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Characterization by immunocytochemistry of ionic channels in *Helix aspersa* suboesophageal brain ganglia neurons

M.J. Azanza[1], C. Pérez-Castejón[1], N. Pes[1], R.N. Pérez-Bruzón[1,2], J. Aisa[1], C. Junquera[1], C. Maestú[2], M. Lahoz[1], C. Martínez-Ciriano[1], A. Vera-Gil[1] and A. del Moral[3]

[1]Laboratory of Magnetobiology, Department of Human Anatomy and Histology, Medical School, University of Zaragoza, Zaragoza, Spain, [2]Humanism and Science Foundation (Madrid, Spain), [3]Laboratory of Magnetism, Department of Solid State Physics & ICMA, University of Zaragoza & Spanish Research Council (CSIC), Zaragoza, Spain

Summary. The aim of this work was to characterize several ionic channels in nervous cells of the suboesophageal visceral, left and right parietal, and left and right pleural brain ganglia complex of the snail *Helix aspersa* by immunocytochemistry. We have studied the immunostaining reaction for a wide panel of eleven polyclonal antibodies raised against mammal antigens as follows: voltage-gated-Na^+ channel; voltage-gated-delayed-rectifier-K^+ channel; SK2-small-conductance-Ca^{2+}-dependent-K^+ channel apamin sensitive; SK3 potassium channel; charybdotoxin-sensitive voltage-dependent potassium channel; BKCa-maxi-conductance-Ca^{2+}-dependent-K^+ channel; hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; G-protein-activated inwardly rectifying potassium channel GIRK2 and voltage-gated-calcium of L, N and P/Q type channels. Our results show positive reaction in neurons, but neither in glia cells nor in processes in the *Helix* suboesophageal ganglia. Our results suggest the occurrence of molecules in *Helix* neurons sharing antigenic determinants with mammal ionic channels. The reaction density and distribution of immunoreactive staining within neurons is specific for each one of the antisera tested. The studies of co-localization of immunoreaction, on alternate serial sections of the anterior right parietal ganglion, have shown for several recognized mapped neurons that they can simultaneously be expressed among two and seven different ionic protein channels. These results are considered a key structural support for the interpretation of *Helix aspersa* neuron electrophysiological activity.

Offprint requests to: M.J. Azanza, Departamento de Anatomía e Histología Humanas, Facultad de Medicina, Universidad de Zaragoza, C/Domingo Miral s/n 50009 Zaragoza, Spain. e-mail: mjazanza@unizar.es

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Introduction

The mollusc brain *in vitro* preparations have been demonstrated to be a potent tool for neurobiological research, facilitating a number of experiments which would prove either unfeasible or difficult to perform on mammalian brains. Gastropod brain studies have largely yielded experimental data which can be reproduced in mammalian preparations. Thus, work on the snail brain is of potential importance to man, although obviously any significant results should be checked again in mammalian preparations. The structural requirements of receptors for a variety of transmitter substances, their agonists and antagonists, were demonstrated long time ago (Akhtar et al., 1971; Walker et al., 1975; Azanza and Walker, 1975; Leake and Walker, 1980). The experimental evidence that GABA as a neurotransmitter specifically either depolarizes or hyperpolarizes neuron activity, was fundamental in showing the existence of two types of GABA-receptors controlling the conductance to Na^+ and Cl^- respectively (Kerkut and Walker, 1961b, 1962; Walker at al., 1975; Azanza and Walker, 1975). These data led to the extrapolation of such a result to explain the excitatory responses promoted by GABA in mammalian neurons (Levy, 1977). More recently, and similarly to mammals central nervous system, nitric oxide (NO) and NO-synthase mediated functions have been described in invertebrates, being associated with the feeding behaviour in *Helix pomatia* (Tekye, 1996). The use of polyclonal and monoclonal anti-mammals raised antibodies for immunocytochemistry studies on *Helix* species have

clearly shown that molluscs share antigenic determinants with mammals (Elekes and Nässel, 1994; Kaufmann et al., 1995; Kerschbaum et al, 1997; Hernadi and Elekes, 1999). Negative results obtained for antibodies against mammalian specific astrocytes, anti-connexin 43 and anti-glial fibrillary acidic protein (GFAP), are in good agreement with evidence for the differences between mollusc and mammalian glia cells (Radojcic and Pentreath, 1979; Azanza et al., 2007a).

Helix aspersa brain preparation has allowed us to experimentally show the subtle modifications induced on the bioelectric activity of neurons under exposure to magnetic fields (MF) (Azanza and del Moral, 1994). Magnetobiology research needs accurate studies with cell controlled variables for the reproduction of experiments on the same kind of neuron, from different specimens, as well as to be repeated many times in order to explain the results under different conditions of exposure to MF. The intracellular recordings from mollusc neurons are very stable, since they retain their functional activity after dissection for several hours. Gastropods can furthermore overcome slight environmental changes, which often prove disastrous for vertebrate cells. Microelectrodes can easily be impaled into the cell so that the response of a neuron to the addition of a specified drug, either applied iontophoretically or by adding it to the bath, can be studied for long periods of time (hours) and repeatedly. Snail brain surrounding Ringer solutions can easily be changed, in this way controlling either the external ionic concentrations or the application of known concentrations of agonists, inhibitors or toxins to the system (Kerkut et al., 1998). In this way the action of neurotransmitters upon individual cells under simultaneous exposure to MF is investigated thoroughly. Based on these foundations we have consistently explained the responses elicited on *Helix* neurons under extremely low frequency and static applied magnetic fields (OMS, 2004).

We have experimentally shown in *Helix aspersa* suboesophageal brain ganglia that single unit neurons bioelectric activity is modified under exposure to MF, either static (SMF, of 0.1 mT - 0.7 T intensity) or sinusoidal (ELF-MF, of frequencies between 0.1-80 Hz and peak intensity from 0.4 μT up to 15 mT). We have shown that 82% of tested neurons modify their bioelectric activity under applied MF, i.e. showing *magnetosensitivity*. The response induced on a particular mapped neuron is constant, either increasing or decreasing the spike frequency, whatever the kind of applied MF, static or sinusoidal. Stimulation and inhibition are mediated by calcium ions (Azanza, 1989, 1993; Azanza and del Moral, 1994; Calvo and Azanza, 1999) the channels involved, being the Ca^{2+}-dependent-K^+-channels sensitive to d-tubocurarine, but not the TEA-sensitive- Ca^{2+}-dependent-K^+-channels (Azanza and del Moral, 1988; Azanza, 1990).

In order to obtain more information and further support for our electrophysiological experimental results, the aim of the present work is to characterize by immunocytochemistry a group of protein channels specific for Na^+, K^+ and Ca^{2+} ions, on tissue sections of the *Helix* suboesophageal ganglia complex. As above mentioned, giant neurons located at the visceral, right and left parietal ganglia, have been the object of intensive research. Our present study should contribute to increase our current knowledge about plasma membrane neurochemistry of morphologically identified and electrophysiologically characterized neurons. It will be possible in this way to search for a correlation between the kind of ionic protein channels expressed by *Helix* neurons and bioelectric activity, either natural or induced by applied MF.

Materials and methods

We have examined 12 samples of *Helix aspersa* suboesophageal visceral, left and right parietal, and left and right pleural brain ganglia complex. The specimens were bought in the local market. Many of the snails maintained in laboratory conditions are inactive, showing a low neuron bioelectric spontaneous activity (Kerkut and Walker, 1961a). In such conditions a low expression of certain proteins involved in bioelectric activity could be expected so that the animals were maintained active as described elsewhere (Kerkut and Walker, 1961b; Azanza et al., 2007a). The circumoesophageal ring of ganglia were removed and the suboesophageal ganglia complex cut and fixed for 16 hours in 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4), at 4°C, dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial sections of 5μm were routinely stained with haematoxylin-eosin.

Immunocytochemical staining was performed on

Table 1. Relation of the tested antisera indicating the dilution, reference number and acronyms in test and figures.

Channel	Acronyms	Chemicon Ref. Nº	Dilution
Na^+: voltage-gated	Na^+	AB 5206	1/200
K^+:voltage-gated-delayed-rectifier	K^+	AB 5182	1/200
K^+:SK2-small-conductance-Ca^{2+}-dependent	SK2	AB 5356	1/300
K^+:SK3-small-conductance-Ca^{2+}-dependent	SK3	AB5350	1/300
K^+:charybdotoxin-sensitive voltage-dependent	HLK3	AB 5178	1/300
K^+:BKCa-maxi-conductance-Ca^{2+}-dependent	BKCa	AB 5228	1/200
K^+:hyperpolarization-activated cyclic nucleotide-gated	Khy	AB 5808	1/300
K^+:G-protein-activated-inwardly rectifying, GIRK2	GK	AB 5200	1/300
Ca^{2+}:voltage-gated -L	Ca^{2+}-L	AB 5156	1/1000
Ca^{2+}:voltage-gated-N	Ca^{2+}-N	AB 5154	1/300
Ca^{2+}:voltage-gated-P/Q	Ca^{2+}-PQ	AB 5152	1/300

serial paraffin sections 5 µm thick fixed in formaldehyde using the immunocytochemistry EnVision® (Dako) method. We used a panel of polyclonal antibodies raised against mammal antigens (rabbit-antimouse) (Table 1). The antibodies were diluted with Dako diluent (S2022).

The tissue sections were deparaffined in xylene (10 min. twice) and rehydrated in a graded ethanol series up to distilled water. Before all assays, for heat-induced antigen retrieval, the samples were treated during 6 min. in an 800-W microwave oven with 10% citrate buffer (Dako S2031) in distilled water and at 360 W for 5 additional minutes. After washing with PBS, 3 min. twice, the sections were treated with endogenous peroxidase blocking (Dako S2001) for 20 min. washed in distilled water and PBS 3 min. twice. The blocking was repeated for a second time. The sections were incubated with the primary antibodies solution for 30 min. followed by a rinse in blocking buffer (100 ml PBS, 2ml triton X100, 0.25 ml BSA (A4503 SIGMA)), for 3 min. twice. The visualization was made by incubating with Envision® peroxidase-based visualization kit (Dako K5007) during 30 min, washed in PBS, for 3 min. twice according to manufacturer's direction. To confirm the presence of immunocomplexes, 3,3'-diaminobenzidine was used as chromogene and hydrogen peroxide as substrate. The samples were washed twice in distilled water, contrasted with Mayer's haematoxylin for 7 min., washed in tap water for 15 min., dehydrated in a graded series of ethanol, cleared in xylene and cover slipped with DPX. Digital microscope images were captured by means of an Olympus BX 51 microscope. The number of immunoreactive cells was estimated from serial sections by counting immunostained cell bodies. The diameter of a neuron was taken from its cross section containing the nucleus. Both measurements were made with the program Olympus DP-Soft.

The research of possible co-localization of immunoreactive staining for Na^+, K^+, SK2, Ca^{2+}L, Ca^{2+}N and Ca^{2+}P/Q type channels was performed on 5 µm-thick serial tissue sections of the anterior right parietal ganglion.

Positive controls were made by applying the same protocol on Wistar rat brain tissue sections. Negative controls were made by omission of primary antibody on *Helix* and rat brain tissue sections.

Results

All the antisera give positive reaction in neurons of the suboesophageal ganglia, showing the distribution of immunoreactive-like staining for the protein channels studied. The reaction density and distribution of immunoreactive-like staining within neurons have been specific for the different studied protein channels. Glia cells and their processes were negative for the eleven antisera tested.

Immunocytochemistry results are accounted for by the percentage of neurons showing immunoreactive-like staining for the studied antisera (Table 2). The immunostaining reveals immunoreactive structures within the cytoplasm of the soma, in the plasma membrane and in fibres of the neuropil. Up to now there have been no data about any morphological dissimilarity between those nerve cells in the character of their physiological rhythmic activity. The cellular morphology did not reveal any gross structural organization, and in the neuropil, the axons were not predisposed to run in any particular position along any of the axes, instead they seem to do so in a random fashion. According with these characteristics, we have considered the size of neurons as the identification criterion. Therefore, we have distinguished between small (diameter below 25 µm), medium (diameter between 30-50 µm) and large (diameter over 55 up to about 140 µm) sized neurons.

The reaction for the Na^+ channels antiserum shows a fine, light, brown precipitate, widespread in the cytoplasm of large and medium size neurons. In certain portions of the plasma membrane the reaction is of

Table 2. Immunohistochemical results.

Ionic channel	Small size (5-25 µm)			Medium size (30-50 µm)			Large size (55-140 µm)			Total number (5-140 µm)		
	nº	(+)	%	nº	(+)	%	nº	(+)	%	nº	(+)	%
Na^+	1288	512	39.7	530	324	61.1	182	159	87.4	2000	995	49.7
K^+	1475	1160	78.8	377	313	83.0	148	137	92.5	2000	1610	80.5
SK2	1431	1051	73.4	529	268	50.6	40	34	85.0	2000	1353	67.6
SK3	1037	865	83.4	414	333	80.4	57	54	94.7	1508	1252	83.0
HLK3	1103	565	51.2	707	369	52.2	131	98	74.8	1941	1095	56.4
BKCa	1216	330	27.1	665	322	48.4	119	89	74.8	2000	741	37.0
Khy	115	34	3.1	678	56	8.3	207	53	25.6	2000	143	7.2
GK	1684	891	52.9	250	193	77.2	66	54	81.8	2000	1138	56.9
CaL	1490	1111	74.6	186	186	100	34	34	100	1710	1331	77.8
CaN	1811	1378	76.0	175	164	93.7	14	14	100	2000	1556	77.8
CaP/Q	1800	1582	87.8	177	169	95.4	23	22	95.6	2000	1773	88.6

The percentage of neurons showing immunoreactive-like staining for the studied antisera is expressed in function of the neuron size. nº: number of neuron counted; (+): number of positive neuron counted.

stronger density. Small neurons, located near the neuropil and fibres, appear more densely stained (Fig. 1a).

The reaction for the potassium channels antisera is observed as brown spots of variable density, widespread in the neurons cytoplasm. Delayed-rectifier-K^+-channels like-immunostaining is observed as discrete and dense spots widespread in the cytoplasm of large, medium and small neurons (Fig. 1b). The reaction is of moderate intensity for SK2, SK3 (Fig. 1c,d) and HLK3 (Fig. 2a). The distribution of the spots in the cytosol is uniform for SK2, concentrated in certain portions of the cytosol for SK3, and mainly in the axonal cone for HLK3 antisera. Immunoreaction for BKCa antiserum is very light in large and medium size neurons and fibres in the neuropil, but stronger in small neurons, showing a homogeneous distribution in the cytosol (Fig. 2b). The reaction for Khy antiserum is of moderate intensity in the neuropil, the positive neurons show a homogeneous and dense staining, widespread in the cytosol (Fig. 2c). GK antiserum staining is very light with fine spots dispersed in the cytosol and more concentrated in the axonal cone (Fig. 2d). It is interesting to note the observation of immunoreaction for BKCa antiserum in the nucleoplasm of about 14% of neurons counted.

The immunostaining for calcium channels is specific

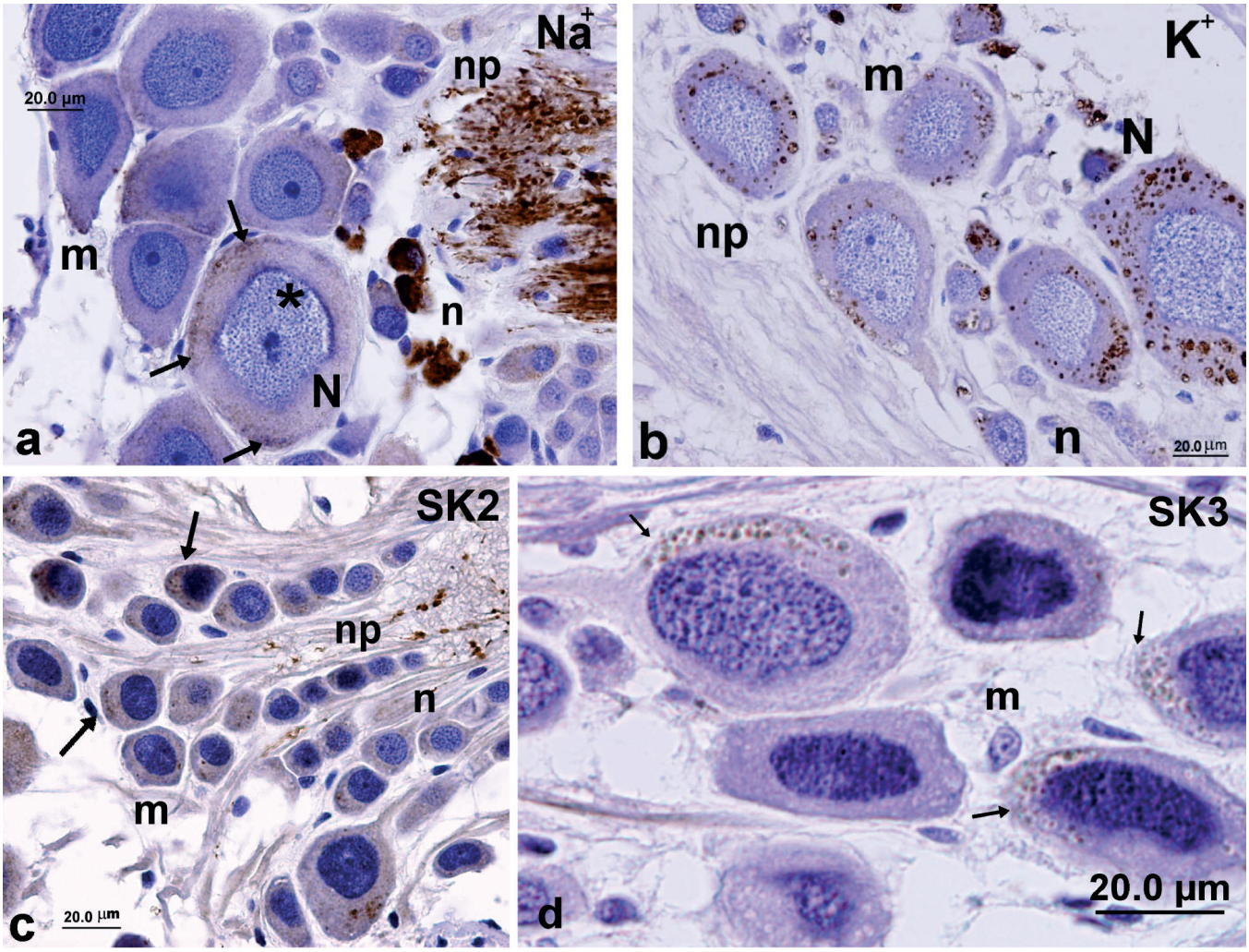

Fig. 1. Immunocytochemical reaction for: **a.** Na^+- channels antiserum is observed as a fine, light reaction widespread in the cytoplasm of large (N) and medium size neurons (m). Small neurons (n) near the neuropil (np) and fibres are densely stained. In some locations, near the membrane, a highest density reaction is observed (arrows) (section of the anterior right parietal ganglion, right-ventral portion) (*neuron F34, Fig. 5a). **b.** K^+- channels antiserum gives discrete, dense brown spots, widespread in the cytoplasm of large (N), medium (m) and small neurons (n) (section of the right pleural ganglion, left-medial portion). **c and d.** SK2- and SK3- channels antisera immunostaining is similar: brown spots of moderate intensity, with a uniform distribution in the cytoplasm of medium (m) and small (n) neurons for SK2, and concentrated in certain portions of the cytosol for SK3 (SK2, section of the left pleural ganglion, right-medial portion; SK3, section of the posterior right parietal ganglion, left-dorsal portion).

for the three studied antisera. The reaction for Ca^{2+}-L-like channels is observed as spreading diffusely in the cytoplasm of big neurons, being more concentrated in the axonal cone and axons, and small neurons appear more densely stained. A positive reaction is observed in the nucleoplasm of medium and small sized neurons (Fig. 3a). The reaction for Ca^{2+}-N and Ca^{2+}-P/Q –like channels is observed as a dense brown precipitate concentrated in discrete portions near the plasma membrane. The Ca^{2+}-N like-immunostaining is also observed in trophospongia structures and in fibres of the neuropil (Fig. 3b). The Ca^{2+}-P/Q immunostaining is also observed in the axonal cone of the large and medium size neurons (Fig. 3c). Clusters of small neurons densely stained are shown near the neuropil, disposed between medium and large neurons (Fig. 3d).

The positive controls made in adult Wistar rat CNS showed a positive reaction for all the tested antisera. In Fig. 4a is shown the immunostaining reaction for Na^+ channels in fibres of the CA3 area of Ammon's horn. Delayed-rectifier-K^+ channels are observed in fibres of the basal Meynert nucleus (Fig. 4b) and in pyramidal neurons of the parietal cortex (Fig. 4c). Ca^{2+}-L channels immunostaining is observed in pyramidal neurons of the

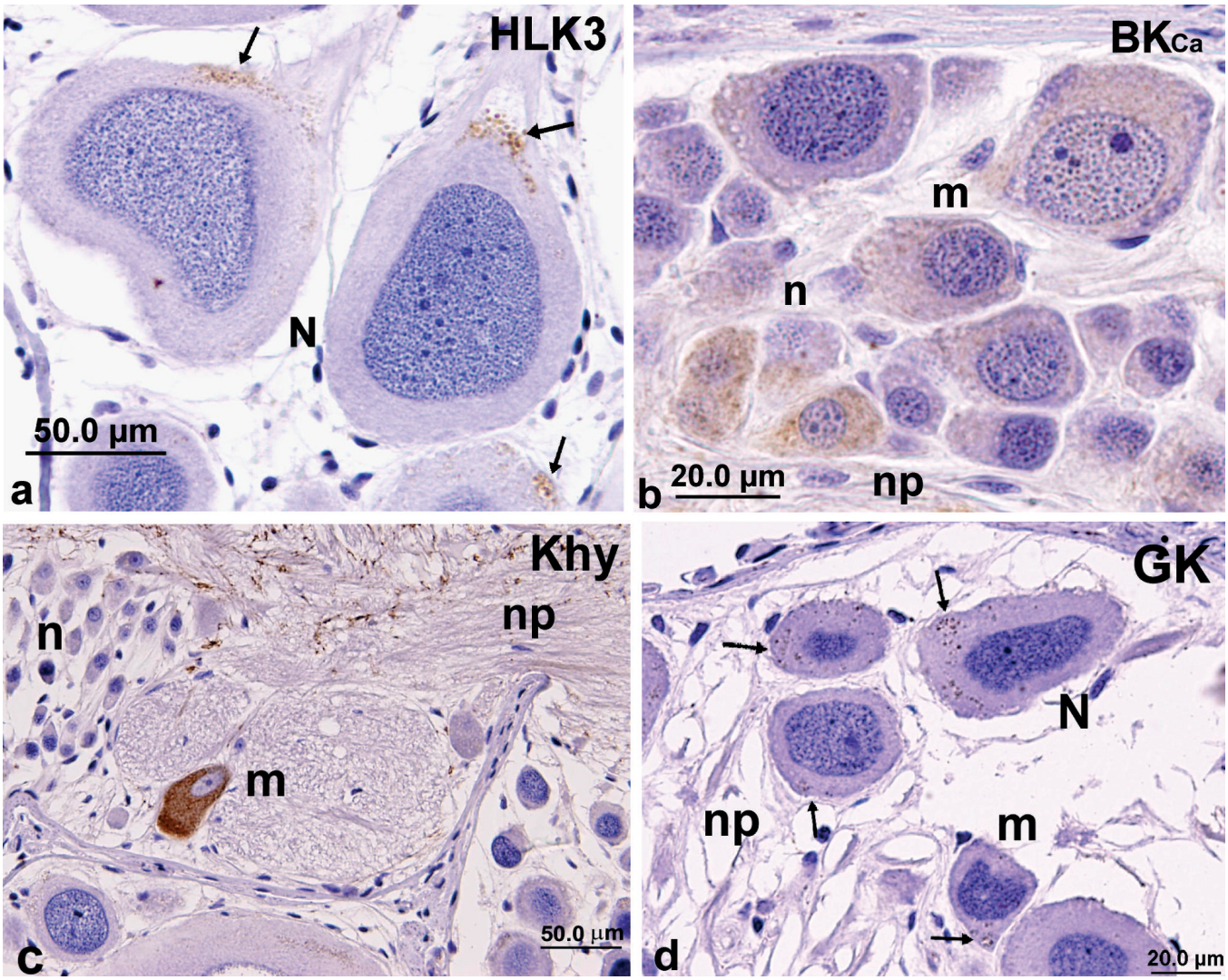

Fig. 2. Immunocytochemical reaction for: **a.** HLK3- channels antiserum in the axonal cone of big neurons (N) (section of the posterior visceral ganglion, dorsal portion). **b.** BK_{Ca}- channels antiserum shows a very light reaction in medium neurons (m) and fibres of the neuropil (np), the reaction is stronger in small neurons (n) with a homogeneous distribution in the cytosol (section of the visceral ganglion, dorsal-anterior portion). **c.** Khy- channels antiserum is observed as a dense, homogeneous brown precipitate, filling the cytoplasm of medium size neurons (m) with a moderate intensity reaction in fibres of the neuropil (np) (section of the posterior visceral ganglion, dorsal-portion). **d.** GK- channels antiserum staining is very light with fine spots dispersed in the cytosol of medium neurons (m), more concentrated in the axonal cone (section of the posterior left parietal ganglion, dorsal portion).

parietal cortex (Fig. 4d). We did not observe a positive reaction in the neurons nucleoplasm for Ca^{2+}-L antiserum, instead, such a reaction has been observed for BKCa in the neurons nucleoplasm of the reticular thalamic nucleus (Fig. 4e).

The data presented in Table 2 give the distribution of the immunoreaction for the studied channels, for neurons of the suboesophageal ganglia complex of *Helix aspersa*, according to the neuron size. It is worth noting that although large neurons are a minority, they give the highest percentage of channel expression. The results for the total number of counted neurons provide an interesting result. The highest number of neurons express: SK3-, delayed rectifier K^+- and P/Q calcium channels-like immunostaining. In a second group we can consider the neurons positive for N- and L- calcium and for SK2-channels like immunostaining. These results corroborate the central role of calcium and potassium ions in conveying the bioelectric activity in *Helix neurons*.

By staining alternate serial sections of *Helix aspersa* right parietal ganglia for various antisera, our immunocytochemistry methods have opened the possibility of studying the colocalization of ionic channels-like proteins expressed in neurons. Fig. 5 show a panoramic view of the right parietal ganglion anterior

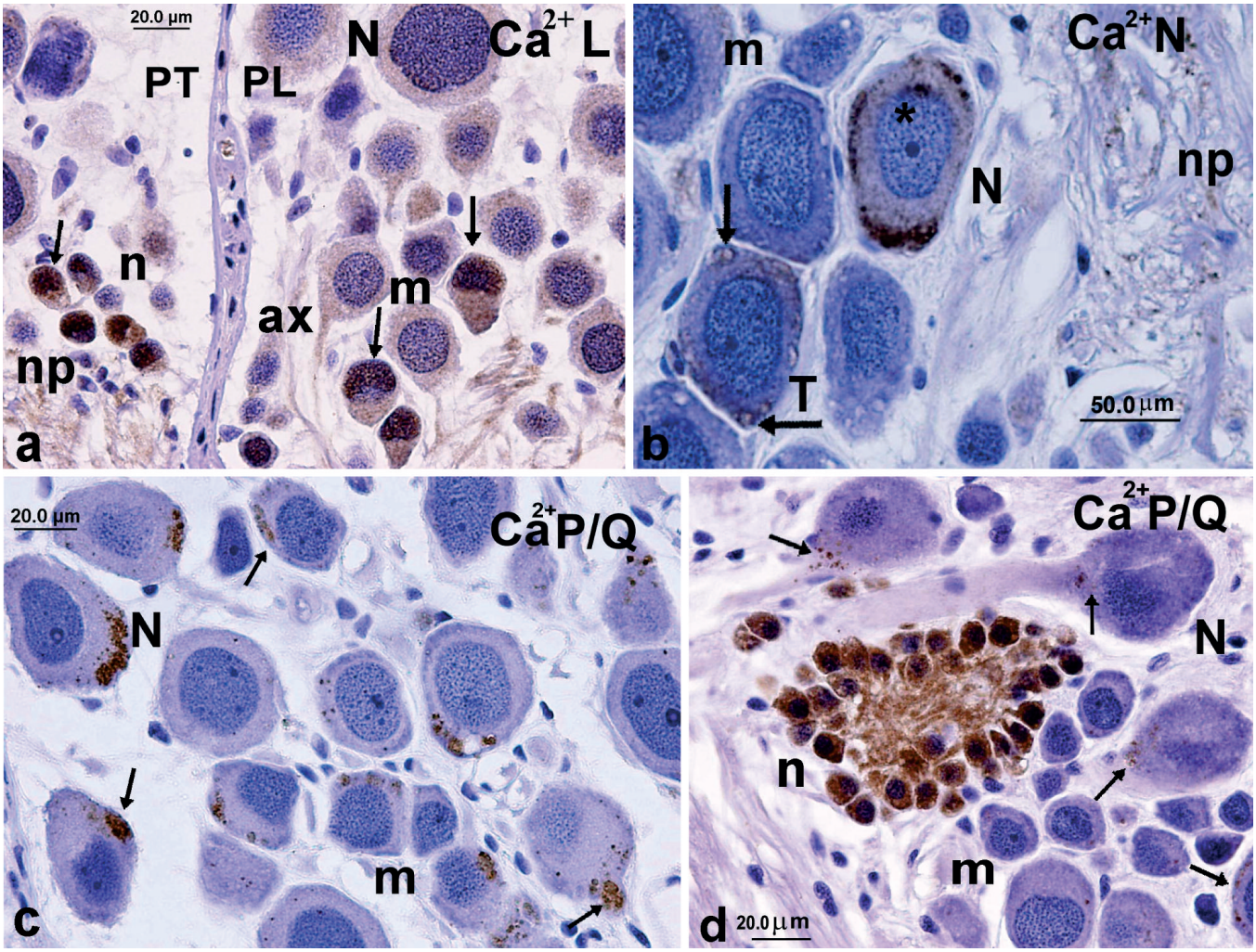

Fig. 3. Immunocytochemistry staining for calcium channels antisera: **a.** L-channels type reaction is observed diffusely spreading in the cytoplasm of big (N) and medium (m) size neurons while small neurons (n), axonal cone and axons are more densely stained. The nucleoplasm is stained in small neurons (section of the posterior right parietal (PT) and anterior pleural (PL) ganglions separated by connective tissue, medial-portions). **b, c.** The reaction for N- (section of the anterior right parietal ganglion, left medial-portion) and P/Q- channels type (section of the anterior right pleural ganglion, medial-portion) is observed as a dense brown precipitate concentrated in discrete portions near the membrane in large (N) and medium (m) size neurons, in structures similar to trophospongia (T), in the axonal cone and in fibres of the neuropil (np) (*neuron F26, Fig. 5d). **d.** Clusters of small neurons (n) densely stained are shown between medium (m) and large neurons (N) (section of the posterior right parietal ganglion, dorsal-portion).

section where the colocalization study has been made for the: Na^+ (Fig.5a); K^+ (Fig. 5b); SK2 (Fig. 5c); BKCa and Ca^{2+} -N-, P/Q-, and L-types (Fig. 5d-f) antisera. Being the anterior portion of the ganglia, these tissue sections are superficially localized, so that we have been able to identify several mapped neurons; the ones denominated as F1, F2, F34, F53, F54 and F77, which have been the object of our electrophysiology recordings. Two groups of small neurons called n1 and n2 have also been identified. It is observed that neuron F1 gives immunoreactive-like staining for all the tested channels antisera; neurons F2 and F34 give positive reaction for Na^+, K^+, SK2 and Ca^{2+}-L antisera. Neurons F53 and F54 show immunostaining for Na^+, K^+, SK2, BKCa and Ca^{2+}-L antisera. Neuron F26 is positive for Na^+, K^+, Ca^{2+}-N and Ca^{2+}-L antisera. In the group of neurons n1 the positive results are for Na^+ and K^+ antisera, and for the group of neurons n2 we observe reaction for Na^+, K^+ and Ca^{2+} N antisera.

Discussion

Among the groups of invertebrates it has been described that there are strong differences with respect to the kind of ionic protein channels expressed in neurons, mollusca being the group with a wider expression of protein channels. They have been studied in great detail with respect to their physiological properties, the conclusion obtained being that molluscs are able to support sophisticated sensory discrimination, learning, communication and social behavior (Hille, 1992).

Na^+ channels are essential for the firing of axon propagated action potentials in all vertebrate and invertebrate animals. Although less diverse than K^+

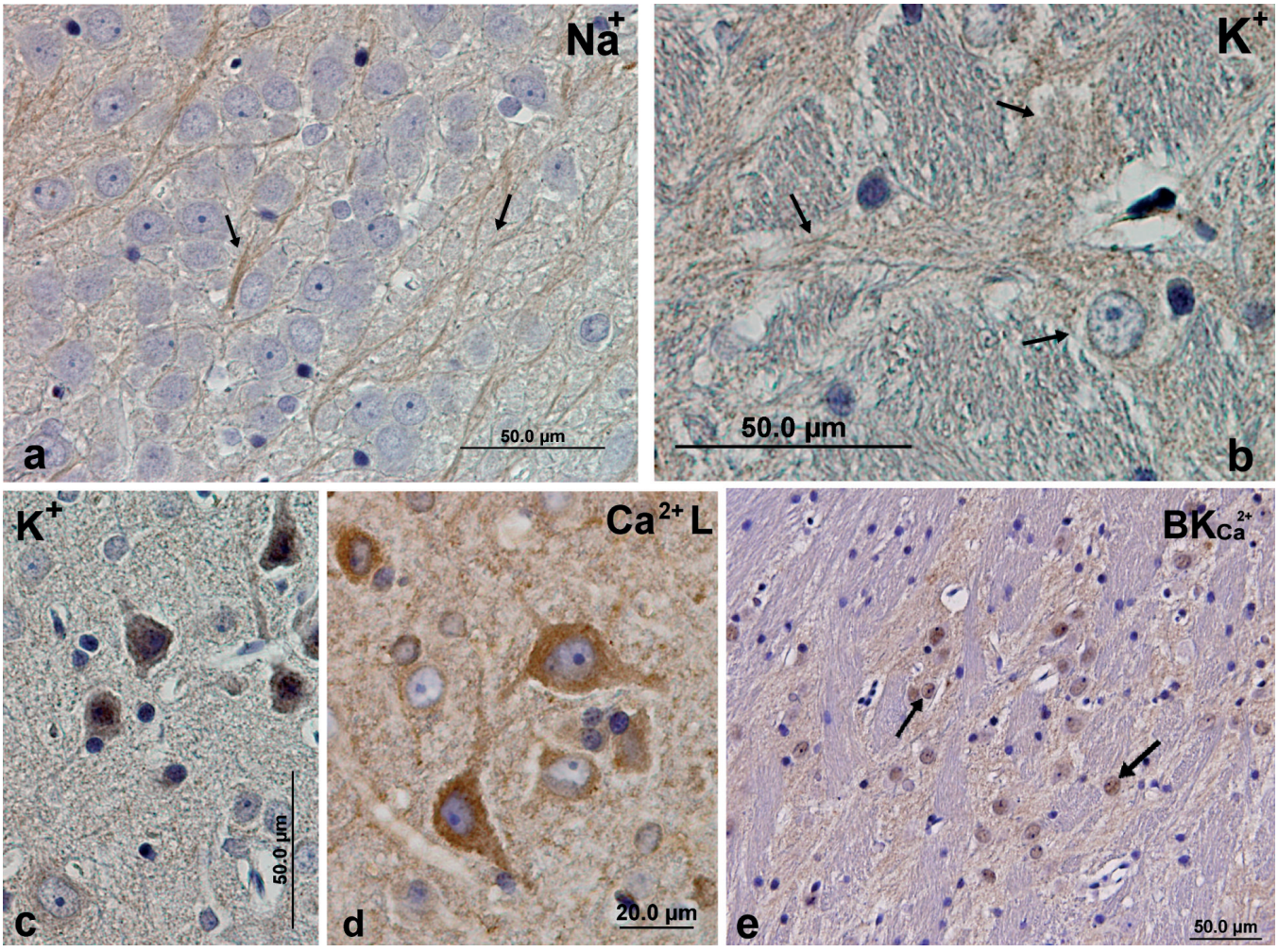

Fig. 4. Positive control made in adult Wistar rat CNS. **a.** Positive reaction for Na^+- channels antiserum is observed in axons of the area CA3 of Ammon's horn. **b, c.** K^+- channels immunostaining is observed in axons of the basal Meynert nucleus **(b)** and in pyramidal neurons of the parietal cortex **(c)**. **d.** Positive reaction for Ca^{2+} L- channels antiserum is observed in neurons and astrocytes of the parietal cortex. **e.** A positive reaction for BKCa- channels antiserum is observed in fibres and in the nucleoplasm of neurons of the reticular thalamic nucleus.

channels, a neuron can simultaneously express several subtypes of voltage-gated Na^+ channels (Barchi, 1988). *Helix* neurons soma has the capability of generating propagated spikes of large amplitude. This aspect is coherent with the wide distribution of voltage-gated Na^+-channels (87.4%) and delayed rectifier K^+-channels (92.5%) like immunostaining in the soma of large size *Helix* neurons (Table 2), which are best characterized by their bioelectric activity. These morphological data are of substantial interest and show that both kinds of channels are co-localized. The percentage of cells expressing Na^+ and K^+ channels is very close; a small difference of about 5.1% is in good agreement with their functional properties for the generation and propagation of bioelectric spikes.

For the eleven antisera tested in the present work, we

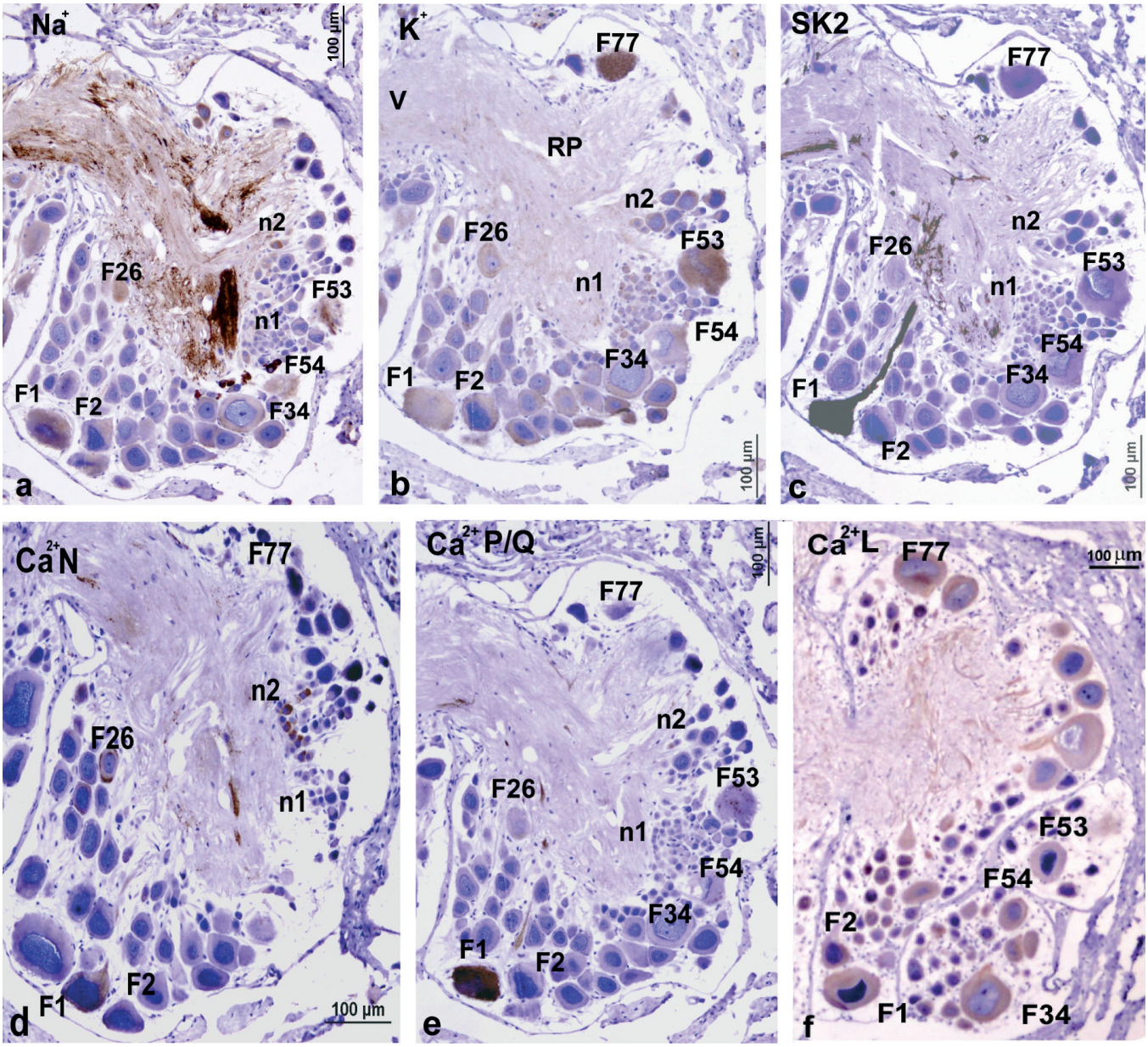

Fig. 5. Colocalization of ionic channels-like immunostaining in serial tissue sections of the anterior right parietal ganglion, a panoramic view. **a.** Na^+-antiserum; **b.** K^+- antiserum; **c.** SK2- antiserum; **d.** Ca^{2+} N- antiserum; **e.** Ca^{2+} P/Q- antiserum; **f.** Ca^{2+} L- antiserum. F1 neuron shows immunostaining for all the tested channels antisera. Neurons F2, F34, F53 and F54 show positive reaction for Na^+, K^+, SK2 and Ca^{2+}-L antisera. Neuron F26 gives a positive reaction for Na^+, K^+, Ca^{2+}-N and Ca^{2+}-L antisera. In the group of neurons n1 the positive results have been for Na^+ and K^+ antisera, and for the group of neurons n2 we have observed reaction for Na^+, K^+ and Ca^{2+} N antisera.

have not observed a positive reaction either in plasmatic or protoplasmic periphery glia and in fibrous glia associated with axonal tracts of the neuropil. We have observed ultrastructural characteristics that define well the molluscs glia (Calvo et al., 2003), also observing a lack of reactivity for glio fibrilar acidic protein immunostaining (Azanza et al., 2007a). Therefore, our current data are a firm support for the structural differences between the glia of molluscs and that of mammals (Radojcic and Pentreath, 1979; Randsom, 1995), which in turn explain our present results.

There is a wide variety of K^+ channels: each excitable membrane uses a different mixing of various K^+-channels to fulfil its need, so that several types are co-expressed in a certain neuron membrane. Most K^+ channels are activated only after the membrane becomes depolarized, but some are activated when the membrane is hyperpolarized. Some channels open rapidly and some slowly. Some ones are strongly modulated by neurotransmitters or by intracellular messengers like Ca^{2+} ions, therefore many cellular functions depend upon changes in the free cytosol Ca^{2+} ionic concentration in response to different stimuli, as we have demonstrated under applied magnetic field (Azanza, 1989, 1993; Azanza and del Moral, 1994). By using the K^+-channels, neurons regulate pacemaker potentials, generate spontaneous trains and bursts, show long plateaus in the action potential, or regulate the overall excitability of the cell. The modification of K^+-channels activation can induce subtle modifications of the neuron firing spontaneous frequency and activity pattern. In turn the biological information transmitted to subsequent group of neurons within a network could be modulated. All these aspects are in good agreement with the high percentage of K^+-channels expressed by *Helix* neurons (Table 2).

The intracellular calcium dependent-K^+-channels are divided into two groups according with their relative conductance: BK of high conductance (280 pS) and SK of small conductance (11 pS). SK channels are highly sensitive to cytosolic fluctuations of calcium ion concentration, being activated with a lower intracellular free Ca^{2+} ion concentration than for BK channels. Furthermore, SK channels remain open several hundred of ms. after an action potential has been induced. BK channels are sensitive to TEA whereas SK channels induced hyperpolarizations are controlled by intracellular calcium concentrations and are bloked by d-tubocurarine. We have studied two kinds of BK channels: BKCa and charybdotoxin-sensitive BK channel (HLK3 channels) and two kinds of SK channels: SK2 and SK3. The observed relative percentages for positive large size neurons immunoreactions (SK3 (94.7%), SK2 (85.0%), BKCa (74.8%) and HLK3 (74.8%)), are in good correlation with our results. We have shown with our electrophysiology recordings that the neurons frequency decrease under applied MF is mediated by calcium ions, through Ca^{2+}-dependent- K^+-channels sensitive to d-tubocurarine, but TEA-sensitive-Ca^{2+}-dependent-K^+-channels were not involved. It is of considerable interest to mention that out of the 82% of neurons which show magnetosensitivity, 55% of studied neurons submitted to MF show this kind of response (Azanza and del Moral, 1988; Azanza, 1990).

Regarding the hyperpolarization-activated potassium channels, Khy (25.6%), they are considered to play an important role in the determination of the resting potential in neurons, since their modulation by neurotransmitters induces variations in the membrane potential. Together with the G-protein-activated potassium channels, KG (81.8 %), they require the previous activation of a G-protein by the binding of a neurotransmitter with its receptor, so that they have been considered nucleotide-gated potassium channels (Tritsch et al., 1998). However we have not tested the activity of these two kinds of channels.

Alongside the central implication of Ca^{2+} ions in the cell-signalling pathways, various mechanisms have evolved for Ca^{2+} mobilization. Voltage-gated Ca^{2+} channels and receptor-mediated Ca^{2+} channels control the two main mechanisms for the increase of cytosolic Ca^{2+} concentration. Or in this other way: the displacement of Ca^{2+} ions from the extracellular space into the cell through plasma membrane, and the release of Ca^{2+} ions from their intracellular storage sites. The types of channels we have studied are located in the plasma membrane, i.e. L- and P/Q-channels of high-threshold, and N-channels of high-threshold and intermediate conductance (Carbone and Lux, 1984; Hess et al., 1984; Nowycky et al., 1985; Tsien et al., 1988). The L-type Ca^{2+} channel seems to be the most common in mammals, and we have also observed that in *Helix* neurons about 100 % of large and medium size neurons give positive immunostainig reaction. In mammals, they are mainly expressed in the soma and proximal dendrites, and it has been considered that Ca^{2+} ions entering through L-channels could work as a link between the surface processes (bioelectric activity) with diverse calcium-dependent cell functions. The high percentage of positive neurons for Ca^{2+} L-channels immunoreaction is in good correspondence with our electrophysiology results. By blocking with verapamil we observed the temporal inhibition of the firing activity, showing that in many *Helix* neurons the membrane activation is simultaneously carried by the entrance of Na^+ plus Ca^{2+} ions (Azanza, 1989). The observed results for L-channels (100%) together with the high percentage of positive reactions for N- (100%) and P/Q- (95.6%) channels-like immunostaining are in favour of the implication of calcium ions in the natural bioelectric activity of *Helix* neurons, their function as second messengers and in the responses elicited by applied MF, as widely shown in our experiments (Azanza, 1989, 1990; Azanza and del Moral, 1994). Also, the results of co-localization of ionic channels for mapped neurons F1, F53 and F2 have been a good approach for establishing a correlation of neuron neurochemistry with the effects induced by applied

magnetic fields as observed in our experiments (Azanza et al., 2007b).

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