Physiol. Res. 49: 463-469, 2000

Neurophysiological and Behavioral Responses to Olfactory Stimuli in the Snail *Helix pomatia* L.

M. VOSS

Institute of Zoology, University of Kiel, Kiel, Germany

Received August 15, 1999
Accepted January 11, 2000

Summary

Some aspects of olfactory sensitivity in the pulmonate *Helix pomatia* L. were studied by means of neurophysiological and behavioral methods. Single fiber recordings were carried out in the olfactory nerve of the posterior tentacles. Olfactory stimulations with different odors were performed by means of a continuous air stream. The order of neuronal sensitivity to different odors was as follows: ethanol ≥ ethyl acetate > pentanol > hexanol > octanol ≥ diethyl malonate > vanillin. Furthermore, the results revealed a relative specificity for some substances. A comparison between neurophysiological and behavioral data shows that those substances, which cause the highest increases in impulse frequency, also evoke a behavioral avoidance reaction.

Key words

Chemosensitivity • Helix pomatia • Odor • Olfaction • Snail

Introduction

Gastropods have proved to be suitable objects for studying the chemosensitivity. Although it was not clear for a long time whether the olfactory sense, separate from the gustatory sense actually exists in snails, detailed studies could answer this question at least for the order *Stylommatophora* (Chase and Croll 1981). The olfactory sense plays a crucial role in orientation and foraging. Early investigations showed that in the Roman garden snail *Helix pomatia* L., as in other *Stylommatophora*, especially the posterior tentacles, housing the eyes, are important for olfaction (Schulz 1938).

Olfaction in *Helix pomatia* L. and other terrestrial pulmonates has mainly been viewed from behavioral aspects (Chase 1982, Hopfield and Gelperin 1989). Moreover, the effects of different olfactory stimuli were also investigated by electrophysiological methods. Intracellular recordings from the tentacle ganglion and extracellular recordings from the whole tentacle nerve of the isolated posterior tentacle of *Achatina fulica* revealed that differentiation between various odors can already take place in the tentacle ganglion (Chase 1981). However, the structure which is thought to be most important for the processing of olfactory stimuli is the procerebrum which is located at the entry site of the olfactory nerve into the cerebral ganglion (Ratté and Chase 1997). In *Limax maximus* neurophysiological recordings from the procerebrum and from the tentacle nerve under stimulation with 2-ethyl-3-methoxypyrazine (potato odor) and air puffs showed stimulus-specific changes in neuronal activity and procerebrum-oscillations, respectively (Gelperin and Tank 1990).

Fax+420 2 293 894
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

The present experiments were designed to examine the effects of a variety of pure substances on the snail olfactory system by means of neurophysiological methods. Most of the substances which were used in these experiments have been reported to be odors for snails or insects. Furthermore, the question was asked whether a specificity for certain substances does exist in single fibers.

Additional behavioral experiments were performed in order to make the interpretation of neurophysiological data easier.

Methods

Adult specimens of *Helix pomatia* L. collected in the field were used in this study. The snails were maintained in the laboratory for at least three weeks at a constant temperature and a 12:12 LD cycle and fed *ad libitum* on fresh lettuce, carrots and cucumber. Fragmented egg-shells were provided as a calcium source.

To prepare the head region for neurophysiological studies, the snails were first decapitated. Care was taken not to damage the CNS. The preparation was then placed in a dish containing Ringer's solution (Tiwari and Woodruff 1992). An incision in the caudal to cranial direction was made in order to expose the CNS and tentacle nerves before the connective tissue sheath around these nerves was removed. After that, all nerves except the three pairs of lip nerves and the tentacle nerves were sectioned. Further the esophagus was laced up with surgical silk. Now the entire preparation was pinned to the bottom of a Plexiglass chamber which was divided into two compartments by means of a thin Plexiglass partition (Fig.1). This insert had two small holes for each posterior tentacle. The tentacles were led through these holes so that they could be pinned down into the back compartment. For this purpose, electrolytically sharpened insect needles were used which allowed fixation of the outermost part of the epidermis without any damage to the nerve. The front compartment of the chamber had two openings in the side walls. One of them contained a tube which served for conveying the tested odors from the outside to the tentacles. The second opening served as an outlet.

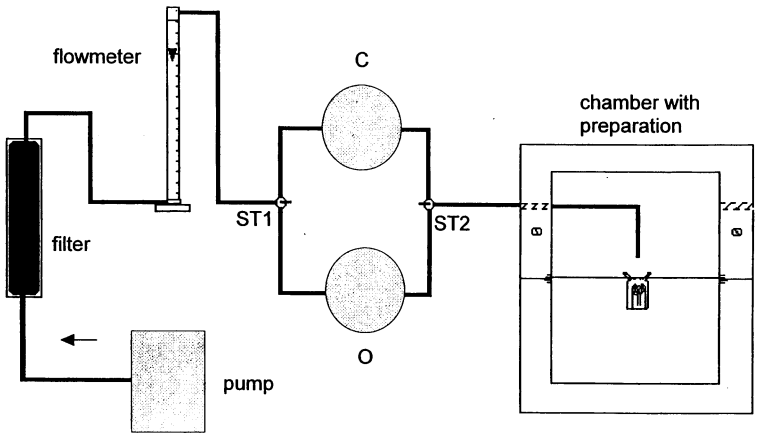

Fig 1. *System for application of odors in the neurophysiological experiments. C – chamber with control (water); O –chamber with odor source; S1, S2: stopcocks; the arrow marks the direction of the air stream.*

The compartments were sealed with vaseline to prevent leakage from one to the other, and the compartment, containing the CNS and the nerves, was filled with Ringer's solution and covered by a layer of liquid paraffin. The front part of the chamber was closed with a Plexiglass cover, and some moistened swabs were placed around the tentacles in order to ensure their high humidity. The cover had an opening which allowed a humidity sensor to be placed above the tentacles. One of the tentacle nerves was placed on a steel microelectrode which had been bent as a hook. The electrode was electrically isolated from the Ringer's solution by pulling it up into the liquid paraffin layer.

The application system for the stimulation with odors is shown in Figure 1. The single components of this system were connected *via* plastic-tubes. An airstream produced by a membrane-pump was first led through an activated carbon filter. The flow rate (40 l/h) was controlled by means of a flowmeter. Air could then be directed through one of two plastic-dishes by means of three-way stopcocks. In these containers, which were connected to the front compartment of the Plexiglass

chamber one Petri dish with the stimulus and a control one were placed into each container. Subsequently the dish was closed airtight by a lid.

The stimuli, used in these experiments were ethanol, pentanol, hexanol, octanol, ethyl acetate, diethyl malonate and vanillin. With exception of vanillin, which was used as an aqueous solution (1 %), all substances were undiluted. The solubility of vanillin does not allow a higher concentration than 1 %.

Each single experiment was performed as follows: For a period of 30 s, air was directed through the control-dish containing a swab which had been moistened with distilled water. Subsequently, the three-way stopcocks were switched in such a way that air now flowed through the second dish. A swab which had been soaked with 1 ml of the stimulus solution was placed in this dish. The stimulation lasted at least 60 s, but was continued if the neuronal activity was still increasing. After each stimulation, moistened air was directed through the Plexiglas chamber for three minutes.

To compensate for any possible adaptation effects and expecting a probable decrease of response over time, the order of stimuli was changed randomly in every experiment. Neuronal responses were recorded using conventional extracellular techniques. By means of window discriminators, the impulse frequencies of three independent single fibers could be assessed. The signals amplified 10 000-fold were registered and stored by a computer.

Table 1. Classes of sensitivity of single fibers from the olfactory nerve: increases in frequency are related to spontaneous activity.

Activity increase	Class	
0-4 Impulses/s	insensitive	S(0)
5-9 Impulses/s	weak sensitivity	S(+)
10-14 Impulses/s	medium sensitivity	S(++)
≥ 15 Impulses/s	strong sensitivity	S(+++)

The frequencies used for the evaluation were means of 10-s periods of the maximum response. Whereas the frequencies of the controls were constant, the rise and duration of the stimulus-induced changes in neuronal activity depended on the stimuli and preparation. Thus, the mean values of the entire stimulation period would have been an inadequate parameter, because a great increase of activity in neurons with fast adaptation would have been leveled out. As a measure of the sensitivity of the investigated single fibers the enhanced activity was classified. For statistical analysis, the standard error of the difference between the means (Lehner 1979) was used. Taking into account the mean frequency intervals and the fact that the spontaneous activity of single fibers was 0.2-5 imp./s, their activity was divided into four classes as shown in Table 1.

Besides the neurophysiological studies, all odors were tested in behavioral studies. These tests were performed in a rectangular plastic chamber 6.5 cm wide and 4.5 cm high) which was open at the top and had an opening at the opposite side of the inlet. The conveyance system for odors and the control air stream was the same as in the neurophysiological experiments. The inlet into the chamber was adjusted so that the air stream flowed onto the bottom of the chamber. Thus, anemotaxis should be prevented. Furthermore, the chamber was illuminated from above to provide constant light conditions. During the experiments, the top of the chamber was covered with parafilm which ensured diffusion of light. Individual snails which had been starved for 5 days were placed 24 cm from the inlet. When the tentacles were evaginated, the air stream was switched from control to odor. The behavior of the snail was then observed for 10 min. If the snail moved at least 5 cm towards the inlet, the substance was evaluated as a positive stimulus. Movement of at least 5 cm into the opposite direction or withdrawal into the shell were assessed as avoidance behavior. If the snail did not change its position but remained outside the shell, the substance was assessed as neutral. As in the neurophysiological experiments, the stimuli were presented in random order. Clean air was passed through the chamber after each stimulation for three minutes.

Results

Behavior

Twenty snails were tested behaviorally. The results are shown in Table 2. Most of the substances used, proved to be inhibitory to *Helix pomatia* L.. Especially ethyl acetate had a strong inhibitory effect: about 50 % of the snails reacted by fast withdrawal into their shells when exposed to this stimulus. The alcohols, particularly ethanol, caused similar effects. Although some animals

moved into the direction of the odor source, avoidance behavior prevailed. On the other hand, stimulation with diethyl malonate and vanillin, had neither a clear positive nor a clear negative effect, respectively.

Sensitivity of single fibers

Most of the neurons produced action potentials with an amplitude of 20 μV, but sometimes the amplitudes were as high as reached a height of 40-50 μV. All of the investigated fibers showed spontaneous activity at frequencies of 0.5-4 Hz. Figure 2 shows the activity of a single fiber response to olfactory stimulation with seven different stimuli. Stimulations in this experiment were performed in the order as shown on the abscissa. This example demonstrates clearly that the application of different stimuli causes different degrees of excitation.

Table 2. Behavioral reactions of 20 snails to olfactoric stimulation with 7 different odors.

Reaction	Ethanol	Pentanol	Hexanol	Octanol	Ethyl Acetate	Diethyl Malonate	Vanillin
positive	0	2	1	2	0	7	5
negative	18	15	14	14	20	5	4
none	2	3	5	4	0	8	11

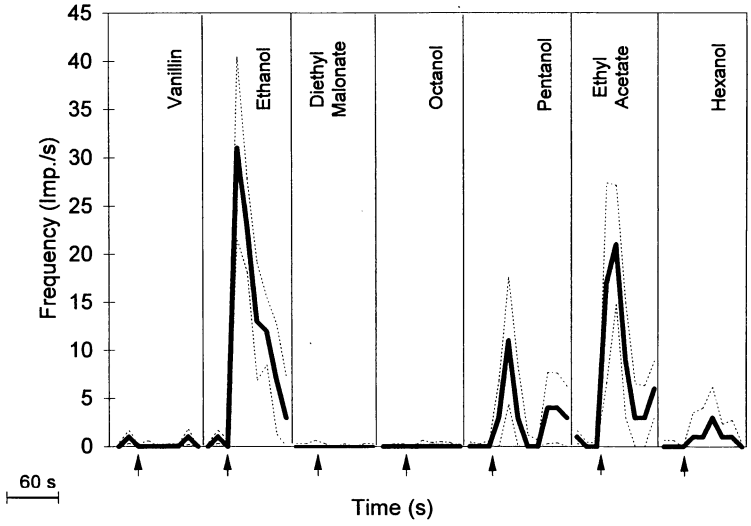

Fig 2. *Frequency increases in a single fiber of the olfactory nerve on sequential stimulation with seven different odors. Each stimulation took place after 30 s (arrows). Dashed lines show the standard deviations.*

Table 3 summarizes the results of all single fiber recordings. Since not all of the seven substances were tested in some experiments, the number of fibers varies between substances. The relation between insensitive and sensitive fibers depends on the substance used for stimulation.

Only a few fibers showed a reaction to vanillin, i.e. 47 out of 52 neurons (=90 %) stimulated with this substance were insensitive to this stimulus. This value does not differ significantly from that in the controls. The proportion of insensitive fibers to stimulation with diethyl malonate and octanol was also quite large. However, the number of sensitive neurons was much higher after stimulation with the remaining substances. Table 3 further shows that the distribution of sensitivity classes may strongly vary among substances. For example, on stimulation with ethanol many neurons (33 %) were highly sensitive whereas 16 % responded weakly and only 7 % showed a moderate increase of activity. Noticeably, out of the alcohols used in these experiments, only octanol did not enhance the frequency by more than 15 Hz. A similar weak excitation was only found on stimulation with diethyl malonate or vanillin solution.

Table 3. Classification of single fiber recordings.

	S(0)	S(+)	S(++)	S(+++)	n
Ethanol	45 % (26)	16 % (9)	7 % (4)	33 % (19)	58
Pentanol	41 % (25)	26 % (16)	18 % (11)	15 % (9)	61
Hexanol	57 % (35)	21 % (13)	10 % (6)	11 % (7)	61
Octanol	76 % (44)	16 % (9)	9 % (5)	0	58
Ethyl Acetate	42 % (26)	23 % (14)	13 % (8)	23 % (14)	62
Diethyl Malonate	84 % (48)	12 % (7)	2 % (1)	2 % (1)	57
Vanillin	90 % (47)	8 % (4)	2 % (1)	0	52

S(0): insensitive; S(+): weakly sensitivity; S(++): medium sensitivity; S(+++): strong sensitivity; n: number of investigated single fibers; Values in brackets are the absolute numbers of fibers per class and substance.

Specificity of single fibers

In those experiments where all odors were successively tested, all neurons (N=40) proved to be sensitive to a number of substances. Twenty-one neurons, however, showed a maximum sensitivity to a single substance and were significantly less excited by all other odors (Table 4). 43 % of these fibers exhibited maximum responses to stimulation with pentanol, while 29 % were most excited by ethanol.

Table 4. Number of single fibers which showed maximal excitability to one of the odors tested.

	Pentanol	Ethanol	Hexanol	Ethyl Acetate
n	9	6	3	3

The total number of all single fibers was N= 21

Table 5. Number of single fibers which showed maximal excitability to more than one odor.

Substances with equal effectivity	n
Ethanol/ Pentanol	6
Ethanol/ Ethyl Acetate	1
Ethanol/ Diethyl Malonate	1
Ethanol/ Hexanol/ Ethyl Acetate	1
Ethanol/ Pentanol/ Vanillin	1

Ten single units responded to two or three test odors with maximally enhanced activity. As is shown in Table 5, some fibers showed equal sensitivities to the combination ethanol/pentanol (n=5).

Obviously, a portion of the fibers investigated exhibited particularly potently enhanced activity to stimulation with one single substance or with several substances.

Discussion

The neurophysiological results show that the activity of single fibers may either remain constant upon chemical stimulation or change according to the applied stimulus. When the results of the behavioral and neurophysiological experiments are compared, a clear correlation emerges: activity increases are particularly potent in response to those stimuli which cause a strong behavioral avoidance reaction of the snails. Especially ethanol and ethyl acetate are most effective. The effect of olfactory stimulation with ethanol on the behavior of *Helix pomatia* L. was already described by Schulz (1938). His studies also revealed different types of avoidance behavior when a capillary containing ethanol at a concentration of 40 % was brought near different sections of the snail's body. The strongest reactions could be evoked by stimulation of the anterior or posterior tentacles. Similarly, the taste sensitivity to bitter substances is particularly high, because these are often toxic and thus should have to be detected as soon as possible (Beidler 1987).

As far as the results of the two strongest stimuli ethanol and ethyl acetate are concerned, it is remarkable that not only the number of highly sensitive neurons "S(+++)", but also the number of neurons with a weak sensitivity "S(+)" is higher than of those neurons with inermediate sensitivity "S(++)". For all other stimuli, the proportion of S(+)-fibers is usually higher than that of the S(++)- and S(+++)-neurons. The fact that a large number of S(+)- and S(+++)-fibers but only a few S(++)-fibers responded to stimulation with particularly effective odors may be due to the activation of neurons with a very high threshold which had not previously been stimulated by less effective substances.

When comparing the results of stimulation with pentanol, hexanol and octanol, it is remarkable that these substances lead to very similar behavioral reactions, although octanol caused a significantly weaker increase of response frequencies. The fact that olfactory stimulation with octanol may be less effective on neuronal activity than stimulation with pentanol or hexanol has also been shown in recordings from trigeminal receptors of the rabbit (Tucker 1963). The observation that behavioral reactions did not differ significantly between stimulations with these substances could be explained by the possibility that the concentrations applied were considerably above the detection thresholds.

All odors mentioned so far induced behavioral avoidance reactions in *Helix pomatia* L.. In contrast, stimulation with the odor of 1 % solution of vanillin had no effect on the behavior of snails, and stimulation with diethyl malonate led to a slight positive effect. This may be explained by the fact that the latter has a strong fruit-like odor and the natural diet of *Helix pomatia* L. also includes different kinds of fruits (Kilias 1985). In both cases the results of the neurophysiological investigations also suggested a weak sensitivity for these substances.

Thus, the relation between neurophysiological and behavioral data can be described as follows: neuronal sensitivity is high for those odors which cause avoidance behavior, while substances which are not potentially dangerous do not lead to a marked increase in activity.

However, there is an exception to this rule as has been shown by the experiments with octanol. It has to be considered that the perception of an odor depends not only on the frequency of neuronal impulses. For example, the quality of gustatory or olfactory sensation is mainly transmitted by a characteristic pattern of neuronal impulses (Erickson 1963, Le Magnen 1963) and the identification of a substance including possible behavioral reactions is a very complex process.

Furthermore, it will cause problems to correlate the chemical structure or other specific properties of an odor source with its olfactory efficacy. Important properties of an odorant substance depend on the form and size of its molecule, but olfactory perception only takes place if the molecule fits a receptor with complementary dimensions. Functional groups are not necessary in this process since a high degree of stereochemical correspondence is sufficient for triggering the neuronal response (Ohloff 1990).

The sensitivity to one or several odors was particularly high in most of the investigated neurons. But all fibers were sensitive to more than one stimulus. Some fibers responded with a maximum increase in activity either to stimulation with ethanol or pentanol. Nevertheless, ethyl acetate and hexanol were also quite effective (Table 3) and could rarely evoke a maximum response. It seems to be remarkable that ethanol which caused a maximum increase of activity in six fibers was equally effective as pentanol in six additional recordings. Single correlations with ethanol did appear in several cases.

Thus, two facts have supplied evidence for the existence of a relative specificity of single fibers in the olfactory nerve. First, all of the investigated neurons responded to stimulation with several different odors and second, the sensitivity to particular substances prevailed in these recordings.

The finding that chemosensitive neurons respond to stimulation with a single substance, or groups of substances, but not to the application of other substances, has been reported previously. Neurophysiological investigations in Syrian hamsters showed that single neurons of the chorda tympani exhibited particularly strong excitation to gustatory stimulation with certain substances (Frank 1973). According to the substances which caused the highest increases in activity the neurons were classified into "best"- types.

Furthermore, recordings from the deutocerebrum of *Periplaneta americana* revealed that olfactory stimulation with certain stimuli, e.g. aliphatic alcohols, was more effective than stimulation with other odors (Boeckh 1974). A comparison of receptors and central neurons showed that the specificity to fruit odors in central neurons is narrower than in receptors. These findings led to a classification into several types like the pentanol-, hexanol- and octanol-type (Boeckh *et al.*

1976). In molluscs, the specificity of the olfactory sense has been studied in *Aplysia californica* (Audesirk and Audesirk 1977). Neurons of the cerebral ganglion receive inputs from the tentacles when these are stimulated by the odor of conspecifics. The odor of food algae does not increase the activity in these cells. Since the odor sources used in the latter study were rather complex, a conclusion on the extent of specificity is not possible.

In the present study, the relative specificity of single fibers has been demonstrated by stimulation with pure substances. But it has to be considered that these, except vanillin, were undiluted. However, it is obvious that stimulation with different concentrations may cause different sequences of sensitivity. Nevertheless, the present results suggest that in *Helix pomatia* L. a classification into different types of neurons according to the "best"- types is also valid.

References

AUDESIRK TE, AUDESIRK G: Chemoreception in *Aplysia californica.* II. Electrophysiological evidence for detection of the odor of conspecifics. *Comp Biochem Physiol* **56A**: 267-270, 1977.

BEIDLER LM: Taste. In: *Encyclopedia of Neuroscience.* G. ADELMANN (ed.), Birkhäuser Verlag, Boston, Basel, Stuttgart, 1987, p 1183.

BOECKH J: Die Reaktionen olfaktorischer Neurone im Deutocerebrum von Insekten im Vergleich zu den Antwortmustern der Geruchssinneszellen. *J Comp Physiol* **90**: 183-205, 1974.

BOECKH J, ERNST K-D, SASS H, WALDOW U: Zur nervösen Organisation antennaler Sinneseingänge bei Insekten unter besonderer Berücksichtigung der Riechbahn. In: *Verh Dtsch Zool Ges,* Gustav Fischer Verlag, Stuttgart, 1976, pp 123-139.

CHASE R: Electrical responses of snail tentacle ganglion to stimulation of the epithelium with wind and odors. *Comp Biochem Physiol* **70A**: 149-155 1981.

CHASE R: The olfactory sensitivity of snails *Achatina fulica. J Comp Physiol* **148**: 225-235, 1982.

CHASE R, CROLL RP: Tentacular function in snail olfactory orientation. *J Comp Physiol* **143:** 357-362 1981.

ERICKSON RP: Sensory neuronal patterns and gustation In: *Olfaction and Taste,* Y ZOTTERMANN (ed), Pergamon Press, Stockholm, 1963, pp 205-213.

FRANK M: An analysis of hamster afferent taste nerve response functions. *J Gen Physiol* **61**: 588-618, 1973.

GELPERIN A, TANK DW: Odor-modulated collective network oscillations of olfactory interneurons in a terrestrial mollusc. *Nature* **345**: 437-440 1990.

HOPFIELD JF, GELPERIN A: Differential conditioning to a compound stimulus and its components in the terrestrial mollusc *Limax maximus. Behav Neurosci* **103**: 329-333 1989.

KILIAS R: Die Weinbergschnecke - Über Leben und Nutzung von *Helix pomatia.* A Ziemsen Verlag Wittenberg, 1985.

LEHNER PN: *Handbook of Ethological Methods.* Garland STPM Press, New York & London, 1979.

LE MAGNEN J: Olfactory identification of chemical units and mixtures and its role in behaviour In: *Olfaction and Taste.* Y ZOTTERMANN (ed), Pergamon Press, Stockholm, 1963, pp 337-345.

OHLOFF G: *Riechstoffe und Geruchssinn Die molekulare Welt der Düfte.* Springer Verlag, Berlin and Heidelberg, 1990,

RATTÉ S, CHASE R: Morphology of interneurons in the procerebrum of the snail *Helix aspersa. J Comp Neurol* **384**: 359-372 1997.

SCHULZ F: Bau und Funktion der Sinneszellen in der Körperoberfläche von *Helix pomatia Z Morphol Ökol Tiere* **33**: 555-581, 1938.

TIWARI SK, WOODRUFF ML: *Helix aspersa* neurons maintain vigorous electrical activity when co-cultured with intact *H aspersa* ganglia. *Comp Biochem Physiol* **101C**: 163-174 1992.

TUCKER D: Olfactory vomeronasal and trigeminal receptor responses to odorants In: *Olfaction and Taste,* Y ZOTTERMANN (ed), Pergamon Press, Stockholm, 1963, pp 45-69.

Reprint requests

Dr. M. Voss, Institute of Zoology, University of Kiel, Olshausenstr. 40-60, D- 24098 Kiel, Germany. Fax: (0431) 880-4197; e-mail: mvoss@zoologie.uni-kiel.de