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HOMEOSTATIC REGULATION OF GLUTAMATE NEUROTRANSMISSION IN PRIMARY NEURONAL CULTURES

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Abstract

Glutamate is the mayor excitatory neurotransmitter in vertebrate nervous system. It has a crucial role in most brain functions under physiological conditions through the activation of both ionotropic and metabotropic glutamate receptors. In addition, extracellular glutamate concentration is tightly regulated through different excitatory amino acid transporters (EAAT). Glutamate neurotransmission is also involved in the neurotoxic effects of many environmental chemicals and drugs. Furthermore, homeostatic changes in glutamate neurotransmission appear in response to prolonged block / enhancement of electrical activity. Here we describe different approaches to evaluate alterations in glutamate neurotransmission regarding glutamate receptors and glutamate transporters by using primary cultures of neurons and of astrocytes. The methods are based on the increased fluorescence of calcium-sensitive probes in response to glutamate agonists, on radioligand binding to glutamate receptors and transport sites and on inmunocytochemistry visualization of glutamate receptors.

Keywords: glutamate; receptor function; receptor binding; transport; intracellular calcium; fluorescence; radioligand; primary cultured neural cells.

1. Introduction

Glutamate is the mayor excitatory neurotransmitter in vertebrate nervous system. Consequently, it has a crucial role in most brain functions under physiological conditions through the activation of both ionotropic and metabotropic glutamate receptors. In addition, extracellular glutamate concentration is tightly regulated through different excitatory amino acid transporters (EAAT). However, excessive glutamate receptor activation leads to neuronal death, also known as excitotoxicity ***(1)***, which is involved in the etiopathology of ischemia and several neurodegenerative disorders, like Alzheimer's or Huntington's diseases. Glutamate neurotoxicity is calcium (Ca^{2+})-dependent and it is mainly mediated through the activation of N-methyl-D-aspartate (NMDA) receptor ***(2)***. In contrast, a reduced glutamatergic synaptic activity has been related to learning and memory deficits ***(3)*** and to the development of schizophrenia ***(4)***. Glutamate neurotransmission is also involved in the neurotoxic effects of many environmental chemicals and drugs. For instance, methylmercury blocks glutamate transporters both in neurons and astrocytes ***(5,6)***, increasing extracellular glutamate concentration. Organochlorine pesticide's acute toxicity can be prevented by glutamate receptor antagonists ***(7)***, whereas long-term exposure to low concentrations of these pollutants reduces glutamate receptors functionalities ***(8,9)***. In addition, the NMDA receptor is the molecular target of several drugs of abuse, like ketamine or phencyclidine ***(10)***.

Homeostatic changes in glutamate neurotransmission appear in response to prolonged block / enhancement of electrical activity. Chronic blockade of neural activity results in increased synaptic clustering and activity of ionotropic glutamate receptors ***(11,12).*** On the contrary, a decrease of functional glutamate receptors is seen when neural activity is enhanced by inhibition of GABAergic neurotransmission ***(8,9)***.

In this chapter, we describe different approaches to evaluate alterations in glutamate neurotransmission regarding glutamate receptors and glutamate transporters by using primary cultures of neurons and of astrocytes. Primary cultures of cortical neurons and of cerebellar granule cells express glutamate receptors and respond to glutamate agonists by increasing intracellular calcium ***(8,13)***. Primary cultures of cerebellar granule cells also express the neuronal EAAT3 ***(6)***, whereas primary cultures of astrocytes express glial EAAT1-2 ***(14)***. These cultures constitute in vitro models that are extensively used in neuropharmacological and neurotoxicological studies involving glutamate neurotransmission, neurodegeneration and neuroprotection mechanisms. Activation of ionotropic NMDA and AMPA/Kainate receptors induces Ca^{2+} influx, either directly through their own channel or through the activation of voltage-gated calcium channels ***(2)***. Furthermore, activation of type-I metabotropic glutamate receptors (mGLUR) also increase $[Ca^{2+}]_i$ by stimulating its release from intracellular stores ***(15)***. Therefore, their functionalities can be evaluated by measuring the increase in $[Ca^{2+}]_i$ in response to agonist's treatment with the fluorescent probe Fluo-3AM. On the other hand, $[^3H]$-MK801 binging assay may be useful to test compounds with potential affinity for the NMDA receptor and moreover to quantify functional NMDA receptor expression. By using $[^3H]$-aspartate uptake we can measure the activity of glutamate transporters. Finally, an example is given on how to measure (qualitatively and quantitatively) NMDA receptor scaling in response to long-term exposure to the organochlorine pesticide dieldrin, by using the combination of Fluo-3AM fluorescence, $[^3H]$-MK801 binging and confocal immunofluorescence for the NR1 subunit of the NMDA receptor.

2. Materials

2.1 Cell cultures

1. Pregnant NMRI mice (16th day of gestation), 7-day-old NMRI mice and newborn NMRI mice are used for the preparation of primary cultures of cortical neurons, of cerebellar granule cells and of astrocytes, respectively.
2. Dulbecco's Modified Eagle medium (DMEM) (Biochrom AG, F0455) pH 7.0 supplemented with 26.2 mM $NaHCO_3$, 25 mM glucose, 0.2 mM L-glutamine, 100 mU/L insulin and 7 µM *p*-aminobenzoic acid. Filter under sterile conditions and add 10% fetal bovine serum (FBS). For cerebellar granule cell cultures DMEM contains 25 mM KCl.
3. Krebs buffer: 120.9 mM NaCl, 4.83 mM KCl, 1.22 mM KH_2PO_4, 25.5 mM $NaHCO_3$, 12 mM glucose, 3 g/L bovine seroalbumin and 0.015 g/L phenol red. Prepare sterile and store at 4 ºC.
4. Trypsin solutions. Prepared in Krebs buffer containing 1.2 mM MgSO4, 50 mg/L deoxyribonuclease I (DNAase), 250 mg/L trypsin. To stop the trypsinization process, prepare a solution containing 500 mg/L soybean trypsin inhibitor and 50 mg/L DNAase in Krebs buffer. Make fresh as required. Prepare sterile.
5. Mitotic inhibitor solution. Prepare a solution containing 250 µM 5-fluoro-2′-deoxyuridine and 1 mM uridine (50X) in distilled water. Prepare sterile.
6. 50 mg/L poly-D-lysine solution in distilled water. Prepare sterile.

2.2 Measurement of $[Ca^{2+}]_i$ by fluo-3AM fluorescence

1. Fluo-3AM fluorescent dye (Molecular Probes, Eugene, OR, USA) is a lyophilized and light sensitive product. Store at -20 ºC. Make fresh as required.
2. Pluronic F-127 (Molecular Probes) is dissolved in dimethyl sulfoxide (DMSO) at 167 mg/ml, by incubating for 10 min at 50 ºC. Make fresh as required.

3. Hank's buffer solution: 137 mM NaCl, 1.3 mM CaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM MgCl, 0.4 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, 5.5 mM glucose (adjusted to pH 7.4 with NaOH if necessary). Store at 4º C. pH is stable at 4 ºC up to 2 weeks.
4. Magnesium-free (Mg-free) Hank's buffer solution: 137 mM NaCl, 1.3 mM CaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, 5.5 mM glucose, adjusted to pH 7.4. Store at 4 ºC. pH is stable at 4ºC up to 2 weeks.
5. L-glycine solution is prepared in water at 5 mM. Store at 4 ºC. (*See* **Note 1**)
6. L-glutamic acid, kainic acid and (R,S)-3,5-dihidroxyphenylglycine (DHPG, Tocris Cookson, Bristol, U.K.) solutions are prepared in Hank's buffer all at 1 mM. Store at 4 ºC. Stable at 4 ºC up to one month.
7. N-methyl-D-aspartate (NMDA) solution is prepared in Magnesium (Mg)-free Hank's buffer at 1mM. Store at 4 ºC. Stable at 4 ºC up to one month. (*See* **Note 2**).
8. (+)-MK-801 hydrogen maleate and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) disodium salt (Tocris) are dissolved in water at 2 mM. Store in aliquots at -20 ºC.
9. 6-methyl-2-(phenylethynyl) pyridine (MPEP, Sigma, St. Louis, MO, USA), 7-(hidroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCOOEt, Sigma) are dissolved in DMSO at 6 mM. Store in aliquots at -20 ºC.
10. A23187 free acid or Calcimycin (Invitrogen, Barcelona, Spain) is reconstituted in DMSO at 25 mM. Store in aliquots at -20 ºC. Working solution is made fresh as required in Hank's buffer at 125µM.
11. $CuSO_4$ solution (5 mM) is prepared in water containing 0,9 % NaCl. Store at 4 ºC.
12. Multi-channel pipette.

13. Fluorimetric plate reader (Ex 485/Em 530; SpectraMax GeminisXS; Molecular Devices, Sunnyvale, CA, USA).

2.3 [^{3}H]-MK801 binding

1. Phosphate buffer solution (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (adjust to pH 7.4 with HCl if necessary). Store at room temperature.
2. Solution containing 100 µM L-glycine and 100 µM L-glutamic acid is prepared in PBS. Store at room temperature.
3. [^{3}H]-MK801 (Perkin Elmer, Boston, MA, USA) (1mCi/ml in ethanol) must be stored at -20 ºC. Prepare fresh [^{3}H]-MK801 solution in PBS at 105 nM for each experiment.
4. (+)-MK-801 hydrogen maleate (Sigma) is dissolved at 100 µM in PBS containing 100 µM L-glycine and 100 µM L-glutamic (*See* **Note 3**). Make fresh as required. Manage with care. Hazard information: harmul by contact, ingestion and inhalation; irritant, slightly neurotoxic, flammable.
5. NaOH is dissolved at 0.2 M in water. Store at room temperature. Manage with care; corrosive.
6. Optiphase 'Hisafe'2 (Perkin Elmer) liquid scintillation cocktail. Store in the unopened container, protected from the light at room temperature.
7. Ultra clear polypropylene tubes of 3ml (Delta Lab, North Huntingdon, PA, USA)
8. Liquid scintillation counter (Wallac 1414 Winspectral™ ;Wallac Oy, Turku, Finland).

2.4 Measurement of glutamate transport by [^{3}H]aspartate uptake

1. HEPES-buffered saline solution (HBSS): 136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2P0_4, 10 mM HEPES and 9 mM glucose, adjusted to pH 7.4.
2. 170 µM glutamate solution: Dilute 60 µL of 2 mM glutamate solution in a total volume of 700 µL with HBSS. This solution is used to prepare the [3H]aspartate solution.
3. [2,3-^{3}H]-D-aspartate (Perkin Elmer, 1mCi/ml) must be stored at 4 ºC. Prepare fresh [^{3}H]-D-aspartate solution at 70 nM in the above 170 µM glutamate solution. Take the solution up and down with a pipette for a complete mixing or use the vortex very gently.

2.5 Immunocytochemistry for the NR1 subunit of the NMDA receptor

1. Permanox chamber slides (Nunc™, Rockilde, Dennamark).
2. Pure methanol pre-tempered at -20 ºC. Manage with care: toxic and highly flammable.
3. Lysis buffer solution: 0.03 % Triton X-100 in PBS. Triton X-100 is irritant and harmful.
4. Blocking buffer solution: 5 % bovine serum albumine (BSA) in PBS.
5. Primary antibody solution: goat polyclonal anti-NMDAR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is diluted in blocking buffer solution at 1:50.
6. Secondary antibody solution: chicken anti-goat Alexa 488 (Molecular Probes) is diluted in PBS at 1:1000.
7. Mowiol 4-88 (Calbiochem, EMD Bioscience Inc., La Jolla, CA, USA).
8. Confocal fluorescence microscope.

3. Methods

3.1 Preparation of primary neural cell cultures

1. Obtain the cerebral cortex from E16 embryos or the cerebellum from postnatal P7 mice to prepare primary cultures of cortical neurons and of cerebellar granule cells, respectively. Glial cells are prepared from cerebral cortex of P1-P2 mice.
2. Mince the tissue and digest the cells with the trypsin solution, under mild agitation at 37 ºC. Stop digestion by adding the trypsin inhibitor solution.
3. Disperse the cells with a cannula or pipette, centrifuge at 1000 rpm for 5 min and resuspend in DMEM.
4. Seed the cell suspension (1.6×10^6 cells/ml for neuronal cultures; $4x10^5$ cells/ml for astrocyte cultures) 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% CO2/95% air atmosphere at 36.8 °C without changing the culture medium (neuronal cultures) or changing the medium every 4 – 6 days.
5. After 24–48 h in culture, add the mitotic inhibitor to halt glial proliferation for neuronal cultures.

3.2 Measurement of $[Ca^{2+}]_i$ by Fluo-3AM fluorescence

Fluo-3AM is a quite sensitive and membrane permeable fluorescent dye, which is cleaved by intracellular esterases. Fluo-3 fluorescence (F) is directly proportional to $[Ca^{2+}]$ present in the cultures. Therefore, it can be used as an indicator of changes in $[Ca^{2+}]_i$ induced by glutamate (non specific endogenous agonist of glutamate receptors) or by specific agonists of either NMDA receptor (NMDA), AMPA/Kainate receptors (kainic acid) or type-I mGLUR (DHPG). (*See* **Note 4**). Figure 1 shows the effects of glutamate receptor agonists and antagonists on $[Ca^{2+}]_i$. Specificity of the assay is

demonstrated by the complete reversion of agonist effects by specific antagonists (Figure 1B). The ionophore calcimycin and the quenching agent $CuSO_4$ are used to obtain the maximum (F_{max}) and the minimum (F_{min}) fluorescence values, respectively. $[Ca^{2+}]_i$ was calculated for each well as: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of Fluo3AM/Ca^{2+} (320nM) ***(16)***.

Procedure:

1. Reconstitute each aliquot of Fluo-3AM in 15 µl of Pluronic-F127 solution and incubate at 37 ºC for 5 min. Transfer it into a new tube (protected from the light) containing 10 ml of Hank's buffer solution (final concentration of Fluo-3AM = 9 µM).
2. Remove the culture media from the 96-well plates where the cells are grown. (*See* **Note 5**). Wash twice with 100 µl/well of Hank's buffer solution and add 80 µl/well of Fluo-3AM solution (*See* **Note 6**). Incubate for 1 h at 37 ºC.
3. Rinse away the excess of Fluo-3AM solution and wash three times with 100 µl/well of Hank's buffer (or Mg-free Hank's buffer).
4. Add 100 µl/well of the agonist solution (*See* **Note 7**) with the multi-channel pipette and immediately read the fluorescence (F) (Ex 485nm/Em 530nm).
5. Add 20 µl/well of calcimycin solution without removing the agonist solution. Incubate for 30 min at room temperature and read the fluorescence (F_{max}).
6. Add 20 µl/well of $CuSO_4$ solution. Incubate for 12 min at room temperature and read the fluorescence (F_{min}).
7. Determine $[Ca^{2+}]_i$ for each well by using the above equation.

3.3 [^{3}H]-MK801 binding

[^{3}H]-MK801 binding can be used to evaluate both the agonist and antagonist properties of several compounds not only within the ion channel but also at allosteric sites on NMDA receptors ***(17,18)***. In addition, it could be used to determine alterations on NMDA receptor activity or cell surface expression after chronic exposure to neurotoxicants or drugs ***(8,19)***. In the first case, a concentration-response curve would be the proper way to test the modulatory effects of the selected compound on [^{3}H]-MK801 binding. The concentration that afford the half inhibition/potentiation on [^{3}H]-MK801 specific binding (IC50/EC50) will indicate the potency of the compound at the NMDA receptor. On the other hand, it is possible to determine the number of functional NMDA receptors from [^{3}H]-MK801 binding assay by fitting the binding values to a competitive one-site binding curve as previously described by De Blasi *et al*. (1989) ***(20)***. (*See* **Note 8**). From apparent K_d and B_{max} calculation we will obtain information about the affinity of MK801 (activity of NMDA receptor) and the number of functional NMDA receptors, respectively.

Procedure:

1. Remove the culture media from the 24-well plates where the cells are grown. (*See* **Note 5**). Wash three times with 500 µl/well of PBS at 37ºC.
2. Add 500 µl/well of PBS at 37 ºC containing 100 µM L-glycine, 100 µM L-glutamic and different concentrations of the selected compound in the case of concentration-response curve or of non-labeled MK-801 (0-10 µM) in order to determine apparent K_d and B_{max}. (*See* **Note 9**). Non-specific binding is determined in the presence of 100 µM non-labeled MK-801.
3. Add 25 µl/well of PBS containing 105 nM [^{3}H]-MK801 (final concentration in the well 5 nM) and incubate for 15 min at 37 ºC.
4. Wash out three times with 1.5 ml/well of ice-cold PBS.

5. Add 250 µl/well of 0.2 M NaOH and incubate 4 h at room temperature with agitation.
6. Collect the disaggregated cells from each well and mix them with 2 ml of Optiphase 'Hisafe'2 in polypropylene tubes. After gently agitation radioactivity is measured in a liquid scintillation counter. (*See* **Note 10**).

3.4 Measurement of glutamate transport by [^{3}H]-D-aspartate uptake

The assay has been reported to be useful in order to evaluate alterations in the functionality of the EAATs in the presence of environmental neurotoxicants such as methylmercury and manganese in primary neuronal and glial cultures ***(6,21)***. EAATs catalyze Na^+- and K^+- coupled transport of L-glutamate as well as L- and D-aspartate with similar affinities. Therefore, the uptake of radioactive labeled D-aspartate (as a tracer of glutamate) can be used to determine the inhibitory potency (IC50) of the selected compound at the glutamate transporter, by performing a concentration-response curve. Primary cultures of cerebellar granule cells are a nearly homogenous population of glutamatergic neurons that express the glutamate transporter EAAT3 ***(6)*** whereas primary cultures of astrocytes express different levels of GLAST (EAAT1) and GLT1 (EAAT2) (***14,26***). (*See* **Note 11**).

Procedure:

1. Remove the culture media from the 24-well plates where the cells are grown. (*See* **Note 5**).
2. Wash out three times with 500 µl/well of HBSS at 37 ºC.
3. Add 250 µl/well of HBSS and 250 µl/well of the test compound (2X). Non-specific uptake is determined in the presence of 1 mM glutamate. Mix solutions gently by drawing a virtual 8 with the plate.
4. Incubate 10 minutes at 37 ºC

5. Add 25 µl/well of [^{3}H]-D-aspartate solution PBS containing (final concentration in the well 5 nM). Mix solutions gently and incubate 5 minutes at 37 ºC.
6. Wash out three times with 1.5 ml/well of ice-cold HBSS.
7. Add 250 µl/well of 0.2 M NaOH and incubate 4 h at room temperature with agitation.
8. Collect the disaggregated cells from each well and mix them with 2ml of Optiphase 'Hisafe'2 in polypropylene tubes. After gently agitation radioactivity is measured in a liquid scintillation counter. (*See* **Note 12**).

3.5 Homeostatic scaling of NMDA receptors visualized by immunocytochemistry

Changes on NMDA receptor localization can be monitored by performing immunolabelling of the NR1 subunit of the NMDA receptor followed by confocal immunofluorescence detection. NR1 immunofluorescence have been used to confirm the reduction of NMDA receptor cell surface expression after long-term exposure to the organochlorine pesticide dieldrin in primary cultures of cortical neurons and of cerebellar granule cells ***(8,9)***. Figure 2 shows an example of how the reduction of NMDA receptor functionality can be observed by three different techniques described here: Fluo-3 fluorescence, [^{3}H]-MK801 binding and immunocytochemistry against the NR1 subunit of the NMDA receptor.

Procedure:

1. Remove the culture media from Permanox chamber slides where the cells are grown and wash twice with 200 µl/well of PBS
2. Fix with methanol at -20 ºC for 10 min.
3. Wash twice with 200 µl/well of PBS. Fixed cells can be stored at 4 ºC for several days.

4. Incubate with 200 µl/well of lysis buffer for 5 min at room temperature and then wash three times (5 min each rinse) with PBS.
5. Incubate with 200 µl/well of blocking buffer for 30 min at room temperature.
6. Incubate over night at 4ºC with 150 µl/well of the primary antibody solution.
7. Wash three times (X 5 min) with PBS and incubate with 150 µl/well of the secondary antibody solution for 1 h at room temperature.
8. Wash three times (X 5 min) with PBS and coverslip the slides with Mowiol.
9. Examine the cells in a confocal fluorescence microscope. (*See* **Note 10**).

4. Notes

1. L-glycine is co-agonist of the NMDA receptor and dramatically potentiates NMDA receptor activation ***(22)***. Therefore, it is convenient that both NMDA and glutamate solutions contain 5 µM L-glycine in order to observe maximal agonist response.
2. NMDA solutions must be prepared in the absence of Mg because it blocks NMDA receptor under basal conditions. In contrast, for glutamate solutions this is not necessary since glutamate-induced depolarization remove Mg from NMDA receptor ***(23)***. Nevertheless, the sensitivity for glutamate-induced $[Ca^{2+}]_i$ rise at low concentrations of agonist could be improved in the absence of Mg.
3. Glutamate and glycine must be present at such high concentration during the binding assay because both neurotransmitters are needed to maintain the open-channel conformation of the NMDA receptor, which allow MK801 to bind to the channel pore ***(24)***. Despite the EC50 for glutamate and glycine binding to NMDAR are around 0.15 µM, it is common to use saturating concentrations of glutamate and

glycine for [^{3}H]-MK801 binding in membrane preparations as well as in cultured neurons ***(8)***.

4. Eventually, the antagonists of NMDA receptor (MK801), AMPA/Kainate receptors (NBQX) or type-I mGLUR (CPCOOEt for mGLUR1 and MPEP for mGLUR5) may be useful to evaluate the contribution of each glutamate receptor to the glutamate-induced $[Ca^{2+}]_i$ rise in the given culture system (see Figure 1C).
5. To evaluate NMDA receptor function in primary neuronal cultures, cells should be grown first for at least 7 days *in vitro* to be sure that they express functional NMDA receptors ***(25)***.
6. From here until the end of the experiment 96-well plates must be protected from the light. Furthermore, it is convenient to work and place the fluorimetric plate reader in the maximum darkness conditions.
7. The proper way to study the functionality of glutamate receptors is by performing a concentration-response curve with at least 4-5 different concentrations of agonist, and fitting the values to a sigmoid curve. An appropriate broad range of concentrations for glutamate, NMDA and DHPG is from 1-100 µM, whereas for kainic acid is from 5-500 µM ***(8)***.
8. In brief, from [^{3}H]-MK801 binding curve we will obtain two parameters: B_0 (specific binding = total binding - nonspecific binding) and IC50. Now, we can calculate apparent K_d and B_{max} as follows: $K_d = IC50 - L$; $B_{max} = B_0 * IC50/ L$, where L is the concentration of [^{3}H]-MK801.
9. If the affinity of the selected compound for the NMDA receptor is unknown, use a broad range of concentrations (from 0 to 1mM) in order to assure whether the compound has effect or not.

10. Before collecting the cells, 10-20 µl can be taken from each well for protein content determination to express radioactivity as pmol of [^{3}H]-MK801/mg of protein, as follow: DPM/(K*A*P). In this equation DPM is the radioactivity in decompositions per minute; K is the conversion factor from DPM to nanocuries (nCi) ($2.24x10^3$ DPM/nCi); A is the specific activity of [^{3}H]-MK801 in Ci/mmol; P is the protein content in mg/well. Eventually, the radioactivity of 25 µl of PBS containing 105nM [^{3}H]-MK801 can be straight measured in order to calculate the actual concentration of [^{3}H]-MK801 in the well, as follows: [[^{3}H]-MK801] (nM) = DPM/(K*A*V], where V is the total volume of the well (0.525 ml).
11. It is worth noting that pure astroglial cultures expressed only GLAST (EAAT1), whereas astrocytes grown in the presence of neurons expressed both GLAST (at increased levels) and GLT1 (EAAT2) ***(14,26)***. Consequently, mixed cultures of astrocytes and cerebellar neurons will express EAAT1, EAAT2 and EAAT3. Thus, mixed cultures may be useful to study general effects on glutamate transport and pure cultures to evaluate the effects of the test compound on one specific EAAT.
12. Since the NMDA receptor is mainly expressed at the cell surface of the cells, the use of confocal fluorescence microscope is needed in order to discriminate the NR1 immunostaing between the plama membrane and the citosol of the neurons.

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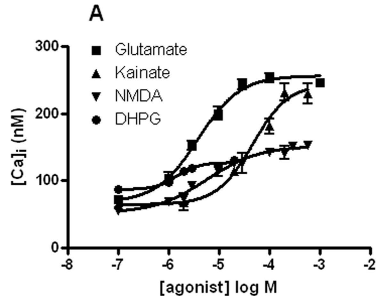
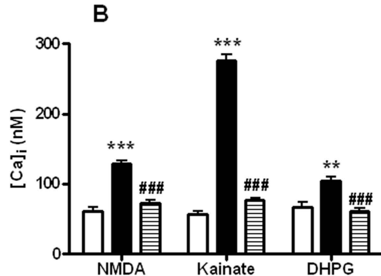
Figure legends

Figure 1. Effects of glutamate receptors agonists/antagonists on $[Ca^{2+}]_i$ measured by fluo-3 fluorescence in primary cultures of cortical neurons. A) Concentration-response curves for glutamate and the specific agonists NMDA, Kainate and DHPG. B) Agonist-induced $[Ca^{2+}]_i$ rise (black bars) is completely reversed by their respective antagonists (stripped bars) (*See* **Note 4**). White bars represent $[Ca^{2+}]_i$ in the absence of agonist. **p<0.01, ***p<0.001 vs. non-treated cultures (white bars); ###p<0.001 vs. agonist-treated cultures. C) Glutamate-induced increase on $[Ca^{2+}]_i$ is reduced by either ionotropic (MK801 and NBQX) or type-I metabotropic (MPEP and CPCOOEt) glutamate receptor antagonists.

Figure 2. Effects of long-term exposure to the insecticide dieldrin (60 nM for 6 days in vitro) on NMDA receptor functionality and cell surface expression in primary cultures of cortical neurons. A) Concentration-response curve for NMDA-induced $[Ca^{2+}]_i$ rise in non treated (squares) and dieldrin-treated cells (triangles). B) The number of functional NMDA receptors is significantly lower (p<0.05) in dieldrin-treated cells with respect to control cells, as measured by determining B_{max} values from $[^3H]$-MK801 binding. C) Confocal immunofluorescence of the NR1 subunit of the NMDA receptor revealed a reduced immunostaining of NR1 at the plasma membrane of the cells after long-term exposure to dieldrin. Bar size = 10 μm.


A
300
200
100
0
-8
-7
-6
-5
-4
-3
-2
Glutamate
Kainate
NMDA
DHPG
[Ca]i (nM)
[agonist] log M


B
300
200
100
0
***
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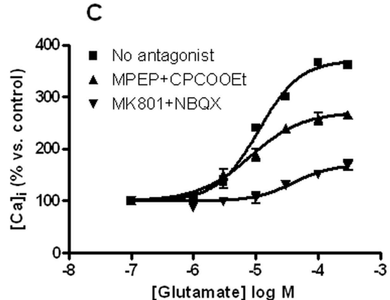
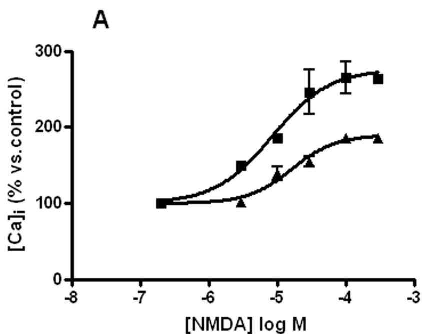
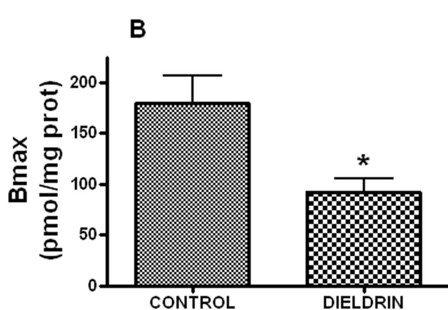
###
###
###
NMDA
Kainate
DHPG
[Ca]i (nM)


C
400
300
200
100
0
-8
-7
-6
-5
-4
-3
No antagonist
MPEP+CPCOOEt
MK801+NBQX
[Ca]i (% vs. control)
[Glutamate] log M





C

CONTROL

DIELDRIN