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Long-Term Exposure to Dieldrin Reduces γ-Aminobutyric Acid Type A and N-Methyl-D-Aspartate Receptor Function in Primary Cultures of Mouse Cerebellar Granule Cells

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The organochlorine pesticide dieldrin is a persistent organic pollutant that accumulates in the fatty tissue of living organisms. In mammals, it antagonizes the $GABA_A$ receptor, producing convulsions after acute exposure. Although accumulation in human brain has been reported, little is known about the effects of long-term exposure to dieldrin in the nervous system. Homeostatic control of the balance between excitation and inhibition has been reported when neuronal activity is chronically altered. We hypothesized that noncytotoxic concentrations of dieldrin could decrease glutamatergic neurotransmission as a consequence of a prolonged reduction in $GABA_A$ receptor function. Long-term exposure of primary cerebellar granule cell cultures to 3 μM dieldrin reduced the $GABA_A$ receptor function to 55% of control, as measured by the GABA-induced $^{36}Cl^-$ uptake. This exposure produced a significant reduction (~35%) of the NMDA-induced increase in $[Ca^{2+}]_i$ and of the [^{3}H]MK-801 binding, which was not accompanied by a reduction in the NMDA receptor subunit NR1, as determined by Western blot. Consistent with the decreased NMDA receptor function, dieldrin-treated cultures were insensitive to an excitotoxic stimulus induced by exposure to high potassium. In summary, we report that the chronic reduction of $GABA_A$ receptor function induced by dieldrin decreases the number of functional NMDA receptors, which may be attributable to a mechanism of synaptic scaling. These effects could underlie neural mechanisms involved in cognitive impairment produced by low-level exposure to dieldrin.

Key words: neurotoxicity; persistent organic pollutant; in vitro

Dieldrin is an organochlorine pesticide that was widely used in agriculture between the 1940s and the 1960s, especially as a soil insecticide. It is also a degradation product of the pesticide aldrin. Although the use of both pesticides was banned in most of the developed countries during the 1970s and the 1980s, humans continue to be exposed to dieldrin because it is highly persistent in the environment and bioaccumulates in the food chain. Dieldrin is now classified as a persistent organic pollutant (POP) by the Stockholm Convention (http://www.pops.int). A recent study reports high levels of dieldrin in farmed salmon, especially in Europe (Hites et al., 2004). Furthermore, there is evidence that daily doses of dieldrin in the diet may exceed the U.S. Environmental Protection Agency (EPA) reference dose for children (Schafer and Kegley, 2002). Other recent studies have reported the presence of dieldrin in human samples of serum, adipose tissue, and milk from all parts of the world (Solomon and Weiss, 2002; Botella et al., 2004).

Acute intoxication with dieldrin induces convulsions in mammals (Bloomquist, 1992). The hyperexcitatory effects of dieldrin can be explained by the antagonism that it exerts on the $GABA_A$ receptor. Several studies have shown that dieldrin inhibits the binding of t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) to the picrotoxinin recognition site and inhibits the GABA-induced Cl^- flux through the $GABA_A$ receptor, without modifying the benzodiazepine or GABA recognition sites (Pomés et al., 1993, 1994; Ikeda et al., 1998; Vale et al., 2003).

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Although dieldrin accumulates in fatty tissues, including the brain, little is known about the effects of prolonged exposure on the central nervous system in animals or humans. Some studies indicate that it could be a risk factor for Parkinson's disease (Corrigan et al., 1998, 2000; Gorell et al., 1998). However, a recent review of epidemiologic and animal studies concludes that there is insufficient evidence to support a causal association (Li et al., 2005). Nevertheless, dieldrin impairs mitochondrial and proteasomal function and induces oxidative stress and cell death in dopaminergic cells in culture (Liu et al., 1997; Sánchez-Ramos et al., 1998; Kitazawa et al., 2001, 2003; Sun et al., 2005).

In addition to these in vitro studies, it has been reported that repeated prenatal exposure to dieldrin reduces both [^{35}S]TBPS binding and the expression of several $GABA_A$ receptor subunit mRNAs in rat (Brannen et al., 1998; Liu et al., 1998). Furthermore, medium-term studies conducted in animals show that dieldrin exposure impairs learning or operant behavior (Burt, 1975; Smith et al., 1976; as cited by Schantz and Widholm, 2001; see also Toxicological profile for aldrin/dieldrin, US ATSDR, 2002, at www.atsdr.cdc.gov), thus suggesting altered neurotransmission in exposed animals. Learning involves excitatory glutamate neurotransmission (Bliss and Collingridge, 1993). Homeostatic control of the balance between excitation and inhibition has been reported when neuronal activity is chronically altered. In other words, glutamatergic and inhibitory GABAergic synapses are scaled up or down in response to long-lasting changes in activity (for review see Pérez-Otaño and Ehlers, 2005).

In this study, we examine the effects of prolonged exposure to noncytotoxic concentrations of dieldrin on the glutamatergic system. We used cerebellar granule cell cultures; a nearly homogenous population of glutamatergic neurons that express functional glutamate and $GABA_A$ receptors and that contain a small proportion of GABAergic neurons (Sonnewald et al., 2004; Babot et al., 2005). The acute effects of dieldrin, and other organochlorine pesticides, on $GABA_A$ receptors and cell viability have been tested in these cell cultures (Pomés et al., 1993; Rosa et al., 1997; Vale et al., 2003). Furthermore, primary cultures of cerebellar granule cells constitute a well-characterized model that is extensively used in neurotoxicological and neuropharmacological studies involving glutamate neurotransmission, neurodegeneration, and neuroprotection mechanisms.

MATERIALS AND METHODS

Materials

Seven-day-old NMRI mice were purchased from Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic multiwell plates were from Costar (Corning Science Products, Acton, MA). Fetal calf serum was from Gibco (Invitrogen, Barcelona, Spain) and culture medium from Biochrom KG (Berlin, Germany). Dieldrin was from LGC (Teddington Middlesex, United Kingdom). $^{36}Cl^-$ (16 mCi/g) was from American Radiolabeled Chemicals (St. Louis, MO) and [^{3}H]MK-801 (22 Ci/mmol) from NEN-Perkin Elmer Life Science, Inc. Optiphase "Hisafe" 2 liquid scintillation cocktail was from Wallac Oy (Turku, Finland). Trypsin, soybean trypsin inhibitor, L-glutamic acid, NMDA, MK-801, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and o-pthaldialdehyde reagent were from Sigma (St. Louis, MO). Fluo-3 AM, anti-goat horseradish peroxidase conjugated, and Alexa 488 secondary antibodies were from Molecular Probes (Leiden, The Netherlands). Goat polyclonal anti-NMDAR1 was from Santa Cruz Biotechnology (Santa Cruz, CA). The 8-isoprostane EIA kit was from Cayman Chemical (Ann Arbor, MI). All other chemicals were of the purest grade available from regular commercial sources.

Neuronal Cultures

Primary cultures of cerebellar granule cells were obtained from cerebella of 7-day-old mice according to the method described by Schousboe et al. (1989). In brief, cells were dissociated by mild trypsinization at 37°C, followed by trituration in a DNAse solution (0.004% w/v) containing soybean trypsin inhibitor (0.05% w/v). The cells were suspended in Dulbecco's modified Eagle medium (DMEM) containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine, supplemented with p-aminobenzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension (1.6×10^6 cells/ml) was seeded in 24-well multiwell plates precoated with poly-L-lysine and incubated for a minimum of 8 days in a humidified 5% CO_2/95% air atmosphere at 36.8°C. A mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added after 48 hr in culture to prevent glial proliferation (<5%; Peng et al., 1991).

Animals were handled in compliance with protocol DMA1852 of the University of Barcelona, approved by the Generalitat de Catalunya, Spain, following the EU guidelines. The University of Barcelona statement of compliance (A5224-01) is held on file by the Office of Laboratory Animal Welfare (OLAW)/U.S. National Institutes of Health (NIH).

Dieldrin Treatment

A stock solution of dieldrin was prepared in dimethyl sulfoxide (DMSO) and used immediately. The final DMSO concentration in the culture medium was 0.2%. Control cells were treated with the same amount of DMSO. To avoid cross-contamination between different wells of the same plate, the different treatments were performed in separate plates. The chemical stability of dieldrin in culture medium at 37°C was analyzed. Two aliquots of culture medium containing 6 μM dieldrin were incubated at 37°C for 1 and 8 days. At the end of the incubation, dieldrin was extracted by adding an equal volume of n-hexane, vortexing, and frozing at −20°C. Aqueous and organic phases were separated by centrifugation, and dieldrin concentration in the n-hexane was determined by gas chromatography-electronic impact mass spectrometry. The concentration of dieldrin in the culture medium did not decay after 8 days at 37°C (dieldrin concentration was 5.70 μM and 5.75 μM, at 1 and 8 days, respectively).

Two different types of dieldrin treatment were performed. The treatment referred to as "long-term" treatment consisted of exposing the cultures to dieldrin for at least

6 days in vitro (DIV). Cultures were treated at 2 DIV by adding the stock dieldrin solution in DMSO to the culture medium. The medium was not changed until the experiments were performed at 8–9 DIV (exposure for 6–7 days). Some experiments were performed at 12 DIV (exposure for 10 days), and the results were pooled with the previous ones because they were not different. On the other hand, in the "acute" treatment, cultures grown for 8–9 DIV without dieldrin were preincubated for 10 min with dieldrin, and the assays were also performed in the presence of dieldrin in the incubation solution.

$GABA_A$ Receptor Function

Cl^- flux through the $GABA_A$ receptor was determined by the $^{36}Cl^-$ uptake assay in intact cultured cells as described by Vale et al. (2003) and García et al. (2006), with minor modifications. Briefly, culture medium was replaced by a prewarmed Earle's balanced salt solution (EBSS: 116 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 15.2 mM $NaHCO_3$, and 5.5 mM glucose, adjusted to pH 7.4), and cell cultures were incubated in a humidified 5% CO_2/95% air atmosphere at 36.8°C. After 30 min of incubation, buffer was replaced by new EBSS solution, with incubation for an additional 15 min. Cells were then rinsed with new EBSS solution and incubated for additional 10 min at room temperature (22–24°C). Cultures were then incubated for 7 sec at room temperature with an HEPES-buffered saline solution (HBSS; 136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, 9 mM glucose adjusted to pH 7.4) containing $^{36}Cl^-$ (0.7 μCi/ml) and different GABA concentrations. At the end of the exposure, cells were rinsed and disaggregated with 0.2 M NaOH. Aliquots were used to determine radioactivity and protein content. Radioactivity was determined by liquid scintillation counting (with Optiphase "Hisafe" 2 cocktail) and proteins were determined by a microtest using the Bradford method, with bovine serum albumin as a standard. Net $^{36}Cl^-$ uptake was determined by subtracting basal $^{36}Cl^-$ uptake in the absence of GABA.

NMDA Receptor Function

NMDA receptor function was determined by measuring the NMDA-induced increase in Fluo-3 fluorescence, as an indicator of NMDA receptor-mediated Ca^{2+} influx. Cultured cells were incubated with Fluo-3 AM (9 μM) for 1 hr at 36.8°C in HBSS. Excess Fluo-3 AM was rinsed away, and cells were treated with different NMDA concentrations in a magnesium-free HBSS. Fluorescence (F) was immediately determined in a fluorimetric plate reader (Ex 485/Em 530; SpectraMax GeminisXS; Molecular Devices, Sunnyvale, CA). To allow calculations of $[Ca^{2+}]_i$, cells were incubated with the ionophore A23187 (10 μM) and thereafter with $CuSO_4$ (5 mM) to obtain the maximum (F_{max}) and the minimum (F_{min}) fluorescent values, respectively. $[Ca^{2+}]_i$ was calculated for each well as: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of Fluo-3/Ca^{2+} (320 nM; Reynolds, 1998).

[^{3}H]MK-801 Binding

The binding assay was performed as described by Marcaida et al. (1995), with modifications. In brief, after the cultures were washed with phosphate-buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), they were exposed for 15 min at 37°C to 4–5 nM [^{3}H]MK-801 in PBS solution containing 100 μM glutamate, 100 μM glycine, and different concentrations of nonlabeled MK-801. After washing in cold PBS containing 100 μM glutamate and 100 μM glycine, cells were disaggregated with NaOH. The disaggregate was used to measure the radioactivity and protein content as before. Apparent B_{max} and K_d were calculated by the adjustment of the binding values to a competitive binding curve as described by DeBlasi et al. (1989). Nonspecific binding was determined in the presence of 100 μM of nonlabeled MK-801 and was <20% of total binding.

Western Blot Analysis

After the cultures had been washed three times in cold PBS, the cells of one 24-well plate were harvested with 0.9 ml of loading buffer [75 mM Tris HCl, pH 6.8, 15% glycerol, 3% sodium dodecyl sulfate (SDS), 150 mM dithiothreitol, 0.02% bromophenol blue] and sonicated briefly. After boiling for 3 min, 50 μl of the homogenate was subjected to SDS-PAGE using 7% polyacrylamide resolving gel for 1.5 hr at 100 V. Proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and incubated for 2 hr in blocking solution containing 5% nonfat dry milk in TBS-T (20 mM Tris HCl, pH 7.6, 140 mM NaCl, 0.1% Tween-20). Membranes were then incubated overnight with a goat polyclonal anti-NMDAR1 (1:1,000; Santa Cruz Biotechnology) in TBS-T solution containing 5% nonfat dry milk. After the membranes were washed, they were incubated for 1 hr at room temperature with an anti-goat horseradish peroxidase-conjugated (HRP) secondary antibody (1:2,000; Molecular Probes). In all membranes a monoclonal anti-actin antibody (1:2,000; Sigma) was used as a control for the amount of protein loaded. Antiactin antibody was incubated overnight at 4°C in TBS-T solution containing 5% BSA. The secondary antibody (HRP-linked anti-mouse, 1:2,000; Cell Signaling Technology Inc, Beverly, MA) was incubated for 1 hr at room temperature in TBS-T.

Membranes were washed and incubated in a substrate solution (Immun-Star HRP Chemiluninescent Kit; Bio-Rad, Hercules, CA) for 4 min. Luminescence was quantified with a Versadoc Imaging System (Bio-Rad). Digital images were then quantified in the Quantity One software (Bio-Rad).

Immunocytochemistry

Cells were seeded in Permanox chamber slides treated with poly-L-lysine, and the immunostaining was performed as described by Crump et al. (2001), with minor modifications. Cultures were rinsed with PBS and fixed with methanol at −20°C for 10 min ,followed by two rinses with 0.02% Triton X-100 in PBS and one with PBS. Cultures were blocked in 5% BSA in PBS at room temperature. After this, cultures were incubated overnight at 4°C with the primary anti-NMDAR1 antibody (1:50) in a solution containing 3% BSA

in PBS, rinsed with PBS, and incubated for 1 hr at room temperature with the secondary antibody rabbit anti-goat Alexa 488 (1:1,000). After rinsing with PBS, the slides were coverslipped with Mowiol. The cells were examined in a confocal fluorescence microscope (Leica Microsystems) using the same excitation laser intensity for control and dieldrin-treated cultures.

Intracellular Amino Acid Analysis

Cultures were rinsed three times with HBSS, and the cells were then scraped with 0.5 ml/well of 0.25 M perchloric acid. The cellular disaggregate was sonicated for 30 min in a water-ice-filled ultrasonic bath and centrifuged at 16,100*g* for 10 min at 4°C. The supernatants were stored at −20°C prior to analysis. The pellet obtained was resuspended in 0.2 M NaOH, and the protein concentration was measured using the Bradford method. Glutamate, aspartate, and GABA content in control and treated cells were determined by HPLC-fluorimetric analysis (Waters, Milford, MA; and Applied Biosystems, Foster City, CA). Samples were neutralized with NaOH and derivatized with o-pthaldialdehyde reagent prior to reverse-phase separation on a C18 column (Tracer Nucleosil C18 5-μm particle size, 10 × 0.4 cm; Teknokroma, Spain) and fluorescence detection at 360/450 nm (Babot et al., 2005). For glutamate detection, a mobile phase of 0.1 M sodium acetate, 5.5 mM triethylamine (pH 5.5) containing 10–70% acetonitrile was used at a flow rate of 0.8 ml/min. For GABA detection, the mobile phase was 0.1 M sodium acetate, 5.5 mM triethylamine (pH 3.15) containing 28.6% acetonitrile at a flow rate of 0.8 ml/min. Neurotransmitter content was calculated using an external standard method with glutamate and GABA as standards.

Cell Viability and Lipoperoxidation

Cell viability was determined by measuring the reduction of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a colored formazan salt by mitochondrial reducing activity, as described previously (Babot et al., 2005). Briefly, cultures were rinsed and incubated for 15 min with a solution of MTT (250 μg/ml) dissolved in HBSS at 37°C. After washing off excess MTT, the cells were disaggregated with 5% SDS and the colored formazan salt was measured at 560 nm in a spectrophotometer plate reader (iEMS Reader MF; Labsystems, Helsinki, Finland).

Lipoperoxidation was analyzed by measuring the concentration of 8-isoprostane in the culture media with an ELISA-based kit (Cayman Chemicals). The samples were collected with 0.005% 2,6-di-tert-butyl-methylphenol and were stored at −80°C until the analysis was performed using the indications given by the manufacturer.

High Potassium Exposure

Cell cultures were incubated for 5 min in HBSS containing 100 mM of K^+ (41.4 mM NaCl, 100 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, 9 mM glucose, and 5 μM glycine, adjusted to pH 7.4) prewarmed to 37°C (0.5 ml/well). At the end of the exposure, the HBSS from each well was replaced with the DMEM conditioned medium previously pooled from the same cultured cells. The cultures were kept in the incubator for 24 hr before measuring cell viability.

TABLE I. Effects of Long-Term Exposure to Dieldrin on $GABA_A$ Receptor-Mediated $^{36}Cl^-$ Uptake†

	EC_{50} (μM)	E_{max} (%)
Control	12.3 ± 3.6	104 ± 4
0.3 μM dieldrin	12.1 ± 7.5	77 ± 15
3 μM dieldrin	8.9 ± 0.8	55 ± 3*

†Net $^{36}Cl^-$ uptake values were normalized to the maximum response induced by 100 μM GABA in control cultures (Vale et al., 2003; Garcia et al., 2006). EC_{50} and E_{max} values are the mean ± SEM of three independent experiments, each performed in quadriplicate, after adjusting them to a sigmoidal curve.
*$P < 0.05$, Dunnett's test after significant ANOVA.

Data Analysis

Data are shown as mean ± SEM. Unless otherwise stated, at least three experiments were performed in triplicate. Statistical comparisons were made by *t*-test and one-way and two-way analysis of variance, followed by post-hoc Dunnett's or Bonferroni's tests in GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Concentration-response data were fitted to a sigmoidal curve in the GraphPad software.

RESULTS

Long-Term Exposure to Dieldrin Reduces GABA-Induced Chloride Influx Without Producing Cytotoxicity

It has been previously reported that dieldrin inhibits both [^{35}S]TBPS binding and GABA-induced chloride influx (Pomés et al., 1993, 1994; Ikeda et al., 1998; Vale et al., 2003). It should be noted that studies of acute $GABA_A$ receptor inhibition are performed in the absence of serum and that binding to serum proteins and partitioning into lipids are major determinants of the potency of chemicals in vitro as well as in vivo. In this sense, Gulden et al. (2006) have recently shown that the cytotoxic potency of dieldrin is dependent on the serum concentration and composition. Therefore, we tested whether long-term exposure to dieldrin in culture medium containing 10% serum reduced GABA-induced Cl^- influx. Table I and Figure 1 show that long-term exposure to dieldrin reduced GABA-induced $^{36}Cl^-$ influx in a concentration-dependent manner, this effect being statistically significant at a nominal concentration of 3 μM dieldrin. The maximum GABA-induced $^{36}Cl^-$ influx was significantly reduced to 55% of control without changing the EC_{50} value, in agreement with the noncompetitive action of dieldrin on the $GABA_A$ receptor (Nagata and Narahashi, 1994).

Prolonged exposure of the cultures to 3 μM dieldrin did not produce a reduction in cell viability (MTT value was 104 ± 5.0% of control) or lipoperoxidation

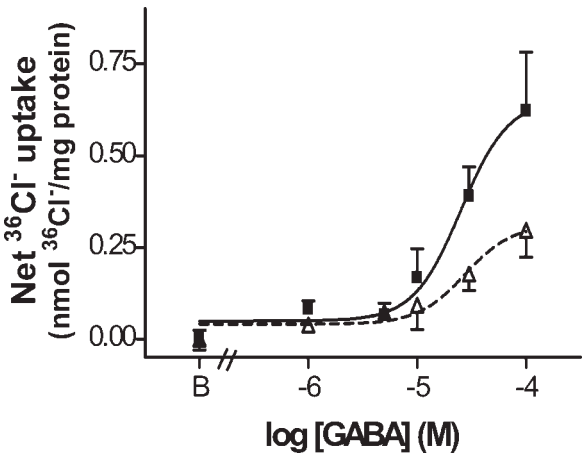

Fig. 1. Net $^{36}Cl^-$ uptake induced by GABA in control cultures (squares) and in cultures long-term exposed to 3 μM dieldrin (triangles). Each point represents mean ± SEM (n = 4) of a representative experiment. B indicates 0 μM GABA.

(concentrations of 8-isoprostane were 84.5 ± 4.0 and 76.9 ± 12.3 pg/ml in control and treated cultures, respectively, n = 2). Therefore, we decided to use a dieldrin concentration of 3 μM to reduce chronically the GABA-induced chloride influx in cerebellar granule cell cultures.

Prolonged exposure to 3 μM dieldrin had no effect on the protein content of the cultures (68.2 ± 6.9 and 66.6 ± 2.1 μg protein/well in a representative control plate and dieldrin plate, respectively) and on the concentration of glutamate or GABA (Table II).

Prolonged Exposure to Dieldrin Produces Changes in the NMDA Receptor

Recent studies indicate that chronically elevated activity, induced by exposure of the cells to bicuculline or picrotoxinin, reduces the number of synaptic NMDA receptors and the amplitude of NMDA receptor-mediated currents (Watt et al., 2000; Crump et al., 2001; Mu et al., 2003). Therefore, we assessed whether the prolonged exposure to 3 μM dieldrin could have altered NMDA receptors. First, we studied the activity of the NMDA receptors by means of changes in intracellular calcium concentration in response to different NMDA concentrations. Figure 2A and Table III show that, after long-term exposure to 3 μM dieldrin, the maximum response induced by NMDA was significantly lower than in control cultures. We discarded the idea that this could be due to a direct action of dieldrin on NMDA receptors, because short exposure to dieldrin did not modify the NMDA-induced increase in $[Ca^{2+}]_i$ (Fig. 2B, Table III). Second, we assessed whether the decreased response of the cultured cells to NMDA could be due to a reduction in the binding of [^{3}H]MK-801 in cerebellar granule cell cultures. Long-term treatment with 3 μM dieldrin significantly decreased the B_{max} for [^{3}H]MK-801 binding by 36% but did not affect the apparent K_d (Table IV). Moreover, we wondered whether the decreased response of the cells to NMDA and the decreased binding of [^{3}H]MK-801 in long-term treated cultures could be due to a reduction of the NMDA receptor protein. Consequently, an immunoblot analysis for the obligatory NR1 subunit of the NMDA receptor was performed. The quantitative determination of the quotient NR1/actin was not different between control and dieldrin-treated cells (Fig. 3). To determine whether the reduced NMDA receptor binding and functionality in dieldrin-treated cultures could be related to different cell receptor localization, we examined NR1 immunocytochemistry. NR1 immunostaining was located mainly at the cell membrane in control cells, whereas a diffuse distribution concentrated mainly in the cytoplasm was observed in dieldrin-treated cultures (Fig. 4).

TABLE II. Effects of Long-Term Exposure to 3 μM Dieldrin on Amino Acid Concentrations (nmol/mg protein) in Cerebellar Granule Cell Cultures*

	Control	Dieldrin 3 μM
Glutamate	85.6 ± 17.0	103.4 ± 15.1
GABA	27.1 ± 0.4	26.1 ± 1.6

*Values represent the mean ± SEM of three experiments performed in quadruplicate.

Long-Term Exposure to Dieldrin Prevents the Excitotoxic Effects Induced by High Potassium Concentration

Primary cultures of cerebellar granule cells release glutamate when briefly exposed to 100 mM K^+. Under these conditions, released glutamate causes excitotoxicity, which can be prevented by using either NMDA or $GABA_A$ receptor antagonists (Babot et al., 2005). No excitotoxicity was observed in long-term dieldrin-treated cultured cells after exposure to 100 mM K^+ for 5 min (Fig. 5). Because dieldrin is a highly lipophilic compound, some dieldrin may remain bound to the cell surface after removing the culture medium (Gulden et al., 2002). Therefore, we rinsed the cells with HBSS containing 2% BSA to wash out dieldrin completely (Llorens et al., 1990) before exposing the cultures to the excitotoxic stimulus. Under these conditions, nonexposed cultures responded to 100 mM K^+ by significantly reducing MTT values to 63% ± 4% vs. control values ($P < 0.001$), whereas MTT values in dieldrin-treated cultures were not reduced after exposure to 100 mM K^+ (110% ± 4%). This indicates that dieldrin had produced long-lasting changes in the cultures over the long-term exposure.

DISCUSSION

In this work, we found that long-term exposure to noncytotoxic concentrations of dieldrin produced a decrease of functional NMDA receptors in cerebellar

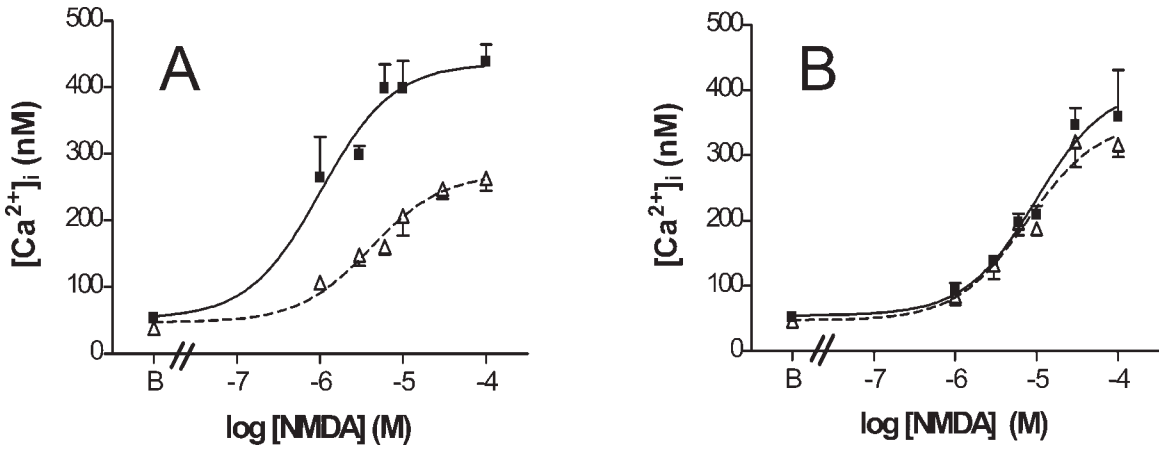

Fig. 2. Concentration-response curves for NMDA-induced changes in intracellular calcium in cerebellar granule cell cultures. **A:** Control cultures (squares) and cultures exposed long term to 3 μM dieldrin (triangles). **B:** Control cultures (squares) and cultures acutely exposed to 3 μM dieldrin (triangles). Values represent the mean ± SEM (n = 3) of a representative experiment. B indicates basal $[Ca^{2+}]_i$ in HBSS.

TABLE III. Effects of Dieldrin on NMDA Receptor-Induced Increase in $[Ca^{2+}]_i$[†]

	EC_{50} (μM)	E_{max} (%)
Long-term exposure		
Control	4.5 ± 2.0	108 ± 7
3 μM dieldrin	6.6 ± 1.6	67 ± 5★
Acute exposure		
Control	12.4 ± 4.9	110 ± 4
3 μM dieldrin	13.6 ± 3.0	110 ± 24

[†]The values of intracellular calcium concentration for each individual experiment were normalized to the maximum response induced by 100 μM NMDA (Babot et al., 2005) in control cultures. EC_{50} and E_{max} values are the mean ± SEM of three independent experiments, each performed in triplicate, after adjusting each experiment to a sigmoidal curve.
★Statistical significance between control and dieldrin-treated cultures was determined with a paired *t*-test ($P < 0.05$).

TABLE IV. Apparent K_d and B_{max} Values for the [^{3}H]MK-801 Binding in Intact Cerebellar Granule Cell Cultures[†]

	Control	Dieldrin
K_d (μM)	1.9 ± 0.4	1.0 ± 0.1
B_{max} (pmol/mg prot)	138.5 ± 17.5	88.7 ± 18.3★

[†]Values represent the mean ± SEM of five independent experiments each preformed in triplicate. The values were obtained by adjusting data of each experiment to a one site competition curve as explained by DeBlasi et al. (1989).
★Statistical significance between control and dieldrin-treated cultures was determined with a paired *t*-test ($P < 0.05$).

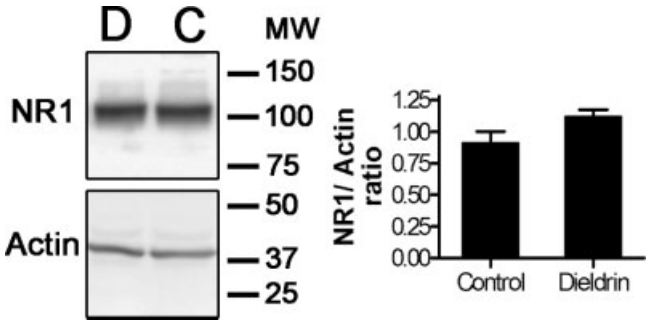

Fig. 3. **Left:** Representative immunoblot for the NMDA receptor NR1 subunit of control cultures (C) and long-term dieldrin-treated cultures (D). The corresponding immunoblot for actin is shown at bottom. Molecular weights are represented at right. **Right:** Densitometric analysis of the immunoblots. Data are mean ± SEM of four independent experiments.

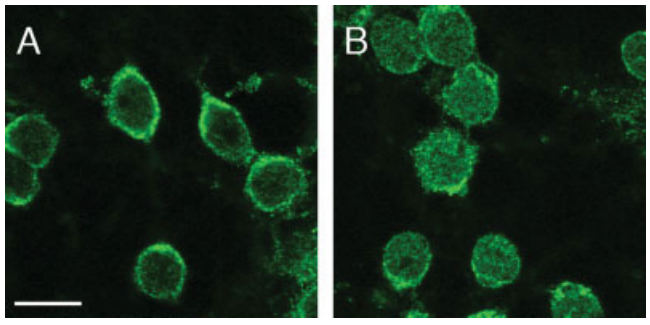

Fig. 4. Confocal micrograph of the immunostaining for the NMDA receptor NR1 subunit of a representative culture of control cells (**A**) and long-term dieldrin-treated cells (**B**). Scale bar = 10 μm.

granule cell cultures that can be accounted for by an adaptative response to the reduction of $GABA_A$ receptor function produced by dieldrin. The chronic reduction of the $GABA_A$ receptor function was afforded by the use of a nominal concentration of 3 μM dieldrin during the long-term exposure of the culture. Dieldrin binds to serum proteins and lipids, and the free concentration of dieldrin would be reduced by this effect (Gulden et al., 2002, 2006). For this reason, although the potency of dieldrin to inhibit the GABA-induced chloride influx in cerebellar granule cells was previously reported (IC_{50} = 0.2 μM; Vale et al. 2003), we had to test which concentration of

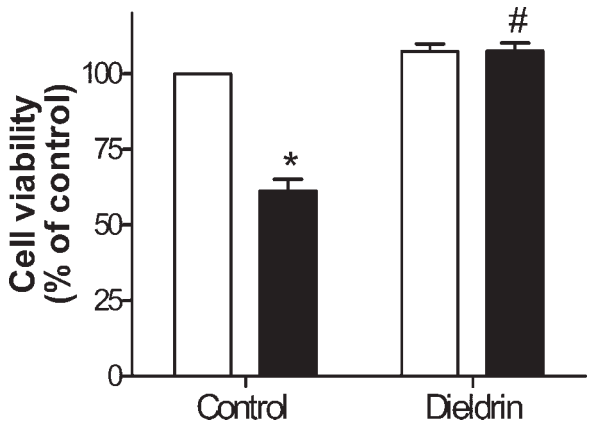

Fig. 5. Cell viability of control and long-term 3 μM dieldrin-treated cultures after 5 min of exposure to 25 mM K^+ (open bars) or 100 mM K^+ (solid bars). Viability was determined with the MTT method and is represented as the percentage over the control cultures after exposure to 25 mM K^+. Data are mean ± SEM of four independent experiments. Statistical significance was determined using two-way ANOVA with Bonferroni's post-hoc test. $^{\star}P < 0.001$ vs. the same culture treatment condition with regard to K^+ exposure; $^{\#}P < 0.001$ vs. the same K^+ concentration in control cultures.

dieldrin would significantly reduce $GABA_A$ receptor function in the continuous presence of 10% fetal bovine serum. In our hands, long-term exposure to 3 μM dieldrin produced an inhibitory effect that was similar to that produced by acute exposure to 0.3 μM dieldrin. This long-term exposure to 3 μM dieldrin did not reduce mitochondrial activity (MTT assay) nor decrease the content of glutamate and GABA, indicating that the treatment did not produce cell toxicity. Amino acid concentrations agree with those previously reported for these cell cultures (Sonnewald et al., 2004).

Long-term exposure to 3 μM dieldrin resulted in a significant reduction of the NMDA receptor function, as measured by NMDA-induced increase in $[Ca^{2+}]_i$. The reduction in the maximum response elicited by NMDA in long-term treated cultures could be accounted for by a reduced amount of effective NMDA receptors in the cell membrane; consequently, we measured the binding of [^{3}H]MK-801 in intact cultured cells, thus determining externalized receptors. A significant reduction of the B_{max} of 36% was found in long-term dieldrin-treated cultures, whereas the apparent K_d was not modified. The apparent K_d value obtained in this work was roughly similar to that previously reported in intact cultured cerebellar granule cells (~0.4 μM; Marcaida et al., 1995) but different from that reported in membranes from cultured cerebellar granule cells (~2 nM; Cebers et al., 1999). It has been reported that inhibition of NMDA function by MK-801 in cerebellar granule cells results from two binding sites for MK-801 with inhibitory IC_{50} values of 5 nM and 3 μM (Perrier and Benavides, 1995). These IC_{50} values would correspond to the different K_d values reported for the [^{3}H]MK-801 binding in cerebellar granule cell cultures (this work; Marcaida et al., 1995; Cebers et al., 1999).

The effects of the long-term treatment with dieldrin are consistent with those obtained using the $GABA_A$ receptor antagonists picrotoxinin and bicuculline to increase the activity in cell cultures chronically. In this sense, both the amplitude of the NMDA receptor-mediated currents and the clustering of the NMDA receptors at the synapse are reduced in neuronal cultures long-term exposed to bicuculline or picrotoxinin, whereas chronically reduced activity induced by tetrodotoxin or NMDA antagonist produces the opposite effect (Rao and Craig, 1997; Watt et al., 2000; Crump et al., 2001). However, the total amount of NR1 protein determined by Western blotting was equivalent in cultures with increased/reduced activity and in control cultures (Rao and Craig, 1997; Watt et al., 2000; Crump et al., 2001). Other authors have also found a reduced binding of [^{3}H]MK801 without changes in the amount of NR1 subunit in hippocampal synaptosomal membranes from rats with chronic hyperammonemia (Marcaida et al., 1995). These findings are in agreement with our results that show that, in spite of the reduction observed in NMDA receptor function and [^{3}H]MK-801 binding in long-term dieldrin-treated cells, the Western blot for the obligatory NMDA subunit NR1 showed no differences between control and dieldrin-treated cells, indicating that the total amount of NMDA receptor protein is probably the same in both cases. Recently, Mu et al. (2003) reported that, upon increased activity, the C2 splice cassette of the NMDA-NR1 subunit predominates over the C2′. Since the C2 domain decreases the rate of export of the NR1 subunit from the endoplasmic reticulum, the synaptic accumulation of NMDA receptors is decreased. This mechanism of action would explain why, after the long-term treatment of cultured cerebellar granule cells with dieldrin, we observed a reduced activity of NMDA receptors and a decreased binding of [^{3}H]MK801 without observing a decrease in the overall quantity of NR1 protein, because we used an antibody that recognizes both splice variants. The reduced NR1 immunostaining at the cell surface shown in Figure 4B would support this hypothesis. Furthermore, the immunocytochemistry of the NR1 NMDA receptor subunit revealed an increased immunostaining in the cytoplasm of dieldrin-treated cells, which might account for the small, although not statistically significant, increase of NR1 expression shown in the Western blot analysis. Whether the reduced NMDA receptor binding sites and activity induced by dieldrin treatment could result in an overall increased expression of the receptor deserves further investigation.

Cultured cerebellar granule cells undergo an excitotoxic process when exposed to 100 mM K^+ for 5 min. It has been described that protection of this effect is afforded either by reducing calcium influx through NMDA receptors or chloride influx through $GABA_A$ receptors and niflumic acid-sensitive channels (Babot et al., 2005). In this work, primary cultures of cerebellar granule cells that were exposed long term to 3 μM dieldrin did not undergo an excitotoxic process, indicating

that the reduction in functional NMDA receptors observed after dieldrin exposure has functional consequences. The possibility that the protection afforded by long-term exposure to dieldrin in response to high K^+ was due to the reduced chloride influx through the $GABA_A$ receptor can be discarded, because a similar magnitude of the $GABA_A$ receptor inhibition induced by acute exposure to 0.3 μM dieldrin (~55% inhibition; Vale et al., 2003) did not protect cells from excitotoxicity (MTT values were 79% of control; unpublished results). Therefore, the absence of an excitotoxic response to high K^+ exposure observed in long-term dieldrin-treated cultures must be accounted for by the reduction of NMDA receptor functionality. Other studies have shown that the reduction of functional NMDA receptors in the cell membrane can result in protection from excitotoxicity. In this sense, it has been reported that down-regulation of NMDA receptors makes cultured cerebellar granule cells insensitive to excitotoxicity (Cebers et al., 1999). Moreover, Fong et al. (2002) have reported that dispersal of NMDA receptors from synaptic to extrasynaptic sites reduced the excitotoxicity induced by high potassium exposure in hippocampal cultures. In contrast, an increased sensitivity to an excitotoxic insult induced by high potassium exposure has been reported in cultures chronically treated with NMDA receptor antagonists (Crump et al., 2001).

NMDA receptors have a defined role in neuronal differentiation, memory formation, and learning (Bliss and Collingridge, 1993). Some studies have shown a relation between a decreased surface expression of NMDA receptor subunits and impairment of learning and memory (Cheli et al., 2006; Son et al., 2006). Therefore, the long-term effects of dieldrin observed in this work, although affording protection against an excitotoxic lesion, might also be deleterious for cognitive and high neural functions. This mechanism might explain why in animals exposed to dieldrin there is an impairment of learning or operant behavior (Burt, 1975; Smith et al., 1976; as cited by Schantz and Widholm, 2001). Although these functions do not directly involve the cerebellum, it is reasonable to assume that the dieldrin effects reported in this work may occur in other types of neurons, as synaptic scaling has also been reported to occur in cortical and hippocampal cultures (Rao and Craig, 1997; Watt et al., 2000; Crump et al., 2001). It should be taken into account that dieldrin accumulates in the brain. Dieldrin concentrations found in samples of human brain regions of control and neurological patients are 0.3–0.9 μg/g lipid (Corrigan et al., 1998, 2000), which would correspond to a concentration of 0.02–0.06 μM. The concentration of dieldrin used in this work (nominal 3 μM) to produce a decrease in NMDA receptor function is lower than concentrations described to produce cellular alterations related to Parkinson's disease (30–300 μM; Kitazawa et al., 2001, 2003; Sun et al., 2005). Therefore, the results of our study emphasize the importance of the $GABA_A$ receptor as a preferential target of dieldrin effects, not only under acute exposure but also after long-term exposure.

In conclusion, we have found that long-term exposure to noncytotoxic concentrations of dieldrin alters glutamatergic neurotransmission as a consequence of prolonged reduction in $GABA_A$ receptor function. This decreased functionality of the NMDA receptor might underlie mechanisms of chronic dieldrin neurotoxicity, manifested as learning and behavioral deficits in experimental animals.

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