Changes in the ultrastructure of the amphids of the potato cyst nematode, *Globodera rostochiensis*, during development and infection

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Summary – The structure of the amphids of *Globodera rostochiensis* was examined at different stages of its life cycle. After hatching, the structure of the amphids does not change. The amphids of hatched second stage juveniles (J2), juveniles in the root and adult males appear similar in structure to each other and to those of other plant parasitic nematodes previously examined. However, in most unhatched J2 the structure of the amphid differs; the receptor cavity is devoid of secretory material and the sheath cell does not contain secretory granules seen in hatched juveniles and adults. The change from the apparently inactive state to that observed in the hatched J2 is not directly influenced by prior stimulation with potato root diffusate. There was no evidence that the feeding plug originated in the amphids.

Résumé – Modifications de l'ultrastructure des amphides du nématode à kystes de la pomme de terre, *Globodera rostochiensis*, pendant son développement et l'infestation de l'hôte – La structure des amphides de *Globodera rostochiensis* a été observée aux différents stades de son cycle. Après l'éclosion, la structure des amphides ne change pas. Les amphides des juveniles de deuxième stade (J2) venant d'éclore, des juveniles présents dans les racines et des adultes mâles sont identiques et semblables à celles précédemment décrites chez d'autres nématodes phytoparasites. Cependant, chez la plupart des J2 non éclos, cette structure est différente : la cavité réceptrice ne contient pas de matière sécrétée et la cellule-gaine ne contient aucun des granules sécrétaires présents chez les J2 éclos et les adultes. Le passage d'un état apparemment inactif à celui observé chez les J2 éclos n'est pas directement influencé par une stimulation préalable à l'aide de diffusat de racines de pomme de terre. Il n'existe aucune évidence que le bouchon de prise de nourriture tire son origine des amphides.

Key-words : nematode, *Globodera rostochiensis*, amphid, sense organ, ultrastructure, microwave fixation, secretion, feeding plug.

The sense organs of nematodes have been studied for many years. As early as 1903 the anatomy of the sense organs and other parts of the nervous system of *Ascaris* was studied using light microscopy (Goldschmidt, 1903). Since then, use of the electron microscope has provided detailed ultrastructural studies of the sense organs of many nematodes (e.g. for reviews see McLaren, 1976; Coomans, 1979; Wright, 1980). The sensory structures of plant parasitic nematodes have been reviewed by Coomans and De Grisse (1981).

The amphids are the largest and most complex of the sense organs and their structure is remarkably conserved in a wide range of plant parasitic nematodes including second stage juveniles (J2) of *Meloidogyne incognita* (Wergin & Endo, 1976), *Heterodera glycines* (Endo, 1980) and adults of *Pratylenchus* species (Trett & Perry, 1985a). Males of some of these species, including *M. incognita* (Baldwin & Hirschmann, 1973) and *H. glycines* (Baldwin & Hirschmann, 1975) have been examined; these also have amphids similar in structure to those of the J2.

Each amphid of a plant parasitic nematode comprises a glandular sheath cell, a supporting socket cell and a number of dendritic processes. The number of dendritic processes differs between species although, in most, seven processes enter the amphid itself and between two and five processes originating from the same bundle of nerves pass through the amphid and form other structures at the anterior tip of the nematode which may or may not be associated with amphid function (Wergin & Endo, 1976; Trett & Perry, 1985a).
The basic structure of the amphid is also conserved in other groups of nematodes. For example, the amphids of the free living nematode Caenorhabditis elegans are similar to those of plant parasitic nematodes except that there are more dendritic processes than in most plant parasitic nematodes and the nerve process which gives rise to the microvilli projecting into the sheath cell does not pass through the receptor cavity in C. elegans as it does in the amphids of plant parasitic nematodes (Ward et al., 1975).

It has been suggested, on the basis of structural observations, that the amphids are the main chemoreceptors in plant parasitic nematodes. Another role for the amphids of plant parasitic nematodes has also been postulated by Wergin and Endo (1976) who considered that the dendritic processes of the amphids of J2 of M. incognita could function as kinocilia and move material secreted by the sheath cell out of the amphids. The feeding plug, secreted once a cyst nematode has established a feeding site, may originate from the amphids; the main evidence to support this came from electron micrographs which indicated that feeding plug material had continuity with material in the amphidial canals (Endo, 1978).

The exact role of the amphids in plant parasitic nematodes remains uncertain. Little work has been done on the development of the amphids as nematodes, especially unhatched J2, are difficult to prepare for transmission electron microscopy (TEM). The amphids are also one of the few structures in the nematode other than the cuticle which are in contact with the external environment; since the nematode encounters different environments during its life cycle, the amphids may respond to these changes.

Using microwave assisted fixation (Jones & Ap Gwynn, 1991), which enables rapid processing of nematodes, we have examined the amphids of G. rostochiensis at various stages of its life cycle to find out more about the development of these organs and their role in the host-parasite relationship.

Materials and methods

Nematode material

Cysts of G. rostochiensis Ro1, from a single generation cultured on potato cv. Désirée in pots, were stored dry at room temperature (20 °C) after extraction. To obtain hatched J2, cysts were soaked for one week in glass distilled water (GDW) and then transferred to potato root diffusate (PRD) (Fenwick, 1949); J2 which hatched within a week were used as experimental material. Unhatched J2 were obtained by cutting soaked cysts in half to release the eggs.

For studies on juveniles in the roots, potato tuber pieces approximately 3 cm in diameter (cv. Désirée) with single sprouts were potted into 9 cm diameter plastic pots containing steam sterilised loam and kept in an incubator (18 °C; 15 h day-length; 25 000 lux light intensity). After 3–4 weeks, a suspension of freshly hatched J2 was poured into a disposable pipette tip (200–1 000 μl) inserted into the soil near to the roots. After a period of time (which varied according to which stage was being examined) the plants were removed, soil was carefully washed from the roots and pieces of root containing the developing nematodes were prepared for TEM.

To obtain adult males, potato plants were removed from the pots three weeks after inoculation, soil was washed from the roots and the plants were placed in supports with the roots in a plastic bowl containing continuously aerated water. Adult males were syphoned daily from the bottom of the bowl and used within 72 h of collection.

For studies on changes in amphid structure occurring before, during and after hatching, J2 at different stages in the hatching process were used as detailed previously (Jones et al., 1993). Briefly, the stages used and methods to obtain them were:

i) Unhatched, unstimulated J2: eggs were soaked in double distilled water (DDW) for 7 days before preparation for TEM.

ii) Unhatched, stimulated J2: eggs were soaked in DDW for 5 days and then in PRD for a further 4 days before preparation for TEM.

iii) Artificially hatched J2: eggs were soaked in DDW (unstimulated J2) or DDW and PRD (stimulated J2) as above then placed into fixative and the J2 were released by gently crushing the eggs; the freed J2 were immediately prepared for TEM.

iv) J2 hatched naturally within 1 h: eggs were soaked in DDW for 5 days and PRD for 4 days; all hatched J2 were removed and J2 which hatched within the next hour were prepared for TEM.

v) J2 hatched naturally 23–24 h previously: J2 were obtained as in iv and were then transferred to fresh PRD for 23 hours before being prepared for TEM.

Different stages of G. rostochiensis required different methods of fixation, dehydration and embedding as described below. More than ten nematodes in each category were examined.

TEM preparation of unhatched J2, hatched J2 and adult males

Nematodes in eggs were fixed for 30 s in 1 % acrolein in 0.05 M phosphate buffer at pH 7.2 in a microwave oven (Jones & Ap Gwynn, 1991). All buffers and fixatives contained 10 % sucrose to match the osmotic pressure present in the egg (Clarke et al., 1978). The eggs were rinsed briefly in buffer before being fixed for 30 s in 4 % glutaraldehyde in the same buffer in the microwave oven. After 15 min rinsing in buffer, the eggs were post fixed for 20 s in 1 % OsO4 in buffer in the microwave oven. Eggs were cut in half and the anterior por-
tions of the nematodes were dehydrated using acidified 2,2-dimethoxypropane (DMF) (Jones & Ap Gwynn, 1991) and embedded in EMix resin (Biorad Laboratories Ltd.), used according to the manufacturer’s instructions. Artificially hatched nematodes were prepared for TEM by a different method: eggs were placed into 4% glutaraldehyde in 0.05 M phosphate buffer at pH 7.2 containing no sucrose and were immediately ruptured in the fixative by applying pressure to the egg. The freed J2 were then fixed, dehydrated and embedded as above.

All hatched J2 and adult males were prepared for TEM using microwave oven fixation and DMP for dehydration; nematodes were embedded in EMix resin as above.

TEM Preparation of Nematodes in Roots

Infected roots were cut into small pieces in a Petri dish containing cold (4 °C) 0.05 M sodium phosphate buffer at pH 7.2. The pieces of root were transferred immediately into a glass vial containing 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) and then fixed in the microwave oven as above. After 30 min rinsing in several changes of cold (4 °C) phosphate buffer, the specimens were post-fixed for 20 s in 1% OsO4 in the microwave oven. The roots were then placed in cold (4 °C) buffer and examined under a stereomicroscope. Nematodes in root pieces were clearly visible at this stage since they took up more osmium than the root tissue and hence appeared darker. Pieces of root containing nematodes were dehydrated in an acetone series (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 100%, 10 min each) and then infiltrated with and embedded in Spurr’s resin (Spurr, 1969).

Sectioning and Staining

Procedures for sectioning and staining were the same for all nematode stages. Blocks were sectioned on a Reichert Ultracut microtome. Silver-grey sections were cut at a speed of 1 mm s⁻¹ using a knife angle of 6°. Serial sections were collected on formvar coated 75 mesh copper grids (Agar Aids Ltd.). Grids were stained in 4% uranyl acetate for 10 min and Reynolds’s lead citrate for 5 min and were viewed in a JEOL Tescan 100 CX TEM operated at 100 kV. Micrographs were taken on Kodak EM film and printed on Ilford Multigrade 3 paper.

Results

Naturally Hatched J2s

Fig. 1 shows an L.S. of the anterior end of a J2, including one of the amphids. Dendritic processes, surrounded by secretions, are located in the receptor cavity. The sheath cell surrounding the receptor cavity contains secretory granules which contain material with similar staining affinities to the secretions in the receptor cavity. Microtubules run along the length of the dendritic processes and basal bodies are present at the bases of the microtubules in the dendritic processes. The dendritic processes are exposed to the external environment through the amphidial opening in the cuticle.

Figs 3-5 show the amphid in T.S. at various distances from its tip; reference to the schematic diagram of an amphid (Fig. 2) will aid in structural interpretation. Just posterior to the base of the receptor cavity (Fig. 3 A), the twelve nerve processes, which become dendritic processes further anterior, are visible adjacent to the posterior extension of the sheath cell, which contains microvillar processes and secretory granules (Fig. 3 B). Mitochondria are present in the nerve processes. The nerve processes enter the receptor cavity (Fig. 4 A) and form tight junctions with each other and with the sheath cell, isolating the receptor cavity from the rest of the body. Further anterior, most of the nerve processes in the base of the receptor cavity are now dendritic processes (Fig. 4 B), which contain doublet and singlet microtubules and are surrounded by large quantities of secretions. Secretory granules and microvillar processes are present in the sheath cell surrounding the receptor cavity. Further anterior to this, seven of the dendritic processes enter the amphidial canal. In Fig. 5 A they are visible at the base of the amphidial canal, which is surrounded by the sheath cell. At this level the accessory cilia (or accessory dendritic processes of the sheath cell) are still surrounded by secretions. Further anterior the amphidial canal is surrounded by the socket cell (Fig. 5 B) and the accessory cilia branch, giving rise to a large number of processes. Also visible at this level are the nerve processes associated with other anterior sense organs, such as the inner labial sensilla, and structures resembling Golgi bodies, although these appear not to be associated with the amphid itself (Fig. 5 B). The Golgi bodies are swollen with material whose granular appearance is similar to the material in the receptor cavities of the amphids and other sense organs. The amphidial canal of one J2 contained eight dendritic processes (Fig. 5 C); in all other respects the appearance of this nematode was normal and its other amphidial canal contained the usual seven dendritic processes.

Unhatched and Artificially Hatched J2s

The structure of the amphid of unhatched and artificially hatched J2 was the same but differed from that of the naturally hatched J2. Comparison between the amphids of naturally hatched J2 (Fig. 4) and unstimulated, artificially hatched J2 (Fig. 6) shows that, in the artificially hatched J2, the sheath cell appears shrunken and contains little cytoplasm and no granular material is visible in the receptor cavity. Sections just posterior to the receptor cavity of an artificially hatched, unstimulated J2 (Fig. 6 B) show that, although the nerves and the microvillar processes have the same appearance as the corresponding structures in the naturally hatched J2, the sheath cell surrounding the microvillar processes contains no cytoplasm; similarly, no material is visible in the...
Fig. 1. A: Longitudinal section (L.S.) through the anterior end of a naturally hatched second stage juvenile (J2) of Globodera rostochiensis showing the structure of the amphid. The sheath cell contains microvillar processes and secretory granules. Towards the anterior end of the nematode, the amphidial canal is surrounded by the socket cell. The receptor cavity and amphidial canal contain dendritic processes which are exposed to the environment through the amphidial opening; B: High power of the dendritic processes in the region of the receptor cavity. Microtubules are visible in the dendritic processes, and basal bodies are present at the bases of the microtubules. Secretory granules are clearly visible in the sheath cell surrounding the receptor cavity.

ABBREVIATIONS USED IN FIGURES: a: amphidial canal; ac: accessory cilia; ao: amphidial opening; bb: basal body; cs: cephalic sensilla; dp: dendritic process; g: Golgi body; ils: inner labial sensilla; mt: mitochondrion; mt: microtubule; mv: microvillar process; n: nerve process; ols: outer labial sensilla; rc: receptor cavity; s: secretions; sc: sheath cell; sg: secretory granule; so: socket cell; st: stylet; tj: tight junction.
receptor cavity (Fig. 6 C). Occasionally, however, the sheath cell of unstimulated, artificially hatched J2 contained cytoplasm and secretory granules (Fig. 6 D). Examination of a large number of nematodes subjected to such treatments showed that change in amphidial structure was not related to the prior application of PRD. Nematodes which had hatched naturally always contained secretions within the receptor cavity, whereas nematodes which were unhatched or artificially hatched, sometimes had secretions in the receptor cavity and sometimes did not, irrespective of exposure to PRD.

Fig. 6 E shows another feature associated with unhatched J2: the Golgi bodies observed at the anterior tip of the nematode are not swollen with material as observed in naturally hatched J2, J2 in the roots and adult males. Golgi bodies with this appearance were frequently observed in individuals whose amphids appeared to contain no material.
Fig. 3. A: T.S. of a naturally hatched J2 of Globodera rostochiensis through the amphidial nerves and sheath cell slightly posterior to the beginning of the amphid. Twelve nerve processes are present for each amphid and the posterior extensions of the sheath cells are visible containing microvillar processes and secretory granules; B: T.S. through one of the bundles of nerve processes. Mitochondria are present and the sheath cell contains microvillar processes and secretory granules and is clearly bounded by a membrane. (Abbreviations, see Fig. 1.)
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Fig. 4. A: T.S. through the amphid at the base of the receptor cavity in a naturally hatched J2 of Globodera rostochiensis. Tight junctions are formed between adjacent nerve processes and between nerve processes and the sheath cell, thus isolating the receptor cavity from the rest of the nematode's body; B. T.S. through the receptor cavity of a naturally hatched J2 where the nerve processes are dendritic processes which contain microtubules. The processes are situated in the receptor cavity surrounded by secretions which are similar in appearance to material in the secretory granules in the sheath cell which surrounds the cavity. (Abbreviations, see Fig. 1.)
Fig. 5. A: T.S. through the amphidial canal of a naturally hatched J2 of Globodera rostochiensis. At this level, the amphidial canal is formed by the sheath cell and contains seven dendritic processes surrounded by secretions. The sheath cell contains secretory granules and accessory cilia; B: T.S. through the amphidial canal near the anterior tip of a naturally hatched J2. The canal, containing seven dendritic processes, is surrounded at this level by the socket cell. Some of the nerve processes (n) which give rise to other sensory organs are also visible. C: The amphid of this naturally hatched J2 is unusual in that it contains eight dendritic processes. (Abbreviations, see Fig. 1.)
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**Fig. 6.** T.S. through unstimulated artificially hatched J2 of Globodera rostochiensis. A: The receptor cavity does not contain secretions and the sheath cell contains little cytoplasm and no secretory granules, although microvillar processes are present; B: At the base of the receptor cavity the nerve processes and the microvillar processes appear as in naturally hatched J2. However, the sheath cell contains little cytoplasm and no secretory granules; C: In the receptor cavity the dendritic processes are not surrounded by secretions; D: The amphid of this nematode corresponded to naturally hatched J2 with cytoplasm and secretory granules in its sheath cell. Unlike Golgi bodies in naturally hatched J2, the membranes of Golgi from unhatched J2 (E) appear close together with no material present between them. (Scale bars: A, B and C = 1 μm; D = 0.5 μm; E = 0.25 μm. Abbreviations, see Fig. 1.)
Fig. 7. A: T.S. through the receptor cavity of a juvenile of Globodera rostochiensis 10 days after inoculation at its feeding site in the root. Dendritic processes are surrounded by secretions as in the invasive J2. The sheath cell contains microvillus processes; B: T.S. through the amphid of a juvenile at its feeding site. Five dendritic processes are present in the amphidial canal surrounded by secretions. Accessory cilia are present in the tissue surrounding the amphidial canal. (Abbreviations, see Fig. 1.)

**Juveniles feeding in roots**

Obtaining good micrographs of the amphids of juveniles at the feeding site in the roots was extremely difficult. The pictures obtained indicated that there are few differences between the structure of the amphid of the juvenile in the root and that of the invasive J2. A section through the receptor cavity of a juvenile (10 days after inoculation) feeding in the root (Fig. 7 A) shows that the dendritic processes in the receptor cavity contain microtubules and are surrounded by secretions with a similar appearance to those observed in the receptor cavity of the invasive J2. Microvilli are present in the sheath cell but secretory granules could not be observed. Fig. 7 B shows a transverse section through the amphidial canal very close to the tip of the nematode. Only five processes
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are visible at this level and, as in the invasive J2, these processes are surrounded by secretions. This material appears to be the same as that observed in the invasive J2. No continuity between the amphid secretions and feeding plug material was observed in any of the specimens examined, even though associated feeding cells were present.

**ADULT MALES**

Examination of micrographs of amphids of adult males indicated that there was little or no difference from the amphids of the naturally hatched J2. The receptor cavity of the adult male shows features common to that of the naturally hatched J2, including dendritic processes containing microtubules, secretions surrounding the dendritic processes, and secretory granules. The microvillar processes in the sheath cell (Fig. 8 A). In the area where the amphidal canal is surrounded by the socket cell, the dendritic processes contain only singlet microtubules (Fig. 8 B, C), in contrast to the dendritic processes in the receptor cavity which contain doublet and singlet microtubules. This, too, is in common with the structures observed in the naturally hatched J2s.

**Discussion**

The amphids of *G. rostochiensis* were found to be similar in structure to those of other plant parasitic nematodes studied, such as *Heterodera glycines* (Baldwin & Hirschmann, 1975), *Meloidogyne incognita* (Baldwin & Hirschmann, 1973; Wergin & Endo, 1976) and *Pratylenchus penetrans* (Trett & Perry, 1985 a). Whilst the amphids of *G. rostochiensis*, *H. glycines* and *P. penetrans* appeared almost identical, minor differences in structure were found between the amphids of *G. rostochiensis* and *M. incognita*. In *G. rostochiensis* J2, the microvillar processes project individually into the sheath cell, each one being surrounded by the sheath cell membrane (although there may be a small amount of secretions between the membranes of the microvillar processes and the sheath cell). By contrast, in J2 of *M. incognita*, many microvillar processes project into cavities formed by the sheath cell and are surrounded by secretions; thus, in this species, the microvillar processes are not in such intimate contact with the sheath cell itself. Using EM techniques, it was not possible to determine the role of the microvillar processes and hence it is difficult to speculate on the significance of these differences. The microvillar processes probably monitor the activity of the sheath cell, given their close contact with this cell and its secretions. However, it is unclear why such a large surface area of contact is required for this function.

After hatching, the structure of the amphid changes very little in *G. rostochiensis* and the amphid may have a similar role throughout the life cycle of the nematode. Given the studies on behavioural mutants of *C. elegans*, in which nematodes with structurally altered amphids respond differently to stimulants compared to those with normal amphids (Lewis & Hodgkin, 1975), it seems likely that the amphid is responsible for chemoreception throughout the life cycle. Further evidence for a chemoreceptive role for the amphids was provided by Trett and Perry (1985 b), who demonstrated that aldicarb, a nematicide known to affect nematode orientation, caused changes in the ultrastructure of the sheath cell in this species.

Ul structural studies on animal parasitic nematodes, such as *Syngamus trachea* (Jones, 1979) and *Necator americanus* (McLaren, 1974; McLaren et al., 1974), show how the amphids of some nematodes become greatly altered at certain stages of the life cycle in order to fulfill a modified role. In these nematodes the sheath cell (referred to as the gland cell by the authors) becomes enlarged and shows ultrastructural changes, such as formation of large quantities of endoplasmic reticulum and Golgi bodies, which are often associated with an increase in secretory activity. These changes occur as the nematode enters its primary host and molts to the adult parasitic stage and are thought to be associated with the onset of production of anticoagulants.

These studies demonstrate that, in some nematodes, the amphids may become altered to serve a functional role other than chemoreception. Endo (1978) considered that a similar process may occur in plant parasitic nematodes with the amphids involved in producing the feeding plug formed at the feeding site in the root. Little evidence was found in the present study or by Endo and Wyss (1992) to support this theory. Although some material resembling the feeding plug material was found near the amphid openings, no continuity between the amphid secretions and the feeding plug was observed in any of the specimens examined and the material present in the amphids did not resemble that of the feeding plug. It is possible that the material in the amphids is modified once it is passed to the outside and that the amphids may, after all, be the source of the feeding plug. We have found, in a previous study (Jones et al., 1993), that material resembling the feeding plug is found in intimate contact with the striae of the cuticle at the anterior tip of *G. rostochiensis* juveniles at their feeding sites indicating that the feeding plug may be secreted through the cuticle. Without the use of markers, such as monoclonal antibodies raised against amphidial secretions, it is not possible using electron microscopy alone to determine the origin of the feeding plug.

Interesting changes in the structure of the amphids were observed during the hatching process. The absence of secretions and the shrunken state of the sheath cell in unhatched nematodes indicate that the amphids may not be functional before hatching. Thus, they may have no role in the detection of stimuli involved in initiating the hatching process. The change from this apparently inactive state into the normal condition (where a normal refers to the amphidial structure observed in naturally hatched J2s) does not appear to be a direct
Fig. 8. T.S. Adult male of Globodera rostochiensis. A: In the receptor cavity dendritic processes are present surrounded by secretions. Secretory granules and microvillus processes are present in the sheath cell surrounding the receptor cavity; B: Where the amphidial canal is surrounded by the sheath cell, secretory granules and accessory cilia are present; the amphidial canal contains seven dendritic processes; C: Slightly further anterior the amphidial canal is surrounded by the socket cell. Accessory cilia are visible nearby and the dendritic processes in the amphidial canal contain singlet microtubules. A and C same magnification as B. (Abbreviations, see Fig. 1.)
response to PRD stimulation. Nematodes which had been exposed to PRD were found to have amphids in the inactive and normal states and, more significantly, nematodes which had not been exposed to PRD were also found to have amphids in both states. Experiments using interference microscopy to determine juvenile water content, followed by electron microscopical observations of the amphids of juveniles at specific hydration levels are required. It was noted that in all naturally hatched nematodes examined (even those hatched less than 1 h previously), the amphids appeared normal. This may indicate that the amphids are used in the later stages of natural hatching, perhaps during widespread exploration of the eggshell (Doncaster & Seymour, 1973), or that once water content increases sufficiently for hatching to occur (Ellenby & Perry, 1976), the amphids change to their normal appearance in preparation for a functional role of host location.

Electron microscopical observations have provided information about the development of the amphids and some inferences can be made on their function and role. However, such observations alone cannot be used to determine precisely how the amphids function or their exact role in the life cycle. These problems can only be solved by the application of other techniques, such as electrophysiology (Jones et al., 1991) and immunocytochemistry (Stewart et al., 1993a, b), which examine the functioning of the sense organs more directly.

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