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Atypical co-localization of snail neurons: impact of season or impaired nerve system?

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Abstract
Hemolymph of snail *Helix pomatia* was analyzed in this study to determine the presence of neurons. The animals were sampled at different localities from April to June 2018. Locations differ in many ecological features, especially in insolation, humidity and altitude. Neurons were recorded in snail's hemolymph from one locality (neurone presence in hemolymph in 40% of specimens). Two types of neurons were identified: unipolar and multipolar. This physiological phenomenon is characterized by the presence of different forms of neurons, both individual and related, as well as the formation of a neuronal network. Neurons are very heterogeneous both in terms of number and size, as well as morphological characteristics. Some neurons did not have dendrites or had short axon, while some had giant axons. The presence of neurons in hemolymph still does not have physiological explanation, but the interaction of hemolymph and nervous system as well as seasonal influences may have great significance for this physiological phenomenon which opens up a significant topic for future research.

Keywords: *Helix pomatia*, hemolymph, neuron's network, unipolar and multipolar neurons

Introduction
Neurobiological studies have shown that behavior is an emerging feature of intricate mutual connections among neurons that operate under the influence of hormones and neuromodulators. However, according to the evolutionary conception, selective pressure in order to achieve a certain adaptive behavior is actually the force that shaped the nervous system (Pinsker 1980) [1]. Gastropods and Cephalopods are the main objects for the research of molluscans neurobiology for many reasons. Gastropods are used because of their relative simplicity and their large neurons such as in *Helix*, *Heliosoma*, *Limax* and *Lymnae* genera (Audersik and Audersik 1985) [2].

Hemostimuli induced to the olfactory epithelium of posterior tentacles elicit signal in central cerebral neuron which than modulates feeding in many gastropods (Egan and Gelperin 1981) [3]. Today, snails are intensively used in non-physiological studies aimed in describing neuronal neural network functioning, identifying neurons by giving a detailed description of the nervous system, its structure and mapping of recognizable neurons. Various types of Heliocidea have been used and many of them are used to analyse their behavior and organization of locomotion (Ierusalimsky *et al*. 1994) [4].

Today, maps for the location of the neurons and precise description of neurons are developed in many species (Kerkut *et al*. 1975) [5]. However, certain neurons are recognizable in each species and those are giant and large neurons mostly. These neurons expose individual variability that includes visual, electrophysiological, morphological and functional properties (Sakharov, 1974) [6]. Neuronal identification is a very complex process, especially in context of their function.

Special group of gigantic neurons are detected and they are very important for snail behavior in withdrawal of command for avoidance (Maksimova and Balaban 1983) [7], while others are called polyfunctional neurons (Arakelov and Shekhter 1981) [8]. Many aspects of neuronal linkage in *Helix* have been studied because of the simplicity of their nervous system, neurite growth, synapse formation and their plasticity (Fiumara *et al*. 2007) [9].

So far, neurons have not been detected in hemolymph of any animal species from the group of invertebrates, nor the function of such neurons in the organism is known. During investigation of snail hemolymph and hemocyte analysis, we encountered cells that by their characteristics did not correspond to hemocytes, but neurons.

Here we report the first results regarding the presence of neurons in invertebrate hemolymph, their types, morphological characteristics and time of appearance in hemolymph.

Material and Methods

Locations

Hemolymph analysis was performed on 60 Roman snails (*Helix pomatia*) samples collected from April to June in the wider area of Sarajevo, Bosnia and Herzegovina. Animal sampling was carried out on five locations, which can be divided in two groups according to ecological characteristics: vegetation of dry and vegetation of wet habitats. All locations are different in insolation, altitude and anthropogenic impact. Most locations are rural areas. Sampling was done by individual collection of snails, after which all animals were transported to the laboratory and the same day the analysis was started. All analyses were carried out according to the Universal Declaration of Animal Health (UDAW). All animals were returned to their natural habitats after non-invasive hemolymph sampling.

Hemolymph sampling

Shell was disinfected with 70% isopropyl-alcohol prep pads (Romed, Netherlands). A part of shell 10 mm^2 size was removed by using monoscope magnifier (Voyager 10-25x42). This is a non-invasive method which includes the aspiration of 0.5 ml hemolymph from pericardial area. Hemolymph collection was done with needle (0.90x38 mm, 20Gx11/2, Romed, Netherlands) through the aperture and was transferred to ependoff tubes.

Slide preparation and hemolymph staining

A total of 50 µl of hemolymph is transferred to microscope slide and the sample was smeared evenly all over slide with a glass rod. The slide was dried on room temperature for 30 minutes. The fixation of slide was done with 99.8%, p.a. methanol (Semikem, Sarajevo, Bosnia and Herzegovina) for five minutes. After methanol evaporation, the excess fluid had been removed by turning slides at an angle of 45º for 15 minutes. Giemsa staining (2:10) was performed for another 20 minutes. The slide was washed gently with distilled water and dried.

Hemolymph analysis and neuron identification

Hemolymph staining analysis was performed using a lightfield and darkfield microscopy (Olympus BX41, Japan) and neuron identification was performed using the Olympus DP12 camera. All photos were imported into Olympus DP Software.

Results

The research was conducted on five different localities. Since neurons were detected in hemolymph of snails at only one locality (location V1), the study was repeated at the same site. Results are presented in Table 1.

Table 1: Locations, characteristics and presence of neurons in snail hemolymph

Locality	Date of collection	Elevation (m)	Characteristics	Presence of neurons in hemolymph
I	09.04.2018.	511	Vegetation of wet habitats with anthropogenic influence, urban place	-
II	09.04.2018.	511	Vegetation of dry habitats with anthropogenic influence, urban place	-
III	19.04.2018.	511	Vegetation of dry habitats with anthropogenic influence, urban place	-
IV	25.04.2018.	697	Vegetation of wet habitats with anthropogenic influence, urban place	-
V1	08.05.2018	850	Meadow, vegetation of wet habitats without anthropogenic influence	+ (40%)
V2	01.06.2018.	850	Meadow, vegetation of wet habitats without anthropogenic influence	-

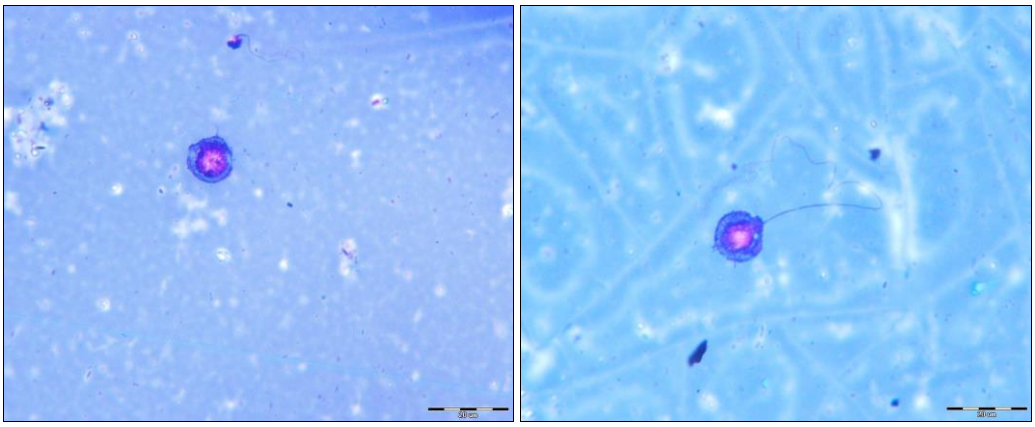
Out of the five analyzed sites during different periods, neurons in hemolymphs were determined at only one locality (V1). Among total number of analyzed animals at that location, the presence of neurons was recorded in 40% of individuals. Repeated sampling and hemolymph analysis at the same site (V2) did not confirm the presence of neurons in hemolymphs.

The presence of neurons has been detected at only one locality, with a smaller number of animals. Characteristics and types of identified neurons are given in Table 2.

Table 2: Types of neurons and general characteristics

Type	Nucleus	Cytoplasm	Processes	Shape
Unipolar	centrally placed with prominent nucleolus; pale rose vesicular staining; occupies ⅔ to ¾ of perikaryon area	basophilic with granulates (Nissl bodies)	single short (1 µm) or long neurite (58 µm) with growth cone and visible axon terminate	round, irregular edges
Multipolar	centrally placed with prominent nucleolus; pale rose vesicular staining; occupies ¾ of of perikaryon area	basophilic with granulates (Nissl bodies)	single long neurite with growth cone (1-235 µm); dendrites consisted of 2-3 main branches coming out from perikaryon, further divided into finer dendrites with small protrusions	stellate, irregular edges

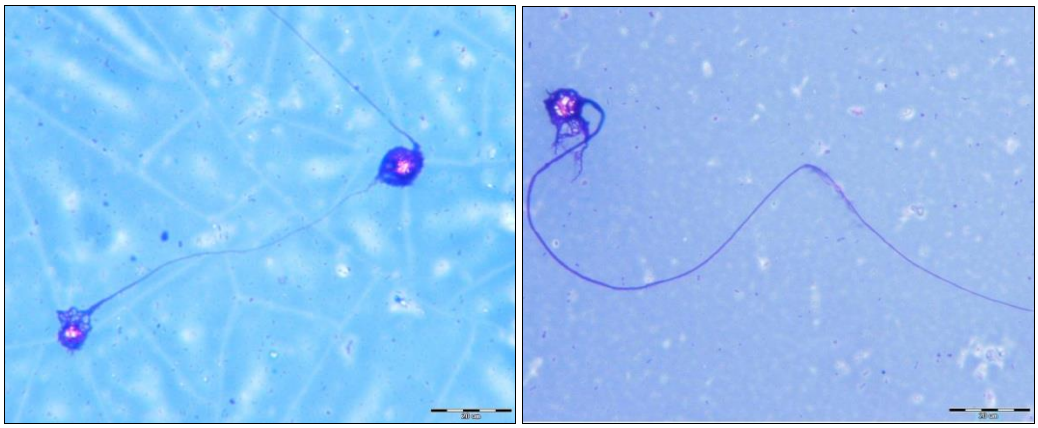
Only two types of neurons, unipolar and multipolar (Figure 1. and Figure 2.), have been identified in snail hemolymph. The extensive observation of hemolymph in animals with determined neurons is very heterogeneous as their number, as well as types of neurons. In some individuals, a very high number of neurons is evident, while in others are not. Most of the neurons were single, while sporadic neurons were recorded.



a **b**

Fig 1: Unipolar neuron with short axon (left) and long axon (right)

The axon length is also a variable character: some neurons have very short axons (Figure 1.), in some are very long (Figure 2.), while in rare cases they are too short so it is possible to see only the conception. Most of the neurons had short dendrites or they are hardly noticed. We found neurons that are connected in hemolymph (Figure 3.) in the way that distal part of first axon was forming synapse with dendrites of second axon.



a b

Fig 2: Connected neurons (left) and multipolar neuron (right)

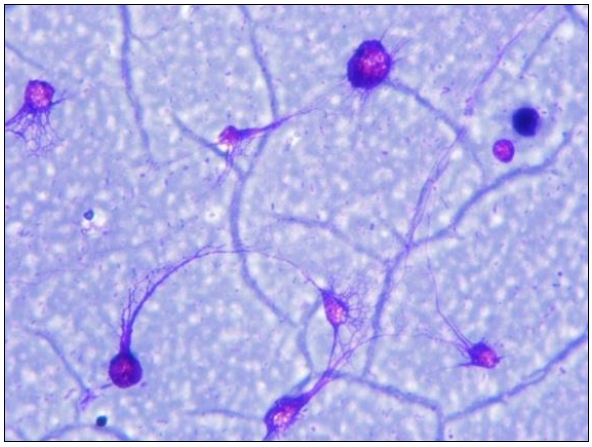


Fig 3: Neural network

Discussion

The emphasis is on three important facts related to this research. First of all the types of neurons of *Helix pomatia*. A large number of invertebrates including snails from genus *Helix* are used as a model for studying of synapse formation and the specificity of neural connectivity. Snail's neurons are individually identified and isolated in cell culture, but entire characterizaton of synaptic connectivity and its dynamics during the development of neural network is not well understood (Massobrio *et al*. 2014) [10]. Large number of neurons can be individually identified and isolated in cell culture. Analysis of mutual neural interactions started in 1990's using the neurons from *Aplysia* (Kleinfeld *et al*. 1990) [11]. Recent studies were based on analysis of neural networks, pairs of synaptically connected neurons whose activity is measured by intercellular techniques of sharp electrodes, as well as various large networks (Massobrio *et al*. 2009) [12]. There are many studies about neural identification but until now they are not identified in hemolymph. Contrary to that, unipolar and multipolar neurons are researched and classified within vertebrates. One of the reasons is fact that generally only certain body regions are conducted with research, such as procerebelum and ganglia. Identified neurons can be classified as unipolar and multipolar based on morphological characteristics. However, the differences in neural structure are obvious, such as neurons with very short or extremly long synapses, with or without dendrites, presence or absence of large or small neural network, huge or small body size of neurons and also presence of extensions on axons. Everything points to complexed physiological phenomenon and heterogenous neurons in hemolymphs. In the literature data different types of neurons have been recorded, based on

applied techniques, functions and isolation sites. Serotonergic C1 neurons were detected on ventral side of cerebral ganglion and it is synaptically connected to B2 neurons of buccal ganglia, togetherthey participate in the regulation of nutritional behavior of Helix species (Cottrell and Macon 1974) [13]. When it comes to the size and number of neurons inside *Helix* species, recent researches discovered the size of neurons which is 100-150 µm. The number of neurons in every procerebral lobe is estimated at about 20.000 in *Achatina fulica* (Chase 1986) [14] and 100.000 inside *Limax maximus* (Gelperin and Tank 1990) [15]. In both cases, this is more than the total number of known neurons in the animal kingdom. Ratté and Chase (1997) [1] revealed two types of neurons inside *Helix aspersa* procerebrum. Neuron C carries long neurite without any branches in procerebrum, neurons H are short and possess abundant and dense arborization. Morfology of those two cells is different so they are refereed as two different subpopulations, intermediate forms were also identified. Neurons B and G owns neurites which stretch far away from soma, but their arborize is longer than their length, with the branching less expressed compared to H neurons. Depending on site of arborizations neurons can be define in three groups: neurons with internal arborization, neurons with internal and external arborization and neurons with extrinsic arborization. When we consider mentioned studies our neurons could belong to two subpopulations, but future research will give better answers. Certainly, it is about large neurons and relatively huge number of them in hemolymph. It was noticed that those snails that had a large number of neurons also had low number of hemocytes.

Another important fact is the reason for presence of neurons in hemolymph. Numerous gastropod's neurons are localized peripheral in the skin, legs and other organs (Schmalz 1914) [17]. These peripheral parts of the nervous system are unexplored and most of studies are physiological analysis on central and visceral ganglion. It should be noted that we have found neurons only on one location in several specimens. This group of snails were sampled immediately after hibernation. The location is at a higher altitude outside residential and rural settlements (43,80º N, 18,55º E). Many years ago, the idea of the existence of some neurons responsible for complex behavior was recorded during an invertebrate escape analysis (Wiersma 1938) [18]. Whether this reaction occurred during the collection of animals, as a form of stressful reaction, is unlikely, because the repeated analysis at the same location later did not result in the presence of neurons in hemolymph. It is considered that there are individual "decision-making" cells which initiates a particular type of behavior or can delay the start of behavior based on seasonal information. These cells are called command neurons or interneurons as the final phase of integration of seasonal information (Wiersma and Ikeda 1964) [19]. The discussion of command neurons still exists in literature since it is a classic neurobiological problem of localization and function. A command neuron is a single neuron (or small set of neurons) whose stimulation results in the evocation of an endogenous and specific occurring behavior pattern (Carew 2000) [20]. Today, there are many questions, such as mechanisms that lead to certain behavior and whether are there centers or individual neurons that start a predetermined behavior after activation. It is believed that the concept of command neurons gives a clear answer in the context of activation set of "neural buttons" that initiates certain behavior and exists in the nervous system (Ierusalimsky *et al*. 1994) [4]. Alternative hypothesis (Weiss 1967) [21] says that behavior is the net outcome of a network and can only be controlled by the entire neuronal network. Whether these are command neurons or post-hybrid period activated signals during waking is not full understandable. However, today two types of command neurons are described: one type which elicits behavior, while the other type initiates a certain pattern of behavior. Actually, command neurons are different from neurons that generate action potential which controls duration and stages of the neural connection (McClellan 1986) [22]. They are considered to be motor neurons (Kandel and Kupfermarm 1970) [23], whose function is not limited only to muscle activation yet consists of activating the motoneuron set and which can obtain and integrate convergent sensory information (Kennedy 1971) [24]. The activity after hibernation starts quite incomprehensible physiological interactions of hemolymph and nervous system.

Many studies have shown that hemolymph has a major influence on nerve cells growth and reparation of damaged neurons. But is there really a dismission of neurons in the hemolymph? Hemolymph of snail *Aplysia sp*. has been investigated for the source of neurotrophic factors recently. Since hemolymph circulates in the open system, it directly comes into contact with internal organs, including ganglia and connectives. Rosenbluth (1963) [25] has been reported that there are sinuses within the abdominal ganglia that allow direct contact between hemolymph and neurons. The nervous system releases certain peptides directly into the hemolymph, where serotonin as neuromodulator is present. This indicates the exchange of substance between circulatory and nervous system (Hatcher and Sweedler 2008) [26]. Some studies have confirmed that hemolymph positively affects on the regeneration of isolated neurons. Several studies have found that hemolymph may affect the regeneration of isolated neurons. Growth factors associated with hemolymph, stimulated neuronal growth and synaptogenesis in neural abdominal ganglia *in vitro* (Schacher and Proshansky 1983) [27]. The main effect on neuron growth is credited to acetiholinesterase present in hemolymphs (Srivatsan *et al*. 1992) [28]. Hyland *et al*. (2014) [29] have proven a strong synergistic effect of the substrate containing hemolymph and laminin proteins on growth and neurite branching during *in vitro* conditions. In fact, the addition of hemolymph accelerated the growth of individual cones over ten times in relation to the laminin itself. This suggests that high molecular weight proteins in hemolymph are responsible for rapid growth which are still undiscovered. Did the sampling method lead to the damage of nerve tissue that would allow the release of neurons into the hemolymph? In terms of damage of the nervous tissue, neurons would appear in the hemolymph of snails in several locations and in several animals. Repeated sampling at the same location did not show the presence of neurons. The presence of neurons is associated with complex mechanisms that are triggered after hibernation. If there was damage of the nerve tissue during the sampling, the released neurons could notform neural network in a short time. Is the interaction between hemolymph and nervous tissue relevant in the physiological context? Observing the sinuses indicates that hemolymph may come in direct contact with the nervous system or with an extracellular matrix around the ganglia (Rosenbluth 1963) [25]. The injury of the nervous system can expose neurons with

hemolymph if there was a barrier between hemocell and nervous system during normal development (Sànchez *et al*. 2000) [30]. Since most animals have open circulatory system, the role of hemolymph and its effect on the nervous system is even more important.

Consequently, functional classification of individual neurons is rather loose and reflectsthe level of our knowledge. The identification of free neurons, as well as the formation of the neuronal network in hemolymph is novelty which makes this physiological phenomenon an important topic for future studies. Mechanisms of hemolymph effect on the nervous system, as well as the presence of free neurons and neuronal networks in hemolymph during certain periods have functional and physiological significance and may suggest new strategies of synergistic nerve reparation reactions, *in vitro* and *in vivo* conditions.

References

1. Pinsker H. Neuroethological analysis of information processing during behavior. In Pinsker HM, Willis WD ed. Information processing in the nervous system. Raven, New York, 1980, 285-312.
2. Audersik T, Audesirk G. Behavior of gastropod molluscs. The mollusca: Neurobiology and behavior. Denver Colorado: Academic Press Inc, 1958, 3.
3. Egan M, Gelperin A. Olfactory inputs to a bursting serotonergic interneuron in a terrestrial Mollusc. Journal of Molluscan Studies. 1981; 47:80-88.
4. Ierusalimsky VN, Zakharov IS, Palikhova TA, Balaban PM. Nervous system and neural maps in gastropod Helix lucorum. International Neuroscience Behavior Physiology. 1994; 24:13-22.
5. Kerkut GA, Lambert JDC, Gayton RJ, Loker JE, Walker RJ. Mapping of nerve cells in the suboesophageal ganglia of *Helix aspersa*. Comparative Biochemistry and Physiology. 1975; 1:1-25.
6. Sakharov DA. Genealogy of neurons, Nauka, Moscow, 1974.
7. Maksimova OA, Balaban PM. Neural mechanisms of behavioral plasticity, Nauka, Moscow, 1983.
8. Arakelov GG, Shekhter ED. Giant polyfunctional neuron in terestrial snails. Zh Vyssh Nervn Dyat. 1981; 31:96-105.
9. Fiumara F, Milanese C, Corradi A, Giovedì S, Leitinger G, Menegon A *et al*. Phosphorylation of synapsin domain A is required for post-tetanic potentiation. Jornal of Cell Science. 2007; 120:3228-3237.
10. Massobrio P, Giachello CNG, Ghirardi M, Martinoia S. Selective modulation of chemical and electrical synapses of Helix neuronal networks during *in vitro* development. BMC Neuroscience. 2014; 25:14-22.
11. Kleinfeld D, Raccuia-Behling F, Chiel HJ. Circuits constructed from identified Aplysia neurons exhibit multiple patterns of persistent activity. Biophysical Journal. 1990; 57:697-715.
12. Massobrio P, Tedesco M, Giachello C, Ghirardi M, Fiumara F, Martinoia S. Helix neuronal ensembles with controlled cell type composition and placement develop functional polysynaptic circuits on Micro-Electrode Arrays. Neuroscience Letters. 2009; 467:121-126.
13. Cottrell GA, Macon JB. Synaptic connexions of two symmetrically placed giant serotonin-containing neurones. Journal of Physiology. 1974; 236:435-464.
14. Chase R. Lessons from snail tentacles. Chemical Senses Journal. 1986; 11:411-426.
15. Gelperin A, Tank DW. Odour-modulated collective network oscillations of olfactory interneurons in a terrestrial mollusk. Nature. 1990; 345:437-440.
16. Ratté S, Chase R. Morphology of interneurons in the procerebrum of the snail *Helix aspersa*. Journal of Comparative Neurology. 1997; 4:359-372.
17. Schmalz E. Zur morphologie des nerven system yon *Helix pomatia*. Zeit Wissenshaften Zoology. 1914; 111:509-568.
18. Wiersma CAG. Function of the giant fibers of the central nervous system of the crayfish. Proceedings of the Societi for Experimental Biology and Medicine. 1938; 38:661-662.
19. Wiersma CAG, Ikeda K. Interneurons commanding swimmeret movements in the crayfish *Procambarus clarkii* (Girard). Comparative Biochemistry and Physiology. 1964; 12:509-525.
20. Carew TJ. Behavioral Neurobiology: The cellular organization of natural behavior. Sinauer Associates, US, 2000.
21. Weiss P. 1+1 = 2. When one plus one does not eqaul two. In The Neuroscience: A Study Program. Rockefeller Press, New York, 1967, 801-832.
22. McClellan AD. Command systems for initiating locomotion in fish and amphibians: Parallels to initiation systems in mammals. In Grillner H and Stein S ed., Neurobiology of vertebrate locomotion. MacMillan Press, New York, 1986, 3-20.
23. Kandel ER, Kupfermarm I. The functional organization of invertebrate ganglia. Annual Review Physiology. 1970; 32:193-258.
24. Kennedy D. Crayfish interneurons. Physiologist. 1971; 14:5-50.
25. Rosenbluth J. The visceral ganglion of *Aplysia californica*. Zeitschrift für Zellforschung und Mikroskopische Anatomie. 1963; 60:213-236.
26. Hatcher NG, Sweedler JV. Aplysia bag cells function as a distributed neurosecretory network. Journal of Neurophysiology. 2008; 99:333-343.
27. Schacher S, Proshansky E. Neurite regeneration by Aplysia neurons in dissociated cell culture: modulation by aplysia hemolymph and the presence of the initial axonal segment. Journal of Neuroscience. 1983; 3:2403-2413.
28. Srivatsan M, Peretz B, Hallahan B, Talwalker R. Effect of age on acetylcholinesterase and other hemolymph proteins in Aplysia. Journal of Comparative Physiology B Biochemical Systemic Environment Physiology. 1992; 162:29-37.
29. Hyland C, Dufresne ER, Forscher P. Regeneration of aplysia bag cell neurons is synergistically enhanced by substrate-bound hemolymph proteins and laminin. Science Reports. 2014; 4:1-9.
30. Sànchez JA, Li Y, Kirk MD. Regeneration of cerebral-buccal interneurons and recovery of ingestion buccal motor programs in Aplysia after CNS lesions. Journal of Neurophysiology. 2000; 84:2961-2974.