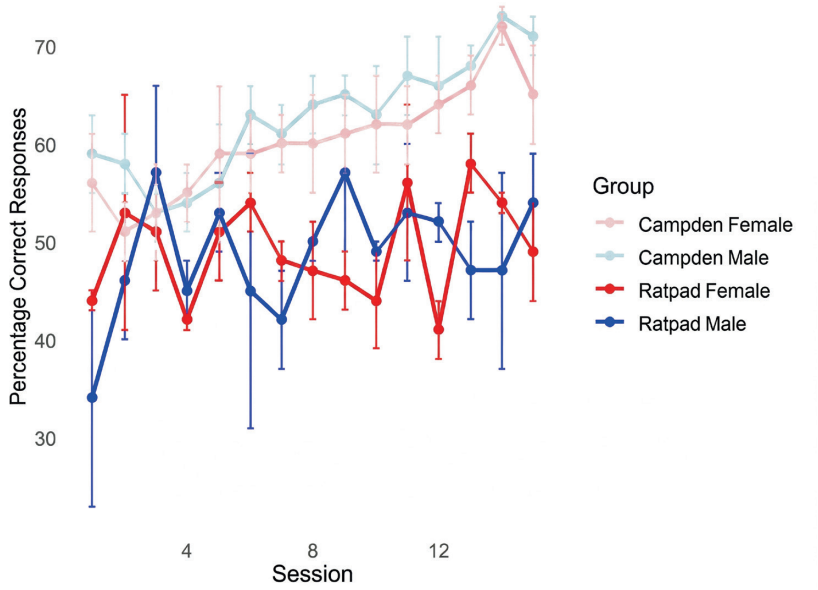

Figure 8. Percentage of correct responses in the TUNL Task across 15 Sessions for rats tested in the RatPad and Campden systems, grouped by sex. Lines represent mean performance per group (Campden Female, Campden Male, RatPad Female, RatPad Male), with error bars indicating standard deviation across sessions.

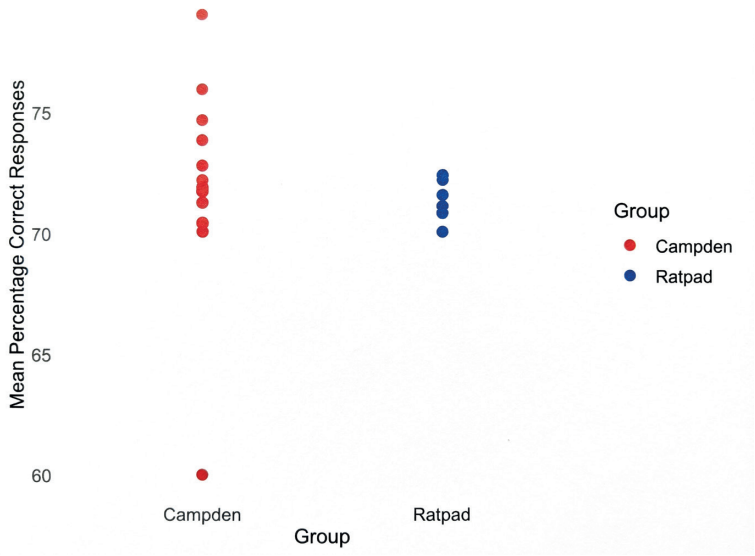

Figure 9. Mean percentage of correct responses to reach the 70% criterion in the TUNL task for rats tested in the RatPad and Campden systems. Each point represents an individual animal's mean performance at criterion, grouped by system.

Following the evaluation of learning progression under different conditions, the correct responses of rats in the TUNL task was assessed using both the RatPad and Campden systems under short and long delay conditions, requiring the rats to remember the target location across varying time intervals. The results are depicted in figure 10, which includes both individual rat data points and system-wide averages for each delay condition. For the RatPad system, a significant difference was found between the short and long delay conditions ($t = 5.139$, $p < 0.001$), with a mean difference of 15.96 (95% CI: 9.53, 22.38). Similarly, for the Campden system, a significant difference was observed ($t = 10.49$, $p < 0.001$), with a larger mean difference of 26.48 (95% CI: 21.24, 31.71). This indicates that both systems successfully detect the expected decline in performance with increasing delay intervals.

In addition to memory retention across different delays, we also assessed how variations in spatial distance affected task performance (Figure 11), providing further insight into the systems' sensitivity to changes in cognitive load. Continuing from the previous analyses, we evaluated whether the RatPad and Campden systems similarly detect performance difficulties when the distance between squares varies. To achieve this, we combined and averaged the percentage of correct responses from both the TUNL B and TUNL C tasks, as these tasks address the same research question. The combined data was organized into three distance clusters: 1/2/3 (closest distances), 4/6/7 (intermediate distances), and 8/9/10 (furthest distances).

The analysis revealed a significant main effect of the distance cluster ($F(2, 36) = 5.555$, $p = 0.00789$), indicating that rats' performance varied significantly across the different distance clusters. However, there was no significant main effect ($F(1, 36) = 0.044$, $p = 0.83492$), suggesting no overall difference in performance between the RatPad and Campden systems. Additionally, the interaction effect between the group and the distance cluster was not statistically significant ($F(2, 36) = 1.296$, $p = 0.28603$), indicating that the difference in performance between the systems did not change significantly across the different distance clusters.

These findings suggest that both systems are capable of detecting performance differences related to varying distances, with a general trend of decreased performance as the squares become closer together.

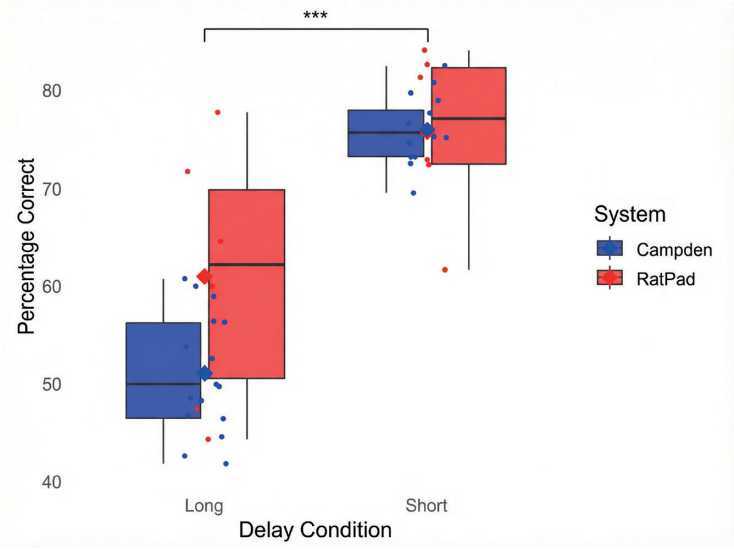

Figure 10. Performance in the TUNL task under short and long delay conditions for rats tested in the RatPad and Campden systems. Boxplots show percentage of correct responses by system (RatPad in red, Campden in blue) and delay conditions, with individual data points and error bars indicating variability across subjects. Asterisks indicate statistically significant differences between groups: $p < 0.001$ (***).

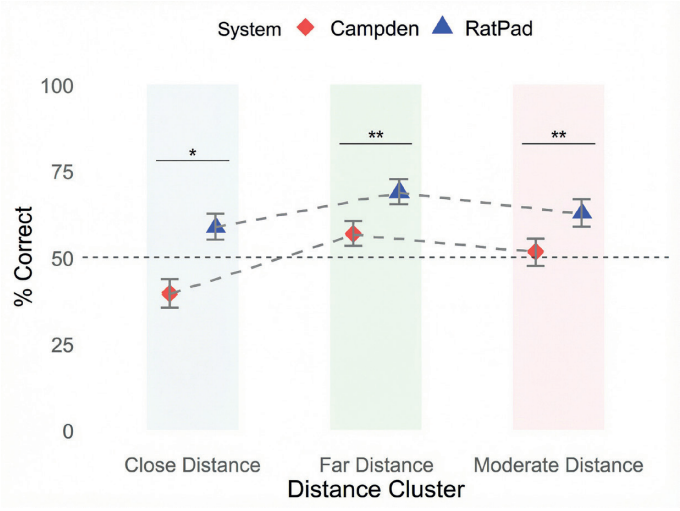

Figure 11. Percentage of correct responses in the TUNL task across different distance clusters (Close, Far, Moderate) for rats in the RatPad and Campden systems. Significant differences in % correct performance reflects comparisons between distance conditions within each system (Campden in red, RatPad in blue), not between systems. Asterisks indicate significant pairwise differences between Close and Far distances and between Moderate and Far distances. $p < 0.05$ (*), $p < 0.01$ (**)

Social Ratpad

As shown in Figure 12, for the Must-touch stage preceding visual discrimination, all rats achieved criterion within 10 sessions, except for Rat 2, which took 13 sessions. Rat 2 also had the longest duration (19 sessions) for the Visual Discrimination task. Several other rats, including Rat 5, Rat 9, and Rat 10, took more than 10 sessions to meet criteria for the Visual Discrimination task. The other rats completed the task within 5-6 sessions. With regards to the Reversal task, only Rat 6 met the criteria within 5 sessions, while all other rats took more than 10 sessions. The longest duration was recorded for Rat 1, who took 32 sessions to meet criteria. Rat 5 and Rat 9 took 20 or more sessions to complete the visual discrimination task.

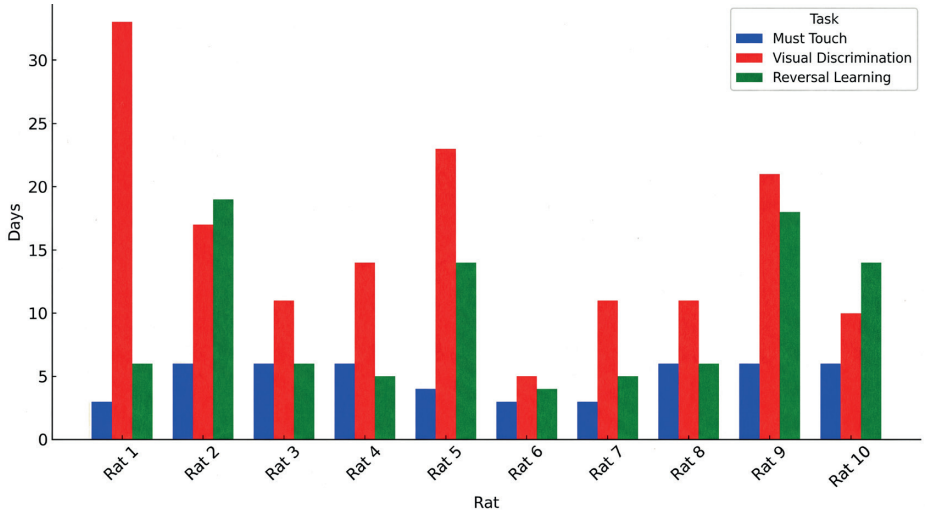

Figure 12. Days required for individual rats to reach 80% criterion in Must-Touch, Visual Discrimination, and Reversal Learning Tasks. Bars represent performance per rat across tasks, with colors indicating task type (Must Touch = Blue, Visual Discrimination = Red, Reversal Learning = Green).

As depicted in the accompanying graph (Figure 13), a general trend of progressive improvement in performance was observed in the Visual Discrimination task performed by the rats. However, two rats, Rat 5 and Rat 9, displayed an unexpected sinusoidal progression pattern. These rats also required the greatest number of days to meet the criteria.

In the context of Reversal Learning (Figure 14), it was observed that the rate of learning was slower in comparison to the Visual Discrimination task. Despite this, the majority of the rats (excluding Rat 1, Rat 5, and Rat 9) demonstrated

a progressive learning pattern. Similar, fluctuating patterns observed for Rat 5 and Rat 9, suggesting that motivation may be a potential factor influencing their performance.

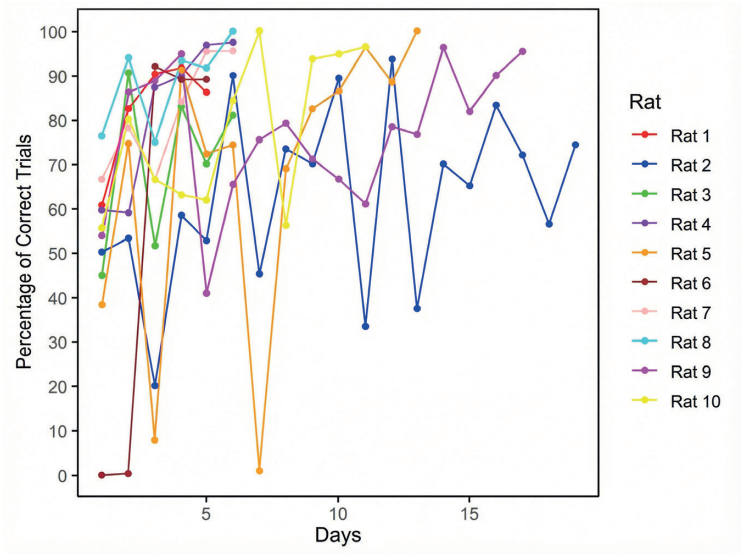

Figure 13. Learning curves for individual rats in the visual discrimination task. Lines represent daily percentage of correct trials across training days for each rat, showing individual performance trajectories throughout the task.

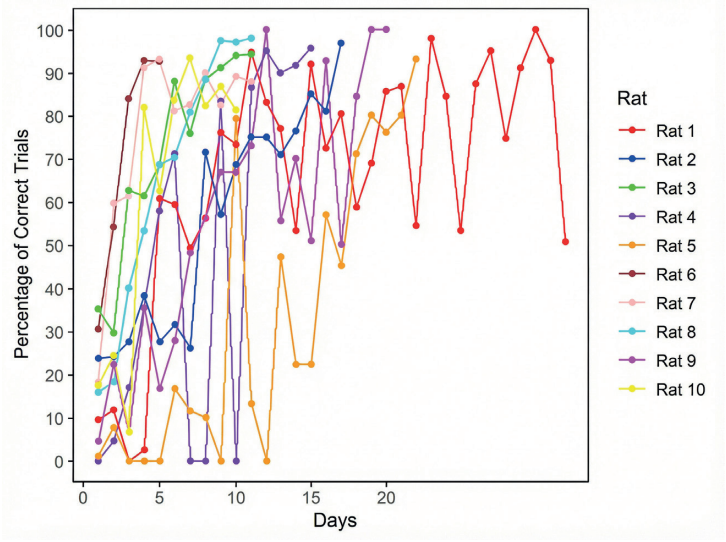

Figure 14. Learning curves for individual rats in the Reversal Learning task. Lines show daily percentage of correct trials across training days for each rat, highlighting individual performance patterns and adaptation over time.

In summary, the results indicate that the rats were able to meet the established criteria of 70% within the projected time frame (completing the tasks within 20 days), with a few noted exceptions. While a comparative study with similar conditions could not be conducted to compare the performance of Social RatPad with other methods, the data suggests that the rats demonstrated appropriate performance within expected time frames, with no recorded instances of prolonged delays.

General Discussion

This study aimed to improve the ecological validity and animal welfare of rodent cognitive assessment by developing the Social RatPad system - an automated, touchscreen-based homecage testing environment. Through three stages of development, we evaluated standalone prototypes using capacitive screens and ultimately built a socially housed system enabling autonomous testing. Our results demonstrate that the system can effectively deliver core touchscreen paradigms such as visual discrimination, reversal learning, and working memory tasks.

Interpretation of Findings in Context

Our findings indicate that capacitive touchscreen technology can serve as a viable alternative to traditional infrared-based setups in rodent cognitive testing. In the first prototype, animals trained on the capacitive RatPad reached the learning criterion significantly faster than those trained in the Campden infrared system. This suggests that, despite longstanding concerns about capacitive screens missing light touches (Bussey and Saksida 2007), with appropriate configuration they can match or even surpass infrared frames in training efficiency while offering practical advantages such as ease of cleaning and reduced false positives in bedded environments.

In the TUNL working memory task, prototype 2 showed broadly comparable learning curves and final performance levels relative to the Campden system. Although minor differences in delay sensitivity were observed - consistent with both systems capturing delay-dependent memory decline - there were no significant differences in overall accuracy at criterion. This suggests that capacitive-screen-based systems can reliably detect working memory demands similar to infrared systems, supporting their utility in translationally

relevant cognitive assays designed to mirror human neuropsychological tasks (Talpos et al. 2010).

A critical advance in our work is the Social RatPad, which allows socially housed animals to voluntarily enter testing chambers linked to a shared central cage. Performance trends showed that rats were able to meet learning criteria in standard stages such as Must-Touch, Visual Discrimination, and Reversal Learning, although with individual variation in acquisition speed. This individual variability is important: it reflects the reality of testing animals in a less constrained, more naturalistic environment. Such variation has also been observed in primate in-cage testing systems, where animals engage with cognitive kiosks on their own schedule (Fagot and Bonté 2010; Fizet et al. 2017). Importantly, no food restriction was applied in our paradigm and aligns with home-cage voluntary testing standards. However, it is important to note that ad libitum food availability may also reduce the rodents' exploratory motivation, potentially contributing to lower levels of task engagement or diminished incentive to seek out food-driven rewards.

Our design reduces the need for daily relocation and handling, factors which have been shown in other work to modulate cognitive outcomes. Prior studies demonstrated that even gentle handling can elevate stress hormones and alter behavior in rodents (Towers et al. 2019). By allowing animals to self-initiate trials in a connected chamber, the Social RatPad minimizes experimenter interaction, aligning with homecage testing innovations shown to improve data quality by reducing human-induced variability (Berger et al. 2018; Gouveia and Hurst 2017).

Moreover, enabling testing during the animals' active phase respects their circadian rhythms - a factor known to influence motivation and cognitive performance.

Technological Considerations

Our results also highlight important trade-offs in touchscreen hardware selection. While infrared systems have set the standard due to their sensitivity, they are vulnerable to false positives from debris and bedding in homecage contexts (Wolf et al. 2014). Capacitive screens, when well-calibrated, offer a sealed, robust, and cost-effective alternative that can perform comparably in both simple discrimination and complex working memory tasks. This finding supports broader efforts to democratize access to touchscreen testing by reducing hardware costs without sacrificing data quality.

Limitations

Several limitations should be acknowledged. First, our studies employed small sample sizes consistent with pilot-scale validation, limiting statistical power. Replication in larger, more diverse cohorts is needed to confirm the replicability and generalizability. Second, while our design aimed to reduce stress by enabling voluntary, homecage-based testing, we did not directly measure stress biomarkers such as corticosterone levels. Future studies should empirically validate these welfare benefits. Third, the observed variability in learning rates in the Social RatPad highlights challenges inherent to autonomous testing. Factors such as individual motivation, social hierarchy, and circadian preferences likely contributed and should be systematically examined in follow-up work. Moreover, other factors such as unrestricted feeding (ad libitum), fixed session durations, and limited physical interaction due to cage dividers may have influenced individual differences in task performance. While the absence of food restriction aligns with welfare principles, it could reduce exploratory drive or motivation to seek food rewards. Future designs should consider implementing controlled feeding schedules, adaptive session timing, and socially enriched environments to better capture and standardize natural behavior across animals.

It is important to notice that during this phase the RatPads were still under development while the Campden systems are established production systems and environmental conditions were not the same during the test.

The RatPads were significantly improved over time to solve software bugs and to increase hardware reliability with respect to touchscreen sensitivity, reward pickup detection and pellet dispenser performance.

Moreover, in the Campden system, each rat was tested in a fully enclosed cabinet, isolated from its surroundings - without visual or auditory contact with other animals. In contrast, the standalone RatPad provided an open testing environment where the rat remained exposed to ambient laboratory sounds and visual cues, even though no other animals were present. These contextual differences are crucial to note, as sensory isolation in the Campden setup likely alters the animal's motivational and attentional state compared to the perceptually open conditions of the RatPad.

Future Directions

Based on these findings, several clear directions emerge: One important step will be scaling up validation with larger, more diverse animal groups to ensure generalizability of results. Another is the incorporation of direct welfare assessments through physiological and behavioral stress markers. The platform can also be advanced by integrating neural recording technologies, which would enable real-time correlation of cognitive performance with brain activity in freely moving animals. Moreover, extending to more complex cognitive paradigms will enhance the translational relevance of the system. Finally, longitudinal assessments will be critical to determine how sustained homecage-based cognitive testing influences learning, welfare, and neurodevelopment.

Conclusion

Overall, the Social RatPad represents a meaningful advance in rodent cognitive assessment methodology, combining the translational power of touchscreen-based tasks with the welfare benefits of autonomous, socially housed, homecage testing. By reducing human intervention, maintaining social housing, and supporting voluntary participation aligned with animals' natural rhythms, this system offers a promising platform for producing high-quality, reproducible, and ethically sound data in preclinical neuroscience.

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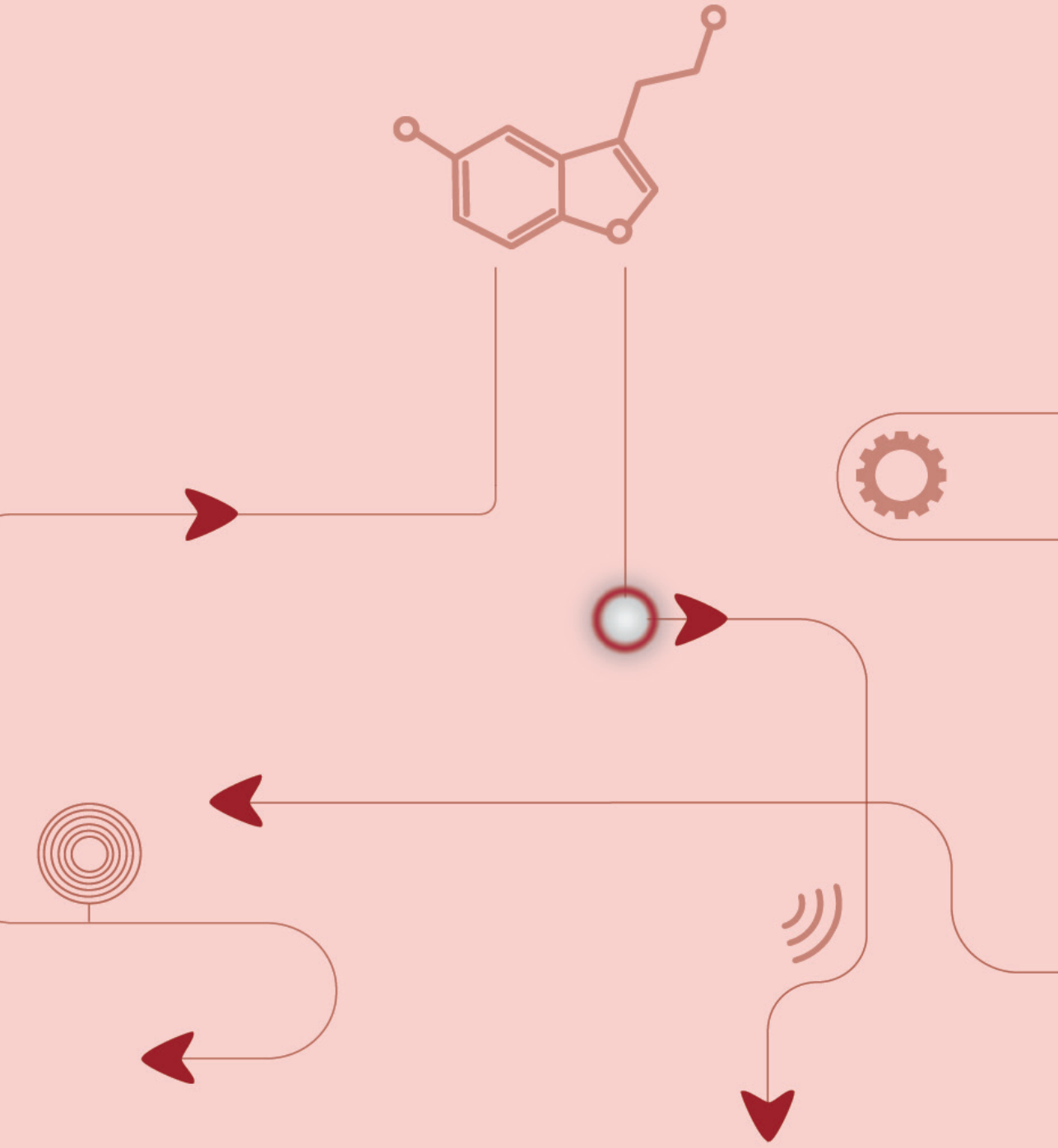

Chapter 3

Reducing Human Intervention in Rodent Cognition Studies: Automating the Social RatPad System

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Abstract

Understanding cognition is crucial for advancing effective treatments for human diseases. Rodent studies are key to uncovering the complex mechanisms underlying cognitive function, yet traditional testing methods often introduce stress and variability that confound results. Hence, high-quality, automated systems that accurately measure cognition while minimizing human interference are urgently needed. This need underpins our study's approach.

The Social RatPad is a cognitive assessment device consisting of two standalone testing chambers (RatPads) connected via tubes to a shared cage where animals can socialize and access food and water. Previously, the system allowed testing of animals at specific time points. A divider was inserted into the central cage, which enabled one rat to enter a designated testing chamber. This method required daily manual intervention by researchers, often involving animal handling during the divider placement, which could potentially cause stress.

To reduce human intervention, we automated the socialization component of the Social RatPad by integrating sensor-driven tubes, a modified One-Rat Turnstile (ORT) gate with size-adjusting wings, weight measurement stations, and self-made gates that selectively allow or block access to the testing chambers. Moreover, by modifying the testing protocols so that the touchscreen-based tasks on the RatPads run continuously, the system now empowers the animals to control when they engage in testing.

This study details the design and implementation of components and compares the data obtained from the automated system with human-based observations to assess its accuracy. Preliminary data demonstrates the effectiveness of the automated approach in identification and sorting procedures. Furthermore, the results indicate that, given the appropriate motivation, the animals rapidly adapt to the environment, exercise control over their testing schedule, and participate consistently. The new solution also allows researchers to observe socializing tendencies among different strains by providing the opportunity to precisely measure the time spent in one part of the complex chamber.

The automation procedures help reduce human interventions, thereby increasing animal welfare and reducing the impact of researchers on testing procedures.

Introduction

The Need for Automation in Animal Research

Animal studies have long been conducted to advance our understanding of challenging questions in cognition, behavior, and other topics. These studies provide researchers with significant flexibility in interventions, allowing for manipulations that would be impossible with human participants. However, limitations and ethical considerations have become topics of discussion and debate. Human physical interventions during studies have been shown to be a significant source of stress for the animals and can also lead to lower ecological validity in the translation of results. To address this gap, researchers have aimed to develop increasingly automated systems that allow tasks to be conducted without human intervention or physical presence in the lab.

Automation of animal scientific research is one of the key aspects in striving for studies with higher ecological validity and translational power. It has been demonstrated that research data are significantly influenced by various factors related to personnel, including researchers' gender working with animals (Sorge et al. 2014). Reducing human intervention is not only crucial for obtaining results with more consistent and replicable data but also for researchers' well-being (Ahn, Park, and Roh 2022; Kang et al. 2018).

Automation also can foster the well-being of animals (Richardson 2015). As such, the automated homecage method allows for the reduction of disturbances and interventions while enabling animals to undergo tests at their own pace. In such settings, animals have the opportunity to voluntarily participate in experiments, which is crucial for translating results effectively.

To further enhance cognitive assessment tasks in our lab we created a system called the Social RatPad. The first step was to develop the Ratpad system (see chapter 2) in which cognition could be assessed in a homecage type system where animals can stay 24 hours or more to reduce animal stress and human interference during the tests and allow faster learning. The RatPad system is equipped with a capacitive touchscreen, reward dispensers, and a reward collection station.

To test the animals in a social setting and further reduce human interference, the Social-RatPad was system was developed. Initially, the Social RatPad consisted of a central home cage connected through tubes to two testing

centers (RatPads). In the central home cage (a Type-4 European standard cage), the animals were physically separated by a perforated divider to secure identification of the animals. Although this approach provided a limited form of social interaction between the rats, further automation and reassessment of the protocols was needed to enable full social interaction .

The main challenges during automation were related to two areas: animal identification and sorting mechanisms. Reliable identification was essential to track individual rats moving between the central cage and the testing centers. Several approaches were considered, including camera-based recognition with unique markers and RFID-based identification. While camera-based methods appeared attractive because of their non-invasive nature, they raised concerns about accuracy under low-light conditions and with fast-moving animals. RFID, by contrast, has been widely applied in laboratory settings and offers continuous, automated identification. Based on these considerations, the available options and their respective advantages and limitations were reviewed to determine the most suitable solution for the Social RatPad. In parallel, sorting mechanisms were required to ensure that only one rat could access a testing center at a time. For this purpose, the One-Rat Turnstile (ORT) system was adapted, preventing simultaneous entry and enabling accurate data collection. The design was made size-adjustable to accommodate the rapid growth of juvenile rats during the month-long experiments, without disconnecting the system or interrupting testing.

The aim of this section is to outline the process by which we selected, evaluated, and adapted animal identification and sorting methods for the automated Social RatPad system. Our goal was to integrate technologies that allow continuous, accurate tracking of socially housed rats with minimal human intervention. To achieve this, we reviewed existing one-time and continuous identification approaches used in breeding laboratories, research facilities, and commercial systems. Based on this evaluation, we initially developed a visual identification method, later replaced by RFID technology, and implemented targeted adaptations to the One-Rat Turnstile (ORT) system, a gate mechanism that lets only one animal pass from a chamber to another one. These developments addressed two key challenges: reliably identifying individual rats and ensuring controlled, single-entry access to testing chambers.

Identification of Laboratory Animals

Animal tracking and identification remarkably vary based on the species being tracked and identified. Numerous variables, such as body shape, size, or the level of intelligence, may impact the chosen method of identification. In aquatic species such as fish, the surrounding environment allows continuous video-tracking to monitor movement patterns (Walter and Couzin 2021). In larger animals such as cats or primates, identification can rely on visible tags or trained behaviors (Fizet et al. 2017; Eagan, Eagan, and Protopopova 2022). By contrast, rodents present greater challenges due to their small size and inability to self-identify. Identifying and tracking rodents poses greater challenges, due to their small size. Various identification methods have been developed for rodents. Almost any type of identification widely used before in laboratory settings has been refined over time.

Ear Tags

Colored ear tags are one of the most common methods for rodent identification. Examples include Stoelting's non-metallic, multi-color ear tags or RapidLab's ear tags (figure 1). Those tags are lightweight. With RapidLab ear tags, it is possible to generate an enormous number of unique identifiers as the tags utilize a data matrix type of code in addition to the color, which includes a six-digit code. Likewise, the tattooing method, which has been widely utilized, has progressed.

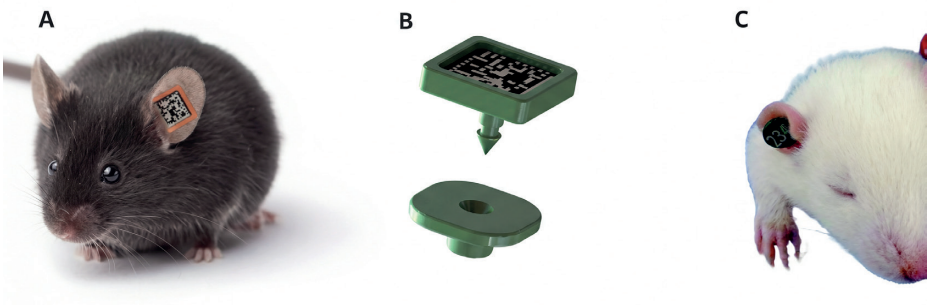

Figure 1. Examples of ear tags used for mouse identification. (a) RapID Ear Tag attached to a mouse's ear (manufacturer: RapidLAB); (b) components of the RapID Ear Tag; (c) stoelting's non-metallic, multi-color ear tag attached to a mouse's ear. Sources: https://rapidlab.com and https://stoeltingco.com.

Tattoos

Another identification method, tail marking, initially being a simple method also advanced. There are tattoo machines available, such as Labstamps' tattoo machine to make a mark on the tail, or some other methods have been tested to make a permanent ink-based mark on the ears (Chen et al. 2016).

Radio frequency identification

With the advancement of technology, various modern methods have been incorporated to achieve higher accuracy (within automated systems) in the identification of animals, especially when dealing with large stocks (Congdon et al. 2022). For this reason, RFID (radio frequency identification) methods have become popular. The RFID method operates as follows: a small battery-free transmitter in a tiny glass tube that is injected into the rodent will be charged and activated as soon as it enters an electromagnetic (EM) field in the RFID frequency range. When the RFID transmitter is activated, it will transmit a unique predefined code. RFID receiver modules that are connected to special antennas are used to generate the EM field and pick up he transmitted unique code to identify the rat passing through the EM field.

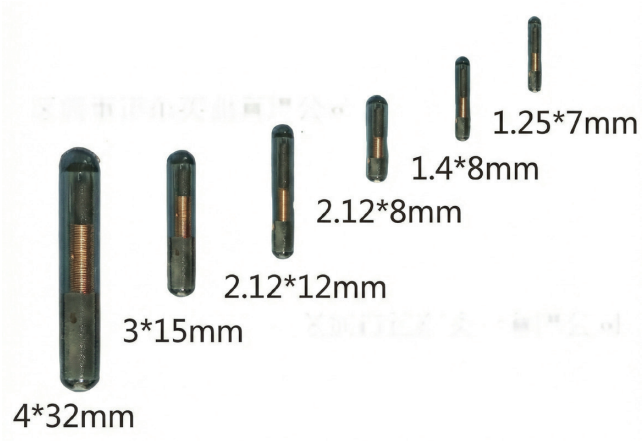

Figure 2. Various dimensions of animals' RFID micro-transponders. Source: https://alibaba.com

RFID technology has been utilized in numerous experiments and for large-scale stock management. Starting from the smallest microchip available in the market (p-chip: 250 μm × 250 μm × 100 μm) to larger RFID tags that are utilized. The effects of the magnetic field on certain components of biological organisms have been examined indicating no-observable adverse effects to embryos injected with p-chips (Mandecki, Rodriguez, and Drawbridge 2016) or without significant observable effects to mice injected with larger microchips (Bains et al. 2020).

The biological impact of continuous exposure to electromagnetic fields (EMFs) used for RFID activation is not yet fully understood. While 134.2 kHz and 125 kHz frequencies are commonly used for animal identification, the available

scientific evidence on their biological effects remains inconclusive. Potential effects depend on multiple factors, including the exact frequency, field strength, and exposure duration. Some studies, such as Kim et al. (2021), suggest that certain frequencies (915 MHz in this case) may influence physiological functions.

According to the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) Opinion "Potential health effects of exposure to electromagnetic fields (EMF): Update with regard to frequencies between 1 Hz and 100 kHz" (SCHEER, 2024a) and its accompanying factsheet (SCHEER, 2024b), scientific knowledge on EMF exposure effects is increasing but still contains major gaps. The committee notes that although typical environmental exposure levels are well below current safety limits, evidence remains insufficient to draw firm conclusions about long-term biological impacts, especially concerning neurodegenerative processes, melatonin production, DNA integrity, and oxidative stress. It further highlights the need for more consistent, real-world-based studies addressing both human and environmental effects.

Therefore, comprehensive evidence on the biological effects of continuous RFID electromagnetic exposure is still lacking (SCHEER, 2024a; SCHEER, 2024b).

Video Monitoring

The second advanced technology widely used comorbid with RFID- based identification in laboratory animal tracking and identification is video surveillance. Researchers utilize video surveillance to capture the movement of animals within cages and employ trained vision detection models to identify specific behavioral patterns. When applying such technologies in shared areas with multiple animals, precise identification is crucial to ensure that the detected data pertains to individual animals and that data mixing does not occur. Achieving this requires either attaching extra markers to the animals, as previously demonstrated, or accurately distinguishing biological markers belonging to each animal (Vidal et al. 2021). Among these markers, theoretically, color patterns in the fur could be used, but in certain cases, such as with albino rats, there may be no major differences for differentiation. It seems effective to combine RFID methods with video monitoring (Arica 2015).

Sorting of Socially Housed Animals

Historically, there have not been many attempts to create systems where socially housed rodents could travel to testing (or other functional) centers and then return to a shared common area. This lack of development contrasts

with primate research, where several systems have already been designed and tested. One of the few notable attempts in rodents was developed by Winter and Schaefers (Winter and Schaefers 2011) in collaboration with PhenoSys. Their design consisted of a tube through which an animal could move between the home cage and experimental compartments, while transponder readers placed along the tube transmitted position data to software controlling two motorized doors (Figure 3). There is also a weight measurement station on the tube. The animal waits on the tube until the system makes sure no other animal is there and goes through the sections one by one.

TSE systems further developed Winters' and Shaefers' systems, and offer a product called "Animal Gate", which is a selective passage tube that allows lab animals to pass from their housing cages to different experimental areas. "Animal Gate" uses a set of Transponders, connected to software developed by TSE, which allow or deny the passage of selected animals. The RFID antennas read the transponder code, then 3 motor controlled movable doors allow the passage of select subjects through the gate. Infrared sensors also monitor the movement of animals within the tube ("AnimalGate," n.d.). In an experiment conducted by (Brenneis et al. 2017) to test an automated tracking method for rats in colony housing (shown in Figure 4), transponders were installed to monitor the rats' movements. The tracking system included two transponder positions, referred to as TP1 (lower) and TP2 (upper), located at the entrance and exit of the staircase equipped with antennas. Each time a rat passed by these transponder points, an event was recorded with its location ID, rat ID, detection time, and potentially its body weight. The collected data were then stored in a database for further processing. The staircase transit tracking system, including TP1 and TP2 (shown in part of figure X), comprises a tube that allows a single rat to enter or exit the staircase through the antenna-equipped entrance.

Apparatus Development and Testing

An Attempt to Identify Rats Through Visual Detection

To develop an effective identification system for tracking animals in the Social RatPad, it was essential to explore various strategies that could meet our specific requirements. We examined multiple potential methods, considering aspects across different domains, such as accuracy, invasiveness, cost, and suitability for rodents.

Figure 3. IDsorter automated sorting system developed by Winter and Schaefers. Designed for identifying and separating individually tagged rodents in laboratory settings.
Source: https://www.phenosys.com.

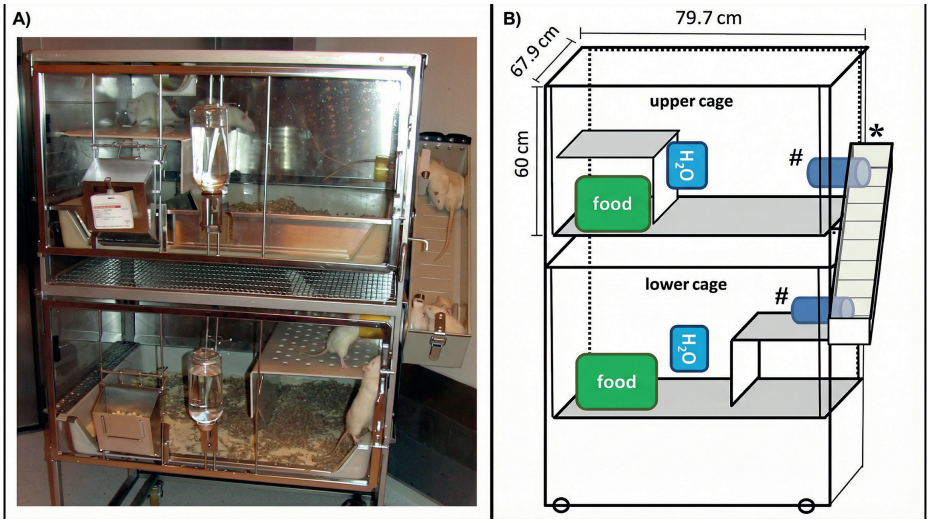

Figure 4. Prototype rat colony cage with integrated RFID-based motion and body weight tracking system. The modular design allowed housing of up to 48 rats across multiple functional areas, with antennas positioned at staircase entrances, jump holes, and weighing stations to enable continuous individual tracking. Panel A shows a photograph of the physical prototype of the colony cage, while panel B provides a schematic overview of its design, including the custom staircase and transit tubes connecting upper and lower cages. Source: Brenneis et al., 2017 (Journal of the American Association for Laboratory Animal Science, 56(1), 18–31)

Table 1. Domains and targeted points considered during system development. Factors include animal well-being (invasiveness, discomfort, possible complications), cost (component costs), and research needs (study characteristics, species-specific requirements).

Domain	Targeted Points
Animals Wellbeing	Invasiveness Level of discomfort Possible complications
Cost	Cost of the components
Research	Characteristics of research Specialties of the species

Based on the criteria mentioned, some possible mechanisms were shortlisted. Camera-based identification was preferred, as it could potentially be non-invasive. However, two challenges were obvious with this method. First, available trained databases lacked rodent-specific and, specifically, rat recognition components, meaning that a potential database should be developed by us. Second, the size of the rodents did not allow for large enough markers to be easily detected by cameras, unlike in the case of the cats mentioned earlier.

Nonetheless, a decision was made to prioritize the camera-based identification method. Therefore, since albino rats were utilized, it was evident that an additional marker should be applied to provide a basis for differentiation. For this reason, after careful consideration, RapidLab's RapidID was selected. These tags were small (0.5 cm x 0.5 cm), containing a data matrix code inside and a color frame pattern around the edges. Made of plastic, they allowed for the potential application of FMRI or similar techniques with this identification method (if needed at any time during the research), which would not be feasible with, for example, metal-based identification methods. Being lightweight (0.07 g), it was assumed that they would not significantly impact the animals by adding extra weight to their ears.

To develop a visual identification system for tracking individual rats, we created a program to detect unique markers on the animals using computer vision techniques. The program was developed using the CV2 library, a popular open-source computer vision library in Python that provides tools for image processing and analysis. The program was designed to detect frame-shaped contours and apply color markers to identify the currently visible colors (See Appendix B).

We utilized the HSV (Hue, Saturation, Value) color space, which is a way of representing colors in terms of their shade (hue), vibrancy (saturation), and brightness (value). This color space is often used in computer vision because it closely aligns with how humans perceive and distinguish colors compared to the standard RGB (Red, Green, Blue) model. Using HSV values allowed us to effectively distinguish frame shapes and recognize contrasting colors, such as blue and yellow, even in varied lighting conditions.

For hardware, we used a low-cost laser reanuder (Grow GM67, Hangzhou Grow Technology Co., Ltd, China) for reading data matrix codes and a low-cost camera (ELP 1080P Camera Module) that could operate under low-light (low lux) conditions.

Despite initial efforts to implement a visual identification system, several challenges emerged that hindered its effectiveness. Firstly, the program struggled to recognize frame-shaped patterns accurately when the rats moved rapidly. Secondly, color mixing occurred depending on the distance between the rat and the camera, suggesting a need for better cameras and more training data to distinguish distance-based differences. Thirdly, the program was not very effective in low-light environments, which is crucial for identifying rats during night recordings. However, it is worth mentioning that new technologies have become available, such as Sony's Starlight cameras, which allow color detection under very low light conditions.

To address these challenges, we propose incorporating more advanced laser-reader modules and cameras, gathering data from various rat positions to improve program training, implementing localized lighting activated by movement, and developing a more accurate identification method to serve as a benchmark for comparison.

Designing the Apparatus

In seeking to develop a less mechanically complex solution for sorting the animals, we opted to incorporate turnstile logic gates into our system. Our research revealed that a research team at North Texas University had already created and successfully tested such a gate design. After reaching out to them, they generously agreed to share their design and offered exceptional support during our subsequent mockup testing phase. This collaboration not only streamlined the developmental process but also ensured that our approach would be informed by prior, empirically validated expertise.

The mechanism that allows only one rat to pass from the main living area to the testing chamber is called ORT (One-Rat Turnstile). ORT is a mechanical design that can be printed using a 3D printer (Butcher et al. 2022). An ORT is built for the size of experimental animals. It is made from a plastic tunnel, turnstile, ramp, locking mechanism, and a plexiglass roof. The turnstile is placed within a tunnel, which a rat could enter. When the rat goes through the tunnel, the turnstile rotates 45 degrees and reveals another opening to the experimental chamber. Full rotation of the turnstile also allows a spring-loaded ramp and locking mechanism to pop into the upright, locked position. The locking mechanism prevents other rats from gaining access to the experimental chamber. The turnstile is only unlocked when the rat leaves the experimental chamber and steps onto a ramp within the ORT. In our experiment, the ORT was situated between a basic experimental chamber and a colony cage.

The main difference between the sluice and ORT concepts lies in how animal movement is managed between compartments. In the sluice design, animals are temporarily stopped for identification before being allowed to proceed, which can introduce waiting periods. In contrast, the ORT concept enables continuous movement by allowing animals to walk through without forced stops, providing a more natural and less restrictive flow. This distinction may influence behavioral outcomes.

While testing the turnstile with the rats, we conceived the idea to make it size adjustable. Grayson Butcher and the team proposed size adjustments by incorporating a piece of plexiglass into the turnstile. While effective, we desired a mechanism that could offer greater scalability. Consequently, we decided to attach additional wings to the turnstile, which could be adjusted from the outside with minimal effort.

They are elevated from the ground, ensuring that both the rectangular component, which secures the turnstile in place (once it's open past a certain point), and the triangular unlockers remain untouched. So, they are hovering over those parts. When opened these wings reduce the space. They can be manually moved back step by step to create more room.

Based on the discussed findings, we decided to create a mockup to test the identification systems, improve research usability, and address components with potential to enhance animal welfare. Particular attention was given to maintenance and cleaning requirements.

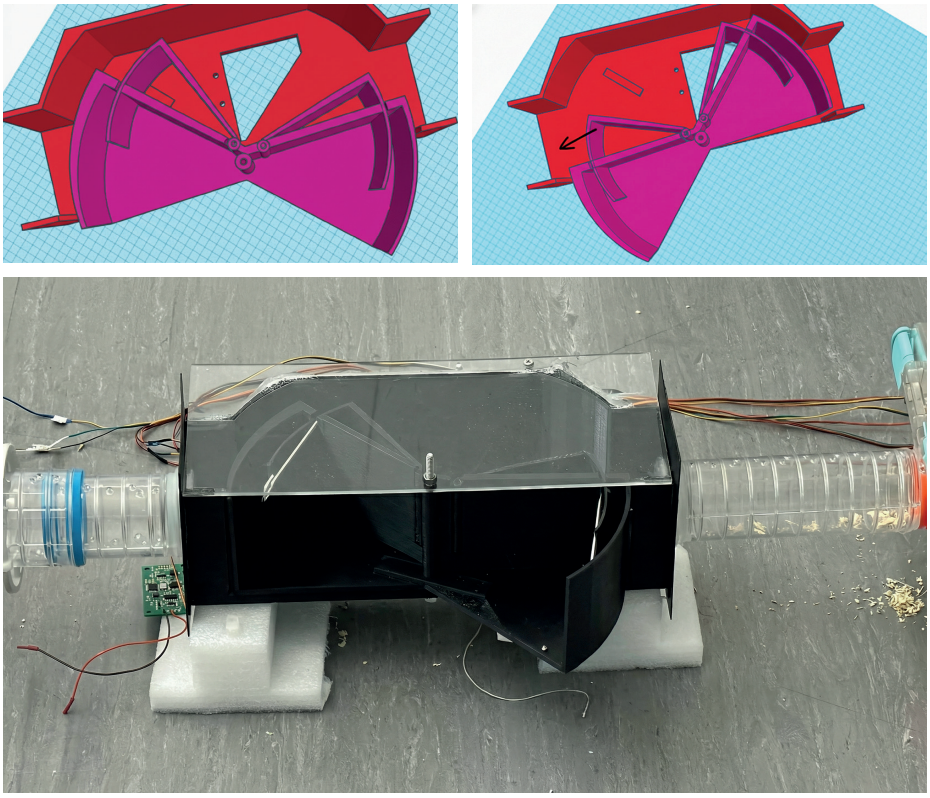

Figure 4: ORT gate design featuring adjustable wings integrated into the turnstile mechanism. Top images show schematic diagrams highlighting wing positions for size adjustment, while the bottom image displays the physical prototype with transparent cover for visualization.

First Mock-Up for Automated Isolation and Identification Testing

The mockup was designed to test methods that would make the overall system much easier to assemble and disassemble for cleaning and maintenance purposes, thereby making it more researcher-friendly. For this purpose, tubes with a diameter of 60 mm and lengths ranging from 125 mm to 202 mm, made of transparent plastic (Ferplast, Italy) previously utilized in the pet industry, were employed. These tubes can be separated into two half-moon-shaped pieces, facilitating easier cleaning. Additionally, by employing a simple mechanism for connection, known as FPI tube-to-cage connectors, these tubes do not require additional force for quick assembly. The remaining components of the system were unchanged, except for the cages, which were also specialized for FPI connections (NX-36, dimensions 495 x 350 x 250 mm, transparent plastic, Jiaxing Nomoy Intelligent Household Products Co., Ltd., China).

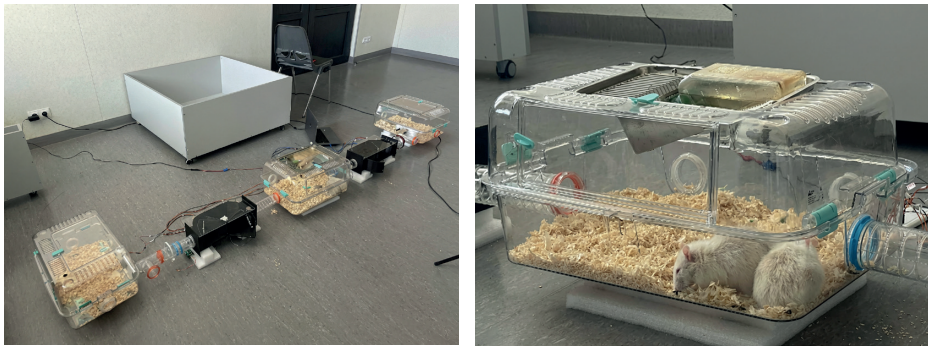

Figure 5. Social RatPad mockup featuring transparent, easy-to-assemble plastic tubes with FPI connectors. Images show central housing and connection to ORT gates designed for improved maintenance, cleaning, and research efficiency.

From the central cage, plastic tubes extended to ORT gates, where the animals were sorted, and other sections of plastic tubes continued from the ORT gates to the testing centers. Prior to entrance into the testing centers, additional automated gates were installed. These gates are controlled by servomotors. RFID magnetic coil antennas (diameter :100mm, inductance: 2.8mH, resistance: 42ohm, material: isolated 0.125mm copper wire) are inserted into self-made PLA enclosures. RFID antennas with a diameter of 100 mm, made of coil material from priority1design in Australia, were inserted into self-made PLA enclosures. The RFID reader module decodes the ID locally and outputs the code over a TTL-level UART serial interface (TX/RX) to the microcontroller. The microcontroller then transfers the data to the PC via a USB-serial connection. Based on the predefined conditions – specifically, the user's assignment of each code to a particular chamber in the GUI before the experiment start – the secondary gates either opened or remained closed (permitting or blocking access to the testing chamber for a specific rat).

These enclosures were mounted over the tubes at specific locations. Each side that extended from the central cage to the testing center had two antennas mounted. The rats were injected with RFID microchips (bio-glass, 1.4*8mm, 134.2 kHz) from Quanzhou Hecere Electronic CO.,LTD. The antennas were connected to RFID-TTL modules from Priority1Design in Australia, which were in turn connected to Arduino modules.

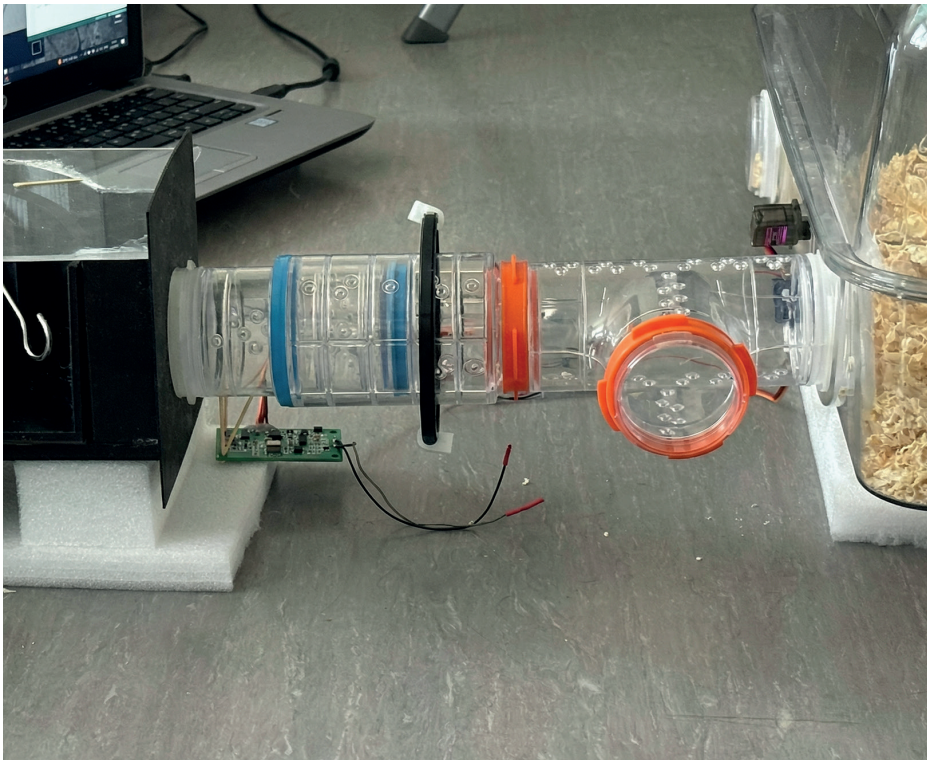

Figure 6. Integrated FPI Tube configuration with T-Shaped junction and embedded RFID antenna. setup enables reliable animal tracking and system navigation, with RFID Coil embedded in custom 3D-printed PLA enclosures.

System Integration and Advanced Prototyping

After the mockup testing, the prototype has been improved to meet specific requirements, such as enclosed electronic modules and a more animal-friendly design, for example by incorporating smoother tube connections and reducing sharp edges, which minimized the risk of injury and made navigation more natural for the animals. The assembly process was also considered to facilitate easier cleaning. From a technical perspective, the ORT gates have been further enhanced to be height-adjustable, with closed or open state detection now performed using reed switches (as opposed to the previously utilized limit switches). This change allows for the detection of a wider range of closed and open states of the triangular rotating part. The ORT gates have also been upgraded with custom-made entry and exit modules that are compatible with 3D-printed FPI gate connectors.

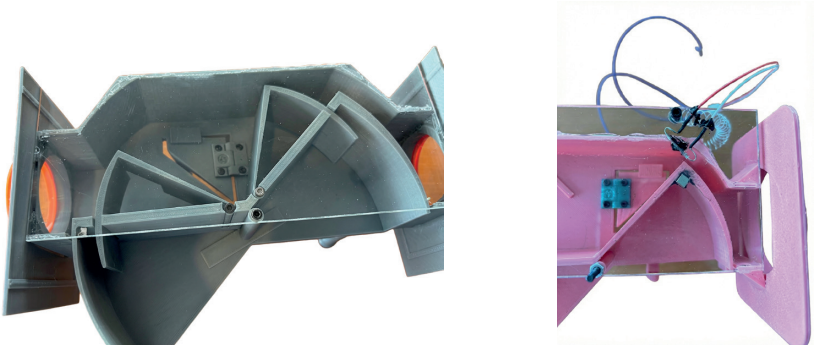

Figure 7. Evolution of the ORT gate design showing integrated additional wings (grey design on left) and reed switches (pink design on right). Images illustrate improved internal layout for size adjustment and enhanced detection capabilities within the system.

The advanced prototype consists of a Type 4 (Eurostandard) cage (UNO B.V., the Netherlands) in the center, serving as the central home cage. FPI binocular tubes (Ferplast, Italy) are connected to each side of the cage. The length of these binocular tubes can be adjusted, allowing them to be easily detached and reattached to the rest of the system. The binocular tubes are connected to 3D-printed connectors specifically designed to match the FPI tubes. These tubes are further connected to the ORT gate.

A custom-made tube follows the ORT gate. At the beginning of this tube, there is a widening where a specially designed (Figure 9) and 3D-printed RFID module enclosure (housing both the module and antenna) is pressure-fitted to the tube. The middle part of the tube is open at the bottom to accommodate a scale underneath for weight measurement. T-shaped tubes follow the weight measurement station and lead to a secondary gate. The secondary gate acts as a barrier to the testing center.

A program functioning as a state machine is integrated into the system, operating with the following logic: When one of the animals passes through the ORT gate, the machine's state changes to "locked" for that specific animal. Subsequently, if the rat exits the ORT gate, the RFID antenna reads the information from the implanted transponder and compares it to the researcher-provided code. If there is a match, the system first opens the secondary gate, granting the animal access to the testing chamber, and second, initiates weight measurements at the scale. All data is recorded in a CSV file for further analysis. Once the animal leaves the testing chamber and the ORT gate, the

secondary gate closes slowly, and the system returns to the idle state for that specific animal.

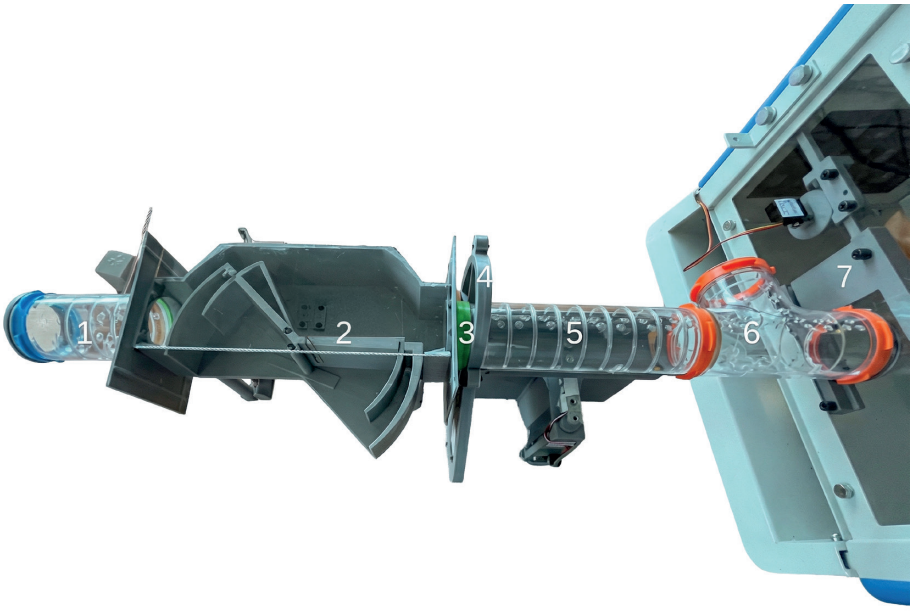

Figure 8. Final View of the socializing system connecting the central cage and standalone RatPad. Labeled components: 1 - Binocular tube; 2 - ORT Gate with size-Adjusting wings; 3 - Custom-made FPI-to-tube connector; 4 - RFID antenna; 5 - Weight measurement station; 6 - T-shaped tube for U-turn; 7 - Secondary gate controlling access to the testing chamber.

In the context of this system, the term "corridor" was introduced. A corridor refers to the physical tunnel connecting two compartments (Figure 9), which animals traverse to reach a goal area or return to their starting point. A full corridor traversal is defined as the sequence between the ORT_Lock (entry into the corridor) and ORT_Unlock (exit from the corridor). These transitions are logged automatically by the system, including associated RFID readings.

Initially, these corridor entries and exits were manually annotated from video recordings and compared one-to-one with system-generated logs. This allowed us to confirm whether individual events detected by humans were correctly logged by the system. Following this step, a dedicated script was developed to automatically extract and compile system-generated corridor events, allowing faster comparison against the human dataset.

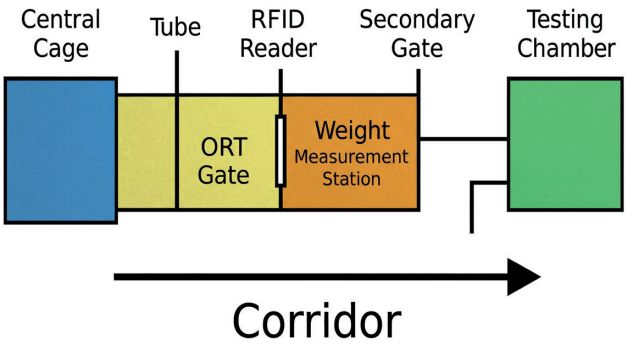

Figure 9. Schematic layout of the automated rat testing apparatus including all functional zones from central cage to testing chamber

Animals

Mockup Testing

Two Sprague Dawley rats (3 months old) were used. The animals were bred in Cobrain lab. Animals were housed in a mockup of the main device with food and water provided ad libitum. The rats experienced a standard day-night cycle (lights on at ~ 7:00 AM, off at ~ 7:00 PM). At the beginning of testing, animals were implanted with RFID microchips. The primary objective of this phase was to validate the basic functionality of the automated entrance and exit mechanisms. Ethical approval was granted by the Yerevan State Medical University Ethics Committee, Yerevan, Armenia.

Prototype Testing

Four in-house bred male rats were used during this phase: three were SERT (the serotonin transporter) wild-type (WT) and one was SERT heterozygous (HET), all 50 days old at the start of testing. The serotonin transporter (SERT) is a key regulator of serotonergic signaling, and alterations in SERT function have been linked to emotional and cognitive phenotypes. SERT mutant rats are environment-sensitive, meaning that they show exaggerated responses to both positive and negative stimuli. This increased sensitivity makes the model valuable for testing the strength of automated behavioral system. Animals experienced a standard day-night cycle (lights on at 7:00 AM, off at 7:00 PM). Animals lived in the system continuously from experimental day 1. The setup consisted of a Eurostandard Type IV central cage (598 mm × 380 mm × 200 mm), a corridor including an ORT gate, RFID reader, weight measurement station, a T-shaped tube, and a servo-driven secondary gate, leading to a Eurostandard

Type III testing chamber (425 mm × 266 mm × 185 mm). Food and water were available ad libitum in the central cage. The total floor area of the combined system was approximately 4,553 cm^2. RFID microchips were implanted before introducing the animals to the testing apparatus. All experimental procedures carried out were approved by the Central Committee Animal Experiments (Centrale Commissie Dierproeven, CCD) and conducted according to the Dutch Animal Experiments Act.

Final Validation and Post-Improvement Testing

In the final validation phase, four animals of the tryptophan-hydroxylase-2 knockout (TPH2-KO) strain were used. The tryptophan hydroxylase 2 (TPH2) enzyme is the rate-limiting step in brain-specific serotonin synthesis. TPH2 knockout (KO) rats lack central serotonin almost entirely and are widely used to study the neurobiological basis of emotional regulation, stress reactivity, and cognitive functioning. These animals serve as a translational model for serotonergic dysfunction observed in affective and neurodevelopmental disorders. Because the main experiments on the device were intended to be conducted using this strain, TPH2 KO rats were also included in the validation phase to ensure system compatibility and behavioral tracking performance with this model. The animals were aged between 33 and 50 days. This stage focused on evaluating the functionality of the system after several physical improvements were made to enhance detection reliability and mechanical resilience. Animals experienced a standard day-night cycle (lights on at 7:00 AM, off at 7:00 PM). Animals lived in the system continuously from experimental day 1. The setup consisted of a Eurostandard Type IV central cage (598 mm × 380 mm × 200 mm), a corridor including an ORT gate, RFID reader, weight measurement station, a T-shaped tube, and a servo-driven secondary gate, leading to a Eurostandard Type III testing chamber (425 mm × 266 mm × 185 mm). Food and water were available ad libitum in the central cage. The total floor area of the combined system was approximately 4,553 cm^2. RFID microchips were implanted before introducing the animals to the testing apparatus. All experimental procedures carried out were approved by the Central Committee Animal Experiments (Centrale Commissie Dierproeven, CCD) and conducted according to the Dutch Animal Experiments Act.

Procedures

Testing Protocol

The testing protocol aimed to evaluate multiple components of the system, including functionality and adjustment, reading accuracy and distance, and overall performance evaluation. During the mockup testing, the system was validated for basic operational integrity and adjusted for local experimental conditions. In the prototype and post-improvement testing stages, the focus was on assessing RFID reading performance, particularly the spatial detection range and the reliability of readouts under dynamic animal behavior. Performance evaluation included monitoring several key parameters: the success rate, defined as the number of sessions in which RFID readings and event detections occurred successfully; traversal detection accuracy, which measured how precisely the system tracked the time spent inside the corridor; and system robustness, referring to the mechanical resistance of the setup to physical interactions by the animals.

Improvements for Device Fine-Tuning

To improve behavioral segmentation and RFID detection, an extension tube was installed between the ORT_Lock state and the RFID antenna zone. This design adjustment created a buffer area, which allowed the animal to fully enter the RFID detection zone before triggering any event-related actions. As a result, this change significantly reduced "no scan" events and increased the consistency of RFID-based entry registration. Additionally, the access gates were mechanically reinforced to prevent physical breaches after one of the animals repeatedly bypassed a weaker design during initial testing.

Validation Analysis

To evaluate the performance of the automated corridor detection system, we developed an approach that compared automatically logged outputs against human-annotated observations from synchronized video recordings. Key analysis components included:

One-to-One Matching: Manual annotation of corridor entries and exits by humans was matched against system-detected transitions based on timestamps.

Event Duration Comparison: Duration of each corridor traversal (entry to exit) was measured independently by both human observers and the system and compared for consistency.

Timestamp Shift Assessment: Differences between human and system entry timestamps were calculated to detect systematic delays due to logging delay.

RFID Detection Success Rate: For each corridor event, the presence or absence of a valid RFID read was evaluated to calculate detection accuracy.

Exclusion of False Events: Any corridor detections linked to exploratory behaviors like gate-shaking without full crossings were excluded from analysis.

To evaluate the performance of the automated corridor detection system, we compared its logged outputs against human-annotated observations from video recordings. A total of 58 rat corridor traversal sessions were analyzed.

Results

Corridor Validation with Automated System Matching

To quantify system performance, we first extracted all corridor transitions directly from the system logs and counted how many included valid RFID reads. Out of 58 events, 55 had corresponding RFID identifications, resulting in a preliminary success rate of 94.8%. This initial calculation was based solely on raw counts without yet considering potential timing discrepancies or exclusion of non-behavioral triggers. This initial success rate was calculated by manually comparing timestamp counts between human and system logs using a ±10-second matching window. However, this rough estimate did not account for potential system-related delays in logging (e.g., hardware or processing lag), which may have caused some valid events to fall just outside the matching window. Additionally, no filtering was yet applied to exclude possible non-behavioral triggers or noise events.

Findings After Re-Evaluation

To provide a more refined estimate, we subsequently re-evaluated the events through manual timestamp alignment and applied additional filters, as detailed below.

Table 2. Details from system-human comparison data annotations

Metric	Value
Total Human-Annotated Traversals	14
System-Detected Events with RFID	17
Time-Matched Events (within ±10 sec window)	13 out of 14
Matching System Timestamps (rounded to min)	22:16, 22:24, 22:25, 22:28, 22:29,22:31, 22:32, 23:06, 23:21, 23:30,23:33, 23:34, 23:35, 23:39, 23:41, 23:44, 23:56

These results demonstrate that while absolute timestamps may occasionally misalign due to fast movements and write-delay artifacts, the overall behavioral sequence and number of crossings is consistently detected by the system. Specifically, both the number of crossings and the sequence in which they occurred were reliably captured when comparing system detections with human annotations. Thus, the interpretation is not based on the raw event timestamps alone, but rather on the accurate alignment of behavioral events across methods. In some cases, the system even detected subtle transitions that were not identified by human annotators, further underscoring its sensitivity.

Final RFID Identification Accuracy

Each event was expected to be logged with a valid RFID code, linking the animal to the crossing behavior. After reviewing all recorded corridor events, scored:

- **Human-Annotated Corridor Traversals:** 58
- **RFID Identifications Detected by the System:** 57
- **Final RFID Read Success Rate (Figure 10):** 98.28%

One corridor traversal was correctly detected by both the human annotator and the system in terms of behavioral events, but the animal's RFID tag was not read as it passed through the antenna. This represents the only truly missed RFID event in the validated dataset.

Figure 10 illustrates the preliminary rate of 94.8%, which did not account for time distortions or gate-shaking events. The updated analysis shows that, with one true missed event, the system performs at nearly full accuracy in identifying animals during corridor transitions.

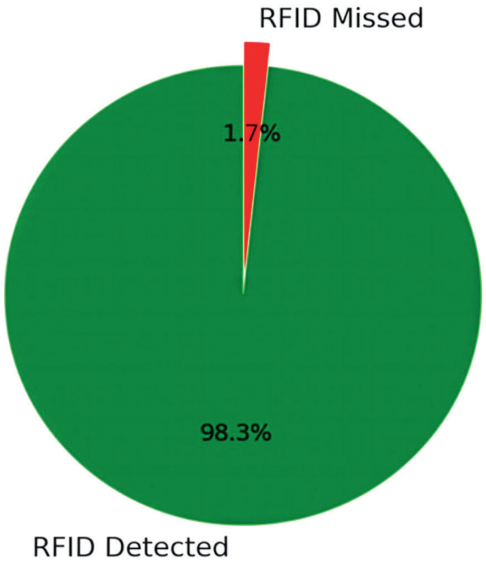

Figure 10. Proportion of corridors with successful RFID detection versus missed identification events. Pie chart illustrates the relative number of corridors where RFID tags were correctly detected compared to those with missed reads during animal movement

This re-analysis confirms that the automated corridor detection system offers a strong and accurate representation of behavior and supports its application in long-duration and high-frequency testing scenarios, especially when used in combination with RFID identification.

Extended Capabilities of the Improved System

In addition to reliably detecting corridor traversals and RFID identification, we improved the system. The improved version now offers enhanced behavioral quantification by enabling the differentiation of tube usage patterns based on logged data post processing. For example, the system can now track how much time each rat spends in assigned vs. non-assigned-side tubes, allowing for fine-grained behavioral analysis that was not possible in the earlier version. This is illustrated in Figure 11, where daily tube times are plotted separately for assigned and non-assigned-side entries for individual rats. Notably, rat 215005869605 consistently spent more time in the assigned tube, while rat 215005869610 increasingly visited the non-assigned-side tube, potentially reflecting strategy shifts or confusion. These types of behavioral dynamics can now be automatically detected and quantified, providing richer insights into task engagement and potentially socializing tendencies. The improved version of the system now offers enhanced behavioral quantification.

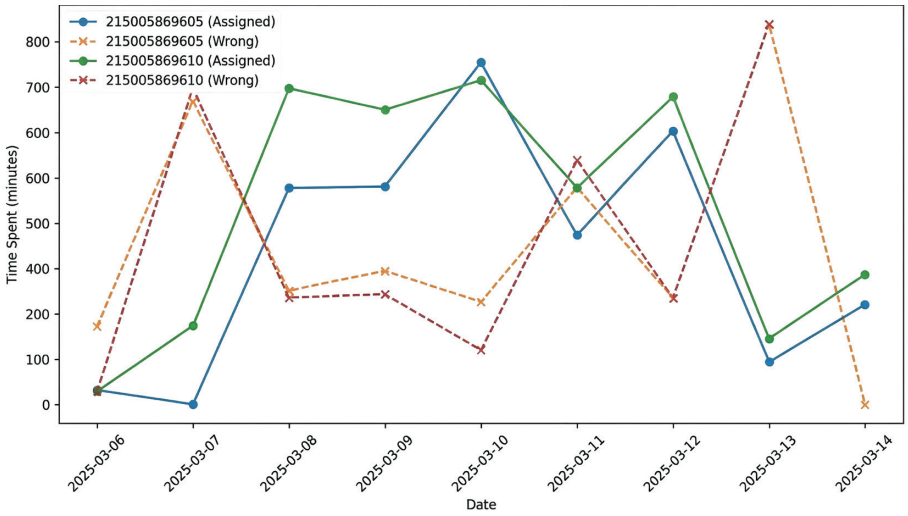

Figure 11: Daily tube time (Assigned vs. Non-Assigned) showcasing the paired rats' cumulative spent time in one of the sides of the automated Social RatPad.

Initial data showed us the opportunity of visualizing the daily activity of rats within the Automated Social RatPad, within what we termed the "Socializing Carpet." This representation integrates continuous RFID detections into a color-coded timeline, showing for each animal how a full 24-hour day is distributed between the assigned testing side, the non-assigned side, and the central shared cage. The woven pattern of colors resembles a carpet, reflecting the interplay between engagement, exploration, and social presence. Although the example here depicts two animals for 3 days, the method can be scaled to larger groups by assigning each rat its own timeline. The plots are generated automatically from the RFID event logs using a custom script, thereby avoiding manual scoring and enabling high-throughput analysis. This approach provides a comprehensive yet intuitive overview of behavioral dynamics, allowing researchers to examine side preferences, social encounters, and natural circadian rhythms in socially housed animals (Figure 12).

The system tracks (Figure 12) how long each animal spends in the corridor and distinguishes whether the rat enters the side it was supposed to access (the only side, where the animal could enter the testing chamber - assigned) or the opposite (non-assigned). This allows for several behavioral insights. First, it enables the assessment of task compliance, reflected in how frequently and consistently rats follow the expected route. Second, it makes it possible to identify individual biases, such as a persistent tendency to visit the non-assigned side, which may indicate either a side preference or a misunderstanding of the

task. Finally, it permits the analysis of temporal trends, capturing changes in strategy and the development of learning curves across days.

This capacity is critical for paradigms requiring goal-directed navigation, reinforcement learning, or social decision-making.

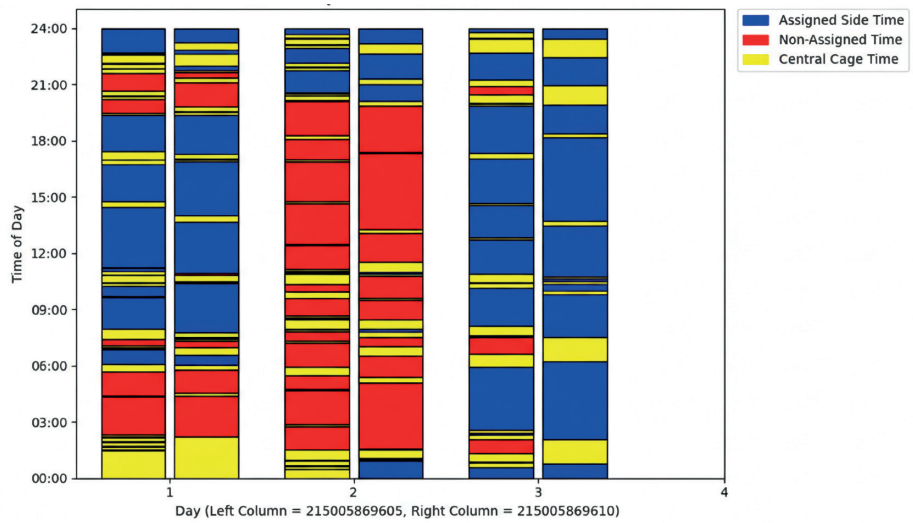

Figure 12. "Socializing Carpet" visualization of continuous 24-hour activity patterns of two RFID-tagged rats in the Automated Social RatPad. Each vertical column represents one rat across a given day, with colors indicating time spent on the assigned testing side (blue), the non-assigned side (red), or in the central shared cage (yellow).

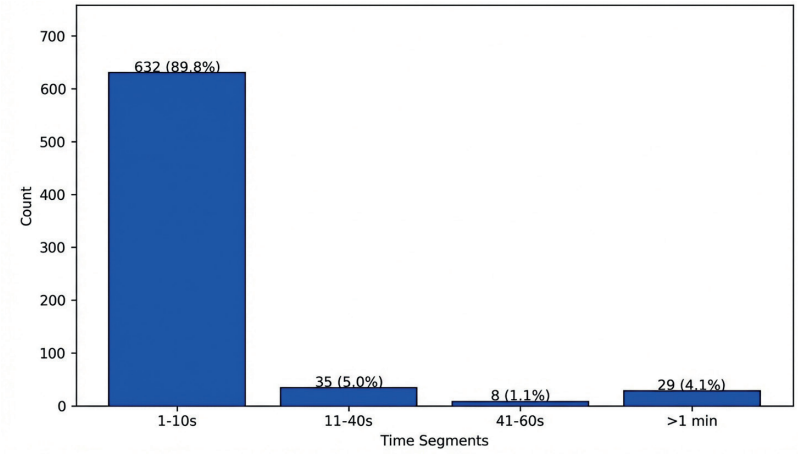

Figure 13. Distribution of RFID-scanned sessions divided into time segments. Bar chart shows counts of sessions grouped by time intervals reflecting when identification occurred after rats entered the corridor (1-10 s, 11-40 s, 41-60 s, and >1 min), highlighting detection timing patterns.

The histogram of RFID event durations (e.g., 1-10s, 11-40s, etc.) reveals how long animals typically remain within the corridor zone. A notable result is that **nearly 90% of all RFID-scanned sessions lasted under 10 seconds**, suggesting most rats moved quickly through the detection zone - indicative of a well-structured environment with minimal friction or confusion.

General Discussion

This chapter presented the design, development, and validation of the automated Social RatPad system, created to reduce human intervention in rodent cognitive testing while preserving animals' natural behaviors and social housing. Our aim was to improve ecological validity, and data quality by enabling continuous, individualized cognitive testing with minimal researcher involvement.

A central innovation was the integration of RFID-based individual identification, which replaced the less reliable visual tagging approach. RFID technology for rodents has proven highly effective, with reading accuracies in between 95 - 98% (Congdon et al. 2022; Walter and Couzin 2021). Our own tests achieved a 98.3% RFID success rate, confirming RFID's suitability for tracking socially housed animals without repeated handling. Implanting RFID microchips is a brief, one-time procedure, after which animals experience no ongoing discomfort, making it superior to repeated manual identification methods that elevate stress and reduce welfare (Bains et al. 2020).

Another major advancement was the voluntary, automated testing paradigm. Traditional cognitive tasks often require restraining animals and placing them into novel environments, introducing confounds from stress and context-switching (Richardson 2015). By contrast, our system allows rats to choose when to engage with touchscreen-based tasks directly from their home cage. This approach aligns with evidence showing that voluntary participation improves learning rates, data throughput, and welfare by leveraging animals' intrinsic motivation (Lopez-Cruz et al. 2021). Automated home-cage touchscreens have enabled 24/7 testing schedules, capturing nocturnal activity patterns and supporting complex paradigms like reversal learning with minimal experimenter bias (Horner et al. 2013).

Our validation demonstrated that the automated corridor logging matched human-annotated events with high fidelity. Small discrepancies in timestamps

were traced to logging delays rather than detection errors. Importantly, system data also captured subtle, rapid transitions that human observers sometimes missed. These findings "match" other RFID-enabled systems like PhenoSys's AnimalGate and TSE's IntelliCage, which have proven capable of enabling individualized operant testing within socially housed groups by selectively granting access to testing chambers (Winter and Schaefers 2011; Brenneis et al. 2017). Such systems have consistently shown that automation reduces human-induced variability and enables high-throughput cognitive phenotyping under more naturalistic conditions.

These findings also suggest that corridor activity data may provide additional behavioral readouts beyond simple transition counts. For instance, differences between brief and prolonged engagements could reflect distinct behavioral patterns; unusually long stays in the corridor may indicate stress or confusion; and repeated gate approaches could signal hesitation or bottlenecks in the system. Such parameters may therefore offer valuable insights into both task engagement and welfare-related aspects of behavior.

Beyond reliable identification and sorting, our system's design emphasizes animal-friendly features, such as smooth, low-friction passages (most crossings <10 s) and easy-to-clean modular tubes. Avoiding forced isolation or extended waits, issues noted with some commercial gates, preserves rodents' sense of control and reduces stress, a known factor influencing behavioral data (Kang et al. 2018; Ahn, Park, and Roh 2022). The use of size-adjustable ORT gates ensures compatibility with juvenile animals whose size changes over time, addressing a common practical challenge in long-term studies.

While RFID proved reliable, it is inherently invasive due to implantation and exposure to 125 kHz electromagnetic field. Recent research highlights the potential of computer vision (CV) systems to further refine automated tracking. CV methods like DeepLabCut or idTracker.ai can achieve high-accuracy pose estimation and multi-animal tracking without physical tags (Mathis et al. 2018; Walter and Couzin 2020). Combining RFID with CV can compensate for each method's limitations, enabling both positive identification and rich behavioral classification (Arica 2015; Vidal et al. 2021). Future improvements to the Social RatPad could integrate such hybrid approaches, allowing for non-invasive identification and even automated analysis of social interactions.

Despite these advantages, our validation was limited by small sample sizes (n=2-4 in different phases), mixed genotypes, and age ranges, limiting generalizability. Broader testing across strains, ages, and social contexts will be crucial for confirming robustness. Furthermore, while the current system logs detailed corridor metrics, expanding software support for complex task design and longitudinal analysis would further increase its research utility.

In conclusion, our work demonstrates that automated, socially housed cognitive testing is not only feasible but achievable with affordable implementations. By reducing handling-induced stress, aligning testing with animals' natural activity patterns, and ensuring reliable individual identification, the Social RatPad advances the 3Rs principles (Replacement, Reduction, Refinement). It joins a growing landscape of automated home-cage systems that enable high-throughput, high-quality behavioral neuroscience while respecting animal welfare.

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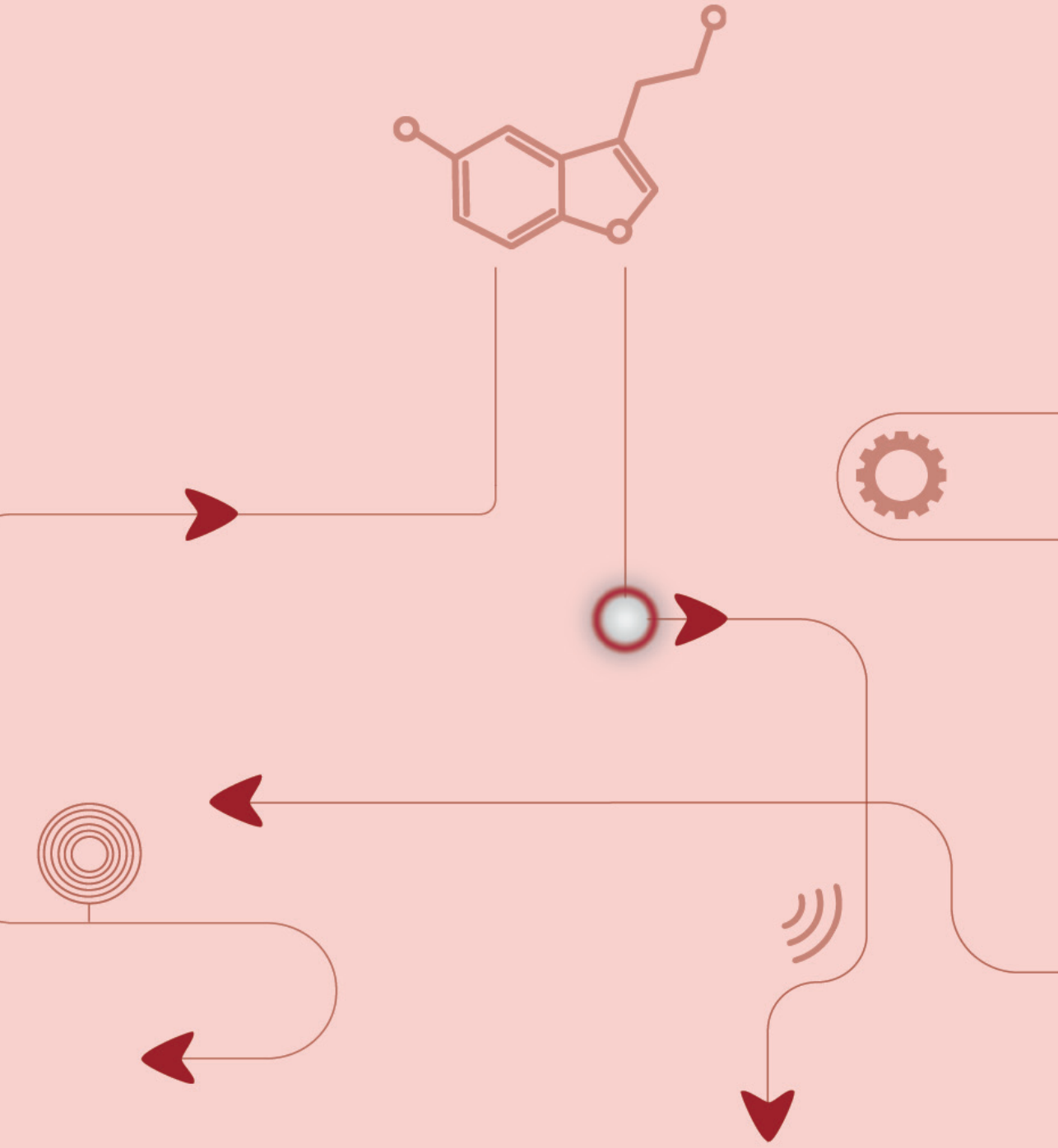

Chapter 4

Behavioral And Cognitive Development During Adolescence In Transgenic Rat Models Characterized By Low Serotonin Levels

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Abstract

Cognition is a core domain affected in many neuropsychiatric disorders, and impairments in cognitive processes often contribute strongly to disease burden. Serotonin plays a critical role in cognition, both by influencing neurodevelopment early in life and by modulating brain signaling throughout adulthood. Alterations in serotonergic signaling therefore have the potential to disrupt normal cognitive function across the lifespan. Despite its importance, there are still limited methods to directly monitor how serotonin impacts cognition in specific domains, such as attention, cognitive flexibility, and reward learning. To address this, we investigated how lifelong serotonin deficiency affects cognitive and behavioral development during adolescence using a new homecage system allowing cognitive assessment from adolescence. More specifically, adolescent tryptophan-hydroxylase-2 knockout (TPH2-KO) rats - lacking central serotonin - and wild-type controls were tested in the Automated Social RatPad, a home-cage touchscreen system assessing learning and motivation, and in LABORAS, which quantified spontaneous behavior. In the RatPad task, TPH2-KO rats showed delayed engagement, fewer completed trials, and higher variability, although those that participated reached discrimination accuracies similar to controls. LABORAS data revealed that KO rats were initially less active and more immobile, differences that diminished with familiarization. Social RFID tracking further showed lower exploratory behavior in most KO rats, while more active individuals learned faster. Together, these findings demonstrate that central serotonin depletion mainly impairs motivational and exploratory drive and highlights the value of automated systems for studying serotonin's developmental role in cognition and behavior.

Introduction

Cognition is a fundamental domain disrupted in many neuropsychiatric disorders, and deficits in cognitive processes substantially contribute to the overall disease burden. (Nordquist and Oreland 2010; Marini et al. 2011). Serotonin plays a crucial role in cognition, influencing both early neurodevelopment and adult brain signaling (Gaspar, Cases, and Maroteaux 2003; Mosienko, Bader, and Alenina 2020). Alterations in serotonergic function therefore have the potential to disrupt cognitive performance across the lifespan (Alonso et al. 2023; Hyun et al. 2023). Although serotonin's role in cognition is well recognized, experimental tools to directly assess its effects on specific domains - such as attention, memory, cognitive flexibility, and reward learning - remain limited. To address this, a wide range of preclinical models have been employed, including tryptophan depletion paradigms, optogenetic stimulation, and genetic manipulations and tryptophan hydroxylase (TPH2) knockout models (Blokland, Lieben, and Deutz 2002; Olivier et al. 2008).

Tryptophan hydroxylase catalyzes the rate-limiting step in serotonin synthesis, and manipulating tryptophan availability alters brain serotonin levels. Dietary tryptophan depletion or supplementation has therefore been widely used to assess serotonergic effects on cognition. In rats, depletion did not affect spatial discrimination learning or sustained attention (Blokland, Lieben, and Deutz 2002), while in humans it caused impairments in declarative and working memory (Park et al. 1994; Jans et al. 2010) and in executive functions such as verbal fluency (Smith et al. 1999). Healthy participants under low-dose depletion conditions showed reduced attention and memory without mood changes (Hayward et al. 2005). Furthermore, Alzheimer's patients displayed impaired global cognition and attention following acute depletion (Porter et al. 2000). Interestingly, rapid tryptophan depletion also improved decision-making performance in probabilistic learning tasks (Talbot et al. 2006). Conversely, tryptophan supplementation enhanced attention and memory (Jenkins et al. 2016) and improved emotion-recognition accuracy and social cognition, accompanied by stronger activation in the superior temporal sulcus, fusiform gyrus, and medial prefrontal cortex (Zamoscik et al. 2021). Nevertheless, these effects are influenced by amino-acid competition, dietary composition, and individual variability (Hulsken et al. 2013; Silber and Schmitt 2010), complicating the interpretation of the findings in terms of low and high central serotonin level.Genetic models further illuminate serotonin's developmental and functional roles. Serotonin production depends on two tryptophan

hydroxylase isoforms: TPH1 (peripheral) and TPH2 (neuronal). Knocking out TPH2 abolishes central serotonin while leaving peripheral levels intact (Walther et al. 2003; Alenina et al. 2009; Kaplan et al. 2016), providing a clean framework to investigate the cognitive consequences of serotonin absence. TPH2-deficient animals, despite relatively preserved serotonergic wiring, were found to exhibit altered behavioral reactivity and subtle changes in learning performance (Mosienko, Bader, and Alenina 2020). Furthermore, our lab found that juvenile TPH2 knockout rats display sex-dependent impairments in recognition memory and exploratory behavior in the novel object recognition (NOR) test. At postnatal day 21 (P21), male TPH2-/- rats spent less time exploring the objects compared to their wild-type and heterozygous littermates. This reduced exploration persisted through adolescence (P35). These rats also showed lower discrimination ratios, indicating deficits in distinguishing familiar from novel objects, whereas females were less affected. Altogether, these findings suggest that the absence of central serotonin during development disrupts early recognition memory and cognitive processing (Samina et al. Submitted).

Together, these diverse findings show that serotonin depletion influences attention, memory, and decision-making across multiple experimental systems (Booij et al., 2005; Riedel et al., 1999; Mendelsohn et al., 2009; Talbot et al., 2006). Yet none fully capture how lifelong, centrally restricted serotonin deficiency shapes cognition over time during development.

To address this gap, we employed the TPH2 knockout (KO) rat model. To capture behaviour and cognition during development we used two complementary platforms: i. the Social RatPad, which enables automated assessment of learning, attention, and flexibility in a social home-cage setting, and ii. The LABORAS system providing detailed, standardized behavioral profiling. This integrated approach allowed us to quantify the cognitive and behavioral consequences of lifelong serotonin deficiency in an ecologically valid manner.

The Social RatPad represents the innovative aspect of this study. Unlike traditional touchscreen or operant-box paradigms, the Social RatPad allows continuous, self-initiated testing under group-housed conditions. Through RFID-based identification, automated gating, and real-time logging of touchscreen interactions, the system maintains individual data streams without disrupting natural social organization and flow granting animals control over the environment and chance voluntary engagement in testing. This design

captures both cognitive and motivational dimensions in a low-stress setting, providing a more realistic measure of spontaneous learning and engagement compared to traditional touchscreen box paradigms. Importantly, because the animals have up to eight hours of daily access to the touchscreen, learning can progress more rapidly, allowing cognitive testing during the juvenile and adolescent phases - periods that together last only about three to four weeks. This represents a major advantage over standard operant box procedures, which typically require one-hour daily sessions across two to three months, extending well beyond the pre-adolescent window. The integration of these technologies enabled us to quantify how lifelong serotonin deficiency shapes cognition, motivation, and social behavior within a unified, fully automated framework.

Experimental Procedures

Subjects

A total of thirteen male Dark Agouti rats were used, comprising seven wild-type (TPH2+/+) and six tryptophan hydroxylase 2 knockout (TPH2-/-) animals, tested between postnatal days 21 and 35. We chose a total of thirteen rats based on power analyses and prior work demonstrating reliable visual discrimination deficits in TPH2-KO animals. All breeding was carried out in our Nijmegen facility: heterozygous parents, themselves derived from heterozygous TPH2-knockout matings, ensured a consistent genetic background. Rats lacking Tph2 (Tph2-/-) were established using a truncation mutation. To produce experimental cohorts, heterozygous animals (Tph2+/-) on a dark agouti background were bred with DA/OlaHsd wild-type rats. Offspring from these pairings included knockout (Tph2-/-), heterozygous (Tph2+/-), and wild-type (Tph2+/+) genotypes. The founder lines were generated at the Jacob Human and Molecular Genetics Center, Medical College of Wisconsin (Milwaukee, USA). At three weeks of age, each rat received a subcutaneous RFID transponder to enable individual tracking within the Automated Social RatPad system, where they remained for the duration of behavioral testing.

Throughout the experiments, rats were pair-housed under a controlled 12-hour light/dark cycle (lights on from 19:00 to 07:00), at a stable ambient temperature of 21 ± 1 °C and 50-60% relative humidity. Food and water were available ad libitum in the home-cage compartments of the RatPad, preserving natural foraging and social interaction patterns. All procedures, including transponder implantation, and continuous home-cage monitoring, were conducted in

accordance with the Dutch Experiments on Animals Act and were approved by the Centrale Commissie Dierproeven (CCD, The Hague).

Apparatus

Cognitive Testings

The animals were housed and tested using a homecage automated social system known as the Automated Social RatPad. This system comprised a central home cage, also referred to as a common area, which was a Type 4 (Eurostandard) cage measuring 530 x 330 mm at the bottom inside, with a height of 200 mm and a floor area of 1219 cm^2. The central cage was connected to Standalone RatPads via tubes (60 mm outer diameter x 54 mm inner diameter, Ferplast, Italy). The home cage was equipped with a wire lid containing a food hopper and water bottles, ensuring continuous access to food and water during housing. Between the central cage and the testing cages, One-Rat turnstile gates (ORT) were positioned to allow only one animal to pass through to the testing centers.

The animals were initially isolated in the ORT gate and gained access to the testing center upon identification by RFID microchips. RFID antennas with a diameter of 100 mm, made of coil material (priority1design, Australia), were inserted into self-made PLA enclosures and mounted over the tubes. The cage would open when the microchip was recognized.

The stand-alone RatPad was equipped with a touchscreen, which was covered by a mask to divide the screen into rectangular sections for the tasks. Additionally, the testing center featured a reward dispenser and a spot for collecting rewards. The rats were provided with sucrose pellets (45 mg, chocolate flavored, TestDiet, USA) as rewards for correct trials. Furthermore, the center was equipped with a punishment light that was activated during incorrect trials.

Behavioral Testings

For behavioral pattern recognition, each rat was placed for 2-hour sessions on days 1 and 7 into a LABORAS cage-its standard Makrolon type II home cage mounted directly atop a precision triangular sensor platform (approximately 690 × 690 × 976 mm, 13 mm thickness). The platform itself comprised a heavy Corian baseplate in which precision single-point load cells are embedded, and a very lightweight, stiff carbon-fiber measurement plate that transmits

even the subtlest movements to an integrated pre-amplifier and signal-conditioning unit. Gain and offset are then automatically calibrated by the accompanying software based on the animal's weight, and analog signals are filtered and digitized at high sampling rates for downstream analysis (Bulthuis et al., 1998).

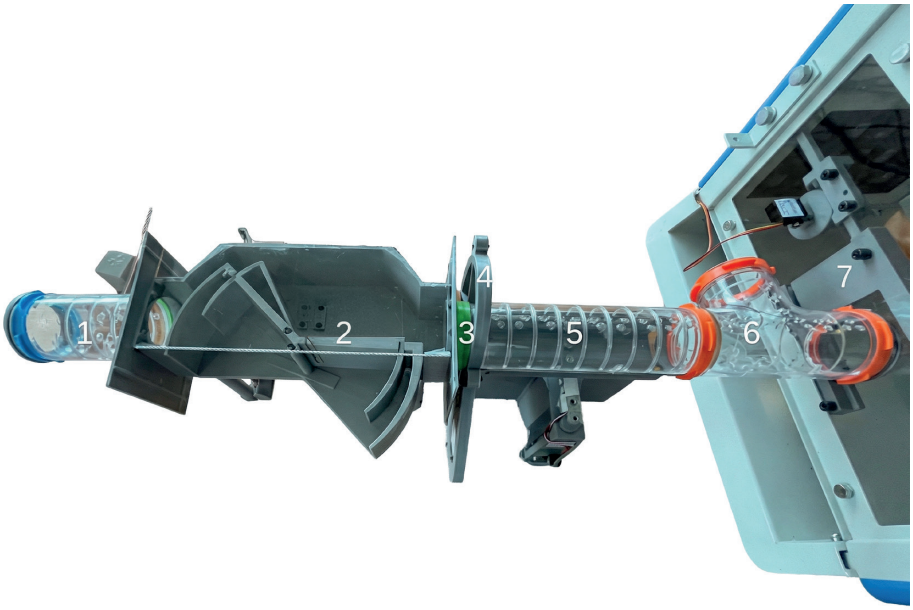


Figure 1. The final view of the socializing system implemented in between the central cage and the standalone RatPad: 1 - Binocular tube, 2 - ORT Gate, enriched with size-adjusting wings, 3 - Custom-made FPI to tube connector, 4 - RFID antenna, 5 - Weight measurement station, 6 - T-shaped tube for the U-turn, 7 - Secondary gate, granting or limiting access to the testing chamber.

The carbon measurement plate is placed on two orthogonally placed force transducers and a third fixed support point enabling precise XY position tracking and automated behavior recognition. The platforms are placed on spikes that isolate the system from external vibrations, all resting on three height-adjustable rubber feet that isolate the system from external vibrations. As the animal moves-whether climbing, grooming, rearing or simply shifting-those mechanical deflections produce unique frequency-amplitude signatures. LABORAS's proprietary pattern-recognition algorithms then classified behavior into eight validated domains (immobility, locomotion, rearing, climbing, grooming, eating, drinking, and "other" activity bursts), providing precise timestamps, bout counts, durations and inter-bout intervals at user-defined epochs.

In parallel, the same vibration signals were processed to extract continuous tracking metrics-X-Y position coordinates, instantaneous and average speeds, maximum speed, total distance traveled and circling parameters-completely independent of lighting conditions or video recording. This force-based approach ensured fully automated, non-invasive, observer-independent quantification of spontaneous and novelty-induced behaviors, ideal for high-throughput, longitudinal phenotyping in juvenile rats under standardized home-cage conditions.

Figure 2. LABORAS system for automated behavioral recording. Image shows the vibration-sensitive platform with home cage setup designed to detect and classify rodent behaviors automatically. Source: Metris B.V., Netherlands.

Experimental Design

RFID implantation and baseline assessment (LABORAS)

On Day 0, each rat underwent subcutaneous implantation of an ISO FDX-B 134.2 kHz RFID micro-transponder, followed by immediate body-weight recording (±1 g). On the following day (Day 1), animals were singly housed for a two-hour spontaneous-behavior session in the LABORAS system (Metris, NL). The same two-hour LABORAS protocol was repeated on Day 7 to probe longitudinal changes.

Housing in the Automated Social RatPad

Following each LABORAS session, animals were relocated to a dedicated Automated Social RatPad where they remained for the rest of the experiment. Rats could voluntarily shuttle through the tubes 24 h day, irrespective of whether an operant task was running.

RFID based identification & gating logic

1. Each ORT gate first enclosed the animal so that only one rat entered the identification zone at a time.
2. Dual antennas read the transponder; the controlling software compared the code with a preloaded allocation table (each rat permanently assigned to *one* of the two chambers).
3. If the ID matched the chamber's list, the secondary sliding door opened, granting entry.
4. Every event (antenna read, door state, gate activation, cage entry/exit, weight measurement on the integrated scale) was timestamped and written to the SocialData log.

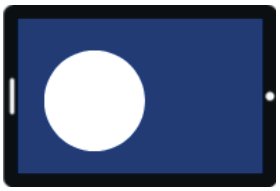
Daily operant task schedule

- Start time: 19:00 h daily (lightsoff at 19:00).
- Stop criterion: whichever occurred first - 40 correct trials or 8 h of elapsed time (i.e. automatic shutdown at 03:00 next day).
- Intertrial interval (ITI): fixed 4 s (blank screen).

Tasks & protocol parameters

In the present study, rats were trained on two touchscreen-based tasks: "Must Touch" and "Visual Discrimination." The Must Touch task functioned as a shaping stage in which animals learned to respond to a single visual stimulus on the screen. The second stage, the Visual Discrimination task, required animals to differentiate between two distinct stimuli and respond selectively to the one designated as correct (S+). Table 1 summarizes the main parameters of both tasks, including stimulus characteristics, response contingencies, reward delivery, and the criteria for advancement.

Table 1. Detailed operant touchscreen protocol parameters for "Must Touch" and "Visual Discrimination" tasks

Parameter	**"Must Touch - long 2023"**	**"Visual Discrimination - long 2023"**
Outlook		
Stimulus set	1 white circle (Ø 6 cm)	2 distinct geometric icons (counter-balanced) - composed of three vertical white bars vs. three horizontal white bars, each measuring approximately 4.5 × 4.5 cm and presented on a dark background.
Correct response	Single nose-poke on stimulus	Touch on the stimulus assigned as S+
Incorrect response	Touch on blank zone (no consequence)	Touch on S- → 5 s blackout
Reward	1 × 45 mg sucrose pellet	1 × 45 mg sucrose pellet
Advancement criterion	Individual: switch when a rat completed > 10 Must Touch sessions within any 3 consecutive days ; or > 5 days of inactivity	≥ 60 % accuracy across >10 trials during three consecutive sessions

Rats began on Must Touch and progression to Visual Discrimination was determined per animal: advancements made either once they had logged > 10 trials across 3 days, or if they failed to initiate any session for > 5 consecutive days. Intriguingly, this task change reengaged most previously inactive animals.

Each animal was given one training session per day, starting and ending at predefined times. A session was terminated either when the animal completed 40 correct trials or when the time window elapsed. If fewer than 40 correct trials were completed, the session was still considered valid provided that the rat performed more than 10 trials. Because the primary goal of the study was to assess Visual Discrimination, animals that remained inactive during Must

Touch sessions for more than five consecutive days were switched forward to Visual Discrimination. In several cases, this transition unexpectedly increased the animals' willingness to engage in the task, although the underlying reason remains unclear. Once animals entered Visual Discrimination, they were not switched back to Must Touch.

In the Must Touch task, a white circle stimulus was presented in randomized positions on the touchscreen to prevent side bias. In the Visual Discrimination task, two fixed stimulus locations were used (left and right), and the stimuli appeared in these positions in a randomized order across trials.

In both tasks, the animal's required action was to touch the visual stimulus on the touchscreen. A correct touch was immediately rewarded with a chocolate-flavored sucrose pellet delivered to the food tray. In the Must Touch task, incorrect touches were simply not rewarded and the trial ended without consequence. In contrast, in the Visual Discrimination task, an incorrect touch triggered the activation of a light stimulus as a mild punishment before the next trial could begin. The test phase was initiated once the Must Touch training criterion was reached - defined as completing more than 10 trials within three consecutive days or, alternatively, following five days of inactivity. For the Visual Discrimination task, learning was considered achieved once a rat reached at least 60% correct responses across more than 10 trials during three consecutive sessions (Table 1).

Experimental timeline

At the start of the experiment, rats underwent RFID implantation, weighing, and baseline behavioral monitoring in LABORAS before being transferred to the RatPad. During the following days, they received daily Must Touch training sessions in the evening. On day 7, animals were exposed to a second LABORAS session in the afternoon, after which they were returned to the RatPad. From day 8 onward, training continued with daily Visual Discrimination sessions. The full timeline of these procedures is shown in Table 2.

Table 2. Complete experimental timeline: RFID implantation, LABORAS baseline sessions, and daily automated social RatPad touchscreen testing

Day	Procedure
0	RFID implantation → weigh → 2 h LABORAS → transfer to RatPad
1-6	Daily Must Touch sessions (19:00 h)
7	Second 2 h LABORAS (afternoon, ~17:00) → return to RatPad
8- Until the End*	Daily Visual Discrimination sessions
*Advancement criterion	Must Touch: switch when a rat completed > 10 Must Touch sessions within any 3 consecutive days (no incorrect/blank-zone trials possible); or switch after > 5 days of inactivity Visual Discrimination: When the rats completed 60% of correct trials within any 3 consecutive days or did not show progress for >20 days.

Data acquisition & automatic logging

Data acquisition and automatic logging were performed across three integrated sources. RatPad operant files recorded the state transitions of every trial, including stimulus onset, response latency, reward delivery, inter-trial intervals, touch coordinates, and cumulative session statistics such as session duration. RFID Data logs provided per-animal measures of social interaction, including time spent in central versus testing cages, visit counts, and gate latencies. Finally, LABORAS data provided detailed ethograms over the test duration (2 hours), consisting of durations and counts for each measured behavior (locomotion, immobility, rearing) and distance travelled in user defined time bins of 120 minutes.

Statistical Analysis

All statistical analyses were conducted in Python (version 3.10) using the pandas, statsmodels, and SciPy libraries. Throughout, a two-tailed significance threshold of $\alpha = 0.05$ was applied.

Learning Stability

To determine which rats achieved stable discrimination learning, we required three consecutive touchscreen sessions with at least >60 % correct responses. The proportion of "learners" (those meeting this criterion) was compared between WT and KO groups using Fisher's exact test on a 2×2 contingency table of Learners vs. Non-learners.

Trial Engagement

Session-level engagement was quantified as the total number of trials completed per session. We fit a linear mixed-effects model with a random intercept for each rat to assess the effect of genotype:

$$TotalTrials_{ij} = \beta_0 + \beta_1 \cdot Group_i + u_i + \varepsilon_{ij}$$

$$Group_i \quad : 0 = WT,\ 1 = KO$$

$$u_i \quad : rat\text{-}specific\ random\ intercept$$

$$\varepsilon_{ij} \quad : residual\ error$$

The key hypothesis test examined whether beta1 differs significantly from zero (i.e. whether KO rats perform fewer trials than WT). As a secondary analysis, we also ran a simple ordinary least squares regression of each rat's mean trials per session against Group.

Age Dependence

We investigated whether trial engagement changed with postnatal day (PND). A pooled mixed-effects model regressed TotalTrials on PND, again including random intercepts for each rat. To explore genotype-specific trends, separate ordinary least squares regressions of TotalTrials on PND were also fitted for the WT and KO subsets.

Session Consistency

To assess the stability of responding across training sessions, we quantified within-rat variability using three descriptive metrics: (1) the mean number of trials per session, (2) the standard deviation of trials across sessions, and (3) the coefficient of variation (CV), defined as the standard deviation divided by the mean. These measures capture both the overall engagement level and the consistency of task participation over time.

Side Bias

We computed each rat's overall side bias as the sum of right-side touches divided by the total touches (right + left). The relationship between this MeanBias and the average discrimination accuracy (AvgCorrect) was tested using both Pearson's correlation coefficient r and Spearman's rank correlation ρ.

Additionally, we compared performance between rats classified as "right-biased" versus "left-biased" using the Mann-Whitney U test on AvgCorrect.

Chamber Travel Behavior

Social-log data were merged by RFID tag to assign each corridor crossing event to a specific rat. We then aggregated the daily number of corridor crossings (CorridorsPerDay). First, we created a binary indicator for whether each rat ever traveled to the chamber (TraveledAny) and compared WT vs. KO using Fisher's exact test. Second, among rats that did travel, we compared the distribution of CorridorsPerDay between genotypes with the Mann-Whitney U test.

Social-Cognitive Associations

For each rat we calculated the mean number of RFID-verified corridor crossings per day (Mean Corridors / Day) and the mean number of touchscreen trials per session (Mean Trials). Because only one animal achieved the original "three consecutive ≥ 60 %" accuracy criterion, learning speed was re-defined as the First-60 index (F60)-the session number on which a rat first reached ≥ 60 % correct with > 10 trials. Spearman's rank correlation coefficient (ρ) and Pearson's product-moment correlation coefficient (r) were used to quantify: (i) the association between Mean Corridors / Day and Mean Trials for all rats with non-zero trial counts, and (ii) the association between Mean Corridors / Day and F60 (First 60 - the session number on which a rat first reached at least 60 % correct responses) among rats that attained at least one qualifying ≥ 60 % session. Owing to the very small number of animals that achieved two consecutive ≥ 60 % sessions (N = 3), correlations involving that stricter stability measure were treated as descriptive only.

Corridor-Travel Dynamics

The daily use of the one-rat corridor was indexed as Corridors per Day, the total number of RFID-logged crossings for a given rat on a given calendar day. To test whether travel activity increases (or decreases) with age, and whether this trajectory differs by genotype, we fitted the following linear mixed-effects model:

$$\text{CorridorsPerDay}_{ij} = \beta_0 + \beta_1 \cdot \text{DayIndex}_{ij} + \beta_2 \cdot \text{Group}_i + \beta_3 \cdot (\text{Group}_i \times \text{DayIndex}_{ij}) + u_i + \varepsilon_{ij}$$

DayIndex_{ij} : calendar days since that rat's first RFID entry (Day 0)

u_i : rat-specific random intercept.

ε_{ij} : residual error term

The primary hypothesis test evaluates a significant interaction indicating that the slope of Corridors per Day differs between WT and KO. In addition, each rat's individual slope (from an ordinary least-squares regression of Corridors per Day on DayIndex) was extracted and the distribution of these slopes was compared between genotypes with a Mann-Whitney U test.

Behavioral Traits Analysis (LABORAS)

The following standard LABORAS domains (Distance travelled, Rearing events, Grooming time, Immobility, Locomotion) were recorded during two two-hour sessions: Session 1 on experimental Day 1 and Session 2 on Day 7.

Between-Group Comparisons (per session)

For each domain, WT and KO group means were compared separately on Session 1 and Session 2. Normal data were analysed with unpaired t-tests; non-normal data with Mann-Whitney U tests.

Within-Rat Change (Session 1 → Session 2)

For every rat, the change in each domain between sessions was assessed with paired t-tests (normal) or Wilcoxon signed-rank tests (non-normal).

Overall Session-Averaged Activity

An overall activity index was calculated for each rat and domain:
OverallMean = (Session 1 value + Session 2 value) / 2.
WT and KO OverallMeans were compared with the same test scheme as in 3.5.1.

Cross-Modal Associations

The LABORAS summary (Session 1, Session 2, and OverallMean values) was merged with the social-travel summary (Corridors per Day) and the cognitive summary (mean number of touchscreen trials per session; sessions-to-criterion) to yield a single master table.

Results

Learning Stability

In the "Must touch" protocol, overall performance revealed clear group differences. The wild-type (WT) group exhibited limited but measurable success: three of seven WT rats achieved at least one qualifying session (more than 10 trials), with one animal (473268) demonstrating two qualifying

sessions in a row, indicating minimal evidence of consistent task acquisition. In contrast, the knockout (KO) group showed no evidence of learning under these conditions; none of the KO rats recorded any valid sessions exceeding 1 trial.

We then screened 13 animals (Figure 3 and Figure 4) -seven wild-type (WT) and six TPH2 knockout (KO)-using a stringent validity rule: only visual-discrimination sessions with more than ten trials were retained. For each rat we asked how many consecutive valid sessions exceeded the 60 % accuracy threshold.

Only one WT rat (Figure 3; ID 474477) completed three consecutive eligible sessions ≥ 60 % correct, thus satisfying the full stability criterion. No KO rat achieved such a three-day run. Two WT rats maintained exactly two consecutive ≥ 60 % sessions, whereas none of the KO rats managed even a two-day streak. An additional two WT rats and four KO rats produced single "peak" sessions but failed to maintain performance into the next eligible day (figure 4).

Two KO animals (Figure 3; IDs 476600 and 477548) and two WT animals (Figure 4; 473207 and 476590) never logged any visual-discrimination session with more than ten trials. These subjects are classified as having zero qualifying sessions-either because they rarely engaged or because they disengaged before delivering ten trials. KO rats therefore concentrate at the lower end of the stability spectrum: 67 % reached no more than one qualifying session, and a further 33 % never reached even a single valid high-performance session.

Fisher's exact test (Figure 5) on the simple Learner (≥ 3) vs non-learner classification yields $p \approx 1.00$. With only one stable learner in the sample, statistical power is far too low to detect modest genotype effects. Relaxing the trial-count criterion (e.g., to ≥ 10 trials or removing the filter) slightly altered the distribution of short streaks but does not change the main finding: stable, three-day high-accuracy performance is infrequent in both genotypes and numerically - but not significantly-favours WT.

Under conditions that demand both robust engagement (> 10 trials) and accuracy (≥ 60 %) across multiple days, TPH2 KO rats show a clear motivational or consistency deficit relative to WT: they rarely reach together more than one qualifying session and never achieve full stability. However, because overall learner counts are extremely low, the group difference does not reach statistical significance.

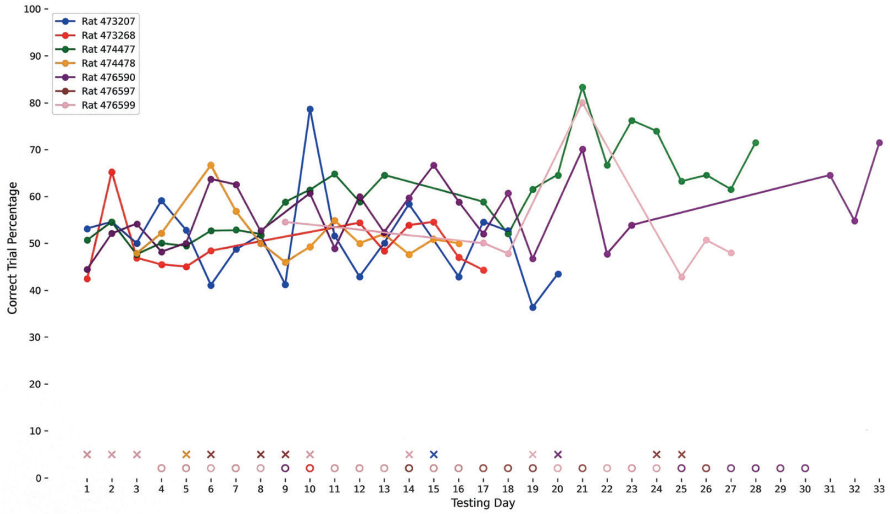


Figure 3. Session-by-session percentage of correct trials for individual rats across testing days. Colored lines represent individual rats (blue = 473207; red = 473268; green = 474477; orange = 474478; purple = 476590; brown = 476597; pink = 476599). Circles on the x-axis indicate sessions with 0 trials; crosses indicate sessions with 1–9 trials. The dashed horizontal line marks the 60% correct learning criterion.

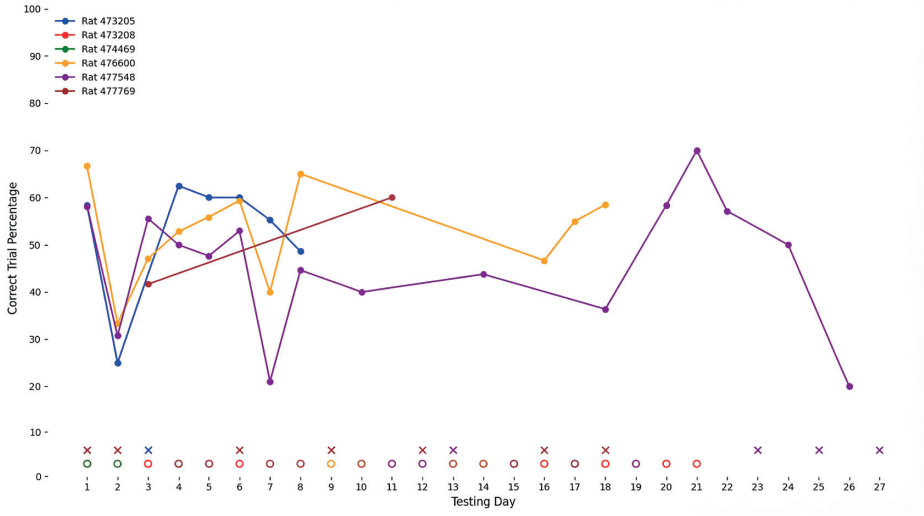


Figure 4. Session-by-session percentage of correct trials for individual KO rats across testing days. Colored lines represent each KO rat (blue = 473205; red = 473208; green = 474469; orange = 476800; purple = 477548; brown = 477769). Circles on the x-axis indicate sessions with 0 trials; crosses indicate sessions with 1–9 trials.

Trial Engagement and Age Dependency

In the visual discrimination phase of the touchscreen task, we first analyzed the session in which each animal produced any valid trials (TotalTrials > 0). There are differences in the timing of task engagement between WT and KO groups. In the wild-type (WT) group, all animals began responding within the first four sessions of testing: specifically, first engagement occurred at session 1 for three rats (476590, 476597, 476599), session 2 for one rat (474477), session 3 for two rats (473268, 474478), and session 4 for one rat (473207). The corresponding postnatal days (PNDs) at first engagement in WT animals ranged from 25 to 37, indicating relatively early developmental stages. In contrast, the knockout (KO) group exhibited much greater variability and generally delayed engagement. While some KO rats showed early responding-one at session 1 (477769) and another at session 3 (477548)-others required substantially more exposure before initiating any trials: 476600 at session 17 (PND 41), 473205 at session 21 (PND 54), and 473208 only at session 28 (PND 61). One KO rat (474469) failed to engage altogether, producing no sessions with any trials.

Mean session engagement differed markedly by genotype (Figure 6). Using all valid visual-discrimination sessions (≥ 10 trials), a linear mixed-effects model with a random intercept for each rat revealed a strong genotype effect. WT animals performed on average 19.4 ± 3.8 trials per session (95 % CI: 12.0-26.7, $z = 5.14$, $p < 0.001$). KO animals completed on average 15.2 fewer trials per session than WT (estimate = −15.2 ± 5.8 trials, 95 % CI: −26.7 to −3.8, $z = -2.60$, $p = 0.009$), after accounting for repeated measures within animals. A confirmatory ordinary least-squares regression on each rat's mean number of trials per session produced an almost identical effect to that observed in the mixed-effects model ($\beta = -15.17$ 5.82 trials, $p = 0.026$; adjusted $R^2 = 0.35$), underscoring the engagement deficit in the TPH2 knockout group.

WT animals show a modest but statistically significant increase in the number of trials (Figure 7) they performed across development (roughly one additional trial every three days). KO animals, in contrast, do not display a reliable age-related change-their slope is shallow and non-significant. It is important to note again that KO animals start doing any trials mostly in adolescence, while WT animals get engaged almost immediately. Therefore, as shown in Figure 7, WT groups total trials dependency on postnatal days is more obvious. The number of trials performed per visit was numerically higher in WT animals (mean = 2.57) compared to KO animals (mean = 1.07), but this difference did

not reach statistical significance (p = 0.131), likely due to high inter-individual variability and the limited sample size.

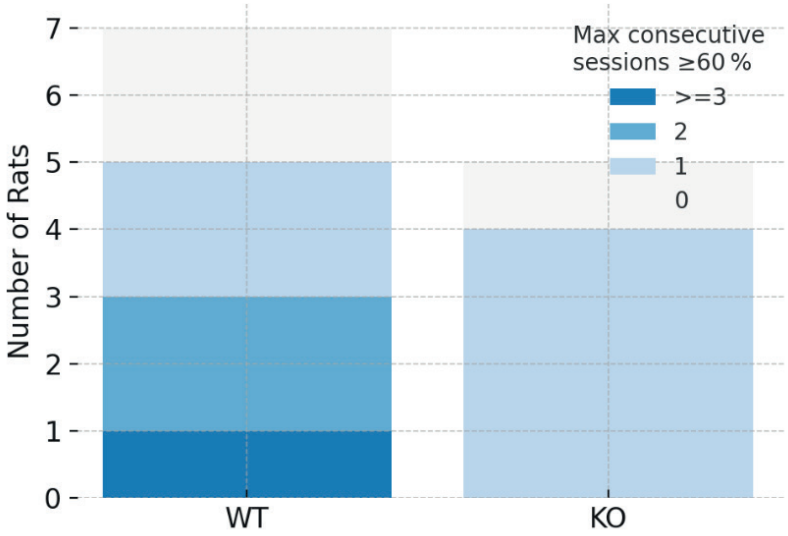


Figure 5: Proportion of learners in WT and KO groups. Bar chart shows the percentage of rats reaching a certain number of consecutive days / sessions of the 60% correct learning criterion in the visual discrimination task, comparing wild-type (WT) and knockout (KO) animals.

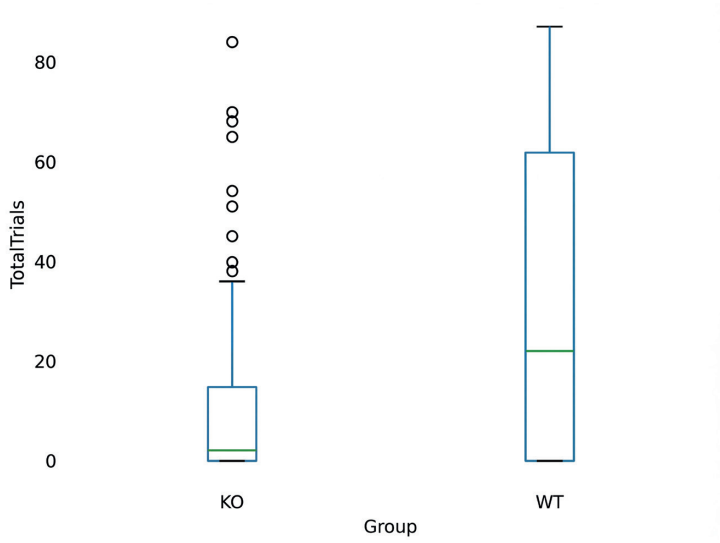


Figure 6. Distribution of total trials completed by genotype. Boxplots compare the number of total trials performed by KO and WT rats in the visual discrimination task, illustrating group differences in overall task engagement and performance variability.

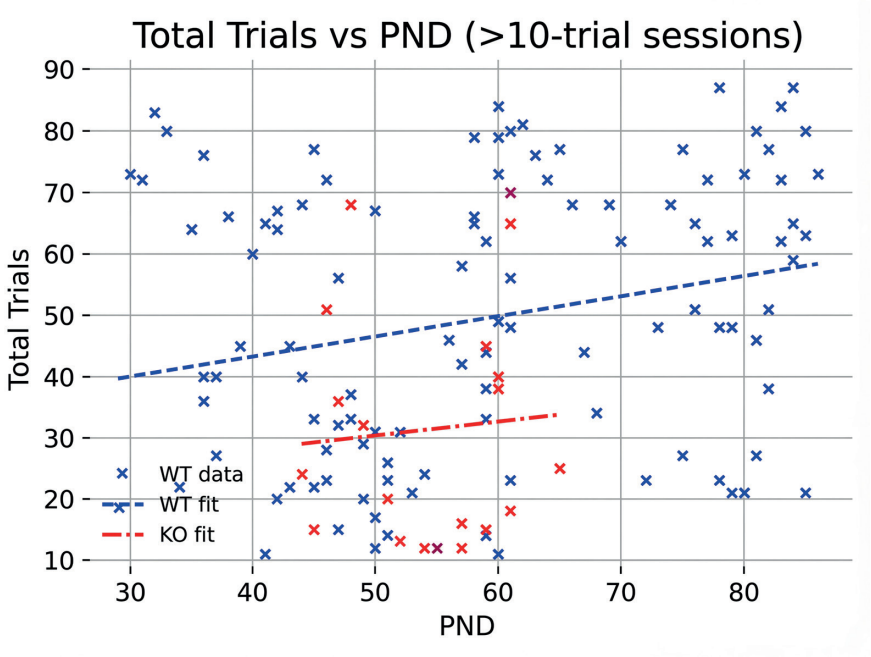


Figure 7. Relationship between total trials and postnatal day (PND) with separate WT and KO regression fits. Scatterplot shows individual data points for WT (blue) and KO (red) rats, with group-specific trend lines highlighting differences in how age (PND) relates to total trials completed in the TUNL task.

Session-to-Session Consistency

In the visual discrimination phase, session-to-session variability in trial engagement was quantified for each rat using the mean number of trials per session, standard deviation (SD), and coefficient of variation (CV). Wild-type (WT) animals generally demonstrated substantially higher average trial counts per session compared to TPH2 knockout (KO) rats. WT means ranged broadly from approximately 16.96 to 36.30 trials per session, while TPH2 KO rats often averaged fewer than 11 trials, with some individuals engaging minimally (e.g., 474469 with 0 trials). Variability within animals, indexed by SD, tended to increase with overall engagement level, but the relative consistency assessed by CV highlighted group differences: WT animals typically exhibited CV values between 0.86 and 1.73, indicating relatively stable responding across sessions, whereas TPH2 KO animals displayed more variable CVs, some exceeding 2.0 or even 5.29 in low-engagement cases. These findings suggest that WT rats not only completed more trials on average but also showed more consistent session-level engagement, while KO animals demonstrated reduced and more erratic participation in touchscreen tasks.

Touchscreen Side Bias

Mean side-bias (TouchRight ÷ TotalTouches) varied between 0.39 and 0.75 across rats. No meaningful association emerged between MeanBias and average accuracy ($r = 0.07$, $p = 0.84$), nor did dominant-side groups differ in performance (Figure 9).

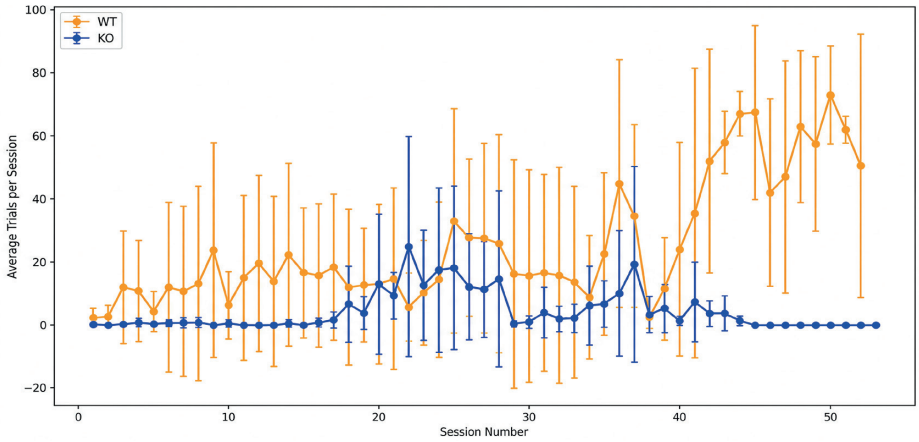


Figure 8. Daily average number of trials per session for WT and KO rats. Lines represent group means across sessions with error bars indicating standard deviation, illustrating differences in mean trials, variability (stdTrials), and coefficient of variation (CV) between genotypes over time.

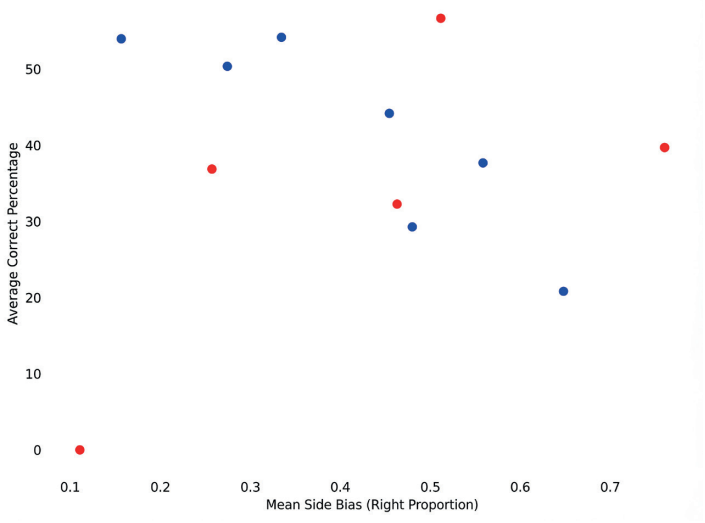


Figure 9. Relationship between mean side bias and average correct performance. Scatterplot shows individual rats' mean side bias (proportion of right choices) versus average percentage of correct trials, with points colored by genotype (WT in blue, KO in red). Reported correlation coefficient and p-value indicate no significant association.

Chamber Travel Behavior

All animals in both genotypes exhibited at least one corridor crossing event, precluding a Fisher's exact test for differences in TraveledAny. After mapping social-log RFID entries to Animal IDs and aggregating daily corridor crossings (Table 4), we found that although mean total corridor crossings did not differ by genotype, KO rats showed far greater inter-individual variability (SD = 437.6; CV ≈ 1.15) than WT rats (SD = 225.2; CV ≈ 0.54). This high variance in the KO group is driven by (for example) one animal with 996 events versus another with only 12. Median and IQR remain comparable, underscoring that a subset of KO rats travel as much as WT, while others barely visit.

Social-Cognitive Relationships

Across the ten rats that performed regular touchscreen trials, Mean Corridors / Day showed no relation to overall touchscreen engagement (Spearman $\rho = 0.29$, $p = 0.39$; Pearson $r = 0.20$, $p = 0.55$). This analysis specifically compared Mean Corridors per Day (daily social travel activity) with Mean Trials per Session (touchscreen engagement), and no significant association was found. By contrast, when learning speed was indexed as the first session on which a rat attained ≥ 60 % accuracy (F60, N = 7), a clear correlation emerged (Spearman $\rho = 0.71$, $p = 0.047$): animals that travelled through the corridor system more frequently reached criterion accuracy earlier in training. Here, the correlation was between Mean Corridors per Day and learning speed, defined as the first session in which a rat reached ≥ 60% accuracy. Too few animals met the two-consecutive ≥ 60 % requirement (N = 3) to draw any reliable inference for that stricter measure. Thus, while the amount of daily social travel did not predict the sheer volume of touchscreen responding, it was associated with faster onset of accurate performance.

Corridor-Travel Dynamics

Across the 13 rats monitored, corridor use showed a clear and systematic temporal pattern (Figure 10). Animals began the study with a relatively high frequency of crossings-median ≈ 11-12 events on Day 0-but travel declined steadily thereafter, falling to single-digit counts by the second experimental week and often to near-zero levels in the final days. Linear mixed-effects modelling confirmed a significant main effect of testing day ($\beta \approx -0.13$ crossings $\cdot$ day^{-1}, $p < 0.001$), while the Group × Day interaction was non-significant ($p = 0.88$), indicating that wild-type (WT) and TPH2-knock-out (KO) animals shared essentially parallel downward trajectories. Individual ordinary least-squares slopes corroborated this group similarity: WT rats

Table 4. Comprehensive per-rat summary of social chamber travel metrics and touchscreen trial engagement. Includes each rat's genotype, total and per-day corridor crossings, overall and per-session touchscreen trial counts, variability measures, and number of sessions.

RatID	473205	473207	473208	473268	474469	474477	474478	476590	476597	476599	476600	477548	477769
Genotype	KO	WT	KO	WT	KO	WT	WT	WT	WT	WT	KO	KO	KO
CorridorEvents	58	168	12	250	97	290	202	770	451	565	872	996	251
DaysTested	29	29	29	29	52	52	52	39	39	39	39	44	44
CorridorsPerDay	2	5.8	0.4	8.6	1.9	5.6	3.9	19.7	11.6	14.5	22.4	22.6	5.7
TotalTrialsOverall	209	686	18	602	0	1485	824	1343	48	238	392	476	99
MeanTrials	7.5	24.5	0.6	21.5	0	28.6	15.9	36.3	1.3	6.4	10.6	10.8	2.3

(n = 7) showed a median slope of -0.37 crossings · day^{-1} (IQR -0.52 to -0.21), and KO rats (n = 6) a median of -0.26 crossings · day^{-1} (IQR -0.39 to -0.09); the distributions did not differ (Mann-Whitney U = 18, p = 0.49). Although KO animals displayed wider between-subject variability in absolute totals (e.g., 14 vs. 996 crossings), their day-by-day decay rate was indistinguishable from WT. Taken together, these results indicate that corridor travel is most frequent immediately after the animals gain access to the apparatus and then wanes with continued exposure, and that this habituation-like decline occurs at the same pace in both genotypes.

Although mean corridor counts did not differ between genotypes, this was driven by two hyper-active KO animals (476600, 996 events; 477548, 872 events). When central-tendency is assessed with the median, WT rats travelled more than KO rats (median 290 vs 174 crossings), and WT activity was far more uniform (IQR 202-565 vs 58-251). Thus, the knockout cohort is best described as heterogeneous, containing a minority of very active travellers but a majority of low-activity animals.

Behavioral Traits Analysis (LABORAS)

The standard LABORAS behavioral domains (Distance travelled, Rearing events, Grooming time, Immobility, Locomotion) were assessed during two two-hour sessions (Session 1 on experimental Day 1 and Session 2 on Day 7).

Between-Group Comparisons (per session)

For each session, WT and KO group means were compared using unpaired t-tests. In Session 1, when rats were postnatal day 21-35, significant genotype differences were observed in several LABORAS domains. Specifically, KO rats showed reduced Distance travelled, $t(14) = 2.10$, $p = 0.0369$, fewer Rearing events, $t(14) = 2.43$, $p = 0.0291$, and greater Immobility, $t(14) = -3.53$, $p = 0.0033$, compared to WT rats. Grooming, $t(14) = 2.08$, $p = 0.0560$, and Locomotion, $t(14) = 1.70$, $p = 0.1112$, did not differ significantly between groups. In Session 2, however, genotype effects were not evident, as none of the domains reached significance. The t-test outcomes were as follows: Distance, $t(14) = 1.09$, $p = 0.293$; Rearing, $t(14) = 1.64$, $p = 0.1233$; Grooming, $t(14) = 1.49$, $p = 0.1575$; and Immobility, $t(14) = -2.05$, $p = 0.0601$. Locomotion differences were assessed with a Mann-Whitney U test, which also did not yield significance ($U = 47$, $p = 0.0727$).

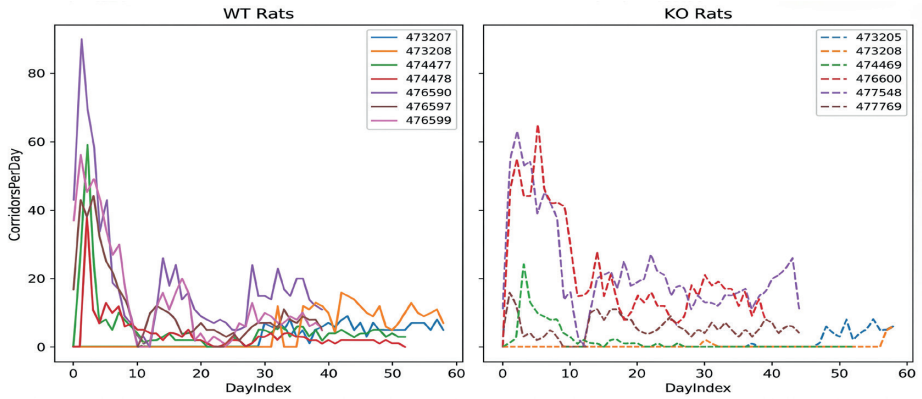


Figure 10. Individual daily corridor-crossing trajectories across the full experimental period: Wild-type (WT) rats on the left panel and knockout (KO) rats on the right panel, with Day 0 marking each animal's first RFID-logged entry.

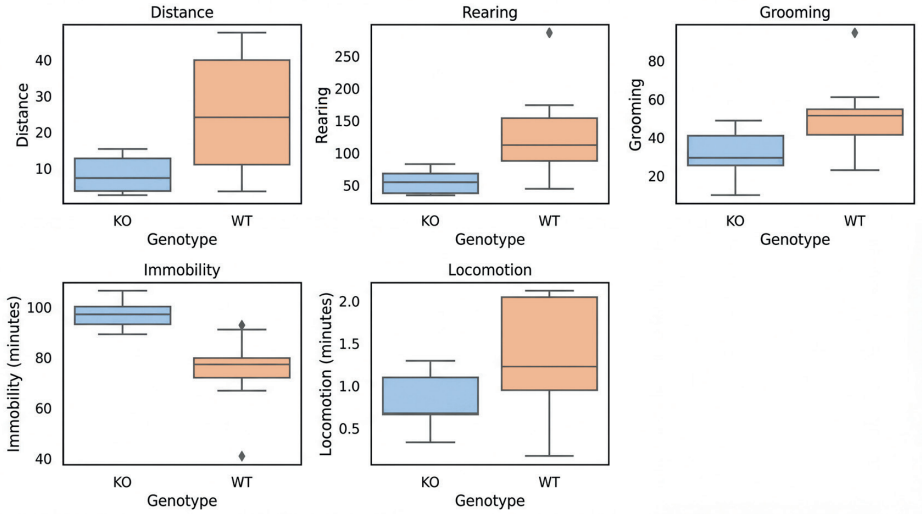


Figure 11. Distribution of LABORAS-measured behavioral domains (distance travelled in meters, rearing and grooming event counts, immobility and locomotion times in minutes) in wild-type (WT) and knockout (KO) genotypes recorded during the first 2-hour testing session on experimental day 1. Boxplots represent group distributions with median and interquartile range, highlighting genotype differences at baseline exposure. Dots represent individual rats whose scores fall outside the whisker range of the boxplot.

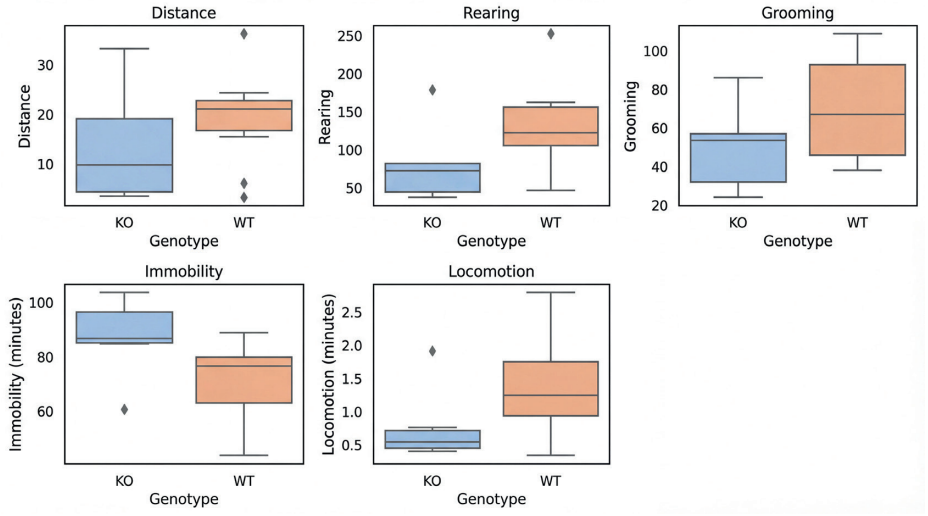


Figure 12. Distribution of LABORAS-measured behavioral domains (distance travelled in meters, rearing and grooming event counts, immobility and locomotion times in minutes) in WT and KO genotypes during the second 2-hour testing session on experimental day 7. Boxplots display group-level behavioral measures after one week of familiarization, illustrating changes in genotype differences over time. Dots represent individual rats whose scores fall outside the whisker range of the boxplot.

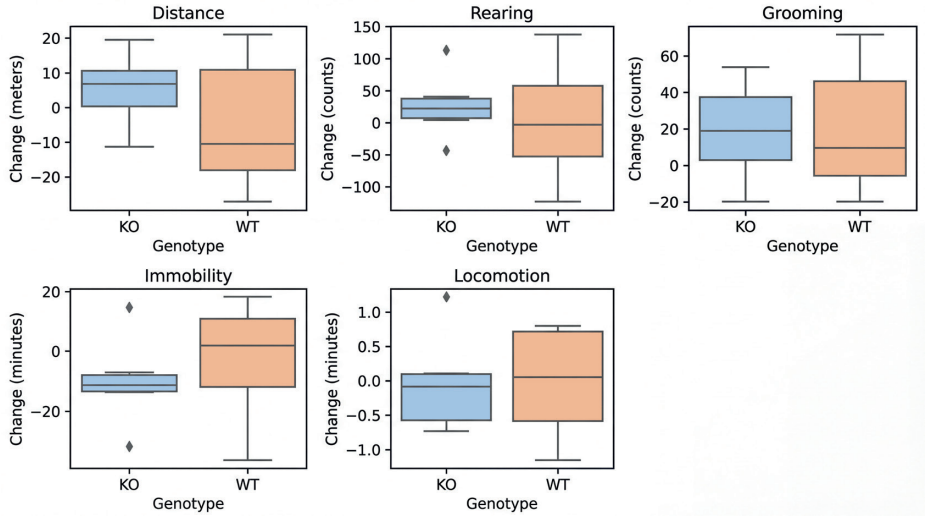


Figure 13. Within-subject change in LABORAS behavioral domains for WT and KO genotypes, representing individual animal differences from the first 2-hour session on day 1 to the second 2-hour session on day 7. Distance is reported in meters; rearing and grooming are counted events; immobility and locomotion are expressed in minutes. Boxplots display the distribution of session-to-session change scores across genotypes, highlighting experience-dependent adaptations.

Within-Rat Change (Session 1 → Session 2)

To complement between-group analyses, we also examined within-subject change, which captures how each rat's behavior evolved from the first to the second LABORAS session. Within-subject change across sessions was assessed (Figure 13) using paired t-tests. A significant within-subject change was observed in Grooming, $t(15) = 2.58$, $p = 0.0209$. No significant changes were detected for Distance, $t(15) = -0.37$, $p = 0.7136$; Rearing, $t(15) = 0.65$, $p = 0.5244$; Immobility, $t(15) = -1.33$, $p = 0.2032$; or Locomotion, $t(15) = -0.06$, $p = 0.9510$.

Cross-Modal Associations

We examined associations between automated home-cage behavioral measures (LABORAS Overall Session-Averaged Activity), social corridor travel (CorridorsPerDay), and touchscreen task engagement (MeanTrials) using Spearman's rank correlations. No statistically significant associations emerged between LABORAS domains and either CorridorsPerDay or MeanTrials (all $p > 0.05$). Although some domains showed positive trends with MeanTrials (e.g., Rearing ρ $rs = 0.410$, $p = 0.1858$; Locomotion $rs = 0.403$, $p = 0.1942$), these did not reach significance, suggesting that overall locomotor and exploratory activity were not tightly coupled with touchscreen task engagement or social travel frequency in this sample.

To assess whether cross-modal behavioral relationships differed between genotypes, Spearman's rank correlations were computed separately for WT and KO rats. No statistically significant associations were observed in either group (all $p > 0.05$). WT animals showed a non-significant negative trend for a correlation between Rearing and CorridorsPerDay ($rs = -0.714$, $p = 0.1108$) and modest positive trends for a correlation between Grooming and MeanTrials ($rs = 0.486$, $p = 0.3287$). KO animals generally exhibited weaker and more uniform non-significant correlations across domains.

General Discussion

This study aimed to examine the effects of congenital serotonin deficiency-via TPH2 knockout-on behavioral and cognitive development during adolescence using a fully automated, low-stress, homecage system. We found that TPH2 knockout (KO) rats were significantly less motivated to engage in cognitive tasks, performed fewer trials per session, and exhibited more variability across

sessions compared to wild-type (WT) controls. Only one WT animal reached a stable learning threshold (≥60% accuracy across three consecutive sessions).

Socially, KO animals showed a notable reluctance to explore novel chambers, with only a minority visiting the touchscreen test cage at all. Despite these deficits in motivation and engagement, KO rats who participated in the tasks were capable of achieving learning milestones similar to their WT counterparts. No significant associations were observed between social travel behavior and cognitive performance.

Interpretation and Integration with Existing Literature

Motivation

Our findings provide strong support for the notion that serotonin plays a critical role in motivational drive and behavioral initiation. TPH2 KO rats demonstrated reduced exploratory behavior and spontaneous task engagement, consistent with prior studies in which serotonin-depleted rodents exhibited a diminished inclination to respond to environmental novelty or initiate effortful actions. Similarly, in our study, the fact that most KO animals failed to enter the testing chamber during the juvenile and early adolescence stages at all underscores a motivational impairment that appears especially pronounced under voluntary, low-pressure conditions.

It is also important to consider potential confounding factors that could have influenced motivational performance. First, the rats were maintained on ad libitum feeding. Given that TPH2 KO rats display reduced body weight at young ages, differences in metabolism and satiety signals may have reduced their drive to engage with the task, independent of serotonin-related motivational deficits. Second, the duration of daily testing sessions may also have played a role. Extended sessions could reduce sustained engagement, while shorter sessions might limit the opportunity for animals with lower baseline motivation to initiate task-directed behavior. Supporting research is needed to further explore and confirm the impact of these factors on motivational performance in serotonin-deficient animals.

These results align with findings from severe serotonin depletion studies using para-chlorophenylalanine (PCPA), which showed that while rodents may retain the ability to exert effort for rewards under compulsion, they often fail to initiate behavior when given a choice (Izquierdo et al., 2012). This suggests

that serotonin deficiency may blunt proactive behavior without disrupting consummatory reward mechanisms, pointing to a dissociation between reward valuation and behavioral activation.

However, starting from postnatal days 65-70, the rats began to exhibit normalized behavior, including a noticeable increase in motivation and reduced anxiety (versus increased anxiety in the juvenile stage). This aligns with the early physiological shift reported by Kaplan et al. (2016), which also occurs during this developmental stage. At this point, body temperature, respiratory rate, and weight gain in TPH2 knockout rats surprisingly and rapidly recover. Interestingly, the same pattern is observed in their motivation and willingness to explore or cross chambers. While several factors may contribute to this recovery, the most likely explanation is that another neurotransmitter system partially compensates for the absence of serotonin.

Learning

In contrast to motivational impairments, our data suggest that basic learning mechanisms may be relatively intact in TPH2 KO rats-provided that the animals engage with the task since those KO rats that did perform a sufficient number of trials reached discrimination accuracies comparable to WT animals (see Results 4.1 and 4.2). However, they were unable to maintain this performance across consecutive sessions, indicating deficits in consistency rather than in the learning process itself. While the overall proportion of learners was low in both groups, those KO animals that did perform consistently across sessions were not able to reach the learning criterion (not even two consecutive sessions), contradicting some of studies that show normal learning capacity in serotonin-deficient *adult* rodents (Alonso et al. 2023).

Other TPH2 KO studies have reported no major deficits in standard cognitive tests (Alonso et al., 2023), and even acute tryptophan depletion often fails to impair learning in rats unless depletion levels are extreme (Blokland, Lieben, and Deutz 2002). However, severe serotonin loss can impair cue-reward learning (Izquierdo et al. 2012), particularly when reward salience or predictive learning is involved, suggesting that 5-HT might be more critical in attentional or incentive processes than in memory encoding per se.

Social And Explorative Behavior

Serotonin's influence on social and explorative behavior is well documented, and our findings further corroborate this role. Serotonin not only modulates

diverse forms of social behaviour, including attachment, play, cooperation, and aggression, but is itself strongly shaped by social experiences. This reciprocal interaction means that serotonin can amplify both positive and negative social influences (for-better-and-for-worse'), creating a bidirectional relationship between 5-HT and the social environment (Kiser, Steemers, Branchi, & Homberg, 2012). KO rats were significantly less likely to explore new environments, which may indicate social clinging or avoidance of isolation. This behavior is consistent with prior studies where TPH2 KO rodents exhibited excessive aggression, reduced grooming, and impaired social communication (Kane et al. 2012; Peeters et al. 2019; Meng et al. 2022).

For example, TPH2-deficient mice and rats often show elevated impulsive aggression and autism-like behaviors, including reduced social sniffing, lower ultrasonic vocalizations, and aberrant social preference (N. Alenina et al. 2009; Kane et al. 2012). Such phenotypes appear early and persist in adulthood, implicating serotonin as a key modulator of affiliative behavior and social cognition. Our use of a semi-naturalistic testing environment may have amplified these differences, as the KOs could choose to remain in social proximity rather than explore, potentially reflecting heightened separation anxiety or novelty aversion.

Cognitive Flexibility

Although our study could not fully assess cognitive flexibility, prior literature suggests that TPH2 KO rodents-and serotonin-deficient animals more generally-struggle significantly with reversal learning and behavioral adaptation (Clarke et al. 2005). Tph2 mutant mice have shown marked perseverative behavior in rule-shifting tasks, and similar impairments have been noted in tryptophan-depleted primates and humans (Rogers et al. 1999).

Recent findings also suggest individual variability in flexibility among TPH2 KOs. For example, (Alonso et al. 2023) reported that only the "poor decision-making" subset of TPH2 KO rats exhibited deficits in flexibility and social memory, indicating that additional behavioral traits may moderate the effects of serotonin deficiency on executive function.

Methodological Contributions and Limitations

A novel aspect of this study lies in the implementation of a fully automated, multimodal testing environment that integrates social, behavioral, and cognitive data within the same animals. This ecologically valid approach

enables the detection of subtle phenotypes that traditional isolated operant testing might overlook. In particular, our setup was sensitive to motivational and exploratory deficits that emerge only when animals are allowed to self-initiate task engagement.

However, some limitations should be acknowledged. The relatively low number of learners-partly due to the naturalistic setup-limits our ability to analyze complex task performance such as cognitive flexibility. Additionally, female rats were not included, and the study's focus on juvenile period and adolescence, while important, leaves open questions about how these phenotypes evolve into adulthood.

Conclusion

In summary, this study suggests that lifelong serotonin deficiency-via TPH2 knockout-significantly impairs motivational drive, task consistency, and social exploration during adolescence. While basic learning mechanisms appear relatively preserved, KO animals show strong reluctance to engage with cognitively demanding environments, particularly when participation is voluntary. These findings align closely with previous research showing serotonin's role in facilitating goal-directed behavior, social communication, and cognitive flexibility. The integration of automated, low-stress assessment methods allowed for the emergence of nuanced behavioral differences, emphasizing the value of ecological testing paradigms in preclinical models of psychiatric vulnerability.

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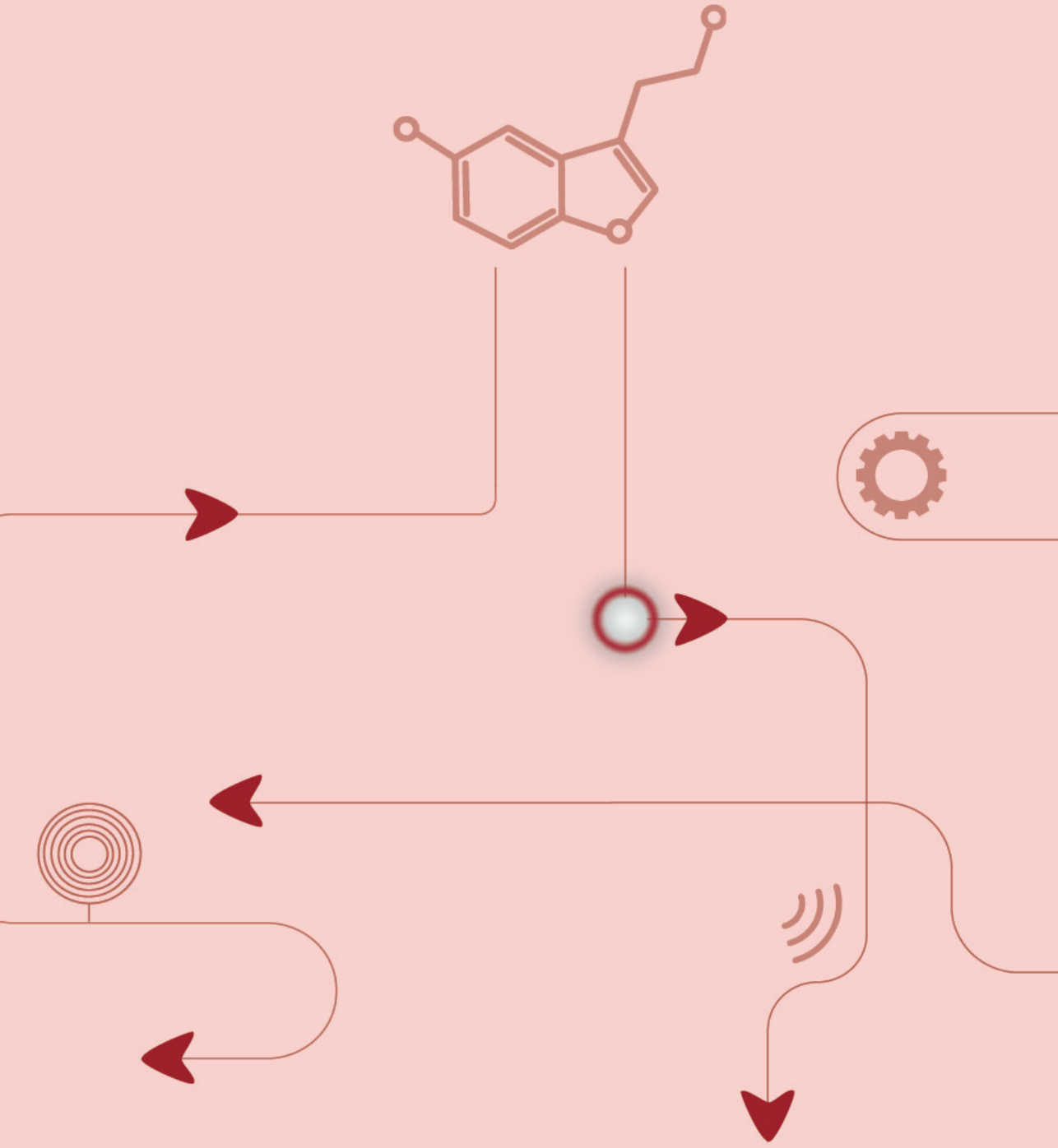

Chapter 5

General Discussion

General Discussion

This thesis set out to advance preclinical cognitive research by (1) developing and validating an automated, low stress, homecage touchscreen system (the Social RatPad) and (2) applying it to probe the role of serotonin in adolescent cognitive and behavioral development. Chapters 2-4 each contributed a distinct piece of this overarching aim: Chapter 2 demonstrated that capacitive touchscreens in a social homecage enables learning; Chapter 3 showed that full automation - via RFID identification, self-adjusting turnstiles, and around-the-clock testing - accurately identifies and sorts the animals as well as may remarkably reduce human involvement during the data acquisition; and Chapter 4 revealed that congenital serotonin depletion (TPH2 knockout) markedly impairs task engagement and learning stability during adolescence. In this General Discussion, I integrate these findings, reflect on strengths and limitations, consider their implications for cognitive-neuroscience research, and propose future directions.

Integration of Findings

Homecage Touchscreen Validity (Chapters 2 & 3)

Together, Chapters 2 and 3 establish that the Social RatPad provides a reliable platform for rodent cognitive testing. In Chapter 2, direct comparison between capacitive and traditional infrared touchscreens showed that capacitive systems yield acquisition of visual discrimination and reversal tasks comparable to market-available counterparts, presumably introducing more precise touch detection and fewer spurious responses (conscious choices). Chapter 3 then built on this by automating all aspects of social housing, animal identification, and access control. The fully automated RFID-gated turnstiles and self-paced testing sessions meant that animals could engage during their natural peak activity periods (primarily during the dark phase), preserving the integrity of social dynamics and eliminating confounds introduced by handling or relocation. Crucially, these two chapters demonstrate that it is technically feasible - and behaviorally valid - to merge complex touchscreen tasks with socially enriched, stress-minimized environments.

Serotonin's Role in Adolescent Cognition (Chapter 4)

Chapter 4 leveraged the validated Social RatPad to interrogate how lifelong central serotonin deficiency affects adolescent rodents. TPH2 knockout rats performed fewer trials per session, exhibited greater trial-to-trial variability,

and were less likely to meet stable learning criteria, despite intact motor abilities. These deficits align with serotonin's known roles in motivational, orientational and attentional processes. By enabling continuous, voluntary engagement, the Social RatPad uncovered motivational impairments that might have been masked in forced, once-daily sessions. Thus, this work provides compelling evidence that adolescent serotonin depletion compromises not only neural circuitry but also the drive to engage with cognitive challenges.

Synthesis.

The findings from Chapter 2-4 paint a cohesive picture: a complex, animal-friendly testing environment reveals subtler cognitive-motivational deficits linked to neurotransmitter perturbations. Social RatPad's combination of technical precision and ecological validity allows researchers to observe how neuromodulatory systems influence both capacity (i.e., ability to learn) and willingness (i.e., engagement patterns). This integrative approach moves beyond binary measures of success/failure to richer behavioral phenotyping, capturing nuances of adolescent cognition and motivation (Rivalan et al., 2017).

The integration of technical and biological findings presented here reveals that cognition and motivation cannot be meaningfully separated when studying adolescent brain function. In the context of the Social RatPad, learning is no longer a static measure of accuracy or latency but a dynamic function of voluntary engagement. This insight places serotonin at the intersection of two levels of control: one developmental, governing how the brain matures to support flexible learning, and one motivational, determining whether those capacities are actively expressed. The observed deficits in trial initiation and persistence among TPH2 knockout rats are therefore not isolated impairments in attention or memory but indicators of a more fundamental disruption in motivational regulation. In line with theoretical models such as the "expected value of control" framework (Shenhav et al., 2013), serotonin appears to modulate the subjective cost–benefit analysis that governs cognitive effort. Its absence reduces the perceived value of exerting effort toward uncertain goals, leading to reduced persistence and exploratory drive - the behavioral signatures captured in this study's automated, self-paced design (Meyniel et al., 2016; Salamone et al., 2016).

Importantly, the Social RatPad made these patterns visible by removing external enforcement and allowing learning to occur spontaneously. In traditional paradigms, where animals are tested at fixed times and reinforced

under controlled schedules, motivational variation is artificially compressed. Here, by contrast, trial numbers, initiation times, and engagement frequencies become meaningful behavioral data. The system thus uncovers an often-overlooked domain of individual differences - how animals choose to learn - providing insight into the motivational dimension of cognition (Rivalan et al., 2017).

Beyond individual behavior, the synthesis of findings demonstrates a convergence between technological design and theoretical progress in behavioral neuroscience. Ecologically valid systems such as the Social RatPad, IntelliCage (Lipp & Wolfer, 2024), and Eco-HAB (Puścian et al., 2016) are reshaping the methodological landscape by linking spontaneous, self-paced behavior with high-precision data capture. The RatPad extends this approach to touchscreen-based tasks, offering the computational structure of classical human paradigms (Bussey et al., 2021) within a social, low-stress environment. This step enables a more direct translation between rodent and human cognition, strengthening the bridge from animal models to developmental psychiatry. Moreover, by integrating RFID and automated gating, the Social RatPad uniquely quantifies motivation as a measurable variable - how frequently animals initiate tasks, how consistently they persist, and how they modulate performance under different internal states. These metrics extend beyond cognitive capacity to encompass the behavioral energy that underlies adaptive function, a domain central to the study of disorders like depression, apathy, and obsessive–compulsive behavior (Salamone et al., 2016).

The synthesis also underscores that adolescence represents a particularly sensitive window for serotonergic modulation. During this period, the prefrontal cortex receives inputs from serotonergic nuclei (Caballero et al., 2017). The TPH2-KO findings - reduced trial persistence and inconsistent engagement - suggest that serotonin deficiency during adolescence weakens the motivational scaffolding necessary for stable cognitive development. These patterns parallel the behavioral features of adolescent mood and anxiety disorders in humans, where reduced serotonin transmission contributes to long-lasting impairments (Leiser et al., 2015; Ogelman et al., 2024).

By capturing these features in an automated, ethologically valid framework, the Social RatPad provides a translationally relevant tool for understanding how neurochemical imbalances translate into altered behavioral rhythms and learning trajectories. Nevertheless, further validation and systematic testing

across additional paradigms and strains will be essential to fully establish its generalizability and reliability.

Finally, this synthesis illustrates a conceptual shift from measuring performance as an endpoint to understanding behavior as a continuous process shaped by neuromodulatory tone. The Social RatPad bridges the artificial divide between "learning" and "motivation," showing that they are dynamically intertwined facets of adaptive function. Under natural conditions, animals decide when to engage, how long to persist, and when to withdraw - decisions that mirror human patterns of cognitive effort allocation. By designing a system that respects these natural decision processes, this work not only enhances experimental realism but also enriches the theoretical framework through which cognition is studied. It redefines success not as the number of correct responses but as the ability to sustain engagement under conditions requiring integration and control over the environment - the capacity that serotonin modulations alter (Westbrook & Braver, 2015).

Strengths and Limitations

Strengths

A major strength of this study is its providing the animals agency to decide when to engage in the task and eliminating human interference enabled high-fidelity data collection with minimal noise. In addition, the system provided rich behavioral readouts, as voluntary session initiation and trial counts offered insights not only into cognitive performance but also into motivational states. Finally, the translational potential of the paradigm should be emphasized. By implementing touchscreen-based tasks in the home cage environment, this approach parallels human tablet-based assessments and may therefore enhance cross-species comparability.

Limitations

Several limitations should be noted. First, the automation infrastructure, including RFID gates, sensors, and custom software, requires considerable initial investment and technical expertise. Second, the current findings are based on two genetic models, which may limit generalizability, as other strains or knockout lines could yield distinct outcomes. Third, although the system is designed to minimize stress, physiological stress markers such as corticosterone were not measured to confirm this assumption. Finally, while the Social RatPad reduces handling-related stress, animals still require

a familiarization and training period before they can reliably perform the homecage tasks and pass through the automated gates.

Implications for the Field

Low-stress, self-paced paradigms enable a rethinking of traditional behavioral models by allowing more nuanced phenotyping of motivational and cognitive deficits across psychiatric and neurodevelopmental conditions. Second, continuous and high-throughput testing opens the possibility for accelerated drug screening, modernizing preclinical evaluation of cognitive enhancers or neuromodulatory compounds while reducing time and animal use. However, as reflected in our RatPad experiments, training speed varied across individuals, sometimes faster and sometimes slower, indicating that efficiency gains are not uniform but may depend on context and individual traits. Third, the system facilitates longitudinal monitoring across developmental windows, enabling researchers to study critical periods of brain maturation under different genetic or environmental conditions. Finally, by employing tablet-style interfaces similar to those used in traditional touchscreen boxes, the Social RatPad enhances cross-species harmonization and extends cognitive testing into the homecage context. This provides a more ecologically valid and translational bridge between rodent and human studies.

Future Research Directions

Building on this thesis, several immediate and practical directions can refine and expand the Social RatPad framework. A first step is to systematically test how temporal parameters - such as session length, access scheduling (e.g., the current 8-hour testing slots), or circadian timing - influence animals' motivation, attention, and persistence. Recent automated home-cage studies have shown that time-restricted protocols can enhance engagement and reduce omissions compared to unlimited access designs (Bruinsma et al., 2019), suggesting that motivational dynamics strongly depend on task availability. Likewise, adaptive self-adjusting paradigms have proven effective for characterizing impulsivity and reward valuation under naturalistic conditions (Carr et al., 2025). Applying similar adaptive or time-restricted approaches in the Social RatPad could clarify how serotonin-related mechanisms interact with motivational drive across the day and developmental stages.

An important next step is the quantification of stress and arousal states in real time to confirm that the Social RatPad indeed minimizes experimenter-induced distress. Continuous physiological monitoring using wireless telemetry or

infrared sensors could provide moment-to-moment data on heart rate, body temperature combined with locomotor activity (which could be achieved via LABORAS integration) during task engagement. Such measures have been shown to sensitively track affective state and welfare in rodents (Lezak et al., 2017). By aligning these physiological indicators with task-specific timestamps - such as trial initiation or pauses - it would be possible to determine whether reduced engagement reflects transient physiological stress or true motivational decline. Such multimodal data integration could also help distinguish adaptive pauses from fatigue-induced disengagement, providing a more comprehensive picture of internal states during autonomous learning.

Parallel to physiological expansion, genetic diversification represents a powerful route to mapping serotonergic and dopaminergic contributions to motivation. Beyond TPH2 knockouts, models targeting serotonin transporter (SERT) or receptor subtypes (e.g., 5-HT1A, 5-HT2C, or 5-HT7) could be employed to examine receptor- or circuit-specific influences on persistence, effort allocation, and decision-making (Carhart-Harris & Nutt, 2017). Conditional knockouts or chemogenetic manipulations confined to the medial prefrontal cortex or dorsal raphe could further clarify circuit-level specificity, distinguishing whether motivational deficits arise from cortical control, subcortical drive, or disrupted top-down integration. Developmentally restricted manipulations would also allow testing whether serotonergic disruption during adolescence produces qualitatively different motivational outcomes compared to adult-onset alterations, topics that are not fully yet understood (Teissier et al., 2017).

Another promising approach involves pharmacological manipulations conducted under fully automated, homecage conditions. Such interventions could dissect state-dependent and trait-dependent components of serotonergic function. Acute administration of selective serotonin reuptake inhibitors (SSRIs), 5-HT receptor agonists, or tryptophan depletion protocols could reveal transient, reversible shifts in engagement, latency, and persistence. In contrast, chronic SSRI treatment (Jiang et al., 2019) could induce long-term shifts in PFC consequently in cognitive style or learning trajectories, simulating trait-level effects akin to genetic modifications. The ability of the Social RatPad to continuously track voluntary behavior enables the detection of both rapid pharmacodynamic changes and slower behavioral plasticity, bridging mechanistic psychopharmacology with ecologically valid behavioral neuroscience.

A fourth direction involves extending the platform with multi-domain task batteries that probe higher-order executive functions. Traditional touchscreen paradigms can be used to assess attentional set-shifting, probabilistic reversal learning, delayed alternation, and foraging-based exploration–exploitation trade-offs. These tasks would enable precise assessment of how serotonergic tone modulates flexibility, working memory, and strategy switching. By embedding these tasks in an autonomous, low-stress context, it becomes possible to capture the natural variability of cognitive engagement, rather than constraining animals to rigid session schedules. Embedding such complex batteries within the Social RatPad environment also allows for multi-day and cross-context analysis, overcoming the limitations of short, human-enforced training sessions. Because animals initiate trials voluntarily and operate within a stable social and environmental setting, one can examine how executive functions fluctuate across circadian cycles, social hierarchies, and internal motivational states. This approach enables the measurement of not only how well but also when and why animals engage in cognitive effort.

Extending this paradigm into a fully living cognitive ecology would allow exploration of domains that are rarely accessible in standard laboratory paradigms - namely, the integration of social cognition, spatial navigation, and executive flexibility within a shared behavioral space. Future versions of the Social RatPad (synchronized with LABORAS) could incorporate multi-room environments connected by RFID-controlled corridors, in which touchscreen-based decision points are embedded as cognitive checkpoints between zones for feeding, social contact, and exploration. Such a configuration would enable continuous tracking of not only learning and memory but also goal planning and adaptive travel behavior - how animals structure their movements and choices when balancing social proximity, resource access, and cognitive challenges. For instance, an animal might choose to interrupt a social interaction to complete a touchscreen task that grants a food reward, or vice versa, revealing the dynamic prioritization processes underlying real-life decision-making.

A particularly promising complex paradigm could combine spatial foraging and social decision-making into a multi-agent exploration–exploitation task. In this setup, each cage would host several rats with partially overlapping access to cognitive stations. The touchscreen could present location-specific cues that correspond to different “virtual zones” in the environment - food, social contact, or neutral exploration. Success in one task might temporarily close another, forcing animals to dynamically allocate effort across competing

goals. By continuously monitoring RFID movement and trial initiation across these zones, researchers could quantify how individuals negotiate resource competition, delay gratification, and shift strategies when social contingencies change. This integrative approach mirrors current trends in systems neuroscience, where cognition is increasingly viewed as distributed and embodied rather than localized. Combining touchscreen challenges with spatial navigation and social choice would allow the modeling of real-world cognitive trade-offs - how humans juggle work, exploration, and social connection under variable motivational states. Moreover, these paradigms could simulate developmental transitions: adolescent animals might display exploratory overcommitment or social distraction, whereas adults could show stabilized strategies.

In addition, future work should explore social network dynamics and group-level behavioral phenomena within the automated environment. The RFID and gating structure of the Social RatPad lends itself to quantifying interaction frequency, spatial proximity, and co-engagement patterns. Applying social network analyses to these datasets can reveal how individual motivational phenotypes shape group structure and information flow. For instance, low-motivation individuals may occupy peripheral network positions, engage less in cooperative tasks, or exhibit reduced responsiveness to group contingencies. Longitudinal monitoring could further test whether motivational recovery correlates with reintegration into the social core, paralleling human findings where social withdrawal and amotivation co-occur in depressive states. Moreover, by designing two- or three-rat cooperative touchscreen tasks, researchers could examine how reward sharing, social facilitation, and competition influence engagement persistence, thereby bridging cognitive neuroscience and ethology.

Finally, the integration of computational modeling will be essential to formalize behavioral dynamics observed in these complex datasets. Reinforcement learning models incorporating effort costs, drift-diffusion frameworks for decision speed, and hierarchical Bayesian estimation of latent motivational states could all be fitted to individual time-series data (Shenhav et al., 2017; Kool & Botvinick, 2018; Westbrook et al., 2013). Integrating physiological indices (e.g., heart rate, body temperature) as regressors into these models could further connect subjective cost computation with embodied states of arousal or stress. Through such models, the Social RatPad could evolve into a platform that not only measures behavior but infers the underlying decision mechanisms and internal motivational states.

In summary, future work should push the Social RatPad towards a multi-level, interdisciplinary system linking physiology, genetics, pharmacology, cognition, and social behavior through continuous monitoring and computational modeling. This expansion will enable the exploration of how serotonin (and other neuromodulators) shape motivation and cognition not as isolated processes but as dynamically coupled components of adaptive behavior, offering a robust bridge between mechanistic neuroscience and translational psychiatry.

Conclusion

This thesis demonstrates that merging cutting-edge touchscreen technology with a socially enriched, automated homecage environment leads to a powerful platform for preclinical cognitive research. The Automated Social RatPad enables high-precision, ecologically valid assessments of both cognitive capacity and motivational engagement - qualities essential for understanding neurodevelopmental disorders and advancing translational neuroscience. Application of this system to the TPH2 knockout model revealed that adolescent serotonin deficiency profoundly disrupts cognitive engagement, underscoring serotonin's central role in motivation and learning. As a flexible, scalable tool, the Social RatPad smoothens the way for future studies that will deepen our understanding of neuromodulator influences on behavior and accelerate the development of therapeutic interventions.

An important implication of these findings is that ecological validity does not automatically translate into efficiency gains. While some rats adapted more quickly in the Social RatPad than in traditional paradigms, others required extended training, highlighting that variability is an inherent feature of less invasive testing. This variability, however, may itself be scientifically valuable, as it captures individual differences in motivation and coping strategies that are often lost in forced paradigms. In this sense, the RatPad does not simply accelerate testing but enriches the behavioral phenotype available for analysis.

When compared to existing automated systems the Automated Social RatPad advances the field by integrating RFID-based identification, adjustable gating, and continuous social housing into a single platform. This combination allows not only for cognitive readouts but also for parallel measures of motivation, exploration, and social interaction. As such, the RatPad extends beyond cognitive testing into a more holistic characterization of animal behavior, providing unique opportunities for translational neuroscience.

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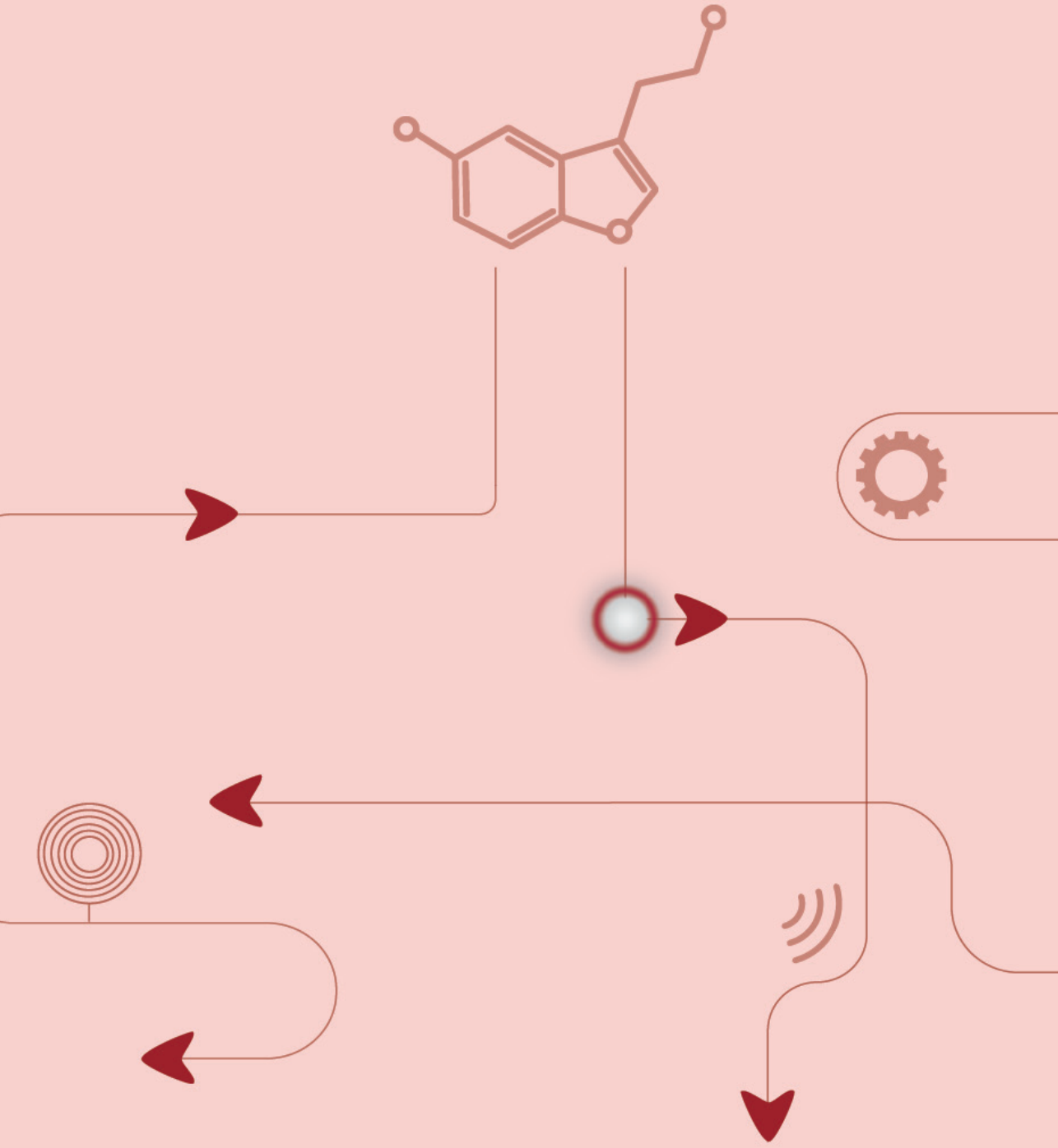

Appendix

Nederlandse Samenvatting

Inleiding

Dit proefschrift richt zich op de ontwikkeling en toepassing van een geautomatiseerd, sociaal-verrijkt Social RatPad systeem. Traditionele methoden voor cognitief onderzoek bij knaagdieren brengen vaak stress met zich mee doordat dieren uit hun vertrouwde omgeving worden gehaald en regelmatig door onderzoekers worden verwerkt. Hierdoor ontstaan meetfouten en verliezen in de bevindingen en transleerbaarhied naar de mens dat een capacitief touchscreen in een sociale thuiskooi de mogelijkheid van leren introduceert, maar ook de variabiliteit tussen dieren verkleint, in vergelijking met conventionele infrarood-touchscreens.

In Hoofdstuk 2 is het systeem verder geoptimaliseerd door volledige automatisering: rats worden met RFID-microchips individueel gepositioneerd via zelfinstellende toegangspoortjes (ORT), en kunnen hun testmomenten zelf kiezen. Door 24/7 toegang tot taken, gecombineerd met sociale huisvesting, wordt transportstress geëlimineerd en blijft de sociaal-motivationele context behouden.

Hoofdstuk 3 toont de toepassing van de Social RatPad op een genetisch model voor serotoninetekort, de TPH2-knockout rat. Hieruit blijkt dat adolescenten met een blijvende centrale serotonine deficiëntie niet alleen verslechterde leercapaciteit, maar vooral ook verminderde vrijwillige taak deelname en verhoogde trial-by-trial variabiliteit vertonen. Dit onderstreept de dubbele rol van serotonine in zowel neuro-ontwikkeling als motivatie.

Methode

Het experiment werd uitgevoerd in een Eurostandard thuiskooi (Type IV) die was gekoppeld aan twee touchscreen-testkooien (RatPads). De toegang tot de testkooien werd geregeld door 3D-geprinte ORT-poortjes met RFID-gelezen roterende segmenten. Capacitieve touchscreens registreerden lichte aanrakingen nauwkeurig, terwijl pellet dispensers automatisch beloningen verstrekten bij correcte responsen.

RFID-antennes en weegschalen (RatPad) maten continu welke rat deelnam aan de test en hoeveel lichaamsgewicht deze had. De software startte op vaste tijdstippen - of op vrijwillige basis door de rat zelf - cognitieve taken, waardoor dieren in hun eigen ritme konden deelnemen zonder menselijke tussenkomst.

De cognitieve taken omvatten onder meer de Must Touch en visuele discriminatie (leertaak). Daarnaast werden de dieren blootgesteld aan korte LABORAS-sessies van twee uur, waarin hun spontane locomotorische en gedragsactiviteit werd geregistreerd.

De proefdieren waren jonge Dark Agouti-ratten, inclusief wildtype en TPH2-knockouts, van beide geslachten. Hun prestaties werden gedurende de adolescentie continu vastgelegd.

Resultaten

1. **Preliminaire data**: In de eerste vergelijkingen tussen verschillende typen touchscreens (preliminaire data) werd bij capacitieve touchscreens waargenomen dat ratten discriminatie en aanpassing in gemiddeld 13 dagen leerden (tegenover 18 dagen bij infrarood), met een significant lagere standaarddeviatie.
2. **Volledige automatisering**: Volledige automatisering: RFID-poortjes en zelfgestuurde sessies elimineerden handelingseffecten. Dieren namen gemiddeld deel aan 30 trials per sessie, met weinig overgeslagen sessies en stabiele prestaties.
3. **Serotonine tekort (TPH2-KO)**: adolescenten misten gemiddeld 40 % meer trials, toonden 50 % meer prestatie variatie en minder dieren haalden het 60 %-criterium voor drie opeenvolgende sessies. Dit suggereert een mogelijk motivatie- en stabiliteit verschil dat in conventionele paradigma's deels verborgen kan blijven; verder onderzoek is nodig om dit te bevestigen.

Discussie en Conclusies

Het Social RatPad-systeem combineert technische precisie (capacitive touchscreens, RFID-tracking) met hoge ecologische validiteit: thuis kooihuisvesting, sociale interactie en vrijwillige taak keuze. Hierdoor worden zowel cognitieve capaciteit als motivatie nauwkeurig gemeten. De bevindingen bij TPH2-knockouts bevestigen de rol van serotonine als neurotrofisch en motivatie-modulator tijdens de adolescentie.

Sterke punten van dit werk zijn de minimalisatie van handelingseffecten, continue datacollectie en direct vergelijkbare taken met menselijke tablet tests. Beperkingen liggen in de technische complexiteit, de initiële trainingsperiode en het ontbreken van directe stress-biomarkers (corticosterone). Toekomstig onderzoek kan dit adresseren door parallelle hormoontests, uitbreiding naar

andere gen-modellen (bijv. SERT-KO) en implementatie van geavanceerde executieve taken (multichoice shifting). Bovendien zou farmacologische modulatie (SSRI's) in dezelfde setup kunnen aantonen of motivatie tekorten reversibel zijn.

Eindconclusie

De Social RatPad biedt een baanbrekend platform voor preklinische cognitieve hersen en gedrag studies. De combinatie van geautomatiseerde, stressarme thuiskooi tests met touchscreen-taken maakt real-time monitoring van leren en motivatie mogelijk. De toepassing bij rat modellen met veranderingen in serotonine niveaus belicht fundamentele neuromodulator effecten en zal de ontwikkeling van therapeutische interventies voor ontwikkelingsstoornissen en psychiatrische aandoeningen aanzienlijk versnellen

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English Summary

Introduction

This thesis focuses on the development and application of an automated, socially enriched Social RatPad system. Traditional cognitive testing methods for rodents often induce stress because animals are removed from their home cage and frequently handled by researchers. These factors introduce measurement errors and reduce translational validity. In contrast, this work demonstrates that a capacitive touchscreen integrated into a social home-cage environment not only enables learning but also reduces inter-animal variability compared with conventional infrared touchscreens.

In Chapter 2, the system was further optimized through full automation: individually identified rats were guided by RFID-controlled One-Rat Turnstile (ORT) gates and could initiate testing sessions voluntarily. With 24/7 access to the tasks and continuous social housing, transport stress was eliminated and the socio-motivational context preserved.

Chapter 3 applies the Social RatPad to a genetic model of serotonin deficiency, the TPH2-knockout rat. Adolescents with lifelong central serotonin depletion showed not only impaired learning, but also markedly reduced voluntary task engagement and increased trial-by-trial performance variability. This highlights the dual role of serotonin in both neurodevelopment and motivation.

Methods

The experiment was conducted in a Eurostandard Type IV home cage connected to two touchscreen-based testing chambers (RatPads). Access was controlled by 3D-printed ORT gates equipped with RFID-read rotating segments. Capacitive touchscreens recorded light touches with high accuracy, while pellet dispensers automatically delivered rewards after correct responses.

RFID antennas and RatPad-integrated scales continuously monitored which rat was participating and recorded its body weight. The software initiated cognitive tasks at fixed times or whenever a rat voluntarily entered the testing chamber, enabling fully autonomous participation without human handling.

The cognitive tasks included Must Touch and visual discrimination paradigms. In addition, all animals took part in short LABORAS sessions, which captured spontaneous locomotor and behavioral activity over two hours.

The study used young Dark Agouti rats—both wildtype and TPH2-knockouts, males and females—whose cognitive and behavioral performance was monitored continuously throughout adolescence.

Results

1. **Preliminary Data (Touchscreen Comparison):** Rats trained on capacitive touchscreens learned discrimination and reversal in an average of 13 days, compared with 18 days on infrared screens, showing significantly lower variability.
2. **Full Automation:** RFID-controlled gates and self-initiated sessions eliminated handling effects. Rats completed around 30 trials per session with few missed sessions and stable overall performance patterns.
3. **Serotonin Deficiency (TPH2-KO):** KO adolescents skipped ~40% more trials, displayed ~50% higher performance variability, and fewer animals reached the 60% learning criterion across three consecutive sessions. These findings suggest motivational and behavioral stability differences that conventional paradigms may overlook. Further research is needed to confirm these patterns.

Discussion and Conclusions

The Social RatPad system integrates technical precision (capacitive touchscreens, RFID tracking) with high ecological validity through home-cage testing, social housing, and voluntary participation. This allows simultaneous measurement of cognitive ability and intrinsic motivation. Findings in TPH2-knockout rats support the role of serotonin as both a neurotrophic and motivational modulator during adolescence.

Strengths of this work include minimal handling, continuous data collection, and direct comparability to human tablet-based tests. Limitations include technical complexity, an initial training period, and the absence of physiological stress markers. Future research could incorporate hormone sampling, expand to additional gene models (e.g., SERT-KO), and introduce more complex executive-function tasks. Pharmacological manipulations (e.g., SSRIs) could further reveal whether motivational deficits are reversible.

Final Conclusion

The Social RatPad provides a groundbreaking platform for preclinical studies of cognition, motivation, and brain development. By combining automated, stress-free home-cage testing with touchscreen-based tasks, the system

enables real-time assessment of learning and motivational processes. Application to serotonin-related genetic models highlights fundamental neuromodulatory mechanisms and will substantially advance the development of therapeutic interventions for developmental and psychiatric disorder

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Research Data Management Statement

Ethics and Privacy

All animal procedures described in this thesis were approved by the Central Committee on Animal Experiments in the Netherlands (Centrale Commissie Dierproeven, CCD) and were conducted in accordance with the Dutch Animal Experiments Act and European Directive 2010/63/EU (project licence AVD10300 2020 11424). Animals were socially housed and individually identified using subcutaneous RFID microchips, enabling automated tracking without repeated handling. Animal welfare was prioritized through minimal human intervention, social housing, and environmental enrichment. No human participants or personal data were involved in this research.

Data Collection and Storage

Data for this thesis were generated through laboratory experiments involving animals, using custom-built automated Social RatPad systems. These systems recorded voluntary touchscreen task performance and RFID-based access events. The resulting raw data include trial-level behavioural responses, timestamps, session participation logs, and system-generated records. Data were created and collected by the author. All raw data files were archived in their original form in a Data Acquisition Collection (DAC) in the Radboud Data Repository (RDR). The collection is stored with closed access, ensuring long-term preservation and protection of confidential and proprietary information related to the experimental system. The data are retained for 15 years in accordance with Radboudumc policy.

Data Sharing According to the FAIR Principles

The data underlying this thesis comply with the FAIR principles of Findability, Accessibility, Interoperability, and Reusability, with access levels tailored to the nature of the data.

The raw, trial-level datasets are archived with closed access in the Radboud Data Repository as a Data Acquisition Collection (DAC), due to proprietary system design and collaboration-related constraints. While the data files themselves are not publicly accessible, the metadata of this collection are publicly available and indexed, ensuring findability. The DAC has been assigned a persistent identifier (DOI):

https://doi.org/10.34973/bm3h-tz55In addition, group-level aggregated data underlying the main results of this thesis have been deposited in the Radboud Data Repository as a Data Sharing Collection (DSC). This collection is published with restricted access, ensuring that data access requests and reuse are handled through the repository in accordance with FAIR principles, institutional policies, and applicable legal or contractual obligations. The aggregated dataset is findable via its DOI:

https://doi.org/10.34973/mb81-3390Requests can be addressed to the corresponding author and will be evaluated based on scientific purpose and compliance with institutional and contractual obligations.

All shared data are stored in interoperable and sustainable file formats (e.g. CSV and JSON) and can be accessed using commonly available software tools.

Curriculum Vitae

Armen Andreasyan was born in 1993 in Yerevan, Armenia. After completing secondary school at the PhysMath School after Artashes Shahinyan, he began his studies in Mathematics before realizing his passion for Psychology. He applied his math skills to statistical analysis and completed a Bachelor's degree in Psychology at Yerevan State University in 2015, writing his thesis on Emotional Intelligence.

He went on to earn a Master's degree in Applied Psychology at Yerevan State University in 2018, specializing in both Clinical and Educational Psychology. After this period, he co-founded the Institute of Cyberpsychology, deepening his engagement in clinical practice, neuroscience, and immersive technologies.

Armen gained professional experience as an Educational Psychologist through work with Teach For Armenia, as well as experience in neuroscience and engineering through research roles at the Orbeli Institute of Physiology and with Metris B.V. in the Netherlands.

In 2021, he began his PhD at Radboud University Medical Center in Nijmegen. Supervised by Prof. Judith Homberg and Ronadl Bulthuis, his research focused on developing and validating the Automated Social RatPad - an automated, socially enriched homecage system for stress-free cognitive testing - and applying it to study behavioral and cognitive development during adolescence in transgenic rat models characterized by low serotonin levels.

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Portfolio

The PhD candidate participated in the following educational activities as part of the Donders Graduate School and Radboudumc Doctoral Education Program.

Courses & Workshops

Activity	Organizer	Year	Hours
Project Management for PhD Candidates	Radboud University	2025	52
Analytic Storytelling	Radboud University	2025	20
Basics of Python for Data Analysis	Radboud University	2025	54
Design and Illustration	Radboud University	2025	26
Career Guidance for PhD Candidates and Postdocs	Radboud University	2025	20
Art of Presenting Science	Radboud University	2025	36
Magnetic Resonance Imaging: From Physics to Physiology	University of Copenhagen	2025	92
Communication with Patients	Serotonin and Beyond Network	2024	14
Graduate School Day	Radboud University	2025	7
Graduate School Day 2	Radboud University	2025	7
GS Introduction Day	Radboud University	2025	7
Scientific Integrity Course	Radboud University	2025	7
Laboratory Animal Science	Radboudumc	2022	84
Communication to Media and Public	Serotonin and Beyond Network	2022	14
BCI & Neurotechnology Spring School	International BCI Community	2025	140

Conferences & Meetings

Activity	Organizer	Year	Hours
Donders Day	Donders Institute	2025	7
The Leiden International (Bio)Medical Student Conference (LIMSC)	Leiden University	2025	6
Donders Discussions	Donders Institute	2024	7

Teaching & Supervision

Activity	Organizer	Year	Hours
Internship Supervision in Cognitive Neuroscience	Radboud University	2022	14
Supervision of Nane Marutyan (Engineering City, Bagrevand21)	Engineering City	2024	-
Supervision of Levong Soghomonyan (Engineering City, Bagrevand21)	Engineering City	2024	-
Supervision of Aram Poghosyan (Yerevan State Polytechnic University)	NPUA	2024	-

Outreach & Public Engagement

Activity	Organizer	Year	Hours
Different Approaches in Psychotherapy: Choosing the Right One	Cobrain Center	2024	-
3D Video Demonstration for School Children	Radboud University	2023	-
1st Prize Winner of Brain-Computer Interface Designers Hackaton	International BCI Community	2025	-

Donders Graduate School

For a successful research Institute, it is vital to train the next generation of scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School in 2009. The mission of the Donders Graduate School is to guide our graduates to become skilled academics who are equipped for a wide range of professions. To achieve this, we do our utmost to ensure that our PhD candidates receive support and supervision of the highest quality.

Since 2009, the Donders Graduate School has grown into a vibrant community of highly talented national and international PhD candidates, with over 500 PhD candidates enrolled. Their backgrounds cover a wide range of disciplines, from physics to psychology, medicine to psycholinguistics, and biology to artificial intelligence. Similarly, their interdisciplinary research covers genetic, molecular, and cellular processes at one end and computational, system-level neuroscience with cognitive and behavioural analysis at the other end. We ask all PhD candidates within the Donders Graduate School to publish their PhD thesis in de Donders Thesis Series. This series currently includes over 750 PhD theses from our PhD graduates and thereby provides a comprehensive overview of the diverse types of research performed at the Donders Institute. A complete overview of the Donders Thesis Series can be found on our website: https://www.ru.nl/donders/donders-series

The Donders Graduate School tracks the careers of our PhD graduates carefully. In general, the PhD graduates end up at high-quality positions in different sectors, for a complete overview see https://www.ru.nl/donders/destination-our-former-phd. A large proportion of our PhD alumni continue in academia (>50%). Most of them first work as a postdoc before growing into more senior research positions. They work at top institutes worldwide, such as University of Oxford, University of Cambridge, Stanford University, Princeton University, UCL London, MPI Leipzig, Karolinska Institute, UC Berkeley, EPFL Lausanne, and many others. In addition, a large group of PhD graduates continue in clinical positions, sometimes combining it with academic research. Clinical positions can be divided into medical doctors, for instance, in genetics, geriatrics, psychiatry, or neurology, and in psychologists, for instance as healthcare psychologist, clinical neuropsychologist, or clinical psychologist. Furthermore, there are PhD graduates who continue to work as researchers outside academia, for instance at non-profit or government organizations, or in pharmaceutical companies. There are also PhD graduates who work in

education, such as teachers in high school, or as lecturers in higher education. Others continue in a wide range of positions, such as policy advisors, project managers, consultants, data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals.

For more information on the Donders Graduate School, as well as past and upcoming defences please visit:

http://www.ru.nl/donders/graduate-school/phd/

Appendix A: Detailed Protocols

A.1 Animal Husbandry and Ethical Approval

Purpose:
To ensure the welfare of animals and compliance with legal and ethical standards during all experimental procedures.

Materials and Methods:
- Species/Strain: Wistar, Long Evans, or Sprague Dawley rats (specify per experiment).
- Source: Charles River, Janvier Labs or own breeding.
- Housing:
  - o Cages: Eurostandard Type IV (central cage) and Type III (testing chambers - RatPads).
  - o Bedding: Corncob, changed weekly.
  - o Enrichment: Tunnels, nesting material, red retreat tubes.
  - o Environmental Conditions: 12:12 light-dark cycle (lights off 7:00-19:00), temperature 21-23°C, humidity 45-65%.
  - o Social Housing: Housed in pairs unless protocol requires single housing.
- Food and Water: Ad libitum unless food restriction is part of the behavioral protocol.
- Acclimatization: Minimum 1 week before experiments if the animals come from a breeding company.
- Health Monitoring: Daily visual health check (by Team1), weekly weighing (by the protocol main researcher).
- Ethical Approval: All procedures approved by [Centrale Commissie Dierproeven, CCD], protocol number [2020-0022-013].

A.2 RFID Microchip Implantation Protocol

Purpose:
To enable individual identification of animals for automated behavioral tracking.

Materials:
- Sterile 1.4 × 8 mm 134.2 kHz bio-glass RFID microchips (Team1 storage room, askTeam1)
- Microchip injector

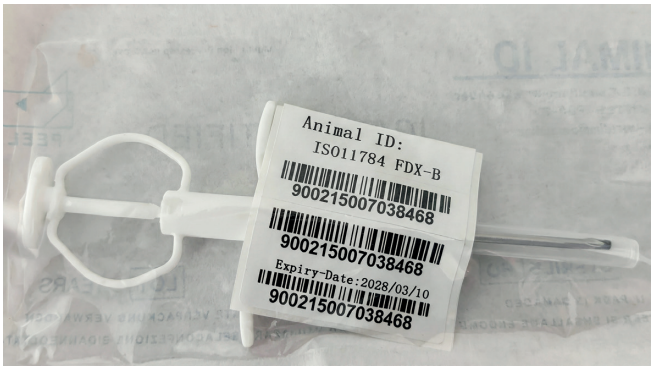


Figure 1. Sterile 134.2 kHz RFID microchip injector with integrated barcode, used for individual animal identification.

- Isoflurane vaporizer and anesthesia setup (optional)
- Sterile gloves
- Alcohol swabs
- Recovery cage with soft bedding

Procedure:

1. Preparation: Ensure all equipment is sterile and labeled with animal IDs.
2. Anesthesia (optional): Induce light anesthesia with isoflurane (3% induction, 1-2% maintenance).
3. Implantation:
   - Place the rat in sternal recumbency.
   - Swab the area between the scapulae with alcohol.
   - Insert the microchip subcutaneously using the injector.
   - Gently massage the site to help close the wound.
4. Recovery: Place animal in a recovery cage. Monitor until fully ambulatory (usually 5-15 minutes).
5. Verification: Scan with handheld RFID reader to confirm chip function and record chip ID.
6. Post-op care: Monitor the site for infection for 3 days.
7. Recordkeeping: Document date, chip code, and animal ID.

A.3 Apparatus Assembly and Calibration (Automated Social RatPad System)

Purpose:

To assemble the automated ASR and ensure accurate function before experiments.

Materials:

- Central Type IV Eurostandard cage
- Two standalone RatPad chambers (Type III)
- FPI connectors and transparent tubes (55 mm inner/60 mm outer diameter)
- ORT gates with adjustable wings
- RFID antennas (100 mm diameter) and modules
- Weight measurement station
- Secondary gates (servo-controlled) attached to side windows of the RatPad chamber
- Cleaning supplies

Assembly:

1. Connect the central cage to FPI binocular tubes.

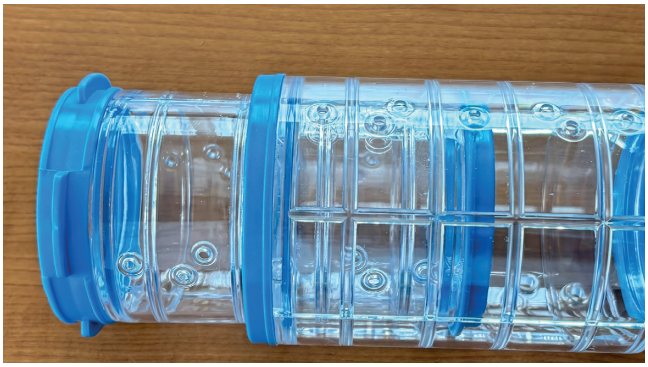


Figure 2. Binocular FPI connector tube, designed for secure and transparent passage between cages in the automated system.

2. Install ORT gate between binocular FPI tube and weight measurement station. Adjust wings to prevent two rats entering at once; verify smooth rotation.

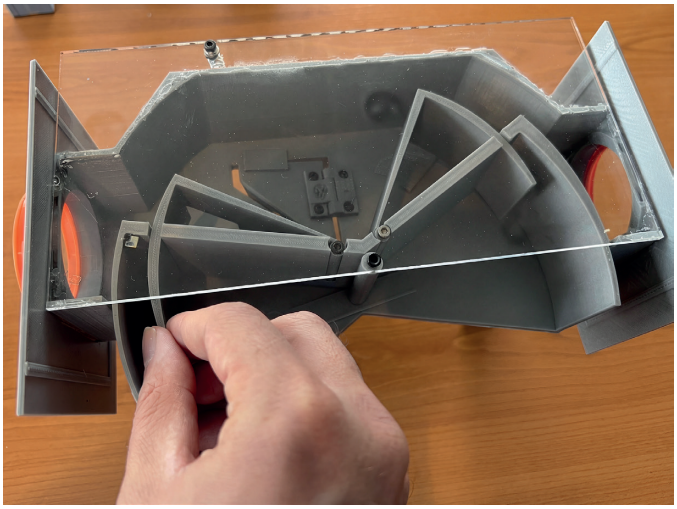


Figure 3. Adjustable One-Rat Turnstile (ORT) gate, featuring size-adaptive wings to ensure single animal passage.

3. Embed RFID antennas at designated points (tube entry). Place the RFID antenna over the weight measurement station tube horizontally and rotate 90 degrees. The antenna will fit the tube (pressure fit).

Figure 4. RFID antenna mounted horizontally and pressure-fitted over the FPI tube for optimal signal detection at tube entry.

4. Install weight measurement station in tube after ORT gate; test with calibration weights. Please note that the weight measurement systems should be installed beforehand as it is required for them to stick to the table.
5. Mount secondary gates between T-shaped tubes end and RatPad chamber, connect servos to Arduino.
6. Test software: Power on and run self-check. Confirm that RFID codes are logged, gates respond. When you run the software "V2" the program will calibrate the secondary gates automatically. When this happens you can be assured that connection exists in between the PC and the microcontrollers.
7. Clean system before introducing animals.

A.4 System Cleaning and Maintenance Protocol

Purpose:

To maintain animal health and data integrity by ensuring all system parts are clean and functional.

Frequency:

- Tubes, gates, cages: Weekly or after each cohort.
- Touchscreens, sensors: Before each experiment.

Procedure:

1. Disassemble tubes, gates, and connectors.
2. Disassemble the tubes into two halves. Wash all plastic tubes with warm water and animal-safe detergent. Rinse thoroughly.

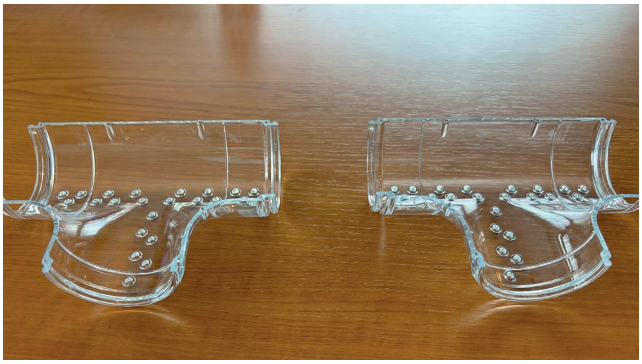


Figure 7. Example of a T-shaped tube fully disassembled, illustrating proper cleaning and maintenance procedures.

3. Sanitize with 70% ethanol or H2O2 tissues, especially frequently contacted surfaces.
4. Dry parts completely before reassembly.
5. Inspect RFID antennas, weight stations, and servo motors for debris or malfunction.
6. Document cleaning date and any maintenance performed.
7. Make a video after the reassembly, checking for proper connections between the tubes, ORT gates, and testing chambers. Ensure that the top part of the weight measurement system is slightly elevated and does not touch the bottom part. Make sure the tops of the RatPad chambers are securely closed. Confirm that there is enough water, food, and sucrose pellets in place. Ensure that both the V2 and RatPad software run smoothly, and that the RatPad tasks are properly initiated. Manually open and close the ORT gates to verify detection by the V2 software.

A.5 Touchscreen Cognitive Task Protocol

Purpose:
To assess cognitive performance using automated touchscreen tasks.

Stages:

1. Habituation: Place rats in the chamber for 10-20 min/day for 2-3 days with no task, rewards dispensed at intervals.
2. Must Touch: A visual stimulus (e.g., white circle) appears. Rat must touch stimulus for reward.
   - Criterion: ≥80% correct in two consecutive sessions.
3. Visual Discrimination: Two stimuli appear; one correct, one incorrect. Touching correct yields reward, incorrect yields no reward (with or without punishment light).
   - Criterion: ≥80% correct in two consecutive sessions.
4. Reversal Learning: Reward contingencies are reversed (previous S+ becomes S–).
   - Criterion: ≥80% correct in two consecutive sessions.
5. TUNL Task (if performed): After pretraining, sample stimulus shown, then after delay, choice phase. Rat must select non-matching location for reward.
   - Criterion: ≥70% correct over three sessions.

Data Collected:

- Number of trials, correct/incorrect choices, omissions, session time, side preference.

A.6 Automated Testing Schedule Protocol

Purpose:

To standardize timing and availability of cognitive testing in the Automated Social RatPad.

Procedure:

1. Programming: Automated system programmed to unlock access to each RatPad chamber at scheduled times.
2. Session Start: Animal entry detected by RFID; trial initiates automatically.
3. Session End: Session ends after a set number of correct trials (e.g 40) or time expires (e.g. 8 hours).
4. Overnight monitoring: System logs all chamber entries/exits and trial outcomes 24/7.

A.7 RFID Animal Identification Protocol

Purpose:

To ensure precise identification and logging of individual animal behavior.

Procedure:

1. Each time a rat enters a tube or testing chamber, RFID antennas read and log its unique code.
2. If the RFID code matches the authorized user for that session, secondary gates open and the trial sequence begins.
3. If a code is not read, the event is flagged for manual review; repeated failures trigger system alert for maintenance.
4. All entries/exits and trial outcomes are stored in a database, cross-referenced with RFID codes.

A.8 ORT Gate Operation and Adjustment Protocol

Purpose:

To allow safe, controlled, and one-at-a-time passage from central cage to testing chamber.

Procedure:

1. Before each week, check the wings on each ORT gate and adjust to rat size.
2. Confirm that only one rat can pass at a time by simulating passage with a model or gentle animal handling.
3. Monitor microswitch status to ensure proper detection of open/closed states; correct any error codes before session starts.

A.9 Social Interaction Monitoring Protocol

Purpose:

To assess social motivation and activity in the Social RatPad.

Procedure:

1. Automated system logs animal presence in each zone (central cage, tube, testing chamber) via RFID antennas and corridor sensors.
2. Data collected:
   - Time spent in each compartment
   - Frequency and duration of social contact (if co-housed)
   - Number of voluntary entries into test chambers
3. Analyze trends in zone preference and transitions as markers of social behavior and motivation.

A.10 Data Logging, Export, and Backup Protocol

Purpose:

To ensure secure, complete, and analyzable data for each experiment.

Procedure:

1. All apparatus events (RFID reads, trial outcomes, zone entries, weights) are automatically timestamped and logged to .csv files.
2. Daily backups of raw data files to a secure server and external hard drive.
3. Before analysis, visually inspect files for missing or anomalous values; flag for manual review if discrepancies are found.
4. Data preprocessing is performed in Python or R according to a standardized pipeline.

A.11 Statistical Analysis Protocol

Purpose:
To analyze and interpret behavioral and cognitive data.

Procedure:
1. Data cleaning: Remove sessions with <5 trials (unless analyzed separately as non-engagement).
2. Primary comparisons:
   - o Mixed-effects models for repeated measures (rat as random effect, genotype as fixed effect).
   - o Fisher's exact test for categorical outcomes (e.g., "learner" vs "non-learner").
   - o Mann-Whitney U or t-tests for group comparisons where appropriate.
   - o Pearson or Spearman correlations for continuous variables.
3. Software: Python (pandas, scipy, statsmodels), R, or SPSS.
4. Reporting: Present mean ± SD or median (IQR), effect sizes, and exact p-values.
5. Visualization: Use matplotlib or seaborn (Python) or ggplot2 (R) for figures; label axes and include sample sizes in captions.

A.12 Emergency and Troubleshooting Protocol

Purpose:
To ensure animal safety and data integrity in case of equipment malfunction or emergency.

Procedure:
1. For equipment malfunction:
   - o Pause experiment, record time and nature of issue
   - o Troubleshoot according to equipment manual
   - o Contact technical support if unresolved
2. Document all emergencies and corrective actions in the experiment logbook.
3. Review data after any incident for possible loss or corruption and note in results/discussion.

A.13 Operation of the RatPad Program

1. Initial Experiment Setup

- Launch the RatPad application.

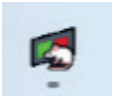


Figure 8. Logo of the RatPad experimental software program.

- Make sure the "Experiment" folder is created in C drive. Click the new experiment. Check the list of available experimental protocols in the interface.

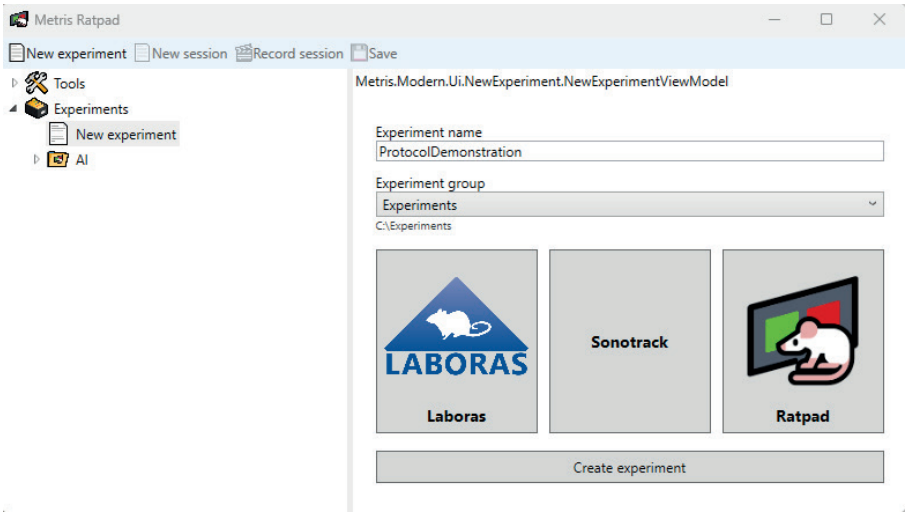


Figure 9. Initial setup screen of the RatPad program for starting a new experiment.

- Review protocol variables (e.g., trial number, task type, reward, session duration) and confirm they match the requirements for your current experiment.

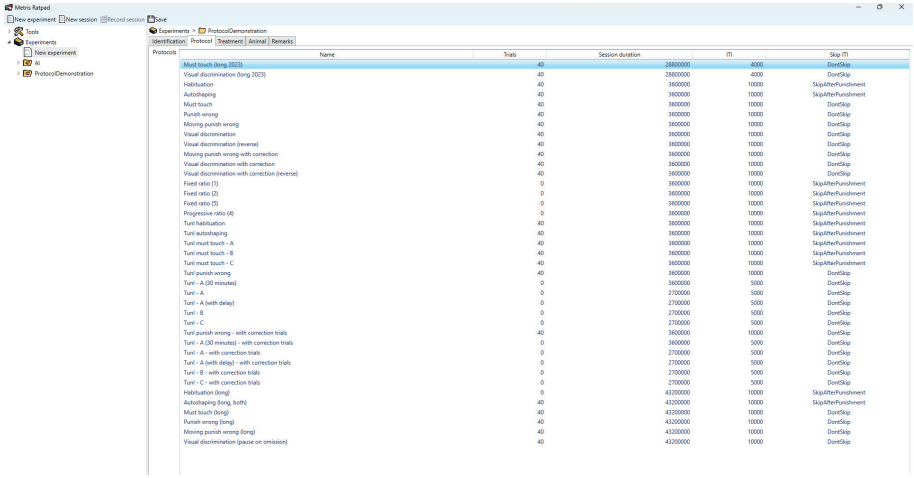


Figure 10. Interface of the RatPad program displaying detailed experimental protocol parameters.

Table 1: Protocol-related terms' explanations

Column	Meaning
Name	Task/protocol name
Trials	Number of correct trials to end the session
Session Duration	Maximum length of session (ms)
ITI	Inter-trial interval (ms) (the pause in between the trials)
Skip ITI	ITI handling for certain outcomes (e.g., after punishment)

- To adjust a protocol, contact Metris.
- Add treatment

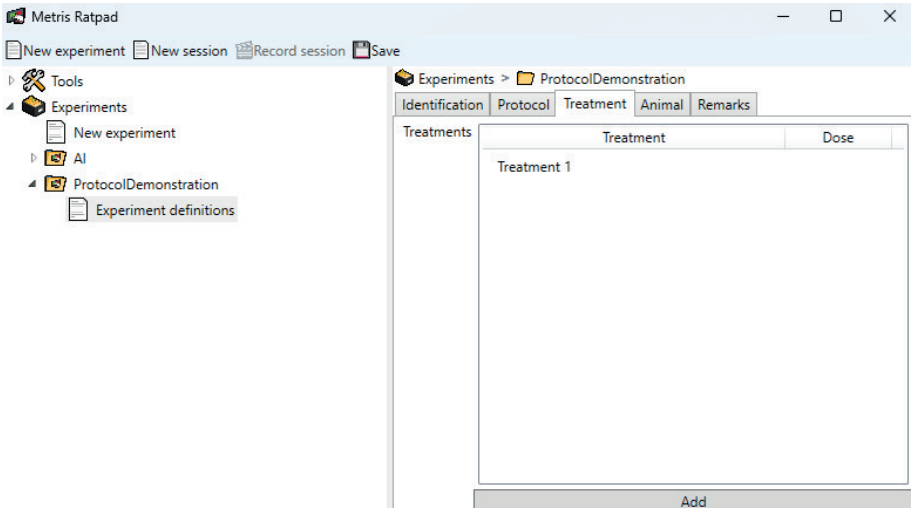


Figure 11. RatPad program screen for entering experimental treatment information.

- Add animals

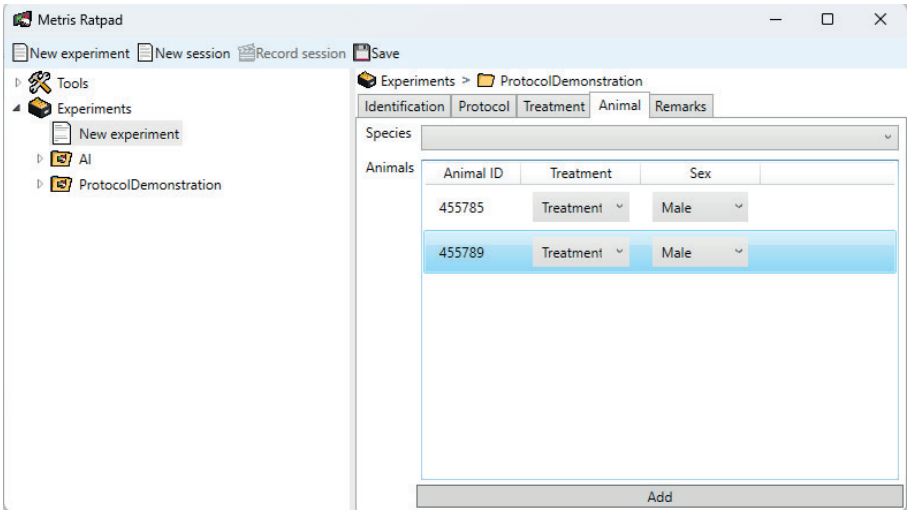


Figure 12. RatPad program interface for adding animals with identification, sex, and treatment assignment.

It is recommended to use CDL-provided IDs for the animals. This will keep the ids everywhere the same and will smoothen data comparison procedures afterward. Choose the treatment and animal sex.

- Add remarks (optional) that will help you to memorize things regarding the experiment-related parts (e.g. animals) and click SAVE.

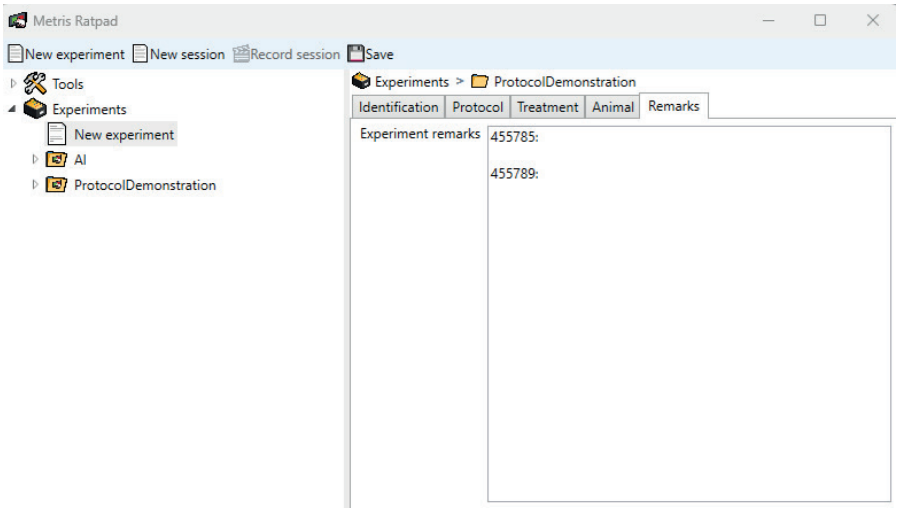


Figure 13. Input section of the RatPad program for adding remarks or important experiment notes.

2. Session Initiation

- Assign rats to chambers according to the protocol. Make sure you first click "Cages", properly assign all of the animals and only then proceed to the "Subsessions" tab. In case the order is not kept, issues may arise.

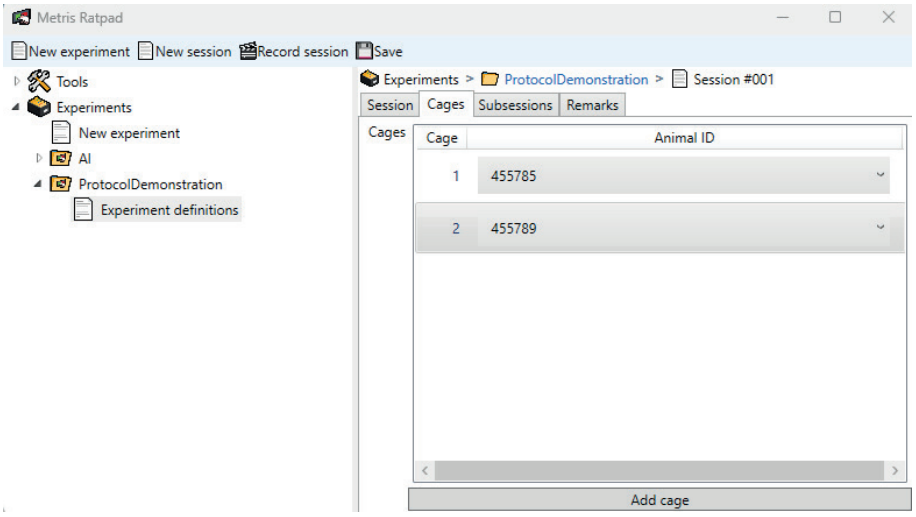
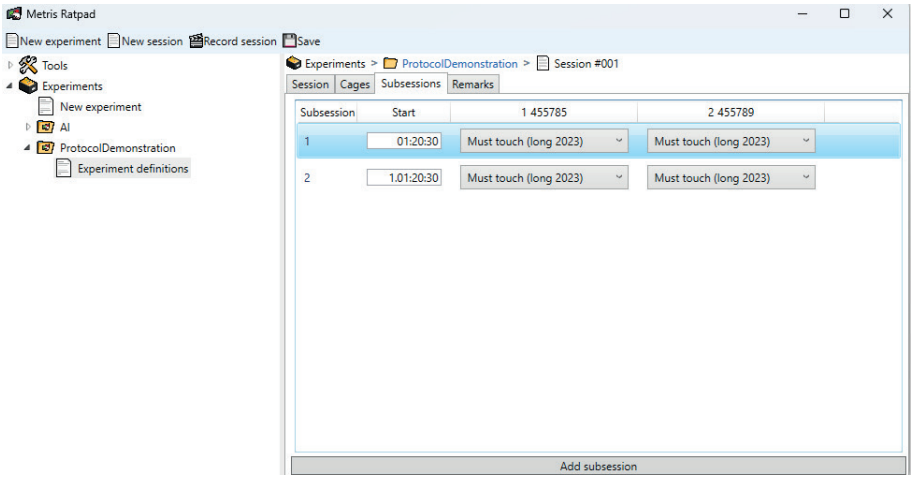


Figure 14. RatPad program interface for initiating the experiment session.

- Initiate the experiment session in the software. Click Save -> Record session.



When scheduling subsessions in the RatPad software, the "Start" column uses a specific time format to indicate when each subsession will begin. Understanding this format ensures accurate timing of behavioral tasks, especially in automated or multi-day protocols.

Time Format Used

- The countdown time is displayed as either:
  - o hh:mm:ss (hours : minutes : seconds)
  - o d.hh:mm:ss (days . hours : minutes : seconds)

Examples from Your Screenshot

1. Subsession 1
Start: 01:20:30

o Meaning:
- The subsession will begin after a countdown of 1 hour, 20 minutes, and 30 seconds from the Record Session start.

o Breakdown:
- 01 = 1 hour
- 20 = 20 minutes
- 30 = 30 seconds

2. Subsession 2
Start: 1.01:20:30

o Meaning:
- The subsession will begin after a countdown of 1 day, 1 hour, 20 minutes, and 30 seconds from the experiment/session start.

o Breakdown:
- 1. = 1 day
- 01 = 1 hour
- 20 = 20 minutes
- 30 = 30 seconds

o Note:
- If you see 2.00:00:00, it means exactly 2 days after the Record Session.

- The software will display real-time monitoring of session progress, including countdown timer, task stage, and chamber status.

If everything is functioning correctly, you will see the chamber indicators turn green.

- This means all chambers are detected, communication is established, and you can proceed by clicking Start Session.

If any indicator appears red (as shown in the example image), it means there is a connection or communication problem with that chamber.

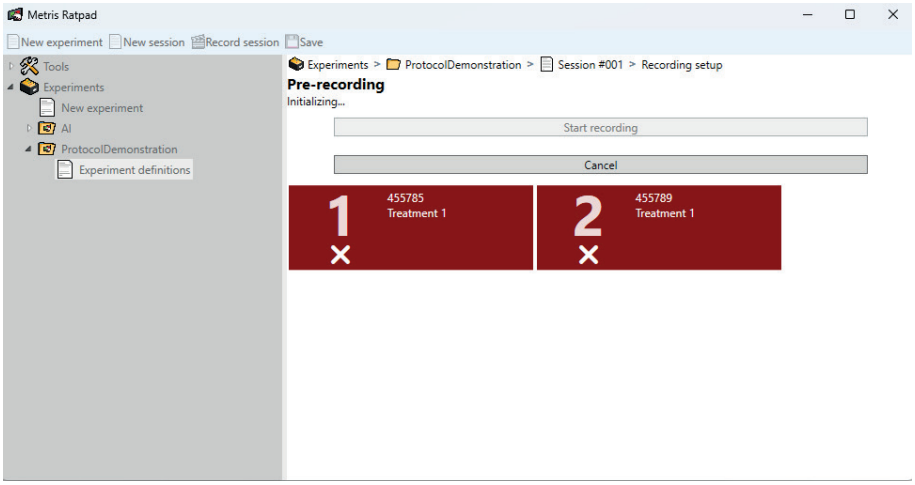


Figure 16. RatPad program error message indicating connection failure with one or more devices.

Possible Causes and Troubleshooting Steps

1. Check LAN Cable Connection
   a. Ensure the Ethernet/LAN cable is securely connected to both the computer and the RatPad system.
2. Verify Router Socket
   a. Confirm the LAN cable is plugged into the correct socket/port on the router (not a wrong port or loose connection).
3. Disable WiFi on the Laptop
   a. If you are using a wired LAN connection, make sure the laptop's WiFi is disabled to prevent network conflicts.
4. Restart the Hardware and Software
   a. Turn off all RatPad devices and completely close the RatPad program on your computer.
   b. Wait a few seconds, then turn the RatPad units back on.
   c. Reopen the RatPad software and try to record the session again.
5. Running the Experiment
   a. The RatPad system will automatically present tasks/trials on the touchscreen and log all relevant data (responses, times, errors, omissions, rewards).

b. Monitor the session via the software dashboard to ensure smooth operation.
c. Intervene only if the system displays an error or if animal welfare requires.

6. Data Download and Export
   a. At the end of the session, navigate to the Data Export tab.
   b. Download raw data files. Click download. Afterward click export.
   c. Save and backup the exported data to secure storage for analysis.
   d. If needed, review logs for any system warnings or anomalies during the session.

A.14 Operation of the Identification Program

1. Initiating an Experiment

- Open the Identification program on your PC (V2).
- Select 'Start New Experiment' in the menu.

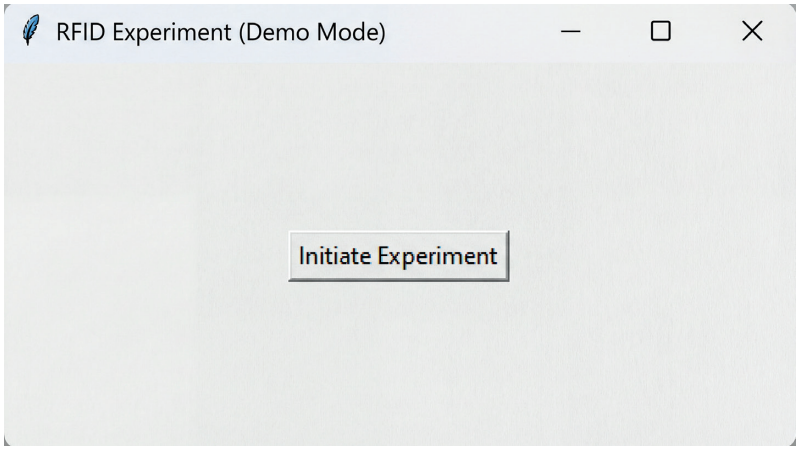


Figure 17. Start screen of the RFID identification program for beginning a new experimental session.

- Double-check that each scanned or entered code matches the animal assigned to the session. Click "Confirm Tags".

2. Inputting Identification Codes

- Manually type in the rat identification codes (RFID, animal number) or
- Use a laser barcode scanner to scan animal ID barcodes (ensure the cursor is placed in the correct field before scanning).

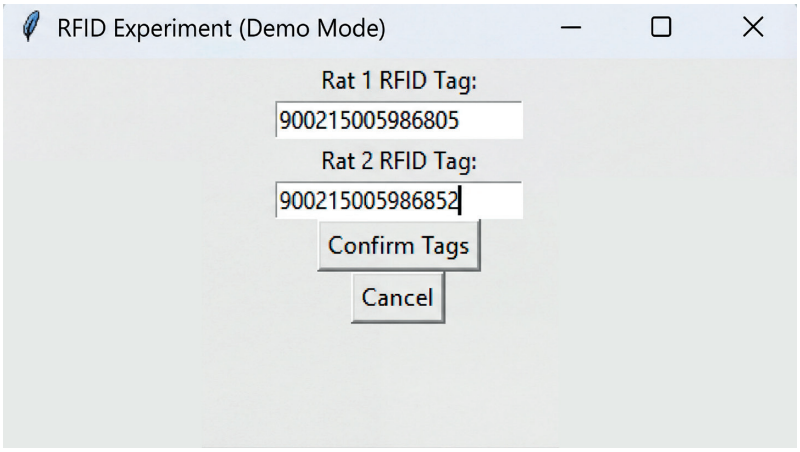


Figure 18. RFID program interface for manual entry or scanning of animal RFID codes.

Double-check that each scanned or entered code matches the animal assigned to the session. Click "Confirm Tags".

3. Verification

- After all IDs are entered, verify the codes once in the software and click "Next."
- The program will display a summary - double-check for typos or mismatches.
- If correct, click "Start Experiment."

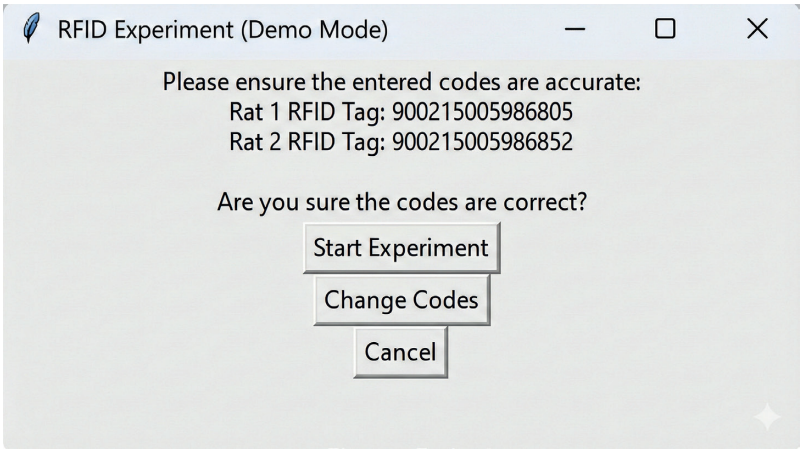


Figure 19. RFID program confirmation screen for verifying animal codes prior to session initiation.

4. System Running

The program should now display "Running." This means logging is active.

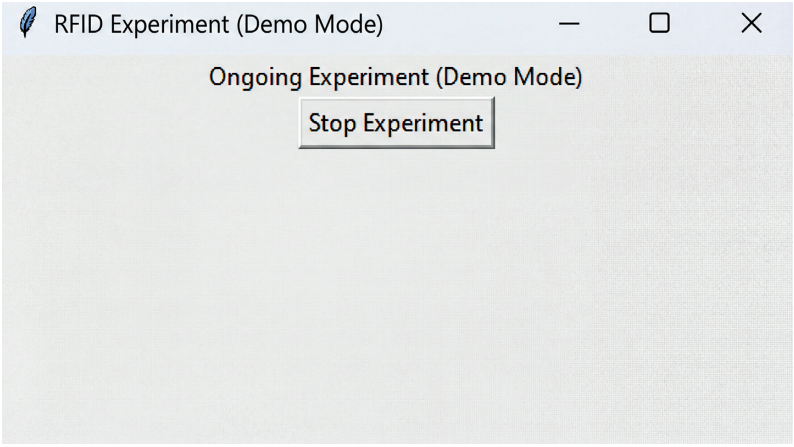


Figure 20. Real-time display of the RFID identification program during an ongoing experiment.

- You may manually open/close the ORT gates and observe in the software if feedback is correctly detected and recorded for each gate operation.

Figure 21. Terminal output example from the RFID identification program, displaying live event logging.

5. Data Logging and Export

The identification program will automatically save logged data (System ID, gate status, timestamps etc.) to a CSV file in the same directory where V2 is located.

System	Date	Time	Event	RFID Code	Weight (grams)
System 1	2024-11-13	12:41:47	System 1&Button pressed1s		
System 1	2024-11-13	12:41:48	System 1 state is ORT_Lock		
System 1	2024-11-13	12:42:44	Received RFID number	215005869684	
System 1	2024-11-13	12:42:45	System 1 state is AGO		
System 1	2024-11-13	12:42:46	Successful RFID Attempt	215005869684	
System 1	2024-11-13	12:43:15	Object detected on scale 1.		
System 1	2024-11-13	12:43:22	Object has exited the scale 1.		
System 1	2024-11-13	12:43:23	Highest Weight Recorded		151.7
System 1	2024-11-13	12:43:24	Object detected on scale 1.		
System 1	2024-11-13	12:43:56	Button released		
System 1	2024-11-13	12:43:57	System 1 state is ORT_Unlock		
System 1	2024-11-13	12:43:59	System 1 state is Idle		

Figure 22. Example of exported RFID program data as stored in a spreadsheet (.xlsx) file.

Explanation of the Event Log Data (Sample Sheet)

This table records the sequence of events detected by the Social RatPad system during a typical experimental session. Each row documents a timestamped event associated with animal movement, identification, or weighing.

Column Descriptions:

Table 2: Explanation of terms from RFID program-created .xlsx file

Column	Description
System	Identifier for the physical system/testing chamber (e.g., "System 1").
Date	Date the event occurred (YYYY-MM-DD format).
Time	Exact time of the event (HH:MM:SS).
Event	Description of what occurred (e.g., button press, RFID scan, animal detected, scale event).
RFID Code	The unique RFID tag code detected for a specific rat.
Weight (grams)	The recorded weight of the object/animal on the scale at the given time, if measured.

Event Sequence Example (as in image):

1. System 1&Button pressed1s:

A button or sensor in System 1 was pressed (this means the ORT gate got closed, a rat passed through it).

2. System 1 state is ORT_Lock:

The One-Rat Turnstile (ORT) is now in the locked state.

3. Received RFID number / Successful RFID Attempt:

The system detected and read the RFID tag, identifying the animal.

- If the event reads "Successful RFID Attempt," this means the rat accessed its assigned chamber (i.e., the system expected this rat to enter this chamber).

4. System 1 state is AGO:

The system advanced to the "AGO" (Access (Secondary) Gate Open) state, meaning the rat is now granted access or is in the process of moving through the corridor to the testing chamber (RatPad).

5. Object detected on scale 1 / Highest Weight Recorded:

The rat stepped onto the scale, and its weight was measured and recorded (e.g., 151.7 grams).

6. Object has exited the scale / Button released:

The animal left the scale and, ending the weighing phase.

7. System 1 state is ORT Unlock:

The ORT is now unlocked, the animal exited the system.

8. System 1 state is Idle:

The system is waiting for the next event or animal.

RFID Identification - Assigned vs. Non-Assigned Chamber

- **Successful RFID Attempt:**

Indicates that the rat was identified and allowed access to its designated (assigned) chamber.

This means the system logic confirmed the rat was in the correct location.

- **Unsuccessful RFID Attempt:**

Would indicate that the rat attempted to enter a non-assigned chamber (i.e., a chamber not designated for that rat at this time).

In such cases, access is denied, and this is logged for behavioral analysis (e.g., for studying errors, spatial memory, or motivation). In this case weight measurement does not take place.

How This Data Is Used

- Tracks precise animal movements and actions in the system.
- Verifies whether rats use their assigned chamber or make mistakes.
- Records RFID code and weight for identity verification and phenotyping.
- Allows for the reconstruction of the animal's activity sequence, trouble-shooting, and statistical analysis.
- Avoid opening the xlsx file ("microswitches_time_elapsed.xlsx") in Excel or another program during the experiment, as this may disrupt logging. It is

recommended to copy the file and then have a look at data on the copied file during the experiment.

6. Troubleshooting Common Issues

- If the microcontroller is not connected, the program will not run. Ensure USB/serial connection is active; restart if needed.

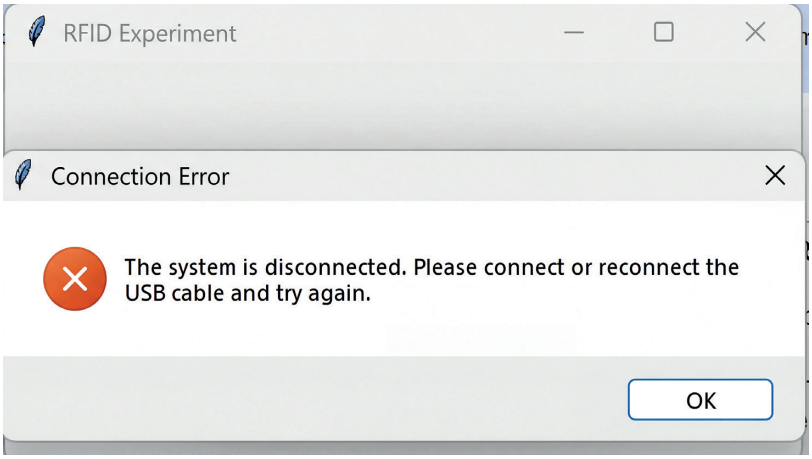


Figure 23. Error notification in the RFID identification program, indicating device or connection issues.

- If you receive a warning that the CSV file is open, close it in any spreadsheet editor before starting.
- After correcting any issues, restart the Identification program and repeat steps above.

Appendix B: Visual (Color-Based) Identification Method

B.1 Overview

This appendix documents the development and operation of the color-based identification module designed to recognize individual rats by colored tags. The system combines OpenCV-based computer vision, a Tkinter graphical interface, and automated Excel/JSON data logging. Its goal was to implement a self-contained tool that performs real-time tag recognition, provides calibration flexibility, and records detections with traceable precision.

Although RFID identification was later preferred for routine use, this module demonstrates the feasibility and workflow of low-cost optical recognition and can serve as a reference for future researchers.

B.2 System Architecture

The system is built from four modular Python scripts that communicate through shared data structures and function calls.

Table 1. Overview of the Python modules constituting the color-based identification system and their primary functions.

Module	Function
application2.py	Handles the graphical interface, user commands, and detection control.
tag_shape.py	Executes image capture, preprocessing, contour extraction, color classification, and confidence estimation.
json_module.py	Manages the database of color - code pairs and supports quick look-ups.
excel_sheet.py	Logs detection events with timestamp, color, code, and notes into a date-labelled Excel workbook.

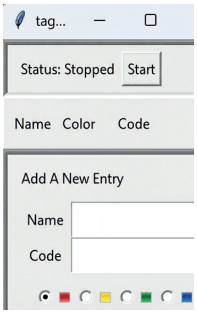


Figure 1. System architecture of the color-based identification software showing data flow between GUI, image-processing, and storage modules.

B.3 Graphical User Interface

The interface is divided into three logical sections:

1. **Control Panel** - Start/Stop button and system-status indicator.
2. **Registered Entries** - Displays existing tags (name, color, code) with delete options.
3. **Entry Form** - Fields for rat name and tag code with color-selection buttons (red, yellow, green, blue).

During live detection, input widgets are temporarily disabled to prevent accidental modification. Color options appear as small squares representing each hue.

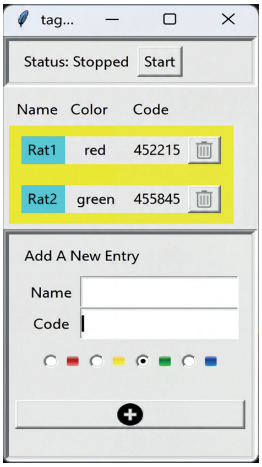


Figure 2. Main user interface of the color-recognition application showing control panel, registered tags, and color-selection fields.

B.4 Real-Time Detection Pipeline

Once started, the system continuously processes camera frames through the following stages.

Step 1 - Frame PreprocessSing

Frames are captured at 640 × 480 px resolution.
Noise is reduced and intensity normalized:

```
imgBlur = cv2.GaussianBlur(img, (7,7), 1)
imgGray = cv2.cvtColor(imgBlur, cv2.COLOR_BGR2GRAY)
```

Edge boundaries are extracted with the Canny algorithm:

imgCanny = cv2.Canny(imgGray, threshold1, threshold2)

Threshold values can be tuned live via the *Parameters* window.

Figure 3. Processing stages: (a) raw image, (b) grayscale conversion, (c) Canny edge output.

Step 2 - Contour Detection and Filtering

Closed contours are located in the binary edge map and filtered by:

- Area: areaMin < area < 4 × areaMin
- Shape regularity: w/h ≈ 1
- Polygon vertex count: len(approx) < 6

Valid contours are considered potential tags. The largest suitable contour is selected for color analysis.

Figure 4. Example of contour selection and bounding-box detection on a colored tag.

Step 3 - Cropping and Alignment

To ensure orientation-independent analysis, the contour is enclosed in a minimum-area rectangle and cropped upright:

```
croppedRotated = cv2.getRectSubPix(
  cropped, (int(croppedW), int(croppedH)), (size[0]/2, size[1]/2))
```

This guarantees consistent region sampling regardless of tag rotation.

Step 4 - Color Classification

The cropped region is converted to HSV space, and each hue is masked with predefined thresholds:

```
HSV_RANGES = {
 'red':   [(0,100,150),(15,255,220)],
 'yellow': [(20,100,150),(30,255,220)],
 'green':  [(40,20,70),(70,255,220)],
 'blue':   [(70,20,70),(120,255,220)],
}
```

The color with the highest proportion of masked pixels is accepted if:

- The colored area ≥ 25 % of the crop, and
- The dominant color ≥ 60 % of the total mask.

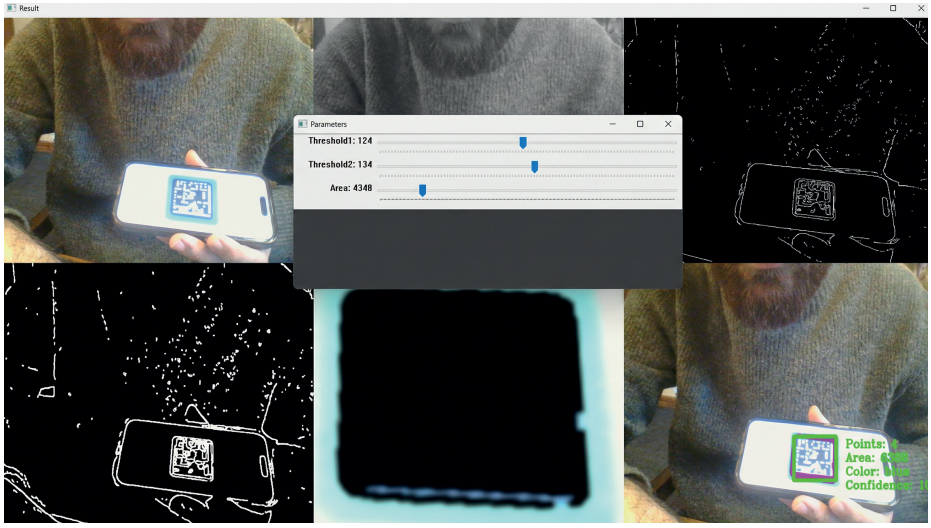


Figure 5. Correctly detected tag displaying color label and confidence value.

Confidence appears on screen (e.g., *Color: Blue - Confidence: 100%*).

Step 5 - Decision and Logging

When a color remains stable for ≈ 1 s, the program retrieves its record:

```
obj = json_module.find_entry(color = majority_col)
```

and logs it into Excel:

```
excel_sheet.insert_data(obj, mode='camera')
```

Each record includes timestamp, rat name, color, code, mode ("camera"), and confidence. A new Excel sheet is created automatically each day.

B.4.1 Adjustable Parameter Control (Real-Time Calibration Bar)

A dedicated *Parameters* window enables live tuning of the three key detection variables.

Table 2. Adjustable parameters available in the "Parameters" window with typical value ranges and descriptions.

Parameter	Typical Range	Function
Threshold 1	0 - 255	Lower Canny limit - controls edge sensitivity.
Threshold 2	0 - 255	Upper Canny limit - confirms strong edges.
Area	1 000 - 30 000 px^2	Minimum contour area accepted for color analysis.

Each frame updates values:

```
threshold1 = cv2.getTrackbarPos("Threshold1","Parameters")
threshold2 = cv2.getTrackbarPos("Threshold2","Parameters")
areaMin  = cv2.getTrackbarPos("Area","Parameters")
```

Threshold 1 - Edge Sensitivity

Controls how faint gradients are treated as edges. Lower = more edges (more noise); higher = cleaner edges.

Threshold 2 - Edge Confirmation

Pairs with Threshold 1 to define edge quality; higher values retain only strong boundaries.

Area - Contour Filter

Rejects shapes smaller than the defined pixel area, preventing false detections.

Together, these parameters determine the clarity of tag borders and the number of valid contours passed to color classification.

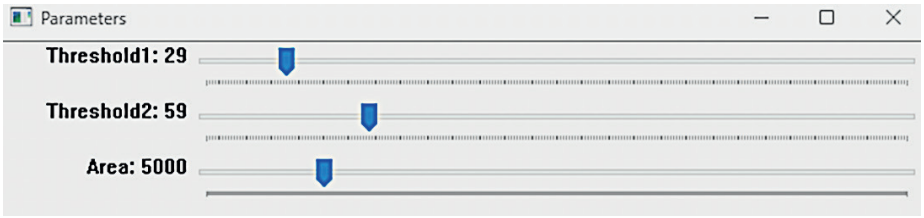


Figure 7. "Parameters" window with adjustable sliders for Threshold 1, Threshold 2, and Area, used for real-time tuning of detection performance.

B.5 Data Management

Two synchronized data stores ensure comprehensive record keeping:

1. **JSON file (data.json)** - Holds tag registry (name - color - code).
2. **Excel file (data.xlsx)** - Logs every detection with timestamp and confidence.

Time	Name	Color	Code	Mode	Notes
15:43:33	Rat 1	blue	PP6302	camera	Confidence: 99.81%
15:43:34	Rat 1	blue	PP6302	camera	Confidence: 100.00%
15:43:35	Rat 1	blue	PP6302	camera	Confidence: 100.00%
15:43:36	Rat 1	blue	PP6302	camera	Confidence: 99.99%
15:44:39	Rat 1	blue	PP6302	scanner	
15:44:42	Rat 1	blue	PP6302	scanner	

Figure 8. Example Excel output showing timestamped detections with associated metadata.

B.6 Recommendations for Future Development

- Implement adaptive HSV thresholding responsive to lighting.
- Use high-dynamic-range cameras, AI cameras or constant LED illumination.
- Apply matte, non-reflective pigments on tags to stabilize hue.
- Combine this visual module with RFID for multimodal redundancy and accuracy checking.
- Add batch-analysis utilities for automated accuracy assessment.

B.7 Summary

Table 3. Main technical specifications and adjustable features of the color-based identification system, summarizing its core functionality and performance characteristics.

Feature	Description
Programming Environment	Python 3.8 + OpenCV + Tkinter
Input	USB camera
Adjustable Parameters	Threshold 1, Threshold 2, Area
Output	Excel + JSON
Typical Frame Rate	20 - 30 fps
Principle	HSV color segmentation with confidence filtering
Strength	Fully adjustable real-time calibration interface

The color-identification module provides a transparent and reproducible workflow that links computer-vision processing, user interaction, and automated data capture.

It demonstrates how low-cost optical sensing can achieve structured and timestamped behavioral data.

Despite its sensitivity to lighting variation, the system validates the concept of using dynamic HSV thresholding and real-time calibration as part of automated behavioral platforms. Future hybrid systems can combine this approach with RFID or infrared sensing for more robust identification accuracy checking, while still relying on the same modular codebase and calibration interface.

List of Abbreviations

Abbreviation	Full Term	Notes
5-HT	5-Hydroxytryptamine (Serotonin)	Neurotransmitter
5-HTT	Serotonin Transporter	Also SERT
5-HTTLPR	Serotonin-Transporter-Linked Polymorphic Region	Genetic polymorphism
ANOVA	Analysis of Variance	Statistical test
CCD	Centrale Commissie Dierproeven	Dutch Animal Experiments Committee
EU	European Union	
FPI	Ferplast-to-cage Plastic Interface	Tube connector in apparatus
KO	Knockout	Genetically modified (e.g., TPH2 KO)
LABORAS	Laboratory Animal Behaviour Observation, Registration and Analysis System	Automated behavioral analysis
ORT	One-Rat Turnstile	Size-adjustable gate in RatPad system (Introduced by Grayson Butcher)
PND	Postnatal Day	Age in days after birth
RFID	Radio-Frequency Identification	Used for animal tracking/ identification
SERT	Serotonin Transporter	Same as 5-HTT
TPH2	Tryptophan Hydroxylase 2	Enzyme for serotonin synthesis in brain
TUNL	Trial-Unique Non-Matching to Location	Working memory task
WT	Wild-Type	Non-mutant/control animal
SD	Standard Deviation	Statistical term
CV	Coefficient of Variation	Statistical term
SD	Sprague Dawley	Rat strain
RDM	Research Data Management	

A

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