Reduction of Glutamatergic Neurotransmission by Prolonged Exposure to Dieldrin Involves NMDA Receptor Internalization and Metabotropic Glutamate Receptor 5 Downregulation

Victor Briz,*† Mireia Galofré,*† and Cristina Suñol*†‡

*Department of Neurochemistry and Neuropharmacology, Institut d’Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, CSIC–IDIBAPS, Rosselló 161, E-08036 Barcelona, Spain; and ‡CIBER Epidemiología y Salud Pública (CIBERESP), Spain

1 To whom correspondence should be addressed at Department of Neurochemistry and Neuropharmacology, Institut d’Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, CSIC–IDIBAPS, Rosselló 161, Barcelona E-08036, Spain. Fax: +34 93 363 83 01. E-mail: csenqi@iibb.csic.es.

Received August 4, 2009; accepted September 23, 2009

Dieldrin was previously used as a pesticide. Although its use has been discontinued, humans are still exposed to it due to its high environmental persistence and because it accumulates in the adipose tissue of animals. Acute exposure to dieldrin provokes convulsions due to its antagonism on the gamma-aminobutyric acid-A (GABA A) receptor. However, little is known about the effects of low chronic exposure to this pollutant. In the present work, we use primary cultures of cortical neurons to study the mechanisms involved in the toxic action of dieldrin. We found that 2 and 6 days in vitro (DIV) exposure to a subcytotoxic concentration (60 nM) of dieldrin reduced the increase in intracellular calcium concentration ([Ca 2+]i) and the excitotoxicity caused by glutamate. Exposure to dieldrin for 6 DIV induced N-methyl-D-aspartate receptor (NMDAR) internalization and reduced metabotropic glutamate receptor 5 (mGLUR5) levels. Double immunostaining for NMDAR and mGLUR5 showed that these receptors lose colocalization on the cell membrane in neurons treated with dieldrin. No changes were observed in receptor functionalities or receptor levels after 2 DIV of exposure to dieldrin. However, the increase in [Ca 2+]i induced by coactivation of NMDAR and mGLUR5 was significantly reduced. Thus, a functional interaction between the two receptors seems to play an important role in glutamate-induced excitotoxicity. We confirm that permanent blockade of the GABA A receptor by this persistent pesticide triggers adaptive neuronal changes consisting of a reduction of glutamatergic neurotransmission. This might explain the cognitive and learning deficits observed in animals after chronic treatment with dieldrin.

Key Words: dieldrin; neurotoxicity; in vitro; glutamate; receptor.

Dieldrin is detected at higher levels than in wild ones (Hites et al., 2004). It has also been found in horticultural soils and the plants grown in them (Hilber et al., 2008) and in the adipose tissue of 30% of women who live near agriculture areas (Botella et al., 2004). For the last few years, dieldrin has been 1 of the 12 “persistent organic pollutants” assessed in accordance with the Stockholm Convention (http://www.pops.int). This organochlorine pesticide is also a derivate of the epoxidation of the cyclodiene pesticide aldrin. It blocks the chloride flux through both the gamma-aminobutyric acid-A (GABA A) receptor (where it inhibits t-[ 35S]butylbicyclophosphorothionate ([ 35S]TBPS) from binding to it) and the glycine receptor (Pome’s et al., 1993; Vale et al., 2003). Acute exposure to dieldrin produces convulsions in mammals (Bloomquist, 1992). However, the effects of chronic exposure to this compound have yet to be established.

Several studies suggest that dieldrin could be a risk factor in the development of Parkinson’s disease (PD) (Kanthasamy et al., 2005; Sanchez-Ramos et al., 1998). Moreover, Corrigan et al. (2000) found higher levels of dieldrin in PD brains than in control brains. The pesticide causes mitochondrial damage in dopaminergic neurons in culture, and these neurons appear to be sensitive to dieldrin-induced oxidative stress and cell death (Kitazawa et al., 2001; Sanchez-Ramos et al., 1998). In addition, prenatal exposure to dieldrin had deleterious effects on the GABAergic-dependent development of the embryonic brainstem (Liu et al., 1997).

The main target for acute lethal toxicity of cyclodienes is the GABA A receptor for which dieldrin presents a high affinity (Narahashi et al., 1995; Pomés et al., 1993; Vale et al., 2003). We have previously reported that the blockade of the GABA A receptor reduces the synaptic release of glutamate from cerebellar granule cells and also protects these neurons from excitotoxic insults (Babot et al., 2005). A link between inhibitory GABA and excitatory glutamate neurotransmission, which compensate each other to maintain brain electrical activity, is now fully acknowledged. Furthermore, long
timescale changes in both neurotransmitter systems finally leads to regulation of receptor expression and trafficking (Pérez-Otaño and Ehlers, 2005; Turrigiano, 2008). In a previous study, long-term exposure of cerebellar granule cells to dieldrin resulted in a significant reduction of activity and in internalization of N-methyl-D-aspartate receptor (NMDAR) (Babot et al., 2007).

Here, we study the effects of long-term exposure to dieldrin on cultured cortical neurons in order to further characterize the long-term effects of dieldrin on GABA and glutamate neurotransmission. It is accepted that both GABA<sub>A</sub> and glutamate receptor subunits are differently expressed in the cortex and cerebellum (Garrett et al., 1990; Llansola et al., 2005), and therefore, different effects of dieldrin could be envisaged. We use primary cultures of cortical neurons that are mainly composed of GABAergic neurons (around 40%) and also glutamatergic and cholinergic neurons, whereas primary cultures of cerebellar granule cells contain predominantly glutamatergic neurons (> 90%). Both the cell types express functional GABA<sub>A</sub> and glutamate receptors (Sonnewald et al., 2004; Šufol et al., 2008). It is also well known that human cortex is responsible for higher brain functions, such as working memory, and that GABA and glutamate play a crucial role in these processes.

This work strongly supports the effects found in cerebellar granule cells by showing that partial inhibition of the GABA<sub>A</sub> receptor induced by continuous (but not acute) dieldrin exposure triggers adaptive neural changes consisting of NMDAR internalization. Furthermore, we report a novel target altered by dieldrin, the metabotropic glutamate receptor 5 (mGLUR5). Thus, the effect of dieldrin on GABAergic and glutamatergic neurotransmission in cortical neuronal cultures might indicate that the cortex is a highly susceptible region of the brain against chronic organochlorine pesticide exposure.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16 gestational day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l’Arbresle, France). Plastic culture multiwell plates were from Nunc (Roskilde, Denmark). Fetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain), and Dulbecco’s modified Eagle’s minimum essential medium (DMEM) was from Biochrom (Berlin, Germany). Trypsin, soybean trypsin inhibitor, DNAse, dimethyl sulfoxide (DMSO), t-glutamic acid (NMDA), kaicid acid, 6-methyl-2-(phenylethyl)pyridine (MPEP), 7-(hydroxyimino)cyclopropane-1-carboxylate ethyl ester (CPCOOEt), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dieldrin, picrotoxinin (PTX), L-pyruvic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and soya bean trypsin inhibitor, DNAse, dimethyl sulfoxide (DMSO), L-glycine, adjusted to pH 7.3) containing 36Cl<sup>-</sup> were from American Radiolabeled Chemicals, Inc. (St Louis, MO). [35S]TBPS and [3H]-MK-801 were from PerkinElmer (Boston, MA). Fluo-3 AM was from Molecular Probes (Leiden, The Netherlands).

**Materials and Methods**

**Cell Cultures**

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mice fetuses according to Frandsen and Schousboe (1990) with minor modifications. Briefly, pregnant animals were anesthetized with isoflurane (FORANE; Abbott Laboratories SA, Madrid, Spain), killed by cervical dislocation, and the fetuses extracted. Cortices were dissected with forceps, mechanically minced, and cells were then dissociated by mild trypsinization (0.02% [wt/vol]) at 37°C for 10 min followed by trituration in a DNase solution (0.004% [wt/vol]) containing soybean trypsin inhibitor [0.05% (wt/vol)]. The cells were then suspended in DMEM (5mM KCl, 31mM glucose, and 0.2mM glutamine) supplemented with p-amino benzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension (1.5 × 10<sup>5</sup> cells/ml) was seeded in 6-, 24-, or 96-well plate precoated with poly-<i>n</i>-lysine and incubated for at least 8 days in a humidified 5% CO<sub>2</sub>/95% air atmosphere at 36.8°C. A mixture of 5μM 5-fluoro-2-deoxyuridine and 20μm uridine was added after 2 days in vitro (DIV) in culture to prevent glial proliferation.

Animals were handled in compliance with protocol DMA1852 of the University of Barcelona, approved by the Generalitat de Catalunya, Spain, following the European Union guidelines.

**Dieldrin Treatment**

A stock solution of dieldrin was prepared in DMSO and frozen in aliquots of 100 μl. The final concentration of DMSO in the culture medium was 0.5%. To avoid cross-contamination between different wells in the same plate, the different treatments, DMSO or dieldrin, were performed on separate plates. Cultures were treated at 2 or 6 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 DIV (exposure for 6 or 2 DIV, respectively). The dieldrin concentration was stable in culture medium for at least 8 DIV at 37°C (Babot et al., 2007). In some experiments, neurons were acutely exposed to dieldrin (less than 5 min).

**Chloride Influx**

Chloride influx was determined as 36Cl<sup>-</sup> uptake in intact cell cultures of cortical neurons grown in 24-well plates (Vale et al., 2003). Briefly, the culture medium was replaced by prewarmed Earle’s balanced salt solution (EBSS: 116mM NaCl, 1.8mM CaCl<sub>2</sub>, 0.8mM MgSO<sub>4</sub>, 1mM NaH<sub>2</sub>P<sub>0</sub><sub>4</sub>, 10mM HEPES, and 9mM glucose, pH 7.4). The final concentration of DMSO in the culture medium was 0.5%. To avoid cross-contamination between different wells in the same plate, the different treatments, DMSO or dieldrin, were performed on separate plates. Cultures were treated at 2 or 6 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 DIV (exposure for 6 or 2 DIV, respectively). The dieldrin concentration was stable in culture medium for at least 8 DIV at 37°C (Babot et al., 2007). In some experiments, neurons were acutely exposed to dieldrin (less than 5 min).

**Materials and Methods**

**Chloride Influx**

Chloride influx was determined as 36Cl<sup>-</sup> uptake in intact cell cultures of cortical neurons grown in 24-well plates (Vale et al., 2003). Briefly, the culture medium was replaced by prewarmed Earle’s balanced salt solution (EBSS: 116mM NaCl, 1.8mM CaCl<sub>2</sub>, 0.8mM MgSO<sub>4</sub>, 1mM NaH<sub>2</sub>P<sub>0</sub><sub>4</sub>, 10mM HEPES, and 9mM glucose, pH 7.4). The final concentration of DMSO in the culture medium was 0.5%. To avoid cross-contamination between different wells in the same plate, the different treatments, DMSO or dieldrin, were performed on separate plates. Cultures were treated at 2 or 6 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 DIV (exposure for 6 or 2 DIV, respectively). The dieldrin concentration was stable in culture medium for at least 8 DIV at 37°C (Babot et al., 2007). In some experiments, neurons were acutely exposed to dieldrin (less than 5 min).

**Materials and Methods**

**Materials**

Pregnant NMRI mice (16 gestational day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l’Arbresle, France). Plastic culture multiwell plates were from Nunc (Roskilde, Denmark). Fetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain), and Dulbecco’s modified Eagle’s minimum essential medium (DMEM) was from Biochrom (Berlin, Germany). Trypsin, soybean trypsin inhibitor, DNAse, dimethyl sulfoxide (DMSO), t-glutamic acid (NMDA), kaicid acid, 6-methyl-2-(phenylethyl)pyridine (MPEP), 7-(hydroxyimino)cyclopropane-1-carboxylate ethyl ester (CPCOOEt), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dieldrin, picrotoxinin (PTX), L-pyruvic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and β-nicotinamide adenine dinucleotide-reduced (NADH) disodium salt were from Sigma (St Louis, MO). 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoloxine-7-sulfonamide disodium salt and (R.S)-3,5-dihydroxyphenylglycine (DHPG) were from Tocris Cookson (Bristol, UK). 36Cl<sup>-</sup> was from American Radiolabeled Chemicals, Inc. (St Louis, MO). [35S]TBPS and [3H]-MK-801 were from PerkinElmer (Boston, MA). Fluo-3 AM was from Molecular Probes (Leiden, The Netherlands).
according to Babot et al. (2007). This is an indicator of changes in intracellular calcium. It is used to measure the Ca\(^{2+}\) influx through the ionotropic receptors or the metabotropic receptor–induced mobilization of intracellular Ca\(^{2+}\) stores in response to the glutamate or specific agonists. Supplementary figure 1 shows that the agonist-induced increase in intracellular Ca\(^{2+}\) is completely blocked by specific glutamate receptor antagonists. Cultured cells grown in 96-well plates were incubated with Fluo-3 AM (9μM) for 1 h at 37°C in Hank’s solution (1.3mM CaCl\(_2\), 5.4mM KCl, 0.4mM KH\(_2\)PO\(_4\), 0.5mM MgCl\(_2\), 0.4mM MgSO\(_4\), 137mM NaCl, 4.2mM NaHCO\(_3\), 0.3mM Na\(_2\)HPO\(_4\), 8mM HEPES, and 5.5mM glucose, adjusted to pH 7.4). Excess Fluo-3 AM was rinsed away, and the cells were treated with different agonist concentrations (from 1μM to 1mM) in Hank’s solution (or magnesium-free Hank’s solution when using NMDA) in the presence of 5mM glycine (when using glutamate or NMDA). Fluorescence (F) was immediately determined in a fluorimetric plate reader (Em 485/Exc 530, SpectraMax GeminiXS, Molecular Devices, Sunnyvale, CA). In order to calculate the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)), the cells were incubated with the ionophore A23187 (10μM) for 30 min and thereafter with CuSO\(_4\) (5mM) to obtain the maximum (F\(_{\max}\)) and the minimum (F\(_{\min}\)) fluorescence 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 AM/Ca\(^{2+}\) (320nM).

\[\text{[Ca}^{2+}]_{\text{i}}\text{= }K_d\text{ (F - F}_{\text{min}}\text{)} / (F}_{\text{max}}\text{ - F},\text{ where }K_d\text{ is the dissociation constant of Fluo-3 AM/Ca}^{2+}\text{ (320nM).}\]

**Western Blot**

Cultures grown in 6-well plates were washed twice with cold Hank’s solution, and cells were harvested with 0.2 ml of loading buffer (62.5mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, and 50mM dithiothreitol) and briefly sonicated. After boiling for 5 min and being centrifuged at 16,100 g for 5 min, 25 μl of the homogenate was subjected to SDS-polyacrylamide gel electrophoresis using 8–10% polyacrylamide resolving gel at 60 mA for 1.5–2 h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% nonfat dry milk in Tris-Buffered Saline Tween-20 (TBS-T) (20mM Tris-HCl [pH 7.6], 140mM NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4°C with a goat polyclonal anti-NMDAR1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal anti-NMDAR1 and anti-NMDAR2B antibodies (1:4000; Molecular Probes). On all the membranes, a monoclonal anti-actin (1:10,000; Sigma) and a secondary HRP-linked anti-rabbit (1:10,000) antibodies were used as a control of the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP kit; Bio-Rad, Hercules, CA). Luminescence was quantified with a Versadoc Imagine System (Bio-Rad). Digital images were then quantified in the Quantity One software (Bio-Rad).

**Immunocytochemistry**

Cells were seeded in Permanox chamber slides (Nunc) treated with poly-D-lysine, and immunostaining was performed as previously described by Babot et al. (2007) with minor modifications. Cultures were rinsed with PBS and fixed with methanol at −20°C for 10 min. Cells were incubated with 0.03% Triton X-100 in PBS for 5 min, and after three rinses with PBS, they were blocked in 5% BSA in PBS for 5 min at room temperature. Subsequently, cultures were incubated overnight at 4°C together with the primary anti-NMDAR1 (1:50) and anti-NMDAR2A (1:500) antibodies in solutions containing 5% BSA in PBS, rinsed three times with PBS for 5 min, and incubated for 1 h at room temperature with both secondary antibodies, chicken anti-goat Alexa 488 (1:1000; Molecular Probes) and donkey anti-rabbit Alexa 594 (1:1000; Molecular Probes) simultaneously. After rinsing with PBS, the slides were coverslipped with Mowiol. The immunostained cells were examined in a confocal fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL) using the same excitation laser intensity for control and dieldrin-treated cells.

**Cell Viability and Cytotoxicity**

Cell viability was determined by measuring the reduction of MTT, whereas cytotoxicity was assessed by quantifying the release of the cytosolic enzyme lactate dehydrogenase (LDH). The methods were used simultaneously for every experiment.

The treatment with glutamate was always performed at 7–8 DIV when functional ionotropic glutamate receptors are completely expressed (Frandsen and Schousboe, 1990). Cells grown in 96-well plates were exposed to different concentrations of glutamate (from 10μM to 10mM) in Hank’s solution for 1 h. After rinsing twice with Hank’s solution, fresh DMEM supplemented with gentamicin (1 mg/ml) was added. Cell viability/cytotoxicity was determined 24 h later.

**MTT assay.** The substrate MTT, when exposed to cells, is reduced by mitochondrial activity to a colored formazan salt, which can be quantified spectrophotometrically. The cells were incubated with MTT (500 μg/ml) dissolved in Hank’s solution at 37°C for 1 h protected from light. After washing off the excess MTT, the cells were disaggregated with 5% SDS and the amount of colored formazan salt formed was measured at 560 nm in a spectrophotometer plate reader (iEMS Reader MF; Lab Systems, Helsinki, Finland).

**LDH assay.** The measurement of LDH released by damaged cells was performed as described previously by Rosa et al. (1997) with slight modifications. Briefly, after glutamate treatment, the solution was replaced with fresh DMEM in order to determine only the LDH released in the 24 h after glutamate exposure. Therefore, 50 μl of the medium from each well was placed into another 96-well plate and mixed with 200 μl of Sorensen’s phosphate buffer solution (53.4mM Na\(_2\)HPO\(_4\) and 13.4mM KH\(_2\)PO\(_4\), adjusted to pH 7.4) containing 2.08mM pyruvic acid, 0.375mM NADH, and 3.97mM NaHCO\(_3\). Immediately, the mixture was mixed and incubated for 1 min at 37°C before reading the absorbance. Twenty readings of the absorbance of the reaction mixture at 340 nm, an index of NADH concentration, taken every 15 s using a spectrophotometer plate reader (iEMS Reader MF; Lab Systems), were recorded as the kinetic curve of the coenzyme’s disappearance. LDH changes were automatically calculated from the slope of the absorbance curve, fitted by linear regression to the initial portion of the curve. We used 50 μl of DMEM plus 200 μl of the NADH solution as a blank for this measure. Cytotoxicity was determined as % of released LDH with respect to control cells. Total death value (% of LDH release) was obtained from cells treated with 0.3% Triton for 30 min.

**Data Analysis**

Data are shown as mean ± SE. Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. Statistical comparisons were made by t-test when comparing control and dieldrin-exposed groups (E\(_{\text{max}}\) values from Figs. 3–5 and 8), one-way ANOVA followed by Dunnett’s multiple comparison test when comparing more than two groups (different exposure time or dieldrin concentrations; Fig. 1, Tables 1 and 2), and two-way ANOVA followed by Bonferroni posttest when comparing two factors (dieldrin and glutamate/antagonists exposures; Figs. 2 and 7) using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).
RESULTS

Effects of Dieldrin Exposure on GABA_A Receptor and Cell Viability in Cortical Neurons

Exposure to dieldrin for 2 DIV inhibited [35S]TBPS binding in intact cultured cortical neurons with an inhibitory concentration 50 (IC50) value of 140 ± 9nM (N = 3) (Fig. 1A). Similar results were obtained after 6 DIV of exposure (IC50 = 145 ± 46nM, N = 3) (Fig. 1B). Long-term exposure to 60nM dieldrin inhibited the 30µM GABA-induced 36Cl– uptake by 55 ± 11% (N = 3, p < 0.05). Since 60nM of dieldrin was the lowest-observed-effect concentration in the [35S]TBPS binding for both 2 and 6 DIV of treatment (Fig. 1), we decided to choose this concentration for all subsequent experiments. This is a noncytotoxic concentration since exposure to up to 200nM dieldrin for 6 DIV did not produce a reduction in cell viability (MTT value was 91 ± 6% with respect to control, N = 4), while exposure to 600nM dieldrin significantly reduced cell viability by 28 ± 8% (p < 0.01).

Effects of Dieldrin on Glutamate-Induced Excitotoxicity and Intracellular Calcium Increase

We previously reported that a short pulse of high K+ concentration (> 70mM) releases glutamate ([glutamate]o > 2µM) from cultured cerebellar granule neurons producing excitotoxic cell death (Babot et al., 2005). Treatment of primary cultures of cortical neurons with 100mM KCl for 15 min induced a lower release of endogenous glutamate ([glutamate]o = 0.85 ± 0.13µM, after the insult) without producing cell death (data not shown). Therefore, we developed a model of excitotoxicity using different concentrations of glutamate (from 10µM to 10mM) at different exposure times (from 15 min to 24 h). Table 1 shows LC50 values for glutamate-induced loss of cell viability. Treatment with glutamate for 15 or 30 min was significantly less toxic than exposure for 1 h, whereas exposures for 2 or 24 h were not significantly different with respect to 1-h exposure. Therefore, an exposure time of 1 h was selected for the subsequent studies of glutamate-induced excitotoxicity in cultured cortical neurons.

Cells were exposed to 60nM dieldrin for 2 or 6 DIV and subsequently exposed to glutamate. The glutamate-induced reduction in cell viability led to cell membrane damage, as determined by the significant release of LDH. This effect was significantly attenuated by exposure to dieldrin for 2 and 6 DIV (Fig. 2). Similar results were obtained from the MTT assay (Supplementary fig. 2).

![FIG. 1. Concentration-dependent inhibition of [35S]TBPS binding after long-term exposure to dieldrin. Primary cultures of cortical neurons were exposed to dieldrin at concentrations ranging from 0 to 200nM for 2 DIV (A) and 6 DIV (B). Thereafter, the cells were incubated with [35S]TBPS (1.5–3nM in TCBSS) for 30 min at 25°C. The data correspond to mean ± SE of three independent experiments determined in triplicate. (A) *p < 0.05 and **p < 0.01. (B) **p < 0.01 with respect to control.](http://toxsci.oxfordjournals.org/)

**TABLE 1**

<table>
<thead>
<tr>
<th>Time</th>
<th>LC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>12.1 ± 0.6***</td>
</tr>
<tr>
<td>30 min</td>
<td>6.6 ± 2.2*</td>
</tr>
<tr>
<td>1 h</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>2 h</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>24 h</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

Note. Cell viability was measured 24 h after transient exposure to glutamate for the specified time using MTT assay. Values are at least mean ± SE of (N) experiments performed in triplicate.

*p < 0.05 and ***p < 0.001 versus 1 h treatment.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>K_d (µM)</th>
<th>B_max (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9 ± 1.6</td>
<td>180.0 ± 26.2</td>
</tr>
<tr>
<td>Dieldrin 60nM</td>
<td>1.9 ± 0.4*</td>
<td>92.3 ± 12.9*</td>
</tr>
<tr>
<td>Dieldrin 200nM</td>
<td>2.4 ± 0.8*</td>
<td>75.5 ± 11.5*</td>
</tr>
</tbody>
</table>

Note. Values represent the mean ± SE of three independent experiments, each performed in triplicate. The values were obtained by adjusting data from each experiment to a one-site competition curve.

*p < 0.05 with respect to control.
We further analyzed whether the increase in \([\text{Ca}^{2+}]_i\) caused by glutamate was affected by long-term exposure to dieldrin. Six DIV of treatment with dieldrin did not modify the basal intracellular calcium (values were 56.6 \(\pm\) 5.9 nM and 71.2 \(\pm\) 6.9 nM for control and dieldrin-exposed cells, respectively, \(N = 12\)). Results are expressed as % of LDH release corresponding to the total cell death (mean \(\pm\) SE, \(N = 3–4\)). Two-way ANOVA shows statistical differences between glutamate and control (\(*p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\)). Exposure to dieldrin significantly reversed the effect of glutamate: (A) \(*p < 0.05\) and ***\(p < 0.001\); (B) ***\(p < 0.001\).

We further analyzed whether the increase in \([\text{Ca}^{2+}]_i\) caused by glutamate was affected by long-term exposure to dieldrin. Six DIV of treatment with dieldrin did not modify the basal intracellular calcium (values were 56.6 \(\pm\) 5.9 nM and 71.2 \(\pm\) 6.9 nM for control and dieldrin-exposed cells, respectively, \(N = 12\)) but significantly reduced glutamate-induced increase in \([\text{Ca}^{2+}]_i\) (Fig. 3). Figure 3 shows the concentration-response curves for the increase in \([\text{Ca}^{2+}]_i\) due to glutamate. Exposure to dieldrin for 2 DIV led to a significant reduction of the \(E_{\text{max}}\) values (463 \(\pm\) 46% and 307 \(\pm\) 48% for control and dieldrin-exposed cells, respectively, \(N = 3, p < 0.05\)) (Fig. 3A). Likewise, longer exposure to dieldrin up to 6 DIV resulted in reduction of \(E_{\text{max}}\) values (403 \(\pm\) 14% and 261 \(\pm\) 23% for control and dieldrin-exposed cells, respectively, \(N = 4, p < 0.01\)) (Fig. 3B).

Effects of Dieldrin on Ionotropic Glutamate Receptors

The next step was to elucidate which glutamatergic target was altered by exposure to dieldrin in order to explain the reduced responsiveness to the glutamate excitotoxic insult in these neurons. We first studied the function of the NMDAR, which is known to be the main receptor involved in glutamate-triggered excitotoxicity (Choi, 1987; Michaels and Rothman, 1990). Surprisingly, we did not observe a reduction in NMDAR functionality after 2 DIV of exposure to dieldrin (Fig. 4A). However, prolonging exposure to dieldrin up to 6 DIV resulted in a significant reduction in the \(E_{\text{max}}\) values, which were 286 \(\pm\) 40% and 195 \(\pm\) 12% for control and dieldrin-exposed cells, respectively, \(N = 3, p < 0.05\)) (Fig. 4B). This reduction in NMDAR function was confirmed by a significant reduction of the \(B_{\text{max}}\) value for the GABA_A receptor antagonist PTX (100 µM) for 6 DIV. PTX mimicked the effect of dieldrin on the glutamate-induced rise in \([\text{Ca}^{2+}]_i\), and cell death (Supplementary fig. 3).
to five independent experiments, each in triplicate. Nonlinear regression fit with respect to cells incubated with Hank’s solution and are mean

\[ \text{[Ca}^{2+}\text{]} \]

immediately after NMDA treatment, for neurons exposed to DMSO (\( \text{E}_{\text{max}} \) values were 353 \( \pm \) 32\% and 242 \( \pm \) 8\% in control and MK-801-treated cells, respectively, \( p < 0.05 \)), this reduction was even greater in dieldrin-treated cells (\( E_{\text{max}} = 219 \pm 10\% \), \( p < 0.01 \)), although not statistically different from cells treated with MK-801 (Fig. 7B). Taking into account that the excitotoxic response to glutamate when all NMDARs were blocked by MK-801 was reduced to a higher extent in cells exposed to dieldrin with respect to control cells, we wonder if long-term exposure to this pollutant might be affecting other glutamate receptors.

In addition, the reduced glutamate excitotoxicity observed after 2 DIV of exposure to dieldrin was not accompanied with a reduced NMDAR function. Therefore, we sought to determine whether AMPA/kainate receptors could be targeted by dieldrin exposure. However, neither the kainate-induced increase in \([\text{Ca}^{2+}]_i\) nor the kainate-induced depolarization were affected by 2 DIV of exposure to dieldrin (Supplementary fig. 4).

**Effects of Dieldrin on Metabotropic Glutamate Receptors**

Next, we studied the group-I ionotropic glutamate receptors (mGLUR1 and mGLUR5) since their activation increases \([\text{Ca}^{2+}]_i\) through the mobilization of intracellular \(\text{Ca}^{2+}\) stores (Nakanishi, 1994). The antagonists for both mGLUR5 and mGLUR1 reduced the increase in \([\text{Ca}^{2+}]_i\), induced by glutamate with similar efficacy (\( E_{\text{max}} \) values were 361 \( \pm \) 13\%, 255 \( \pm \) 18\%, and 266 \( \pm \) 25\% for control, MPEP-treated, and CPCOOEt-treated cells, respectively; \( p < 0.05 \)) (Supplementary fig. 5A). However, only the antagonist of mGLUR5, MPEP, significantly prevented glutamate-induced excitotoxicity (\( p < 0.05 \), \( N = 3 \), for MPEP with respect to control) (Supplementary fig. 5B). When cortical neurons were exposed to dieldrin for 6 DIV, the increase of \([\text{Ca}^{2+}]_i\), induced by the specific agonist of group-I metabotropic glutamate receptor, DHPG, was significantly reduced (\( E_{\text{max}} \) values were 157 \( \pm \) 10\% and 132 \( \pm \) 4\% in control and dieldrin-exposed cells, respectively; \( p < 0.05 \), \( N = 3 \)) (Fig. 8A). However, this effect was not observed neither after exposure to dieldrin for 2 DIV nor after acute exposure (data not shown). Since exposure to dieldrin for 2 DIV reduced the glutamate-induced rise in \([\text{Ca}^{2+}]_i\) (Fig. 3A) but not that induced by NMDA (Fig. 4A) or DHPG, we wondered whether coexposure to both agonists would be needed to observe a similar reduction to that observed with glutamate on the increase of \([\text{Ca}^{2+}]_i\). The simultaneous treatment with 100\(\mu \text{M}\) of NMDA and 100\(\mu \text{M}\) of DHPG induced an increase in \([\text{Ca}^{2+}]_i\) in control cells (\( [\text{Ca}^{2+}]_i = 213 \pm 7\% \)), which was significantly

![FIG. 4. Effect of exposure to dieldrin on the NMDA-induced increase in \([\text{Ca}^{2+}]_i\) in cultured cortical neurons. Fluo-3 fluorescence was measured immediately after NMDA treatment, for neurons exposed to DMSO (■) or dieldrin (▲) for 2 DIV (A) or 6 DIV (B). Values obtained are expressed as % with respect to control. The 95% confidence intervals are shown by the error bars. The data are mean ± SE of three to five independent experiments, each in triplicate. Nonlinear regression fit shows statistical differences between the \( E_{\text{max}} \) values of the curves shown in (B) two-tailed \( t \)-test, \( p < 0.05 \).](https://doi.org/10.1093/toxsci/kfz033)
reduced in cells exposed to dieldrin for 2 DIV ([Ca\(^{2+}\)]_i = 177 ± 10\%, N = 3, p < 0.05) (Fig. 8B).

In order to discriminate which of the group-I metabotropic glutamate receptors was affected by the exposure to dieldrin, we used specific antibodies against mGLUR1 and mGLUR5. Quantification of the immunoblots showed a reduction in the total amount of mGLUR5 protein after 6 DIV of exposure to dieldrin (Fig. 5B). The reduction in the amount of mGLUR5 was confirmed by immunostaining. mGLUR5 colocalizes with the NR1 subunit of NMDAR on the cell membrane of the soma in control cultures (Fig. 6A–C). In contrast, in neurons exposed to dieldrin for 6 DIV, the mGLUR5 immunostaining was reduced overall (Fig. 6H) and the colocalization with the NR1 subunit disappeared (Fig. 6I). No significant changes were observed in the mGLUR1 protein; however, the immunoblot signal was very faint in our cultures (Fig. 5C). Contrarily, exposure to dieldrin for 2 DIV did not modify either mGLUR5 (Fig. 5D) or mGLUR1 protein levels (data not shown), which is in agreement with the lack of effect on the functionality of these receptors after 2 DIV of exposure to dieldrin. Nevertheless, mGLUR5 immunostaining was mainly located at the cell membrane in control cells (Fig. 6B), as mentioned, whereas in cells treated with dieldrin for 2 DIV, a diffuse distribution was observed (Fig. 6E) and moreover the colocalization with NMDAR almost completely disappeared (Fig. 6F). This effect may explain why after 2 DIV of dieldrin exposure the individual

**FIG. 5.** Long-term exposure to dieldrin decreased mGLUR5 protein but not mGLUR1 or NMDAR. Left: representative immunoblots for the indicated proteins from control cultures (Cnt) and cultures treated with dieldrin for 6 DIV (A–C) or 2 DIV (D). The corresponding immunoblot for actin is shown at the bottom. Molecular weights are given on the right. Right: densitometric quantification of the immunoblots. Data are mean ± SE of three to four independent experiments. (B) Exposure to dieldrin for 6 DIV reduced the levels of mGLUR5 protein with respect to control (p < 0.001, N = 4).
functionalities of both NMDAR and mGLUR5 were not significantly different from control, but the physiological consequences of their simultaneous activation were affected.

**DISCUSSION**

In this work, we show that long-term exposure to a non-cytotoxic concentration of dieldrin produced both an internalization of NMDAR and a downregulation of mGLUR5 in primary cultures of cortical neurons. These effects were secondary and a consequence of the continuous blockade of GABA_α_ receptor by dieldrin. This compensatory balance between excitation and inhibition explains the reduced responsiveness to glutamate in long-term dieldrin-treated cells, as measured by both the increase in [Ca^{2+}]_i and the excitotoxicity induced by glutamate (Figs. 3 and 4). Swann et al. (2007) have recently found that chronic inhibition of GABA_α_ receptor with bicuculline reduces glutamate receptor–mediated synaptic activity and several components of the glutamatergic synapse, such as the post synaptic density-95 (PSD-95) and Homer. As opposed to toluene, which is known to acutely enhance glycine and GABA_α_ receptor–mediated currents, NMDA-mediated (but not AMPA/Kainate-mediated) neurotransmission is enhanced after chronic exposure to this solvent (Bale et al., 2005). Moreover, inhibition of synaptic activity using NMDAR antagonists also induces upregulation of NMDAR expression (Slikker et al., 2007). Thus, increasing evidence shows an activity-dependent trafficking and expression of membrane receptors that accurately regulate synaptic excitability.

The concentration of dieldrin used in this work (60nM) inhibited the binding of [35S]TBPS after 2 and 6 DIV of exposure to a similar extent to that observed after acute exposure (Cole and Casida, 1986; Pomés et al., 1993). Accordingly, we also report that long-term exposure to a non-cytotoxic concentration of dieldrin inhibited the GABA-induced chloride influx by around 50%. Again, this effect is similar to that observed after acute exposure in neuronal cultures (Vale et al., 2003), which is consistent with the reported stability of this compound in serum-containing culture medium (Babot et al., 2007). Some work reports transient effects on [Ca^{2+}]_i fluxes and prolactin release at very low concentrations (picomolar to nanomolar) of dieldrin in a pituitary tumor cell line (Wozniak et al., 2005). However, to the best of our knowledge, this is the first study in which such low concentrations of dieldrin are shown to cause
prolonged alterations to synaptic transmission. A similar reduction of NMDAR function was observed in cultured cerebellar granule cells exposed to dieldrin (Babot et al., 2007), however, the concentration used in that work was much higher than here. Other works have also found that micromolar concentrations of dieldrin strongly inhibit serotonergic neurons (Liu et al., 1997) and cause oxidative stress and apoptosis in dopaminergic neurons (Kitazawa et al., 2001; Sanchez-Ramos et al., 1998). Our results show that cortical neurons are highly sensitive to prolonged exposure to dieldrin and therefore probably to other permanent organochlorine pesticides that share a similar mechanism of acute toxicity.

Glutamate-induced neuronal damage is predominantly not only Ca dependent but also Cl dependent (Babot et al., 2005; Choi, 1987). It is also accepted that activation of NMDAR is the main cause of glutamate-triggered cytotoxicity (Michaels and Rothman, 1990). For these reasons, the effect of long-term exposure to dieldrin on glutamate-mediated cell death found in the present study cannot be accounted for only by partial inhibition of GABA\(_A\) receptor. It probably indirectly affects some of the components of the glutamatergic system. Therefore, we first studied the possible action of long-term exposure to dieldrin on NMDAR. As shown in Figure 4, 2 DIV of exposure to dieldrin is not long enough to significantly affect the functionality of NMDAR; longer treatments are needed to observe an effect on this receptor. This is at variance with the rapid changes in the expression or localization of the NR1 subunit of NMDAR that has been found after excitotoxic stimulation in cortical neurons (Gascón et al., 2005; Nakamichi and Yoneda 2006). However, in our context, we expect that...
partial blockade of the GABA<sub>A</sub> receptor would produce slight but continuous hyperactivation of the glutamatergic system, the so-called "slow excitotoxicity", that probably requires longer periods of time to cause changes in NMDAR cell surface expression.

It has been demonstrated that several proteins are needed for cell membrane localization of NMDAR, such as PSD-95 (Kornau et al., 1995). For instance, the administration of NMDAR antagonists upregulates SAP90/PSD-95 and SAP97 in the rat cortex (Linden et al., 2001), whereas treatment with bicuculline reduces the expression of PSD-95 and Homer proteins (Swann et al., 2007). Therefore, the fact that prolonged exposure to dieldrin (> 2 DIV) is needed to modify NMDAR function suggests that this pesticide may affect the expression of some transport or anchorage proteins needed for NMDAR clustering, which in turn can result in NMDAR internalization. Further studies are required to determine which of these proteins are primarily affected by dieldrin exposure.

Prolonged exposure to dieldrin specifically induced mGLUR5 downregulation, as observed by Western blot and immunocytochemistry, whereas mGLUR1 remained unaffected. This selective effect on mGLUR5 has also been observed in hippocampal slices after transient global ischemia and after status epilepticus (Kirschstein et al., 2007; Yeh and Wang, 2005). Although there is a consensus concerning the major contribution of NMDAR to glutamate-induced cell death, the involvement of metabotropic glutamate receptors is still controversial. Dual role in neurotoxicity and neuroprotection has been proposed for type-I mGLUR (Nicoletti et al., 1999). For instance, the activation of type-I mGLUR causes neurotoxic damage and enhances NMDA- (but not AMPA-) mediated degeneration in rat brain and in cultured cortical neurons (Bruno et al., 1995; McDonald and Schoepp, 1992). Although the mGLUR1 antagonist CPCOOEt is neuroprotective against NMDA toxicity by enhancing GABA release (Battaglia et al., 2001), it fails to prevent cell death induced by coadministration of NMDA and DHPG in cortical neurons, whereas MPEP is effective in the same conditions (Bruno et al., 2001). In agreement with these data, here we show that both CPCOOEt and MPEP reduced the glutamate-induced [Ca<sup>2+</sup>]<sub>i</sub> increase but only MPEP was able to prevent the neuronal loss induced by glutamate (Supplementary fig. 5). The lower expression of mGLUR1 with respect to mGLUR5 in cortical neurons (Fig. 5, this work; Bruno et al., 1995) might explain these observations. However, a specific involvement of mGLUR5 in NMDAR-mediated neurotransmission cannot be ruled out since several studies show a direct association between mGLUR5 and NMDAR. For instance, it has been demonstrated that mGLUR5 knock-out (K.O.) mice show impaired NMDAR-dependent long-term potentiation (LTP) but not NMDAR-independent LTP in the hippocampus (Lu et al., 1997). Furthermore, bicuculline-induced persistent bursting from the hippocampus to the entorhinal cortex is mediated by both mGLUR5 and NMDAR activation (Stoop et al., 2003). In addition, Yang et al. (2004) have recently proved a synergistic effect on extracellular regulated kinase phosphorylation and gene expression by simultaneous mGLUR5 and NMDAR activation via PSD-95 and Homer-1b/c. This in turn could directly cross-link the two receptors, through the Shank family of proteins, to their respective intracellular signaling pathways (Tu et al., 1999). After just 2 DIV of exposure to dieldrin, reduced colocalization in the cell soma and a more diffused pattern in the immunolabeling of mGLUR5 and NMDAR were observed. This may explain the reduced responsiveness to glutamate in these cells since at this time dieldrin was able to reduce both neuronal death and [Ca<sup>2+</sup>]<sub>i</sub> increase that were induced by glutamate. These observations lend weight to the importance of simultaneous activation of the two receptors in glutamate excitotoxicity.

Activation of type-I mGLURs has been shown to promote NMDAR trafficking to the cell membrane (Lan et al., 2001). We therefore hypothesize that the internalization of the NMDAR observed after 6 DIV of dieldrin exposure might be explained at least in part as a consequence of the reduced expression of mGLUR5 in these cells. Nevertheless, a more detailed time-course monitoring study is needed to confirm this hypothesis.

It is well known that NMDAR plays a crucial role in learning and memory, whereas the involvement of type-I mGLURs is currently not so clear. Increasing evidence suggests that there is a functional interaction between NMDAR and mGLUR5 in these processes (Gravius et al., 2006; Homayoun et al., 2004). It has also been found that K.O. mice lacking NMDAR1 or mGLUR5 show impaired learning (Cheli et al., 2006; Lu et al., 1997). Thus, the reduced functionality of NMDAR and mGLUR5 found in the present study might explain the impairment of learning and cognition observed in animals after chronic treatment with dieldrin (Gesell and Robel, 1979; Smith et al., 1976; Topinka et al., 1984).

In conclusion, here we report that long-term exposure to a noncytotoxic concentration of dieldrin causes NMDAR internalization and mGLUR5 downregulation in cultured cortical neurons. Therefore, neuronal hyperactivation, such as that produced by GABA<sub>A</sub> receptor antagonists, may disrupt normal network activity and maturation of glutamatergic neurotransmission (Stoop et al., 2003; Swann et al., 2007; this work), and it is expected that these neurons have reduced responsiveness to physiological glutamate. Rather than presenting this pollutant as a neuroprotective drug against diseases in which glutamate excitotoxicity is involved, such as cerebral ischemia or Alzheimer’s disease, here we emphasize the neurophysiologic alterations that may underlie the behavioral and learning deficits observed after prolonged exposure to persistent pesticides such as dieldrin (Gesell and Robel, 1979; Smith et al., 1976; Topinka et al., 1984).

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.
FUNDING

This work was funded by the Instituto de Salud Carlos III, Ministry of Health, Spain (grant number PI 061212); Generalitat de Catalunya (grant number 2005/SGR/00826). V.B. is recipient of a predoctoral fellowship from Institut d’Investigacions Biomèdiques August Pi i Sunyer.

ACKNOWLEDGMENTS

The authors would like to thank Sara Sánchez-Redondo for her technical assistance.

REFERENCES


