Statocyst is necessary for negative gravitaxis behavior of the terrestrial slug *Limax valentianus*

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INTRODUCTION

Gravity is an environmental cue available to all living organisms on earth. Gravitaxis, also termed geotaxis, is an innately equipped behavioral preference to move in a fixed direction with respect to gravity. Positive and negative gravitaxes mean the preference to move downward and upward, respectively. *Drosophila* exhibits negative gravitaxis, and its neuronal and genetic bases have most extensively been investigated because this model animal is amenable to a large scale genetic screening and genetic manipulations (Erlenmeyer-Kimling and Hirsch, 1961; Hirsch and Erlenmeyer-Kimling, 1961; Armstrong et al., 2006; Kamikouchi et al., 2009; Desroches et al., 2010).

In other animals, less studies have been conducted, but there are several reports on the gravitaxis-related behavioral traits intrinsic to each animal species. The dauer larva of the nematode *Caenorhabditis japonica* exhibits negative gravitaxis (Okumura et al. 2013), whereas L4 larva of *Caenorhabditis elegans* exhibits positive gravitaxis in aqueous environment (Chen et al., 2021). Infant rats have been reported to show a negative gravitaxic behavior although the interpretation of this observation is still open to controversy (Crozier and Pincus, 1926; Motz and Alberts, 2005). Even the protists *Chlamydomonas* and *Paramecium* show negative gravitaxis in water (Roberts, 2006; 2010).

In gastropod mollusk, several studies have been conducted on gravitaxis long before. The pond snail *Lymnaea stagnalis* exhibits negative gravitaxis in O_2-poor water, and positive gravitaxis in air and in O_2-rich water (Kanda, 1916; Lever and Geuze, 1965; Janse, 1981). The freshwater snail *Pomacea* moves upward on a vertical plane in water (McClay, 1966). The terrestrial slug *Agriolimax laevis* orients its head to the upper direction in an inclination angle-dependent manner when it is placed on a slope (Wolf, 1927).

The gravity is thought to be sensed by a pair of statocysts, which are located in the pedal ganglia of the brain of gastropods. This organ has the shape of a sphere, within which several particles of statoconia consisting of calcium carbonate reside (Wiederhold et al., 1986). Like an otolith organ of mammals, gravity or inertia imposed on statoconia crystals moves them, and the hair cells lining the internal surface of statocyst are stimulated through the displacement of their cilia, resulting in action potential generation.

Requirement of statocysts for gravitaxis has been demonstrated in *Lymnaea* and *Pomacea* by lesion experiments (Lever and Geuze, 1965; Wolf, 1976). In the pond snail *Lymnaea*, downward movement in air was abolished by bilateral ablation of statocyst, and unilateral ablation resulted in the deviation of the trail path to the side of an intact statocyst on a slope (Lever and Geuze, 1965). Negative gravitaxis in low-Po_2 water was also abolished by bilateral ablation of statocysts in *Lymnaea*, with no sign of functional compensation or spontaneous recovery up to 120 days following the surgery (Janse, 1982). Negative gravitaxis behavior was also abolished by bilateral ablation of the statocysts in *Pomacea*. However, the author reported the recovery of the negative gravitaxis behavior within a week (Wolf, 1976).

Therefore, in the aquatic gastropods, the statocyst is undoubtedly required for gravitaxis. However, it has not been sufficiently confirmed whether the statocyst in terrestrial gastropods is necessary for their graxitaxis behavior. Moreover, it has not been demonstrated whether there is any functional recovery or compensatory mechanisms following the ablation of statocysts in *Limax*.

In the present study, we developed a behavioral experimental system to analyze the gravitaxis behavior of *Limax*, and examined the effect of bilateral or unilateral ablation of statocysts. We also investigated the recoverability of the statocyst and gravitaxis behavior 7 days after the surgery.

MATERIALS AND METHODS

Animals

The terrestrial slugs *Limax valentianus* were maintained for 35-37 generations as a closed colony. They were kept in our laboratory in an incubator (19°C), and supplied with a diet of humidified powder mixture consisting of 500 g of potato starch, 520 g of rat chow (Oriental Yeast, Tokyo, Japan), and 21 g of vitamin mixtures (ANI-76, Oriental Yeast). All slugs used in this study were 3-4-month post-hatchling adults (0.4-0.8 g body weight).

Surgery

The slugs were deeply anesthetized by an injection of cold Mg^{2+} buffer (57.6 mM $MgCl_2$, 5.0 mM glucose, and 5.0 mM HEPES, pH 7.0) into the body cavity, and laid on the ice flake for several minutes to ensure anesthetization. Small incisions were made bilaterally on the sides of the head under a stereo microscope. Bilateral or unilateral statocysts were physically removed using fine forceps. To promote the recovery from anesthesia, approximately 500 μl of physiological saline (70.0 mM NaCl, 2.0 mM KCl, 4.7 mM $MgCl_2$, 4.9 mM $CaCl_2$, 5.0 mM glucose, and 5.0 mM HEPES, pH 7.0) was injected into the body cavity. The slug was then returned to a plastic container, and maintained in an incubator (19°C). The slugs were transferred to a clean plastic container supplied with a diet of humidified powder mixture twice (every 3 or 4 days) until the behavioral experiment that was performed 7 days after the surgery.

Toluidine-blue staining

The slugs were deeply anesthetized by an injection of cold Mg^{2+} buffer into the body cavity, and the brain was isolated. The brain was rapidly frozen in Tissue-Tek O.C.T compound (Sakura-Finetek, Torrance, CA, USA) using liquid nitrogen. Cryostat sections (20 μm-thick) were cut and mounted onto CREST adhesive glass slides (Matsunami, Osaka, Japan). The sections were fixed in neutralized 10% formalin (Nacalai Tesque, Kyoto, Japan) for 30 min. Following a brief wash in water, the sections were stained with 0.02% toluidine-blue dissolved in water for 5 min. The sections were then washed twice in water, and coverslipped with Permount (Thermo Fisher Scientific, Waltham, MA, USA). The images were obtained using a microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a DP70 CCD camera (Olympus, Tokyo, Japan).

Behavioral experiment

Seven days after the surgery, gravitaxis behavior was analyzed. A glass plate (40×240×5 mm) was placed vertically, and a sheet of grid paper (spaced 10 mm apart) was attached on the back side. To exclude the effect of air flow, the glass plate was covered with a transparent plastic tray (350×250×40 mm). In a dark room (22°C), the slug was gently placed at the baseline (0 cm) on the surface of the glass plate with its head directing to the right or left (i.e. horizontally) under a dim safety light. The initial head direction (right or left) was assigned randomly. Then the safety light was turned off, and the behavior of the slug was recorded for 10 min using an infrared video camera HDR-PJ790V (Sony, Tokyo, Japan). All the behavioral experiments were performed in a blind manner, where the experimenter was not informed as to whether the slug had undergone statocyst ablation or sham operation. The behavior of the slugs was analyzed off-line. The direction of the head at 10 sec was categorized into either “upward”, “horizontal”, or “downward”. The displacement (cm) was quantified based on the displacement of the head from the baseline at 10 min.

Confirmation of the ablation of statocyst

Immediately following the behavioral experiment, we confirmed the removal of the statocyst for all the slugs in the bilateral or unilateral ablation groups. The slug was deeply anesthetized by an injection of cold Mg^{2+} buffer into the body cavity, and the brain was isolated. The absence of the statocyst(s) was checked under a stereo microscope. For some slugs, the isolated brain was sectioned and stained with toluidine-blue as described above to better check the statocyst removal. The behavioral data of the slugs with a failure in the ablation were omitted from the data analysis.

Statistical analysis

Differences in the body weight were statistically analyzed using a paired t-test. Differences of the head direction (at 10 sec) were statistically analyzed using χ^2-tests. Differences in upward or downward displacements of the head position (at 10 min) were analyzed using a one-way ANOAV and post-hoc Scheffe tests. A P value less than 0.05 was considered statistically significant. The displacements of the head position (cm) were expressed as mean ± SE (s.e.m.).

RESULTS

Effect of surgical operation on the body growth

Too see whether the surgical ablation of statocyst has any adverse effect on the health of the slugs, we checked the body weight of all the slugs used in the present study. As shown in Table 1, the body weight of the sham-operated, bilateral statocysts-ablated, left statocyst-ablated, and right statocyst-ablated slugs at 7 days were all larger compared to those just before operation (P = 0.013, 0.010, 0.021, 0.012, respectively, by paired t-tests). This indicates that the slugs in all the groups exhibited body growth during 7 days and that the surgery itself did not seriously affect the health of the slugs.

Table 1 Body weight of the slugs used in the present study

	before surgery (g)	day-7 (g)
sham operated (n=26)	0.54 ±0.020	0.60 ±0.036
bilaterally ablated (n=26)	0.62 ±0.019	0.69 ±0.026
left ablated (n=25)	0.56 ±0.019	0.61 ±0.027
right ablated (n=24)	0.53 ±0.018	0.59 ±0.029

Direction of the head at 10 sec

Because the slugs usually move their heads before the start of crawling, the head direction of the slug was analyzed at 10 sec after the slug was placed on the vertical glass plate. Most of the sham-operated slugs (22 of 26) moved their heads upward. However, the majority of the bilateral statocyst-ablated slugs (17 of 26) turned their heads to the other directions (Table 2). The χ^2-test (d.f.=3) followed by residual analyses revealed statistically significant difference between the sham operated and bilateral statocysts-ablated slugs (χ^2=14.08, P < 0.01). The unilateral statocyst-ablated group exhibited intermediate tendency between the sham-operated and the bilateral statocyst-ablated groups, and statistically significant difference was detected when compared among the three groups (bilateral statocyst-ablated, left statocyst-ablated, and right statocyst-ablated groups), where a significantly larger number of slugs could direct their heads upward in the unilateral statocyst-ablated group (d.f.=2, χ^2=5.82, P < 0.05). However, there was no statistically significant difference among the sham-operated, left statocyst-ablated, and right statocyst-ablated groups (d.f.=2, χ^2=4.00).

Table 2 Direction of head at 10 sec

		statocyst ablation		
	sham	bilateral	left	right
upward	22[a]	9[b]	15	16
other	4[b]	17[a]	10	8

"a" and "b" denote significantly higher and lower (p < 0.01) than expected, respectively.

Displacement at 10 min

We then analyzed the displacement of the head position 10 min after placement of the slug on the vertical glass plate. The head position of the sham-operated slugs were +5.56 ±1.17 cm from the baseline (n=26), whereas that of the bilateral statocyst-ablated slugs was -1.15 ±0.70 cm (n=26). The head positions of the left- and right-statocyst ablated slugs were -0.40 ±0.49 cm (n=25) and +0.58 ±0.69 cm (n=24), respectively. A one-way ANOVA with post-hoc Scheffe tests revealed that the displacement of the head position was significantly larger in the sham-operated slugs compared to all other groups (P < 0.01, Fig. 1), while no difference was detected among the other three groups.

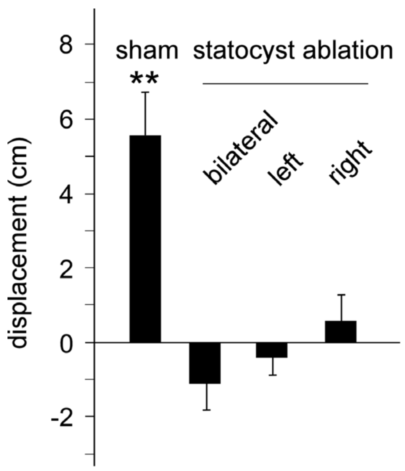

Fig. 1 Displacement of the head position at 10 min. Bilateral or unilateral statocyst ablation abolished the displacement in an antigravity direction. Error bars: ±SE. **P < 0.01 vs. the other groups by post-hoc Scheffé tests.

DISCUSSION

In the present study, we clearly demonstrated the requirement of the statocyst in the brain of *Limax valentianus* for their negative gravitaxis behavior by using our newly developed behavioral experimental system. Ablation of bilateral statocysts completely abrogated both upward turning of the head just following the placement on a vertical plane and the succeeding negative gravitactic movement.

Unilateral ablation resulted in a mild but not statistically significant defect with respect to the head direction at 10 sec (Table 2), whereas the displacement at 10 min was completely abolished (Fig. 1). These results suggest that a single statocyst is not sufficient to support the negative gravitaxis behavior, although there remains a possibility that a longer recovery period would make it possible to accomplish gravitaxis behavior by using only the remaining single statocyst (see below). In an intact animal, some interactions between the bilateral statocysts may be necessary for proper behavioral outputs during negative gravitactic behavior, like in the case of the photo-tropotaxis of *Limax* that relies on the crosstalk between the bilateral eyes through the cerebral commissure of the brain (Matsuo et al. 2014).

Wolf (1976) demonstrated the spontaneous recovery of the negative gravitaxis behavior 7 days following the surgical ablation of a unilateral statocyst in the freshwater snail *Pomacea*. However, we did not find any clear evidence of compensation of the statocyst's function at least at 7days following the surgery in the present study. There is no denying a possibility that the better score in the head direction (at 10 sec) in unilaterally ablated slugs compared to bilaterally ablated slugs (Table 2) is a reflection of the functional compensation exerted by the remaining intact statocyst. Far longer recovery period would ensure better functional compensation, and it awaits a further investigation in the future.

We also did not find any sign of regeneration of the ablated statosyst at the histological level. Figure 2 exemplifies a toluidine-blue-stained coronal section of the brain 7 days following the bilateral statocyst ablation, which proved to have an almost intact right statocyst (arrow) possibly due to the failure in the surgery and thus the behavioral data of this slug was excluded from the analysis of gravitaxis behavior. In contrast, the left statocyst was absent from the pedal ganglia, suggesting the lack of spontaneous regeneration as well as the successful ablation during the surgery. This is consistent with the report by McClay (1966), where no regeneration of statocyst was found even more than 14 days after the surgical ablation in *Pomacea*. Lack of regeneration of statocyst shows a clear contrast to the neurogenesis-mediated spontaneous regenerability of the procerebrum in the cerebral ganglia reported previously (Matsuo et al. 2010), and may be explained by the absence of adult neurogenesis near statocyst (Matsuo and Ito 2011).

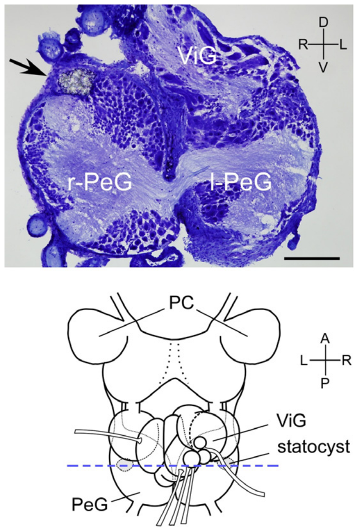

Fig. 2 A section of a brain 7 days after the surgery. An arrow indicates a remaining right statocyst that evaded the surgical ablation. Below is a cartoon of the dorsal side of a brain explaining the cutting plane. Scale bar: 200 μm. PC, procerebrum; ViG, visceral ganglion; PeG, pedal ganglion; D, dorsal; V, ventral; R, right; L, left; A, anterior; P, posterior.

Future studies would clarify whether a unilateral statocyst is sufficient for the head turning behavior if a sufficiently long recovery period is provided. It would also be intriguing to see how the two statocysts crosstalk in an intact slug's brain.

SUMMARY

Gravity sensation is an important ability for the animals living terrestrial lives. The terrestrial mollusks (snails and slugs) are equipped with a pair of statocysts in the brain, and this organ serves as a gravity sensor. Thus far, however, the precise role of the statocysts has not been investigated in the terrestrial slugs. In the present study, we monitored the behavior of the slugs *Limax valentianus* on a vertical plane 7 days after the surgical removal of bilateral or unilateral statocysts. When placed with their heads directing horizontally, most of the sham-operated slugs turned their heads upward (i.e. in the opposite direction to the gravity) after 10 sec. Furthermore, most of them exhibited a displacement to the upper position after 10 min, confirming the negative gravitactic nature of the terrestrial slugs. In contrast, the slugs with bilateral statocyst ablation directed their heads to random directions at 10 sec, whereas those with unilateral statocyst ablation could direct their heads to the anti-gravity direction with a better score compared to the bilateral

statocyst-ablation group. At 10 min, the slugs with both bilateral and unilateral statocyst ablation exhibited a displacement to random positions. These results suggest the critical role of statocyst in the negative gravitaxis of the terrestrial slugs, and the absence of any compensatory mechanism with respect to the negative gravitaxis within 7 days following the statocyst ablation.

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