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RESEARCH ARTICLE

Regional modulation of the response to glutathione in *Hydra vulgaris*

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ABSTRACT

In the presence of prey, or upon exposure to reduced glutathione (GSH), Hydra polyps open a mouth to ingest the captured prey and close it after feeding; at rest the mouth is not evident. In previous papers we have shown that GABA, glycine and NMDA modulate the mechanisms of mouth closure through ligand-gated-ion-channel receptors that are similar to their mammalian analogues in terms of biochemical and pharmacological properties. In order to study the regional distribution of these receptors, we have applied the GSH assay to polyps amputated at different levels of the body column. The response to 1–10 μmol l^{-1} GSH of polyps lacking either peduncle and foot or the entire body columns (heads) was not different from control, whole animals. In the presence of GABA or muscimol, duration of the response was significantly decreased in heads; the decrease was suppressed by the GABA antagonists gabazine and bicuculline. By contrast, in animals lacking peduncle and foot, duration of the response did not vary upon GABA administration. Conversely, in the presence of glycine, duration of the response in heads preparations was similar to control, whereas in footless polyps, it was significantly reduced. The decrease was mimicked by the glycine agonists taurine and β-alanine, and counteracted by strychnine. These results suggest a regional distribution of receptors to GABA and glycine in the neuromuscular circuitry modulating the feeding behaviour.

KEY WORDS: GABA receptors, Feeding response, Glycine receptors

INTRODUCTION

In the fresh-water polyp *Hydra vulgaris* (Cnidaria, Hydrozoa) neurons are connected to one another to form a net spreading homogeneously throughout the body, except at the head and foot regions, where the fibres are condensed into a circular nerve ring (Koizumi et al., 1992). Different types of synapses, with their complement of clear and dense-cored vesicles, have been described in Hydra and in all cnidarian classes (Westfall, 1996). The apparent lack of centralized ganglia and the occurrence of diffuse epithelial conduction via gap junctions have long favoured the view that in Hydra the electrical signal may pass from contractile myoepithelium to unpolarized nerve net to non-contracting epithelium (Anderson, 1980). Current knowledge indicates that in Hydra species the nerve net, one of the most primitive nervous systems to have evolved, shows a greater structural and functional complexity than previously acknowledged, modulating different behavioural responses through a variety of cellular effectors (Mackie, 1990; Koizumi, 2007; Koizumi et al., 2004, 2015; reviewed in Kass-Simon and Pierobon, 2007). Neuronal signalling relies largely on neuropeptides; recently, a peptide-gated ion channel has been cloned and functionally characterized (Assmann et al., 2014; Durrnagel et al., 2010; Golubovic et al., 2007; reviewed in Grunder and Assmann, 2015).

Hydra vulgaris feeds on live prey. Their tentacles sense vibrations of nearby swimming prey through mechanoreceptors. This leads to activation of the stinging cells, or nematocytes, which discharge the nematocyst tubule into the prey, capturing and paralyzing it onto the tentacles. A sequence of events follows prey capture, namely tentacle writhing, opening of a mouth (Campbell, 1987; Technau and Holstein, 1995), ingestion of prey, closing of the mouth. The feeding response is initiated by the association of reduced glutathione (GSH), flowing out of the wounded prey, with an external chemoreceptor (Grosvenor et al., 1992; Venturini, 1987).

Part of the response, tentacle writhing and mouth opening, can be produced *in vitro* by exposure of polyps to GSH, which is the specific stimulant of the feeding behaviour in several cnidarian species (Loomis, 1955; Lenhoff, 1961). GSH is the specific activator of the feeding response in Hydra; intensity and duration of the GSH-induced response are dose-dependent, saturable and antagonized by L-glutamic acid (Lenhoff, 1974; Lenhoff and Heagy, 1977). Measurement of response duration, i.e. the time interval between mouth opening and mouth closure, in basal conditions or in the presence of additional drugs, provides a quantitative assay for determining the activity of different substances on the feeding response.

By using the GSH assay, we have shown that amino acid neurotransmitters, GABA, glycine, NMDA and related ligands, acting through their ionotropic receptors, are able to modulate the duration of the response to GSH by delaying or anticipating, respectively, the time of mouth closure (Concas et al., 1998; Pierobon et al., 1995, 2001, 2004a). In contrast to glutamate (Bellis et al., 1991), these ligands do not modify times of mouth opening, suggesting that their effect is exerted on the neuromuscular circuitry underlying the feeding behaviour, rather than on the GSH receptor itself (reviewed in Pierobon, 2012). Therefore, neurotransmitter modulation of the feeding behaviour seems to be attained by multiple complex chemical and/or cellular pathways.

In order to obtain functional evidence on the regional distribution of receptors to GABA and glycine in hydra tissues, I studied the effects of these ligands in amputated polyps exposed to GSH. Two types of preparations were used: isolated hypostomes with their tentacles (heads), or polyps amputated of peduncle and foot (footless). In a preliminary series of experiments, the response to GSH was examined in heads and footless polyps at different times after cutting. In this paper, I present the results obtained upon exposure to GSH in the absence or in the presence of the different drugs.

RESULTS

The GSH response

In experimental conditions, the duration of mouth opening in response to GSH varied from about 10 min at 1 μmol l^{-1} GSH to

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List of symbols and abbreviations

B_{max}	binding site density
DMSO	dimethylsulfoxide
Ftl	footless
GABA	γ-amino butyric acid
GABAR	GABA receptor
GlyR	glycine receptor
GSH	γ-glutamyl-cysteinyl-glycine (reduced glutathione)
Hds	heads
LGIC	ligand-gated ion channel
NMDA	*N*-methyl-D-aspartic acid
T_f	time of mouth closure
T_i	time of mouth opening

about 20 min at 10 μmol l^{-1} GSH in whole polyps. Ablated heads obtained identical results upon GSH administration, provided the test was effected within 3–5 min after cutting: both times of mouth opening and response duration were comparable to control, i.e. whole animals (Table 1; Fig. 1A). However, duration of the response of heads treated at different times after cutting decreased significantly, starting at 15 min and for the following 2 h, returning to control values 2 h after the cut (Fig. 1C). The decrease depended on a shortened duration of the response, while times of mouth opening were not different from control. Finally, the duration of mouth opening in heads undergoing the GSH test 20 h after cutting was equal to the control value (Table 1; Fig. 1A). On the basis of these results, in the following experiments we only used heads either immediately after cutting (Hds0) or 20 h after cutting (Hds20).

Similarly, duration of the response to GSH in animals lacking peduncle and foot (footless) was equal to that in control, whole polyps (Table 1; Fig. 1B). In this case, however, duration of the response to GSH remained equal to control when the assay was performed at different times after cutting (Fig. 1C). In order to maintain comparable parameters, in the following experiments we used footless polyps either immediately (Ftl0) or 20 h after cutting (Ftl20).

GABA, agonists and antagonists

Administration of 100 μmol l^{-1} GABA to isolated heads significantly reduced duration of the response to all GSH concentrations, by anticipating times of mouth closure (∼ −35% of control, with a maximum of −38.8% at 10 μmol l^{-1} GSH; supplementary material Fig. S1). The effects of GABA were dose dependent: 10 μmol l^{-1}, 50 μmol l^{-1} and 100 μmol l^{-1} GABA reduced response duration, whereas 1 μmol l^{-1} GABA was not effective (Fig. 2A). Conversely, in footless polyps, GABA did not modify duration of the response in a 1–100 μmol l^{-1} concentration range (Fig. 2A). In whole animals, 50 μmol l^{-1} and 100 μmol l^{-1} GABA produced a significant increase in response duration (+21.5% and +31.5%, respectively, at 10 μmol l^{-1} GSH), as

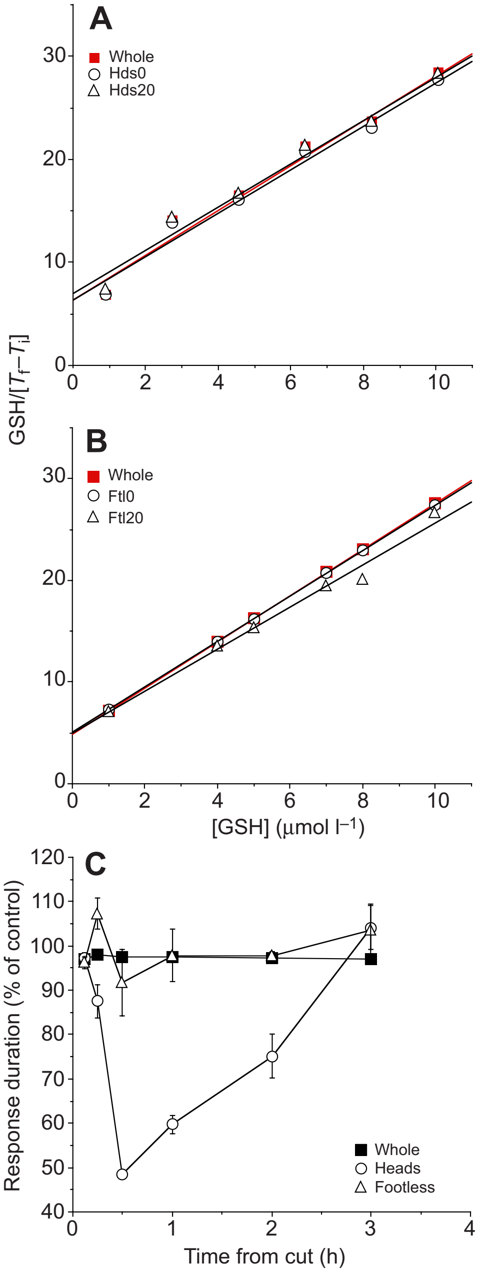

Fig. 1. Response of whole Hydra, footless polyps and heads to GSH. Linear regression analysis of the response to 1–10 μmol l^{-1} GSH concentrations in heads (A) and footless polyps (B). The kinetics of the response are calculated by a modified Lineweaver–Burk equation of [GSH]/(T_f–T_i) ratios, where [GSH] represents the stimulus concentration and T_f–T_i is the time measured at the corresponding GSH dose. (A) Control and isolated heads 3–5 min after cutting (Hds0) or 20 h after cutting (Hds20). Data are from 10 experiments. (B) Control and footless polyps 3–5 min after cutting (Ftl0)or 20 h after cutting (Ftl20). Data are from eight experiments. (C) Time course of response to 10 μmol l^{-1} GSH. Duration of response is shown in isolated heads and footless polyps at 15 min, 30 min and 1, 2 and 3 h after cutting. Data are expressed as the percentage variation in response duration relative to respective whole control value (10 μmol l^{-1} GSH) and are the means±s.e.m. of four separate experiments for each time setting (heads) and three separate experiments for each time setting (footless). ANOVA followed by Scheffé's test.

Table 1. Times of mouth opening and closing after 10 μmol l^{-1} GSH administration

Drug	T_i	T_f	T_f–T_i
Whole (control)	46±1″	22′5″±1′13″	21′4″±1′4″
Heads, 5 min after cut	40±14″	22′19″±1′31″	21′39″±1′27″
Heads, 20 h after cut	47±13″	22′±1′14″	21′13″±1′16″
Footless, 5 min after cut	22±9″	20′53″±28″	20′31″±36″
Footless, 20 h after cut	34±14″	21′49″±38″	21′15″±37″

Data are expressed in minutes (′) and seconds (″), and are the means±s.d. from a typical experiment for each time setting. T_i, time of mouth opening; T_f, time of mouth closure. Duration of the response, i.e. the time interval (T_f–T_i), was calculated for each polyp in all sample groups. Average values were used for linear regression analysis or as a percentage of maximal control value (ANOVA).

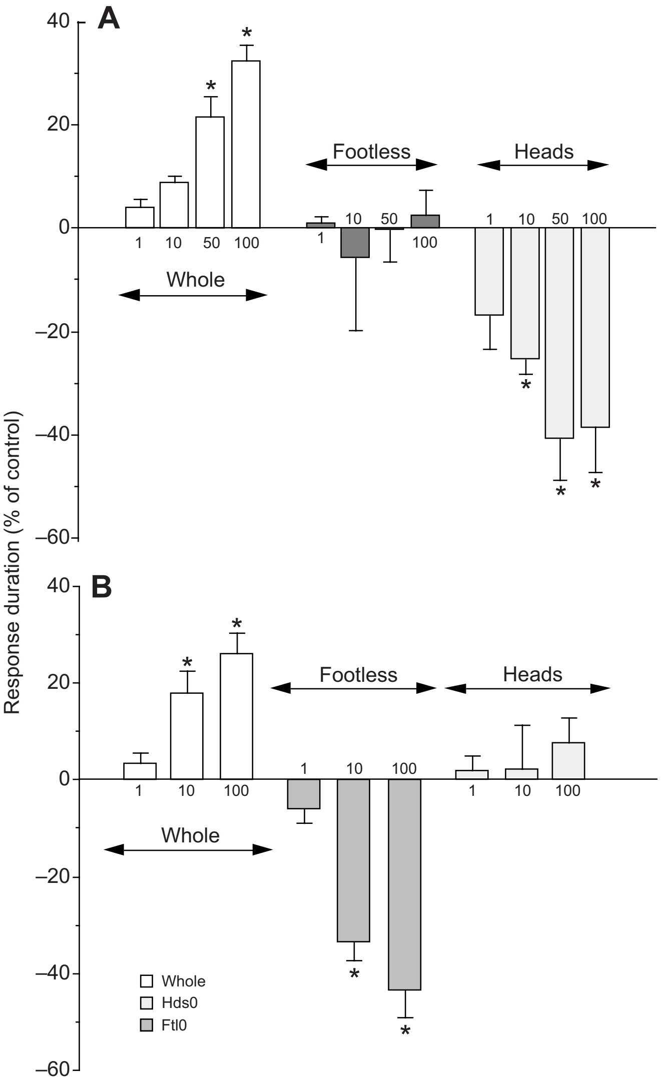

Fig. 2. Effects of different GABA and glycine doses on response duration in whole Hydra, footless polyps and heads. (A) 50 μmol l^{-1} and 100 μmol l^{-1} GABA significantly increased response duration in whole animals; 1 μmol l^{-1} and 10 μmol l^{-1} GABA were not effective. Conversely, 10 μmol l^{-1}, 50 μmol l^{-1} and 100 μmol l^{-1} GABA significantly reduced response duration in isolated heads (Hds0); the decrease produced by 50 μmol l^{-1} and 100 μmol l^{-1} GABA was also significantly greater than that obtained by 1 μmol l^{-1} and 10 μmol l^{-1} GABA. GABA did not significantly modify response duration in footless polyps at any of the concentrations. Here, and in the following figures, data are expressed as the percentage variation in response duration relative to respective whole control value (10 μmol l^{-1} GSH) and are the means±s.e.m. of 9–10 separate experiments. ANOVA followed by Scheffé's test; $^{*}P<0.05$. (B) 10 μmol l^{-1} and 100 μmol l^{-1} glycine significantly increased response duration in whole animals, whereas 1 μmol l^{-1} glycine was not effective. Conversely, 10 μmol l^{-1} and 100 μmol l^{-1} glycine significantly reduced response duration in footless polyps (Ftl0). Glycine did not significantly modify response duration in isolated heads at all concentrations. Data are the means±s.e.m. of 9–10 separate experiments. ANOVA followed by Scheffé's test; $^{*}P<0.05$.

expected. 1 μmol l^{-1} and 10 μmol l^{-1} GABA did not significantly modify response duration.

The GABA$_A$ antagonist gabazine reduced response duration at 10 μmol l^{-1} and 5–10 μmol l^{-1} doses, respectively, in heads as well as in whole animals and suppressed the decrease in response duration produced by 100 μmol l^{-1} GABA in head preparations (supplementary material Fig. S2). Co-administration of 10 μmol l^{-1} bicuculline methiodide, another GABA$_A$R antagonist, completely counteracted the decrease of response duration produced by 100 μmol l^{-1} GABA (Fig. 3A). The Cl^{-} channel blocker picrotoxin at 1 μmol l^{-1} concentration, as well as reducing response duration, partially antagonized the GABA-induced decrease of the response (Fig. 3A).

The action of GABA was mimicked by the GABA$_A$ agonist muscimol, which also decreased duration of the response in isolated heads at 10 μmol l^{-1} and 100 μmol l^{-1} doses. The effects of muscimol were counteracted by 5–10 μmol l^{-1} gabazine (Fig. 3B).

Finally, the specific GABA$_B$R agonist baclofen was able to reduce response duration at 10 μmol l^{-1} and 100 μmol l^{-1} doses. The decrease was antagonized by the GABA$_B$R antagonist phaclofen, which was ineffective at 10 μmol l^{-1} or 100 μmol l^{-1} concentrations (Fig. 3C). In the presence of 100 μmol l^{-1} GABA, 10 μmol l^{-1} phaclofen caused a significant reduction of the decrease in response duration; conversely, it did not modify the decrease produced by 100 μmol l^{-1} muscimol (Fig. 3D). In whole animals, neither baclofen in a 0.05–100 μmol l^{-1} concentration range, nor 1–100 μmol l^{-1} phaclofen modified duration of the response to GSH (data not shown; see Pierobon et al., 1995).

Glycine, agonists and antagonists

Administration of 10 μmol l^{-1} or 100 μmol l^{-1} glycine to amputated heads did not obtain significant differences in duration of the response to all GSH doses (99.5±8.9% and 105.6±5.3% of control, respectively, at 10 μmol l^{-1} GSH). In these experiments, administration of glycine to whole animals resulted in a dose-dependent increase of response duration, as expected (Fig. 2B).

Conversely, in the presence of 10 μmol l^{-1} or 100 μmol l^{-1} glycine, response duration of footless polyps was significantly reduced in a dose-dependent manner, with a maximum of ~43% at 10 μmol l^{-1} GSH for 100 μmol l^{-1} glycine (Fig. 2B). Co-administration of the GlyR antagonist strychnine, which was inactive at 1 μmol l^{-1} concentration, and 1–100 μmol l^{-1} glycine, suppressed the agonist-induced decrease and the duration of the response returned to control values (Fig. 4A). The GlyR agonists taurine and β-alanine at 10 μmol l^{-1} doses mimicked the effects of glycine (–48% and –26%, respectively); the decrease was antagonized by 1 μmol l^{-1} strychnine (Fig. 4B). Table 2 summarizes the results of GABA and glycine administration in intact, footless polyps and isolated heads.

DISCUSSION

Our results show that amputated Hydra polyps react to GSH stimulation similarly to intact animals. Both polyps lacking peduncle and foot (footless) and heads lacking the entire body column open the mouth in response to GSH: times of mouth opening and response duration do not differ from controls. These findings suggest that (1) the cellular GSH transduction pathway is localized in the hypostome and tentacles; (2) the neuronal and neuromuscular circuitry involved in mouth opening and closing also resides in the head; (3) the body column does not apparently contribute to the GSH response.

In fact, early electrophysiological experiments provided evidence that, upon GSH stimulation, both body column and tentacle contractions are inhibited, as well as the corresponding electrical coordinates, namely tentacle pulses and contraction burst pulses (Rushforth and Hofman, 1972); at the same time, monophasic potentials associated with asymmetric, GSH-induced movements start in the tentacles (Rushforth and Burke, 1971). These data suggest that the sequence of cellular events prompted by feeding is regionally restricted to the head. Further experiments directed to

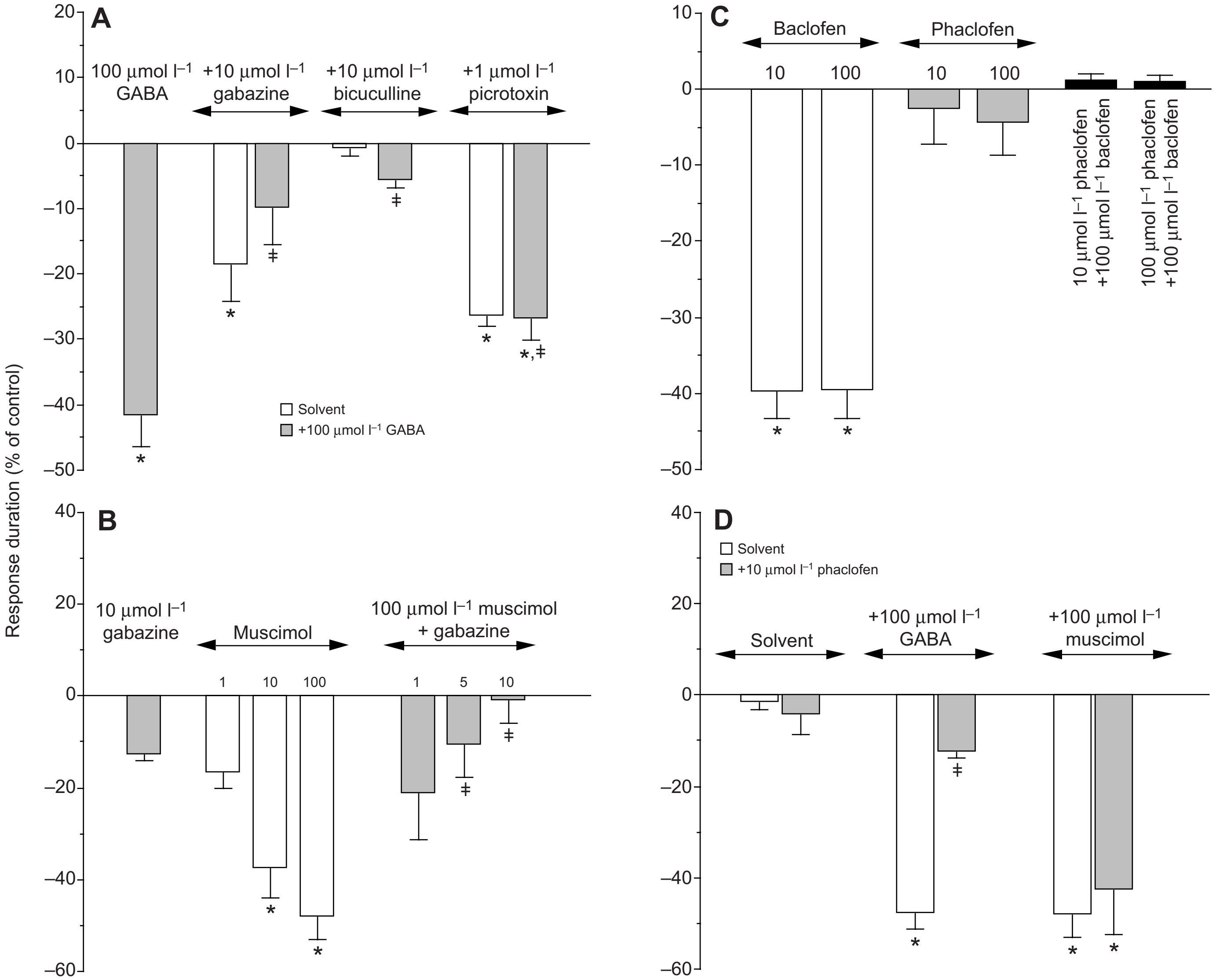

Fig. 3. Effects of GABA agonists and antagonists on response duration in Hydra heads. (A) The GABA$_A$R antagonists gabazine (10 µmol l^{-1}), bicuculline (10 µmol l^{-1}) and picrotoxin (1 µmol l^{-1}) suppressed the decrease in response duration produced by 100 µmol l^{-1} GABA in isolated heads (Hds0). Data are the means±s.e.m. of three separate experiments for each drug. ANOVA followed by Scheffé's test. *$P<0.05$ vs control; $^{‡}P<0.05$ vs 100 µmol l^{-1} GABA-treated heads. (B) The GABA$_A$ agonist muscimol at 10 µmol l^{-1} and 100 µmol l^{-1} doses mimicked the effects of GABA on heads (Hds0). The muscimol-induced decrease was suppressed by 5–10 µmol l^{-1} gabazine. Data are the means±s.e.m. of 4–6 separate experiments. ANOVA followed by Scheffé's test. *$P<0.05$ vs control; $^{‡}P<0.05$ vs 100 µmol l^{-1} muscimol-treated heads. (C) In the presence of 10 and 100 µmol l^{-1} baclofen, a specific GABA$_B$R agonist, duration of the GSH response significantly decreased in heads preparations (Hds20). The decrease was completely abolished by concomitant administration of 10 µmol l^{-1} phaclofen. Data are the means±s.e.m. of 4 separate experiments. ANOVA followed by Scheffé's test. *$P<0.05$ vs control. (D) The specific GABA$_B$R antagonist phaclofen at 10 µmol l^{-1} concentration significantly reduced the GABA-induced decrease of response duration in isolated heads (Hds20) but did not counteract the muscimol-induced decrease. Data are the means±s.e.m. of 6 separate experiments. ANOVA followed by Scheffé's test. *$P<0.05$ vs control; $^{‡}P<0.05$ vs 100 µmol l^{-1} GABA-treated heads.

studying the roles of neuromuscular and epithelial conduction in Hydra electrical activities again indicate a regional distribution of pacemakers and their conducting systems (Kass-Simon, 1973, 1976). The mechanisms underlying the feeding behaviour in Hydra could be explained as 'linked sequences of local responses, each component being initiated by the results of the preceding one' (Josephson, 1965).

The decrease of response duration observed in heads, but not in footless polyps, during the 2 h after the cut could depend on amputation. In fact, the wound may cause loss of signal molecules, nutrients, ions, cAMP etc. As a consequence, the electrical coordinates of the neuromuscular circuitry involved in mouth opening and closure would change. This hypothesis could tentatively explain the shortening of response duration in heads, but not in footless polyps, in the first 2 h after cutting: in footless polyps, in fact, the wound distance from the hypostomal region may be sufficient to prevent perturbation of the response. It is interesting to note that wounding triggers stretching of endodermal and ectodermal epithelial layers to close the wound within 2 h; the wound-healing process requires the contractile activity of myoepithelial cells (Wenger et al., 2014), thus contributing to further alteration of the conducting systems involved in modulation of mouth closure. In addition, regeneration and reorganization of the neuromuscular circuitry following amputation per se may affect the extant excitable structures on which the GSH response relies. Further studies are needed in order to clarify this issue.

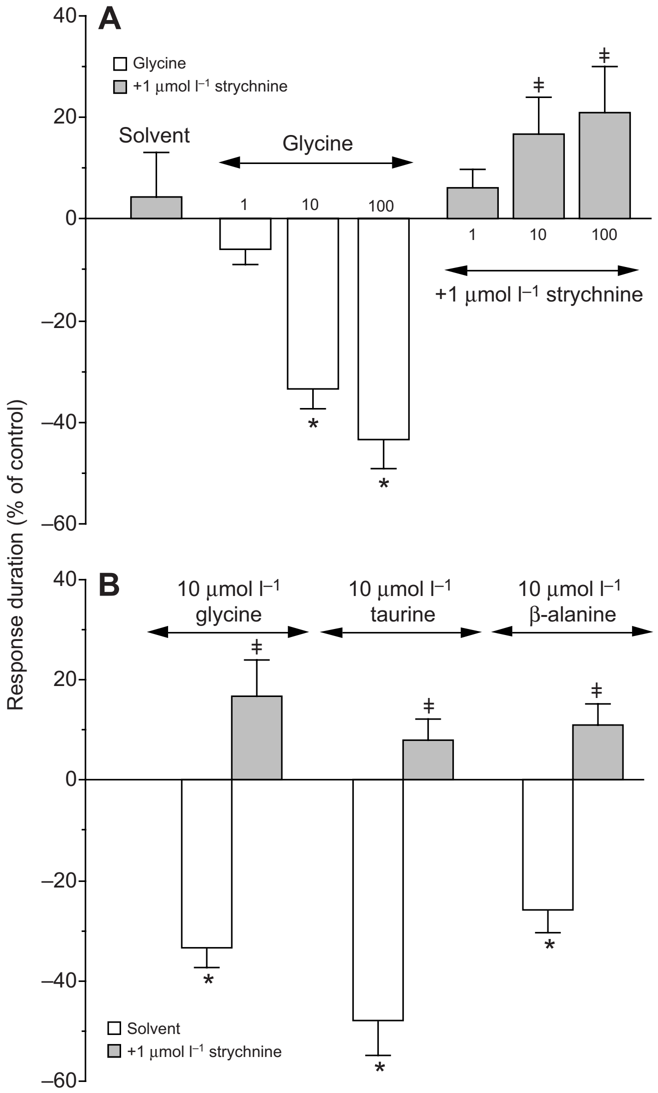

Fig. 4. Effects of glycine agonists and antagonists on response duration in footless Hydra. (A) The GlyR-specific antagonist strychnine, which is inactive at 1 μmol l^{-1} concentration, suppressed the decrease in response duration produced by 10–100 μmol l^{-1} glycine in footless polyps (Ftl0). Data are the means±s.e.m. of 4 separate experiments. ANOVA followed by Scheffé's test; *$P<0.05$ vs control; $^{‡}P<0.05$ vs respective glycine-treated footless polyp value. (B) The GlyR agonists taurine and β-alanine significantly reduced response duration in footless polyps (Ftl0) at 10 μmol l^{-1} concentration. The relative potency of ligands was taurine>glycine>β-alanine, similarly to results obtained in whole animals. 1 μmol l^{-1} strychnine completely suppressed the decrease produced by 10 μmol l^{-1} glycine, taurine or β-alanine. Data are the means±s.e.m. of 4–6 separate experiments. ANOVA followed by Scheffé's test; *$P<0.05$ vs control. $^{‡}P<0.05$ vs respective solvent-treated footless polyp value.

In Hydra, the classical amino acid neurotransmitters GABA and glutamate exert an inhibitory and excitatory action, respectively, on the pacemaker systems (Kass-Simon et al., 2003). In previous papers, we have shown that the response to GSH is finely tuned by inhibitory and excitatory amino acid neurotransmitters, indicating that the cellular components leading to mouth closure are modulated by the nerve net (Concas et al., 1998; Pierobon et al., 1995, 2001, 2004a, 2004b). However, GABA and glycine prolong response duration, while NMDA reduces it; this finding was tentatively explained with the hypothesis of potentiation or inhibition, respectively, of a chain of multiple sequential inhibitory loci, which modulate contraction and relaxation of ectodermal and endodermal myofibrils (reviewed in Pierobon, 2012).

Here, I show that administration of GABA to isolated heads obtains an opposite effect to that in whole animals, in that it significantly reduces duration of the response to GSH, with a dose-dependent effect in a 10–100 μmol l^{-1} GABA concentration range (Fig. 3). Conversely, GABA administration does not modify times of the GSH response in footless polyps. A working hypothesis to understand these results could be that part of the GABAergic inhibitory circuit localizes into the gastric region, peduncle and/or foot; the interruption of neural circuits obtained by ablation of the body column and/or peduncle may result in removing one or more inhibitory loci, thus reversing or suppressing the local action of GABA.

In polyp heads, the pharmacology of GABA was consistent with previous findings (Concas et al., 1998; Pierobon et al., 1995). Muscimol, the specific GABA$_A$R agonist, mimicked the effects of GABA in the same concentration range. The GABA$_A$R antagonist gabazine suppressed the GABA-induced or the muscimol-induced decrease of the response, the latter in a dose-dependent manner. Bicuculline completely antagonized the action of GABA, while picrotoxin was the least-effective antagonist. These findings provide further evidence that the action of GABA depends on activation of the specific ionotropic receptors, blocked by the corresponding receptor antagonists; they also indicate that different types of GABARs by their subunit structure may be involved in modulation of the response to GSH.

The finding that baclofen, the specific GABA$_B$R agonist, and its antagonist phaclofen are able to modulate the feeding response of amputated heads, although surprising, is not entirely unexpected. In previous works, we failed to find an action of baclofen either on GABA binding or on the feeding behaviour (Pierobon et al., 1995, 2004b). However, more recent studies have now provided evidence that putative GABA$_B$ receptors are present in *Hydra vulgaris*, where they modulate nematocyst discharge (Scappaticci and Kass-Simon, 2008) and in tentacles, nematocytes and ganglion cells of another cnidarian, the sea fan *Eunicella cavolini* (Girosi et al., 2007). It is tempting to speculate that the relative abundance of receptors to GABA of the LGIC superfamily in Hydra tissues (4.75 pmol mg^{-1} of protein) may contribute to masking the activity of GABA$_B$ receptor ligands in whole animals, which only becomes evident upon surgical removal of major body portions. Studies directed at investigating the issue are currently in progress.

The results of glycine administration to heads and footless polyps, though quite preliminary, still point to a diversified regional distribution of receptors to glycine in Hydra tissues. In footless polyps, 10 μmol l^{-1} and 100 μmol l^{-1} glycine significantly reduces duration of the response to GSH; 1 μmol l^{-1} strychnine, the specific GlyR antagonist, reverses the decrease. The GlyR agonists taurine and β-alanine also decrease response duration, with taurine being more potent than glycine and β-alanine. Again, the pharmacological findings are in keeping with previous results (Pierobon et al., 2001), but the effects of glycine administration are reversed in footless with respect to whole animals. In heads, glycine administration does not significantly modify response duration. The lack of an effect could depend on the removal of glycinergic loci, or on the presence of an insufficient receptor density in the hypostome and tentacles. In fact, the estimated B_{max} of the Hydra strychnine-sensitive GlyR population is quite low (79 fmol mg^{-1} of protein) compared with that of GABA$_A$Rs. The data suggest localization of GlyRs in the gastric region and/or in the peduncle or foot. In this case also

Table 2. Effects of GABA and glycine on the response to GSH in Hydra polyps

	Whole		Heads		Footless	
Drug	Solvent	+10 µmol l^{-1} gabazine	Solvent	+10 µmol l^{-1} gabazine	Solvent	+10 µmol l^{-1} gabazine
100 µmol l^{-1} GABA	**+32.2±3.0**	**−28.5±0.3**	**−38.8±8.7**	−9.8±4.9	+2.4±4.9	−18.3±5.6
100 µmol l^{-1} muscimol	**+22.2±2.9**	**−23.9±7.4**	**−48.0±8.2**	−0.9±4.8		
	Solvent	+1 µmol l^{-1} strychnine	Solvent	+1 µmol l^{-1} strychnine	Solvent	+1 µmol l^{-1} strychnine
10 µmol l^{-1} glycine	**+18.4±1.5**	+0.1±1.9	+7.5±5.3	−8.0±15.2	**−33.5±3.7**	+20.9±9.9
10 µmol l^{-1} taurine	**+18.9±3.6**	+3.3±5.7			**−48.1±6.7**	+7.9±4.2
10 µmol l^{-1} β-alanine	**+17.7±4.9**	+14.5±3.3			**−26.1±4.0**	+10.8±4.3

Data are expressed as percentage variations of the response to 10 µmol l^{-1} GSH (control). Figures in bold represent significant differences (ANOVA followed by Scheffé's test; *P<0.05 vs control).

interrupting the circuitry would result in an opposite effect with respect to whole animals.

In conclusion, the amputation of different body regions of Hydra polyps shows that the complex behavioural response to GSH is positioned in the head. The effects of GABA or glycine administration are reversed in heads and in footless animals, respectively, when compared with control, whole polyps. These findings hint at a possible modulation of the response by the gastric and foot neural circuitry that actively participates in the feeding behaviour through different LGIC receptor populations. Studies directed at investigating the contribution of different types of LGICs to the electrical activity of Hydra conducting systems in intact and regenerating animals could help to better understand this controversial subject.

MATERIALS AND METHODS

Animals

Hydra vulgaris (Pallas 1766) were originally obtained from Prof. P. Tardent (University of Zurich, Switzerland) and cultured asexually in our laboratories by the method of Loomis and Lenhoff (1956), with minor modifications. GSH assays were carried out on animals that were kept at 18±1°C under an artificial 12 h:12 h light:dark cycle in physiological solution (1 mmol l^{-1} $CaCl_2$, 0.1 mmol l^{-1} $NaHCO_3$, pH 7.3 to 7.4) and fed three times a week with freshly hatched nauplii of the brine shrimp *Artemia salina*; culture solution was changed 1 h after feeding. Homogeneous sample populations were obtained from freshly detached buds collected on the same day and cultured in separate dishes until use.

The GSH assay

The feeding reaction was studied by the procedure described by Lenhoff et al. (1983), with minor modifications. Polyps from homogeneous populations, ~3 weeks old and carrying one or two buds, were starved for at least 3 days before the trial. On the day of the experiment polyps were transferred in physiological solution buffered with 1 mmol l^{-1} Tris-HCl (pH 7.4) and equilibrated at room temperature for 1 h. Either 4 or 3+3 animals at a time were placed in 3.5-cm-diameter Falcon dishes divided into four chambers by glass partitions and allowed to relax under the stereo microscope (2 to 3 min). The test was initiated by removing the physiological solution and gently pipetting 1 ml of buffered physiological solution containing GSH (1 to 10 µmol l^{-1}) or GSH plus ligands at different concentrations. Animals were then monitored for mouth opening and mouth closure times. As usual in behavioural experiments, independent observers monitored mouth opening and closing, recorded the corresponding times, and dispensed the different solutions. This procedure permits simultaneous testing and recording of individual mouth opening (T_i) and closing (T_f) times for each animal. Scoring of mouth opening and closing was performed with a cold-light Wild stereo microscope. All experiments were performed in an air-conditioned environment at 22°C.

In order to obtain heads, namely hypostomes and tentacles, groups of 5–6 polyps were allowed to relax briefly under the microscope light before head excision; the cut was effected by transverse section immediately below tentacle insertion. Heads were then collected, rinsed in buffered physiological solution and either used immediately (within 3–5 min after cutting) or stored at 18±1°C and used 20 h later. In some experiments, heads were tested at different times after cutting, i.e. 15 min, 30 min, 1 h, 2 h, 3 h; between these intervals, the physiological solution was changed repeatedly. The same procedure was used for preparing footless samples: in this case, the cut was made immediately below the gastric region, i.e. along the distinct border between dense (budding region) and clear (peduncle) tissues.

All the drugs were dissolved in distilled water at a 100× concentration and used immediately. Lipophilic molecules (phaclofen) were initially dissolved in dimethylsulfoxide (DMSO) so that its final concentration did not exceed 1 µl ml^{-1}; in these experiments, the equivalent amount of DMSO was added to the controls. Six to eight animals were tested per group and per GSH concentration. Mouths opened within 1 to 2 min of GSH administration, and healthy animals were able to respond repeatedly. T_i and T_f were always measured for the first response. A control series of 4–5 groups treated with GSH only was performed in all experiments, which were repeated three to several times for each substance tested. GSH, GABA, muscimol, gabazine, bicuculline methiodide, picrotoxin, baclofen, phaclofen, glycine, taurine, β-alanine and strychnine were obtained from Sigma (Milan, Italy) or from Tocris Cookson, Inc. (Ballwin, MO, USA).

Data analysis

Behavioural data were analysed as follows: in each experiment the duration of the response to different GSH doses in the absence or in the presence of the various drugs was measured. The kinetics of the response was determined by linear regression analysis of all the data obtained in different experiments, using a modified Lineweaver–Burk equation.

Since only a limited number of animals could be tested in a single experiment (100–120 polyps), in the assays where several groups were required (direct comparison of two or more drugs, drug association, etc.), both the number of polyps and of GSH doses had to be reduced, thus preventing linear regression analysis. In order to compare data from these experiments, percentages of decrease or increase versus the maximal control value were calculated for each treatment at all GSH doses. Differences were then analyzed by ANOVA followed by Scheffé's test. Data are presented as means±s.e.m. from experiments repeated at least three times. Since the number of *post hoc* comparisons did not exceed the degrees of freedom, no correction in the α level was made, and a P value of <0.05 was considered statistically significant. The software used was StatView 4.5 (Abacus).

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Competing interests

The author declares no competing or financial interests.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.120311/-/DC1

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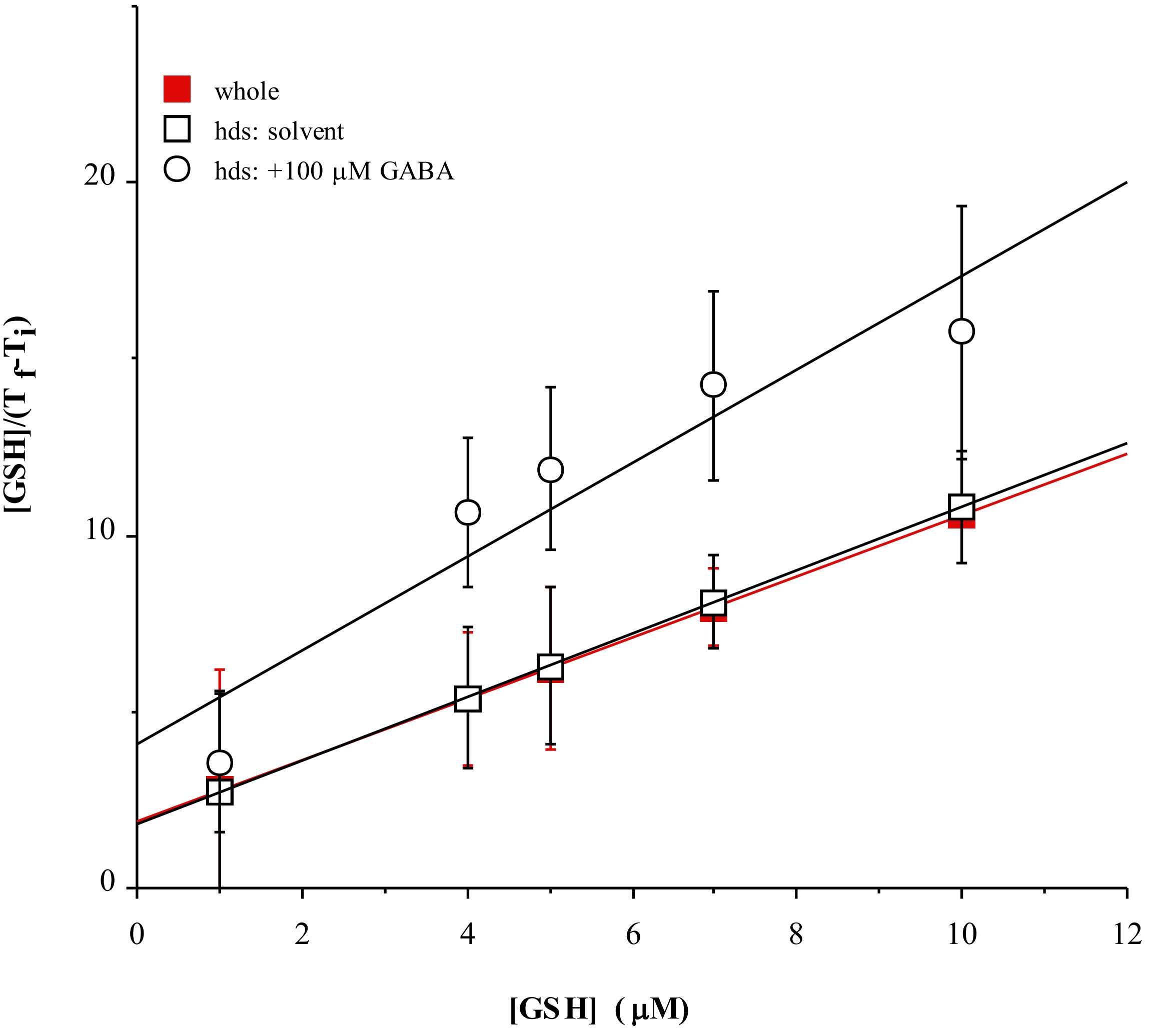

Fig S1. Effects of 100 μm GABA on heads: linear regression analysis

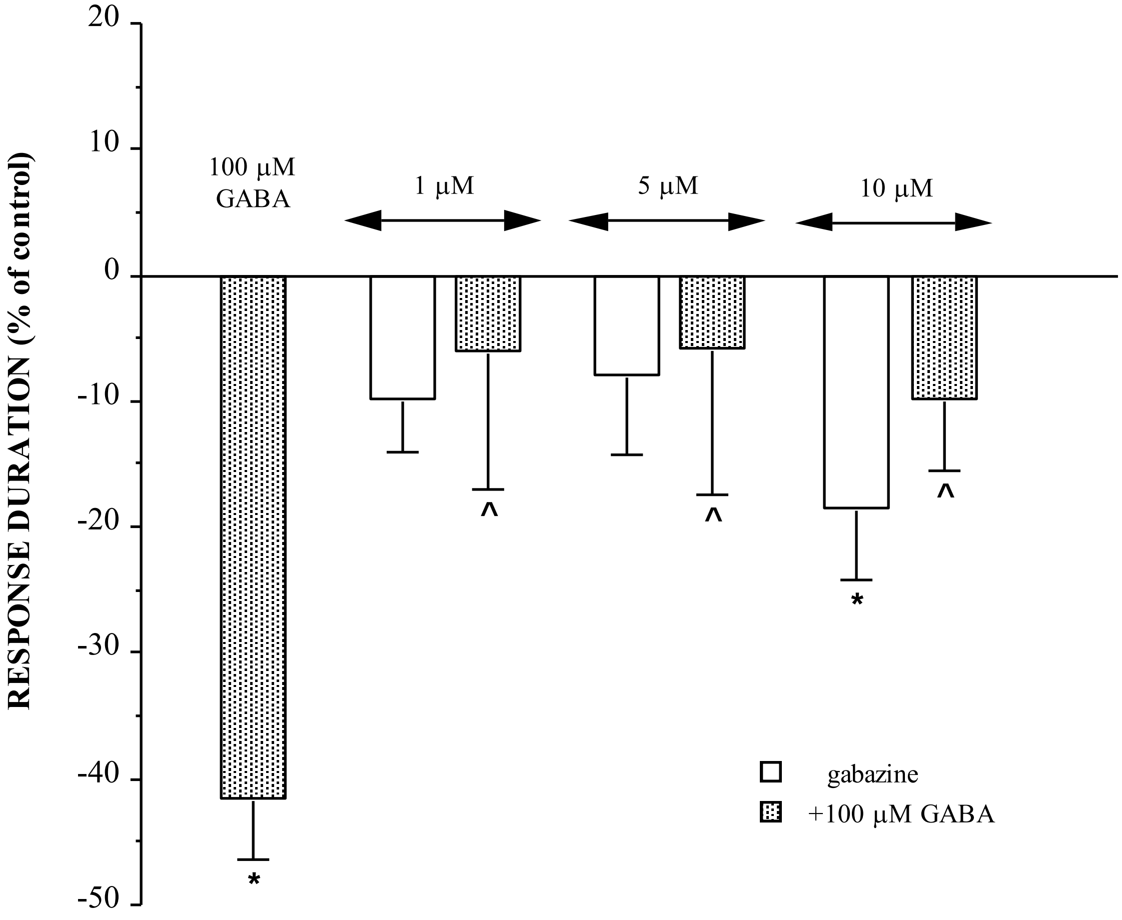

Fig. S2. Effects of gabazine: dose-response, heads