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The Neuronal $GABA_A$ Receptor as Target for Therapeutic and Toxic Chemical Compounds. In Vitro Studies Using Cultured Cortical Neurons

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RESUMEN

Los neurotransmisores son sustancias químicas liberadas por las neuronas y reconocidas por proteínas o receptores específicos situados en la membrana celular. El control de la neurotransmisión excitadora e inhibidora es fundamental para el funcionamiento correcto del sistema nervioso. El neurotransmisor ácido γ-aminobutírico (GABA) es el principal responsable de las señales inhibidoras de la transmisión entre las neuronas. Actúa en el receptor $GABA_A$, complejo proteico que conforma un canal iónico permeable a iones Cl^-. El receptor $GABA_A$ es una diana neuronal para productos químicos extensamente usados. Entre ellos fármacos benzodiacepínicos, esteroides y anestésicos neuroactivos y los pesticidas de la familia de los policlorocicloalcanos. Los estudios sobre la actividad de productos químicos en el receptor $GABA_A$ se realizaron en neuronas que fueron crecidas in vitro. Las neuronas fueron obtenidas de la corteza del cerebro de embriones de ratón y crecidas in vitro durante 6-8 días. Estas neuronas corticales sintetizaron GABA y lo liberaron en respuesta a un estímulo de despolarización. Se determinaron las características del receptor $GABA_A$ en este sistema in vitro. La función del receptor $GABA_A$ fue determinada mediante la afluencia de $^{36}Cl^-$ y los sitios de unión para GABA y las benzodiacepinas fueron determinados usando los radioligandos [^{3}H]muscimol y [^{3}H]flunitrazepam. El diazepán, el fenobarbital, análogos del neuroesteroide pregnanolona y el timol aumentaron la función del receptor $GABA_A$, activando directamente el receptor o potenciando el efecto del neurotransmisor GABA. Los pesticidas α-endosulfan, γ-hexaclorociclohexano (lindano) y la dieldrina bloquearon totalmente la acción de GABA en su receptor neuronal, por lo tanto produciendo síntomas excitadores y convulsiones en mamíferos. En conclusión, los cultivos primarios de neuronas corticales son un modelo in vitro que está enriquecido en neuronas gabaérgicas y que expresan los receptores $GABA_A$. Estas neuronas cultivadas son útiles para la evaluación de la actividad farmacológica y la neurotoxicidad de compuestos químicos.

Palabras clave: **Neuronas. Neurotransmisión inhibidora. Actividad farmacológica. Neurotoxicidad. In vitro.**

SUMMARY

Neurotransmitters are chemical substances that are released by the neurons and recognized by specific proteins or receptors located in the cellular membrane. The right control of excitatory and inhibitory neurotransmission is fundamental for the proper function of the nervous system. The neurotransmitter γ-aminobutyric acid (GABA) is in charge of inhibitory transmission signals between neurons. It acts on the $GABA_A$ receptor, which operates an ionic channel permeable to Cl^-. The $GABA_A$ receptor is the target for widely used chemicals. Among them the therapeutic drugs benzodiazepines, neuroactive steroids and anaesthetics and the polychorocycloalkane pesticides. Studies on the activity of chemicals on the $GABA_A$ receptor were performed in neurons that were grown in vitro. Neurons were obtained from the brain cortex of embryo mice and grown in vitro for 6 -8 days. These cultured cortical neurons synthesized GABA and released it in response to a depolarizing stimulus. We determined the characteristics of the $GABA_A$ receptor in the in vitro system. The function of the $GABA_A$ receptor was measured as $^{36}Cl^-$ influx and the binding sites for GABA and benzodiazepines were determined using the radioligands [^{3}H]muscimol and [^{3}H]flunitrazepam. Diazepam, phenobarbital, a B-nor analogue of the neurosteroid pregnanolone and thymol increased $GABA_A$ receptor function, either by direct activation of the receptor or by potentiating the effect of the neurotransmitter GABA. The polychlorocycloalkane pesticides α-endosulfan, γ-hexachlorocyclohexane (lindane) and dieldrin completely blocked the action of GABA on its neuronal receptor, therefore producing excitatory symptoms and convulsions in mammals. In conclusion, primary cultures of cortical neurons are an in vitro model that are enriched in GABAergic neurons and express functional $GABA_A$ receptors. These cultured neurons are useful for the evaluation of pharmacological activity and neurotoxicity of chemical compounds.

Key words: **Neurons. Inhibitory neurotransmission. Pharmacological activity. Neurotoxicity. In vitro.**

RESUM

Els neurotransmissors són substàncies químiques alliberades per les neurones i reconegudes per proteïnes o receptors específics situats en la membrana cel·lular. El control de la neurotransmissió excitadora i inhibidora és fonamental per al funcionament correcte del sistema nerviós. El neurotransmissor àcid γ-aminobutíric (GABA) és el principal responsable dels senyals inhibidors de la transmissió entre les neurones. Actua en el receptor $GABA_A$, complex proteic que conforma un canal iònic permeable a ions Cl^-. El receptor $GABA_A$ és una diana neuronal per a productes químics extensament usats. Entre ells fàrmacs benzodiacepínics, esteroids i anestèsics neuroactius i els pesticides de la família dels policlorcicloalcans. Els estudis sobre l'activitat de productes químics en el receptor $GABA_A$ es van realitzar en neurones que van ser crescudes in vitro. Les neurones van ser obtingudes de l'escorça del cervell d'embrions de ratolí i crescudes in vitro durant 6-8 dies. Aquestes neurones corticals van sintetitzar GABA i ho van alliberar en resposta a un estímul de despolarització. Es van determinar les característiques del receptor $GABA_A$ en aquest sistema in vitro. La funció del receptor $GABA_A$ va ser determinada mitjançant l'afluència de $^{36}Cl^-$ i els llocs d'unió per a GABA i les benzodiazepines van ser determinats usant els radiolligands [^{3}H]muscimol i [^{3}H]flunitrazepam. El diazepán, el fenobarbital, anàlegs del neuroesteroide pregnanolona i el timol van augmentar la funció del receptor $GABA_A$, activant directament el receptor o potenciant l'efecte del neurotransmissor GABA. Els pesticides α-endosulfà, γ-hexaclorciclohexà (lindane) i la dieldrina van bloquejar totalment l'acció de GABA en el seu receptor neuronal, per tant produint símptomes excitadores i convulsions en mamífers. En conclusió, els cultius primaris de neurones corticals són un model in vitro que està enriquit en neurones gabaérgicas i que expressen els receptors $GABA_A$. Aquestes neurones cultivades són útils per a l'avaluació de l'activitat farmacològica i la neurotoxicitat de compostos químics.

Mots clau: **Neurones. Neurotransmissió inhibidora. Activitat farmacològica. Neurotoxicitat. In vitro.**

INTRODUCTION

The human brain consists of about 100,000 million neurons that can carry out about 1,000 synaptic contacts each one. Neurotransmitters make these synaptic contacts. Neurotransmitters are chemical substances that are released by the neurons and that are recognized by specific proteins or receptors located in the cellular membrane. Binding of neurotransmitters to their specific receptors produce several effects, among them, the activation of ionic channels (ionotropic receptors), or the activation of a cascade of second messengers (metabotropic receptors). The main chemical mediators in the Central Nervous System (CNS) are amino acids, like glutamate, γ-aminobutyric acid (GABA) and glycine, which are in charge of excitatory (glutamate) and inhibitory (GABA and glycine) transmission signals between neurons. The neuronal gabaergic system is widely distributed in the CNS, where around 30- 40 % of the neurons release the neurotransmitter GABA. The gabaergic system mediates a series of physiological functions and neurological and psychiatric alterations. Due to the wide distribution and use of GABA in the CNS, the pharmacology based on this neurotransmitter has been thoroughly studied, given rise to the development of numerous drugs and other neuroactive compounds; some of the latest compounds are however neurotoxic agents (for review of GABA neurotransmission see references 1 - 6).

The development of selective pharmacological agents allowed to identify and to characterize two different types of GABA receptors, $GABA_A$ and $GABA_B$, which differ in their pharmacological, biochemical and electrophysiological properties. The $GABA_A$ receptor is a member of the superfamily of receptors ionic channels operated by ligand binding. The activation of the $GABA_A$ receptor generally induces a rapid inhibitory potential in the neuronal postsynaptic membrane.

The activation of the $GABA_A$ receptor by GABA or their agonists leads to the opening of a channel permeable to Cl^-. These Cl^- flux is reduced by the convulsant agents bicuculline and picrotoxinin and increased by several types of depressing drugs, fundamentally benzodiazepines, barbiturates, steroids and classes of anaesthetics. Other substances interact with the $GABA_A$ receptor which mediates their neurotoxic effects. Some of these substances are: alcohols, polychlorocycloalkane pesticides, convulsant β-carbolines, and metals (such as Zn^{2+}, mercury, lanthanum). In the present work we show the gabaergic characteristics of mice cortical neurons that have been grown in vitro (cultured neurons). These primary cultures of brain cortical neurons constitute an in vitro model that is extensively used in neuropharmacological and neurotoxicological studies involving GABA and glutamate neurotransmission, neurodegeneration and neuroprotection mechanisms. Evidences are shown that these neurons synthesize and release the neurotransmitter GABA and that express $GABA_A$ receptors. The pharmacological/neurotoxic activity of different chemical compounds with respect to the activity of the $GABA^A$ receptor is analyzed in this in vitro system.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16th gestational day) were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic culture multiwell plates were purchased from CoStar (Corning Science Products, Acton, MA, USA). Foetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's modified Minimum Essential Medium (DMEM) from Biochrom (Berlin, Germany). [^{3}H]Flunitrazepam (88 Ci/mmol) and $^{36}Cl^-$ (0.1 Ci/mol) were procured from Amersham, Life Sciences (Buckinghamshire, UK); [^{3}H]muscimol (36.5 Ci/mmol) from PerkinElmer (Boston, MA, USA). Liquid scintillation cocktail Optiphase Hisafe 2 was obtained from Wallace Oy (Turku, Finland). GABA, diazepam, phenobarbital, γ-hexachlorocyclohexane, α-endosulfan, dieldrin, trypsin, soybean trypsin inhibitor, DNAse, amino acids and poly-L-lysine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Rabbit anti-glutamic acid decarboxylase (GAD_{67}) polyclonal antibody was from Chemicon International Inc. (Temecula, CA, USA) and goat anti-rabbit IgG Alexa 488 was from Molecular Probes (Leiden, The Netherlands). All the other chemicals were of analytical grade. When needed, stock solutions were prepared in DMSO, light protected, and stored at 4 ºC. Stock solutions were diluted before each experiment in buffered solution, maintaining a 0.25% (v/v) DMSO final concentration.

Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mice foetuses according to described methodology[7]. Pregnant animals were killed by cervical dislocation and foetuses extracted. Neocortices were dissected with forceps, mechanically minced and the cells were dissociated by mild trypsinization (0.02 %-w/v) at 37 ºC for 10 min followed by trituration in a DNAse solution (0.004%-w/v) containing a soy-

bean trypsin inhibitor (0.05%-w/v). The cells were re-suspended in a DMEM (5 mM KCl, 31 mM glucose and 0.2 mM glutamine) supplemented with insulin, penicillin, and 10% foetal calf serum. The cell suspension (1.6×10^6 cells/ml) was seeded in 24x-multiwell plates pre-coated with poly-L-lysine, and incubated for 6-9 days in a humidified 5% CO_2 / 95% air atmosphere at 36.8 ºC. A mixture of 5 µM 5-fluoro-2'-deoxyuridine and 20 µM uridine was added after 48 h in culture to prevent glial proliferation.
Animals were handled in compliance with protocols of the University of Barcelona, 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) (identification number A5224-01).

$GABA_A$ receptor binding and function

Chloride influx was determined as $^{36}Cl^-$ uptake in intact cellular cultures as previously described[8, 9]. [^{3}H]muscimol and [^{3}H]flunitrazepam binding to intact cell cultures were perfomed as previously described[9, 10].

GABA determination and immunocytochemistry

The concentration of GABA in the exposure medium was determined by high performance liquid chromatography (HPLC) as previously described[11, 12]. Glutamic acid decarboxylase (GAD) immunocytochemistry was performed as previously described[12].

RESULTS AND DISCUSSION

GABAegic characteristics of primary cultures of cortical neurons

Figure 1A shows a photomicrograph of a primary culture of cortical neurons. Cells are profusely linked by neurites (prolongations that extend between neurons) and both cells and neurites were labelled with an antibody against glutamic acid decarboxylase (GAD) (Figure 1B). GAD immunostaining confirms the ability of cultured cortical neurons to synthesize the neurotransmitter GABA, since the enzyme GAD converts the precursor L-glutamic acid into GABA[13]. The content of GABA in these cultures has been reported to be around 35 nmol/mg protein[14]. Futhermore, primary cultures of cortical neurons released endogenous GABA when exposed to a depolarizing stimulus of a high concentration of K^+ (figure 2).
Primary cultures of cortical neurons express $GABA_A$ receptors, which operate a channel permeable to Cl^-. Figure 3 shows the binding of [^{3}H]muscimol and [^{3}H]flunitrazepam at the GABA and benzodiazepine recognition sites, respectively, of the $GABA_A$ receptor. The binding of the radiolabeled muscimol, the ligand of choice for the GABA recog-

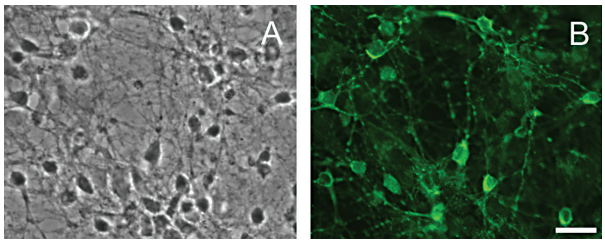

Figure 1. Microphotography of primary cultures of mice cortical neurons. Cultured neurons were grown for 8 days in vitro. A) Phase contrast microscopy. B) Immunostaining with glutamic acid decarboxylase (GAD) antibody. A and B microphotographs represent the same field of the culture. Bar size: 20 µm.

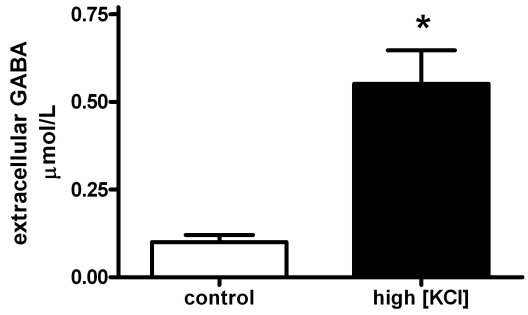

Figure 2. Release of γ-aminobutyric acid (GABA) from primary cultures of cortical neurons. Cultured cells were exposed to an extracellular medium containing 5 mM KCl (control) or 90 mM KCl (high KCl) for 10 minutes. The release of cellular GABA to the extracellular medium is produced by KCl-induced cell membrane voltage changes. $P < 0.001$, Student's t-test.

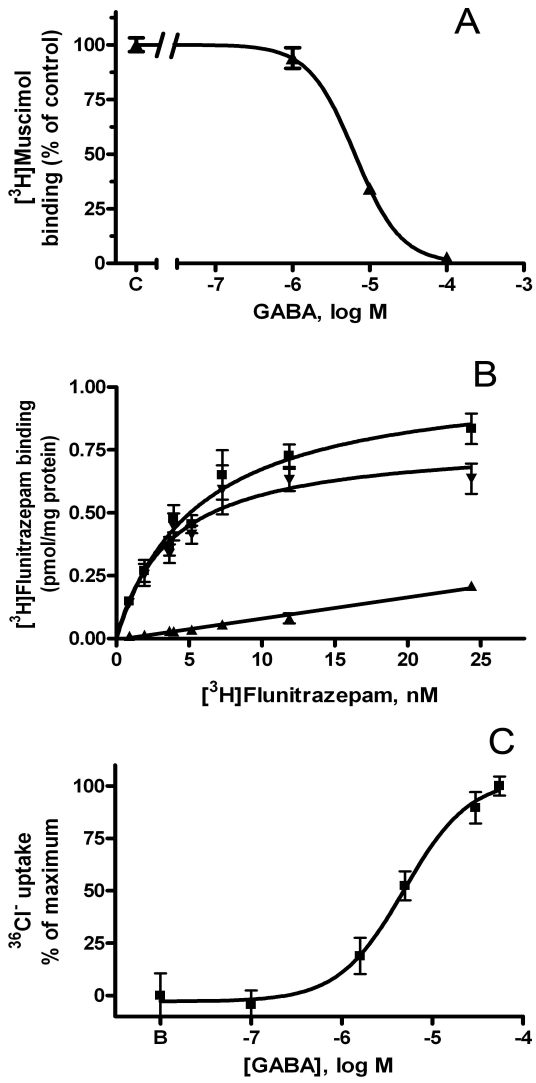

Figure 3. $GABA_A$ receptor binding and function in primary cultures of mice cortical neurons. A) Binding of [^{3}H]muscimol and inhibition by the neurotransmitter γ-aminobutyric acid (GABA). B) Binding of [^{3}H]flunitrazepam. ■ Total binding; ▲ non-specific binding; ▼ specific binding. C) Concentration-response curve for GABA-induced $^{36}Cl^-$ uptake in intact cultured cortical neurons. $^{36}Cl^-$ uptake was normalised to maximum response induced by GABA over basal (B) $^{36}Cl^-$ uptake. Reproduced from reference 9.

nition site at the $GABA_A$ receptor, was inhibited in a concentration-dependent manner by the endogenous neurotransmitter GABA (figure 3A). The binding of [^{3}H]flunitrazepam to the benzodiazepine recognition site in primary cultures of cortical neurons gave Kd and Bmax values of 7.4 nM and 731 fmol/mg protein, respectively (Figure 3B). These binding parameter values agree with those obtained using brain membranes[15]. Figure 3C shows the concentration-response curve for the effect of GABA on Cl^- influx, determined as $^{36}Cl^-$ uptake, in primary cultures of cortical neurons. Micromolar concentrations of GABA were effective at the $GABA_A$ receptor (GABA potency on the [^{3}H]muscimol binding assay was 6.4 E-6 M and against the activation of the Cl- channel was 5.0 E-6 M).

Positive modulatory effects of drugs on the $GABA_A$ receptor

Several drugs act as positive modulators of the GABA action on the $GABA_A$ receptor, like benzodiazepines, barbiturates, neurosteroids and the anaesthetic propofol. Furthermore, some of them directly act on the receptor by opening its associated Cl^- channel. Figure 4A shows the effect of the benzodiazepine diazepam and the barbiturate phenobarbital. While phenobarbital induced a Cl^- influx by itself, diazepam only increased the Cl^- influx in the presence of GABA, without directly inducing a Cl^- influx by itself. The Cl^- assay was used to determine whether synthetic analogues of the endogenous neurosteroids had biological activity mediated by the $GABA_A$ receptor. Figure 4 B shows the effect of a B-nor analog of the neurosteroid allopregnanolone. 3α-Hydroxy-7-nor-5α-pregnan-20-one increased, in a concentration dependent manner, the influx of Cl^- induced by GABA. Similarly, thymol, a phenolic compound closely related to the anaesthetic propofol, acted in a similar way (figure 4C). Based on these studies we have proposed a pharmacophoric model that describes the molecular essential features of compounds like thymol and propofol to interact with $GABA_A$ receptors. The pharmacophoric model includes a hydrogen bond donor group as well as an aromatic ring with two aliphatic substituents[9]. Likewise, by means of computational studies a pharmacophoric hypotheses has been proposed to account for the activiy of neurosteroids on the $GABA_A$ receptor. It includes three essential requirements, specifically one hydrophobic group, one hydrogen bond donor, and one hydrogen bond acceptor, three or four additional hydrophobic features and up to six exclusion volumes, all of them contributing to the binding of neurosteroids at the $GABA_A$ receptor[16].

Blocking effects of neurotoxic compounds on the $GABA_A$ receptor

Several polychlorocycloalkane pesticides belonging to the family of cyclodienes and γ-hexachlorocycloalkane (lindane) act on the $GABA_A$ receptor by blocking the Cl^- channel. They interact with the convulsant picrotoxinin recognition site at the $GABA_A$ receptor[17-20]. Figure 5 shows the effect

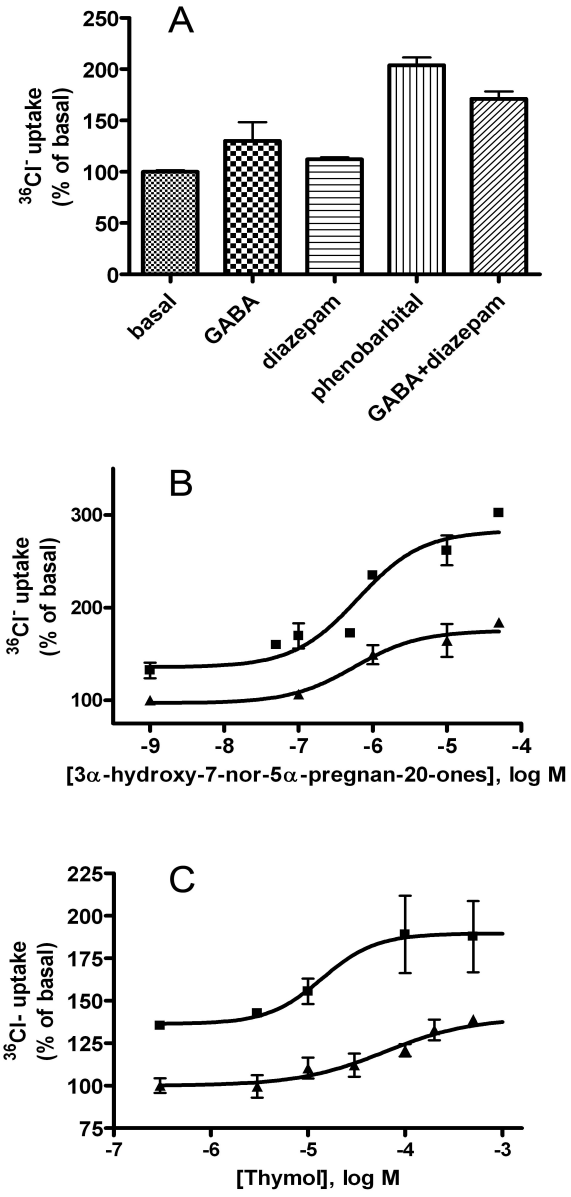

Figure 4. Positive modulation of the $GABA_A$ receptor. A) The pharmaceutical benzodiazepine and barbiturate drugs increase $GABA_A$ receptor function (Cl^- influx) by itself (the barbiturate phenobarbital) or in the presence of γ-aminobutyric acid (GABA) (the benzodiazepine diazepam). B) 3α-hydroxy-7-nor-5α-pregnan-20-one (a synthetic analogue of the neurosteroid allopregnanolone) increased $GABA_A$ receptor function; ■ in the absence of GABA (direct effect) and ▲ in the presence of GABA (positive modulator effect). Adapted from data included in references 9 and 16.

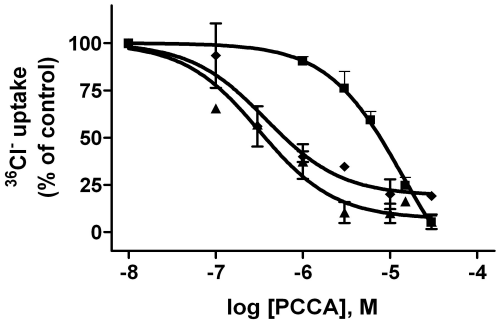

Figure 5. Inhibition of the $GABA_A$ receptor by polychlorocycloalkane (PCCA) pesticides. The organochlorine pesticides lindane (■), α-endosulfan (▲) and dieldrin (♦) inhibit the Cl^- influx induced by γ-aminobutyric acid (GABA). Adapted from data included in reference 8.

of γ-hexachlorocyclohexane (lindane) and of the cyclodienes α-endosulfan and dieldrin on GABA-induced Cl^- flux. While lindane had similar potency on Cl^- influx induced by both GABA or glycine, α-endosulfan and dieldrin were more potents against Cl^- influx through $GABA_A$ receptor than through glycine receptor[8]. Computational studies allowed us to establish a 3D pharmacophore model for the activity of polychlorocycloakane pesticides on the $GABA_A$ and the glycine receptors, consisting of five hydrophobic regions and one hydrogen bond acceptor site in a specific three-dimensional arrangement. The hydrogen bond acceptor moiety and the hydrophobic region were responsible for the affinity of these compounds at the $GABA_A$ receptor whereas only the hydrophobic region of the molecules was responsible for their interaction with the glycine receptor[8].

CONCLUSIONS

Primary cultures of cortical neurons are an in vitro model that allows the evaluation of chemical compounds on the neural transmission mediated by the amino acid GABA. They are enriched in GABAergic neurons and express $GABA_A$ receptors, resembling the native structure of the $GABA_A$ receptor found in vivo. GABAergic pharmacological activity and neurotoxicity of chemical compounds can be assessed using these cultured neurons. Here we demonstrate that primary cultures of cortical neurons can recognize chemical compounds that both positively and negatively interact with the $GABA_A$ receptor. Benzodiazepines, barbiturates, neurosteroids, and thymol act as positive allosteric modulators at the $GABA_A$ receptor, while polychlorocycloalkane pesticides block or negatively modulate the $GABA_A$ receptor. The inhibition of GABA and glycine neurotransmission produces symptoms of neuronal hyperexcitability. In fact, the polychlorocycloalkane pesticides induce convulsions in mammals[21-23]. Furthermore, the potency values for the blocking activity of polychlorocycloalkane pesticides on the $GABA_A$ receptor function are in good relationship with their toxicity in mammals.

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