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ORIGINAL PAPER

Immunochemical Localization of $GABA_A$ Receptor Subunits in the Freshwater Polyp *Hydra vulgaris* (Cnidaria, Hydrozoa)

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Abstract γ-aminobutyric acid (GABA) receptors, responding to GABA positive allosteric modulators, are present in the freshwater polyp *Hydra vulgaris* (Cnidaria, Hydrozoa), one of the most primitive metazoans to develop a nervous system. We examined the occurrence and distribution of $GABA_A$ receptor subunits in *Hydra* tissues by western blot and immunohistochemistry. Antibodies against different $GABA_A$ receptor subunits were used in *Hydra* membrane preparations. Unique protein bands, inhibited by the specific peptide, appeared at 35, 60, ~50 and ~52 kDa in membranes incubated with α3, β1, γ3 or δ antibodies, respectively. Immunohistochemical screening of whole mount *Hydra* preparations revealed diffuse immunoreactivity to α3, β1 or γ3 antibodies in tentacles, hypostome, and upper part of the gastric region; immunoreactive fibers were also present in the lower peduncle. By contrast, δ antibodies revealed a strong labeling in the lower gastric region and peduncle, as well as in tentacles. Double labeling showed colocalization of α3/β1, α3/γ3 and α3/δ immunoreactivity in granules or cells in tentacles and gastric region. In the peduncle, colocalization of both α3/β1 and α3/γ3 immunoreactivity was found in fibers running horizontally above the foot. These data indicate that specific $GABA_A$ receptor subunits are present and differentially distributed in *Hydra* body regions. Subunit colocalization suggests that *Hydra* GABA receptors are heterologous multimers, possibly sub-serving different physiological activities.

Keywords $GABA_A$ receptor subunits · *Hydra* · Western blot · Immunohistochemistry

Introduction

Our present knowledge on the structure and function of ionotropic $GABA_A$ receptors ($GABA_A$Rs) is based essentially on studies of mammalian receptor proteins. To date, considerable progress has been made towards a better understanding of the nature and mechanisms of action of these receptors. $GABA_A$Rs are heteropentameric complexes formed by five different subunits, usually two α, two β and one γ subunit, belonging to the Cys-loop family of Ligand-Gated Ion Channels (LGIC). The subunit composition determines different degrees of affinity for various ligands, based on the stoichiometry of the receptor binding sites (see [1] for an updated review). Cloning of $GABA_A$R genes has shown a substantial homology of extracellular and transmembrane domains in vertebrate receptor proteins [2]. A recent study by Miller and Aricescu [3] presents a three-dimensional crystal structure of a human β3 homopentamer GABA receptor, opening new perspectives into the signalling mechanisms of LGICs.

By contrast, systemic knowledge about invertebrate GABA receptors is still in progress. Receptors to GABA

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have been described and/or characterized in many species of different phyla, nematodes, mollusks, insects, crustaceans, tunicates, as well as in bacteria [4]. In *Caenorhabditis elegans* [5], and in *Drosophila melanogaster* [6, 7], the corresponding genes have been cloned. Despite the considerable variability of results, it is becoming clear that the biological activity and the pharmacological properties of GABA receptors in many invertebrate species are similar to that of mammalian ones, though not fitting precisely into the classification developed for mammalian brain. Furthermore, even though amino acid identity can be very low, multiple sequence alignments show a high degree of conservation of critical residues, thereby supporting the inclusion of invertebrate GABA receptors in the Cys-loop family [8, 9]. Invertebrate pentameric LGICs, in fact, include receptors to GABA, acetylcholine, serotonin, and histamine, as well as excitatory GABA receptors [4, 8]. In addition, glutamate-gated chloride channels, also belonging to the LGIC receptor superfamily, are typically present in many invertebrates [10–12].

We have previously shown the presence of high affinity $GABA_A$Rs in *Hydra vulgaris* (Cnidaria, Hydrozoa), among the most primitive metazoans to develop a nervous system. In membrane preparations the binding of [^{3}H]GABA was specific, reversible and saturable. A Scatchard analysis of saturation data indicated the presence of one population of binding sites with high affinity and low capacity. [^{3}H]GABA binding was completely inhibited by the GABA agonist muscimol but not by the $GABA_A$ receptor antagonist bicuculline [13]. These data suggested the temporary conclusion that *Hydra* GABA binding proteins represented a primitive receptor type in an early-evolved nervous system [14].

Later studies revealed that *Hydra* receptor proteins exhibit a rather complex pharmacological profile. Sensitivity to Cl^- channel ligands suggested that these receptors belong to the superfamily of ionotropic GABA receptors. Neuroactive steroids, benzodiazepines and general anaesthetics increased [^{3}H]GABA binding to *Hydra* membranes with nanomolar potency and high efficacy, effects abolished by the respective specific antagonists [15]. These findings indicate that multiple binding sites for different ligands exist on the *Hydra* GABA receptors, suggesting that they may be comprised of different subunits, namely α, β, γ, or δ subunits, whose biochemical and pharmacological properties compare with those of their mammalian counterparts.

In the whole polyp, 100 µM GABA increased the duration of mouth opening in response to reduced glutathione (GSH); this effect was suppressed by the classical $GABA_A$R antagonists gabazine and bicuculline, and by the Cl^- channel blockers picrotoxin or t-butylbicyclophosphorothionate (TBPS) in a 1–10 µM concentration range. 100 µM muscimol, diazepam, general anaesthetics and neuroactive steroids at 1–10 µM concentrations also increased the duration of GSH-induced mouth opening, mimicking the effect of GABA [13, 15, 16]. These findings indicate that, in vivo, GABA and its allosteric modulators are able to regulate the feeding behaviour, possibly by fine tuning the contractile elements involved in mouth opening and closing (reviewed in [17]).

In a recent study we showed that GABA administration to *Hydra* polyps amputated just below tentacle insertion, i.e. to heads comprised of hypostome and tentacles but lacking the entire body column, produced a significant decrease in duration of mouth opening with respect to control, intact polyps; in animals cut just below the budding region, i.e. comprised of head and gastric region, but lacking peduncle and foot, GABA administration did not modify duration of the response to GSH. Thus, the action of GABA and GABA agonists was reversed or abolished in amputated polyps, depending on the apical or basal level of the cut [18]. These data suggest that in *Hydra* multiple GABAergic loci exist in different body regions, possibly contributing to sequential modulation of the neuronal circuitry that controls motility and feeding. A neurochemical map of receptors to neurotransmitters should help to gain a better understanding of the functional organization of the conducting systems.

In this paper we report the occurrence and differential distribution of $GABA_A$R subunits in *Hydra* labeled by western blot and immunohistochemical analysis.

Materials and Methods

Animals

Hydra vulgaris were originally obtained from Prof. P. Tardent (University of Zurich, Switzerland), and cultured asexually in our laboratories by the method of Loomis and Lenhoff [19], with minor modifications. Polyps were kept at 18 ± 1 °C under artificial 12 h-light, 12 h-dark cycle in physiological solution [1 mM $CaCl_2$, 0.1 mM $NaHCO_3$ (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. Experiments were carried out on animals starved 3–4 days before use.

Immunoblot Analysis

Groups of 3000–4000 *Hydra* polyps starved for 3 days were collected in physiological solution (1 mM $CaCl_2$, 0.1 mM $NaHCO_3$; pH 7.3–7.4), washed once or twice to remove debris and food particles by low-speed centrifugation, resuspended in ice-cold distilled water and homogenized on ice with a Teflon pestle and a glass homogenizer. The homogenate was immediately centrifuged at 48,000×*g* for 10 min at 4 °C; the resulting pellet, resuspended in ice-cold

distilled water (1000 *Hydra* per ml), was frozen at −80 °C until use (48 h to 3 months later).

On the day of assay, *Hydra* membranes were thawed, diluted in a final volume of 7 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, bacitracin (200 µg/ml), and aprotinin (1 µg/ml) and centrifuged at 1000×*g* for 20 min at 4 °C. The resulting supernatant was collected and further centrifuged at 12,000×*g* for 20 min at 4 °C; the resulting pellet was suspended in homogenization buffer (2.5 ml) and then stored at −20 °C. For western blots, equal amounts of protein (40 µg) were heated for 5 min at 70 °C, and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4–12 % Bis-Tris Midi gels (NuPAGE Novex; Life Technologies, Milan, Italy). The separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane and then subjected to immunoblot analysis with polyclonal antibodies against the $GABA_AR$ subunits α1, α3, (Alomone Labs, Jerusalem, Israel, 1:300 dilution), and α2, β1, β2, β3, γ1, γ2, γ3, δ, ε, ρ (Santa Cruz Biotechnology, Heidelberg, Germany, 1:200 dilution). The specificity of the reactions was shown by preincubation of the specific antibody with the respective peptide at 0.5 pM final concentration. Blocking peptides were obtained from Alomone Labs, Jerusalem, Israel (anti-$GABA_AR$ α1 and α3 subunits), and from Santa Cruz Biotechnology, Heidelberg, Germany (anti-$GABA_AR$ α2, β1, β2, β3, γ1, γ2, γ3, δ, ε, and ρ subunits).

Immune complexes were detected with the use of an ECL Plus detection kit (GE Healthcare, Milan, Italy) and the presence of each subunit was assessed by the visualization of immunoreactive bands with the use of a Geliance Imaging System (Perkin Elmer, Monza, Italy).

Immunohistochemistry

About 100 specimens of *H. vulgaris* were used for all experiments: five–six polyps were used for each antibody, both for immunoperoxidase and immunofluorescent labeling. Experimental animals were starved 3–5 days before use. The immunohistochemical localization of $GABA_AR$ subunits was performed on whole polyps.

On the day of the experiment the animals were relaxed in 2 % urethan (Carlo Erba Reagents, Milan, Italy) in physiological solution ($CaCl_2$, 1 mM, $NaHCO_3$, 0.1 mM; pH 7.3–7.4), for 2–5 min, and then fixed by immersion in 4 % paraformaldehyde (wt/vol)/0.1 M phosphate buffer (PB, pH 7.4) freshly prepared before use, for 1 h at 4 °C [20]. After fixation, the animals were rinsed thoroughly in PB and incubated for 1 h at room temperature in PB containing 0.3 % Triton X-100, to permeabilize the cells, and 10 % normal donkey serum (Sigma Aldrich, Saint Louis, USA), to minimize non-specific binding. The animals were then incubated overnight at 4 °C with the primary antibodies (diluted 1:200).

Two different methods were used: 3,3′-diaminobenzidine (DAB) staining and immunofluorescence. For confocal or light microscopy the primary polyclonal antibodies used were: rabbit anti-$GABA_AR$ α3, (AGA-003, Alomone Labs, Jerusalem, Israel), goat anti-$GABA_AR$ β1, [(R-20), sc-31426], goat anti-$GABA_AR$ γ3 [(P-19), sc-31434], and goat anti-$GABA_AR$ δ [(N-20), sc-31436], (Santa Cruz Biotechnology, Heidelberg, Germany). For bright-field microscopy, the biotinylated secondary antibodies used were goat anti-rabbit and rabbit anti-goat (Vector Laboratories, UK), followed by ABC reagent and DAB Peroxidase Substrate (Vector Laboratories, UK). For double immunofluorescence the animals were incubated overnight at 4 °C with the following couples of primary antibodies: $GABA_AR$ α3/$GABA_AR$ β1; $GABA_AR$ α3/$GABA_AR$ γ3 and $GABA_AR$ α3/$GABA_AR$ δ. In these experiments all primary antibodies were used at 1:100 dilution.

Multiple immunofluorescence was revealed by specific Alexa −488 or −546 secondary donkey anti-IgGs (Invitrogen Life Technologies, Paisley, UK). The sections processed for immunofluorescence were observed by confocal microscope (Zeiss 710) and acquired from one airy unit pinhole, and emission spectra for each dye were limited as follows: Alexa Fluro 488 (505–540 nm) and Alexa 546 (560–580 nm). Pictures were acquired from orthogonal z-stack with a depth interval of 1.0 µm through 30 µm thickness with the 40× water objective (N.A. 1.40). Images were processed using the ZEN2012 software (Zeiss). Single DAB immunohistochemistry was acquired by a digital camera DFC 340FX (Leica, Germany) connected to the microscope DMI6000 equipped with appropriate filters and deconvolution software MetaMorph (Leica, Germany).

Control incubations of the immunosignals were performed (a) without addition of each specific primary antibody, and (b) with addition of the primary antibody, at 1:200 or 1:100 dilution, plus the corresponding blocking peptide at 1:100 or 1:50 dilution. Blocking peptides were obtained from Alomone Labs, Jerusalem, Israel (anti-$GABA_AR$ α3: AGA-003 P34903), and Santa Cruz Biotechnology, Heidelberg, Germany (anti-$GABA_AR$ β1 [(R-20), sc-31426p], anti-$GABA_AR$ γ3 [(P-19), sc-31434p], anti-$GABA_AR$ δ [(N-20), sc-31436p]).

Results

Immunoblotting

In order to evaluate the presence of $GABA_AR$ subunits in *H. vulgaris* membrane preparations, different antibodies against α, β, γ, δ, ε and ρ subunits were tested. For each

antibody tested in western blot experiments, only those bands that were specifically inhibited by preincubation with the respective peptide were considered suitable. In the membranes incubated with the N-terminus (29–43) anti-α3 antibody, the unique band inhibited by the specific peptide appeared in the location of the 35 kDa marker (Fig. 1a). Protein bands blotted with the cytoplasmatic domain (R-20) anti-β1 antibody occurred at 60 kDa (Fig. 1b). Immunoblot analyses with the cytoplasmatic domain P-19 anti-γ3 antibody, and N-terminus (N-20) anti-δ antibody revealed protein bands in the locations of approximately 50 and 52 kDa markers, respectively (Fig. 1c, d). Finally, several additional antibodies, namely anti- α1, α2, β2, β3, γ1, γ2, ε and ρ2 subunits of the human $GABA_AR$ were also tested. None of the other antibodies examined revealed specific protein bands in *H. vulgaris* membrane preparations.

Immunohistochemistry

The anatomy of the freshwater polyp *H. vulgaris* is very simple: the body is shaped as a tube made of two epithelial cell layers, the ectoderm and the endoderm, surmounted by a ring of tentacles around a cone (the hypostome) where the mouth opens when feeding. Tentacles are comprised of battery cells, i.e. modified epithelio-muscular cells in which one or two neurons, and several stinging cells, the nematocytes, are embedded. The battery cell complex represents the functional unit of the tentacle [21].

The nervous system is arranged in a net spreading through the epithelial layers of the body, from head, namely tentacles and hypostome, to the foot. Neurons are connected by chemical synapses or gap junctions to other neurons, muscle fibers or effector cells [22], the nature of neurotransmitter molecules being yet under study [23, 24]. Current knowledge indicates that in *Hydra* species the nerve net is not homogenous throughout the body, neuronal density being higher in the head region and in the lower peduncle [25, 26]. Electrophysiological studies have shown that, far from being an unpolarized fiber array, the net exhibits a greater functional complexity than previously acknowledged [27].

Immunohistochemical experiments were carried out on whole mounts preparations, using anti-α3, anti-β1, anti-γ3 and anti-δ subunit antibodies, selected among those giving clearly defined bands in western blots. Both immunoperoxidase and immunofluorescent labeling were used in order to study the regional distribution of positive immunoreactive elements and to check by the appropriate combination of primary specific antibodies the occurrence of subunit colocalization. The results described below were observed repeatedly in all the specimens examined.

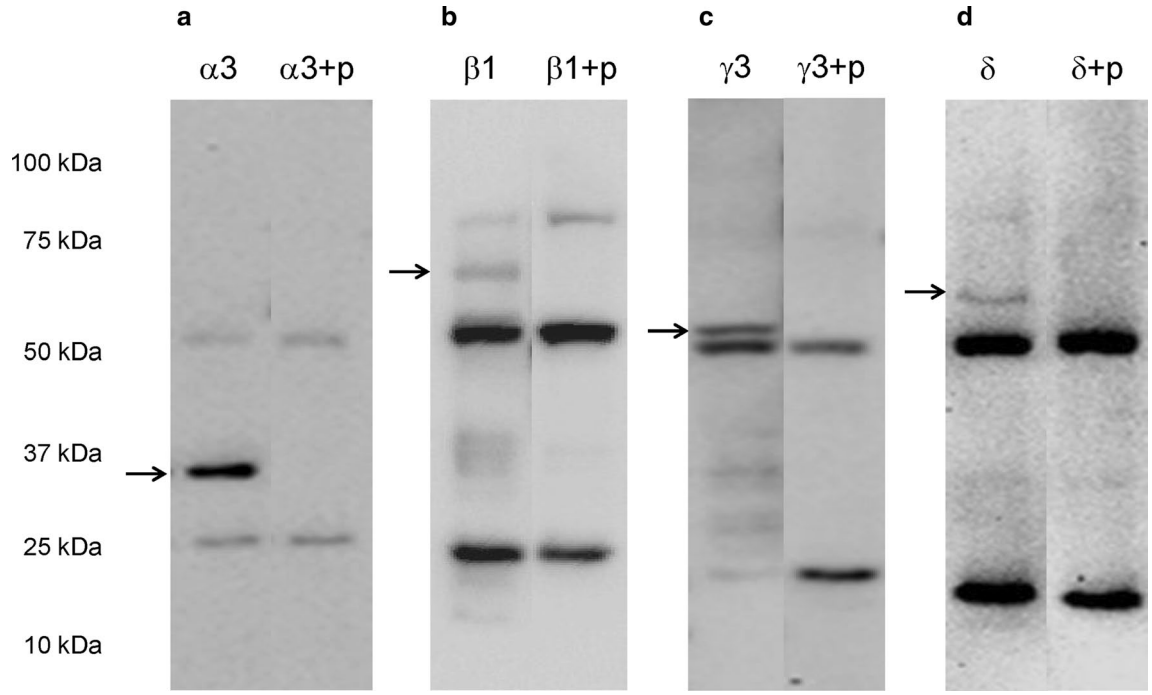

Fig. 1 Western blot analysis of putative $GABA_A$ receptor subunits in *Hydra vulgaris*. Representative immunoblots showing qualitative signals in membranes incubated with antibodies to **a** α3, **b** β1, **c** γ3, and **d** δ subunits. The same amount of total *Hydra* membrane proteins was loaded in each well (40 µg). The molecular sizes of the immunoreactive band are indicated in kilodalton, and are referred to those obtained with the Precision Plus Protein WesternC Marker (Bio-Rad, Milan, Italy). The specificity of the reactions was shown by preincubation of the specific antibody with the respective peptide (p). For all antibodies tested, each immunoblot experiment was repeated 3–4 times and qualitative data were confirmed

The α3-like subunit was revealed as intensely immunoreactive by immunoperoxidase staining in the head, mainly in the hypostome, around tentacle insertion, and in the gastric region; in the tentacles, immunopositive components of battery cells were also observed. Sparse labeling was found in the lower part of the peduncle. Immunopositive elements were observed mainly as granules or patches localized on cell membranes (Fig. 2, second row). Immunofluorescence revealed diffuse labeling, more prominent in the gastric region but also evident in the hypostome and lower peduncle. In the tentacles, distinct immunopositive components of battery cells were visible, thus confirming the results obtained by immunoperoxidase staining (Fig. 3).

A comparable distribution was found for the β1-like subunit. Immunoperoxidase revealed specific intense labeling for anti-β1 antibody in the tentacles, where immunopositive fibers were also observed at the tentacle insertion on the hypostome, and diffused immunoreactivity throughout the gastric region. In the peduncle, immunopositive fibers were observed running horizontally above the foot (Fig. 2, third row). Immunoreactivity occurred primarily in the form of granules or patches apparently found on fibers, or surrounding cell membranes. Immunofluorescent labeling provided the same overall picture, i.e. labeling was less intense and diffused quite homogeneously throughout all body regions. Immunopositive fibers and cell bodies above the foot were clearly visible, again confirming the immunoperoxidase findings. Double labeling clearly showed colocalization of α3 and β1 immunoreactivity on the fiber structure above the foot; in the tentacles, sparse cell bodies also exhibited double staining in the form of patches (Fig. 3a, c, upper row).

By contrast, γ3-like subunits were found mainly in the upper part of the gastric region, in the budding region and in the peduncle; few immunoreactive cells were observed in the hypostome, while tentacles were intensely stained (Fig. 2, fourth row). Punctate labeling, occurring primarily on cell bodies and/or cell membranes, appeared to be prevalent either by immunoperoxidase or by immunofluorescent staining. Double immunofluorescent labeling showed colocalization of α3/γ3 immunoreactivity in granules or cells in the peduncle, where circular fibers above the foot were clearly double-stained (Fig. 3a, middle row).

Finally, the δ-like subunit exhibited a different pattern of staining: anti-δ immunopositive granules were abundant in the peduncle and in the tentacles, less evident in the gastric region and practically absent in the hypostome by DAB staining (Fig. 2, fifth row); immunofluorescent staining revealed colocalization of α3 and δ immunoreactivity in cells of the peduncle, budding region and primarily in the tentacles (Fig. 3a–c, lower row).

It is important to note that immunoreactivity to all four antibodies was completely suppressed in negative controls. i.e. in experiments performed without the primary antibody (Fig. 2, first row), as well as in preparations incubated with the corresponding blocking peptide for each primary antibody examined (Fig. 2, sixth and seventh rows).

Discussion

The results of immunohistochemical analysis reported in this paper provide further indications of the differential distribution and composition of GABA receptor complexes in the excitable structures, nerves and myofibrils, of *Hydra* conducting systems. While the cellular localization of immunopositive elements cannot be clearly determined, owing to the experimental preparation used, their regional distribution appears different: in fact, α and β immunoreactivity, though diffused throughout the body, is more evident in the tentacles, hypostome, and upper part of the gastric region, while γ and δ immunoreactivity is more pronounced in the lower part of the body column and in the foot. α3/β1 subunits and α3/γ3 subunits colocalize in tentacles, hypostome and peduncle, but not in the gastric region; conversely, α3/δ subunit colocalization is very pronounced in the lower part of the gastric region and in the tentacles. It is interesting to note that α3, β1, and γ3 also colocalize on nerves running horizontally above the foot. To our knowledge, this finding provides new evidence of the existence of a nerve ring in the peduncle, a structure previously suggested by histochemical studies [28], but not yet identified.

The results of immunoblotting indicate that at least one $GABA_AR$ subunit for each of α, β, and γ or δ subfamilies is present in *Hydra* tissues, suggesting that the *Hydra* $GABA_AR$ complex is comprised of different subunits. It should be noted that the western blots were run on crude membrane preparations, given the impossibility to separate nerve cells from the polyp tissues. This constraint may explain (a) the relative small amounts of immunoreactive bands, (b) the presence of nonspecific bands, some of which may co-migrate with the proteins of interest, thereby masking their characterization. Thus, the inability to identify other $GABA_AR$ subunits in *Hydra* may be a false negative result. Further studies will help to clarify this issue.

The slight differences in molecular weight of β, γ and δ peptides suggest the occurrence of different subunit isoforms in *Hydra*, compared to the corresponding human subunits (59 kDa, 43–46 kDa, and 51 kDa, respectively), while the α3 subunit, considerably smaller than the reference peptide (55–57 kDa), could be a truncated peptide. The putative α3-like protein was the only α subunit to be clearly identified by the specific antibody among the many different anti-α antibodies examined. In mammals the α3 subunit is formed by mRNA editing of transcript of GABRA3, the gene coding for the specific protein, and it is important in brain development [29] and in

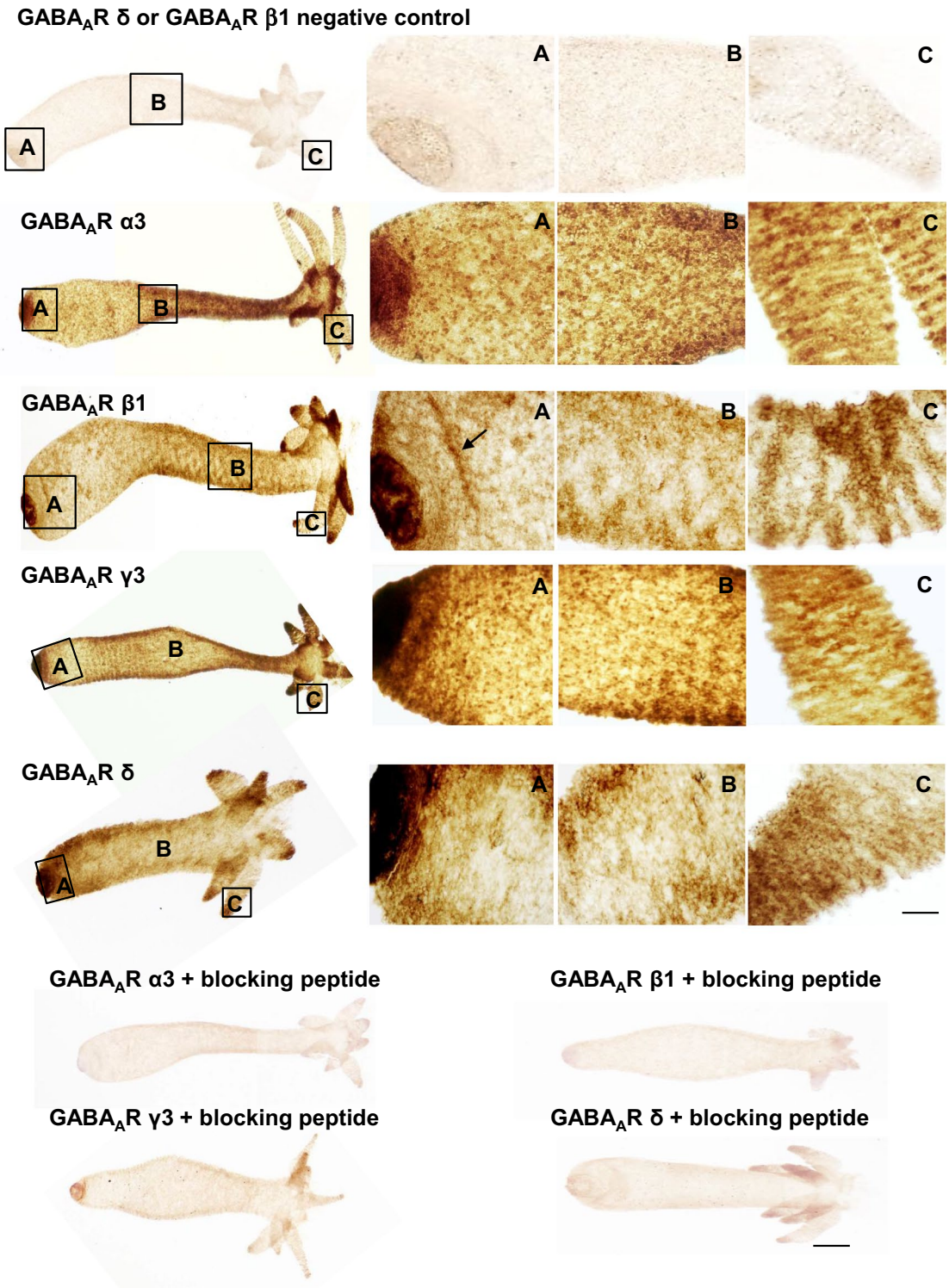

Fig. 2 Regional distribution of putative α3, β1, γ3 and δ $GABA_A$ receptor subunits. Immunoperoxidase staining reveals distribution of anti-α3 antibodies, mainly as granules or patches localized on cell membranes, in the lower part of the peduncle (**a**), along the upper part of the gastric region (**b**) and in the tentacles (**c**). Anti-β1 antibody specific labeling is found in the peduncle, where immunopositive fibers run horizontally above the foot (see *arrow*) (**a**), throughout the gastric region, primarily in the form of granules or patches (**b**), and in the tentacles (**c**). Anti-γ3 antibody specific labeling, occurring primarily on cell bodies and/or cell membranes, is found in the peduncle (**a**), in the upper gastric region (**b**) and tentacles (**c**) which are stained homogeneously. Anti-δ immunopositive fibers and granules are abundant in the peduncle (**a**) and in the tentacles (**c**), but not in the upper gastric region (**b**). Note that immunoreactivity to all four antibodies is suppressed in negative controls without the addition of primary antibody (*first row*), or with addition of the primary antibody plus the respective blocking peptide (*sixth and seventh row*). *Scale bar* 200, 50 μm

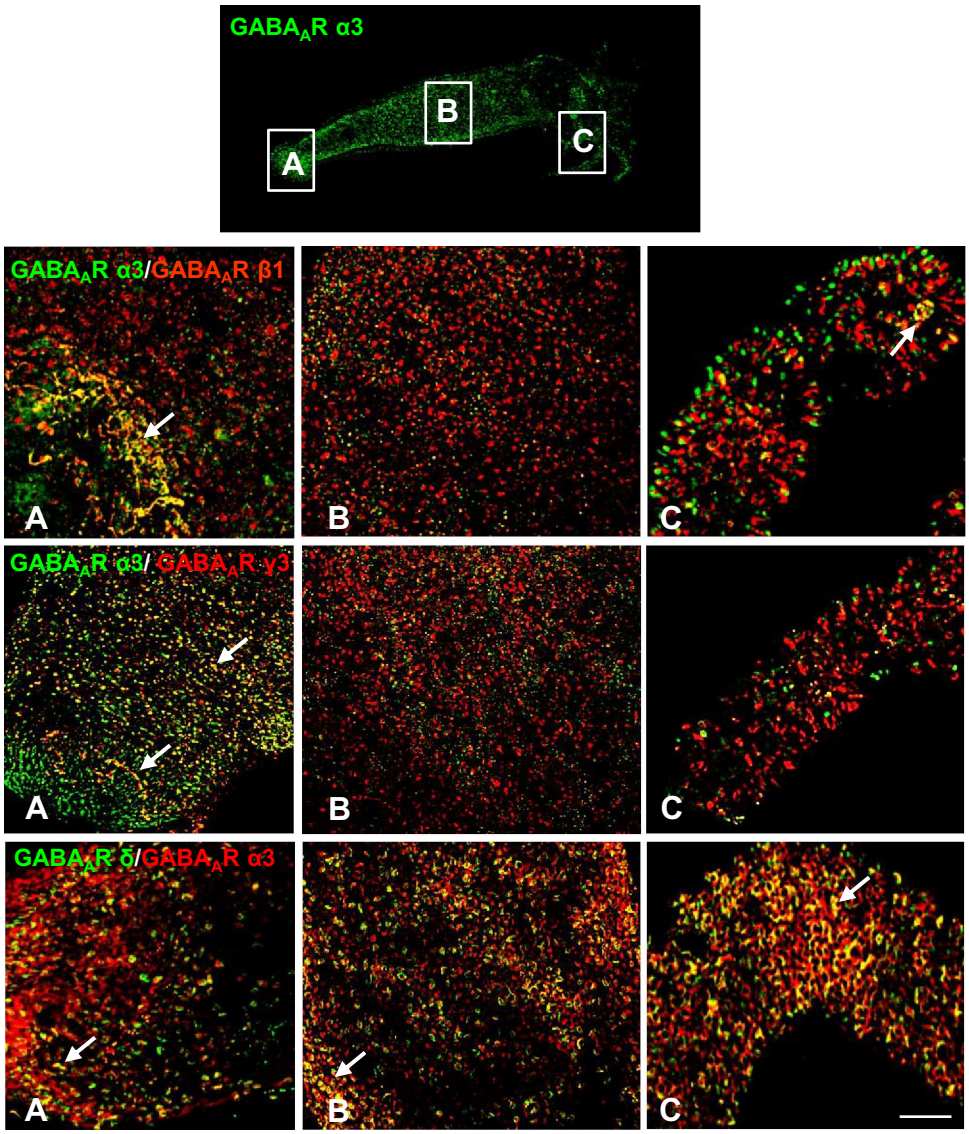

Fig. 3 Colocalization of putative α3, β1, γ3 and δ $GABA_A$ receptor subunits. Whole polyp labeled for the α3 subunit showing diffuse immunofluorescence (*green*) in the lower peduncle (**a**), in the gastric region (**b**) and in some tentacles, where immunopositive components of battery cells are visible (**c**). *Upper row* double α3/β1 immunoreactivity (*green*/*red*, respectively) shows pronounced colocalization (*yellow* fibers indicated by *arrow*) in a circular structure above the foot (**a**) and in sparse α3/β1 positive cell bodies in the tentacles (*yellow* puncta indicated by *arrow*) (**c**). No colocalization is found in the gastric region (**b**). *Center row* circular fibers above the foot are double-stained for anti-α3/γ3 antibodies (*green*/*red*, respectively), as well as granules or cells in the peduncle (*yellow* fibers and puncta indicated by *arrows*) (**a**). No colocalization is found in the gastric region (**b**) or in the tentacles (**c**). *Lower row* strong colocalization of α3 (*red*) and δ (*green*) immunoreactivity is observed in cells of the peduncle (**a**), in the budding region (**b**) and is most abundant in the tentacles (**c**) (*yellow dots* indicated by *arrow*). *Scale bar* 200, 50 µm (Color figure online)

benzodiazepine binding [30]. Furthermore, $GABA_A$Rs comprising the α3 subunit exhibit low sensitivity to GABA [31]. It is interesting to note that in *Hydra* GABA is only effective at high micromolar doses both in behavioral and electrophysiological experiments [32], while benzodiazepines, neuroactive steroids and general anesthetics act at 1–10 µM concentrations.

The occurrence of a putative β subunit, a β1 isoform, is in keeping with the hypothesis that the β subfamily is a primitive subunit population, present in the last common bilaterian ancestor protein(s) before divergence of $GABA_A$R subunits [8, 33]. Its regional distribution in *Hydra* tissues suggests that the anti-β1 immunoreactive protein is a major component of the $GABA_A$Rs. The finding of a putative γ3 subunit provides additional molecular evidence for the diazepam sensitivity of *Hydra* receptors, shown in vitro and in vivo in previous studies [13, 15]. In fact, besides α3 subunits, γ subunits, notably γ2 isoforms, are needed for

benzodiazepine binding by vertebrate $GABA_A$Rs, and γ3 subunits rescue sensitivity to benzodiazepines in receptor clustering after deletion of the γ2 subunit [34–36]. Finally, the occurrence of a putative δ subunit, required for neuroactive steroid binding in mammalian CNS [37, 38] further supports the strong affinity of *Hydra* $GABA_A$Rs for neuroactive steroids [15].

It is interesting to note that the putative subunits found in *Hydra*, namely α3-, β1- and γ3-like subunits, compare with the $GABA_A$R subunit isoforms highly expressed both in rodents and humans during early brain development, but not in adult brain [39–41]. It is well known that *Hydra* tissues, besides their ability to regenerate, are highly plastic: in the steady state, both epithelio-muscular and nerve cells are continuously migrating towards the oral and aboral polyp ends, to be eliminated; they are replaced by new cells derived by division and differentiation of different stem cell populations [42]. Our findings suggest the hypothesis of a possible involvement of some $GABA_A$R populations in cell development and/or neurogenesis, the evolution of neurogenetic processes currently being the object of intense investigation [43, 44].

In conclusion, these results indicate that different subpopulations of $GABA_A$Rs are present in *Hydra*, comprised of α-, β-, and γ- or δ-like subunits; their structure, based on putative subunit composition, appears to be that of heteromeric/multimeric complexes. The presence of a circular fiber structure, possibly a nerve ring, in the peduncle, so far undescribed, provides new evidence of the nerve net polarization. The different regional localization of these receptors may contribute to a better understanding of the modulation of cellular signalling in *Hydra*.

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