



GABA_A receptor subunits identified in *Paramecium* by immunofluorescence confocal microscopy

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Abstract

The presence of opioid, β -adrenergic and cholinergic receptors has been demonstrated in ciliated protozoa, but little is known about γ -aminobutyric acid (GABA) receptors. In this study we have analyzed the distribution of GABA_A-type receptor subunits in *Paramecium*. Confocal laser microscopy using antibodies specific for α_1 -, α_2 -, α_3 -, α_6 -, $\beta_{2/3}$ -, γ_2 -, ϵ -, λ -, and θ -subunits showed that most receptors are aggregated in clusters and are distributed both on cell surface and in the cytoplasm. The intensity of labelling of the α_6 -, $\beta_{2/3}$ - and γ_2 -subunits was more intense than the α_1 -, ϵ -, and θ -subunits, suggesting that the former are present in higher concentrations than the latter.

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1. Introduction

The presence of opioid [1], β -adrenergic [2], cholinergic [3] and purinergic receptors [4] controlling phagocytosis, swimming behaviour and cell recognition has been shown in ciliated protozoa. However, little is known about γ -aminobutyric acid (GABA) receptors [5,6]. The aim of this paper is to study the distribution of GABA_A subunits in *Paramecium*.

GABA_A receptors are members of a ligand-gated chloride channel super family, which includes the nico-

tinic acetylcholine receptors, the strychnine sensitive glycine receptors and the 5-HT₃ receptors. They are heteropentameric complexes assembled from 21 subunits. Four families of subunits have several members, six α -type (α_{1-6}), four β -type (β_{1-4}), four γ -type (γ_{1-4}) and three ρ type (ρ_{1-3}) subunits, while other families have a single representative subunit ϵ -, λ -, π -, and θ -type, respectively (reviewed in [7–10]). Some authors [11] have also classified the ρ -type subunits as GABA_C because of their unusual pharmacology. The vast majority of native GABA_A receptors are composed of at least one type of α - and β -subunits in combination with the γ_2 -subunit and the major isoforms in vertebrate brain contain two α - and two β -subunits. The γ_2 -subunit is essential for the benzodiazepine (BZ) high-affinity

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binding, regular channel gating, clustering of receptors and clustering of the protein gephyrin at postsynaptic sites *in vivo* [7–10]. The role of the δ -subunit remains controversial: it could be a possible replacement of a γ -subunit. Human ϵ - and θ -subunits share 50% sequence identity with γ - and β -subunits, respectively. A functional expression of recombinant subunits suggested that $\alpha\beta\epsilon$ -containing receptors are insensitive to benzodiazepines and the related ion channels are spontaneously active. θ -subunits apparently only produce functional receptors when co-expressed (and probably co-assembled) with α -, β - and γ -subunits. The type of α -, β -, γ -, or δ -subunit determines the pharmacological and functional properties of the receptor [12], and influences the cellular domain to which the receptor can be targeted during intracellular trafficking [13].

In this study we have analyzed the major α -subunit variants because they represent a receptor population with distinct pharmacological profiles, γ_2 - and $\beta_{2/3}$ -subunits because they are widely distributed in the brain, as well as δ - and ϵ -subunits which may substitute the γ -subunit and θ - the β -subunits.

2. Materials and methods

2.1. Cell and culturing conditions

Experiments were carried out on *Paramecium primaurelia*, stock 90, cultured at 25 °C in lettuce medium (pH 6.9) supplemented with *Enterobacter aerogenes* as a source of food. Cells were harvested in the mid-exponential phase of growth.

2.2. Antibodies

Rabbit anti-GABA_A receptors α_1 (1:250), α_6 (1:1000), γ_2 (1:2000) and mouse anti-GABA_A receptor $\beta_{2/3}$, clone bd17 (16:100), were purchased from Chemicon International (Germany), rabbit anti-GABA_A receptors α_2 (1:100) and α_3 (1:100) by Alomone Labs (Jerusalem, Israel) and goat anti-GABA_A receptor δ (1:200): by Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-GABA_A receptors ϵ (1:5000) and θ (1:5000) were a generous gift from Dr. Garrett (University of Bordeaux, France). Anti-mouse, anti-rabbit, anti-goat and streptavidin conjugated with Alexa Fluor 488 were obtained from Molecular Probes (Eugene, OR), goat anti-rabbit conjugated with biotin from Sigma (Germany).

2.3. Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS buffer (0.01 M, pH 7.4) for 30 min, washed three times with PBS and incubated for 60 min with 3% bovine serum albumin (BSA) in PBS plus 1% Triton X-100. The

blocking buffer was removed and the cells were incubated overnight at 4 °C with primary antibodies diluted in PBS containing 1% BSA and 0.1% Triton X-100. Secondary antibodies coupled to Alexa Fluor 488 (1:300) were applied for 2 h at 37 °C after three washes in 1% BSA and 0.1% Triton X-100 in PBS for 10 min each. Cells were mounted in glycerol/buffer on a glass slide after extensive washing with PBS. The specificity of primary antibodies was tested by preincubation with the control peptide antigens. Secondary antibodies were controlled in cells processed as described above but in the absence of primary antibodies.

2.4. Immunofluorescence staining with signal amplification

ϵ and θ immunofluorescence staining were amplified. Cells removed from blocking buffer were incubated for two days at 4 °C with primary antibodies. A biotin-labelled goat anti-rabbit (1:15) was applied for 30 min at 37 °C after three washes in 1% BSA in PBS plus 0.1% Triton X-100 for 10 min each. Cells were then washed for three times with 1% BSA in PBS plus 0.1% Triton X-100 and incubated for 60 min at 37 °C with streptavidin Alexa Fluor 488 (1:200). Cells were mounted in glycerol/buffer after extensive washing with PBS.

2.5. Image acquisition

Images (1024 × 1024 × 8 bit) were acquired by an Olympus FluoView 300 confocal laser scanning microscope mounted on an inverted optical microscope Olympus IX71. Alexa Fluor 488 was excited using an Argon-ion laser and the fluorescence was collected at 515 nm. Serial optical sections were taken through the cell at a z-step of 0.5 μ m. An Olympus PLAN-APO oil immersion objective 100×/NA1.3 was used.

Olympus FluoView software was used for acquisition, storage and visualization.

Labelling experiments were repeated three to four times and images represent observations for an average of 30 cells per sample.

2.6. Image analysis

Image processing was performed by a home-made analysis software realized in the form of routines (macro), integrated into the NIH-Image public domain platform (US National Institutes of Health). Fluorescence images were processed and a threshold algorithm was used to assess the amount of green fluorescence per unit area. All data was calculated as means \pm standard errors and represented as % of total fluorescence.

Fluorescence analysis was carried out in five serial sections from 10 cells. Pictures were prepared using Pho-

toShop Pro 7. The original images only had to be adjusted to remove dust and scratches.

3. Results

To detect the presence and distribution of GABA_A receptors in *Paramecium* we used monoclonal and polyclonal antibodies raised against subunits found in mammalian cells. Here we show that α_1 -, α_2 -, α_3 -, α_6 -, $\beta_{2/3}$ -, γ_2 -, ϵ -, θ - and δ -subunits are expressed in *P. primaurelia*. In all the cases examined most of receptors are aggregated in clusters. Their fluorescence is localized in spots, varying in size and amount, distributed on the plasma membrane and throughout the cytoplasm.

membrane and, due to both endocytosis from the cell membrane and exocytosis from the Golgi apparatus, in the cytoplasm (Fig. 1). Analysis of different optical sections revealed that most of the α_1 -subunit immunoreactivity is located inside the cytoplasm; a few spots are seen on cell membrane (Fig. 1, arrow). A similar distribution but higher concentrations of α_2 - and α_3 -subunits were observed both inside the cell and on the plasma membrane (Fig. 1). The α_6 -subunit was both highly abundant (Fig. 2) and uniformly distributed throughout both the cytoplasm and on cell membrane (Fig. 1).

The $\beta_{2/3}$ -subunit immunoreactivity (Fig. 1), indicated by monoclonal bd-17 antibody labelling, is present in large quantities in the analysed cells (Fig. 2). As the

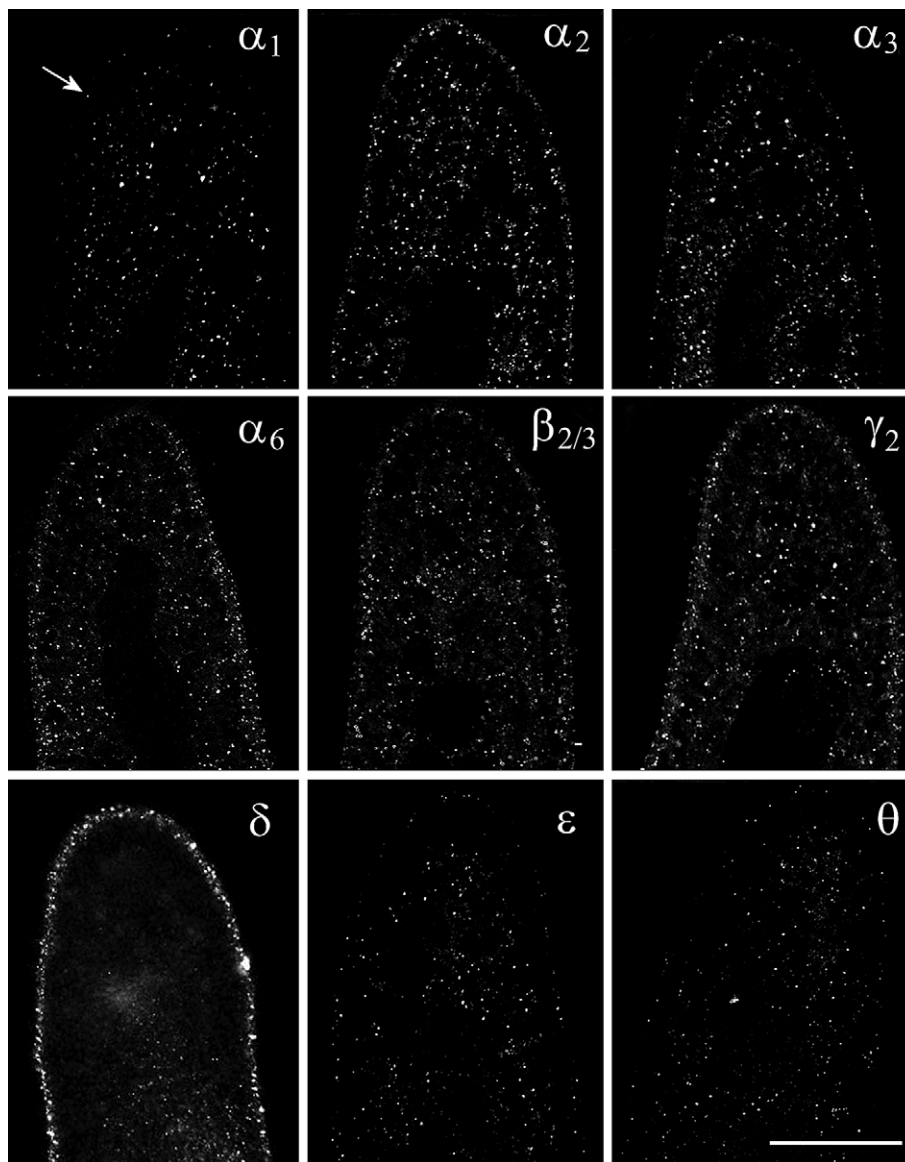


Fig. 1. Immunological localization of GABA_A-like receptor subunits in *Paramecium primaurelia*: α_1 -, α_2 -, α_3 -, α_6 -, $\beta_{2/3}$ -, γ_2 -, δ -, ϵ - and θ -subunits. In all the cases examined most receptors are aggregated in clusters and their fluorescence is localized in spots, varying in size and amount, distributed on the plasma membrane and throughout the cytoplasm. An optical section collected in the middle plane of the cell is shown. Bar, 20 μ m.

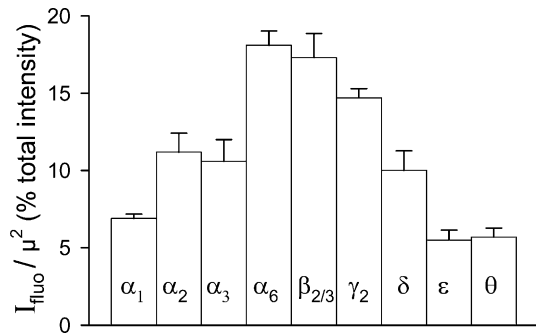


Fig. 2. Normalized intensity of the GABA_A receptor subtype fluorescence representatively shown in Fig. 1.

bd-17 antibody recognizes both β_2 - and β_3 -subunits, it was not possible to demonstrate whether either just one isoform or both of them are present in *Paramecium*.

The γ_2 -subunit, which is ubiquitous in the central nervous system (CNS) of vertebrates, is also present in large quantities in *Paramecium*. The labelling is uniformly distributed inside the cytoplasm and is abundant on the cell membrane (Fig. 1). In contrast, δ -subunit immunoreactivity is mainly localized on the cell membrane and around the buccal cavity (Fig. 1).

Consistent with their presence only in restricted regions of mammalian CNS, only small quantities of ε - and θ -subunits are present in *Paramecium* (Fig. 1). Immunoreactivity is localized almost exclusively in the cytoplasm, with only rare spots visible on the plasma membrane.

No immunostaining was observed in control experiments in which the primary antibodies were omitted (data not shown).

The presence of each type of subunit was quantified calculating the related total fluorescence intensity per unit area (Fig. 2). The most abundant subunits are α_6 , $\beta_{2/3}$ and γ_2 , representing about 80% of the total fluores-

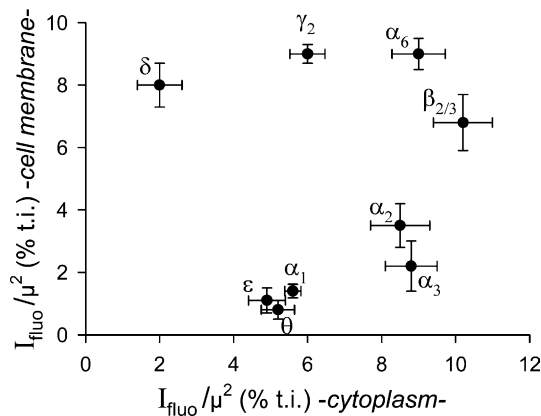


Fig. 3. Normalized intensity of the GABA_A receptor subtype fluorescence plotted according to its distribution in cell membrane vs. cytoplasm.

cence. The α_1 -, ε - and θ -subunits are much less abundant. The distribution of subunits in plasma membrane vs. cytoplasm is shown in Fig. 3.

4. Discussion

The results presented here provide the first demonstration of GABA_A subunit immunoreactivity in ciliated protozoa. Using specific antibodies and confocal microscopy to visualize in situ different GABA_A-receptor subunits, we have shown that α_1 -, α_2 -, α_3 -, $\beta_{2/3}$ -, γ_2 -, δ -, ε - and θ -subunits are expressed in *P. primaurelia*. Among the GABA_A receptor subunits found in *Paramecium*, α_1 -, α_2 -, α_3 -, $\beta_{2/3}$ - and γ_2 -subunits are commonly expressed in neurones of the cerebral cortex, hippocampus, amygdala and olfactory bulb [14–16]. The δ -subunit is abundant in cerebellar granule cells, in the hippocampal dentate gyrus and thalamus, whereas the expression of α_6 -subunit appears to be restricted to cerebellar granule cells and dorsal cochlear nucleus [17]. The ε - and θ -subunits, previously identified only in humans, were recently also found in rodent brain [18,19]. Most receptors are aggregated in clusters. A clustering of GABA_A receptors has been documented in different cerebellar granule cells at extrasynaptic sites [20].

The majority of GABA_A subunits expressed in *Paramecium* are intracellular. This pool of intracellular subunits includes both subunits available for receptor assembly and subunits undergoing endocytosis. This observation appears to endorse the studies of Connolly et al. [13] who showed that GABA_A α -subunits are retained in an intracellular compartment, presumably the endoplasmic reticulum (ER), through an ER retention signal, awaiting association with β -subunits. Interaction of α - with β -subunits is proposed to confer a conformational change, which presumably masks the retention signal and prompts trafficking to the cell surface.

The evidence of the presence of different GABA_A receptor subunits allows inference that structurally different GABA_A receptors may be expressed in *Paramecium*. The multi-subunit composition of GABA_A receptors, which produce the receptor subtypes, is very important because the specific properties of the receptors depend on their subunit composition. The α_1 -, α_2 -, α_3 -, and α_5 -GABA_A receptors correspond to diazepam-sensitive receptors, whereas the α_4 - and α_6 -GABA_A receptors are insensitive to diazepam [12]. The α_1 -, α_2 -, α_3 -GABA_A receptors are distinguished even further by their affinity to zolpidem and various β -carbolines [12]. Positive modulation by most benzodiazepine-type agonists is reduced when γ_2 - is replaced by the γ_1 -subunit, and inverse agonists (β -carbolines) then become agonists [21]. New benzodiazepine-insensitive subtypes are created if γ - is replaced by the δ - or ε -subunit [22]. Native

subtypes exist in the rat cerebellum, which contains the δ -subunit and has no high-affinity benzodiazepine binding [23]. Furthermore, the type of α -subunit determines the kinetics of receptor deactivation [24], and the presence of the δ -subunit results in markedly increased agonist affinity and apparent lack of desensitization [25].

In conclusion, even if we have not yet determined which GABA_A receptor subtypes are present, we have shown that GABA_A receptor subunits typical of cells of different areas of mammalian CNS are expressed in the single-celled organism *Paramecium*. The presence of a GABA receptor system, typical of mammalian neuronal cells, in the motile *Paramecium* suggests that this system was developed early in excitable cells and has been largely conserved throughout the evolution.

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