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Research report

3D video analysis of the novel object recognition test in rats

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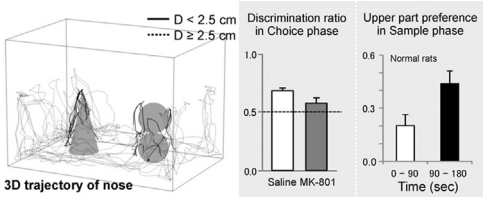
HIGHLIGHTS

- A 3D video analysis system was developed for the novel-object recognition test.
- The 3D system scored the test reproducibly and precisely.
- The 3D system also enabled investigation of the 3D trajectory of object exploration.
- Exploration shifted from the lower to the upper parts of objects during the sampling phase.
- The 3D system is useful for studying the neural processes of object exploration.

GRAPHICAL ABSTRACT



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ABSTRACT

The novel object recognition (NOR) test has been widely used to test memory function. We developed a 3D computerized video analysis system that estimates nose contact with an object in Long Evans rats to analyze object exploration during NOR tests. The results indicate that the 3D system reproducibly and accurately scores the NOR test. Furthermore, the 3D system captures a 3D trajectory of the nose during object exploration, enabling detailed analyses of spatiotemporal patterns of object exploration. The 3D trajectory analysis revealed a specific pattern of object exploration in the sample phase of the NOR test: normal rats first explored the lower parts of objects and then gradually explored the upper parts. A systematic injection of MK-801 suppressed changes in these exploration patterns. The results, along with those of previous studies, suggest that the changes in the exploration patterns reflect neophobia to a novel object and/or changes from spatial learning to object learning. These results demonstrate that the 3D tracking system is useful not only for detailed scoring of animal behaviors but also for investigation of characteristic spatiotemporal patterns of object exploration. The system has the potential to facilitate future investigation of neural mechanisms underlying object exploration that result from dynamic and complex brain activity.

1. Introduction

Rodents have a tendency to interact more with a novel object than with a familiar object [1]. Utilizing this tendency, the novel object recognition (NOR) test was developed to test memory functions in rodents [2,3]. The NOR test has been widely used because

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it does not require extensive training, exposure to aversive stimuli, or water or food deprivation, and the test can be conducted in one session [3].

Two types of method are presently used to score object exploration in the NOR test: visual observation [3] and computerized 2D video analysis (e.g., Ethovision, Noldus; ObjectScan, Clever-sys). Scoring object exploration based on visual observation by an experimenter is sensitive, but it is dependent on the skills of the experimenter and has problems with reproducibility. On the other hand, the computerized 2D video analyses give reproducible scores, but the scores are susceptible to confounding by behaviors other than object exploration. The 2D video analyses were based on a 2D color video camera mounted on the ceiling. The object exploration was counted if the distance between the animal's nose and an object was less than a certain small length in the 2D horizontal plane. Therefore, the nose did not necessarily contact the object when the score was incremented; for example, the score was also incremented when the animal stood on an object without nose contact. To overcome this problem, we developed a 3D computerized video analysis system to estimate the actual distance between the nose and the object surface in 3D space, based on our 3D video analysis system for social interactions (3DTracker [4], http://matsumotoj.github.io/). The primary purpose of this study is to validate the novel 3D scoring method by investigating how the accuracy of the estimated parameters compares with visual observation, and by testing whether the system could capture novel object preference in normal rats and impairment of the preference in rats systematically injected with MK-801 (an NMDA receptor antagonist) [5].

The 3D system also provides a 3D trajectory of the nose, enabling detailed analyses of spatiotemporal patterns of object exploration. As far as we know, no previous system can estimate the 3D trajectory of the nose during object exploration. In the present study, we applied the 3D system to analysis of object exploration in normal rats and in rats injected MK-801. Here we show that the data provided by the 3D system enables not only detailed scoring of animal behaviors but also investigation of characteristic spatiotemporal patterns of object exploration.

2. Materials and methods

2.1. Subjects

Thirty-six adult 8-week-old male Long Evans rats (SLC) were used (twelve rats for experiment 1 and 24 rats for experiment 2). Rats were kept at a temperature of $25 \pm 1\,^{\circ}C$ and a 12 h light:dark cycle (light on at 7:00 A.M.), and food and water were available ad libitum. Behavioral procedures were conducted between 1:00 P.M. and 5:00 P.M. All rats used in this study were treated in strict compliance with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals, National Institutes of Health Guide for the Care and Use of Laboratory Animals, and Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. All experiments were reviewed and approved by the Committee of Animal Research, University of Toyama.

2.2. Experimental setup

Fig. 1A shows the experimental setup. The testing chamber ($60 \times 40 \times 40$ [height] cm) consisted of transparent acrylic walls and a black acrylic floor. The chamber was placed on a 23-cm high base. The testing chamber was located in the center of a metal frame ($150 \times 125 \times 60$ [height] cm), where four depth cameras (Microsoft Kinect for Windows) were mounted at 3, 6, 9, and 12 o'clock positions. Two objects were symmetrically placed 15 cm distant from

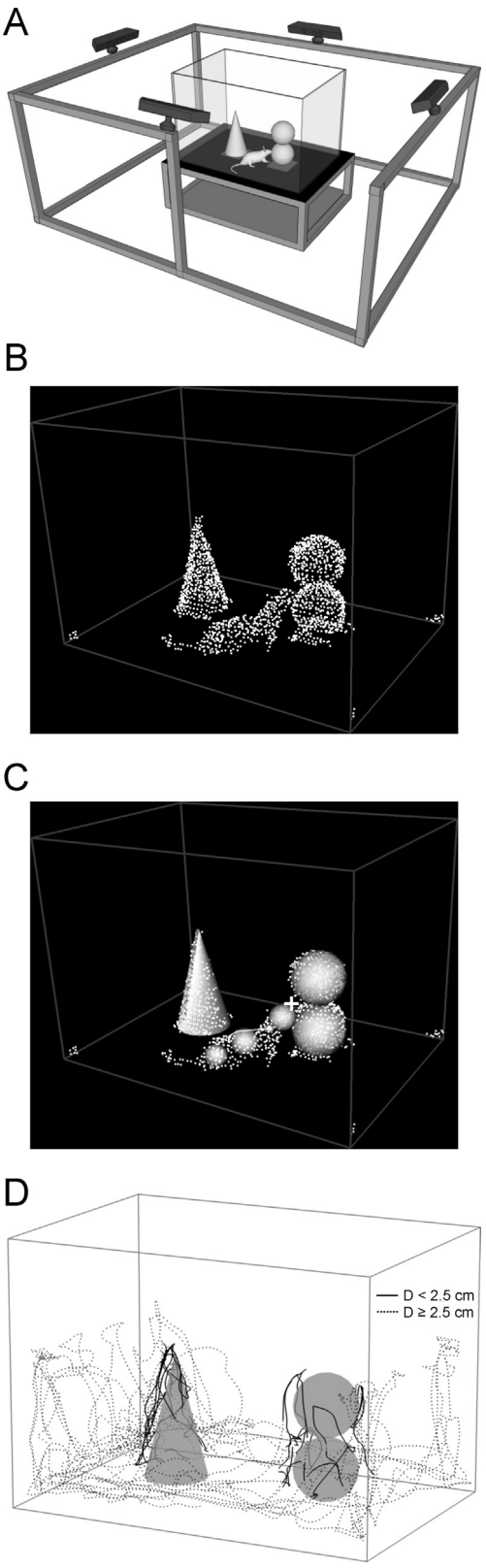

Fig. 1. Experimental setup and algorithms for nose contact detection based on 3D video. (A) Experimental setup. (B) An example of a captured 3D image. Points represent the surface of objects and a rat. Two types of objects (cone-shaped and snowman-shaped) were used. (C) An example of estimated positions of a rat and objects. The rat model consists of four connected spheres, corresponding to head, neck, trunk, and hip. The white cross indicates the estimated position of the nose. See Supplementary video 1 for a movie. (D) An example of a trajectory made up of estimated nose positions occurring in a trial. Solid and dotted lines indicate trajectories with and without contact with an object, respectively.

the three walls. Two types of white-colored objects, snowman-shaped (Fig. 1A right) and cone shaped (Fig. 1A left), were used in this study. The size of both objects was 10 (maximum diameter) × 20 (height) cm.

2.3. Estimating the 3D positions of body parts and objects

Three-dimensional video was acquired by integrating images captured by four depth cameras (see [4] for details). Each frame of the 3D video represented 3D points on the surfaces of the objects and rat (Fig. 1B). To estimate the 3D positions of the objects, the points corresponding to each of the objects were extracted from the video frames without the rat by using the EuclidianClusterExtraction function in Point Cloud Library, version 1.6.0 (open-source software, http://pointclouds.org/). The object location was then estimated by fitting the corresponding object model to the extracted points with a least-squares method (Fig. 1C). To estimate the body-part positions of the rat, the 3D image of the rat in each frame was acquired by subtracting the images of the objects from the frame. Then the positions of head, neck, trunk, and hip were estimated by fitting a rat skeleton model consisting of four connected spheres to the 3D rat image (Fig. 1C, see [4] for details). The position of the nose was estimated by averaging the five points in the 3D image farthest from the estimated head center along the vector from the neck to the head (Fig. 1C). Points located more than 5 cm distant from the head center were not used for the nose position estimation. Finally, the trajectories of the body parts were filtered with a loess filter (time window: 0.5 s) using the "smooth" function of Matlab version R2012a. The nose was considered to contact an object when the nose-object distance (the distance between an object model surface and the estimated nose) was less than 2.5 cm. The duration of the nose contact with an object was defined as the time interval over which the nose-object distance was continuously less than 2.5 cm. The number of nose contacts with an object was defined as the number of occurrences of the nose-object distance becoming more than 5 cm after the distance became less than 2.5 cm. The duration per nose contact was calculated by dividing the total nose contact duration by the number of nose contacts. Fig. 1D shows an example of the 3D trajectory of the estimated nose positions.

2.3.1. Experiment 1: NOR test using normal rats

Twelve rats were used in this experiment. Prior to the NOR test day, the rats were habituated for 20 min in the testing chamber without any object on each of three consecutive days. The NOR test consisted of three consecutive phases: the habituation phase, the sample phase, and the choice phase, in that order. In the habituation phase, the rats were again habituated for 3 min to the testing chamber without any object being present. In the sample phase, the rats were put in the testing chamber that now contained two identical objects (both either snowman-shaped or cone-shaped) and were allowed to explore for 3 min. In the choice phase, the rats were put in the testing chamber that now contained a familiar and a novel object, and were allowed to explore for 3 min. The interval between the habituation and sample phases was 2–3 min (immediately after cleaning and setting the objects). The interval between the sample and choice phases was 10 min. During each phase, a 3D video was captured. The positions of the objects and the rat's body parts during the three phases were estimated offline according to the procedure described above.

2.3.2. Experiment 2: A NOR test using rats injected with MK-801

Twenty-four rats were used in this experiment. The NOR tests were conducted in the same way as in experiment 1, except that the interval between the sample and choice phases was 1.5 h in this experiment, to conform to the procedure used in a previous study investigating the effect of MK-801 on the NOR test [5]. Rats received an i.p. injection of either saline (1.0 mL/kg) or MK-801 (Sigma-Aldrich Co. LLC, St Louis, MO, USA; 0.1 mg/mL in a 1.0 mL/kg injection volume), 20 min before the sample phase.

2.3.3. Validation of the 3D system

To check the accuracy of the parameters estimated by the system, the parameters were compared with the corresponding data obtained by visual observation. First, the nose positions estimated by the system were compared with those estimated by two blinded experimenters. For the comparison, 30 frames were sampled from each of 4 randomly selected videos of the choice phases in experiment 1; a total of 120 frames were sampled. The frames sampled from a given video were taken at regular intervals every 6 s of play time. For each of the sample frames, the experimenters were asked to select the 3D point closest to the nose. The averaged distances between the nose positions estimated by the present system and those estimated by the experimenters in the sample frames were calculated. Second, to validate the estimated nose contact events, we calculated the correlation between the durations of nose contact to each object estimated by the system and those estimated by the experimenters observing 2D video captured from the ceiling. Ten randomly selected videos captured during the sample (five videos) and the choice (five videos) phases of experiment 1were used for the calculation. In addition, to confirm the 3D system is more reproducible than visual observation, the nose contact durations in the 10 videos were measured by two experimenters with the aide of this 3D system and without the 3D system (i.e., by visual inspection). The difference in nose contact duration between the two experimenters was separately computed with and without the system. Then, the differences were compared between the 3D system and visual inspection by a paired *t*-test.

To test whether the system could reproduce the results of previous studies [3,5], object exploration parameters were scored by the 3D system and compared between objects in each of the two experiments. The duration and number of nose contacts and duration per contact were calculated as indexes of object exploration. The scores were compared between identical left and right objects in the sample phase and between familiar and novel objects in the choice phase by paired *t*-test. In addition to the parameters, the discrimination ratio of each of the parameters [(the value of a novel object)/(total value of a novel and a familiar object)] was calculated and compared with the value that would be expected assuming that there was no difference between the objects (i.e., 0.5), by one-sample *t*-test. In addition, for experiment 2, the discrimination ratios of the parameters were compared between rats injected with saline (saline group) and rats injected with MK-801 (MK-801 group) by unpaired *t*-test. The *t*-tests and the regression analyses were conducted with Microsoft Excel 2010.

2.3.4. Detailed analysis of the spatiotemporal pattern of object exploration

Capturing the 3D trajectory of the nose enables detailed analyses of the spatiotemporal pattern of object investigation.

First, the normal pattern of object exploration was analyzed based on data from experiment 1. Because both types of object were solids of revolution, height was the only geometrical independent variable of an object that needed to be captured. The durations of nose contact for each height of each object in the 1st half (0–90 s) and 2nd half (90–180 s) in the sample and in the choice phases were calculated. Because the ratios of nose contact duration in the upper part to those on the lower parts seemed to be different between 1st and 2nd halves of the sample phase, this aspect was further analyzed. The objects were divided into two parts; lower (0–7 cm high; "L") and upper (13–20 cm high; "U"). Preference for the upper part, calculated as [(nose contact duration at the upper part)/(sum

of nose contact duration at the lower and upper parts)] were compared between 1st and 2nd halves of the phase by paired *t*-test. To clarify the pattern of exploration, "LU curves" were drawn by plotting the percentage of cumulative nose contact duration on the upper part (U) on the *y*-axis versus the percentage of cumulative duration of contacts on the lower part (L) on the *x*-axis (Fig. 3C and F). P_{50} was defined as the *x*-value (lower-part cumulative duration) when *y*-value (upper-part cumulative duration) reached 50% on the LU curve (Fig. 4B, inset). P_{50} was used as an index of the order of exploration. If P_{50} was higher than 50%, there would be a "from lower to upper" tendency, and if P_{50} was lower than 50%, there would be a "from upper to lower" tendency. P_{50} was compared with 50% by a one-sample *t*-test. The averaged P_{50} of two objects was compared between the sample phase and the choice phase by paired *t*-test. The subjects that did not show nose contact with the upper part were excluded from the LU curve analysis.

Second, we analyzed how the exploration pattern was affected by MK-801 injection based on the data of experiment 2. The LU curves and P_{50} were similarly calculated and compared with 50% by a one-sample *t*-test. In addition, the averaged P_{50} in each phase were compared between the saline and MK-801 groups by unpaired *t*-test.

To test the possibility that the exploration pattern and its alteration by MK-801 could be ascribed to general changes in behavioral activity or anxiety, the following behavioral parameters were analyzed. Three indexes of behavioral activity were defined: the path length (the length of the trajectory of the trunk center in the horizontal plane), the total duration of rearing (i.e., the height of the head center > 13 cm and the angle between floor, the vector from the hip center to the head center > 45°; [4]) without object contact, and the total nose contact duration. As the index of anxiety, the averaged stretch length (the length from the hip center to the nose in the horizontal plane) during nose contact with an object was calculated. It has been previously reported a rat shows the "stretch approach posture" during approach-avoidance conflict [6,7]. Thus, the averaged stretch length was used as an index of anxiety in this study. To prevent counting a short stretch length when the rat was exploring the upper part of an object, only the stretch lengths during nose contact with the lower part not followed by contact with the upper part was used for the calculation of average stretch length.

For experiment 1, the path lengths, the total duration of rearing, the total nose contact durations, and the stretch length were compared with 2-way repeated measures ANOVA among 4 conditions: 2 phases (the sample and the choice phases) × 2 periods (1st and 2nd halves of the phase). For experiment 2, these parameters were compared with 3-way repeated measures ANOVA among eight conditions: two groups (saline and MK-801 groups) × 2 phases (the sample and the choice phases) × 2 periods (1st and 2nd halves of the phase). Subsequent multiple post-hoc comparison analyses were performed with a Bonferroni correction. The *t*-tests were conducted with Microsoft Excel 2010. The ANOVAs and the post hoc comparisons were conducted with SPSS Statistics 19.

3. Results

3.1. Validation of the 3D system

Table 1 shows the averaged nose position errors estimated by the present system, calculated by comparing the system's output with the nose position estimates of two blinded experimenters. The averaged errors were less than 2 cm. Given that the resolution of the 3D image was 1 point/cm^3 and the error between the two experimenters was around 1 cm, the error is not particularly high. Furthermore, the nose contact durations estimated by the system

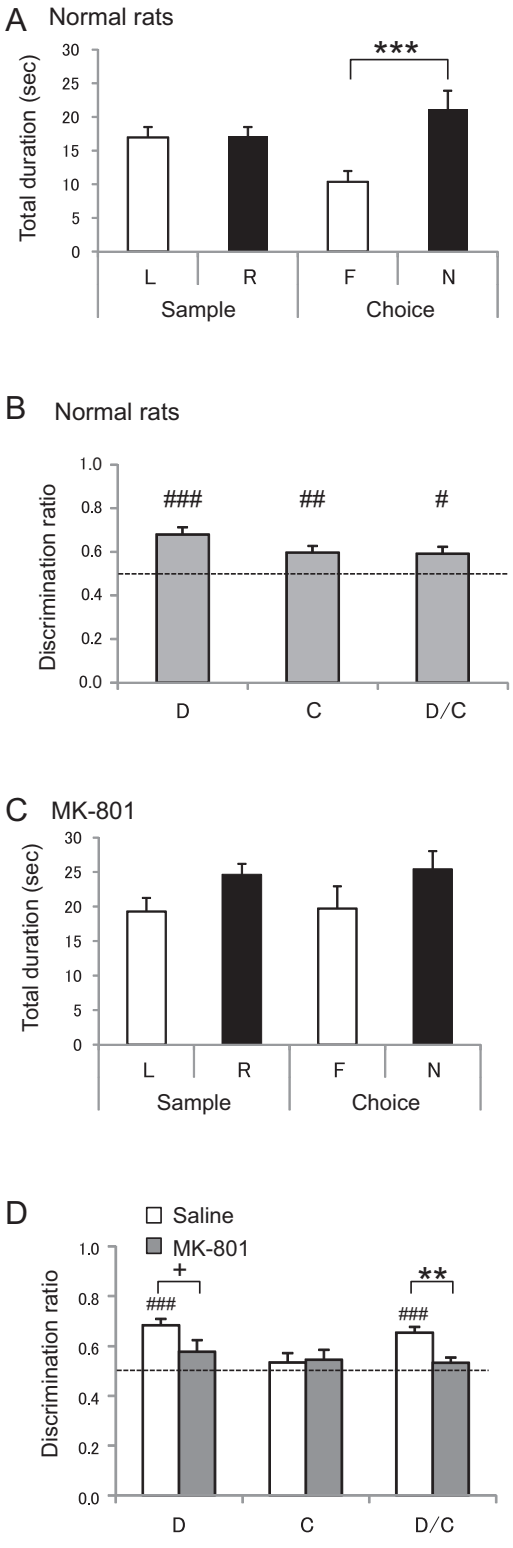

Fig. 2. Analysis of object discrimination. (A) Total nose contact duration of left (L) and right (R) objects in the sample phase, and of familiar (F) and novel (N) objects in the choice phase in normal rats (experiment 1). *** $p < 0.001$ (paired *t*-test). (B) Discrimination ratios [(the value of a novel object)/(value of novel + familiar object)] for nose contact duration (*D*), number of nose contacts (*C*) and duration per contact (*D*/*C*) in normal rats (experiment 1). # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ (one sample *t*-test, compared with 0.5, the dotted line). (C) Total nose contact duration of the MK-801 group (experiment 2). (D) Discrimination ratios of the saline and MK-801 groups (experiment 2). + $p < 0.1$; ** $p < 0.01$ (unpaired *t*-test); ### $p < 0.001$ (one sample *t*-test, compared with 0.5).

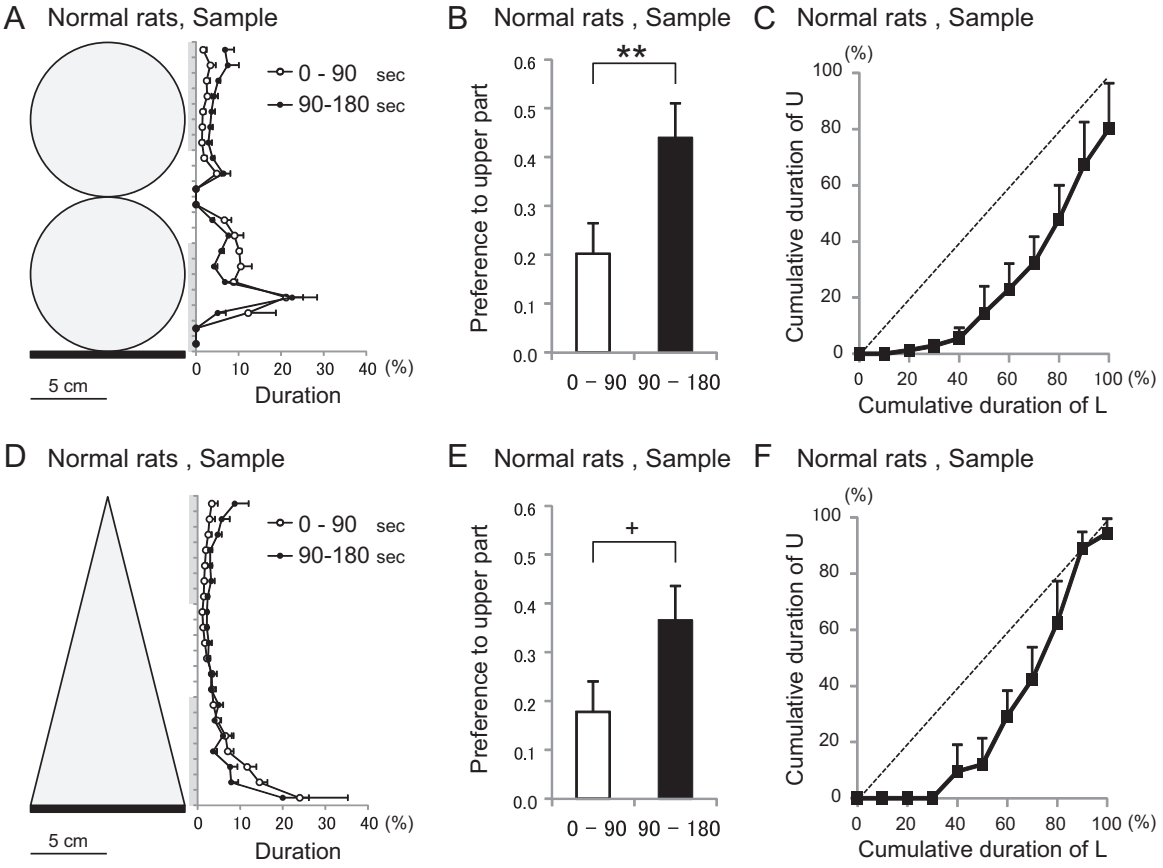

Fig. 3. Spatiotemporal pattern of nose contacts during the sample phase in normal rats (experiment 1). (A)–(C) Analysis of nose contacts with snowman-shaped objects. (A) Nose contact duration at each height on the object during the 1st half (0–90 s) and 2nd half (90–180 s) of a phase. The data from left and right objects were averaged. The shaded ranges along the height axis illustrate our working definitions of the upper and the lower parts. (B) Comparison of preferences for the upper part of the object between the 1st and 2nd halves of the phase. ** $p < 0.01$ (paired t-test). (C) The averaged LU curve. The horizontal axis indicates the percentage of cumulative nose contact duration on the lower part (L). The vertical axis indicates that of the upper part (U). The dotted line indicates the expected value. $^{\#\#}$ $p < 0.01$ (one sample t-test, compared with 50%). (D)–(F) The same analyses of nose contact, but with the cone-shaped objects. (B) $^{+}$ $p < 0.1$ (paired t-test); (C) $^{\#}$ $p < 0.01$ (one sample t-test).

Table 1
Errors in nose-position estimates for the present system and two experimenters.

	System vs. experimenter 1	System vs. experimenter 2	Experimenter 1 vs. 2
Error (cm)	1.7 ± 1.7	1.6 ± 1.4	0.8 ± 0.9

Data are expressed as mean ± SD.

were highly correlated with those estimated by the experimenters ($r^2 > 0.85$, $p < 0.001$; see Supplementary Fig. 1 for details). These results indicate that the present system can detect a nose contact as accurately as can the visual observation method. Generally, a computational video analysis is useful because it outputs more reproducible scores than those estimated with visual observation. Consistent with this idea, the average differences in nose contact durations between the two experimenters were significantly lower with the aide of this 3D system than by visual observation (average difference by the 3D system, 0.12 ± 0.05 s; average difference by visual observation: 2.45 ± 0.52 s; mean ± SEM; $p = 2.4 \times 10^{-4}$, paired t-test).

Fig. 2A shows comparisons of the total nose contact duration among objects in normal rats (experiment 1). The value toward novel objects in these parameters were significantly higher than that toward familiar objects were ($p < 0.001$, paired t-test), while no significant difference was found between left and right objects during the sample phase. The same tendency was observed in the analyses of the number of nose contacts and the duration per contact (Supplementary Fig. 2A and B). Furthermore, the discrimination ratios of all of three parameters were significantly higher than 0.5 (Fig. 2B, $p < 0.05$, one-sample t-test). These results indicate that the novel object preference in the normal rats is clearly detected by analyzing the nose contacts estimated by the present system.

Lima et al. [5] reported that an i.p. injection of MK-801 diminished the investigation preference for novel objects in the choice phase. Consistent with the previous study, in the MK-801 group in experiment 2 there was no difference between the familiar and the novel objects in the total nose contact duration (Fig. 2C, $p > 0.05$, paired t-test). Also, there was no significant difference in the number of nose contacts and duration per contact (Supplementary Fig. 2C and D). Additionally, there was no significant difference in these parameters between left and right objects during the sample phase ($p > 0.05$, paired t-test). Fig. 2D shows comparisons of the discrimination ratios of the three parameters between the saline and the MK-801 groups. The total nose contact duration of the saline group tended to be higher than that of the MK-801 group ($p = 0.058$, unpaired t-test). The duration per contact of the saline group was significantly higher than that of the MK-801 group ($p = 0.0014$, unpaired t-test). These results suggest that the present system effectively demonstrated the effects of MK-801 reported in the previous study.

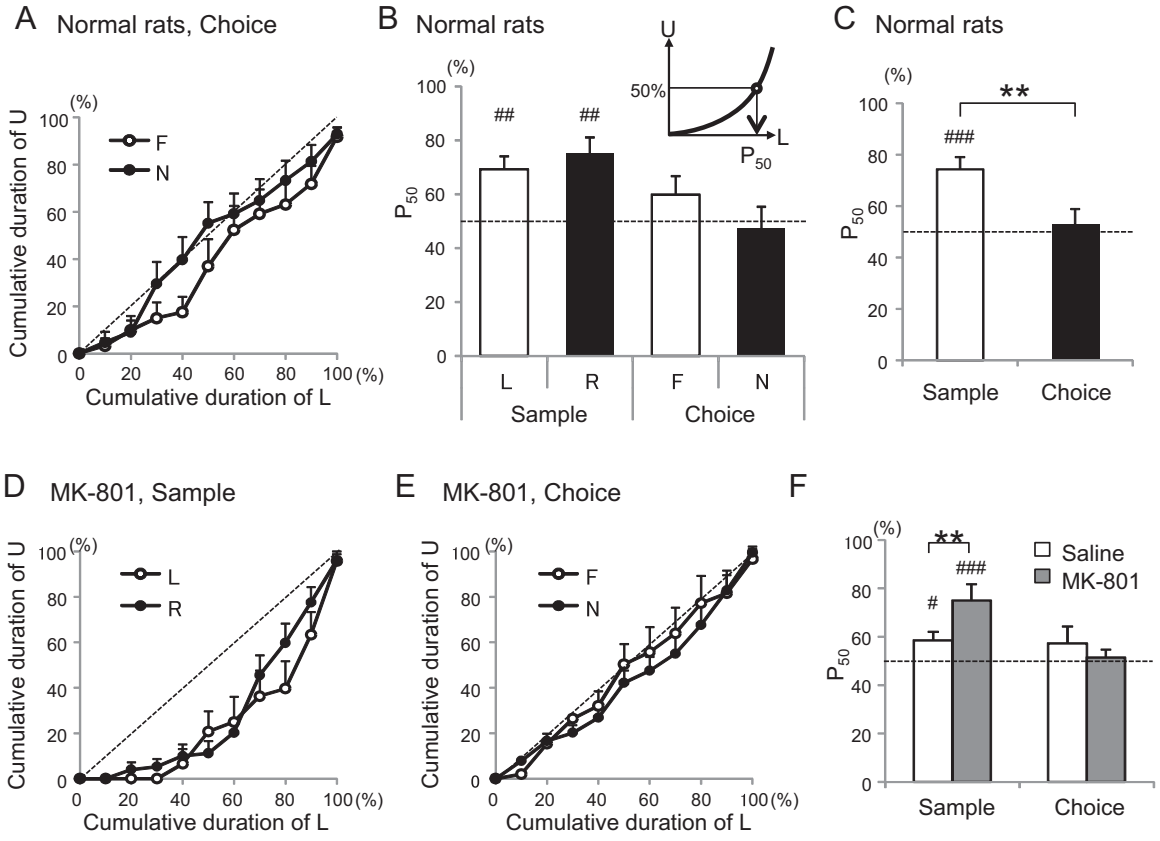

Fig. 4. The "Lower to upper" tendency was not observed during the choice phase and MK-801 strengthened the tendency. (A) The LU curves for familiar (F) and novel (N) objects during the choice phase in normal rats (experiment 1). The tilted dotted line indicates expected values from a random exploration. (B) P_{50} for each object (L, left; R, right; F, familiar; N, novel) in each phase in normal rats (experiment 1). Inset: definition of P_{50}. The dotted line indicates the expected value (50%). $^{\#\#}$, $p < 0.01$ (one sample t-test, comparing with 50%). (C) Comparison of averaged P_{50} between the sample and the choice phases in normal rats (experiment 1). ** $p < 0.01$ (paired t-test); $^{\#\#\#}$ $p < 0.001$ (one sample t-test, comparing with 50%). (D) The LU curves of the MK-801 group during the sample phase (experiment 2). (E) The LU curves of the MK-801 group during the choice phase (experiment 2). (F) Comparison of P_{50} between the saline and MK-801 groups (experiment 2). $^{**}p < 0.01$ (unpaired t-test);$^{\#}$ $p < 0.05$;$^{\#\#\#}$ $p < 0.001$ (one sample t-test, comparing with 50%).

3.2. Detailed analysis of the spatiotemporal patterns of object exploration

The present system outputs the 3D trajectory of the nose (Fig. 1D). These data could be applied to a detailed analysis of the spatiotemporal pattern of object exploration. First, the normal pattern of object exploration was analyzed based on data from normal rats obtained in experiment 1. Fig. 3A and D shows the ratios of nose contact duration for each height of each object in the 1st half (0–90 s) and 2nd half (90–180 s) in the sample phase in the normal rats. For both types of object, the ratio of upper-part contact duration seemed to be larger in the 2nd half. To confirm this impression, preferences for the upper part were calculated and compared between the 1st and the 2nd halves. The preference was significantly higher in the 2nd half than in the 1st half for the snowman-shaped object (Fig. 3B, $p = 0.0021$, paired t-test). The same tendency was found for the cone-shaped object (Fig. 3E, $p = 0.072$, paired t-test). These results suggest that the rats seem to explore the objects "from lower to upper." To test this idea more directly, we calculated the LU curve by plotting the percentage of the cumulative nose contact duration on the upper part (U) on the y-axis versus that on the lower part (L) on the x-axis (Fig. 3C and F). The increase in the value for the upper part seemed to be delayed relative to the increase in the value for the lower part, corresponding to the idea of a "lower to upper" tendency. The P_{50} (Fig. 4B inset; defined as the x-value [lower-part cumulative duration] when the y-value [upper-part cumulative duration] reached 50% on the LU curve) for both types of object was significantly higher than 50% (Fig. 4B left, $p < 0.05$, unpaired t-test), confirming the idea. These results suggest a "lower to upper" tendency in object exploration during the sample phase. We analyzed the spatiotemporal pattern of the normal rats in the same way during the choice phase. Fig. 4A shows the LU curves for familiar and novel objects. Interestingly, neither the lower to upper nor the upper to lower tendency was observed for either the familiar or the novel object in the choice phase (Fig. 4B right). Furthermore, averaged P_{50} was significantly lower in the choice phase than in the sample phase (Fig. 4C, $p = 0.0045$, paired t-test). These results suggest that the lower to upper tendency was specific to the sample phase.

Second, the effect of MK-801 injection on exploration pattern was analyzed based on data from experiment 2. Fig. 4D and E shows the LU curves of the MK-801 group in the sample and the choice phases, respectively. The lower to upper tendency was observed only in the sample phase in the MK-801 group (Fig. 4F), similarly to experiment 1 (Fig. 4B) and to the saline group (Fig. 4F). Interestingly, the P_{50} of the MK-801 group was significantly larger than that of the saline group ($p = 0.0025$, unpaired t-test, Fig. 4F). These results suggest that MK-801 delayed the shift from the lower-part exploration to the upper-part exploration in the sample phase.

To test whether the exploration pattern and its alteration by MK-801 could be accounted for by changes in behavioral activity or anxiety, the behavioral parameters relating to activity (path length, total rearing duration, and total nose contact duration) and anxiety (stretch length) were analyzed. Table 2 shows these behavioral parameters in normal rats (experiment 1). The path length was significantly shorter in the choice phase than in the sample

Table 2
Behavioral parameters associated with behavioral activity and anxiety in experiment 1.

	Habituation All	Sample			Choice		
		1st half	2nd half	Mean	1st half	2nd half	Mean
PL (m/min)[a,b]	3.8 ± 0.3	5.7 ± 0.2	4.1 ± 0.3	4.9 ± 0.2	5.1 ± 0.4	3.5 ± 0.3	4.3 ± 0.3
Rearing (s/min)	9.4 ± 1.3	8.1 ± 0.8	8.5 ± 1.4	8.3 ± 1.0	8.3 ± 1.2	10.3 ± 1.9	9.3 ± 1.4
NCD (s/min)[b]	–	12.3 ± 1.0	10.5 ± 1.5	11.3 ± 0.9	12.7 ± 1.8	8.3 ± 1.3	10.5 ± 1.3
SL (cm)[a,b]	–	14.0 ± 0.2	13.0 ± 0.2	13.6 ± 0.2	13.0 ± 0.3	12.7 ± 0.3	12.8 ± 0.2

PL, path length; rearing, total rearing duration; NCD, total nose contact duration; SL, averaged stretch length.
[a,b] Show results of the 2-way repeated measures ANOVA among 4 conditions: 2 phases (the sample and the choice phases) × 2 periods (1st and 2nd halves of the phase); [a] significant main effect of phase ($p < 0.05$); [b] significant main effect of period ($p < 0.05$). No significant interaction between the phase and the period was found in any parameter. Data are expressed as mean ± SEM.

phase ($F[1,33] = 7.261$, $p = 0.011$)and was significantly shorter in the 2nd half than in the 1st half($F[1,33] = 39.73$, $p = 4.0 \times 10^{-7}$). The total nose contact duration was significantly shorter in the 2nd half than in the 1st half ($F[1,33] = 6.114$, $p = 0.019$). The decrease in the path length and total nose contact duration suggests a decrease in exploration as the environment became familiar to the rats. Similarly, the stretch length (the averaged length from the hip center to the nose in the horizontal plane during nose contact with an object, used as an index of anxiety; see Section 2 for details) was significantly shorter in the choice phase than in the sample phase ($F[1,32.7] = 5.424$, $p = 0.026$]) and was significantly shorter in the 2nd half than the 1st half ($F[1,32.7] = 6.871$, $p = 0.013$), suggesting that the anxiety elicited by the objects decreased as the environment became familiar to the rats. The lower to upper tendency might be ascribed to a general increase in rearing. However, neither the main effect of the period nor the interaction between the period and the phase on the total rearing duration were significant by ANOVA (main effect: $F[1,33] = 1.111$, $p = 0.229$; interaction: $F[1,33] = 1.713$, $p = 0.20$). Table 3 shows the behavioral parameters measured in experiment 2 (saline- vs. MK-801-injected rats). As observed in experiment 1, there were significant main effects of the phase ($F[1,66] = 14.592$, $p = 3.0 \times 10^{-4}$) and the period ($F[1,66] = 26.514$, $p = 3.0 \times 10^{-6}$)on the path length, a significant main effect of period ($F[1,66] = 7.050$, $p = 0.01$)on nose contact duration, and significant main effects of the phase ($F[1,66.3] = 14.212$, $p = 3.5 \times 10^{-4}$) and the period ($F[1,66.3] = 9.086$, $p = 0.0036$) on the stretch length. Note that for stretch length, there was no significant main effect of group ($F[1,21.7] = 0.071$, $p = 0.79$), or interactions involving group ($p > 0.1$). Unlike experiment 1, there was a significant main effect of period ($F[1,66] = 7.318$, $p = 0.0087$) and a significant interaction between phase and period ($F[1,66] = 7.253$, $p = 0.0090$)on rearing duration. There was also a significant interaction between group, phase, and period ($F[1,66] = 4.380$, $p = 0.04$). The effects could be ascribed to the much longer rearing duration in the 2nd half of the choice phase than in the 1st half, in the saline group. Post-hoc analyses indicated a significant difference between the 1st and 2nd halves, choice phase only, saline group ($p = 1.4 \times 10^{-4}$ in this condition, $p > 0.05$ in the other conditions). The interval between the sample and the choice phases is longer in experiment 2 (1.5 h) than in experiment 1 (10 min). The long interval might have decreased the arousal level of the rats and therefore decreased rearing at the beginning of the choice phase. Consistent with this idea, there was a significant interaction between phase and period in path length ($F[1,66] = 7.892$, $p = 0.0065$) and post hoc analysis indicated that the path length in the 1st half was significantly shorter in the choice phase than in the sample phase ($p = 1.4 \times 10^{-5}$), while that in the 2nd half was not significantly different between phases ($p = 0.47$). Long rearing time in the 2nd half of the choice phase might be compensatory to the reduction in the 1st half. The effect was weaker in the MK-801 group. MK-801 might have increased arousal, because the path length and nose contact duration were generally longer in the MK-801 group than in the saline group (a significant main effect of group on path length, $F[1,22] = 7.225$, $p = 0.013$; and a significant main effect of group on nose contact duration, $F[1,22] = 5.626$, $p = 0.027$, were found). There was a significant interaction between group and phase in nose contact duration ($F[1,66] = 5.267$, $p = 0.025$). Post-hoc analysis indicated that nose contact duration was significantly longer in the MK-801 group than the saline group in the choice phase ($p = 0.019$), but not in the sample phase ($p = 0.64$). This might reflect an impairment of memory for objects caused by MK-801, as indicated in Fig. 2.

4. Discussion

4.1. Effectiveness of the 3D video analysis system as a new scoring method for the NOR test

The proposed system estimated the 3D position of the nose of a rat and counted nose contacts with an object as an index of object exploration. The new system evolved from the previous system that

Table 3
Behavioral parameters associated with behavioral activity and anxiety in experiment 2.

		Habituation All	Sample			Choice		
			1st half	2nd half	Mean	1st half	2nd half	Mean
PL (m/min)[a,b,c,e,f]	S	3.1 ± 0.3	5.4 ± 0.2	3.5 ± 0.2	4.4 ± 0.1	3.9 ± 0.2	3.5 ± 0.2	3.7 ± 0.1
	M	4.3 ± 0.6	5.8 ± 0.4	5.1 ± 0.5	5.4 ± 0.4	5.0 ± 0.4	4.7 ± 0.6	4.9 ± 0.4
Rearing (s/min)[a,c,f,g]	S	9.3 ± 1.2	8.5 ± 1.0	8.4 ± 1.2	8.4 ± 0.8	6.4 ± 0.8	12.2 ± 1.7	9.3 ± 1.1
	M	5.4 ± 1.2[*]	3.0 ± 0.6	3.1 ± 0.6	3.0 ± 0.6	3.0 ± 0.7	3.8 ± 0.8	3.5 ± 0.6
NCD (s/min)[a,c,d]	S	–	15.5 ± 0.8	12.4 ± 1.1	14.0 ± 0.9	11.6 ± 1.1	9.1 ± 1.1	10.3 ± 0.7
	M	–	16.1 ± 1.2	13.2 ± 1.3	14.6 ± 0.7	15.4 ± 2.1	14.7 ± 1.4	15.0 ± 1.5
SL (cm)[b,c]	S	–	13.7 ± 0.2	13.3 ± 0.1	13.5 ± 0.1	12.8 ± 0.3	12.8 ± 0.4	12.7 ± 0.2
	M	–	13.8 ± 0.1	12.8 ± 0.3	13.5 ± 0.1	13.1 ± 0.2	12.6 ± 0.2	12.8 ± 0.2

PL, path length; Rearing, total rearing duration; NCD, total nose contact duration; SL, averaged stretch length; S, saline group; M, MK-801 group.
[a–g] Show results of the 3-way repeated measures ANOVA among four conditions: 2 groups (saline and MK-801 groups) × 2 phases (the sample and choice phases) × 2 periods (1st and 2nd halves of the phase); [a] significant main effect of group ($p < 0.05$); [b] significant main effect of phase ($p < 0.05$); [c] significant main effect of period ($p < 0.05$); [d] significant interaction between group and phase ($p < 0.05$); [e] significant interaction between group and period ($p < 0.05$); [f] significant interaction between phase and period ($p < 0.05$); [g] significant interaction between group, phase and period ($p < 0.05$); [*] significant difference from saline group ($p < 0.05$, unpaired *t*-test). Data are expressed as mean ± SEM.

could estimate the 3D positions of head, neck, trunk and hip [4]. There were two main additional features in the new system: (1) robust tracking of the nose by searching a 'tip' in a 3D image of a rat along with the neck-head axis, and (2) estimating object locations by fitting the object models in a 3D image. We confirmed that the present system could detect a nose contact as accurately as visual observation could, i.e., the averaged errors of estimated nose position were less than 2 cm (Table 1) and the nose contact durations were highly correlated with those estimated by visual observations (Supplementary Fig. 1). We calculated the nose contact duration, the number of contacts, and the duration per contact. By comparing these parameters between exploratory behavior directed to a novel and a familiar object, we could confirm the phenomenon of novel object preference in normal rats (Fig. 2A and B). Furthermore, we could similarly reproduce the known impairment of novel object preference by MK-801 (Fig. 2C and D; [5]). The successful reproduction of the results of previous studies suggest the usefulness of the 3D system for scoring NOR tests, as well as confirming the accuracy of the system.

Scoring object exploration based on visual observation is dependent on the skills of the experimenters and thus has a reproducibility problem. Although the computerized video analyses give reproducible scores (independent of the skills), the 2D video analysis used in the previous systems had a significant limitation in detecting nose contact with an object; the distance between the nose and an object in the 2D video image does not necessarily indicate the actual distance in the real 3D space. The present 3D system got over this problem, suggesting that the score by this system is more accurate than by the previous 2D systems. We also confirmed that the scores by the present system were more reproducible than those by visual observation (see Section 3). The results suggest that the present system inherits high reproducibility of the computational video analyses.

The present system still has some limitations because of the low resolution of the 3D image captured by the depth camera (1 point/cm^3). In some visual observation methods, paw contact and sniffing the object are also counted in addition to nose contact as object exploration [3,5]. These behaviors were difficult to detect in the present system because of the low resolution. This limitation may result in lower sensitivity to object exploration in the present system than in the visual observation methods. The low resolution of the present system also prevents its application to mice. Depth cameras with higher resolution (e.g., SR4000, MESA Imaging AG; CamBoard nano, PMD Technologies GmbH) are available, although they are more expensive. A recent study succeeded in capturing 3D images of a mouse in an open field using 10 color video cameras [8]. Thus, by using better depth cameras or many color cameras, the above limitations may be overcome.

4.2. Possible causes of the lower to upper tendency

We found a lower to upper tendency in object exploration during the sample phase (Figs. 3 and 4). The tendency was not due to a general increase in rearing (Tables 2 and 3). Interestingly, previous studies using an open field or a small cylinder also reported a similar sequence of exploration, i.e., from horizontal to vertical exploration, although the cause of the behavior is not clear [9,10]. We suggest two possible causes of this behavioral pattern. First, the tendency may be caused by neophobia. It was reported that novel environments elicit increases in arousal, heart rate, defecation, and plasma corticosteroid levels in rodents [11]. In the sample phase in this study, the objects suddenly appeared in the familiar open field. The rats might have been frightened by the new situation, causing them to cautiously approach and explore the objects gradually. This behavioral pattern could result in the lower to upper tendency. Moreover, it was previously reported that mice avoided a novel object that suddenly appeared in a familiar open field [12,13]. Consistent with this idea, we also found longer stretch lengths, which might reflect cautious approaches, in the 1st half than in the 2nd half of the phase. Although the lower to upper tendency was not observed in the choice phase where a novel object was presented, the lack of the tendency might be due to the relatively small change from the sample to the choice phases. Furthermore, another previous study reported that strains of mice showing stronger avoidance from an object in the initial phase of a trial showed longer exploration of the object in the latter phase, than strains of mice showing weaker avoidance from an object in the initial phase [14]. This suggests that neophobia positively correlates with novelty-seeking trait, and further suggests that neophobia may affect performance in the NOR test. However, some of the present results seemed inconsistent with the neophobia hypothesis. MK-801 strengthened the lower to upper tendency (Fig. 4D) but has been reported to show an anxiolytic effect [15]. In addition, the stretch length was not significantly changed by MK-801 (Table 3). The neophobia hypothesis should be further examined by testing whether anxiolytic or amygdala lesions, which are known to reduce cautious approach [7,16], can attenuate the lower to upper tendency.

Second, the tendency may reflect changes from spatial learning to object learning. It is suggested that the NOR test in an open field involves two distinct learning processes: hippocampus-dependent spatial learning and perirhinal cortex-dependent object learning [17]. Activity of hippocampal place cells is reported to be affected by changing the layout of objects but not by substitution of one object for another [18]. This finding suggests that the hippocampus is more important for learning spatial layout. Thus, if the two types of learning occur in sequence, the lower to upper tendency could be explained as follows: rats first explore the field layout by walking around the bottom parts of objects, and then explore the objects themselves. This hypothesis is consistent with the fact that the lower to upper tendency was not observed in the choice phase, where there is no change in the layout of the objects. In the choice phase, rats may not need to relearn the layout and so explore objects from the first. MK-801 blocks NMDA receptors, which play an important role in learning [19]. MK-801 might slow learning and thus delay the shift of exploration from the lower to the upper part. If this learning sequence hypothesis is correct, a change of object layout would induce the lower to upper tendency and inactivation of the hippocampus would diminish the lower to upper tendency. These predictions should be verified in future studies.

Although exact mechanisms underlying lower to upper tendency are unknown (see above), this pattern of exploration might be essential for performance in the NOR test. A previous study reported that mice explored objects that can be climbed over significantly longer than objects that can only be touched, and that object discrimination indexes were higher in the climbable objects [20]. This suggests that exploration of an upper part of objects is important for object recognition. We also observed that discrimination index in the choice phase tended to negatively correlate with the P_{50} in the sample phase in normal rats ($r^2 = 0.26$, $p = 0.09$; data not shown). These results suggest that 3D exploration of objects enhances memory of those objects. Taken together, the present results suggest that the lower to upper tendency is one of the important behaviors that affect performances in the NOR test, and that the 3D analysis of exploration pattern of objects is useful to analyze the important behavioral characteristics in the NOR test.

4.3. Possible applications of the 3D analysis

Modified versions of the NOR test have been used for basic research on cognitive functions, such as episodic-like memory

[21] and cross-modal cognition [22] in rodents. The detailed spatiotemporal pattern here measured through 3D analysis may be useful for research into cognitive functions. For example, capturing the pattern of exploration that occurs in the dark may be useful for studying how the animal recognizes an object through tactile stimuli (the depth camera uses infrared light to capture the 3D image; therefore it can function in the dark). Moreover, the availability of the 3D trajectory of the nose allows researchers to calculate the neural correlates of exploration of a specific area of the object surface or with a specific pattern of exploration, to study how the object is represented in memory. The detailed spatiotemporal pattern may also be useful for characterizing the effects of drugs or transgenic manipulations, as previous studies have shown that exploration patterns in an open field are useful for such characterization [23]. The object exploration pattern of normal rats revealed in the present study can serve as a control for these further applications.

Tracking of body parts or the whole body is useful for capturing dynamic changes in attention or intention of a subject. Thus, analysis of the trajectory is useful for investigating processes underlying the spontaneous behaviors that result from dynamic and complex brain activity. For example, eye tracking has contributed to investigation of the process underlying gaze control during scene perception [24], and body tracking in an open field revealed the exploration sequence in mice [9]. We believe that capturing the 3D trajectory of the nose could similarly contribute to investigation of processes underlying object exploration.

5. Conclusion

We proposed a novel computerized 3D video analysis system for analyzing object exploration. The proposed 3D system provided reproducible and relatively accurate scores of NOR tests, whereas these two properties had been incompatible in previous scoring methods. Furthermore, the 3D system enabled investigation of the 3D trajectory of object exploration for the first time. By analyzing the 3D trajectory, we found an interesting pattern of exploration in the sample phase of the NOR test, in which rats first explored the lower parts of objects and then gradually explored the upper part. Future work using the system should facilitate investigation of complex mechanisms underlying object exploration, analogously to the way previous studies have used eye-tracking systems to reveal the complex mechanisms underlying human gaze control [24].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.06.047.

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