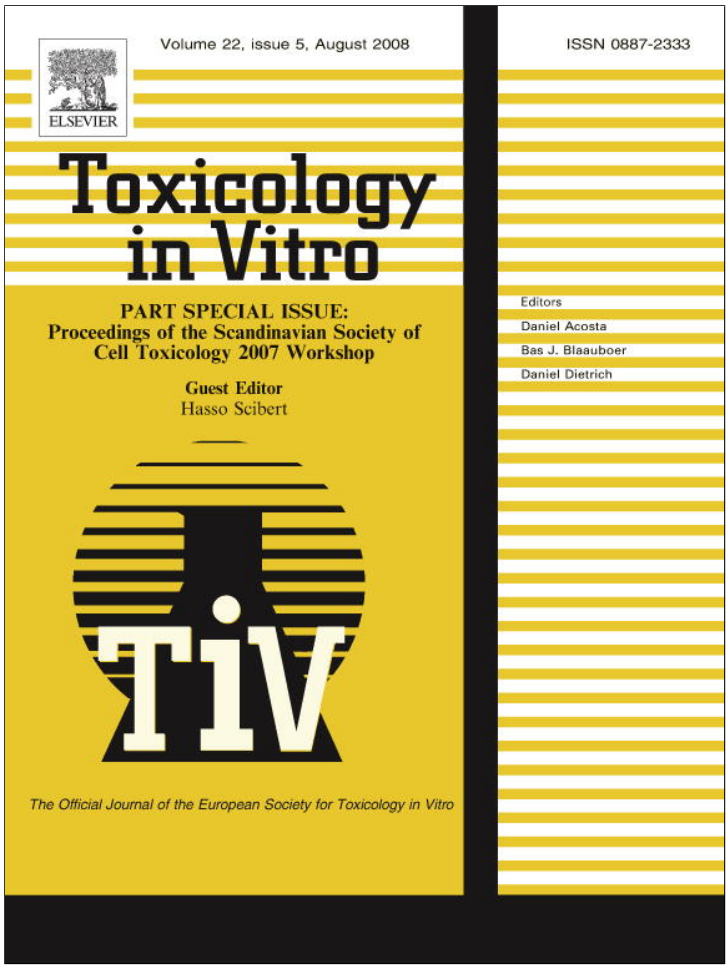

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Studies with neuronal cells: From basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity

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ABSTRACT

Neurotoxicology considers that chemicals perturb neurological functions by interfering with the structure or function of neural pathways, circuits and systems. Using *in vitro* methods for neurotoxicity studies should include evaluation of specific targets for the functionalism of the nervous system and general cellular targets. In this review we present the neuronal characteristics of primary cultures of cortical neurons and of cerebellar granule cells and their use in neurotoxicity studies. Primary cultures of cortical neurons are constituted by around 40% of GABAergic neurons, whereas primary cultures of cerebellar granule cells are mainly constituted by glutamatergic neurons. Both cultures express functional $GABA_A$ and ionotropic glutamate receptors. We present neurotoxicity studies performed in these cell cultures, where specific neural targets related to GABA and glutamate neurotransmission are evaluated. The effects of convulsant polychlorocycloalkane pesticides on the $GABA_A$, glycine and NMDA receptors points to the $GABA_A$ receptor as the neural target that accounts for their *in vivo* acute toxicity, whereas NMDA disturbance might be relevant for long-term toxicity. Several compounds from a list of reference compounds, whose severe human poisoning result in convulsions, inhibited the $GABA_A$ receptor. We also present cell proteomic studies showing that the neurotoxic contaminant methylmercury affect mitochondrial proteins. We conclude that the *in vitro* assays that have been developed can be useful for their inclusion in an *in vitro* test battery to predict human toxicity.

1. Introduction

In the field of the neurobiology, *in vitro* techniques based on cellular cultures have been developed and used successfully for the study of specific questions of cellular biology and function of the nervous system. These *in vitro* methods can also be used to analyze the neurotoxic effects of chemical substances and to study the underlying mechanisms of neurotoxicity. This way, the cellular or functional changes examined by means of *in vitro* models can be related with mechanisms associated with toxic effects. These studies should result in the development of systems and methods for the evaluation of the neurotoxic potential and potency of chemical compounds, drugs, environmental pollutants or chemical products of industrial use.

The approaches for the development and standardization of appropriate systems for the evaluation and detection of neurotoxic compounds should include general cellular targets and specific targets indicative of the functionalism of the nervous system (Stacey and Viviani, 2001; Hartung et al., 2004). Such approach has been undertaken in the European-funded project ACuteTox, aimed to develop *in vitro* strategies for the assessment of acute human toxicity (http://www.acutetox.org). Previous *in vitro* approaches have demonstrated that general cytotoxicity assays may reasonably predict mammal acute systemic toxicity attaining a level of prediction of 70–80% (Halle, 2003; Ekwall, 1999; Kitagaki et al., 2006; Clemedson et al., 2006). However, neurotoxic events may underlie acute human toxicity that may not be predicted by using an *in vitro* test that exclusively relies on cell death, contributing to the observed 20–30% lack of predictability. Primary neuronal cultures, reaggregate cultures, and cell lines are being used in this project, to evaluate specific functions of the nervous system (uptake and release of neurotransmitters, voltage-operated ion channels, ionotropic receptors – $GABA_A$, glutamate NMDA and AMPA/KA, and nicotinic acetylcholine –), neural gene expression and general cellular functions (plasmatic and mitochondrial membrane potential, intracellular Ca^{2+} homeostasis, energy metabolism, red-ox status).

In the present review we show the characteristics of primary cultures of mice cortical neurons and of cerebellar granule cells

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with respect to the GABAergic and glutamatergic neurotransmission and to the cell proteome. The neurotoxicity of different chemical compounds, organochlorine pesticides, methylmercury, and compounds included in the ACuteTox project (a reference list from the Multicentre Evaluation *In vitro* Cytotoxicity (MEIC) study that includes therapeutic and abuse drugs, pesticides and industrial chemicals; Ekwall, 1999) as analyzed in these *in vitro* systems is reviewed.

2. Primary cultures of cortical and cerebellar granule neurons

Neuronogenesis takes place during the embryonic period from E14 to E18 in the rat neocortex while it occurs postnatally from P0 to P15 for the granule cells in the rat cerebellum (Bayer et al., 1993). Tissue from the neocortex or the cerebellum is taken at defined developmental stages of neurogenesis to assure that non-differentiated neurons, at an age close to the last mitotic division of the cells, are used for the preparation of primary neuronal cultures. Therefore, primary cultures of cortical neurons are prepared from the cerebral cortices of 16-day-old mice foetuses, whereas those of cerebellar granule cells are prepared from 7-day-old mice pups (García et al., 2006; Babot et al., 2007). About 120 million cells are obtained from either the embryos of one pregnant mouse or the pups of a litter. In our hands, this leads to the preparation of 6–7 plates of 24 wells or 12–14 plates of 96 wells.

Primary cultures of cortical neurons are mainly constituted by GABAergic and cholinergic neurons (White et al., 1980; Thomas, 1985), while primary cultures of cerebellar granule cells are mainly constituted by glutamatergic neurons (Gallo et al., 1982). We used the immunocytochemistry for the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) to identify GABAergic neurons. Fig. 1 shows a microphotography of the phase contrast and the immunolabeling with GAD antibody in a primary culture of cortical neurons, where 39 ± 2% of the cells were GAD positive. On the contrary, in primary cultures of cerebellar granule neurons a minority of the cells (6%) are GAD immunoreactive (Sonnewald et al., 2004). In both cases, the astrocytic content is maintained low by the addition of antimitotic agents.

3. Neurochemical endpoints in primary cultured cortical and cerebellar granule neurons

Transport of the neurotransmitters γ-aminobutyric acid (GABA) and glutamate can be evaluated as the uptake of [^{3}H]GABA and [^{3}H]aspartate, respectively, aspartate being an analogue of glutamate that is taken by the cells through the glutamate transport system. Fig. 2A shows that [^{3}H]GABA uptake in primary cultures of cortical neurons is completely inhibited by guvacine and nipecotic acid, while β-alanine only partially inhibits [^{3}H]GABA uptake and glycine does not have any effect. On the other hand, [^{3}H]aspartate uptake in primary cultures of cerebellar granule cells (Fig. 2B) is completely inhibited by L-*t*-PDC (IC_{50}: 46 μM) and TBOA and partially by dihydrokainate (DHK) (Fonfría E; Doctoral Thesis 2002; http://www.tdx.cesca.es/TDX-0213103-133431/index_an.html). Furthermore, cultured cerebellar granule cells express the neuronal glutamate transporter EAAT3, as it has been demonstrated by western immunoblotting (inset Fig. 2B). This pharmacology is consistent with the major expression of neuronal GABA and glutamate transporters in these cellular models since β-alanine and DHK, which preferentially inhibit glial transport, have lower effects (Soudijn and Van Wijngaarden, 2000; Bridges and Esslinger, 2005; Gether et al., 2006).

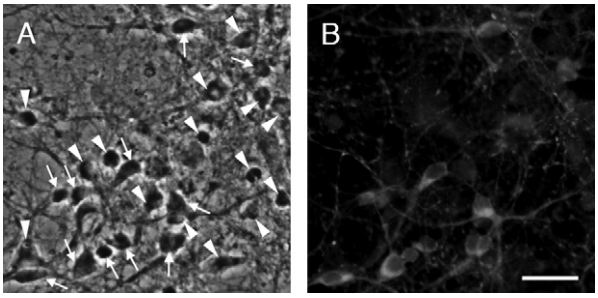

Fig. 1. Phase contrast microscopy (A) and immunostaining with glutamic acid decarboxylase (GAD) antibody (B) in the same field from a representative primary culture of mice cortical neurons grown for 8 days *in vitro*. Arrows and arrowheads indicate GABAergic and non-GABAergic cells, respectively, identified by their GAD immunostaining. Bar size: 25 μm. For methodology, see Babot et al. (2005).

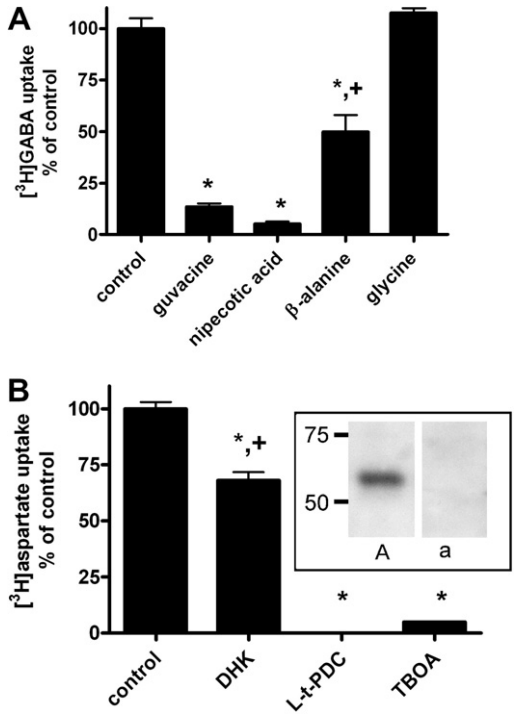

Fig. 2. (A) [^{3}H]GABA uptake in primary cultures of neocortical neurons. Data are extracted from Vale et al. (1999). (B) [^{3}H]aspartate uptake in primary cultures of cerebellar granule cells. Inhibitors were used at 1 mM. For methodology, see Fonfría et al. (2005). Inset in (B): Expression of neuronal EAAT3 glutamate transporter in primary cultures of cerebellar granule cells. Cell homogenates were immunoblotted with an anti-EAAT3 antibody (lane A) or with the peptide-preabsorbed antibody (lane a), showing the specificity of the signal for EAAT3. Reproduced from Fonfría et al. (2005), with permission. $^*p < 0.05$ with respect to control; $+p < 0.05$ with respect to guvacine and nipecotic acid for the [^{3}H]GABA uptake and to L-*t*-PDC and TBOA for the [^{3}H]aspartate uptake.

Brief exposure of primary cultures of cerebellar granule cells to high concentrations of K^+ induces a calcium-dependent release of endogenous glutamate. Thereafter, released glutamate acts on postsynaptic NMDA receptors present in these cultures producing excitotoxic neuronal death when extracellular levels of glutamate rise up to micromolar concentrations (Babot et al., 2005). Since neurodegeneration after brain trauma and stroke may have an excitotoxic component, this assay might constitute a useful *in vitro* model for studying neuroprotectant drugs against excitotoxic-mediated neurodegeneration.

Postsynaptic events in primary cultures of cortical and of cerebellar granule neurons are mainly due to activation of ionotropic GABA and glutamate receptors. Both cultured cortical neurons and cerebellar granule cells express $GABA_A$ receptors, as it has been demonstrated by the binding of the specific radioligands [^{3}H]GABA and [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{35}S]*t*-butyl bicyclo-

phosphorothionate (TBPS) at the GABA, benzodiazepine and picrotoxinin recognition sites, respectively (Pomés et al., 1993; Vale et al., 1997; García et al., 2006). The functionality of the $GABA_A$ receptor protein in both types of primary cultured neurons can be determined by measuring the $^{36}Cl^-$ influx induced by GABA (Figs. 3A and B). Furthermore, the presence of the strychnine-sensitive glycine receptor has also been demonstrated in primary cultures of cerebellar granule cells by measuring the $^{36}Cl^-$ influx induced by glycine (Fig. 3B). Similarly, glutamate receptors are also expressed in these cultured neurons. The binding of [^{3}H]MK801 and the immunolabeling of the NR1 subunit of the NMDA receptor demonstrates the presence of this receptor in cultured cerebellar granule cells (Babot et al., 2007). Likewise, the activation of the NMDA receptor in primary cultures of cortical neurons is shown in Fig. 3C, where NMDA produced an increase of intracellular [Ca^{2+}], as determined by the increase in the fluorescence of Fluo-3, like it occurs in cultured cerebellar granule cells (Babot et al., 2005, 2007). While glutamate activates a Ca^{2+}-channel operated by the NMDA receptor when the membrane is depolarized, the activation of the ionotropic glutamate receptors AMPA and kainate (KA) produces an influx of Na^+, which leads to membrane depolarization. In our laboratory, we determined the activity of AMPA/KA receptors by determining cell membrane depolarization after exposure to glutamate and kainate. This is accomplished by means of a fluorescent probe that allows determination of changes in membrane potential by using a fluorescence microplate reader (Fig. 4B). Fig. 4 also shows the changes in membrane potential in primary cultures of cortical neurons after depolarization induced by high concentrations of KCl (Fig. 4A) and by activation of neuronal Na^+ channels with veratridine (Fig. 4B). This assay, based on fluorescence measurements using microwell plates, might be relevant in determining neurotoxicity. It should be taken into account that some drugs and toxicants act on Na^+ channels (e.g., the antiepileptic drug carbamazepine and the natural toxins tetrodotoxin, saxitoxin and ciguatoxins) and on the AMPA/KA receptor (e.g., domoic acid).

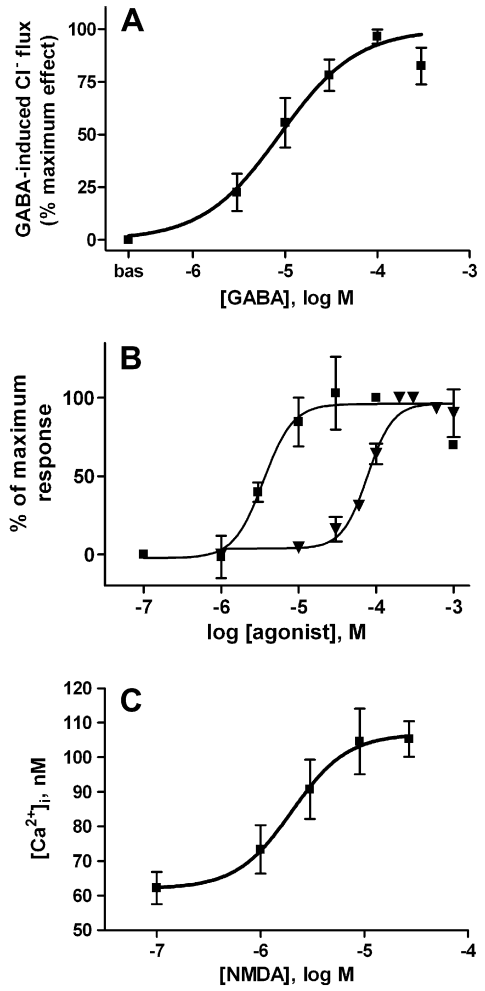

Fig. 3. $^{36}Cl^-$ uptake in primary cultures of cortical neurons (A) and in primary cultures of cerebellar granule cells (B) induced by GABA (squares) or by glycine (triangles). Reproduced from García et al. (2006) and Vale et al. (2003), with permission. (C) Increase in intracellular calcium induced by activation of the NMDA-glutamate receptor in primary cultures of cortical neurons. Data are mean ± sem, $n = 3$. For methodology, see Babot et al. (2007).

4. Studies on mechanisms of neurotoxicity

Neurons, using neurotransmitter receptor systems, are mainly excited by the neurotransmitter glutamate and inhibited by the neurotransmitter GABA. Glutamate is the excitatory neurotransmitter most widely used in the central nervous system and excessive activation of ionotropic glutamate receptors results in degeneration of neurons through a process known as excitotoxicity (Leist and Nicotera, 1998; Olney, 2002). On the other hand, the major receptor of γ-aminobutyric acid ($GABA_A$ receptor) is known to be modulated by therapeutic drugs (benzodiazepines, barbiturates, neurosteroids, and steroidal anesthetics) as well as by toxic agents (MacDonald and Olsen, 1994). The neurotoxic organochlorine pesticide γ-hexachlorocyclohexane (lindane) induces in mammals a hyperexcitability syndrome that can progress until the production of convulsions (Tusell et al., 1987; Suñol et al., 1989). We have thoroughly studied the interaction of lindane and cyclodiene-related compounds (dieldrin and α-endosulfan) with the $GABA_A$ receptor using neuronal *in vitro* systems. These pesticides inhibit [^{35}S]TBPS binding at the

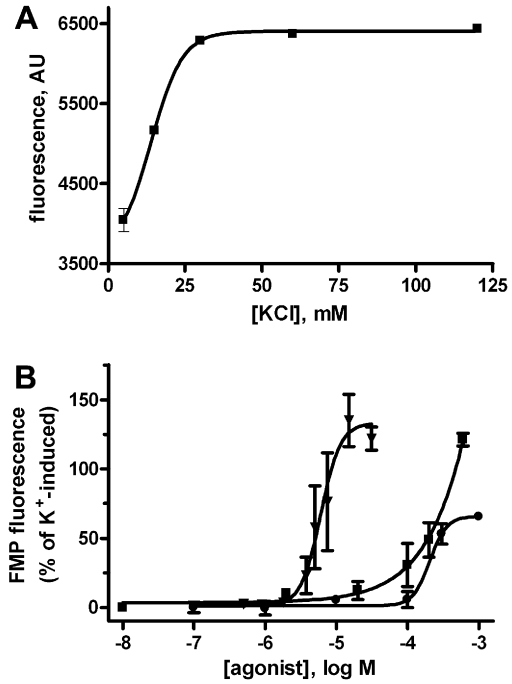

Fig. 4. Depolarizing effects of KCl (A), and of veratridine and glutamate agonists (B) in primary cultures of cortical neurons. Cells were preloaded with FLIPR® voltage sensor probe and exposed to the test agents for 5 min. Changes in membrane potential in B were normalized to the maximum depolarization induced by 60 mM KCl. Symbols: ▼, veratridine; ■, kainic acid; ●, L-glutamic acid. Data are mean ± sem, $n = 3$. The FLIPR® probe was used as indicated by the commercial supplier (Molecular Devices), according to Baxter et al. (2002).

Table 1
In vitro neuronal/*in vivo* parameters of the effects of polychlorocycloalkane pesticides

Compound	$GABA_A$ receptor		Glycine receptor	Rat LD_{50}
	[^{35}S]TBPS binding Ki (nM)[a]	$^{36}Cl^-$ influx IC_{50} (μM)[b,c]	$^{36}Cl^-$ influx IC_{50} (μM)[c]	(mg/kg)[d]
α-Endosulfan	4.2	0.1; 0.4	3.5	20–40
Dieldrin	57.1	0.2; 0.2	3.0	24–87
Endrin	3.8	n.d.	n.d.	5–43
Lindane	166.0	10; 6.1	5	88–200

n.d.: not determined.
[a] Pomés et al. (1993).
[b] Pomés et al. (1994).
[c] Vale et al. (2003).
[d] Smith (1991).

$GABA_A$ receptor but have no effect on [^{3}H]GABA or [^{3}H]flunitrazepam binding (Pomés et al., 1993; Vale et al., 1997). $GABA_A$ receptor function, measured as the influx of $^{36}Cl^-$ induced by GABA, is inhibited by lindane and cyclodienes (Pomés et al., 1994; Vale et al., 2003). Both inhibitory effects confirm these compounds as non-competitive antagonists of the $GABA_A$ receptor. Additionally, these compounds inhibit the glycine receptor (Vale et al., 2003). Based on the effects of these polychlorocycloalkane pesticides on $GABA_A$ and glycine receptors we have proposed a pharmacophoric hypothesis for this class of compounds, where a lipophilic zone and a polar zone on the drug molecules differentially contribute to their binding to $GABA_A$ and glycine receptors. We have proposed that both the hydrogen bond acceptor moiety and the hydrophobic region are responsible for the affinity of these compounds at the $GABA_A$ receptor whereas only the hydrophobic region of the molecules accounts for their interaction with the glycine receptors (Vale et al., 2003). Comparison of IC_{50}/Ki values for these compounds against the $GABA_A$/glycine receptors with respect to their oral toxicity (rat LD_{50} values) (Table 1) shows that mammal toxicity correlates better with GABA parameters than with glycine parameters, pointing to the $GABA_A$ receptor as the neural target that accounts for the *in vivo* toxicity of these compounds. The cyclodienes α-endosulfan, dieldrin and endrin inhibit both [^{35}S]TBPS binding and GABA-induced $^{36}Cl^-$ influx with higher potency than lindane, in agreement with the higher *in vivo* acute toxicity of cyclodienes with respect to lindane. Besides an inhibition of the $GABA_A$ receptor, an overactivation of NMDA-glutamate receptors could also produce acute excitatory toxic effect. We have reported that acute exposure of primary cultures of cerebellar granule cells to dieldrin lacks to modify the NMDA-induced increase in $[Ca^{2+}]_i$. However, the NMDA receptor function is reduced after long-term exposure to dieldrin, and this effect may be related to the permanent reduction in the $GABA_A$ receptor function, which in turn induces a down-regulation of glutamate neurotransmission (Babot et al., 2007). A reduced function of the NMDA receptor might underlie mechanisms of chronic dieldrin neurotoxicity, manifested as learning and behavioral deficits (Son et al., 2006). The relevance of this finding for neurotoxicity assessment lies in the fact that dieldrin may pose problems for the human health. Dieldrin is a highly persistent compound in the environment and animals, including the man; and it is one of the toxic compounds included in the Stockholm Convention on Persistent Organic Pollutants (POPs) (http://www.pops.int). Therefore, attention should be paid to the long-term effects of dieldrin on neural endpoints.

5. The use of neural endpoints to predict acute human toxicity

For the nervous system, acute toxicity correlating to adverse neuronal function is mainly a result of over-excitation or depression of the CNS or PNS, generating symptoms such as lethal convulsions, respiratory arrest or unconsciousness *in vivo*. The $GABA_A$ receptor accounts for most of the inhibitory signals in the CNS and, therefore, tightly controls neural excitability. We have determined the effects on the $GABA_A$ receptor of a set of 22 reference compounds included in the MEIC study (Multicentre Evaluation *In vitro* Cytotoxicity), as part of the neurotoxicity testing strategy undergone in the ACuteTox project. Fig. 5 shows the concentration–response curves for atropine, carbamazepine, lindane, methadone and pentachlorophenol, which inhibited the $GABA_A$ receptor in primary cultures of cortical neurons. It should be noticed that severe human poisoning with these compounds results in seizures (Ada Kolman, privileged communication within the ACuteTox consortium, to be published), which could correlate with the observed inhibition of the $GABA_A$ receptor. On the other hand, the therapeutic drugs diazepam and phenobarbital increased the GABA-induced $^{36}Cl^-$ uptake in agreement with their known mechanism of action as positive modulators of the $GABA_A$ receptor. Also, isopropanol and mercury II chloride increased $^{36}Cl^-$ influx. Other compounds, like acetaminophen, acetyl salicylic acid, cycloheximide, sodium valproate, glufosinate ammonium and nicotine did not modify the $GABA_A$ receptor function when tested up to 1E-3 M. No cytotoxicity was observed in cultures similarly exposed to the compounds at concentrations shown to have inhibitory effects on the $GABA_A$ receptor, clearly supporting a neurotransmission-mediated toxicity for these compounds.

6. Omics in *in vitro* studies of neurotoxicty

Neurotoxicity is also the consequence of gene or protein changes. To address these questions, genomic and proteomic approaches can be undertaken. Differential proteomics give information on changes in protein expression, by comparing the protein spots in two-dimension electrophoresis gels and analyzing these spots by mass spectrometry, resolving the whole array of cell proteins in a unique assay. We have analyzed the cell proteome of cultured cerebellar granule cells, where more than 800 spots are visualized in a two-dimensional electrophoresis gel (Vendrell et al., 2007). According to the mass spectrometric identification of the 100 most abundant spots, 43% corresponded to cytosolic, 26% to mitochondrial, 8% to nuclei, 4,5% to lysosomal and endoplasmic reticulum proteins and 3% to membrane proteins. The latest group of proteins were poorly recovered, due to the general buffer used to prepare total protein extracts, which is not appropriate to extract specifically membrane proteins. In these cells, exposure to a low concentration of methylmercury (60 nM) for 10 days *in vitro* resulted in a decrease of the amount of the mitochondrial protein 3-ketoacid-coenzyme A transferase I (Vendrell et al., 2007), reinforcing other works that indicate the mitochondria as one of the targets for methylmercury neurotoxicity (Fonfría et al., 2002).

7. Concluding remarks

We present evidences that primary cultures of cortical neurons and of cerebellar granule cells are useful *in vitro* models to evaluate GABA and glutamate neurotransmission. It should be taken into account that neurons, using neurotransmitter receptor systems, are mainly excited by the neurotransmitter glutamate and inhibited by the neurotransmitter GABA, both systems being developed in the *in vitro* neural models as demonstrated by their neurochemical properties. Primary cultures of cortical neurons contain an important proportion of GABAergic neurons like in the neocortex, where GABAergic neurons are thoroughly distributed forming an inhibitory neuronal network. Primary cultures of cerebellar granule cells are representative of the largest homogeneous neuronal popula-

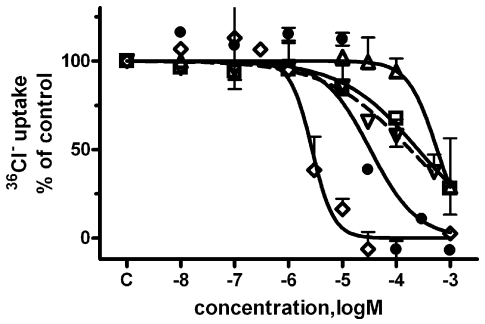

Fig. 5. Testing of a set of compounds for their inhibitory effect on the $GABA_A$ receptor in primary cultures of cortical neurons. The selected compounds are part of a list of compounds included in the European project ACuteTox (http://www.acute-tox.org). Results are mean ± sem of 3–4 experiments, each concentration determined in triplicate. For methodology, see Vale et al. (2003). Symbols: □, atropine; Δ, carbamazepine; ●, lindane; ◇, pentachlorophenol; Δ, methadone.

tion in the cerebellum, where more than 90% of the neurons in the cerebellum are granule neurons, and have been used for years for many neurobiology laboratories (Contestabile, 2002). The characterization of presynaptic and postsynaptic events in the primary cultured neurons herein reviewed is in agreement with the fact that these neurons, when differentiated *in vitro*, develop neural properties that are representative of the GABAergic interneurons in the mature cortex and of the granule neurons in the cerebellar granule layer. Therefore, both presynaptic (neurotransmitter transport) and postsynaptic (ionotropic receptor activation/blocking) GABA and glutamate events susceptible to be targeted by chemical compounds can be evaluated using these *in vitro* neural systems.

Mechanisms of neurotoxiciy have been fully validated using primary neuronal cultures. Block of the GABA-induced Cl^- flux by lindane and polychlorocycloalkane pesticides is in agreement with their binding on the picrotoxinin recognition site at the $GABA_A$ receptor using rat brain homogenates (Llorens et al., 1990). Furthermore, the inhibition of the $GABA_A$ receptor is in agreement with the reported excitatory and convulsant effects of polychlorocycloalkanes in mamals. Twelve from the twenty-two compounds tested in the frame of the ACuteTox project were active at the $GABA_A$ receptor, either inhibiting or potentiating its function. Only lindane, isopropyl alcohol, diazepam and phenobarbital were known to interact with the $GABA_A$ receptor. It might be that the $GABA_A$ receptor is not the main target for all the compounds that inhibited Cl^- influx in the present assay, like for methadone that preferentially inhibited noradreline uptake (A. Forsby, privileged communication within the ACuteTox consortium, to be published). However, the fact that compounds inhibiting the $GABA_A$ receptor produce convulsions in humans when severely intoxicated supports the inclusion of the $GABA_A$ receptor assay in an *in vitro* neurotoxicity testing strategy. We also propose that primary cultured neurons are useful *in vitro* systems to address studies of long-term toxicity, where different neural mechanisms can be involved. We have demonstrated that the long-term reduction of the $GABA_A$ receptor function by dieldrin induces a down-regulation of glutamate neurotransmission. Other authors have also reported that there is a homeostatic control of the balance between excitation and inhibition 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 neural activity (for review see Pérez-Otaño and Ehlers, 2005). Finally, other compounds produce neurotoxicity by targeting both specific neurotransmission endpoints and general cellular functions. For example, methylmercury inhibits glutamate transport (Fonfría et al., 2005) and produces oxidative stress (Vendrell et al., 2007), but it is still unknown the processes underlying its toxicity. For these compounds, proteomic studies using primary neuronal cultures are a promising tool to unravel their mechanisms of neurotoxicity.

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