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Programvezető: Dr. Szökő Éva, egyetemi tanár

Témavezető: Dr. Lévay György, egyetemi tanár

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BEHAVIORAL ANALYSIS OF PHARMACOLOGICALLY TREATED RODENTS IN AUTOMATED HOMECAGES

PhD thesis

Péter Pelsőczi

Semmelweis University

Doctoral School of Pharmacological Sciences

Supervisor:	György Lévay CSc
Official reviewers:	István Gyertyán PhD
	József Halász PhD
Head of the Complex Examination Committee:	Éva Szökő DSc
Members of the Complex Examination Committee:	Péter Petschner PhD
	Klára Felszeghy PhD

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Table of Contents

DOI:10.14753/SE.2021.2523

DOI:10.14753/SE.2021.2523

1. LIST OF ABBREVIATIONS

DOI:10.14753/SE.2021.2523

T : thalamus 27
USV : ultrasonic vocalization..... 22
VPA : sodium valproate / valproic acid..... 17

2. INTRODUCTION

2.1. Challenges of Behavioral Studies

Almost two decades ago, Robert Gerlai predicted the birth of a new field he named 'phenomics' [1]. The growth of the number of genetic models induced in mice generated an elevated need for phenotypic analysis. In parallel with the revolution of molecular biology - especially with CRISPR/Cas9 system – instrumentation and bioinformatics evolved significantly. These developments stimulated behavioral sciences as well.

Behavioral studies have been slow and labor-intensive; often narrowly focused on one specific readout. These experiments are also space- and time-intensive. Rodents are usually kept in facilities that are separated from the experimental laboratories. Behavioral studies often use sizable mazes, which are needed to be placed in separate rooms.

Traditional behavioral tests are often based on observation (scoring) by one experimenter, hence increasing the chance of being subjective [2]. This could lead to unrepeatable or incomparable results by different laboratories. To overcome these obstacles, researchers suggested that a battery of tests should be conducted to have a good understanding of a new phenotype [3]. Such batteries have gained recognition like SHIRPA protocol [4] or CANTAB [5]. Batteries enable the standardization of behavioral data, making it comparable between laboratories. A pharmaceutical company benefits from a battery optimized to a specific disease target. For example, a test battery aimed at studying Alzheimer's disease (AD) is needed to analyze major domains of cognition: attention, short- and long-term memory and executive function.

Another solution to tackle observer bias can be achieved using video tracking softwares to record and analyze rodent behavior such as EthoVision [6], or TSE's VideoMot2 [7].

To target the demand for more and better-quality data in behavioral science, different strategies can be applied. First, scalability could solve the problem (more units of the apparatus collect information on the behavior which run in parallel). Second, to increase the information density of the test by increasing the number of behavioral measures of the brain function of interest. Third, to increase the flexibility of the tests; a single apparatus could measure a broad spectrum of behaviors, e.g. anxiety, social behavior, learning, impulsivity, etc.

Novel advancements in computational speed and memory capacity allow several units of apparatus run by a single computer which monitors and simultaneously records numerous variables in the same time.

There are now some commercially available instruments which meet the aforementioned expectations. MED Associates (Vermont, USA) created an equipment using force-transducer technology, which monitors eight chambers at a time and records several behavioral outputs [8]. Moving and posture is analyzed, the software creates an ethogram. This is a complex data set, from which many types of behavior can be extracted.

PsychoGenics Inc. (Terrytown, NY, USA) developed SmartCube, NeuroCube and PhenoCube systems. SmartCube captures a large number of behavioral and physiological readouts, including video recording, measures frequency, duration and intensity of numerous behavioral parameters and heart rate, on a high-throughput scale. This cage uses actors such as food pellet or water reward, levers and even punishment which allows it to carry out complex Pavlovian or operant conditioning behavioral paradigms [9]. The modular design allows to flexibly change the setup, adding e.g. a shock grid if needed. The density of information it produces makes it especially applicable in pharmaceutical industry. Their equipment covers cognitive, motor, circadian, social, anxiety-like, gait and other domains, using custom-built computer vision software and machine learning algorithms for analysis.

2.2. Learning, Memory and Cognition

What is learning, cognition and memory? “Learning is referred to as a more or less permanent change in behavior that occurs as a result of practice” [10]. Kandel defined learning and memory “Learning is the process by which we acquire knowledge about the world and memory is the process by which that knowledge of the world is encoded, stored, and later retrieved” [11]. Cognition is a much broader term: includes processes such as memory, association, language, attention, concept formation and problem solving [12].

Memory can be divided into short- and long-term memory. Long-term memory can be further divided into declarative or explicit memory or non-declarative (implicit) memory. Declarative memory answers the question “what”, non-declarative memory answers “how”. Declarative memory is further sub-divided into episodic and semantic memory.

The major brain structures involved in declarative memory is the hippocampus and other medial temporal lobe structures [13]. Formation of a new declarative memory is a sequential process that includes acquiring new knowledge (encoding), retaining the information (storage), and bringing it back (retrieval). Furthermore, memories are continually being consolidated in the neocortex [14].

2.3. Cognitive Impairment and Its Rodent Models

Cognitive impairment or dementia is a symptom of various neurodegenerative diseases, but the majority of cases are linked to AD. It can be characterized by progressive cognitive, functional and behavioral impairment, which evolves into a significant loss of cortical and subcortical functions and ultimately leads to death[15](Van Dam & De Deyn, 2006). The successful use of animal models in drug discovery relies on both the development of valid disease models and the availability of adequate testing paradigms for evaluating the effects of different therapeutic approaches.

There are a number of transgenic knockout/in (e.g. APP23 [16], APP/PSEN [17]) or chemical knockout models. One can induce certain aspects of the disease with surgical lesion procedures (bulbus olfactorius or the nucleus basalis magnocellularis, the rodent analogue of the human nucleus basalis of Meynert), or with pharmacological interventions (e.g. scopolamine). It is also possible to induce some aspect of the disease with intracerebral injection of amyloid precursor protein, or - to induce neuroinflammation - lipopolysaccharide. Perhaps one of the most valid model is aged animals, although the process is costly and obviously time consuming [18]. Models based on many different species including invertebrates, zebrafish, primates, or other mammals have contributed to the field of AD research, rodent models are predominant.

A valid animal model resembles the human condition in aetology, pathophysiology, symptomology and response to therapeutic interventions. In reality, most animal models are partial models and focus on restricted aspects of a disease. Modelling the complete condition is hardly possible in AD research [15]. For example ageing rodents do not spontaneously develop amyloid plaques and neurofibrillary tangles, but they show behavioral alterations and cognitive decline [19].

2.4. Traditional Behavioral Methods to Study Learning & Memory

Historically, mazes have been heavily used methods to assess hippocampal-based spatial memory mainly in mice but also in rats. They are usually on dry land such as T-maze, Y-maze, Cheeseboard maze tests or Radial Arm maze [20]. Aquatic version of mazes are also exists, one of the most widely used is Morris Water Maze test [21]. Barnes maze is basically a land version of the Morris water maze, which was developed to avoid the stress induced by swimming [22]. Hole board discrimination test was originally used to study exploratory behaviors [23]. Later it was modified to study learning where the subjects had to locate and retrieve food pellets. Novel Object Recognition Test is also an often-used paradigm to assess declarative memory [24].

There are many further paradigms using shuttle box (passive or active avoidance tests) or operant conditioning chambers (Skinner boxes). In the latter one, one can train animals to press retractable levers based on rewarded learning. For example, delayed matching to position tasks are carried out in such chambers and allows to study the working memory of the animals. Newer methods including Touchscreen based learning methods allow to harness rodents' visual abilities and is also usually rewarded by food pellets [25].

Regardless of the type of all these aforementioned tests, they all have certain disadvantages. The short maze tests do not allow a long period of habituation, hence increasing the sensitivity to environmental factors. Food pellet-based tests often need food deprivation not only prior to experiment but throughout an extended period of time, causing unknown behavioral changes to the animals. Water based mazes are highly stressful to the animals [15]. Morris Water Maze Test is also confounded by motor impairments and ceiling effects (i.e., swimming velocity) that reduce its sensitivity in detecting improvements in cognition [26]. Shuttle box tests and certain type of mazes can be done in a high-troughput manner, but on the other hand can be done only once on a given animal. Almost all of these methods are also labor intensive. Operant conditioning paradigms (either in Skinner box or Touchscreen methods) can have a very long training period, even several months. All of these tests require the experimenter to handle the animals; remove them from their home-cage. This is a known source of stress, which is a factor negatively affect cognition [27].

Conflicting results are common in behavioral studies. Seemingly, especially in the last decade, the field is moving towards translational cognitive tests, automatization and robust and reproducible protocols.

2.5. Automated Home-cage Systems

San Diego Instruments (USA) created a Photobeam Activity System Home Cage, where mainly the movements (horizontal and vertical) are measured and recorded. Tecniplast (Italy) has its own home cage recording system combined with their digital ventilated cage technology. Metris (Netherlands) manufactures the LABORAS system, which identifies tiny movements made by the animals and translates them into behaviors e.g. locomotion, grooming, climbing, eating/drinking etc. Some system uses automated video analysis, e.g. Clever Sys Inc.' (USA) HomeCageScan, Boca Scientific Inc.'s (USA) Home Cage Analyzer or the NOLDUS (Netherlands) developed PhenoTyper which uses Ethovision software. There are now even open-source equipment which could be relatively easily assembled [28,29].

2.6. IntelliCage

The creators of IntelliCage (Figure 1) (distributed by TSE Systems GmbH) stepped even further, allowing the researchers to keep a group of animals in the same cage, where the experiment is carried out [30].

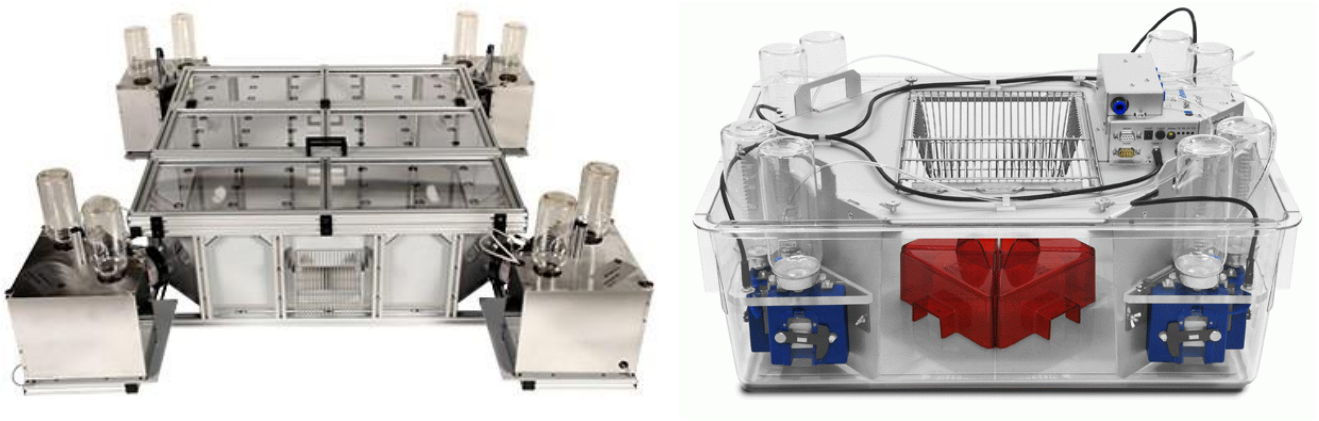

Figure 1 Rat and mouse IntelliCages

This design has many advantages. It saves laboratory space (no separate animal facility and experimental rooms are needed) and gives the opportunity to keep the rodents in a social environment. The latter is very important, because rodents, just like humans, are highly social animals. In the vast majority of the behavioral assays the animal is taken out by the experimenter from its homecage, causing handling stress which distorts data (Balcombe et al., 2004; Gärtner et al., 1980). Stress caused by handling can be explained by rodents' ecological role in nature as prey animals. Automated monitoring often takes place in the absence of the experimenter, which is key when studying prey species such as rodents where immobility may be adaptive and the presence of the experimenter may mask behavioral indicators of animal discomfort, particularly when pathological changes are mild to moderate [33].

Using IntelliCage, the experimenter can minimize human interference, hence providing a relatively stress free and socially enriched environment for the animals, increasing their welfare. If assessment of the animals using automated technologies is carried out in an enriched and complex environment, this is likely to encourage a broad range of species-typical behaviors as well as allowing animals to maintain some control over which resources they invest in [34].

The system employs a transponder-based technology to monitor the location of several animals in the cage. Another benefit of automatization is that animals can perform the preprogrammed tasks voluntarily in their active phase. Rodents are nocturnal, and they often are experimented during their inactive phase, severely impacting behavioral results. The effect of the circadian rhythm on cognitive performance is long known [35].

Another example for the instruments' advantage is that it makes possible to collect data for an extended period of time. One can follow a disease progression, and find the best timing for an intervention based on the actual phenotype [36].

Laws on animal experimenting are more and more rigorous in the EU. The 3Rs (reduce, refine and replace) are an important consideration when designing a new experiment [37]. In traditional behavioral assays, researchers usually do one type of experiment on a group of animals. However, automated cages allow us to design a set of consecutive paradigms - with an increasing complexity – on the same batch of animals, reducing greatly the number of animals needed.

A consecutive set of paradigms - or batteries as mentioned before - gather huge amount of data. Bioinformatics tools, such as Bayesian statistical simulations, multivariate statistical methods or principal component analysis are required to interpret information from all these data. Even deep learning can be harnessed for pattern recognition in the data pool. Sorting, filtering and analyzing this amount of data is a challenging and pioneering effort, behavioral scientists have to apply analytical procedures which was never used before in the field.

2.7. ASD

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by social and communicative impairments and excessive repetitive behaviors [38]. A 2012 review commissioned by WHO estimated the global prevalence of ASD was about 1% [39].
Although many consider the pioneer study on autism "Autistic disturbances of affective contact" by Leo Kanner [40], the disorder was first described by Hans Asperger in the 1930s in Vienna, while he named it autistic psychopathy. His work remained unrecognized until the 1980s. His contribution to the field is undeniable, but it has been overshadowed by recent findings on his strong ties to the eugenic program on autistic children of the Nazi Germany [41]. In the past 50 years, autism spectrum disorder (ASD) has gone from a narrowly defined, rare disorder of childhood onset to a well-publicized, advocated, and researched lifelong condition, recognized as fairly common and very heterogeneous [42]. ASD is believed to result from early altered brain development and neural reorganization [43]. Because there are no approved and reliable biomarkers, diagnosis must be done on the basis of behavior [42]. Although the pathogenesis of ASD is not fully understood, several factors have been identified as possible contributors such as genetic [44–46] or environmental factors [47,48]. Several studies also suggest advanced maternal (≥40 years) and paternal (≥50 years) age as an aggravating factor to ASD [49]. During pregnancy, maternal admission to hospital due to bacterial or viral infections have also been associated with a mildly increased risk of ASD and developmental delay, combined [50]. To date, there is no pharmacotherapy proven to be effective in treating core symptoms of ASD [51]. There are two major scientific goals concerning ASD research: clarify the still unexplored neurological basis of the disease and develop effective drugs against symptoms of ASD. A reasonably

sound way to achieve such advances in the field is to study and use rodent models of ASD. Hallmarks of autistic-like symptoms in rodents can be measured with several behavioral assays such as the three-chamber social interaction, self-grooming, ultrasonic vocalization, tube dominance or social play tests. However, in most of these tests the subject is removed from its home cage and must quickly adapt to a novel environment. In these situations, animals are exposed to excessive human handling that can cause unnecessary stress and might have a serious impact on the natural behavior of the experimental animals. This stress can lead to an elevated level of anxiety which could introduce a strong bias in the results.

2.8. Scopolamine

The cholinergic muscarinic antagonist scopolamine is generally found to increase locomotor activity [52–54]; with evidence that cholinergic signaling in the hippocampus, striatum and frontal cortex is positively correlated with scopolamine-induced hyperactivity [55]. In contrast, studies with scopolamine in learning tasks have identified either decreased locomotor activity[56,57], or no effect [58]. In addition to the effects on activity, the blockade of muscarinic receptors with the non-specific antagonist scopolamine prior to a learning task has been consistently reported to impair spatial learning and memory in both rats and mice [59,60]. In our study we investigated activity, drinking behavior and reversal learning of C57BL/6J mice treated with various doses of scopolamine in the IntelliCage system.

2.9. Valproic Acid

Prenatal exposure to various factors can lead to neurodevelopmental disorders such as maternal immune activation, stress, poor nutrition, etanol or thalidomide [61,62]. Valproic acid or valproate (VPA) is an anti-epileptic drug and used clinically in other neuropsychological disorders as well. However, it has been reported in a study where they involved more than 650.000 children over ten years, that maternal use of VPA during pregnancy significantly increased the risk of ASD in the offspring [63]. This and earlier results suggesting VPA's effect during pregnancy inspired scientists to use the compound to successfully mimic the main hallmarks of ASD in rodents [64–70].

2.10. Social Agonistic Behaviors in Rats

Social behaviors can be divided into subsequent categories: agonistic behavior (social- and maternal aggression), play-fighting, allogrooming and communication (vocal and olfactory). Rat is a social species; they establish dominance hierarchies through social agonistic behavior. Social agonistic behavior includes avoidance, appeasement and aggression behavior that occurs between members of a social group [71].

Agonistic behaviors are a complex sequence of behaviors with various intensity and duration. The lowest intensity encounter is chasing. During chasing, the chased rat flees, and either outruns the chaser or the chaser stops chasing it any further. If the pursuer catches up, it attempts to engage the other in an encounter. If the chased rat resists, an encounter occurs. As intensity increases, stand-offs occur and boxing or sidling continues. This rarely escalates into fights. These fights in most cases are non-lethal. The goal is for one rat to deliver a bite to the other rat's rump and prevent from getting bitten as well at the same time.

The defender may show off its teeth, do long squeaks and hisses. The tails often repetitively hit the ground and both rats' fur may show piloerection. Most of the time the encounter stops here, finished with one of the rats fleeing or hiding. If neither rat flees, the stand-off escalates into physical contact. It continues with boxing or sidle and they may push or kick each other when in range [72].

Aggressive neck grooming is also a type of agonistic behavior. Grooming consists of rapid nibbles in which the groomer (the dominant male) seizes folds of the neck skin between his teeth [73]. The groomed remains immobile and may peep or squeak softly. Any sudden movement of the groomed is punished by a kick or a bite from the dominant male. The intricate complexity of these behavioral sequences clearly indicates the necessity of intact social skills to decode all these interactions, which would be expected to be impaired in rats with ASD-like features.

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3. OBJECTIVES

Our main objective was to harness the power of huge amounts of data when analyzing rodents' behavior in IntelliCage. We aimed to establish a new standard methodology, accompanied by developing new statistical procedures to analyze appropriate parameters to follow behavioral changes.

First, we aimed to establish a reward driven place preference learning paradigm in mice. We plan to apply an already well studied and documented mouse model of cognitive impairment, using an anticholinergic agent, scopolamine, to disturb the formation of long-term memory. To increase the challenge for the subjects, sequentially we plan to introduce reversal learning in a form of changing the rewarded corner regularly. We study not only the learning abilities of the mice during the process but the general activity, the nosepoking and the drinking behavior as well.

In a separate study, we wanted to further characterize the rat VPA model, with a new equipment and with a new way of collecting and analyzing data, hoping we detect previously unknown behaviors which could be utilized as potential new biomarkers. Our aim was to discover behavioral patterns when animals are kept in a social environment, relatively undisturbed while they are observed during their natural, undisturbed circadian cycle. We planned to establish a phenotype first with conventional methods used to study various symptoms associated with Autism Spectrum Disorder (ASD) of rats Using the phenotype data, we preselected a group of animals and designed a set of experiments using the IntelliCages.

Communication is a pivotal part of social interaction and social cognition. As the latter domain is highly affected in ASD, it is an often-targeted area of study in ASD. However, adult animals' communication is hardly measurable. The process of forming hierarchy within a group of rats is shaped by social agonistic behaviors. It includes chasing, boxing, nipping, biting which behaviors are certainly the result of audio-visual communication between the animals. Hierarchy can be interpreted as a cumulative result of all these agonistic interactions. Studying the dynamics of the hierarchy, one could deduce the communicative skills of the autistic animals. We have run the experiment for

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an extended period with relatively minimal challenge. The last part of the study was an amplified competitive situation utilizing partial water deprivation, which urged the rats to establish a strict hierarchical structure within each group.

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4. MATERIAL & METHODS

4.1. Animals

32 male C57BL/6JOlaHsd mice (25–30 g) were purchased from a commercial vendor (ToxiCoop, Budapest, Hungary) and acclimated for two weeks prior to testing. Mice were implanted with microchips under isoflurane anesthesia 1 week prior to placing them into the IntelliCages. At 8–12 weeks of age, mice were group housed (16 per cage) and kept in our animal facility with a 12 h light/dark cycle (lights off at 3 p.m.), ambient room temperature was maintained at 22 ± 2ºC and 40–50% relative humidity. Food and water were freely available. Food was freely available; water access was related to specific tasks.

Twenty eleven-week-old male Wistar rats were chosen from the F1 generation (n=10 in control and n=10 in VPA group, selected from 4 litters in each treatment group) and were placed separately in two IntelliCages. Animals were implanted with microchips (UNO PICO-ID ISO transponder, UNO BV, Netherlands) under isoflurane anesthesia 1 week prior to the experiments. Rats were kept in the animal facility with 12 h light/dark cycle (lights off at 4 p.m.), while ambient room temperature was maintained at 22 ± 2ºC and 40–50% relative humidity. All efforts were made to minimize the suffering of experimental animals. Experimental procedures were reviewed and approved by the Local Animal Care and Use Committee (PE/EA/2885-6/2016) and were carried out in accordance with the European Animal Protection Directives (Directive 2010/63/EU).

4.2. Pharmacological Treatments: Acute Scopolamine and Prenatal Valproate Treatments

Scopolamine-HBr was purchased from Tocris (UK). The formulations were prepared freshly on each experimental day in PBS solution, to avoid scratching due to acidity. Scopolamine and its vehicle were injected subcutaneously (s.c.) at a volume of 5 ml/kg body weight. The following doses and groups were tested: scopolamine (0.05, 0.17 and 1 mg/kg n=16, each); vehicle (PBS, n=16). The drug doses used in this study were previously found to be effective in studies of spatial learning and locomotor activity. Two

IntelliCages were used for the experiment, where 8 mice of each treatment group were treated with either vehicle or scopolamine. Administration was scheduled approximately 15 minutes prior to the start of the dark phase of testing (3 p.m.). Due to rapid onset of the drug's pharmacodynamic effects (occurs as soon as 20 minutes following s.c. treatment), to decrease the time needed for administration, only 16 mice were injected in a given experiment. The next day the other 16 mice were injected, then data were pooled. Altogether, results of 12 experiments are analyzed. After each experiment, the animals had 4-6 days to recover from the scopolamine effect, to avoid any behavioral change in vehicle treated animals due to within-subject design.

Timed-pregnant Wistar rats (outbred stock, Janvier, France) kept on soy-free diet (Teklad soy protein-free rodent diet, ENVIGO, Madison, WI, USA) and tap water received a single dose of 300 mg/kg sodium valproate (VPA, cat. P4543-10G, Sigma, UK) intraperitoneally in a volume of 2.5 ml/kg physiological saline on gestational day 12. The pregnant rats were transported from Janvier to our animal facility. Control dams received an injection of physiological saline of identical volume at the same gestational time-point. The size of litter was adjusted to 10 for each dam (by removing female pups) and then left undisturbed until the time of weaning on postnatal day 21 when the male offspring were housed in groups of 3 or 4 until behavioral testing.

4.3. The IntelliCage Apparatus

The IntelliCage system (Figure 1) (TSE Systems, Bad Homburg, Germany; https://www.tse-systems.com/product-details/intellicage) allowed group-housed rats to be assessed for spontaneous behavior and various other behavioral tasks. The size of the central arena was 100x100x35 cm. As bedding material wood shavings were used (OSAFE, J. Rettenmeier & Söhne GmbH, Rosenberg, Germany). In order to enrich the environment two black plastic shelters were placed in each cage, allowing the animals to hide and climb (TSE Systems, Bad Homburg, Germany). The IntelliCage has four recording corners. Water was only available in the corners behind remotely controlled doors. When a rat entered a corner, an antenna detected its unique transponder and recorded its visit. It needs to be emphasized that the corner design allowed the entry of only one animal at a time. Each corner housed two drinking bottles, while left and right

sides could be distinguished. The activity of the rats within the corners was monitored by using a tracking software (IntelliCage Plus 3.1.1.0, TSE Systems). The principal parameters were: the number of visits to the corners, initiated nosepokes, lick numbers and the durations of all these parameters.

4.4. Training Phases in the Mouse IntelliCage

In the first phase of the mouse IntelliCage study, mice were allowed to habituate to the new environment for 7 days. The preprogrammed protocol allowed them to visit any corner any time during the day. They could also choose in a corner any of the two bottles to drink from. The number of trials was not limited and mice voluntarily visited the corners. Cohort sizes in both cages were n=8 per treatment group. In the second phase of the study mice had to learn to perform a nosepoke within the corners to gain access to the drinking bottle for 7 seconds. During any visit, only the first nosepoke results in door opening. In the third phase, the place preference learning paradigm, mice were evenly assigned to all four corners: for any individual mouse one corner was correct, while all three other corners were incorrect. Mice were allowed to drink in any of the four corners (incorrect ones included) but in the incorrect corners they received a 5-sec air-puff (2 bar) as punishment. In the fourth and final phase, the reversal learning paradigm, the position of the correct corner was rotated clockwise daily (Figure 2A).

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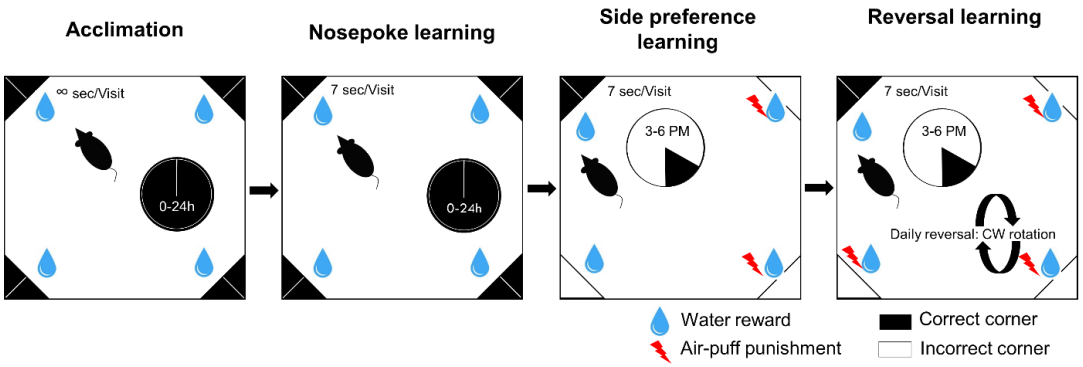
A



B

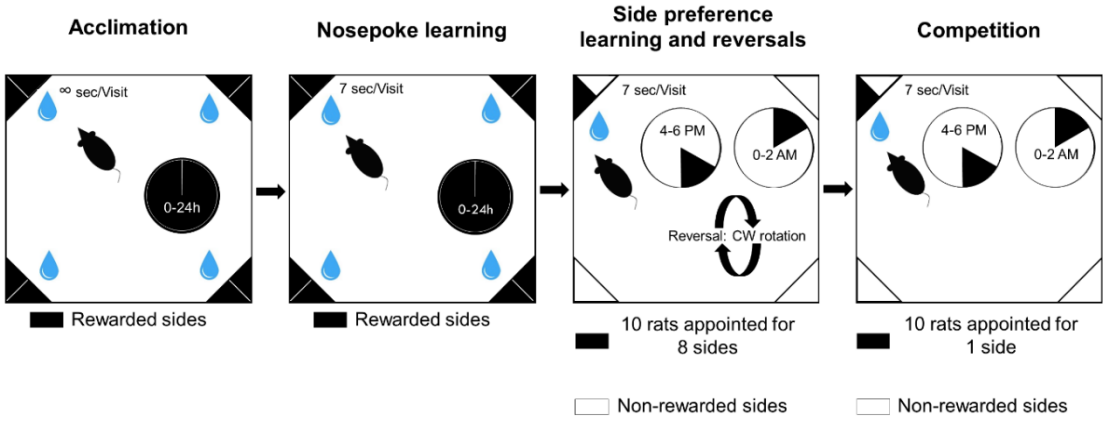


Figure 2 Schematic representation of the mouse (A) and rat (B) IntelliCage studies experimental phases and visual description of the tasks

4.5. Training Phases in the Rat IntelliCage

Control and VPA groups were tested in IntelliCages for 41 days. Rats were challenged with gradually more and more complex tasks. The time spent in the IntelliCages was divided into four phases: acclimation, nosepoke learning, place preference learning and competition (Figure 2B).

In the first phase of the study, rats could habituate to the new environment for 3 days (acclimation). They were allowed to visit any corner any time and choose any bottle to drink from throughout the day. They could also drink from the bottles ad libitum. The number of trials was not limited; thus, rats voluntarily visited the corners. The second phase of the study was nosepoke learning for 20 days when rats had to initiate trials with a nosepoke to gain access to the water bottles for 7 seconds. The third phase of the study was to train the animals to develop a side preference for an appointed corner in order to study learning behavior for 15 days. One side was rewarded by providing access to water for 7 seconds, whereas the remaining 7 sides did not open after a nosepoke. Nosepokes at the 7 remaining sides were recorded as incorrect choices. Ten rats were allotted to eight sides. Two rats were allotted to bottles 1 and 2, and one rat to each of the remaining bottles. Reversals were achieved with randomly changing the position of the correct corner to another corner (excluding the current one), and the side was interchanged as well. Reversal was carried out on day 3, 7 and 11. In contrast to the mouse study, rats did not received negative reinforcement (air-puff) in the incorrect corners. The last phase of the study included the competition task. In this phase, all ten rats were assigned to only one corner in both groups. In side preference and competition water access was available only for 2 periods of 2 hours each day (4:00-6:00 pm and 0:00-02:00 am). During any visit only the first nosepoke resulted in door opening.

4.6. Collection of Blood Samples

Following decapitation, trunk blood was collected rapidly into 0.5 ml plastic tubes and put on ice. Tubes were then centrifuged at 10.000 rpm for 2 minutes at room temperature. The serum was separated and divided into aliquots of ~300 µl and stored at -80 °C. Serum samples (n=6 for each groups) were analyzed using a Beckman Coulter AU480 Chemistry Analyzer instrument (Beckman Coulter, Inc., Brea, California, USA).

4.7. Rat Aldosterone and Anti-Diuretic Hormone ELISA Assays

Concentrations of aldosterone (ALD) and anti-diuretic hormone (ADH) were measured by sandwich ELISA kits (MyBiosource, San Diego, CA, USA) specific for rat ALD and

ADH protein, respectively, according the manufacturer's instructions. Standards and samples were measured in duplicate.

ALD and ADH levels were calculated plotting the optical density (OD) of each sample against the seven points standard curve. Absorbance was measured at 450 nm. High standard of 1000 pg/ml (ALD) and 800 pg/ml (ADH) were used. Assays detection limits of rat ALD and ADH were 5 pg/ml and 2pg/ml, respectively. ALD and ADH levels were expressed in pg/ml.

4.8. Gene Expression Assay

Each removed tissue sample (n=10, hippocampus, cerebellum, prefrontal cortex, thalamus) was immersed in RNALater and stored at 4°C overnight, then stored at −20°C. The tissue was homogenized and RNA was extracted using an RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The RNA was stored at −80°C in RNase/DNase-free water. All RNA preparations were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK) to determine the RNA concentration and the quality of the RNA using the RNA integrity number (RIN). cDNA was synthesized from total 1 μg RNA in a 20-μL reaction mixture by using a Superscript VILO cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol. Quantitative PCR was carried out using the Applied Biosystems (Carlsbad, CA, USA) Quantstudio 12K Flex Real-Time PCR System, according to the manufacturer's instructions. Primers and probes for quantitative PCR were purchased from ThermoFisher (Cry1: Rn01503063_m1; Per1: 01325256_m1; Npas2: Rn01438223_m1; Arntl: Rn00577590_m1; Clock: Rn00573120_m1; Mtnr1a: Rn01488022_m1; beta-actin (ACTB), 4352340E). The cycle conditions for quantitative PCR were 95 °C for 20 sec, followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec. All data were normalized to ACTB expression. Data were calculated using 2-ΔΔCT method. RQ mean values are normalized to 1 for the control.

4.9. Spontaneous Locomotor Activity

Spontaneous locomotor activity was measured in male rats (n=10 in each group) at postnatal days 26-28 by a six-channel activity monitor manufactured by Experimetria (Hungary). The apparatus consisted of acrylic cages (48.5cm x 48.5cm x 40cm) equipped with 2 x 30 pairs of photocells along the bottom axis of the cage. Additional arrays of photocells (30 pairs) were placed along two opposite sides of the cage at different heights (6.5, 12, 18 and 23 cm) in order to detect rearing responses. The photocell beam, when broken, signaled a count which was then recorded by a computer. The signals were processed by a motion analyzing software that determined the spatial position of the animal with 1 Hz sampling frequency, and computed the distance travelled and the time spent by the rats with ambulation, local movement (e.g. grooming), immobility, rearing, etc. Animals were individually placed in the photocell cages; horizontal movements (ambulation time) as well as vertical rearings were determined for one hour. Data are expressed as means ±SEM.

4.10. Juvenile Social Play

Pinning as most characteristic parameter of social play behavior was scored for each pair of male rats (n=10 in each group) on postnatal days 33-36. The testing arena of juvenile social play was a plexiglass cage (42 × 42 × 32 cm) with approximately 2 cm of wood shavings covering the floor. Pairs of rats (from the same treatment group) were assigned for social interaction by using unfamiliar partners (i.e., not a cage mate or litter mate). Animals in a test pair did not differ more than 10 g in body weight. On postnatal day 34 and 35 each animal was introduced to the testing arena for a period of 5 minutes individually. On the third day (postnatal day 36), the motivation for play was enhanced by isolating the animals for 4 h before the test. Animals that had been unfamiliar to each other were placed simultaneously into the opposite corners of the previously discovered arena and their behavior was recorded for 15 minutes. Behavioral elements were assessed using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). The frequency of pinning as the most characteristic parameter of social play

behavior was scored for each pair of animals and expressed as means ± SEM [74,75]. Data are expressed as means ±SEM.

4.11. Maternal Deprivation-Induced Ultrasonic Vocalization

Impairments in communication between pups and their mothers were measured by recording ultrasonic vocalizations. To induce calls, pups (n=10 in each group) were separated from their mothers and placed individually into a cage for 10 minutes, while calls were being recorded with bat microphones. Calls were digitized with an audio filter and ultrasonic vocalization was recorded and quantified with SonoTrack software (Metris bv., The Netherlands). Vocalization was measured at age of 12 days for 10 minutes. Statistical analysis included the Kruskal Wallis non-parametric test and the post hoc Dunn test. Data are presented as means ± SEM of USV calls count /10 minutes.

4.12. Von Frey Test

Von Frey test was used for estimating paw withdrawal thresholds (expressed in grams) with a series of filaments, which uses a constant number of five stimuli per test. It was conducted with simplified up-down method as previously described [76] (n=10 in each group).

4.13. Statistical Analysis

In the mouse IntelliCage study, data are presented as group mean+SEM and were analyzed using GraphPad Prism 7.01 (GraphPad Software, San Diego, CA, USA). Subjects were taken as repeated measures. Non-parametric and parametric one-way analysis of variance (ANOVA) with Friedman-test, Dunn's multiple comparison post-hoc test, and Kruskal-Wallis test was applied for statistical analysis of the data. As we had different trial numbers in each group - due to the limitations of the chosen statistical method - we had to disregard some data, to have an equal number of data points in every group. This could slightly deteriorate the results. For all comparisons, $p<0.05$ was considered reliable. Asterisks indicate: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

In the rat IntelliCage study, exploratory visits (i.e. visits without nosepoke or drinking) were aggregated for each day of the experiment by subjects. Generalized linear models (GLM) with log link using negative binomial distribution were constructed. Differences in initial exploratory activity were also compared between groups. To show how exploratory activity changed during the day, visits were aggregated for each four-hour period. For nosepoke learning, a cosinor analysis was conducted to see how daytime exploratory activity patterns differed between groups [77]. Two main estimates were considered: mesor (mean activity) and amplitude (difference of peak and midline activity). Mean values for groups were compared using t-test. Daytime mean activity was calculated for the first 72 hours, groups were compared using bootstrapped Watson's test. Drinking volume were estimated by assessing the number of licks and lick duration. For acclimation, the number of licks for each animal was calculated for four-hour periods which also revealed how drinking behavior changed over the course of the day. When competing, animals were restricted both in their access to water bottles, and maximum lick duration for each visit. Therefore, mean lick duration was calculated for each day of the experiment. In addition, linear mixed effects models were used to compare groups with subjects as random factors [78]. Acclimation was treated separately from the other phases because of the difference in underlying data distribution due to the time limit introduced after acclimation.

In side preference learning, the proportion of correct nosepokes to all nosepokes were calculated. This response variable was put in a binomial generalized mixed effects model (GLMM) with treatment as the fixed effect. Binomial distribution was used because the number of correct responses out of all trials was measured. The proportion of correct nosepokes is expected to change during time due to the initial period and the reversals. Therefore, the drink session was included as a random factor within the model. As dispersion was high, cumulative lick duration was included as a random factor. Differences in drinking volume changed the proportion of visits to the correct corner that was not related to learning. This addition to the model dropped the dispersion to near 1, while mean of all random factors was close to 0 (-0.02). Type II Wald chi-square test was used to assess the treatment effect. Hierarchy was estimated by differences in lick duration within groups. Total lick duration was calculated for each day of the experiment

and evenness of the values was used as a community measurement. Evenness is most often used to describe distribution of individuals within a community using Pielou's evenness index ranging from 0 to 1. This measure was adapted to reflect evenness in drinking among individuals, because the lower the evenness, the stronger the hierarchy that is expected in the community. Hierarchy could be best observed during competition; therefore, rank abundance curves were fitted to the cumulative lick number per hour values of each subject of the groups. Models were compared using Akaike Information Criterion (AIC) to find the shape of the best fitting model.

Reentering visits (‘guarding’) were defined as visits after which the same subject entered the corner. The number of reentering visits were calculated for each subject and day of the experiment. The maximum divided by mean values for groups were calculated to express the distribution of reentering visits within the groups. Experimental phases were merged based on whether water access was unlimited (acclimation, nosepoke learning) or limited (side preference learning, competition) during the given phase. Population level values were compared by using a linear model. Calculations were carried out by using R (https://www.R-project.org/).

Statistical evaluation was performed by unpaired t-tests to analyze spontaneous locomotor activity (ambulation and rearing), von Frey test, ultrasonic vocalization, and juvenile social play (pinning) results in GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla California USA).

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5. RESULTS

5.1. Place Reversal Learning in Mice

We looked at the temporal pattern of the pharmacodynamic effects of scopolamine following administration of the drug. Behavioral effects were seen predominantly in the first three hours, when analyzing data by hourly bins. In the fourth hour no differences between the treatment groups in any of the measured parameters were found (data not shown). Using the three-hour long-time window, at the treatment doses of 0.17 and 1 mg/kg animals showed a trend towards increased activity levels (Figure 3A).

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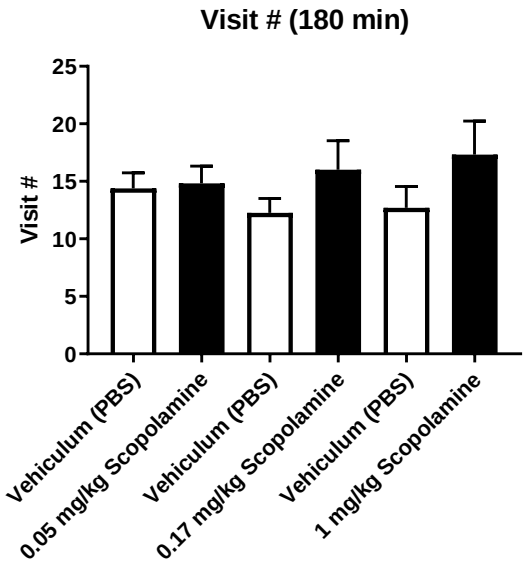

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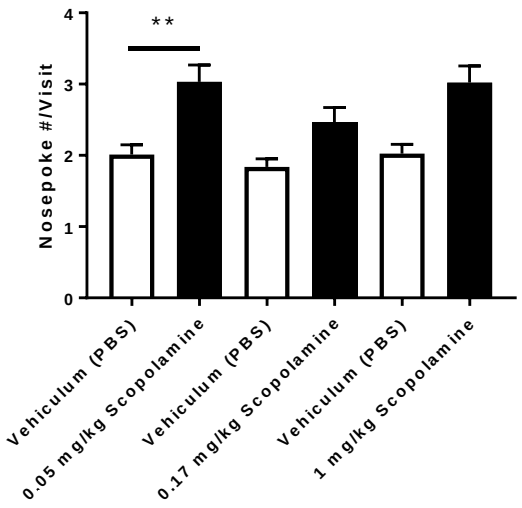

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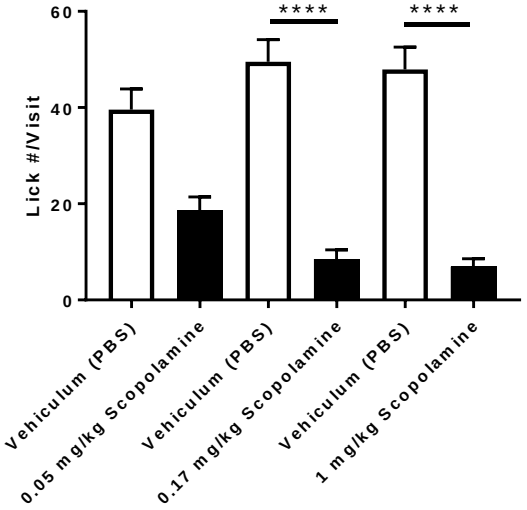

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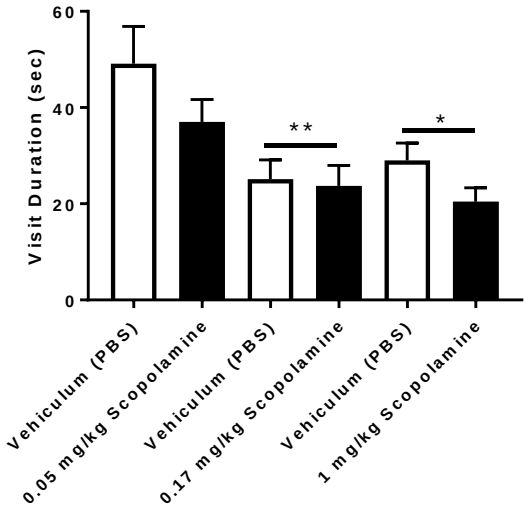

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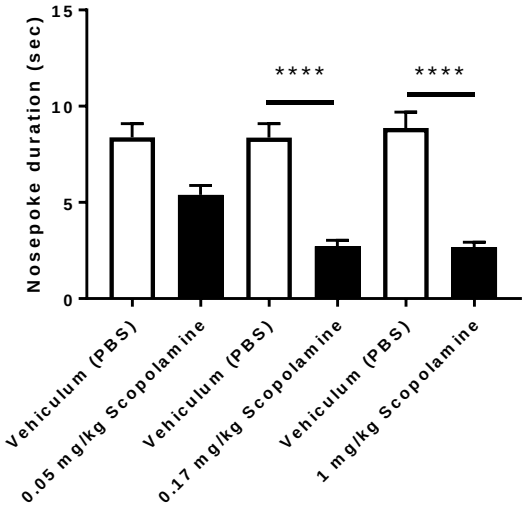

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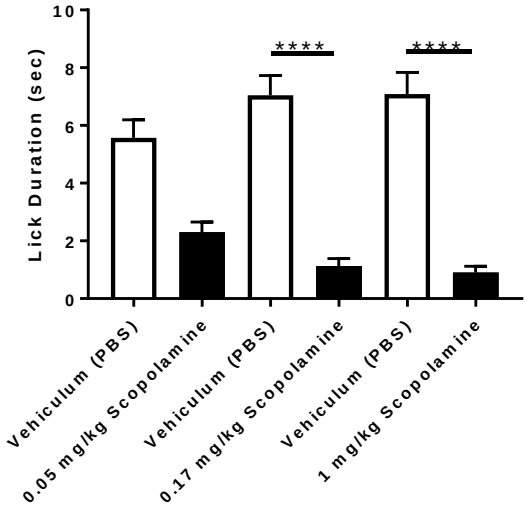

Figure 3 Effect of 0.05, 0.17 and 1 mg/kg scopolamine in untrained C57BL/6JOlaHsd mice in IntelliCage in the first 180 minutes (n=16, each). Main parameters of the drinking behavior and locomotor activity are shown (a-f). White columns represent vehicle group mean value, black columns show the different doses of scopolamine. Each drug treatment were compared to its vehicle group result in the same experiment. Errors are shown as mean+SEM. Asterisks indicate significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Interestingly, analyzing only the first 20 minutes, we found significant increase in visit numbers in all applied doses (Figure 4A).

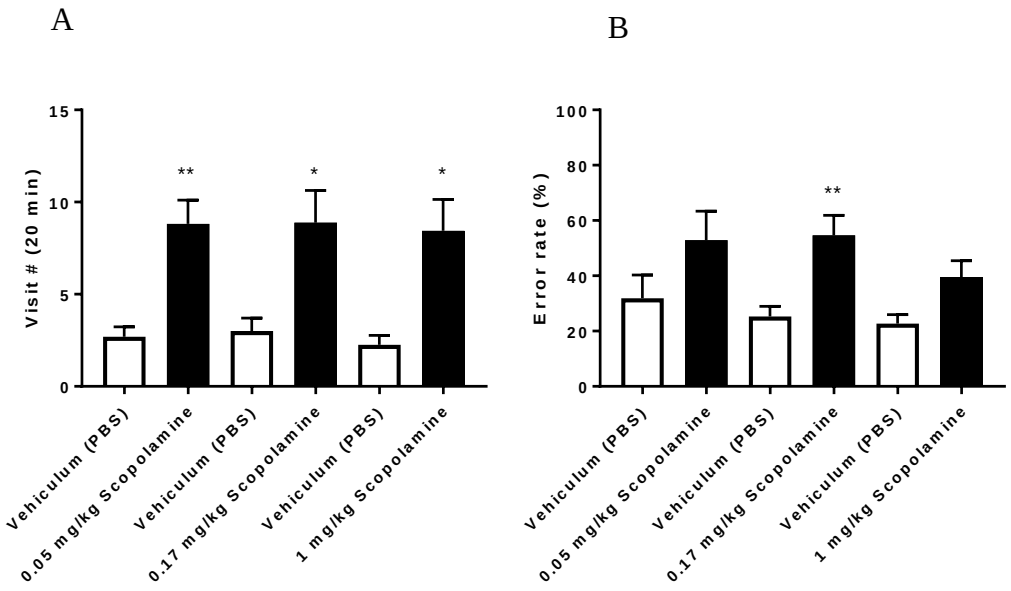

Figure 4 Effect of scopolamine in 0.05/0.17/1 mg/kg dose range in trained C57BL/6JOlaHsd mice in IntelliCage in the first 20 minutes. Drug treatment effect on general activity measured as visit numbers (A). Scopolamine effect on reversal learning paradigm indicated as error rates, the ratio of incorrect visits and all visits (A). Errors are shown as mean+SEM. Asterisks indicate significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Nevertheless, using small time window means less data to analyze, which makes it hard to interpret. Therefore, we decided to analyze the 0-180 minute time window of the

dataset, where 0 min indicates drug injections. In accordance with Robinson's earlier study [79], we did not find significant difference between vehicle and scopolamine-treated groups in general activity measured as visit numbers (Figure 3A). In our initial paradigm (first phase), mice could drink freely from any bottles without time restraint. Scopolamine-treated mice showed significant increase in nosepoke number per visit at 0.05 mg/kg dose ($p=0.0072$) and showed a similar trend in the two higher doses as well, but the effect is not significant (Figure 3B). Nosepoke duration in a given visit showed significant decrease ($p<0.0001$) when treated with 0.17 and 1 mg/kg scopolamine (Figure 3E). In accordance with the decreased nosepoke duration in the scopolamine-treated group, lick duration and lick number per visit were also decreased significantly in the 0.17 and 1 mg/kg doses ($p<0.0001$), compared to the vehicle groups (Figure 3C, F). In these dose groups, visits were significantly shorter as well ($p=0.0039$ and $p=0.0217$; Figure 3D). In our reversal learning paradigm, scopolamine significantly decreased the ability of mice to adapt to the new rule in 0.17 mg/kg dose ($p<0.01$, Figure 4B) indicated by elevated error rates. A trend towards increased error rates is also apparent in the doses of 0.05 mg/kg and 1 mg/kg, however, it is not statistically significant.

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5.2. Characterization of Autistic Phenotype

Autistic behavior phenotype was characterized by a series of assays before the beginning of the IntelliCage study (Figure 5).

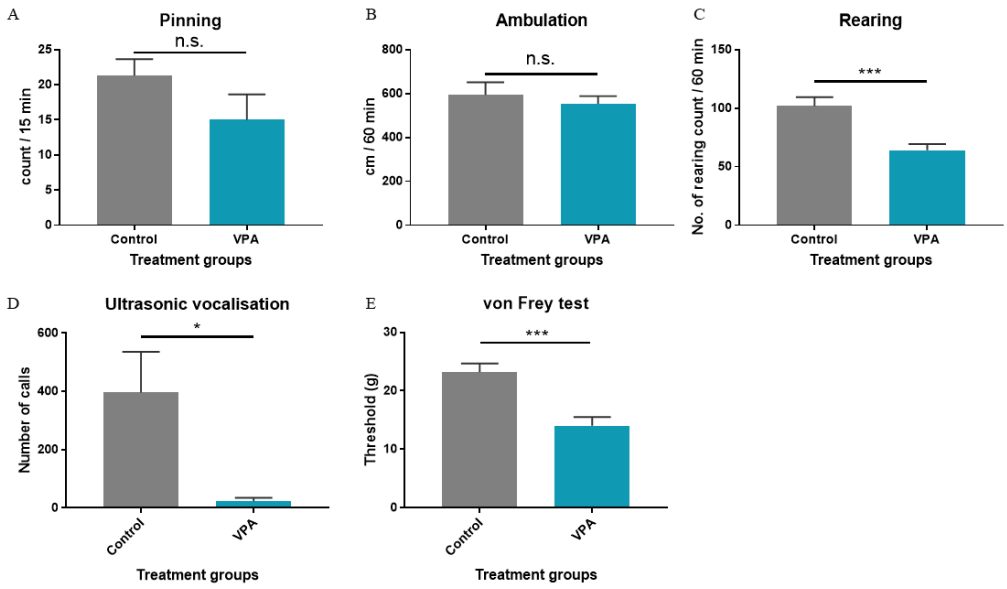

Figure 5 Summary of phenotype characterization tests on control and VPA rats. Means (±SEM) are shown. ("n.s." indicates non significant, *** $p<0.001$, * $p<0.05$)

Juvenile social play, spontaneous locomotor activity, ultrasonic vocalization and von Frey test were carried out. VPA rats showed a non-significant decrease in pinning numbers (21.3±2.343 vs. 15±3.639 n=10,10). In spontaneous locomotor activity test ambulation did not differ significantly between the groups (593.4±57.91 vs. 553.7±34.83). VPA rats showed a significantly lower rearing count compared to the control group (101.7±7.765 vs. 64±5.348 n=10,10; p=0.0008). The number of calls in ultrasonic vocalization test was also significantly lower in VPA group (396.4±139.2 vs. 23.2±11.31 n=10,10; p=0.0155). The threshold was significantly lower of the VPA group (23.2±1.489 vs. 14±1.506 n=10,10; p=0.0004) in the von Frey test.

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5.3. Decreased Initial Exploration in VPA Rats

The exploratory activity was assessed by calculating the number of exploratory visits defined as visits without nosepoke and lick. Control rats tended to explore the novel environment of the IntelliCage, represented by high numbers of exploratory visits during acclimation (Figure 6A and B) especially in the first 24 hours (5.45±0.40, 2.03±0.17 exploratory visits/h for the control and VPA group, respectively).

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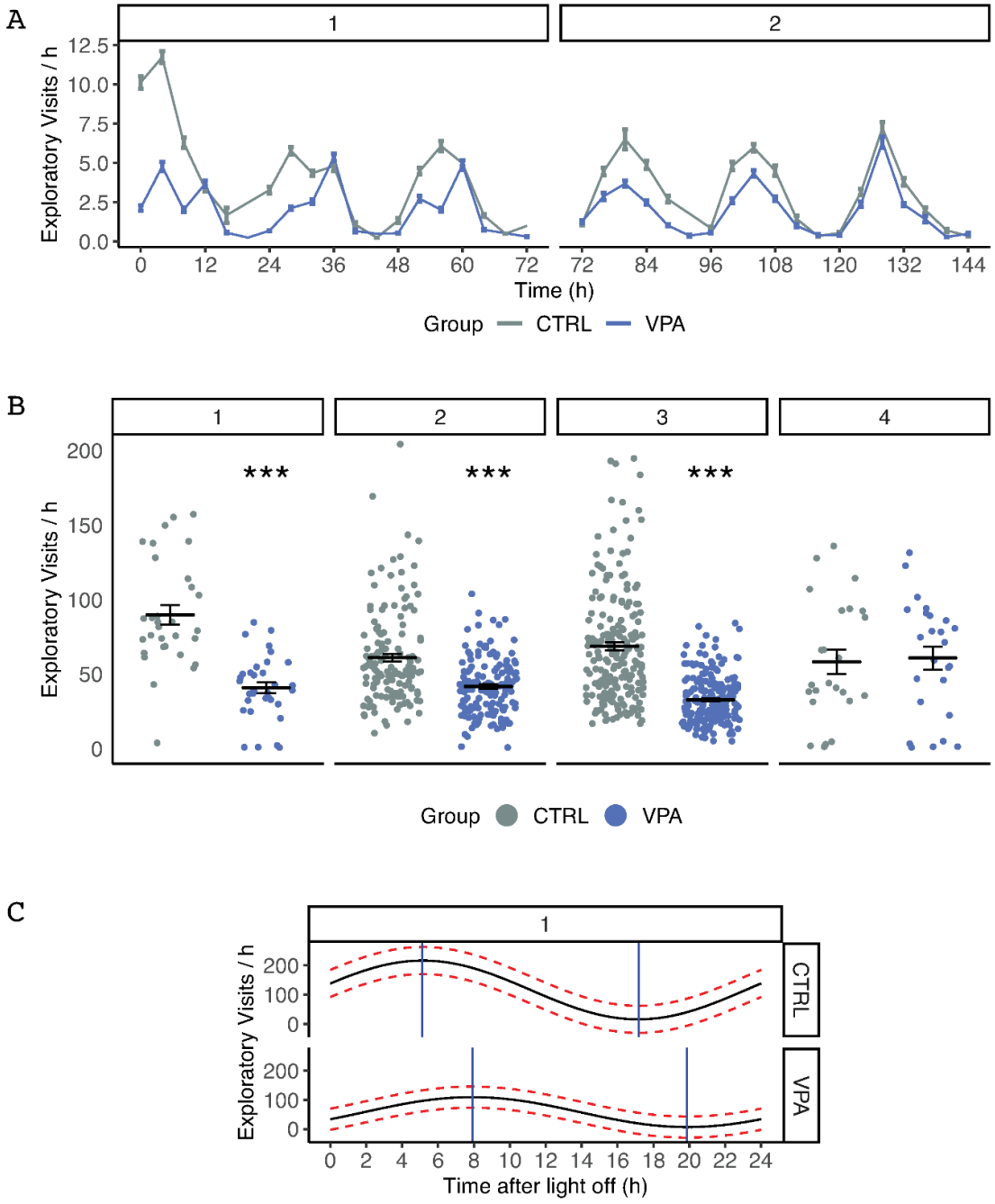

Figure 6 (A.) Number of exploratory visits in control and VPA groups. 1, 2, indicate phases: Acclimation, Nosepoke learning. Time scale of exploratory visits summed in four-hour periods. Means (±SEM) are shown ($p<0.001$). (B.) Number of exploratory visits in control and VPA group 1, 2, 3, 4 indicate phases: Acclimation, Nosepoke learning, Side preference learning, Competition, Number of exploratory visit in control and VPA group of rats. In acclimation, nosepoke learning and side preference learning VPA group showed a significant decrease in explorative visits ($p<0.001$) (C.) Cosinor

analysis of circadian rhythm during acclimation. Mean (black line) and confidence interval (red dotted lines) are drawn for pooled group data. Time after light off values associated with maximum and minimum activity are shown with blue lines.

The VPA rats showed a significant decrease of exploratory visits in the first 24 hours ($p<0.001$). Later, this initial exploratory activity decreased in the control group and showed a stable daily pattern (Figure 6A). The VPA group showed significantly lower initial exploratory activity in nosepoke learning (cosinor regression, difference in mesor, average activity; 33.87 vs. 22.94 visits/h for control and VPA group, respectively; $p<0.05$). The reduction of exploratory visits in the VPA group was robust and highly significant (GLM, $p<0.001$) throughout acclimation, nosepoke- and side preference learning phases (VPA group -55% vs. control) (Figure 6B).

5.4. Circadian Rhythm Disturbance in VPA Rats

The daily activity pattern was assessed during the acclimation phase when water was freely available. Circadian activity was estimated based on an analysis of daytime mean activity. The circadian rhythm of the VPA group showed an approximately two-hour shift after light off compared to the control group ($p<0.05$; 7:00 for VPA compared to 5:01 for control) (Figure 6C). Cry1, Per1, Arntl, Npas2, Clock and Mtnr1a gene expression was measured but did not reach two-fold change between the treatment groups, hence it cannot be interpreted as biologically relevant change. VPA group data are presented in the order of the following brain regions: cerebellum (C), hippocampus (HPC), prefrontal cortex (PFC) and thalamus (T).

Cry1 (C) 1.07, (HPC) 1.16, (PFC) 0.998, (T) 0.764; Per1 (C) 1.007, (HPC) 1.224, (PFC) 0.723, (T) 1.166; Arntl (C) 0.712, (HPC) 0.639, (PFC) 0.813, (T) 1.07; Npas2 (C) 0.717, (HPC) 0.924, (PFC) 0.828, (T) 1.126; Clock (C) 0.89, (HPC) 1.069, (PFC) 0.735, (T) 1.058; Mtnr1a (C) 0.697, (HPC) 0.886, (PFC) 1.497, (T) 0.706.

5.5. Normal Place Preference and Reversal Learning in VPA Rats

Control and VPA groups did not differ in place and side preference learning abilities, nor was a difference in their reversal learning (Figure 7).

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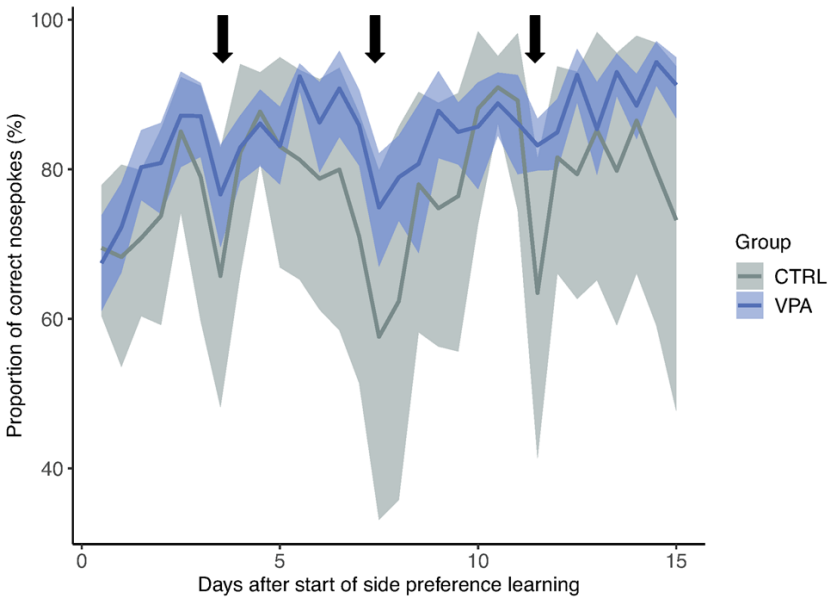

Figure 7 Side preference learning of ctrl and VPA group of rats. Proportion of correct nosepokes to all nosepokes is expressed in percentage. Arrows indicate side reversals. Lines denote mean, while ribbons show confidence interval for treatment groups

The proportion of correct nosepokes was identified: 89.2% for the control and 88.1% for the VPA group (fitted value Chi^2=0.898, p=0.3).

5.6. Altered Drinking Behavior in VPA Rats

During acclimation, the daily pattern of drinking behavior showed frequent but short bouts for the control group, while VPA rats drank in rare but long bouts (Figure 8).

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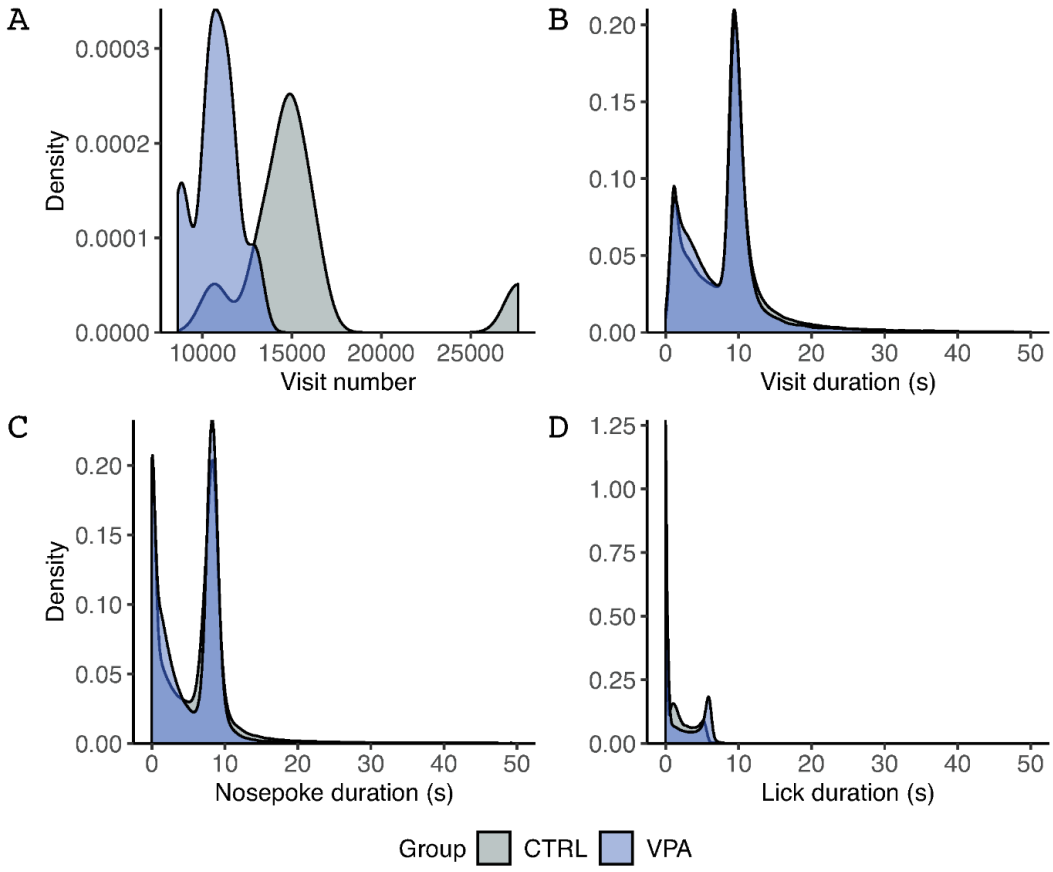

Figure 8 Descriptive density plots for (A.) visit number, (B.) visit duration, (C.) nosepoke duration and (D.) lick duration.

Consequently, control rats showed a well-balanced, stable pattern of lick number per hour distribution (Figure 9A).

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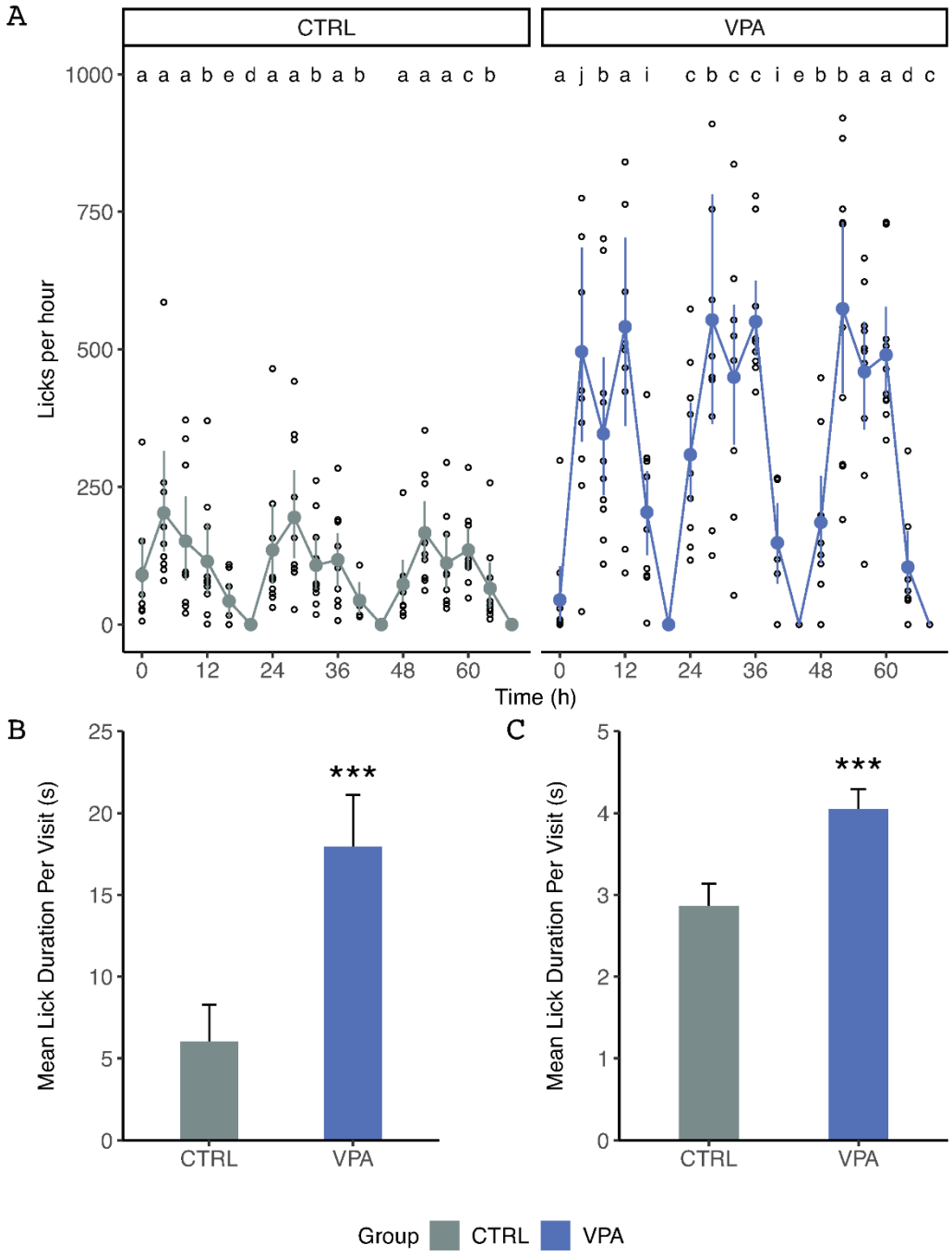

Figure 9 Differences in drinking behavior. (A.) Number of licks grouped for four-hour periods during acclimation. Mean, confidence interval and individual values are shown. Number of lick values are transformed for 1hour. Letters indicate the individual with the highest number of licks for each four-hour period. (B.) Mean lick duration (±SEM) per

visit during acclimation ($p<0.001$). (C.) Mean lick duration (±SEM) per visit during later phases (pooled).

In the control group, 56% of the 4-hour-long period was dominated by one subject ("a") who drank the most during this time. In contrast, in the VPA group the individual animal with the highest number of licks ("a") dominated only 24% of the 4h periods.
Such distribution could arise from the differences in the number of visits or that of lick duration during visits. Therefore, we had investigated the mean lick duration per visit and found that there was an almost threefold difference between control and VPA rats for the mean lick duration during acclimation (6.0 s vs. 17.9 s linear mixed effects model $p<0.001$; Figure 9B). We pooled these data for nosepoke learning, side preference learning and competition, as well (Figure 9C). Although each protocol after acclimation was set up to limit lick duration to 7 s, there was a significant difference between groups (2.9 s vs. 4.1 s, linear mixed effects model $p<0.001$; Figure 9C). Sera of the groups were analyzed for blood chemistry parameters (Figure 10, Table 1).

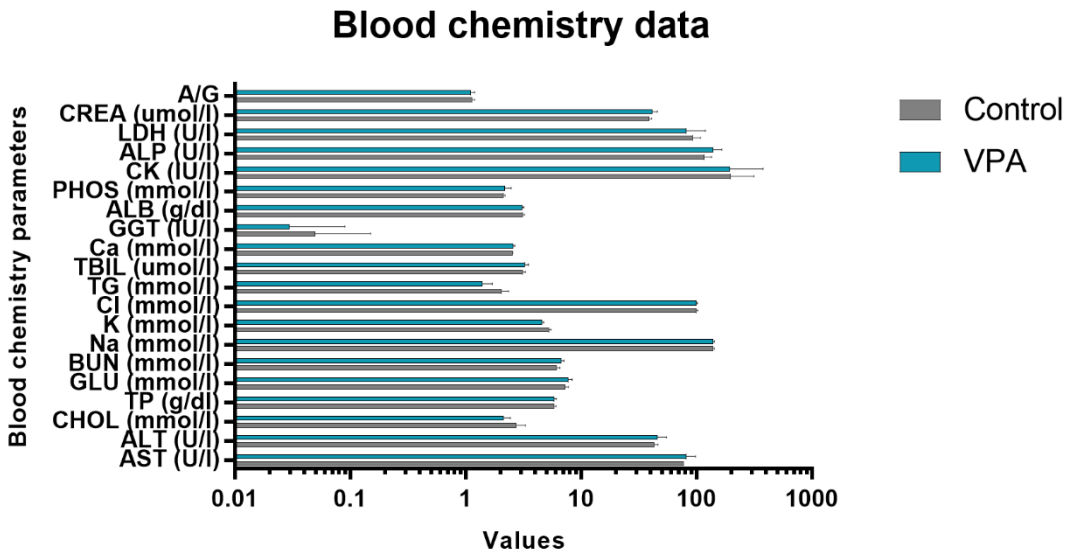

Figure 10 Blood chemistry parameters of treatment groups. Means (±SD) are shown. Only the potassium decreased significantly in the VPA group.

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Table 1 Abbreviations of the blood parameters in Figure 10.

Abbreviation	Blood parameters
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CHOL	Cholesterol
TP	Total Protein
GLU	Glucose
BUN	Blood Urea Nitrogen
Na	Sodium
K	Potassium
Cl	Chloride
TG	Triglycerides
TBIL	Total Bilirubin
Ca	Calcium
GGT	Gamma-Glutamyl Transferase
ALB	Albumin
PHOS	Phosphate
CK	Creatine Kinase
ALP	Alkaline Phosphatase
LDH	Lactate Dehydrogenase
CREA	Creatinine
A/G	Albumin to Globuline ratio

Out of 20 parameters only the potassium showed a slight increase compared to the reference values (5.29 for control and 4.64 for the VPA groups; reference values are for the potassium 4-5.9 mmol/liter [80]. ALD and ADH serum levels were not significantly different of the VPA group relative to the control group (Figure 11).

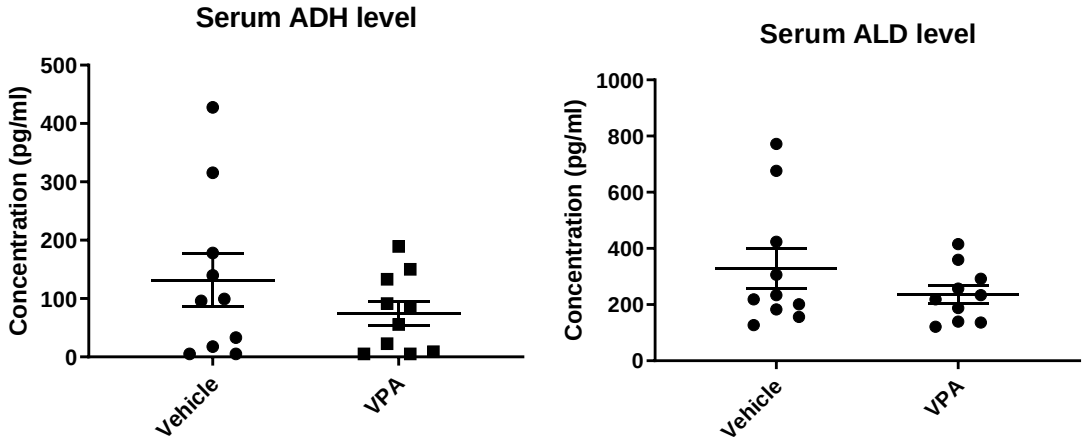

Figure 11 Aldosteron (ALD) and anti-diuretic hormone (ADH) concentration in sera of VPA and control group of rats. Means (±SEM) are shown.

5.7. Disturbed Hierarchy in VPA Rats

To reveal differences in group dynamics and adaptive behavior within the groups, a deeper analysis of drinking and visiting pattern was performed. During the competition phase of the study three control subjects showed apparent competitive dominance over the rest of the animals which was manifested in the larger cumulative lick number (Figure 12A).

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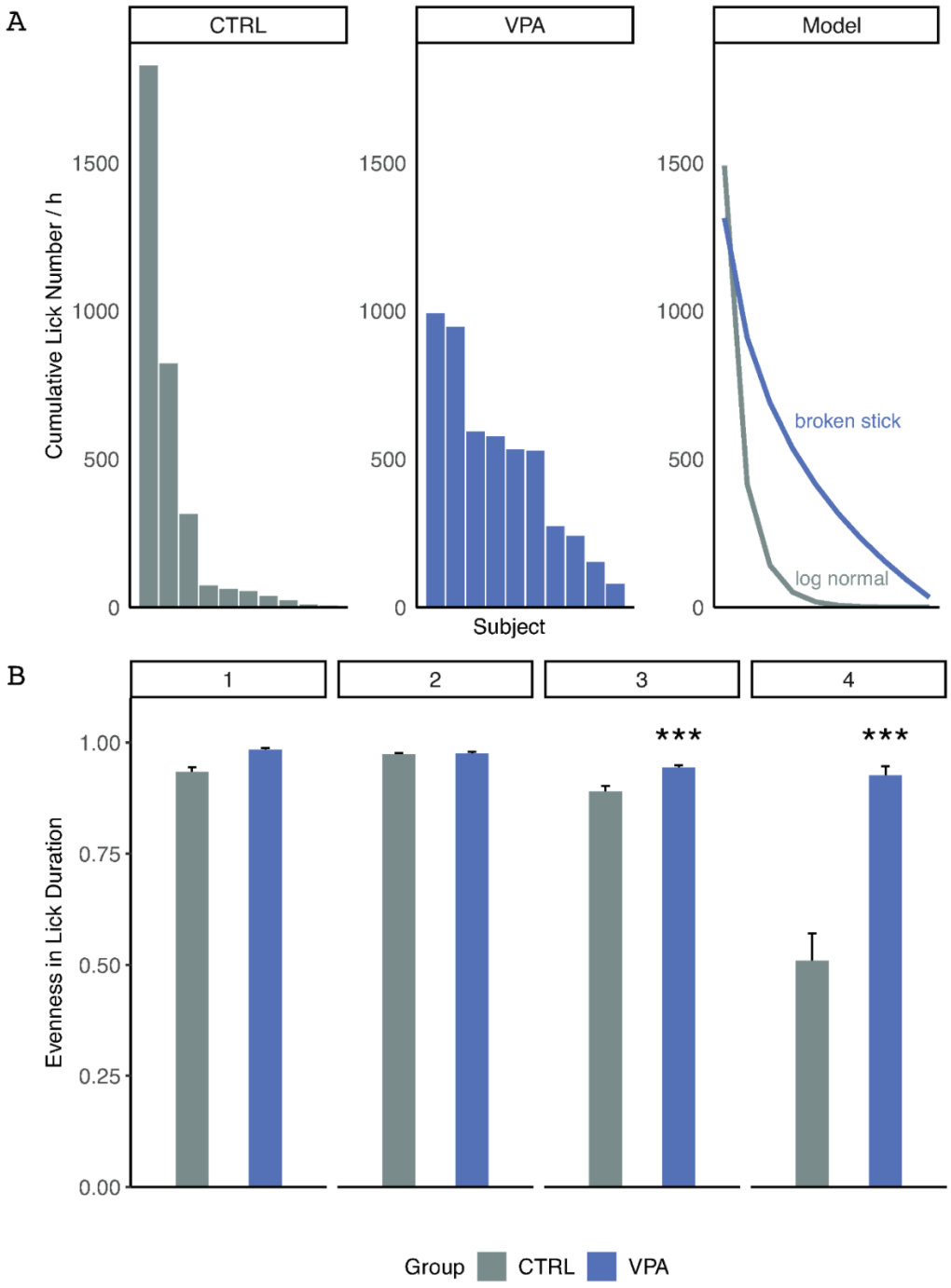

Figure 12 Inter-individual drinking behavior of treatment groups. (A.) Distribution of cumulative lick number per hour. Rank abundance curves are drawn according to the model best fit the data: log-normal for control (AIC=171.5) and broken stick for VPA group (AIC=184.9). (B.) Evenness (diversity divided by maximum diversity) values for groups and phases (daily mean ±SEM; $p<0.001$)

In contrast, such clear formation of subgroups was not observed within the VPA group (Figure 12A). When tested this in rank-distribution models, the best fit for the control group was log-normal (AIC=171.5), whereas for the VPA group followed a broken-stick model (AIC=184.9). This difference only appears when access to water is strictly limited in time and space. High evenness values were observed through acclimation and nosepoke learning phases, without significant difference between the groups (Figure 12B). As water became less accessible in side preference learning and even more so in competition phases, the control group showed significantly lower evenness in lick duration, while in the VPA group it remained stable indicating an intensifying hierarchy for the control animals (Figure 12B, $p<0.001$).

Representative subjects' with most, median and least number of consecutive visits are shown to the same corner during competition (Figure 13A).

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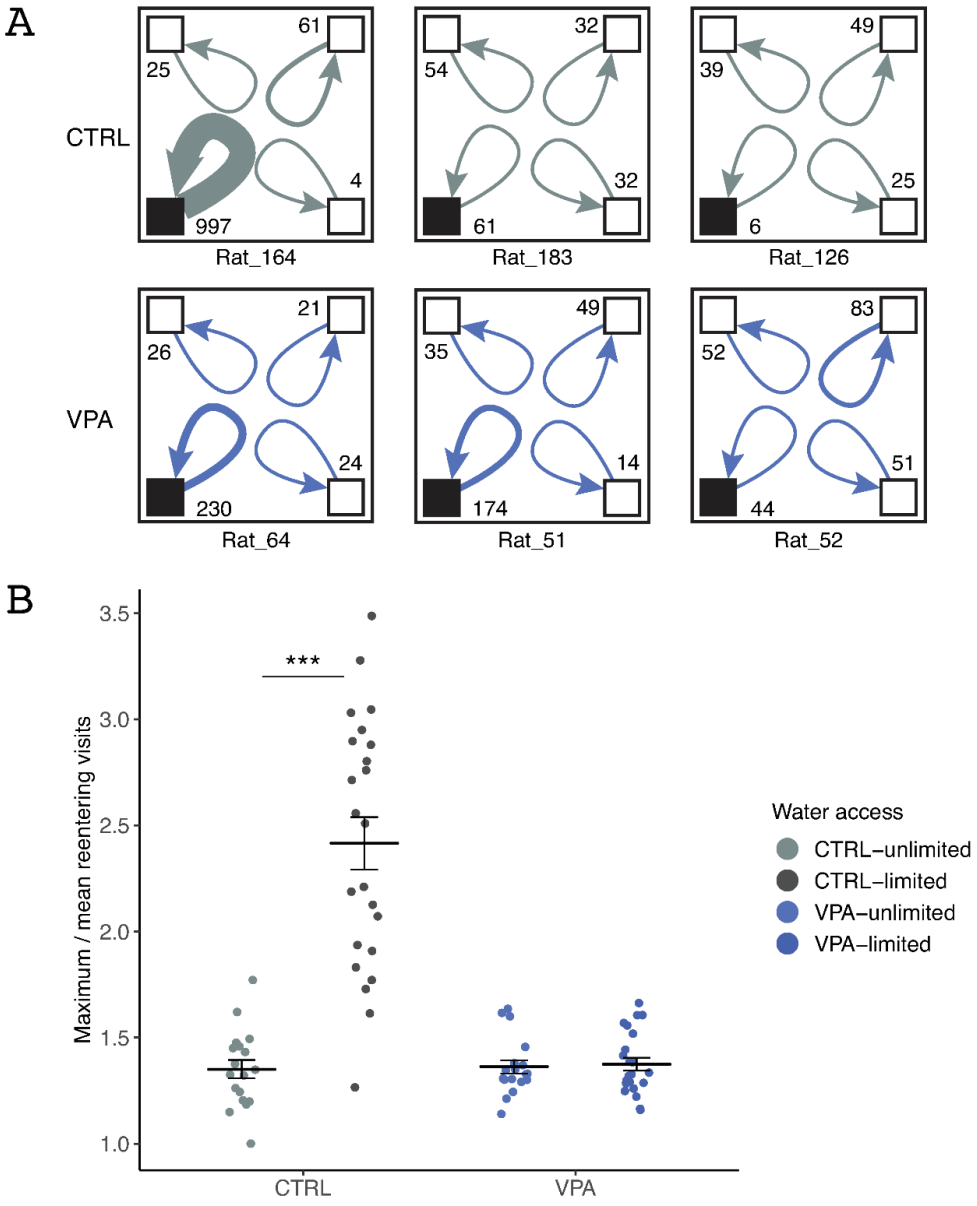

Figure 13 Lack of social dominance in VPA group. (A.) Representative cage layouts for 3 individuals (ID presented at the bottom of the schematic cage) with the highest, median and lowest numbers of reentering visits for each treatment group (rows) during competition. Reentering visits are calculated for each subject as visits to the same corner when the subject left without other subjects entering the corner (numbers shown at the corners). The thickness of the arrows indicates the strength of the reentering visits. (B.) Social dominance within the group is characterized by using a ratio of the maximum visits of an individual divided by the mean values of the group (±SEM) for daily cumulative

reentering visits. Water limitation provoked the elevation of social dominance in the control group as compared to condition with unlimited water access (effect size: 78.8%, $p<0.001$), whereas no significant effect was shown within the VPA group that remained at a dominance value below 2.

In the control group, one animal had substantially higher number of consecutive visits compared to the rest. VPA group's subjects showed much more converging numbers. The entire population was thus described using maximum over mean consecutive visits calculated for each day of the experiment.

There was a significant difference in the limited or unlimited conditions of water access within the control group (Figure 13B) when the daily maximum/mean reentering visits (i.e subject reentering the same corner) were calculated. Control rats visited the same corner 78% more frequently when water access was scarce (competition phase), compared to the water unlimited condition ($p<0.001$). In contrast, the limitation of water access did not change the behavior of VPA rats; they did not change the frequency to reenter the same corner in any condition (Figure 13B).

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6. DISCUSSION

Harnessing a novel concept in behavioral neuroscience, automated home-cage systems allow us to develop more advanced methods to study psychiatric disorders. Applying a powerful statistical analysis was also necessary to uncover new findings from a sizable amount of data. Thanks to continuous data-collecting, our method can be well fitted to study pharmacological interventions as well. One can follow the dynamic changes of the behavior affected by a drug of interest.

Here we studied the effects of a single dose of scopolamine in mice in the IntelliCage, mouse IntelliCage. Our results indicate that finding the right time window for data collection is crucial in an experimental design where animal activity is registered throughout the day. We established that given the limitation of an acute administration, this window has to coincide with meaningful pharmacodynamic effects, and at the same time it should be sufficiently long to have the necessary amount of visit and nosepoke data. As published earlier, 3 hours of access daily with 21 hours of water deprivation is sufficient and motivating for different learning paradigms in IntelliCage, such as place preference learning, reversal learning and place avoidance tasks [81].

Scopolamine, a frequently used anticholinergic cognitive impairing agent, is a promising tool compound in learning paradigms conducted in the IntelliCage. Scopolamine administration has an impact on esophageal function as well. The esophagus is composed of both smooth and striated muscle. Smooth muscle function and coordination are dependent on cholinergic innervation. Therefore, drugs with anticholinergic activity have the potential to cause dysphagia as well [82]. As acute scopolamine treatment is used in rodents to cause temporary cognitive impairment, we were interested whether it causes any alteration in the registered behavioral parameters in the IntelliCage. The apparatus offers a certain time span of water access as positive reinforcement. Due to known side-effects of scopolamine in humans [82] (dysphagia, dry mouth) and the known effect on locomotor activity, we hypothesised that it may change drinking behavior, and general activity following drug treatment. Robinson and coworkers reported a trend towards elevated visit numbers after 0.5 mg/kg scopolamine treatment in IntelliCage; however it was not significant [79].

Earlier publications showed contradictory results of locomotor activity in different behavioral methods using scopolamine [55,56]. Our results can partly explain this phenomenon. Before using scopolamine in actual learning paradigms, we first aimed to study the simpler behavioral alterations due to its known side-effects in naïve, untrained mice (dry mouth, dysphagia in humans) [82]. These data could help us develop specific learning paradigms in IntelliCage, considering scopolamine's effect on locomotor activity and drinking behavior. We found that depending on the time window chosen, the effect of scopolamine on locomotor activity shows remarkable differences. In the first 20 minutes after injection, the visit number indicates increased locomotor activity of the scopolamine group (Figure 4A) but using all data of the first 180 minutes these differences are not significant (Figure 3A). Increased nosepoke number per visit (Figure 3B) can be interpreted as increased general activity, or even decreased attention, as it was reported in an earlier study, where mice treated with scopolamine showed a disruption in accuracy and increased number of omissions in 5CSRTT paradigm [58].

As in humans, we revealed behavioral traits (decreased lick number and lick duration) suggesting difficulties in swallowing in mice (Figure 3C, F). These data suggest that lick number and lick duration cannot be interpreted as cognitive parameters when using a pharmacological agent causing difficulties in swallowing. Based on these data, in case using scopolamine in IntelliCage studies as a memory disrupting agent, we suggest to use a learning paradigm, in which the positive reinforcement of the correct choices is not water access, but e.g. the absence of a negative reinforcement (i.e. the absence of air-puff), whereas water is available at all corners. This way undesired side-effects can be avoided which could deteriorate learning behavior, and even result in false positive results.

In the mouse IntelliCage experiments, our place preference and reversal learning paradigms were designed with the abovementioned insights in mind. Access to drinking bottles upon nosepoke was allowed in all corners, however, incorrect corners were punished. This way the lick number and lick duration were not considered as cognitive parameters. Our results suggesting scopolamine effect in reversal learning (Figure 4B) confirm Robinson and coworkers' results in IntelliCage [79]. However, their protocol was slightly different. Unlike in our paradigm, in the Robinson *et al.* study mice had access to water in correct corner only, while incorrect corners simply did not respond to nosepokes

and did not punish with airpuffs either. In our laboratory, scopolamine did not alter place preference learning in the same dose range (data not shown). We only found significant increase of error rate of the scopolamine treated group in our reversal learning paradigm. It might suggest that an already acquired knowledge becomes so stable over time, that it cannot be altered with a cognitive impairing agent. However, in an environment, where one has to adapt to an everchanging rule, the learning process can be more easily disrupted.

With rotating the correct corner position on a daily basis our results represent reversal learning, which can be interpreted as rather cognitive flexibility compared to Robinson's spatial learning paradigm [79]. It is also worthwhile to mention that they analyzed 6 hours after drug treatment while according to our results, data analysis for 3 hours is sufficiently enough. There were no differences found after the third hour between treatment groups.

Gathering detailed information in a social environment may provide novel insights on fundamental elements of behavior that cannot be deduced from conventional behavioral observations that deliver relatively small amounts of data. In the present study, data mining of the pattern of simple animal actions; visiting, nosepoking and drinking allowed us to investigate various behaviors in communities of prenatally VPA-exposed autistic-like and control rats. Autistic behavior of rats prenatally treated with VPA includes social components resembling the symptoms of human Autism Spectrum Disorder [70]. Here, we focused on more sophisticated aspects of behavior beyond the well-known social effects described in VPA animals.

VPA-treated rats showed a drastic decrease in exploratory visits in the first 24 hours compared to the control group, which was maintained at some level in all phases except competition. One can speculate, that the reason behind decreased exploratory behavior during competition is that both groups optimized their behavior in a way that they did not 'waste' visits only for exploration and took every chance to drink whenever possible. The overall decrease of exploration may derive from neophobia, anxiety or alternatively a shifted circadian rhythm could also play a role.

Indeed, our results showed a 2-hour shift in the circadian rhythm of the VPA group. As earlier reported, VPA exposure alters core circadian rhythm transcription factors *in vitro* and *in vivo* [69,83]. In both studies, altered expression of known circadian rhythm genes were

reported. Interestingly, our results showed no significant change in a selected set of circadian rhythm genes (Cry1, Per1, Arntl, Npas2, Clock and Mtnr1a) of the VPA rats. This may suggest that our behavioral findings in the VPA group are not the consequence of the differentially expressed mRNA levels of the circadian genes, rather on the level of proteins, single nucleotid polymorphisms or changed regulation of certain transcription factors. On the other hand, we only analysed a small set of genes, hence we must be careful when drawing conclusions. Circadian changes due to different pattern of melatonin expression were studied in human ASD patients [84]. Indeed, sleep disorders are very common comorbidity of ASD (25-40%) [85]. In parallel with these studies, our results confirm the circadian rhythm disturbance *in vivo*. We report a non-significantly slightly lower ADH serum level of the VPA group. Circadian profiles of ADH in the suprachiasmatic nucleus (SCN) suggests its pivotal role as a circadian pacemaker in mammals [86]. Intriguingly, ADH levels in SCN showed circadian rhythmicity with a peak at early light phase. Although we did not measure ADH levels in SCN, this early light phase peak could overlap with our result where we showed a 2-hour shift in VPA group. Consequently, lowered ADH level in SCN could explain the altered circadian rhythm of the VPA rats. A slightly altered ADH regulation in VPA rats can be also associated with reduced ultrasonic vocalizations, as we showed (Figure 12). In parallel with our results, vasopressin 1b (Avpr1b) knockout mice showed also reduced ultrasonic vocalizations [87]. During the acclimation phase, we have unexpectedly found a substantial difference on general drinking behavior of the VPA group that appeared as an almost threefold increase in lick duration per visit. Control rats tend to visit very often but drank only in short bouts while VPA rats visited rarely but once they entered the corner, they drank considerably more. In the following phases this difference in behavior stayed significant despite the limitation of water access (7s). Altered drinking behavior was most likely not due to any underlying mechanism of the VPA treatment, as we did not see major anomalies in blood chemistry results (Figure 11, Table 1). Only the potassium level showed a slight increase in the control group compared to the upper limit of the reference value. However, the extent of the difference seems irrelevant (0.49 mmol/liter) that could be only a consequence of the higher water intake, as well. We also measured two major water balance regulating hormones, ALD and ADH. Both hormones showed a non-significant but slightly decreased sera level in the VPA group. ADH has been associated with social

functioning in children with ASD [88]. They even proposed ADH blood level as a potential biomarker of theory of mind ability in children with ASD and they also suggested ADH may be a promising therapeutic target by which to improve social cognition in individuals with ASD. In case of ALD, ASD is usually associated with elevated level of aldosterone (aldosteronism). It has been even suggested for therapeutic purpose to use spironolactone, an aldosterone antagonist especially in hyperandrogenic children with autism [89]. All the analyzed blood parameters and the water balance hormones suggest an intact water balance physiology of the VPA rats. Altered drinking behavior in VPA rats could be explained by increased perseveration (represented by mean lick duration increase) that might result from the prenatal VPA exposure. The increase in water consumption could be most likely explained by psychogenic polydipsia (PPD). Psychogenic polydipsia is a clinical symptom, which has a common occurrence in patients with psychiatric disorders, most commonly schizophrenia [90]. PPD is characterized by polyuria and polydipsia [91]. We believe that this is the first preclinical study showing the appearance of psychogenic polydipsia in conditions that can be associated with human ASD. This phenomenon was already shown in autistic children, which further strengthens the translational value of the prenatal VPA rat model of ASD [92].

Puścian and coworkers reported earlier impaired place preference learning of prenatally VPA treated C57BL/6 and BALB/c mice using automated home cages [93], while we found no difference in side preference and reversal learning of VPA rats. Because of the difference in spatial learning paradigms between the two studies, it is difficult to compare results. The differing results of the two studies may be explained by the application of different species (mice vs. rats). In contrast to the mouse IntelliCage study, the rat place preference and reversal learning paradigms did not have punishment, only positive reinforcement (water access). The length and timing of the experimental phases in the rat study was based upon the experiences gathered during the mouse IntelliCage study.

The use of the automated home cage for behavioral experiments has the remarkable potential to reveal the naturally occurring spontaneous hierarchy and social structure within the small rat population of the cage. The interaction of multiple social and cognitive skills is needed to organize a functioning hierarchy that in nature increases the chance of survival of a population in gregarious species. It has been even suggested that

social organization is one of the biggest evolutionary driver of mammals [94]. We hypothesized that impaired social and communicative skills typical of the VPA model can result in unstable or disorganized hierarchy of a group with autistic-like symptoms. The IntelliCage allows us to examine not only the behavior of individual rats but also enables us to analyze the inter-individual interactions that can lead to a better understanding of how a group behaves in certain situations. We designed a competitive task in which water availability was spatially and temporally limited. Spontaneous hierarchy and its alteration in a highly competitive task were assessed using rank abundance curves and evenness. During competition, hierarchy in the control group is characterized by unevenness and log normal distribution of lick duration, both features lacking in the VPA group. Supporting our results, a recent study reported disturbed hierarchy in Fmr1 KO rats, revealed by dominance tube tests [95]. Fmr1 KO is a model of Fragile X syndrome, frequently co-diagnosed with ASD.

In a competitive situation, subjects have essentially two distinct ways of keeping others from drinking: repelling others and occupying or guarding the rewarded corner. In the first situation, inter-visit intervals would be higher after dominant subjects exit the corner compared to subordinate animals. However, this was not the case: the distribution of inter-visit intervals did not depend on subjects (data not shown). On the other hand, guarding behavior would increase the number of reentering visits by dominant subjects. Our study revealed a guarding behavior of the control rats, represented by the increased reentering visit number when water was limited. Strict hierarchy and guarding the water source was not necessary when it was more available. In contrast, guarding behavior did not evolve in VPA rats when water became limited. Therefore, VPA rats could not develop stronger hierarchy upon water scarcity. The most striking discovery of the present study is that failure in changing to uneven resource sharing and lack of guarding can be interpreted as social/supra-individual rigidity in VPA rats. The occurrence of unevenness (representing strength of hierarchy) was already apparent in side preference learning but even more pronounced during competition. We suspect that in control rat's strength of hierarchy is inversely proportional with water availability. They were able to adapt to the changing environment by changing their behavior.

It has been previously reported that vasopressin 1b receptor gene knockout mice show mild impairments in social recognition and they have reduced aggression [96]. Decreased

ADH serum levels may reveal a correlation of ADH with aggression or the ability to form hierarchy. The study showed that they can form hierarchy, but they employ alternate strategies. It is very similar to our finding of VPA rats alternate way of distributing a scarce resource.

As resource distribution becomes patchy, the members of a group are forced into more direct competition with each other for better utilization of each patch of resource [97]. In nature, more subordinate individuals will leave the group or will not survive [97]. To put differently, when resources are scarce, uneven resource sharing serves the survival of the fittest. Limiting a vital resource, water and studying its utilization among the group members gave us an insight into the altered social economy of autistic-like rats.

On the contrary, VPA group did not adapt to decreasing resources by uneven sharing. This maladaptive behavior could be strongly correlated with behavioral or cognitive rigidity as reported earlier in prenatally VPA treated mice [93]. Cognitive rigidity is a known hallmark of autistic-like traits on the level of individual subjects, as well [66]. Even though 'awkwardness' in social situations, struggling to recognize social rank in society are considered core symptoms of ASD, there is very limited scientific literature covering the topic of social dominance in ASD. However, a recent study shows that subjects living with ASD tend to judge dominance in a social interaction slower, indicating malfunctioning nonverbal processing [98]. There are some other indirect examples which stem from dysfunctional social hierarchy recognition in ASD. Sterzing et al. found that children with ASD are bullied nearly five-times more often than their neuro-typical schoolmates [99]. This latter finding may derive from the fact that children with ASD are less able to acknowledge or respect someone with higher social rank in their community. Taken these results together with our finding, the disrupted dominance hierarchy among autistic rats can be related to clinical manifestation.

Our approach of investigating the social and non-social behaviors of VPA-treated rats in automated home cages led us to detect novel characteristics of the prenatal VPA rat model of ASD in an etiologically more relevant design. However, we did not find a significantly altered ADH regulation of the VPA group, scientific literature suggests that ADH seems to be a key player regarding many aspects of autistic behaviors. Our results showed psychogenic polydipsia, decreased exploration and altered circadian rhythm of autistic rats. The most salient finding is that prenatally VPA-exposed rats as a group show an

inability to adapt their behavior in a changing environment. Since substantial impairments of adaptive behavior are a hallmark feature of ASD with serious consequences on everyday functioning of individuals, this finding further increases the translational value of the prenatal VPA model and may indicate its potential usefulness in ASD drug discovery.

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7. CONCLUSION

Using automated home-cages, we established different rodent models of psychiatric disorders to study the underlying mechanisms. We analyzed huge amount of data gathered through a sophisticated, unique statistical approach. We also created new behavioral tests in two rodent species that allow us to investigate compounds to alleviate symptoms of various psychiatric disease models. These results will further validate our findings and will open the opportunity to test our own synthetized compounds as medium-throughput robust behavioral methods with high translational value. We led a pioneering work to harness the advantages of relatively stress-free environment for rodents and subjective bias-free data collection made possible by automated home-cages in pharmacological studies.

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8. SUMMARY

Using automated home-cages to study behavior is a relatively new concept. Rodents are kept in a social, relatively natural environment. These conditions allowed us to reveal new findings in psychogenic rodent models. We established mouse place preference learning and reversal learning methods and studied the effect of a known memory disrupting anticholinergic agent, scopolamine in mice. We found not only impaired reversal learning ability when treated with scopolamine but found difference in activity and lick behavior as well. Partly based on these findings, we designed a more complex set of methods to study autistic rats. Autism spectrum disorder (ASD) is characterized by impaired socio-communicational function, repetitive and restricted behaviors. Valproic acid (VPA) was reported to increase the prevalence of ASD in humans because of its use during pregnancy. VPA treatment also induces autistic-like behaviors in the offspring of rats after prenatal exposure; hence it is a preclinical disease model with high translational value. In the present study our aim was to characterize ASD relevant behaviors of socially housed, individually identified male rats in automated home cages. Natural behavior of rats was assessed by monitoring their visits to drinking bottles in an environment without human influence aiming at reducing interventional stress. Although rodents normally tend to explore their new environment, prenatally VPA-treated rats showed a drastic impairment in initial and long-term exploratory behavior throughout their stay in the automated cage. Furthermore, VPA rats displayed psychogenic polydipsia as well as altered circadian activity. In the competitive situation of strict water deprivation controls switched to an uneven resource sharing and only a few dominant animals had access to water. In VPA animals similar hierarchy-related changes were completely absent. While the control rats secured their chance to drink with frequent reentering visits, thereby ‘guarding’ the water resource, VPA animals did not switch to uneven sharing and displayed no evidence of guarding behavior.

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10. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

10.1. On the topic of the dissertation

Pelsőczi, P., & Lévay, G. (2017). Effect of Scopolamine on Mice Motor Activity, Lick Behavior and Reversal Learning in the IntelliCage. *Neurochemical Research, 42*(12), 3597–3602. https://doi.org/10.1007/s11064-017-2408-4

Pelsőczi, P., Kelemen, K., Csölle, C., Nagy, G., Lendvai, B., Román, V., & Lévay, G. (2019). Disrupted Social Hierarchy in Prenatally Valproate-Exposed Autistic-Like Rats. *Frontiers in Behavioral Neuroscience, 13,* 295. https://doi.org/10.3389/fnbeh.2019.00295

10.2. Other, dissertation independent publications

Pecze, L., Pelsoczi, P., Kecskés, M., Winter, Z., Papp, A., Kaszás, K., Letoha, T., Vizler, C., & Oláh, Z. (2009). Resiniferatoxin mediated ablation of TRPV1+ neurons removes TRPA1 as well. *The Canadian Journal of Neurological Sciences. Le Journal Canadien Des Sciences Neurologiques, 36*(2), 234–241. https://doi.org/10.1017/s0317167100006600

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Disrupted Social Hierarchy in Prenatally Valproate-Exposed Autistic-Like Rats

*Péter Pelsőczi[1,2], Kristóf Kelemen[1], Cecília Csölle[3], Gábor Nagy[1], Balázs Lendvai[4], Viktor Román[3] and György Lévay[1,5]**

[1]Laboratory of Cognitive Pharmacology, Division of Pharmacology and Drug Safety, Gedeon Richter Plc., Budapest, Hungary, [2]Faculty of Pharmaceutical Sciences, Semmelweis University School of PhD Studies, Budapest, Hungary, [3]Laboratory of Neurodevelopmental Biology, Division of Pharmacology and Drug Safety, Gedeon Richter Plc., Budapest, Hungary, [4]Division of Pharmacology and Drug Safety, Gedeon Richter Plc., Budapest, Hungary, [5]Department of Morphology and Physiology, Faculty of Health Sciences, Semmelweis University, Budapest, Hungary

Autism spectrum disorder (ASD) is characterized by impaired socio-communicational function, repetitive and restricted behaviors. Valproic acid (VPA) was reported to increase the prevalence of ASD in humans as a consequence of its use during pregnancy. VPA treatment also induces autistic-like behaviors in the offspring of rats after prenatal exposure; hence it is a preclinical disease model with high translational value. In the present study, our aim was to characterize ASD relevant behaviors of socially housed, individually identified male rats in automated home cages. The natural behavior of rats was assessed by monitoring their visits to drinking bottles in an environment without human influence aiming at reducing interventional stress. Although rodents normally tend to explore their new environment, prenatally VPA-treated rats showed a drastic impairment in initial and long-term exploratory behavior throughout their stay in the automated cage. Furthermore, VPA rats displayed psychogenic polydipsia (PPD) as well as altered circadian activity. In the competitive situation of strict water deprivation controls switched to an uneven resource sharing and only a few dominant animals had access to water. In VPA animals similar hierarchy-related changes were completely absent. While the control rats secured their chance to drink with frequent reentering visits, thereby "guarding" the water resource, VPA animals did not switch to uneven sharing and displayed no evidence of guarding behavior.

Keywords: rats, valproate, autism spectrum disorder, exploratory behavior, hierarchy, prenatal exposure delayed effects, polydipsia, psychogenic

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Neza Grgurevic,
University of Ljubljana, Slovenia
Luigia Trabace,
University of Foggia, Italy

****Correspondence:***
György Lévay
gy.levay@richter.hu

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by social and communicative impairments and excessive repetitive behaviors (American Psychiatric Association, 2013). Although the pathogenesis of ASD is not fully understood, several factors have been identified as possible contributors such as genetic (Sebat et al., 2007; Klei et al., 2012; De Rubeis et al., 2014) or environmental factors (Brown, 2012; Hertz-Picciotto et al., 2018). Hallmarks of autistic-like symptoms in rodents can be measured with several behavioral assays such as the three-chamber social interaction, self-grooming, ultrasonic vocalization, tube dominance or social playtests. However, in most of these tests the subject is removed from its home-cage and must

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adapt to a novel environment. In these situations, animals are exposed to excessive human handling that can cause unnecessary stress and might have a serious impact on the natural behavior of the experimental animals. This stress can lead to an elevated level of anxiety which could introduce a strong bias in the results.

With automated home-cages, the experimenter can reduce human interference to a minimum. In automated home-cages animals are kept in their familiar environment, while the social structure of the group is intact for the entire duration of the experiment. This virtually undisturbed environment could reveal more natural spontaneous behaviors that in turn may result in more sensitive assessment methods compared to traditional behavioral assays. Because in ASD social behavior and structure are so crucial, we focused our research interest more on group dynamics rather than only on the individual behavior of subjects.

Prenatal exposure to valproic acid (VPA), a frequently used anticonvulsant medication (Löscher, 2002), is a major non-genetic risk factor of ASD (Bromley et al., 2013; Christensen et al., 2013). VPA treatment also induces autistic-like behaviors in the offspring after prenatal exposure in rats (Schneider and Przewocki, 2005), including impaired social interaction, excessive repetitive behaviors (Kim et al., 2013; Roullet et al., 2013; Dai et al., 2018; Nicolini and Fahnestock, 2018), reduced ultrasonic communication (Gandal et al., 2010) as well as increased anxiety (Mehta et al., 2011). The well-grounded etiopathology of the model and its symptomatic similarity to the human condition confer this animal model of ASD a high translational value.

Although it is a widely accepted model of ASD, the behavior of groups of prenatally VPA-exposed animals has not been investigated extensively. As described above, automated home-cages offer an excellent means to study non-apparent/underlying behaviors, hierarchy and group dynamics in socially-kept rodents, by avoiding rats even recognize that they are under surveillance. Given the scarcity of studies investigating autistic-like rodents in an automated environment, here we aim at studying the behavior of communities of autistic-like and control rats with as little human intervention possible, besides trying to minimize any stress or subjective bias.

MATERIALS AND METHODS

Prenatal Valproate Treatment

Timed-pregnant Wistar rats (outbred stock, Janvier, France) kept on soy-free diet (Teklad soy protein-free rodent diet, ENVIGO, Madison, WI, USA) and tap water received a single dose of 300 mg/kg sodium valproate (VPA, cat. P4543-10G, Sigma, UK) intraperitoneally in a volume of 2.5 ml/kg physiological saline on gestational day 12. The pregnant rats were transported from Janvier to our animal facility. Control dams received an injection of physiological saline of identical volume at the same gestational time-point. The size of litter was adjusted to 10 for each dam (by removing female pups) and then left undisturbed until the time of weaning on postnatal day 21 when the male offsprings were housed in groups of three or four until behavioral testing.

Subjects and Husbandry

Twenty 11-week-old male Wistar rats were chosen from the F1 generation (n = 10 in control and n = 10 in the VPA group, selected from four litters in each treatment group) and were placed separately in two IntelliCages. Animals were implanted with microchips (UNO PICO-ID ISO transponder, UNO BV, Netherlands) under isoflurane anesthesia 1 week prior to the experiments. Rats were kept in the animal facility with a 12 h light/dark cycle (lights off at 4 p.m.), while ambient room temperature was maintained at 22 ± 2°C and 40–50% relative humidity. Food was freely available; water access was related to specific tasks. All efforts were made to minimize the suffering of experimental animals. Experimental procedures were reviewed and approved by the Local Animal Care and Use Committee (PE/EA/2885-6/2016) and were carried out in accordance with the European Animal Protection Directives (Directive 2010/63/EU).

The IntelliCage Apparatus

The IntelliCage system (TSE Systems, Bad Homburg, Germany[1]) allowed group-housed rats to be assessed for spontaneous behavior and various other behavioral tasks. The size of the central arena was 100 × 100 × 35 cm. As bedding material wood shavings were used (OSAFE, J. Rettenmeier and Söhne GmbH, Rosenberg, Germany). In order to enrich the environment, two black plastic shelters were placed in each cage, allowing the animals to hide and climb (TSE Systems, Bad Homburg, Germany). The IntelliCage has four recording corners. Water was only available in the corners behind remotely controlled doors. When a rat entered a corner, an antenna detected its unique transponder and recorded its visit. It needs to be emphasized that the corner design allowed the entry of only one animal at a time. Each corner housed two drinking bottles, while the left and right sides could be distinguished. The activity of the rats within the corners was monitored by using tracking software (IntelliCage Plus 3.1.1.0, TSE Systems). The principal parameters were: the number of visits to the corners, initiated nose pokes, lick numbers and the duration of all these parameters.

Training Phases in the IntelliCage

Control and VPA groups were tested in IntelliCages for 41 days. Animals were challenged with gradually more and more complex tasks. The time spent in the IntelliCages was divided into four phases: acclimation, nose poke learning, place preference learning and competition (**Figure 1**).

In the first phase of the study, rats could habituate to the new environment for 3 days (acclimation). They were allowed to visit any corner any time and choose any bottle to drink from throughout the day. They could also drink from the bottles ad libitum. The number of trials was not limited, thus rats voluntarily visited the corners. The second phase of the study was nose poke learning for 20 days when rats had to initiate trials with a nose poke to gain access to the water bottles for 7 s. The third phase of the study was to train the animals to develop a side preference for an appointed corner in order to study learning

[1] https://www.tse-systems.com/product-details/intellicage

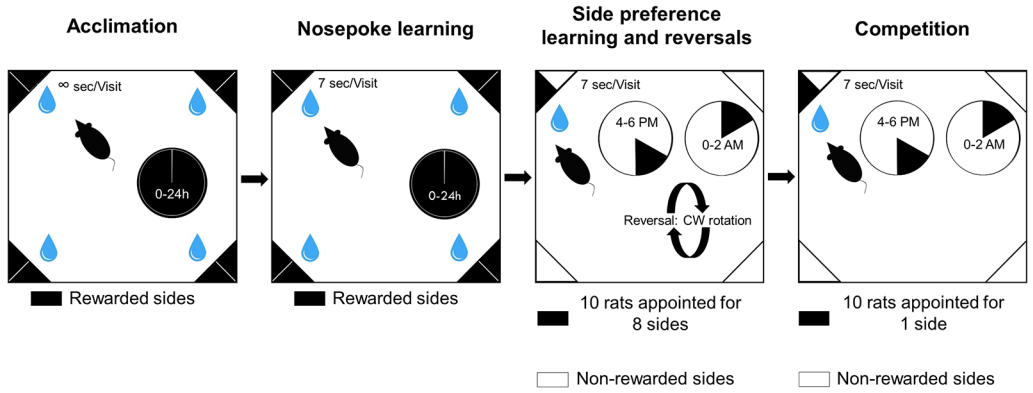

FIGURE 1 | Schematic representation of the experimental phases and visual description of the tasks.

behavior for 15 days. One side was rewarded by providing access to water for 7 s, whereas the remaining seven sides did not open after a nose poke. Nose pokes at the seven remaining sides were recorded as incorrect choices. Ten rats were allotted to eight sides. Two rats were allotted to bottles 1 and 2, and one rat to each of the remaining bottles. Reversals were achieved with randomly changing the position of the correct corner to another corner (excluding the current one), and the side was interchanged as well. The reversal was carried out on day 3, 7 and 11. The last phase of the study included the competition task. In this phase, all ten rats were assigned to only one corner in both groups. In, side preference and competition water access was available only for two periods of 2 h each day (4:00–6:00 pm and 0:00–02:00 am). During any visit only the first nose poke resulted in door opening.

Collection of Blood Samples

Following decapitation, trunk blood was collected rapidly into 0.5 ml plastic tubes and put on ice. Tubes were then centrifuged at 10,000 rpm for 2 min at room temperature. The serum was separated and divided into aliquots of ∼300 μl and stored at −80°C. Serum samples (n = 6 for each group) were analyzed using a Beckman Coulter AU480 Chemistry Analyzer instrument (Beckman Coulter, Inc., Brea, CA, USA).

Gene Expression Assay

Each removed tissue sample (n = 10, hippocampus (HPC), cerebellum, prefrontal cortex, thalamus) was immersed in RNA *Later* and stored at 4°C overnight, then stored at −20°C. The tissue was homogenized and RNA was extracted using an RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The RNA was stored at −80°C in RNase/DNase-free water. All RNA preparations were analyzed on an Agilent 2,100 Bioanalyzer (Agilent Technologies, Berkshire, UK) to determine the RNA concentration and the quality of the RNA using the RNA integrity number (RIN). cDNA was synthesized from total 1 μg RNA in a 20-μl reaction mixture by using a Superscript VILO cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol. Quantitative PCR was carried out using the Applied Biosystems (Carlsbad, CA, USA) Quantstudio 12K Flex Real-Time PCR System, according to the manufacturer's instructions. Primers and probes for quantitative PCR were purchased from Thermo Fisher Scientific, Waltham, MA, USA (Cry1: Rn01503063_m1; Per1: 01325256_m1; Npas2: Rn01438223_m1; Arntl: Rn00577590_m1; Clock: Rn00573120_m1; Mtnr1a: Rn01488022_m1; b-actin (ACTB), 4352340E). The cycle conditions for quantitative PCR were 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s All data were normalized to ACTB expression. Data were calculated using the $2^{-\Delta\Delta CT}$ method. RQ mean values are normalized to 1 for the control.

Spontaneous Locomotor Activity

Spontaneous locomotor activity was measured in male rats (n = 10 in each group) at postnatal days 26–28 by a six-channel activity monitor manufactured by Experimetria (Hungary). The apparatus consisted of acrylic cages (48.5 cm × 48.5 cm × 40 cm) equipped with 2 × 30 pairs of photocells along the bottom axis of the cage. Additional arrays of photocells (30 pairs) were placed along two opposite sides of the cage at different heights (6.5, 12, 18 and 23 cm) in order to detect rearing responses. The photocell beam, when broken, signaled a count which was then recorded by a computer. The signals were processed by a motion analyzing software that determined the spatial position of the animal with 1 Hz sampling frequency and computed the distance traveled and the time spent by the rats with ambulation, local movement (e.g., grooming), immobility, rearing, etc. Animals were individually placed in the photocell cages; horizontal movements (ambulation time), as well as vertical rearings, were determined for 1 h. Data are expressed as means ± SEM.

Juvenile Social Play

Pinning as the most characteristic parameter of social play behavior was scored for each pair of male rats (n = 10 in each group) on postnatal days 33–36. The testing arena of juvenile social play was a plexiglass cage (42 × 42 × 32 cm) with approximately 2 cm of wood shavings covering the floor. Pairs of rats (from the same treatment group) were assigned for social interaction by using unfamiliar partners (i.e., not a cage mate or littermate). Animals in a test pair did not differ more than 10 g in body weight. On the postnatal day 34 and

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35, each animal was introduced to the testing arena for a period of 5 min individually. On the third day (postnatal day 36), the motivation for the play was enhanced by isolating the animals for 4 h before the test. Animals that had been unfamiliar to each other were placed simultaneously into the opposite corners of the previously discovered arena and their behavior was recorded for 15 min. Behavioral elements were assessed using the Observer 5.1 software (Noldus Information Technology B.V., Netherlands). The frequency of pinning as the most characteristic parameter of social play behavior was scored for each pair of animals and expressed as means ± SEM (Panksepp et al., 1984; Trezza et al., 2010). Data are expressed as means ± SEM.

Maternal Deprivation-Induced Ultrasonic Vocalization

Impairments in communication between pups and their mothers were measured by recording ultrasonic vocalizations. To induce calls, pups (n = 10 in each group) were separated from their mothers and placed individually into a cage for 10 min, while calls were being recorded with bat microphones. Calls were digitized with an audio filter and ultrasonic vocalization was recorded and quantified with SonoTrack software (Metris BV., Netherlands). Vocalization was measured at age of 12 days for 10 min. Statistical analysis included the Kruskal–Wallis non-parametric test and the *post hoc* Dunn test. Data are presented as means ± SEM of USV calls count/10 min.

Von Frey test

Von Frey test was used for estimating paw withdrawal thresholds (expressed in grams) with a series of filaments, that uses a constant number of five stimuli per test. It was conducted with the simplified up-down method as previously described (Bonin et al., 2014; n = 10 in each group).

Statistical Analysis

Exploratory visits (i.e., visits without nose poke or drinking) were aggregated for each day of the experiment by subjects. Generalized linear models (GLM) with log link using negative binomial distribution were constructed. Differences in initial exploratory activity were also compared between groups. To show how exploratory activity changed during the day, visits were aggregated for each 4-h period. For nose poke learning, a cosinor analysis was conducted to see how daytime exploratory activity patterns differed between groups (Cornelissen, 2014). Two main estimates were considered: mesor (mean activity) and amplitude (difference of peak and midline activity). Mean values for groups were compared using the t-test. The daytime mean activity was calculated for the first 72 h, groups were compared using bootstrapped Watson's test.

Drinking volume was estimated by assessing the number of licks and lick duration. For acclimation, the number of licks for each animal was calculated for 4-h periods which also revealed how drinking behavior changed over the course of the day. When competing, animals were restricted both in their access to water bottles, and maximum lick duration for each visit. Therefore, the mean lick duration was calculated for each day of the experiment. In addition, linear mixed-effects models were used to compare groups with subjects as random factors (Bolker et al., 2009). Acclimation was treated separately from the other phases because of the difference in underlying data distribution due to the time limit introduced after acclimation.

In, side preference learning, the proportion of correct nose pokes to all nose pokes was calculated. This response variable was put in a binomial generalized mixed-effects model (GLMM) with treatment as the fixed effect. Binomial distribution was used because the number of correct responses out of all trials was measured. The proportion of correct nose-pokes is expected to change during time due to the initial period and the reversals. Therefore, the drink session was included as a random factor within the model. As dispersion was high, cumulative lick duration was included as a random factor. Differences in drinking volume changed the proportion of visits to the correct corner that was not related to learning. This addition to the model dropped the dispersion to near 1, while the mean of all random factors was close to 0 (−0.02). Type II Wald chi-square test was used to assess the treatment effect. The hierarchy was estimated by differences in lick duration within groups. Total lick duration was calculated for each day of the experiment and evenness of the values was used as a community measurement. Evenness is most often used to describe the distribution of individuals within a community using Pielou's evenness index ranging from 0 to 1. This measure was adapted to reflect evenness in drinking among individuals, because, the lower the evenness, the stronger the hierarchy that is expected in the community. Hierarchy could be best observed during competition; therefore, rank abundance curves were fitted to the cumulative lick number per hour values of each subject of the groups. Models were compared using Akaike information criterion (AIC) to find the shape of the best fitting model.

Reentering visits ("guarding") were defined as visits after which the same subject entered the corner. The number of reentering visits was calculated for each subject and day of the experiment. The maximum divided by mean values for groups was calculated to express the distribution of reentering visits within the groups. Experimental phases were merged based on whether water access was unlimited (acclimation, nose-poke learning) or limited (side preference learning, competition) during the given phase. Population-level values were compared by using a linear model. Calculations were carried out by using R[2].

Statistical evaluation was performed by unpaired t-tests to analyze spontaneous locomotor activity (ambulation and rearing), von Frey test, ultrasonic vocalization, and juvenile social play (pinning) results in GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

VPA Rats Show Autistic Phenotype

Preceding studies confirmed autistic behavior of the VPA group (**Supplementary Figure S4**). In spontaneous locomotor

[2]https://www.R-project.org/

activity they showed impaired rearing ($p < 0.001$), whereas ambulation was unaffected by the treatment. Pinning remained also unchanged. In ultrasonic vocalization ($p < 0.05$) and von Frey test ($p < 0.001$) VPA rats showed significant impairment compared to the control group.

Decreased Initial Exploration in VPA Rats

The exploratory activity was assessed by calculating the number of exploratory visits defined as visits without nose-poke and lick. Control rats tended to explore the novel environment of the IntelliCage, represented by high numbers of exploratory visits during acclimation (**Figures 2A,B**) especially in the first 24 h (5.45 ± 0.40, 2.03 ± 0.17 exploratory visits/h for the control and VPA group, respectively). The VPA rats showed a significant decrease in exploratory visits in the first 24 h ($p < 0.001$). Later, this initial exploratory activity decreased in the control group and showed a stable daily pattern (**Figure 2A**). The VPA group showed significantly lower initial exploratory activity in nose-poke learning (cosinor regression, difference in mesor, average activity; 33.87 vs. 22.94 visits/h for control and VPA group, respectively; $p < 0.05$). The reduction of exploratory visits in the VPA group was robust and highly significant (GLM, $p < 0.001$) throughout acclimation, nose-poke- and side preference learning phases (VPA group −55% vs. control; **Figure 2B**).

Circadian Rhythm Disturbance in VPA Rats

The daily activity pattern was assessed during the acclimation phase when water was freely available. The circadian activity was estimated based on an analysis of daytime mean activity. The circadian rhythm of the VPA group showed an approximately 2-h shift after light off compared to the control group ($p < 0.05$; 7:00 for VPA compared to 5:01 for control; **Figure 2C**). Cry1, Per1, Arntl, Npas2, Clock and Mtnr1a gene expression was measured but did not reach a 2-fold change between the treatment groups, hence it cannot be interpreted as biologically relevant change. VPA group data are presented in the order of the following brain regions: cerebellum (C), HPC, prefrontal cortex (PFC) and thalamus (T).

Cry1 (C) 1.07, (HPC) 1.16, (PFC) 0.998, (T) 0.764; Per1 (C) 1.007, (HPC) 1.224, (PFC) 0.723, (T) 1.166; Arntl (C) 0.712, (HPC) 0.639, (PFC) 0.813, (T) 1.07; Npas2 (C) 0.717, (HPC) 0.924, (PFC) 0.828, (T) 1.126; Clock (C) 0.89, (HPC) 1.069, (PFC) 0.735, (T) 1.058; Mtnr1a (C) 0.697, (HPC) 0.886, (PFC) 1.497, (T) 0.706.

Normal Place Preference and Reversal Learning in VPA Rats

Control and VPA groups did not differ in place and side preference learning abilities, nor was a difference in their reversal learning (**Supplementary Figure S1**). The proportion of correct nose-pokes was identified: 89.2% for the control and 88.1% for the VPA group (fitted value $\chi^2 = 0.898$, $p = 0.3$).

Altered Drinking Behavior in VPA Rats

During acclimation, the daily pattern of drinking behavior showed frequent but short bouts for the control group, while VPA rats drank in rare but long bouts (**Supplementary Figure S2**). Consequently, control rats showed a well-balanced, stable pattern of lick number per hour distribution (**Figure 3A**). In the control group, 56% of the 4-h-long period was dominated by one subject ("a") who drank the most during this time. In contrast, in the VPA group the individual animal with the highest number of licks ("a") dominated only 24% of the 4 h periods.

Such distribution could arise from the differences in the number of visits or that of lick duration during visits. Therefore, we had investigated the mean lick duration per visit and found that there was an almost threefold difference between control and VPA rats for the mean lick duration during acclimation (6.0 s vs. 17.9 s linear mixed-effects model $p < 0.001$; **Figure 3B**). We pooled these data for nose-poke learning, side preference learning and competition, as well (**Figure 3C**). Although each protocol after acclimation was set up to limit lick duration to 7 s, there was a significant difference between groups (2.9 s vs. 4.1 s, linear mixed-effects model $p < 0.001$; **Figure 3C**). The sera of the groups were analyzed for blood chemistry parameters (**Supplementary Figure S3**, **Supplementary Tables S1, S2**). Out of 20 parameters only the potassium showed a slight increase compared to the reference values (5.29 for control and 4.64 for the VPA groups; reference values are for the potassium 4–5.9 mmol/l (Stender et al., 2007).

Disturbed Hierarchy in VPA Rats

To reveal differences in group dynamics and adaptive behavior within the groups, a deeper analysis of drinking and visiting patterns was performed. During the competition phase of the study, three control subjects showed apparent competitive dominance over the rest of the animals which was manifested in the larger cumulative lick number (**Figure 4A**). In contrast, such a clear formation of subgroups was not observed within the VPA group (**Figure 4A**). When tested this in rank-distribution models, the best fit for the control group was log-normal (AIC = 171.5), whereas for the VPA group followed a broken-stick model (AIC = 184.9). This difference only appears when access to water is strictly limited in time and space. High evenness values were observed through acclimation and nose-poke learning phases, without significant difference between the groups (**Figure 4B**). As water became less accessible in, side preference learning and even more so in competition phases, the control group showed significantly lower evenness in lick duration, while in the VPA group it remained stable indicating an intensifying hierarchy for the control animals (**Figure 4B**, $p < 0.001$).

Representative subjects' with most, median and least number of consecutive visits are shown to the same corner during the competition (**Figure 5A**). In the control group, one animal had a substantially higher number of consecutive visits compared to the rest. VPA group's subjects showed much more converging numbers. The entire population was thus described using maximum over mean consecutive visits calculated for each day of the experiment.

There was a significant difference in the limited or unlimited conditions of water access within the control group (**Figure 5B**) when the daily maximum/mean reentering visits (i.e subject reentering the same corner) were calculated. Control rats visited

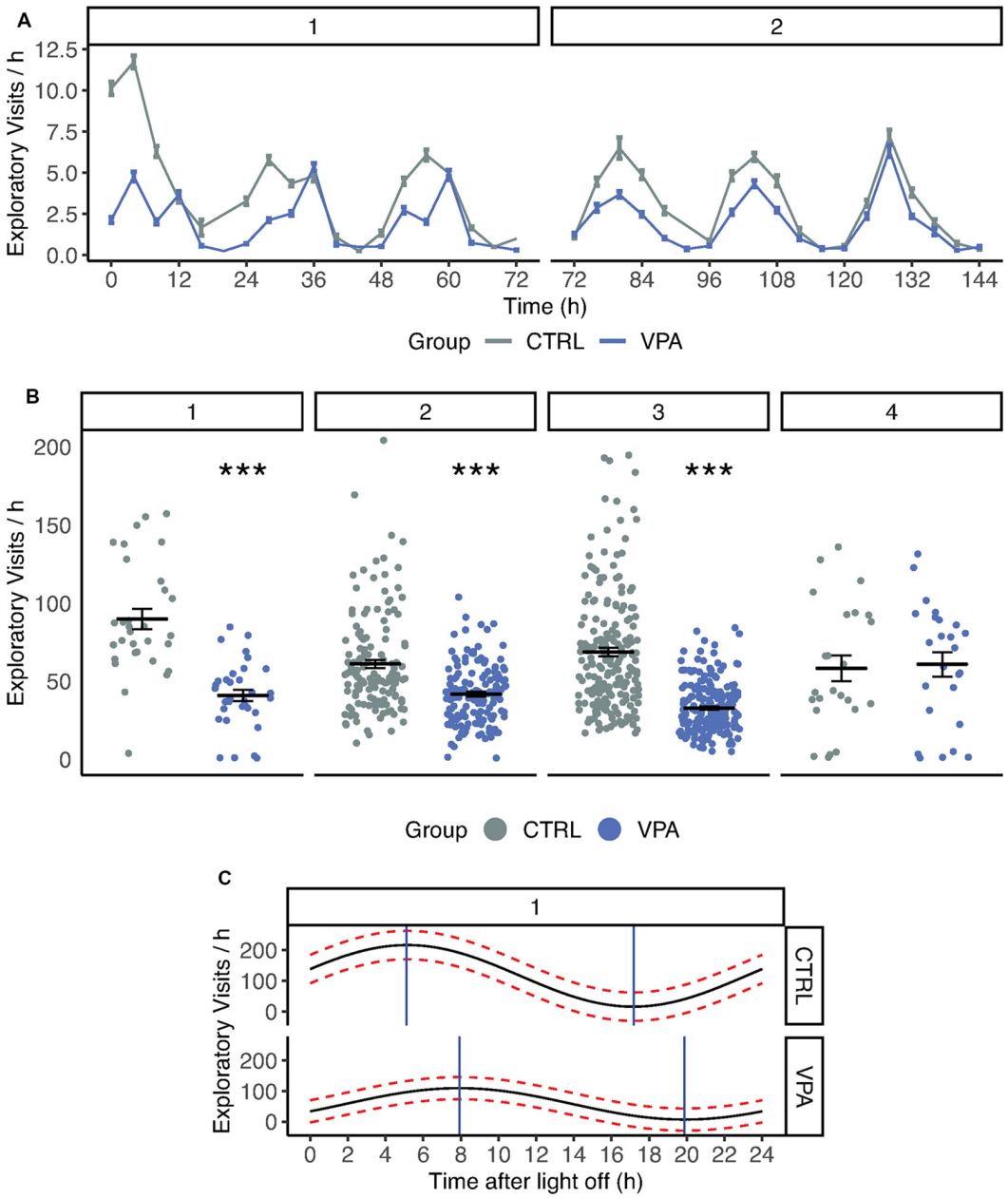

FIGURE 2 | (A) Number of exploratory visits in control and VPA groups. 1, 2, indicate phases: Acclimation, Nosepoke learning. Time scale of exploratory visits summed in four-hour periods. Means (±SEM) are shown ($p < 0.001$). **(B)** Number of exploratory visits in control and VPA group. 1, 2, 3, 4 indicate phases: Acclimation, Nosepoke learning, Side preference learning, Competition. In acclimation, nosepoke learning and side preference learning VPA group showed a significant decrease in explorative visits ($p < 0.001$). **(C)** Cosinor analysis of circadian rhythm during acclimation. Mean (black line) and confidence interval (red dotted lines) are drawn for pooled group data. Time after light off values associated with maximum and minimum activity are shown with blue lines. ***$p \leq 0.001$.

the same corner 78% more frequently when water access was scarce (competition phase), compared to the water unlimited condition ($p < 0.001$). In contrast, the limitation of water access did not change the behavior of VPA rats; they did not change the frequency to reenter the same corner in any condition (**Figure 5B**).

DISCUSSION

Collecting very detailed information in a social environment may provide novel insights on fundamental elements of behavior that cannot be deduced from traditional behavioral observations that deliver only limited amounts of data. In

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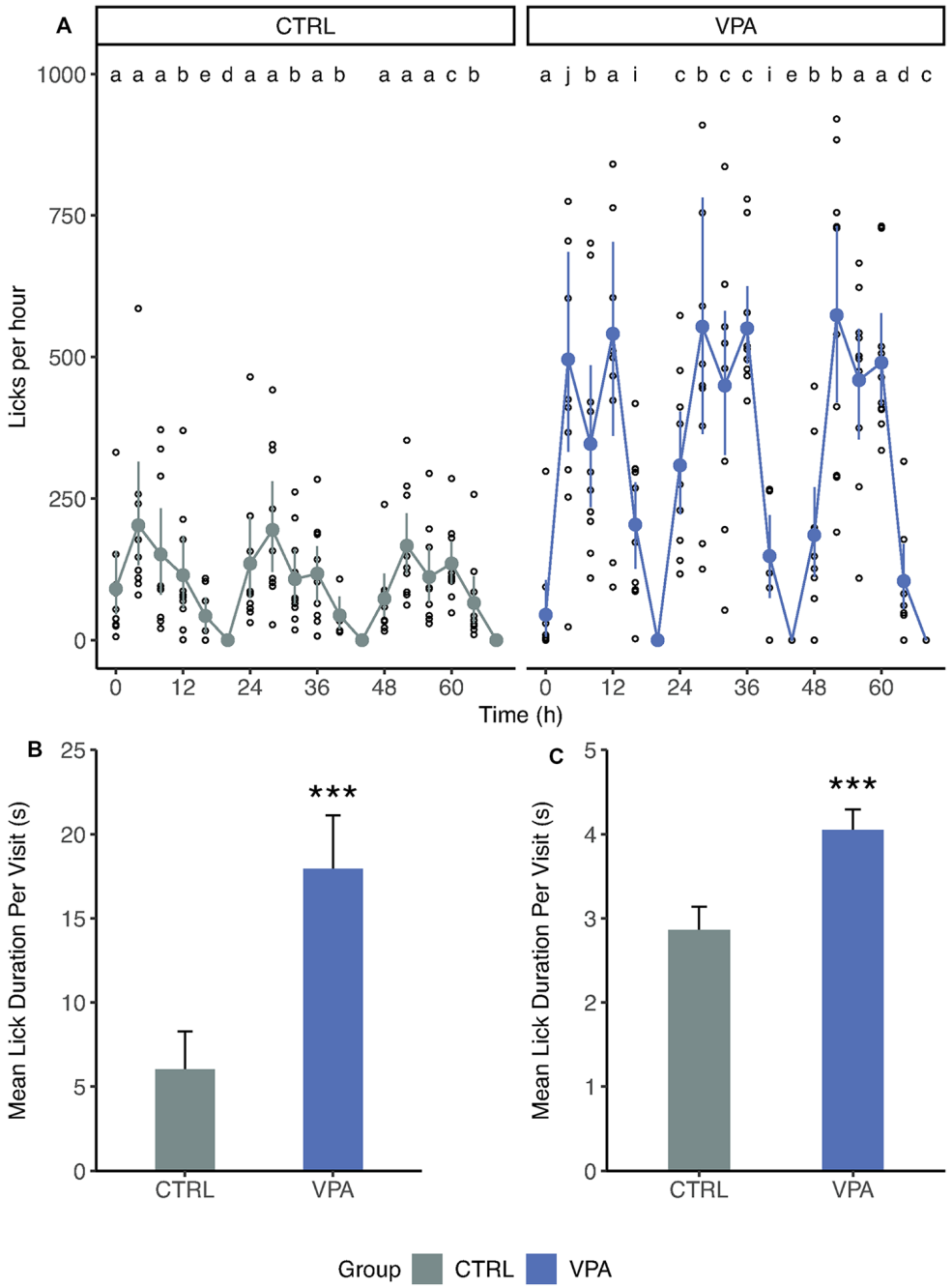

FIGURE 3 | Differences in drinking behavior. **(A)** The number of licks grouped for 4-h periods during acclimation. Mean, confidence interval and individual values are shown. The number of lick values is transformed for 1 h. Letters indicate the individual with the highest number of licks for each 4-h period. **(B)** Mean lick duration (±SEM) per visit during acclimation ($p < 0.001$). **(C)** Mean lick duration (±SEM) per visit during later phases (pooled). ***$p \leq 0.001$.

the present study, data mining of the pattern of simple animal actions; visiting, nose-poking and drinking allowed us to investigate various behaviors in communities of prenatally VPA-exposed autistic-like and control rats. The autistic behavior of rats prenatally treated with VPA includes social components resembling the symptoms of human ASD (Schneider and Przewocki, 2005). Here, we focused on more sophisticated aspects of behavior beyond the well-known social effects described in VPA animals.

VPA-treated rats showed a drastic decrease in exploratory visits in the first 24 h compared to the control group, which was maintained at some level in all phases except competition. One can speculate, that the reason behind decreased exploratory behavior during competition is that both groups optimized their

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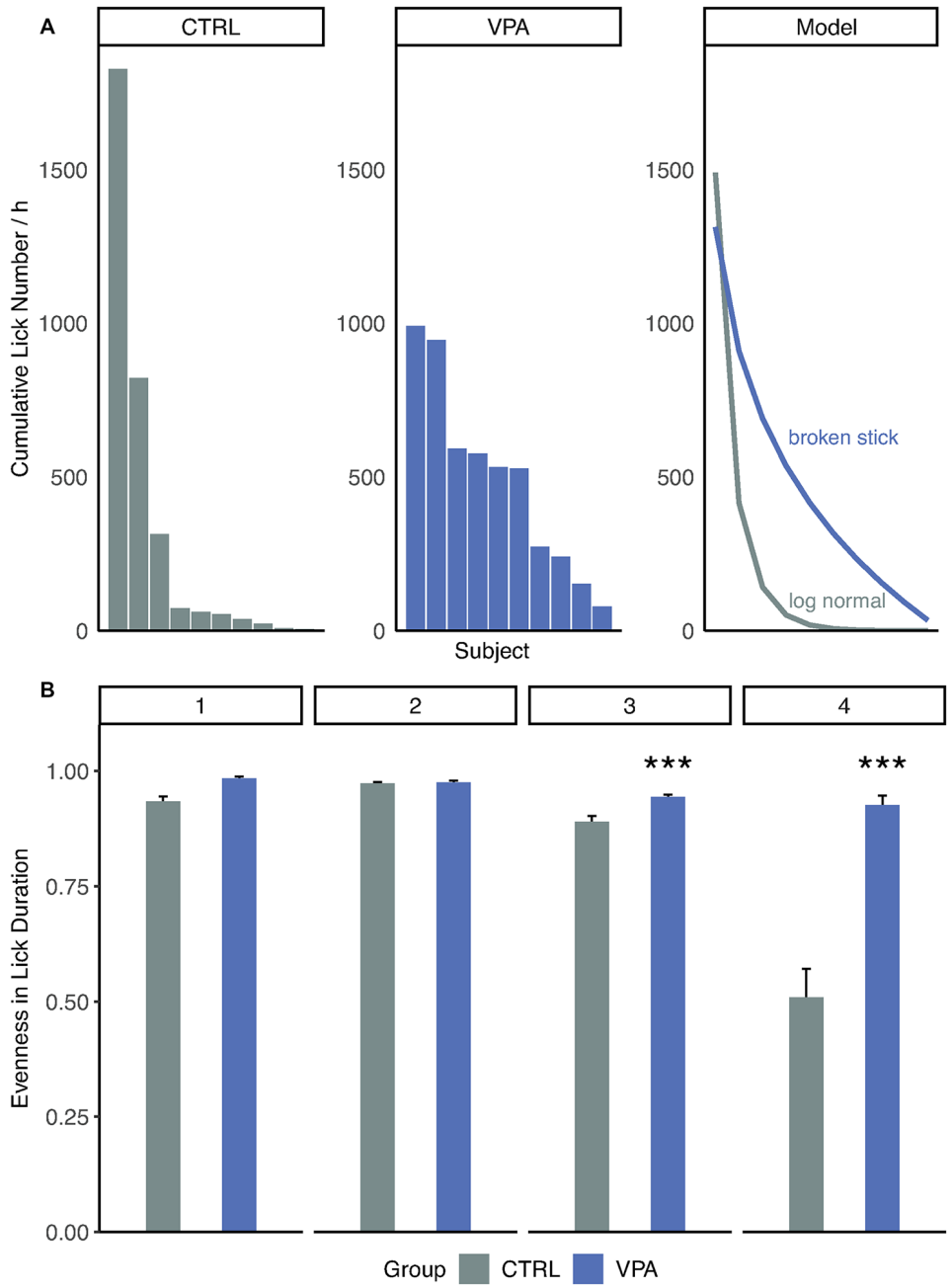

FIGURE 4 | Inter-individual drinking behavior of treatment groups. **(A)** Distribution of cumulative lick number per hour. Rank abundance curves are drawn according to the model best fit the data: log-normal for control Akaike information criterion (AIC = 171.5) and broken stick for VPA group (AIC = 184.9). **(B)** Evenness (diversity divided by maximum diversity) values for groups and phases (daily mean ± SEM; ***$p \leq 0.001$).

behavior in a way that they did not "waste" visits only for exploration and took every chance to drink whenever possible. The overall decrease of exploration may derive from neophobia, anxiety or alternatively, a shifted circadian rhythm could also play a role.

Indeed, our results showed a 2-h shift in the circadian rhythm of the VPA group. In parallel with our result, an earlier report showed that VPA shortened circadian period *in vivo* and *in vitro* and suppressed behavioral activity across species (Landgraf et al., 2016). ASD patients often have sleep problems and altered social timing, and it has been correlated with identified single nucleotide polymorphisms (SNPs) of Per1 and Npas2 genes (Nicholas et al., 2007). VPA exposure alters core circadian rhythm transcription factors *in vitro* and *in vivo* (Olde Loohuis et al., 2017; Griggs et al., 2018). In both studies, altered expression of known circadian rhythm genes was reported. Interestingly, our

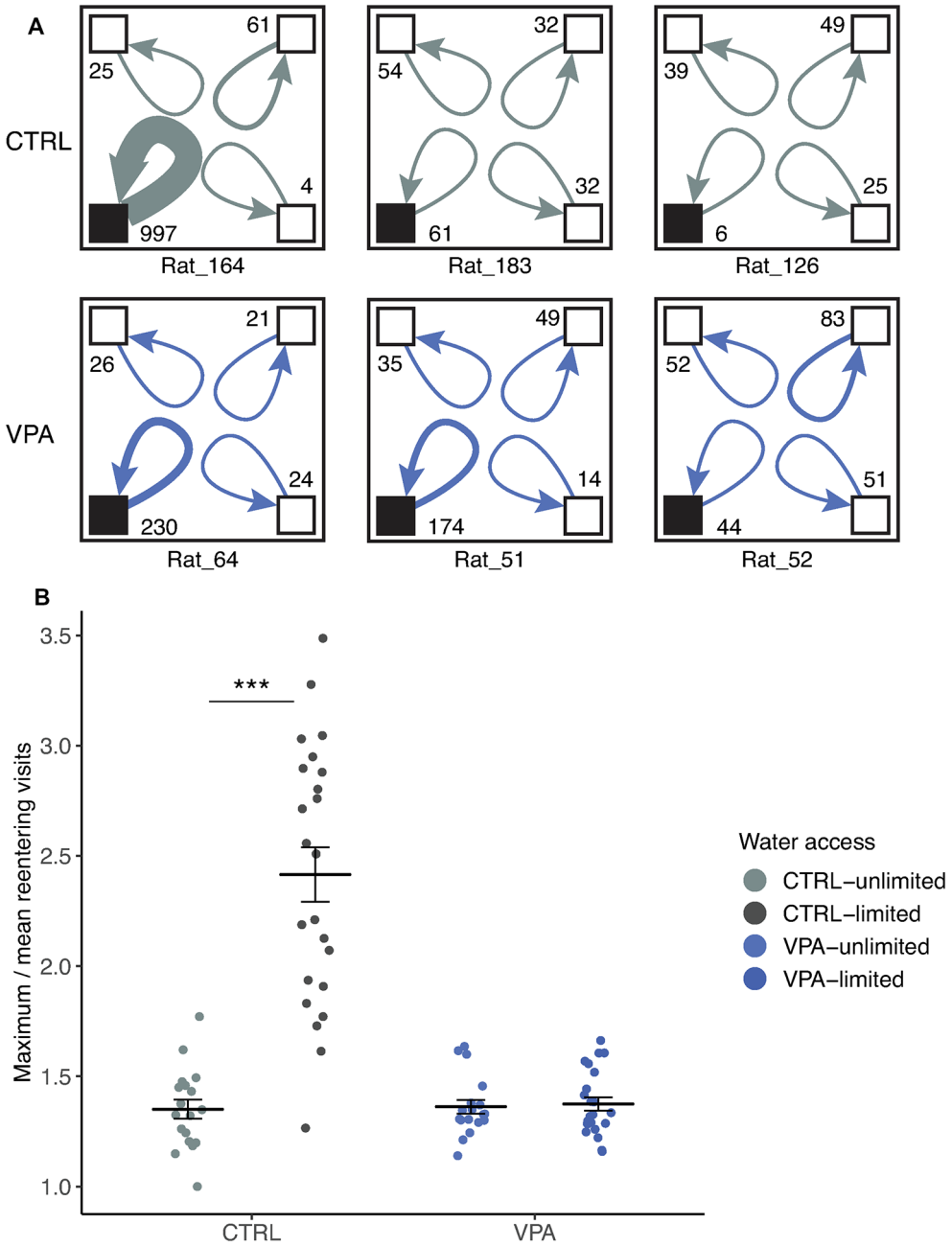

FIGURE 5 | Lack of social dominance in the VPA group. **(A)** Representative cage layouts for three individuals (ID presented at the bottom of the schematic cage) with the highest, median and lowest numbers of reentering visits for each treatment group (rows) during competition. Reentering visits are calculated for each subject as visits to the same corner when the subject left without other subjects entering the corner (numbers shown at the corners). The thickness of the arrows indicates the strength of the reentering visits. **(B)** Social dominance within the group is characterized by using a ratio of the maximum visits of an individual divided by the mean values of the group (±SEM) for daily cumulative reentering visits. Water limitation provoked the elevation of social dominance in the control group as compared to conditions with unlimited water access (effect size: 78.8%, $^{***}p \leq 0.001$), whereas no significant effect was shown within the VPA group that remained at a dominance value below 2.

results showed no significant change in a selected set of circadian rhythm genes (Cry1, Per1, Arntl, Npas2, Clock and Mtnr1a) of the VPA rats. This may suggest that our behavioral findings in the VPA group are not the consequence of the differentially expressed mRNA levels of the circadian genes, rather on the level of proteins, SNPs or changed regulation of certain transcription factors. On the other hand, considering the highly complex and multifactorial nature of the circadian regulation, we only analyzed a small set of genes, hence we must be careful when drawing conclusions. Circadian changes due to different patterns

of melatonin expression were studied in human ASD patients (Nir et al., 1995). In parallel with these studies, our results confirm the circadian rhythm disturbance *in vivo*.

During the acclimation phase, we have unexpectedly found a substantial difference in general drinking behavior of the VPA group that appeared as an almost threefold increase in lick duration per visit. Control rats tended to visit very often but drank only in short bouts while VPA rats visited rarely but once they entered the corner, they drank considerably more. In the following phases, this difference in behavior stayed significant despite the limitation of water access (7 s). Altered drinking behavior was most likely not due to any underlying mechanism of the VPA treatment, as we did not see major anomalies in blood chemistry results (**Supplementary Figure S3**, **Supplementary Table S1**). Only the potassium level showed a slight increase in the control group compared to the upper limit of the reference value. However, the extent of the difference seems irrelevant (0.49 mmol/l) that could be only a consequence of the higher water intake, as well. Therefore, we concluded, that there was no apparent water balance issue that could explain the high duration of licks. This altered drinking behavior in VPA rats could be explained by increased perseveration (represented by mean lick duration increase) that might result from the prenatal VPA exposure. The increase in water consumption could be also explained by psychogenic polydipsia (PPD). PPD is a clinical symptom, which has a common occurrence in patients with psychiatric disorders, most commonly schizophrenia (de Leon et al., 1994). PPD is characterized by polyuria and polydipsia (Dundas et al., 2007). We believe that this is the first preclinical study showing the appearance of PPD in conditions that can be associated with human ASD. This phenomenon was already shown in autistic children, which further strengthens the translational value of the prenatal VPA rat model of ASD (Terai et al., 1999).

Puścian et al. (2014) reported earlier impaired place preference learning of prenatally VPA treated C57BL/6 and BALB/c mice using automated home cages, while we found no difference in side preference and reversal learning of VPA rats. Because of the difference in spatial learning paradigms between the two studies, it is difficult to compare results. The differing results of the two studies may be explained by the application of different species (mice vs. rats).

The use of the automated home cage for behavioral experiments has the remarkable potential to reveal the naturally occurring spontaneous hierarchy and social structure within the small rat population of the cage. The interaction of multiple social and cognitive skills is needed to organize a functioning hierarchy that in nature increases the chance of survival of a population in gregarious species. It has been even suggested that social organization is one of the biggest evolutionary driver of mammals (Christian, 1970). We hypothesized that impaired social and communicative skills typical of the VPA model can result in unstable or disorganized hierarchy of a group with autistic-like symptoms. The IntelliCage allows us to examine not only the behavior of individual rats but also enables us to analyze the inter-individual interactions that can lead to a better understanding of how a group behaves in certain situations. We designed a competitive task in which water availability was spatially and temporally limited. Spontaneous hierarchy and its alteration in a highly competitive task were assessed using rank abundance curves and evenness. During the competition, hierarchy in the control group is characterized by unevenness and log-normal distribution of lick duration, both features lacking in the VPA group. Supporting our results, a recent study reported disturbed hierarchy in Fmr1 KO rats, revealed by dominance tube tests (Saxena et al., 2018). Fmr1 KO is a model of Fragile X syndrome, frequently co-diagnosed with ASD.

In a competitive situation, subjects have essentially two distinct ways of keeping others from drinking: repelling others and occupying or guarding the rewarded corner. In the first situation, inter-visit intervals would be higher after dominant subjects exit the corner compared to subordinate animals. However, this was not the case: the distribution of inter-visit intervals did not depend on subjects (data not shown). On the other hand, guarding behavior would increase the number of reentering visits by dominant subjects. Our study revealed a guarding behavior of the control rats, represented by the increased reentering visit number when water was limited. Strict hierarchy and guarding the water source was not necessary when it was more available. In contrast, guarding behavior did not evolve in VPA rats when water became limited. Therefore, VPA rats could not develop stronger hierarchy upon water scarcity. The most striking discovery of the present study is that failure in changing to uneven resource sharing and lack of guarding can be interpreted as social/supra-individual rigidity in VPA rats. The occurrence of unevenness (representing the strength of hierarchy) was already apparent in, side preference learning but even more pronounced during competition. We suspect that in control rat's strength of hierarchy is inversely proportional with water availability. They were able to adapt to the changing environment by changing their behavior.

As resource distribution becomes patchy, the members of a group are forced into more direct competition with each other for better utilization of each patch of resource (Meurant, 1988). In nature, more subordinate individuals will leave the group or will not survive (Meurant, 1988). To put differently, when resources are scarce, uneven resource sharing serves the survival of the fittest. Limiting a vital resource, water and studying its utilization among the group members gave us an insight into the altered social economy of autistic-like rats.

On the contrary, the VPA group did not adapt to decreasing resources by uneven sharing. This maladaptive behavior could be strongly correlated with behavioral or cognitive rigidity as reported earlier in prenatally VPA treated mice (Puścian et al., 2014). Cognitive rigidity is a known hallmark of autistic-like traits on the level of individual subjects, as well (Karvat and Kimchi, 2014). Even though "awkwardness" in social situations, struggling to recognize social rank in society are considered core symptoms of ASD, there is very limited scientific literature covering the topic of social dominance in ASD. However, a recent study shows that subjects living with ASD tend to judge dominance in a social interaction slower, indicating malfunctioning nonverbal processing (Kuschefski et al., 2019). There are some other indirect examples which stem

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from dysfunctional social hierarchy recognition in ASD. Sterzing et al. (2012) found that children with ASD are bullied nearly five times more often than their neuro-typical schoolmates. This latter finding may derive from the fact that children with ASD are less able to acknowledge or respect someone with higher social rank in their community. Taken these results together with our findings, the disrupted dominance hierarchy among autistic rats can be related to clinical manifestation.

Our approach of investigating the social and non-social behaviors of VPA-treated rats in automated home cages led us to detect novel characteristics of the prenatal VPA rat model of ASD in an etiologically more relevant design. Our results showed PPD, decreased exploration and altered circadian rhythm of autistic rats. The most salient finding is that prenatally VPA-exposed rats as a group show an inability to adapt their behavior in a changing environment. Since substantial impairments of adaptive behavior are a hallmark feature of ASD with serious consequences on the everyday functioning of individuals, this finding further increases the translational value of the prenatal VPA model and may indicate its potential usefulness in ASD drug discovery.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Local Animal Care and Use Committee (PE/EA/2885-6/2016) and were carried out in accordance with European Animal Protection Directives (Directive 2010/63/EU).

AUTHOR CONTRIBUTIONS

PP, BL, VR and GL conceived the study and wrote the manuscript. PP and GN implemented the experiments. PP and KK analyzed the data and participated in the interpretation of the results. CC participated in the experimental design and conceiving the study. All authors read and approved the final version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00295/full#supplementary-material.

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ORIGINAL PAPER

Effect of Scopolamine on Mice Motor Activity, Lick Behavior and Reversal Learning in the IntelliCage

Péter Pelsőczi[1] · **György Lévay**[1]

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Abstract Automated homecage monitoring systems are now widely recognized and used tools in cognitive neuroscience. However, few of these studies cover pharmacological interventions. Scopolamine, an anticholinergic memory disrupting agent is frequently used to study learning behavior. We studied the impact of scopolamine treatment in a relevant dose-range on activity, drinking behavior and reversal learning of C57BL/DJ mice in a homecage-like, social environment, using the IntelliCage. Naïve mice were first habituated to the IntelliCage, where they learned to nosepoke in any of the four corners in order to gain access to the water reward. Visits, nosepokes, lick numbers and durations were recorded. Mice were then trained to distinguish between a rewarded correct corner and punished, incorrect corners. Later, in the reversal learning phase, the assigned correct corner was rotated clockwise every 24 h. Upon s.c. administration of scopolamine general activity represented by visit and nosepoke numbers increased, but their durations were shorter. Surprisingly, general activity and lick behavior were drastically altered. Scopolamine also significantly reduced the ability to perform a reversal learning task. We not only found significant decline in reversal learning due to scopolamine treatment, but studied the method specific underlying behaviors: the general activity and lick behavior as well.

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✉ György Lévay
gy.levay@richter.hu

Péter Pelsőczi
pelsoczip@richter.hu

[1] Laboratory of Cognitive Pharmacology, Gedeon Richter Plc., Gyömrői út 19-21, Budapest 1103, Hungary

Keywords Natural activity · Reversal learning · Cognitive impairment · Scopolamine · IntelliCage

Introduction

Learning and memory functions are widely investigated in rodents using a number of well-known simple behavioral paradigms such as novel object recognition [1], Morris water maze [2] or fear conditioning [3, 4]. More complicated operant instruments such as Skinner Box [5] or Touch Screen systems [6] are used to evaluate higher cognitive functions in specially designed experimental environment. However, all these models and systems share one common feature, i.e. the test animals are kept in regular home cages which are distinct from the experimental instrument(s). Animals have to be removed from their home cage for treatment and placed in the experimental instrument to perform their task. This leads to an extra source of stress, and consequently manifests as an unspecific increase in variance of their behavioral parameters [7, 8]. To circumvent this stress factor, automated home cage monitoring systems have recently become recognized and used tools in cognitive neuroscience. The IntelliCage enables non-stop automatic monitoring of mice's behavior over an extended period of time in a homecage-like social environment [8]. All mice are subcutaneously microchipped, so the cage corners recognize and appropriately respond to the behavior of the visiting animals according to the preprogrammed paradigm. The system is capable of teaching conditioned learning paradigms to mice, using positive or negative reinforcements and cues. The sensors record visits, nosepokes, licks and all data are time-stamped. IntelliCages provide an experimental system where subjects need minimal contact with the experimenter, in contrast with

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traditional behavioral methods. Also, animals can visit the corners according to their own circadian rhythm.

The cholinergic muscarinic antagonist scopolamine is generally found to increase locomotor activity [9–11]; with evidence that cholinergic signaling in the hippocampus, striatum and frontal cortex is positively correlated with scopolamine-induced hyperactivity [12]. In contrast, studies with scopolamine in learning tasks have identified either decreased locomotor activity [13, 14], or no effect [15]. In addition to the effects on activity, the blockade of muscarinic receptors with the non-specific antagonist scopolamine prior to a learning task has been consistently reported to impair spatial learning and memory in both rats and mice [16, 17]. Scopolamine administration has an impact on esophageal function as well. The esophagus is composed of both smooth and striated muscle. Smooth muscle function and coordination are dependent on cholinergic innervation. Therefore, drugs with anticholinergic activity have the potential to cause dysphagia [18]. As acute scopolamine treatment is used in rodents to cause temporary cognitive impairment, we were interested whether it causes any alteration in the registered behavioral parameters in the IntelliCage. The apparatus offers a certain time span of water access as positive reinforcement. Due to known side-effects of scopolamine in humans [18] (dysphagia, dry mouth) and the known effect on locomotor activity, we hypothesised that it may change drinking behavior, and general activity following drug treatment. Robinson and coworkers reported a trend towards elevated visit numbers after 0.5 mg/kg scopolamine treatment in IntelliCage; however it was not significant [19]. In our study we investigated activity, drinking behavior and reversal learning of C57BL/DJ mice treated with various doses of scopolamine in the IntelliCage system.

Materials and Methods

Subjects

32 male C57BL/6JOlaHsd mice (25–30 g) were purchased from a commercial vendor (ToxiCoop, Budapest, Hungary) and acclimated for 2 weeks prior to testing. Mice were implanted with microchips under isoflurane anasthesia 1 week prior to placing them into the IntelliCages. At 8–12 weeks of age, mice were group housed (16 per cage) and kept in our animal facility with a 12 h light/dark cycle (lights off at 3 p.m.), ambient room temperature was maintained at 22 ± 2 °C and 40–50% relative humidity. Food and water were freely available. All animals were housed and tested in accordance with our institutional regulations. All experiments were conducted with approval from the local ethical committee and were in accordance with both local and international regulations and principles (86/609/EEC Directive).

Drug Treatments and Groups

Scopolamine-HBr was purchased from Tocris (UK). The formulations were prepared freshly on each experimental day in PBS solution, to avoid scratching due to acidity. Scopolamine and its vehicle were injected subcutaneously (s.c.) at a volume of 5 ml/kg body weight. The following doses and groups were tested: scopolamine (0.05, 0.17 and 1 mg/kg $n = 16$, each); vehicle (PBS, $n = 16$). The drug doses used in this study were previously found to be effective in studies of spatial learning and locomotor activity. Two IntelliCages were used for the experiment, where eight mice of each treatment group were treated with either vehicle or scopolamine. Administration was scheduled approximately 15 min prior to the start of the dark phase of testing (3 p.m.). Due to rapid onset of the drug's pharmacodynamic effects (occurs as soon as 20 min following s.c. treatment), to decrease the time needed for administration, only 16 mice were injected in a given experiment. The next day the other 16 mice were injected, then data were pooled. Altogether, results of 12 experiments are analyzed. After each experiment, the animals had 4–6 days to recover from the scopolamine effect, to avoid any behavioral change in vehicle treated animals due to within-subject design (Fig. 1).

Apparatus

The IntelliCage system (TSE Systems, Bad Homburg, Germany) allows group-housed mice to be assessed for spontaneous behavior and spatial learning [20, 21]. Each IntelliCage is able to house and simultaneously record up to 16 mice. A unit consists of an open communal space with shelters to provide comfort and environmental enrichment, and four recording chambers, which are located at the corners. Within the IntelliCage, mice have free access to food in the center, and water is available only in the corners behind remotely controlled doors. When a mouse enters a corner an antenna detects the mouse's unique transponder (microchip) and records the visit. Importantly, corner design allows the entry of only one mouse at a time. Each corner houses two drinking bottles so that left and right sides can be distinguished. To open the controlled doors the animals have to perform a nosepoke. The behavior of the animals and activity within the corners of the cage is monitored using the PC based tracking software (IntelliCage Plus, TSE Systems). The principal parameters reflecting activity were number of visits to any of the four corners, the initiated nosepokes, the lick number, and the durations of these parameters. Based on preliminary experiments, we recorded the first 180 min of these parameters following drug treatment.

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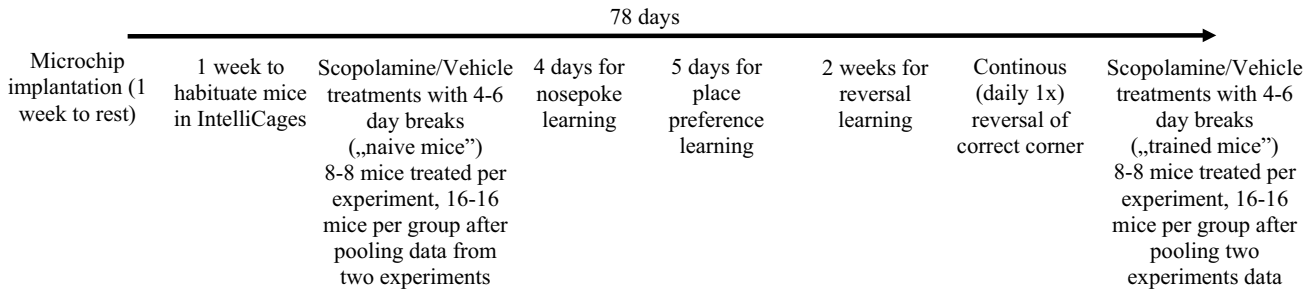


Fig. 1 Full timeline of the experiments

Behavioral Test in the IntelliCage

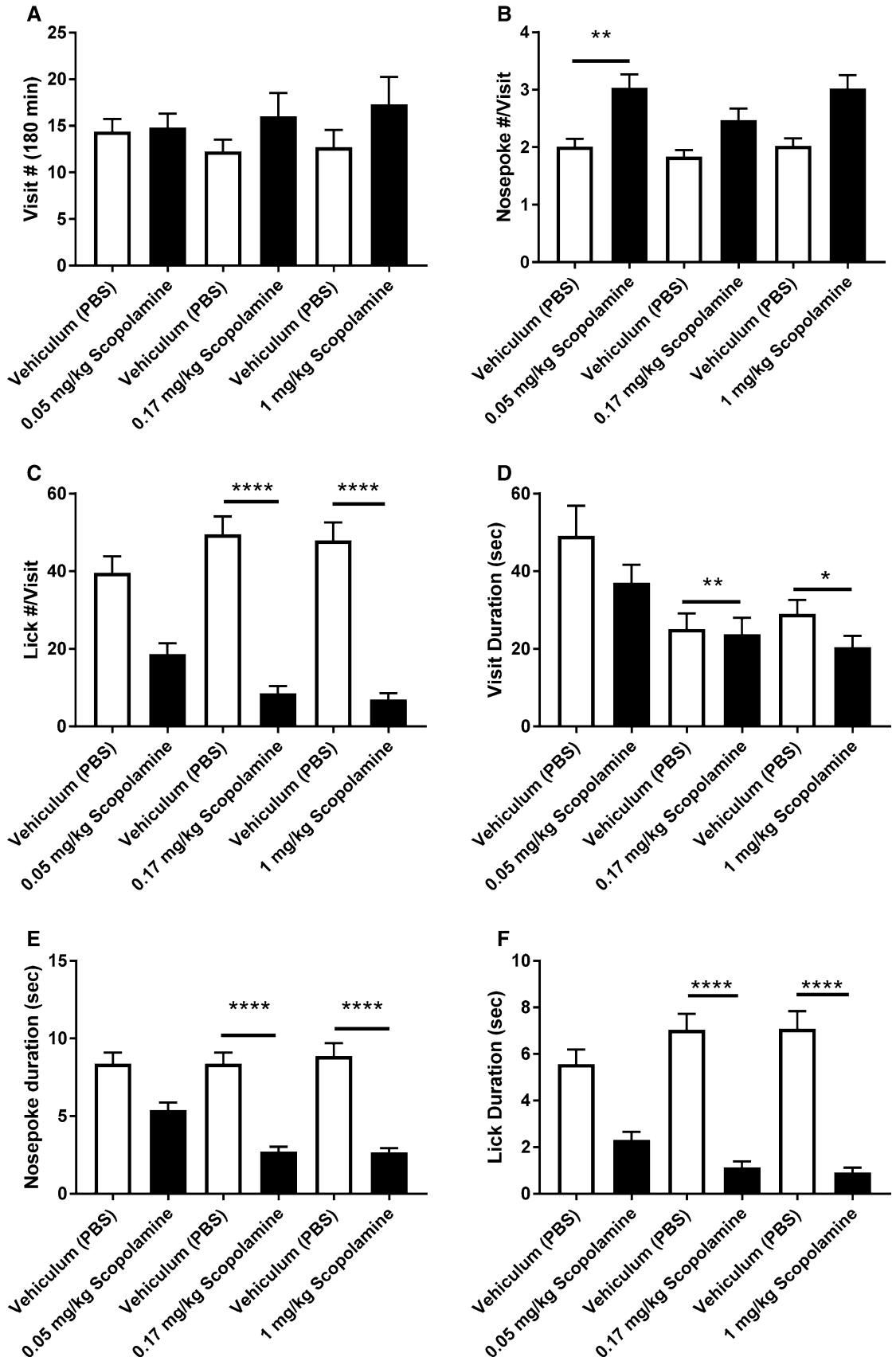
In the first phase of the study, mice were allowed to habituate to the new environment for 7 days. The preprogrammed protocol allowed them to visit any corner any time during the day. They could also choose in a corner any of the two bottles to drink from. The number of trials was not limited and mice voluntarily visited the corners. Cohort sizes in both cages were $n = 8$ per treatment group. In the second phase of the study mice had to learn to perform a nosepoke within the corners to gain access to the drinking bottle for 7 s. During any visit, only the first nosepoke results in door opening. In the third phase, the place preference learning paradigm, mice were evenly assigned to all four corners: for any individual mouse one corner was correct, while all three other corners were incorrect. Mice were allowed to drink in any of the four corners (incorrect ones included) but in the incorrect corners they received a 5-s air-puff (2 bar) as punishment. In the fourth and final phase, the reversal learning paradigm, the position of the correct corner was rotated clockwise daily (Fig. 1).

Statistical Analysis

Data are presented as group mean + SEM and were analyzed using GraphPad Prism 7.01 (GraphPad Software, San Diego, CA, USA). Subjects were taken as repeated measures. Non-parametric and parametric one-way analysis of variance (ANOVA) with Friedman-test, Dunn's multiple comparison post-hoc test, and Kruskal–Wallis test was applied for statistical analysis of the data. As we had different trial numbers in each group—due to the limitations of the chosen statistical method—we had to disregard some data, to have an equal number of data points in every group. This could slightly deteriorate the results. For all comparisons, $p < 0.05$ was considered reliable. Asterisks indicate: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Results

First, we looked at the temporal pattern of the pharmacodynamic effects of scopolamine following administration of the drug. Behavioral effects were seen predominantly in the first three hours, when analyzing data by hourly bins. In the fourth hour no differences between the treatment groups in any of the measured parameters were found (data not shown). Using the three hour long time window, at the treatment doses of 0.17 and 1 mg/kg animals showed a trend towards increased activity levels (Fig. 2a). Interestingly, analyzing only the first 20 min, we found significant increase in visit numbers in all applied doses (Fig. 3a). Nevertheless, using small time window means less data to analyze, which makes it hard to interpret. Therefore, we decided to analyze the 0–180 min time window of the dataset, where 0 min indicates drug injections. In accordance with Robinson's earlier study [19], we did not find significant difference between vehicle and scopolamine-treated groups in general activity measured as visit numbers (Fig. 2a). In our initial paradigm (first phase), mice could drink freely from any bottles without time restraint. Scopolamine-treated mice showed significant increase in nosepoke number per visit at 0.05 mg/kg dose ($p = 0.0072$), and showed a similar trend in the two higher doses as well, but the effect is not significant (Fig. 2b). Nosepoke duration in a given visit showed significant decrease ($p < 0.0001$) when treated with 0.17 and 1 mg/kg scopolamine (Fig. 2e). In accordance with the decreased nosepoke duration in the scopolamine-treated group, lick duration and lick number per visit were also decreased significantly in the 0.17 and 1 mg/kg doses ($p < 0.0001$), compared to the vehicle groups (Fig. 2c, f). In these dose groups, visits were significantly shorter as well ($p = 0.0039$ and $p = 0.0217$; Fig. 2d). In our reversal learning paradigm, scopolamine significantly decreased the ability of mice to adapt to the new rule in 0.17 mg/kg dose ($p < 0.01$, Fig. 3b) indicated by elevated error rates. A trend towards increased error rates is also apparent in the doses of 0.05 and 1 mg/kg, however, it is not statistically significant.


A
Visit # (180 min)
25
20
15
10
5
0
Vehiculum (PBS)
0.05 mg/kg Scopolamine
Vehiculum (PBS)
0.17 mg/kg Scopolamine
Vehiculum (PBS)
1 mg/kg Scopolamine
B
Nosepoke #/Visit
**
4
3
2
1
0
C
Lick #/Visit
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D
Visit Duration (sec)
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*
E
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◂**Fig. 2** Effect of 0.05, 0.17 and 1 mg/kg scopolamine in untrained C57BL/6JOlaHsd mice in IntelliCage in the first 180 min (n = 16, each). Main parameters of the drinking behavior and locomotor activity are shown (**a**–**f**). White columns represent vehicle group mean value, black columns show the different doses of scopolamine. Each drug treatment were compared to its vehicle group result in the same experiment. Errors are shown as mean + SEM

Discussion

Here we studied the effects of a single dose of scopolamine in inbred mice in the IntelliCage, a simple and robust homecage system [8]. Our results indicate that finding the right time window for data collection is crucial in an experimental design where animal activity is registered throughout the day. We established that given the limitation of an acute administration, this window has to coincide with meaningful pharmacodynamic effects, and at the same time it should be sufficiently long to have the necessary amount of visit and nosepoke data. As published earlier, 3 h of access daily with 21 h of water deprivation is sufficient and motivating for different learning paradigms in IntelliCage, such as place preference learning, reversal learning and place avoidance tasks [22]. To target this problem, one solution could be to use an implanted osmotic or mechanical pump, in order to have a constant drug release [23]. This way we could analyze much longer periods of time, one could even study correlation between drug effect and circadian rhythm.

Scopolamine, a frequently used anticholinergic cognitive impairing agent, is a promising tool compound in learning paradigms conducted in the IntelliCage. Earlier publications showed contradictory results of locomotor activity in different behavioral methods using scopolamine [12–15]. Our results can partly explain this phenomenon. Before using scopolamine in actual learning paradigms, we first aimed to study the simpler behavioral alterations due to its known side-effects in naïve, untrained mice (dry mouth, dysphagia in humans) [18]. These data could help us develop specific learning paradigms in IntelliCage, taking into account scopolamine's effect on locomotor activity and drinking behavior. We found that depending on the time window chosen, the effect of scopolamine on locomotor activity shows remarkable differences. In the first 20 min after injection, the visit number indicates increased locomotor activity of the scopolamine group (Fig. 3a), but using all data of the first 180 min these differences are not significant (Fig. 2a). Increased nosepoke number per visit (Fig. 2b) can be interpreted as increased general activity, or even decreased attention, as it was reported in an earlier study, where mice treated with scopolamine showed a disruption in accuracy and increased number of omissions in 5CSRTT paradigm [15].

As in humans [18], we revealed behavioral traits (decreased lick number and lick duration) suggesting difficulties in swallowing in mice (Fig. 2c, f). These data suggest that lick number and lick duration cannot be interpreted as cognitive parameters when using a pharmacological agent causing difficulties in swallowing. Based on these data, in case using scopolamine in IntelliCage studies as a memory disrupting agent, we suggest to use a learning paradigm, in which the positive reinforcement of the correct choices is not water access, but e.g. the absence of a negative reinforcement (i.e. the absence of airpuff), whereas water is available at all corners. This way undesired side-effects can be avoided which could deteriorate learning behavior, and even result in false positive results.

Our place preference and reversal learning paradigms were designed with the abovementioned insights in mind. Access to drinking bottles upon nosepoke was allowed in all corners, however, incorrect corners were punished. This way the lick number and lick duration were not considered as cognitive parameters. Our results suggesting scopolamine effect in reversal learning (Fig. 3b) confirm Robinson and coworkers results in IntelliCage [19]. However, their protocol was slightly different. Unlike in our paradigm, in Robinson et al. mice had access to water in correct corner only, while incorrect corners simply did not respond to nosepokes and did not punish with airpuffs either. In our experiments, scopolamine did not alter place preference learning in the same dose range (data not shown). We only found significant increase of error rate of the scopolamine treated group in our reversal learning paradigm. It might suggest that an already acquired knowledge becomes so stable over time, that it cannot be altered with a cognitive impairing agent. However, in an environment, where one has to adapt to an ever changing rule, the learning process can be more easily disrupted.

With rotating the correct corner position on a daily basis our results represent reversal learning, which can be interpreted as rather cognitive flexibility compared to Robinson's spatial learning paradigm [19]. It is also worthwhile to mention that they analyzed 6 h after drug treatment while according to our results, data analysis for 3 h is sufficiently enough. There were no differences found after the third hour between treatment groups.

Conclusion

Our results confirmed earlier findings and suggests a deeper understanding of anti-cholinergic effect of scopolamine on learning behavior in IntelliCage. By fine tuning data collection and analysis, we were able to show impact of scopolamine on general activity using different time windows for analysis and showed significant effect on drinking behavior and reversal learning in naturally behaving mice.

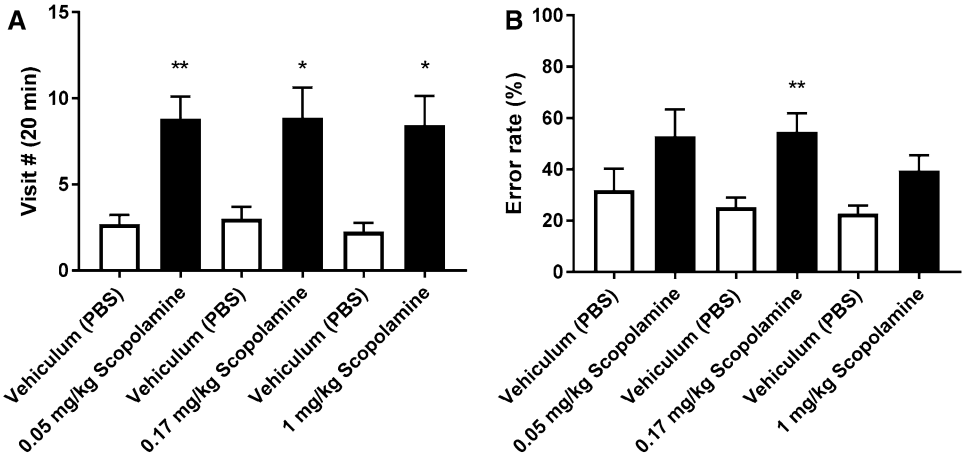

Fig. 3 Effect of scopolamine in 0.05/0.17/1 mg/kg dose range in trained C57BL/6JOlaHsd mice in IntelliCage in the first 20 min. Drug treatment effect on general activity measured as visit numbers (**a**). Scopolamine effect on reversal learning paradigm indicated as error rates, the ratio of incorrect visits and all visits (**b**). Errors are shown as mean + SEM

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