THE ROLE OF INTERNEURONAL CONNECTIONS IN THE REGULATION OF HEART BEATS IN THE SNAIL *HELIX POMATIA* L.

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Similarly to other myogenic hearts, the heart of Gastropoda is supplied with external and central nervous regulation manifest in the excitation or inhibition of heart rate (KRIJGSMAN and DIVARIS, 1955; RIPPLINGER, 1957; S.-RÓZSA and GRAUL, 1964). This central system serves as a crossing-place for reflexes influencing the heart rate (ZUBKOV, 1935) and it provides the adequate feed-back regulation in response to the informations coming from the heart (McKAY and GELPERIN, 1972; S.-RÓZSA, 1972; S.-RÓZSA and SALÁNKI, 1973a). According to the results obtained on the *Aplysia*, the neurons taking part in the central regulation of heartbeats are under the influence of different excitatory and inhibitory stimuli (MAYERI et al., 1971). Analysing the effect of heart afferents at the neuronal level both excitatory and inhibitory reactions could be discovered (S.-RÓZSA and SALÁNKI, 1973a,b). This shows, that in the reflex and feed-back regulations of the heart both monosynaptic as well as polysynaptic interneuronal connections are involved. In the present paper the results referring to these latter questions obtained at the cellular level are summarized.

Material and methods

Experiments were carried out on brain-heart preparation of *Helix pomatia* L. (S.-RÓZSA and SALÁNKI, 1973a). The circumoesophageal ganglionic ring and the heart are connected by the intestinal nerve, insuring nerve connections between the two organs and the heart was perfused by physiological solution.

Electrical activity was recorded extracellularly from the heart-branch of the intestinal nerve with bipolar AgAgCl electrodes while all other branches of the nerve were cut off. The membrane and action potentials of the neurones were recorded intracellularly using 3 mol KCl-filled glass microelectrodes. The same microelectrode was used for the polarization of the cell membrane.

In the experiments high input impedance amplifier (VÉRÓ, 1971) and polarizator, while for amplification and recording the potentials ALVAR instruments were used.

For the identification of single neurones and their axon pathways 1 per cent solution of $CoCl_2$ was injected into the cell (PITMAN et al., 1972) for 30—60 minutes at $10^{-6} — 10^{-7}$ A. To obtain black precipitate in the cell 1 per cent solution of $(NH_4)_2S$ was used in 20—30 minutes after injection of $CoCl_2$.

The heart receptors were excited by tactile and chemical stimuli. Tactile stimulation was performed at the external surface of the heart with a fine brush while 5-hydroxytryptamine (10^{-5} M) was given intracardially.

Results

Among the nerve cells studied without selection in the visceral, right and left parietal ganglia at least twenty undoubtedly different neurones were found, showing changes in activity as a response to the heart stimulation. The reaction was either an increase or a decrease in the frequency of the cell activity, however, there occured also double or altering effects especially to a prolonged stimulation (S.-RÓZSA and SALÁNKI, 1973b).

Part of the cells responded to the heart stimulation comparatively quickly, almost instantaneously *(Fig. 1)*, while others by changing the activity after a few seconds *(Fig. 2)*. This is not a direct evidence, nevertheless, it permits to suggest that the neurones showing fast reaction receive direct inputs from the heart, while those responding with some delay are influenced by the heart afferents through interneurones. To value this correctly is rather difficult, since in most cases no synaptic potentials were registered from the cell body and therefore any correlation between the afferent impulses of heart nerve and PSPs eliciting the neurone response could not be analysed. There was only a single case, when the impulsation registered from the heart nerve evoked by the tactile stimulation of the heart, obviously elicited spikes in 1 : 1 proportion in a certain neuron of the visceral ganglion *(Fig. 3)*, so that this cell may be activated either in a monosynaptic way or it may well be a sensory neuron. In other cases, however, when PSPs not generating spikes could be registered, it seems more likely that these are neurones excited or
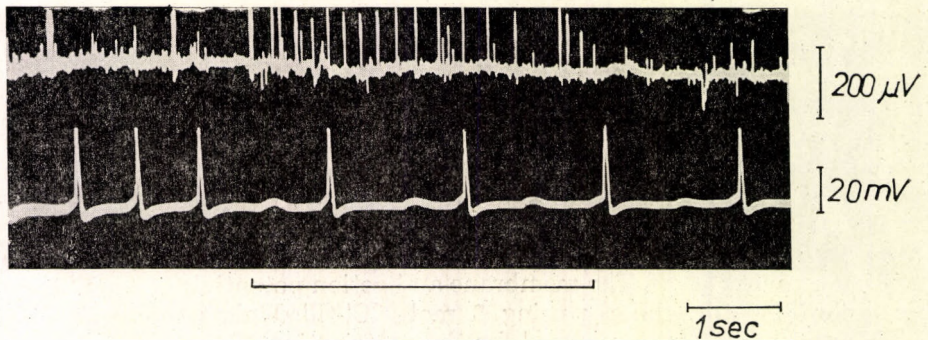

$200 \mu V$

$20 mV$

$1 sec$

Fig. 1. Neuron responding immediately to the tactile stimulation of the heart. *Above:* the electrical activity registered from the heart nerve and increasing in frequency during the heart stimulation. *Below:* the activity of the neurone registered intracellularly and decreasing at the heart stimulation
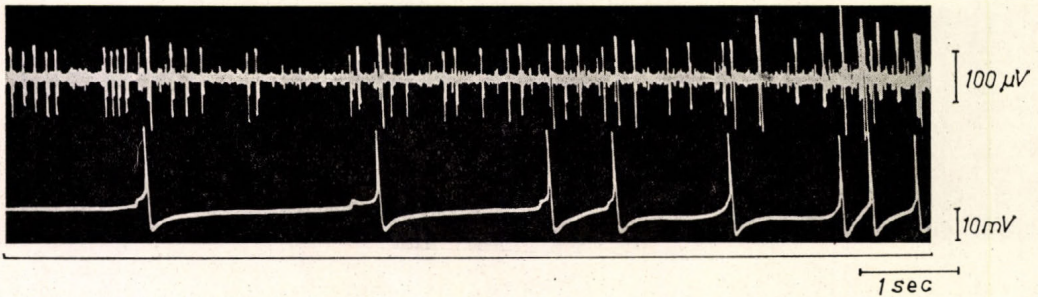
Fig. 2. Neuron showing delayed reaction to the tactile stimulation of the heart. *Above:* electrical activity of the heart nerve increasing at heart stimulation. *Below:* the activity of the neuron increasing in frequency in 6—8 sec after the beginning of the heart stimulation
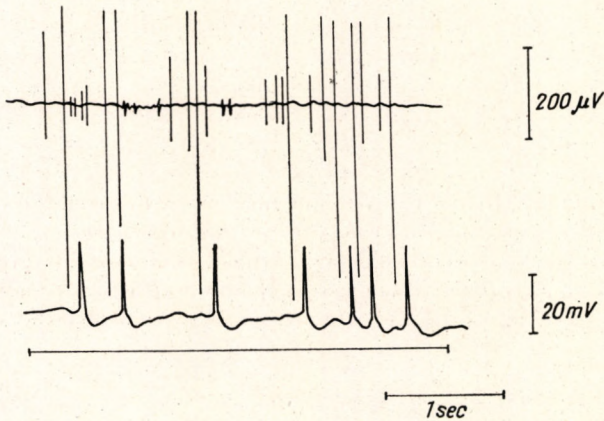

Fig. 3. Neuron responding by firing *(below)* at 1 : 1 proportion to the afferent input *(above)* of heart stimulation

inhibited through interneurones *(Fig. 4)*. This is the case especially in neurons when the PSPs appear with some delay after the heart stimulation.

Consequently, when studying the changes at the cellular level to the afferent influences, it is difficult to decide, whether a mono-, or polysynaptic reaction takes place. In order to clear up the interneuronal connections more exact information was provided by investigations of the efferent influences running to the heart. In this case, the activity of the cell was modified by the polarization of the membrane across the microelectrode and changes in the pattern of extracellular potentials registered from the heart nerve were observed during and after polarization. In most cases, the neurones responding to the excitation of heart afferents was investigated but in a limited number of cases nerve cells not reacting to the heart stimulation were also involved in the analysis.

According to the observed effects the cells could be divided into three groups:
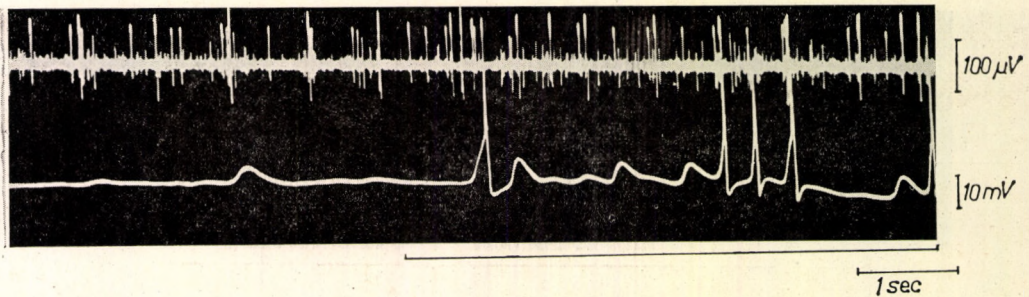
Fig. 4. Above: extracellular activity registered from the heart nerve and its increase in frequency during the stimulation of the heart. *Below:* simultaneous increase in EPSPs and firing of the soma of the neuron (intracellular registration)

1. *Neurones sending direct axon to the heart*

In certain cases there is no doubt that one of the components of the activity registered from the heart nerve is synchronous with the action potentials of the neuron. In our earlier studies neurones RPa5, V12, V13 and V15 proved to be such type and among them cell V12 had also direct effect on the contractile activity of the heart (S.-RÓZSA and SALÁNKI, 1973a). However, it has not been proved that the axon of these neurons is running to the heart, so it was also considered that the neuron in question is not a motoneuron but an interneuron forming input to one of the motoneurones of the heart. This doubt seemed to be justified especially in the case of cell V13, where the extracellular firing registered from the heart nerve comprised always several components. In the present experiments after intracellular staining with CoCl$_2$ it was directly verified that the axons of V12 and V13 neurones are running to the heart and so these neurons cannot be regarded as interneurons.

The observed picture *(Fig. 5)* proves for both cells that their axons are directed into the intestinal nerve, although only comparatively short section of the axon can be tracked down following the origin of the nerve. In the case of cell V12 the doubling of the axon is striking phenomenon. However, since the extracellular sign being synchronous with the cell activity, comprises only one component it is suggested that only one of the branches is running to the heart, while the other is in connection with some other organ. At the same time, the firing of cell V13 resulted in an extracellular sign of the two components referring to the peripheral branching of the axon or showing that each firing of the neuron (V13) triggers an other neuron sending fiber to the heart, too.

Calculations were made also for the conduction velocity of the axons taking into account the distance between the electrodes situating intra- and extracellularly and the time passed between the generation of the potentials under the two electrodes. The conduction velocity for the axon of cell V12 proved to be 50 cm/sec. The conduction velocity for the axon of cell V13 was 41 cm/sec for the first component while for the second component it was 33 cm/sec. Similarly to the above two neurones cells RPa5, V14 and V15 on the basis of their functional properties can also be regarded as efferent neurones sending direct branches to the heart without interneurones.
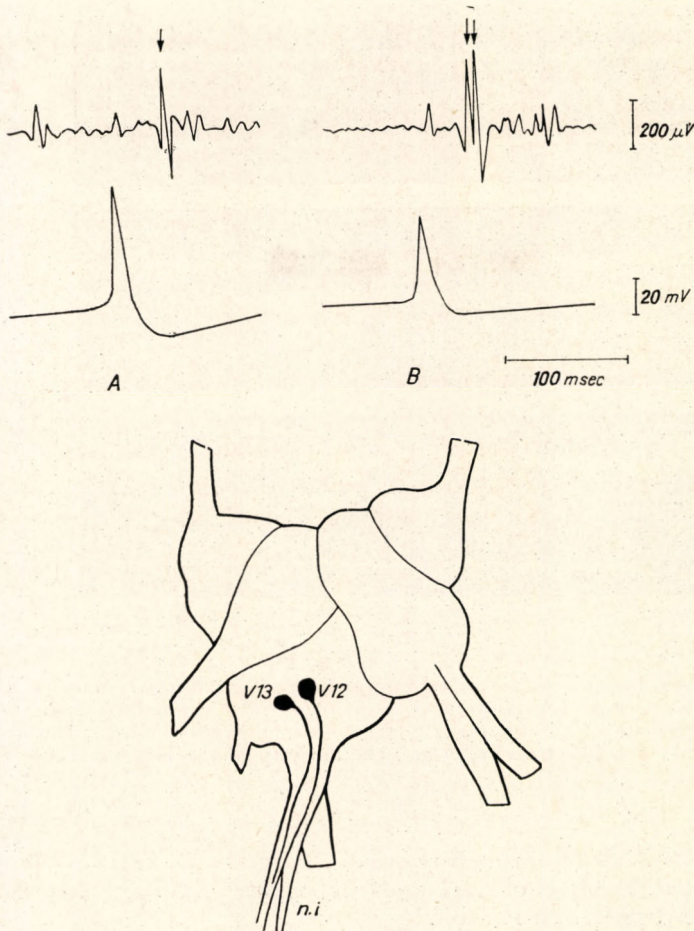
Fig. 5. *A* — firing of cell V12 *(below)*, and the extracellular potentials registered synchronously from the heart nerve *(above)*; *B* — the firing of neuron V13 *(below)* and the synchronous potentials comprising two components registered from the heart nerve *(above)*; *C* — staining of the neurones V12 and V13 after injection with $CoCl_2$. n. i. — intestinal nerve

2. *Interneurons adjoining heart motor neurons*

There were found nerve cells showing no synchronous firing with the nerve activity, nevertheless, their polarization, namely, the increase of their spiking caused characteristic and repeatable changes in the activity of the heart nerve *(Fig. 6)*. The extracellular potentials arising under the depolarization of these neurones not only are asynchronous with the cell activity but sometimes they are much lower in frequency. On the other hand, the nerve activity evoked by depolarization of the neuron may be prolonged during the transient stopping of the cell following the repolarization. This showed that the excited neuron can act as a command cell by the aid of summarizing
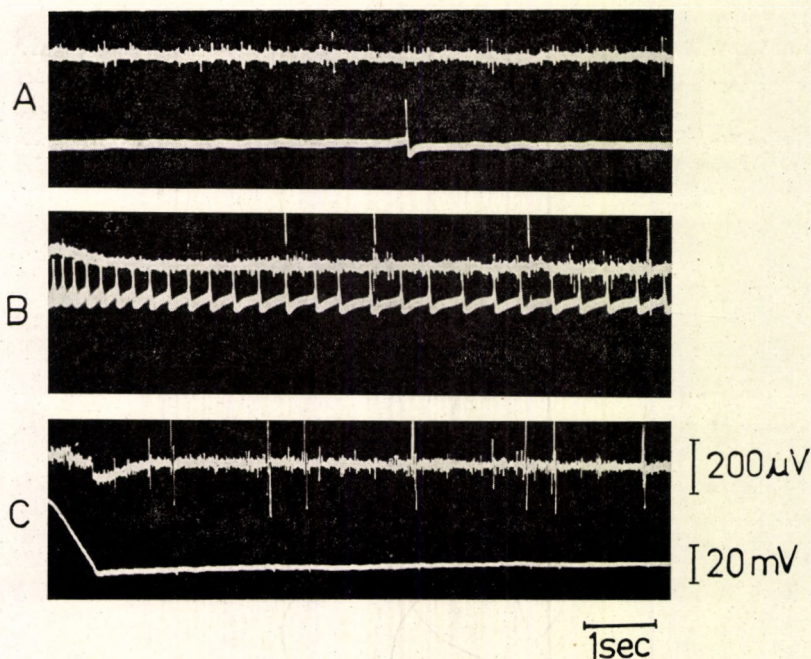
Fig. 6. Asynchronous increase in the frequency of heart nerve activity as a response to the depolarization of a neuron. *A* — control; *B* — depolarization of the neuron; *C* — repolarization of the neuron. In all three registrations *above*: the activity of the heart nerve, *below*: the activity of the neuron

synaptic potentials or through another neuron to the cell connected with the heart and that the effect of the latter can be more prolonged than the excitatory state of the depolarized neuron.

3. *Command neurons producing delayed asynchronous reaction*

In most cases the increased activity of neurons of the visceral ganglia appearing to depolarization for 5—10 sec did not cause any change in the activity of the heart nerve. However, in certain cases by repeated depolarization of similar duration with intervals 20—30 sec a characteristic delayed reaction could be seen *(Fig. 7)* at the extracellular record. The delayed response gradually appearing and disappearing had comparatively large amplitudes and high frequency, comprising the firing of three or four neurones as it is shown by the amplitudes. This reaction lasting for 10—20 sec can be observed during or at the interval of the polarization, however, it may also reappear periodically several times. Nevertheless, no synchronous firing between the activity of the excited cell and heart nerve was observed, i. e. no pathway is running to the heart from the excited neuron. With the repeated excitation of the cell, the firing in the nerve became more and more prolonged, however, after some minutes the control activity was restored.
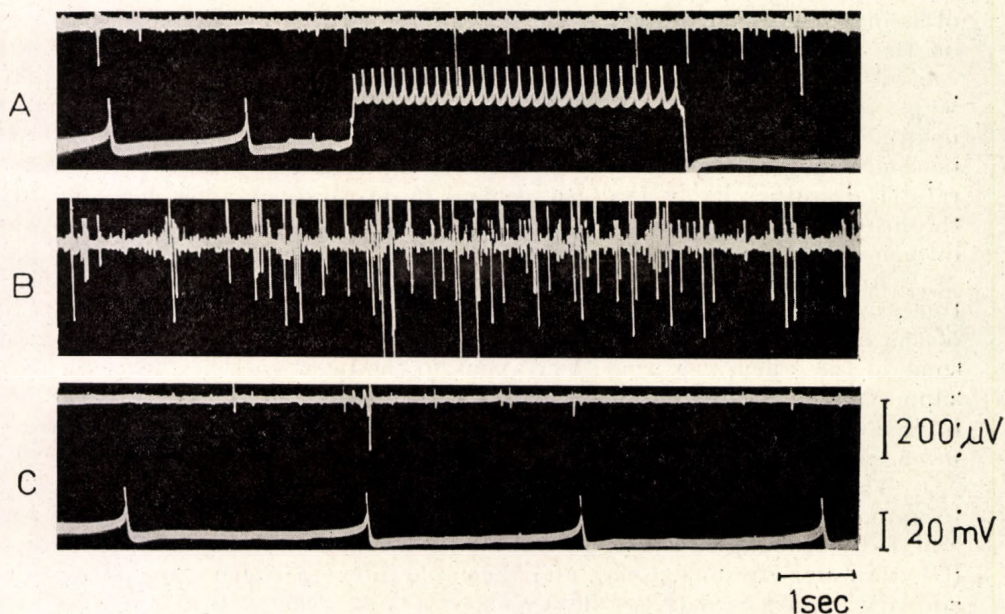
Fig. 7. Increase in the activity of the heart nerve *(B)* appearing 20 sec after the depolarization of the neuron *(A)*. *C* — restoration of the control activity at 60 sec. At registration *B* the neuron is silent, it is strongly hyperpolarized and the resting level of the membrane potential is not shown

In the delayed reaction the role of interneurones have to be assumed and/or the activation of a pacemaker neuron commanding several other neurones having direct effect on the heart. As the effect appeared somewhat delated after the repeated stimulation it can also be supposed that the effect is connected with the liberation of a large amount of transmitter or it is the result of interconnections of neurones coupled to each other within the central, extracardial regulatory system.

Discussion

The effect of the central nervous system reaches the heart through the fibers running in the intestinal nerve while the cell bodies are found in the circumoesophageal ganglionic ring. Morphological data proved that neurones innervating the heart have scattered distribution in the central nervous system of Gastropoda (GUBICZA and S.-RÓZSA, 1969). According to the double innervation of the heart (KRIJGSMAN and DIVARIS, 1955) both excitatory and inhibitory neurones must exist, because from the heart muscle cells EPSPs and IPSPs were registered (KUWASAWA and HILL, 1973; KISS and ELEKES, 1972). Pharmacological investigations proved the inhibitory transmitter role of acetylcholine (RIPPLINGER, 1957), while the excitatory transmitter, serotonin was detected in the heart perfusate after the stimulation of the intestinal nerve (S.-RÓZSA and PERÉNYI, 1966). Recently it was found that the excitation

of a single neurone can modify the heart rate: McKay and Gelperin (1972) on the *Limax* observed excitation of the heart under the stimulation of a central neurone, while S.-Rózsa and Salánki (1973a) found more than 50 per cent decrease in the heart rate during the depolarization of an identified neurone of *Helix*. This proved that no close connection between the scattered neurones innervating the heart is necessary in order to influence the heart rate. It is rather likely that the central heart-neurones are able to modify the heart rate independently from each other and each neuron is under the influence of a separate reflex area.

Among the reflex areas, the own receptor area of the heart takes distinctive place. According to our experiments the tactile and chemical stimulation of the heart can evoke excitation or inhibition of the central neurones and some of these neurones send their axons to the heart so that they take part supposedly in the regulation of heart beat as motoneurones. Other neurones influenced by heart stimulation can afford input or output to other regulatory mechanisms or to their modifications.

The excitatory and inhibitory reactions to the excitation of different receptor areas were found also in the nervous system of *Aplysia* (Hughes and Tauc, 1962), *Buccinum* (Bailey and Laverack, 1966) and *Anisodoris* (Gorman and Mirolli, 1969), and their role in the peripheral regulation was similarly emphasized (Kupferman and Kandel, 1969). However, it is well known also that the excitation running down along the axon not always occupies the soma (Tauc and Hughes, 1963; Arakelov, 1973), so it is possible that at the intracellular registration from the soma we obtain only partial information about the influences, reaching the neurones under the effect of afferent impulsation. The fact, however, that as a response to the afferent stimulation PSPs were only rarely registered, while the changes in the activity of neurones were noticed regularly, proved that the activity of the soma can indicate the afferent influence even in those cases when PSPs were not registered from the cell body. For this reason it seems to be verified that those neurones can be regarded as target cells of heart afferents whose activity changes during the stimulation of heart receptors. Among the latter there are neurones which are able to modify the heart beat owing to their feed-back signalization and neurons sending their activity modified under the input coming from the heart to various other organs.

The extracardial regulation of the heart beat is the funcion of the cells sending axons into the intestinal nerve. The excitation of the receptor areas influencing directly or through interneurones the activity of these cells may have decisive role in the regulation of the heart rate. This suggestion is supported by experiments demonstrating different types of reactions from the heart nerve after the depolarization of single central neurones. Different types of connections in the neuronal network can be suggested whether the electrical activity of the heart nerve was synchronous, asynchronous or delayed as compared to the single cell activity.

When the activity of the investigated neuron was modified under the influence of heart stimulation and the firing of the cell could be registered synchronously from the heart nerve, the monosynaptic connection might be suggested as well (see: cell V13), but under all circumstances only a limited number of interneurones are involved *(Fig. 8)*. When the firing of the investigated cell cannot be recognized in the heart nerve activity but the depolari-

zation of the cell resulted in the increase of the latter *(Fig. 8)*, in this case at least one but probably several synapses are involved in the pathway on to the efferent neuron *(Fig. 8)*. At the same time, the investigated neuron may have input from the heart or from other receptor areas. When after repeated stimulation of the cell only a delayed reaction was induced in the heart nerve, what seemed to be as an explosive activity, the connection is suggested:
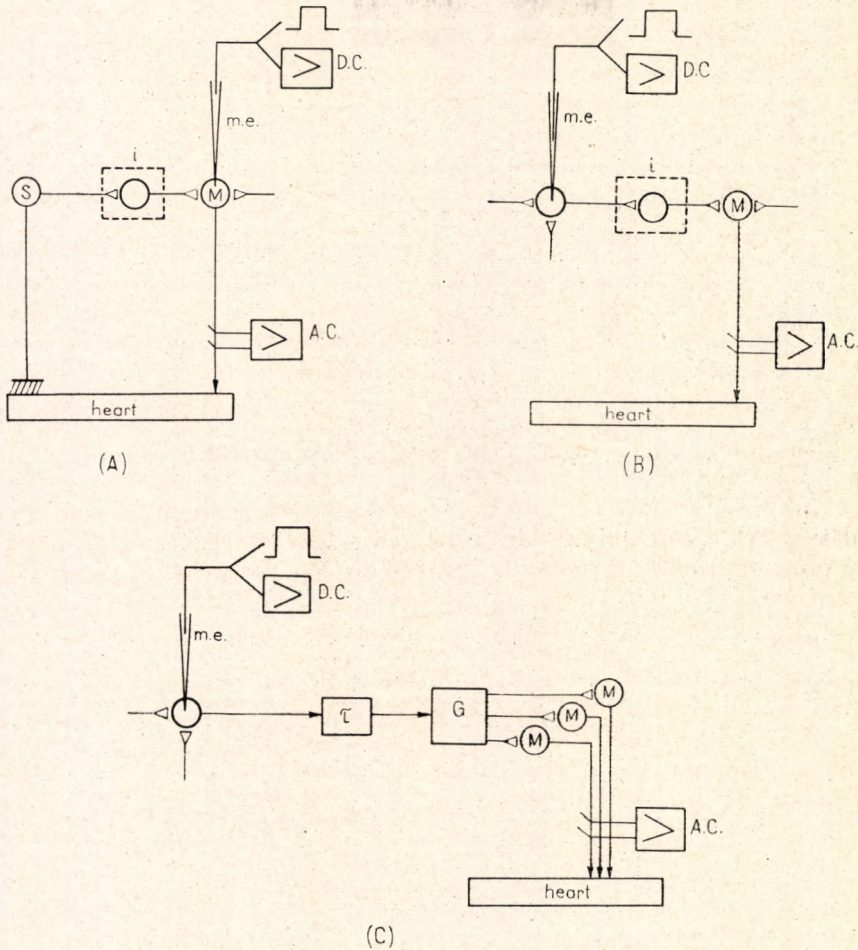

Fig. 8. The possible models of connections in the neuronal net built up according to the reactions observed at different conditions of registration and stimulation. M — the motoneuron of the heart, S — sensory neuron of the heart, m. e. — the investigated neuron marked with microelectrode, D. C. — intracellular registration from the neuron, A. C. — extracellular registration from the heart nerve, i — the suggested interneurons. *A* — neurones responding to heart stimulation and sending branches to the heart; *B* — unit reacting comparatively quickly to the depolarization of the neuron in increasing the asynchronous extracellular activity; *C* — unit reacting to the depolarization of neuron with delayed and unpattered activity of several motor cells. τ — coupling unit with delay; G — the net of generators

several coupled neurones are able to produce self-going activity *(Fig. 8)*. Of course, besides the excitatory synaptic connections inhibitory ones may also be exist determining the specificity and time limitation of the response. The possibility cannot be excluded either that the delayed effect is connected by the local liberation of a large amount of transmitter and is not bound only to the specificities of neuronal connections.

Summary

The role of interneuronal connections in the regulation of heart beats was studied at neuronal level in the brain-heart preparation of *Helix*. Three types of neurones were described.

1. Neurons sending direct branches to the heart whose axons can be detected both by physiological and morphological methods in the intestinal nerve;

2. interneurons joining to the motoneurons of the heart, the excitation of which cause asynchronous increase in the activity of the heart branch of the intestinal nerve;

3. neurons producing delayed asynchronous reaction, the stimulation of which is manifested in increased excitation of the heart nerve activity in 20—30 sec, or causing excitation only at repeated stimulation.

According to the types of neurons different neuronal connections are suggested, building up the complex system of the extracardial regulation of heart beats.

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INTERNEURONÁLIS KAPCSOLATOK SZEREPE A SZIVMŰKÖDÉS SZABÁLYOZÁSÁBAN CSIGÁN (*HELIX POMATIA* L.)

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Összefoglalás

Agy-szív preparátumon neuronszinten tanulmányozták interneuronális kapcsolatok szerepét a szívműködés szabályozásában. Három neuron típust írtak le:

1. Szívhez közvetlen rostot adó neuronok, melyek szív felé haladó axonja fiziológiai és morfológiai úton egyaránt detektálható.

2. Motoros idegekre kapcsolódó interneuronok, melyek serkentése aszinkron aktivitásfokozódást okoz az intestinális ideg szívhez menő ágán.

3. Késői aszinkron választ kiváltó neuronok, melyek ingerlése a szívideg aktivitásában 20—30 sec múlva, vagy csak ismétléskor okoz serkentést.

A sejttípusok szerint eltérő neuronális kapcsolás tételezhető fel, ami együttesen az extrakardiális szív-szabályozás komplex rendszerét alkotja.