Journal of Cellular Biochemistry 85:561–571 (2002)

Acrylamide Disturbs the Subcellular Distribution of $GABA_A$ Receptor in Brain Neurons

Wen-Hsin Ho, Seu-Mei Wang, and Hsiang-Shu Yin*

Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Abstract Mechanisms underlying the action of acrylamide on neurons were studied by monitoring the expression of $GABA_A$ receptor (R) in cultured brain neurons derived from chicken embryos. In situ trypsinization of the neurons and ^{3}H-flunitrazepam binding assay were employed to examine the subcellular distribution of $GABA_AR$. A 3-h exposure of the cultured neurons to 10 mM of acrylamide raised reversibly the proportion of intracellular (trypsin-resistant) ^{3}H-flunitrazepam binding sites by about 48% and decreased cell surface binding 24% from respective control values, without altering total cellular binding and the affinity of the ligand. Moreover, the acrylamide treatment induced more intense perikaryal immunostaining of $GABA_AR$ α subunit proteins than that in control neurons but did not change the total level of cellular α immunostain, in accordance with the binding data. In the cell bodies of acrylamide-treated neurons, the level of neurofilament-200 kDa proteins was similar to control, whereas the tubulin protein content was significantly lowered approximately 51% from control, as revealed by quantifying the immunostained cytoskeletal elements. In addition, electron microscopic observations found reductions in the numbers of microtubules and neurofilaments in the perikarya of acrylamide-treated neurons. As exhibited by the ^{3}H-leucine and ^{3}H-monosaccharide incorporation experiments, the exposure to acrylamide inhibited the rate of general protein synthesis in the culture by 21%, while the rate of glycosylation remained unaltered. Furthermore, in situ hybridization analysis showed that acrylamide did not modify the expression of $GABA_AR$ α subunit mRNAs. Taken together, these data suggest that acrylamide may downregulate the microtubular system and disintegrate neurofilaments, and thereby block the intracellular transport of $GABA_AR$, resulting in the accumulation of intracellular receptors. J. Cell. Biochem. 85: 561–571, 2002.

Key words: neurofilaments; tubulin; intracellular transport; ^{3}H-flunitrazepam binding; trypsin treatment; immunocytochemistry

Acrylamide monomer is a neurotoxicant to humans and experimental animals. The neurotoxicity generated by the agent is characterized by the distal neurofilamentous swellings and degeneration of large axons in peripheral and central nervous systems [Spencer and Schaumburg, 1974a,b]. Moreover, chromatolysis-like responses were described in the neuronal cell bodies [Jones and Cavanaugh, 1984; Sterman, 1984]. However, the behavioral and functional abnormality can develop in the absence of axonal swelling and degeneration [Takahashi et al., 1995; Stone et al., 1999; LoPachin, 2000]. Thus, the mechanisms underlying the action of acrylamide require further clarification. Hypotheses to explain the acrylamide-induced axonal changes include inhibition to axonal energy production, changes in axonal transport, disruption of cytoskeletal structure and function, and alterations in the interaction between perikarya and axons [Miller and Spencer, 1985; Lopachin and Lehning, 1994 for reviews; Harris and Gulati, 1994]. Recent studies proposed that the inhibition to the Na^+/K^+-ATPase activity of the tibial nerve could account for, at least in part, the neuropathic conditions of swollen tibial axons in acrylamide-treated rats [LoPachin and Lehning, 1997; LoPachin, 2000].

Acrylamide-induced changes in perikarya have been thought to precede the axonal abnormality [Jones and Cavanaugh, 1984]. Previous

Grant sponsor: National Science Council, Taiwan; Grant number: NSC-86-2314-B-002-151.

*Correspondence to: Dr. Hsiang-Shu Yin, Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, 1-1, Jen-Ai Road, Taipei, Taiwan 100, ROC. E-mail: hsyin@ha.mc.ntu.edu.tw

Received 3 December 2001; Accepted 30 January 2002

DOI 10.1002/jcb.10159

studies recorded decreased numbers of neurofilaments, and modifications in mitochondria and rough endoplasmic reticulum in the perikarya of spinal ganglion neurons of rats and quails repeatedly injected with acrylamide [Jones and Cavanaugh, 1984; Sterman, 1984; Takahashi et al., 1994]. There were increased numbers of microtubules in cell bodies of both spinal ganglia and ventral horn cells in quails intoxicated with acrylamide [Takahashi et al., 1994]. By contrast, another study on acrylamide-treated rats described the depletion of microtubule-associated protein 1 (MAP1) and MAP2 in the perikarya of neurons in the caudate-putamen [Chauhan et al., 1993]. Moreover, differential alterations were found in the binding levels of dopamine, muscarinic acetylcholine, serotonin, glycine, GABA, and benzodiazepine receptors in various brain regions of rats treated with acrylamide [Agrawal et al., 1981; Bondy et al., 1981]. Nevertheless, whether acrylamide affects the subcellular distribution of the receptors is unknown.

The maintenance of regular subcellular distribution of neurotransmitter receptors is crucial to proper neurotransmission. Cellular events involved in receptor targeting or clustering are probably related to the organization of cytoskeletal elements. $GABA_AR$ is a ligand-gated Cl^- channel and the major inhibitory neurotransmitter receptor in the vertebrate brain. By combining with GABA, $GABA_AR$ mediates fast inhibitory transmission via increasing the Cl^- conductance [Macdonald and Olsen, 1994]. The activity of $GABA_AR$ can be modulated by a variety of clinically important compounds, such as the benzodiazepines, barbiturates, and steroids. The receptor is presumably a hetero-pentameric glycoprotein composed from at least 20 distinct subunits, α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε, π, and ρ_{1-3} [Rabow et al., 1995; Barnard et al., 1998].

Linking proteins were discovered between $GABA_AR$ and microtubules or microfilaments, and play important roles in the postsynaptic localization of the receptor [Yang et al., 1997; Hanley et al., 1999; Wang and Olsen, 2000]. In addition, we have previously demonstrated increased intracellular and decreased cell surface proportions of the $GABA_AR$ in cultured brain neurons, after the destruction of microtubules with colchicine, as revealed by using in situ trypsinization and binding assay with ^{3}H-flunitrazepam for the benzodiazepine modulator sites [Ho et al., 2001]. Thus, it is suggested that the microtubular system participates in the intracellular transport of the receptor because microtubules are key in neural growth and intracellular transport of proteins [Aliva, 1992; Hirokawa, 1998]. Furthermore, the organization of immunostained neurofilaments was also somewhat disturbed by colchicine [Ho et al., 2001]. Neurofilaments are believed to participate in maintaining the cell shape and interact with other cytoskeletal components such as microtubules [Robinson and Anderton, 1988]. However, the relationship is unclear between neurofilaments and the subcellular distribution or intracellular transport of $GABA_AR$. Therefore, acrylamide was used in the present study, as it is thought to affect neurofilaments.

In order to further understand the pathogenesis of acrylamide-induced neuropathy and the relation between neurofilaments and $GABA_AR$, the goal of the present study was to examine the effect of acrylamide on the expression and the subcellular distribution of $GABA_AR$ in cultured brain neurons. Trypsinization of acrylamide-treated cultured neurons in situ and ^{3}H-flunitrazepam binding were performed to determine the fractions of cell surface and intracellular receptors, because the macromolecule, trypsin, could only digest surface $GABA_AR$ [Czajkowski and Farb, 1986; Yin and Yang, 1992]. The cellular expression of neurofilament proteins, tubulin, and $GABA_AR$ α subunits was also investigated in the treated neurons by using immunocytochemistry.

MATERIALS AND METHODS

Neuron Culture and Drug Treatment

Dissociated neuron culture was prepared from the brains of 7-day old chick embryos as described previously [Yin and Yang, 1992]. In brief, the neurons were plated 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 1, 10, or 20 mM of acrylamide (Sigma) for 3 h in the incubator followed by the experiments depicted below. The viability of cultured neurons was examined by Trypan Blue incorporation [Lin et al., 1998].

Radioligand Binding

Reversible binding assay and trypsinization of cells in situ were conducted according to earlier procedures [Czajkowski and Farb, 1986; Yin and Yang, 1992]. Aliquots of the cell homogenates, prepared from homogenization and centrifugation of the cultured cells, were incubated with 2–25 nM ^{3}H-flunitrazepam (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 of the reaction mixtures through glass-fiber filters (Whatman GF/B). The radioactivity remaining on the filters was determined by liquid scintillation counting. A number of control and drug-treated cultures were incubated with or without 0.5 mg/ml bovine trypsin (Sigma) in 25 mM HEPES buffer in the presence or absence of the drug for 90 min at 37°C. Subsequently the cells were 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, Amersham) 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 cultures 2 h before terminating the drug treatment [Yin and Yang, 1992]. After washing with PBS, each dish was incubated with 1 ml SDS/Nonidet P-40/urea (0.02%/2%/8 M) 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 the samples through the glass fiber filters. The radioactivity associated with the filter was measured by liquid scintillation counting.

All biochemical determinations were done at least in triplicate, and normalized to control. The results are presented as means ± standard deviation (SD)/culture dish with $n = 6–10$. Scatchard plots were analyzed by using the ligand program [Munson and Rodbard, 1980] and used to determine the maximal binding sites, B_{max} and apparent affinity constant, K_D of the radioligand to the receptor. The significance of differences among data was analyzed using two-tail Student's *t*-test.

Electron Microscopy

Cultured neurons were subjected to electron microscopy according to a previous protocol [Lin et al., 1999]. Cells grown on 35 mm culture dishes were fixed with 2.5% glutaraldehyde in PBS for 30 min and then post-fixed with 1% OsO_4 for 10 min at room temperature (RT). After dehydration with a graded series of ethanol, the cells were embedded in Epon. Ultrathin sections of about 70 nm in thickness were obtained, stained with 1% uranyl acetate and lead citrate, and observed under a Jeol JEM-2000EXII electron microscope.

Immunocytochemistry

Immunocytochemical staining was carried out as described before [Yin and Fan, 1990]. Neuron cultures kept on dishes 35 mm in diameter 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 incubating with the anti-α-tubulin, anti-NF200 kDa subunit, anti-α_1 subunit of $GABA_AR$ [Fan et al., 1997] or anti-α_1 and 51 kDa ($\alpha_1 + 51$ kDa) antibody [Yin and Fan, 1990] at 4°C for 16 h. Afterward, the receptor immunoreactivity was visualized with a biotinylated secondary antibody and the ABC-peroxidase (Vector) method using 3,3′-diaminobenzidine tetrahydrochloride (DAB).

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 complementary to the unique nucleotide segment 1356–1400 of the mRNA of chicken $GABA_AR$ α_1 subunit [Bateson et al., 1991], and 1504–1548 of rat receptor α_2 subunit 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 as described previously [Lin et al., 1998]. Briefly, cultured 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 in 42°C for 1 h. Thereafter, the cells

were incubated with 0.6 pmol/μl of the DIG-labeled probe in the buffer at 42°C for 18 h. Posthybridization 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. The samples were then blocked with 2% normal sheep serum for 30 min at RT and incubated with an anti-DIG antibody coupled to alkaline phosphatase (1:500 dilution) (BM) at 30°C for 3 h, followed by incubation 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.

Image Analysis

Quantification of morphological data was performed as depicted before [Lin et al., 1998]. The levels of the labeled receptor proteins and mRNAs, and NF200 kDa and α-tubulin proteins were measured in the somata of cultured neurons with a computer-based image-analysis system (MCID, Image Research). The specific integrated optical density (IOD) was obtained for each labeled neuron in 9–14 evenly distributed fields of each culture dish 35 mm in diameter under a 40 × objective (400 × magnification). At least 750 labeled cells were measured from each culture dish of control or drug-treated group in every experiment. Results are presented as mean IODs ± SD/neuron/culture dish with n = 6–10 culture dishes derived from 3 to 5 experiments. The significance of difference 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 in cultures exposed to 3 h incubation with 1, 10, and 20 mM of acrylamide, the levels of specific ^{3}H-FNZ binding were 116 ± 2, 116 ± 5, and 110 ± 6% (n = 7) of respective control (untreated) values, and were insignificantly different from the controls. In control cultures, Trypan Blue was incorporated by approximate 4.0% (n = 2,910 cells counted) of the neurons, representing a normal rate of cell death in the neuronal culture. The 3-h treatment with 10 mM of acrylamide resulted in 4.3% (n = 2,890) of cells that collected the dye. This suggests that the drug treatment does not induce apparent membrane leakiness or cell death.

As revealed by saturation binding assay and Scachard analysis, the B_{max} of ^{3}H-FNZ was 0.23 ± 0.02 pmol/mg protein (n = 5) of the cultures following the 3 h incubation with 10 mM of acrylamide, which was similar to the control value, 0.25 ± 0.04 pmol/mg protein. Similar values of K_D, 3.2 ± 0.05–3.4 ± 0.01 nM of the ligand to the cells were also seen for the control, and the drug-treated groups. In another experiment, aliquots of cell homogenates of control cultures were subjected to reversible binding assay using 5 nM ^{3}H-FNZ; 10 mM of acrylamide was included in some of these incubations. The levels of binding were analogous between the control, and acrylamide (10 mM)-treated, implicating the lack of interaction between the agent and ^{3}H-FNZ or the receptor.

Subcellular Levels of Receptor Binding

Following trypsin-induced exhaustive proteolysis of control cultures, the remaining trypsin-resistant binding of ^{3}H-FNZ was 33 ± 3% (n = 10) of the total binding density, which denoted the intracellular fraction of the total number of receptors in the neurons. The 3-h exposure to 10 mM acrylamide increased the trypsin-resistant binding to 49 ± 2% (n = 10) (Fig. 1). Thus the acrylamide treatment induced an elevation of about 48% in the intracellular portion of $GABA_AR$ and a decrease of 24% on the cell surface receptor. However, this subcellular change was reversible, because the trypsin-resistant binding was restored to the control level after removal of the agent. When the acrylamide-treated cultures were first incubated with trypsin and then exposed to trypan Blue, the ratio of cells, 96% (n = 2,890), that excluded the dye, was similar to that of the cultures without trypsinization. Hence the trypsin incubation did not appear to generate cell death.

General Protein Synthesis and Glycosylation

Effects of acrylamide were examined on the rates of general protein synthesis and glycosylation of the cultured neurons by performing the incorporation experiments of ^{3}H-leucine or sugars. The 3-h treatment with 10 mM of acrylamide resulted in 79 ± 3% of, about 21% lower than, the control incorporation extent (100%) of ^{3}H-leucine into TCA-precipitable proteins of the cultures (n = 5, $P < 0.05$). Nevertheless, the uptake level of ^{3}H-mannose or

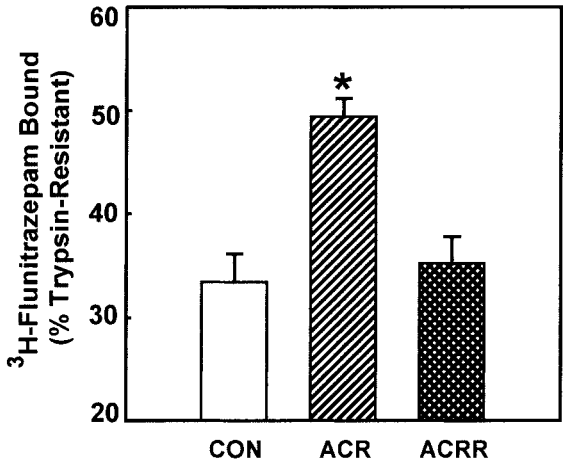

Fig. 1. Acrylamide reversibly elevates the intracellular fraction of $GABA_AR$. Cultured neurons were treated without (CON) or with 10 mM of acrylamide (ACR) for 3 h, followed by in situ trypsinization and reversible binding using 5 nM of ^{3}H-flunitrazepam (FNZ), according to the protocol in Materials and Methods. The acrylamide-containing medium of a number of cultures was replaced by fresh medium only, after the 3-h incubation with 10 mM of acrylamide; these cultures were further incubated in the incubator for 3-h and then subjected to the trypsinization and binding experiment (ACRR). Percentages of trypsin-resistant binding of ^{3}H-FNZ were determined in the cultures, which represents the intracellular fraction of ^{3}H-FNZ binding. Data points are means ± SD. $*P < 0.05$ vs. control value. N = 10.

^{3}H-galactose by the acrylamide-treated culture was 102 ± 8 or 94 ± 6% of that of control culture and not significantly different from the control value.

Immunocytochemical Localization of NF200, α-Tubulin, and $GABA_AR$

Almost all cells, 96%, in culture were positively immunostained by the anti-neurofilament 200 kDa subunit (NF200) antibody in the absence or presence of acrylamide. The NF200-immunoreactive substance, dark brown in color, was observed throughout the perikarya and processes of the control and drug-treated neurons (Fig. 2A,B). No positive staining signals were detected in cells stained without the primary antibody. The treatment did not seem to change the shape and size of the neurons. In the perikarya, the level of NF200 immunoreactivity was quantified by image analysis (Table I). The incubation with acrylamide resulted in a mean integrated optical density (IOD), 228 ± 65/neuron/culture dish, insignificantly lower than 272 ± 100 of control (Fig. 2B). However, the immunostain of NF in the distal parts of processes appeared somewhat lower than that of control.

The α-tubulin immunoreactivity was seen in the cytoplasm of cell bodies and processes of control neurons (Fig. 2C). Notably, in the perikarya of the acrylamide-treated neurons, the mean intensity of α-tubulin immunoreactivity, 110 ± 23/neuron/dish, was about 51% weaker than 225 ± 47 of the control cells ($P < 0.01$) (Fig. 2D). Moreover, in the processes of treated cells, the content of the immunostained tubulin was also lower than control. Compared with control, acrylamide-induced ultrastuctural changes included relatively more indented nucleus, less rough endoplasmic reticulum, as well as reduced numbers of microtublubes and neurofilaments, as shown by electron microscopy (Fig. 3). Shorter microtubules were frequently seen, while the neurofilaments were rarely present in the perikarya of the treated cells.

Immunocytochemical localization of $GABA_AR$ was done by using two distinct antibodies raised against the α subunits of the receptor, the anti-α_1 (50 kDa), and the anti-[$\alpha_1 + 51$ kDa]. The receptor was present in the perikarya of most control and acrylamide-treated neurons (Fig. 4). Acrylamide did not change the percentages of neurons that expressed the α_1 or the $\alpha_1 + 51$ kDa. The α_1 or $\alpha_1 + 51$ kDa immunoreactivity was present in approximate 86 ± 2%–86 ± 3% of neurons/culture dish (n = 8) or 89 ± 3–91 ± 2% of the control and acrylamide-treated neurons. Furthermore, acrylamide did not alter the total cellular content of the subunits, as revealed by measuring the integrated optical density (IOD) of each immunostained neuron. For cultures exposed to acrylamide, the mean IODs of α_1 and $\alpha_1 + 51$ kDa were 107 ± 9/neuron/culture dish and 392 ± 66 (n = 4), respectively, similar to 101 ± 18 and 380 ± 58 in control. Immunoblotting [Lin et al., 1998] was also conducted and showed that the molecular weights and levels of the α subunits were not modified by acrylamide (data not shown).

In positively labeled control neurons, the α_1 immunoreactivity was seen as dark-brown substance distributed in the perikarya and cell membrane (Fig. 4A). The presence of the receptor in the cytoplasmic region and on the cell membrane has been confirmed by immuno-EM previously in cultured chicken neurons [Lin, 1999]. In many acrylamide-treated cells, the intensity of α_1 immunoreactivity became

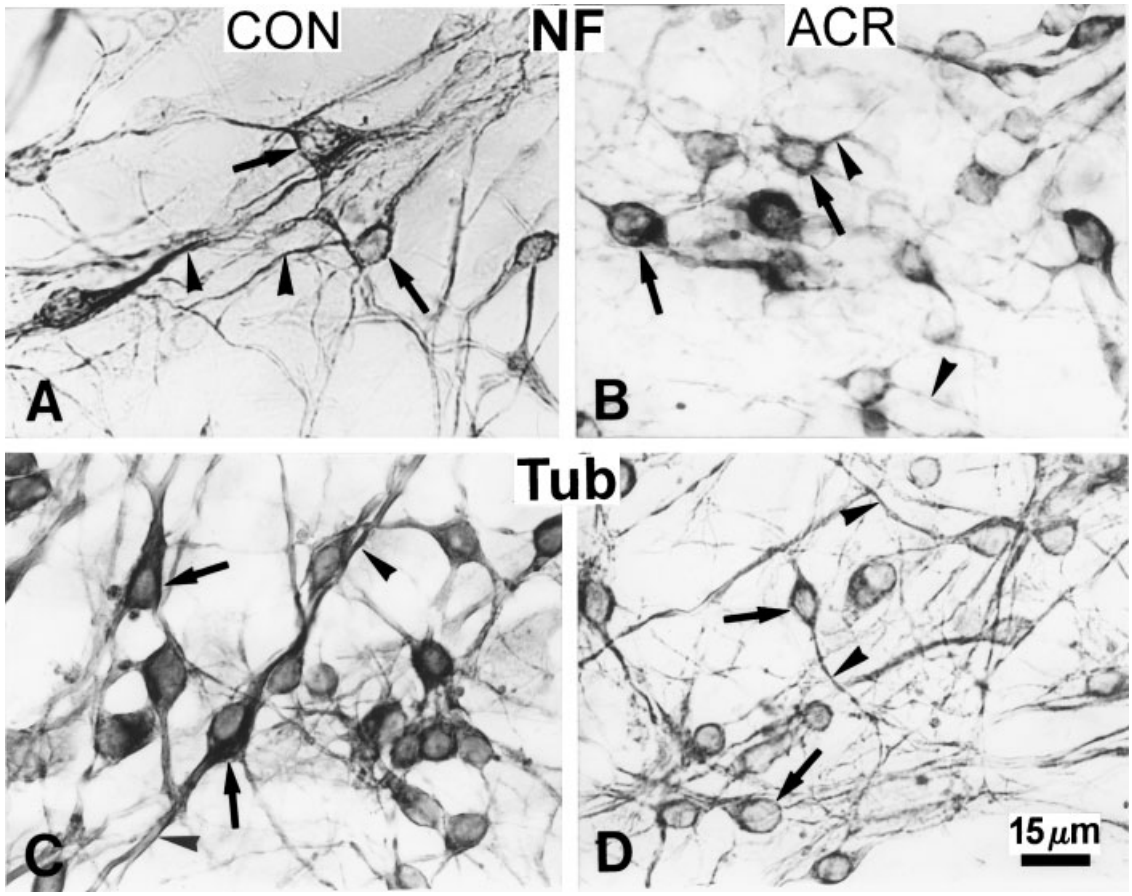

Fig. 2. Representative micrographs illustrating acrylamide-induced changes in the distribution of the NF200 and α-tubulin proteins in cultured neurons. Immunostaining using an anti-NF200 (**A** and **B**) or an anti-α-tubulin (**C** and **D**) antibody was performed on cultures treated without (CON) or with 10 mM of acrylamide (ACR) for 3 h. There is abundant NF200 (A) or α-tubulin (C) immunoreactivity in the cell bodies (arrows) and processes of control neurons (arrow heads). Compared with control, the NF200 immunoreactivity appears downregulated in the processes and unaltered in the somata by acrylamide (B). Most acrylamide-treated cells possess weaker α-tubulin immunostain in their perikarya and processes (D, arrows) than that of control (C). The levels of the perikaryal NF200 and α-tubulin immunoreactivity were quantified and the data are summarized in Table I.

TABLE I. Effect of Acrylamide on the Perikaryal Content of NF200 and α-Tubulin in the Cultured Neurons

	Control	Acrylamide
Immunoreactivity		
NF200 (IOD)	272 ± 100 (100%)	228 ± 65 (84%)
α-Tubulin (IOD)	225 ± 47 (100%)	110 ± 23* (49%)

The cultured neurons were treated without (Control) or with acrylamide, 10 mM, for 3 h (Acrylamide). Subsequently the cells were immunostained with the anti-NF200 or anti-α-tubulin antibody as described under Materials and Methods. The levels of the NF200 and α-tubulin immunoreactivity were quantified in the perikarya of the neurons with an image-analysis system. Data are presented as mean integrated optical densities (IODs) ± SD/neuron/culture dish.
*Significantly different from control, $P < 0.05$. N = 5. The mean IODs of NF200 and α-tubulin of Acrylamide are 84 and 49% of respective Controls (100%).

stronger in the perikaryal region than control (Fig. 4B). This implicates an increased intracellular fraction of the receptor and a decrease in the level of cell membrane receptors.

In the labeled control neurons, immunostained punctate aggregates of $\alpha_1 + 51$ kDa occupied the perikaryal region and cell membrane (Fig. 4C). Resembling the response of α_1 subunit, the intracellular immunostaining of the $\alpha_1 + 51$ kDa seemed to be intensified by acrylamide, and the cell membrane staining weakened in many cells (Fig. 4D).

Expression of $GABA_AR$ α_1 and α_2 Subunit mRNAs

Mechanisms underlying the action of acrylamide on $GABA_AR$ were further examined by in situ hybridization cytochemistry using DIG-

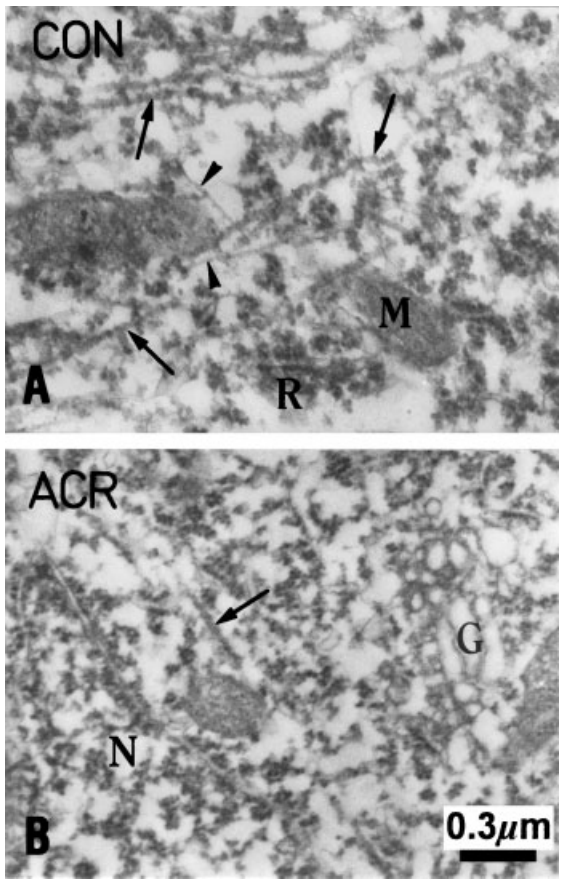

Fig. 3. Electron micrographs illustrating reductions in numbers of perikaryal microtubules and neurofilaments of acrylamide-treated neurons. Control (CON) and acrylamide (10 mM, 3 h)-treated (ACR) neurons were subjected to fixation and embedding in Epon as depicted in Materials and Methods. Photographs taken from ultrathin sections illustrate that in the perikarya of neurons exposed to acrylamide, there are decreased numbers of micotubules (arrows) and neurofilaments (arrowheads), compared with control. G, Golgi apparatus; M, mitochondrion; N, nucleus; R, rough endoplasmic reticulum.

labeled probes for the α_1 and α_2 subunit mRNAs of the receptor, respectively. The dark purple α_1 and α_2 mRNAs were seen in $71 \pm 2\%$ of neurons/culture dish ($n = 6$) and in $73 \pm 3\%$ in acrylamide-treated culture respectively, in analogy with $74 \pm 3\%$ of neurons/culture dish and $71 \pm 2\%$ ($n = 6$) of control cultures (Fig. 5). The acrylamide treatment resulted in mean IODs of 184 ± 75/neuron/culture dish of α_1 mRNA and 122 ± 17 of α_2 mRNA, similar to 148 ± 25 of α_1 and 118 ± 17 of α_2 in control neurons.

DISCUSSION

We have found that the acrylamide treatment reduced the level of tubulin proteins, and the numbers of microtubules and neurofilaments in the neuronal perikarya, while increasing the intracellular proportion of $GABA_AR$. The subcellular redistribution of the receptor is likely to be associated with the downregulated microtubules and possibly disintegrated or disorganized neurofilaments. This study provides the first evidence for the influence of an environmental neurotoxicin on the subcellular distribution of a major neurotransmitter receptor in the central nervous system.

In the present study, the exposure of cultured neurons to acrylamide increased reversibly the intracellular fraction of $GABA_AR$ binding sites by around 48% and decreased the cell surface binding by 24%, 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. In accordance with the binding results, stronger cytoplasmic immunostaining of the α subunits of $GABA_AR$ and unaltered total cellular α immunostain were observed in the perikarya of acrylamide-treated neurons. An earlier investigation has revealed that chronic treatment of rats with acrylamide produced increased ^{3}H-muscimol binding in the frontal cortex and unchanged benzodiazepine binding in the cerebellum [Agrawal et al., 1981]. Electrophysiological changes of the neurons can be predicted, because the number of cell surface receptors was reduced. In our previous work, a decrease in the amplitude of GABA-evoked current was recorded in the tunicamycin-treated neurons, which contained a lower level of cell surface $GABA_AR$ than control [Lin et al., 1999].

The subcellular redistribution of $GABA_AR$ may arise from changes in the intracellular trafficking, which includes the delivery of new receptors, and transportation of the receptor during its internalization, recycling, and degradation. These presumed intracellular modifications could come from altered cytoskeletal organization, and/or other relevant cellular events. Acrylamide did not significantly decrease the level of neurofilament subunit 200 kDa proteins in the perikarya. However, under electron microscopic observation, neurofilaments were rarely seen in the perikarya. This probably indicates a disintegration or disorganization of the neurofilaments, regardless that a near control level of the NF200 proteins was still present in the perikarya. Thus, it is possible that the disintegrated

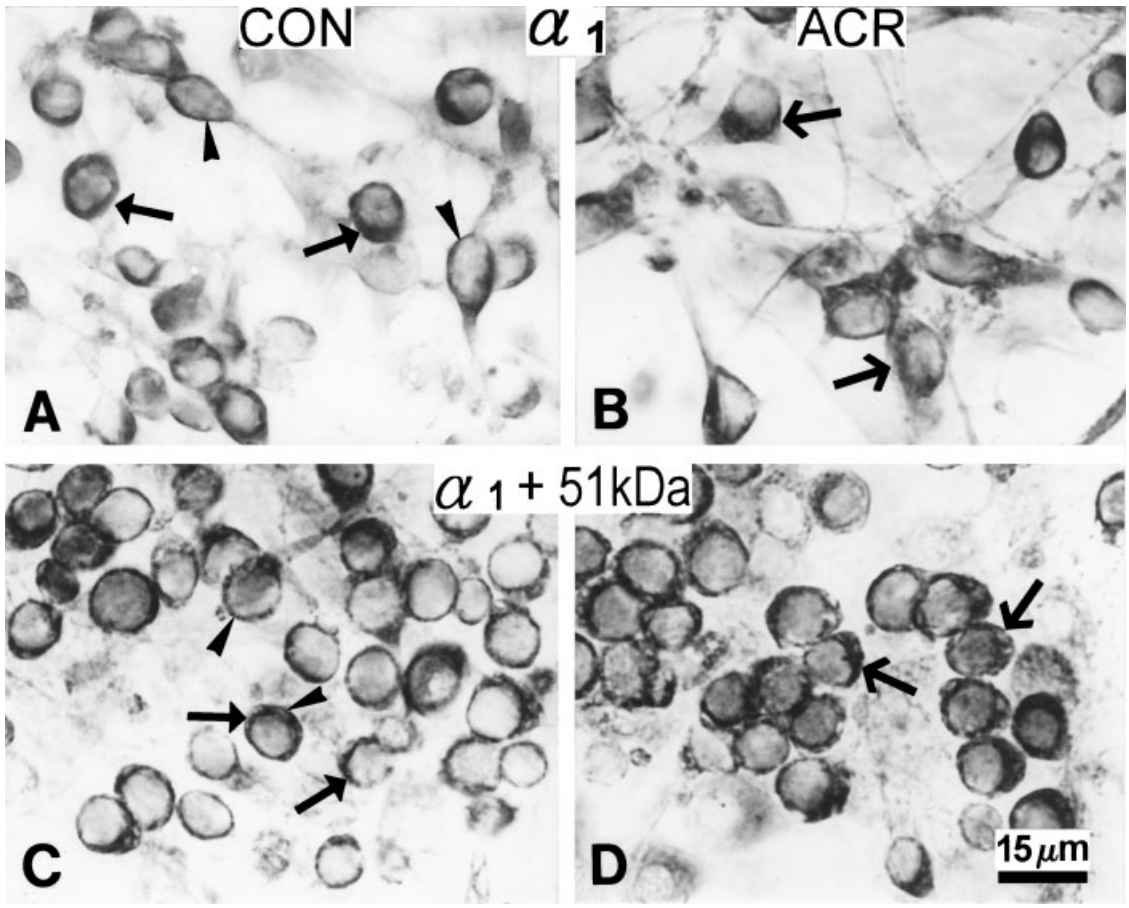

Fig. 4. Effect of acrylamide (ACR) on $GABA_AR$ α subunit immunoreactivity in cultured neurons. Immunostaining was performed using either an anti-α_1 subunit, or anti-$\alpha_1 + 51$ kDa subunits of $GABA_AR$ antibody on control cultures (CON), and cultures treated with 10 mM of acrylamide for 3 h. Immunoreaction products are present in the perikarya (arrows) and cell membrane (arrow heads) of most neurons. The intensity of α_1-immunoreactivity seems stronger in the perikarya of many ACR-treated neurons than that of control cells (**A**, **B**). It appears that ACR likewise induces a more intense immunostain of $\alpha_1 + 51$ kDa in the perikarya of many neurons, compared with control (**C**, **D**).

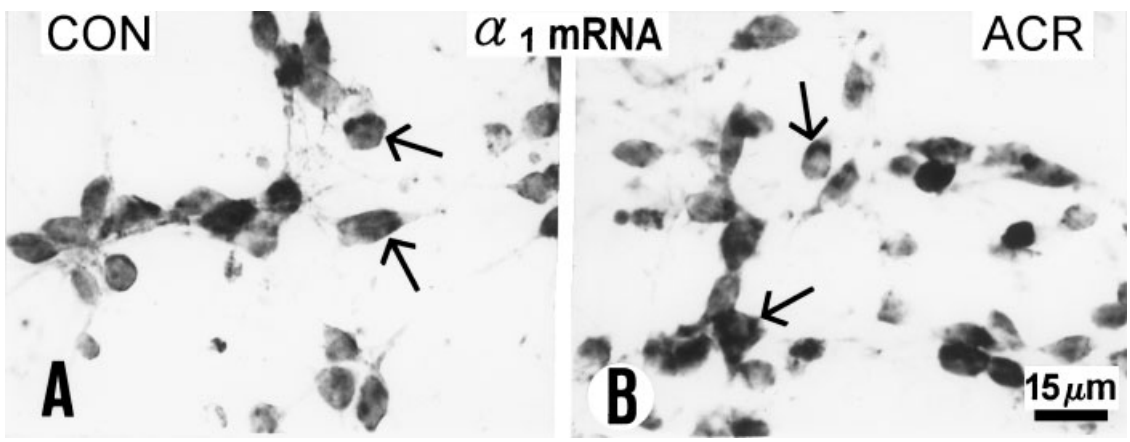

Fig. 5. The expression of the mRNA of $GABA_AR$ α_1 subunit in acrylamide-treated neurons. Cultures treated without (CON) or with 10 mM of acrylamide for 3 h (ACR) were subjected to in situ hybridization cytochemistry, by using a digoxigenin-labeled oligonucleotide probe to the $GABA_AR$ α_1 subunit mRNA. The signal of labeled mRNA is present in the perikarya of most neurons (arrows). Acrylamide does not seem to alter the expression of the α_1 subunit mRNA (**B**).

neurofilaments are related to the redistribution of the receptor. Loss of neurofilaments occurred in cell bodies of spinal ganglia and ventral horn cells of rats and quails repeatedly injected with acrylamide [Jones and Cavanaugh, 1984; Takahashi et al., 1994]. In our data, NF200 content was generally downregulated in the processes of acrylamide-treated cells, in contrast to the acrylamide-induced neurofilamentous accumulation seen in many central and peripheral nerves [Schaumburg and Spencer, 1977]. Moreover, elevated NF protein synthesis occurred in rat cerebral cortex and PC12 cells in response to acrylamide [Endo et al., 1994; Lin et al., 2000].

In our report, the level of perikaryal tubulin proteins was markedly downregulated about 51% by acrylamide. This corresponds with the decreased number of microtubules in the cell body detected from electron micrographs. It is likely that a reduction in microtubules might hinder the intracellular transport of the receptor, leading to an accumulation of the receptor intracellularly. It has been suggested that acrylamide interferes with axonal and intradendritic transport by acting on microtubules as well as MAPs [Lapadula et al., 1989; Sager, 1989; Chauhan et al., 1993]. In our previous study, the colchicine treatment likewise caused an enhanced intracellular level of $GABA_AR$ in the neurons [Ho et al., 2001]. Although colchicine did not seem to significantly decrease the tubulin content in the neuronal perikarya, the depolymerization of microtubules evidently interfered intracellular transport to some extent [Ho et al., 2001].

It is possible that acrylamide produced a stronger and more rapid effect on microtubules than neurofilaments in the perikarya because the content of tubulin protein was lowered, whereas the neurofilament protein was not by the agent. However, an assumed interaction between the NF200 tail domain and the carboxyl-terminal region of tubulin might account for the corresponding responses, i.e., reductions in the numbers of microtubules and neurofilaments to acrylamide [Miyasaka et al., 1993]. By contrast, the number of microtubules was increased in cell bodies of spinal ganglia and ventral horn cells of quails repeatedly injected with acrylamide [Takahashi et al., 1994]. In our present report, tubulin immunostain in the neuronal processes was weaker than control. An early study has shown profound decreases in the binding of ^{3}H-colchicine to tubulin in sciatic nerve and spinal cord, but not in cerebellum or brain, following prolonged exposure to acrylamide [Tanii and Hashimoto, 1983].

Following the acrylamide treatment in the present examination, the 21% decrease in the rate of general protein synthesis did not seem capable to significantly modify the regular level of the total number of $GABA_AR$, for newly synthesized receptors make up merely 4% of the total receptors, as shown previously [Czajkowski and Farb, 1989]. The 51% decrease of tubulin protein content in the perikarya might originate from an accelerated rate of tubulin degradation, rather than the decrease in the rate of general protein synthesis. By contrast, the exposure to acrylamide did not alter the rate of galactosylation and mannosylation of the cultured cells, implicating the independence of the glycosylation to the acrylamide-induced accumulation of intracellular $GABA_AR$. Nevertheless, the intracellular proportion of the de-N-glycosylated $GABA_ARs$ was lower than that of the normal receptors, as shown previously [Lin et al., 1998].

In conclusion, the acrylamide treatment generated an increase in the intracellular fraction of $GABA_AR$, in addition to a reduced level of tubulin proteins and decreased numbers of microtubules and neurofilaments in the perikarya. Acrylamide may not only act on the neurofilaments, but also the microtubular system to affect the subcellular expression of the receptor. Our findings support that the pathogenesis for the acrylamide-induced abnormality in the nervous system involves modifications in the expression of neurotransmitter receptors.

REFERENCES

Agrawal AK, Seth PK, Squibb RE, Tilson HA, Uphouse LL, Bondy SC. 1981. Neurotransmitter receptors in brain regions of acylamide-treated rats. I: Effects of a single exposure to acrylamide. Pharmacol Biochem Behav 14:527–531.

Aliva J. 1992. Microtubule functions. Life Sci 50:327–334.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ. 1998. International union of pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291–313.

Bateson AN, Harvey RJ, Wisden W, Glencorse TA, Hicks AA, Hunt SP, Barnard EA, Darlison MG. 1991. The chicken $GABA_A$ receptor α_1 subunit: cDNA sequence and localization of the corresponding mRNA. Mol Brain Res 9:333–339.

Bondy SC, Tilson HA, Agrawal AK. 1981. Neurotransmitter receptors in brain regions of acrylamide-treated rats. II: Effects of extended exposure to acrylamide. Pharmacol Biochem Behav 14:533–537.

Chauhan NB, Spencer PS, Sabri MI. 1993. Acrylamide-induced depletion of microtubule-associated proteins (MAP1 and MAP2) in the rat extrapyramidal system. Brain Res 602:111–118.

Czajkowski C, Farb DH. 1986. Transmembrane topology and subcellular distribution of the benzodiazepine receptor. J Neurosci 6:2857–2863.

Czajkowski C, Farb DH. 1989. Identification of an intracellular pool of γ-aminobutyric acid$_A$/benzodiazepine receptors en route to the cell surface of brain neurons in culture. Mol Pharma 35:183–188.

Endo H, Kittur S, Sabri MI. 1994. Acrylamide alters neurofilament protein gene expression in rat brain. Neurochem Res 19:815–820.

Fan SS, Chang NC, Chang AA, Yin HS. 1997. Differential distribution of the GABA$_A$ receptor α_1 subunit in developing chicken brain. NeuroReport 8:2399–2404.

Hanley JG, Koulen P, Bedford F, Gordon-Weeks PR, Moss SJ. 1999. The protein MAP-1B links GABA$_C$ receptors to the cytoskeleton at reticnal synapses. Nature 397:66–69.

Harris CH, Gulati AK. 1994. Toxic neurofilamentous axonopathies and fast axonal transport. V. Reduced bi-directional vesicle transport in cultured neurons by acrylamide and glycidamide. J Toxicol Environ Health 42:343–356.

Hirokawa N. 1998. Kinesin and dynein superfamily proteins and mechanism of organelle transport. Science 279:519–526.

Ho W-H, Wang S-M, Yin H-S. 2001. Regulation of the subcellular distribution and gene expression of GABA$_A$ receptor by microtubules and microfilaments in cultured brain neurons. J Cell Biochem 83:291–303.

Jones HB, Cavanaugh JB. 1984. The evolution of intracellular responses to acrylamide in rat spinal ganglion neurons. Neuropath Appl Neurobiol 10:101–121.

Lapadula DM, Bowe M, Carrington CD, Dulak L, Friedman M, Abou-Donia MB. 1989. In vitro binding of 14C-acrylamide to neurofilaments and microtubule proteins in rats. Brain Res 481:157–161.

Lin T-Y. 1999. The N-glycosylation inhibitors interfere the functional expression and subcellular distribution of GABA$_A$ receptor, and induce cell death in cultured neurons. Ph. D. Thesis, National Taiwan University.

Lin T-Y, Wang S-M, Yin H-S. 1998. Downregulation and subcellular redistribution of the γ-aminobutyric acid$_A$ receptor induced by tunicamycin in cultured brain neurons. J Cell Biochem 70:38–48.

Lin T-Y, Wang S-M, Fu W-M, Chen Y-H, Yin H-S. 1999. Toxicity of tunicamycin to cultured brain neurons: ultrastucture of the degenerating neurons. J Cell Biochem 74:638–647.

Lin WW, Friedman MA, Wang X-F, Abou-Donia MB. 2000. Acrylamide-regulated neurofilament expression in rat pheochromocytoma cells. Brain Res 852:297–304.

LoPachin RM. 2000. Redefining toxic distal axonopathies. Toxicol Letters 112-113:23–33.

LoPachin RM, Lehning EJ. 1994. Acrylamide-induced distal axon degeneration: a proposed mechanism of action. Neurotoxicology 15:247–260.

LoPachin RM, Lehning EJ. 1997. Mechanism of calcium entry during axon injury and degeneration. Toxicol Appl Pharmacol 143:233–244.

Macdonald RL, Olsen RW. 1994. GABA$_A$ receptor channels. Annu Rev Neurosci 17:569–602.

Miller MS, Spencer PS. 1985. The mechanisms of acrylamide axonophathy. Ann Rev Pharmacol Toxicol 25:643–666.

Miyasaka H, Okabe S, Ishiguro K, Uchida T, Hirokawa N. 1993. Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by tau protein kinase II. J Biol Chem 268:22695–22702.

Munson PJ, Rodbard D. 1980. Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239.

Rabow LE, Russek SJ, Farb DH. 1995. From ion currents to genomic analysis: recent advances in GABA$_A$ receptor research. Synapse 21:189–274.

Robinson PA, Anderton BH. 1988. Neurofilament probes—a review of neurofilament distribution and biology. Rev Neurosci 2:1–40.

Sager PR. 1989. Cytoskeletal effects of acrylamide and 2,5-hexaedione: selective aggregation of vimentin filaments. Toxicol Appl Pharmacol 97:141–155.

Schaumburg HH, Spencer PS. 1977. Ultrastructural studies of the dying-back process. IV. Differential vulnerability of PNS and CNS fibers in experimental central-peripheral distal axonopathies. J Neuropathol Exp Neurol 36:300–320.

Spencer PS, Schaumburg HH. 1974a. A review of acrylamide neurotoxicity. Part I. Properties, uses and human exposure. Can J Neurol Sci 1:143–152.

Spencer PS, Schaumburg HH. 1974b. A review of acrylamide neurotoxicity. Part II. Experimental animal neurotoxicity and pathologic mechanisms. Can J Neurol Sci 1:152–169.

Sterman AB. 1984. Acrylamide-induced remodeling of perikarya in rat superior cervical ganglia. Neuropathol Appl Neurobiol 10:221–234.

Stone JD, Peterson AP, Eyer J, Oblak TG, Sickles DW. 1999. Axonal neurofilaments are nonessential elements of toxicant-induced reductions in fast axonal transport: video-enhanced differential interference microscopy in peripheral nervous system axons. Toxicol Applied Pharmacol 161:50–58.

Takahashi A, Mizutani M, Agr B, Itakura C. 1994. Acrylamide-induced neurotoxicity in the central nervous system of Japanese quails. Comparative studies of normal and neurofilament-deficient quails. J Neuropathol Exp Neurol 53:276–283.

Takahashi A, Mizutani M, Itakura C. 1995. Acrylamide-induced peripheral neuropathy in normal and neurofilament-deficient Japanese quails. Acta neuropathol 89:17–22.

Tanii H, Hashimoto K. 1983. Neurotoxicity of arylamide and related compounds in rats: effects on rotorod performance, morphology of nerves and neurotubulin. Arch Toxicol 54:203–213.

Wang H, Olsen RW. 2000. Binding of the GABA$_A$ receptor associated protein (GABARAP) to microtubules and microfilaments suggests involvement of the cytoskeleton in GABARAP-GABA$_A$ receptor interaction. J Neurochem 75:644–655.

Wisden W, Laurie DJ, Monyer H, Seeburg PH. 1992. The distribution of 13 $GABA_A$ receptor subunit mRNA in the rat brain. I. Telecephalon, diencephalon, mesencephalon. J Neurosci 12:1040–1062.

Yang SH, Armson PF, Cha J, Phillips WD. 1997. Clustering of $GABA_A$ receptors by rapsyn/43 kDa protein in in vitro. Mol Cell Neurosci 8:430–438.

Yin HS, Fan SS. 1990. Monoclonal antibodies recognizing the benzodiazepine receptor of chick embryo brain. J Recept Res 10:249–267.

Yin HS, Yang MF. 1992. Effect of monensin on the neuronal ultrastructure and endocytic pathway of macromolecules in cultured brain neurons. Cell Mol Neurobiol 12:297–307.