

## A13–40 YEARS OF INVERTEBRATE NEUROPHARMACOLOGY – A TRIBUTE TO PROF. ROBERT J. WALKER

Organised by L. Holden-Dye and R. Williamson for the Neurobiology Group and co-sponsored by the Physiological Society, Syngenta and Pfizer

### A13.1–Modulation of cholinergic events in insect neurones

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Ligand-gated ion channels normally mediate rapid chemical synaptic transmission. However, it is becoming clear that the function of most if not all of these receptors may be modulated by other neurotransmitters. The ability to uniquely identify neurones within known neural circuits responsible for specific behaviours renders insect preparations useful in understanding how channel modulation may operate at a behavioural level. ACh is a major excitatory neurotransmitter in the insect nervous system, in which many of its actions involve nicotinic receptors. We have evidence that activation of muscarinic ACh receptors can modulate nicotinic cholinergic transmission between the locust forewing stretch receptor (fSR) and the first basilar motoneurone (BA1) by both presynaptic and postsynaptic mechanisms, leading to a reduction in EPSP amplitude. Postsynaptic modulation in at least some neurones is achieved by way of second messengers that mediate changes in nicotinic receptor function. Nicotinic cholinergic synaptic transmission between the fSR and BA1 can also be down-regulated by the biogenic amines octopamine 5-hydroxytryptamine and dopamine. Evidence from a cockroach motoneurone indicates that 5-HT mediates its effects upon nicotinic indirectly via a second messenger system distinct from that activated by muscarinic receptors.

It appears, therefore that modulation of nicotinic receptors may be complex and mediated by different mechanisms. A challenge ahead is to understand how this operates in the context of a specific behaviour such as insect flight.

### A13.2–Nicotinic acetylcholine receptors – snails overtake in the fast lane

David Sattelle (MRC Functional Genetics Unit, Dept of Human Anatomy and Genetics, University of Oxford, UK)

Nicotinic acetylcholine receptors (nAChRs) mediate the fast actions of acetylcholine at cholinergic synapses.

They are key targets for novel analgesics and other drugs. Invertebrate nAChRs are targets for anthelmintics and insect control agents. Electrophysiological experiments on the large diameter neurons of snails and sea-slugs provided important early evidence for a rich diversity of neuronal nicotinic receptors. Research in this area was pioneered in the UK by Robert Walker. The largest nicotinic receptor gene family currently known is that of another invertebrate *Caenorhabditis elegans*. In this genetic model organism, chemistry-to-gene screens have identified, a discrete subset of subunit-encoding genes targeted by the drug levamisole, along with novel genes encoding proteins acting upstream and downstream of nicotinic receptors. Another genetic model, *Drosophila melanogaster*, has provided evidence of extensive alternative splicing and RNA editing of neuronal nAChR subunits. Mutant analysis and gene silencing by double-stranded RNA interference are helping to add to our understanding of subunit functions. In 2001 Titia Sixma and colleagues crystallised an acetylcholine binding protein from the snail, *L. stagnalis*, from which the first homology models of the ACh binding site are now being constructed. Thus, the impact on neural signalling studies of invertebrate models extends from behaviour to atomic structure. In the context of this Symposium, it is fitting that snails are again helping to accelerate our understanding of neuronal nicotinic acetylcholine receptors.

### A13.3–Peptide-gated Na<sup>+</sup> channels

G. Cottrell (University of St Andrews)

ENa/Deg channels have many roles. Some regulate Na<sup>+</sup> absorption in epithelia, are 'constitutively active' and are important in regulating blood pressure. Others, in mammalian peripheral neurones, are activated mechanically or by mild acidification, the latter being implicated in pain perception. Yet others occur in the CNS and elsewhere, e.g. pituitary, where their roles are unknown; not all are activated by acid. ENa/Deg channel subunits have two transmembrane sections (TM1 and TM2) with cytoplasmic N- and C-terminal domains. Each subunit possess a large extracellular loop with sections rich in cysteines. Most mammalian members are hetero-multimers. Some of the acid-sensing channels (ASICs) are *modulated*, apparently directly, by peptides. The peptide(FMRFa)-gated Na<sup>+</sup> channel of snail *Helix*

*aspersa* neurones was the first ENa/Deg channel shown to be ligand-gated. It is a very useful experimental model, because it occurs in large identified neurones, its natural ligand is known, and is probably a homo-multimer. The properties of the *Helix* neuronal channel and the heterologously expressed clone, *HaFaNaC*, are similar. The FMRFamide-gated Na<sup>+</sup> channel of the pond snail *Helisoma trivolvis* has also been cloned. Message for FaNaCs has been detected in several neurones that exhibit the fast Na<sup>+</sup> response to FMRFamide. The *Helisoma* neuronal channel and its heterologously expressed clone, *HtFaNaC*, are less sensitive to FMRFamide. Furthermore, unlike *HaFaNaC*, amiloride enhances the action FMRFamide. These differences are allowing some information to be obtained about active sites of these FaNaCs. The major question remains, however: what are their physiological roles?

#### **A13.4—Does NO have a role in long-term memory formation?**

P.R. Benjamin, Sussex Centre for Neuroscience, School of Biology, University of Sussex

The nitric oxide (NO)-cGMP pathway is implicated in a number of experimental models of plasticity underlying learning and memory. In a behavioural analysis of long-term memory (LTM) formation in the feeding system of the pond snail *Lymnaea* using a one trial appetitive conditioning paradigm we showed that there was a critical requirement for this pathway for up to 5 hours following training. Transient depletion of NO by PTIO injection blocked LTM formation as did the injection of L-NAME and ODQ indicating that the intact NO-cGMP pathway was necessary for LTM. An electrophysiological correlate of LTM was also blocked by PTIO injection showing that the dependence of LTM on NO is amenable to analysis at the neural level. One candidate nitrenergic neuron is the Cerebral Giant Cell of the feeding circuit. This neuron expresses the nNOS gene and electrophysiological recording showed that it was part of the electrical correlate of the LTM trace. It responded to the CS in reduced preparations by firing more strongly after behavioural training and was persistently depolarised post-training revealing a cellular correlate of LTM. At the molecular level, we have evidence that the level of nNOS gene expression increases transiently after conditioning suggesting that de novo NO synthesis may be important in LTM formation.

#### **A13.5—Forgetting in *Lymnaea* Is a Form of Learning Dependent On Altered Gene Activity**

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A 3-neuron central pattern generator, whose sufficiency and necessity has been directly demonstrated, mediates aerial respiratory behaviour in the pond snail, *Lymnaea stagnalis*. In the intact, freely moving snail this behaviour can be operantly conditioned, and the associative learning is consolidated into long-term memory (LTM) that can depend on the training procedure used last days to weeks. We have now directly established that one of the 3 CPG neurons, RPeD1, is a site of LTM formation and storage. We accomplished this by surgically removing the soma (and thus the nucleus) of RPeD1, leaving behind a fully functional primary neurite, where all synaptic interactions occur. If RPeD1's soma is removed before training, learning occurs, but LTM does not. We then showed that the persistence of LTM is extended (i.e. forgetting is inhibited) by ablating RPeD1's soma after memory consolidation occurred. Following RPeD1 soma ablation, memory does not wane. These and other data show that forgetting is thus dependent on altered gene activity and new protein synthesis in RPeD1. Forgetting can also be prevented by blocking the occurrence of interfering events post-learning can also prevent forgetting. Finally, we have now been able to show that similar learning and memory can occur in semi-intact preparations whilst we record from RPeD1. Together our results show that forgetting has many of the attributes of learning and memory and that there are directly attainable neural correlates of learning, memory and forgetting.

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#### **A13.6—Calcium stores used during muscarinic receptor activation in a holothurian smooth muscle**

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Responses of a holothurian smooth muscle (longitudinal muscle of the body wall, LMBW) to a range of mus-

carinic (M1 to M5) acetylcholine receptor (mAChR) agonists and antagonists were surveyed using calcium ( $\text{Ca}^{2+}$ )-selective electrodes and a mechanical recording technique. 4-DAMP, himbacine, oxotremorine, pilocarpine, pirenzepine, and telenzepine caused a net efflux of  $\text{Ca}^{2+}$  ions whereas only QNX hemioxalate caused  $\text{Ca}^{2+}$  influx. Carbachol, oxotremorine and oxotremorine M induced tonic muscle contractions. Agonist muscarine and antagonists, himbacine, pirenzepine, telenzepine and tropicamide increased the force of phasic contractions. 4-DAMP and gallamine acted as relaxants. Most of the antagonists tested caused phasic contractions of the LMBW possibly by a block of a non-specific, inhibitory pre-synaptic mAChR. M1-specific agents like oxotremorine M were the most potent at altering contractility indicating a primary role of a M1-like mAChR that was probably located post-synaptically in this preparation. We used a series of agents that disrupted intracellular  $\text{Ca}^{2+}$  ion sequestration and/or release (cyclopiazonic acid, CPA; caffeine; ryanodine), the phosphoinositide signaling pathway (LiCl) and L-type  $\text{Ca}^{2+}$  channels (diltiazem and verapamil) to investigate the possible  $\text{Ca}^{2+}$  sources tapped during mAChR activation. Oxotremorine M-induced contractions were significantly blocked by treatment with CPA, caffeine, LiCl, and by channel blockers, diltiazem and verapamil. These data suggest that the LMBW used an M1-like receptor that was linked to the phosphoinositide signaling pathway. This pathway primarily relied on intracellular  $\text{Ca}^{2+}$  stores (that were caffeine—but not ryanodine—sensitive) but also tapped extracellular  $\text{Ca}^{2+}$  via the opening of L-type channels.

### **A13.7—Pharmacological characterization of 5-hydroxytryptamine-induced contraction of the locust extensor tibiae muscle, using selective agonists, antagonists and second messenger manipulators, reveals the presence of 5-HT2-like and 5-HT1-like receptors**

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A range of selective agonists, antagonists and second messenger manipulators was used to attempt a pharmacological classification of the 5-HT receptors causing contraction of extensor tibiae muscles, incubated in Evan's locust Ringer (Evans, 1984a), isolated from adult *Schistocerca gregaria*. 5-HT ( $10^{-8}$  M –  $10^{-4}$  M) caused dose-dependent contraction with an EC<sub>50</sub> value of  $2 \times 10^{-6}$  M. The potency of the agonists tested was: 5-HT, 1; alpha-methyl 5-HT, 2.6; 5-methyltryptamine, 4.6; 5-methoxytryptamine, 6.2; 2-methyl 5-HT, 32; methysergide, 61; 8-OH DPAT, 76.6 and 5-carboxami-

dotryptamine, 1119. The 5-HT3 antagonist tropisetron and the 5-HT4 antagonist RS 23597-190 had no effect on 5-HT-induced contraction. In comparison, the 5-HT2 antagonists cinanserin, cyproheptadine, ketanserin, ritanserin and mainserin caused significant inhibition of contraction at  $10^{-6}$  M and  $10^{-5}$  M but were unable to abolish the effects of 5-HT. The 5-HT1/5-HT2 antagonists metergoline and methysergide reduced the effects of 5-HT by 90% at  $10^{-5}$  M while the mammalian 5-HT1 agonists RU24969 and buspirone were competitive antagonists. The protein kinase C inhibitor H7 ( $10^{-5}$  M) and  $\text{L}^{1+}$  ( $10^{-3}$  M) an inhibitor of IP3 recycling, potentiated the effects of 5-HT. However 5-HT-induced contraction was attenuated in the presence of the phosphodiesterase inhibitor theophylline ( $10^{-5}$  M), the L-type  $\text{Ca}^{2+}$ -channel blocker verapamil ( $10^{-5}$  M), the IP3 receptor modulator 2-aminoethoxydiphenylborate ( $10^{-5}$  M) and the SERCA ATPase inhibitor *tert*-butylbenzohydroquinone ( $10^{-5}$  M). These data suggest that the tissue contains at least two 5-HT receptors subtypes which have properties in common with mammalian 5-HT2 and 5-HT1 receptors.  
P.D. Evans (1984a), J. Exp. Biol. 110, 231–251.

### **A13.8—Crustacean cardioactive peptide (CCAP)-like molluscan peptide (M-CCAP) modulates the neuronal activity of the feeding network in the pond snail *Lymnaea stagnalis***

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The crustacean cardioactive peptide (CCAP) is a potent cardioexcitatory substance, originally identified in the pericardial organs of the shore crab, *Carcinus means*. It also modulates the neuronal activity in other arthropods. A CCAP-related new peptide family, the molluscan CCAP (M-CCAP) has been isolated and characterized from the snail *Helix pomatia* (Muneoka et al. 1994). Structural differences between the crustacean CCAP and the molluscan peptides are restricted only to the amidated end of the molecules.

Electrophysiological tests were carried out on identified buccal neurons of the isolated CNS of *Lymnaea stagnalis*. M-CCAP with a 0.1 micromolar threshold concentration modulated the activity pattern, while 1 micromolar M-CCAP occasionally evoked regular bursting activity (pattern of fictive feeding) from both motoneurons and the modulatory interneuron (SO) of the buccal feeding network. CCAP immunostaining revealed no immunopositive cells bodies, only fibers in the buccal ganglia, which terminate in the buccal neu-

ropile. Part of these fibers belong to the axonal processes of the cerebro-buccal interneurons since some of them showed CCAP immunopositivity.

Considering the present observations we suggest that the CCAP immunopositive cerebro-buccal interneurons modulate the activity of the buccal feeding network by liberating CCAP-like substance in the buccal neuropile.

Reference:

Y. Muneoka, T. Takahashi, M. Kobayashi, T. Ikeda, H. Minataka, K. Nomoto (1994). In: Perspectives of Comparative Endocrinology (eds. Davey, K.G., Peter, R.E., Tobe, S.S.) Nat. Res. Council, Canada. pp 109–118.

Acknowledgements:

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### A13.9–S. Modulation of *C. elegans* feeding by neuropeptides

Papioannou, L. Holden-Dye and R.J. Walker (University of Southampton).

Neuropeptides have a widespread distribution in the nematode nervous system (Sithigorngul et al., 1990) and more than 150 neuropeptides have either been biochemically isolated from, or predicted from the genomes of nematodes (Nelson et al., 1998; Nathoo et al., 2001). However, the biological relevance of many of these peptides has not yet been investigated. Here we report a preliminary assessment of the biological activity of these peptides on the somatic muscle of the parasitic nematode *A. suum* and on the pharyngeal muscle of *C. elegans*. Three of these are encoded by *flp* (FMRFamide-like peptides) genes and three by *nlp* (neuropeptide-like precursor) genes. Two of the latter show some sequence similarity to myomodulin, and one to buccalin, molluscan neuropeptides.

The effect of the peptides on the body wall muscle of *A. suum* was determined using an in vitro preparation of dorsal muscle strips as described in Trim et al., (1997). The excitatory neuromuscular junction transmitter acetylcholine (ACh; 10 micromolar) was added for 1 min and then washed out. This elicited a rapid and reversible contraction. This was repeated twice to obtain contractions of reproducible amplitude. Subsequently the neuropeptide was added to the preparation 2 min prior to ACh, and the amplitude of the contraction to ACh in the presence of the peptide was expressed as a % of the average amplitude of the three contractions prior to the addition of neuropeptide. None of the neuropeptides had a marked effect on basal muscle tension. However, 3 had a potent effect on the contraction elicited by ACh, with 2 acting as inhibitors and one as a potentiator.

The effect of peptides on the pharyngeal muscle of *C. elegans* was determined by extracellular recordings of

muscle activity. FLP15A (GGPQGPLRFamide) and FLP11 (AMRNALVRFamide) both had an effect. Further studies are in progress to assess their mechanism of action in *C. elegans*.

Nathoo, A.N., Moeller, R.A., Westlund, B.A. & Hart, A.C. (2001). Proceedings Of The National Academy Of Sciences Of The United States Of America **98**, 14000–14005.

Nelson, L.S., Kim, K.Y., Memmott, R.E. & Li, C. (1998). Molecular Brain Research **58**, 103–111.

Sithigorngul, P., Stretton, A.W. & Cowden, C. (1990). Journal of Comparative Neurology **294**, 362–376.

Trim, N., Holden-Dye, L., Ruddell, R. & Walker, R.J. (1997). Parasitology **115**, 213–222.

### A13.10–Characterisation of G-protein-coupled receptors in *C. elegans*

N. Kriek and D. Marsden, University of Southampton; C. Keating and M. Daniels, University of Sussex; R.J. Walker, University of Southampton; J.F. Burke, Genetix Ltd; L. Holden-Dye, University of Southampton

This is a systematic analysis of the functional role of a family of GPCRs in *Caenorhabditis elegans*. We describe progress in the classification of *C. elegans* GPCRs, their functional analysis using reverse genetics, and definition of the cognate ligands.

The study focused on sixty-five rhodopsin-like GPCRs in *C. elegans*. Their functional role was assessed by RNA interference (RNAi) and indicates a non-redundant role for seven neuropeptide-like GPCRs in locomotion, and for four other receptors in the regulation of brood size.

More than 70 neuropeptide precursor genes have been identified in *C. elegans*, encoding more than 150 putative neuropeptides (Li et al., 1999, Ann. N. Y. Acad. Sci., 897: 239–252). These represent candidate ligands for GPCRs. To define receptor/ligand pairs we have taken three complementary approaches. First, pharmacological responses to neuropeptides were determined in an isolated pharyngeal preparation taken from worms treated with RNAi for specific GPCRs to test for a reduction in response compared to wildtype. Second, responses to neuropeptides were measured in worms overexpressing GPCRs in the pharynx. Third, we used reporter constructs to determine whether the receptor and ligand are expressed in a physiologically relevant synaptic context. This will facilitate pairing *C. elegans* orphan GPCRs with cognate ligands to provide further insight into structure–function relationships for this gene family.

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### A13.11–Inhibitory glutamate receptors and the control of *C. elegans* pharyngeal muscle

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The target of the anthelmintic, ivermectin, is a glutamate-gated chloride channel (GluCl). Little is known about the physiological roles and subunit stoichiometries of native GluCl receptors in nematodes. We are using *C. elegans* to address these issues, employing immunohistochemistry and electrophysiological analysis of mutant strains or worms treated with RNAi for specific GluCl subunits. Here we evidence for the role of GluCl subunits in receptor regulation within the pharyngeal muscle of *C. elegans*.

The pharynx exhibits a desensitising depolarisation to glutamate, mediated by at least two GluCl subunits. GluCl $\alpha$ 2 confers both a high affinity for glutamate and rapid desensitisation (1). GluCl $\beta$  is also expressed in pharyngeal muscle (2). GluCl $\beta$  RNAi further reduces the affinity for glutamate in *avr-15* mutants. Wild-type worms treated with GluCl $\beta$  RNAi display significantly faster desensitisation kinetics across a range of glutamate concentrations.

Pharmacological evidence suggests the possibility that further GluCl subunits are expressed in pharyngeal muscle. However, immunohistochemical data, and the results of electrophysiological experiments performed on mutant or RNAi treated strains for the remaining known GluCl subunits are not consistent with their involvement in the pharyngeal GluCl receptor. This may suggest the existence of further, as yet undiscovered GluCl subunits in the *C. elegans* genome.

Refs:

(1) Pemberton et al. (2001) Mol. Pharmacol. 59: 1037–43

(2) Dent et al. (1997) EMBO J. 16: 5867–79

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### A13.12–Sodium-dependent currents in the pharyngeal muscle of *C. elegans*

I. Vinogradova, R.J. Walker and L. Holden-Dye, University of Southampton

To make full use of *C. elegans* for investigating gene function it is imperative that physiological approaches for phenotypic analysis are made available. Our contribution to this effort has been to establish patch-clamp recordings for *C. elegans* in a semi-intact worm prepa-

ration which includes muscle and a simple enteric nervous system consisting of 20 neurones. We have used this preparation to study determinants of muscle excitability by manipulating the ionic environment, using pharmacological agonists and blockers and by comparing the properties of wild-type with mutant worms. Evidence points to the involvement of a voltage-gated Na<sup>+</sup> current in the control of muscle excitability. This is intriguing as there are no obvious candidate genes for voltage-gated Na<sup>+</sup> channels in the *C. elegans* genome. To establish whether the Na<sup>+</sup> dependence derived from the presence of a Na<sup>+</sup>-dependent channel in the muscle itself, rather than from a Na<sup>+</sup>-dependent neurogenic event, we recorded voltage-activated currents directly from muscle. In whole-cell recordings depolarizing steps activated inward currents that apparently consisted of two components; a fast activating and inactivating component that peaked at –50mV, and a slowly activating, non-inactivating component that peaked at 0 mV. We studied the effect of various channel blockers on the inward current. The most striking finding, however, was that although the inward current was not significantly affected by removal of Ca<sup>++</sup> from the external solution, the inward current elicited by steps to –50mV was reduced by 75% in the absence of external Na<sup>+</sup>, whilst the inward current elicited by steps to more depolarized potentials were unchanged. To provide a detailed analysis of the channels that may contribute to this voltage-dependence we made single channel recordings from the muscle. We characterised a unitary inward current at –80 mV that was selectively reduced in the absence of external Na<sup>+</sup> and for which mean open time increased with depolarising steps. Taken together, our data suggest the presence of a Na<sup>+</sup> selective channel with unusual properties, pivotal to muscle excitability. Re-evaluating data from homology searches has suggested candidate gene(s) that may confer this Na<sup>+</sup> sensitivity.

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### A13.13–The mechanism of action of the anthelmintic emodepside

Willson, J., Amliwala, K., Holden-Dye, L., Harder<sup>1</sup>, A. & Walker, R.J. (University of Southampton, UK; <sup>1</sup>Bayer, Germany)

Resistance of parasitic nematodes to existing anthelmintics has encouraged the search for novel compounds. A new compound, emodepside, a 24 membered cyclic depsipeptide, acts as a potent, broad-spectrum anthelmintic. It causes fast onset paralysis of nematodes, favouring

the view that it is neuropharmacologically active. The aim of this project is to define and characterise the site of action of emodepside.

The action of emodepside on the body wall muscle of the nematode *Ascaris suum* was investigated. Dorsal muscle strips (DMS) were bathed in artificial perienteric fluid (APF). The DMS was contracted using acetylcholine (ACh; 30  $\mu$ M, 30 secs). The amplitude of this contraction after pretreatment of the DMS with emodepside (10  $\mu$ M, 10 mins) was expressed as a percentage of the control contraction (i.e. without emodepside pretreatment). Emodepside reduced the contraction by  $39\% \pm 4$  (n=9).

To elucidate whether emodepside acts pre-or post-synaptically at the neuromuscular junction, the effect of emodepside was investigated on a denervated muscle strip preparation. Application of emodepside (10  $\mu$ M) to the denervated muscle strip caused no reduction in ACh induced contraction (n=7).

These data show that emodepside has an inhibitory action on locomotion and that its action in the nematode motor nervous system is likely to be pre-synaptic to the neuromuscular junction. Recent work on the model organism *C. elegans* has shown that emodepside potently inhibits pharyngeal pumping ( $IC_{50}$  5.9nM) and locomotion ( $IC_{50}$  4.1nM). Further experiments are in progress to define the signaling pathway for emodepside.

#### **A13.14–**

Abstract withdrawn

#### **A13.15–Pharmacology and second messengers of octopamine receptors in the pond snail *Lymnaea stagnalis***

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We recently showed a major role for octopamine as a transmitter in the buccal ganglia of the snail *Lymnaea stagnalis* (Vehovszky & Elliott, 2001, J Neurophysiol. 86:792–808). Most octopamine responses in insects are mediated by adenylyl cyclase-dependent mechanisms (see, Roeder 1999, Prog Neurobiol 59: 533–561 for review). We have recorded simultaneously the responses in the B1, B2 and B3 motoneurons to bath-applied octopamine and examined their cellular signalling mechanisms.

The B1 response is excitatory and is reduced in low sodium saline. It is enhanced by preincubation with IBMX and reduced by the cyclase blocker SQ 22536. Application of the direct cyclase activator forskolin also depolarises the B1 motoneuron. Taken together, these observations suggest that the B1 has an octopamine receptor that activates adenylyl cyclase.

The B2 and B3 neurons are hyperpolarised by octopamine, and their responses are not affected by IBMX or SQ 22536. Forskolin has a weak excitatory effect on the B2 motoneuron, producing a bursty pattern. No inhibition was seen. We conclude that the inhibitory octopamine receptor in the B2 and B3 neurons, does not use cAMP for signalling, and must use a different pathway.