INNOVATIVE METHODOLOGY

Spatial cognition in a virtual reality home-cage extension for freely moving rodents

Kay Thurley, 2,3 Katja Frei, 1 Francesco Bagorda, 1 Alexej Schatz, 1
Gilad Tocker, 4,5 Sophie Rapoport, 5 Dori Derdikman, 5 and 6 York Winter 1, 6

1 Cognitive Neurobiology, Humboldt–Universität zu Berlin, Berlin, Germany; 2 Department Biology II, Ludwig-Maximilians-Universität München, München, Germany; 3 Bernstein Center for Computational Neuroscience Munich, München, Germany; 4 Gonda Multidisciplinary Brain Research Center, Bar Ilan University, Ramat-Gan, Israel; 5 Neuroscience Department, Rappaport Faculty of Medicine and Research Institute, Technion–Israel Institute of Technology, Haifa, Israel; and 6 NeuroCare Center of Excellence, Charité–Universitätsmedizin Berlin, Germany

Submitted 8 August 2016; accepted in final form 4 January 2017

TO UNDERSTAND THE ROLE of a specific mechanism in the control of behavior, testing must be performed in a behavioral context. This requires experimental manipulation of the mechanism in ways that meaningfully influence behavior. Virtual reality (VR) experimental environments have become a powerful tool for investigating the neural basis of behavior (Bohil et al. 2011; Dombbeck and Reiser 2012; Harvey et al. 2009; Hölscher et al. 2005; Thurley and Ayaz 2016), as they overcome the limitations of experimental physical arenas. VR allows behaviors to be investigated through closed-loop manipulations of the environment and even permit the generation of physically impossible environments (Bohil et al. 2011; Dombbeck and Reiser 2012; Thurley and Ayaz 2016). Air-floating track spheres for rodents permit optical imaging or intracellular recordings of neurons in awake, behaving animals (Domnisoru et al. 2013; Harvey et al. 2009; Harvey et al. 2012; Keller et al. 2012). However, the required head fixation impedes the extent of voluntary behavior, which limits the control of self-motion cues and distorts vestibular inputs.

Mismatch between optic flow and proprioceptive inputs may disrupt hippocampal space representation (Aghajan et al. 2015). It is therefore desirable to have a VR experimental environment that does not require body fixation to allow unperturbed place coding in VR. This is also pertinent in the context of recently developed technologies that permit the brain imaging of freely behaving animals (Anikeeva et al. 2011; Ghosh et al. 2011; Iyer et al. 2014; Sieu et al. 2015). In some systems, rotation around the vertical body axis is possible despite body fixation, leaving vestibular information regarding rotational movement unperturbed (Aronov and Tank 2014; Hölscher et al. 2005; Thurley et al. 2014). This is a first step toward more natural sensory stimulation and behavior in VR, in contrast to that provided by head fixation. Nevertheless, a more comprehensive approach should permit freely moving animals.
Here, we show the results from using such a VR experimental system in which animals can move freely and that uses neither head nor body fixation. In short, the animal is placed in a circular arena, the bottom of which consists of a large sphere that rests on an arrangement of rollers and servomotors. Animal movements are video tracked and the position signal is used for closed-loop position compensation by counterrotating the sphere via the servomotors as the animal attempts to move from its apex, keeping the animal in the center of the arena. This treadmill is surrounded by a circle of monitors that display the visual environment from the animal’s current position in the VR scene. Eight retractable liquid reward devices located at the periphery permit the delivery of food reinforcement at experimentally defined locations. VR scenes may include linear alleys, where compensation is restricted to the long axis so that animals can approach and touch the side walls of the experimental enclosure. In contrast to systems employing head or body fixation (Aghajan et al. 2015; Aronov and Tank 2014; Hölscher et al. 2005; Thurley et al. 2014), the Servoball treadmill stops at the end of an alley and the animal is then able to walk to the reward at the perimeter. In this situation, translational acceleration is available. Thus the freely moving animal on the Servoball can obtain touch information from the physical walls of the arena and receive vestibular information when its body rotates. Regarding translational movement, vestibular encoding will likely be perturbed by the artificial acceleration of the ball when it repositions the rat. Such repositioning may also lead to a mismatch between visual and motor signals.

However, at the end of an alley, where compensation is suspended and VR is stationary, the rat should experience physiological vestibular encoding.

A general difficulty involved in complex behavioral paradigms is the large amount of time required to train the animals. Our VR system also presents a novel solution to this challenge. The VR arena can be accessed directly from the animals’ home cage through a tunnel with an radio-frequency identification (RFID)-controlled gate system. Rats tagged with ID chips voluntarily and individually enter from their group home cage into the VR treadmill experimental arena to perform fully automated experimental sessions. This operator-independent system makes self-determined training sessions possible 24/7, with individuals completing up to 10 self-timed training sessions during each 24-h period.

The rodent VR maze can simulate any environmental scale by adapting the simulation to natural habitat dimensions. We here show that rats in this virtual environment use visual and acoustic cues to reach goal locations. In a series of experiments, we presented rats with visual cues that served as beacons marking a goal location or that served as landmarks defining the angular position of a goal. Furthermore, we tested whether acoustic cues (pure tones differing in frequency or in pulse rate) are sufficient for rat orientation. Our results show that animals learn to use this operator independent system within 3 wk and that rats subsequently solve spatial orientation tasks in this VR environment. Moreover, we successfully combined behavioral testing with recordings from single units in the entorhinal cortex. This makes the VR Servoball attached to the home cage a perfectly controllable experimental environment for freely moving animals that permits highly time-efficient experimentation for a wide range of applications.

METHODS

Animals

Experiments were performed with 12 male and 3 female Long-Evans rats (Rattus norvegicus). Training commenced at an age of 2 mo when animals weighed between 300 and 445 g. Experiments followed national guidelines regarding animal experimentation. The experimental protocol was approved by the local authority Regierung von Oberbayern. We used four groups in succession. In the first group (three females), we established the gating procedure (below). Two groups of four animals passed pretraining and beacon orientation, with one individual per group excluded from the final experimental phase, as these two animals remained passive and barely moved after having been enclosed. By staying within the central area, they did not activate the treadmill function and thus avoided walking on the moving sphere. The four rats in group 3 and one rat in group 2 passed all experiments, including orientation along an acoustic gradient. A group size of four to six rats allowed an adequate balance to be obtained between utilizing experimental capacity and having sufficient individual time slots available for self-determined entering of the Servoball arena for an experimental session (below). For the recording experiments, five male Long-Evans rats (aged 3 mo; 350–500 g) were housed in pairs, maintained on a 12-h light/12-h dark schedule, and tested during the dark phase. During training stages, the rats received water both from the reward system in the experimental arena and during two daily 60-min periods with ad libitum access to water within the home cage.

Servoball VR Home-Cage Environment

The complete experimental system consisted of a home cage, the attached individual sorter that allowed rats to autonomously and individually enter the experimental arena, and the Servoball, a spherical treadmill system for freely moving animals (Fig. 1, A–C). Experiments took place within a 490-mm experimental arena enclosed by a transparent cylinder that limited the movement of the animal to the central part of a 600-mm ball (Fig. 1, A and B). A 100-Hz camera tracked the position and movement of the animal, and this information was used for compensatory movements of the ball and the updating of the virtual environment. The closed-loop feedback control system was driven by two perpendicularly arranged servomotors, with tachometers that counterrotated the sphere underneath the animal to keep it within a circular area close to the apex of the sphere (Fig. 1, A–C). When nearing a wall in the VR environment, the compensation mechanism was suspended and the animal could approach and physically touch the wall of the enclosing cylinder. The position of the animal within the VR scene was the result of its movement in the x and y directions and the compensation distance, which was sensed by two x/y optical encoders directed at the surface of the ball. Visual stimuli and VR were presented as a 360° panorama from an octagon of eight 19-ft TFT monitors (408 × 255 mm, 670-mm internal diameter of the octagon) connected to a graphics card. From the vertical perspective, the field of view spanned 56.5° if viewed from the apex of the sphere; however, this varied between 78.2 and 36.6° depending on the rat’s position within the 490-mm arena. Viewed from the apex, the screens covered 48.8° above and 7.7° below the horizon. Acoustic stimuli were presented from four loudspeakers positioned above the cylinder; all loudspeakers provided the same output. An animal was motivated to explore and exploit the VR environment by the possibility of obtaining water rewards delivered from the eight devices located at the arena’s perimeter. These devices were retractable and could be activated at any experimentally defined locations within the VR environment. On arriving at a feeding location within the VR scene, treadmill compensation was suspended so that the animal could move to the peripheral feeder and obtain a 100-μl liquid reward.
The Animal Management System, with Sorting and Shutting-In Procedures

The home cage was connected to the Servoball via an individual separation and gating system, the sorter (Fig. 1, D–F), which was equipped with two electronic guillotine gates (Winter and Schaefers 2011). A rat entering the sorter was identified at ID sensor 1. If the Servoball or sorter were unoccupied and the rat’s individual schedule permitted the next experimental session to commence, gate 1 opened for the rat to enter the gated area. On detection at ID sensor 2, gate 1 was closed. On being detected at ID sensor 3, and if no other animal was detected at ID sensors 2 or 3 during a 30-s waiting interval, gate 2 opened and the rat could proceed to the experimental arena. The rat entered from below onto a circular platform located between monitors and the ball while the acrylic glass cylinder was raised by 8–10 cm. When the rat was within the central region of the ball, the shut-in mechanism commenced, whereby the cylinder slowly descended to 8 mm above the ball’s surface. Movement of the rat during the shut-in procedure reversed cylinder movement, and this process repeated until the rat was shut in or returned to the home cage.

Servoball, Optical Tracking, and Movement Compensation

The 60-cm ball was composed of 6-mm polyethylene weighing 6,500 g and had a roundness precision of ± 4 mm. The color was
translucent white and transmitted infrared illumination from below (Fig. 1 A and B). To increase surface friction, the ball was sandblasted before its first use. The ball rested on a multidirectional roller system.

The animal’s movement on the sphere was restricted by a transparent acrylic glass cylinder (outer diameter/inner diameter: 500/486 mm, height: 500 mm, Plexiglas XT) suspended from a motorized linear slide at ~8 mm above the ball’s surface. The cylinder provided tactile information regarding boundaries and prevented a rat from leaving the Servoball during a session.

The horizontal position of the freely moving animal was detected by a camera (Basler 602F, 100 frames/s; Basler, Ahrensburg, Germany) with a 6-mm lens (F = 1.2) and firewire connection to the PC. Long-Evans rats, with their sharp color transition from a dark hood to white body fur, are well suited to tracking. We used the optical center of mass in the black areas of fur to determine two-dimensional coordinates. Before each experiment, a background image was taken and later subtracted from the images taken during a session. This increased the contrast between the animal and the ball surface. The image was cropped to the area within the cylinder and then segmented, resulting in one or several objects. An erosion mechanism eliminated very small objects and smoothed edges. A dilatation mechanism filled holes in the segmented object. Normally, only one object remained, but for multiple objects, the largest was selected. Its center of gravity was taken as the position of the animal. Detection was performed at 100 Hz using the Halcon machine vision library (MV Tec). The deviation of the animal’s positional signal to the sphere’s top center was used to generate feedback signals that drove the motors to compensate displacement. The speed of the motors was proportional to the deviation between the center and the position of the animal. Two optical sensors (ADNS-3080, 100 Hz; Agilent Technologies) placed above the servomotors sensed the ball’s rotation.

In contrast to air-cushioned systems, where a treadmill is moved by the animal’s own power (Hölscher et al. 2005), our system used an active compensating mechanism via tracking of the animal’s movement, and activating motors that counterrotated the Servoball so that the animal stayed within the top central region of the treadmill. The technical complexity is higher than in the air-cushioned systems, causing technical challenges such as systematic errors in tracking (e.g., small movements of a rat sitting on the top of the ball produced a random walk-like drift of the tracked position). Such systematic errors accumulated over the duration of a trial but could be removed online or post hoc from tracking data. The mechanical powering of the system resulted in slight vibrations of the Servoball. This may be a reason for the overly cautious behavior of some animals. Optical sensors tracked the length of travel paths during the compensation mode. Animal movement within the arena while no compensation occurred was tracked by the camera.

The sphere was rotated by two feedback-controlled position servos arranged at right angles and controlled by four-quadrant controllers driven from the PC using an analog voltage. Integrated motor tachometers provided velocity feedback for damping acceleration as a component of controller integrated velocity control. The motors transmitted force via nitrile butadiene rubber rollers (NBR/SBR 80° ± 5 Shore A, r = 15 mm). The drive rollers provided two of the three supports on which the ball rested, and were located slightly below its equator. The orthogonal position of the servos permitted pitch and roll rotation. A ball bearing supported the ball at a counterpart position to the rubber rollers, forming a triangular support at angles of 90°/135°/135°, 190 mm below the level of the motors’ drive rollers.

**Calculation of Position Compensation**

Compensation was here defined as the adaptation of the ball’s rotary movement in response to the animal’s position in the circular arena. The experimental arena was subdivided into three circular areas. Within the 144-mm diameter apex area, no compensation occurred (Fig. 2, A and B, area 4). Within the ring between the 72- and 215-mm radii, the rotational speed was a linear function of the distance to the apex (Fig. 2, A and B, area 2) with a maximum acceleration of 0.78 m/s². With the animal within the ring between the 215-mm and 243-mm radii, the system could accelerate to the maximum rotational speed of 0.31 m/s (Fig. 2, A and B, area 3). Values for maximal acceleration and velocity were set to lower values at the beginning of habituation. The conversion of camera pixels to metric used the 486-mm inner diameter of the cylinder as a reference.

**Calculation of Minimal Distances**

To reach the target feeder, a rat was minimally required to move from the inner circle (radius: 72 mm) to the cylinder wall (radius: 245 mm). The rat’s tracking point, however, never quite reached the wall, as this was ~60 mm behind the tip of the snout. Thus, when the compensation mode was deactivated, the minimal distance was 0.11 m. This distance increased during the compensation mode by the length of an arm or corridor, e.g., 1.11 m for a 1.00-m arm (see Figs. 4E and 6C, broken line).

**Simulation of the Virtual Environments**

The simulation of the virtual environment was implemented using DirectX. The texture of the virtual walls consisted of dark and light

---

Fig. 2. Schematic of compensation. Zoning of the experimental arena as used for the compensation algorithm. Top view of the setup with the octagonal structure of the TFT-screens, positions of feeders (black dots), and the cylinder surrounding the experimental arena. The animal is located at the right end of a virtual corridor that extends to the left (1). A: the rat is in the center of the inner circle (4) and the compensation mechanism is inactive. B: the rat has left the inner circle (4), crossed the acceleration zone (3), and entered the outer circle (2), where counterrotation of the sphere is set to the maximum of 0.31 m/s. Directions of movement are indicated by arrows representing the animal (gray arrow) and the ball (black arrow). Note that in the example given, the animal is located at the right end of a virtual corridor. Therefore, compensation is only activated when the animal enters the left quarter zone (marked in gray). In the remaining three quarters of the arena (5), compensation is inactivated, so that the animal can walk up to and touch the surrounding wall without initiating movement of the sphere. Black horizontal lines indicate the accessible zone of the path.

*J Neurophysiol* • doi:10.1152/jn.00630.2016 • www.jn.org

Downloaded from www.physiology.org/journal/jn by ${individualUser.givenNames} ${individualUser.surname} (146.009.022.227) on January 30, 2018. Copyright © 2017 American Physiological Society. All rights reserved.
Training Procedures

During habituation, gates were open and rats could move freely through the tunnel between the home cage and the Servoball, where all eight feeders were activated. Rats were identified on passing the ID sensors. Thereafter, the gating system was activated and the rats could only pass individually.

In the next training stage, rats learned to remain in the center of the Servoball arena. This triggered the lowering of the arena’s acrylic glass cylinder. However, if a rat prematurely left the central area, the cylinder again moved upwards. This back and forth process lasted until the rat was finally enclosed or returned to the home cage. The retractable feeders became available only after the arena was enclosed. The next phase involved alternation training, during which a rat was required to shuttle back and forth between two feeders located opposite to each other to obtain water rewards. During this phase, the compensation mechanism remained deactivated. After the acquisition of alternation, we activated the servo-driven locomotion compensation for the first time, which turned the ball into a treadmill. In this mode, the treadmill was used to compensate for rat movement such that the animal remained physically in place but could still move through virtual space. The compensating distance was gradually increased from 0.1 to 4.0 m.

For experiments that tested spatial cognition with visual cues, a rat was placed in an octagonal arena and a 12 × 12 cm black square was displayed above the target feeder. During initial training for a new task, the treadmill function was inactivated. Each trial began with the presenting of a black square; after a delay of 5 s, the target feeder was advanced for the rat to collect a reward, while the other seven feeders remained retracted. Next, after the cue (black square) was presented, all eight feeders were advanced. A feeder visit was detected from the nose poke into a photo-gate sensor of a feeder. A trial ended with the first choice made by the rat. After an incorrect choice, a 30-s timeout was given that commenced once the rat returned to the center.

Subsequently, the compensation mode was activated. Initial arm length (compensation distance) was 0.1 m, which increased to 0.5 m on the second day of task acquisition and to 1.0 m during testing. In the experiment involving compensation, the cues were presented in the central crossing of the four arms above the entrance to the reward arm. On choosing the incorrect, unbaited arm, the trial was terminated after a compensation distance of 110 mm, which corresponded to a 1.0 m advance. In the following trial, the rat was required to remain in the center of the ball. After a correct choice and reward collection, feeders were retracted and the VR scene disappeared as the monitors turned black. The trial procedure immediately stopped, compensation was deactivated, and the cylinder moved upwards. The rat could then leave the sphere and walk through the connecting tube and through the open gate (right) into the gating system. On detection by an ID sensor, the right gate opened and the rat returned to the home cage. In the home cage, the rat could either return to the gating system for the following session or remain in the home cage.

Sequence of a Typical Experimental Session

Figure 3 explains the sequence of a typical experimental session. An individual rat living in the home cage (Fig. 3A) voluntarily approached the gating system where it was sensed by the first ID sensor and the VR scene disappeared as the monitors turned black. The trial procedure immediately stopped, compensation was deactivated, and the cylinder moved upwards. The rat could then leave the sphere and walk through the connecting tube and through the open gate (right) into the gating system. On detection by an ID sensor, the right gate opened and the rat returned to the home cage. In the home cage, the rat could either return to the gating system for the following session or remain in the home cage.
Electrophysiological Recordings

Behavioral training in an open-field virtual environment. During the first stage (2-3 days), the rats were trained to associate feeders with water rewards. All feeders were in the advanced position, and the servo-driven locomotion compensation was turned off. The rats were required to explore the 49-mm arena for 20 min. During this time, the rats learned to poke their noses into the nose poke sensors and collect the rewards. During the second stage (3 wk), rats were trained to associate a black beacon with a reward feeder. During the first week, the rats could collect rewards from only one feeder marked by the beacon on the wall behind it, and every 5 min the position of the beacon and the reward randomly changed. This procedure was repeated for 2 wk, during which the duration of the beacon’s position was reduced to 3 min and then 1 min. In the third stage (4–6 days), the rats could collect a reward from only one feeder, which was marked by the beacon on the wall and the position of the beacon, and the reward position was randomly changed subsequent to the rat collecting a reward. During the fourth stage (4 wk), the environment was gradually enlarged and the servo-driven locomotion compensation was activated. Rats learned to adapt to the compensation of the ball. Each time the environment was enlarged, the rat was required to reach distances further from the beacon to collect the reward. After 9 wk, the rats were trained to move freely in the 1.2 × 1.2 m virtual environment and to collect rewards each time from one of the eight randomly appearing beacons.

Electrode implantation and surgery. Tetrodes were constructed from four twisted 17-µm diameter HM-L coated platinum-iridium wires (90/10%; California Fine Wire) and mounted in groups of four into a microdrive with a single turning screw and no separation between tetrodes. The electrode tips were plated with platinum to reduce electrode impedances to between 200 and 300 kΩ at 1 kHz. Anesthesia was induced by placing the animal in an induction cage with isoflurane 5% vapor. Following this, the rats were rapidly moved into a digital stereotaxic frame (Kopff), which had a mask connected with isoflurane 5% vapor. Anesthesia was maintained at approximately 36°C using a closed-loop temperature controller (FHC) connected to a rectal temperature probe and a heating-pad placed under the rat. Local anesthetic (Lidocain) was applied to the skin before making the incision. A hole was drilled into the dorsal skull anterior to the transverse sinus to reach the entorhinal cortex. The coordinates for the entorhinal implant were 4.5 mm medilateral relative to the lambda and 0.3 mm anterior to the border of the transverse sinus. The tetrodes were slightly angled in the sagittal plane (10°, pointing in the anterior direction). A bone screw (shaft diameter: 1.17 mm; length: 4.70 mm) with a soldered wire was fixed to the skull in the frontal plate and served as a ground screw. An additional set of three to five bone screws (shaft diameter: 0.85 mm; length: 4.00 mm) were fixed to the skull and served as anchor screws for the mechanical stability of the implanted microdrive. The screws were then covered with dental acrylic that was used to secure the microdrive to the screws and to the skull.

Data collection. During data collection, each rat was connected to the recording equipment (Neuralynx) via a unity-gain preamplifier (HS-16 or HS-18; Neuralynx) that was attached on one side to a connector on top of a custom-built microdrive and on the other side to a cable that allowed the animal to move freely within the VR cylinder space. The recorded signal was amplified (1,400–5,000 times) and band-pass filtered (6,000 – 6,000 Hz, Lynx-8; Neuralynx). A voltage threshold (70 µV) was used for collecting 1-ms spike waveforms, which were sampled at 30.3 kHz (0.25 ms before the peak of the spike and 0.75 ms after; Neuralynx Cheetah).

Tracking the position of the animal was performed using the Servoball optical tracking. The position of the rat in the 49-mm diameter cylinder was detected by a camera located above the cylinder (Xcomp, Ycomp), and the ball’s rotation as an indication of rat movement was detected by the two optical sensors placed above the servomotors (Xcomp, Ycomp); see Servoball, Optical Tracking, and Movement Compensation.

The position of the rat in the virtual environment (in units of real world mm) was computed by the following equations:

\[
X = \frac{X_{\text{cam}}}{\alpha_x} + \frac{X_{\text{comp}} \cdot G}{\beta_x}
\]
where $\alpha_y = 0.557$, $\alpha_x = 0.543$ were used to map the camera units into real world millimeter units, $G$ is the gain between the optic flow and the ball rotation, and $\beta_x = 111$, $\beta_y = 124$ were used to map the ball rotation units into real world millimeters. $\alpha_x$, $\alpha_y$, $\beta_x$, and $\beta_y$ were measured in a calibration session.

The two types of data (neuronal spikes and rat position) were time-stamped using different clocks. To synchronize the two clocks, the Servoball system sent 0-V/5-V-TTL signals to the Neuralynx system each 10 s with a jitter of 0–1 s. Synchronization was performed offline by matching between the two reports of the TTL trains in the two systems.

Spike sorting. Spike sorting was manually performed offline using graphical cluster-cutting software (SpikeSort3D; Neuralynx). Each spike was graphically represented as a point in a two- or three-dimensional parameter space consisting of the energy or the height (peak to trough distance) of the spike on two or three of the tetrode’s four channels. To ensure that the well-separated clusters indeed contained spikes of a single unit, we checked that a refractory period (2 ms) was present in the interspike interval histogram of the cluster spikes (see Fig. 8E).

Data analysis. SPATIAL RATE MAP. The firing rate map of the cell was computed by partitioning the arena into 35 equally spaced bins and dividing the number of spikes fired in each bin by the total amount of time spent in that bin. The rate map was then smoothed using a Gaussian kernel with an SD of 1.5 bins.

RESULTS

Individual Gating to the VR Arena

When with the group, even cautious individuals explored quickly. Within 4–6 days, the overall number of individual visits to feeders reached 374 ± 143 (means ± SE, day 5) per day (Fig. 4A, $n = 4$ groups with 3–4 individuals per group). After individual gating was switched to the VR arena, the animals passed the sorter without additional training (Fig. 4B). Each animal visited the Servoball arena $7 ± 1$ times (means ± SE, $n = 15$) for drinking sessions per day. After activating the “shutting-in” procedure, rats required 5 days to adapt and reach a level of approximately six self-activated sessions per 24 h (Fig. 4C). Rats required two sessions (less than a day) to learn alternation (Fig. 4D) and then completed 38 ± 7 trials (means ± SE) per session with inter-reward intervals of 9.5 ± 2 s (means ± SE) ($n = 8$ subjects from sessions 3 to 8).

Treadmill Training

In a first series of experiments, compensation extended the locomotion distance between two opposing feeders. Rats required ~100 trials (1-3 days) to adapt to the treadmill function. This included becoming accustomed to the compensation mechanism and learning to walk on the moving substrate. Performance during training on a 62-cm corridor is shown in

Fig. 4. Duration of pretraining phases. A–E: different functions of the Servoball home-cage system are successively activated. A: habituation of group-living rats to entering the Servoball VR arena and using the liquid feeders. Initially, all ID-sorter gates are open and the cylinder is permanently raised, giving all animals simultaneous access to the eight activated liquid reward feeders. Data show the number of rewards collected per individual during 24 h during the 6-day habituation phase. B: learning individual admission to the Servoball arena. After the ID-sorter function is activated, only single animals can access the Servoball arena from the home cage. Data show the number of passages through the ID-sorter and to the Servoball per individual and per 24-h period during the four days of this phase. C: habituation to becoming enclosed by the cylinder before receiving rewards. In this phase, feeders only become active after the cylinder has been lowered to shut in the rat. The cylinder moves downwards when the rat is in the center of the sphere. Left: individual shutting-in events; right: individual rewards per 24 h. D: alternation training. This training occurred within the stationary 49-cm Servoball arena without compensation. Data show successful individual trials per 10-min session (left) and time intervals between rewards (right). E: treadmill training with locomotion compensation activated. Alternation between the 2 ends of a corridor. Lines to the left and right show moving averages from 15 trials, and the gray area shows SE; $n = 15$ for $A$, $B$, and $C$; $n = 8$ for $D$ and $E$. For calculation of the minimum distance in $E$, see the APPENDIX.
Fig. 5. Exemplary tracking paths. Exemplary tracking paths from a single session of one individual moving between the ends of a 2.0-m alley. The lines in A–C are plotted in the VR coordinate system and show data from trials 1, 5, and 10. In the sessions before the trials shown here, the rat was exposed to a 1.4 m alley. The actual distances moved and durations of the trials were 10.1 m and t = 71 s (T1, A), 3.8 m and 25.5 s (T5, B), and 2.6 m and 10.7 s (T10, C). The dashed circle indicates the diameter of the Servoball arena (49 cm) in relation to the dimensions of the alley. During training, the length of the virtual alleys was successively extended (from 1.4 to 2.0 m) and the rats adapted to this quickly, usually during the first session after the implementation of a change. Gray square top right: target feeder.

Fig. 4E. In subsequent sessions, the length of the corridor was gradually increased to 4.0 m (data not shown). Figure 5 shows a training example of a rat that adapted within the first 10 trials of a session to the extension of the virtual path from a length of 1.4 to 2.0 m.

Beacon Orientation

Animals had passed all phases of pretraining within 3 wk. The following experiments tested the use of visual cues for navigation in VR (Fig. 6, A and B). In the first experiment, we investigated beacon-based orientation. Rats required 100–150 trials to learn the task (Fig. 6C, top left). After 100 trials, the locomotion distance traveled by an animal to reach the reward feeder had dropped from an initial 1.0 ± 0.6 m (means ± SE) to <0.5 m. During subsequent tests, all eight feeders were activated. All animals acquired the task (binomial test with 12.5% chance level, P < 0.001, n = 12, see Fig. A1). As a final step, the treadmill function was activated. During this function, we only presented a four-arm radial maze (Fig. 6B, bottom) to reduce complexity. Entering a maze arm by one body length was designated a decision. Six of 10 animals learned the task within 50 trials (binomial test, chance level 25%, P < 0.05, n = 6; Fig. 6C, bottom left).

Landmark Orientation

During training, only the feeder at the target location was presented. Within 100 trials, rats had reduced the locomotion distance to reward from 0.8 ± 0.21 m (means ± SE) to less
As a final step, we aimed to provide evidence of the unique possibilities of VR, such as convenient, controllable sensory cues, environments of arbitrary size or physically impossible environments, and closed-loop interactions with the animal, all possible.

The great advantage of our system is that the rat is not head fixed. This may be considered a flaw in a number of applications, such as those involved in calcium imaging. However, the usual head fix implemented in common VR setups dramatically restricts behavior, so that any complex behavior, which surely requires head movement, is impossible to achieve. Moreover, the head fix is stressful for rats, and from our experience, unlike mice, it is dangerous to head fix rats without their consent, as they are much stronger than mice. When performing electrophysiological extracellular recordings from awake, behaving rats, there are cases in which head fixation dramatically alters the results. The most prominent example is perhaps the recording of place cells and grid cells, which clearly differ when the rat is running on a linear track. This can be simulated in the head-fixed VR setup without creating significant changes to the properties of the cells (compare Aronov et al. 2014, in which the head is not fixed, to other studies with head or body fixation, such as Ravassard et al. 2013). Thus the use of the Servoball in open-field scenarios is in Servoball VR. We implanted tetrodes into the medial entorhinal cortex (MEC). After pretraining, rats were connected to the recording equipment and moved freely in a virtual open field measuring $1.2 \times 1.2$ m. One of the eight feeders was randomly marked by a beacon, indicating the position of a reward. The trajectory of the rat in the virtual world and the positions where the single unit fired are shown in Fig. 8.

**DISCUSSION**

Behavioral experiments with rodents in VR seem to allow for the perfect control of sensory cues while investigating neural mechanisms (Dombeck and Reiser 2012; Thurley and Ayaz 2016). However, stimulation within most VR systems is limited to the visual system to which vestibular, tactile and proprioceptive inputs may be incongruent. Fixing the head or body unavoidably leads to mismatch, and this may perturb neural processing, e.g., altered hippocampal place cell activity in VR as compared with real world situations (Aghajani et al. 2015; Ravassard et al. 2013). A major challenge for rodent VR systems is controlling for the presence of experimentally intended sensory stimuli and the absence of unintended sensory stimuli and their consistent interplay. In the Servoball VR system, rodents move freely, and stimulation is therefore closer to natural conditions regarding the touch sensation of the walls and rotational vestibular encoding. However, while the treadmill is in the rotary compensation mode, the translational movement itself is only virtual. Furthermore, the artificial acceleration of the ball when it repositions the rat may lead to a mismatch between visual and motor signals when feedback correction is imperfect. With our 100-Hz camera, we had a 10-ms latency for detecting movement and an additional 10- to 25-ms latency for the servomotors to accelerate, adding to a minimum delay of 35 ms before initiating compensatory movement in reaction to animal displacement. Therefore, we do not consider this system suitable for compensating for small movements; however, it is well able to adapt to the steady pace of an animal moving down a corridor (see Supplemental Video S1). Thus the unique possibilities of VR, such as convenient, controllable sensory cues, environments of arbitrary size or physically impossible environments, and closed-loop interactions with the animal, are all possible.

As a final step, we aimed to provide evidence of the feasibility of recording neural activity during spatial behavior than 0.5 m (Fig. 6C, top right). In subsequent tests, all feeders were presented, and rats chose the correct feeder location at a level above that of chance alone (binomial test with chance level $12.5\%$, $P < 0.001$, $n = 10$). After activating compensation mode with a four-arm maze, four rats successfully learned to enter the correct arm by using the landmark information (binomial test with chance level $25\%$, $P < 0.001$, $n = 4$, see Fig. A2).

**Orientation Along an Acoustic Gradient**

In natural environments, acoustic cues emanating from stationary sources or from fixed directions can also provide spatial information useful for orientation. We examined whether rats were able to use acoustic cues (Fig. 7A) instead of visual cues for orienting and locating a goal in VR. Preliminary data are reached significance level (binomial test, $P < 0.001$, $n = 12.5\%$, $n = 10$). After its presentation, either a frequency gradient or a pulse rate gradient was used. Trials commenced at the 1- or 3-m corridor location ($s1$ and $s2$). The visible virtual corridor was seemingly endless and absent visual cues indicating position. Positional information was contained in the frequency or pulse rate of the acoustic signal. From its starting location, the animal was required to move to the end of the closest corridor to obtain a reward. After ~100 trials, they reached asymptotic performance for the frequency gradient and two of the four individuals learned to use the pulse rate signal to identify their start location along the 4-m track and choose the correct feeder position. At the end of the closest corridor to obtain a reward. The trajectory of the rat in the virtual world and the positions where the single unit fired are shown in Fig. 8.

**Recordings of Neural Activity**

As a final step, we aimed to provide evidence of the feasibility of recording neural activity during spatial behavior...
Fig. 8. Recording of neural activity in a cell of the entorhinal cortex. One 30-min trial in which the rat was running freely in the environment and collecting water rewards from one of the eight possible reward sites in the virtual environment. A, left: trajectory of the rat and spikes in the virtual environment; the black line represents the trajectory of the rat in the virtual environment. Each red dot marks the location of the rat when the cell fired a spike. A, right: rate maps of the trajectory and spikes. B, left: trajectory and spikes according to the compensation of the ball; the black line represents the rotation of the ball during the trial (Xcomp, Ycomp). Each red dot marks the degree of rotation of the ball when the cell fired a spike. B, right: rate maps of the trajectory and spikes. C, left: trajectory and spikes according to the 50-cm diameter cylinder (Xcam, Ycam). The black line represents the trajectory of the rat according to the cylinder (Xcam, Ycam). Each red dot marks the locations of the rat when the cell fired a spike. C, right: rate maps of the trajectory and spikes. D: nMean spike shape: The mean spike shapes of each electrode in recording tetrode (red, green, blue, and black). E: interspike interval (ISI) histogram: Histogram of the ISI of the spike train. The red dashed line marks the 2-ms interval (refractory period). The x (time)-axis is in logarithmic scale.

of great value for allowing the combination of open-field behavior with recordings of nerve cells, while providing all the flexibility that a virtual environment can give.

Rats learned to use the automated experimental setup and VR within a few weeks, with 8-wk-old rats learning faster than those 6 wk of age. Most demanding for the animals to learn was the shutting-in process using the downward-closing cylinder. All rats learned to alternate between the ends of a linear track on the actively rotating sphere, comparable to alternating on a linear treadmill with one-dimensional compensation. Approximately 25% of animals showed cautious behavior during the compensation mode. Cautious rats did not leave the central circle for prolonged periods of time, thus avoiding a moving substrate. This was overcome by repeatedly and gradually introducing compensation. As an additional feature, we connected the VR arena to the animal’s home cage and obtained a fully automated, operator-independent behavioral training and operant experimental system that functioned 24 h per day. This was made possible by inserting an automated animal sorter that functioned as the mechanical replacement of the experimenter. For a similar approach with a touch screen setup, automation reduced daily experimental time for training rats by up to 80% (Rivalan et al. 2017). This versatile VR platform is well prepared to be combined with recordings of neural activity for investigating spatial navigation (Hölscher et al. 2005; Thurley et al. 2014; Youngstrom and Strowbridge 2012) and underlying neural mechanisms (Aronov and Tank 2014; Domnisoru et al. 2013; Harvey et al. 2009; Heys et al. 2014; Schmidt-Hieber and Häusser 2013), as well as perceptual decision making (Garbers et al. 2015; Harvey et al. 2012; Kautzky and Thurley 2016). We have included our preliminary results from electrophysiological extracellular recordings as proof of principle to demonstrate that despite the proximity of strong servomotors, such recordings are possible. While the automated access mechanism cannot be used with animals connected to a cable, even these animals can initially proceed through fully automated pretraining while carrying an unconnected (or wireless) head stage. In this case, multiple cages containing single housed animals may need to be connected to a multiple gate system.

The closed-loop manipulations of our VR environment enable multisensory control of multimodal behavior for a freely moving animal. In comparison to VR systems with head (Ravassard et al. 2013; Taube et al. 2013) or body (Hölscher et al. 2005; Thurley et al. 2014) fixation, our system, which permits free movement, provides a wider range of motion cues. The vestibular system and self-motion perception are stimulated by walking and turning on the treadmill. The monitors present a moving background pattern for optic flow and can display spatial cues indicating the locations of hidden rewards. Since an animal can change both its distance and angular point of view from the monitors, it obtains more natural visual information. This is relevant in the context of using more complex two-dimensional virtual space instead of simple straight paths in VR (Aghajan et al. 2015; Aronov and Tank 2014; Harvey et al. 2012; Thurley et al. 2014). Beyond vision, rats can receive tactile information from the surrounding transparent cylinder representing the virtual walls by using direct touch. This is possible at the ends of maze arms and within linear sections of maze arms if the animal moves sideways toward the wall. With its additional acoustic cues, the system...
allows for the investigation of multisensory control of multimodal behavior during food searches (Cushman et al. 2013).

The results from the virtual radial-maze learning tasks with visual beacon orientation and landmark orientation demonstrated that rats were able to perform allothetic spatial orientation in Servoball VR. Rats learned that a cue indicated the entrance to the VR goal arm and walked down that arm to collect rewards. Rats demonstrated their ability for allothetic orientation by using the angular relationship to a landmark to locate a goal. In VR setups with air-cushioned treadmills, boundaries cannot be physically implemented. Thus, an animal will reach a goal even if its vector of translation occasionally touches or even crosses a virtual wall. In contrast, rats within Servoball VR are confronted with a physical barrier when they reach the boundary of an open area.

Rats also oriented successfully using acoustic gradient cues where a cue was specific to a location in VR and changed either in pulse rate or pitch. Beyond directional acoustic cues (Cushman et al. 2013) that are comparable to a visual beacon, rats are expected to use both qualitative and quantitative components of acoustic cues to derive spatial information in VR. Our experiments show how this type of acoustic stimulation can be integrated into VR. Building on this by using two-dimensional stimulation or free field acoustics would strongly enhance the spectrum of VR system applications.

The electrophysiological data that we obtained provide a proof-of-concept that in this environment with its high-power active servomotors it is possible to perform electrophysiological recordings. Of course, some advantages of our home-cage environment are lost if investigators need to manually tether. However, automated training performed before tethering is still possible with rats carrying a head stage but yet without cable. Even if instrumented rats have to be singly housed, multiple cages with individual gates can be connected to the access tube for an automated sequencing of individual training.

As a tool, the Servoball VR with 24/7 automated training can be combined for cellular and physiological research with optogenetic techniques (Boyden et al. 2005; Zhang et al. 2006) or miniature microscopic applications (Ghosh et al. 2011; Ziv et al. 2013). This would open up new avenues of research using freely moving animals. As an animal does not require physical strength to move the servo-powered ball, the system would also be suited for smaller species, such as mice (see Supplemental Video S2) and gerbils, small primates such as mouse lemurs, or small cursorial birds such as quails, or even insects as done since the 1970s on the predecessor of the Servoball (Kramer 1975; 1976).

APPENDIX

Further Information on Experimental Procedures Using Acoustic Cues

The rat commenced at one of two points (at 0.25 or 0.75 of the track’s length, designated as s1 or s2 in Fig. 7, respectively) selected at random. The animal commenced a trial by entering the central region of the ball (the “inner circle”). The monitors showed the landscape with striped walls along the corridor, and the compensation mode was activated. Compensation occurred for movement along the direction of the corridor until an invisible wall within the visually endless corridor was reached. The sound associated with each location along the corridor was repeatedly emitted. As the rat moved along the corridor, the direction of the change in pitch or pulse rate allowed the rat to determine the orientation of the VR corridor.

The task was to visit the end closest to the starting location, where the rat could obtain a water reward. On collecting the reward and after a 3-s intertrial-interval (ITI) with monitors turned off, a new trial was initiated as soon as the rat had returned to the center position. On selecting an incorrect feeder, an additional timeout (15 s of monitors off) preceded the ITI. A trial started with teleportation to a randomly selected location (s1 or s2). In the pitch experiment, the frequency changed from 1,000 to 16,000 Hz between the two ends of the corridor, and was presented as pulses with a duty cycle of 100 ms on and 233 ms off. At the experimental start locations, the frequencies were 4,750 Hz (s1, Fig. 7) and 12,250 Hz (s2, Fig. 7). In the pulse experiment, the frequency was constant at 10 kHz and was presented as a pulse with a changing duty cycle of 100 ms on and between 50 and 1,000 ms off. At the experimental start locations, the pulse off times were 288 ms (s1, Fig. 7) and 763 ms (s2, Fig. 7).

Figure A1 shows beacon-based navigation. Figure A2 shows landmark-based navigation. Figure A3 shows navigation with acoustic cues.

VR and Experimental Control Software

The last software version used before changing to PhenoSoft Control (PhenoSoft) is openly available on Github, at https://github.com/Servoball.

ACKNOWLEDGMENTS

We thank physicist Dr. Hans-Ulrich Kleindienst from the Max-Planck Institute for Behavioural Physiology in Seewiesen for dedicated support while developing this technology from 1970s precursors invented at that very Institute.

![Fig. A1. Beacon-based navigation. The columns show data for training, test phase, and test with compensation. Top: cumulative correct decisions. Middle: time to reward for all animals (light gray lines) and average (black line). Data from single animals are gliding averages with a 50-trial window. Bottom: distance to reward. Dashed line shows the minimum distance.](https://example.com/figA1.png)
Fig. A2. Landmark-based navigation. The columns show data for training, test phase and test with compensation. Top: cumulative correct decisions for all animals (different animals may have performed different numbers of trials). The dashed line shows the level of random choice. Middle: time to reward for all animals (light gray lines) and average (black line). Data from single animals are gliding averages with a 50-trial window. Different animals completed different numbers of trials.

Fig. A3. Navigation with acoustic cues (pitch experiment). Gray lines are time to feeder (A) and distance (B) to feeder for n = 5 animals, and a corresponding mean (black line) and standard error (gray area). Behavior became more directed within the first 100 trials, leading to a reduction of total locomotion distance and time to reach a feeder. Data from single animals are gliding averages with a 50-trial window. Different animals completed different numbers of trials. C: performance in (cumulative decisions, coded with + or −1, respectively) shows positive learning behavior for 1 individual.

REFERENCES


