

Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, *Ephydatia muelleri* (Demospongiae, Spongillidae)

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SUMMARY

The view that sponges lack tissue level organisation, epithelia, sensory cells and coordinated behaviour is challenged by recent molecular studies showing the existence in Porifera of molecules and proteins that define cell signalling systems in higher order metazoans. Demonstration that freshwater sponges can contract their canals in an organised manner in response to both external and endogenous stimuli prompted us to examine the physiology of the contraction behaviour. Using a combination of digital time-lapse microscopy, high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis, immunocytochemistry and pharmacological manipulations, we tested the role of the diffusible amino acids glutamate and γ -aminobutyric acid (GABA) and a short-lived diffusible gas, nitric oxide (NO), in triggering or modulating contractions in *Ephydatia muelleri*. We identified pools of glutamate, glutamine and GABA used to maintain a metabotropic glutamate and GABA receptor signalling system. Glutamate induced contractions and propagation of a stereotypical behaviour inflating and deflating the canal system, acting in a dose-dependent manner. Glutamate-triggered contractions were blocked by the metabotropic glutamate receptor inhibitor AP3 and by incubation of the sponge in an allosteric competitive inhibitor of glutamate, Kynurenic acid. Incubation in GABA inhibited glutamate-triggered contractions of the sponge. Nitric oxide synthase, involved in the formation of the diffusible gas NO, was localised using NADPH-diaphorase to mesenchyme cells in the osculum and pinacoderm. A cGMP assay showed the same cells were labelled suggesting that the NO system is functional. Our findings suggest sponges coordinate behaviour using chemical messenger systems common to other animals.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/213/13/2310/DC1>

Key words: coordination, evolution of nervous systems, conduction, nitric oxide, GABA, glutamate.

INTRODUCTION

Sponges are benthic suspension feeders that are often described as lacking a tissue level of organisation, sensory cells and coordinated behaviour – features that are characteristic of eumetazoans. The absence of these features is usually attributed to the early branching of sponges from other metazoans, yet intriguingly, recent molecular and physiological studies generated by the new genome project on the encrusting demosponge *Amphimedon queenslandica* suggest that Porifera possess many of the molecules that are involved in cell signalling systems in higher metazoans (for a review, see Adamska et al., 2007; Richards et al., 2008; Sakarya et al., 2007). The genomic findings force us to ask whether these components reflect a primitive pre-nervous system or whether sponges have a signalling and coordination system similar to that of other animals but without the use of conventional nerves and muscle.

While the absence of nerves and of rapid behaviour in sponges has intrigued researchers for over a century, at the same time it has posed difficulties for testing hypotheses using conventional techniques that require observation of responses to stimuli. The general activities of cellular sponges include slow contractions across part and occasionally all of the body, closure of the osculum (the vent for water and the most distinct morphological region for observation) and slowing or cessation of pumping (filtering). Three mechanisms of signalling have been proposed to explain these behaviours: electrical, mechanical or chemical (Jones, 1962). Electrical signalling is only known from glass sponges, presumably because they are syncytial, which means that action potentials can

move throughout the animal unimpeded by cell boundaries (Leys, 1995; Leys and Mackie, 1997; Leys and Meech, 2006). Mechanical signalling, through tugging on each cell by neighbour cells (Emson, 1966), and chemical signalling, through diffusible molecules either in the aquiferous canal system or through paracrine signalling in the mesohyl have been investigated extensively in cellular sponges with few concrete results except to show that sponges respond by slow contraction to touch, electric shock, temperature change, sediment and chemical agents (reviewed in Leys and Meech, 2006). It is unlikely that contractions propagate by mechanical tugging of cell-on-cell because controlled inflation and contraction responses can still propagate through sponges whose tissue is torn by the application of stimulants or whose osculum has been removed (this paper; and S.P.L., personal observations). However, two observations strongly suggest that paracrine chemical signalling is the most likely mechanism of signal transduction. First, cells in the mesohyl stop crawling as contractions pass over a region (de Vos and Van de Vyver, 1981; Elliott and Leys, 2007), and second, the choreography of a stereotypical behaviour of inflating and deflating the aquiferous system in response to clogging or agitation [called the inflation–contraction response (Elliott and Leys, 2007)] involves the coordination of regions hundreds of micrometres apart at the same moment. Paracrine chemical signalling mechanisms in sponges rely on a molecule binding to a ligand–receptor system that controls the movement of ions directly (ionotropic) or indirectly (metabotropic) into and out of the cells *via* G-protein coupled

pathways, possibly by an amino acid [e.g. glutamate, γ -aminobutyric acid (GABA)], a biogenic amine (e.g. epinephrine), a peptide (e.g. RF-amides, melatonin) or a short-lived gas [e.g. nitric oxide (NO), carbon monoxide].

Glutamate, GABA and NO are important chemical messengers that are found in plants, protists and metazoans where they function in feeding, sensory systems, development and also act as neuro-active compounds (Lam et al., 1998; Moroz, 2001; Bouche et al., 1999). Molecular evidence suggests that receptors for glutamate, GABA and NO were present before plants and animals diverged (Lam et al., 1998; Moroz, 2001). The first characterisation of any of these receptors from a sponge was a putative dual Glutamate/GABA receptor from *Geodia cydonium* (Perovic et al., 1999). However, the recent work from the genome of the demosponge *A. queenslandica* (<http://www.jgi.doe.gov/sequencing/why/3161.html>) has shown there to be metabotropic, but no ionotropic, glutamate receptors, as well as GABA and NO receptors (Sakaraya et al., 2007). The post-synaptic scaffolding proteins required for signalling have also been found in sponges, yet morphological and behavioural demonstration of 'proto-synaptic' complexes is still lacking (Sakaraya et al., 2007; Richards et al., 2008). Glutamate, GABA and NO have been found to play a role in stimulating and modulating contractions in the demosponge *Tethya wilhelma* (Ellwanger and Nickel, 2006). Furthermore, GABA-immunoreactive proteins are said to be localised in cells associated within the aquiferous canal system in *Chondrilla nucula* (Ramoino et al., 2007), and NO has been implicated in temperature stress activation in *Axinella polypoides* and *Petrosia ficiformis* (Giovine et al., 2001). In the absence of more direct mechanisms of signalling between sponge cells, the slowness of the coordinated behaviour shown by sponges makes glutamate/GABA signalling systems and modulation by a NO system a most likely hypothesis.

Ephydatia muelleri has coordinated contractions of the aquiferous canal system that function to expel wastes and to flush the canal system on a regular basis (Elliott and Leys, 2007). We hypothesised that these sponges are able to coordinate or modulate contractions by the use of small diffusible molecules such as amino acids (glutamate, GABA) or a short-lived diffusible gas (NO). Using a combination of microscopy techniques, immunocytochemistry and pharmacological manipulations we provide a description of possible signalling systems of the freshwater sponge *E. muelleri*. We have found that glutamate triggers the inflation-contraction cycle of the sponge in a dose-dependent manner that varies with amplitude and duration. Glutamate-triggered contractions can be inhibited by general metabotropic glutamate blockers and also by incubation of the sponge in GABA. Nitric oxide synthase (NOS) is found in choanocytes, in dendritic cells of the apical pinacoderm, osculum and in cells that line the excurrent canal system. Upon stimulation by a NO donor, cGMP was localised to cells in the osculum, which also contracted. These results confirm a role for a chemical messenger signalling system in sponges and offer insight into the mechanisms by which coordination of contractile behaviour occurs in the absence of a conventional nervous system.

MATERIALS AND METHODS

Collection and culturing of sponges

Pieces of the freshwater sponges *Ephydatia muelleri* (Lieberkuhn 1855) and *Spongilla lacustris* (Linnaeus 1759) consisting of a spicule scaffold containing gemmules were collected in winter months from sunken trees at a depth of 0–3 m in Frederick and Rousseau Lakes, BC, Canada (48°47'51.7559°N, 125°2'58.5600°W), and stored in sealed bags of unfiltered lake water at 4°C in the dark until ready

to use. The water was aerated once a month and gemmules kept in this way were viable for up to one year. The gemmules were mechanically dissociated from the dead sponge skeleton, sterilised with 1% hydrogen peroxide, rinsed well in distilled water and plated on glass coverslips as described previously (Elliott and Leys, 2007). Sponges were grown in M-medium (Funayama et al., 2005), which was replaced every 48 h. Whole-mount preparations consisted of a single gemmule on an ethanol flamed sterilised 22 mm² coverslip in 60 mm diameter Petri dishes, which allowed for easy transfer of the sponge into test substances and rinses. Only sponges 7–10 days post-hatching (d.p.h.) with a fully functional aquiferous canal system were used for experimentation, and care was taken not to use sponges if they had begun to regress due to lack of feeding.

High-performance liquid chromatography (HPLC)

For HPLC, 150–200 sponges were grown in mass cultures on 6 cm diameter Petri dishes. The growth medium was changed every 24 h. At 7 d.p.h. the medium was aspirated off and tissue was scraped from the bottom of the Petri dishes with a sterile, plastic tissue scraper, and transferred using a sterile glass pipette to 1.6 ml microfuge tubes. The tissue was centrifuged for 1 min at 1000g to remove excess M-media. The final mass of each preparation was ~200 mg. The sponge tissue was immediately frozen in liquid nitrogen, weighed and stored at –80°C until analysed.

HPLC coupled with fluorimetric detection and a derivitisation procedure (Grant et al., 2006) was used to separate and detect amino acids at the Neurochemical Research Unit of the University of Alberta. Sponge tissue samples of both *E. muelleri* and *S. lacustris* were homogenised in 5 volumes of distilled water. The homogenate was diluted 1:3 in methanol to precipitate proteins, immersed in ice for 10 min and centrifuged (13,000g) at 4°C. Stock solutions of amino acid standards [glutamate, L-glutamine, L-aspartate, L-asparagine, L-alanine, D-serine, L-serine, L-serine-*O*-phosphate, L-tryptophan, glycine, GABA, L-threonine, L-tyrosine, L-valine; Sigma-Aldrich, Oakville, ON, Canada) were prepared in 20% (v/v) methanol at a concentration of 1.0 mg ml^{–1} and were used to identify peaks in the samples based on retention time and to quantify amino acids in the samples based on a 9-point calibration curve. The actual standard concentrations of glutamate, aspartate, serine, glutamine, alanine, glycine, tyrosine and tryptophan curves were 6, 4, 2, 1, 0.5, 0.2, 0.1 and 0.04 µg ml^{–1}; asparagine and GABA curves were 3, 2, 1, 0.5, 0.25, 0.1, 0.05 and 0.02 µg ml^{–1}; threonine and valine were 1.5, 1, 0.5, 0.25, 0.125, 0.05, 0.025 and 0.01 µg ml^{–1}. Derivitisation reagent solutions were prepared by dissolving 1 mg of α -phthalaldehyde (OPA; Sigma-Aldrich) and 2 mg of *N*-isobutyl-L-cysteine (IBC; Sigma-Aldrich) in 0.1 ml of methanol followed by the addition of 0.9 ml 0.2 mol l^{–1} of sodium borate buffer (pH 10). Automated pre-column derivitisation was carried out on 5 µl of sample, standard or blank and 5 µl of derivitising agent that was injected into a HPLC system for analysis. The HPLC system consisted of a Waters Alliance 2690XE instrument equipped with a Waters 474 programmable fluorescence detector (Waters Corporation, Milford, MA, USA) that pumped a mobile phase containing 15% methanol in 0.04 mol l^{–1} sodium phosphate buffer (pH 6.2) through a Waters Symmetry C18 column (3.9 mm × 20 mm, 5 µm). Samples were detected with an excitation wavelength of 344 nm and an emission wavelength of 443 nm; spectra were depicted by change in voltage.

Digital time-lapse and data acquisition

For all pharmacology experiments, digital images of whole-mount sponges were viewed on a stereomicroscope (Olympus SZX-12,

Markham, ON, Canada) captured with a QI-Cam monochrome CCD camera (QImaging, Surrey, BC, Canada) with a colour filter. Images were captured using Northern Eclipse version 7 (Empix Imaging Inc., Mississauga, ON, Canada) from live video feed every 5 s. For image analysis, changes in the diameter of canals for every first, fifth, tenth or twentieth image of the aquiferous canal system (perpendicular to the long axis of the canal) were measured in triplicate using either Northern Eclipse or Image J 1.31 (NIH, Washington, MA, USA) and data were logged into MS Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Centre, middle and peripheral canals were classed based on location and resting diameter as characterised in Elliott and Leys (Elliott and Leys, 2007). Graphs were created using Sigma Plot (Systat Software, Inc., San Jose, CA, USA).

Test substance application

The contractile mechanism, response and coordination of the aquiferous canal system in *E. muelleri* was characterised and tested by the use of calcium and magnesium-free media [CF (calcium-free) and CMF (calcium–magnesium-free) media] (M-media without MgSO_4 and CaCl_2 but with 1 mmol l^{-1} NMDG–Cl, 0.5 mmol l^{-1} NMDG– SO_4 , 0.5 mmol l^{-1} EGTA and/or 0.005 mmol l^{-1} EDTA), glutamate (Sigma-Aldrich) and GABA (Sigma-Aldrich) with an antagonist of metabotropic glutamate receptors (m-GluR), 2-amino-3-phosphonopropionic acid (AP3, Sigma-Aldrich) (Young et al., 1994), and a non-competitive inhibitor of m-GluR, Kynurenic acid (KYN, Sigma-Aldrich). In order not to move sponges prior to experiments, sponges in 60 mm diameter Petri dishes containing 20 ml of M-medium were left on the microscope stage to relax for one hour prior to each experiment. Stock solutions of 20 mmol l^{-1} glutamate, 50 mmol l^{-1} GABA, 50 mmol l^{-1} AP3 and 50 mmol l^{-1} KYN were prepared in distilled water or dimethylsulphoxide (DMSO) and were added directly to the medium to reach the final concentrations listed in each experiment. Great care was taken to always add solutions at the side of the Petri dish opposite to the sponge and to mix by gently pipetting 5–6 times for each test substance; equivalent mixing of fluorescein dye confirmed the uniform distribution of the test substances in the dish. The sponges did not respond to equivalent additions of M-medium and mixing. For blockers, sponges were placed into the final diluted medium in a Petri dish and allowed to sit undisturbed for 60 min. For each experiment sponges were filmed for 10 min prior to any treatment to ensure that canals were at resting diameter immediately prior to addition of the test substance.

NADPH-diaphorase histochemical detection of NOS

Juvenile sponges were fixed by direct immersion into a mixture of 3.7% paraformaldehyde and 0.3% glutaraldehyde in 0.1 mol l^{-1} phosphate buffered saline (PBS) for 24 h at 4°C (Elliott and Leys, 2007). After fixation, preparations were rinsed three times in cold PBS, twice in 0.1 mol l^{-1} Tris-HCl buffer (pH 8.0) for 5 min each and permeabilised for 10 min in 0.1 mol l^{-1} Tris-HCl buffer (pH 8.0) with 0.25% Triton X-100. For histochemical detection of NOS, sponges were incubated with 1 mmol l^{-1} β -NADPH (Sigma-Aldrich), 0.5 mmol l^{-1} Nitro Blue Tetrazolium (Sigma-Aldrich), 0.1 mmol l^{-1} dicumarol (Sigma-Aldrich), 0.25% Triton X-100 in 0.1 mol l^{-1} Tris-HCl buffer (pH 8.0) in the dark until colour development was reached. The reaction was stopped by rinsing in cold PBS four times. Sponges were post-fixed in 4% paraformaldehyde in methanol for 1 h at 4°C and rinsed in 100% dry ethanol. The ethanol was allowed to evaporate, and coverslips with sponges were inverted, mounted slightly raised on Vaseline® legs in 100% glycerol. Edges were sealed with nail polish. Images were captured as indicated above.

cGMP assay for NO reaction

Juvenile sponges were incubated in 1 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX: phosphodiesterase inhibitor; Sigma-Aldrich) in Strekal's medium, an equivalent culture medium to M-medium (Strekal and McDiffett, 1974), diluted from a 0.5 mol l^{-1} stock solution in DMSO for 30 min. Sponges were exposed to 0.1 mmol l^{-1} *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP: NO donor; Sigma-Aldrich) for 2 min and fixed in 4% paraformaldehyde in PBS for 2 h at 4°C . Coverslips with sponges were rinsed (30 min) and inverted on a solution containing primary rabbit anti-cGMP (1:3000) (Millipore-Chemicon, Billerica, MA, USA) with 3% goat serum (Sigma-Aldrich) and 0.1% Triton X-100 in cold PBS and placed on a shaker at 4°C overnight. Preparations were rinsed in PBS and labelled with a solution containing Alexa 488 goat anti-rabbit (1:100) (Molecular Probes-Invitrogen, Carlsbad, CA, USA) with 10% goat serum in cold PBS on a shaker for 3 h. Sponges were rinsed in PBS, mounted in 100% glycerol, sealed with nail polish and imaged with a Zeiss Axioskop epifluorescence microscope with a QIcam CCD camera operated by Northern Eclipse.

RESULTS

Evidence for neurotransmitter molecules in sponge tissues:

HPLC–MS analysis

Samples of sponge tissue analysed by HPLC revealed chromatographs of molecules that co-eluted with standards for the putative amino acid neurotransmitters aspartic acid, glutamic acid, asparagines, serine, glutamine, glycine, threonine, taurine, alanine, GABA, tryptophan and valine (Fig. 1). All amino acids were found in each sample with a detection limit ranging from 0.01 to $0.04\text{ }\mu\text{g ml}^{-1}$ of supernatant. As shown in Table 1, the amino acid levels were low except for glutamic acid, glutamine, aspartic acid, glycine and valine. Similar high concentrations were found for glutamate in *S. lacustris*; however, all other amino acids in that sponge were low compared with *E. muelleri* (Table 1, Fig. 1). When single sponges were exposed to 30 mmol l^{-1} KCl in an attempt to activate release of possible transmitters, no detectable amino acids were released into the supernatant.

Evidence for a metabotropic glutamate signalling system

The juvenile freshwater sponge has four regions that can be monitored by stereomicroscopy for a response to stimulants: the osculum (vent), the tent (dermal tissues that lie above a space which surrounds the choanosome), the choanosome (housing the choanocyte chambers which are the pumping and filtration units), and the incurrent and excurrent canal systems (the aquiferous canal system). In the following description we refer to each of these in turn.

Glutamate triggered both an instant contraction of the osculum and tent and also stimulated a full inflation and contraction of the aquiferous canal system with the same characteristics as those provoked by vigorous mechanical stimulation (Fig. 2) (Elliott and Leys, 2007). Both the initial contraction of the tent and triggering of the full inflation–contraction cycle were dependent on the concentration of glutamate. The inflation–contraction cycle was triggered at an agonist concentration of $70\text{--}90\text{ }\mu\text{mol l}^{-1}$ of glutamate (Fig. 3; supplementary material Movies 1 and 2). All sponges were undisturbed for 1 h and then filmed for 10 min prior to application of glutamate in order to make sure that an inflation–contraction cycle was not spontaneously occurring. Concentrations of agonist below $30\text{ }\mu\text{mol l}^{-1}$ had no effect on the sponge, and concentrations above $100\text{ }\mu\text{mol l}^{-1}$ were fatal. A sub-threshold agonist concentration ($30\text{--}60\text{ }\mu\text{mol l}^{-1}$) application of glutamate triggered a gradient of behaviours. The first response was an initial lowering of the dermal tissues or tent, pushing down on the subdermal cavity and

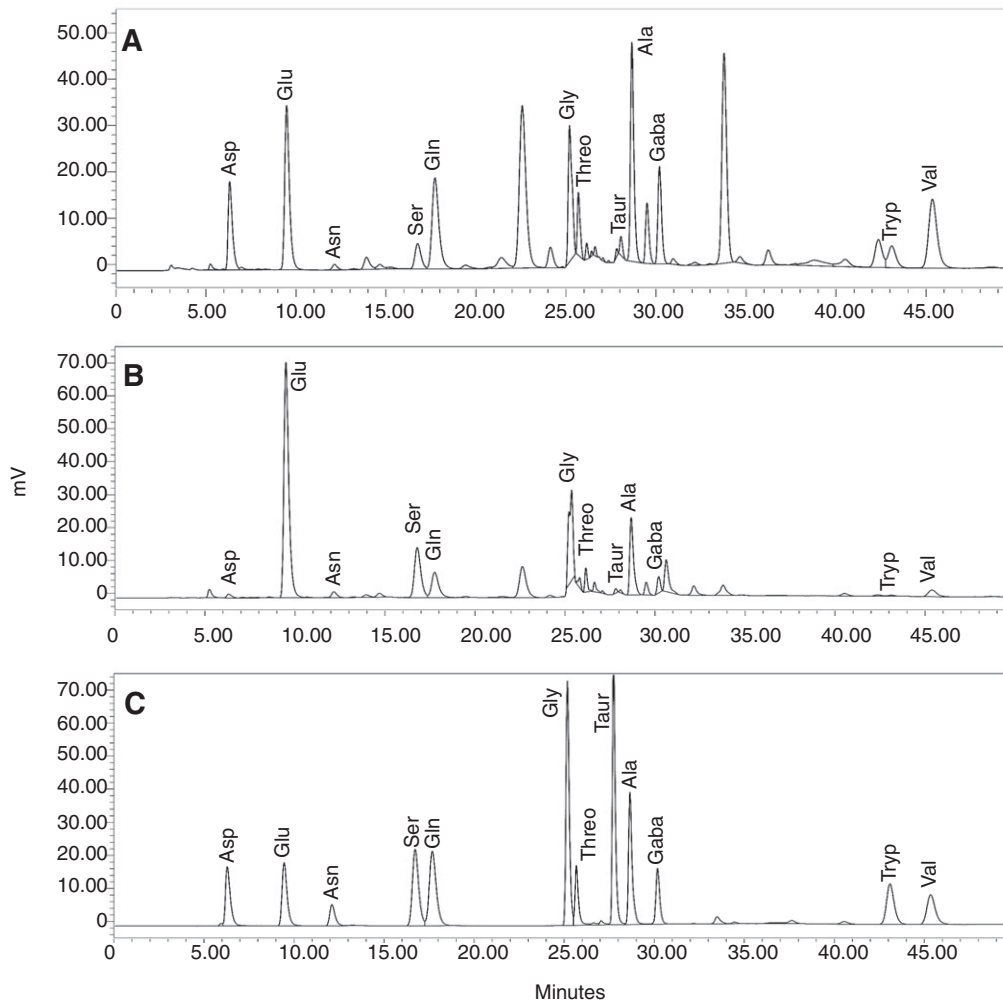


Fig. 1. High-performance liquid chromatography–mass spectrometry (HPLC–MS) chromatographs of amino acids in tissue of *Ephydatia muelleri* and *Spongilla lacustris*. Peaks represent putative active neuronal amino acids; the baseline indicates the mobile (carrier) phase.

(A) *Ephydatia muelleri*, (B) *S. lacustris*, (C) amino acid standards. Glu, Asp, Ser, Gln, Ala, Gly, Taur and Trp were used at $2\mu\text{g ml}^{-1}$, Asn and GABA were used at $1\mu\text{g ml}^{-1}$, and Threo and Val at $0.5\mu\text{g ml}^{-1}$. Aspartate (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), threonine (Threo), taurine (Taur), alanine (Ala), tryptophan (Trp), γ -aminobutyric acid (GABA) and valine (Val).

choanosome, and its return to normal diameter (supplementary material Movie3). Following this the incurrent canals contracted thereby expanding the excurrent canals; however, it was not until the application of $70\mu\text{mol l}^{-1}$ of glutamate that a consistent inflation of the excurrent canals was generated by the contraction of the incurrent canals, and at that threshold level of $70\mu\text{mol l}^{-1}$ of glutamate a full inflation–contraction cycle occurred (Fig. 3), which was similar to the response of sponges stimulated by ink and agitation (Elliott and Leys,

2007), except that the plateau phase was much reduced. At an agonist concentration of $80\text{--}100\mu\text{mol l}^{-1}$ of glutamate the dermal tissues contracted so severely that often either the tent or choanosome tore (supplementary material Movie4); interestingly however the canals and osculum could still inflate and contract normally even without the intact structure of the overlying dermal tissues. At an agonist concentration of $80\mu\text{mol l}^{-1}$ of glutamate, two types of events occurred: either the sponge choanosome began to fibrillate and then fully contracted with no inflation–contraction cycle, or it began an inflation–contraction cycle ending with contraction of the excurrent canals and did not recover for a long period. At an agonist concentration of $100\mu\text{mol l}^{-1}$ of glutamate it appeared as if both the incurrent and excurrent canals contracted at the same time thus preventing the normal inflation event (see Movie4 and Fig. S1 in supplementary material).

As the agonist concentration increased from $30\mu\text{mol l}^{-1}$ to $100\mu\text{mol l}^{-1}$ the amplitude and duration of the inflation–contraction cycle increased (Fig. 4A). The rate of contraction of both incurrent and excurrent canals (i.e. during the inflation and contraction phases, respectively) was similar (Fig. 4B) but both phases contracted faster than when stimulated by agitation (Elliott and Leys, 2007).

Evidence for function of glutamate receptors

The maximum concentration that elicited a full inflation–contraction response was $80\mu\text{mol l}^{-1}$ of glutamate, and so concentrations of

Table 1. Amino acid levels for tissue from *Ephydatia muelleri* and *Spongilla lacustris* analysed by HPLC

| Amino acid | <i>E. muelleri</i> | <i>S. lacustris</i> |
|-----------------------------|--------------------|---------------------|
| Aspartic acid | 24.98 ± 3.23 | 4.67 ± 0.33 |
| Glutamic acid | 48.21 ± 6.03 | 116.94 ± 3.94 |
| Asparagine | 1.58 ± 0.14 | 2.76 ± 0.20 |
| Serine | 4.67 ± 1.09 | 16.40 ± 2.39 |
| Glutamine | 23.16 ± 2.59 | 10.40 ± 1.38 |
| Glycine | 12.38 ± 0.98 | 14.77 ± 1.24 |
| Threonine | 9.88 ± 0.90 | 3.28 ± 0.13 |
| Taurine | 2.97 ± 0.57 | 1.97 ± 0.26 |
| Alanine | 28.16 ± 3.84 | 19.56 ± 1.43 |
| γ -Aminobutyric acid | 6.09 ± 1.28 | 2.67 ± 0.16 |
| Tryptophan | 7.13 ± 1.94 | 2.59 ± 0.11 |
| Valine | 18.58 ± 3.06 | 4.86 ± 0.17 |

Values are expressed in $\mu\text{g ml}^{-1}$ of sample as means \pm s.e. ($N=5$ colonies of 150–200 sponges). HPLC, high-performance liquid chromatography.

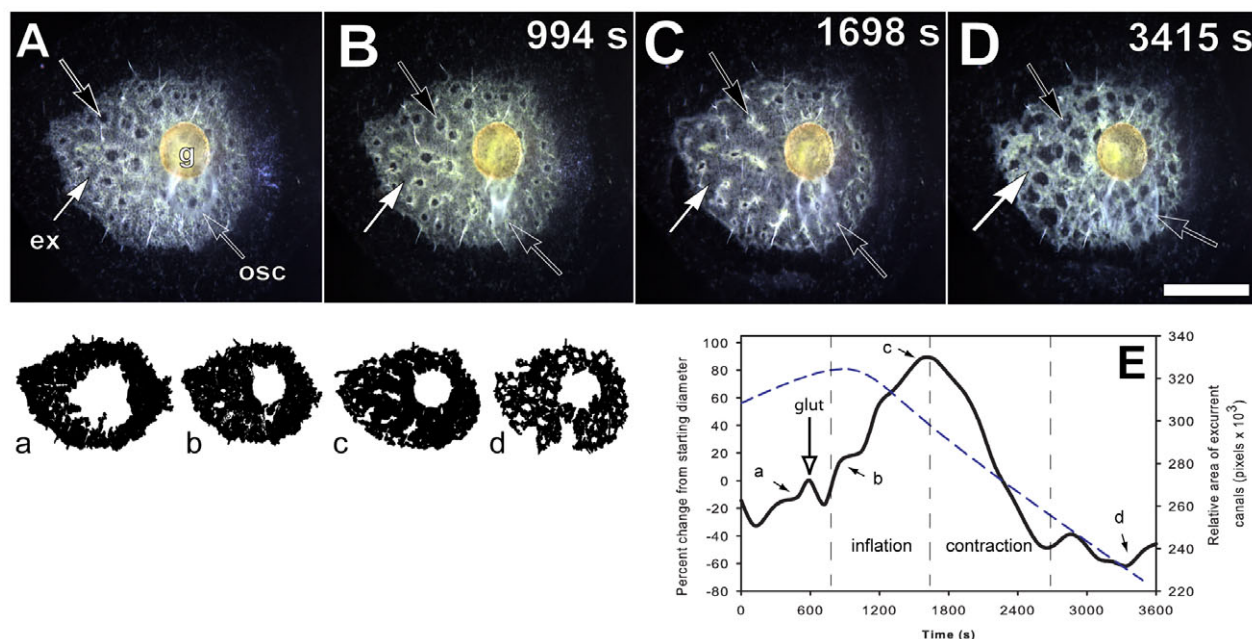


Fig. 2. Response of *Ephydatia muelleri* to application of $80\mu\text{mol l}^{-1}$ of glutamate. (A–D) Images showing the stages of contraction and the (a–d) corresponding areas of the excurrent aquiferous canal system. (E) A plot of the change in diameter of the largest excurrent canal (solid line) and the area of the excurrent aquiferous canal system (dashed line) in response to a stimulus of $80\mu\text{mol l}^{-1}$ of glutamate. The application of glutamate is shown as are the periods of inflation and contraction and times at which each image (A–D) are indicated. Gemmule (g), osculum (osc), glutamate (glut), excurrent canals (ex, white arrow) and incurrent canals (black arrow). Scale bar, 1 mm.

$75\mu\text{mol l}^{-1}$ and $80\mu\text{mol l}^{-1}$ were used in the following experiments to investigate the type of receptor involved, its competitive ability and the role of calcium stores. The receptor type was evaluated by using AP3, widely used as a general m-GluR antagonist. When sponges were treated in control experiments with $75\mu\text{mol l}^{-1}$ and $80\mu\text{mol l}^{-1}$ of glutamate, they exhibited normal glutamate-induced inflation–contraction cycles (Fig. 5A,C solid lines). AP3 either reduced or eliminated the inflation–contraction cycle depending on the concentration of antagonist and agonist. When sponges were incubated in either $50\mu\text{mol l}^{-1}$ or $100\mu\text{mol l}^{-1}$ of AP3 for 30 min, stimulation with $75\mu\text{mol l}^{-1}$ of glutamate caused waves of inflation to ripple across the sponge body in different directions (Fig. 5A); after the antagonist was washed out for 3 h, sponges were able to both inflate and contract in a more normal manner (Fig. 5B). When stimulated by $80\mu\text{mol l}^{-1}$ of glutamate, sponges pre-incubated in $100\mu\text{mol l}^{-1}$ of AP3 also carried out irregular inflation of the excurrent canal but did not contract; however, sponges in the lower concentration of AP3 were able to carry out a much reduced but complete inflation–contraction event when stimulated by the higher concentration of agonist (Fig. 5C). After the AP3 was washed out for 3 h, both sponges carried out a normal inflation–contraction cycle in response to $80\mu\text{mol l}^{-1}$ of glutamate (Fig. 5D). Sponges that were incubated in either $50\mu\text{mol l}^{-1}$ or $100\mu\text{mol l}^{-1}$ of AP3 and then stimulated by vigorous agitation, exhibited convulsions or twitches but no inflation–contraction cycle (Fig. 5E).

To further test the functionality of the m-GluR system a non-competitive inhibitor KYN was applied to the sponges (Fig. 6). KYN is thought to bind to an allosteric site of m-GluR changing the structure of the molecule, and as such it should reduce the receptor availability to glutamate. Sponges were incubated in 25, 50, 100, 150, $200\mu\text{mol l}^{-1}$ of KYN, and $80\mu\text{mol l}^{-1}$ of glutamate was added after 10, 20 or 30 min to test for competition of the two molecules; in one set of experiments sponges in all concentrations of KYN were stimulated by vigorous

shaking. Concentrations of KYN above $200\mu\text{mol l}^{-1}$ were cytotoxic and concentrations below $25\mu\text{mol l}^{-1}$ had little to no effect. After each experiment sponges were washed with fresh M-media and left for 24 h to confirm viability after experiments took place.

KYN eliminated the sponges' inflation–contraction response in a pattern that showed dependence on the concentration of inhibitor and length of incubation. The longer the incubation and the higher the concentration of inhibitor, the less likely it was that an inflation–contraction response could be stimulated by glutamate. Whereas 10 min incubation in $25\mu\text{mol l}^{-1}$, $50\mu\text{mol l}^{-1}$ and $100\mu\text{mol l}^{-1}$ of KYN did not prevent an inflation–contraction response, 10 min in $150\mu\text{mol l}^{-1}$ and $200\mu\text{mol l}^{-1}$ KYN concentrations were sufficient to block any response (Fig. 6A). After 20 min incubation in KYN, glutamate was only able to trigger an inflation–contraction response in sponges incubated at the lower concentrations ($25\mu\text{mol l}^{-1}$ and $50\mu\text{mol l}^{-1}$) of KYN; no response occurred in preparations incubated in $100\mu\text{mol l}^{-1}$, $150\mu\text{mol l}^{-1}$ or $200\mu\text{mol l}^{-1}$ KYN (Fig. 6B). A 30 min incubation in all concentrations eliminated all inflation–contraction responses from sponges with the exception of $50\mu\text{mol l}^{-1}$, which allowed an inflation–contraction response of slightly lower amplitude and of lower duration (Fig. 6C). However, all sponges incubated in KYN for 30 min began to show small ripples of contractions in different regions of their choanosome. Sponges incubated in all concentrations of KYN and stimulated by shaking immediately contracted and then gradually relaxed; there was no inflation–contraction behaviour (Fig. 6D).

Role of calcium in contractions

CF media greatly reduced the inflation–contraction response stimulated by $80\mu\text{mol l}^{-1}$ of glutamate, and CMF media abolished all inflation–contraction responses stimulated by $80\mu\text{mol l}^{-1}$ of glutamate (Fig. 7). Sponges that were shaken in either CF or CMF media showed no inflation–contraction behaviour (data not shown).

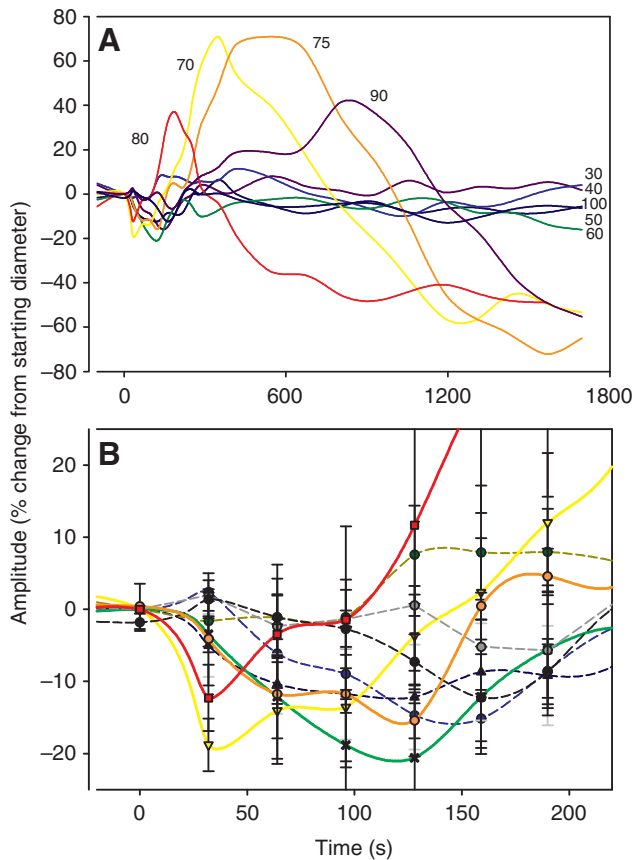


Fig. 3. Graphs showing the dose-dependent response of the inflation-contraction behaviour in *Ephydatia muelleri* to glutamate. (A) Concentrations of 70–90 $\mu\text{mol l}^{-1}$ of glutamate trigger a 20–30 min-long inflation and contraction of the aquiferous system, the 'inflation-contraction' response. The response is calculated as the percentage change in diameter of the largest excurrent canal over time, and each line represents the mean of three experiments (see Fig. S1 in supplementary material for each independent graph, and Movies 1–4 in supplementary material for example experiments). (B) The initial contraction of the tent (reflected by the quick contraction of the large excurrent canal) is also dependent on the concentration of glutamate; solid lines show the response to concentrations of 60–80 $\mu\text{mol l}^{-1}$; dashed lines show the response to lower and higher concentrations.

Evidence for a GABA signalling system

In contrast to glutamate, application of GABA had barely any effect at concentrations as high as 1 mmol l^{-1} . In 3/18 sponges application of 250 $\mu\text{mol l}^{-1}$ and 500 $\mu\text{mol l}^{-1}$ of GABA triggered a contraction of the incurrent canals (e.g. Fig. 8A). In two of these cases the drug also triggered minor contractions of the choanosome, suggesting the sponge tent had contracted; however, because this thin dermal tissue strung across the surface of the sponge is both taut and flat when viewed from the microscope above, this movement was not easily quantifiable in the images captured.

The absence of any direct effect of GABA prompted us to examine whether the molecule had an inhibitory role in the sponge. We used 1 mmol l^{-1} of GABA because it had no detrimental effect on the sponges (they were perfectly normal the following day after washing out the drug). Remarkably, sponges soaked in 1 mmol l^{-1} of GABA for 20 min showed no response at all to application of 75 $\mu\text{mol l}^{-1}$ of glutamate or to multiple applications of glutamate,

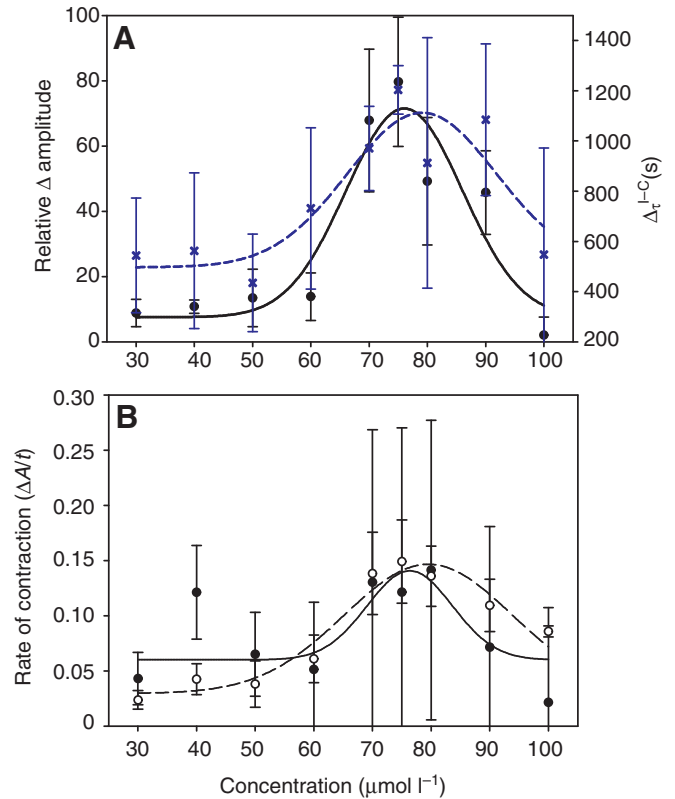


Fig. 4. (A) Relative amplitude (left axis, circles and solid line) and duration (right axis, crosses and dashed line) of the inflation response stimulated by different concentrations of glutamate. (B) Rate of contraction of the incurrent canals during inflation (solid circles and solid line), and contraction (open circles, dashed line) of excurrent canals during deflation. Data analysed from Fig. 3.

which brought the final concentration to 225 $\mu\text{mol l}^{-1}$, a concentration that would normally cause dramatic contractions and tearing of the dermal tissues in sponges not soaked in GABA (Fig. 8B). After the drug was washed out overnight, the sponges responded to challenge by 75 $\mu\text{mol l}^{-1}$ of glutamate with a normal inflation-contraction cycle (Fig. 8B).

Evidence for a NO signalling system

NOS activity was visualised by the use of NADPH-diaphorase staining and cGMP accumulation assay (Fig. 9). Using the NADPH-diaphorase staining protocol, cells that stained blue, indicating possible sites of active NOS, were found in the choanocyte chambers, osculum, excurrent canals and apical pinacoderm (Fig. 9A). Cells that lined the osculum stained the darkest and showed a gradient of lighter staining at the base as compared with the tip of the osculum by average grey level in that region. The pinacocytes of the excurrent canals stained for NOS activity as well. Within the dermal tissues only a few cells that formed a loose network in the mesohyl and the outer epithelium stained darkly for NOS activity (Fig. 9B).

Using a cGMP live assay, cells immunoreactive for cGMP were found within the osculum and in the dermal tissues, indicating these are active targets for NO. Young sponges were treated with 1 mmol l^{-1} of IBMX to prevent the breakdown of cGMP produced in cells responsive to NO and then challenged by the NO donor SNAP. When exposed to SNAP cells in the osculum accumulated

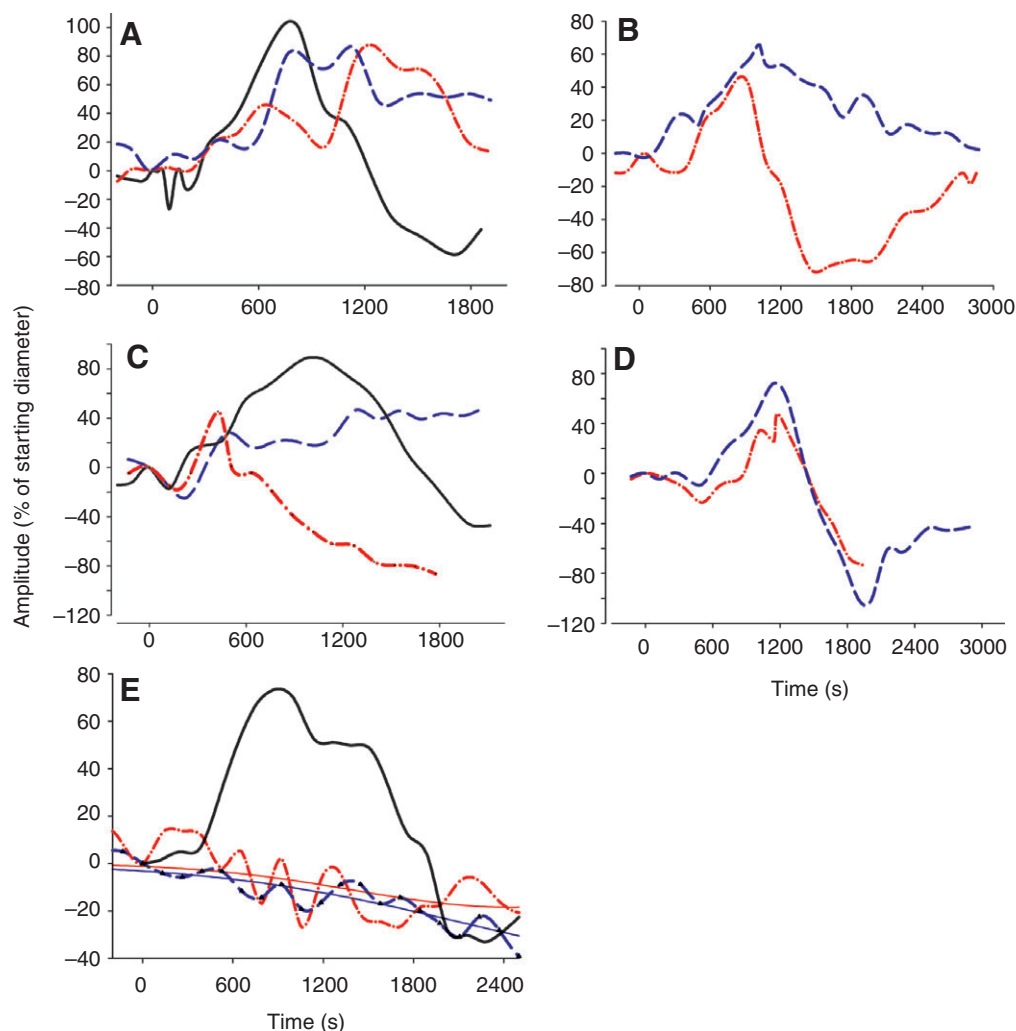


Fig. 5. The effect of 2-amino-3-phosphonopropionic acid (AP3), a competitive inhibitor of metabotropic glutamate receptors, on glutamate-triggered contractions. Sponges were stimulated by treatment with 75 $\mu\text{mol l}^{-1}$ (A,B) or 80 $\mu\text{mol l}^{-1}$ (C,D) of glutamate or shaken (E) at $t=0$ s. (A,C,E) In each graph the normal response of sponges to glutamate or shaking (solid line) is shown in comparison with the response of sponges pre-incubated in 50 $\mu\text{mol l}^{-1}$ (dashed-dotted line) or 100 $\mu\text{mol l}^{-1}$ (dashed line) of AP3; (B,D) The response of the same sponges to glutamate after washing out AP3 for 3 h. (A,B) Stimulus with 75 $\mu\text{mol l}^{-1}$ of glutamate causes an inflation but no proper contraction in sponges incubated in AP3; after washing out the inhibitor, both sponges can undergo a moderate inflation-contraction event. (C,D) Stimulus with 80 $\mu\text{mol l}^{-1}$ of glutamate can trigger a small inflation contraction in sponges incubated at the lower dose of AP3 but not in sponges incubated in 100 $\mu\text{mol l}^{-1}$ of AP3. After washing out the inhibitor, both sponges can undergo normal inflation-contraction events. (E) Sponges incubated in either concentration of AP3 were unable to carry out an inflation-contraction event when stimulated by shaking. (Solid lines plot the regression of each treatment.)

cGMP and the osculum contracted (Fig. 9C). No detectable staining was observed within the choanosome (Fig. 9D). However, when the pinacoderm was exposed to SNAP diffuse cytoplasmic staining was found even after the incubation period (data not shown).

DISCUSSION

Neuroactive molecules are defined by their action in the nervous system and specifically by their role in the chemical synapse (Kandel et al., 1991) but what about activity in an organism without a conventional nervous system, like sponges? Pavans de Ceccatty (Pavans de Ceccatty, 1974b) suggested that sponges have the ability to form different adjoining contacts, i.e. close appositions giving visible exchange areas, press-button-like articulations and punctate tight junctions, which could carry out directional spreading of signals from cell to cell. Now molecular analysis has shown that demosponges possess the proteins involved in forming a post-synaptic scaffold in other animals (Sakarya et al., 2007). Although there is still no morphological evidence for true chemical synapses in sponges, it is important to determine what molecules could be active at a potential 'proto'-synapse and whether these molecules play a role in controlling sponge behaviour.

In the present study we report evidence for the presence of signalling molecules in the tissues of the freshwater sponge *E. muelleri* and for their role in triggering and mediating contractions of the sponge body. Of the great array of signalling molecules

possibly acting in the sponge we selected three molecules known to be in sponges and known to have a common role in signalling in animals and plants and for which a known (stimulatory or inhibitory) response would be expected and thus the behavioural response might be monitored. Our results show that glutamate is not only able to trigger contractions of the sponge but is also a propagating signal, spreading contractions along both the incurrent and excurrent canal system. We show that GABA has an inhibitory effect on contractions, and that many cells of the osculum, tent and canal epithelia show NOS activity. Together our results provide the first behavioural evidence for synaptic-like transmission of signals in a sponge.

Neuroactive amino acids in sponge tissue

Our first approach was to screen *E. muelleri* tissue lysate for all available molecules using HPLC-MS. This analysis revealed 12 free amino acids that are considered neuroactive compounds in the vertebrate nervous system. However, analysis of whole sponge lysate does not differentiate between possible metabolically active molecules (e.g. osmolytes) and neuroactive substances (neurotransmitters, neuromodulators or their precursor molecules), and as osmolytes can be dominant amino acids that are mobilised from intracellular pools in response to a hyposmotic or hyperosmotic stress or for maintenance of cellular osmolarity, it is possible that these sponges use amino acids for osmotic protection in freshwater

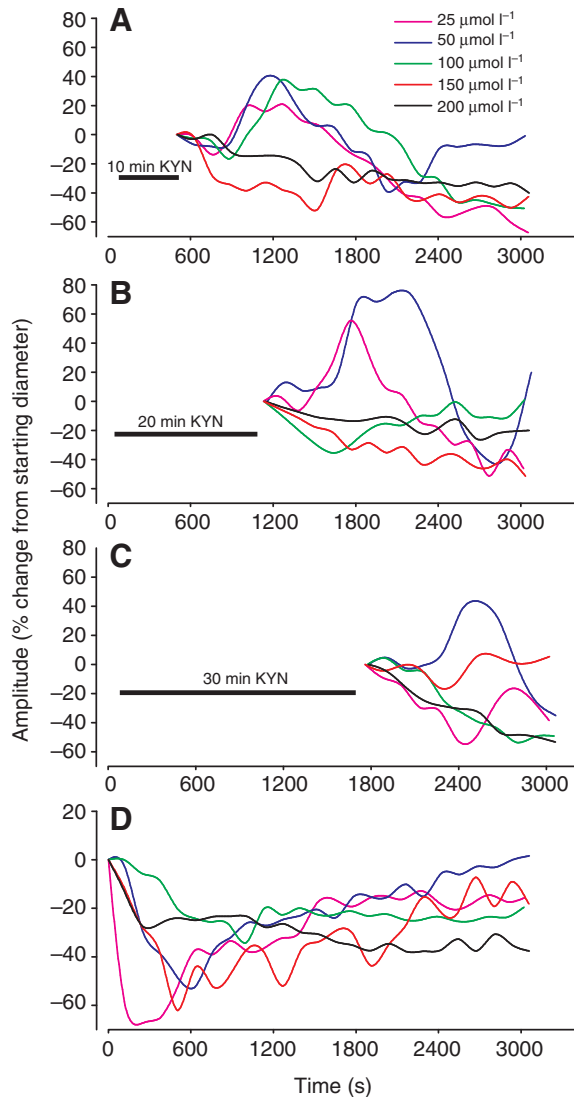


Fig. 6. The effect of Kynurenic acid (KYN), a non-competitive (allosteric) inhibitor of glutamate receptors, on contractions triggered by glutamate (A–C) or shaking (D). (A–C) Sponges were incubated in 25–200 $\mu\text{mol l}^{-1}$ of KYN for 10 (A), 20 (B) and 30 (C) minutes and then treated with to 80 $\mu\text{mol l}^{-1}$ of glutamate; (D) sponges were treated similarly with KYN and shaken immediately for one minute. (A) When glutamate was applied 10 min after incubation in KYN, the inflation–contraction response was delayed by 2, 5, and 10 min with a respective increase in the concentration of KYN, and no inflation–contraction response occurred at the two highest concentrations of KYN. (B) After 20 min incubation in KYN the inflation–contraction response was delayed by 5 and 10 min in 25 and 50 mmol l^{-1} KYN, respectively, but no contractions occurred in concentrations of 100 mmol l^{-1} and above. (C) After 30 min incubation in KYN, an inflation–contraction response only occurred in 50 $\mu\text{mol l}^{-1}$ of KYN, all other preparations could not respond normally.

systems; however, some of the free amino acids may also be used for signalling.

Eight potential neuroactive amino acids and metabolites were found with the HPLC screen: glutamate, GABA, glycine, taurine, serine (D-serine), tryptophan (biogenic amines), aspartate (precursor for NMDA: N-methyl-D-aspartic acid) and glutamine (precursor for GABA and glutamate) (Fig. 1; Table 1). Glutamate and GABA are neuroactive amino acids that are involved in both the central nervous system (CNS) and peripheral nervous system of vertebrates and in

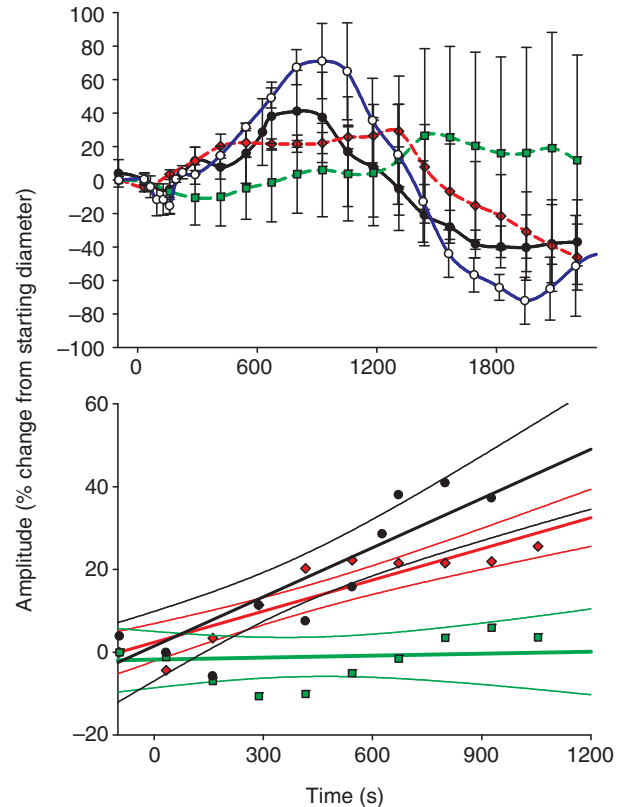


Fig. 7. The effect of the absence of calcium and of calcium and magnesium on the response of *Ephydatia* to glutamate and to shaking. (A) The inflation–contraction response to stimulation by 75 $\mu\text{mol l}^{-1}$ of glutamate (open circles, blue) and 80 $\mu\text{mol l}^{-1}$ of glutamate (solid circles, black) in normal medium, in calcium-free medium (diamonds, red), and in calcium–magnesium-free medium (squares, green). The mean change \pm s.e. of three different sponges is shown. (B) The slopes of the inflation phase of 80 $\mu\text{mol l}^{-1}$ of glutamate treatment in normal (black), Ca-free (red) and Ca–Mg-free (green) media plotted with 95% confidence intervals.

invertebrates are typically associated with control of muscle contraction in either an excitatory action (glutamate) or an inhibitory action (GABA). Glutamine is the precursor molecule or reserve store for glutamate and the GABA metabolic pathway. The high activity of GABA and glutamate as signalling molecules requires storage of an inactive glutamine for proper function within a cell. Glycine is a major inhibitory amino acid neurotransmitter in the vertebrate CNS that works by inducing a hyperpolarising chloride current when bound to a post-synaptic receptor but it can also be a modulator in excitatory ionotropic glutamate receptors. In *Hydra*, glycine has been identified and localised to the nerve net where it functions in pacemaker activity of peristaltic contractions (elongations and contractions) of the muscle in the body column; it is also involved in the chemosensory response during feeding by inhibiting the closure of the mouth upon stimulation by glutathione (Pierobon et al., 2001; Ruggieri et al., 2004; Kass-Simon and Pierobon, 2006). Similar responses are observed by the application of alanine and taurine in *Hydra* (Kass-Simon and Pierobon, 2006). In the demosponge *Tethya wilhelma*, glycine has been shown to stimulate a contraction, increase contraction rhythm and decrease contraction amplitude (Ellwanger and Nickel, 2006). It was proposed that this action occurs *via* a metabotropic glycine receptor but unfortunately no antagonists (e.g. strychnine) were used to block the response to confirm receptor–ligand binding.

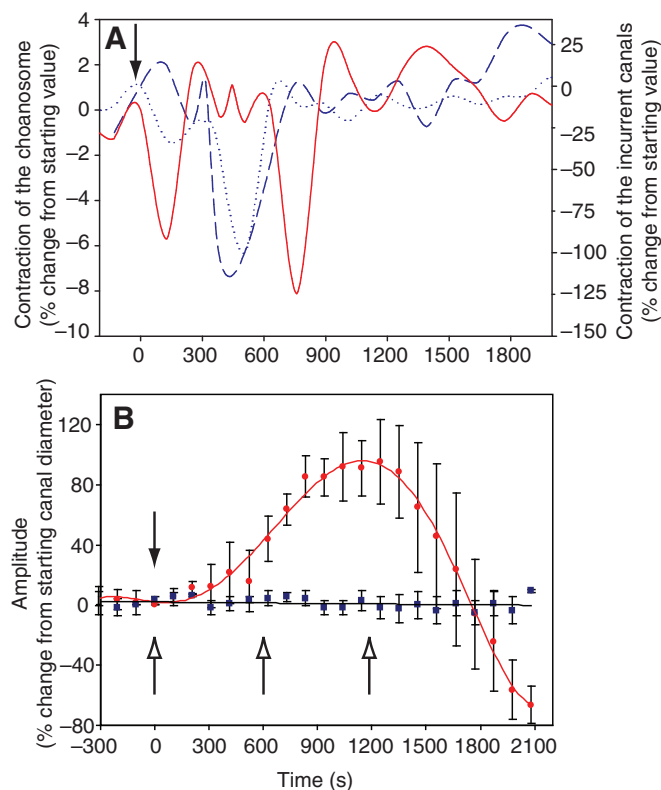


Fig. 8. Inhibition of contractions by γ -aminobutyric acid (GABA). (A) GABA (250 and 500 $\mu\text{mol l}^{-1}$, applied at the black arrow) caused a quick contraction of the choanosome (solid red line, left axis) and of the incurrent canals (dotted and dashed blue lines, right axis) in *E. muelleri*. (B) When sponges were incubated in 1 mmol l^{-1} GABA for 20 min (squares, black line) they did not respond to application of 75 $\mu\text{mol l}^{-1}$ of glutamate at $t=0$ or to additional doses of the same concentration of glutamate 10 and 20 min afterwards (white arrows). After 24 h washout in M-medium (circles, red line), all sponges responded normally to 75 $\mu\text{mol l}^{-1}$ glutamate (black arrow). The mean (\pm s.e.) of three sponges is shown.

Taurine is increasingly acknowledged to be a biologically active substance in both invertebrate and vertebrate nervous systems (Strang et al., 1990; Pirvola and Panula, 1992). Carlberg and colleagues showed that taurine was abundant in many cell types that function both neurally and metabolically in cnidarians (Carlberg et al., 1995). Anctil and Minh have shown that taurine is the dominant amino acid found in endodermal contractile cells in the sea pansy *Renilla* but within the spicule cells they suggest it functions as an osmolyte (Anctil and Minh, 1997). In marine flatworms, taurine had the highest free amino acid concentration indicative of an osmolyte (Barrett, 1981). Taurine was detected in *E. muelleri* but no functional data is available to suggest if it has a signalling or osmolyte function in the Porifera.

Other interesting metabolites found in the sponge tissue are tryptophan and aspartate both of which are used as precursor molecules for biogenic amine metabolism (Kandel et al., 1991). Tryptophan is the precursor molecule for the production of serotonin, which one report suggests may be in sponge larvae (Weyerer et al., 1999). High concentrations of serotonin were found to induce contractions in *Tethya* but there was no dose dependence of the effect (Ellwanger and Nickel, 2006). Aspartate is a modulator of NMDA glutamate receptors and its involvement as a chemical transmitter is tentative (Kandel et al., 1991), but because it is also

a major product of the Krebs cycle its role in chemical transmission in sponges is uncertain without further experimentation. We did not examine the amount of free arginine but its presence would indicate a pool that could be used for NO production.

Although presence/absence data cannot confirm the function of amino acids as chemical transmitters in sponges, the role of amino acids as transmitter molecules has probably arisen from the simple gustatory behaviour of protists to control feeding. Cellular responses to specific amino acids are speculated to have evolved into triggers for feeding in cnidarians – stimulating the gut and entrances to the feeding system; in sponges this is equivalent to the aquiferous canal system, i.e. ostia, canals and choanocytes. We focused our experimental work on two principal amino acids found in *Ephydatia* and *Spongilla* tissue lysate, glutamate and GABA, because of their ubiquity in signalling in plant and animal systems.

Evidence for paracrine signalling by glutamate and GABA

Glutamate is a chemical messenger that acts both through metabotropic and ionotropic receptors in sensory systems (Fagg and Foster, 1983) and is involved signalling in plant, protists, invertebrates and vertebrates (Van Houten, 1998; Nedergaard et al., 2002; Filleur et al., 2005). In *Tethya wilhelma* glutamate was found to regulate body contractions in a dose-dependent manner (Ellwanger and Nickel, 2007) but desensitisation and spasm-like behaviour were also observed; however, because *Tethya* is opaque and the canals of that sponge cannot be viewed by light microscopy, it was impossible for those authors to determine the precise effects on dermal tissues, osculum and canals. We found that in *E. muelleri* glutamate triggered contractions of the tent and also, more interestingly, stimulated a propagated contraction along the entire incurrent and then excurrent canal systems to carry out a stereotypical behaviour that we call the inflation–contraction cycle. Both types of contraction – the quick contraction of the tent and the inflation–contraction response – were sensitive to glutamate in a dose-dependent manner. Contractions increased in duration and amplitude with increasing concentration of glutamate and could be inhibited by blockers and competitive agonists of glutamate receptors, AP3 and KYN.

The blockers/inhibitors did not affect contraction of the osculum or tent but did prevent propagation of the inflation–contraction response; that they had long enough to act was demonstrated by their action in a time and concentration-dependent manner. The action of AP3 was to disrupt or at higher concentrations to prevent the inflation–contraction response but not to prevent all inflation of excurrent canals (contraction of incurrent canals). This suggests to us that control of the ‘inflation–contraction’ response is separated regionally not only into primary (tent and osculum) and secondary (in- and excurrent canals) systems but that the incurrent and excurrent canal epithelia probably have different populations of receptors. The addition of glutamate after the sponge was incubated in AP3 caused the primary system to contract and some contraction of the incurrent canal but not of the excurrent canals, suggesting that receptors in the excurrent canal system were blocked.

The action of KYN was even more distinct; lower concentrations with short incubation times had no effect on the inflation–contraction response but longer incubations of low concentrations reduced the likelihood there would be an inflation–contraction response and high concentrations inhibited the inflation–contraction after only 10 min in the competitive antagonist. The slightly delayed inflation–contraction response to glutamate at lower concentrations and shorter incubation periods of KYN suggests that glutamate eventually is able to out compete KYN and bind to its receptor. The complete

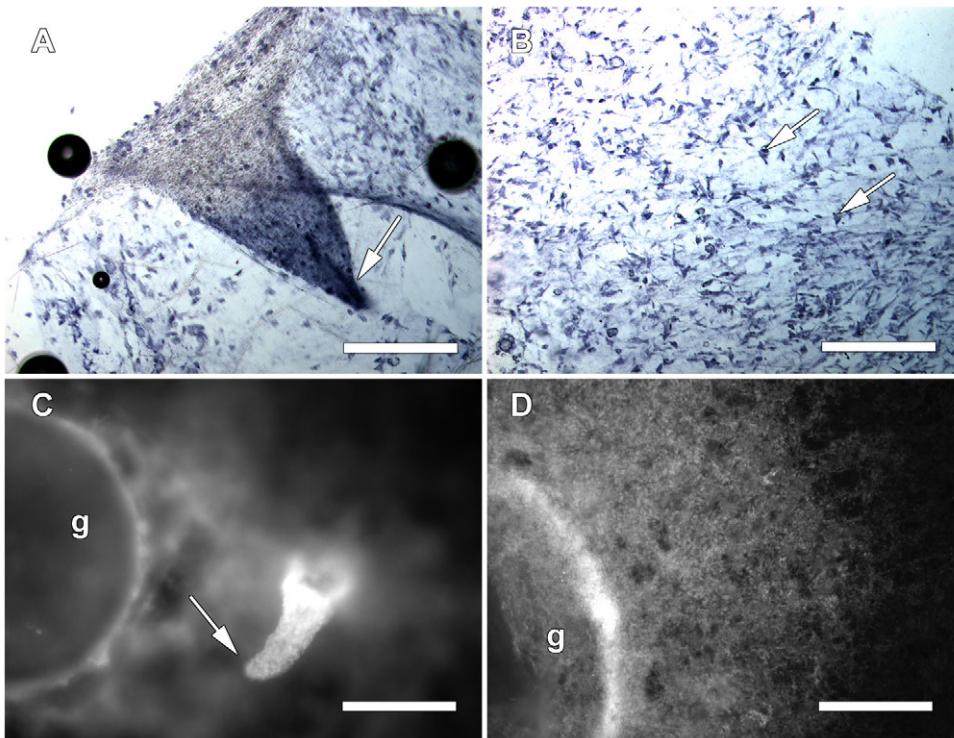


Fig. 9. Localisation of nitric oxide synthase (NOS) in *Ephydatia muelleri* by NADPH-diaphorase staining (A,B) and cGMP antibody assay (C,D). Cells showing NOS activity (blue stained cells, white arrows) were located in the osculum (A), and in the epithelium lining the excurrent canals and the mesohyl of the dermal tissues (B). (C) Many cells in the osculum (arrow) were immunoreactive for cGMP in the presence of the nitric oxide donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). (D) Control: in the absence of the nitric oxide donor SNAP there was fluorescence in the choanosome but no cGMP accumulation in the pinacoderm. Scale bars, A,B,D: 500 μ m; C, 100 μ m. g, gemmule.

block of propagation of the inflation–contraction response at higher concentrations of KYN however indicates that all sites for glutamate binding were inhibited and therefore the sponge must depend on the release of glutamate between cells to propagate contractions, generating a pool that eventually is able to bind to receptors on neighbouring cells, much like a chemical synapse.

Our findings with GABA are even more interesting. Whereas in *T. wilhelma* GABA was found to be 100-fold more potent than glutamate in triggering contractions, we found that it in fact acts as an inhibitory molecule, preventing contraction of the osculum, tent and of any inflation–contraction events. At first we were surprised that no concentration of GABA could trigger a contraction – which we had expected based on the results from *Tethya*. However, our results are much more in line with what we would expect for the function of GABA in other animals as an inhibitory signal in feeding, growth, metamorphosis and as an inhibitory neurotransmitter causing relaxation of muscle (Fagg and Foster, 1983; White et al., 1986; Chebib and Johnston, 1999; Bouche et al., 2003).

Emson (Emson, 1966) also showed that GABA treatment had no effect on the behaviour of the demosponge *Cliona celata*, and suggested that its role was inhibitory, so it is unclear why GABA might trigger a contraction in *Tethya*. The authors of that work (Ellewanger et al., 2007) suggested that since a sequence with putative affinity to both metabotropic glutamate and metabotropic GABA receptor families was cloned from the demosponge *Geodia cydonium* (Perovic et al., 1999), the glutamate/GABA system might not have yet diverged in sponges. However, the new genome project on *A. queenslandica* has identified eight separate sequences of m-GluR and one GABA receptor (Sakarya et al., 2007), and given that demosponge families are equally primitive it is likely that early sponges already had distinct glutamate and GABA receptors. It would be interesting to assess the action of GABA on internal tissues of *Tethya*.

Our work is the first to identify physiologically distinct regions in the sponge body and suggests that sponges may have different receptor populations on different tissues. Given the variety of

glutamate receptors now known to be in demosponges it would be interesting to determine if they have regionalised expression boundaries in *Ephydatia*, some on the osculum, others on the tent and yet others on the incurrent and excurrent canal systems. GABA receptors may be more limited in type and more ubiquitous in distribution. One report shows that antibodies to mammalian GABA are localised to most cells of the sponge *Chondrilla* (Ramoino et al., 2007). Although cross-reactivity of the antibodies was confirmed by western blotting, the implication of the rather vague immunostaining obtained is difficult to interpret. It was shown that *Chondrilla* possesses the enzymes to synthesise and transport GABA, and HPLC–MS analysis showed that GABA was released from cells stimulated by KCl but as no experimental work was carried out it cannot be speculated what function GABA might have in that sponge.

Requirement for calcium and magnesium

We also tested for the ability of glutamate-triggered contractions to propagate in the absence of calcium and of all divalent cations. In CF media, the effective dose of glutamate (75 μ mol l⁻¹) caused the osculum to contract and the incurrent canals to inflate but no further events occurred. In CMF media the incurrent canals did not even inflate. These results confirm that Mg²⁺ can substitute for Ca²⁺ in contractions, as shown by Prosser and colleagues (Prosser et al., 1962), but that an external calcium store is required for propagation of contractions. A link between the glutamate receptor and an internal calcium store is suggested by the fact that an initial contraction of the osculum and apical pinacoderm can occur in CMF media. However, no blockers have been identified that will block the internal calcium stores in the Porifera (Lorenz et al., 1996).

A potential role for NO signalling

We also found using NADPH staining that in *E. muelleri*, NO activity is located in the cells of the osculum, canals and choanocytes

and particular cells in the mesohyl of the tent. Although NO activity in these cells could be involved in a stress (immune type) response we did find that the osculum in *E. muelleri* contracted with application of the NO donor SNAP but no inflation–contraction response was triggered. In *T. wilhelma* NO (produced by the NO donor NOC-12) induced contractions and at the same time modulated endogenous contraction rhythm and amplitude (Ellwanger and Nickel, 2006). These results are similar to the involvement of NO in the feeding response in *Hydra* (De Petrocellis et al., 1999) and in molluscs (Korneev et al., 1998), in the control of swimming in *Aglantha* (Moroz et al., 2004) and in peristaltic contractions in *Renilla* (Anttil et al., 2005).

Another method to detect if a NO system is active is to use an assay to induce production of cGMP by activation of guanylate cyclase by a NO donor (SNAP). In *E. muelleri* the osculum labelled strongly for cGMP, as did some mesohyl cells of the tent. Thus, in *E. muelleri* NO may function in modulating contraction of cells of the dermal tissues and the osculum. In *A. queenslandica* a gene for NOS has been found but it does not share similarities with either neuronal or immune NO signalling (Sakarya et al., 2007). Future work is required to develop a protocol for a behavioural assay that will allow the dissection of the NO signalling system in sponges to determine whether it is directly involved in, is responsible for or modulates the propagation of contractions across the sponge.

Evolution of ligand-based coordination pathways

Our results showing precise activation and inhibition of physiologically distinct regions of a sponge help to provide a clearer picture of the properties and mechanisms of cell–cell signalling in sponges. We can now say with certainty that glutamate and GABA work as transmitters in excitatory–inhibitory roles to coordinate distinct types of contractions in this sponge. Cells (some or many but presumably not all) in the epithelia of the osculum, tent and canal system can release and receive signals in a precise manner, functioning in an analogous manner to a suite of synapses across these tissues. Because sponges lack a conventional nervous system this confirms opinions that the evolution of ligand-based receptor systems predated the evolution of a nervous system (Parker, 1910; Jones, 1962; Pavans de Ceccatty, 1962; Mackie, 1970; Pavans de Ceccatty, 1974a; Pavans de Ceccatty, 1974b; Mackie, 1979; Pavans de Ceccatty, 1979; Mackie, 1990; Nickel, 2004). As most sponges are cellular (only glass sponges are syncytial), it would probably have been easier for a mechanical or chemical messenger system to evolve by adapting existing chemical molecules and membrane receptors instead of an electrical system that would require completely novel proteins such as connexins or innexins to allow rapid transmission of signals between cells. Our results show that the inflation and contraction of the sponge aquiferous system (the inflation–contraction response) can continue despite tissues being torn apart by high doses of stimulant; this rules out the possibility that signals are simply transmitted mechanically from cell to cell. Rather we show that a chemical messenger (paracrine) system is capable of providing discrete information transfer over long distances and at the speed necessary to allow an effective behavioural response by the animal. This system functions very effectively without electrical signalling, the gain of which would have been a massive addition but one required by the co-evolution of rapidly contracting (striated) muscle.

LIST OF ABBREVIATIONS

| | |
|-----|-----------------------------------|
| AP3 | 2-amino-3-phosphonopropionic acid |
| CF | calcium free |

| | |
|---------|--|
| CMF | calcium–magnesium free |
| DMSO | dimethylsulphoxide |
| EDTA | ethylene diamine tetraacetic acid |
| EGTA | ethylene glycol tetraacetic acid |
| GABA | γ-aminobutyric acid |
| HPLC–MS | high-performance liquid chromatography–mass spectrometry |
| IBMX | 3-isobutyl-1-methylxanthine |
| KYN | Kynurenic acid |
| m-GluR | metabotropic glutamate receptor |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| SNAP | S-nitro-N-acetyl-DL-penicillamine |

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