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$GABA_A$ receptor and cell membrane potential as functional endpoints in cultured neurons to evaluate chemicals for human acute toxicity

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ABSTRACT

Toxicity risk assessment for chemical-induced human health hazards relies mainly on data obtained from animal experimentation, human studies and epidemiology. *In vitro* testing for acute toxicity based on cytotoxicity assays predicts 70–80% of rodent and human toxicity. The nervous system is particularly vulnerable to chemical exposure which may result in different toxicity features. Acute human toxicity related to adverse neuronal function is usually a result of over-excitation or depression of the nervous system. The major molecular and cellular mechanisms involved in such reactions include GABAergic, glutamatergic and cholinergic neurotransmission, regulation of cell and mitochondrial membrane potential, and those critical for maintaining central nervous system functionality, such as controlling cell energy. In this work, a set of chemicals that are used in pharmacy, industry, biocide treatments or are often abused by drug users are tested for their effects on $GABA_A$ receptor activity, GABA and glutamate transport, cell membrane potential and cell viability in primary neuronal cultures. $GABA_A$ receptor function was inhibited by compounds for which seizures have been observed after severe human poisoning. Commonly abused drugs inhibit GABA uptake but not glutamate uptake. Most neurotoxins altered membrane potential. The $GABA_A$ receptor, GABA uptake and cell membrane potential assays were those that identified the highest number of chemicals as toxic at low concentrations. These results show that *in vitro* cell assays may identify compounds that produce acute neurotoxicity in humans, provided that *in vitro* models expressing neuronal targets relevant for acute neural dysfunctions are used.

1. Introduction

Toxicity risk assessment for chemical-induced human health hazards relies mainly on data obtained from animal experimentation, human studies and epidemiology. The nervous system is particularly vulnerable to chemical exposure; its complexity results in multiple potential target sites with different toxicity features. Acute human toxicity related to adverse neuronal function is mainly a result of over-excitation or depression of the peripheral or central nervous system (CNS). The major molecular and cellular mechanisms involved in such effects include GABAergic, glutamatergic and cholinergic neurotransmission, regulation of cell and mitochondrial membrane potential, and those critical for maintaining CNS functionality, such as controlling cell energy. Severe disturbance of these mechanisms may result in convulsions, fatal central depression and cell death. Compared to other tissues, nerve cells have little ability to replace themselves or regenerate, which limits full recovery when cell damage occurs. Therefore, it is crucial to develop models that predict and can detect neurotoxic chemicals. Recently implemented regulations such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in the EU (EC 1907/2006 directive) (http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm), and the High Production Volume (HPV) Program in the USA (http://www.epa.gov/HPV), are designed to manage the risks from chemicals and provide safety information on them. In this context, non-animal testing allowing high-throughput analysis of a huge number of chemicals is foreseen and promoted in the REACH program.

In vitro testing for acute toxicity based on general cytotoxicity assays, such as the Registry of Cytotoxicity (RC) and the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC), can predict 70–80% of rodent and human toxicity [8,19,22]. There are many reasons for the 20–30%

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failure: i) chemical metabolism, ii) barrier passage, iii) biokinetics, and iv) organ-specific toxicity (neurotoxicity, hepatotoxicity, nephrotoxicity, etc.). Alternative testing strategies, including biokinetic models and endpoints for organ specific toxicity, aim to define alerts and correctors to improve this failure rate in prediction when using exclusively cytotoxicity-based assays. Such strategies would either reveal factors that improve the correlation between *in vitro* cytotoxic data and acute human toxicity, or define alerts that identify outliers (compounds for which the cytotoxicity *in vitro* data give a false evaluation of their acute human toxicity).

Neurotoxic events are the consequence of the failure of one or several molecular mechanisms (see [29,39]) such as the functioning of ion channels (voltage- or ligand-operated ionotropic receptors, such as $GABA_A$, NMDA, AMPA/kainate, and nicotinic acetylcholine), the transport of amino acidergic and aminergic neurotransmitters, neurotransmitter synthesis and degradation involving enzymes, cell energy control, the regulation of cell and mitochondrial membrane potential, intracellular calcium homeostasis and control of the production and inactivation of reactive oxygen species. The amino acids γ-aminobutyric acid (GABA) and glutamate are, respectively, the most common inhibitory and excitatory neurotransmitters in the CNS. Once released into the synapse they act on ionotropic receptors of GABA ($GABA_A$) and glutamate (NMDA and AMPA/kainate). Whereas excessive potentiation of the $GABA_A$ receptor activity results in central depression, inhibition of $GABA_A$ receptor activity results in overall excitatory symptoms and convulsions in mammals [26,28]. Conversely, excessive activation of ionotropic glutamate receptors results in excitatory symptoms and in degeneration of neurons through a process known as excitotoxicity [23,31].

In vitro evaluation of these neuronal specific and general endpoints requires well-characterized cell culture systems. Primary neuronal cultures of cortical and cerebellar granule cells are enriched in GABAergic and cholinergic neurons [41,42,48], and in glutamatergic neurons [14,38], respectively. Transport of the neurotransmitters GABA and glutamate can be evaluated by determining the uptake of [^{3}H]GABA in cortical neurons and of [^{3}H]aspartate or [^{3}H]glutamate in cerebellar granule neurons, respectively (aspartate being an analogue of glutamate that is taken up by the cells through the glutamate transport system). Furthermore, both cultured cortical neurons and cerebellar granule cells express functional ionotropic $GABA_A$ and glutamate receptors, which are neural targets for depressant, convulsant and excitotoxic compounds. For a review of the functional characteristics of these *in vitro* systems see [41].

In the present work we select a set of compounds (Table 1 in Supplementary Material) based on the European project ACUTETOX (www.acutetox.org). We then test the effect of these compounds on several neuronal functional endpoints and on cell viability in primary cultured neurons. Most of the reference compounds were chosen from the MEIC project [10] and the NICEATM/ECVAM *In Vitro* Cytotoxicity Validation Study [1] and are pharmaceuticals, pesticides, and industrial chemicals, for which data on their acute human toxicity exist. Both compounds whose acute systemic toxicity was well predicted by general cytotoxicity tests and compounds identified as outliers (i.e., their toxicity was poorly predicted) were included in the list. In addition, some of the reference compounds were selected as positive control compounds for specific target endpoints in the nervous system, kidney and liver. The ACuteTox project aims to improve predictability by combining a handful of simple and robust tests that measure complementary parameters such as absorption, distribution and metabolism, as well as organ specific toxicity. The objective of these research activities is to increase knowledge of the mechanisms by which these compounds cause toxicity and to identify corrector/alert assays in order to improve the *in vivo*/*in vitro* correlation with the aim to use the in vitro test strategy for regulatory classification and risk assessment. The compounds selected have been tested by different research groups using relevant *in vitro* assays and *in silico* approaches including basal cytotoxicity, barrier passage, metabolism, hepatotoxicity, renal toxicity and neurotoxicity. Here we present results concerning GABAergic and glutamatergic neurotransmission (as the main depressant and excitatory neural systems) and cell membrane potential (which determines neural electrical excitability) in primary cultures of cortical neurons and of cerebellar granule cells.

2. Methods

2.1. Materials

Pregnant NMRI mice (16th day of gestation) and 7-day-old NMRI mice were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic multi-well culture plates were purchased from Nunc™ (Roskilde, Germany). Foetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain) and Dulbecco's modified minimum essential medium (DMEM) from Biochrom (Berlin, Germany). $^{36}Cl^-$ (111–532 Mbq/g), [^{3}H]-flunitrazepam (≈3 TBq/mmol), [^{3}H]-GABA (≈3 TBq/mmol) and [^{3}H]-D-aspartate (1 TBq/mmol) were obtained from Amersham Life Science (Buckinghamshire, UK). Optiphase Hisafe 2 liquid scintillation cocktail was obtained from Wallace Oy (Turku, Finland). The FMP membrane potential assay kit (blue) was from Molecular Devices (Sunnyvale, CA, USA). 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan (MTT) was from Sigma Chemical Co. (St. Louis, MO, USA) and the lactate dehydrogenase (LDH) cytotoxicity kit was from Roche (Manheim, Germany). Fluo-3/AM was obtained from Molecular Devices.

2.2. Chemicals

Of the first set of reference compounds: acetaminophen, acetylsalicylic acid, carbamazepine, cycloheximide, diazepam, digoxin, mercury (II) chloride, phenobarbital, sodium lauryl sulphate (SDS), verapamil hydrochloride, nicotine, (±)methadone hydrochloride, d-amphetamine sulphate, sodium valproate, pentachlorophenol and isopropyl alcohol were obtained from Sigma Chemical Co; atropine sulphate monohydrate, caffeine, colchicine and ethanol were obtained from Fluka (St. Louis, MO, USA); and malathion, lindane and glufosinate ammonium were obtained from Riedel de Haen (St. Louis, MO, USA).

Of the second set of reference compounds: 5-fluorouracil, tert-butylhydroperoxide, rifampicine, tetracycline hydrochloride, cadmium (II) chloride, cyclosporin A, 17α-ethynylestradiol, lithium sulphate, warfarin, 2,4-dichlorophenoxyacetic acid, strychnine, pyrene, hexachlorobenzene, amiodarone hydrochloride, parathion, dichlorvos, physostigmine, cis-diamminiumplatinum (II) dichloride, (−)-epinephrine bitartrate, ochratoxin A, sodium chloride, thallium sulphate, sodium selenate, dimethylformamide, amitriptyline hydrochloride, ethylene glycol, methanol, lithium sulphate, arsenic trioxide, chloral hydrate, acetonitrile and propanolol hydrochloride were obtained from Sigma Chemical Co; acryaldehyde, orphenadrine hydrochloride and diethylene glycol were obtained from Fluka; and diequat dibromide was obtained from Riedel de Haen.

The chemicals were dissolved and diluted in HEPES-buffered saline solution or in DMSO. When dissolved in DMSO, a 200× concentration was prepared, thus the concentration of DMSO in the testing solution was 0.5%. Controls contained the same amount of DMSO, when so required.

2.3. Neuronal cultures

Primary cultures of cortical neurons were obtained from neocortices from 16-day-old mice foetuses, using 10–12 foetuses from one pregnant mouse for each culture preparation. Pregnant animals were anesthetized with isofluorane (FORANE®, Abbott Laboratories SA, Madrid, Spain) and killed by cervical dislocation. Primary cultures of cerebellar granule cells were obtained from 7-day-old pups, using a litter of 10 pups per culture. Pups were killed by decapitation.

Table 1
Concentrations of reference chemicals that were effective on the $GABA_A$ receptor in primary cultured cortical neurons.

Compound	IC_{20} (M)	IC_{50} (M)	IC_{80} (M)	I_{max} (%)
Inhibition>80% at the maximal concentration tested				
17α-Ethynylestradiol	6.3E-7±3.0E-7	2.5E-6±1.2E-6	1.1E-5±0.4E-5	89±11
Atropine	3.5E-5±2.3E-5	2.1E-4±1.4E-4	1.3E-3±0.8E-3	85±10
2,4-Dichlorophenoxyacetic acid	6.0E-4±4.0E-4	1.8E-3±1.2E-3	5.8E-3±3.9E-3	100±0
Lindane	2.6E-5±0.1E-5	3.1E-5±0.2E-5	3.7E-5±0.2E-5	100±0
Malathion	2.5E-6±0.7E-6	3.9E-6±1.9E-6	1.1E-4±0.3E-4	100±0
Parathion	1.3E-6±0.4E-6	5.3E-6±1.6E-6	2.1E-5±0.6E-5	94±6
Pentachlorophenol	2.2E-6±0.8E-6	3.9E-6±1.5E-6	7.8E-6±2.6E-6	99±1
Rifampicine	1.2E-6±0.3E-6	8.9E-6±2.7E-6	7.0E-5±2.1E-5	84±8
Sodium lauryl sulphate	1.4E-6±0.5E-6	2.6E-6±0.9E-6	5.2E-6±1.8E-6	98±2
Strychnine	9.5E-6±7.3E-6	3.8E-5±2.9E-5	1.5E-4±1.2E-4	100±0
Verapamil	2.4E-5±0.9E-5	1.7E-4±0.6E-4	1.2E-3±0.4E-3	81±6
Warfarin	2.0E-4±1.5E-4	8.0E-4±6.0E-4	3.1E-3±2.3E-3	91±10
50%<Inhibition at the highest concentration tested<80%				
Acrylaldehyde	2.7E-3±0.3E-3	1.1E-2±0.1E-2		75±5
t-Butylhydroperoxide	9.5E-3±4.8E-3	3.8E-2±1.9E-2		68±11
Carbamazepine	1.1E-5±0.2E-5	2.3E-4±0.4E-4		52±12
Colchicine	1.5E-4±0.3E-4	4.8E-4±0.8E-4		72±5
Cyclosporine A	7.7E-6±3.7E-6	3.1E-5±1.5E-5		64±13
(−)-Epinephrine bitartrate	2.6E-3±1.4E-3	1.2E-3±0.5E-3		67±17
5-Fluorouracil	7.1E-5±3.5E-5	2.8E-4±1.4E-4		61±3
Lithium sulphate	2.1E-2±0.6E-2	8.5E-2±2.6E-2		55±3
Methadone	3.1E-4±1.1E-4	7.4E-4±2.7E-4		69±16
20%<Inhibition at the highest concentration tested<50%				
Cadmium chloride	3.0E-4±0.8E-4			41±6
Digoxin	1.0E-4±0.3E-4			39±2

	EC_{50} potentiation (M)	E_{max} (%)
Diazepam	2.5E-8±1.1E-8	54±13
Phenobarbital	7.2E-5±3.7E-5	133±42
Isopropyl alcohol	2.6 E-5	52±8
Mercuric chloride	1.4E-4±1.3E-4	218±27
Chloral hydrate	8.7E-6±0.9E-6	89±12

$GABA_A$ receptor activity was determined as percentage of $^{36}Cl^-$ uptake induced by 100 μM GABA (inhibition experiments) or 5 μM GABA (potentiation experiments). Values are mean±S.E.M. ($n=3$–4). I_{max} refers to the percentage of inhibition. E_{max} refers to the percentage of potentiation with respect to that induced by 100 μM GABA.

Primary cultures were prepared as previously described [3,15]. In brief, the neocortices from foetuses or cerebella from pups were minced, with cells then dissociated by mild trypsinization (0.02–0.025% w/v) at 37 °C for 10 min followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were re-suspended in modified DMEM solution (31 mM glucose and 0.2 mM glutamine), supplemented with insulin, penicillin and 10% foetal calf serum, containing 5 mM KCl for cortical neurons and 25 mM KCl for cerebellar granule cells. The cell suspension (1.6×10^6 cells/ml) was seeded in 24-well plates (0.5 ml/well) and 96-multi-well plates (0.1 ml/well), pre-coated with poly-D-lysine, and incubated for 6–9 days in a humidified 5% CO_2/95% air atmosphere at 36.8 °C without changing the culture medium. After 24–48 h in culture, the primary cultures were treated with the mitotic inhibitor (5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine) to prevent glial proliferation. Cells were fully differentiated after 6–8 days *in vitro* and ready to use for the testing assays. Usually, 6 plates of 24 wells or 12 plates of 96 wells (filling the external wells with water) were obtained from each culture preparation.

Animals were handled in compliance with the protocols of the University of Barcelona, as approved by the *Generalitat de Catalunya*, Spain, in accordance with EU guidelines, and in compliance with the Office of Laboratory Animal Welfare (OLAW)/National Institutes of Health (NIH).

2.4. Chloride influx

Chloride influx was determined as $^{36}Cl^-$ uptake in intact cell cultures of cortical neurons grown in 24-well plates [15,45]. Briefly, the culture medium was replaced by a pre-warmed 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 incubation, the buffer was replaced by new EBSS solution and incubation continued for an additional 15 min. Cells were pre-incubated with test agent solutions prepared in EBSS for 10 min at room temperature. After this, the cells were incubated for 10 s with 225 μl HEPES-buffer (HBSS: 136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2P0_4, 10 mM HEPES and 9 mM glucose, adjusted to pH 7.3) containing $^{36}Cl^-$ (0.4 μCi/ml), 5 μM or 100 μM GABA and different concentrations of the test agents. 100 μM picrotoxinin was used as a positive control of the assay. Plates where picrotoxinin did not inhibit GABA-induced $^{36}Cl^-$ uptake were discarded. After removing the $^{36}Cl^-$ solution, each well was immediately rinsed four times with 1.5 ml cold HBSS solution. Cells were lysed by water-induced hyposmotic shock, and radioactivity was determined by liquid scintillation counting in a Wallac 1414 Winspectral™.

2.5. Flunitrazepam binding assay

[^{3}H]-Flunitrazepam binding to intact cultured cells of cortical neurons was performed in 24-well plates [15]. In brief, attached cells were rinsed with pre-warmed (37 °C) HBSS solution and pre-incubated together with this solution containing different concentrations of the test agents for 10 min at 25 °C. The HBSS solutions were then replaced with fresh solutions containing different concentrations of the compounds in the presence of 100 μM GABA and 2–4 nM [^{3}H]-flunitrazepam. Basal flunitrazepam binding performed in the absence of GABA was subtracted to obtain GABA-enhanced

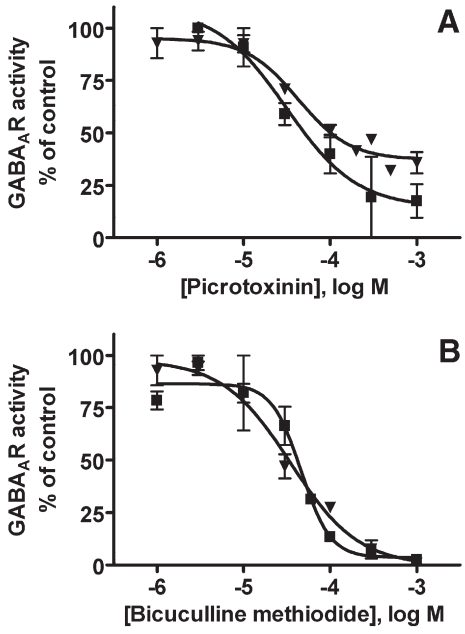

Fig. 1. $GABA_A$ receptor antagonists picrotoxinin (A) and bicuculline (B) inhibit the $^{36}Cl^-$ influx and the increase of [^{3}H]-flunitrazepam binding induced by 100 μM GABA in primary cultures of cortical neurons. ■, $^{36}Cl^-$ influx assay; ▼, [^{3}H]-flunitrazepam binding assay. Values are mean±S.E.M of 3 independent experiments, each concentration point determined in triplicate.

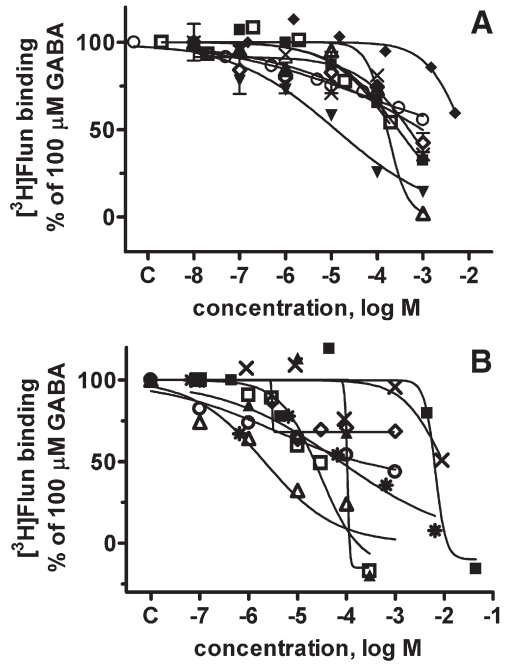

Fig. 2. Effect of compounds that inhibit GABA-induced $^{36}Cl^-$ influx on the allosteric response of [^{3}H]-flunitrazepam binding induced by GABA in primary cultures of cortical neurons. A) pharmaceutical chemicals: ▼, 17-α-ethynylestradiol; ■, methadone; ♦, epinephrine bitartrate; △, atropine; ○, rifampicine; ◇, colchicine; □, verapamil; ✖, carbamazepine. B) industrial and pesticide chemicals: ▲, sodium lauryl sulfate; ✱, warfarin; ■, acrylaldehyde; ✖, 2,4-dichlorophenoxyacetic acid; △, pentachlorophenol; ◇, parathion; ○, malathion; □, strychnine. Values are mean ± S.E.M ($n = 3$). Error bars are omitted for clarity.

flunitrazepam binding. Following 30 min incubation at 25 °C, the medium was aspirated and the cells were quickly rinsed four times with cold HBSS solution. Cells were digested in 0.2 M NaOH overnight and bound radioactivity was determined by liquid scintillation counting.

2.6. GABA uptake

GABA transport was determined as [^{3}H]-GABA uptake in primary cultures of cortical neurons. Intact cortical cultured cells were rinsed with pre-warmed HBSS solution and incubated for 10 min at 35 °C in the same buffer containing 4 nM [^{3}H]-GABA, 10 µM GABA (approximate Km for GABA in cortical neurons [44]) and different concentrations of the test agents. The assay was terminated by rapid washing with ice-cold buffer. The cells were digested with 0.2 M NaOH overnight and incorporated radioactivity was determined by liquid scintillation counting. Specific uptake was determined by subtracting radioactivity measurements carried out in the presence of 5 mM GABA.

2.7. Glutamate uptake

Glutamate transport was determined as [^{3}H]-D-aspartate uptake, since D-aspartate is a non-metabolized substrate for glutamate transporters, as previously reported [12]. Primary cultures of cerebellar granule cells were washed three times with HBSS solution and incubated with HBSS buffer containing [^{3}H]-D-aspartate (3 nM), 60 µM glutamate (Km for glutamate transport in cerebellar granule cells [47]) and different concentrations of the test agent for 10 min at 36 °C. Cells were digested in 0.2 M NaOH overnight and incorporated radioactivity was determined by liquid scintillation counting. Specific uptake was determined by subtracting radioactivity measurements carried out in the presence of 1 mM glutamate.

2.8. Intracellular calcium in Mg^{2+}-free buffer

The increase of intracellular calcium in cultured cortical neurons in Mg^{2+}-free buffer was used to determine the activity of chemicals on *N*-methyl-D-aspartic acid (NMDA) receptors, as previously described [2,3]. In brief, NMDA receptor function was determined by measuring the increase in Fluo-3 fluorescence in Mg^{2+}-free buffer to relieve the physiological blocking of the NMDA receptor by Mg^{2+}. Cultured cells were incubated with 9 µM Fluo-3 AM for 1 h at 36.8 °C in HBSS solution. Excess Fluo-3 AM was rinsed away and the cells were treated with test agents in the absence and in the presence of 5 or 100 µM NMDA. Fluorescence (Ex 485/Em 530) was immediately determined in a fluorimeter plate reader (SpectraMax GeminisXS; Molecular Devices Corporation, Sunnyvale, CA, USA).

2.9. Cell membrane potential

The FMP Membrane Potential Assay kit-BLUE (Explorer kit) (FMP) (Molecular Devices Corporation) was used to detect changes in the voltage across cell membranes. Cultured cortical neurons grown for 6–9 days in 96-well plates were rinsed with pre-warmed Hanks solution (136.9 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, 5.5 mM glucose) and incubated with Hanks solution for 20 min at 37 °C in darkness. After this, the cells were loaded with FMP and incubated for 30 min at 37 °C. After measuring basal fluorescence, Hanks solution containing different concentrations of the test agent (5×) was added and fluorescence read after 5 min. Fluorescence was determined in a bottom-reader fluorimeter plate reader (Cytofluor 2350, Millipore) at an Ex/Em of 530/590 nm. 30 mM KCl was used as a positive control of cell membrane depolarization and to normalize fluorescence values.

2.10. Cell viability

The LDH assay was used to determine cell membrane leakage. Cultured cortical neurons grown in 96-well plates were rinsed and incubated for 30 min at 36 °C with HEPES buffered saline solution containing different concentrations of the test agents. Control wells contained the vehicle, and Triton X-100 was added to three wells to obtain maximum cytotoxicity. The activity of the released LDH was determined in an enzymatic test by using the Cytotoxicity Detection Kit from Roche, according to the manufacturer's instructions. In brief, after removing the incubation solution, 100 µl of the LDH Reagent Solution was added to each well. The cells were incubated in the dark

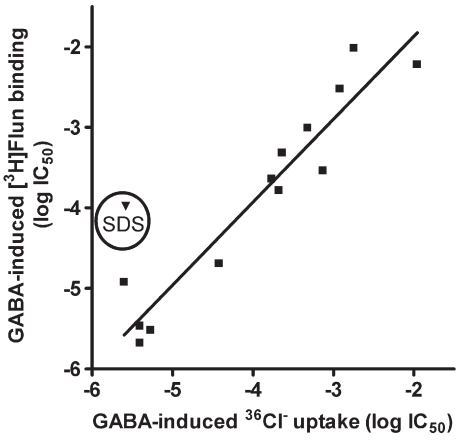

Fig. 3. Correlation between IC_{50} values for [^{3}H]-flunitrazepam binding and ^{36}-Cl^- uptake in the presence of 100 µM GABA in primary cultures of cortical neurons for compounds that inhibited $GABA_A$ receptor activity. Sodium lauryl sulphate (SDS) value was excluded from the correlation.

Table 2
Concentrations of the 1st set of reference compounds that inhibited amino acid transport in cultured neurons.

	GABA transport			Glutamate transport		
Compound	IC_{20} (M)	IC_{50} (M)	IC_{80} (M)	IC_{20} (M)	IC_{50} (M)	IC_{80} (M)
Mercuric chloride	4.1E-6 ± 1.0E-6	6.8E-6 ± 1.6E-6	1.1E-5 ± 0.3E-5	1.2E-6 ± 0.5E-6	1.6E-6 ± 0.6E-6	2.2E-6 ± 0.9E-6
Pentachlorophenol	1.4E-4 ± 0.5E-4	2.0E-4 ± 0.5E-4	3.0E-4 ± 0.5E-4	4.8E-6 ± 2.2E-6	1.6E-5 ± 0.7E-5	5.1E-5 ± 2.4E-5
Sodium lauryl sulphate	8.8E-5 ± 0.3E-5	9.9E-5 ± 0.4E-5	1.1E-4 ± 0.04E-4	4.3E-5 ± 0.5E-5	7.0E-5 ± 0.9E-5	1.2E-4 ± 0.1E-4
Methadone	5.9E-5 ± 0.6E-5	1.7E-4 ± 0.2E-4	4.7E-4 ± 0.5E-4	>1E-3		
Verapamil	4.7E-5 ± 0.6E-5	1.4E-4 ± 0.2E-4	3.9E-4 ± 0.5E-4	>1E-3		
Digoxin	2.1E-5 ± 0.2E-5	1.4E-4 ± 0.1E-4	>5E-4	3.1E-6 ± 1.8E-6	1.2E-5 ± 0.7E-5	4.7E-5 ± 2.9E-5
Diazepam	3.9E-5 ± 0.3E-5	1.3E-4 ± 0.1E-4		>1E-3		
Amphetamine	8.2E-5 ± 3.0E-5	1.4E-3 ± 0.5E-3		>1E-3		
Malathion	>1E-3			9.2E-4 ± 0.1E-4	>1E-3	
Isopropyl alcohol	8.9E-3 ± 4.1E-3			>1E-3		
Carbamazepine	8.1E-4 ± 3.5E-4			>1E-3		

GABA and glutamate transport were determined as [^{3}H]GABA uptake in cultured cortical neurons and [^{3}H]-aspartate uptake in cultured cerebellar granule cells, respectively. Values are mean ± S.E.M. ($n = 3$).

at room temperature for 30 min, in an orbital shaker. Thereafter, the enzymatic reaction was stopped by adding 50 µl 1 M HCl to each well. After removing the bubbles, the absorbance was measured at 492 nm in a spectrophotometer plate reader, using a reference wavelength of 620 nm (iEMS Reader MF; Labsystems, Helsinki, Finland).

The reduction of MTT to a coloured formazan salt by mitochondrial reducing activity, was measured as previously described [15]. Briefly, cells grown in 96-well plates were rinsed and incubated for 30 min at 36 °C with HEPES buffered saline solution containing different concentrations of the test agents or vehicle. Following the incubation period, the cells were rinsed with HBSS solution and then incubated for 20 min at 37 °C with 100 µL MTT reagent solution (0.25 mg/ml). After removal of the MTT solution, 100 µl of solubilization solution (SDS 5% w/v) was added to each well and the cells were kept overnight at 37 °C in darkness. Absorbance was measured at a wavelength of 560 nm using a spectrophotometer plate reader (iEMS Reader MF; Labsystems).

2.11. Data analysis

Results are expressed as mean ± S.E.M of at least 3 independent experiments. Concentration-response curves were fitted to sigmoid curves using the GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). 5–7 concentration points, each in duplicate or triplicate, were used in each experiment. Concentration values are given in mol/L (M). The two-tailed Student's *t*-test was used to compare mean values.

2.12. Human data

Human blood 50% lethal concentrations (LC50), expressed in moles per litre (M), were adapted from recently published data [37]. The data for the chemicals used in this study were taken from Acutoxbase which is a part of the integrated ACuteTox project (2005–2009) under the EU 6th Framework Programme [7]. At present, Acutoxbase includes 97 reference chemicals of different origin (drugs, industrial chemicals, biocides, etc.). Acutoxbase will be made accessible for the scientific community after the completion of the ACuteTox project.

Human blood concentration data, at acute poisoning, was collected from clinical and forensic sub-lethal and lethal case reports. The information was obtained from the MEIC project [9], MEIC monographs (www.cctoxconsulting.a.se), papers in international journals, poison information centres in several countries, and several on-line databases [7].

3. Results

The first set of reference compounds were evaluated against the different neuronal assays used in this work. After completion of the study, the $GABA_A$ receptor assay was selected (see below) for the testing of the additional compounds included in the second set of reference compounds.

3.1. In vitro assays of GABA neurotransmission

We determined the effects on the $GABA_A$ receptor of the whole set of reference compounds. 23 out of the 58 compounds tested inhibited $GABA_A$ receptor activity, as determined by measuring the $^{36}Cl^-$ uptake induced by 100 µM GABA. Table 1 shows the IC_{20}, IC_{50}, IC_{80} and I_{max} values. Diazepam, phenobarbital, mercuric chloride, chloral hydrate and isopropyl alcohol increased GABA-induced Cl^- influx (Table 1). All together, twenty-eight out of the fifty-eight compounds tested positive against the $GABA_A$ receptor. The assay was developed as a non-robotic, medium-throughput, screening assay using 24-well plates, performing a whole test agent concentration–response curve in each plate. Throughout this work, the intra-assay variability (repeatability) of $^{36}Cl^-$ influx for the control wells in each individual plate accounted for 12 ± 0.6% (3 replicates in each plate; $n = 105$ plates). The intra-assay variability of $^{36}Cl^-$ influx for the control

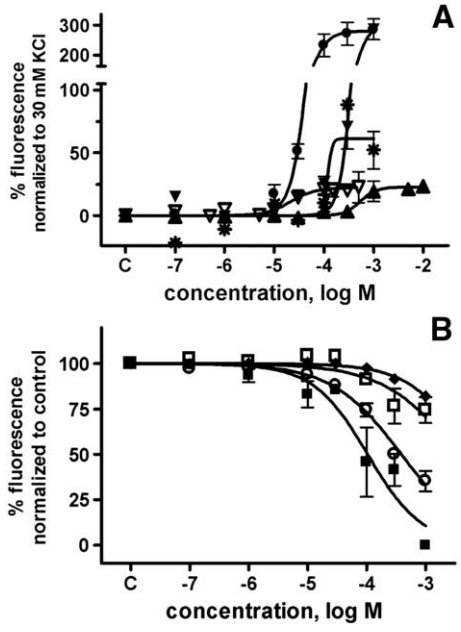

Fig. 4. Concentration–response curves for the effect of selected chemicals on cell membrane potential in primary cultures of cortical neurons. A) ✱, colchicine; ▼, methadone; ▽, digoxin; ●, mercury II chloride; ▲, nicotine. B) ○, caffeine; □, verapamil; ♦, pentachlorophenol; ■, sodium lauryl sulphate. Values are mean ± S.E.M of 3 independent experiments, each concentration point was determined in triplicate.

Chemical	human blood LC50[a]	$GABA_AR$-IC50	GABA uptake-IC50	Glutamate uptake-IC50	CMP-IC20	Cytotoxicity 3T3[a]	Neurotoxicity ALERTS
Digoxin							
Colchicine							
Cyclosporine A			nt	nt	nt		
Cadmium (II) chloride			nt	nt	nt		
methadone							
Lindane							
Malathion							
Atropine sulfate monohydrate							
Parathion			nt	nt	nt		
Amitriptyline hydrochloride			nt	nt	nt		
Hexachlorobenzene			nt	nt	nt		
Arsenic trioxide			nt	nt	nt		
Verapamil hydrochloride							
Strychnine			nt	nt	nt		
Thallium sulphate			nt	nt	nt		
Nicotine							
Propranolol hydrochloride			nt	nt	nt		
Amiodarone hydrochloride			nt	nt	nt		
Mercury (II) chloride							
Cis-diammineplatinum (II) dichloride			nt	nt	nt		
Orphenadrine hydrochloride			nt	nt	nt		
Sodium selenate			nt	nt	nt		
Diazepam							
Rifampicine			nt	nt	nt		
Warfarin			nt	nt	nt		
Carbamazepine							
5-fluorouracil			nt	nt	nt		
Diquat dibromide			nt	nt	nt		
Phenobarbital							
Caffeine							
Sodium fluoride			nt	nt	nt		
Chloral hydrate			nt	nt	nt		
Pentachlorophenol							
Dichlorvos			nt	nt	nt		
Acetonitrile			nt	nt	nt		
Acetaminophen							
2,4-dichlorophenoxyacetic acid			nt	nt	nt		
Lithium sulfate			nt	nt	nt		
Dimethylformamide			nt	nt	nt		
Acetylsalicylic acid							
Sodium valproate							
Glufosinate-ammonium							
Ethylene glycol			nt	nt	nt		
Sodium chloride			nt	nt	nt		
Methanol			nt	nt	nt		
Isopropyl alcohol							
Ethanol							
Cycloheximide	nd						
Sodium lauryl sulfate	nd						
Tert-butylhydroperoxide	nd		nt	nt	nt		
Acrylaldehyde	nd		nt	nt	nt		
Pyrene	nd		nt	nt	nt		
Tetracycline hydrochloride	nd		nt	nt	nt		
Physostigmine	nd		nt	nt	nt		
Diethylene glycol	nd		nt	nt	nt		
Ochratoxin A	nd		nt	nt	nt		
17a-ethynylestradiol	nd		nt	nt	nt		
Epinephrine bitartrate	nd		nt	nt	nt		

wells corresponding to different plates from 25 culture preparations (3–6 plates for each preparation) accounted for $17 \pm 3\%$.

We have previously shown that [^{3}H]-flunitrazepam binding in primary cultured neurons is increased by GABA, neurosteroids and depressant compounds that act on the $GABA_A$ receptor, and that this increase is reduced in the presence of $GABA_A$ receptor antagonists [15,40,43,44]. We wanted to test whether this indirect assay of the $GABA_A$ receptor activity, based on the allosteric increase by GABA of the binding of benzodiazepines, could be used as an alternative testing assay for the $GABA_A$ receptor function. Fig. 1 shows the concentration–response curves for picrotoxinin (Fig. 1A) and bicuculline (Fig. 1B) against [^{3}H]-flunitrazepam binding and $^{36}Cl^-$ uptake in the presence of 100 μM GABA in primary cultures of cortical neurons. The two assays gave IC50 values that were not statistically significantly different (44 ± 6 μM and 36 ± 7 μM for bicuculline, and 76 ± 21 μM and 37 ± 6 μM for picrotoxinin, against $^{36}Cl^-$ uptake and [^{3}H]-flunitrazepam assays, respectively; $p > 0.1$). Reference compounds that were found to inhibit the $^{36}Cl^-$ influx assay were tested using the [^{3}H]-flunitrazepam binding assay. Fig. 2A and B shows the concentration–response curves for some of the compounds listed in Table 1. Fig. 3 shows the correlation between IC_{50} values for [^{3}H]-flunitrazepam binding and $^{36}Cl^-$ uptake in the presence of 100 μM GABA in primary cultures of cortical neurons. A high correlation ($r^2 = 0.9108$; $p < 0.0001$) and a slope value of 1.03 was found.

We also tested whether reference compounds could inhibit [^{3}H]-GABA uptake, since a defect in the GABA transport system would result in an excess of extracellular GABA and lead to increased activity of neuronal GABA receptors, which would produce depression. Ten out of the twenty-three chemicals tested from the 1st set of reference compounds inhibited [^{3}H]-GABA uptake in primary cultured cortical neurons. Table 2 shows the IC_{20}, IC_{50} and IC_{80} values for these compounds.

3.2. In vitro assays of glutamate neurotransmission and of cell membrane depolarization

We have previously reported that primary cultures of cerebellar granule cells express NMDA- and AMPA/kainate-glutamate receptors and the neuronal transporter EAAT3 [2,3,12,41]. Activation of ionotropic glutamate receptors results in a Ca^{2+} influx through the NMDA receptor when the membrane is depolarized or in a Na^+ influx through AMPA and kainate receptors leading to cell membrane depolarization.

We tested the 1st set of reference chemicals against [^{3}H]-aspartate uptake and against the increase of intracellular calcium ($[Ca^{2+}]_i$) in Mg^{2+}-free buffer (Mg^{2+} blocks NMDA receptor). Five out of the twenty-three chemicals tested from the 1st set of reference compounds inhibited [^{3}H]-aspartate uptake in cultured cerebellar granule cells. Table 2 shows the IC_{20}, IC_{50} and IC_{80} values for these compounds. Complete inhibition of glutamate transport by digoxin, mercuric chloride, pentachlorophenol and SDS resulted in a net accumulation of extracellular glutamate that was even higher than that produced by exposing the cells to a high depolarizing stimulus of 100 mM KCl (data not shown). This extracellular glutamate accumulation was not induced by malathion, which did not completely inhibit [^{3}H]-aspartate uptake. When assessing $[Ca^{2+}]_i$, three out of the twenty-three chemicals tested from the 1st set of reference compounds increased intracellular calcium as determined by Fluo-3 fluorescence

Fig. 5. Summary of *in vivo*/*in vitro* toxicity datafor the reference compounds. *In vivo* data represent human blood lethal concentrations (LC50), whereas *in vitro* data represent effective concentration values for the different neural cell assays performed in primary neuronal cultures and for the cytotoxicity assay performed in the 3T3 cell line. Colours indicate the range of concentrations (M) for the selected parameters: black, <1E-6; blue, 1E-6–1E-5; pink, 1E-5–1E-4; yellow, 1E-4–1E-3; green: 1E-3–1E-2; white, 1E-2–5E-1 (human) or up to the highest concentration tested (*in vitro*; see Table 1, SI). Nd: not data; nt: not tested. a: extracted from reference 37.

(digoxin, EC_{50}: 29 μM; mercuric chloride, EC_{50}: 9 μM, and pentachlorophenol, EC_{50}: 38 μM).

We tested the effect of the 1st set of reference compounds against cell membrane potential in primary cultures of cortical neurons. Nine out of the twenty-three chemicals tested modified basal FMP fluorescence. Fig. 4 shows the concentration–response curves for the compounds that modified basal cell membrane potential. We also tested whether this assay could be used to identify compounds that inactivate neuronal Na^+ channels, as this mechanism underlies the effects of some animal toxins, anaesthetics and anticonvulsant drugs. Veratridine specifically causes persistent activation of Na^+ channels and increases FMP fluorescence in cultured neurons [41]. The increase in fluorescence induced by 20 μM veratridine was inhibited by carbamazepine ($IC_{50} = 120 \pm 16$ μM, $n = 3$) in agreement with its mechanism of action inactivating Na^+ channels [27]. Carbamazepine did not modify FMP fluorescence, indicating that it does not modify cell membrane potential by itself.

3.3. Cell viability

The first set of reference compounds were analyzed for their effects on cell viability after 30 min exposure. This exposure time was slightly longer than that used in the neurofunctional assays (5–20 min). Only sodium lauryl sulphate (SDS) released intracellular LDH ($IC_{50} = 2.2E\text{-}4 \pm 0.3E\text{-}4$ M) indicating cell membrane leakage. SDS also reduced mitochondrial activity at similar concentrations. Lower concentrations of SDS (≤1E-4 M) did not release LDH or reduce mitochondrial activity. Pentachlorophenol reduced mitochondrial activity ($IC_{50} = 6.9E\text{-}5 \pm 0.7E\text{-}5$ M) without releasing intracellular LDH, suggesting that mitochondria failure preceded cell membrane damage. Mercuric chloride also reduced mitochondrial activity ($IC_{50} = 4.7E\text{-}6 \pm 0.3E\text{-}6$ M), however, the effect of mercuric chloride on cell membrane integrity could not be determined due to the interference of Hg^{2+} with the LDH assay. The 2nd set of reference compounds did not produce cell cytotoxicity at the concentrations used in the functional $GABA_A$ receptor, as visualized by phase-contrast optical microscopy (data not shown).

3.4. Predictive value of the in vitro neurofunctional assays

Fig. 5 shows a mosaic colour diagram that summarizes human toxicity data [37] and *in vitro* neural data for the compounds analyzed. Basal cytotoxicity data on the non-neural cell line 3T3 [37] are also included, to compare *in vitro* neuronal endpoints with basal cytotoxicity. This comparison allowed us to establish neurotoxic alerts for the compounds that were more active at the neuronal endpoints determined in the primary neuronal cultures than at the basal cytotoxicity endpoints in the 3T3 cell line. The $GABA_A$ receptor and the cell membrane potential assays were the tests that identified most compounds. For the most toxic compounds analyzed (human blood lethal concentration – LC50-values <1E-5 M; black and blue colours in the diagram), the $GABA_A$ receptor and the FMP assays produced a neurotoxic alert with respect to the cytotoxicity 3T3 assay for methadone, lindane, malathion, parathion, strychnine and digoxin. Atropine and nicotine were recognized by the neurotoxic assays at concentrations similar to those producing cytotoxicity in the 3T3 cell line, whereas colchicine and verapamil did not produce a neurotoxic alert. For the compounds causing medium levels of toxicity (human blood LC50 values in the range 1E-5 M to 1E-3 M; pink and yellow colours), the neuronal $GABA_A$ receptor and the FMP assays gave a neurotoxic alert for diazepam, rifampicine, caffeine, phenobarbital and pentachlorophenol, while warfarin, carbamazepine and mercuric chloride were recognized by the neural assays at concentrations similar to those producing cytotoxicity in the 3T3 cell line. Mercuric chloride produced a neurotoxic alert in the GABA uptake and glutamate uptake assays. Compounds whose toxic blood concentrations were <1E-3 M (green and white colours) did not modify any of the neural endpoints studied. Isopropyl alcohol was an exception, since it was detected in the $GABA_A$ receptor assay as a neuroactive compound.

4. Discussion

In this work we provide evidence that *in vitro* cell-based functional neuronal tests can be used to identify chemical compounds that interfere with excitatory and inhibitory neurotransmission. Fifteen out of the twenty-one compounds in the first set of chemicals tested were identified as toxic by at least one of the five neuronal endpoints assayed in primary cultured neurons. Acetaminophen, acetylsalicylic acid, sodium valproate, glufosinate ammonium, ethanol and cycloheximide did not modify the assayed endpoints up to 1 mM. However, these compounds are known to be of low acute toxicity in humans and rats. The $GABA_A$ receptor assay identified the highest number of agents tested from the 1st set of reference compounds, among them atropine, lindane, malathion, pentachlorophenol, carbamazepine, and methadone, which result in seizures after severe human intoxication (Clemedson and Kolman, private communication within the AcuteTox consortium, forthcoming). Furthermore, diazepam, phenobarbital, chloral hydrate and isopropyl alcohol were recognized as $GABA_A$ receptor potentiators; they are CNS depressants at therapeutic and toxic doses. Because of the high capacity of the $GABA_A$ receptor to recognize neurotoxic compounds (even when the receptor was not their primary neuronal target) we extended this assay to the second set of 36 reference compounds. The pesticide parathion, which shares with malathion a phosphothiol group, was also identified as toxic by the $GABA_A$ receptor. Strychnine, a competitive antagonist of the glycine receptor-operated Cl^- channel, also inhibited the $GABA_A$ receptor-operated Cl^- channel; however, it was less potent on the $GABA_A$ than the glycine receptor [45]. The oral contraceptive hormone 17α-ethynylestradiol was also identified as toxic by the $GABA_A$ receptor assay. It has been reported that ß-estradiol and its derivative 17α-ethynylestradiol reduce the effect of GABA on human recombinant rho-1-subunit GABA receptors [24], in agreement with a recent report suggesting that the proconvulsant-like effects of testosterone could be mediated by increased synthesis of 17 β-estradiol [33]. According to our results, 17α-ethynylestradiol might produce a toxic response in the form of seizures, however at blood concentrations much higher than those required for its therapeutic use (95–160 ng/L) (Clemedson and Kolman, private communication within the AcuteTox consortium, forthcoming). A literature search confirms the $GABA_A$ receptor effects for 61% of the compounds shown to be active in the present work, while there were no data for 36% of the compounds and one compound (epinephrine) was reported not to act on the $GABA_A$ receptor, although at concentrations far lower than those used in the present work (see Table 1 for the effects of compounds on the $GABA_A$ receptor and Table 1 in Supplementary Material for related references). With respect to the 27 compounds tested that did not show activity on the $GABA_A$ receptor in our assay, there are reports for 4 of them confirming our results and no data for 20 compounds. Three compounds (valproate, caffeine and ethanol) have been demonstrated to be active, however, at higher concentrations than those used in this study. Only one compound, amitryptyline has been reported to inhibit $GABA_A$ receptor function and was not recognized in our assay.

The $GABA_A$ receptor has binding and recognition sites for a multiplicity of drugs and chemicals, including benzodiazepines, barbiturates, neurosteroids, anaesthetics, avermectin, polychlorocycloalkane pesticides, Zn^{2+} and lanthanides [25,36,49]. It is structurally composed of 5 subunits (two α, two β and one γ), the interface between the α and β subunits and between the α and γ subunits allocate the binding sites for GABA and benzodiazepines, respectively. Each subunit has extracellular C- and N-terminus domains and four transmembrane domains (TM1–4). While TM2 faces the lumen of the Cl^- channel and TM4 is anchored in the lipid membrane, the

transmembrane segments TM1 and TM3 interact with the neighbouring subunit. It has been proposed that such an arrangement would allow conformational flexibility and would provide the receptor with space or cavities for putative binding sites. This model allows for new drug binding sites to be proposed [20]. Therefore, the $GABA_A$ receptor has a high capacity to be targeted by a wide range of chemicals. In fact, it has been reported that several nicotinic antagonists also inhibited $GABA_A$ receptor function [35].

In this work, the $GABA_A$ receptor activity was determined by measuring the uptake of $^{36}Cl^-$ induced by GABA in primary neuronal cultures. As we previously reported, this assay recognizes both chemicals that potentiate and those that inhibit the $GABA_A$ receptor [15,32,44,45]. However, the high energy of the radionuclide ^{36}Cl (710 keV) requires the use of acrylamide screens and tube-holders to protect the analyst against the high beta-radiation emitted by ^{36}Cl. Furthermore, ^{36}Cl has a long disintegration half-life (3×10^5 years). All of this poses technical and environmental drawbacks for the acceptance of this test in high-throughput screening frameworks. Therefore, developing an assay for the $GABA_A$ receptor using ^3H (19 keV; 12-year half-life) instead of ^{36}Cl will result in a clear improvement. It has been demonstrated that binding to a site at the $GABA_A$ receptor exerts allosteric modulation on the other independent recognition sites [11,15,43,44]. In this work we provide evidence that the binding of [^{3}H]-flunitrazepam in the presence of GABA can be used as a functional assay for the $GABA_A$ receptor. The good correlation between IC_{50} values for GABA-induced Cl^- influx and GABA-induced increase of flunitrazepam binding for compounds that inhibited the $GABA_A$ receptor suggests that both assays can be used indistinctly to test for inhibition or blocking of $GABA_A$ receptor activity. This binding assay, although still using tritium radioactivity, is more user-friendly than the radiometric $^{36}Cl^-$ influx assay and much more prone to be automated. Standardization of this method and further validation could give rise to a high-throughput assay for the activity of chemicals on the $GABA_A$ receptor. Electrophysiology is the technique that provides most information on $GABA_A$ receptor function; however, this methodology requires very specific equipment, high researcher expertise and is not amenable to high throughput. The development of multielectrode chambers where primary neuronal cultures can be grown is also creating expectations for their use in functional neurotoxicity screening [4,46].

Chemical compounds may produce hyperexcitability by directly activating ionotropic glutamate receptors or by increasing extracellular glutamate, which in turn will activate these receptors, resulting in degeneration of neurons through a process known as excitotoxicity [23,31]. Inactivation of glutamate released from neural cells is accomplished by efficient uptake through glutamate transporters expressed in glial cells and neurons. Few compounds from the 1st set of reference compounds were demonstrated to be active on the NMDA-glutamate receptor. Since these compounds also completely inhibited [^{3}H]-aspartate uptake and increased the extracellular concentration of glutamate, the NMDA receptor could have been activated as an indirect result of the accumulation of extracellular glutamate. The EC_{50} values for the increase in $[Ca^{2+}]$ were similar to the IC_{50} values for glutamate transport. Neither was glutamate transport a sensitive endpoint, since it recognized very few compounds. In contrast, GABA transport was inhibited by 10 chemicals from the 1st set of reference compounds, including some commonly abused drugs, such as methadone and amphetamine. These are more potent inhibitors of monoamine transport (IC_{50} values: 0.3–90 μM; [5,13,17,34]) than of GABA transport (170–1400 μM; this work). However, since GABA and monoaminergic transporters, but not glutamate transporters, belong to the same slc6 protein family [6], the inhibition of the GABA transport in cultured cortical neurons could be used as a specific alert for neurotoxicity. Furthermore, compounds like tiagabine that inhibit GABA uptake might have anticonvulsant properties [16].

In this work we used the cell membrane potential fluorescent probe FMP as a non-specific assay to identify compounds that modify neuronal excitability, whatever the mechanism. We recently reported that the FMP fluorescence assay for cell membrane potential responds to different depolarizing stimuli mediated by voltage- and receptor-operated ion channels in cultured neurons. KCl, veratridine, and glutamate and kainate increase FMP fluorescence at concentrations compatible with activation of Na^+ and AMPA/kainate receptors, respectively [41]. Nine out of the twenty-three chemicals in the 1st set of reference compounds modified FMP fluorescence. The assay was able to recognize compounds acting at different neuronal endpoints, such as nicotine at the nACh receptor and digoxin which inhibits Na^+/K^+/ATPase. The increase in FMP fluorescence by the microtubule disruptor agent colchicine is in agreement with the effects observed in cardiac myocytes, where colchicine increases sodium but not calcium currents [30]. The decrease of FMP fluorescence caused by caffeine could be indicative of a hyperpolarizing shift of membrane potential after ryanodine receptor activation, as demonstrated in mammalian neurons [18]. Neither lindane nor malathion, which inhibited $GABA_A$ receptor activity, modified membrane potential. The effect of SDS on FMP fluorescence could be attributed to membrane disruption since EC_{50} values for FMP fluorescence and cytotoxicity in cortical neurons were similar (1–3 E-4 M). Furthermore, the compounds that showed sensitivity to glutamate endpoints (mercuric chloride, pentachlorophenol and digoxin) were also recognized by the cell membrane potential assay. The advantages of this method are that it can recognize compounds interfering with different neuronal targets that modify neuronal excitability and that the assay can be easily brought to high-throughput standards.

A crucial point when validating alternative methods is the comparison/correlation with tests in current use. Although the majority of traditional animal test methods have not been validated, they are routinely used as reference test methods for validation. As stated in the workshop on “Points of Reference in the Validation Process” of alternative test methods held at the ECVAM (European Centre for Validation of Alternative Methods) in 2006 [21], the ideal situation when validating alternative methods addressing human health (as in toxicology testing) would be to compare *in vitro* data with human data. This would allow an optimal relevance of assessment. Lethal human blood concentrations, LC_{50}, for the tested chemicals have recently been calculated [37] from reported human toxicity data. Comparison of the *in vitro* neural results obtained in this work with basal cytotoxicity in the mouse fibroblast cell line 3T3 and with human *in vivo* data [37] shows that neurotoxic alerts have been established for compounds known to be neuroactive, causing hyperexcitability or depression of the nervous system (lindane, malathion, parathion, strychnine, methadone, digoxin, diazepam, phenobarbital and caffeine). Except for phenobarbital and caffeine, these compounds were outliers when their toxicity was predicted from *in vitro* basal cytotoxicity assays [37]. We can conclude that a better prediction of acute human toxicity has been obtained by using *in vitro* neuronal functional assays. Nicotine, also an outlier in the basal cytotoxicity assay prediction, was recognized by the neuronal FMP assay, however, its toxicity was not better predicted. The FMP assay should be refined to detect compounds acting on the nicotinic acetylcholine receptor. In this work, the $GABA_A$ receptor, the GABA uptake and the cell membrane potential assays were those that recognized the highest number of chemicals at low concentrations. Furthermore, we have previously reported that the organophosphate pesticides dichlorvos and malathion inhibited AChE activity in primary cultures of cortical neurons [4], a mechanism that results in central and peripheral nervous system excitability.

In this work we used primary cultures of cortical and of cerebellar granule neurons because they are widely used in neuroscientific research and they are produced in high yields. They are also well characterized in terms of pre-synaptic and post-synaptic amino acid

and acetylcholine neurotransmission; the major neurotransmitter systems involved in acute neurotoxicity. However, it can be argued that these cultures may not recognize chemicals that exhibit regional and cellular specificity such as, for example, dopaminergic toxins. The use of primary cultures obtained from the whole cerebral tissue could overcome this problem once they are fully characterized. Neurogenesis occurs at different embryonic ages for the different cell types in the brain, thus determining the viability and differentiation of the cells in culture.

We conclude that *in vitro* evaluation of neural endpoints in primary neuronal cultures may identify compounds that produce acute neurotoxicity in humans, provided that *in vitro* models expressing neuronal targets relevant for acute neural dysfunctions are used. We provide evidence that test methods based on the measurement of the activity of $GABA_A$ receptors, GABA transport, AChE and on the measurement of the cell membrane potential are potent candidates to be included in an *in vitro* testing strategy for neurotoxicity. Further studies and validation of these methods are needed to progress in the development and implementation of medium- to high-throughput alternative methods for the assessment of human neurotoxicity.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ntt.2009.01.010.

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