Regulation of the Subcellular Distribution and Gene Expression of $GABA_A$ Receptor by Microtubules and Microfilaments in Cultured Brain Neurons

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Abstract Mechanisms underlying the intracellular transport of γ-aminobutyric $acid_A$ receptor ($GABA_AR$) were examined in the cultured neurons derived from chicken embryo brains. In situ trypsinization of the cultures and ^{3}H-flunitrazepam (FNZ) binding assay were employed to determine the cell surface and intracellular distribution of the receptor. A 3-h treatment of the cells with 1 μM of colchicine, a microtubule depolymerizer, reversibly raised the proportion of intracellular $GABA_AR$ density by about 36% and decreased that of the cell surface receptors by 18% from respective control values, whereas the 3-h incubation with 2 μM of cytochalasin D, a microfilament disrupter, did not cause significant changes. These treatments failed to alter the total number of the ^{3}H-FNZ binding sites of the neurons and the affinity of the ligand. Moreover, the exposure to colchicine seemed to produce a stronger cytoplasmic immunostaining of the $GABA_AR$ α subunits in many neurons without affecting the total cellular level of the proteins, in accordance with the increased fraction of intracellular ^{3}H-FNZ binding. However, in the neurons exposed to cytochalasin D, there was an increase of around 28% in the total content of α_1+51kDa proteins. In addition, the colchicine or cytochalasin D treatment inhibited approximately 21 or 18% of the rate of general protein synthesis in the culture. Notably, in situ hybridization assay showed that the $GABA_AR$ α_1 or α_2 mRNA was present in 92±2% or 94±2% of the cytochalasin D-treated neurons, both of which were higher than 71±2–74±3% of the control and colchicine-treated cells. The data suggest that by regulating the intracellular transport, the microtubular system participates in the maintenance of normal subcellular distribution of $GABA_AR$ in the neurons. By contrast, the organization of microfilaments may play a role in modulating the gene expression of $GABA_AR$ subunits. J. Cell. Biochem. 83: 291–303, 2001. © 2001 Wiley-Liss, Inc.

Key words: ^{3}H-flunitrazepam binding; trypsin treatment; colchicine; cytochalasin D; immunocytochemistry; in situ hybridization

The maintenance of normal expression and subcellular distribution of neurotransmitter receptors is crucial to proper neurotransmission. However, it remains to be elucidated concerning the regulatory mechanisms for the subcellular expression of the receptors. The principle inhibitory neuroreceptor, γ-aminobutyric $acid_A$ receptor ($GABA_AR$), is a ligand-gated Cl^- channel and mediates fast inhibitory transmission by increasing the Cl^- conductance [Macdonald and Olsen, 1994]. The activity of $GABA_AR$ can be modulated by a variety of therapeutically important compounds, such as the benzodiazepines, barbiturates, and steroids. Based on molecular biological studies, there are at least 20 distinct subunits, α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε, π, and ρ_{1-3} for the composition of a presumed hetero-pentameric $GABA_AR$ [Rabow et al., 1995; Barnard et al., 1998].

We have previously demonstrated that regular glycosylation of $GABA_AR$ is key to preserving a steady total number, and constant cell surface and intracellular fractions of the receptor binding sites in cultured brain neurons. It appears that the receptor protein passes through the Golgi apparatus for maturation of its oligosaccharides [Yin, 1992; Yin and Yang, 1992; Lin et al., 1998]. Moreover, cytoskeletal

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elements could be involved in regulating the subcellular organization of $GABA_AR$ in the cells. An increasing body of evidence indeed attests that microtubules and microfilaments play significant roles in the clustering or targeting of neuroreceptors.

Earlier investigations have suggested an interaction between $GABA_AR$ and microtubules or microfilaments [Item and Sieghart, 1994; Kannenberg et al., 1997]. A microtubule-associated protein (MAP)-like $GABA_AR$ associated protein (GABARAP) may link the γ_2 subunit of the receptor to microtubules by making direct interaction with them. Though the GABARAP colocalizes with microfilaments as well, the two components do not directly interact [Wang et al., 1999; Wang and Olsen, 2000]. In addition, MAP-1B probably connects the ρ subunit of the $GABA_C$ receptor to the cytoskeleton in retinal neurons [Hanley et al., 1999]. Another recent study recorded that the changes in the organization of microfilaments altered the distribution of somatodendritic $GABA_AR$ of cultured hippocampal neurons [Meyer et al., 2000]. Nevertheless, the relationship is unclear between the cytoskeletal elements and the sustaining of adequate cell surface and intracellular expression of the receptor.

The structure of microtubules and microfilaments are also apparently related to the functional property of $GABA_AR$ channel. The treatment with colchicine was able to block the uptake of muscimol-stimulated $^{36}Cl^-$ by cerebral cortical microsacs, and inhibit GABA-mediated Cl^- currents in *Xenopus laevis* oocytes expressing the $GABA_AR$ subunits [Whatley et al., 1994]. Moreover, the destruction of microtubules or microfilaments lowered the amplitude of muscimol-induced currents in the cultured hippocampal neurons [Meyer et al., 2000].

It is known that microtubules critically participate in the neural growth, and intracellular transport of proteins [Aliva, 1992]. Many studies support that microtubular pathways transport newly synthesized proteins from the Golgi cisternae to the plasma membrane of the soma, dendrite or axon, and carry endosomal vesicles to the Golgi compartment [Allan et al., 1991; Wood et al., 1991; Hirokawa, 1998]. Furthermore, microtubules are thought to be associated with the translocation of certain mRNAs [Hesketh, 1996]. Additionally, colchicine modified differentially the levels of mRNAs and proteins of the β1- and β2-adrenergic receptors in C6-glioma cells, as well as the levels of muscarinic(m)2 and m3 acetylcholine receptors in rat cerebellar granule cells [Fukamauchi et al., 1991; Hough et al., 1994].

Microfilaments have been shown to co-localize with specific mRNAs, including those of cytoskeletal proteins; this co-localization may be connected with translational events [Litman et al., 1994; Hesketh, 1996]. A morphological observation declared that the δ glutamate receptors anchored along the actin cytoskeleton of Purkinje cells, because actin-disrupting agents decreased the number of the receptor clusters on the dendritic spines [Hirai, 2000]. Furthermore, there was an association between the preservation of microfilament architecture and the mRNA levels of the subtypes of β-adrenergic receptors in C6 glioma cells [Hough et al., 1994]. Nonetheless, whether the mRNAs of $GABA_AR$ interact with microtubules or microfilaments is unknown.

Thus, the goal of the present study was to examine the significance of microtubules and microfilaments to maintaining the proper subcellular expression of $GABA_AR$ in cultured brain neurons, by disrupting the cytoskeletal components with colchicine and cytochalasin D. ^3H-flunitrazepam (FNZ) was used as a probe for the benzodiazepine modulator sites on the receptor. The subcellular distribution of the receptor was investigated by a macromolecule, trypsin, in combination with the radioligand-binding assay, because the sensitivity of surface $GABA_AR$ to trypsin digestion allows the determination of the proportion of the receptors located intracellularly or on the cell surface [Czajkowski and Farb, 1986; Yin, 1992]. The cellular expression of $GABA_AR$ α subunits was also studied by immuncytochemistry as well as in situ hybridization assays.

METHODS

Neuron Culture and Drug Treatment

Dissociated neuron culture was prepared from the brains of 7-day chicken embryos as described previously [Czajkowski and Farb, 1986; Yin and Yang, 1992]. In brief, the neurons were plated at a density of 10^6 cells/ml onto collagen-coated culture dishes, and then incubated in a 95% air/5% CO_2 incubator at 37°C. Cytosine arabinoside (1 μM) was added to the culture medium to inhibit the proliferation of

non-neuronal cells. The cultures maintained in the incubator for 7 days were treated with or without each of the following: 0.1, 1, 10, and 50 μM of colchicine (Sigma), 1 μM of the inactive colchicine analog β-lumicolchicine (a control for 1 μM of colchicine), and 0.2, 2, and 10 μM of cytochalasin D (Sigma), in the incubator for 3 h followed by the experiments depicted below. To access the viability of cultured neurons, the cultures were incubated with 0.4% trypan blue solution (0.4% trypan blue, 0.8% NaCl, 0.06% KH_2PO_4, and 0.05% methyl-p-hydroxybenzoate, pH 7.2) for 4 min at room temperature (RT) and washed with PBS.

Radioligand Binding

Reversible binding assay and trypsinization of cells in situ were conducted according to earlier procedures [Czajkowski and Farb, 1986; Yin, 1992]. The cells kept on 100 mm culture dishes were washed, scraped from the dishes, homogenized, and centrifuged. The pellets were resuspended in PBS. Aliquots of the cell homogenates were incubated with 2–25 nM ^{3}H-FNZ in PBS for 1 h at 4°C. In half of the incubations, 1 mM flurazepam (a gift from F. Hoffman-La Roche, Switzerland) was included to determine the non-specific binding. The incubation was terminated by filtration through glass-fiber filters (Whatman GF/B). The radioactivity remaining on the filters was measured by liquid scintillation counting. The non-specific binding value was subtracted from the total binding to yield the specific component.

A number of control and drug-treated cultures were incubated with or without 0.5 mg/ml bovine pancreas trypsin (Sigma) in 25 mM HEPES buffer in the presence or absence of the agent for 90 min at 37°C. To halt the trypsin activity, the dishes were placed on ice and the soybean trypsin inhibitor (Sigma) was added to the solution. Cells were scraped from the dishes and centrifuged. The pellet was homogenized in PBS and then subjected to reversible binding using 5 nM ^{3}H-FNZ as described above.

^{3}H-Leucine, ^{3}H-Mannose, and ^{3}H-Galactose Incorporation Assays

The extent of amino acid or sugar incorporation by the cultures was assayed by the addition of ^{3}H-leucine (specific activity: 153 Ci/mmol) at 0.4 μCi/ml, ^{3}H-mannose (13.9 Ci/mmol) at 10 μCi/ml, or ^{3}H-galactose (25.5 Ci/mmol) at 5 μCi/ml to the control and drug-treated cultures 2 h before terminating the drug treatment. Thereafter, the cultures were washed four times with PBS and then each of the cultures was incubated with 1 ml SDS/Nonidet P-40/urea (0.02%/2%/8M) for 1 h on a shaker. Ten-percent trichloroacetic acid (TCA) was used to precipitate the proteins in the sample on ice for 1 h. Following the addition of 1 N NaOH, TCA-precipitable proteins were collected by filtering through the glass fiber filters, and washing with PBS containing 5% TCA. The radioactivity associated with the filter was measured by liquid scintillation counting. For ^{3}H-mannose, and ^{3}H-galactose incorporation experiments, the culture medium was replaced by glucose-free medium 1 h before the addition of ^{3}H-mannose or ^{3}H-galactose.

All biochemical determinations were done in triplicate, and normalized to control. The results are presented as means±standard deviations. Cells from at least two 100 mm dishes were used in each group of every experiment. Scatchard plots were analyzed by using the ligand program [Munson and Rodbard, 1980] and were utilized to estimate the maximal binding sites, B_{max} and apparent affinity constant, K_D of the radioligand to the receptor. The significance in differences among data was analyzed using two-tail Student's *t*-test.

Immunocytochemistry

Immunocytochemical staining was carried out as described before [Yin and Fan, 1990; Lin et al., 1998]. The cultures kept on 35 mm dishes were fixed in 4% paraformaldehyde at RT for 20 min. They were then treated with 0.3% H_2O_2 in 0.1 M PBS for 5 min, and blocked with 1.25% normal serum and 0.2% Triton X-100 for 20 min at RT, before being incubated with the anti-α-tubulin, anti-200kDa neurofilament (NF), or anti-$GABA_AR$ α subunit(s), α_1 or both α_1 and 51 kDa (α_1+51 kDa) [Yin and Fan, 1990; Fan et al., 1997] antibodies in the blocking solution at 4°C for 16 h. Afterward, the immunoreaction products were visualized with a biotinylated secondary antibody and the ABC-peroxidase (Vector) method using 3,3′-diaminobenzidine tetrahydrochloride (DAB).

Phalloidin Staining

The cultures were rinsed with PBS and fixed with 10% Formalin (Kanto) in PBS containing 0.2% Triton X-100 for 15 min. After washing in PBS, they were incubated in PBS containing

FITC-conjugated phalloidin, 2 μg/ml (Sigma) for 30 min. The samples were then rinsed in PBS, coverslipped with the mounting medium (2% n-propyl gallate in 60% glycerol and 0.1 M PBS, pH 8.0), and observed under a Leica microscope equipped with epifluorescence.

In Situ Hybridization Cytochemistry

Two 45-mer antisense oligonucleotide probes were custom-synthesized to tag the α_1 and α_2 subunit mRNAs of $GABA_AR$ respectively. Their sequences are 5′-TTT CTG GCT TAA CTT CTT TGG GCT CTA TCG TTG CAC TTT TAG CAA-3′, and 5′-TCT CTG GCT TCT TGT TTG GTT CTG GAG TAG TTG CAC TTT TGG AAA-3′, complementary to the unique nucleotide segment 1356–1400 of the α_1 mRNA of chicken $GABA_AR$ [Bateson et al., 1991], and 1504–1548 of the rat receptor α_2 mRNA [Wisden et al., 1992]. The probes were 3′ end-labeled with digoxigenin (DIG)-11-ddUTP by terminal transferase (Boehringer Mannheim: BM).

In situ hybridization cytochemistry was performed on the cultures as depicted previously [Lin et al., 1998]. The neurons were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 20 min at RT. Prehybridization was done by incubating the cells with the hybridization buffer (50% deionized formamide, 4× SSC, 1X Denhart's solution, 500 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, and 10% dextran sulfate) at 42°C for 1 h. Thereafter, the cells were incubated with 0.6 pmol/μl of the DIG-labeled probe in hybridization buffer at 42°C for 18 h. Post-hybridization washes included 2× SSC, 15 min at RT, 1× SSC, 15 min at RT, 0.5× SSC, 30 min at 60°C, and 0.5× SSC, 15 min at RT. Subsequently the samples were blocked with 2% normal sheep serum for 30 min at RT. The cells were then incubated with an anti-DIG antibody coupled to alkaline phosphatase (BM) at 30°C for 3 h, before being subjected to color development using the substrates (nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate) for the enzyme. Negative controls were obtained from hybridization by a DIG-labeled sense probe under the same conditions as described above.

Image Analysis

To eliminate variations among data, the experimental procedures were conducted as consistently and uniformly as possible, including the plating numbers of the neurons, and the processes of immunostaining and in situ hybridization. The labeling intensity by various probes was measured in the neurons with a computer-based image-analysis system (MCID, Image Research, Ontario, Canada). Under a fixed level of illumination, the specific integrated (total) optical density (IOD) was obtained for each labeled soma in 9–14 evenly distributed fields of each culture dish under a 40× objective (400× magnification). At least 750 labeled cells were measured from each dish of control and drug-treated group in every experiment. Results are presented as mean integrated optical densities (IODs)±standard deviation (SD)/dish from 6 to 10 dishes derived from 3 to 5 experiments. The significance in differences between control and experimental groups was analyzed by using Student's *t*-test.

RESULTS

Total Cellular Radioligand Binding

Reversible binding using 5 nM ^{3}H-FNZ showed that the levels of specific ^{3}H-FNZ binding to the cultures were 104±2–114±4% (n = 7) of control values after 3-h exposure to 0.1–50 μM of colchicine or 0.2–10 μM of cytochalasin D. No significant statistical differences were found between the control and drug-treated values. The Trypan blue experiment revealed approximately 4.0% (n = 2,910 cells counted) of the neurons that incorporated Trypan blue in control cultures, representing a normal rate of cell death in the neuronal culture. Under the treatment with colchicine or cytochalasin D, around 4.2% (n = 2,868), or 3.6% (n = 3,003) of the cells collected the dye. This suggests that the treatment does not induce apparent membrane leakiness or cell death.

The B_{max} of ^{3}H-FNZ was 0.22±0.01 or 0.29±0.06 pmol/mg protein (n = 5) for the cultures exposed to the colchicine or cytochalasin D incubation, which were insignificantly different from the control value, 0.25±0.04 pmol/mg protein. Similar values of K_D, 3.0±0.03–3.4±0.05 nM of the ligand to the cells were also seen for the control, and the drug-treated groups. In another set of experiment, cell homogenates of control cultures were subjected to reversible binding assay using 5 nM ^{3}H-FNZ; 1 μM colchicine or 2 μM cytochalasin D was included in some incubations. The levels of

binding were alike from these three groups of samples, control, colchicine (1 μM)-treated, and cytochalasin D (2 μM)-treated, implicating the lack of interaction between either of the agents and ^{3}H-FNZ or the receptor. In contrast, a previous study had found that 100 μM colchicine blocked substantially the GABA-elicited current in L(tk$^-$) cells expressing $GABA_AR$ [Weiner et al., 1998].

Subcellular Distribution of Radioligand Binding

Following trypsin-induced exhaustive proteolysis of control cultures, the remaining trypsin-resistant binding of ^{3}H-FNZ was 33±3% of the total binding (n = 10), which delineated the intracellular fraction of receptors in the neurons. For the colchicine-treated cultures, the trypsin-resistant binding increased to 45±4% (n = 10) (Fig. 1). Thus, exposure to colchicine generated an elevation of about 36% in the intracellular portion of $GABA_AR$ and an 18% decrease in cell surface receptors. However, this modification was reversible, since after removal of the agent, the trypsin-resistant binding was restored to the control level. By contrast, the incubation of the culture with cytochalasin D did not significantly alter the subcellular distribution of ^{3}H-FNZ binding. When the colchicine-treated cultures were first incubated with trypsin and then exposed to Trypan blue, the ratio of cells that excluded the dye, 96% (n = 2,919), was similar to that of the cultures without trypsinization. Hence the trypsin incubation did not appear to provoke cell death.

General Protein Synthesis and Glycosylation

Effects of the cytoskeleton-disrupting agents were examined on the general protein synthesis and glycosylation of the cultured neurons by incubating the control or drug-treated cultures with ^{3}H-leucine or sugars. The incorporation extent of ^{3}H-leucine into TCA-precipitable proteins of the cultures was significantly inhibited by about 21 or 18% ($P < 0.05$) by a 3-h exposure to 1 μM of colchicine or 2 μM of cytochalasin D. Nevertheless, the uptake of ^{3}H-mannose or ^{3}H-galactose by the cultures was unaffected by the agents (Table I).

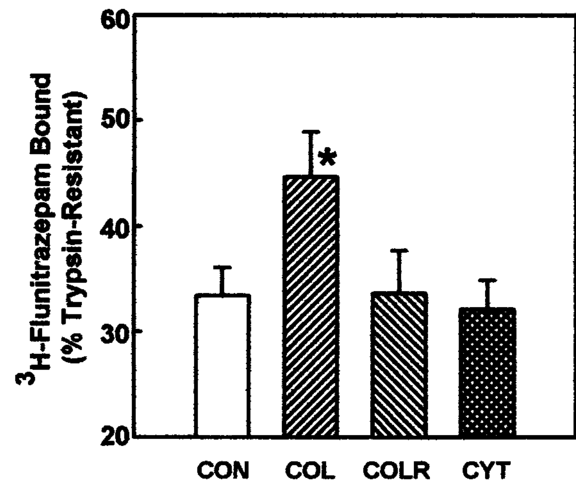

Fig. 1. Colchicine reversibly elevates the intracellular proportion of $GABA_AR$. Cultured neurons were treated without (CON) or with 1 μM of colchicine (COL) or 2 μM of cytochalasin D (CYT) for 3 h, followed by in situ trypsinization and 5 nM of ^{3}H-flunitrazepam binding, according to the protocols in Methods. After 3-h exposure, the colchicine-containing medium of a number of cultures was replaced by fresh medium only; these cultures were further incubated in the incubator for 3-h and then subjected to the trypsinization and binding experiment (COLR). Percentages of trypsin-resistant binding of ^{3}H-FNZ were determined in the cultures, which represents intracellular ^{3}H-FNZ binding. Data points are means±SD with n = 10. * $P < 0.05$ vs. control value.

Morphology of α-Tubulin, NF 200kDa, and Actin

The α-tubulin-immunoreactivity appeared as dark brown substance in the cytoplasm of cell bodies and processes of cultured neurons (Fig. 2A). The exposure to colchicine evidently

TABLE I. Effect of Colchicine or Cytochalasin D on the Rates of General Protein Synthesis and Glycosylation of the Neuron Culture

	Control (%)	Colchicine (%)	Cytochalasin D (%)
Incorporation			
^{3}H-Leucine	100	79±3*	82±7*
^{3}H-mannose	100	98±4	105±3
^{3}H-galactose	100	99±2	103±6

The neuron cultures were treated without (control) or with colchicine, 1 μM, or cytochalasin D, 2 μM, for 3 h. Subsequently the cells were subjected to the experiment of the ^{3}H-leucine or ^{3}H-monosaccharide incorporation as described under Methods. The level of TCA-precipitable ^{3}H-leucine or sugar was measured in the cells. Data are shown as mean percentages±SD/culture dish of the control values (100%) with n = 5. *Significantly different from control. $P < 0.05$.

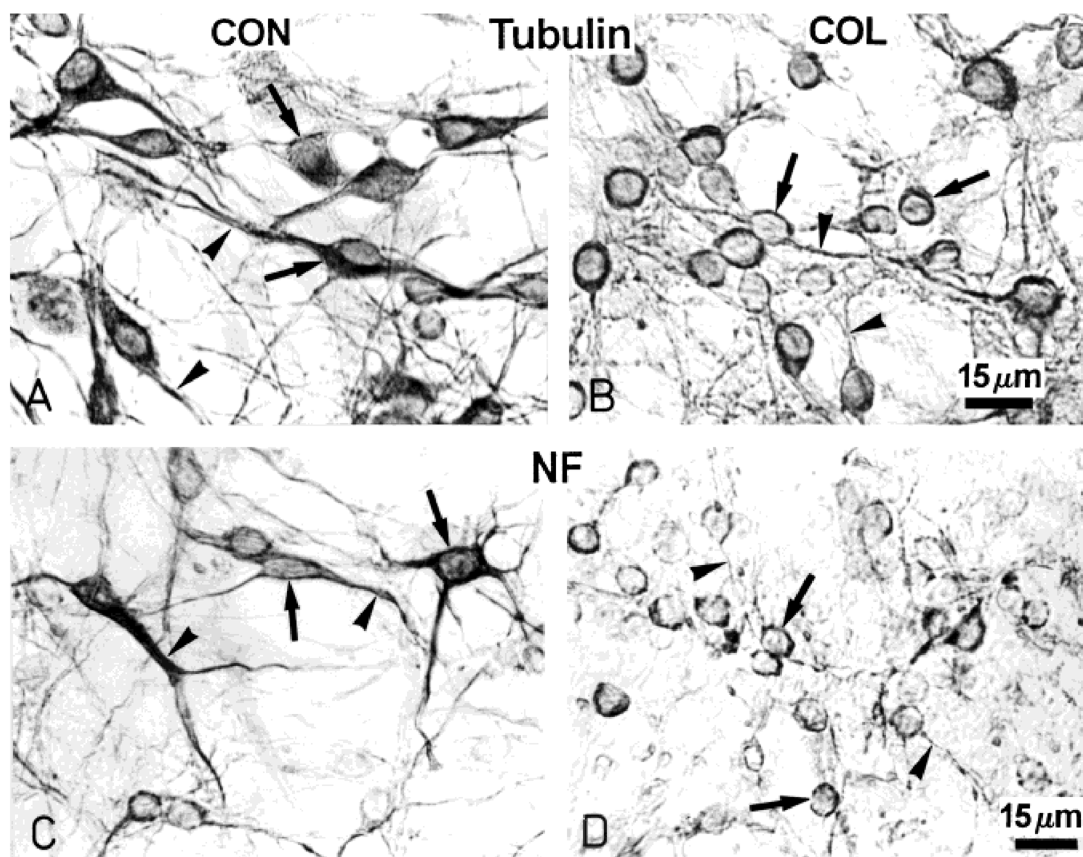

Fig. 2. Representative micrographs illustrating colchicine-induced changes in the distribution of the α-tubulin and NF 200kDa immunoreactivity in cultured neurons. Immunostaining using an anti-α-tubulin (**A** and **B**) or an anti-NF 200kDa (**C** and **D**) antibody was performed on control cultures (CON) or cultures treated with 1 μM of colchicine for 3 h (COL). In many control neurons, the α-tubulin (A) or NF 200kDa (C) immunoreactivity occupies the perikarya (arrows) and processes (arrowheads). Most colchicine-treated cells possess round or oval-shaped cell bodies (arrows in B,D), compared with polygonal-shaped somata of many control cells (A,C), and display weaker α-tubulin or NF 200kDa immunostain in their processes (arrowheads in B,D) than that of control (A,C).

transformed the polygonal shape of most somata into round or oval (Fig. 2B,D). For tubulin-positive neurons of control culture, about 70% (n = 2,000) possessed polygonal-shaped somata, and 30% oval or round. The colchicine treatment resulted in an increased percentage of round or oval neurons, 85% (Fig. 2B). Down-regulated tubulin-immunoreactivity was observed in the processes of many neurons (Fig. 2B).

Almost all cells in culture (96%) were positively immunostained by the anti-NF 200kDa antibody in the presence or absence of colchicine or cytochalasin D. The NF-immunoreactivity was seen in the perikarya and processes of control neurons (Fig. 2C). In colchicine-treated neurons, the NF-immunoreactivity generally became weaker in the processes than control (Fig. 2D). The exposure to cytochalasin D did not seem to alter the cellular distribution of α-tubulin and NF-immunoreactivity in the neurons (data not shown).

The FITC-conjugated phalloidin staining revealed peripheral localization of actin filaments in the cell bodies and processes of control neurons, which often appeared as thin linear lines in the processes (Fig. 3A). In cells exposed to cytochalasin D, the normal configuration of microfilaments was apparently disrupted and became irregular aggregates in the somata and processes (Fig. 3B).

Immunostained $GABA_AR$ α Subunit Proteins

Immunocytochemical localization of $GABA_A$ R with anti-α_1 or anti-α_1+51kDa antibody demonstrated the presence of the receptor

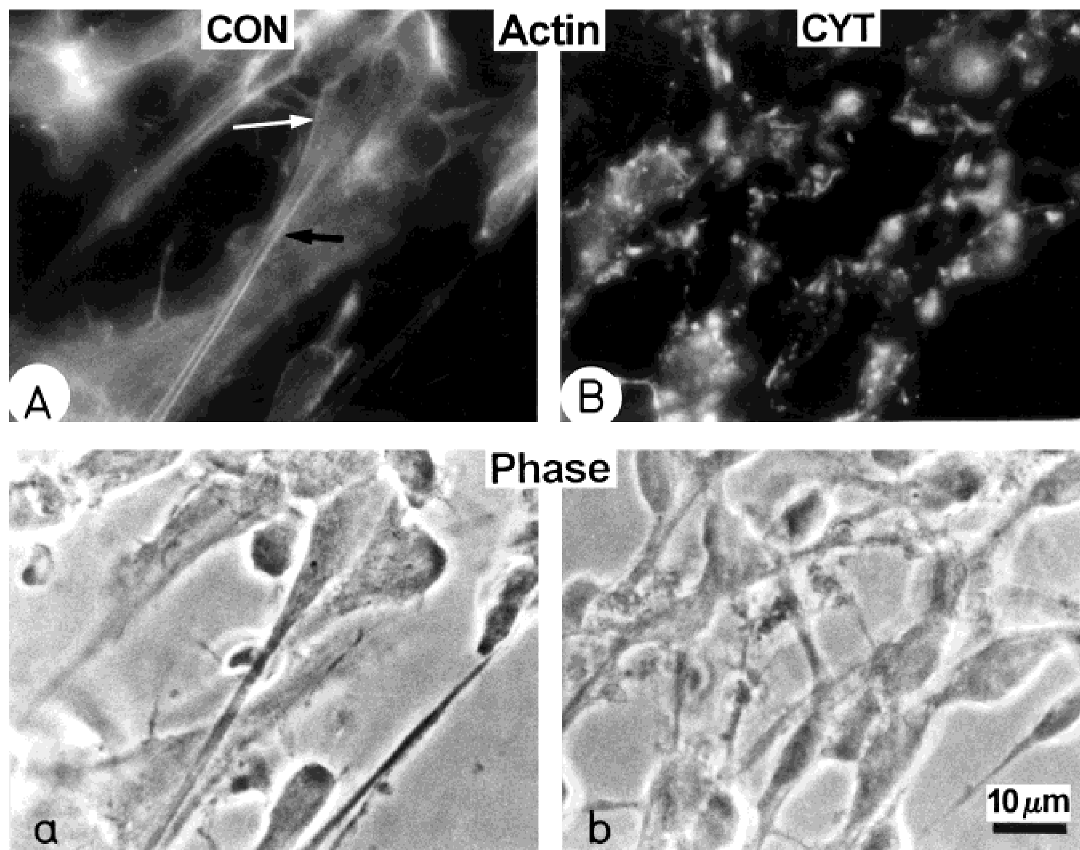

Fig. 3. Cytochalasin D discomposes the organization of actin filaments in cultured neurons. Immunofluorescent staining of actin using FITC-phalloidin was conducted on cultures treated without (CON, **A**,**a**) or with 2 μM of cytochalasin D for 3 h (CYT) (**B**,**b**). In control neurons, the phalloidin staining is seen in the peripheral somata and continuous with the thin linear organization in the peripheral part of the processes (A, arrows). In the CYT-treated culture, the staining pattern becomes discontinuous irregular aggregates in the somata and processes (B, arrows). Phase pairs are shown in a and b for respective fluorescent images.

in the perikarya of many neurons (Fig. 4). In the control, and colchicine- or cytochalasin D-incubated cultures, the α_1 subunit was observed in 85±3%–86±3% of the neurons/dish (n = 8), whereas 90 ± 2–91±2% (n = 8) contained the α_1+51kDa subunits. The staining intensity by the antibodies was also quantified by measuring the IOD of each neuron. Values from the quantification are summarized in Table II.

In many control neurons, the α_1 immunoreactivity was seen as dark-brown products, distributed fairly evenly in the perikaryal regions, including cell membrane and cytoplasm (Fig. 4A). Previous observations had confirmed the cell membrane and intracellular localization of the α subunits, by the method of immuno-electron microscopy [Lin, 1999]. Many colchicine-treated cells displayed a somewhat disorganized pattern of the α_1 immunoreactivity, which often manifested condensation and intensification of the signal to certain parts of the somata (Fig. 4B). The immuno-labeled α_1+51kDa frequently appeared as dark punctate aggregates occupying the cytoplasm and cell membrane of control neurons (Fig. 4D). Colchicine seemed to converge these punctate dots into coarser structures in the cytoplasm of many cells, resulting in a relatively stronger cytoplasmic staining (Fig. 4E). By contrast, cytochalasin D did not significantly affect the cellular distribution of the α subunits in most cells (Fig. 4C,F).

Analogous levels of the α_1 were detected in control cells, 101±18 (IOD)/neuron/dish, colchicine-treated, 105±17, and cytochalasin D-treated, 107±20. However, in cells exposed to cytochalasin D, the level of α_1+51kDa elevated around 28% from the control value 380±58 to 488±33, whereas colchicine caused a near-

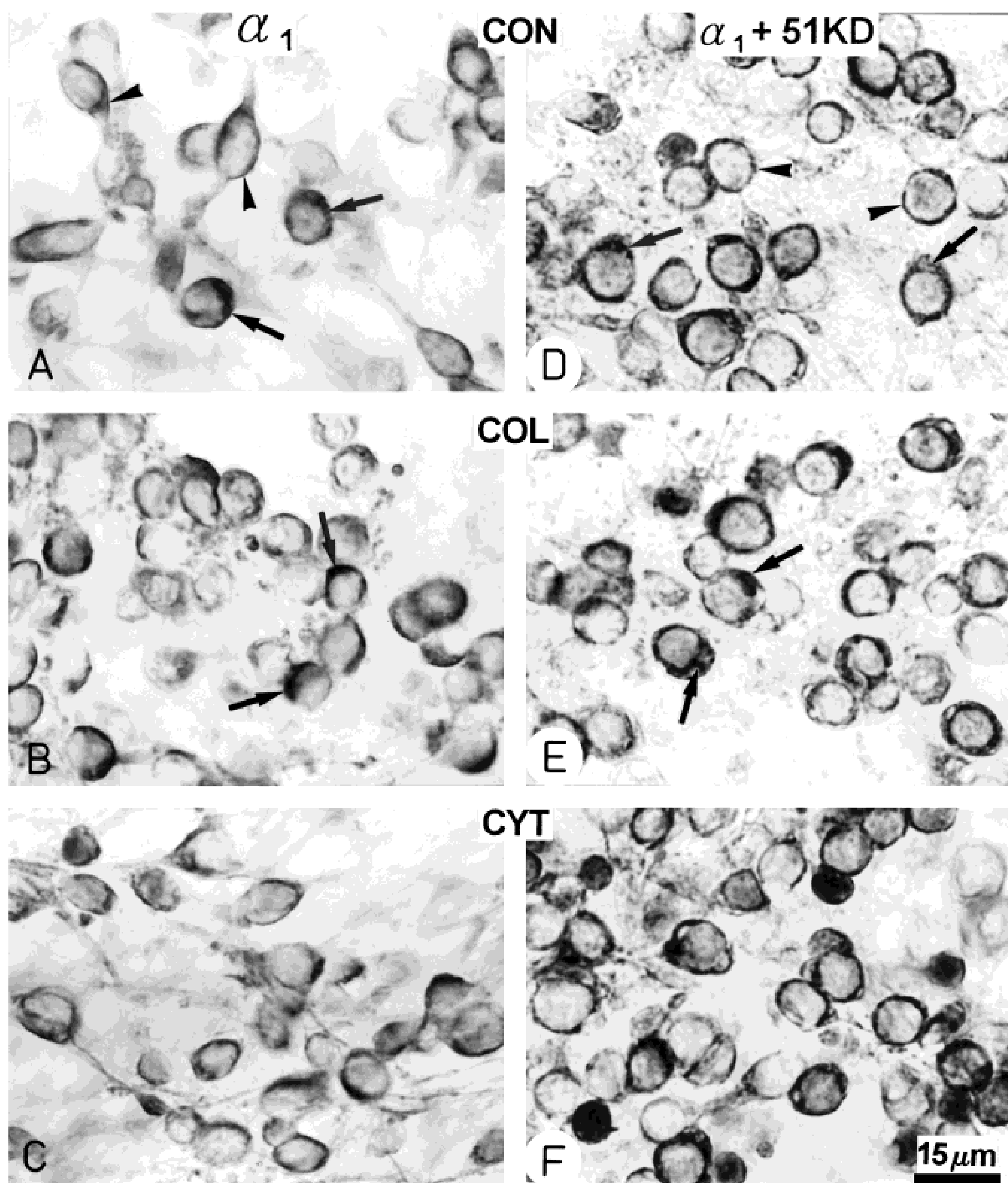

Fig. 4. Effect of colchicine or cytochalasin D on the expression of $GABA_AR$ α subunit proteins in cultured neurons. Immunostaining was performed using either an anti-α_1 subunit of $GABA_AR$, or anti-α_1+51kDa subunit antibody on control cultures (CON), and cultures exposed to 3 h of 1 μM of colchicine (COL) or 2 μM of cytochalasin D (CYT). Immunoreaction products are present in the perikarya of many neurons, including the cytoplasm (arrows) and cell membrane (arrow heads). The α_1-immunoreactivity is fairly evenly distributed in the somata of control neurons (**A**). In COL-treated cells, the labeling of α_1 is usually concentrated to certain parts of perikaryon (**B**, arrows). Punctate immunostaining of α_1+51kDa is seen in many control neurons (**D**). In COL-treated cells, the α_1+ 51 kDa immunostain appears as larger and denser forms in the cytoplasm (**E**, arrows) than control. The α_1+51kDa in the CYT-treated cells generally is more intense (**F**) than control (D).

control level, 419±59. Immunoblotting of the cultures was conducted according to earlier procedures [Yin and Fan, 1990]. The exposure to colchicine did not induce apparent alterations in the molecular weights and the levels of the α subunit proteins (data not shown).

Expression of $GABA_AR$ α_1 and α_2 Subunit mRNAs

Mechanisms underlying the actions of colchicine and cytochalasin D on $GABA_AR$ were further examined by in situ hybridization

TABLE II. The Levels of GABA$_A$R Subunit Proteins and mRNAs in Cultured Neurons Treated With Colchicine or Cytochalasin D

	Control	Colchicine	Cytochalasin D
α_1 protein			
Positive neurons	86±2%	85±3%	86±3%
IOD/neuron	101±18	105±17	107±20
α_1+51kDa proteins			
Positive neurons	91±2%	90±3%	90±2%
IOD/neuron	380±58	419±59	488±43*
α_1 mRNA			
Positive neurons	74±3%	73±2%	92±2%*
IOD/neuron	148±25	135±24	202±53
α_2 mRNA			
Positive neurons	71±2%	74±3%	94±2%*
IOD/neuron	118±17	125±25	134±14

The neuron culture was incubated without (control) or with 1 μM of cytochalasin D for 3 h. Immunocytochemistry and in situ hybridization were performed on the cells to localize the α_1 or α_1+51 kDa proteins, and the α_1 or α_2 mRNA. The percentages of neurons that expressed the protein or mRNA were determined in the cultures (positive neurons). The integrated optical density (IOD) in each labeled neuron was measured by an image analysis system as described in Methods. Data are presented as mean percentags of IODs±SD/dish with n = 6–10. * Significantly different from control. $P < 0.05$.

cytochemistry using DIG-labeled probes for the α_1 and α_2 subunit mRNAs of the receptor. The positively labeled mRNAs appeared as dark purple substance localized to the perikaryal and proximal parts of processes of the neurons (Fig. 5A–D). Cultures exposed to colchicine displayed similar percentages of neurons expressing the α_1 mRNA or α_2 mRNA to control,

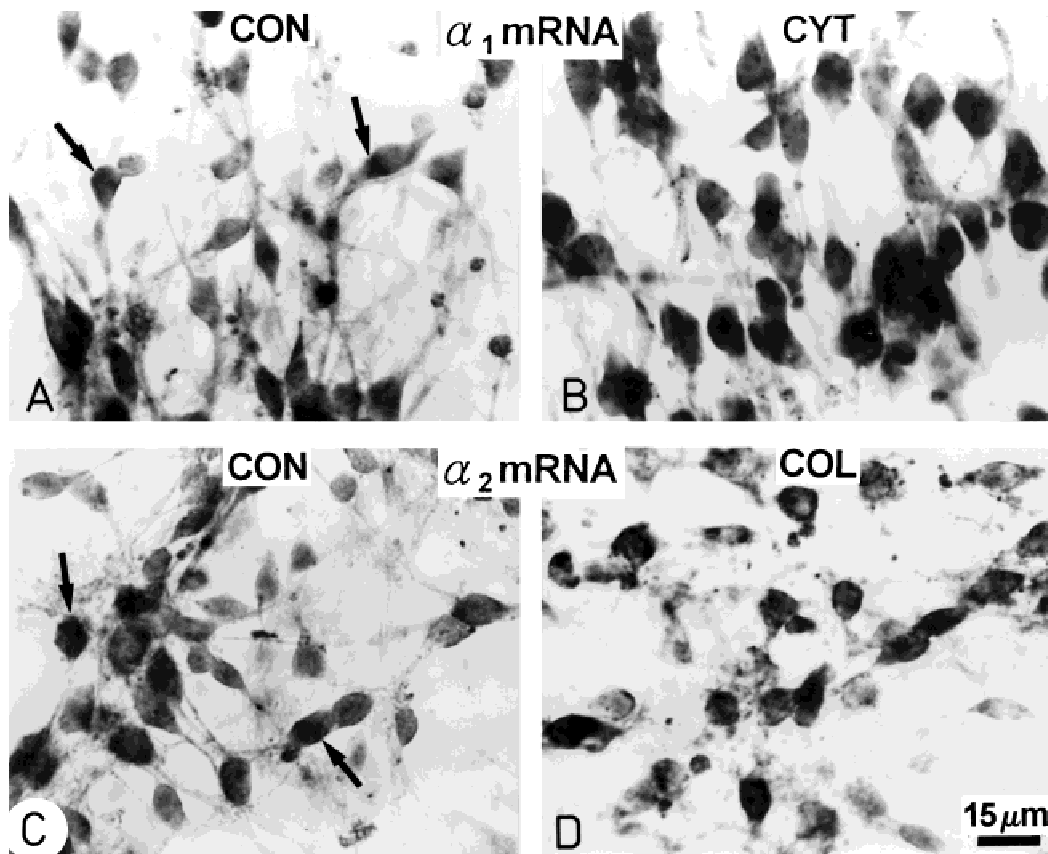

Fig. 5. Effect of colchicine or cytochalasin D on the mRNA expression of GABA$_A$R α_1 or α_2 subunit. Control cultures and cultures following a 3-h incubation with 1 μM of colchicine (COL) or 2 μM cytochalasin D (CYT), were subjected to in situ hybridization cytochemistry, by a digoxigenin-labeled oligonucleotide probe to GABA$_A$R α_1 (**A** and **B**) or α_2 (**C** and **D**) subunit mRNA. The signal of the mRNA is observed in the perikarya of many neurons (arrows). It seems that more cells express α_1 subunit mRNA in CYT-treated cultures (B) than that in control (A).

which were 73±2%/dish (n = 6) for α_1 and 71±2% (n = 10) for α_2, compared with 74±3% and 74±3% of control. Notably, the cytochalasin D treatment produced higher percentages, 92±2% (n = 8) and 94±2% (n = 6), that expressed the α_1 mRNA and α_2 mRNA, respectively (Fig. 5). Nonetheless, the drug treatment did not modify significantly the level of α_1 mRNA or α_2 mRNA. In colchicine-incubated cultures, the α_1 mRNA attained 135±24 (IOD)/neuron/dish and α_2 125±25. The levels of 202±53 of α_1 mRNA and 134±14 of α_2 occurred in the cells treated with cytochalasin D. These values resemble control levels of 148±25 of α_1 and 118±17 of α_2.

DISCUSSION

We have found that intact microtubules are important in maintaining the appropriate subcellular distribution of the $GABA_AR$ in the cultured brain neurons. In addition, microfilaments may be associated with the regulation to the expression of the receptor subunit proteins and mRNAs.

$GABA_AR$ and Microtubules

The treatment of cultured neurons with colchicine increased reversibly the intracellular fraction of $GABA_AR$ to 45% from 33% of control, as shown by trypsinization and ^{3}H-FNZ binding, despite that the agent did not change the total density of the receptor (B_{max}) in the cells and the affinity of ^{3}H-FNZ to the receptor. Hence, the level of intracellular receptors increased around 36% and the cell surface receptors decreased 18% from the control. In many colchicine-treated cells, the more intense immunostain of $GABA_AR$ in the cytoplasm presumably parallels the raise in the intracellular receptor binding sites. This subcellular redistribution implicates an association between microtubules and the receptor. The redistribution could arise from a blockade to the transport between cell membrane and intracellular region, such as deliverance of new receptor, or receptor recycling or degradation, suggesting that microtubules participate in regulating the intracellular transport of $GABA_AR$. An early study also described an accumulation of plasma proteins in the Golgi-derived secretory vesicles of hepatocytes in response to microtubule depolymerization [Redman et al., 1975]. In addition, the viral integral membrane glycoproteins were retained in the somata of hippocampal neurons by nocodazole treatment [Cid-Arregui et al., 1995].

A recent report proclaimed that the distribution of the $GABA_AR$ associated protein (GABARAP) corresponded with that of the microtubules in CHO cells and the protein bound microtubules directly [Wang and Olsen, 2000]. Furthermore, biochemical data suggest a connection between the $GABA_AR$ and tubulin via gephyrin, a well-known MAP linking the glycine receptor to post-synaptic localization [Kirsch et al., 1991; Sassoe-Pognetto et al., 1995; Essrich et al., 1998]. These findings support our hypothesis that microtubules transport the receptor intracellularly. By contrast, depolymerization of microtubules did not alter the transport of acetylcholine receptors in embryonic myotube, though the number of cell surface receptors was reduced [Connolly, 1985].

Changes might be anticipated in the electrophysiological properties of $GABA_AR$, because of the 18% decline in the cell surface receptors, as detected from our present report. A recent study indeed observed that the muscimol-induced currents showed a rundown by 78% in cultured hippocampal neurons, after destruction of their microtubules with a 4-h treatment of 10 μM of nocodazol [Meyer et al., 2000].

In the present study, the 3-h exposure to 1 μM of colchicine failed to change the total number of the $GABA_AR$ binding sites in the neurons. Similarly, a 4-h treatment of 10 μM of colchicine had little effect on the total number of radioligand binding sites of the β-drenergic receptor in C6 glioma cells [Hough et al., 1994]. In the colchicine-treated neurons, the control level of the immunostained $GABA_AR$ α_1 and 51 kDa subunits is consistent with the regular binding level of ^{3}H-FNZ.

Following the colchicine exposure in the present examination, the 21% decrease in the rate of general protein synthesis did not seem capable to affect significantly the total number of $GABA_AR$, for newly synthesized receptors make up merely 4% of the total receptors, as shown previously [Borden et al., 1984; Czajkowski and Farb, 1989]. Moreover, the colchicine treatment did not modify the rate of galactosylation and mannosylation of the cultured cells, pointing to the independence of glycosylation to the microtubule polymerization.

Our previous study has declared that N-linked glycosylation is required for a normal subcellular distribution of $GABA_AR$, because tunicamycin, an inhibitor to N-linked glycosylation, down-regulated the intracellular receptors [Lin et al., 1998]. The different responses of $GABA_AR$ to colchicine and tunicamycin indicate that microtubules and the glycosylation process of neurons probably participate in diverse regulatory mechanisms for the subcellular expression of $GABA_AR$. After the treatment with colchicine, the distribution of NF-immunoreactivity became somewhat disorganized in the neurons. An interaction has been proposed between the 200 kDa-NF tail domain and the carboxyl-terminal region of tubulin [Miyasaka et al., 1993]. Whether the change of NF relates to the increase in the intracellular fraction of the receptor awaits further investigation.

$GABA_AR$ and Microfilaments

In contrast to the effect of colchicine, the incubation with cytochalasin D had not caused significant changes in the subcellular distribution of $GABA_AR$. This implies the relative independence of the organization of microfilaments to the preservation of subcelluar distribution or intracellular transport of $GABA_AR$. The microfilaments of the neurons were disrupted by cytochalasin D, but the normal pattern of tubulin and NF-immunoreactivity persisted in the treated cells, and thereby confirms indirectly that the microtubules play a more major role in the subcellular expression of $GABA_AR$. However, cytochalasin D increased the level of $GABA_AR$ α_1+51 kDa-immunoreactivity by approximately 28%, regardless that the total receptor binding level was unaffected and the protein synthesis was inhibited by about 18%. ^{3}H-FNZ could presumably bind to all α subunits and thus the binding results do not necessarily correlate with the immunostaining data. The up-regulation of the receptor subunit proteins may be explained by post-translational and/or other relevant mechanisms. Conversely, the cytochalasin D perturbation decreased the level of myelin proteins of Schwann cells [Fernandez-Valle et al., 1997].

A number of studies have demonstrated a connection between microfilaments and neuroreceptors. Rapsin, an actin binding protein, may involve in the clustering of the acetylcholine receptor and $GABA_AR$ on the cell membrane [Hill, 1992; Yang et al., 1997]. The GABARAP binds indirectly with actin filaments and therefore connects indirectly the $GABA_AR$ with the microfilaments [Wang and Olsen, 2000]. Furthermore, morphological evidence indicates a link between the δ glutamate receptor and actin-cytoskeleton [Hirai, 2000]. Nonetheless, the maintenance of post-synaptic localization of $GABA_AR$ did not depend on F-actin in the post-synaptic density, even though the F-actin cytoskeleton seemed to stabilize clusters of NMDA receptors at post-synaptic membranes and AMPA receptor-containing spines of hippocampal neurons [Allison et al., 1998].

It is interesting to note that cytochalasin D increased the number of neurons expressing the α_1 or α_2 subunit mRNA of $GABA_AR$, as revealed by in situ hybridization. Possible mechanisms for this elevation include an enhancement in the gene transcription and/or mRNA stability, and a reduction of mRNA degradation. A rise in the level of α_1 mRNA did occur in the cytochalasin D-treated neurons, yet it was insignificantly different from the control level. According to another report using Northern blotting, cytochalasin B up-regulated the mRNAs of the β_1- and β_2-adrenergic receptors in C6 glioma cells [Hough et al., 1994]. On the other hand, cytochalasin D might have induced a certain population of cells to express the $GABA_AR$ subunit mRNAs, which in control condition did not contain detectable amount of the mRNAs. The disturbance to microfilaments might produce a series of intracellular events to elicit the expression of the mRNAs. Better understanding of the mechanisms underlying these changes probably requires a treatment with higher concentration/longer exposure time of the agent and more molecular approaches. It has been exhibited that cytochalasin activated actin gene transcription, resulting in elevated rates of actin mRNA and protein syntheses in murine erythroleukemia cells [Sympson et al., 1993].

Thus, there is a possible connection between microfilaments and the expression of specific mRNAs in the cells. An mRNA binding protein p50 has been shown to bind actin filaments and could be important in mRNA transport, anchoring, and localization on actin filaments in the cell [Ruzanov et al., 1999]. In regard to potential interaction between microtubules and microfilaments, some MAPs were found to interact with both elements. For example, MAP2 and τ use the same domain for actin and tubulin

interactions [Correas et al., 1990]. In our experiment, the two components responded independently to respective disrupters, while a longer and/or stronger treatment may generate different consequences.

In conclusion, the colchicine treatment induced an increase in the intracellular content of $GABA_AR$, suggesting a role of microtubular system in the intracellular transport of $GABA_AR$. By contrast, the organization of microfilaments seemed to be irrelevant with the subcellular distribution of the receptor. However, cytochalasin D produced changes in the expression of $GABA_AR$ subunit proteins and mRNAs, and thereby further indicates the importance of cytoskeletal elements to sustaining the regular expression of the receptor. Our findings provide the evidence for cytoskeletal association with the intracellular transport and gene expression of $GABA_AR$ in neurons.

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