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Analogous Effects of Nicotine and Muscarine on the Curare-Sensitive/ Atropine-Insensitive Discharges of Identified *Lymnaea* Neurons

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Abstract

The effects of acetylcholine (ACh), its orthodox agonists and antagonists were tested on identified neurons associated with respiratory behaviour in the intact brain of *Lymnaea stagnalis* (L.) using intracellular microelectrodes. Drugs were bath applied. With one exception, ACh caused depolarization and increased spike frequency in the cell types studied. In most cells nicotine and muscarine usually had similar effects, exciting some and inhibiting others. Further unorthodox results were also found in that succinylcholine (SCh) acted more like a nicotinic agonist rather than an antagonist, as did arecoline. The muscarinic antagonist, atropine, had little or no effect on any of the cells studied. Sensitivity to ACh, nicotine and muscarine differ for different cells, and comparison of the effects of ACh, nicotine and muscarine suggest that some of the cholinoceptors studied resemble either conjoint receptors or multiple cholinoceptors on the same cell.

Keywords: *Acetylcholine; Curare; Atropine; Nicotine; Muscarine; Succinylcholine; Arecoline; Identified Neurons; Lymnaea stagnalis (L.)*

Introduction

It was in 1914 that Dale [1] first suggested that acetylcholine (Ach) might have actions imitated either by nicotine or muscarine, giving rise to modern ideas of nicotinic and muscarinic acetylcholine receptors (AChRs) [2], particularly in mammals. Nicotinic acetylcholine receptors (nAChRs) divide into N1 and N2 subclasses with many subtypes, all of which are ionotropic and ligand gated [3], while the G-protein coupled muscarinic receptors are currently divided into five subtypes (M1 to M5).

Background Review

In gastropod molluscs, acetylcholine (ACh) is known to be a natural neurotransmitter, responsible for excitatory motor control of the columellar muscle, as well as inhibitory control of the heart [4], both of which are constituents of whole-body withdrawal behaviour. Furthermore, Elliott., *et al.* [5] demonstrated that acetylcholine excited some of the motoneurons and inhibited others in the feeding system of *Lymnaea* stagnalis, in which Elliott and Kemenes [6] reported that the N1 neurons of the feeding pattern generator are multiaction, premotor cholinergic interneurons. Elliott [7] also demonstrated that nicotinic and muscarinic antagonists, including atropine, slowed or blocked the feeding rhythms, implying the possibility of muscarinic-type receptors. However, earlier work on the marine gastropod *Aplysia californica* [8] on three

different cell types indicated that there were two types of nicotinic ACh receptors and another with unique properties that did not fit the classical scheme of cholinergic receptors. Since then two subtypes of nicotinic acetylcholine receptors (nAChRs) controlling chloride conductance have been described in giant neurons on the ventral surfaces of the right and left parietal ganglia of *Lymnaea* (i.e. LP1, 2 3 and RPV2) [9]. These receptors are thought to have common features with cation selective α7 AChRs of vertebrates [10]. Subsequently both cation and anion selective nAChRs were described in *Lymnaea* neurons [11] and at least 10% of central neurons in *Lymnaea* were shown to express nAChR subunits "comparable with vertebrate species, but with a functional complexity that may be much higher" [12].

Another multi-action neuron, VD4 [13], is a constituent part of the of the central respiratory generator [13,14] which co-releases ACh and a FMRFamide-like peptide [15-17]. To date, muscarinic receptors have not been described in the respiratory system and current evidence indicates a preponderance of several types of nAChRs on neurons within the CNS of *Lymnaea* [9,12], but there is little firm evidence for muscarinic-like ACh receptors. Given that muscarinic receptors have been shown to occur in other pulmonates such as *Helix aspersa* [18] and in the marine opisthobranch gastropods *Aplysia californica* [19] and *Pleurobranchaea* sp. [20], it is surprising that such receptors have not been clearly described in *Lymnaea*. Perhaps this is a chance omission given the systems that have been investigated up to the present time or perhaps due to the complexity of molluscan AChRs.

Typically, muscarinic receptors are blocked by atropine and nicotinic receptors are blocked by curare, but it is already clear that the cholinergic antagonists used in vertebrate preparations cannot be assumed to have similar actions in molluscs [21]. Previous authors have shown that arecoline acts as a weak cholinergic agonist on H-type ACh receptors of *Aplysia* [8] and in the *Lymnaea* feeding system [7]. We previously demonstrated that ACh, arecoline (Arec) and succinylcholine (SCh), a cholinergic antagonist, all modified the firing frequencies of neurons associated with the respiratory pattern generator (rCPG) of *Lymnaea* [21]. However, the data we obtained were thought, at that time, to be unorthodox in that Arec appeared to act more like a cholinergic antagonist, while SCh acted more like a cholinergic agonist, although this did imply that cholinergic receptors of some type were present on the neurons studied (RPeD1, LPeD1, VD1/RPD2). Arecoline also acts as an agonist on two types of nicotinic receptors of *Xenopus laevis* oocytes [22] and is a partial agonist of mammalian M1 to M4 muscarinic receptors [23-25]

Current investigation

Here we describe a more detailed investigation of the actions of "orthodox" cholinergic agonists and antagonists, mainly on neurons associated with respiration in *Lymnaea*. We recorded from the giant neurons associated with respiration in *Lymnaea* the giant neurons RPeD1 (part of the (rCPG) and LPeD1 (which has excitatory connections from rCPG neurons VD4 and the input 3 interneuron [27-29] of the pedal ganglia and the electrically connected giant neurons VD1 and RPD2, both of which are hypoxia sensitive, and both part of the pneumostome control system [30,31]. They lie in the visceral and right parietal ganglia, respectively. Additional, but incomplete data is also included on the A and B groups (both parts of pneumostome control system) of neurons of the right parietal ganglion as well as the J cells (pneumostome opener motor neurons [31]) and giant neurons VV1/2 (of the visceral ganglion). The exact functions of VV1/2 are unknown, but they are responsive to osmotic stimulation of the osphradium, from which RPeD1 receives excitation under hypoxic conditions [32]. For locations of neurons see Winlow and Polese [31].

Materials and Methods

Animals were obtained from suppliers, maintained at 10 - 16°C in aerated pond water and fed on lettuce supplemented with tropical fish food. Intracellular recordings were made from identified respiratory neurons of *Lymnaea* using standard preparatory and electrophysiological recording techniques [33], as follows. Several hundred specimens of *Lymnaea stagnalis*, weighing 2 - 5g, were obtained from animal suppliers (Blades Biological, Cowden, Kent). Brains were removed and maintained in a flow of HEPES buffered saline (pH 7.9) of the following composition (mM): Na^+, 59.4: K^+, 2.0; Mg^{2+}, 2.0; Ca^{2+}, 4.0; Cl^-, 38.00; HPO_4^-, 0.1; glucose, 0.3 and HEPES buffer 50.0 (4-(2-hydroxyethyl)-I-piperazine-ethansulfonic acid) supplied by Boehringer Corp. (London). Brains were prepared for experimentation according to the methods of Winlow and Benjamin (1976). Fibre-filled glass (Clark Electromedical instruments, Reading) microelectrodes

were manufactured with a Searle Bioscience Vertical electrode puller and were filled with the supernatant from a saturated solution of K_2SO_4 (10-30 MΩ). Electrophysiological signals were amplified using Neurolog NL102G bridge balance preamplifiers and were displayed and recorded by conventional means. If required up to four neurons could be recorded simultaneously.

HEPES snail saline continuously superfused the brain at a rate of a 2 - 5 ml per minute. Acetylcholine chloride, its agonists and antagonists were superfused over the brain at concentrations of 10^{-4}M in HEPES saline and alterations in neuron firing frequency were determined. The following vertebrate nAChR antagonists were applied: SCh, d-tubocurarine chloride (curare) and hexamethonium. Nicotine and muscarine were also applied, as were the supposedly muscarinic agonist arecoline and the muscarinic antagonist atropine (all chemicals from Sigma-Aldrich, UK). In terms of the actions of cholinergic agonists and antagonists, an increase in firing frequency was described as an excitatory effect and a decrease in firing frequency was described as an inhibitory effect. No attempts were made to block synaptic transmission in these preparations.

Results

Differential effects of cholinergic agonists and antagonists on identified neurons

The data collected in these experiments are summarised in table 1 and expressed both in percentages and numerically. Although the data from VV1/2, A group and B group cells are incomplete they provide some additional insights on the differential effects of the applied cholinergic agonists and antagonists. On application of ACh, the majority responses of all cell types, except VV1/VV2, were excitation, i.e. an increase in firing frequency. VV1/VV2 were inhibited by ACh. However, application of the nicotinic antagonist SCh had the unexpected effect of mimicking the excitatory responses of ACh in RPeD1 (See figure 1), LPeD1 (See figure 2) and RPD2/VD1 and also mimicked the inhibitory effect of ACh on VV1/VV2. The majority responses of these cells to the orthodox nicotinic antagonists curare and hexamethonium were different from one another. The majority response to curare was a decrease in firing frequency, while most cells sampled were insensitive to hexamethonium. Thus, the most potent antagonist of the ACh excitatory responses was curare.

Cells	RPeD1			LPeD1			RPD2& VD1			A group			B group			VV1/2		
Firing Frequency	N	E	I	N	E	I	N	E	I	N	E	I	N	E	I	N	E	I
10^{-4} Acetylcholine	24%	71%	5%	26%	53%	21%	26%	48%	26%	33%	56%	11%	29%	57%	14%	0%	14%	86%
	5/21	15/21	1/21	5/19	10/19	4/19	5/19	9/19	5/19	3/9	5/9	1/9	2/7	4/7	1/7	0/7	1/7	6/7
10^{-4} Succinylcholine	20%	70%	10%	18%	64%	18%	18%	64%	18%							10%	0%	90%
	2/10	7/10	1/10	4/22	14/22	4/22	4/22	14/22	4/22							2/21	0/21	19/21
10^{-4} Curare	12%	12%	76%	19%	19%	62%	14%	14%	72%									
	2/17	2/17	13/17	3/16	3/16	10/16	1/7	1/7	5/7									
10^{-4} Hexamethonium	80%	0%	20%	75%	0%	25%	100%	0 %	0%									
	4/5	0/5	1/5	3/4	0/4	1/4	4/4	0/4	0/4									
10^{-4} Nicotine	0%	0%	100%	20%	80%	0%	0%	83%	17%	50%	0%	50%	17%	67%	17%			
	0/5	0/5	5/5	1/5	4/5	0/5 -	0/6	5/6	1/6	2/4	0/4	2/4	1/6	4/6	1/6			
10^{-4} Muscarine	20%	0%	80%	25%	75%	0%	33%	67%	0%				75%	0%	25%			
	1/5	0/5	4/5	1/4	3/4	0/4	2/6	4/6	0/6				3/4	0/4	1/4			
10^{-4} Arecoline	18%	18%	64%	0%	33%	67%	11%	0%	89%	25%	75%	0%						
	2/11	2/11	7/11	0/6	2/6	4/6	1/9	0/9	8/9	1/4	3/4	0/4						
10^{-4} Atropine	100%	0%	0%	100%	0%	0%	75%	25%	0%	100%	0%	0%	100%	0%	0%			
	4/4	0/4	0/4	4/4	0/4	0/4	3/4	1/4	0/4	4/4	0/4	0/4	4/4	0/4	0/4			

Table 1: *Effect of cholinergic agonists and antagonists on the spontaneous firing frequencies of identified Lymnaea neurons. N, no effect; E, increased firing frequency; I, decreased firing frequency. Fresh cells were studied in each case. Green, red and purple boxes indicate similarities' of changes in firing frequencies induced by pharmacological agents. In neurons RPeD1, LPeD1 and RPD2/VD1 (green boxes), the majority of cells studied showed increased frequencies of firing in the presence of ACh and SCh, whereas VV1/2 (purple box) exhibited decreased firing frequencies in the presence of both substances. Similarities of firing were again observed in these cells when treated with nicotine and muscarine (red boxes), but RPeD1 exhibited a decline in frequency, which was also mimicked by arecoline, while the frequency of firing of LPeD1 and RPD2/VD1 increased. In both these latter cases arecoline induced inhibition. B group cells were different again, with nicotine usually causing excitation, while muscarine had little effect. Yellow background for the effects of ACh and its orthodox antagonists on cell firing frequencies; Pink background for the effects of nicotine and muscarine; Green background for arecoline, an orthodox muscarinic agonist (but with known agonistic actions on nAChRs) and atropine an orthodox muscarinic antagonist. To obtain the data reported here, the firing patterns of 305 neurons were studied.*

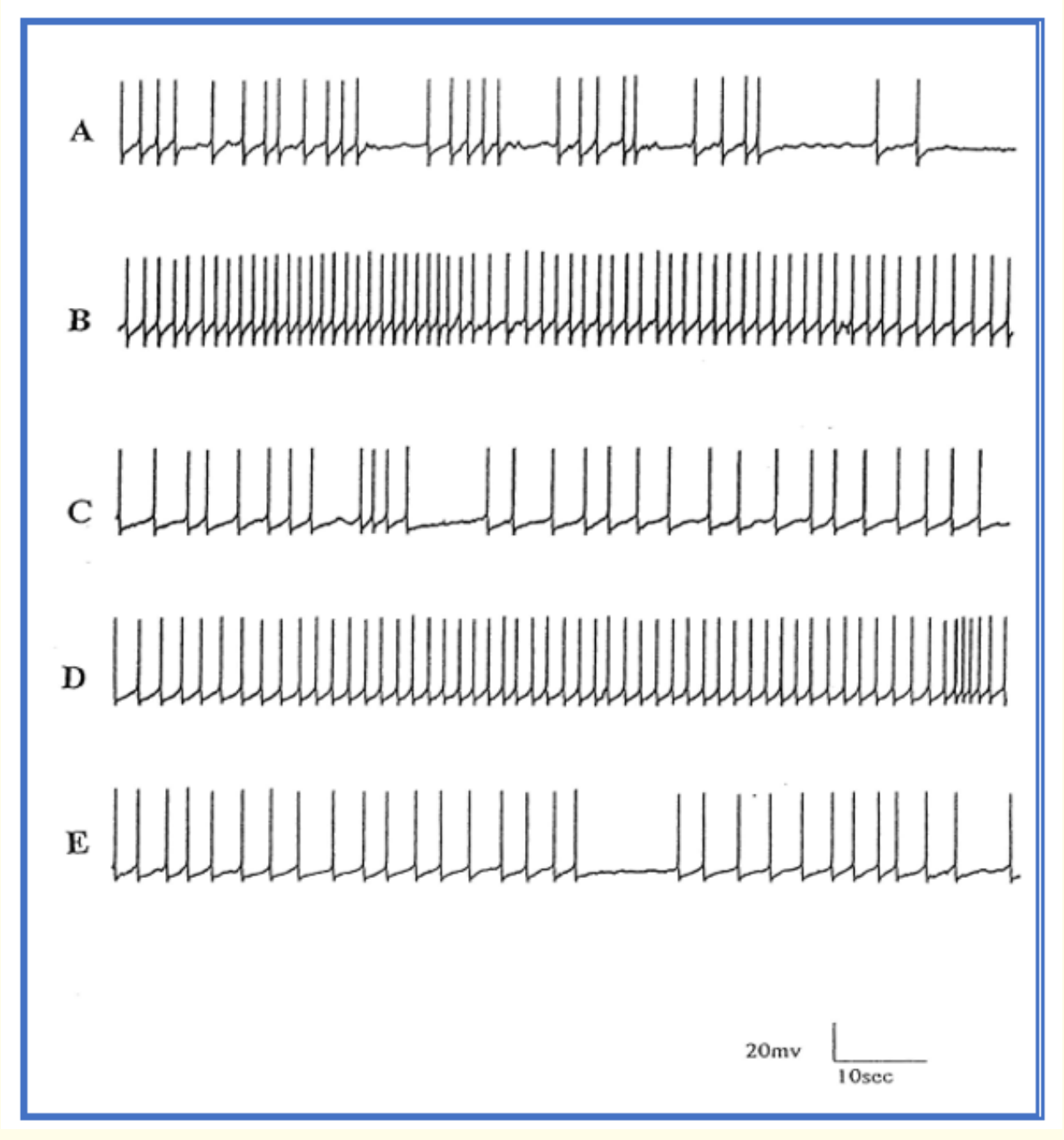

Figure 1: *A comparison of the effects of ACh and SCh on RPeD1. A) Control; B) Effect of 10-4 M ACh on firing frequency; C) Continuous wash out after 6 min; D) Effect of 10-4 M SCh E) Continuous wash out after 6 min. As can be seen from traces B and D, the effects of ACh and SCh on RPeD1 are not dissimilar.*

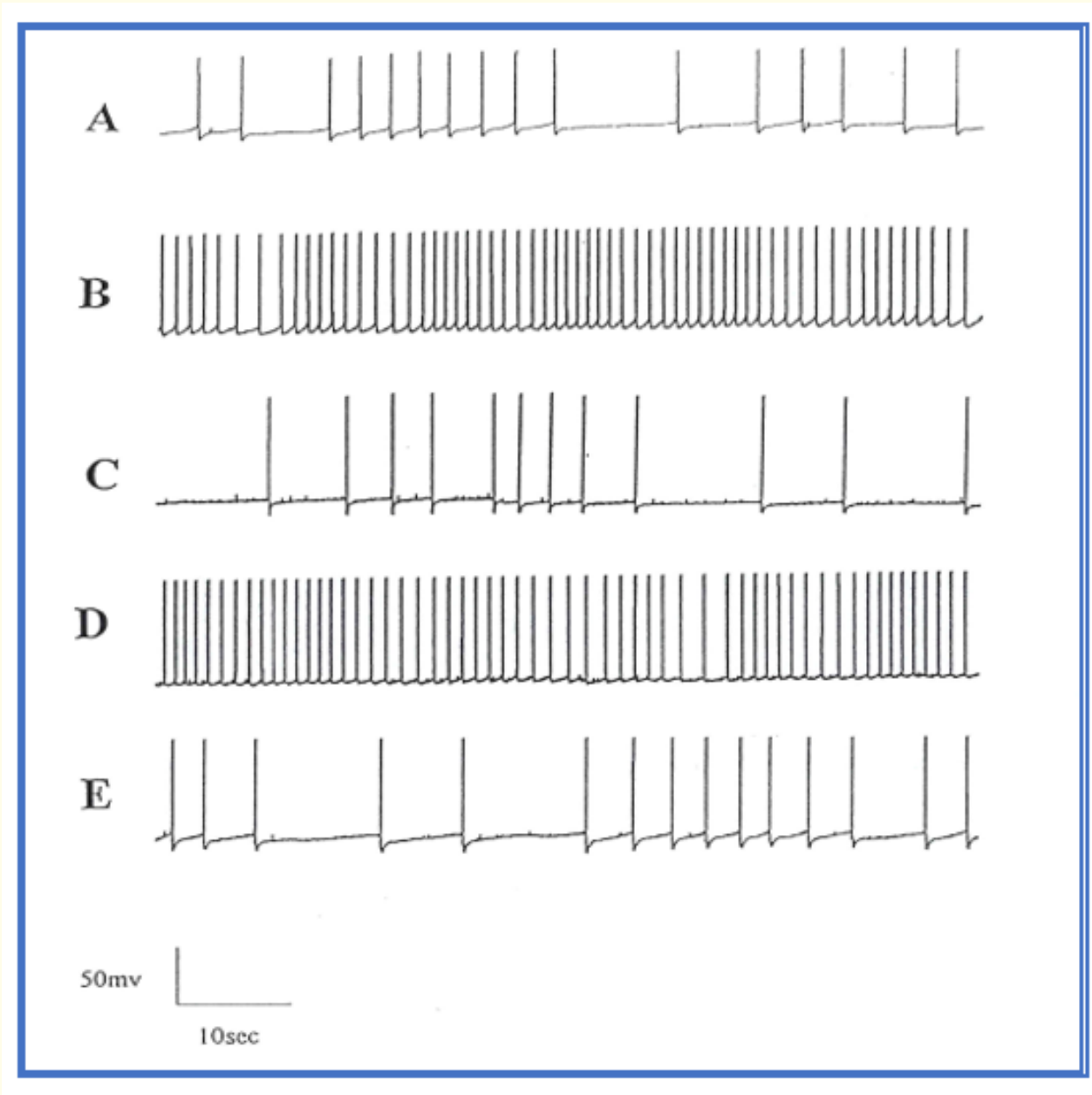

Figure 2: *A comparison of the effects of ACh and SCh on LPeD1. A) Control; B) effect of 10-4 M ACh on firing frequency; C) continuous wash out after 6min; D) effect of 10-4 M SCh on LPeD1; E) continuous wash out after 6 min. As is clear from trace B and trace D ACh and SCh have similar effects on LPeD1.*

In our experiments arecoline hyperpolarized and inhibited the RPeD1 VD1, RPD2 and LPeD1, but excited A group neurons. Observations on RPeD1 showed that arecoline mimicked the effects of both nicotine and muscarine, but this was not the case in the remaining neurons. Although the predominant effect of Arecoline was to cause a reduction in the firing frequency in R/LPeD1 it induced an irreversible cessation of firing in RPeD2/VD1 (Figure 3). Thus, arecoline had similar effects to nicotine and muscarine on RPeD1, but opposite effects to these compounds on LPeD1 and RPD2/VD1, which were all excited by nicotine and muscarine as well as ACh (Figure 4) and SCh. Atropine, an orthodox muscarinic blocking agent, had little effect on any of the neurons studied. Thus, the effects of these agents were cell specific, varying from one cell type to another, but did not fit into orthodox concepts of cholinergic agonists and antagonists.

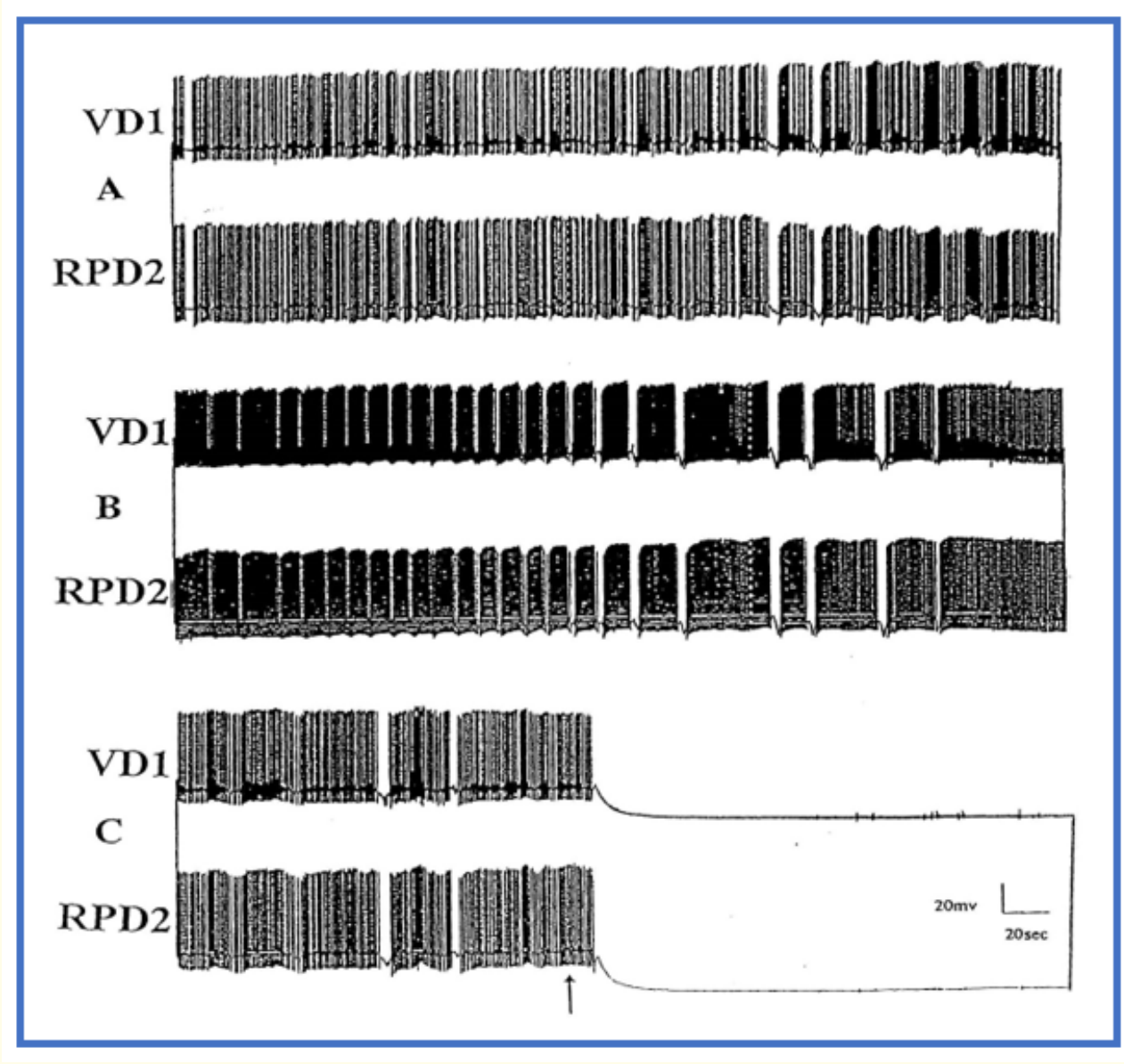

Figure 3: *A comparison of the effects of ACh and Arecoline on the firing frequency of VD1 and RPD2. A) control; B) effect of 10-4 M ACh; C) effect of 10-4 M Arecoline. Continuous wash out for more than 30 min (not shown) did not change the situation, which shows that the inhibition caused by arecoline is not reversible.*

Actions of nicotine and muscarine

Nicotine and muscarine were also tested against these cell types (Table 1) and in the majority of cases found to increase the firing frequency of LPeD1, RPD2/VD1, but both substances decreased the firing frequency of RPeD1. However they had opposite effects on B group cells, nicotine causing mainly excitation and muscarine most often having no effect or occasionally an inhibition of spiking.

Are there conjoint nicotinic/muscarinic ACh receptors on some identified neurons?

With the exception of VV1/2, the majority effect of ACh on the neurons studied here is excitation (Table 1), but differences in firing patterns are shown in figure 4, presumably due in part to the membrane properties of the individual neurons and in part to excitation within the respiratory network. However, if we consider the effects of ACh, muscarine and nicotine on the spontaneous activity of J cells (which are pneumostome motor neurons) the differential effects of the three agents are clear (Figure 5). All of them produce excitation, but the patterned activity differs from agent to agent, with nicotine inducing the typical discharge pattern of these cells [33]. The three different excitatory patterns may suggest that conjoint nicotinic/muscarinic receptors occur on these neurons or that nicotine and muscarine excite other neurons within the respiratory network. The data presented in table 1 (red boxes) suggest that the conjoint cholinergic receptors may occur on RPeD1, LPeD1 (See also figure 6) and RPD2/VD1, but not on B group cells.

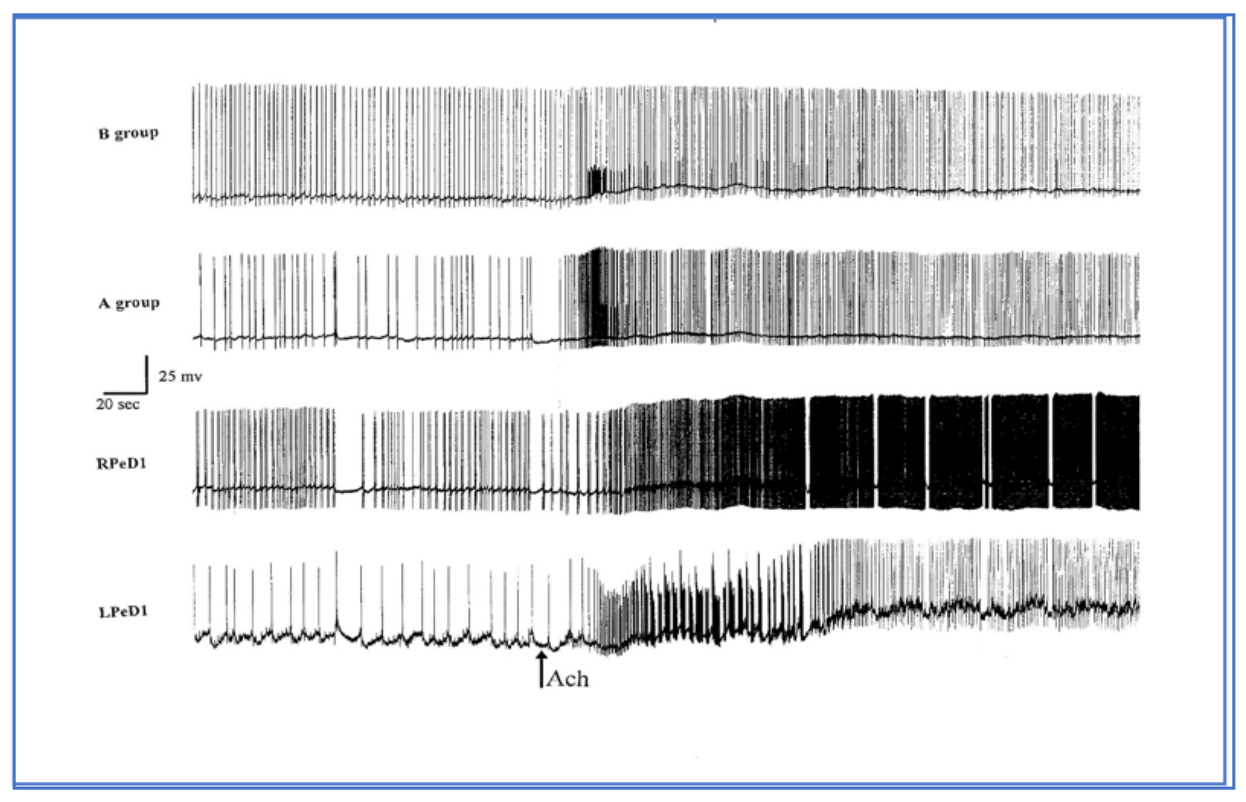

Figure 4: *Effect of ACh on A and B group, RPeD1 and LPeD1. Simultaneous recording of spontaneous activity from four identified cells. As arrow shows bath application of ACh excited all four cells and caused different patterns of excitatory discharge.*

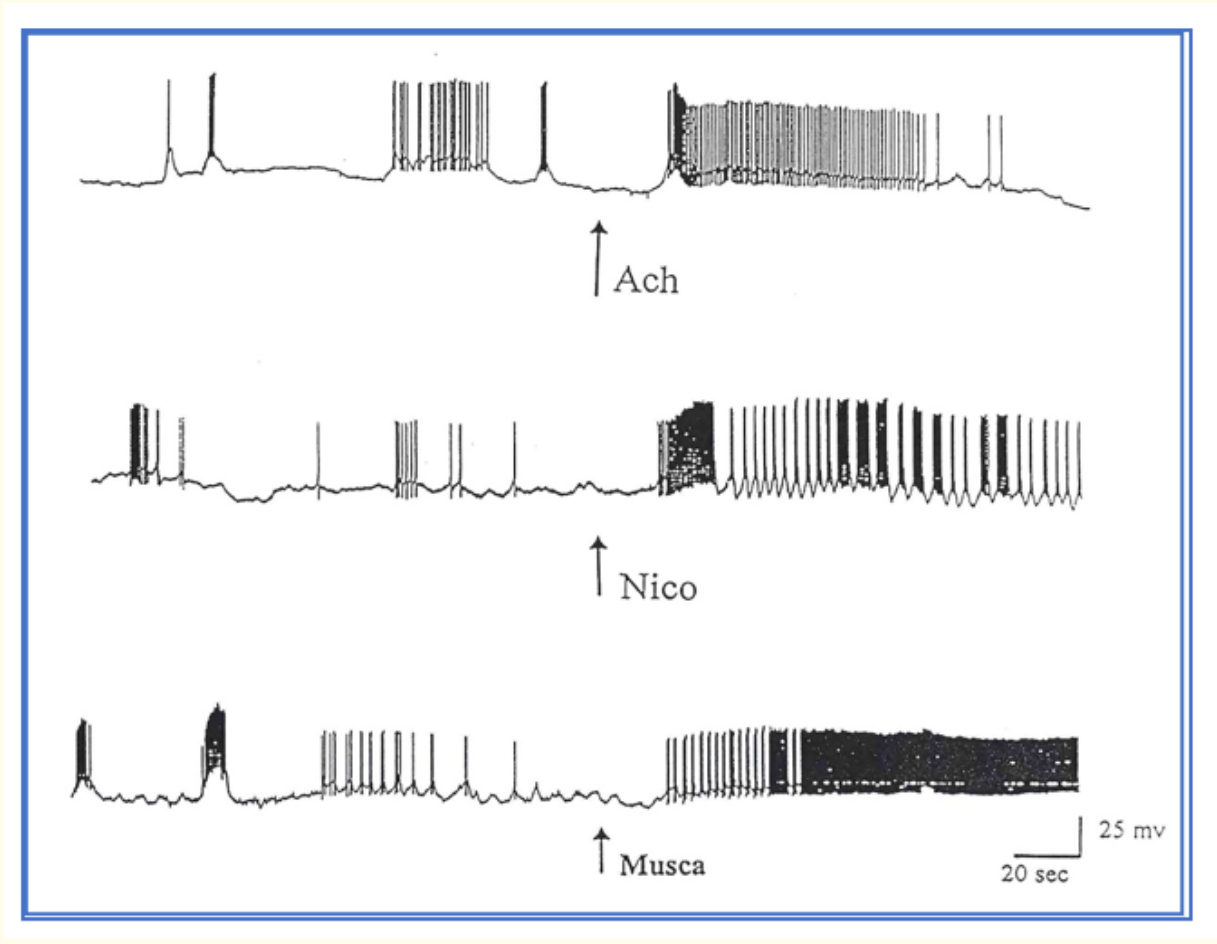

Figure 5: *A comparison of the effect of ACh, nicotine and muscarine on a J cell. A) Spontaneous recording from a J cell. Bath applied 10-4 M ACh excited these cells and increased firing frequency for a short time and they then returned to normal activity. B) Application of 10-4 M nicotine excited and caused bursting activity in these cells and then changed its pattern of activity with post burst afterhyperpolarization which is a typical discharge pattern in these neurons [37]. C) 10-4 M muscarine excited J cells strongly, causing a different discharge pattern compared to ACh and nicotine.*

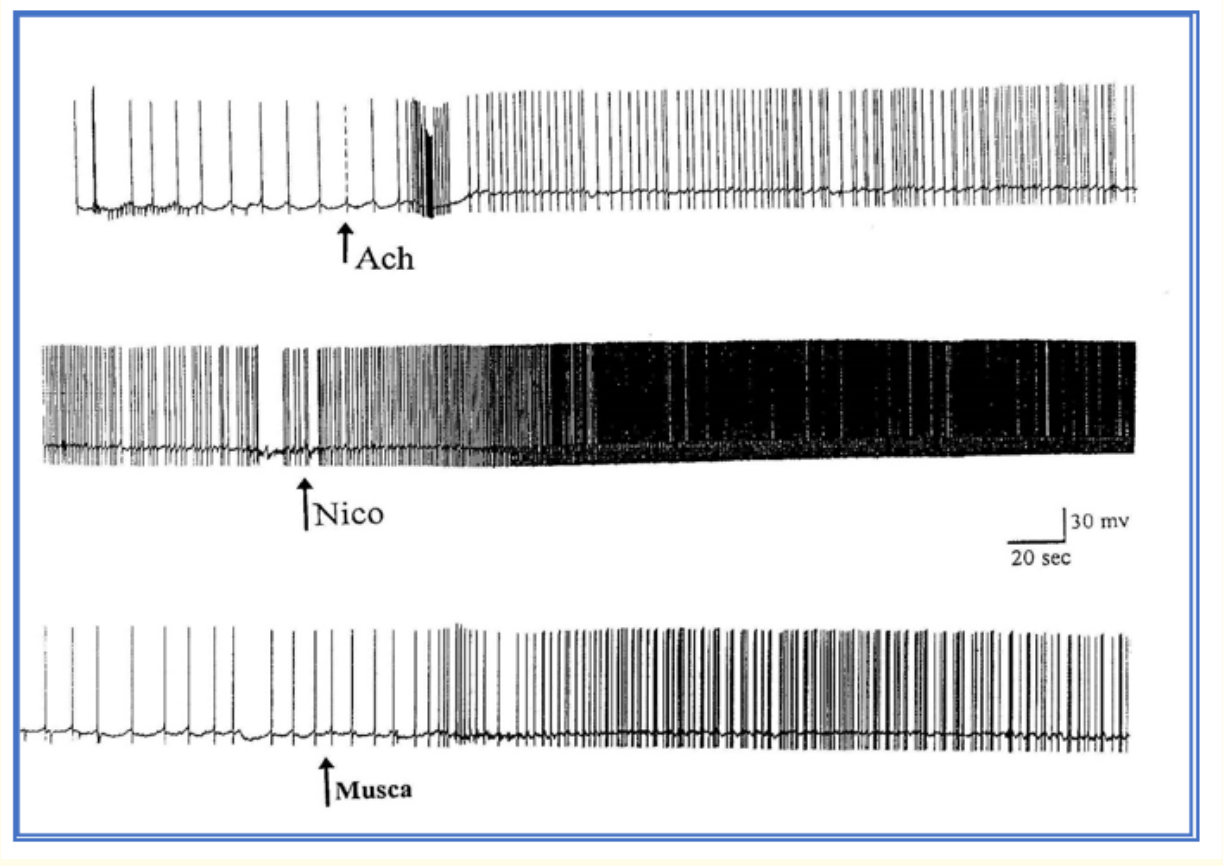

Figure 6: *Excitation of LPeD1 by bath applied of ACh, nicotine and muscarine (all 10-4 M). These recordings of spontaneous activity in examples of LPeD1 show the variable actions of the three substances, which may reflect either their actions within the neural network or their direct effects on the cell.*

Discussion

The data provided here and in previous studies clearly support the view that acetylcholine is an important neurotransmitter in *Lymnaea*, and other related molluscs including *Aplysia californica* [8,34] and *Helix aspersa* [18]. However, the responses to cholinergic agonists and antagonists are unorthodox, indicating that a variety of ACh receptors exist in *Lymnaea,* but that they do not fit into the standard nicotinic and muscarinic categories found in the vertebrates. This is unsurprising given the previous findings of Kehoe [8] and Elliott [7]. However, although atropine was ineffective in the neurons associated with the respiratory system, this was not the case in neurons of the feeding system as reported by Elliott [7], which suggests that more orthodox muscarinic receptors may occur in the *Lymnaea* feeding system. In addition, arecoline appears to be specific cholinergic agent on the *Lymnaea* neurons, studied here, but its pharmacology is complex in that it mimics the actions of both nicotine and muscarine on RPeD1, but not the other neurons as shown in table 1. Further studies on its mode of action are therefore required.

There appears to be good evidence for nicotinic receptors in the cells studied here, particularly as curare is most effective at decreasing neural activity, although hexamethonium has little effect. It is also of interest that succinylcholine appears to act as a partial agonist of cholinergic activity (See table 1), while nicotine and muscarine have similar effects to one another, with the exception of their actions on B group cells (Table 1). Furthermore, although muscarine is effective at altering firing frequency, atropine is ineffective as an antagonist on any of the neurons studied here. This implies that such muscarinic receptors as exist in the respiratory system are completely different from those found in vertebrates where all the known muscarinic subtypes are antagonised by atropine [35]. Thus, there appears to be evidence for a new muscarinic subtype in *Lymnaea*.

Some form of conjoint nicotinic/muscarinic receptor may also exist on *Lymnaea* neurons, but this may be due to effects on the respiratory neural network since we did not use synaptic blocking agents to prevent synaptic transmission. Previous studies elsewhere have also suggested the presence of conjoint ACh receptors in invertebrates. At a cholinergic neuro-neuronal synapse of the buccal ganglion of *Aplysia,* curare and atropine were shown respectively to decrease and increase ACh quanta [19,36]. These results were interpreted as indicating the conjoint presence of both nicotinic and muscarinic AChRs on the same terminal. In another study the effects of atropine and d-tubocurarine on a monosynaptic cholinergic connection between identified neurons of *Helix pomatia* [37] were investigated [38] and both d- tubocurarine and atropine were found to supress EPSPs at this monosynaptic cholinergic synapse. Similar results were also obtained for the RPa4 neuron of *Helix lucorum* [39,40], which has an atypical pharmacological profile not unlike those we have described here, except that the cells were inhibited by both d-tubocurarine and atropine. Taken together these findings suggest that a range of atypical cholinoceptors exist in gastropod molluscs, perhaps with multiple atypical receptors on a single neuron, which could be separate and/or conjoint. In evolutionary terms nAChRs are ancient and highly conserved with a massive expansion in molluscs, which may explain their apparent diversity in *Lymnaea* [41]. Molluscan muscarinic receptors may be less diverse.

Further confirmation of our findings requires both studying the effect of cholinergic agonists and antagonists in high Ca^{2+}/low Mg^{2+} solution to block transmission [42,43] and the use of isolated cultured *Lymnaea* neurons as described elsewhere [44].

Conclusion

Pharmacologically atypical nicotinic and muscarinic receptors, some of which may be conjoint receptors, appear to exist within the nervous system of the great pond snail, *Lymnaea stagnalis.*

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Volume 13 Issue 8 August 2021