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EXCITATORY NEUROTRANSMITTERS IN THE TENTACLE FLEXOR MUSCLES RESPONSIBLE FOR SPACE POSITIONING OF THE SNAIL OLFACTORY ORGAN

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Abstract. Recently, three novel flexor muscles (M1, M2 and M3) in the posterior tentacles of the snail have been described, which are responsible for the patterned movements of the tentacles of the snail, *Helix pomatia*. In this study, we have demonstrated that the muscles received a complex innervation pattern via the peritentacular and olfactory nerves originating from different clusters of motoneurons of the cerebral ganglia. The innervating axons displayed a number of varicosities and established neuromuscular contacts of different ultrastructural forms. Contractions evoked by nerve stimulation could be mimicked by external acetylcholine (ACh) and glutamate (Glu), suggesting that ACh and Glu are excitatory transmitters at the neuromuscular contacts. Choline acetyltransferase and vesicular glutamate transporter immunolabeled axons innervating flexor muscles were demonstrated by immunohistochemistry and in Western blot experiments. Nerve- and transmitter-evoked contractions were similarly attenuated by cholinergic and glutamatergic antagonists supporting the dual excitatory innervation. Dopamine (DA, 10^{-5} M) oppositely modulated thin (M1/M2) and thick (M3) muscle

responses evoked by stimulation of the olfactory nerve, decreasing the contractions of the M1/M2 and increasing those of M3. In both cases, the modulation site was presynaptic. Serotonin (5-HT) at high concentration (10^{-5} M) increased the amplitude of both the nerve- and the ACh-evoked contractions in all muscles. The relaxation rate was facilitated suggesting pre- and postsynaptic site of action. Our data provided evidence for a DAergic and 5-HTergic modulation of cholinergic nerves innervating flexor muscles of the tentacles as well as the muscles itself. These effects of DA and 5-HT may contribute to the regulation of sophisticated movements of tentacle muscles lacking inhibitory innervation.

Keywords:

Helix pomatia, olfaction, tentacular muscle, acetylcholine, glutamate, dopamine, serotonin, modulation.

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Introduction

Olfaction in terrestrial snails and slugs is an essential sensory modality and plays a crucial role in orientation and foraging. In Stylommatophora snails, including *Helix pomatia*, the paired posterior tentacles house the main olfactory organ, which is involved principally in localizing distant food source and conspecifics (Chase 2002; Chase et al. 1978; Gelperin 1974; Friedrich and Teyke 1998; Chase and Croll 1981). The posterior tentacles are innervated by the olfactory (on), the external (ePt) and internal peritentacular (iPt) nerves (Peschel et al. 1996; Kerkut et al. 1975). During the exploration of the chemical environment, tentacles display different types of movements such as scanning the environment in three dimensions, quivering and twitching (Lemaire and Chase 1998; Peschel et al. 1996; Nikitin et al. 2005; Friedrich and Teyke 1998). In the course of scanning, the protracted tentacles are moved in different directions around a basal pivot by three tentacle flexor muscles (M1, M2 and M3) described recently (Krajcs et al. 2012; Hernadi and Teyke 2012). The three very thin string-like flexor muscles, present in both posterior tentacles, extend along the entire length of the tentacle from the tip to the base where they are anchored at different sites; thus, their one-by-one contraction results in movements in different directions of the space (Hernádi et al. 2013; Hernadi and Teyke 2012). Twitch is a quick and brief retraction of the tentacle tip, and quivering is a fast external lateral movement of the posterior tentacle tips which is not accompanied by retraction. Each flexor muscle and the tegumental musculature, however, receive both distinct (via the iPt and the ePt nerves) and common innervation (via the olfactory nerve). Therefore, when the odor induces distinct motor output via Pt nerve and it evokes contraction of a flexor muscle, it also induces contraction of a given stripe of the tegumental musculature. The patterned tentacle movements serve to bring the olfactory receptors in an appropriate position maintaining the perception of increasing odor

concentration, which then help the animal explore the source of the odor by adjusting the direction of locomotion. In addition, it has been suggested that quivering enhances the olfactory performance by improving the access of new odor molecules to the receptors meanwhile removing the old ones (Lemaire and Chase 1998; Moore et al. 1991). Until recently, it has been assumed that the motor pathway for quivering and twitching is the same (Lemaire and Chase 1998; Moore et al. 1991) as for tentacle bending during orientation towards the learned food odor (Zakharov 1992; Peschel et al. 1996). Bending and retraction of the tentacle is, however, always accompanied by shortening of the tentacles, which is performed by contraction of the tentacle retractor muscle (TRM) and by interwoven muscular net of the tegument (Rogers 1968). Scanning movements of the protracted tentacles around the basal pivot, twitching and quivering of the tentacles, however, cannot only be explained by the contraction of the TRM and tegumental muscles alone, because during these movements, neither the diameter nor the length of the tentacles is changed, which is otherwise the prerequisite for organs possessing a hydrostatic skeleton (Kier 1992). It has been suggested that the scanning of the environment by protracted tentacles, twitching and quivering movements performed during olfactory orientation are primarily due to the contraction of flexor muscles (M1, M2 and M3) (Krajcs et al. 2012). Bending of the tentacle is more complex and is due to the concerted movement of the string and tegumental muscles, TRM and body wall muscles (Krajcs et al. 2012; Hernadi and Teyke 2012). Applying anterograde neurobiotin tracing via the olfactory and Pt nerves, the innervation pattern of nerves belonging to the different flexor muscles (M1, M2 and M3) was described. Applying parallel retrograde Co/Ni lysine staining via the above nerves, the location of cerebral neurons innervating the flexor and tegumental muscles was also determined (Hernádi et al. 2013). Based on these data, it is suggested that the M1, M2 and M3 muscles are innervated by efferent neurons concentrated in eight groups in the cerebral ganglion (CG). However, these neurons also

innervate distal part of the TRM and the whole tegumentum of the tentacles (Rogers 1968; Peschel et al. 1996). The anatomical basis of neuronal regulation and coordination of the tentacle muscles is described, very little is known about the chemical nature of the neural control of these muscles. Our preliminary study has shown that acetylcholine (ACh) was able to induce contraction of denervated flexor muscles (Krajcs et al. 2012). Therefore, the present study aims both at the detailed physiological and pharmacological analysis of the excitatory innervation and immunocytochemical demonstration of transmitter-containing elements in the flexor muscles of *Helix pomatia*. In addition, the effects of several signal molecules on the muscle contraction were investigated in order to identify the putative neurotransmitter candidate or candidates possibly involved in the regulation of the flexor muscles. Also, the ultrastructural background of the flexor muscle innervation at the level of neuromuscular contacts has been analyzed. Finally, we investigated the modulatory effects of two monoamines, serotonin (5-HT) and dopamine (DA), on the electrically and ACh-evoked contractions. Using these approaches, we wanted to obtain an insight into the physiology of the muscle system, which plays an important role in the space positioning of the main olfactory organs of the snail.

Materials and methods

Recording of denervated and innervated muscle contraction

Adult specimens of the pulmonate snail, *Helix pomatia*, collected locally were used for experiments. In isometric experiments, both ends of the muscles were fixed in a perfusion chamber. One end of the muscle was hung up by a hook attached to a force transducer (WPI, FORT 1000), and the other end was fixed at the bottom of the chamber. In isometric recording regime, both the time course and the amplitude were analyzed. In isotonic experiments, one end

of a single muscle was fixed and the other end was free; the change in muscle length was measured by an ocular micrometer. In isotonic recording regime, only the size of contraction was measured. In both recording configuration for the electrical stimulation of innervated muscles, a pair of silver hooked electrodes was placed under the olfactory nerve. The ganglion was placed in a pit of the recording chamber and separated by a Vaseline gap from the muscle. Electrical pulses of 10 ms, 5-10 V and 1-1.5 Hz were applied. The geometry of the perfusion chamber enabled a fast solution exchange. The scheme of the device constructed for the isotonic contraction measurements was published earlier (Krajcs et al. 2012). Drugs, ACh, glutamate (Glu), 5-HT, histamine (His), glycine (Gly) d-tubocurarine (d-TC), mytolon, succinylcholine chloride (SCh) and 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX, all purchased from Sigma, Budapest, Hungary), were applied in an appropriate concentration to the organ bath in order to obtain the desired concentration. Drugs were dissolved in the physiological solution (or first in DMSO, when it was necessary) containing 80 mM NaCl, 4 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$ and 10 mM Tris-HCL (pH = 7.4) and were applied onto the muscle through a gravity perfusion system.

Anterograde neurobiotin tracing

Posterior tentacles with the flexor muscles and the CNS interconnected via the olfactory nerve were dissected and pinned out in Sylgard-coated plates containing *Helix* saline. The olfactory nerve was cut and placed in a Vaseline cup containing 5 % neurobiotin (Vector Laboratories, Burlingame, USA) diluted in distilled water. The cup was sealed with Vaseline; thus, anterograde tracing via the distal segment of the selected nerve was excluded. The preparation was covered with saline and kept at room temperature for one day. The preparations were then fixed in 4 % paraformaldehyde (Reanal, Budapest, Hungary) diluted in 0.1 M phosphate buffer (PB, pH 7.4)

for 6 h at 4 °C. The neurobiotin-labeled axons were visualized in whole-mount preparations by applying avidine-conjugated Alexa-fluor 488 (Molecular Probes, London, England) diluted 1:1,000 in phosphate-buffered saline containing 0.25 % Triton-X (PBS-TX) for 1 h at room temperature. After washing in PBS-TX, the samples were mounted in PBS-glycerol (2:1) and viewed under a fluorescence microscope equipped the appropriate filters.

Immunohistochemistry

Choline acetyltransferase (ChAT) immunostaining

The CNS as well as the posterior tentacles with the flexor muscles were dissected and fixed in 4 % paraformaldehyde diluted in 0.1 M PB (pH 7.4) for 6 h at 4 °C. The CG were separated from the CNS, and the flexor muscles were isolated from the tentacles. Immunohistochemical procedure was carried out on 40 µm thick cryostat sections made from the CG and the posterior tentacles as well as on whole-mount preparations of the tentacle flexor muscles. Both the sections and the whole mount preparations were incubated 24 h at room temperature with a polyclonal anti-central type of ChAT (cChAT) antibody raised in rabbit (donation by H. Kimura) diluted 1:10,000 in PBS-TX containing 0.25 % bovine serum albumin. After several short wash in PBS-TX, the immunoreaction was visualized using the polymer-HRP-conjugated donkey anti-rabbit secondary antibody (One-step Polymer-HRP IHC Detection System, BioGenex, USA). Specificity of the antibody was tested in absorption control when the antibody was preabsorbed with the control peptide of the antibody (Abcam, Cambridge, UK). Method control was performed by omitting the primary antibody from the incubation solution. No immunolabeling could be observed after these experiments.

Vesicular glutamate transporter (VGlut) immunostaining

Immunostaining was performed on PFA-fixed flexor muscles incubating them with the mixture of VGlut1, VGlut2 and VGlut3 antibodies raised in guinea pig (polyclonal, Millipore-Chemicon) diluted in 1:3,000 in PBS-TX-BSA for 24 h at room temperature. After several short wash in PBS-TX, the samples were incubated with biotinylated secondary antibody (donkey anti-guinea pig, Vector Laboratories, Burlingame, USA) diluted in 1:200 in PBS-TX-BSA for 4 h at room temperature; thereafter, the immunoreaction was visualized using fluorescent-conjugated streptavidin (Alexa-Fluor 488, Molecular Probes) diluted in 1:1,000 in PBS-TX.
Method control was performed by omitting the primary antibody from the incubation solution. No immunostaining could be observed after these experiments.

Western blot

Cerebral ganglia were homogenized in cold 50 mM Tris-HCl buffer (pH 7.4) containing protease inhibitor cocktail (Sigma), centrifuged at 12,000×*g* at 4 °C for 20 min, and the supernatant was collected. After boiling, 200 mM iodoacetamide (Sigma) was added to the sample and incubated for 30 min at room temperature in order to prevent dimer formation. A total of 5 µl of molecular weight marker (ProSieve Quadcolor Protein Markers, Lonza, Basel, Switzerland) and 10 µl aliquots of supernatant were processed on a 10 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and thereafter blotted onto nitrocellulose or PVDF Immobilon-P membrane (Millipore, Billerica, USA). Membranes were blocked by 5 % BSA at room temperature and thereafter incubated with anti-cChAT antibody (1:10,000) or the antibody and its control peptide with 1 % normal goat serum overnight at 4 ºC. After incubation with HRP-conjugated goat anti-rabbit antibody (1:10,000, Sigma), the primary antibody labeled bands were visualized with ECL reagent (Pierce, Rockford, USA).

Electron microscopy

The tentacles were pinned out and covered with a fixative containing a mixture of 4 % paraformaldehyde and 0.1 % glutaraldehyde diluted in 0.1 M PB. Fixation lasted overnight at 4 ^{o}C. The M1, M2 and M3 flexor muscles attached to a small piece of the tentacle were cut off from the rest of the tentacle, postfixed for 1 h at 4 ^{o}C in 1 % OsO_4 diluted in 0.1 M cacodylate buffer, dehydrated in graded ethanol and propylene oxide and embedded in Araldite (Durcupan ACM, Fluka, Buchs, Switzerland). In the course of dehydration block, staining was performed in 70 % ethanol saturated with uranylacetate. After polymerization, 1 μm semi-thin sections were cut, stained with 1 % toluidine blue and used for orientation. For ultrastructural investigations, 50-60 nm ultra-thin sections were taken, stained with lead citrate and viewed in a JEOL 1200EX electron microscope.

Statistical analysis

Comparisons were made by one-sample *t* test. Differences were considered significant at $p < 0.05$.

Results

Neuromuscular contacts and innervation

Ultrastructurally neuro-muscular contacts (NMC) characteristic for molluscan muscles were observed. They were established either between axon varicosities and sarcoplasmic processes (Fig. 1a and 1a') protruding from the contractile part of the muscle fibers, or the varicosities were

embedded in the distal region of the sarcoplasm near the nucleus. At the active region of the NMC parallel running pre- and postsynaptic membranes displayed increased electron density and at the presynaptic (neuronal) membrane segment vesicle clustering was seen, suggesting possible sites of cell-to-cell signaling (Fig. 1b, 1b'). Two types of presynaptic varicosities could be distinguished based on their vesicle and granule content at higher magnification. One type of the axon profiles contacting the muscle fibers contained a large number of clear synaptic vesicles, intermingled with a few clear dense-core vesicles (Fig. 1a'). The other types of varicosities contained clear synaptic vesicles mixed with a large number of electron-dense granules (Fig. 1b'). Tentacle flexor muscles M1, M2 and M3 are innervated by a dense net of neurobiotin-labeled axons, the cell bodies of which are located in the CG (Hernádi et al. 2013). Two or three relatively thick nerve bundles were observed running along the M1 (Fig. 1c) and M3 (Fig. 1d) muscles giving extensive side branches suggesting a polyneuronal innervation pattern. At larger magnification, numerous varicosities can be recognized in both muscles (Fig. 1c' and 1d').

Effect of putative transmitters on isolated muscle

Putative neurotransmitters such as ACh, Glu, Gly, His, 5-HT and DA were tested whether they elicit muscle contraction. At 10^{-4} M concentration, the ACh, Glu, Gly, His and DA were able to elicit consistently an increase in the tension of the denervated muscle (Fig. 2a). 5-HT appeared to have a biphasic effect depending on the applied concentration; at low (10^{-8} to 10^{-6} M) concentrations, 5-HT elicited contraction whereas at high (10^{-6} to 10^{-4} M) concentrations, it relaxed the muscle. The ACh-evoked tonic response was long lasting followed by gradual relaxation, while Glu-elicited contraction terminated faster. The wash-out of the transmitter effect was not immediate, and some preparations needed several minutes to return to the resting state. The peak tension elicited by DA, Gly, His and 5-HT, however, was significantly smaller than that

in response to ACh and Glu at the same concentration. The column diagram summarizes the mean amplitude and integral values of the contractions elicited by putative transmitters (Fig. 2a') and calculated from the records as shown in Fig. 2a.

Experiments were also carried out to see whether there was a similarity between the effects of electric nerve stimulation and externally applied ACh or Glu on the muscle response (Fig. 2b). The nerve-muscle preparation consisted of the olfactory nerve and the M3 flexor muscle. The mean muscle length induced by stimulation of the nerve was 86.2 ± 4.2 % of the control (100 %), which corresponded well to the shortening elicited by ACh (83.6 ± 2.5 %) or Glu (80.5 ± 3.7 %), respectively. Contractions evoked either by the stimulation of one of the innervating nerves or by externally applied 10^{-5} M ACh were effectively attenuated by cholinergic antagonists (mytolon, SCh and d-TC) applied at a concentration of 10^{-4} M, suggesting that one of the main excitatory neurotransmitter released from the olfactory nerve endings was ACh. Contractions evoked by the nerve stimulation or by externally applied 10^{-5} M Glu were significantly decreased by 10^{-4} M CNQX (potent AMPA/kainite receptor antagonist, Fig. 2b') suggesting that Glu could be additional excitatory transmitter.

The dose-response curves of ACh and Glu revealed that evoked contractions were concentration dependent (Fig. 2c). The threshold for the effect of ACh was between 10^{-8} and 10^{-7} M, while the maximum response was observed at 10^{-4} to 10^{-3} M concentrations. The effective concentration of the ACh inducing contraction was sufficiently low to support that ACh acted as neurotransmitter. The EC_{50} and Hill-slope values determined from the concentration response curve were 7 x 10^{-6} M and 0.55, respectively. The threshold concentration of Glu was between 10^{-8} and 5 x 10^{-8} M, and peaked at 10^{-4} M. The EC_{50} and Hill-slope values determined from the concentration response curve were 8 x 10^{-7} M and 0.45, respectively. The Hill-numbers revealed 1:1

stoichiometry, suggesting that the binding of both putative transmitter molecules to their membrane receptors was competitive.

The tentacular flexor muscles M1, M2 and M3 occasionally revealed spontaneous activity regardless whether they were innervated or denervated. Both the spontaneous contractions and those evoked by the stimulation of the innervating nerves (on, iPtn, ePtn) could reversibly be blocked or significantly decreased when the preparation was bathed in 20 mM Ca^{2+}/20 mM Mg^{2+} solution. The high Ca^{2+}/high Mg^{2+} solution raised the threshold of the chemical responses of the postsynaptic membrane; consequently, the same electrical stimulus elicited smaller contraction (Fig. 2c'). Washing the preparation with physiological saline of normal ion content the recovery was complete.

Demonstration and identification of neurotransmitters in the flexor muscles

The nature of the excitatory transmitters in the flexor muscles of the tentacles was further verified by using antibodies raised against the central type of ChAT and the different forms of VGlut. VGlut immunoreactive fibers could be shown in each flexor muscles. VGlut immunolabeled fiber trunks are running along the longitudinal axis of the muscle. The thick labeled axons in the trunks give off fine varicose side-branches, which are well visible on the surfaces of the muscles. (Fig. 3a, b).

cChAT immunoreactivity could be detected in bundles of axonal processes in the tentacle muscles, forming a longitudinal arrangement along the muscle (Fig. 3c, d). The anti-cChAT antiserum also detected immunolabeled cells and processes in the CG (Fig. 3e). Further our attention was focused on the presence and the effects of ACh. The presence of cChAT was further supported by Western blot experiments. Western blot experiments using nitrocellulose

(Fig. 4, lanes 1 and 2) and PVDF membrane (Fig. 4, lanes 3, 4) revealed that cChAT occurs as a mixture of monomers and dimers, since the anti-cChAT antibody made a clear reaction at the 130, 68 and 48 kDa bands in brain homogenate (lanes 1 and 3). The 68 kDa corresponds to the molecular weight of cChAT, while 48 kDa corresponds to the molecular weight of the peripheral type of ChAT. The 130 kDa band may correspond to the dimer form of cChAT. The bands were blocked or became notably lighter when the antibody was preabsorbed with the control peptide (lanes 2 and 4). Addition of the alkylating agent iodoacetamide increased the amount of the monomer form (68 kDa band on lane 3). All these data supported that ChAT was present in axons that innervate the flexor muscles.

Effect of dopamine and serotonin on muscle contractions

Modulators tested were those, namely 5-HT and DA that proved to be physiologically relevant (see Fig. 2). Since 5-HT and DA were less effective in eliciting contractile responses of denervated muscles, we have characterized their effects on parameters of nerve- and ACh-elicited muscle contractions such as the amplitude and integral of the contraction, and the rate of relaxation. Selective application of 10^{-6} M DA over the tentacle muscles M1/M2 and M3 revealed that the muscles were differentially modulated by the monoamine. DA applied at 10^{-6} M had no effect on the muscle tone; however, the nerve-evoked contractions of the M1/M2 muscles were attenuated (Fig. 5a). Both the integrals and the amplitudes of nerve-evoked and ACh-elicited contractions decreased in the presence of DA (Fig. 5c). In the presence of 10^{-6} M DA, both the integral and amplitude of the contractions of the M3 elicited either by stimulation of the olfactory nerve or by external application of 10^{-4} M ACh was increased (Fig. 5b and 5c). Comparing normalized contractions elicited by either nerve stimulation (Fig. 5a, insert) or by externally

applied ACh (Fig. 5b, insert), no change was observed in the relaxing phase, suggesting a presynaptic modulation**.**

The biphasic change in the muscle tone elicited by different 5-HT concentrations suggests that there are two different 5-HT receptors on the muscle or that 5-HT acts at both pre- and postsynaptic sites. Indeed 5-HT at low concentrations (10^{-8} to 10^{-6} M) increased the muscle tone, whereas at high concentrations (10^{-6} to 10^{-4} M) decreased it (Fig. 2). It was demonstrated that 10^{-5} M 5-HT decreased the resting tone of both M1/M2 and M3 muscles, and on this background, the electrical impulse of the similar parameters elicited higher amplitude contraction (Fig. 6a, 6b). In M1/M2 and M3 muscles, the amplitude of the nerve-evoked contractions increased or remained unchanged after 5-HT treatment (Fig. 6a, 6b); meanwhile, the integrals of the contractions decreased or remained unchanged (Fig. 6c) suggesting that the relaxation phase was accelerated (insert on Fig. 6a and 6b). All these changes refer to a possible postsynaptic effect of the 5-HT. The postsynaptic effect of 5-HT at concentrations higher than 10^{-6} M is demonstrated in Fig. 7a, where contraction was evoked by 90 mM KCl (control trace = thin line). During the next KCl-evoked contraction, 5-HT was added at the time shown by arrow (thick line). The direct effect of 5-HT was relaxation on the muscle. When modulation was examined at low 5-HT concentration (10^{-8} M), a slightly different effect was obtained on M3 muscles. The nerve-evoked contractions were modulated similarly to that observed at high 5-HT concentration, i.e., the amplitude of the nerve-evoked contraction remained unchanged and the integral decreased. The amplitude of the ACh-elicited contractions, however, were decreased (Fig. 7b), the relaxation phase remained unchanged suggesting both pre- and postsynaptic modulation by 5-HT.

Discussion

The innervation pattern and possible excitatory transmitters of the flexor muscles

Superior tentacles are supplied by three, recently described flexor muscles the M1, M2 and M3 (Hernadi and Teyke 2012). The muscles are attached to the tip of tentacle in a way that they allow special movements such as scanning, quivering and twitching. Retrograde Ni/Co staining through olfactory, iPt and ePt nerves revealed several groups of neurons in the CG innervating muscles of the tentacles (Hernádi et al. 2013). According to our present findings, the flexor muscles were shown to receive rich innervation by axons of cerebral motoneurons branching extensively along the muscle fibers. The transmitter release can occur at the NMC displaying moderate membrane specialization as it was demonstrated in the muscles at ultrastructural level. Two types of NMC were observed: one contained almost exclusively clear synaptic vesicles, while the other contained mixed, clear and dense vesicles alike raising the possibility of the colocalization of the transmitter and modulator substances. Such NM contacts are observed in different muscle types of *Helix* species (Elekes and Ude 1994; Elekes 2000; Heyer and Kater 1973; Rogers 1969) and the columellar muscle of *Helisoma trivolvis* (Heyer and Kater 1973; Rogers 1969; Sherman et al. 1976). Based on our morphological (neurobiotin tracing and immunolabeling experiments) and physiological data, the innervation is suggested to be polyneuronal. Polyneuronal innervation is important when muscle fibers do not generate propagated action potentials and several motoneurons are necessary to induce full mechanical response through local depolarization. The external stimulus recruits more and more motoneurons which are stimulus intensity dependent. Another explanation could be that by polyneuronal innervation, the release of more than one excitatory transmitter is possible which may contribute to the sophisticated regulation of flexor muscles. Our data suggested that is the situation, and the flexor muscles have a dual excitatory innervation. The excitatory transmitters

are ACh and Glu because they elicited a contraction of the flexor muscles already at 10^{-8} M threshold concentration. The presence of dual excitatory transmitters in regulating muscle contraction is not unique in mollusks because ACh and Glu proved to be excitatory transmitters at several different neuromuscular synapses in gastropods (Yoshida and Kobayashi 1991; Zoran et al. 1989; Kobayashi et al. 1981). Although DA and 5-HT also elicited contraction and/or relaxation of the M1, M2 and M3 muscles, the peak tension in response to these compounds was significantly smaller than that in response to ACh or Glu at the same concentration. The shape (saturating curves) and the shift of the dose-response curve suggest that ACh and Glu exerted their effect at different receptors located at the postsynaptic site with different EC_{50}. Our implication that a part of the excitatory inputs in the tentacle flexor muscles is cholinergic, and/or glutamatergic seems to be supported further by the observation that contraction evoked by the stimulation of nerves innervating the M1, M2 and M3 flexors could be blocked by cholinergic and glutamatergic antagonists and mimicked by exogenous ACh or Glu. Further results obtained by immunohistochemical and WB experiments also strengthen the view that ACh and Glu are excitatory transmitters involved in the regulation of the flexors hence the olfactory orientation of the snail.

Dopamine and serotonin differentially modulate muscle contractions

Monoamines, such as 5-HT and DA, are well-established modulators of neuromuscular transmission both in vertebrates and in invertebrates. The overall role of modulators is to modify the amplitude, speed and duration of muscle contractions evoked by excitatory transmitters released from the motor neurons (Calabrese 1989).We have observed that DA oppositely modulated thin (M1/M2) and thick (M3) muscles, that is, contractions of the M1/M2 elicited either by the stimulation of the nerve or by externally applied ACh were decreased, while those

of M3 were increased. The contraction parameters, the amplitude and the integral were changed parallel, suggesting a presynaptic modulation. It is assumed that the opposite presynaptic modulation by DA was due to an increased or decreased ACh release, which in turn elicited the appropriate change in the contraction (Fig. 8a). Our results agree with those obtained from the NMC of various vertebrates and invertebrates according to which DA exhibits both excitatory and inhibitory effects at the presynaptic nerve terminal by activation of different DA receptors on the cholinergic nerves (Magoski and Bulloch 1999). Despite the evidence that DA can affect motoneuron physiology in mollusks, it is unknown whether DA regulates muscle activity by controlling motoneuron function or acting directly on the muscle (Charnetski et al. 2000). Here, we suggest that activation of different D-receptors on nerves innervating tentacle muscles directly affected presynaptic physiology by facilitating or inhibiting the release of ACh. Preliminary results obtained by immunohistochemistry support our conclusion since D1- and D2-like receptors were observed on axons but not on the muscle cells (Hernadi et al. unpublished).

We have observed that 5-HT applied at high concentration (10^{-5} M) increased the amplitude of both the nerve- and the ACh-evoked contractions. However, in both muscles, the integral of the nerve-evoked contractions was decreased showing that the relaxation rate was facilitated, thereby shortening contraction duration and suggesting clearly the postsynaptic site of action (Fig. 8b). The 5-HTergic modulation was comparable to that described for different buccal muscles of *Aplysia*. These muscles are innervated by cholinergic buccal (B15 and B16) and cerebral serotonergic (MCC) neurons. Activity of the MCC has no direct effect on the buccal muscles; however, the contractions elicited by stimulation of cholinergic neurons B15 and B16 are potentiated through a postsynaptic mechanism (Fox and Lloyd 1997; Weiss et al. 1978; Hurwitz et al. 2000). The postsynaptic site of action was further confirmed by the observation that 5-HT elicited significant decrease in the resting tone of the muscle and also accelerated the relaxing

phase of the contraction elicited by high KCl. At the same time, the relaxation phase of the ACh-evoked contraction remained unchanged because changes in the integral values were statistically not significant. At low 5-HT concentration (10^{-8} M), the modulatory response was more complex: parameters of the nerve-evoked contractions were similar to that obtained at high 5-HT concentration; however, both the amplitude and integral values of the ACh-elicited contraction of M3 muscle decreased. Seemingly at 10^{-8} M concentration, 5-HT could have both pre- and postsynaptic effect to decrease transmitter release and increase the rate of relaxation and amplitude of the contraction.

In summary, analyzing the neuroanatomical and physiological background of the complex behavior of the superior tentacles of *Helix* performed by three flexor muscles, we have shown that their neuronally evoked contraction was mimicked by external ACh and Glu suggesting a dual excitatory innervation. Inhibitory innervation was not observed; therefore, the movements of tentacles during olfactory orientation are achieved by interaction between excitatory transmitters and a delicately balanced modulation by DA and/or 5-HT.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current ethical laws of Hungary.

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Legend to the figures

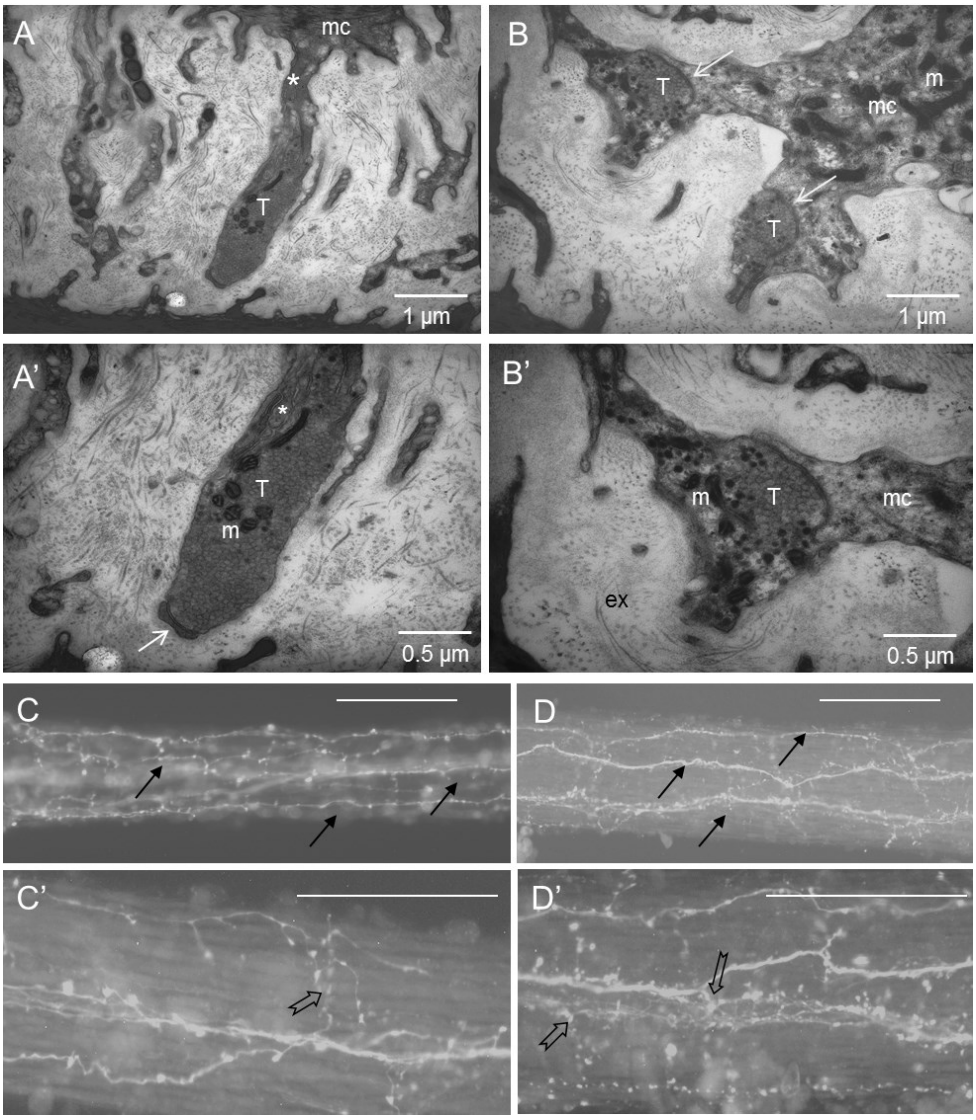

Fig. 1 Innervation and neuromuscular contacts of flexor muscles of the tentacle. Two types of neuromuscular contacts found between the motor nerve and muscle fibers. **a** Nerve terminal (*T*) located in the extracellular space contacts a sarcoplasmic process (*asterisk*) of a muscle fiber (*mc*). **a'** Higher magnification detail of **a** showing that a small distal part of the sarcoplasmic process also contacts the tip of the varicosity (*arrow*). The nerve ending contains mostly clear synaptic vesicles. **b** Two varicosities (*T*) contacting (*arrows*) the non-contractile part of the sarcoplasm of a muscle fiber (*mc*). **b'** - Higher magnification detail of **b** showing one of the

varicosities (*T*) establishing a close membrane contact (*arrow*) with the muscle fiber. Note the increased electron density of the pre- and postsynaptic membrane as well as a number of granular vesicles (*arrowheads*) in the nerve terminal. *m* mitochondria. *Bars* **a, b** 1 µm, **a', b'** 0.5 µm. **c, d** Neurobiotin tracing via olfactory nerve shows that the flexor muscles (**c** M1 and **d** M3) are innervated by well separated axon bundles (*arrows*) running along the longitudinal axes of the muscles. **c'** and **d'** High magnification pictures show that the axons give rise to fine side branches (*open arrows*) with numerous varicosities. *Calibration bar* 100 µm.

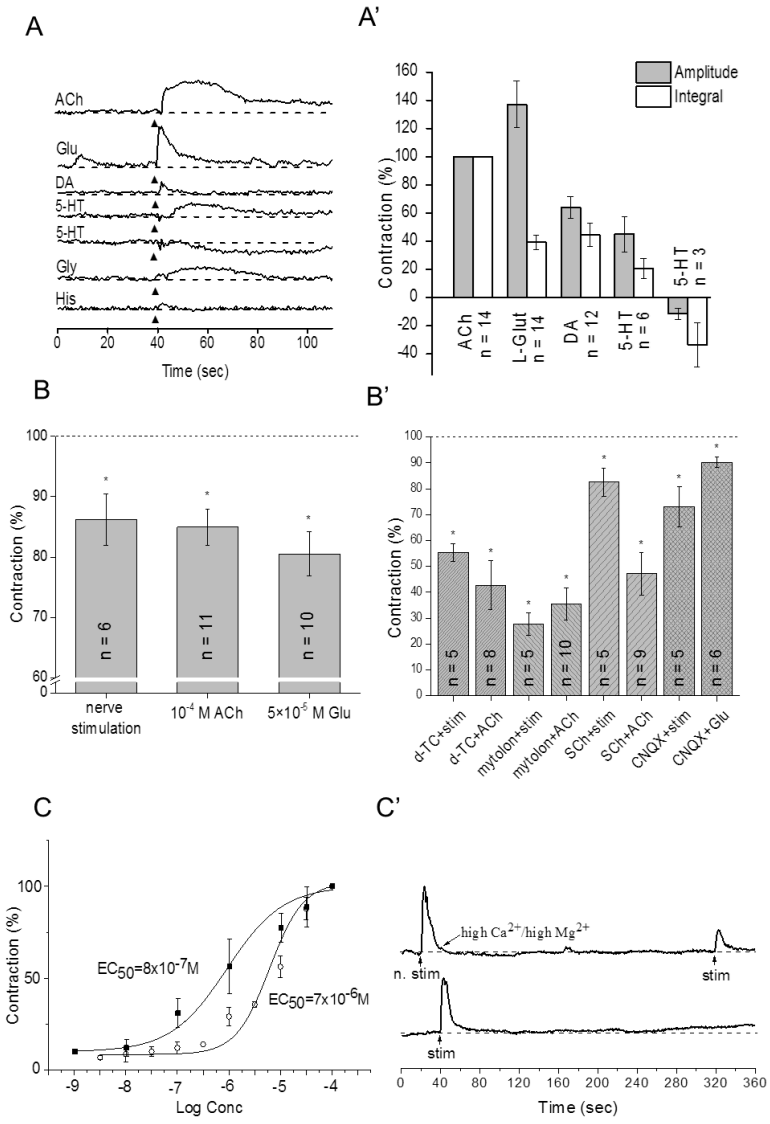

Fig. 2 Potency of putative neurotransmitters applied at 10^{-4} M to elicit change in the flexor muscle tone. **a** Flexor muscle responses to externally applied ACh, Glu, DA, 5-HT, Gly and His recorded in isometric regime. 5-HT was added at 10^{-8} M (upper 5-HT trace) and 10^{-4} M (lower 5-HT trace) concentrations. **a'** Column diagrams represent the mean amplitude and integral of the contractions of flexor muscle obtained following the application of different transmitter candidates. Data were related to ACh contraction taken as 100%. Data obtained on M1, M2 and

M3 muscles were pulled to calculate averages. *Triangle* neurotransmitter application, *n* number of experiments. **b** Comparison of responses evoked by externally applied ACh, Glu and electrical nerve stimulation in the M3 muscle. *Dashed line* shows the muscle length in resting state. **b'** The contractions triggered by electrical stimulation of the olfactory nerve or by externally applied 10^{-5} M ACh or Glu (shown as 100 %, *dashed line*) were attenuated by cholinergic and glutamatergic antagonists added at a concentration of 10^{-4} M. On **b** and **b'** data obtained from isotonic measurements are represented. Results are given as mean ± S.E.M. *Asterisk* shows significant difference at $p < 0.05$ (one-sample *t* test). **c** Concentration-response curves for ACh (*open circles*) and Glu (*closed squares*). Each point represents the mean ± S.E.M. of data obtained from 8-12 muscles. **c'** The neuromuscular transmission is chemical. Contraction evoked by stimulation of the olfactory nerve was significantly decreased in external solution containing 20 mM Ca^{2+}/20 mM Mg^{2+}. Washing the preparation with control solution the restoration was complete (*lower trace*).

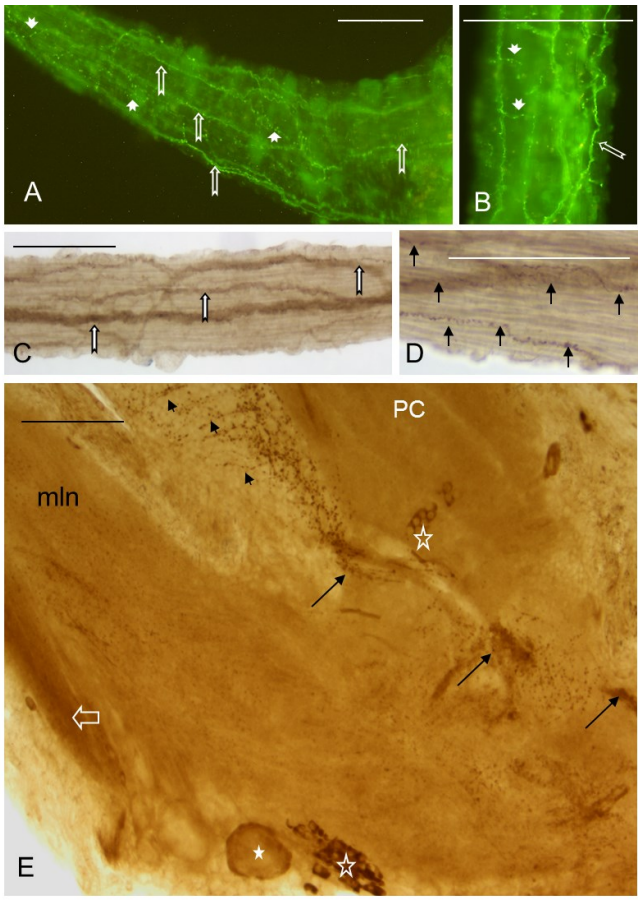

Fig. 3 a, b VGlut immunolabeled fiber trunks are running along the longitudinal axis of the flexor muscle (*open white arrows*). On the surface of the muscle fine varicose axonal side branches can be seen (*white arrowheads*). **c** ChAT immunostaining outline nerve trunks (*solid white arrows*) in the flexor muscle. **d** Higher magnification reveals that only a few of labeled axons (*black arrows*) showing varicosities can be observed in the trunks. **e** Horizontal section of the cerebral ganglion shows the basal procerebrum (*PC*) and the origin of medial lip nerve (*mln*). Both solitary (*asterisk*) and groups of small immune-labeled cell bodies (*empty asterisks*) can be observed in this area of the cerebral ganglion. Labeled axon bundles (*black arrows*) innervate the cell body layer of the PC (*black arrowheads*). Large empty white arrow shows a labeled nerve trunk in the mln. *Calibration bars* 100 μm.

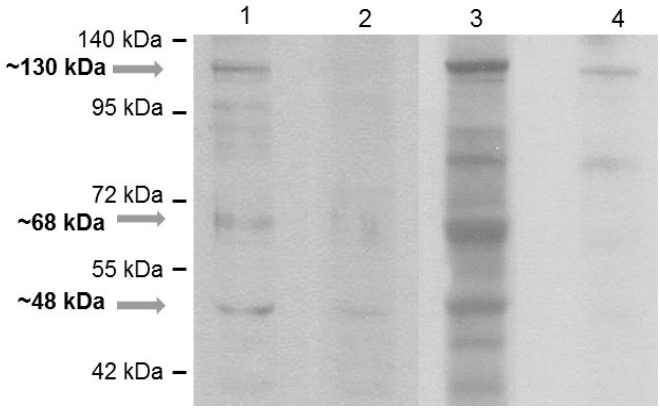

Fig. 4 Identification of choline acetyltransferase by Western blot of cerebral ganglion homogenate using nitrocellulose (*lanes 1, 2*) and PVDF (*lanes 3, 4*) membranes. Anti-ChAT antibody made clear bands at about 130, 68 and 48 kDa (*lanes 1, 3*). The bands were blocked or became lighter when the primary antibody was inactivated combining it with ChAT protein (*lanes 2, 4*).

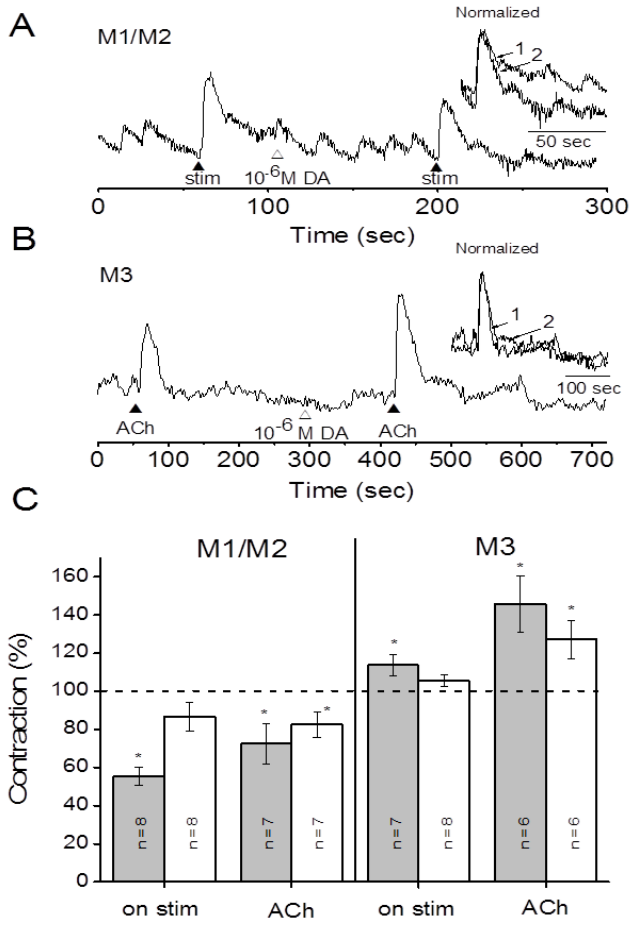

Fig. 5 Dopamine differentially modulates tentacle muscles. **a, b** Contractions of the M1/M2 or M3 evoked by stimulation of the olfactory nerve or by 10^{-4} M ACh were inhibited or augmented in the presence of 10^{-6} M DA. *Inserts* show normalized muscle responses: *1* control, *2* contraction recorded in the presence of DA. **c** The averaged data (mean ± SEM) in column diagrams revealed that the amplitudes and the integrals of contractions of the M1/M2 evoked either by nerve stimulation or by ACh application were inhibited by DA, while responses of the M3 were modulated by DA in opposite direction. *on stim* olfactory nerve stimulation by electrical impulses, *n* number of preparations. *Grey columns* mean integral values, *white columns* mean

amplitude values. *Asterisk* shows significant difference at $p < 0.05$ compared to the contractions evoked by nerve stimulation or externally applied ACh (marked as 100 %). *Open* and *full triangles* in **a** and **b** show the time of chemical manipulation or electrical stimulation (*stim*). Contractions were measured by isometric recording configuration.

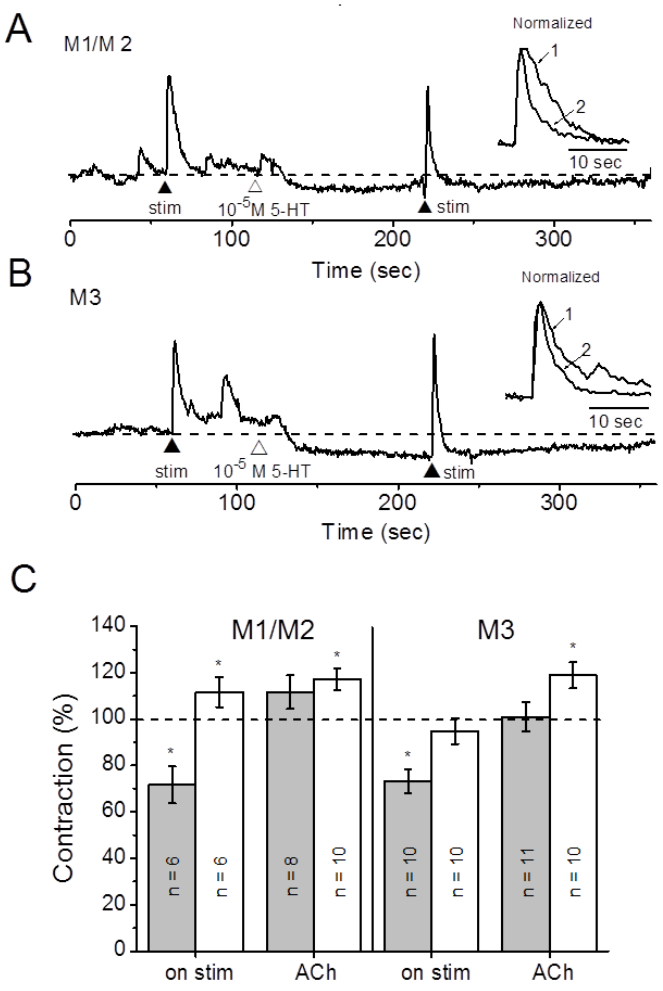

Fig. 6 Effect of 10^{-5} M 5-HT concentration on the muscle responses evoked either by the electrical stimulation of the on or by externally applied ACh. **a, b** Amplitudes of the nerve evoked contractions (*full triangle*) of both M1/M2 and M3 muscles were increased by 5-HT. In

addition 5-HT application (*open triangle*) substantially decreased the resting muscle tone. *Insert* show normalized contractions obtained before (*1*) and after 5-HT treatment (*2*). **c** The column diagram (mean ± SEM) revealed that both in M1/M2 or M3 muscles the nerve-evoked or ACh-evoked contractions were similarly modulated by 5-HT: the integral of nerve-evoked contractions decreased while the amplitude increased or remained unchanged; the integral and the amplitude of the ACh-evoked contractions were stimulated. *n* number of muscles tested. *Grey columns* mean integral values, *white columns* mean amplitude values. *Asterisk* shows significant difference at $p < 0.05$ compared to the contractions evoked by nerve stimulation or externally applied ACh (marked as 100 %). Contractions were measured by isometric recording configuration.

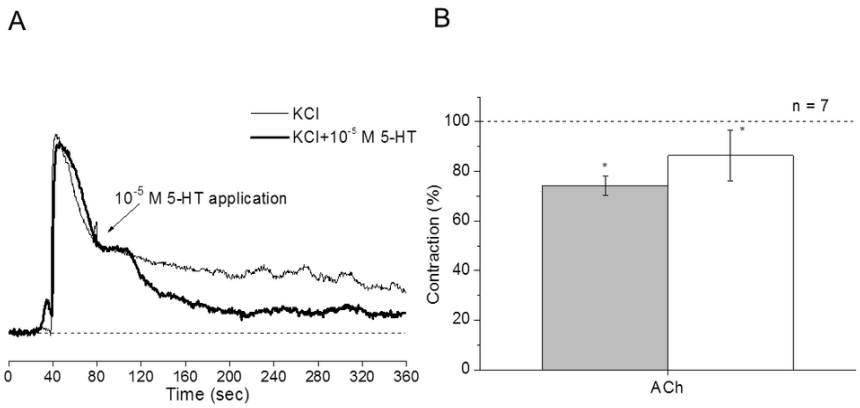

Fig. 7 Effect of 5-HT on the muscle responses evoked by KCl or ACh. **a** Superimposed contraction traces elicited by 90 mM KCl. *Thin line* shows the time course of the control trace. Application of 10^{-5} M 5-HT shown by the *arrow* during the second contraction elicited also by high-KCl application. 5-HT induced acceleration of the relaxing phase. **b** Low 5-HT concentration (10^{-8} M) modulated oppositely the M3 muscle contractions elicited by externally

applied ACh compared to the modulation of high 5-HT concentration (see Fig. 6c). *Grey columns* mean integral values, *white columns* mean amplitude values. *Asterisk* shows significant difference at $p < 0.05$ compared to the contractions evoked by nerve stimulation or externally applied ACh (marked as 100 %). Contractions were measured by isometric recording configuration.

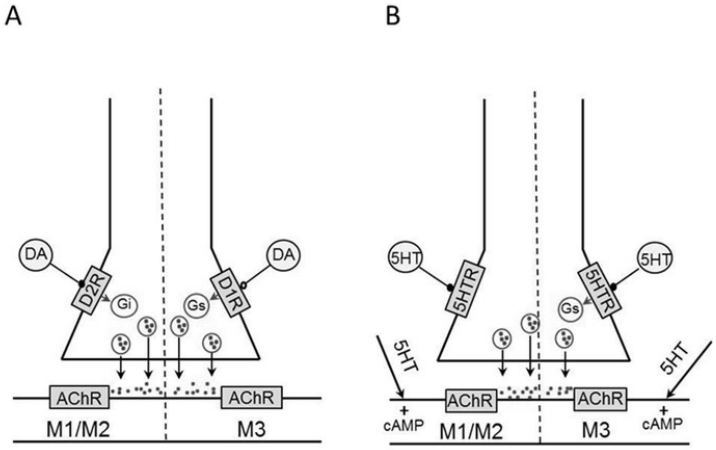

Fig. 8 Scheme of the possible dopaminergic and serotoninergic modulation of the ACh release from motoneuron axon terminal. **a** DA increases or decreases ACh release activating different DA receptors through different intracellular cascades. **b** 5-HT exerts mainly postsynaptic effect directly affecting the contractile properties of muscles. The presynaptic effects is the decrease of the ACh release which could be observed at low (10^{-8} M) 5-HT concentration. G_i and G_s are inhibitory and stimulatory G-proteins, respectively. *R* receptor. *empty circle* excitatory synapse, *filled circle* inhibitory synapse.