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Coordinated modulation of cellular signaling through ligand-gated ion channels in *Hydra vulgaris* (Cnidaria, Hydrozoa)

PAOLA PIEROBON*

Istituto di Cibernetica "E. Caianiello" C.N.R., Naples, Italy

ABSTRACT Cnidarians lack well developed organs, but they have evolved the molecular and cellular components needed to assemble a nervous system. The apparent 'simplicity' of the cnidarian nervous net does not occur at the cellular level, but rather in the organisation of conducting systems. Cnidarian neurons are in fact electrically excitable, show the typical extended morphology and are connected by chemical synapses or gap junctions. They have been regarded as peptidergic, given the wealth of neuropeptides generally distributed along neurites and in cell bodies, supporting the hypothesis of a modulatory role in neurotransmission. However, the presence of clear-cored, as well as dense-cored synaptic vesicles in cnidarian neurons suggests both fast and slow synaptic transmission mechanisms. In fact, biochemical and functional evidence indicates that classical neurotransmitters and their metabolic partners are present in cnidarian tissues, where they are involved in coordinating motility and behavior. We have identified and characterized in *Hydra* tissues receptors to the inhibitory and excitatory amino acid neurotransmitters, GABA, glycine and NMDA, that are similar to mammalian ionotropic receptors in terms of their biochemical and pharmacological properties. These receptors appear to regulate pacemaker activities and their physiological correlates; in the live animal, they also affect feeding behavior, namely the duration and termination of the response elicited by reduced glutathione, with opposite actions of GABA and glycine or NMDA, respectively. These results suggest that modulation of cellular signaling through ligand-gated-ion channels is an ancient characteristic in the animal kingdom, and that the pharmacological properties of these receptors have been highly conserved during evolution.

KEY WORDS: *GABA receptor, glycine receptor, glutamate receptor, feeding response*

Introduction

The freshwater polyp *Hydra vulgaris* (Cnidaria: Hydrozoa) has been a choice experimental model for studies of regeneration, morphogenesis, and development since Trembley's pioneer experiments in 1744 (Tardent, 1963; Gierer, 1977; Bode *and* David, 1978; Bode *et al*., 1986). Modern research focuses on the gene families regulating axial patterning, stem cell biology and neurogenesis (Galliot *and* Schmid, 2002; Holstein *et al*., 2003; Galliot *et al*., 2009, 2011); the recent publication of the genomes of *Nematostella vectensis* (Putnam *et al*., 2007) and *Hydra magnipapillata* (Chapman *et al*., 2010) now adds a basic framework for a comparative approach to the evolution of regulated developmental genetic programs.

Since its discovery, *Hydra* has also proven a fashionable model organism for studies of nervous form and function, as a representative of the earliest phylum in which a nervous system evolved. The

Abbreviations used in this paper: ACh: acetylcholine; AChE, acetylcholinesterase; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BZ, benzodiazepine; CB, contraction burst; ChAT, choline acetyltransferase; CNS, central nervous system; Con A, concanavalin A; D-AP5, D(-)-2-amino-5-phosphonopentanoic acid; DCKA, 5,7-dichlorokynurenic acid; GABA, γ-amino butyric acid; GABAR, GABA receptor; GluR, glutamate receptor; GlyR, glycine receptor; GPCR, G protein-coupled receptor; GSH, γ-glutamil-cysteinyl-glycine (reduced glutathione); LGIC, ligand-gated ion channel; MK-801, dizocilpine hydrogen maleate; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; NMDA, N-methyl-D-aspartic acid; NO, nitric oxide; PTX, picrotoxin; RP, rhythmic potential; TBPS, t-butylbicyclophosphorothionate; THDOC, 3α,21-hydroxy-5-pregnan-20-one; THP, 3α-hydroxy-5α-pregnan-20-one; TP, tentacle pulse.

***Address correspondence to:** Paola Pierobon. Istituto di Cibernetica E Caianiello C.N.R., Via Campi Flegrei 34, 80078 Pozzuoli (Naples), Italy.
e-mail: p.pierobon@cib.na.cnr.it

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simple polyp anatomy allows the view that questions concerning development and functions of conducting systems, movement co-ordination and handling of sensory information can be studied as well as, and perhaps more easily than, in more complex organisms. Furthermore, *Hydra* has been regarded as an ideal model system in which the paradox of co-existing cellular plasticity and defined neural functions could be resolved. In his plenary lecture at the NATO Workshop on "Evolution of the first nervous systems" G. O. Mackie (1989a) could state *'...Speculations about evolution have an irresistible fascination, partly because they are hard to prove wrong, partly because they make us think about origins and look for clues in development. Cnidarians may have been the first metazoans to evolve.... Given however, that of the surviving phyla they alone retain the presumably ancestral (diplobastic) body plan, it becomes especially interesting to look at their nervous systems for clues as how nerves evolved.... Molecular biology may (probably will) eventually help us reconstruct phylogeny, and place the cnidarians in their proper place on the many-branched tree of neural evolution, but we are still far from this point...'*

Mackie's foresight is proving well founded. Other papers in this issue deal with advances in our present understanding of neuronal development in *Hydra* and its contribution to studies on the origin of neurogenesis. This review focuses on a specific topic concerning the molecular machinery of chemical neurotransmission, namely the occurrence and properties of neurotransmitter receptors in *Hydra*, updating current knowledge and exploring future directions for studies of cnidarian neurophysiology. A brief description of *Hydra* and cnidarian neurophysiology is also presented in a historical perspective; students of the field will find this part rather sketchy, a choice made for the purpose of not abusing beyond measure the readers' patience. The last part of the review summarizes data on the biological roles of amino acid receptors, in particular in the modulation of a complex behavior, the feeding response.

The 'elementary' nervous system

The simple body plan of cnidarians is made of columnar epithelia supported by basement membranes, arranged in a sac with radial symmetry; in jellyfish, the sack culminates in an oral structure (the manubrium), surrounded by tentacles; in the case of polyps or anemones, the manubrium and tentacles (often called a head) form the apical part of a column which often rests upon a disc (the 'foot') (see in this issue Martinez *and* Bridge, 2012). Cnidarians lack well developed organs, but they have evolved the molecular and cellular components needed to assemble a nervous system. Polyps, medusae and sea anemones show major differences in neural anatomy and physiology, thus discouraging generalizations about primitive nervous systems. However, a common characteristic is the organization of cells in distributed nerve nets.

Cnidarian nerve nets have been the obvious candidate for comparative and evolutionary studies on the origin of synaptic transmission. Parker's theory (1919) that 'the elementary nervous system' originated in three successive phylogenetic stages, as a means to co-ordinate independent effector cells in response to environmental changes, has prompted innumerable studies and speculations on the origin, structure and physiology of conducting systems (see reviews by Pantin, 1952; Passano, 1963; Josephson, 1974; Shelton, 1982; Satterlie *and* Spencer, 1987). The lack of a recognizable brain encouraged the speculation that in these animals spreading of excitation was attained by way of diffuse, non-polarized conducting systems such as nerve nets. The organization into a net could well represent an adaptive response to ensure distribution and co-ordination of electrical activity in large portions of tissues, or perhaps to the radial symmetry of the body plan (Bullock *and* Horridge, 1965). The discovery of epithelial conduction in hydromedusae (Mackie *and* Passano, 1968; Spencer, 1974; Anderson, 1980), but not in anthozoans, was construed as further evidence for the primitive character of conducting systems in these organisms.

In the middle of last century, the explosion of electrophysiological studies both on vertebrate and invertebrate nerves and neurons, primed by the discovery of the action potential by Hodgkin and Huxley (1939), was rewarded by two Nobel prizes assigned in 1963 to Eccles, Hodgkin and Huxley for the theory of electrical signalling in excitable structures (Eccles, 1957), and in 1970 to Katz, von Euler and Axelrod for the discovery of quantal neurotransmitter release at the synapses (Katz, 1971). These new facts and theories renewed the interest for the functional properties of cnidarian neurons and nerves. In the following decades new techniques, electron microscopy, immunocytochemistry, intracellular and voltage clamp electrophysiology, have drawn an updated picture of cnidarian neurophysiology, based on findings from anatomical, ultrastructural, biochemical and functional studies (Anderson, 1985; Spencer, 1989; Satterlie, 2002; Mackie, 1989a, 1990). There is now ample evidence that the 'simplicity' of the cnidarian nervous net does not occur at the cellular level but rather in the organisation of conducting systems (Kass-Simon *and* Pierobon, 2007). Cnidarian neurons, in fact, are electrically excitable, show the typical extended morphology and are connected by chemical synapses, gap junctions or syncytial bridges (Jha *and* Mackie, 1967; Filshie *and* Flower, 1977; Wood, 1979; Westfall, 1987, 1996; Holtman *and* Thurm, 2001). They produce Na^+-dependent action potentials, interact by chemical or electrical synapses with other neurons or with nematocytes, muscle processes and other effector cells, generate rhythmic electrical activity and coordinate responses to sensory stimulation, integrating information from a variety of conduction pathways (Anderson, 2004). While modern technical advancements have reduced the need for easy-to-handle experimental models, a full understanding of cnidarian neurophysiology could still contribute useful information on conserved properties and functions of nervous systems.

The nervous net of Hydra: anatomy, ultrastructure and physiology

The first description of a medusan nervous system by the Swiss paleontologist and geologist Louis Agassiz in 1850 met considerable skepticism, because of the lack of a central brain (Mackie, 1989b). By the end of the century and in the following decades, however, several studies confirmed the occurrence of a nervous net lacking a recognizable center in many cnidarian species (Horridge *and* MacKay, 1965; Lentz, 1966). Still, in the 1961 Coral Gables workshop dedicated to cnidarian biology, doubts were openly voiced about the predicted neuronal nature of the cells identified in *Hydra* by classical histological methods such as silver impregnation or vital staining by methylene blue, but not yet confirmed by electron microscopy preparations (see floor discussion in Lenhoff *and* Loomis, 1961). The seminal work by Jane Westfall and coworkers, who described in detail the ultrastructure of nerve cells and synapses in *Hydra* sp. and other coelenterates contributed

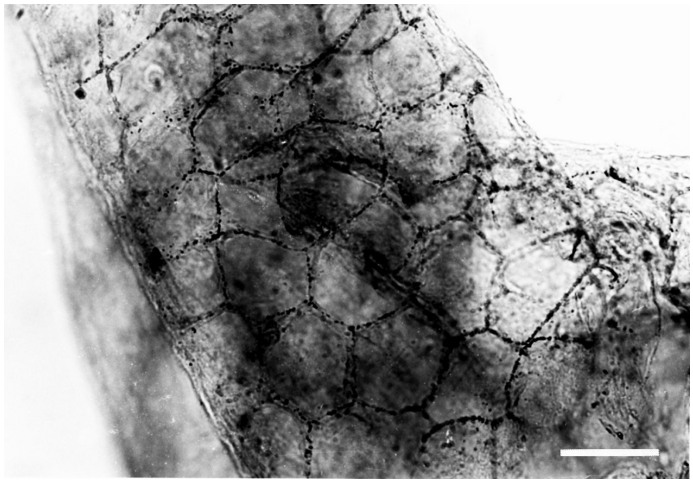

Fig. 1. The nervous net at the hypostome - tentacle junction of *Hydra oligactis*. *Whole mount silver nitrate staining. Scale bar: 50 μM.*

to definitely establish the presence of synaptic connections in the following years, erasing residual doubts on the existence of a nervous system (Westfall, 1970, 1973a, 1973b, 1988, 1996, 2002; see also Kass-Simon *and* Pierobon, 2007 for review).

Among Cnidaria, *Hydra* possesses perhaps one of the anatomically simplest nervous systems: neurons are connected to one another forming a nerve net that extends throughout the body as shown here (Fig. 1), with a concentration of neurons in the regions of the head and the foot. The nerve net is composed of two morphologically distinguishable cell types, sensory cells and interneuronal or ganglion cells. Sensory cells are inserted between epithelial cells perpendicular to the mesoglea; they have elongated somas that project from myonemes to the surface of the cell layer, with a ciliary cone at the apical end. Ganglion nerve cells lie parallel to muscle processes at the basal end of epithelial cells; they are interconnected by synapses with lucent- and dense-core vesicles (Davis *et al.*, 1968; Kinnamon *and* Westfall, 1981; Westall *and* Kinnamon, 1978, 1984). They also impinge on effector cells by way of synaptic foci or neurosecretory endings.

Current knowledge indicates that in *Hydra* species the nerve net is not diffused homogenously throughout the body, but shows a far greater structural and functional complexity (Koizumi, 2007). An immunocytochemical study performed with anti-RFamide antibodies (Koizumi *et al.*, 1992) demonstrated the occurrence of a nerve ring at the base of the hypostome in *Hydra oligactis*, formed by neurites of ganglion cells running circumferentially in a bundle, providing the first anatomical evidence of the existence of a ring structure previously inferred by electrophysiological studies (Kass-Simon, 1972). Labeling with different monoclonal antibodies or antibodies raised against different *Hydra* neuropeptides identified distinct subsets of neurons with specific regional distributions, dependent on axial position (Koizumi *and* Bode, 1986, 1991; Koizumi *et al.*, 2004). A remarkable feature of the net is, in fact, its plasticity: neurons are continually migrating to be eliminated at the apical and basal ends of the polyp together with the adjacent epithelia (Dunne *et al.,* 1985; Bode *et al.*, 1986), thus implying that extensive synapse remodeling must occur in physiological conditions. Furthermore, though *Hydra* polyps have no recognizable organs, a detailed ultrastructural analysis of the epithelial cells of the tentacles, the battery cells, showed that these cells enclose in their cytoplasm sensory and ganglion neurons as well as myonemes and different types of nematocytes, anchored by gap and septate junctions respectively (Hufnagel *et al.*, 1985). The authors suggest that these cell complexes may represent the functional units by which tentacles exert their functions: sensory perception, bending and contraction, prey capture (Hufnagel *and* Kass-Simon, 1988).

Considerable efforts were devoted to the study of *Hydra* electrophysiology, despite the difficulties encountered in recording from small, dispersed nerve cells. The apparent lack of a centralized ganglion or group of ganglia and the existence of diffuse epithelial conduction via gap junctions (Mackie *and* Passano, 1968; Anderson, 1980) initially favored the view that in *Hydra* electrical activity may pass from contractile myoepithelium to unpolarized nerve nets. However, extensive work by many researchers demonstrated that in *Hydra* through-conducting systems exist that exhibit complex patterns of rhythmic electrical activity, generated by pacemaker loci, challenging Parker's theory of independent effectors. Body contraction and elongation are controlled by ectodermal and endodermal pacemaker systems, respectively, while a third pacemaker system controls tentacle contractions (Passano, 1963; Passano *and* McCullough, 1964, 1965; Macklin *and* Josephson, 1971; Rushforth, 1971; Rushforth *and* Burke, 1971; Rushforth *and* Hofman, 1972; Josephson, 1974; Kass-Simon, 1972, 1976; Kass-Simon *and* Passano, 1978). Electron microscopy studies by Lentz (1966, 1968), Westfall and collaborators (1970, 1971, 1987, 1996) and others described both electrical and chemical synapses, bearing clear- and dense-cored vesicles, in *Hydra* neurons. Their importance in the coordination of electrical activity of excitable structures became steadily acknowledged (Anderson *and* Spencer, 1989); conversely, in one study no electrotonic coupling or dye exchange was detected between epithelial cells of *Hydra attenuata* (de Laat *et al.*, 1980). At present, chemical synaptic transmission is regarded as the basis for neuronal signaling in *Hydra*, the question having shifted to what molecules and proteins make the signaling system(s).

The molecular machinery of chemical neurotransmission in eumetazoans

Two types of signals in chemical neurotransmission: neurotransmitters and neuropeptides

Chemically mediated synaptic transmission is one of the mechanisms by which intercellular signaling occurs between neurons, or between a neuron and its target cell(s); it relies on two major components: neurotransmitters, stored in presynaptic terminals and released at need in the synaptic cleft, and postsynaptic receptors, membrane proteins that specifically recognize and bind to released transmitters. These receptors convert chemical into electrical signals, thus ensuring transfer of information.

Neurons use many different molecules to convey information; very often, they make both a conventional neurotransmitter (such as glutamate, GABA or dopamine) and one or more neuropeptides. Neurotransmitters are generally packaged in small, clear synaptic vesicles, clustered in presynaptic terminals, and peptides in large dense-cored vesicles, found in all parts of the neuron (see in this issue Fujisawa *and* Hayakawa, 2012). Rather ironically, some of these substances were first discovered and identified as signaling molecules in lower vertebrates (acetylcholine, Loewi, 1921) or in invertebrates (GABA, Otsuka, 1966); but proof of their status as neurotransmitters and the present extensive knowledge about their

biochemistry, pharmacology and physiology derives from studies on mammalian central nervous systems (CNS).

More recently, new molecules such as nitric oxide (NO), carbon monoxide, and D-serine have been proposed as neurotransmitters; differently from conventional transmitters, they are not stored in synaptic vesicles, are not released by exocytosis, and do not act at postsynaptic membrane receptors. D-serine is synthesized and stored in astrocytes rather than neurons (Bohening *and* Snyder, 2003). The discovery of atypical neurotransmitters opens new exciting perspectives for studies of cellular signalling. It is interesting to note that at least two of these molecules, NO and D-serine, are involved in modulation of biological activities in cnidarians (see Kass-Simon *and* Pierobon, 2007, for review).

Two types of receptors are involved in chemical neurotransmission

Chemical synapses can produce fast, short-term transmission carried by small molecules, the "classical" transmitters such as acetylcholine, amino acids, biogenic amines; or slow, prolonged action often due to secretion of small neuroactive peptides. Neurotransmitters modify the activity of one or a few adjacent cells by binding to a specific membrane receptor; the nature of the receptor, i.e. ionotropic (forming ion-channel pores) or metabotropic (most often G protein-coupled receptors, GPCRs), determines the mode of action of the transmitter. The former can produce brief conductance changes of the postsynaptic membrane whereas the latter induce the production of intracellular second messengers, usually intermediate G-proteins. Both types of receptors exist for the majority of classical transmitters, while neuropeptides use almost exclusively metabotropic receptors. GPCRs are heteromeric protein complexes formed by seven transmembrane domains, activating cellular responses through two main signal transduction pathways: the cAMP signal pathway and the phosphatidylinositol signal pathway (Fig. 2A). The ligands for this large protein family include opsins, odorants, pheromones, hormones, growth factors, besides neurotransmitters; part of these are orphan receptors, i.e, no specific ligand has yet been identified. The size of the GPCR superfamily is unknown, but about 800 genes have been predicted from human genome sequence analysis (Foord *et al.*, 2005).

Ligand-gated ion channel (LGIC) receptors form three superfamilies

In the last thirty years advances in molecular biology and genomics allowed the identification and cloning of the protein components of ligand- and voltage-gated ion channels as well as metabotropic receptors. These studies have discovered a multiplicity of protein subunits, grouped into classes on the basis of amino acid sequence homology; within classes there are further subdivisions into subunit isoforms, some of which exhibit alternative splice variants. This heterogeneity is considered to be responsible for the distinct pharmacological and functional properties displayed by the various subunit configurations. Despite the extraordinary diversity of molecular receptor components, these studies have also revealed a considerable similarity in amino acid sequence and structure, leading to current classification of postsynaptic receptor proteins in different gene families (Alexander *et al.*, 2007).

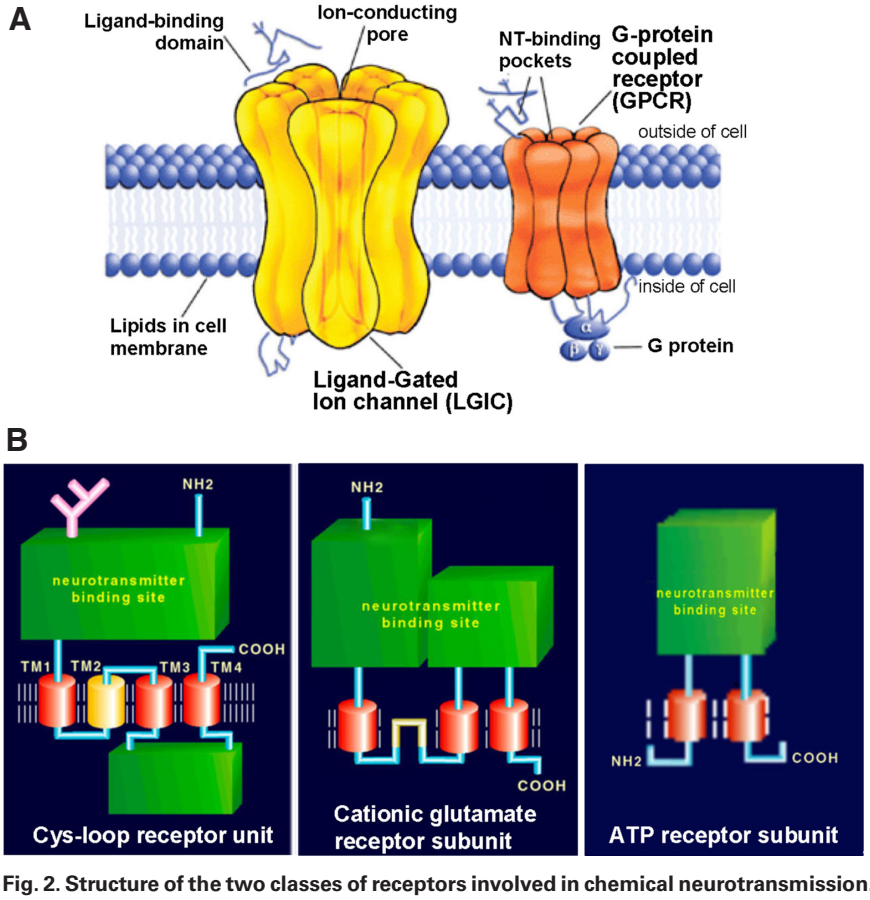

Fig. 2. Structure of the two classes of receptors involved in chemical neurotransmission. **(A)** *Schematic drawing of a ligand-gated ion channel receptor (LGIC, left) and a G protein-coupled receptor (GPCR, right).* **(B)** *Transmembrane subunit arrangement in the three different LGIC classes (schemes reprinted from the Ligand-Gated Ion Channel database EMBL EBI: www.niaaa.nih.gov).*

The prevalent forms of neurotransmitter receptors belong to the superfamily of Ligand-Gated Ion Channel (LGIC) receptors, both excitatory (acetylcholine, glutamate, serotonin) and inhibitory ($GABA_A$, glycine, glutamate-gated Cl^- channels). LGIC receptors are heteromeric or homomeric transmembrane protein complexes that can exist under different conformations, at least one forming a pore through the membrane connecting the two neighbour compartments (Fig. 2A). The equilibrium between the various conformations is affected by the binding of ligands on the channels. Phenomenologically, the ligands "open" or "close" the channel. The allosteric binding site of endogenous ligands on LGIC protein complexes is normally located on a different portion of the protein than the ion pore. The direct link between ligand binding and ion channel opening or closing is characteristic of these receptors. There are three different gene superfamilies of extracellularly activated LGIC subunits: the Cys-loop superfamily, glutamate-activated cationic channels, and ATP-gated channels, or purinergic receptors (Fig. 2B).

The Cys-loop superfamily is comprised of

nicotinic acetylcholine receptors (nAChR), serotonin receptors, $GABA_A$ receptors ($GABA_AR$) and glycine receptors (GlyR). Some glutamate, histamine and serotonin-activated anionic channels also belong to the Cys-loop superfamily. The structure of Cys-loop LGIC receptors has been the object of extensive biochemical and molecular investigation. Current knowledge derives from X-ray crystallography data on the soluble ACh binding protein (Brejc *et al.*, 2001) and subsequently from the water-soluble portion of the nAChR α1 subunit (Dellisanti *et al.*, 2007) that confirmed and resolved previous findings, making the ACh receptor a paradigm for the topology of LGIC.

Cys-loop receptors are heteromeric or homomeric protein pentamers. Each subunit has a N-terminal extracellular domain containing a signature loop formed by a disulfide bond between two cysteine residues separated by 13 highly conserved amino acids, four transmembrane domains of about 20 amino acids that form the central pore, with a large intracellular loop between M3 and M4 α-helices, and a C-terminal extracellular domain, the most divergent part of the sequence; two to five binding sites per receptor are located on the N-terminus domain. In particular, receptors to GABA are assembled from a family of 19 homologous subunit gene products (α 1-6, β 1-3, γ 1-3, δ, ε. θ; three ρ subunits, related to $GABA_C$ receptors also are included in the family) and form different, normally heteromeric, pentamers (Barnard *et al.*, 1998; Olsen *and* Sieghart, 2008). Typically, $GABA_A$ receptors are formed by two copies of α, two copies of β and one copy of either γ, δ or ε subunits, out of the hundreds of theoretically possible combinations. Receptor composition and subunit selectivity for drugs is currently the objective of massive research efforts, given the fundamental role of GABARs in brain function.

Receptors to glycine are homomeric or heteromeric pentamers comprised of five subunits, four α (1-4), and one β subunit. The β subunit is responsible for anchoring GlyRs to the subsynaptic cytoskeleton via the cytoplasmic protein gephyrin (Betz *et al.*, 1999; Lynch, 2004). A distinctive feature of glyRs is the presence of a second cys-loop, 45 residues downstream, that is critical for ligand binding (Rajendra *et al.*, 1995). As GABARs, GlyRs gate a Cl^- channel, thus hyperpolarizing membranes of the target cells. However, glycine also acts as a NMDA co-agonist at the glycine-binding site of the NMDA receptor in vertebrate CNS, thus exerting an excitatory action (Kleckner *and* Dingledine, 1988).

The ionotropic glutamate receptor family (iGluRs) is comprised of at least three receptor subtypes defined by their pharmacological characteristics, namely selective binding to different glutamate agonists, the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, the kainate receptors, and the N-methyl-D-aspartic acid (NMDA) receptors (Dingledine *et al.*, 1999). Receptor topology has been only recently clarified (Mayer *and* Armstrong, 2004): eukaryotic iGluRs are made of four subunits, each with three transmembrane segments. Each subunit contains an extra-cellular amino-terminal domain followed by half of the agonist-binding core, two transmembrane domains separated by a pore loop ion channel, the second half of the agonist-binding core and a third transmembrane segment. The M2 segment does not cross the membrane but forms an intracellular loop. The C-terminus is located within the cell and has a variable length.

AMPA receptors are assembled from GluR1-GluR4 subunits, and kainate receptors are made of GluR5-GluR7 plus KA1/KA2 subunits of a different gene family. NMDA receptors consist of two NR1 and two NR2 subunits, each containing three transmembrane domains The glycine binding site is formed by two distinct extracellular regions, S1 and S2, of the NR1 subunit, whereas the homologous domains of the NR2 subunit mediate glutamate binding. NR1 subunits are also required for receptor trafficking and assembly. Receptor activation opens postsynaptic cation channels with different degrees of permeability, thus depolarizing the postsynaptic membrane. NMDA-gated channels are also highly permeable to Ca^{2+} and to other divalent cations; they are regulated by a voltage-dependent Mg^{2+} block, and their activation requires simultaneous binding of both glutamate and the co-agonist glycine.

Origin and evolution of ligand-gated ion channels

Phylogenetic studies have shown that LGIC receptors probably derive, as a result of divergent evolution, from a common ancestral protoreceptor originated in a unicellular organism (Tasneem *et al.*, 2004) or in a bilaterian ancestor (Xue, 1998; Tsang *et al.*, 2006). This raises the possibility that members of this structurally related protein set might be widely present in living organisms including bacteria and primitive invertebrates. In the final part of last century, many studies provided evidence of the presence of LGIC receptors in various invertebrate species, nematodes, molluscs, insects, often showing unconventional properties. Based on the available data, early studies grouped the Cys-loop LGIC receptors into two clades, the cationic AChR and serotonin receptors, and the anionic GABA/glycine like receptors, whose divergence was estimated to have occurred at least 2500 million years ago (Ortells *and* Lunt, 1995). However, another phylogenetic analysis of the genome sequences of *Caenorhabditis elegans* and *Drosophila melanogaster* (Dent, 2006) only in part confirms these hypotheses. The study shows the existence of several LGIC families unique to invertebrates, suggesting that the major clades of the Cys-loop family likely diverged before the deuterostome/ecdysozoa split, and that many of these disappeared from chordates. Thus, evolution of contemporary phyla reveals a surprising loss of pentameric LGIC diversity in vertebrates (Dent, 2006; Dent, 2010).

The origin of cationic glutamate receptors may be equally ancient: a potassium channel activated by glutamate was discovered in prokaryotes, containing ligand-binding domains homologous to the eukaryotic glutamate receptors and the TVGYG sequence typical of potassium channels (Chen *et al.,* 1999). Based on this and other findings, a recent study advances the hypothesis of a common origin of glutamate receptors and voltage-gated channels, whose ancestor could be traced in prokaryotes (Tikhonov *and* Magazanik, 2009).

The ancient origin of the LGIC receptor superfamily appears to precede the evolution of nervous systems, raising questions related to the functional roles of these proteins and to the mechanisms driving their diversification. Current analyses favor the view that the last common bilaterian ancestor may have evolved a large and diverse family of both metabotropic and LGIC chemoreceptors, later diverging in different phyla to gain additional functions (Cockcroft *et al.*, 1990; Dent, 2006). The increasing availability of molecular data will help to better understand a crucial issue of neural development.

Classical neurotransmitters in *Hydra:* or, absence of evidence is not evidence of absence

Evidence for the presence of classical neurotransmitters in cnidarian nerves or tissues has accumulated from many mor-

phological and functional studies, starting from the early sixties; however, owing to technical difficulties and to contradictory findings, a general disbelief about the occurrence and possible transmitter role of molecules such as acetylcholine, glutamate, GABA and glycine in cnidarians has long prevailed (Martin *and* Spencer, 1983). By contrast, the abundance of biogenic amines and different neuropeptides found in neurons of many hydrozoan and anthozoan species by histochemical, immunocytochemical, biochemical and molecular studies led to the assumption that in cnidarians neurotransmission was essentially aminergic and/or peptidergic, despite the lack of direct evidence of the involvement of these peptides in neurotransmission or neuromodulation. Since the topic has been extensively reviewed elsewhere (Kass-Simon *and* Pierobon, 2007), only two new contributions are briefly discussed here. Instead, given the focus on the occurrence of neurotransmitter ionotropic receptors in *Hydra*, this section will present old and new data on the biochemical and biological activities of selected LGICs and their ligands, namely acetylcholine, glutamate, GABA and glycine. Part of this work has also been reviewed in Kass-Simon *and* Pierobon (2007).

A recent survey of the genome of *Nematostella vectensis* for genes related to chemical transmission and hormonal signaling (Anctil, 2009) has produced a wealth of interesting results. Proteins related to aminergic transmission, receptors, enzymes, transporters, are heavily represented in the genome of *Nematostella*. Tyrosinase, dopa decarboxylase and monoamino oxidase transcripts with varying degrees of similarity to vertebrate sequences were found, together with different types of aminergic receptors. A tentative classification shows that some of the receptor transcripts present more similarity to dopaminergic and serotonergic, others to adrenergic or histaminergic, vertebrate and protochordate orthologues. By contrast, the abundant melatonin receptor transcripts present more similarity to other sea anemone transcripts.

RFamide-related transcripts were found, together with transcripts related to cnidarian peptides of other families and to vertebrate peptides (galanins, tachyinins, vasopressin, melanocortin and more). A larger number of transcripts corresponding to putative peptide receptors, outmatching their potential ligands, were also found. The represented neuropeptide families show a greater diversity than anticipated from current knowledge on cnidarian peptides. Furthermore in the phylogenetic tree, transcripts related to specific cnidarian peptides appear to be closer to invertebrate than to vertebrate counterparts; the opposite is true of transcripts related to non-peptidergic transmitters. It is interesting to note that the survey supports the conclusion of gene diversification and gene loss by descendants of the common ancestors of cnidarians and bilaterians, in agreement with the hypothesis advanced in other studies (Dent, 2006; Dent 2010).

Finally, a novel ion channel, directly gated by the neuropeptides Hydra-RFamides I and II, was recently cloned from *Hydra* and found expressed in epitheliomuscular cells at the base of the tentacles (Golubovic *et al*., 2007; Durrnagel *et al.,* 2010). Homology searches show that this channel relates to members of the DEG/EnaC gene family, that includes the only peptide gated-ionotropic receptor known to date, FaNaC from snails.

Excitatory transmitters and receptors: acetylcholine

Although acetylcholine (ACh) was one of the first substances to be tested for effects on the bioelectric activity in cnidarians (Romanes, 1885), evidence of its presence and biological role is scarce and controversial. ACh was found in desmoneme nematocysts of *Hydra* sp. (Castano *and* Rossi, 1978). Acetylcholinesterase (AChE) reactivity was found in planula larvae of hydromedusae (Falugi *et al*., 1994), in nematocytes and hypostomal ganglion cells of *Hydra* (Lentz *and* Barrnett, 1961). Nicotinic AChR antagonists inhibited contraction bursts (CBs), the train of impulses that precedes ectodermal muscle contraction, while atropine, a muscarinic ACh antagonist, significantly increased them in *Hydra* (Kass-Simon *and* Passano, 1978). Exogenous ACh and cholinergic agonists were found to induce muscle contraction and nematocyst discharge (Lentz *and* Barrnett, 1962); however, the latter result was not confirmed by a later study (Scappaticci *and* Kass-Simon, 2008). AChE inhibitors were found to impair head regeneration (Lentz *and* Barrnett, 1963). ACh and choline esters increased the bioelectric activity of isolated muscle preparations from *Bunodosoma* (Mendes *and* Freitas, 1984); other studies failed to detect ACh, AChE and ChAT activity in other cnidarian species (van Marle, 1977; Scemes, 1989). Conversely, biochemical studies reported isolation and purification of choline acetyltransferase (ChAT) from *Hydra* (Erzen *and* Brzin, 1978) and from hydrozoan and anthozoan tissue homogenates (Talesa *et al*., 1992).

Interest for the biological role of acetylcholine has been revived by recent studies. AChE activity was found in the endoderm of tentacle bulbs, but not in the nervous system of *Clytia hemisphaerica* and three genes from the neuroligin-cholinesterase family were identified by BLAST search of a *Clytia* EST collection; enzyme activity was inhibited by physostigmine (Denker *et al*., 2008). A full length cDNA coding for AChE was cloned from *Hydra magnipapillata* (Takahashi *et al*., 2010); the HyAChE cRNA showed AChE activity in *Xenopus* oocytes and was expressed mainly in epithelial cells of the body column, but not in tentacles or basal disk. The authors conclude that their findings support a morphogenic role for ACh. However, it must be noted that, apart from western blot analysis, the study did not search for expression and localization of the corresponding protein; therefore, in my opinion, their conclusion should be regarded as temporary.

The analysis of the genome of *Nematostella vectensis* has found 20 sequences related to cholinergic functions, 3 ChATs, 5 AChEs and 12 nicotinic receptor subunits (Anctil, 2009). The presence of AChE and ChAT sequences in the *Nematostella* EST database suggests that these enzymes are expressed and potentially functional. The nicotinic receptor sequences show a range of 44-59% similarity to vertebrate and invertebrate nicotinic AChR subunits. The author concludes that all the proteins necessary for nicotinic neurotransmission are apparently present in the sea anemone, while transcripts of the muscarinic class could not be detected. Unfortunately, no data are available to date on the characterization of ACh receptor proteins in cnidarian systems. Although not conclusive, these new findings clearly indicate that research on cnidarian acetylcholine and its metabolic partners still is an open and promising field.

Excitatory transmitters and receptors: glutamate

Receptors to glutamate were discovered in *Hydra vulgaris* tissues twenty years ago. Binding studies show that glutamate binds to crude membrane fractions of *Hydra* (Bellis *et al*., 1991). The binding of L-[^{3}H]glutamate was rapid, reversible and saturable. A Scatchard analysis of the specific binding revealed values of 10

μM for K_D and 170 pmol/mg of protein for B_{max}. About 65% of the specific L-[^{3}H]glutamate binding was inhibited by reduced glutathione (GSH) (Bellis *et al.,* 1992). The remaining 35% of the specific L-[^{3}H]glutamate binding was displaceable by the GluR agonists kainate and quisqualate but not NMDA, giving the first indication of a putative glutamate receptor in *Hydra*. The GSH-sensitive glutamate binding site presumably represents the association of glutamate with a putative GSH receptor, whose existence had been postulated by Lenhoff as early as 1961. Further work has shown specific [^{35}S]GSH binding with K_D values of 3.4 μM and an EC_{50} value of 2.3 μM GSH (Grosvenor *et al*., 1992).

Following the discovery of strychnine-insensitive glycine binding sites in *Hydra* (Pierobon *et al*., 2001; see below), saturation experiments showed the presence of one population of binding sites with nanomolar affinity and low capacity for [^{3}H]MK-801, a potent, noncompetitive antagonist of the NMDA receptor (Pierobon *et al*., 2004a). Before equilibrium, [^{3}H]MK-801 binding was increased by the agonists glutamate and glycine as well as by GSH. These findings indicate that these receptors are functionally activated by their specific ligands; the ability of GSH to potentiate [^{3}H]MK-801 binding is consistent with data obtained in rat (Ogita *et al*., 1995) and *Hydra* (Bellis *et al*., 1991) membrane preparations.

In *Hydra*, $NMDAR_1$ immunoreactivity localised both in nerve and effector cells, epitheliomuscular cells and nematocytes, where NMDA plays a role in nematocyst discharge, through activation of calcium fluxes (Scappaticci *et al*., 2004; Scappaticci and Kass-Simon, 2008). Preliminary reports localize AMPA- and kainate-like immunoreactivity in neurons and nematocytes of the tentacles (Kass-Simon *and* Scappaticci, 2004).

In vivo, glutamate and its agonists AMPA and kainate appeared to be generally excitatory in the ectodermal pacemaker systems of *Hydra vulgaris*, where their administration increased the frequency of contraction bursts (CBs) and tentacle pulses (TPs), the pacemaker systems responsible for body and tentacle contractions, respectively (Kass-Simon *et al*., 2003). The increase in CBs or TPs frequency was counteracted by the GluR antagonist NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide), and potentiated by the lectin Con A, that prevents receptor desensitization in other systems (Evans *and* Usherwood, 1985). Association of the GluR agonist NMDA plus D-serine increased the frequency of TPs *per se* and in the presence of AMPA, but decreased the frequency of endodermal rhythmic potentials (RPs) induced by kainate; the ectodermal pacemaker system was not affected (Kay *and* Kass-Simon, 2009).

Apart from *Hydra*, where the glutamatergic system and its physiological activities have been studied in detail, very few data are available for other cnidarian species. Glutamate immunoreactivity was described in non-neuronal cells of the sea anemone *Metridium senile*, where, however, it has been considered a cell metabolite rather than a transmitter (Anctil and Carette, 1994). Martin (2004) reports that interneurons innervating the photoreceptors of the cubozoan *Carybdea marsupialis* label with anti-glutamate antibodies, as well as with antibodies made against serotonin, GABA and RFamide. A recent study reports glutamate immunoreactivity in the nerve plexus of the sea anemone *Phymactis papillosa*, where immunogold labeling localizes glutamate in neural processes (Delgado *et al*., 2010). Glutamate inhibits the electrical activity of the sphincter muscles in the sea anemone *Tealia felina* (Carlyle, 1974) but failed to affect the response of neurons of the motor nerve net of the scyphozoan jellyfish *Cyanea capillata* (Anderson *and* Trapido-Rosenthal, 2009).

Finally, a genomic analysis of the *Nematostella* database identified a large number of ionotropic GluRs, mainly AMPA-like and NMDA-like receptors. The AMPA orthologues show higher similarity to their bilaterian counterparts than NMDA orthologues. In addition, several members of the metabotropic GluR superfamily, together with calcium-sensing receptors, were detected (Anctil, 2009). The author suggests that the large number of glutamate-related sequences hints at an important role of glutamic acid as a transmitter, with a possible diversity of responses, in the sea anemone.

Inhibitory transmitters and receptors: GABA and glycine

Evidence of the occurrence of γ-amino butyric acid (GABA) and its cellular localization in *Hydra* has proven exceedingly difficult to obtain. A gas chromatography/mass spectrometry analysis of tissue homogenates revealed the presence of three fragmentation peaks perfectly coincident with those obtained from synthetic GABA at the same time of elution; this result was reproduced with two different derivatization methods (Pierobon, unpublished). However, at the time, we considered these findings inconclusive, since we were not able to confirm these data by HPLC and to observe convincing GABA immunoreactivity in *Hydra* cryosections, so that we could not exclude a possible bacterial origin of GABA. Therefore, we chose a different experimental approach.

Receptors to GABA were identified in membrane preparations from *Hydra vulgaris* (Pierobon *et al*., 1995). The binding of [^{3}H] GABA was specific (70% specific binding), reversible and saturable. A Scatchard analysis of saturation data indicated the presence of only one population of binding sites with high affinity (K_D = 76 nM) and low capacity (B_{max} = 4.75 pmol/mg of protein). [^{3}H]GABA binding was completely inhibited by the GABA agonist muscimol and by the $GABA_A$ receptor antagonist gabazine, whereas bicuculline was ineffective; baclofen, a $GABA_B$ receptor antagonist, weakly (>30%) inhibited [^{3}H]GABA binding. The apparent insensitivity to bicuculline and baclofen shown in competition experiments, similar to other invertebrate findings, suggested that these receptors were a primitive, early evolved, protein class.

Later studies revealed that *Hydra* receptor proteins exhibited a complex pharmacological profile (Concas *et al*., 1998; Pierobon *et al*., 2004b). The neuroactive steroids allopregnanolone (3α-hydroxy-5α-pregnan-20-one; THP) and tetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one; THDOC) increased [^{3}H] GABA binding to *Hydra* membranes with nanomolar potency and high efficacy, whereas the 3β-hydroxy epimer of allopregnanolone was ineffective. The benzodiazepine (BZ) receptor ligands diazepam, clonazepam and abecarnil enhanced [^{3}H]GABA binding to hydra membranes by 20% - 24%, effects abolished by the specific central BZ antagonist flumazenil. On the contrary, the peripheral BZ receptor ligand 4'chlorodiazepam failed to affect [^{3}H]GABA binding to *Hydra* membranes. Alphaxalone (3-hydroxypregnane-11,20-dione), barbiturates, and propofol similarly increased [^{3}H]GABA binding. The modulation of $GABA_A$ receptors by these various drugs *in vitro* correlated with their effects on the GSH-induced response in the living animals (see below). These pharmacological findings indicate that multiple binding sites exist on the *Hydra* GABA receptors, suggesting that they may be comprised of different subunits, at least α β γ and δ subunits, whose biochemical and pharmacological

properties compare with those of their mammalian counterparts. *In vivo*, GABA and its positive allosteric modulators were shown to decrease the frequency of CBs, the ectodermal pacemaker system, (GABA) and of RPs, the endodermal rhythmic potentials system (GABA, muscimol, diazepam); none of the ligands affected TPs frequency, i.e. tentacle activity. Interestingly, the inhibitory action of GABA was suppressed by bicuculline, *per se* ineffective (Kass-Simon *et al.*, 2003). GABA and the metabotropic $GABA_B$R agonist baclofen significantly increased the rate of distant desmoneme discharge, by acting through $GABA_B$ receptors in *Hydra* tentacles (Scappaticci *and* Kass-Simon, 2008).

Several authors reported negative results in biochemical, morphological and functional studies of *Hydra* and other cnidarian species, (Gitter *et al.*, 1994; Anctil *and* Minh, 1997; reviewed in Grimmelikhuijzen *et al.,* 1996, 2002). More recently, GABA immunoreactivity was found in interneurons of the cubozoan jellyfish *Carybdea marsupialis* (Martin, 2004) and in the ectodermal nerve net of the anthozoan sea anemone *Nematostella vectensis* (Marlow *et al.,* 2009). GABA immunogold labelling was found in putative neural processes within the neural plexus of the sea anemone *Phymactis papillosa* (Delgado *et al.,* 2010). A recent study reported identification of GABA by HPLC and immunocytochemical localization of related molecules, the synthesizing enzyme glutamic acid decarboxylase, the vesicular GABA transporter, and one of the GABA receptors, the metabotropic $GABA_B$ receptor, in neuronal and non neuronal cells of the sea fan *Eunicella cavolini* (Girosi *et al.,* 2007).

Following the characterization of GABA receptors, the occurrence of receptors to glycine, another inhibitory amino acid neurotransmitter in vertebrate nervous system, was investigated in *Hydra vulgaris*. We reported the identification and characterisation of the first invertebrate glycine receptor, whose biochemical and pharmacological properties also compare with those of vertebrate GlyRs (Pierobon *et al.*, 2001). Saturation experiments revealed the existence of one population of binding sites for [^{3}H]strychnine of nanomolar affinity (K_D = 33 nM) and low capacity (B_{max} = 79 fmol/mg protein). The addition of glycine or taurine (0.1 μM to 1 mM) produced a dose-dependent inhibition of [^{3}H]strychnine binding. β-alanine, a partial glycine agonist, did not significantly affect [^{3}H] strychnine binding at the same concentrations. [^{3}H]strychnine binding was not displaced by D-serine, a glycine agonist at the glycinergic binding site of NMDA receptors (Mothet *et al.*, 2000).

In the living polyp, the administration of D-serine reduced the duration of GSH-induced mouth opening suggesting the presence of strychnine-insensitive binding sites (Pierobon *et al.*, 2001). Extracellular electrophysiological experiments have shown that glycine and taurine, acting on strychnine-sensitive receptors, decreased the endodermal rhythmic potentials (RPs); in addition, glycine *per se* significantly decreased both the rate of CBs and the number of impulses per contraction burst (P/CB). However, in the presence of strychnine-insensitive site blockers, this effect was suppressed, suggesting that glycine acts on the ectodermal pacemaker system through the glycine-binding site of the NMDA receptor (Ruggieri *et al.*, 2004). The dual action of glycine observed in behavioral and electrophysiological experiments led to the characterization of NMDA receptors (see above).

These findings are not isolated. Carlberg *et al.,* (1995) found taurine-like immunoreactivity in the motor nerve net of the jellyfish *Cyanea capillata*. Anctil *and* Minh (1997) demonstrated taurine-like immunoreactivity in the peri-oral subectodermal nerves and the zooid nerve net of the endodermal retractor muscle of *Renilla koellikeri*, suggesting a possible role as a neuromuscular transmitter. Nakanishi *et al.,* (2009) found taurine immunoreactivity in sensory cells of the rhopalial nerve net in *Aurelia*. Taurine and β-alanine, together with GABA, glycine, glutamate and aspartate, are present in the free amino acid pool of neurons of the motor nerve net of *Cyanea capillata*, from which they are released by depolarization; however, when applied on neuron preparations, only taurine and β-alanine produced a response, namely a large depolarization followed by conductance change (Anderson *and* Trapido-Rosenthal, 2009). Thus in *Cyanea*, taurine and partly β-alanine act as excitatory transmitters at bidirectional synapses in the motor nerve net (Anderson *and* Grunert, 1988). The discrepancy between these results and our observations (in our experiments taurine acted as a typical GlyR agonist, both in behavioural and electrophysiological studies) cannot be satisfactorily explained on the basis of the available evidence. However, it is interesting to note that glycine receptors change their binding characteristics by point mutations in a variety of pathological conditions, such as inherited neurological disorders. In fact, substituting arginine 271 with either leucine or glutamine by site-directed mutagenesis transforms taurine and β-alanine from agonists into competitive antagonists (Rajendra *et al.*, 1995).

Finally, both $GABA_A$ and $GABA_B$ receptors, and one glycine receptor were found in the genome of *Nematostella*. $GABA_A$ sequences are more abundant, but they appear to be distantly related to their vertebrate orthologues; the difference in structure is also reflected by the lack of several residues important for GABA binding; by contrast, $GABA_B$ and GlyR transcripts show a higher degree of similarity (Anctil, 2009). These results are not consistent with the biochemical and pharmacological data obtained in *Hydra vulgaris*. It is possible that a survey of the *Hydra* genome might eventually draw a different picture; on the other hand, gene diversity and/or gene loss may be already observable within Cnidaria. Future studies, primarily the molecular characterization of GABA receptor genes in *Hydra*, are clearly needed in order to reach a better understanding of this controversial issue.

In conclusion, ACh and glutamate seem to play an excitatory role in *Hydra,* while GABA and glycine seem to be inhibitory, in keeping with the known biological activity of vertebrate neurotransmitters. At least two molecules, glutamate and GABA, act on nematocyte effectors, by increasing the rate of nematocyst discharge, while glycine and likely ACh are not effective. All substances, ACh, glutamate, GABA and glycine, modulate myoneme effectors, possibly acting on intermediate neurons. Evidence of neuronal localization is at present only available for glutamate and NMDA.

ACh and cholinergic agonists show an excitatory role, in that they induce muscle contraction, acting on both the ectodermal and endodermal pacemaker systems. Glutamate and its agonists AMPA and kainate display an excitatory role on body contraction and tentacle pulses; they also increase the frequency of endodermal rhythmic potentials. However another GluR agonist, NMDA, specifically increases tentacle activity, but inhibits the endodermal pacemaker system.

GABA has an inhibitory role on muscle contraction, by reducing the frequency of both ectodermal CBs and endodermal RPs, but does not significantly affect electrical activity of the tentacles. Finally glycine plays a dual role on contraction and elongation of

the body column, in that it has inhibitory effects on the endodermal pacemakers, and may be potentially excitatory on the ectodermal pacemaker system through its effects on the glycine site of the NMDA receptor (see Ruggieri *et al.,* 2004, for discussion).

Since intracellular recording data are not available, and the actual sites affected by treatment cannot be identified, these results rather reflect a composite effect of drugs on muscular and multiple neuronal effectors. In particular, glutamate and glycine acting on selective receptor types may cause opposing effects on the coordinating systems of body and tentacles. Future studies will help to obtain a more detailed picture of the role of these substances in *Hydra*.

The *Hydra* feeding response

Hydra polyps feed through a complex behavioral pattern prompted by prey capture. Tentacles sense vibrations of nearby swimming prey through mechanoceptors; chemical stimulants also concur to recognition of prey. This leads to activation of nematocytes, which discharge the nematocyst tubule into the prey, capturing and paralyzing it onto the tentacles. GSH, outflowing from the wounded prey, stimulates the opening of a mouth. Prey-loaded tentacles bring the prey into the mouth, where it is finally ingested. Part of the response, tentacle writhing and mouth opening, can be produced *in vitro* by polyps' exposure to GSH, which is the specific stimulant of the feeding behavior in *Hydra* as well as in other cnidarian species (Loomis, 1955; Lenhoff, 1961). Lenhoff (1974, 1977) described in detail the kinetics of the response to GSH, advancing the hypothesis that a specific chemoreceptor mediated this response. Later, two independent groups reported the occurrence and characterization of a GSH receptor population in *Hydra* tissues (Venturini, 1987; Grosvenor *et al*., 1992).

Several studies contributed to confirm and clarify several aspects of this model. Different steps of the feeding response (tentacle concert, tentacle writhing) are suppressed by monoclonal antibodies that bind to the cnidocil complex of tentacle nematocytes, suggesting that these antibodies may be directed against structural components involved in mechano- and/or chemotransduction (Golz *and* Thurm, 1992; Ohta *et al*., 1992). GABA and NMDA respectively increase the rate of discharge of desmoneme and stenotele nematocytes, originally considered independent functional units, suggesting a neural regulation of tubule discharge (Scappaticci *and* Kass-Simon, 2008). Previous studies had shown that discharge of stenotele nematocytes is voltage- and Ca^{2+}-dependent (Gitter *et al*., 1994).

Upon GSH stimulation, CBs pacemaker activity and body contractions are inhibited, tentacle movements become convulsive and TPs are also inhibited, while endodermal RPs are not affected (Rushforth *and* Hofman, 1972); a mouth opens abruptly. Formation and opening of the mouth, that is normally undetectable in non-stimulated polyps, are ultimately attained by coordinated contraction and relaxation of myofibrils embedded in the ectodermal and endodermal epithelial cells of the head (Campbell, 1987; Technau *and* Holstein, 1995) mediated by electrical synapses (Hufnagel *and* Kass-Simon, 1976).

Transduction of the GSH signal relies on second messengers, whose identification is still quite controversial. Cobb *et al.,* (1980) report an increase of both cGMP and cAMP levels in the presence of GSH, but exclude a direct or indirect action of cyclic nucleotides on the feeding behavior. Dopamine increases cAMP levels, prolonging duration of the response to GSH (Venturini and Carolei, 1992). Elevated nitric oxide (NO)-induced cGMP levels are able to trigger inhibition of the response to GSH, presumably via cGMP-activated protein kinases (Colasanti *et al*., 1997). A widespread distribution of NADPH-diaphorase reactivity, a marker of NO synthase, was found in nerves of the body column but not of tentacles of *Hydra vulgaris* (Cristino *et al*., 2008). The NO pathway is also involved in triggering nematocyst discharge in the sea anemone *Aiptasia diaphana*, where NO synthase localizes in acontial tissue surrounding nematocytes (Salleo *et al*., 1996). Furthermore, the anatomical pathways leading from mechano- and chemoreceptors to the effector cells involved are only in part identified. Recent studies show that hydrozoan nematocytes send and receive synaptic signals upon mechano-chemical stimulation, acting as bimodal sensory cells (Thurm *et al*., 2004; Oliver *et al*., 2008).

The feeding behavior is interrupted upon GSH removal, but the mechanisms by which termination of the response is achieved are still poorly understood. Lenhoff (1974) suggested that mouth closure simply derived from receptor desensitization, or unbinding of GSH from its receptor. This hypothesis was challenged by the finding that wounded prey release other substances, as well as GSH, that are able to shorten response duration by competitive inhibition of GSH binding (Grosvenor *et al*., 1996). Our group found that inhibitory and excitatory amino acid transmitters modulate the feeding behavior by prolonging or shortening, respectively, the duration of the response to GSH.

The γ-glutamil-cysteinyl-glycine (GSH) assay

The GSH assay is a simple and reliable model for the study of chemoreception. Onset of response, i.e. opening of the mouth is very fast (seconds) and occurs within 1 - 2 minutes after GSH administration; mouth closure is a slower process, and occurs 20 to 25 minutes at 10 μM GSH. Duration of the response, i.e. the time interval between mouth opening and mouth closure, is linearly related to the stimulus intensity in the low micromolar GSH concentration range and can be easily measured, providing a quantitative parameter for process evaluation.

In the presence of GABA, glycine, and their agonists, the mouth opens in 1 to 2 minutes upon GSH stimulation, with no difference with respect to controls, but mouth closure is delayed, resulting in a significant increase of response duration. Conversely NMDA, a GluR agonist, significantly reduces response duration by anticipating times of mouth closure, but does not affect times of mouth opening; glutamate, AMPA and kainate, that prevent mouth opening, could not be used in the GSH assay. These data suggest that amino acid transmitters, presumably acting on the cellular circuitry involved, regulate part of the response activated by GSH. The action of transmitters is exerted through their specific receptors, identified and characterized by binding experiments.

Administration of 100 μM GABA significantly increased the duration of mouth opening induced by GSH. Prolonged exposure to the ligand suppressed the effect, suggesting receptor desensitization. As predicted from biochemical experiments, $GABA_A$R agonists and positive allosteric modulators mimicked or potentiated these effects of GABA in a dose-dependent manner. The specific $GABA_B$ agonist baclofen did not significantly modify duration of the GSH-induced response (Pierobon *et al*., 1995; Concas *et al*., 1998).

The classical $GABA_A$ receptor antagonist bicuculline, *per se*

inactive, was able to suppress the GABA-induced increase in response duration. Another $GABA_A$ receptor antagonist, gabazine, suppressed the effects of GABA and GABA agonists in a 1-10 μM concentration range; in the absence of GABA, it shortened response duration. The specific Cl^- channel blockers, picrotoxin (10μM) and TBPS (1 μM), which *per se* shortened the duration of the response, also abolished the increase in response duration induced by GABA and counteracted the effects of muscimol, neurosteroids and general anesthetics. These results are summarized in Table 1, Fig. 3.

Taken together, these data indicate that in the living polyp GABA modulates duration of the feeding response acting through $GABA_A$-like receptors: in fact, both ligand binding and channel gating pharmacological properties are typical of canonical $GABA_A$Rs. Furthermore, these results suggest that receptor subpopulations of different subunit composition may be present, with varying degrees of affinity for specific agonists and antagonists. Finally, the finding that the GSH response is shortened by gabazine and by Cl^- channel blockers suggests a role of endogenous GABA in modulation of the response in physiological conditions.

A significant increase in the duration of mouth opening was also obtained in the presence of glycine, taurine or β-alanine. The enhancement of the response was related both to amino acid (10-100 μM) and to GSH concentration (1-10 μM). The effects of glycine or its agonists were suppressed by the GlyR antagonist strychnine (1-10 μM) and by picrotoxin (1-10 μM) (Pierobon *et al.*, 2001). However, glycine, but not taurine, reduced duration of mouth opening when all the strychnine-sensitive binding sites were blocked by 10 μM strychnine; this effect was mimicked by D-serine. The effects of D-serine were suppressed by the antagonist DCKA (5,7-dichlorokynurenic acid) but not by strychnine (Table 2, Fig. 4). In fact, in the presence of DCKA, the administration of glycine plus high strychnine increased the duration of mouth opening by 40%, increase significantly higher than control animals, but also

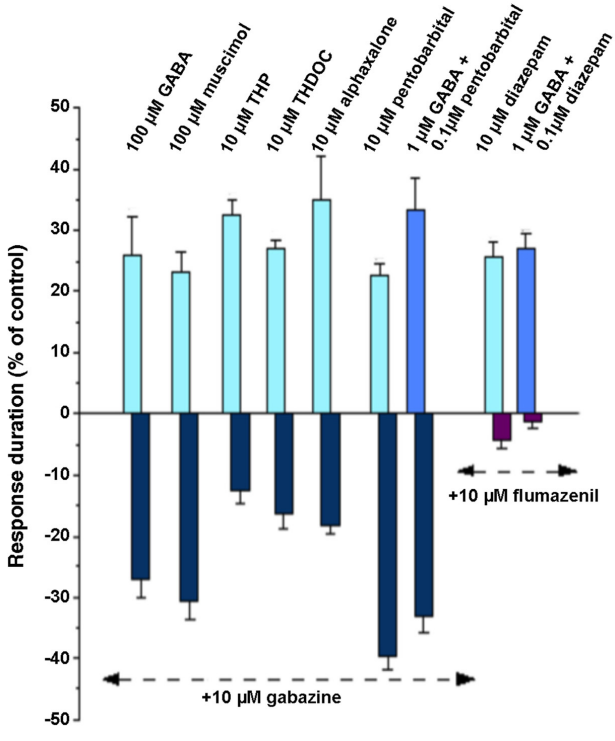

TABLE 1

EFFECTS OF GABA AGONISTS AND ANTAGONISTS ON THE DURATION OF THE RESPONSE TO GSH

AGONISTS		ANTAGONISTS	
		+1 μM TBPS	+10 μM gabazine
solvent	100%	-31.5% ± 4.1	-22.8% ± 3.5
100 μM GABA	+25.7% ± 6.4	-23.5% ± 2.5	-27.1% ± 3.1
100 μM muscimol	+23.1% ± 3.1	-25.7% ± 2.5	-30.7% ± 2.7
10 μM THP	+32.3% ± 2.5	+2.9% ± 1.1	-12.6% ± 2.3
10 μM THDOC	+26.9% ± 1.3	-16.7% ±2.4	-16.5% ± 2.1
10 μM alphaxalone	+34.6% ± 7.5	+4.8% ±1.9	-18.5% ± 1.0
10 μM pentobarbital	+22.4% ± 1.9		-39.8% ± 2.3
1 μM GABA + 0.1 μM pentobarbital	+33.2% ± 5.1		-33.3% ± 2.8
			+10 μM flumazenil
10 μM diazepam	+25.5% ± 2.4		-4.5% ± 1.1
1 μM GABA + 1 μM diazepam	+26.8% ± 2.5		-1.6% ± 1.0

GABA and muscimol significantly increase duration of the response to GSH, the increase is suppressed by gabazine or by Cl^- channel blockers TBPS (GABA) and 10 μM PTX (muscimol). Neurosteroids, pentobarbital, and diazepam, also increase response duration, potentiating the action of GABA. Their effects are suppressed by GABA antagonists. Data are presented as mean percentage variation ± SEM of control (10 μM GSH). See corresponding graph in Fig. 3.

of animals treated with the corresponding dose of glycine alone. Thus glycine also appears to be involved in modulation of the feeding behavior, acting both on ionotropic GlyRs, as shown by picrotoxin, with a presumed inhibitory action, and on the glycine binding site of putative NMDA receptors, whose action could be excitatory.

The dual action of glycine in *Hydra* tissues suggested that NMDA receptors might also be present in *Hydra*, and implicated in the modulation of the feeding behavior. Accordingly, the GluR agonist NMDA markedly decreased the duration of the response to GSH. This effect was linearly related to ligand doses in the nanomolar concentration range and was counteracted by either the NMDAR specific antagonist D-AP5 (D-(-)-2 amino-5-phosphopentanoic acid) or by the D-serine antagonist DCKA. When NMDA concentration was increased to 10 or 100 μM, duration of the response to GSH was no longer affected unless Con A was added to the test medium. Simultaneous administration of ineffective doses of NMDA and strychnine, glycine or D-serine, resulted in a strong reduction of response duration. Both D-AP5 and DCKA suppressed this effect (Table 2, Fig. 4) (Pierobon *et al.*, 2004a).

In order to investigate the contribution of endogenous amino acids to the GSH response, polyps were exposed to depolarizing pulses of 56 mM KCl or 1 μM veratridine. In these conditions, the animals retained the ability to perceive the GSH stimulus and to react appropriately. However, the magnitude of the response was significantly reduced. GABA and its agonist muscimol were able to restore or to enhance duration of the response in stressed

Fig. 3. Effects of GABA agonists (GABA, muscimol, THP, THDOC, alphaxalone, pentobarbital, diazepam) and GABA antagonists (gabazine, flumazenil) on the response to γ-glutamil-cysteinyl-glycine (GSH). *GABA and muscimol significantly increase the duration of the response to GSH, the increase is suppressed by gabazine or by Cl^- channel blockers TBPS (GABA) and 10 μM PTX (muscimol). Neurosteroids, pentobarbital, and diazepam, also increase response duration, potentiating the action of GABA. Their effects are suppressed by GABA antagonists. Data are presented as mean percentage variation ± SEM of control (10 μM GSH, see Table 1).*

TABLE 2

EFFECTS OF GLYCINE, TAURINE, β-ALANINE, STRYCHNINE, NMDA, D-SERINE, AND NMDA ANTAGONISTS, ON THE DURATION OF THE RESPONSE TO GSH

AGONISTS		ANTAGONISTS	
		+10μM PTX	+10μM strychnine
solvent	100%	-18.1% ± 4.7	-4.8% ± 3.4
100μM glycine	+22.1% ± 1.5	-1.8% ± 3.6	-18.6% ± 2.9
100μM taurine	+26.5% ± 3.0	-6.1% ± 7.6	+4.5% ± 4.1
100μM βalanine	+25.0% ± 2.8	+3.5% ± 4.2	-0.2% ± 3.0
100μM glycine + 0.1μM DCKA	+39.7% ± 3.5		+5.2% ± 1.5
		+0.1μM DAP5	+0.1μM DCKA
		+0.1% ± 5.1	-0.4% ± 6.1
100μM D-serine	-35.1% ± 1.6	+2.7% ± 3.3	+0.4% ± 2.7
0.1μM NMDA	-1.2% ± 5.3		
0.1μM NMDA + 0.1μM D-serine	-39.9% ± 4.3	+0.1% ± 5.1	-0.4% ± 6.1
0.1μM NMDA+ Con A	-19.1% ± 1.0	0.0% ± 1.0	
1μM NMDA	-22.7% ± 2.3	-3.4% ± 0.7	-2.5% ± 0.9
1μM NMDA + Con A	-39.8% ± 5.1	+4.9% ± 2.4	

Glycine, taurine, β-alanine significantly increase response duration; the increase is suppressed by PTX and by strychnine. The significant decrease of response duration observed in the presence of 10μM strychnine plus 100 μM glycine is suppressed by the specific NMDA antagonist DCKA. NMDA significantly reduces response duration. The action of NMDA is potentiated by Con A (27 μg/ml) and by the co-agonist D-serine, *per se* also shortening the response at higher doses. Both DAP5 and DCKA counteract the effects of NMDA and D-serine. Data are presented as mean percentage variation ± SEM of control (10 μM GSH). See corresponding graph in Fig. 4.

animals in a dose-dependent manner, while glycine and taurine did not counteract the decrease in response duration. Gabazine suppressed the effects of GABA and muscimol (Pierobon *et al.*, 2004b). These results support the conclusion that GABA appears to be the major inhibitory transmitter involved in the regulation of the feeding response, and that GABA and glycine act by different pathways.

Problems and perspectives

The finding that GABA and glycine do not modify the time interval between GSH administration and opening of the mouth suggests that these molecules are not interfering with the GSH transduction pathway but may rather be acting on myofibril effectors. Alternatively, electrophysiological data support the hypothesis that at least GABA could act synergistically with the GSH stimulus, reinforcing inhibition of ectodermal and endodermal pacemaker systems. In addition GABA, acting through its metabotropic receptor, could serve a chemosensory role in the initial steps of the feeding behavior. Termination of the response, then, could result from of a coordinated interplay of different sets of target cells (neurons?) that regulate muscle contraction and relaxation.

The mechanisms of action of glycine and glutamate are more difficult to understand, and more information should be gathered in order to draw a reliable picture. Glycine, like NMDA, exerts both an inhibitory role on endodermal RPs and an excitatory role on ectodermal pacemakers, but, differently from NMDA, does not affect tentacle activity; its action could be tentatively attributed to differential modulation of neural intermediates involved in the effector response to GSH stimulation or in the GSH transduction pathway. Studies directed at identifying the cellular localization of the different glycine binding sites are clearly needed to begin to solve the riddle.

Glutamate and GluR agonists appear to act in all stages of the feeding behavior, with multiple mechanisms of action. Glutamate and its iGluR agonists modulate the rate of nematocyst discharge, possibly acting with a chemosensory role. Glutamate prevents the GSH-induced mouth opening *in vivo*, but does not displace the specific binding of radiolabeled GSH, and must therefore block the feeding response by a mechanism other than competitive inhibition. An effect of glutamate on metabotropic GluRs, not yet identified in *Hydra*, could not be excluded in principle. It is interesting to note that a taste receptor, the vertebrate umami receptor, is a metabotropic glutamate receptor variant (Chaudhari *et al.*, 2000). Conversely GSH binds to GluRs or potentiates NMDA binding *in vitro*. It is tempting to speculate that the putative *Hydra* GSH receptor may belong to an ancestral class of sensory chemoreceptors, distributed on the ectodermal epithelial cell membranes of the polyp, and later evolved to serve different or additional functions. A possible role of glutathione as a new neurotransmitter and/or neuromodulator, in addition to its well-known cellular anti-oxidant actions, has been repeatedly proposed (Ogita *et al.*, 1995; Shaw, 1998; Janaky *et al.*, 2000). Finally, glutamate and its iGluR agonists modify the electrical activity of ectodermal and endodermal pacemaker systems with effects opposite to GSH; accordingly, at

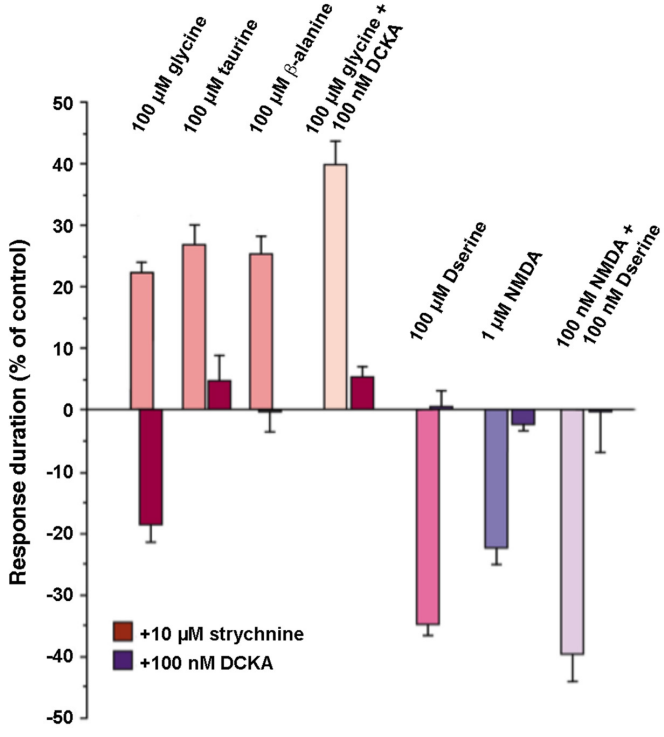

Fig. 4. Effects of glycine, taurine, β-alanine, strychnine, NMDA, D-serine, and NMDA antagonists, on the response to γ-glutamil-cysteinyl-glycine (GSH). *Glycine, taurine, β-alanine significantly increase response duration; the increase is suppressed by PTX and by strychnine. The significant decrease of response duration observed in the presence of 10 μM strychnine plus 100 μM glycine is suppressed by the specific NMDA antagonist DCKA. NMDA significantly reduces response duration. The action of NMDA is potentiated by Con A (27 μg/ml) and by the co-agonist D-serine,* per se *also shortening the response at higher doses. Both DAP5 and DCKA counteract the effects of NMDA and D-serine. Data are presented as mean percentage variation ± SEM of control (10 μM GSH, see Table 2).*

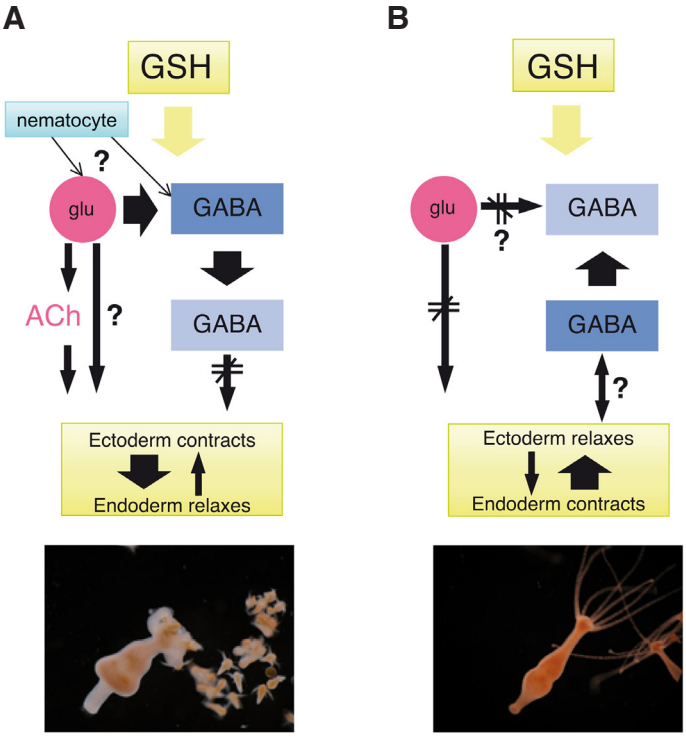

Fig. 5. A working model of the hypostomal circuitry regulating the feeding response and its cessation in *Hydra*. (A) *Mechanisms controlling mouth opening. Upon exogenous GSH stimulation, and possibly upon concurrent afferent synaptic signals from discharged nematocytes, a glutamatergic pathway is activated. Multipolar glutamatergic neurons stimulate the ectodermal pacemakers, either directly or via cholinergic neurons. They simultaneously stimulate a GABAergic pathway comprised of at least two interneurons (ganglion cells?), whose inhibitory input on the mouth ectoderm is thus suppressed. As a result of this combined stimulation, the ectodermal myofibrils of the mouth contract, and the endodermal myofibrils relax: the mouth opens.* **(B)** *Termination of the feeding behavior. In time, the endodermal rhythmic potential (RP) pacemaker overrules the contraction burst (CB) pacemaker, the ectodermal epithelial cells no longer responding to GSH/transmitter stimulation. Furthermore, transmitter reuptake by the GABAergic interneuron(s) blocks the GABAergic pathway, resuming inhibition of the ectoderm muscles. The endodermal myofibrils contract, while the ectodermal myofibrils slowly relax: the mouth closes. In the presence of exogenous GABA, the GABAergic pathway is potentiated: prolonged stimulation of GABAergic interneurons increases duration of the contractions of ectodermal myofibrils, opposing the activation of RP pacemakers, and therefore delaying mouth closure. In the presence of exogenous NMDA, concomitant activation of glutamatergic neurons by GSH signals and NMDA may result in overstimulation, anticipating onset of refractory state and/or receptor internalization, thus reducing the efficacy of the GSH stimulus. As a consequence, the duration of ectodermal contraction and therefore of mouth opening is shortened. Alternatively, a second inhibitory pathway, as yet not identified, may be activated by NMDA. Finally, other neuronal subsets reacting to different gluR agonists might concur to the overall response to GSH.*

least one of the GluR agonists, NMDA, shortens duration of the response. The neuronal localization of NMDAR immunoreactivity supports the hypothesis that glutamatergic neurons play a role in modulation of the feeding response.

Why do inhibitory transmitters prolong the duration of the response to GSH and excitatory transmitters reduce it? A satisfactory answer to the question is hampered by the lack of detailed or sufficient information on basic facts such as cellular and regional receptor localization; neurochemical anatomy of the circuitry involved; identification, localization and timing of response of effectors to GSH and/or of the GSH signal transduction pathway; detailed analysis of the processes leading to mouth opening. Nonetheless, a provisional working hypothesis, based on the assumption that regulation of local excitation and inhibition in the hypostome by neurotransmitters and GSH may produce different results with respect to the body column (shown in Fig. 5), could help to focus on aspects and problems to be addressed in future experiments.

In conclusion, despite the incompleteness of the picture, a growing body of evidence supports the hypothesis that in *Hydra* the coordinated neural activation and inhibition of the contractile elements responsible for mouth closure in response to GSH stimulation is finely tuned by GABA and glutamate with opposite actions, while glycine has a dual role. The receptors subserving the amino acids actions appear to belong to the LGIC superfamily by their pharmacological properties, and may be prototypical molecules, with more than one functional role. The ability to perceive and react to chemical stimuli with appropriate responses is a primary tool for survival of multicellular organisms. Understanding development and evolution of the molecular machinery needed to integrate cell signaling and effector output can take advantage of a comparative approach. A better knowledge of the mechanisms regulating the feeding behavior in *Hydra* would be of interest for cnidarian physiologists, but could also add useful information for understanding the evolution of structure and functions of the basic tools of cellular signaling, the ligand-gated ion channel receptors.

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