Davide Giust

EFFECT OF NANOCOMPOUNDS ON THE MODULATION OF ADENOSINE AND METABOTROPIC GLUTAMATE RECEPTORS, ON DIFFERENT *IN VITRO* MODELS

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"Estudio del efecto de los nanocompuestos en la modulación de los receptores de adenosina y metabotrópicos de glutamato en diferentes modelos *in vitro*."

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Thesis by

DAVIDE GIUST

Doctorate on Chemistry

2007-2010

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Certifican:

Que el trabajo de investigación titulado "*EFFECT OF NANOCOMPOUNDS ON THE MODULATION OF ADENOSINE AND METABOTROPIC GLUTAMATE RECEPTORS ON DIFFERENT IN VITRO MODELS.*" constituye la Memoria de D. DAVIDE GIUST, Licenciado en Farmacia por la Universidad de Trieste (Italia), para optar al **grado de Doctor en Ciencias Químicas**.

Así mismo, certifican que este trabajo ha sido realizado bajo su tutela en el departamento de Química Inorgánica, Orgánica y Bioquímica de la Facultad de Ciencias Químicas de la Universidad de Castilla la Mancha, y que cumple todos los requisitos necesarios para su presentación.

Para que así conste, firman el presente certificado en Ciudad Real a 21 de Mayo de 2010.

Fdo. Dra. Mairena Martín López Fdo. Dr. José Luis Albasanz Herrero

Alla mia grande famiglia, e a coloro che ne custodiscono l'integritá: i miei genitori.

Quo fata trahunt,
retrahuntque sequamur,
quid quid erit superanda
omnis fortuna, ferendo est.

Eneide, Libro V, Virgilio

Honey you're a rock,
upon which I stand.

Green Eyes, Coldplay

The present work was mainly performed in the Neurochemistry Group from *Area de Bioquímica* in the Faculty of Chemistry in University of Castilla-La Mancha and during two non consecutive secondments at the University of Trieste. Davide Giust has been hired as ESR (Early Stage Researcher), during the PRAIRIES European Research Training Network, funded by the VIth Frame Program, as Marie Curie action.

Some of results herein presented have been used in the following manuscripts:

D. Giust, A. Delforge, M. Martín, J. L. Albasanz, D. Bonifazi, 2010, “Biologically active [60]fullerene based surfaces”, *JACS Comm.*, on preparation.

D. Giust, T. Da Ros, M. Prato, J.L. Albasanz and M. Martín, 2010, “[60]fullerene hidrosoluble derivative modulates adenosine receptors endogenously expressed in SK-N-MC cells”, submitted to *Neuroscience Letters.*

D. Giust, T. Da Ros, J.L. Albasanz and M. Martín, 2010, “Protective effect of [60]fullerene hydrosoluble derivative against L-Glutamate toxicity in SK-N-MC cells”. On preparation.

D. Giust, T. Da Ros, J.L. Albasanz and M. Martín, 2010, “Reduced Amyloid-beta peptide toxicity by using [60]fullerene hydrosoluble derivative ”. On preparation.

ABSTRACT

Engineering compounds and materials for interfacing with biology and medicine is actually one of the most interesting challenge of scientists from different areas. In that sense, one of the investigated compounds due to their possible applications in a variety of areas, have been fullerenes and allotropic carbon made related compounds, like nanotubes and nanohorns. The large number of possible applications of this family compounds, in particular [60]fullerenes, is strictly related to the peculiar physical and chemical properties of their unsaturated carbon structures, although implicating a low solubility in the majority of organic solvent, and only partial solubility in aqueous systems, and for that reason demonstrating toxic effects *in vivo*, due to the accumulation in tissues. The most used strategies used to bypass these limitations, have been the modification of the main structure with appendages which increasing the solubility in aqueous solvent, including biological fluids. In the present study we used an hydrosoluble derivative of [60]fullerene bis-adduct trans-3 isomer, demonstrating to own a large number of possible biological applications, from HIV-protease inhibition, antitumor activity, to antibacterial activity. We tested it on different *in vitro* models of neurodegenerative diseases. Furthermore, we tested [60]fullerenes as different gold modified surfaces, to proof biocompatibility and eventually effect of modified surface. The context we used to test [60]fullerene, has been about the neurodegenerative disease, as Alzheimer's disease. In these diseases have been study the relevant role of L-Glutamate and amyloid-beta peptide as responsible of an irreversible degeneration of neuronal cells in CNS (central nervous system). Furthermore as investigated by our group, adenosine receptors and metabotropic glutamate receptors seem to have differential role in neurodegeneration and/or neuroprotection of neuronal cells exposed L-Glu and Aβ toxicity. The modulation of these receptors have been demonstrated to be a promising target to engineering new therapeutics compound for neurodegenerative disease, in that sense [60]fullerene hydrosoluble derivative has proved its habylity to modulate the expression of adenosine and metabotropic glutamate receptors in these models. Furthermore the protective effect of [60]fullerene is preserved also as modified surfaces (SAMs), and demonstrating to be totally biocompatible to biological model used.

RESUMEN

El diseño de materiales que relacionen la Biología y la Medicina es uno de los retos más interesantes en la actualidad para científicos de diferentes áreas. Entre los compuestos con un futuro más prometedor en estos campos debido a sus múltiples aplicaciones encontramos a los fulerenos y los compuestos sintetizados a partir de alótropos de carbono. La gran cantidad de aplicaciones para la familia de compuestos derivados del fulereno está estrictamente relacionada con su particular estructura físico química. Su pobre solubilidad en disolventes acuosos debido a su estructura orgánica le han adjudicado un papel de compuesto tóxico *in vivo*, debido a procesos de bioacumulación en tejidos. Entre las estrategias más empleadas para solventar estas limitaciones encontramos las modificaciones químicas de las estructuras carbonadas encaminadas a aumentar su solubilidad en líquidos acuosos, entre los que incluiríamos los fluidos biológicos. En el presente estudio, hemos empleado un derivado hidrosoluble del fulereno que ya ha demostrado algunas de sus posibles aplicaciones biológicas como inhibición de la proteasa del VIH, acción antitumoral o antibacteriana. En este trabajo, se ha probado la efectividad de este compuesto en diferentes modelos *in vitro* de enfermedades neurodegenerativas. Además, hemos probado el efecto de este compuesto en superficies modificadas, con el fin de estudiar su biocompatibilidad y su posible efecto sobre una superficie modificada. En las enfermedades neurodegenerativas se ha observado el papel del L-glutamato así como del péptido β-amiloide como responsables de la neurodegenración irreversible que se observa en células del sistema nervioso central. Además, se ha publicado por este grupo de investigación el papel de los receptores de adenosina y metabotrópicos de glutamato en los fenómenos de neurodegeneración/neuroprotección en células del SNC expuestas a L-Glu o al péptido amiloide. La modulación de estos receptores resulta una diana prometedora para tratamientos futuros frente a estas enfermedades neurodegenerativas. En este sentido, el derivado de fulereno empleado ha demostrado su habilidad para modular los niveles de estos receptores en modelos de enfermedades neurodegenerativas. Por último, el carácter protector del fulereno ha sido demostrado en superficies modificadas demostrando así ser totalmente biocompatible con los modelos biológicos empleados.

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ABBREVIATIONS

[^{3}H]DPCPX	[Dipropil-2,3-3H(N)]ciclopentil-1,3- dipropilxantine β-D-ribofuranuronamide
AD	Alzheimer's disease
ADA	Adenosine deaminase
Ado	Adenosine
ADP	Adenosine-5´-diphosphate
AIDA	(RS)-1-aminoindan-1,5-dicarboxilic
AMP	Adenosine 3´,5´-monophosphate
AMPA	Amino-3-hidroxi-5-metilisoxazol-4-acido propionic
AMPc	Adenosine 3´,5' monophosphate cyclic
APP	Amyloid precusor protein
ATP	Adenosine 5´-trifosfato
Aβ	amyloid-β
BACE-1	Amyloid-β peptide converting enzyme
Bmax	Maximum union
BSA	Albumine bovine serum
cDNA	Deoxyribonucleic acid complementary
CGS21680	2-[p-(2-carboxietil)pheniletilamin]-5'-N-etilcarboxamide adenosine
CHA	N6-ciclohexiladenosine
CHPG	(RS)-2-Cl-5-hidroxifenilglicine
CPA	N^6-cyclopentyladenosine
CPA	N^6-ciclopentiladenosine
Da	Daltons
DAG	Diacylglicerol
DEPC	Diethilpirocarbonate
DMEM	Dulbecco modified medium
DMSO	Dimethylsufloxide
DNA	Deoxyribonucleic acid
DNasa	Desoxiribonuclease
dNTPs	Deoxinucleotide triphosphate
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
DPCPX	8-ciclopentil-1,3-dipropilxantine
GDP	Guanosine-5´-diphosphate

GNP	Gold Nanoparticle
GPCR	G-protein coupled receptors
GTP	Guanosine 5'-trisphosphate
iGluRs	Ionotropic glutamate receptors
IP3	Inositol-1,4,5-trisphosphate
Kb	Kilobase
Kd	Dissociation constant
kDa	Kilodalton
L-Glu	L-Glutamic acid
mGluRs	Metabotropic Glutamate receptors
mRNA	messengerRNA
MTT	Bromure of 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolium
NH	Nanohorn
PBS	Phosphate saline Buffer
PDE	Phoshodiesterase
PKA	Protein kinase cAMP dependent
PKC	Protein kinase calcium dependent
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Fenilmetilsulfonilfluoruro
PSMF	Fluorure de fenilmetilsulfonil
R-PIA	R-N^6-(phenylisopropyl)-adenosine
RT	Reverse Transcriptase
RT-PCR	Polymerase reverse chain transcription reaction
SAM	Self Assembled Monolayer
SCH 58261	5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SEM	Standard Error of the Mean
WCA	Water contact angle
XPS	X-ray photoelectron spectroscopy
ZM241385	4-(2-[7-amino-2-[2-furyl]-[1,2,4]triazolo[2,3-a]{1,3,5}triazin-5-yl-amino]ethyl)phenol

INTRODUCTION

Introduction

1. Fullerenes and nanotubes

Fullerenes belong to the family of compound allotropes of carbon, and are the third form of pure carbon made compound after diamond and graphite; those are present as elliptic, plane or spherical compounds of fully unsaturated carbons. Spherical fullerenes are called *buckyballs*, instead of those cylindrical ones called nanotubes or *buckytubes*. In 1985, a compound made of 60 carbon atoms was discovered by Robert Curl, Harold Kroto and Richard Smalley, next renamed fullerene because similarity to the geodesic structure engineered by Richard Buckminster Fuller; those same chemists received the Nobel prize for that discover in 1996 (Kroto and al., 1985). About spherical fullerenes it's out of doubt that the most known and investigated is the C_{60}; the C_{60} (illustr. 1) has the smallest stable structure considering the series of the known fullerenes species, C_{70}, C_{76} and C_{84}. Its structure made of 60 carbon atoms has the form of a truncated icosahedra that reminds the form of a soccer ball; that spherical is thus made by 20 hexagon and 12 pentagons carbon-carbon bonded. The Van der Waals diameter for C_{60} is approximately 1 nm, instead the molecule diameter from nucleus to nucleus is 0.7 nm. The molecule of C_{60} has two type of bond: 6:6 bonds between hexagons that may be considered like double carbon bonds and bonds 6:5 shorter than the first ones present between hexagons and pentagons carbon (Li, 2001). Initially it was hypothesized that [60]fullerenes were 'super aromatic' molecules, further analysis clarified that C_{60} possess a polyenic structure, with all the double bonds inside the six-membered rings. The reactivity of C_{60} is typical of an electron deficient olefin, thus reacts readily with nucleophiles and is a reactive component in cycloadditions (Hirsch and Brettreich, 2005). An useful method to obtain C_{60} fullerenes is well established and consist in the generation of a high current between to electrodes of graphite (Scott *et al*., 2002). Fullerenes represent the main example of compounds pure carbon made, soluble in organic solvent, even at low concentration. A typical profile of saturated solubility for C_{60} in some organic solvent: 1-chloronaphtalene (53 mg/ml);

1,2,4-trymethylbenzene (18 mg/ml); carbon disulfide (8 mg/ml); toluene (3 mg/ml); water (1.3 x 10^{-11} mg/ml) (Ruoff *et al*., 1993)

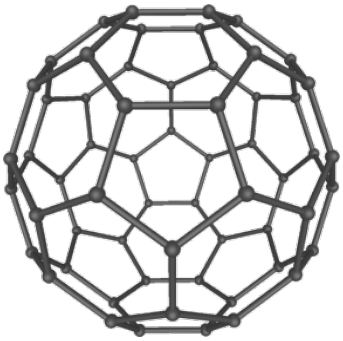


illustration 1. A carbon cage of C_{60} fullerene.

1.1 Fullerenes chemistry

Fullerenes tend to react as electrophiles with a host nucleophiles in nucleophilic additions. Example of these reactions are Grignard reagents and organolithium reagents; a typical reaction is that of C_{60} with methylmagnesium chloride that stops quantitatively with the formation of the penta-adduct with the methyl groups centred around a cyclopentadienyl anion which is subsequently protonated (Matsuo *et al.,* 2006). Other typical nucleophilic reaction of fullerenes is the Bingel reaction (Bingel, 1993). Fullerenes reacts with chlorobenzene and aluminium chloride in a Friedel-Crafts alkylation type reaction; during the reaction an hydroarylation occurs and the product is the 1,2 addition adduct (Ar-C-C-H) (Washita *et al*., 2007). An other interesting reaction occurs when [6,6] bonds of fullerenes react with dienes or dienophiles in cycloadditions, for instance Diels-Alder reactions. Four-membered rings can be obtained by [2+2]cycloadditions for instance with benzyne. An example of a 1,3-dipolar cycloaddition to a 5-membered ring is the Prato reaction (Maggini, Scorrano and Prato, 1993) (illustr. 2). Fullerenes are easily hydrogenated by several methods with $C_{60}H_{18}$ and $C_{60}H_{36}$ being the most studied hydrofullerenes. However, completely hydrogenated $C_{60}H_{60}$ fullerenes is only hypothetical because of large strain. Highly hydrogenated

fullerenes are not stable, prolonged hydrogenation of fullerenes by direct reaction with hydrogen gas at high temperature conditions results in collapse of cage structure with formation of polycyclic aromatic hydrocarbons (Jin *et al.,* 1994). Although not easily, fullerenes can be oxidized, for example it is well known the oxidation with osmium tetraoxide and cerium tetroxide (Akama, 1995). Fullerenes react in electrophilic additions as well. The reaction with bromine can add up to 24 bromine atoms to the sphere; the record holder for fluorine addition is $C_{60}F_{48}$ (Jia *et al*., 2008). An other reaction observed for fullerenes is the reaction with carbenes to have methanofullerenes (Bettinger *et al*., 2006). For an exhaustive review on fullerenes and their properties *see* Diederich's review (Diederich, 1997).

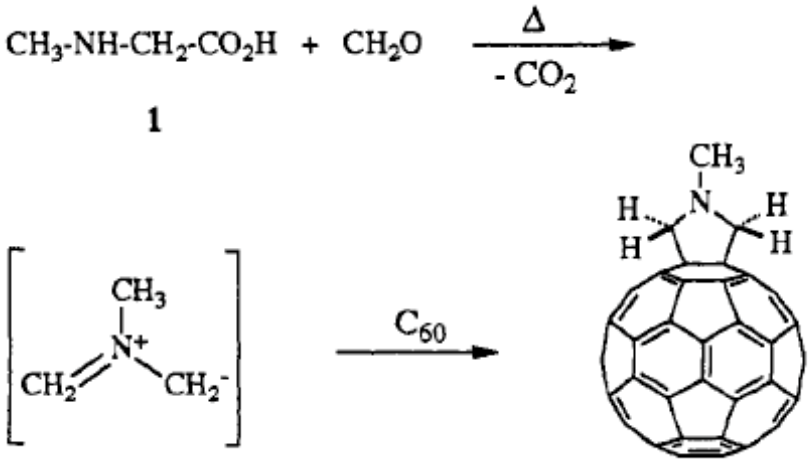


illustration 2. Scheme of Prato reaction (from Maggini, *et al,* 1993).

1.2 Fullerenes applications

From the first years of the XXI century, fullerenes had focused the attention for several possible applications where they could be used. Though it's the only soluble form of carbon, many strategies had been provided to improve the solubility of fullerenes in some of the most useful organic solvent, but especially in water, in order to enlarge the spectra of possible application of fullerenes, for example in biomedicine.

1.2.1 Application of fullerenes to materials science

Fullerenes enter in the formation of electroactive polymers by enhancing the electronic properties of these, obtaining polymers with optical limiting properties. On the other hand, fullerenes embedded in polymers may become more ductile and easy to handle. Resulting materials could be useful for surface coating, photoconducting devices, and to create new molecular networks (Hirsch, 1993, Kojiima, 1995). Recently, it was reported the functionalization of fullerenes with specific molecules electron donor, thus converting fullerene in an electron acceptor-donor in order to transfer electrons to a specific device, as recently reported for fullerenes derivatized porphyrins-carotenoid complex (illustr. 3) (Gust *et al*., 2009).

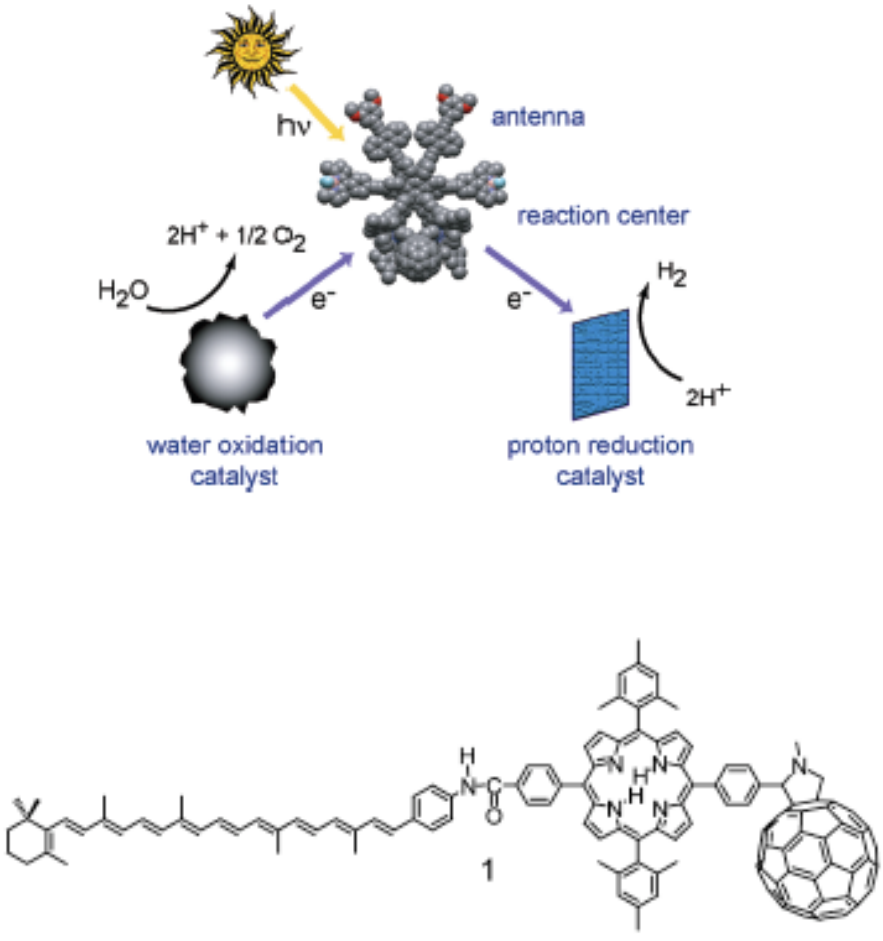


illustration 3. Example of reaction center using fullerene as electron donor-acceptor. (from Gust *et al.,* 2009).

In this sense [60]fullerenes has been demonstrated to be an excellent electron acceptor for studies and design of news systems D-B-A (donor-bridge-acceptor); like in the example above, fullerene received a considerable attention for its properties in photoinduced charge-transfer as dyads and triads. In these cases the singlet or triplet state of photoexcited C_{60} accepts an electron from the linked donor to give the charge separate state. Several fullerene-based donor acceptor containing porphyrins, phtalocyanines, ruthenium complexes, ferrocenes have been examinated as donor-acceptor systems (Lissen *et al.,* 1995; Maggini *et al.,* 1992.; Guldi *et al*., 1997). These fullerene based systems beside the use for the engineering of limiting optical material or electronic device, could take advantage from the photoinduced effect on fullerene and be used for example in biomedicine, as it will be describe later. The use of fullerene in making thin films is currently of high interest; by simple surface coating it is possible to transfer the properties of fullerenes to a material. In this sense, it has been increased the use of fullerenes in the formation of self-assembled monolayers (SAM's) and Langmuir films, to form controlled and organized network on surface. About the use of fullerenes derivatives in optical devices like explained for the donor-bridge-acceptor, we have to consider that not so far was synthesized the first thermotropic liquid crystal containing two cholesterol units attached to a methanofullerene, and its bahaviour was investigated too (Chuard *et al.,* 1996). Furthermore fullerenes have focused the attention for its use in super-resistant material, water resistant material, superconductor, hydrogen storage and many others applications(Tang *et al*., 2001; Wind *et al.*, 2003; Nasibulin *et al*., 2009).

1.2.2 Fullerenes interfacing with biology

Fullerene C_{60} ([60]fullerene) has been investigated furthermore than for its applications to science of materials, for its promising properties on biology. In the recent years an increase of experimental evidence demonstrated much of its biological properties, even if some limitations were represented by its non solubility in biological fluid, especially in water, which generated some problems in studing its activity and eventual toxicity. The hydrophobic nature of [60]fullerene, or better the water repulsion, posed some limitations in its use, nevertheless its hydrophobic properties have been demonstrated to form part of its activity. To overcoming these limitations different strategies have been employed, like encapsulation of C_{60} in cyclodestrin (Andersson *et al.,* 2006) and in calixarenes (Shinkai and Ikeda, 1997) or water suspension preparation (Scrivens *et al*., 1997). Anyway, the most effectiveness technique was to modify the solubility properties of [60]fullerene by covalent attachment of water soluble groups, such as dendrimers (Brettreich and Hirsch, 1998), cyclodestrins (Samai and Geckeler, 2000), or calixarenes (Wang *et al*., 2000), Recently it was synthesized by the group of prof. Prato a bis-adduct *trans*-3 derivative of [60]fullerene (t3ss) containing two amino acidic moieties with two cationic ammonium terminus which make the [60]fullerene derivative high water soluble (illustr. 4).

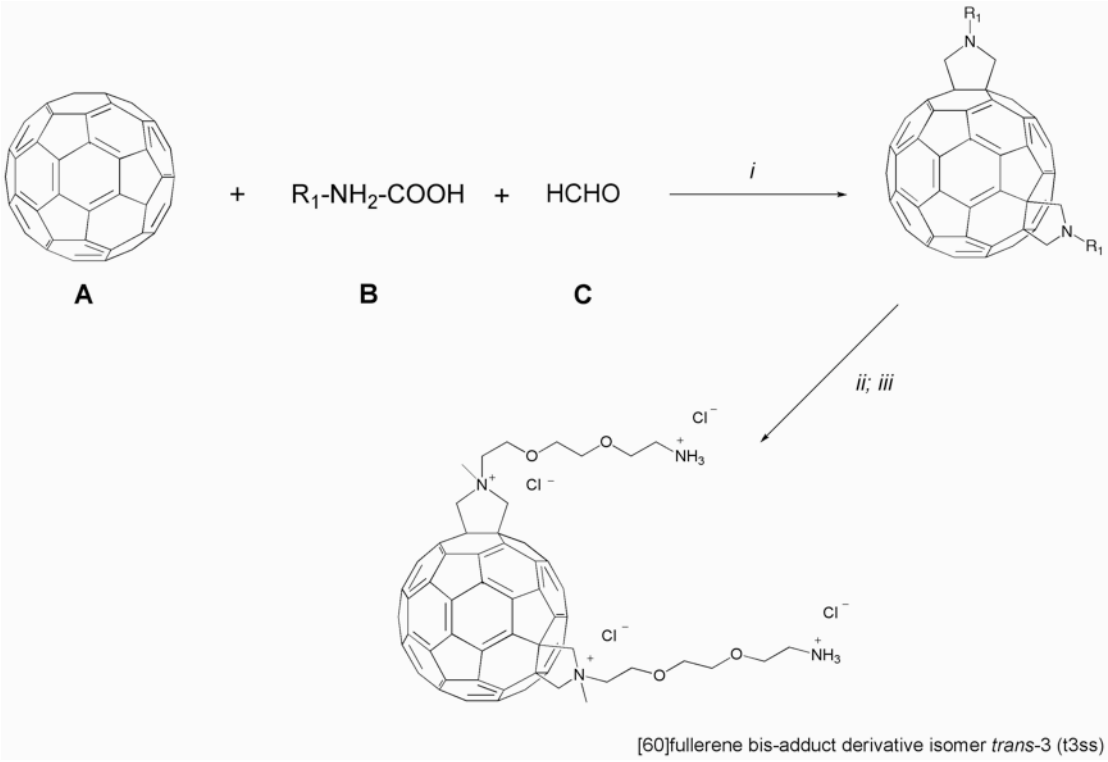


illustration 4. Synthetic scheme for *trans-3* isomer [60]fullerene bis-adduct derivative (t3ss). R_1 = $CH_2CH_2OCH_2CH_2OCH_2CH_2NHBoc$. Reagent and conditions: (*i*) molar ratio A:B:C 1:2:5, toluene, 12 h at reflux; (*ii*) CH_3I, CH_2Cl_2, 24h at 80°C; (*iii*) HCl (g), CH_3OH, 20 min at 0°C. For more details and procedures see the following reference (Bosi *et al*., 2003).

That [60]fullerene hydrosoluble derivative shown interesting activity on HIV-virus, and was thus employed to perform a large part of the experiments described in the present work. More in general, [60]fullerenes shown many biological properties like antibacterial activity (Da Ros *et al.,* 2006), inhibition of HIV-P (HIV protease) (Friedman *et al.,* 1993), photocytotoxicity (Schuster *et al.,* 1996) and for its properties of radical scavenger, like an anti-aging and/or anti apoptotic agent (Bisaglia *et al.,* 2000). One of the most important properties of [60]fullerene confering it biological activity is related to its easy phoexcitation and related properties (Guldi and Prato, 2000). From the ground state [60]fullerene can be excited $^1C_{60}$ by photo irradiation; this short lived species is fast converted to the long-lived $^3C_{60}$ *via* intersystem crossing. By the presence of molecular oxygen, the fullerene can decay from its triple to

the ground state, transferring its energy to O_2, generating $^1O_2$, know to be an highly cytotoxic species. On other hand, like discussed before fullerenes can be excellent acceptors after photo excitation, due to the formation of the two high energy species $^1C_{60}$ and $^3C_{60}$, and in the presence of a donor could be reduced to $C_{60}^{\bullet-}$ by electron transfer. In this case in presence of oxygen the [60]fullerene radical anion can transfer one electron generating $O_2^{\bullet-}$. The excited [60]fullerene can be reduced for example in the presence of guanosine residue present into DNA; the hydrolysis of oxidized guanosine followed by DNA cleavage is a consequence of the electron transfer from G to C_{60}* (An *et al.,* 1996). On the other hand, singlet oxygen and superoxide anion are well know species reactive towards DNA. In that sense many suggestions come from the possibility to intercalate [60]fullerene on DNA by recognizing different sequence with a good selectivity; some evidence have been reported about [60]fullerene linked to an intercalator or minor groove binder (Nakamaura *et al.,* 1996), but the cleavage of guanine residues occur without any significant sequence selectivity (Tokuyama *et al.,* 1993). At the same time, it was observed a major selectivity when [60]fullerene was linked to a oligonucleotide (Boutorine *et al.,* 1994). Like for the photo cleavage of DNA, antioxidant activity of [60]fullerene was demonstrated to be strictly related to its electronic properties; thus, fullerene demonstrated to be a redox-active compound towards reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyperoxide (ROOH), and hydroxyl radical (•OH) produced during oxidative stress and responsible for different type of damages in cells. These properties are due to that fullerene has a low LUMO level and a high HOMO level. For example, a polyhydroxylated C_{60} (fullerenol) was reported to have a quenching activity for $O_2^{\bullet-}$. Furthermore it has been demonstrated that fullerenol loss part of the quenching activity towards radical species respect to the parent form C_{60} since conjugated double bonds are widely broken. Thus, experiments with C_{60}-dimalonic acid, having rather similar properties to C_{60}, demonstrated the major efficacy in quenching radical species. The described activity of [60]fullerene of quenching radical species seem to be related with anti-aging and neuroprotective effect as reported

(Dugan *et al*., 1997; Pellicciari *et al*., 1998; Bisaglia *et al*., 2000). On the other hand, [60]fullerene showed an antibacterial-bacteriostatic activity by inhibiting bacteria growth acting on respiratory chain with a mechanism related to its electronic properties. Moreover [60]fullerene showed a bactericide activity by intercalating into the cells walls and disrupting the cell structure, effect explained by its hydrophobic properties (Da Ros *et al.,* 2001). The hydrophobic property could be used to explain the surprising effect as anti-HIV compound acting on HIV-RT (HIV Reverse Transcriptase) exerted by proline-type fullerene derivative compared with nevirapine (Viramune®) actually used in anti-HIV therapy. An anti-HIV activity of [60]fullerene was also described acting on the inhibition of HIV-P (HIV Protease). In all cases the hypothetic mechanism of action proposed for [60]fullerene involved its accommodation on the hydrophobic cavity of the specific enzyme (Marcorin *et al.,* 2000) (illustr. 5).

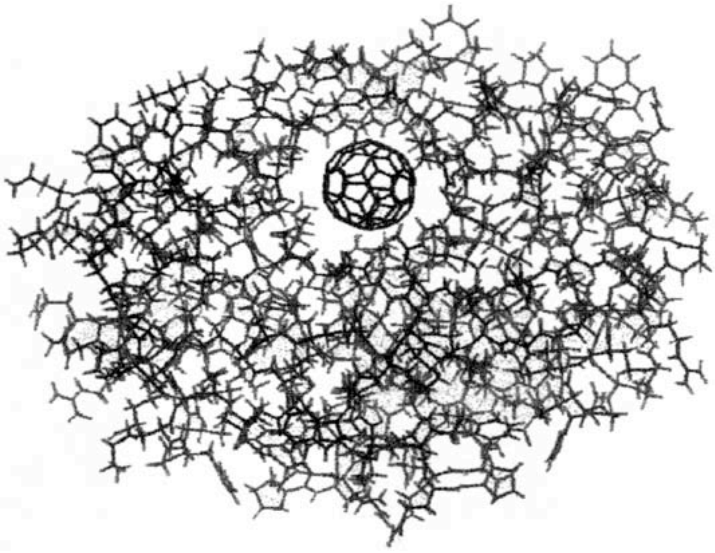


illustration 5. Computer designed accommodation for C_{60} in the HIV protease (HIV-P) hydrophobic cavity (from Da Ros *et al.,* 2001).

1.2.3 Fullerenes related toxicity

Due to its lower solubility in common solvent and high lypophylic, [60]fullerene demonstrated to have acute toxicity related to its accumulation in some organism compartments and relative low elimination. There are examples reported of the presence of [60]fullerenes in 10,000-year old ice core samples (Murr *et al.,* 2004) or dinosaur eggs (Wang *et al.,* 1998) *In vitro* studies reported cytotoxicity in human cells exposed to water soluble form of [60]fullerene due to the formation of reactive oxygen species (Sayes *et al.,* 2004) and lipid peroxidation (Sayes *et al.,* 2005). Baker and co-workers observed acute toxicity of inhaled nanoparticles and microparticles of [60]fullerene in rats and their deposition of these in lung (Baker *et al.,* 2008). In that sense, Oberdorster reported an increase in lipid peroxidation in brain and glutathione depletion in gills of juvenile bass fish exposed for 48 h to 0.5 ppm water soluble [60]fullerene (Oberdorster, 2004). On the other hand there are a lot of evidence of the safety in using [60]fullerene, and its positive action in many cases counterbalance the low acute toxicity, if present. An example is given by Andrievsky and co-workers who first demonstrated that the administration of [60]fullerene hydrates derivatives for in small doses for 3 years to patient with malignancy provided a positive effect by reducing radiotherapy related toxicity (Andrievsky *et al.,* 2002; Andrievsky *et al.,* 2000) Furthermore, preclinical studies performed by Mori and co workers in rats orally administrated with fullerenes demonstrated its low toxicity *in vivo*, with no evidences of mutagenesis in organ and tissues analyzed; no death of treated rats were observed neither reaching the doses of 2000 mg/kg (Mori *et al.,* 2006) thus confirming, once more, the low toxicity of [60[fullerene, even though the scientific community is still divided about the toxicity and safety of C_{60}.

2. Nanocompounds and Nanoneuroscience

Nanoneuroscience is a term recently coined to describe a large field of investigations including different disciplines as nanotechnology, chemistry, engineering and neurobiology. That emerging and complex field is anyway quite limited by the complexity of the mammalian nervous system (Silva *et al*., 2006). On the other hand, the recent results obtained interfacing nanomaterials with neural system confirmed the important challenge for generating new classes of multifunctional devices able, for example, to repair damaged CNS tissue. We will report below some example of nanocompounds interfacing with neuroscience, and their applications.

2.1 Carbon Nanotubes

Carbon nanotubes (CNTs) are cylindrically shaped carbon nanostructures, discovered in the 1990s, with unique electrical, mechanical and chemical properties that allow their use in a variety of applications (illustr. 6). The simplest geometry of a CNT is the single-walled nanotube (SWNT), on which a single graphene sheet is rolled up and closed at each end by a hemispherical fullerene cap; SWNT typical diameter is between 0.8 and 0.2 nm. On the other hand, multi walled nanotubes (MWNT) are formed by numerous concentric graphite cylinders, reaching diameter of up to 100 nm. CNTs can be systematically modified with different chemical group to display for example a variety of surface charges, such as positive, neutral, or negative. There is a variety of application for CNTs in science of materials; they take part in electric circuit (Postma *et al.,* 2001), solar cells, ultracapacitors, batteries and others (Park *et al.,* 2009). Many examples demonstrated the biocompatibility of these compounds, and the possible use of CNTs for drug delivery *in vivo*. At the same time the most attractive application for CNTs was focused on interfacing with neurons and nervous system. Mattson and co-workers in 2000 demonstrated the compatibility of CNT's with rat's neurons, confirmed by a relative long-term survival of cells on a CNT's support (Mattson *et al.,* 2000). Other researchers confirmed these

findings by characterizing neurites outgrowth and branching in rat hippocampal neurons grown on MWNTs (Hu *et al.,* 2004). Furthermore, it was demonstrated that MWNT substrates boosted neuronal network activity under culturing conditions (Lovat *et al*., 2005); similar effects were founded when using SWNTs (Mazzatenta *et al*., 2007). In that sense, a particular attention is focused on generating CNT scaffolds that, for example, could guide nerve tissue regeneration after injury.

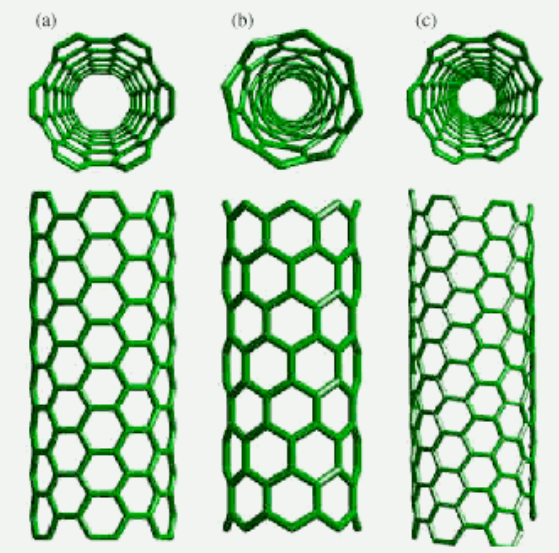


illustration 6. Structure of CNTs (a) armchair, (b) zigzag, (c) chiral.

(from: http://mrsec.wisc.edu/Edetc/nanoquest/carbon/index.html)

2.2 Nanohorns

Nanohorns belong to the family of carbon nanotubes, respect to those maintain chemical, physical properties, and structure similarities although with one capped extremity. Single wall carbon nanohorns (SWNHs) are derived directly from SWNTs (single wall carbon nanotubes) and were discovered first by Iijima and co-workers in 1999 (Iijima *et al.,* 1999). SWNHs are composed by a tube of about 2-5 nm in diameter and 30 to 50 nm long, closed by a cone at one extremity. Usually SWNHs associate forming aggregates of 100 nm in

diameter (illustr. 7); the large surface areas and porosity ensure a great affinity with organic compounds (Yuge *et al.,* 2005) making them promising candidates for hydrogen and methane storage (Bekyarova *et al.,* 2005; Tanaka *et al.,* 2004), and as drug delivery systems (Venkatesan *et al.,* 2005). What enhanced greatly their properties was the possibility to functionalizing them with group able to increase their solubility for example in water. Nanohorns are usually produced by electric arc ablation of graphite and are commercially available. The typical reaction on nanohorns involved for example the functionalization *via* 1,3-dipolar cycloaddition like for the same fullerenes or nanotubes; which allows obtaining different functionalized nanohorns for many applications. In the present work we have used a partially hydrosoluble SWNHs to verify their biocompatibility (for more details see results/materials and methods section).

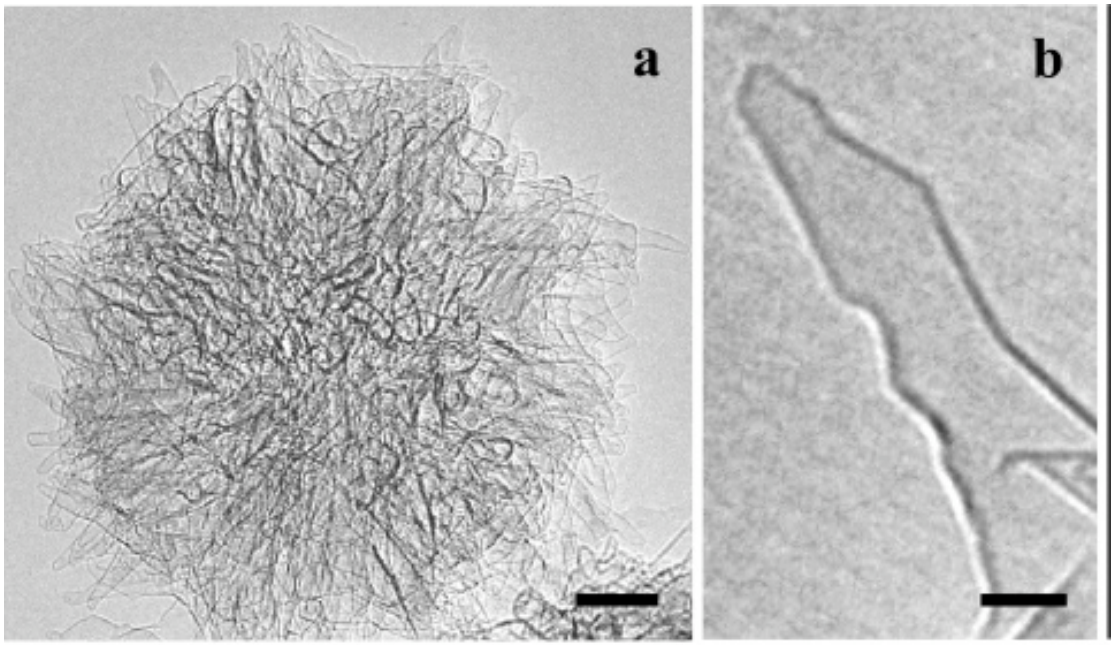


illustration 7. (a, b) HRTEM (high resolution TEM) images of SWNHox (scale bars of 10 and 2 nm, respectively) (from Ajima *et al.,* 2005).

2.3 Gold-nanoparticles

The biomedical use and applications of metal nanoparticles started in the 1970s with use of nanobioconjugates after the discovery of immunogold labeling by Faulk and Taylor (Faulk and Taylor, 1971). In diagnostic the traditional imaging techniques are crucial; in this sense gold nanoparticles (AuNPs) still remain the first choice with respect to the classical chemicals. Furthermore, AuNPs have been used and investigated for a broad range of application like photothermal therapy of cancers and other main diseases (Ferrari, 2005). Gold nanoparticles are usually synthesized from ($H[AuCl_4]$) by adding reducing agent to form Au^{3+} ions then reduced to neutral gold atoms; gold particles of submicrometre-sized are thus dispersed in a fluid, usually water, and functionalized with a variety of designed chemical groups in order to enhancing their solubility in water or modified their biological properties, avoiding for example their toxic side effects or formation of aggregates. The most used functionalized AuNPs are formed following the reaction with a thiol: (RS)nAuNP + mR0SH - (RS)n_m(R0S)mAuNP + mRSH ; that reaction easily provide the majority of functionalization of gold with oligonucleotides, peptides and PEG (polyethylene glycol); in that sense AuNPs could be also used for drug vectorization.

3. Self-Assembled Monolayers (SAM's)

We describe now some essential concepts about the interesting and increasing field of Self-assembled monolayers (SAM's), by focus on specific substrates and structure with particularly reference to models used in the present work, (for more details the reader is invited to the published book "*Ultrathin Organic Films"* by A. Ulman Academic Press, Apr 1991). Self-assembled monolayer (SAM) is an organized layer of amphiphilic molecules formed by a portion "head group" which has a particular affinity for the substrate, by a chain "tail" and an end group "functional group" (illustr. 8). The substrates used to obtain SAM's are many but the most common are gold, glass and silver. The typical molecules used for the preparation of SAM's are

formed by groups with a great affinity for the substrate; usually alkyl chain terminated by adding -OH, -NH_3, -COOH groups to allow further modification of the SAM. The most used molecules for SAMs preparation are alkanethiols, with a $(C\text{-}C)_n$ chain and -SH as the head group, and a common substrate is Au with different spatial orientation of elemental cell. Typical methods for the preparation of substrate are *physical vapor deposition*, *electrodeposition* or *electroless deposition* (Love *et al.,* 2005) at the same time the election method for the preparation of the layer is the absorption of the molecules by immersing the substrate in a solution of the desired compound usually for 12-72 h at room temperature, and dried with nitrogen (Vos *et al.,* 2003). On the other hand some molecules can be adsorbed from the vapor phase, this is the case of chlorosilanes (Knieling *et al.,* 2007). Some examples of suggested and implemented applications for SAM's are molecular recognition, model substrates and biomembrane mimetic in studies of biomolecules at surfaces, selective binding of enzymes to surfaces, chemical force microscopy, metallization of organic materials, molecular crystal growth, pH-sensing devices, electrically conducting molecular wires and photoresists (Smith *et al.,* 2004).

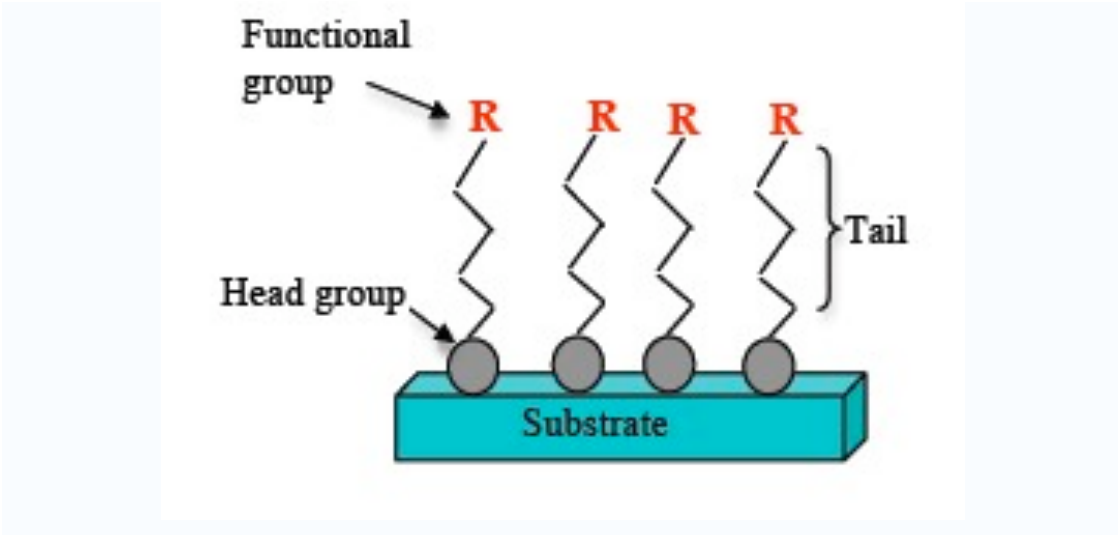


illustration 8. Schematic example of a SAM; usually a functional group exposed on surface is linked, by different length tail, to an head group with high affinity for the substrate, allow the anchorage of compound on material.

3.1 [60]Fullerenes based self assembled monolayers

In the large and promising field of SAM's, have been increasing examples of engineered materials interfacing with chemistry; like described above, there are many reasons to link a particular compound to a surfaces In most of cases, modify the properties of the substrates is one of these reasons, but in other cases it 's to take advantage from the chemicals or physicals properties of a particular compound. In that sense, there are many examples of [60]fullerenes based material in form of SAM's. We first review the methods to obtain [60]fullerenes based SAM's, passing through their applications. From what it is known the first example of [60]fullerene based SAM formation is reported by Mirkin and co-workers in 1994, which demonstrated how a fulleropyrrolidine -derived thiol spontaneously adsorbed onto a Au(111)/mica substrate (Shi *et al.,* 1994), leading to a highly organized monolayer of [60]fullerenes on Au(111) as confirmed by AFM (atomic force microscopy) measures. There are many evidences of studied chemisorption of C_{60} in a variety of substrates like Au(110), Ag(111), GaAs(110), Si(111), Cu(111), mica, KBr(001) or MoS2(0001), and strategies for the formation of [60]fullerenes SAM's. For example like reported for the first thermal evaporation of C_{60} on gold, which led to surfaces with very unusual properties of conductivity, superconductivity and strong optical nonlinearities. In this sense, it has been employed others approaches and strategies to self-assembly C_{60} on surfaces (here not reported), but the functionalization of C_{60} with useful groups undergoing a chemisorption on a specific substrates, is the most used strategy that provides more stable and homogeneous materials. This is the case provided by Mirkin reported above, but there are many more examples (for an extensive review on fullerenes assembly on surfaces *see* Bonifazi, 2007). The many applications of C_{60} based SAM's are strictly related to the properties of the same [60]fullerene we described above, including low-lying level of the excited state and exceptional electron-accepting properties. Due to these properites, [60]fullerene based monolayers have been engineered like light-harvesting devices (Gust *et al.,* 2001) in order to mimic

natural photosynthetic process and charge separation, including donor-functionalised network incorporated in two and three-dimensional network (Guldi and Prato, 2004); the most investigated photoreactive systems are [60]fullerene-porphyrin conjugates (Imahori *et al.,* 2001). [60]fullerene based substrates could be useful to engineering devices able to convert light energy in electrical energy, like for solar energy conversion purpose. Because the system works the molecules have to be confined on a surface of an electrode which in turn should be able to collect the electrons. Once more the most investigated system has been the [60]fullerene-porphyrin-based donor-bridge-acceptor system dyad; in that model first proposed by Imahori and co-workers (Akiyama *et al.,* 1997) the [60]fullerene-porphyrin is linked toward a thiol group to Au surface acting like the second of two electrodes (illustr. 9).

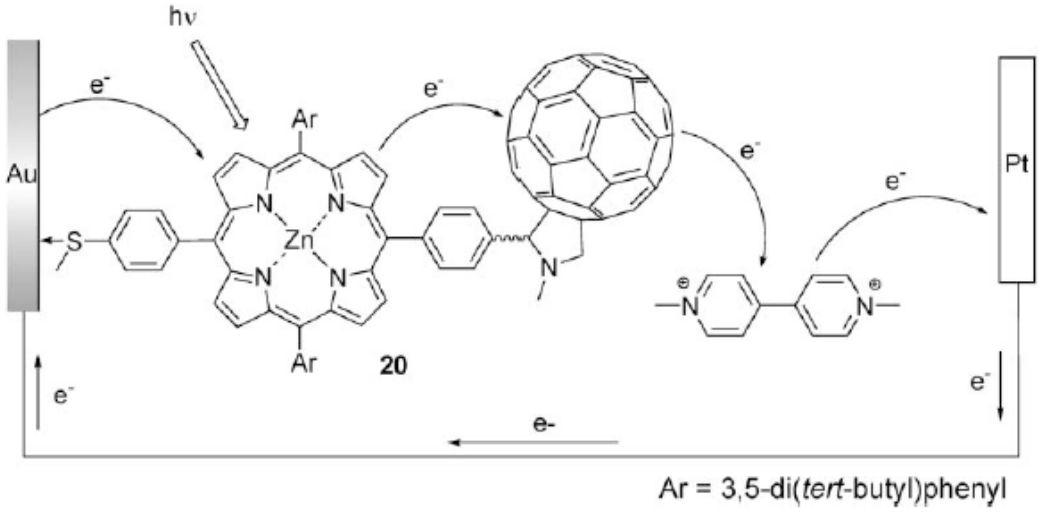


illustration 9. Scheme of the photoinduced electron transfer proposed with a SAM of porphyrin-C_{60} dyad (adapted from Bonifazi, 2007) .

The same authors demonstrated how the same system is useful for photoinduced electron transfer by using [60]fullerene alone bearing a thioalkane moiety toward the Au surface; this system is suitable for the generation of a stable anodic photocurrent under illumination in presence of the ascorbic acid (Imahori *et al.,* 1999). Imahori *and co-workers* extended these systems of modified SAM to electrodes modified with ferrocene-

porphyrin-C_{60} triad which demonstrated under optimal conditions the best quantum yield ever reported for a photosynthetic electron transfers across artificial membranes or at monolayer-modified metal electrodes (Imahori *et al.,* 1999). Recently it was proposed a functionalization of gold coated nanoparticles by porphyrins intercalating C_{60} molecule acting like a sort of photoactive antenna system (Hasobe *et al.,* 2003). Among examples of the applications of [60]fullerene based SAM's there is the one provided by Heeger and co-workers about a large-area image sensors made with organic photodiodes. These sensors can be used as pixel element of large-area, full colour image sensors such as linear or two dimensional digital cameras. These consist in a sandwich devices of thin film with a discontinuos donor/acceptor composite; a polymer blend containing a C_{60} derivative and poly(3-octyl-thiophene) is spin-cast onto ITO(Indium-Tin-Oxide)-glass substrates (Wang *et al.,* 1998) (illustr. 10).

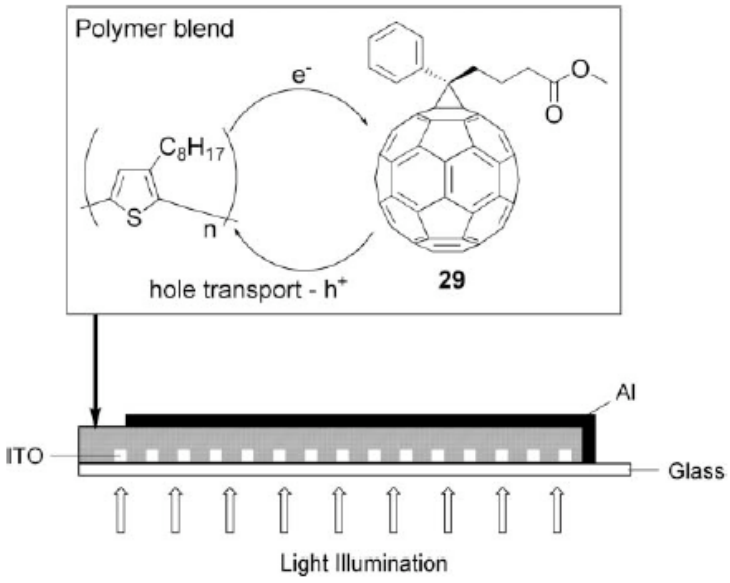


illustration 10. Structure of a linear photodiode device employed as a sensor (adapted from Bonifazi, 2007)

Even though there are many more examples of [60]fullerene based material and SAM's for electronic and special material devices uses, however the most important to the goal and the realization of the present work are the possible

C_{60} based SAM's biological applications. In that sense the biological effect described before for the [60]fullerene on solution or dispersion, could be well applied to the surface. Higashi and co-workers, demonstrated that positively charged SAM's on a gold surface can immobilise double-stranded DNA without disrupting its intrinsic high-order structure, and that site of cleavage is successfully achieved by covalent incorporation of C_{60} into the SAM (Higashi *et al.,* 1997). The authors observed a highly guanidine-selective DNA cleavage, consistent with the involvement of singlet oxygen generated by interaction of the photoexcited fullerene core with molecular oxygen (Bernstein *et al.,* 1999). Electrodes modified with fullerene monolayers offer interesting perspectives in using as substrates acting with coupling redox-active biomolecule or enzymes with electrodes and activate the respective biological functions (Sherigara *et al.,* 2003). In that sense Willner and co-workers described the use of C_{60} as an electron mediator for an electrocatalysed biotransformation. In that work, a methano[60]fullerene carboxylic acid derivative covalently attached to a cysteamine monolayer pre-adsorbed on a gold electrode with the result of a monolayer providing an active interface for mediating the biocatalysed oxidation of glucose to gluconic acid. The same authors proposed that because of the size of the carbon cage of [60]fullerene, the electron mediator cannot penetrate into the protein to have intimate contact with the FAD-site (FAD= flavin adenine dinucleotide) for electron transfer. For that reason the mediated electrical contact is assumed to proceed *via* long-distance tunneling (Patolsky *et al,* 1998)(illustr. 11).

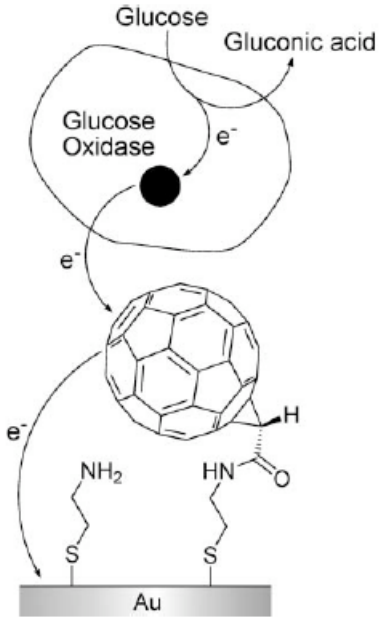


illustration 11. Reduction of glucose by glucose oxidase mediated by a [60]fullerene SAM as reported by Wilner and co-workers (from Bonifazi, 2007).

With the aim to understand the role of the co-enzyme redox couple NAD/NADH in biological redox processes involving electron transfer from a substrate, the group of Zhou and co-workers prepared a C_{60}-glutathione modified Au electrode for the electrocatalytical oxidation of NADH (Cheng 2001). Electrocatalysis studies revealed that NADH form is electrochemically oxidised by the fullerene radical cation ($C_{60}^{\bullet +}$) confined on the surface. The examples reported about fullerene-coated surfaces can represent a new class of functional biologically active materials.

4. Cellular signalling

At the base of complexity of the living systems there is the capacity of relationship between the same organism and their surrounding. In that context, it takes more relevancy if we think of an organism made by million and million of cells, communicating by different chemicals, electrochemicals ways and signals and receiving various stimulus of different nature. There are

events happening in the living system allowing communication between cells, that involve specific target called receptors. Those target proteins have to be able to translate a signal, not always chemical, to cell and translate it to an effect; it is occurring first by an outside signal (first messenger) follow by an inside signal (second messenger); that process is known as cellular transduction pathway. There is a large variety of different type of receptors, with different transduction pathway, however in the present work we focus our attention on transmembrane receptors. Receptors are generally complex amino acidic based molecules, crossing the plasmatic cell membrane; they own in the outside of cell a portion designed for the interaction of a specific ligand; on the intracellular side of the receptor there is the specific effector causing a pathway cascade of event. That is the case of metabotropic receptors, which interact with a specific class of protein located on the intracellular side of cell membrane, called G-protein, which allow the receptor to translate a chemical interaction happening outside cell into a more complex signal inside cells, by activating a cascade of events we will describe later. On the other hand there are the ionotropic receptors type which after the interaction with a ligand are responsible for generating flux of ions causing change in the potential of cell membrane; that is due to the fact that ionotropic receptors are essentially constituting a ionic channel across the cell membrane, regulating the ions flux. The G protein-coupled receptors (GPCRs) have been successfully maintained along the evolution of living systems representing between the 1 and 2% of the total genes of the mammalians (Pin *et al.* 2003); this is due to the capacity of these receptors to translate different type of signals from different origins. For that reason the variability in the response of the receptors depends on the type of the G-protein coupled and the way this coupling take place. A schematic behaviour of the relationship between the receptors and different class of G-protein with the related intracellular responses, is represented in illustration 12.

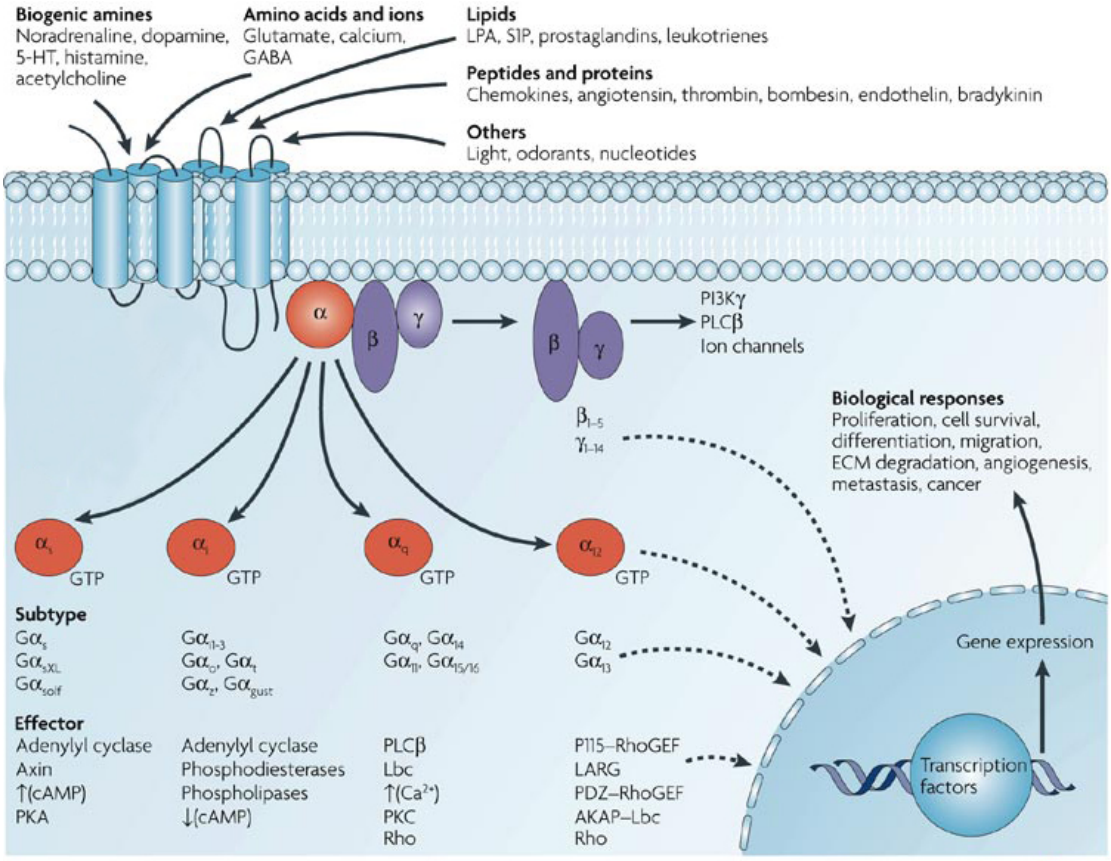


illustration 12. Scheme of the relationship between receptors and different class of G-protein exerting their effect on different effector, which are related to differential processes involving transcription of new factor, while gene expression is involved to physically related effect on cells, including cells proliferation, survival, differentiation and many others. (Modified from Dorsam and Gutkind, 2007).

4.1 G-protein coupled receptors (GPCRs)

The receptors belonging to this family own a common architecture made of a polypeptides chain that cross 7 times the cell membrane; the polypeptides sequence start with an N-terminus extreme on the extracellular space. The transmembrane dominium are formed by sequences from 25 to 35 hydrophobic aminoacids with an α–helix conformation connected by 6 loops, 3 intra and 3 extra cellular of variable size, with the C -terminus inside the cell. The N-terminus is designed for the interaction with the specific ligand, instead the C-terminus has relevancy for the interaction with the G-protein; in particular the C-terminus interacts with intracellular loop 2 and 3 (for a review

see Bourne, 1997), those have specific sequences for a specific G-protein recognition.

4.1.1 Clasification of GPCRs

At present the widely accepted classification for the GPCRs is established on data-base GPCRDB (available on www.gpcr.org/7tm/), also called classification A-F, dividing GPCRs into 6 families (Horn *et al*., 2003) and collecting specific sequences, mutations, genes and specific ligand for the receptor from 1994 (Kolakowsi, 1994). About this classification, the most abundant family, the family A, known as the *rhodopsin like* receptors includes more than 80% of human GPCRs, adenosine receptors belongs to this family. The family B is called *secretin-like* and the family C receives the name from the metabotropic glutamate receptors even though containing also GABA (γ–amino butyric acid) receptors. Families A, B, C and F are present in mammalians, while families D and E belong to receptors for pheromones and are minor represented in fungi.

4.1.2 Role of GTP binding proteins and effectors systems

G-proteins are a family of hetherotrimeric proteins made of three subunit called α, β and γ, having an important role in many physiological and pathological processes. The main role of these proteins are the ability to convert GDP (guanosin di-phosphate) into GTP (guanosin tris-phosphate) after their interaction with an activated receptor, passing from an inactivated state to an activated state in which the subunit α, linked to GTP, dissociates from the dimer $\beta\gamma$ allowing to exert their effect on the related enzyme and activating the cell response (review on Pin *et al*. 2003). The α subunit acts as a catalyst for the hydrolysis of GTP to GDP, turning back the G-protein to its inactivated state. We have to consider that because of the presence of

different subtype of G-protein and the presence of three subunits acting on different types of effectors, we have a multiplicity of possible responses and flexibility, in addition to the type of receptor we consider, and the many possible effectors molecules coupled to. In the present work we have focused the attention on the metabotropic glutamate and adenosine receptors coupled to phospholipase C and adenylyl cyclase respectively. The adenylyl cyclase are a family of enzymes which could catalyze the formation of cyclic AMP (cAMP) an ubiquitous second messenger in animal cells. The subunit $G_{\alpha i}$ inhibits the activation of that enzyme, and the subunit $G_{\alpha s}$ promotes their stimulation. The most important role of cAMP is to activate protein kinase cAMP dependent (PKA); once activated PKA phosphorilates different substrates, by using ATP, as enzymes, receptors, ionic channels, transcription factors etc. On the other hand the subunit $G_{\alpha q}$ activate the phospholipase Cβ isoform, inside the cell membrane, promoting the synthesis of two second messengers, inositol triphosphate (IP_3) and diacylglicerol (DAG), after the conversion of the phosphatidilinosiltol-bisphosphate (PIP_2) placed on the internal side of the cell membrane. The IP_3 is a hydrosoluble molecule that dissolve in the cytosolic space acting on receptors on the endoplasmic reticulum, generating a free calcium imbalance in the cytosolic space, activating different pathways with many related biological effects. Furthermore, DAG is a hydrophobic molecule laying in the cell membrane, reacting with the protein kinase calcium dependent (PKC) also laying inside the cell membrane. PKC may activate, after the increase in cytosolic Ca^{2+} concentration, phosphorilation of proteins with different role and biochemical responses (illustr. 13).

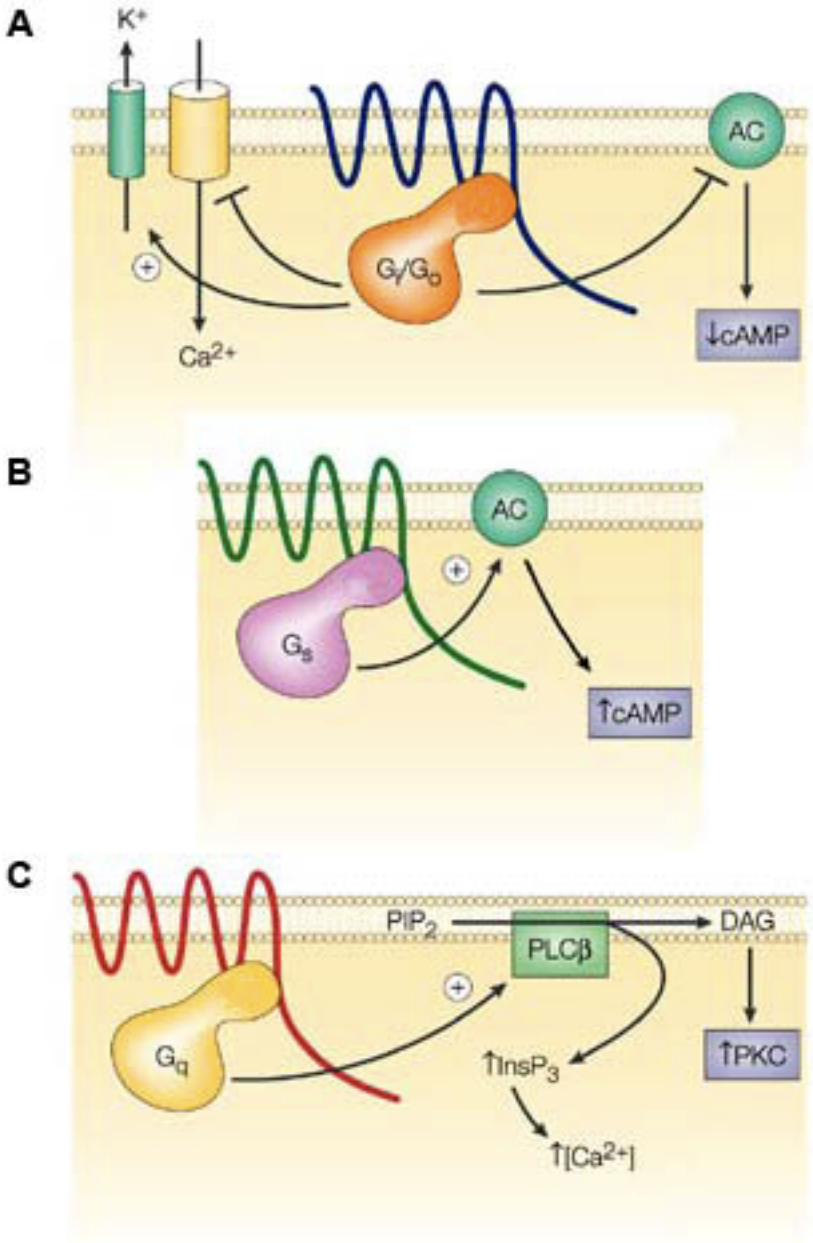


illustration 13. Activation by G-proteins of the effectors pathway related. A. Receptors coupled with $G_{i/o}$-proteins inhibit the AC (Adenylyl Cyclase) enzyme, in some cases activate the exit of K^+ or entrance of Ca^{2+}. B. Receptors coupled with G_s-proteins stimulate de formation of cAMP. C. Some others receptors are coupled to Gq proteins activating PLCβ, that increase the hydrolisis of PIP_2 into IP_3 and DAG with the subsequently increase of Ca^{2+} and activation of PKC (Adapted from *The state of GPCR research in 2004*).

5. The glutamatergic system

Glutamate is the most abundant and important aminoacid in the central nervous system (CNS), where it acts like the main neurotransmitter, being implicated in many physiological and pathological processes. Glutamate acts as an excitatory neurotransmitter in CNS (Bortolotto *et al.*, 1999), taking part in the normal development of CNS, in learning and memory processes, but also in neurodegenerative ones; it has been demonstrated that alterations in the glutamatergic system and equilibrium could bring to degenerative processes like epilepsy (Sloviter, 1991), schizophrenia (Tamminga, 1999), Parkinson's disease (Turski *et al.*, 1991), Huntington dementia (Schwarcz *et al.*, 1983) and Alzheimer's disease (Geddes *et al.*, 1992; Ulas *et al.*, 1992).

5.1 Ionotropic Glutamate receptors

The excitatory effect of glutamate in mammalian brain is well known from decades of the 50's on the last century (Hayashi, 1952). Moreover on the 70's of the 20th century it was generally accepted the role of glutamate as the main neurotransmitter in the CNS. At first glutamate was believed to act only on ionotropic receptors as main agonist. Later it was discovered that glutamate may activate other class of receptors, more complex than the first one, and with many more pathways which could be activated: the metabotropic glutamate receptors (Dingledine *et al*., 1999). Inotropic glutamate receptors are ionic channel present at postsynaptic level (illustr. 14), with different permeability for Na^+ and Ca^{2+} ions depending on its multimeric composition. There are three main family of these receptors, named about the main agonist: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxil-5-methyl-4-isoxazolpropionic acid (AMPA) and kainic acid. Those receptors regulating the flux of ions across the cell membrane changing the potential of cell membrane, and thus inducing an hyperpolarisation of the internal of cells by generating a excited state (review on Madden, 2002).

Furthermore these activated receptors are responsible for the toxicity strictly related with the Ca^{2+} entrance in cytosol, promoting apoptotic events and cell necrosis (Kim *et al*., 2001).

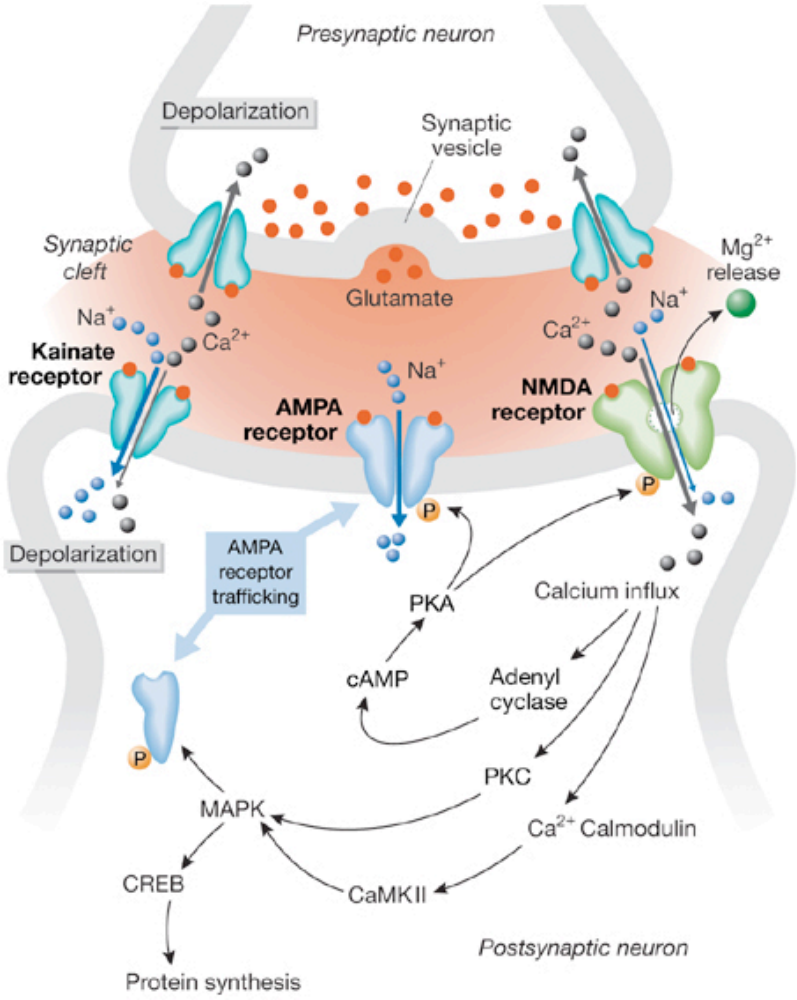


illustration 14. Inotropic gutamate receptors and related activated pathways. The binding of NMDA and AMPA receptors by glutamate, and the related increase of intracellular concentration of calcium, activates many pathways, including adenylate cyclase inducing the activation by cAMP of protein kinase A (PKA) and cAMP response element (CREB), with the consequent activation of the transcription of many genes, including ones for the synthesis of new functional NMDA and AMPA receptors, contributing to increase glutamate toxicity. AMPA, -amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CaMKII, calcium/calmodulin-dependent kinase II; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase (from Voglis and Tavernarakis, 2006)

5.2 Metabotropic glutamate receptors

Metabotropic glutamate receptors belong to the family of G-protein coupled receptors. Those are divided in three groups (I, II and III) and eight subtypes according to their pharmacological profile, transduction mechanism and specific aminoacidic composition. The group I of these receptors include the $mGlu_1$ and $mGlu_5$ receptors and coupled to the transduction of signal through the activation of hydrolysis of phosphadyl inositol/Ca^{2+} pathway. Instead the receptors of group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$, $mGlu_8$) are coupled in a negative way to adenylyl cyclase by inhibiting the synthesis of cAMP (Ferraguti *et al.*, 2006). Even though it is well described the excitotoxic and neurotoxic role of the ionotropic glutamate receptors, it is not still clear the role of the metabotropic ones (illustr. 5). There are some evidences about the activation of group I is related to an amplification in the activity of receptors NMDA and for that reason related to neurodegenerative and excitotoxic process (Skeberdis *et al.*, 2001) While receptors belonging to the group II and III seem to be implicated on neuroprotective mechanisms, by modulating NMDA receptors by the inhibition of adenylyl cyclase (Ambrosini *et al.*, 1995; Allen *et al.*, 1999). At the same time some authors associated the group I receptors to a neuroprotective role (Lee *et al.*, 1996). Furthermore, during the last years metabotropic glutamate receptors have been investigated as possible target for therapeutic advances in some type of neurodegenerative pathologies. The receptors $mGlu_{2/3}$ have been investigated for their therapeutic role in schizophrenia and anxious status, as agonist of those receptors have been demonstrated to improve the general status of the patient (Patil *et al.*, 2007). Recently, it was suggested that receptors $mGlu_4$ could represent a new and valid therapy against Parkinson's dementia (Marino *et al.*, 2003).

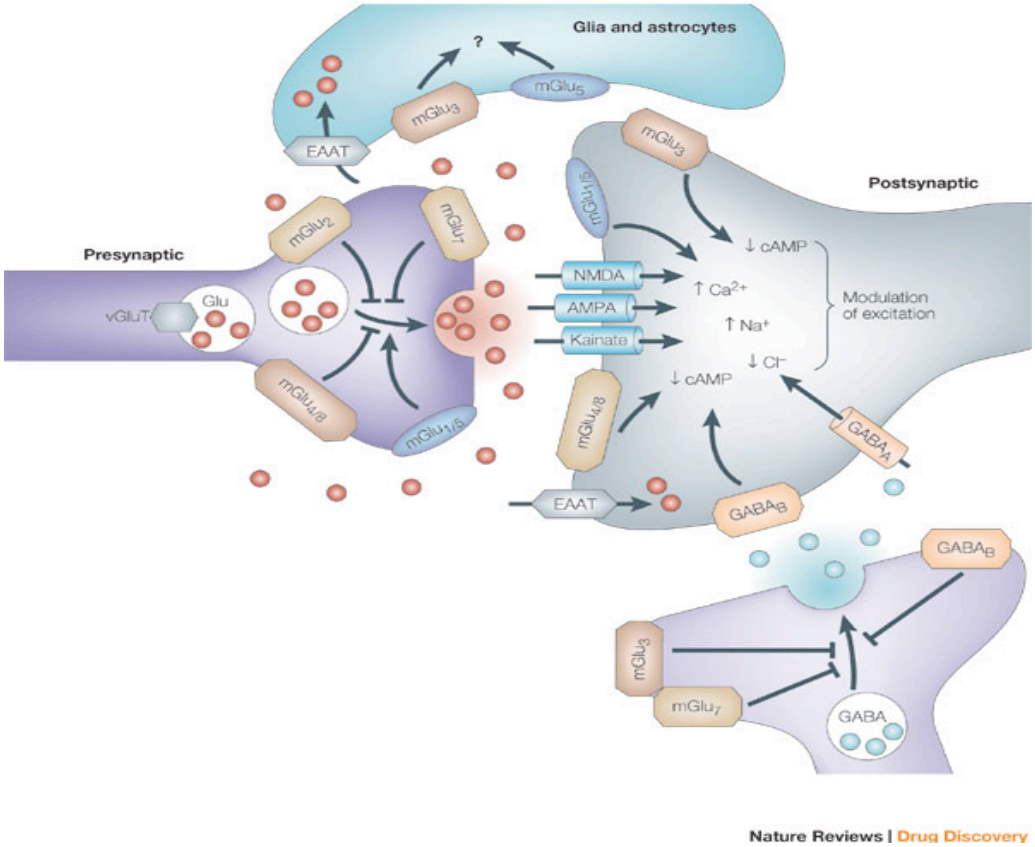


illustration 15. The ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA), kainate and -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtypes, largely function to mediate fast receptor transmission, but also mediate the use-dependent changes required for neuronal plasticity. The vesicular transporters (vGluT1 and vGluT2) load glutamate into vesicles presynaptically. The glial, astrocyte and postsynaptic glutamate transporters (excitatory amino-acid transporters, EAAT1–5) are thought to mediate the uptake of glutamate and therefore termination of synaptic transmission. The metabotropic glutamate receptors have a diverse synaptic localization and function pre- and postsynaptically to modulate neurotransmitter release and postsynaptic excitability, respectively. For instance, the group II mGlu (mGlu2/3) receptor agonists probably serve to stimulate mGlu autoreceptors on glutamatergic terminals to suppress excitatory neurotransmission at selected synapses in the central nervous system. By contrast, $mGlu_5$ receptor antagonists might inhibit $mGlu_5$-mediated potentiation of NMDA receptor ion currents and potentially disrupt the formation of memory processes associated with stressful events. GABA, γ-aminobutyric acid (from Swanson *et al.,* 2005).

6. The adenosinergic system

Adenosine is a nucleoside forming part of the purinergic nucleotides (ATP, ADP); it takes part in many processes of development and cellular communication in all the living systems. The first evidence of an investigation on the physiological role of adenosine was dated on 1933 by Gaddum and co-workers who demonstrated the implication of adenosine in vascular and cardiac system. Since then, many roles of adenosine have been investigated and clarified, like for example its role in inflammation, muscular contraction, the neurotransmission in the peripheral and central nervous system, the immune response, and the modulation of the cardiac function (Burnstock and Kennedy, 1986; Seifert and Schultz, 1989; Burnstock, 1990; Olsson and Pearson, 1990; Relevic and Burnstock 1991; Dubyak and El-Moatassim, 1993; Dalziel y Westfall, 1994; Fredholm, 1995; Burnstock and Wood, 1996; Ongini and Fredholm, 1996; Sebastiao and Ribeiro, 2000). Adenosine is widely distributed on all the CNS, where acts like a neuromodulator of the pre and post-synaptical transmission, by controlling the release of excitatory neurotransmitters, mainly glutamate. Based of this, the possible physiological responses could include the sedative, analgesic and anticonvulsive one.

6.2 Adenosine receptors

Adenosine posses its own receptors where acts as main agonist. The receptors are part of the family of G-protein coupled receptors, and present the same common structure, like described before. They are divided in four subtypes: A_1, A_{2A}, A_{2B} and A_3, all coupled to G-protein but with different localization in the tissue. The receptors subtype A_1 and A_3 are coupled to the protein $G_{i/o}$ inhibiting the adenylyl cyclase, for that reason an activation of these receptors decrease the intracellular levels of cAMP. On the other hand the receptors A_{2A} and A_{2B} are coupled to adenylate cyclase through stimulating proteins G_s, what means that their activation promotes an increase in cAMP concentration (illustr.16). Adenosine receptors are widely distributed over much type of human tissues, taking part in a variety of physiological

function. The most important role of adenosine receptors can be found in brain, where the most abundant A_1, A_{2A} and A_{2B} seem to be related to neurodegenerative and neuroprotective processes (Ralevic and Burnstock, 1998). For example adenosine A_{2A} receptors have been related with a release of Glutamate and related toxicity (Popoli *et al.,* 2003), while adenosine A_1 receptors are widely associated to a neuroprotective effect in brain, against toxicity evoked by a large numbers of compounds and conditions (Kalkan *et al.,* 2009). Nevertheless A_{2B} receptors have been demonstrated to own a specific role probably depending on type of cells they are expressed; in fact they have been found to participating in pro- and at the same time anti-inflammatory processes, by depending on cells model they were expressed (Ham and Rees, 2008).

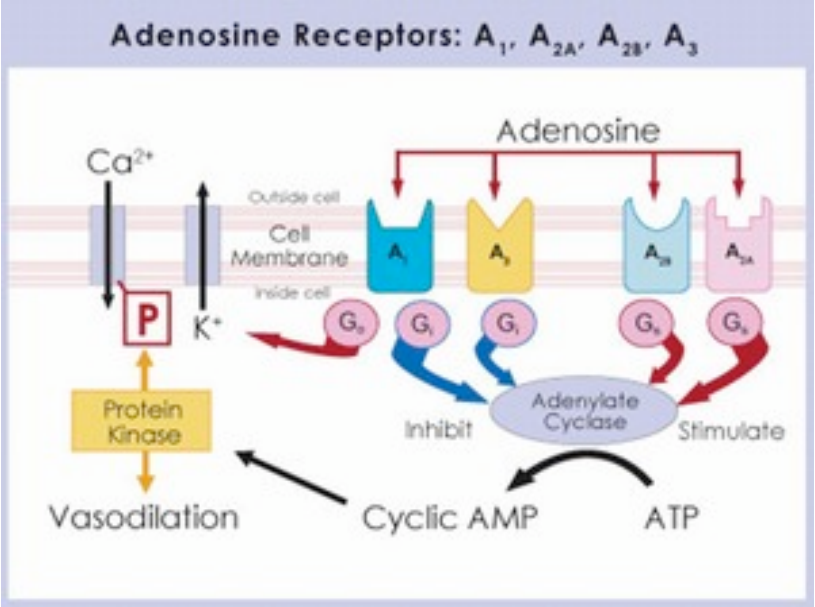


illustration 16. Scheme of the main response pathways, after activation of adenosine receptors. Interaction of agonist with adenosine receptors, involve the activation of different pathway in cells. To the activation of A_1 and A_3 subtype coupled to an inhibiting G-protein, is related a decrease of Adenylate Cyclase activity and subsequently conversion of ATP into cAMP active form. At the same time A_1 receptors are coupled to a stimulatory G-protein, acting directly of Ca^{2+} and K^+ channels, and regulating the ions imbalance into cells. Nevertheless, those receptors are regulated by cAMP converted from ATP by Adenylate Cyclase activated. The stimulation of Adenylate Cyclase is strictly related to the activation of A_{2A} and A_{2B} receptors, and related increasing of cAMP concentration provide an increase activity of Protein Kinase, and the main physiological related effect as vasodilatation.

7. Processes of GPCR regulation

Among all processes, responsible for the regulation of G-protein coupled receptors (GPCRs), we will describe the most important. The modern recently advances in molecular techniques provided a great chance to observe mechanism involved in the regulation of GPCRs. Some of them are well known and accepted by most of the scientific community. Receptors regulation is without doubt, one of the most relevant processes that cells should activate for the own protection from an increased stimulation by a certain agonist, by activating for example an internalization of receptors. In turn, a great decrease of a specific agonist may induce cells to up-regulate certain receptors, as response, in order to have the major availability of active site for lower concentration of agonist. All those mechanisms we will be briefly described in the following section.

7.1 Rapid Desensitization and Resensitization

Studies about the regulation of β_2-adrenergic receptor (β_2AR), allow to understand the general mechanism involved in rapid desensitization and resensitization (Ferguson *et al.,* 1998). It is known to pass seconds between the binding of the main agonist to the receptor, and subsequently activation of adenylyl cyclase via G_s. Nevertheless after a more prolonged activation the ability of G_s to maintain activated the adenylyl cyclase, diminishes greatly. This diminution of signal transduction is generally called *desensitization,* almost mediated by the regulation of the same receptor. On the other hand a process of *rapid desensitization* was named because it occurs within second to minute after agonist-induced activation, it furthermore may be reversed within minute after removal of the main agonist in a process named *resensitization*. Rapid desensitization of receptors is generally not accomplished by a decrease in total number of receptors, but by a change in the *functional activity* of those. Desensitization mechanism of receptors have been related with internalization of receptors by endocytosis mechanism, involving the formation of clathrin coated pits on surface of cells membrane,

subsequently those clathrin originating endosome, which may be disrupted by lysosome formation, or recycling to the membrane, to restore the functional receptor in the resensitization process, as a non phosphorilated endosome (Toews *et al.,* 1984; von Zastrow *et al.,* 1992). Desensitization and consequently formation of clathrin coated pits, has been demonstrated to be promoted by β-arrestin, a cytosolic protein activated by an hyperphosphorilation of receptors, and able in some case to physically linking phosphorylated receptors with clathrin (Goodman *et al.,* 1996).

7.2 Down-regulation and Up-regulation of GPCRs

Processes as previously described may referring mainly to the receptors regulated by catecholamines, as these molecules can be released intermittently by vesicular exocytosis and rapidly removed from extracellular space, by enzyme degradation or membrane transport. Nevertheless exposure of receptors to a ligand, within several hours to days, involve different type of regulation. The process called *down-regulation*, is associated with a great decrease in total number of receptors, in contrast to rapid-desensitization, and typically detected by means of radioligand binding techniques. Further distinguishing between rapid-desensitization and down-regulation, is related to a slow recovery on the signaling activity by receptors involved in down-regulation respect to those involved in rapid desensitization, mainly due to the process require a biosynthesis of new receptor protein (Dass *et al.,* 1981). Ligand involved in down-regulation process are always agonist, on the other hand, certain antagonist can induce an opposite process, named *up-regulation*, by increasing the total number of receptors. However in some cases, certain antagonists demonstrated to induce down-regulation of receptors (Pfeiffer *et al.,*1998). In other cases, up-regulation of receptors may be induced by ligand with partial agonist activity (Zaki *et al.,* 2000). Endocytosis followed by proteolysis is known to be the main regulating process involved in down-regulation of GPCRs, as demonstrated in

mammalian cells and certain type of neurally derived cell types (Law *et al.,* 1984; Ko *et al.,* 1999). However it has been also suggested, another mechanism involved in down-regulation, not involving the endocytic process, but by involving for example the activation of plasma membrane-associated metalloprotease (Kojiro *et al*., 1995). The endocytosis of clathrine-coated pits have been demonstrated to be a common route of internalization during desensitization and down-regulation (Gagnon *et al.,* 1998), nevertheless the molecular sorting of the vesicle proceeding from the two process is different.

Different factor contribute to the different sorting of the internalized endosome, into proteolyitic lysosome for degradation of receptor, or into a recycling endosome for the final recovery (for an exhaustive review see von Zastrow, 2002). All processes discussed until now, are summarize in the following scheme (illustr. 17).

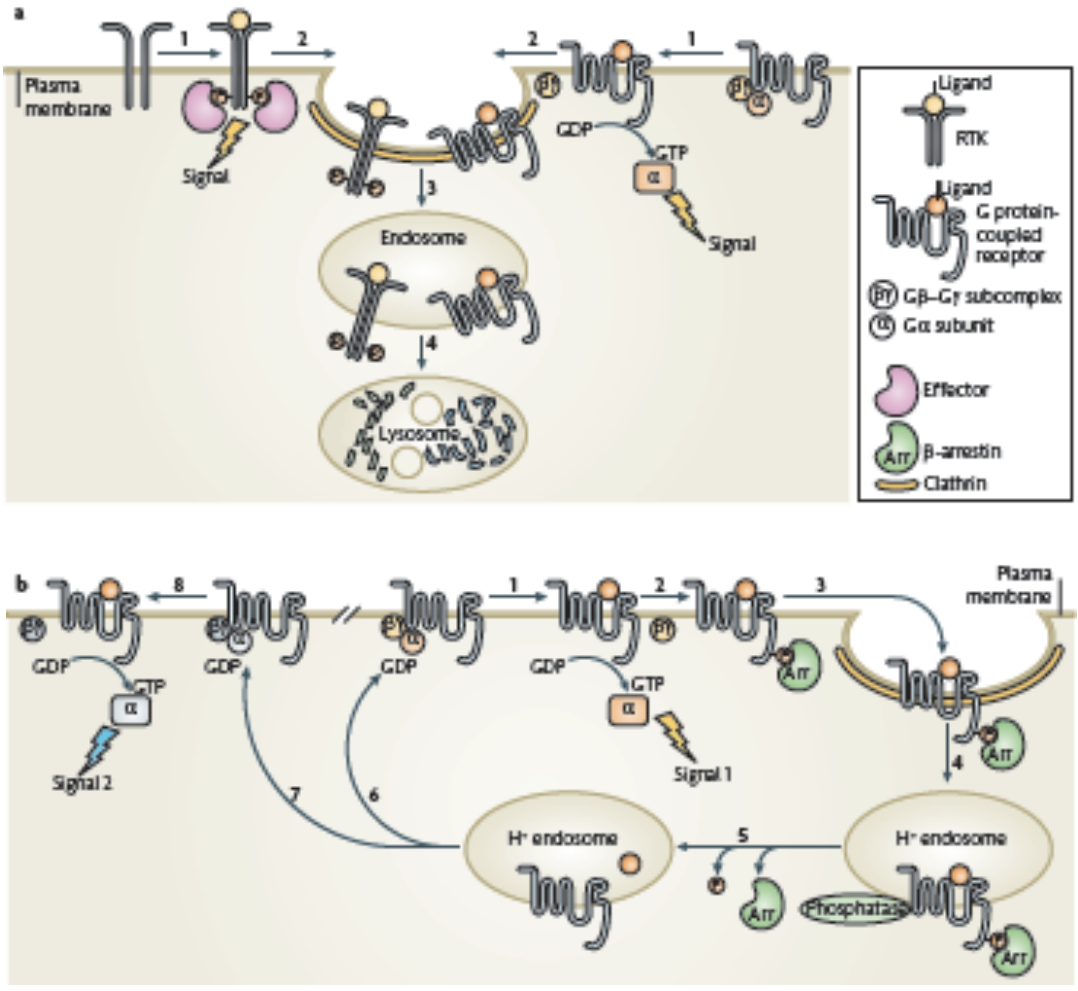


illustration 17. Schematic depicting signal attenuation. **a.**Ligand-induced activation of receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) promotes signalling from the plasma membrane by the receptor-mediated recruitment of signalling effectors to phosphorylated RTKs, or the activation of G proteins associated with GPCRs when the Gα subunit is bound to GTP (step 1). GPCR signalling can be mediated both by the GTP-bound Gα subunit (as depicted) and by the Gβ–Gγ subcomplex. Receptor recruitment into coated pits (step 2) and clathrin-dependent endocytosis (step 3) attenuate signalling by separating the receptors from plasma membrane-delimited substrates and/or mediators. Some receptors traffic to lysosomes after endocytosis, which results in their downregulation by proteolysis and further attenuates signalling (step 4). **b.** Schematic depicting signal desensitization, re-sensitization and pathway switching. The ability of some GPCRs to activate trimeric G proteins (signal 1; step 1) is attenuated before endocytosis by receptor phosphorylation and β -arrestin binding (step 2). Such desensitized receptor–β-arrestin complexes concentrate into clathrin-coated pits (step 3) and are endocytosed into acidic (H+) endosomes (step 4), which promotes various events that may include (depending on the receptor and cell type) ligand dissociation or destruction, dissociation of β -arrestin and phosphatase-catalysed dephosphorylation (step 5). Recycling (step 6) restores the receptors to the cell surface, re-sensitizing the cell for another round of signalling. In some cases GPCR recycling inserts receptors into a different G protein-containing environment (step 7), which produces a switch' in signalling specificity following subsequent receptor activation (signal 2; step 8). (adapted from Sorkin and von Zastrow, 2009).

7.3 Homologous and Heterologous desensitization

Many type of cells express a large number of different GPCRs, nevertheless it has been observed that the prolonged activation of one type of receptor, does not involve the signaling of the other types of receptors. That process is named *homologous* desensitization. On the other hand, in some cases the desensitization of certain receptors, attenuates their own signalling but also other type(s) of GPCRs present in the same cell. That process is called *heterologous desensitization.* Homologous and heterologous process have been demonstrated to play an important role in facilitating functional "cross-talk", between pharmacologically distinct signaling pathways (Krupnick *et al.,* 1998) (illustrat. 18).

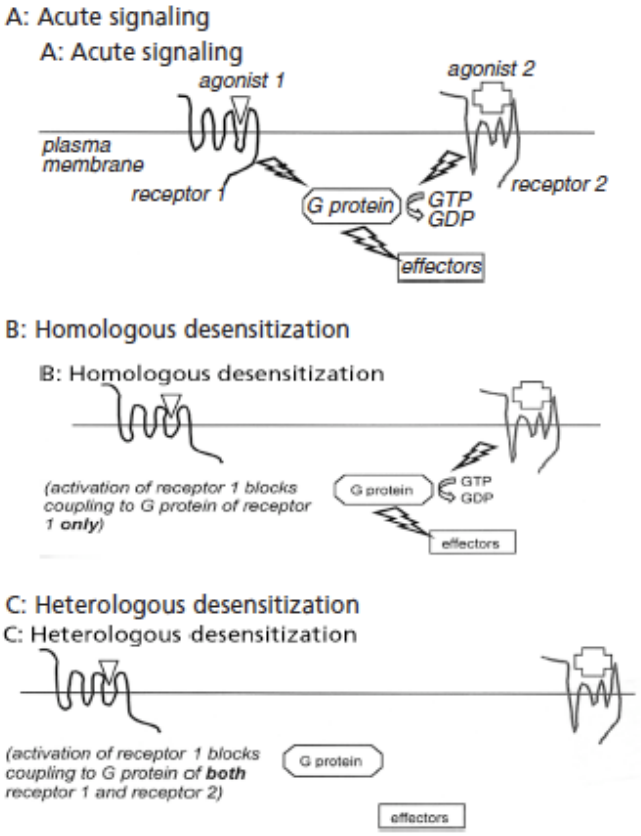


illustration 18. The figure illustrates mechanism involved in homologous and heterelogous desensitization of GPCRs. A. Binding of agonist 1 and 2 on two different receptors may involve the activation of specific effectors for desensitization of receptors. B The binding of the agonist 1 to the receptor 1 involve block the G-coupling to receptor 1 only. C. The binding of the agonist 1 to receptor 1 block the G-coupling of both 1 and 2 receptor (from von Zastrow, 2002).

8. Excitotoxicity

The first proof about the neurotoxic effect of glutamate has been provided at the end of 50s. Lucas and Newhouse demonstrated how by exposing young rats to L-Glutamate, neuronal layers of retina cells appeared to be destroyed (Lucas and Newhouse, 1957). Subsequently John Onley confirmed, after several studies on cell model, the neurotoxicity and neurodegeneration evoked by excitatory aminoacids, by naming it as excitotoxicity (Olney, 1969). Previously, it has been discussed how the activity of glutamate on his own receptors, related to a strong stimulation on

ionotropic receptors, bring to an uncontrolled flux of Na^+ and Ca^{2+} into cells (Coyle and Puttfarcken, 1993). In particular, the flux of calcium into cells, through NMDA receptors, has been strongly related to dramatic, lethal effects in neuronal cells. Nevertheless, the use of compounds able to block NMDA receptors, did not provide satisfactory results in patients exhibiting neuronal death, among the encouraging results obtained on experimental models (Muir and Lees, 1995). In any case, calcium is known to be involved in a large number of cellular processes, as growth, differentiation and synaptic activity; as well as the regulation of calcium imbalance in cells that is determinant in the activation of many pathways (Arundine and Tymiansky, 2003). A fully scheme of the effect of calcium imbalance related with L-Glutamate toxicity, is presented in illustration 19.

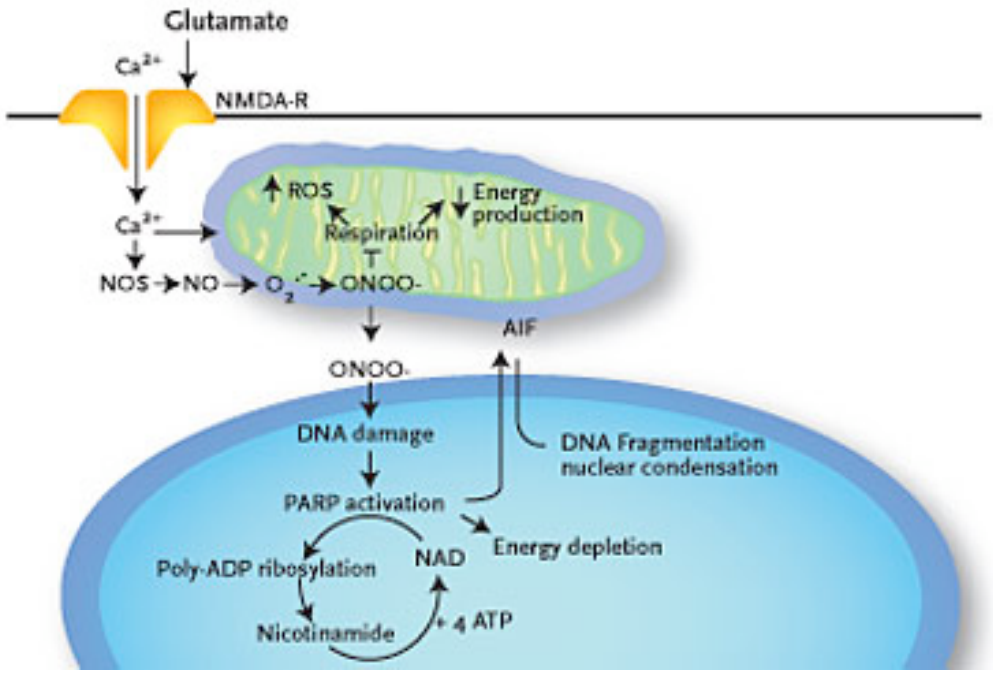


illustration 19. Several pathways seem to be involved to the increase in intracellular concentration of Calcium. Most of them are directly implicated in cell death, by damage of DNA, generation of oxygen and NO reactive species (ROS; NOS), failure of mitochondrial respiration mechanism, induced oxidative stress. Furthermore the increase of intracellular concentrations of calcium, resulted in the activation of apoptosis inducing factor (AIF), inducing cell caspase-dependent and caspase-independent cell death, and PARP (Poly ADP-

rybose polymerase) activation following DNA damage, and implicated in the caspase-independent cell death. (from Hong *et al.,* 2004).

8.1 Glutamate as neurotoxin

Glutamate, the most important excitatory neurotransmitter in the CNS, is responsible for toxicity in brain at higher concentration than the physiological one. It is well known that glutamate is extensively used in drink and food industry as tasting enhancer. Due to its extensive use in a variety of commercial products, it could represent a serious threat to human health, if we think for example to child, unborn child and old people. On the other hand many authors demonstrated that about the glutamate from diet only a 5% is absorbed in the blood system, while the 95% is converted in energy at intestine level. Furthermore, glutamate is believed to not trespassing the hematoencefalic barrier (Bellisle *et al.*, 1999; Flood *et al.*, 2007). Nevertheless, it has been demonstrated how glutamate, from blood, could penetrate and accumulate into circumventricular organs, and from that penetrate into brain (Viña *et al.,* 1997) For sure there are many more researchers who focused the attention of the toxic effect of glutamate related with an extensive consumption (Hermanussen *et al*., 2006).

9. The amyloid β-peptide

The amyloid β-peptide (Aβ) is the main component of senile plaques, typical structures visible in brain affected by Alzheimer disease. Senile plaques accumulate outside neurons all around the soma, avoiding the communication between neurons, thus provoking oxidative stress and cell death. The amyloid β-peptide is constituted by 39-42 aminoacids and the physiological role is still unclear; that peptide is obtained from the proteolysis of a precursor protein called APP (amyloid precursor protein). It is well known

that amyloid β-peptide is implicated in many toxic events in neuronal cells like oxidative stress and cell membrane lipid oxidation. After intercalation into cell membrane it may occur a change in membrane potential by formation of channel across membrane and change in the cell calcium homeostasis (Pearson *et al*., 2002; Drake *et al*., 2003; Klein *et al*., 2004; Butterfield *et al*., 2002) (illustr. 20).

9.1 Alzheimer's disease and the "amyloid hypothesis"

Main causes of Alzheimer's disease are still unclear, nevertheless some theories have been proposed. The mainly existing hypothesis are founded on observations made on post-mortem tissues, as abnormal accumulation of proteins outside and inside neuronal cells, furthermore those macroscopic observations on tissues are strictly related to the severity of the dementia (Blessed *et al.,* 1968.). About 80s years after studies provided by Alzheimer (translate on Alzheimer *et al.,* 1995), the main molecular components of that protein have been identified. At one side, amyloid **β-** peptide, proceeding from proteolysis of a transmembrane protein and main biochemical component of extracellular protein plaques (Glenner and Wong, 1984), while a tau hyperphosphorilated protein results to be the main component of neurofibrillar tangles inside cells (Grundke-Iqbal *et al.,* 1986). Nevertheless, the way those molecular components bring to pathological degeneration, is still unknown. In particular some old age patients presenting proteins plaques have been demonstrated to not suffer pathological degeneration, respect to other patients in the same conditions (Shoji *et al.,* 1992).

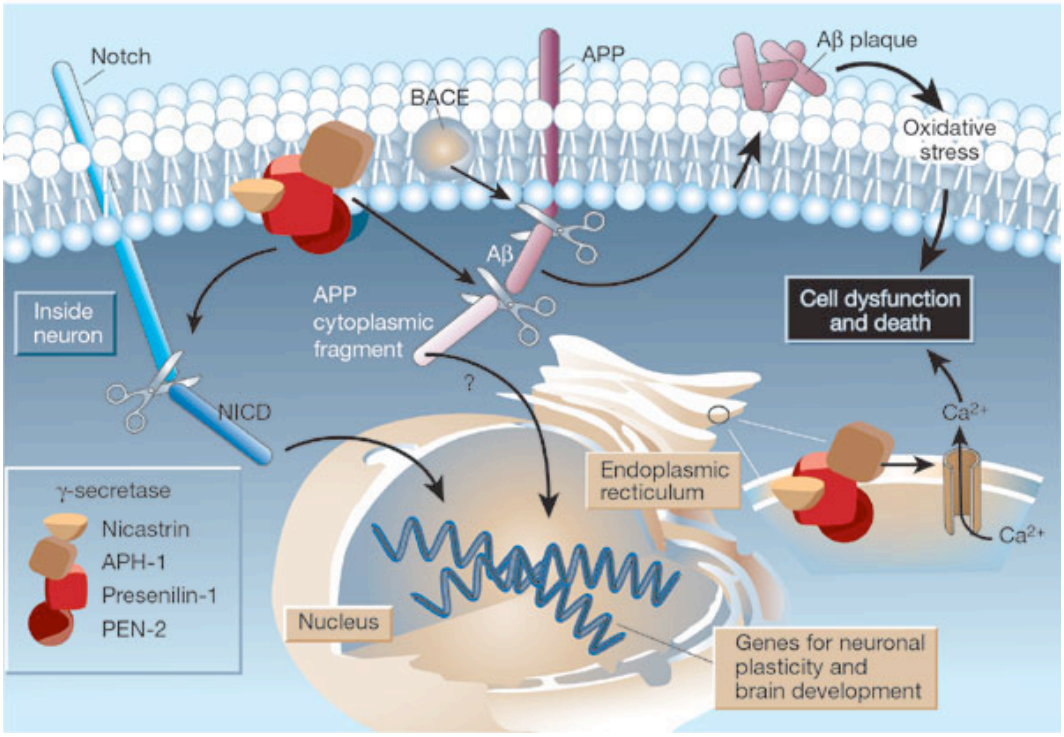


illustration 20. Amyloid β-peptide formation pathway and related effect in neuronal cells. γ–secretase is responsible of the cleavage of the APP fragment acting in cytoplasm, while BACE-1 enzyme is processing the fragment of APP going to intercalate outside cells, and forming amyloid beta-peptide plaques, even intercalating into cells membrane. Those fragment induce oxydative cells, and γ-secretase induce liberation of calcium from endoplasmic reticulum, acting on calcium channels on reticulum membrane, bring cells to death. APP cytoplasmic fragments, may induce specific genes transcription (from Mattson, 2003).

The main accepted hypothesis to explain Alzheimer disease, is the "amyloid hypothesis" (review on Hardy and Selkoe, 2002). The hypothesis explains how the increase of amyloid peptide, is the first pathogenic factor, bringing to the rest of Alzheimer related toxic pathways, including the formation of hyperphosphorilated tau proteins. Different hypothesis have been recently proposed about the exactly sequences of events, bring to the pathologic toxic conditions of disease. Some authors described some intracellular aggregates which may have pathologic role (review on LaFerla *et al.,* 2007), and probably generating previously than the amyloid plaques are formed (Braak and Del Tredici, 2004). These recently results and studies about molecular

Alzheimer's causes, make the investigation of the disease as a continuous review. In the present work, we accepted the "amyloid hypothesis " like an *in vitro* experimental model.

10. The β-site APP cleaving enzyme (BACE-1)

The β-site APP cleaving enzyme (BACE-1) belongs to the family of the secretase, enzymes able to cleave fragment of proteins. The proteinase originally named 'β-secretase', catalyse the first step in the amyloidogenic metabolism of the large transmembrane amyloid precursor protein (APP), by release a soluble APPβ (sAPPβ) form, which is further processed by the γ-secretase enzyme complex that, at the same time generates the APP intracellular domain and releases the 39-42-amino-acid amyloid β-peptide (Aβ). On the other hand there is an alternative pathway of APP metabolism initiating by the metallo-proteinase, α-secretase pathway, predominating in most of cells type (illustr.21). β-secretase is widely distributed in the majority of tissues of human body (Haass *et al.,* 1992) while the maximal activity is found in neural tissue and neuronal cell lines (Seubert *et al.,* 1993). Although the exact physiological role of BACE-1 has not been still clarified, in the last decades attracted the attention of many researcher because its main role in the first and rate-limiting step in amyloidogenic pathway, and like a possible target in neurodegenerative diseases. In that sense it has been demonstrated that its deletion in mice has minimal phenotypic and behavioural consequences (Roberds *et al.,* 2001).

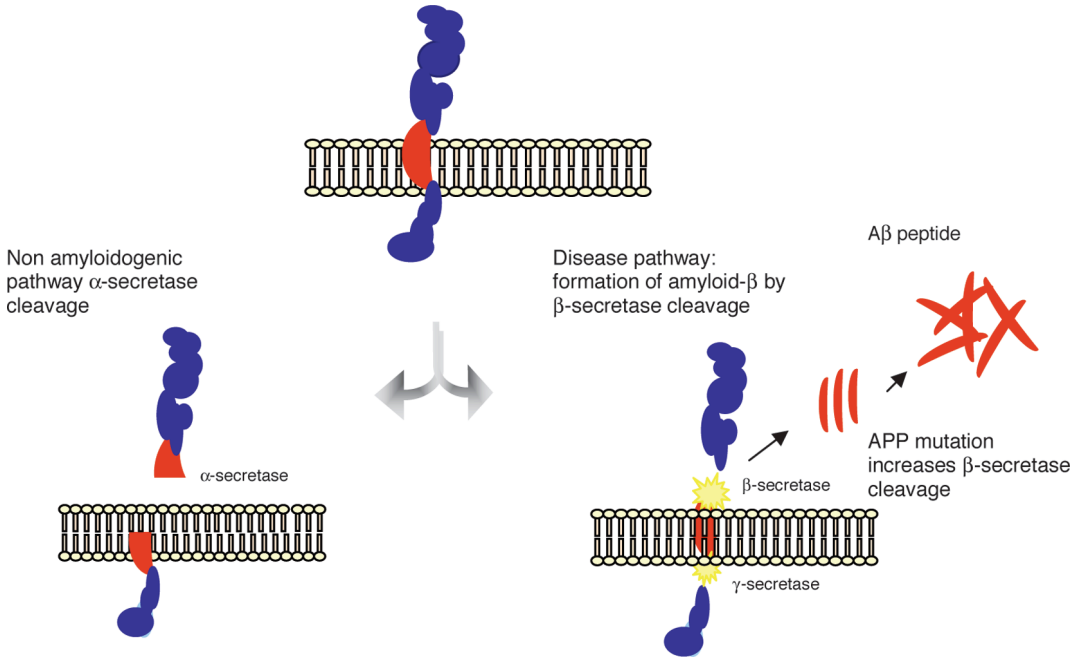


illustration 21. The amyloid precursor protein (APP) is a transmembrane protein cleaved by secretase enzymes. In the non-amyloidogenic pathway, APP is cleaved preferentially by α-secretase. In the amyloidogenic pathway, neurotoxic Aβ peptides are released after sequential cleavage of APP by β and γ-secretases, and further accumulate into oligomeric aggregates (adapted from de Paula *et al.,* 2009).

Recently BACE-1 inhibitors have been proposed as potential therapeutic compounds for the cure of neurodegenerative disease as Alzheimer's. Baxter and co-workers proposed the use of 2-Aminoquinazolines as BACE-1 inhibitors, by structure-activity studies on enzyme structure (Baxter *et al.,* 2007). Many experimental evidences confirm the potential of this therapeutic approach (Singer *et al.,* 2005; Maiorini *et al.,* 2002), but development of safe and effective inhibitors, has been challenging. In that sense, the irreversible inhibition of BACE-1 could bring to unexpected secondary and toxic effects, while the non-pathologic role is still unclear (Zacchetti *et al.,* 2007), as well as it has been reported observations on mice deficient for BACE-1, provided a lower production of amyloid beta-peptide without toxic consequences observed in animals (Luo *et al.,* 2001). Furthermore studies on glutamatergic system, confirmed relationship occuring between glutamate receptors and amyloid peptide processing. Thus it has been reported how mGluR activation,

mainly $mGlu_1$, is related to the activation of nonamyloidogenic APP processing (Lee *et al*., 1995), while an increase in $mGlu_2$ receptors has been related to high production of phosphorilated tau proteins (Lee *et al.,* 2004).

11. Effect of oxygen deprivation on cells

11.1 Hypoxia conditions

The decrease of availability of oxygen in cells, is the main cause of cognitive and physical deficiencies manifested by patient who suffered ischemic toxicity in brain. To low concentrations of oxygen in cells, mainly corresponds the activation of specific factors named HIF (hypoxia inducible-factor), which induce the transcription of specific genes involved in various pathways into cells. Nevertheless other specific factors are involved during the decrease of partial concentration of oxygen in cells (Kenneth and Rocha, 2008). HIF factor is a dimer formed by two subunit, one α- sensible to low concentration of oxygen, and a β- subunit constitutively expressed. There are many type of HIF expressed in mammalian called HIF-1α, HIF-2α and HIF-3α. The first form is ubiquitous in cells and widely distributed; while the two remain present a limited distribution. The activation of that HIF-1α factor involves various steps including the stabilization of α-subunit, its translocation to nucleus and the interaction with others proteins regulating specific genes involved in hypoxia (Brahimi-Horn *et al.,* 2005) (illustr. 22). HIF factors have been related as main responsible in neovascularisation, in glucose metabolism, in cells survival and cancer metastasis (Pouyssegúr *et al.,* 2006).

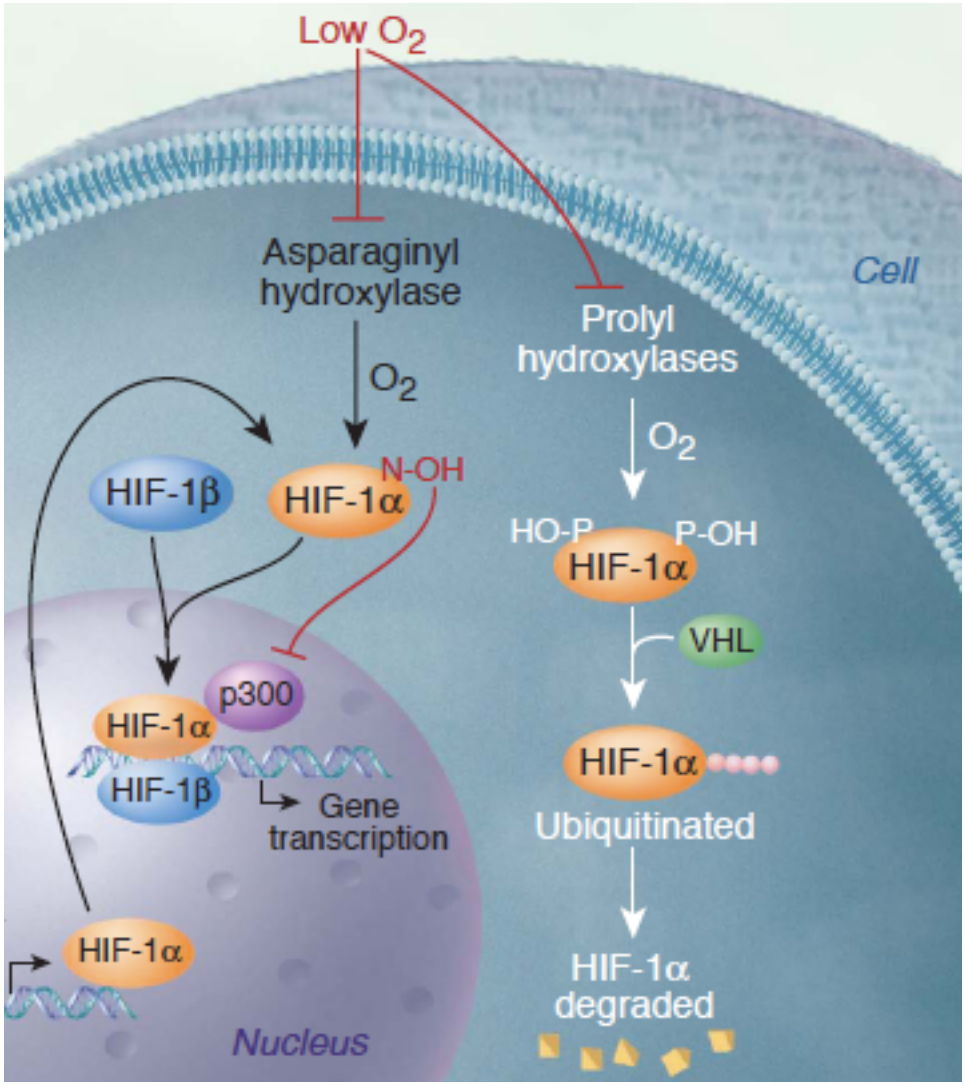


illustration 22. Cells control HIF-1 in two ways. When oxygen is plentiful, hydroxylase enzymes promote degradation of the HIF-1α subunit, which is aided by the VHL tumor suppressor protein, and also block HIF-1α's ability to bind p300 and other proteins needed for gene transcription. Low oxygen concentrations inhibit those activities of the hydroxylases, thus turning up HIF-1 activity (adapted from Marx J., 2004).

11.2 The oxidative stress

The oxidative stress is mainly related with death processes in cells, generally by an apoptotic mechanism, related to the formation of reactive oxygen species (ROS), and subsequently oxidative modifications of lipid, DNA, cell membrane and other targets molecules. The oxidative stress have been furthermore related with the development of some neurodegenerative diseases (Barnham *et al.,* 2004). The oxidative damage by free radical is known originating in mitochondria respiratory chain by complex I and III, as source of superoxide anion (O_2^-) (Jezek and Hlavatá, 2005). Fortunately that organelle owns specific antioxidative enzyme defences, converting reactive species into no toxic ones (Yu, 1994). Those recruiting defence enzymes belong to the family of dismutase superoxide (SOD), converting superoxide anion into H_2O_2, catalase, converting H_2O_2 into water, and other enzymes like glutathione reductase whose expression is induced by ROS. Nevertheless some enzymes may change their function from antioxidant to oxidant mainly due to the increase of iron and copper, related with brain ageing (Barnham *et al.,* 2004). Thus a dramatically increase in ROS species have been observed, and consequently related with cell death by oxidative stress, and with neurodegenerative diseases as Alzheimer's (De Leo *et al.,* 1998). The most common process related to cell death by free radical species generated by mitochondria, is the variation in mitochondria membrane permeability to calcium, with consequently liberation of cytocrome C initiating the apoptotic cascade (Mattson and Kroemer, 2003). In that sense, it is believed that first step of Alzheimer's disease is the oxidative damage originated by ROS (Nunomura *et al.,* 2001). Discussed processes involved in oxidative stress, are summarized in the following scheme (illustr.23)

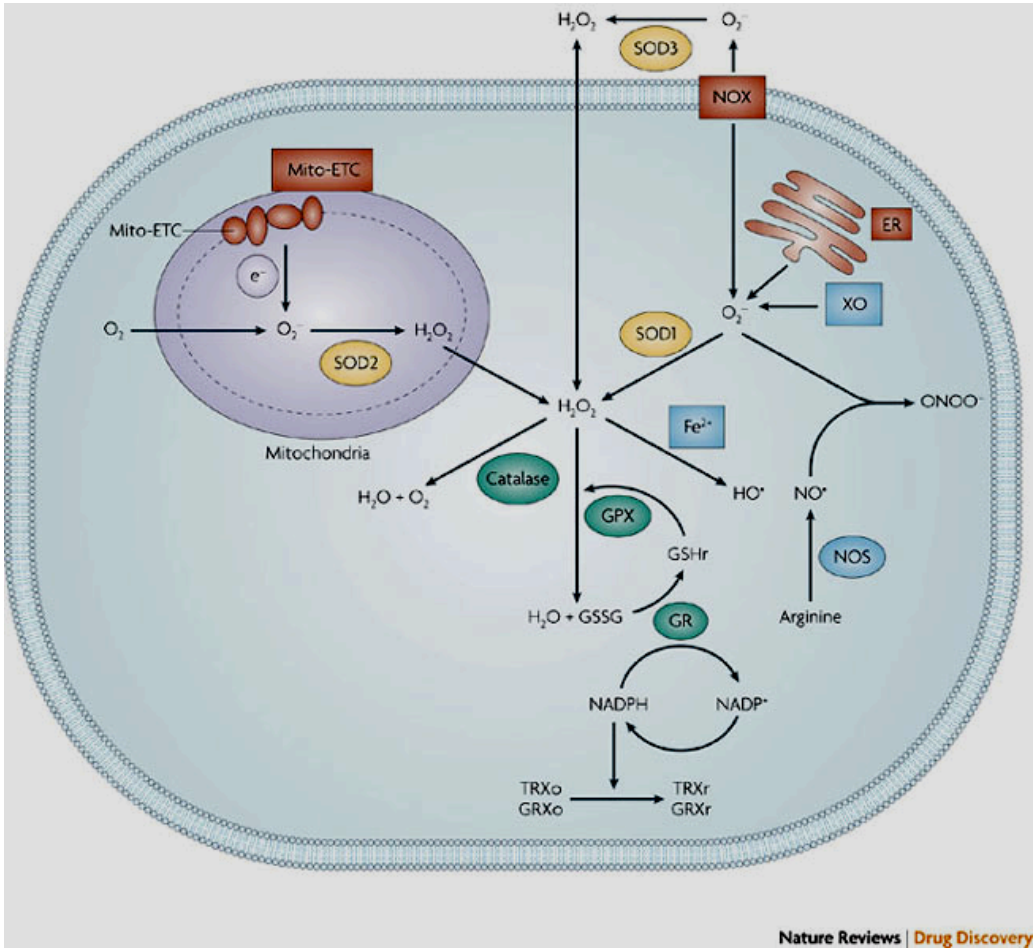


illustration 23. Major sites of cellular reactive oxygen species (ROS) generation (indicated in red) include the mitochondrial electron transport chain (Mito-ETC), the endoplasmic reticulum (ER) system and the NADPH oxidase (NOX) complex. Superoxide (O_2^-) is the main initial free radical species, which can be converted to other reactive species. In the mitochondria, superoxide is generated by the capture of electrons escaping from the electron transport chain by molecular oxygen. Superoxide can be rapidly converted to H2O2 by superoxide dismutases (SOD, yellow), which can be reused to generate superoxide radicals or converted to water by catalase. In the presence of transition metals (such as Fe^{2+}), H_2O_2 can be converted to hydroxyl radicals (HO), which are highly reactive and can cause damage to lipids, proteins and DNA. Nitric oxide (NO) is a reactive radical produced from arginine by nitric oxide synthase (NOS). Nitric oxide has a very short half-life and can react with superoxide to form peroxynitrite (ONOO-), a non-radical species that is capable of modifying the structure and function of proteins. To prevent the harmful effects of ROS, cells regulate ROS levels by maintaining the balance between ROS generation and elimination. Major ROS-scavenging enzymes are shown in green. GPX, glutathione peroxidase; GR, glutathione reductase; GRXo, glutaredoxin (oxidized); GRXr, glutaredoxin (reduced); GSHr, glutathione (reduced); GSSG, glutathione (oxidized); TRXo, thioredoxin (oxidized); TRXr, thioredoxin (reduced); XO, xanthine oxidase (adapted from Trachootham *et al.,* 2009).

AIMS

AIMS

The aim of the present work was to analyze the effect of different nanocompounds on well established models of neurodegeneration, as β-amyloid treatment or glutamate excitotoxicity, and their effect on adenosine and metabotropic glutamate receptors. As nanocompound it was used the hydrosoluble [60]fullerene isomer *trans*-3 (T3SS) in solution or linked to surface used as growing substrate, partially soluble nanohorns and hydrosoluble derived gold nanoparticles. We analyzed the effect of these compounds on different cell types as SK-N-MC, SH-SY5Y and rat cortical neurons by determining cell viability and the expression of adenosine and metabotropic glutamate receptors.

The main goals were:

1. Identification of adenosine (ARs) and metabotropic glutamate receptors (mGluRs) natively expressed in SK-N-MC cells.
2. The study of the possible toxic or protective activity of T3SS on previously cited *in vitro* models.
3. The effect of T3SS on the expression of ARs and mGluRs on cells models and its possible role after different toxic insults.
4. Evaluation of biocompatibilidy of [60]fullerene Self Assembled Monolayers (SAM's) on the same *in vitro* models.
5. The effect of gold nanoparticles and nanohorn hydrosoluble derivatives on cell viability.
6. The study of the possible role of adenosine and metabotropic glutamate receptors in nanocompounds mediated effects.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURES

1. Materials

The hydrosoluble [60]fullerene bis-adduct *trans*-3 (T3SS) was kindly provided and previously synthesized by group of professor Prato at University of Trieste. Cyclopentyl-1,3-dypropylxanthine, 8-[dipropy-2,3-^{3}H(N)] ([^{3}H] DPCPX 120 Ci/mmol) was purchased from Amersham (Madrid, Spain). [2-^{3}H](4-(2-[7-amino-2-(2-fury1) [1,2,4] triazolo [2,3-a] [1,3,5] triazin-5-ylamino]ethyl)phenol ([^{3}H]ZM241385 27.4 Ci/mmol) was from Tocris (Bristol, UK). N^6-cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA) and calf intestine adenosine deaminase (ADA) were obtained from Sigma (Madrid, Spain). The (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles used in the experiments were purchased from Sigma Aldrich (Madrid, Spain), as a stock 2% (w/v) water solution, and diluted in MILLI-Q sterile water to perform the specific assay. Functionalized water soluble Nanohorns used in the showed experiments, were synthesized and kindly donated by the group of Ester Vazquez from Organic Chemistry group, University of Castilla La Mancha, Campus of Ciudad Real, Spain. Amyloid $\beta-$peptide 25-35 fragment was purchased from Sigma Aldrich (Madrid, Spain) and L-Glutamate was from Tocris (Bristol, UK). The compound antagonist for human adenosine A_{2A} receptors (SCH58261) and A_3 (SCHx), were kindly donated by the group of prof. Piero Spalluto from Department of Pharmaceutical Science, University of Trieste, Italy; these compounds were dissolved in a mixture of MILLI-Q sterile water with 1% of DMSO and stored at 4°C until use. The antagonist of A_1 receptors PSB 36 (1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione), the agonist of adenosine A_{2A} receptors CGS 21680 (4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride), the antagonist of adenosine A_{2B} receptors PSB 1115 (4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl) -benzenesulfonic acid potassium salt), the antagonist of $mGlu_1$ receptors JNJ 16259685 (3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)((1s,4s)-

4-methoxycyclohexyl)methanone) and the antagonist of $mGlu_5$ receptors MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride) were all purchased from Tocris (Bristol, UK) stored at 4°C and dissolve in sterile MILLI-Q water or Ethanol just prior to use. All other products used were of analytical grade.

2. Cell culture

SK-N-MC neuroepithelioma cells (purchased from ATCC, HTB-10) (Biedler et al., 1973) were grown in MEM (Modified Eagle's Medium, Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM pyruvate, 1% non-essential amino acids and antibiotics in a humidified atmosphere with 5% CO_2 at 37°C, as previously reported (Seeger et al., 1977). Cells were subcultured on 10 ml Petri dishes (Nunc, Denmark) and when at confluence were detached by using 4 ml of Trypsin (Tryple Express, Gibco, USA), centrifuged and the pellet was resuspended in complete medium and plated onto 24 or 96 wells (Nunc, Denmark) and let grow for 24h before experiment, to have a final density per well of $2{\cdot}10^5$ cells and $3{\cdot}10^4$ cells, respectively. SH-SY5Y (purchased from ATCC) human neuroblastoma cells[5] were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, and 1% of mixture antibiotic-antimycotic (Gibco, USA), humidified atmosphere with 5% CO_2 at 37°C. SH-SY5Y were subcultured on 10 ml Petri plaques (Nunc, Denmark) and when at confluence where detached by using 4 ml of Trypsin (Tryple Express, Gibco, USA), centrifuged and the pellet was resuspended in complete medium of growth and plated onto 24 or 96 plaques (Nunc, Denmark) and let grow for 24h before experiment, to have a final density per well of $2{\cdot}10^5$ and $3{\cdot}10^4$ cells respectively. For the experiments described in the present work SK-N-MC and SH-SY5Y cells were used between the 3 and 12 sub-cultivation passages, avoiding ageing problems of cells. The primary culture of rat's neuron was obtained from cortex region of Wistar rats brain fetuses at 18th days of pregnancy. The fetuses brain was dissected and put on PBS supplemented with glucose 6 mM and BSA 1%. After the separation of the meningis, the

dissected cortex were incubated with 30 U/mL of papain for 5 minutes at 37°C and subsequently dissociated by using a Pasteur pipette. Once the tissue was disaggregated, 10 mg of DNAse was added and the dissociated cells were filtered by gravity on a 70 μm diameter filter (BD Falcon). The obtained filtered material was centrifuged at 300 g for 6 minutes and the pellet was thus suspended in MEM (Minimum Essential Medium) supplemented with $NaHCO_3$ 2,2 g/L, Glutamax I 10 ml/L, HEPES 2,6 g/L antibiotic and atimicotic 10 ml/L, B27 and 10% of horse serum (previously deactivated) at density of 4 x 10^5 cells/mL. Cells were then put on poly-Lysin covered plaques (BD Falcon), at a final density of 2,6 x 10^5 cells per well on 24 wells plaque, 8 x 10^4 cells per well on 96 wells plaque. Cells were maintained at 37°C in humidly atmosphere at 5% of CO_2 and 95% of O_2. The day after the totality of medium was changed with Neurobasal medium (NB), supplemented with B27. At the second day from *in vitro* cells plaqued (DIV), cytosin arabinose was added (AraC) at a final concentration of 5 μM. At day 7 and 14 DIV half of medium was substituted by NB supplemented with B27. All the experiments were performed between the 8 and 15 DIV at least to avoid ageing of rat's neurons.

3. SH-Sy5y cells differentiation

SH-Sy5y human neuroblastoma cells were differentiated into a population of fully human neurons according to literature (Constantinescu *et al*., 2007), and to the following procedure. Briefly, the medium of cells was substituted by complete medium with 10 μM ATRA (*all trans* retinoic acid, Sigma Aldrich). The day after the medium was substituted by fresh medium with 10 μM of ATRA; cells were thus maintained in culture for 72h. At this time the medium was replaced with fresh medium with 10 μM ATRA, and after 48h hours the medium was eliminated and substituted by DMEM without fetal bovine serum, with 1% mixture antibiotic-antimycotic and 50 ng/ml BDNF (Brain Derived Neurons Factor); cells were thus incubated for 3 days in the

presence of that medium and used for the selected experiments at least for one week by using the medium described above with the specific treatment.

4. MTT reduction assay

Cell viability was determined using an *in vitro* toxicology assay kit based on the reduction of 3-[4,5-dimetylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) purchased from Sigma (Madrid, Spain), according to Mosmann (1983). Briefly, SK-N-MC neuroepithelioma and SH-SY5Y neuroblastoma cells were seeded at $3 \cdot 10^4$ cells per well in 96 well plates. At the end of fullerene derivative treatment, cells were incubated in culture medium with MTT solution (5 mg/mL) at 37ºC for 3 h. After incubation, MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to the wells to dissolve formazan crystals. The plates were thoroughly shaken and the absorbance of each well was measured at 570 nm.

5. Plasma membrane isolation

The isolation of plasma membranes was performed as previously described (Albasanz et al., 2002). Cells were homogenized in isolation buffer (50 mM Tris-HCl pH 7.4, containing 10 mM $MgCl_2$ and protease inhibitors) and centrifuged for 5 min at 1000xg in a Beckman JA 21 centrifuge. The supernatant was centrifuged for 20 min at 27000xg and the pellet was resuspended in isolation buffer. The concentration of protein was measured by the method of Lowry, using bovine serum albumin as the standard.

6. Western Blotting assay

Western Blotting assay was performed as previously described (Albasanz et al., 2006) by using 50 micrograms of protein. After transferred to nitrocellulose membranes, proteins were incubated with the rabbit polyclonal anti A_1R (Calbiochem, UK), anti $A_{2A}R$ (Upstate, US) and anti $A_{2B}R$ (Santa Cruz Biotechnologies), all at dilution of 1:1000. The incubation with the secondary antirabbit IgG antibody (Dako, Barcelona, Spain) was carried out at a dilution of 1:5000. The monoclonal anti β-actin antibody (Sigma, Madrid, Spain) was used as a gel loading control (1:5000).

7. Total RNA isolation and preparation of cDNA

Total RNA was extracted using an ABI 6100 Nucleic Acid PrepStation according to the manufacturer's protocol. All chemicals for the ABI 6100 were purchased from Applied Biosystems (Foster City, CA). Total RNA from cells was isolated and stored at -80°C. Ratio of A_{260}/A_{280} (purity of RNA) was in the range 1.9–2.1. RNA concentrations were determined from the A_{260}. One microgram of total RNA was reverse transcribed using Applied Biosystems High-Capacity cDNA Archive Kit according to manufacturer's protocol.

8. Quantitative real time RT-PCR analysis

To assess relative gene expression in SK-N-MC neuroepithelioma cells quantitative real time RT-PCR analysis (Higuchi et al. 1993) was performed with an Applied Biosystems Prism 7500 Fast Sequence Detection System, using TaqMan universal PCR master mix according to the manufacturer's specifications (Applied Biosystems Inc., Foster City, CA). The TaqMan probes and primers for A_1 (assay ID: Hs 00181231; Rn 00567669), A_{2A} (assay ID: Hs 00386497; Rn 00583935), A_{2B} (assay ID: Hs 00169123; Rn 00567697) mGlu1 (assay ID: Hs 00168250; Rn 00566625), mGlu5 (assay ID: Hs 00168275; Rn

00690336), BACE-1 (assay ID: Hs 00201573; Rn 00569988), AC-I (assay ID: Hs 00299832), PLCβ (assay ID: Hs 00248563) and β-actin (assay ID: Hs 99999903; Rn 00667869) were assay-on-demand gene expression products (Applied Biosystems). The TaqMan primer and probe sequences are packaged together in a 20x solution. The sequences are proprietary, so they are not available. The gene-specific probes were labeled using reporter dye FAM. A non-fluorescent quencher and the minor groove binder were linked at the 3´ end of probe as quenchers. The thermal cycler conditions were as follows: hold for 20 s at 95"C, followed by two step PCR for 40 cycles of 95ºC for 3 s followed by 60ºC for 30 s. Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems) according to the $2^{-\Delta\Delta Ct}$ method. Briefly, expression results of a gene were normalized to internal control β-actin relative to a calibrator, consisting of the mean expression level of the receptor gene as follows: $2^{-\Delta\Delta Ct} = 2^{-((Ct\ receptor\ gene - Ct\ actin)\ sample\ -\ (Ct\ receptor\ gene - Ct\ actin)\ calibrator)}$. The results from 4-5 independent repeat assays, performed in different plates each using different cDNA's from the cultures analyzed, were averaged to produce a single mean quantity value for each mRNA.

9. Detection of adenosine A_1 and A_{2A} receptors in intact cells by radioligand binding assay

Radioligand binding assays using intact cells were performed in 24-well plates at 80–90% of confluence ($2 \cdot 10^5$ cells/well) as previously described (Ruiz et al. 2000) with some modifications. Briefly, cells were washed with serum-free MEM pH 7.4 and pre-incubated with 2 U/mL adenosine deaminase at 37ºC for 30 min to remove endogenous adenosine. After incubation, in order to measure adenosine A_1 receptors the indicated concentrations of [^{3}H]DPCPX (1-30 nM) were added in the absence or the presence of 1 mM non-labeled CPA to obtain non-specific binding. Adenosine A_{2A} receptors were quantified by using the radioligand [^{3}H]ZM241385 (0.1-40 nM) and 3 mM theophylline to obtain nonspecific binding. After incubation at

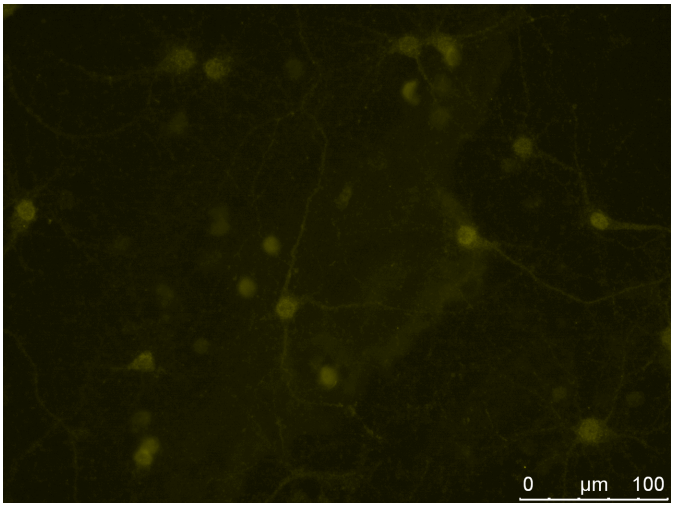
25ºC for 2 h in a final volume of 250 μL, cells were washed with 500 μL of ice-cold medium and disrupted with 0.1% sodium dodecyl sulphate (SDS). Wells content were then transferred to vials and scintillation liquid mixture was added in order to measure radioactivity. At least two wells from each plate were reserved for protein concentration measurement.

10. Fluorescence microscopy

Images for labeled fluorescent antibody of receptors A_{2A} have been obtained with a digital camera Leica DFC350FX, attached to a Leica DMI6000B (Leica Microsystem, Wetzler, Germany) fluorescent inverted microscope. A 20x HCX PL FLUOTAR objective and the LAS AF Lite software were used. The protocol for the fluorescent immune assay was performed as follow: SK-N-MC and SH-SY5Y cells were growth in complete medium for 24 h before assay or specific treatment. For the fluorescence labeling of SK-N-MC the procedure used as follow: complete medium was replaced with phosphate saline buffer (PBS), let 5 min and repeated it for three times and the cells were fixed in 4% paraformaldehyde in phosphate buffer for 15 min. Cells were then incubated with bovine serum albumine (3% BSA) and Triton TX-100 (0.1% in PBS) at room temperature for 10 min, then incubated with primary antibody goat antimouse A_{2A} (Santa Cruz, USA) diluted 1:100 in PBS-3% BSA for 3 hours at 37°C. Cells were then washed three times with PBS at 37°C and incubated with secondary IgG anti goat antibody labeled with fluorescein isothiocyanate (FITC, Invitrogen, USA) at dilution of 1:500 in PBS-3% BSA for 30 min. Cells were then washed to remove not fixed fluorescent antibody. For SH-Sy5y and Rat's neurons the fluorescence labeling procedure was preformed as follow: medium was removed and well washed with PBS three times each for 5 minutes followed by incubating cells with Locke Buffer (134 mM NaCl /4 mM $NaHCO_3$ /25 mM KCl /10 mM HEPES /5 mM glucose /2.3 mM $CaCl_2$ /1 mM $MgCl_2$, pH 7,4) for three times each for 5 minutes. Cells were then incubated for 10 min in presence of paraformaldehyde 4% in 0.1 M phosphate buffer (PB), follow by incubating three times each of 10 minutes with 0.1 M PB, and

one time of 10 min with Triton 0.25% in 0.1 M PB. Cells were then treated with normal goat serum 3% (NGS) in 0.1 M PB, following by incubation for 3.5h with primary antibody goat antimouse NEFH (Sigma Aldrich), MAP-2 (Sigma Aldrich), BACE-1 (Sigma Aldrich), all diluted 1:1000 in 0.1 M PB/ NGS 3%. Subsequently cells were washed with 0.1 M PB, and incubated for 45 min in darkness with secondary IgG anti goat antibody labeled with fluorescein isothiocyanate (FITC, Invitrogen, USA) at dilution of 1:500 in PBS-3% BSA for 30 min. Cells were then washed to remove not fixed fluorescent antibody.

A



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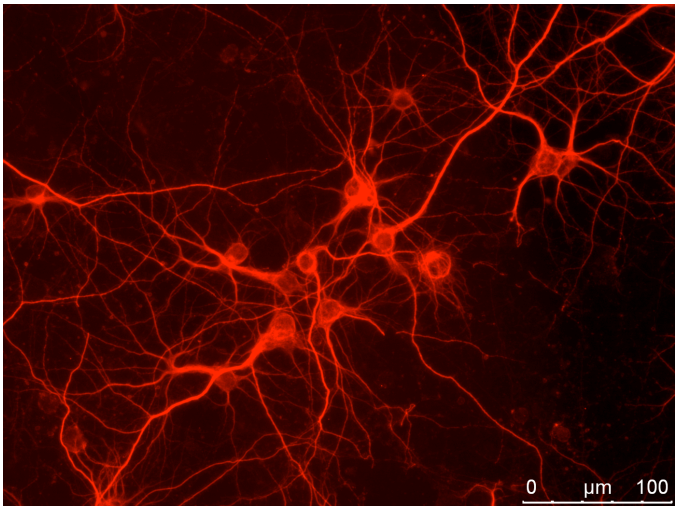


C

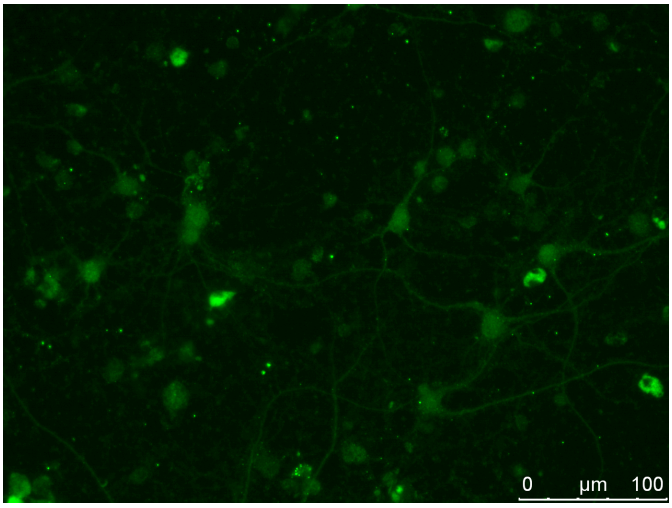


illustration 23. **Example of fluorescence images on different target expressed by neruonal cells from rat's brain.** (**A**) Microscopy confocal image of neuronal rat cells constitutively expressing BACE-1 enzyme. (**B**) Microscopy confocal image of neuronal rat cells constitutively expressing MAP-2 (Microtubules Associated Protein), it should be noticed that the intensity of the expression related to the abundance of MAP-2 rats neurons, is used as fundamental marker in neurogenesis process. (**C**) Microscopy confocal image of neuronal rat cells constitutively expressing NEFH (Neurofilament heavy polypeptide), a relevant marker to distinguish healthy neurons, and more in general, neurons from other type of cells.

11. Protein determination

Protein concentration for radioligand binding assay and Western-blotting was measured by the method of Lowry (1951), using bovine serum albumin as standard.

12. Statistical and data analysis

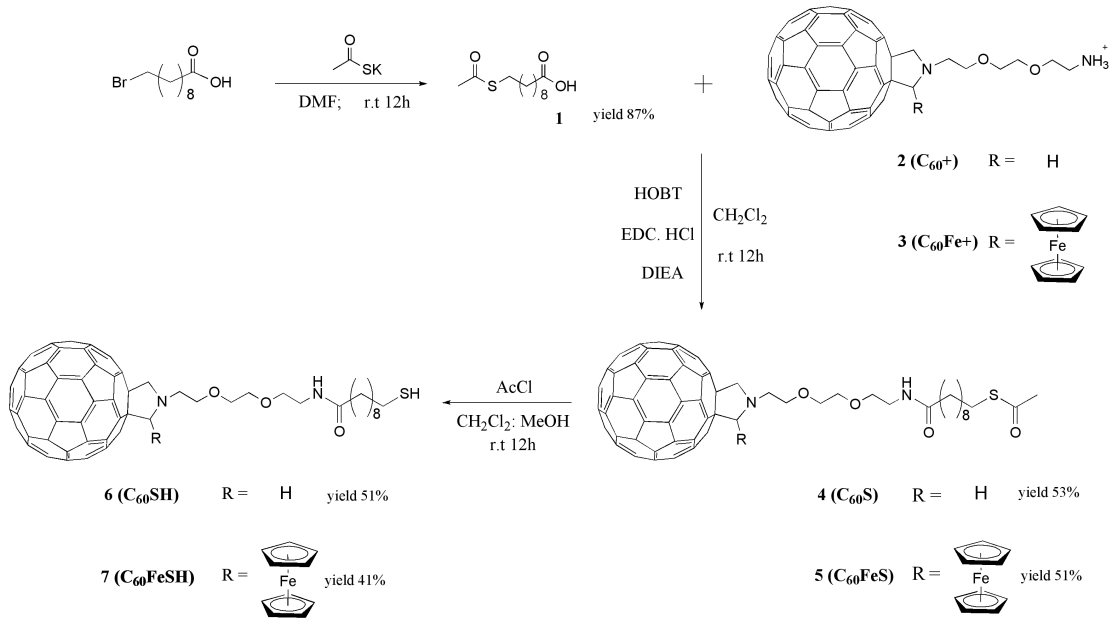
Data statistical analysis was performed using the Student's t-test. Differences between mean values were considered statistically significant at $p<0.05$. Saturation (Bmax, Kd) binding curves were analyzed performing Scatchard

and non-linear regression analysis of binding data with the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA).

13. Synthetic procedures and characterizations

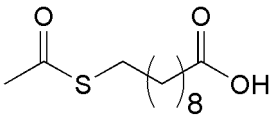
13.1 Synthetic Procedures

All reagents and solvents were used without further purification. Reactions were carried out under nitrogen atmosphere and under anhydrous conditions. The compound 1-dodecanethiol deposed on gold surfaces and used as reference modified substrate, was purchased from Sigma Aldrich (Germany); gold Au(111) 200 nm thickness on mica 10 mm square substrates used for these experiment were purchased from Phasis (Switzerland). Products were purified by flash chromatography on SiO_2 (particle size 0.032-0.063 mm). NMR spectra were obtained at 400 MHz (1H NMR; 13C NMR) using $CDCl_3$ as the solvent. IR-DRIFT spectra were recorded using KBr powder. UV-vis spectra were obtained using toluene as the reference solvent.



Scheme view of synthetic procedure for [60]fullerenes derivatives.

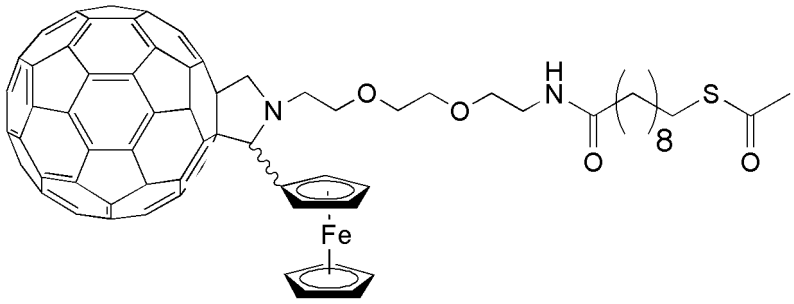
Synthesis of compound **1** (*Decanoic acid 1-thioacetate*)



The synthesis was performed according to the procedure indicated in literature (see references Muller *et al.*, 1997; Fan *et al.*, 2000).To a stirring solution of 1-bromide-decanoic acid (3,98 mmol, 1g) dissolved in 6ml DMF, was added in one portion potassium thioacetate (5,57 mmol, 651 mg) at 0°C and the mixture let stirring overnight at r.t. under Ar. When the solution appeared to be deep orange coloured, the reaction was stopped by evaporating DMF; the residue was then dissolved in 15 ml of CH_2Cl_2 and washed three times with water; the organic phase was then concentrated, let stirring under adding $MgSO_4$, and dry under vacuum to afford desired compound as a deep orange solid (3,5 mmol, 880 mg, 87% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): 2.8 (t, *J* = 7.4 Hz, 2H, S-CH_2); 2.33 (t, *J* = 7.8 Hz, 2H, CO-

CH_2), 2.3 (s, 3H, -C*H*$_3$); 1.6 (m, 2H, -CH_2); 1.5 (m, 2H, -CH_2); 1.3 (m, 10H, -CH_2). 13C-NMR (400 MHz, $CDCl_3$): 194.3, 179.8, 34.2, 32.5, 29.54, 29.22, 29.09, 28.82, 24.5; IR-DRIFT: cm^{-1} 3366, 2852, 1693, 959, 714, 629. ESI-MS: *m/z* 246.13 (MH^+).

Synthesis of compound **5 (C_{60}FeS)**

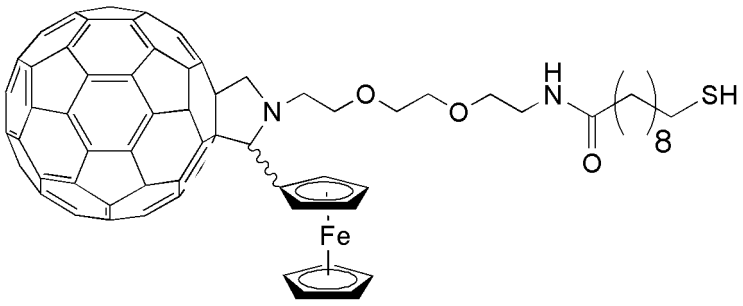


The synthesis of compound **5 (C_{60}FeS)** was performed according to literature, and the complete procedure is reported below. The fullerene mono deprotected derivative **3 (C_{60}Fe+)** used for the follow reactions have been previously synthesized and fully characterized according to literature (Kordatos K., *et al*., 2001).

A mixture containing **1** derivative (0,08 mmol, 20 mg), HOBT (0,16 mmol, 22 mg) and EDC.HCl (0,16 mmol, 31 mg) in 5 ml of distilled CH_2Cl_2, was stirred at r.t. under Ar for 30; to that a solution of **3** (0,08 mmol, 100 mg) and DIEA (0,49 mmol, 90 µl) dissolved in 5 ml of distilled CH_2Cl_2 was added dropwise; the resulting mixture was stirred overnight under Ar at r.t. and protected from light. The reaction was stopped by evaporating the solvent under vacuum and dissolve the residue in a minimal amount of $CHCl_3$ to be subjected to a chromatography column on fine Silica, using Toluene 3: EtoAC 7 as the eluant. The residue was then dissolved in a minimal amount of CH_3Cl_3,

precipitated by using cyclohexane following by trituration with pentane and subsequently dried under high vacuum to afford the pure desired compound (0,042 mmol, 60 mg, 52% yield) as a deep red solid. ^{1}H-NMR 1H NMR (400 MHz, CDCl3) δ5.93 (bs, 1H), 5.11 (mult., 2H), 5.03 (s, 1H), 4.58 (s, 1H), 4.53 (mult., 1H), 4.32 (s, 5H), 4.27-4.19 (m, 4H), 3.99 (mult., 1H), 3.91 (mult., 1H), 3.80 (t, J = 4.56 Hz, 2H), 3.65 (t, J = 5.04 Hz, 2H), 3.49 (t, J = 5.28 Hz, 2H), 3.18 (t, J = 5.96 Hz, 2H), 2.86 (t, J = 7.32 Hz, 2H), 2.33 (s, 3H), 2.13 (t, J = 7.76 Hz, 2H), 1.58 (m, 4H), 1.27 (t, 10H). 13C NMR (100 MHz, CDCl3) δ 196.23, 173.28, 156.44, 156.12, 154.53, 153.95, 153.49, 147.68, 147.41, 147.30, 146.39, 146.28, 146.07, 145.61, 145.38, 145.29, 144.56, 143.15, 142.73, 142.29, 142.20, 141.92, 141.77, 141.57, 140.34, 140.18, 139.55, 136.64, 136.41, 135.98, 135.81, 87.07, 71.00, 70.67, 70.36, 70.25, 69.55, 68.85, 68.53, 68.38, 67.86, 67.67, 67.32, 67.20, 53.51, 39.37, 36.96, 30.83, 29.64, 29.47, 29.30, 29.23, 28.94, 28.79, 25.86; IR-DRIFT cm^{-1} :3430, 2920, 2850, 1734, 1540, 1425, 1340, 1102, 526, 477. UV-vis (toluene) l_{max} nm: 704, 430, 330, 310. HRMS (ESI) calc for $C_{90}H_{46}FeN_2O_4S$ [M+Na]+: 1329.2421; found: 1329.2333.

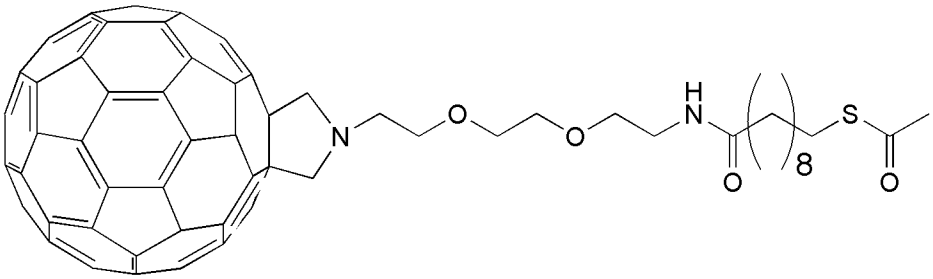
Synthesis of compound **7 (C_{60}FeSH)**



The synthesis of compound **7** (**C_{60}FeSH)** was performed following literature (Fibbioli M., *et al*., 2000) and the procedure as follow. A solution of **5** (0,081

mmol, 100 mg) dissolved in distilled CH_2Cl_2 (15 ml): MeOH (4 ml), was stirred at r.t under Ar; to that solution AcCl (3,6 mmol, 250 µl) was added drop wise at 0° C and the mixture let stirring at r.t. overnight under Ar. The reaction was stopped by drop wise addition of Pyridine (2,5 mmol, 200 µl) under stirring and Ar; the mixture was then dry under vacuum, the residue dissolved in 10 ml of CH_2Cl_2 and washed three time with water. The organic phase was then concentrated, stirred with $MgSO_4$, filtered, and the organic phase dry under vacuum. The residue was dissolved in a minimal amount of $CHCl_3$ to be subjected to chromatography column on fine Silica using Toluene 8: EtoAc 2 as the eluent; the residue was dissolved in a minimal amount of CH_3Cl_3 , precipitated with cyclohexane and then triturated with pentane to afford desired compound (0,032 mmol, 40 mg, 40% yield) as a deep red solid. ^{1}H-NMR 1H NMR (400 MHz, CDCl3) δ 5.93 (bs, 1H), 5.11 (mult., 2H), 5.03 (s, 1H), 4.58 (s, 1H), 4.53 (mult., 1H), 4.32 (s, 5H), 4.27-4.19 (m, 4H), 3.99 (mult., 1H), 3.91 (mult., 1H), 3.80 (t, J = 4.56 Hz, 2H), 3.65 (t, J = 5.04 Hz, 2H), 3.49 (t, J = 5.28 Hz, 2H), 3.18 (t, J = 5.96 Hz, 2H), 2.86 (t, J = 7.32 Hz, 2H), 2.13 (t, J = 7.76 Hz, 2H), 1.58 (m, 4H), 1.27 (t, 10H). 13C NMR (100 MHz, CDCl3) δ 196.23, 173.28, 156.44, 156.12, 154.53, 153.95, 153.49, 147.68, 147.41, 147.30, 146.39, 146.28, 146.07, 145.61, 145.38, 145.29, 144.56, 143.15, 142.73, 142.29, 142.20, 141.92, 141.77, 141.57, 140.34, 140.18, 139.55, 136.64, 136.41, 135.98, 135.81, 87.07, 71.00, 70.67, 70.36, 70.25, 69.55, 68.85, 68.53, 68.38, 67.86, 67.67, 67.32, 67.20, 53.51, 39.37, 36.96, 30.83, 29.64, 29.47, 29.30, 28.94, 28.79, 25.86. IR-DRIFT: cm^{-1} 3295, 3081, 2919, 2360, 1651, 1539, 1461, 1105, 818, 769, 526, 477. UV-vis (toluene) l_{max} nm: 710, 430, 345, 315, 285. ESI-MS: *m/z* 1264.24 (MH^+).

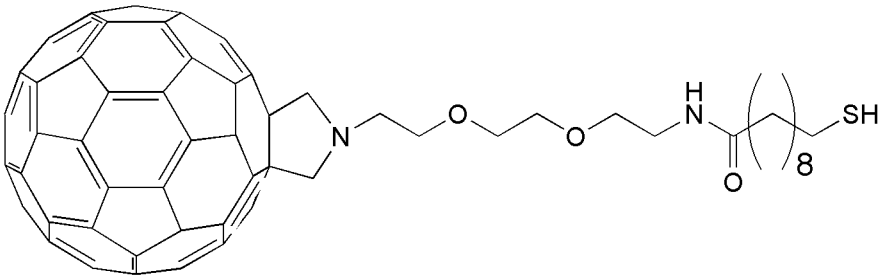
Synthesis of compound **4 (C_{60}S)**



The synthesis of compound **4(C_{60}S)** was performed according to literature [2], and like reported below. The fullerene mono deprotected derivative **2(C_{60}+)** used for the follow reactions have been previously synthesized and fully characterized according to literature (Kordatos K., *et al*., 2001). A mixture containing (0,10 mmol, 25 mg), HOBT (0,20 mmol, 27 mg) and EDC.HCl (0,20 mmol, 38 mg) in 5 ml of distilled CH_2Cl_2, was stirred at r.t. under Ar for 30;to that a solution of **2** (0,10 mmol, 100 mg) and DIEA (0,48 mmol, 90 µl) dissolved in 5 ml of dry CH_2Cl_2 was added dropwise; the resulting mixture was stirred overnight under Ar at r.t. and protected from light. The reaction was stopped by evaporating the solvent under vacuum and the residue dissolved in a minimal amount of $CHCl_3$ to be subjected to a chromatography column on fine Silica, using Toluene 3: EtoAC 7 as the eluent; subsequently the residue was dissolved in a minimal amount of CH_3Cl_3, precipitated by using cyclohexane, subsequently triturated with pentane and dried under vacuum to afford the desired compound (0,48 mmol, 70 mg, 55% yield) as a red brown solid. 1H NMR (400 MHz, CDCl3) δ6.00 (bs, 1H), 4.52 (s, 4H), 4.07 (t, J = 5.52 Hz, 2H), 3.84 (m, 2H), 3.75 (m,2H), 3.63 (t, J = 6.68 Hz, 2H), 3.47 (t, J = 3.16 Hz, 2H), 3.38 (t, J = 4.00 Hz, 2H), 2.85 (t, J = 7.32 Hz, 2H), (s, 3H), 2.18 (t, J = 7.56 Hz, 2H), 1.56 (m, 4H), 1.24 (m, 10H). 13C-NMR (400 MHz, $CDCl_3$):196.65, 173.64, 155.52, 147.86, 146.80, 146.55, 142.11, 142.62, 142.45, 71.08, 70.91, 69.11, 54.87, 39.76, 39.54, 37.39, 29.69, 29.62. IR-DRIFT: cm^{-1} 3435, 2919, 2849, 2361, 1685, 1541, 1420, 1339, 1101, 951,

767, 668, 562, 477. UV-vis (toluene) l_{max} nm: 710, 440, 340, 330, 294. ESI-MS: *m/z* 1122(MH^+).

Synthesis of compound **6 (C_{60}SH)**



The synthesis of compound **6 (C_{60}SH)** was performed according to literature (Fibbioli M., *et al*., 2000) and the procedure as reported below.

A solution of **4 (C_{60}S)** (0,12 mmol, 130 mg) dissolved in distilled CH_2Cl_2 (15 ml): MeOH (5 ml), was stirred at r.t. under Ar; to that solution AcCl (3,6 mmol, 250 µ l) was added drop wise at 0° C and the mixture let stirring at r.t. overnight under Ar. The reaction was stopped by drop wise addition of Pyridine (2,5 mmol, 200 µ l) under stirring and Ar; the mixture was then dry under vacuum, the residue dissolved in 10 ml of $CHCl_3$ and washed three time with water.

The organic phase was then concentrated, stirred with $MgSO_4$, filtered, and the organic phase dry under vacuum. The residue was dissolved in a minimal amount of $CHCl_3$ to be subjected to chromatography column on fine Silica using Toluene 8: EtoAc 2 as the eluent; the residue was then dissolved in a minimal amount of CH_3Cl_3 precipitated with cyclohexane and subsequently triturated with pentane and the residue dried under vacuum to afford desired compound (0,070 mmol, 80 mg, 60% yield) as a brown solid. 1H NMR (400

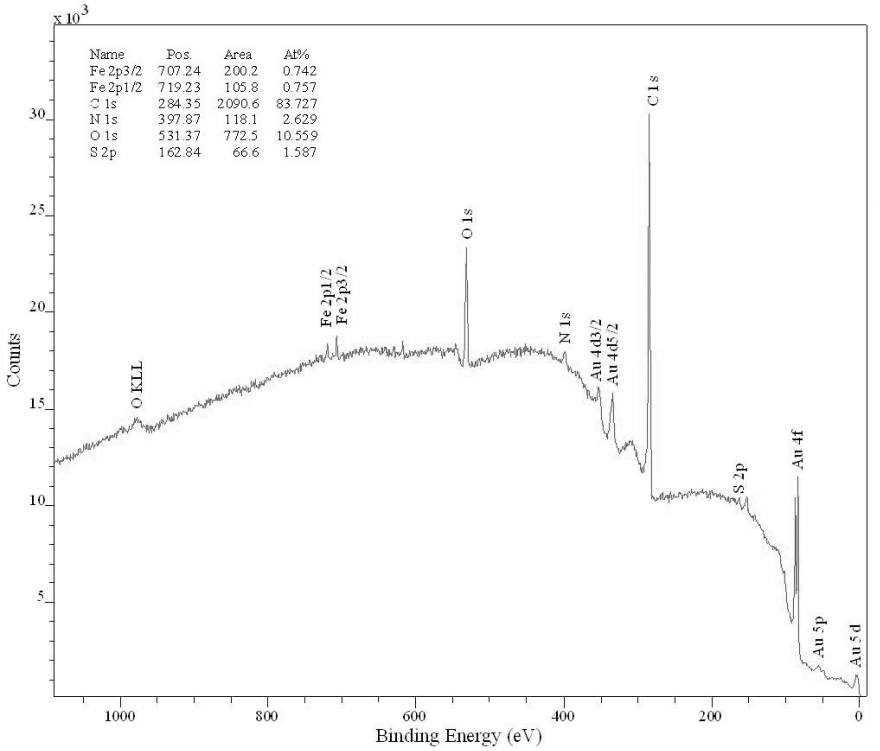
MHz, CDCl3) δ6.00 (bs, 1H), 4.52 (s, 4H), 4.07 (t, J = 5.52 Hz, 2H), 3.84 (m, 2H), 3.75 (m, 2H), 3.63 (t, J = 6.68 Hz, 2H), 3.47 (t, J = 3.16 Hz, 2H), 3.38 (t, J = 4.00 Hz, 2H), 2.85 (t, J = 7.32 Hz, 2H), 2.18 (t, J = 7.56 Hz, 2H), 1.56 (m, 4H), 1.24 (m, 10H). 13C-NMR (400 MHz, $CDCl_3$): 196.65, 173.64, 155.52, 147.86, 146.80, 146.55, 142.11, 142.62, 142.45, 71.08, 70.91, 69.11, 54.87, 39.76, 39.54, 37.39, 29.88, 29.69, 29.62. IR-DRIFT: cm^{-1} 3288, 2921, 2358, 1644, 1538, 1427, 1341, 1230, 1103, 767, 526, 477. UV-vis (toluene) l_{max} nm: 710, 436, 340, 320, 280. ESI-MS: *m/z* 1080.24 (MH^+)

13.2 Preparation of [60]fullerene modified gold surfaces

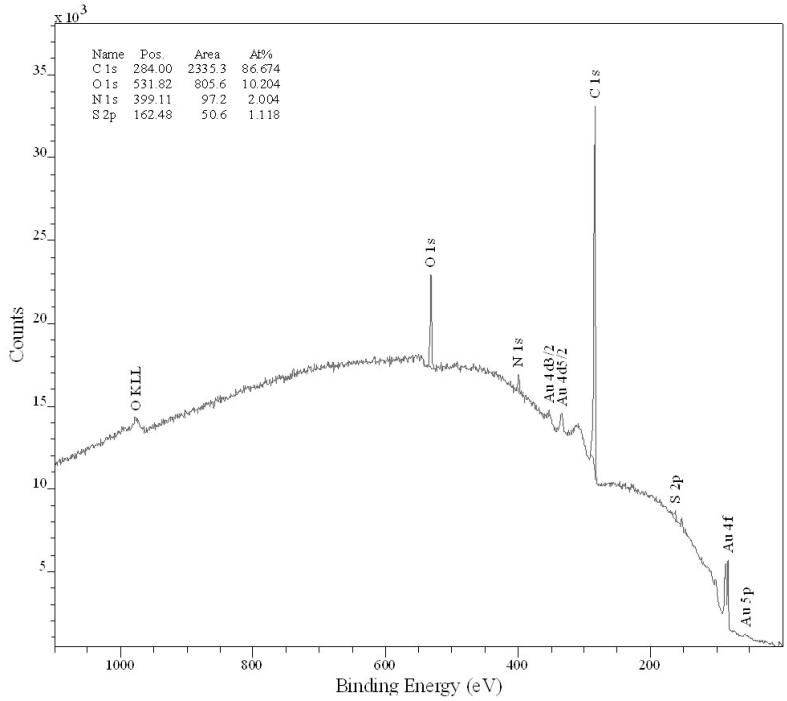
Preparation of fullerene derived gold surfaces was performed by using the LB(Langmuir-Blodgett) method for the formation of thiol monolayers on gold according to literature (Jordan C.E., *et al*. 1994) Gold surfaces of Au(111) 200nm thickness on mica support using in these experiments were purchased at Phasis (Switzerland) and stored at -20°C until use. Before the deposition of fullerene derivatives, gold surfaces were subjected to U.V light and extensively washed with water, ethanol, conditioned with dry CH_2Cl_2 and dried under Ar flux to avoid impurities. Desired modified gold surfaces (SAM's) were obtained by overnight immersion of cleaned gold surfaces into a 1mM dry CH_2Cl_2 solution of the fullerenes alkanethiols monoderivative **7**, **6,** and 1-dodecanethiol derivative used as reference substrate; all the deposition was performed under dark condition. After overnight deposition the obtained surfaces were then extensively washed with dry CH_2Cl_2 and CH_3Cl_3 to avoid the un-reacted fullerene derivatives and impurities. The resulting surfaces were then characterized by using Water Contact angle and XPS techniques and the characterizations are reported below. XPS spectra was recorded on ESCA spectrometer SSX 100 model; water contact angle measurements was performed on a Dataphysic camera using SCA20 as caption software.

XPS analysis of [60]fullerenes Au(111) modified surfaces

XPS measurements for C_{60}FeSH derived surface



XPS measurements for C_{60}SH derived surfaces



Water contact angle measurements of [60]fullerene modified Au(111) surfaces

Angle q values	BLANK	$C_{60}FeSH$	$C_{60}SH$	$CH_3(CH_2)_{11}SH$
	37,7 41,6 37,6 42,0 34,0 41,2 38,5	83,6 83,3 83,5 87,7 83,7	76,9 81,4 78,1 78,3 72,6 75,2	99,5 101,3 100 99,8 99,7
Average angle	38,94	84,36	77,08	100,06
Counts	7	5	6	5
STDEV	2,66	1,68	2,73	0,64

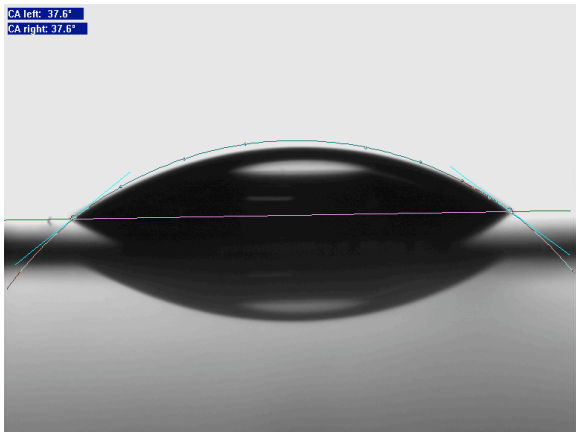


Photo of water drop formation on clean gold surface (BLANK)

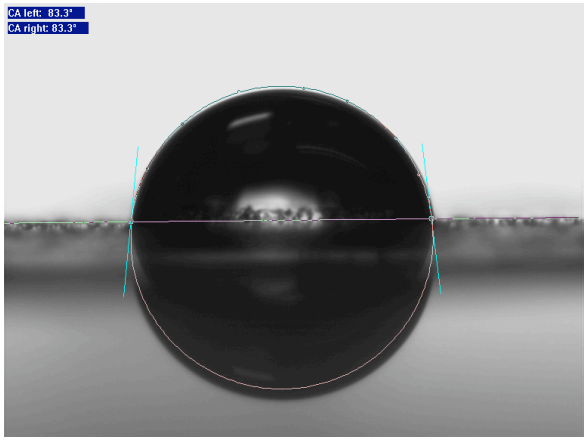


Photo of water drop formation on C_{60}FeSH Modified surface

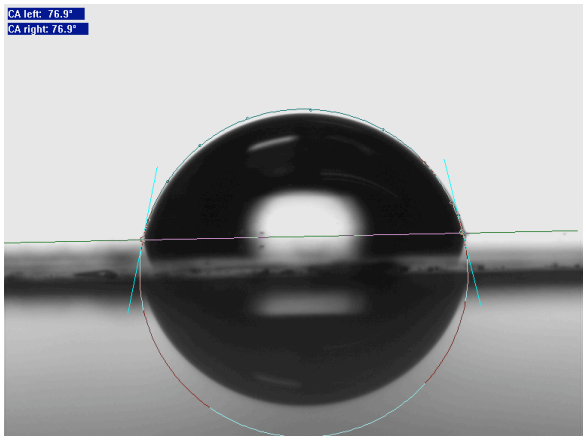


Photo of water drop formation on C_{60}SH modified gold surface

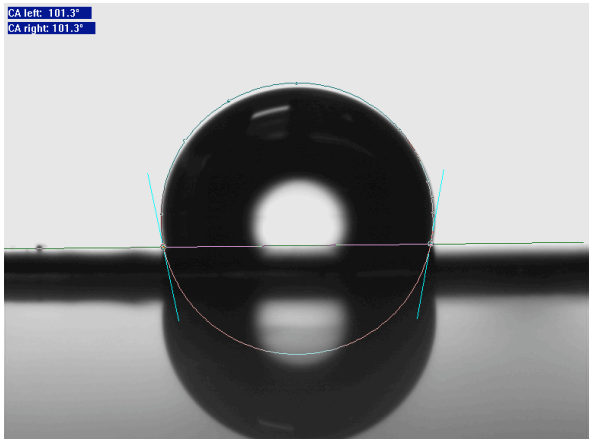


Photo of water drop formation on $CH_3(CH)_{11}$SH modified gold surface

14. Cells viability using MTT reduction assay on gold modified substrates

Cell viability was determined using an in vitro toxicology assay kit based on the reduction of 3-[4,5-dimetylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) purchased from Sigma (Madrid, Spain), according to literature (Mosmann T., 1993). Briefly, SH-SY5Y neuroblastoma cells were seeded at $2 \cdot 10^5$ cells per well in 24 well plates containing modified gold surfaces. At the end of 6h 100 μM L-Glu treatment in 250μl of complete medium, then 25 μl of MTT solution (5 mg/mL) was added and incubated at 37ºC for 3 h. After incubation 250 μl of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to the wells to dissolve formazan crystals. That suspension from each substrate was thus well homogenised by pipetting the entire volume, each volume of sample was then divided in 5 fraction each of 100μl and transferred to a 96 plate wells to be reading on a spectrophotometer avoiding the opacity of gold substrates. The plates were then thoroughly shaken and the absorbance of each well was measured at 570 nm.

15. SH-Sy5y cells growth on substrates

Selected fullerenes modified gold substrates for cells growth were stored in protected and sterile atmosphere at 4°C until use. Before cells seeding, selected modified substrates were extensively washed with PBS (phosphate saline buffer 1M) and conditioned for 30 min with complete cells growth medium at 37°C. SH-SY5Y cells were then seeded at a final concentration of 200.000 cells on the gold substrates in a 24 wells plaque. The cells were let grown for 48h hours on modified surfaces before fixation for SEM imaging and prior L-Glutamate treatment. The differentiation of SH-Sy5y on gold substrates was performed as reported in literature (Constantinescu *et al*.,

2007). The fixation of cells for SEM imaging was performed as reported in literature (Liljie O. and Armati P.J., 1995). SEM images were recorded on a Philips XL30 Scanning Elecetron Microscopy model.

RESULTS

RESULTS

1.1 Receptors endogenously expressed by SK-N-MC

The first part of the present work has been focused on the characterization and investigation of the receptors endogenously expressed by SK-N-MC cells derived from human neuroblastoma which we used as a cell model in the study of excitotoxicity and neurodegenerative processes. First, it was analyzed the gene expression of adenosine and glutamate receptors by using real time RT-PCR. Results showed the presence of adenosine receptors type A_{2A}, A_{2B}, and metabotropic glutamate receptors type $mGlu_1$, $mGlu_2$, $mGlu_3$ and $mGlu_5$; The presence of the effectors system AC-I and PLC-β_1 (fig. 1) has been also detected. Under the conditions described in *Materials and Methods* for the real time RT-PCR asay, we were not able to evaluate the expression of genes for adenosine receptors type A_1 and A_3, even testing different primers sequences (TaqMan Expression Assays) for A_1.

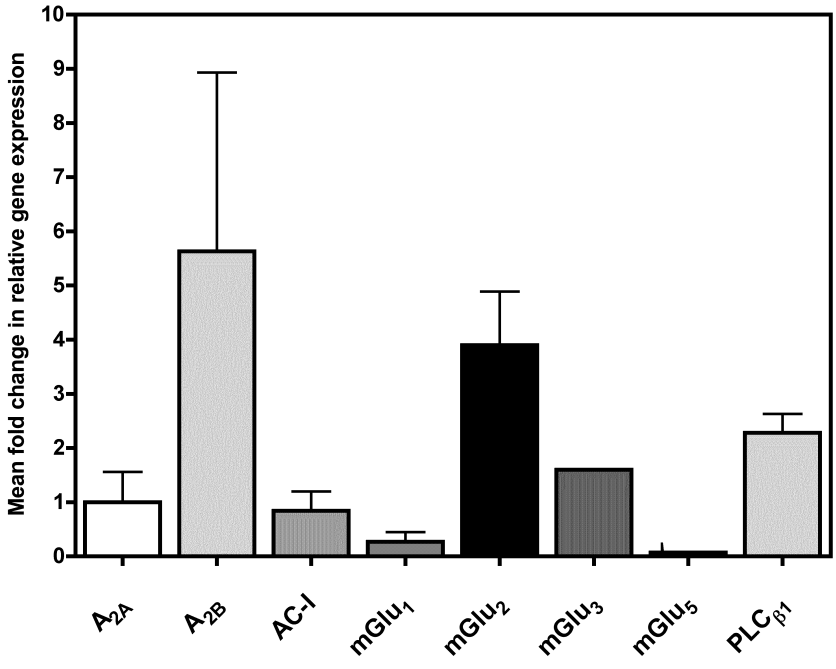


Figure 1. **Detection of genes relative to the expression of adenosine, metabotropic glutamate receptors and effectors system endogenously expressed in SK-N-MC human neuroblastoma cells.** The detection was performed as three independent assays, by RT-PCR technique on entire SK-N-MC cells at 48h of growth and by using TaqMan Expression assays as described in *Materials and Methods*.

The presence of adenosine receptors was confirmed by Western Blotting assay (fig. 2).

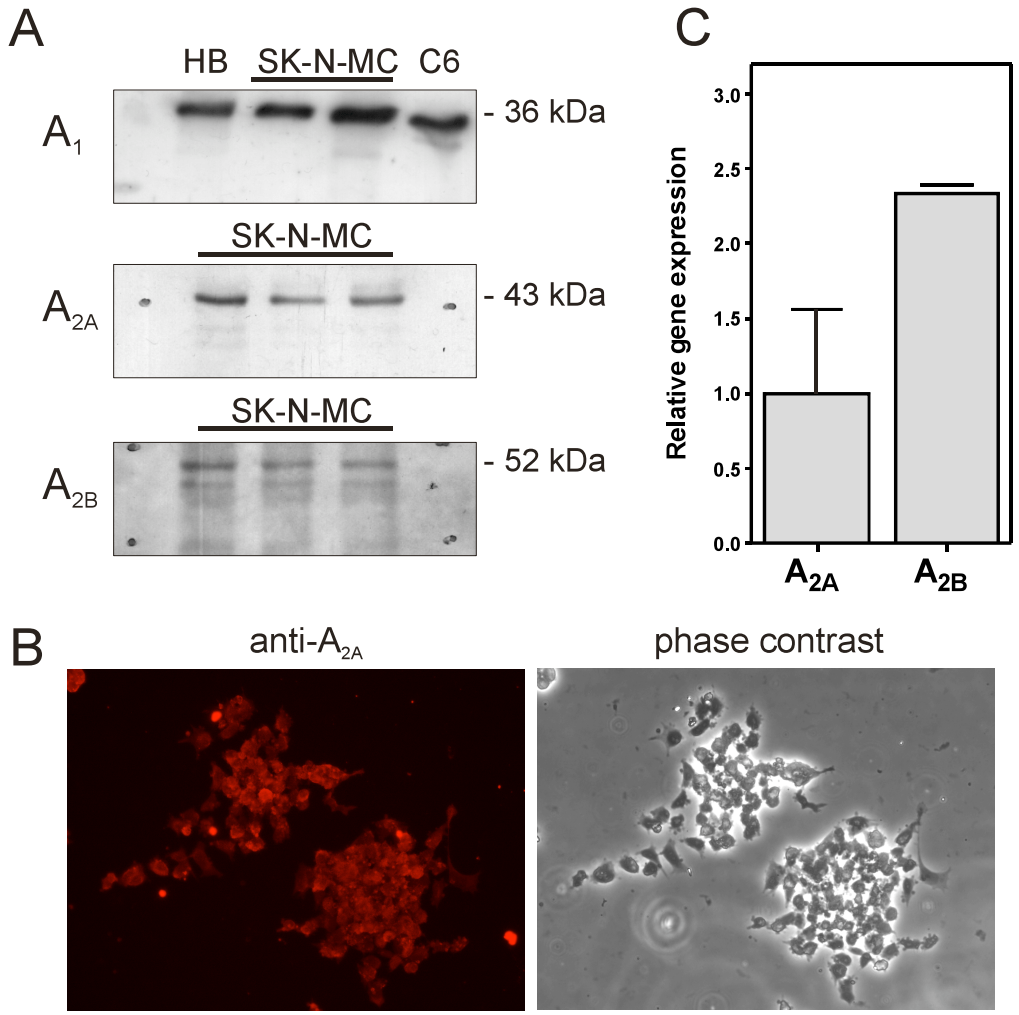


Figure 2. **Adenosine receptors presence in SK-N-MC cells**. Panel A: Adenosine receptors A_1, A_{2A} and A_{2B} were specifically detected by Western-blotting, as stated in *Materials and Methods* section, in plasma membranes from human brain (HB), C6 glioma cells (C6) and SK-N-MC cells (SK-N-MC). Panel B: a representative image showing A_{2A} receptors in intact cells by immunocytochemistry. Panel C: relative expression levels of A_{2A} and A_{2B} receptors in SK-N-MC cells.

Furthermore, it was confirmed the presence of adenosine receptors type A_{2A} by radioligand binding assay using [^{3}H]ZM241385, a specific A_{2A} antagonist, as radioligand in intact cells and plasma membrane preparation (fig. 3).

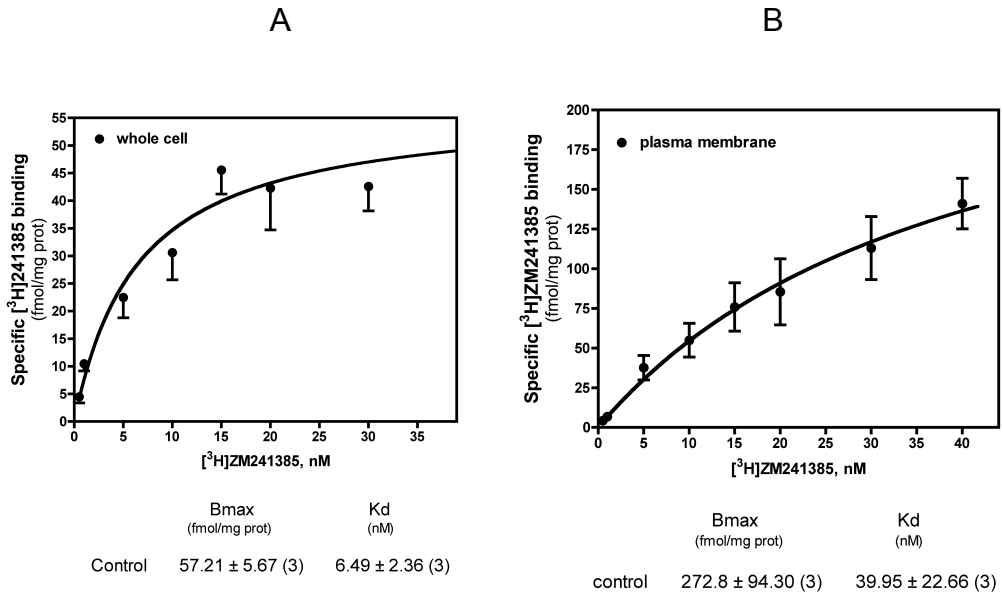


Figure 3.**Detection of A_{2A} receptors by radioligand binding assay.** Adenosine A_{2A} receptors were detected and quantified in whole cells (A) and plasma membrane (B) by using ZM241385 (A_{2A} antagonist) as the radioligand. Results herein presented are from three independent assay, on different cells cultives, each performed in duplicate. Data are mean ± SEM of three independent assays, performed in duplicate using cells at different passage number. The total receptor number (Bmax) and receptor affinity (Kd) are shown in the inset.

Similarly, presence of adenosine A_1 receptors protein observed by Western blotting was confirmed by radioligand binding assay using [^{3}H]DPCPX, specific A_1 antagonist, in intact cells (fig. 4).

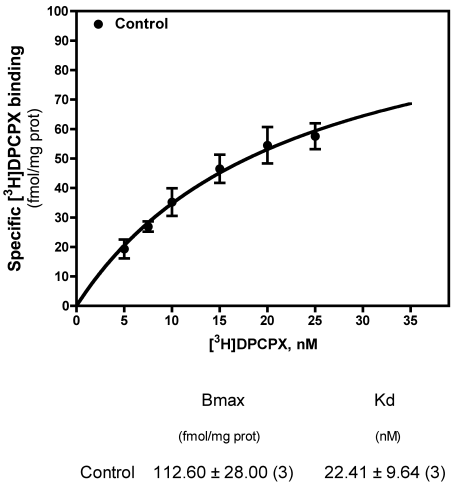


Figure 4. **Expression of A_1 receptors by Radioligand Binding assay of SK-N-MC in intact cells as controls.** Adenosine A_1 receptors were detected and quantified in whole cells by using DPCPX A_1 specific antagonist as the radioligand.

The evidence that adenosine A_1 receptors could be showed only by using radioligand binding and Western Blotting assay, and not by real time RT-PCR, means that probably genes coding for adenosine A_1 receptors are present at very low level or those tested TaqMan expression assays for A_1 gene were not valid for SK-N-MC cells.

1.2 Effect of amyloid-β peptide treatment on viability of SK-N-MC cells and the expression of adenosine and metabotropic glutamate receptors.

The amyloid-β peptide (fragment 25-35), used to create the condition of neurodegeneration usually present in Alzheimer's disease, showed a quite toxic effect on SK-N-MC cells like demonstrated the values analyzed and reported for treatments at 2 and 6 h at different concentrations (fig. 5).

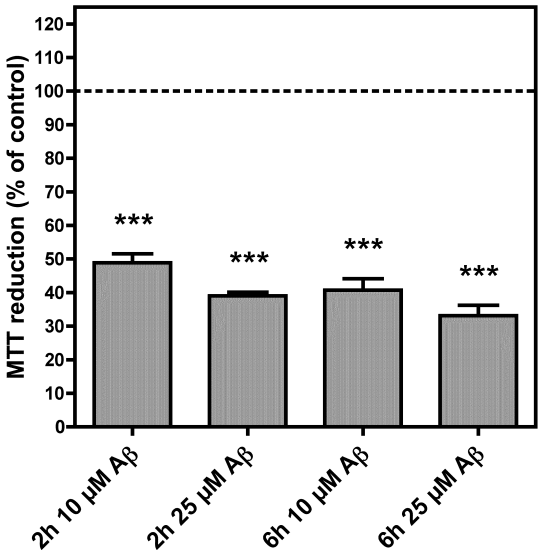
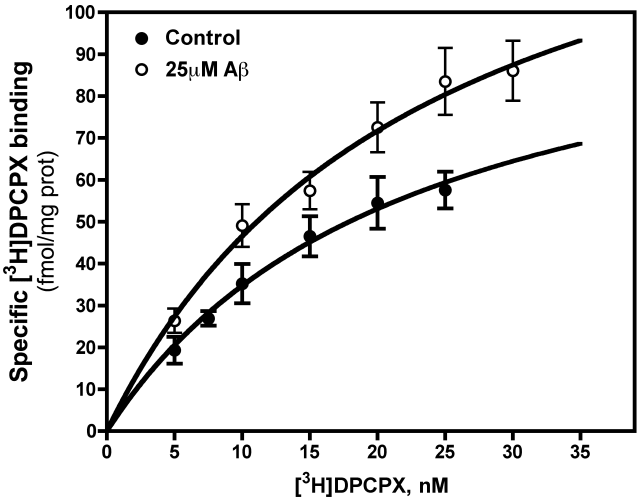


Figure 5. **Profile of viability evaluated by using MTT test reduction in SK-N-MC cells at different concentrations of amyloid-β peptide (Aβ) and exposure time.** The assay was performed by exposing SK-N-MC cells after 48 h growth, to amyloid-beta peptide for 2 and 6h. Values herein presented are from three days independent assays, and each value is the average of 8-10 values of absorbance from metabolized MTT, read on a spectrophotometer as described in *Material and Methods*. Thus values were analyzed by using the *software* GraphPad 5.0, and significance calculated respect 100% of survival (CTR). ***p<0,001 significantly different from control.

On the other hand, the same treatment with amyloid-β peptide demonstrated a slight but not significant increase in Bmax value for adenosine A_1 receptors measured in intact cells by radioligand binding assay. Not variations in Kd values were observed, meaning that the treatment didn't change the affinity of receptors with respect to the specific ligand (fig. 6).



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.60 ± 28.00 (3)	22.41 ± 9.64 (3)
Aβ	155.9 ± 29.53 (3)	23.47 ± 8.15 (3)

Figure 6. **Expression of adenosine A_1 receptors in SK-N-MC cells treated with amyloid β-peptide at 25μM and during 6h of treatment.** The assay were performed on entire SK-N-MC cells, after 48 h of growth and exposed for 6 h to amyloid-beta peptide (Aβ) 25μM. The specific A_1 antagonist DPCPX was used as the radioligand. Values of Bmax and Kd obtained are the average of three independent assays (treatment), performed in duplicate, and analyzed by using the *software* GraphPrisma 5.0.

Although we were unable to detect A_1 mRNA expression in control SK-N-MC cells, we decided to verify the expression of A_1 gene by real time RT-PCR in Aβ treated cells, where radioligand binding assay, as mentioned above, suggested an increase in A_1 protein level. Unfortunately A_1 gene expression was again indetectable. Anyway the same treatment with amyloid-β peptide showed opposite effect on adenosine receptors type A_{2A} and A_{2B} ,

producing a decrease and an increase in mRNA levels, respectively, as figure 7 shows (fig. 7a and 7b).

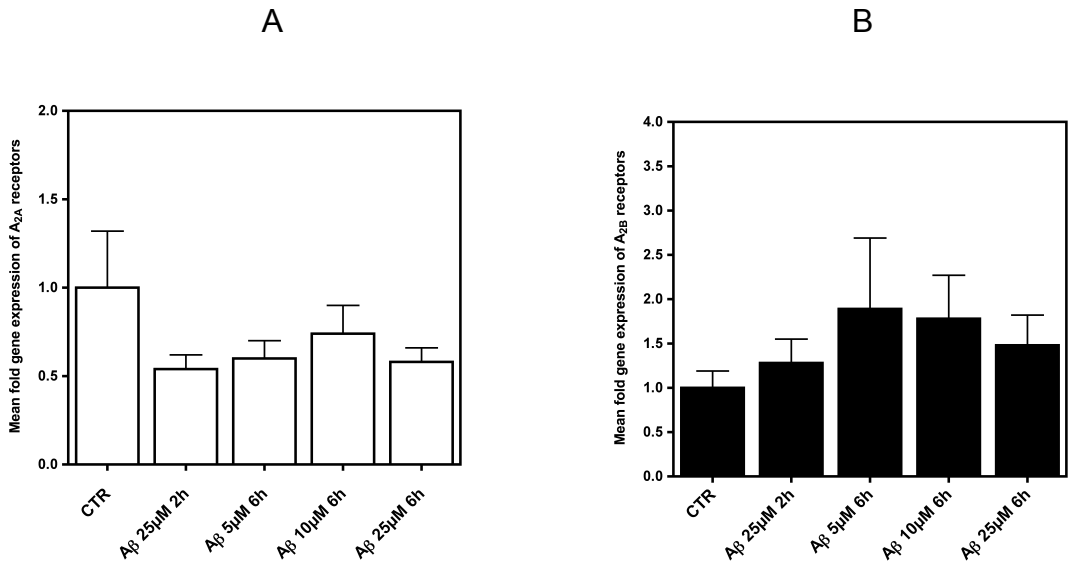


Figure 7. **Relative genes expression evaluated on SK-N-MC cells treated with amyloid-β peptide.** The expression of relative gene for A_{2A} (A) and A_{2B} (B) adenosine receptors, was obtained in control (CTR) SK-N-MC cells and exposed to 2-6 h treatment with amyloid-beta peptide (Aβ) 5-10-25μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0.

Although not significant, data about the expression of adenosine receptors showed a tendency of the amyloid beta peptide to decrease the expression of adenosine A_{2A} receptors and, on the other hand to increase the expression of adenosine A_{2B} receptors. Unfortunately it wasn't possible to confirm these data by using radioligand binding assay, due to a problem with compound used for the displacement of the labelled radioligand. The same treatment with amyloid beta peptide seems to have an effect on metabotropic glutamate receptors, showing a tendency to increase the expression of $mGlu_1$ and by significantly increasing the $mGlu_5$ expression, as showed by real time RT-PCR (fig. 8 and 9).

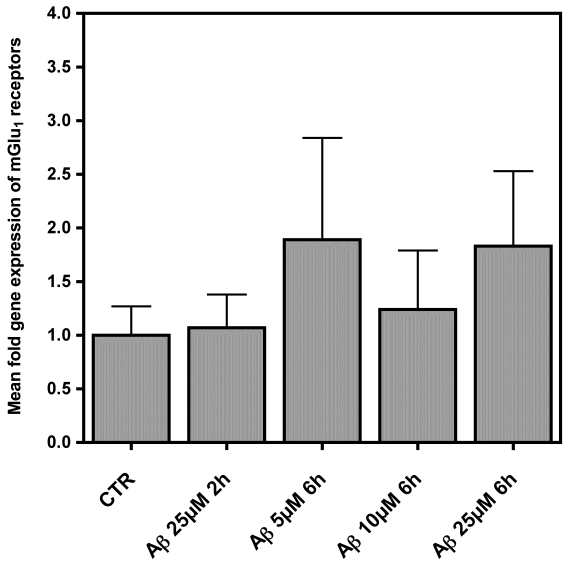


Figure 8. **Relative gene expression of $mGlu_1$ evaluated by real time RT-PCR in SK-N-MC cells treated with amyloid-beta peptide**. The expression of relative gene for $mGlu_1$ metabotropic glutamate receptors, was obtained in control (CTR) SK-N-MC cells and exposed to 2-6 h treatment with amyloid-beta peptide (Aβ) 5-10-25μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, and each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0.

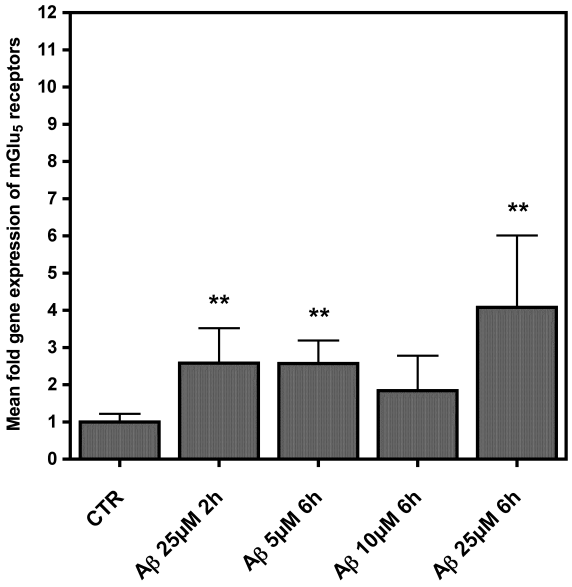


Figure 9. **Relative gene expression of $mGlu_5$ evaluated by real time RT-PCR in SK-N-MC cells treated with amyloid-beta peptide**. The expression of relative gene for $mGlu_5$ metabotropic glutamate receptors, was obtained in control (CTR) SK-N-MC cells and exposed to 2-6 h treatment with amyloid-beta peptide (Aβ) 5-10-25μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. **$p<0,01$ significantly different from control.

1.3 Effect of L-Glutamate treatment on viability and the expression of adenosine and metabotropic glutamate receptors.

The treatment at different time and concentration with L-glutamate in SK-N-MC cells showed results similar to those observed for the treatment with amyloid-beta peptide, inducing in most of conditions assayed more than a 50% of cell death (fig. 10 panel A-B).

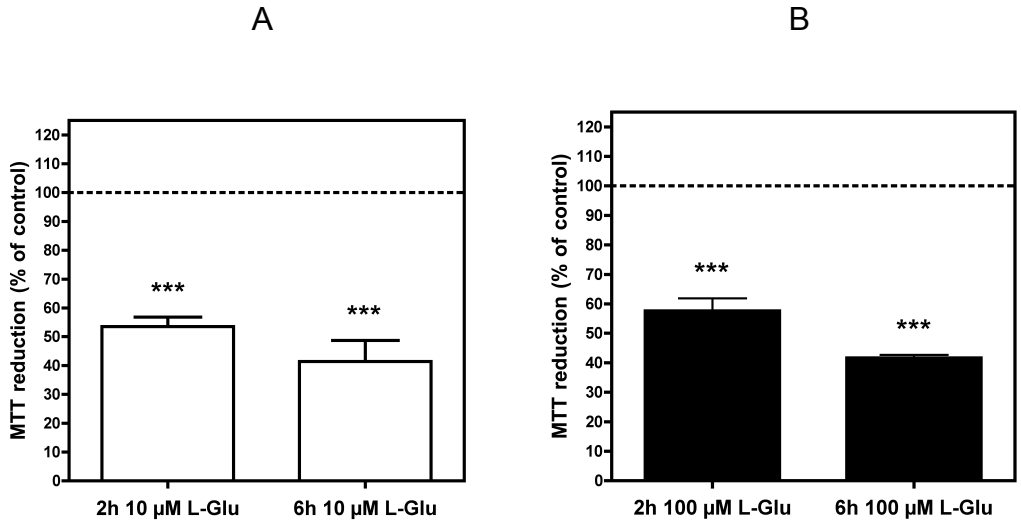
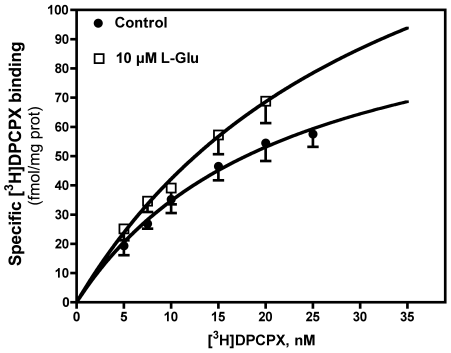


Figure 10. **Viability assay on SK-N-MC cells after exposure to L-Glutamate.** SK-N-MC cells after 48 h growth were exposed to L-Glutamate at 10μM (A) or 100μM (B) for 2 and 6 hours, and MTT reduction assay was performed as stated in *Methods* section. Values of cell survival herein presented, are the average from three days independent assay, and analyzed from at least 8-10 values of absorbance of metabolized MTT. Data were analyzed by using GaphPad 5.0 as the *software*, and significance considered respect to the 100% of cell survival obtained in control. *** $p<0,001$ significantly different from control.

These results suggest that the toxicity evoked by glutamate could be influenced by the time of exposition to that treatment more than the concentration used for the treatment. In fact, the effect of glutamate at short time of exposition (2h) induced a similar toxic response at 10 μM and 100 μM. Next we evaluated the receptors involved in L-Glutamate toxicity. The radioligand binding assay for adenosine A_1 receptors showed a tendency of the treatment with 10 μM L-Glu for 6h to increase the expression of receptors of intact cells (fig. 11a). A similar effect was observed by treating cells with L-

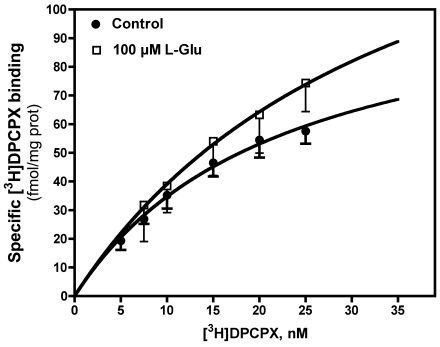
Glu 100 μM at 6h (fig.12b), confirming that glutamate even at low concentration, tend to increase the expression of adenosine A_1 receptors.

A



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.60 ± 28.00 (3)	22.41 ± 9.64 (3)
L-Glu	184.20 ± 86.65 (3)	33.67 ± 22.67 (3)

B



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.6 ± 28.00 (3)	22.41 ± 9.64 (3)
L-Glu	180.9 ± 72.14 (3)	36.24 ± 21.72 (3)

Figure 11. **Radioligand binding assay of adenosine A_1 receptors, on intact SK-N-MC cells after exposure to L-Glutamate for 6 h.** The radioligand binding assay was performed on intact SK-N-MC cells, after growth of 48 h, and exposed for 6h to L-Glutamate (L-Glu) at 10μM (A) and 100μM (B). For the assay, a specific radiolabelled antagonist DPCPX was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays (treatments), and each one obtained as duplicate. The data were then analyzed on GraphPad 5.0 *software*, and corresponding values obtained are reported below each graph.

The expression of mRNA for adenosine A_{2A} and A_{2B} receptors was also analyzed after glutamate treatment. Results obtained by treating cells for 6h with L-Glu at concentrations of 1, 10 and 100μM, suggest a slight but significant increase in the expression of adenosine A_{2A} receptors (fig 12). This effect was significantly higher in the expression level of adenosine A_{2B} receptors, as figure 13 shows.

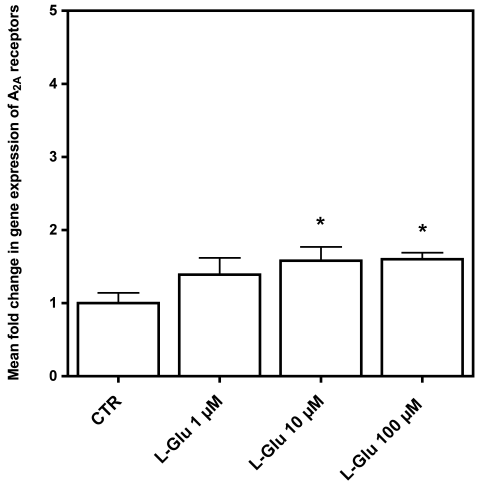


Figure 12. **Relative gene expression of adenosine A_{2A}receptors in SK-N-MC cells evaluated by RT-PCR technique.** The expression of relative gene for adenosine A_{2A} receptor, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with L-Glutamate (L-Glu) 1-10-100μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. *p<0,05 significantly different from control.

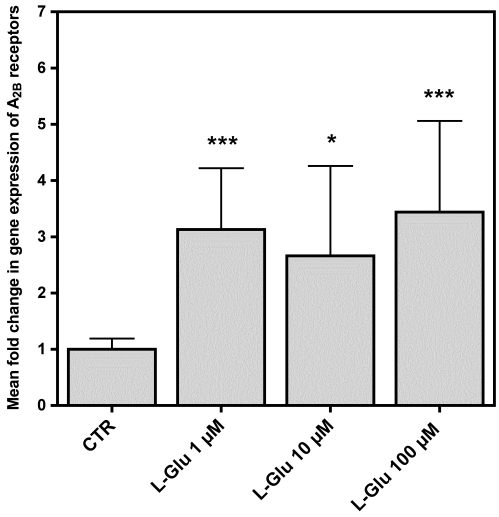
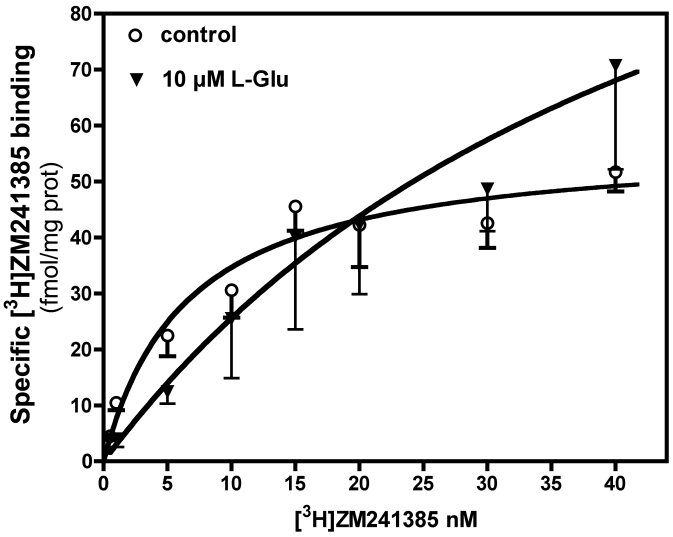


Figure 13. **Relative gene expression of adenosine A_{2B}receptors in SK-N-MC cells evaluated by RT-PCR technique**. The expression of relative gene for adenosine A_{2B} receptor, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with L-Glutamate (L-Glu) 1-10-100μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. *p<0,05 significantly different from control; ***p<0,001 significantly different from control.

Furthermore, adenosine A_{2A} receptors were quantified by using radioligand binding assay after 10 μM L-Glu for 6h, when we observed the most significant effect on real time RT-PCR assay. It's evident that glutamate promotes an increase in the total number of adenosine A_{2A} receptors, in agreement with the observed increased gene expression of transcripted mRNA fragments (fig. 14). At the same time we observed an increase in the Kd value, suggesting a lower receptor affinity after glutamate treatment.



	Bmax (fmol/mg prot)	Kd (nM)
Control	57.21 ± 5.67 (3)	6.49 ± 2.36 (3)
L-Glu	152.1 ± 61.18 (3)*	49.33 ± 31.56 (3)

Figure 14. **Radioligand binding assay of adenosine A_{2A} receptors performed on intact SK-N-MC cells with L-Glutamate.** The radioligand binding assay was performed on intact SK-N-MC cells, after growth of 48 h, and exposed for 6h to L-Glutamate (L-Glu) at 10μM. For the assay, a specific radiolabelled antagonist ZM241385 was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays (treatments), each one obtained as duplicate. The data were then analyzed on GraphPad ver. 5.0 *software*, and correspondent values obtained are reported below graph. *$p<0,05$ significantly different from control.

Gene expression was evaluated for metabotropic glutamate receptors type $mGlu_1$ (fig. 16) and $mGlu_5$ (fig. 17) A significant increase in the expression of both receptors was detected.

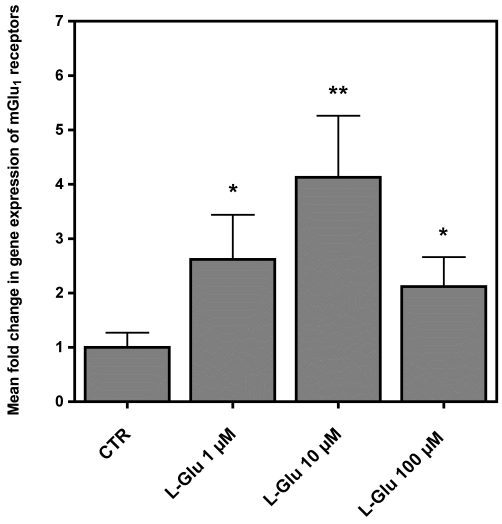


Figure 15. **Relative gene expression of metabotropic $mGlu_1$ glutamate receptors in entire SK-N-MC cells treated with L-Glutamate evaluated by RT-PCR technique**. The expression of relative gene for $mGlu_1$ metabotropic glutamate receptors, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with L-Glutamate (L-Glu) 1-10-100μM as showed in figure. Values of relative gene expression are the average of at least three independent experiments, conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. *p<0,05 significantly different from control; **p<0,01 significantly different from control.

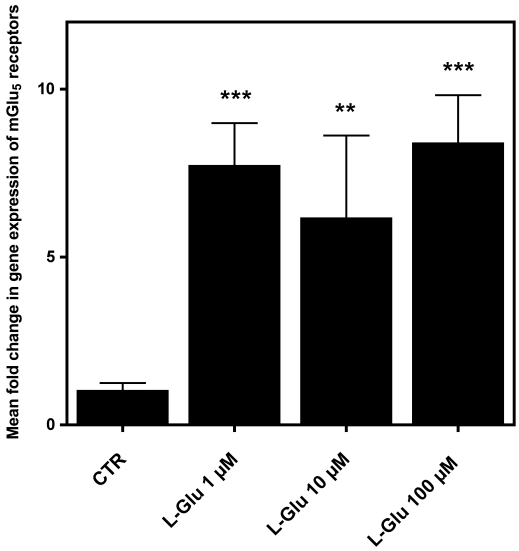
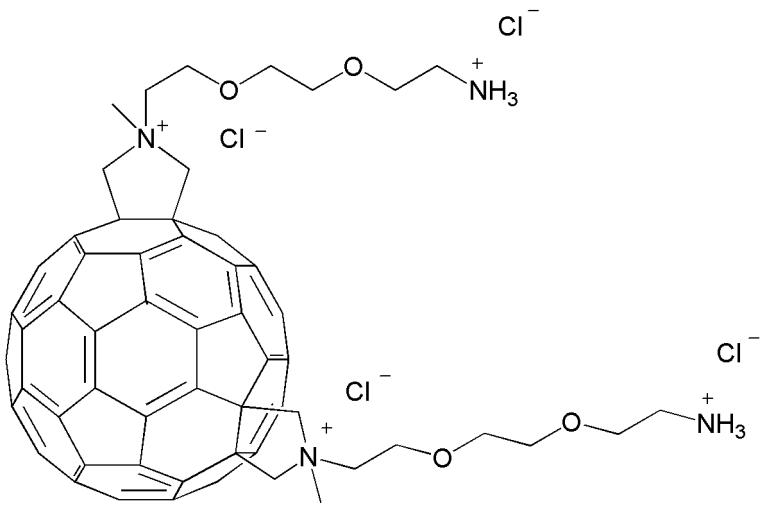


Figure 16. **Relative gene expression of metabotropic $mGlu_5$ glutamate receptors in entire SK-N-MC cells treated with L-Glutamate evaluated by RT-PCR technique**. The expression of relative gene for $mGlu_5$ metabotropic glutamate receptors, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with L-Glutamate (L-Glu) 1-10-

100μM as showed in figure. Values of relative gene expression are the average of at least three days independent experiments, and each conducted in duplicate. Data were analyzed by using the *software* GraphPad ver. 5.0. **p<0,01 significantly different from control; ***p<0,001 significantly different from control.

1.4 Treatment of SK-N-MC cells with a hydrosoluble form of [60]fullerene derivative.

In this section of the present work, the activity of a derived hydrosoluble form isomer of [60]fullerene has been investigated (t3ss, fig. 17), in cell viability and in the expression of adenosine and glutamate receptors.



[60]fullerene bis-adduct derivative *trans*-3 isomer

Figure 17. Schematic structure of hydrosoluble form of [60]fullerene bis-adduct *trans*-3 isomer used in the present work; for details on the synthetic route and biological applications *see* Bosi *et al.*, 2003.

The treatment of SK-N-MC cells with T3SS derivative showed a very low toxicity, except at the concentration of 1 mM, at which it was observed a small toxicity (fig.18). However concentration is out of the range of the acceptable useful concentration for a given treatment.

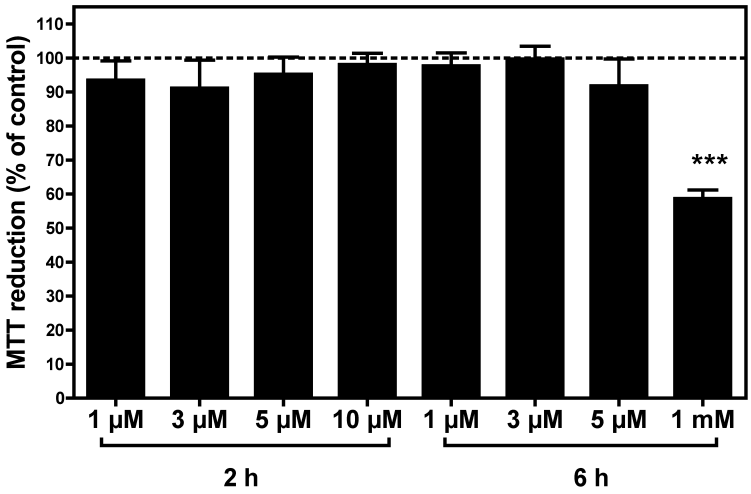
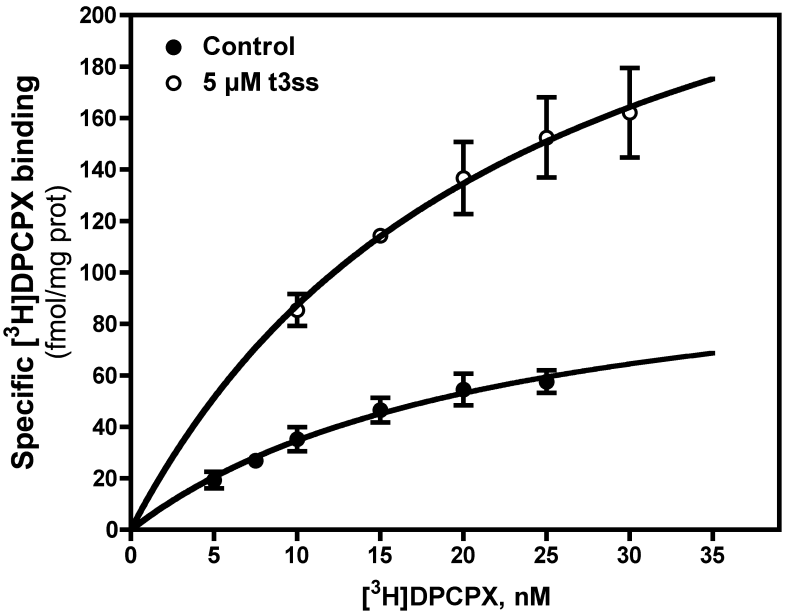


Figure 18. **Profile of SK-N-MC cells survival exposed to t3ss derivative.** The survival of SK-N-MC cells after exposure at indicated time and concentrations to t3ss derivative, has been performed by MTT reduction assay as described in *Methods*. The indicated values are the average from three independent of treatment, each one corresponding as the average of at least 8-10 values of metabolized MTT, read at the spectrophotometer, and analyzed by using GraphPad 5.0 *software*.***$p<0,001$ significantly different from control as 100% of cell survival.

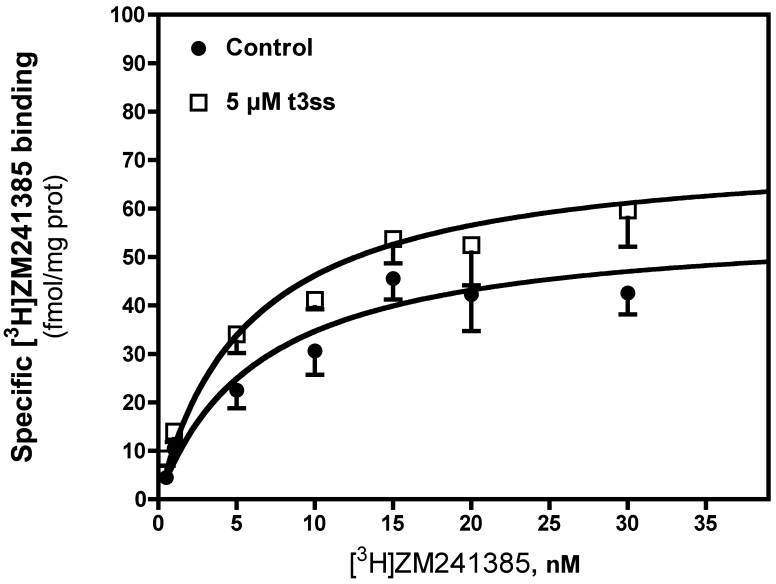
Treatment with 5 μM t3ss at 6 h seem to have effect on the expression of the adenosine A_1 receptors, as confirmed by radioligand binding assay, in which it was observed an important increase in Bmax values (fig. 19). Furthermore we again tried to detect in that condition the gene coding for adenosine A_1 receptors but it was impossible to detect by using real time RT-PCR, even by using different type of specific oligonucleotides (TaqMan probes), as mentioned before.



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.60 ± 28.00 (3)	22.41 ± 9.64 (3)
t3ss	293.70 ± 64.60 (3)*	23.63 ± 9.86 (3)

Figure 19. **Expression of adenosine A_1 receptors evaluated by using radioligand binding assay on entire SK-N-MC cells exposed to t3ss derivative.** The radioligand binding assay was performed on intact SK-N-MC cells, after growth of 48 h, and exposed for 6h to [60]fullerene hydrosoluble derivative (t3ss) at 5 μM. For the assay, a specific radiolabelled antagonist DPCPX was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays (treatments), each one obtained as duplicate. The data were then analyzed on GraphPad 5.0 *software*, and correspondent values obtained are reported below the graph. *p<0,05 significantly different from control.

The same treatment with 5 μM t3ss at 6 h showed to have effect, although with less relevancy, on the expression of adenosine A_{2A} receptors, like confirmed by the results of radioligand binding assay performed in intact cells (fig. 20). However, receptors affinity did not change after treatment as Kd value revealed.



	Bmax (fmol/mg prot)	Kd (nM)
Control	57.21 ± 5.67 (3)	6.49 ± 2.36 (3)
t3ss	72.95 ± 5.80 (3)	5.79 ± 1.75 (3)

Figure 20. **Expression of adenosine A_{2A} receptors evaluated by radioligand binding assay in entire SK-N-MC cells exposed to t3ss derivative.** The radioligand binding assay was performed on intact SK-N-MC cells, after growth of 48 h, and then exposed for 6h to [60]fullerene hydrosoluble derivative (t3ss) at 5 μM. For the assay, a specific radiolabelled antagonist ZM241385 was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays (treatments), each one obtained as duplicate. The data were then analyzed on GraphPad 5.0 *software*, and correspondent values obtained are reported below the graph.

The gene expression of adenosine receptors seems to be affected by the presence of t3ss in the same way as described before, although at greater level in the case of A_{2B} receptors. In any case, a generally tendency to an increased gene expression of adenosine A_{2A} receptors has been observed (fig. 21)

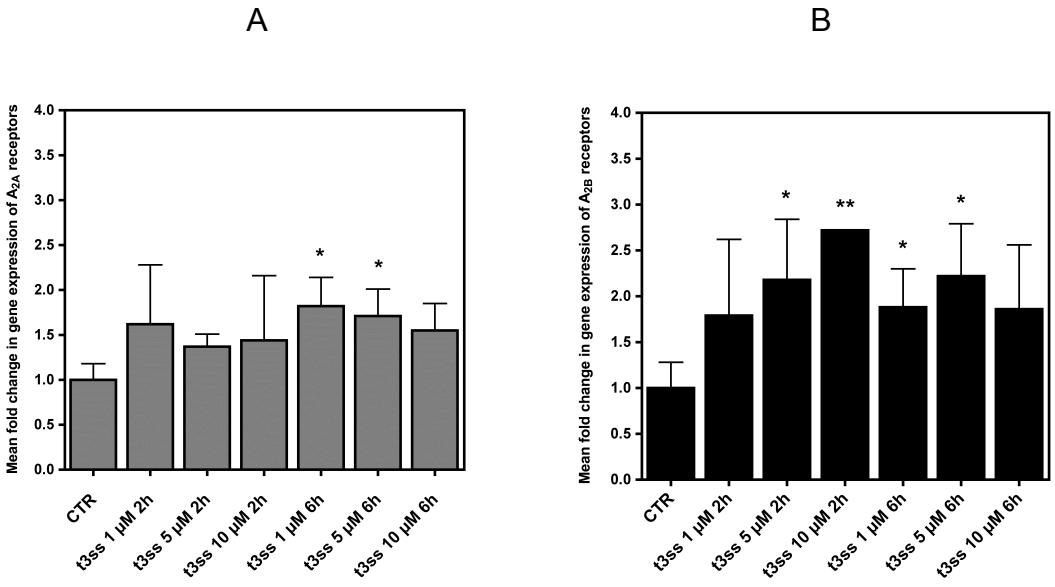


Figure 21. **Relative gene expression of adenosine A_{2A} and A_{2B} receptors in SK-N-MC cells exposed to t3ss derivative and evaluated by RT-PCR technique.** The expression of relative gene for adenosine A_{2A} (A) and A_{2B} (B) receptor, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with [60]fullerene hydrosoluble derivative (t3ss) at concentrations as showed in figures. Values of relative gene expression are the average of at least three independent experiments, and conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. *p<0,05 significantly different from control; **p<0,01 significantly different from control.

The expression of metabotropic glutamate receptors type $mGlu_1$ and $mGlu_5$ has been also evaluated during t3ss treatment at 6h. Results indicated a significant increase in gene expression of $mGlu_1$ and $mGlu_5$ (fig. 22). Surprisingly, this effect at 5 μM, was not observed at 1 and 10 μM.

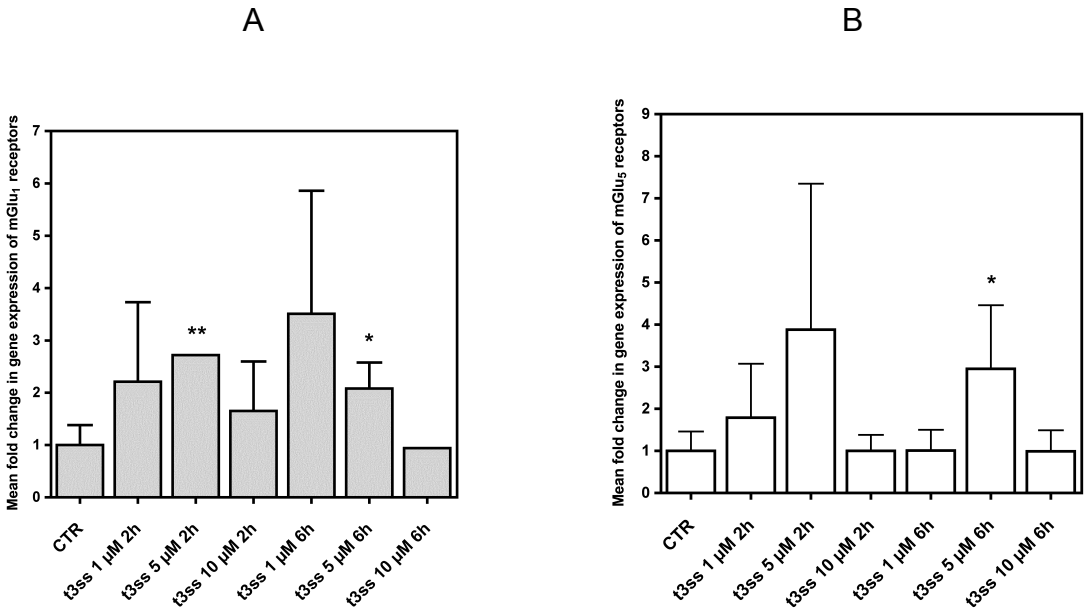


Figure 22. **Relative gene expression of metabotropic $mGlu_1$ and $mGlu_5$ glutamate receptors in SK-N-MC cells exposed to t3ss derivative evaluated by RT-PCR techique**. The expression of relative gene for metabotropic $mGlu_1$ (A) and $mGlu_5$ (B) glutamate receptor, was obtained in control (CTR) SK-N-MC cells and exposed to 6 h treatment with [60]fullerene hydrosoluble derivative (t3ss) at concentrations as showed in figures. Values of relative gene expression are the average of at least three independent experiments, each conducted in duplicate. Data were analyzed by using the *software* GraphPad 5.0. *$p<0,05$ significantly different from control; **$p<0,01$ significantly different from control.

1.5 Effect of the hydrosoluble [60]fullerene derivative t3ss on SK-N-MC cells under toxicity conditions induced by L-Glutamate and amyloid-β peptide.

In this part of the present work it was evaluated the activity of hydrosoluble [60]fullerene derivative t3ss on SK-N-MC cells treated at the same time with L-Glutamate and amyloid-β peptide in order to mimic toxicity conditions typical of neurodegenerative disease. Because of the low toxicity profile of [60]fullerene derivative on SK-N-MC cells and the ability to modulate some receptors involved in neurotoxicity (i.e. adenosine and mGlu receptors), we decided to test its activity under induced "stress" conditions. First we tested the SK-N-MC cells survival in the presence of a toxic stimulus alone or together with the contemporary presence of t3ss at the established non-toxic concentration of 5 μM. Apart from the lack of toxicity, this concentration of t3ss we decided to use for this experimental part, has been demonstrated to

be effective on the modulation of adenosine and metabotropic glutamate receptors. Results of contemporary treatment of SK-N-MC cells with 10-100 μM L-Glu and 5 μM t3ss suggested a protective effect for t3ss due to the observed recovery profile in viability of cells at different time of treatment (fig. 23). The fact that the recovery of cells viability exhibited at 2h of treatment with 100 μM L-Glutamate is less evident (fig. 23b), could be related to the short time of action of t3ss on cells, which would be not enough to evoke a relevant protective effect. This is confirmed for the same treatment with L-Glu at 6h in which the recovery we observed with the contemporary presence of 5 μM t3ss, was significantly higher, against a strong toxic effect elicited by 100 μM L-Glutamate (fig. 23a).

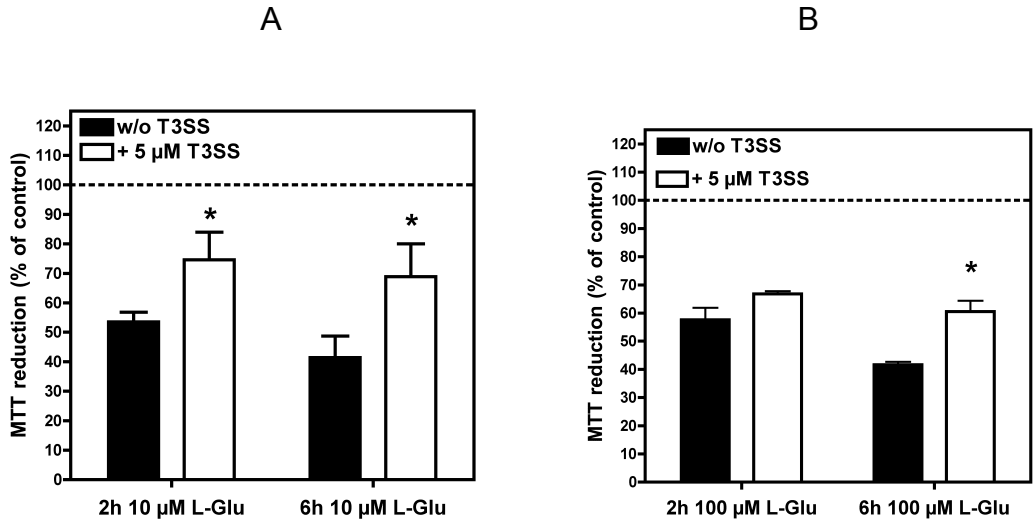


Figure 23. **Survival of SK-N-MC cells exposed to L-Glutamate and t3ss derivative**. After 48 h growth SK-M-NC cells were exposed to L-Glutamate (L-Glu) 10 μM (A) and 100 μM (B), and together with 5μM t3ss derivative for 2-6 h as showed in figures. Values of cells survival have been obtained by MTT reduction assay, from independent experiment and each by analyzing at least 8-10 absorbance values of metabolized MTT as described in *Methods*. Data were analyzed by using GraphPad *software*. *$p<0,05$ significantly different from cell survival in presence of L-Glutamate alone.

The data obtained by treating SK-N-MC cells with amyloid-β peptide in the presence or the absence of 5 μM t3ss suggested a similar protective effect to that observed for L-Glu treatment. The amyloid treatment at 10 and 25 μM

showed high toxicity at different time assayed, causing high mortality in SK-N-MC cells; at the same time the contemporary treatment with 5 μM t3ss showed an effective recovery from amyloid induced toxicity in cells viability (fig. 24), thus confirming a protective effect by t3ss.

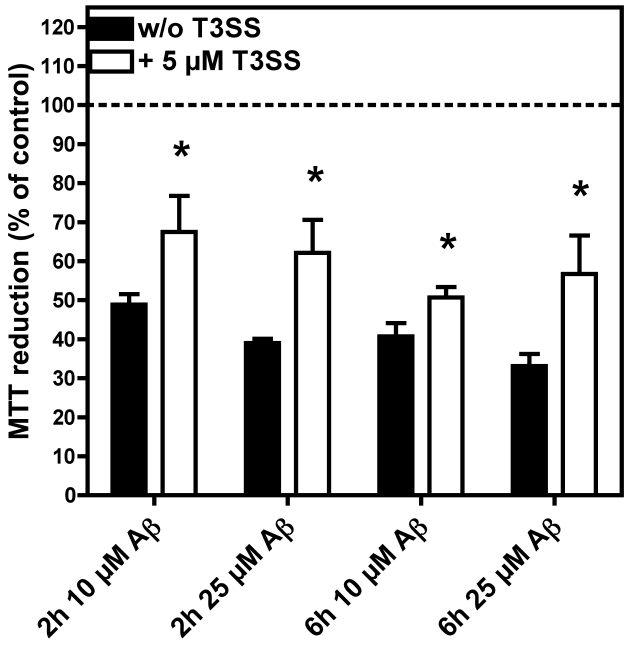
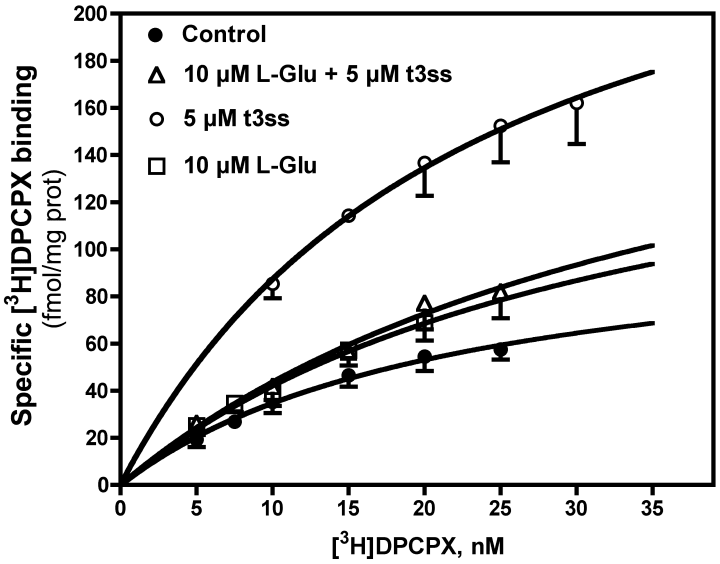


Figure 24. **Survival of SK-N-MC cells exposed to amyloid β-peptide together t3ss evaluated by MTT reduction assay**. SK-N-MC cells 48 h growth were exposed to amyloid-β peptide (Aβ) 10-25 μM and together with 5 μM t3ss derivative (t3ss) for 2-6 h of treatment. Values of cells survival have been obtained by MTT reduction assay, after three days independent experiment (treatment) and each by analyzing at least 8-10 absorbance values of metabolized MTT as described in *Methods*. Data were thus analyzed by using GraphPad ver. 5.0 *software*. *$p<0,05$ significantly different from cell survival in presence of L-Glutamate alone.

Subsequently to the observed protective effect by t3ss on cell viability in the presence of a toxic treatment, we followed by evaluating adenosine A_1 receptors by radioligand binding assay , in order to understand if that effect of t3ss in the presence of L-Glutamate or amyloid-β peptide could have some consequence in the expression of this class of receptors. The data from radioligand binding assay of adenosine A_1 receptors after treating intact cells with 10 μM L-Glutamate at 6 h in the presence or the absence of 5μM t3ss showed a slight increase in the total number of receptors after 10 μM Glu treatment. Furthermore the contemporary use of t3ss seems to potentiate the

increase of adenosine receptors respect to cells treated alone with L-Glutamate (fig. 25).

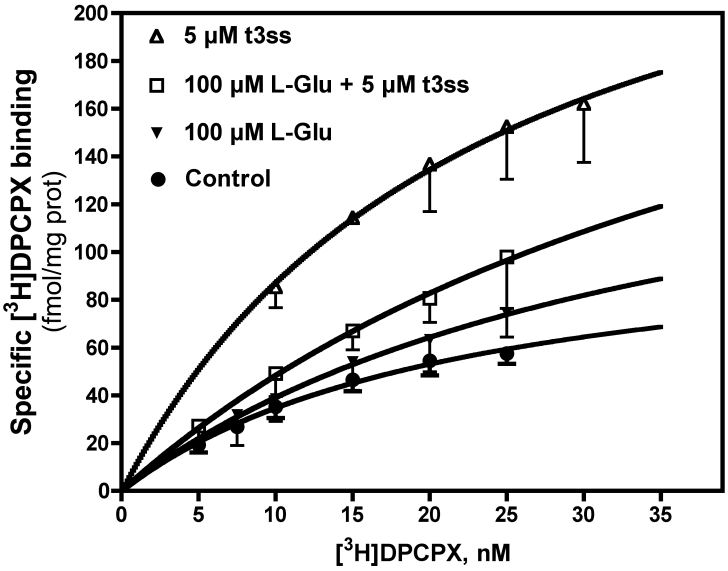


	Bmax (fmol/mg prot)	Kd (nM)
Control	112.60 ± 28.00 (3)	22.41 ± 9.64 (3)
t3ss	293.70 ± 64.60 (3)*	23.63 ± 9.86 (3)
L-Glu	184.20 ± 86.65 (3)	33.67 ± 22.67 (3)
L-Glu + t3ss	217.30 ± 111.80 (3)	39.67 ± 30.13 (3)

Figure 25. **Radioligand binding assay of adenosine A_1 receptors, in intacted SK-N-MC cells exposed to L-Glutamate and together with t3ss derivative**. The radioligand binding assay was performed on intact SK-N-MC cells, after 48 h growth, and exposed for 6h to L-Glutamate (L-Glu) 10 μM and [60]fullerene hydrosoluble derivative (t3ss) at 5 μM. For the assay, a specific radiolabelled antagonist DPCPX was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays, each one obtained as duplicate. The data were then analyzed on GraphPad *software*, and corresponding values obtained are reported below the graph. *p<0,05 significantly different from control.

When cells were treated with 100 μM L-Glu in the presence or the absence of 5 μM t3ss, and the expression of adenosine A_1 receptors was

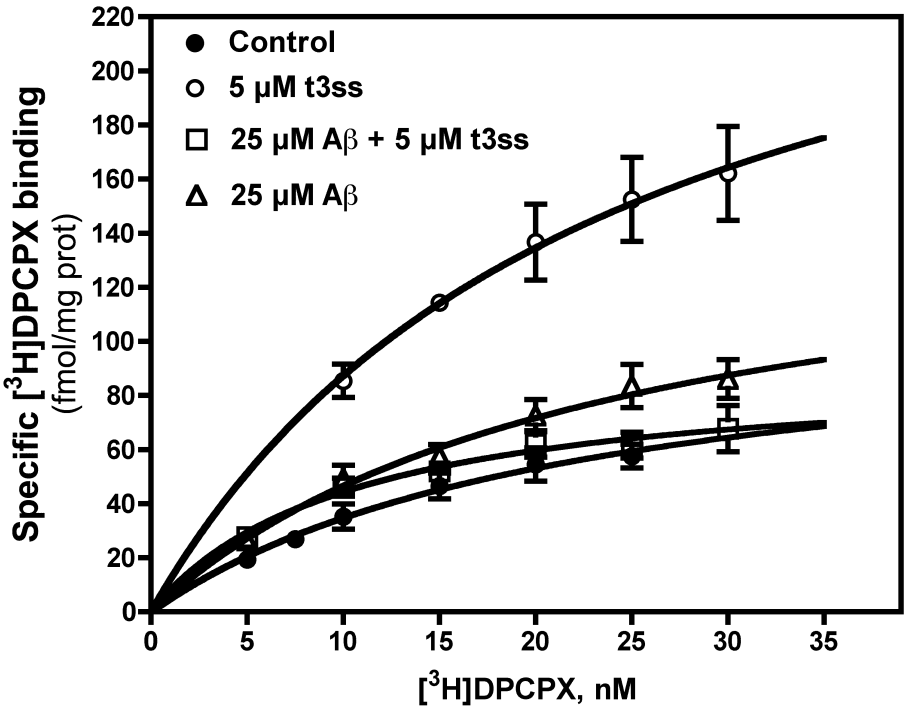
evaluated with the same assay method, a similar but even more evident effect was observed (fig.26), suggesting the protective effect of t3ss could be related to a modulation in the expression of adenosine receptors type A_1.



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.6 ± 28.00 (3)	22.41 ± 9.64 (3)
t3ss	*293.70 ± 64.60 (3)	23.63 ± 9.86 (3)
L-Glu + t3ss	288.3 ± 158.5 (3)	49.63 ± 37.98 (3)
L-Glu	180.9 ± 72.14 (3)	36.24 ± 21.72 (3)

Figure 26. **Radioligand binding assay of adenosine A_1 receptors, in entire SK-N-MC cells exposed to L-Glutamate and together with t3ss derivative**. The radioligand binding assay was performed on intact SK-N-MC cells, after 48 h growth, and exposed for 6h to L-Glutamate (L-Glu) 100 µM and [60]fullerene hydrosoluble derivative (t3ss) at 5 µM. For the assay, a specific radiolabelled antagonist DPCPX was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays, and obtained as duplicate. The data were then analyzed on GraphPad *software*, and correspondent values obtained are reported below the graph. *p<0,05 significantly different from control.

The expression of adenosine A_1 receptors was evaluated with the same assay method treating cells with amyloid-β peptide at 25 μM in the presence or the absence of 5 μM t3ss. Although not significant, amyloid-β peptide treatment showed an increase in the expression of those receptors and the contemporary presence of t3ss seems to limit the observed increase in the receptors, bringing the values to control values of binding parameters (fig.27). These results could indicate that in the case of treatment with amyloid-β the protective mechanism of t3ss is not directly related with adenosine A_1 receptors. Unfortunately, due to some problems during experimental procedure, as described before, it was not possible to confirm these results by using real time RT-PCR.



	Bmax (fmol/mg prot)	Kd (nM)
Control	112.60 ± 28.00 (3)	22.41 ± 9.64 (3)
t3ss	293.70 ± 64.60 (3)*	23.63 ± 9.86 (3)
Aβ+t3ss	91.00 ± 10.13 (3)	10.48 ± 3.07 (3)
Aβ	155.90 ± 29.53 (3)	23.47 ± 8.15 (3)

Figure 27. **Radioligand binding assay of adenosine A_1 receptors, in entire SK-N-MC cells exposed to amyloid-β peptide and together with t3ss derivative**. The radioligand binding assay was performed on intact SK-N-MC cells, after 48 h growth, and exposed for 6h to amyloid-β peptide (Aβ) 25 μM and [60]fullerene hydrosoluble derivative (t3ss) at 5 μM. For the assay, a specific radiolabelled antagonist DPCPX was used, as described in *Methods*. The values of Bmax and Kd herein obtained were an average of three independent assays, and obtained as duplicate. The data were then analyzed on GraphPad 5.0 *software*, and correspondent values obtained are reported below the graph. *p<0,05 significantly different from control.

2.1 Effect of t3ss [60]fullerene hydrosoluble derivative on undifferentiated SH-SY5Y human neuroblastoma cells.

In the following section of results we investigated the activity of the hydrosoluble [60]fullerene derivative t3ss, also used for SK-N-MC cells, in undifferentiated SH-SY5Y human neuroblastoma cells. We used it like treatment alone and in combination with different type of toxic treatment. We first evaluated the viability of SH-SY5Y cells by treating with the same concentration of t3ss derivative previously used for SK-N-MC cells (5μM) and demonstrated to have a protective effect on these cells. We considered different time of exposure to t3ss, including a long-term exposure of 24, 48 and 72 h. The results reported slight but not significant toxicity by treating SH-SY5Y cells with t3ss derivative at the used concentration, even for long-term exposure (fig.28).

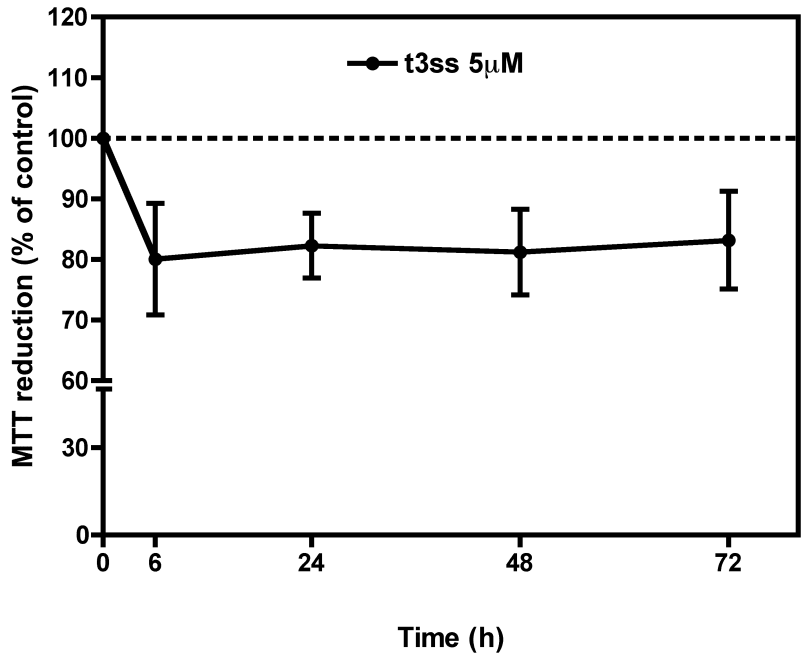


Figure 28. **Profile of viability of SH-SY5Y cells after exposure to t3ss**. Cells SH-Sy5y were exposed to 5 μM [60]fullerene hydrosoluble derivative (t3ss), for 6, 24, 48 and 72h, as showed in figure. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and conducted as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, by using GraphPad 5.0 *software*.

Next we investigated the activity of t3ss derivative on the expression of receptors endogenously expressed by SH-SY5Y cells and known to be involved in neurodegenerative and/or neuroprotective processes in neuronal cells, by using real time RT-PCR. Furthermore we evaluated the effect of t3ss derivative on the expression of the enzyme effectors system named BACE-1 also known to be involved in neurodegenerative process in many neurological diseases (fig. 29).

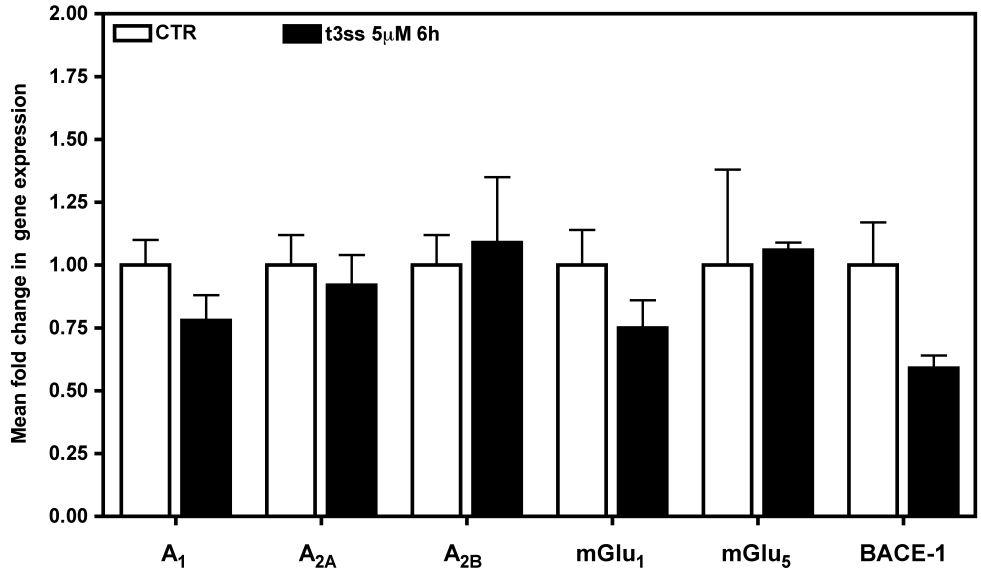


Figure 29. **Relative gene expression in SH-SY5Y exposed to t3ss derivative, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 6h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*.

Although not significant, a tendency of t3ss derivative to decrease the expression of BACE-1 was observed (fig. 30). Furthermore, we observed the loss of that tendency to decrease BACE-1 gene expression after long-time exposure of SH-SY5Y to t3ss derivative. Concerning the receptors we did not observe any significant change in gene expression at 6h (fig. 29), or for longer time of exposure (fig. 30).

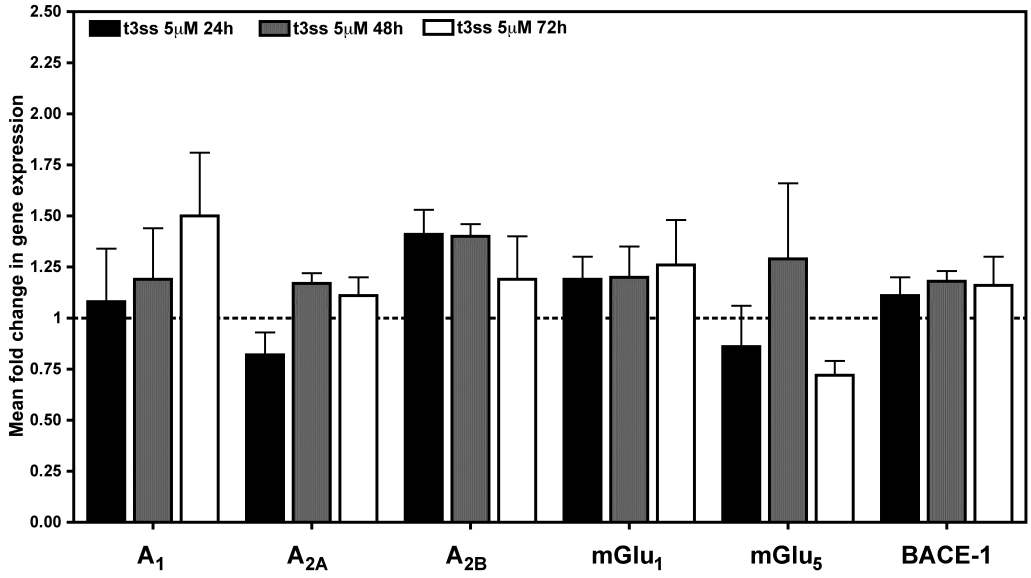


Figure 30. **Relative gene expression in SH-SY5Y exposed to t3ss derivative, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24, 48 and 72 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*.

2.2 Effect of L-Glutamate treatment on undifferentiated SH-SY5Y human neuroblastoma cells.

We investigated the activity of L-Glutamate on undifferentiated SH-SY5Y cells, evaluating first the survival of cells after L-Glutamate exposure. Next we described the effect of these treatments on the gene expression of the same target we described in the previous section, mainly focusing on long-time exposure to the L-Glutamate toxic treatment. About the survival of SH-SY5Y cells in presence of L-Glutamate we observed a time-dependent toxicity by considering the concentration of 100μM, for which we observed an increase in mortality of cells, by increasing the time of treatment. On the other hand, at 1-10 μM L-Glutamate cell survival was less affected. However, 10 μM glutamate elicited cell death was significant even at long time of exposure as 72 h (fig. 31).

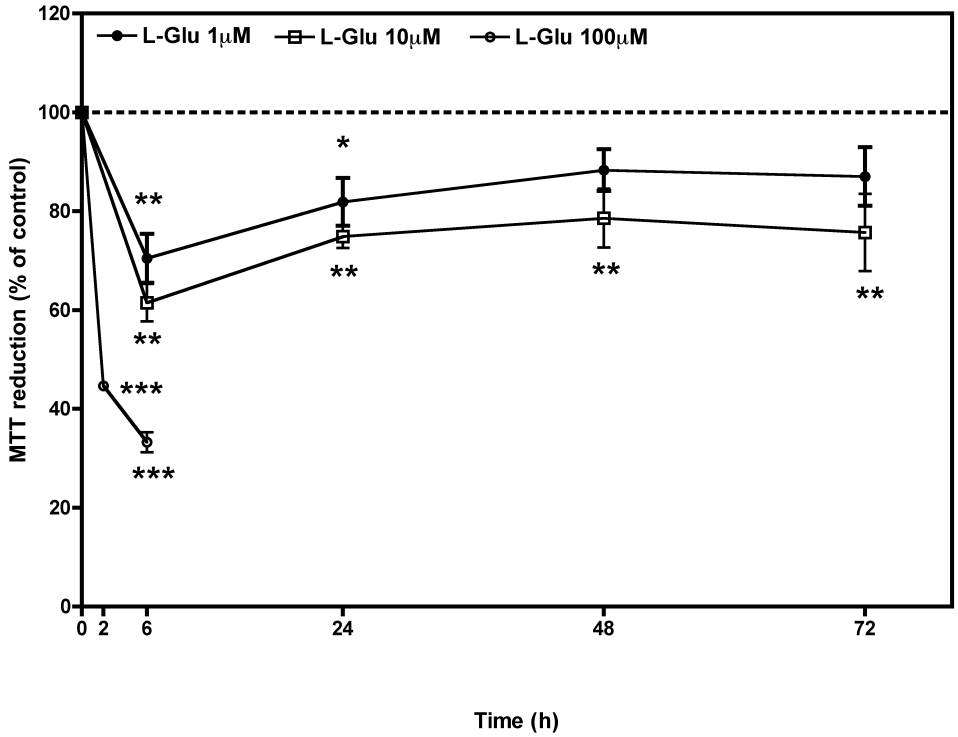


Figure 31. **Profile of viability of SH-SY5Y after exposure to L-Glutamate, evaluated by MTT reduction assay.** Cells SH-Sy5y were exposed to 1-10-100 μM L-Glutamate (L-Glu), for 6, 24, 48 and 72h, as showed in figure. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, by using GraphPad 5.0 as the *software*. *p<0,05, **p<0,01, ***p<0,001 significantly different from control.

In fact, although cells seemed to partially recover from initial toxic insult detected at 6h, during the subsequent 72h, they reached like a "plateau", even maintaining a different profile in the toxicity, related to the employed doses.

We thus evaluated the gene expression of SH-SY5Y cells focusing on the same target previously described for the treatment with t3ss, during short and long-time exposure to L-Glutamate. In that case we set, as described before for the profile of viability, for the short treatment a time of 6h and 100μM as the concentration of L-Glutamate, in order to evaluate the expression of genes affected by toxicity (fig. 32).

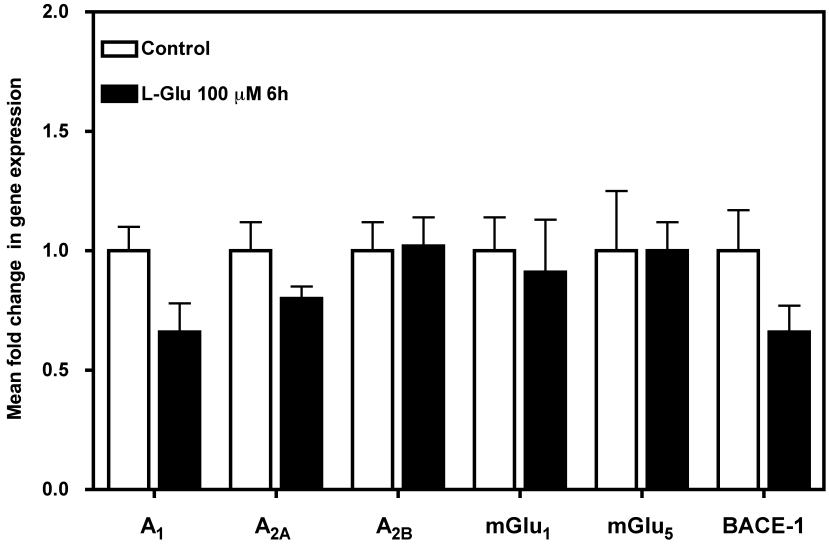


Figure 32. **Relative gene expression in SH-SY5Y exposed to L-Glutamate, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 100 μM L-Glutamate (L-Glu) for 6 h. The obtained extracted total RNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and as described in *Methods*.

Even though we didn't observe any significant change in the expression of the target, it was evident a tendency of the treatment with 100μM L-Glutamate to decrease the expression of certain target like adenosine A_1 and A_{2A} receptors, and the enzyme BACE-1. This tendency could be related to the short time of exposure to 100μM L-Glutamate. Instead, by considering the 1 μM L-Glutamate treatment for long time, we observed a significant increase of the gene expression of adenosine A_1 receptor at 24 and 72 h (fig.33).

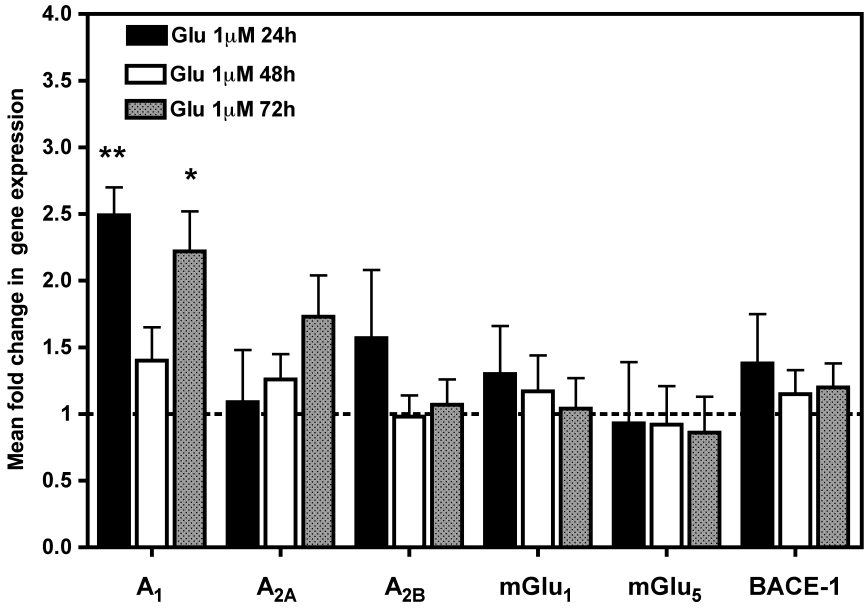


Figure 33. **Relative gene expression in SH-SY5Y exposed to L-Glutamate, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 1 μM L-Glutamate (L-Glu) for 24, 48, 72 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and as what described in *Methods*. *p<0,05 , **p<0,01 significantly different from control. Control corresponding to the dotted line.

Increasing L-Glutamate concentration to 10 μM we observed a significant modulation in the gene expression of metabotropic $mGlu_1$ glutamate receptors, at 48 h of treatment (fig. 34). Respect to the other considered target we observed only a tendency in modulating gene expression of adenosine A_1 and metabotropic $mGlu_5$ receptors, without affect BACE-1, A_{2A} and A_{2B} expression.

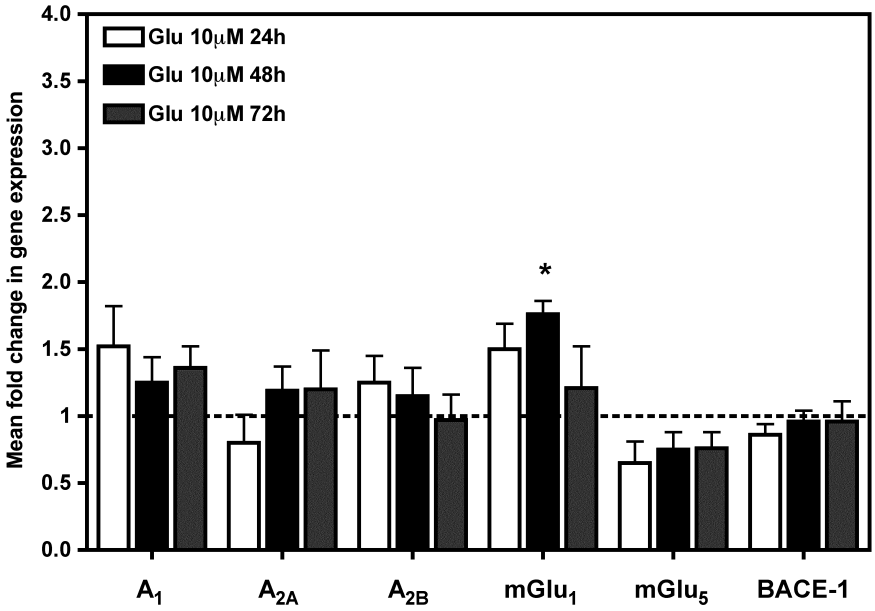


Figure 34. **Relative gene expression in SH-SY5Y exposed to L-Glutamate, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 10 μM L-Glutamate (L-Glu) for 24, 48, 72 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad ver. 5.0 *software* and following what described in *Methods*. *$p<0,05$ significantly different from control. Control corresponding to the dotted line.

2.3 Effect of contemporary use of [60]fullerene hydrosoluble derivative t3ss during treatment of undifferentiated SH-SY5Y cells with L-Glutamate.

We will consider now the results obtained by treating SH-SY5Y cells with L-Glutamate alone and in combination with t3ss derivative. We have mainly focused on viability of cells and gene expression during acute and chronic exposure to both treatments considered. Like described in the previous section we have analyzed a short time treatment with 100μM L-Glutamate at 2 h and 6 h of exposure. We first evaluated the viability of SH-SY5Y in the presence of 100μM L-Glutamate alone and in combination with 5μM t3ss derivative at indicated time (fig. 35).

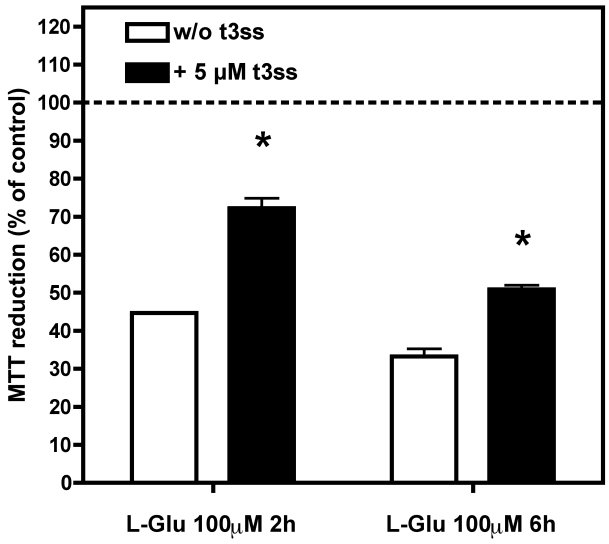


Figure 35. **Viability of SH-SY5Y cells after exposure to L-Glutamate alone and together with t3ss derivative, evaluated by MTT reduction assay.** Cells SH-SY5Y were exposed to 100 μM L-Glutamate (L-Glu) and together with 5 μM t3ss derivative (t3ss), for 2 and 6 h as showed in figure. Cell survival was obtained by MTT assay reduction as described in *Methods*, about three independent days treatment, corresponding each one to at least 8-10 values of MTT absorbance for each considered treatment, calculated as reported in *Methods*. Statistical analysis, and relative conversion, from MTT absorbance to percent of cell survival, were performed by using GraphPad 5.0 as *software*. *p<0,05 significantly different from control. Control corresponding to the dotted line as 100% of cell survival.

The presented results, show that when SH-SY5Y cells were treated in combination with L-Glutamate and t3ss, we noticed a partially but significant recovery of cells from toxicity of L-Glutamate alone elicited by t3ss presence.

Thus we considered an evident protective effect by 5 μM t3ss in SH-SY5Y cells, like those observed for SK-N-MC cells by using the same concentration of t3ss derivative. The observed effect of t3ss on SH-SY5Y cells viability were more evident at 2 h of exposure to L-Glutamate, than 6h. However we have to consider that SH-SY5Y cells subjected to 100 μM L-Glutamate for 6 h presented a more evident loss of viability respect to the treatment at 2 h, therefore the effect of t3ss derivative could be less evident, not significant.

We thus considered the gene expression of target involved in toxicity and/or protection of cells during the described treatment; we evaluated by real time RT-PCR changes in gene expression of SH-SY5Y during 6 h treatment with 100 μM L-Glutamate in combination with 5 μM t3ss, for the same target genes as described in the previous sections (fig.36).

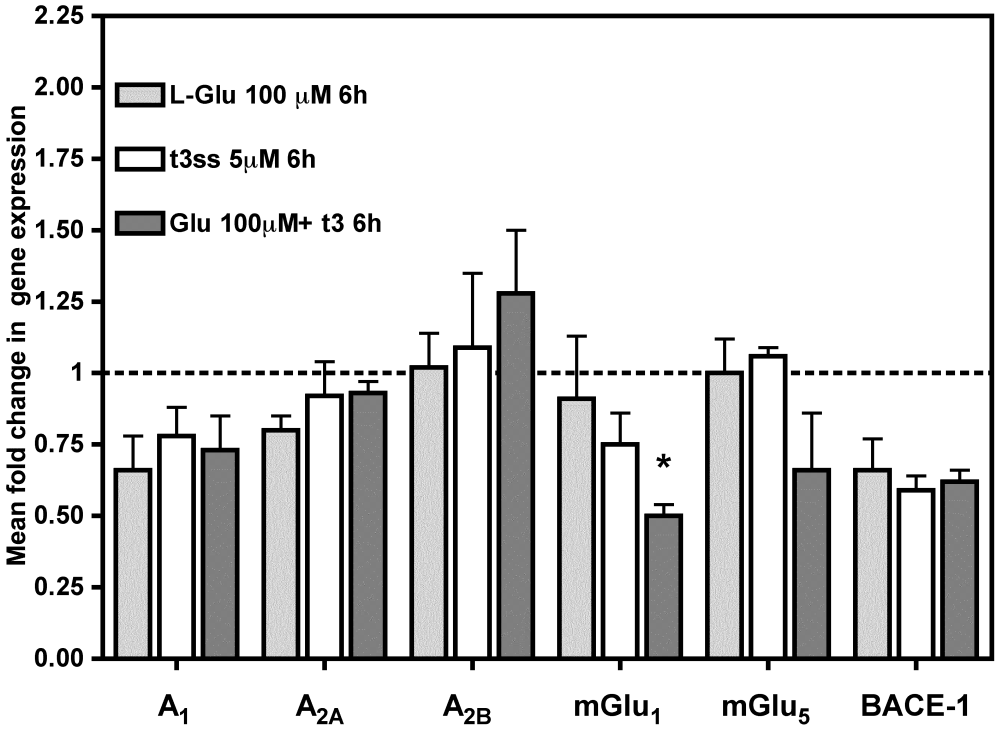
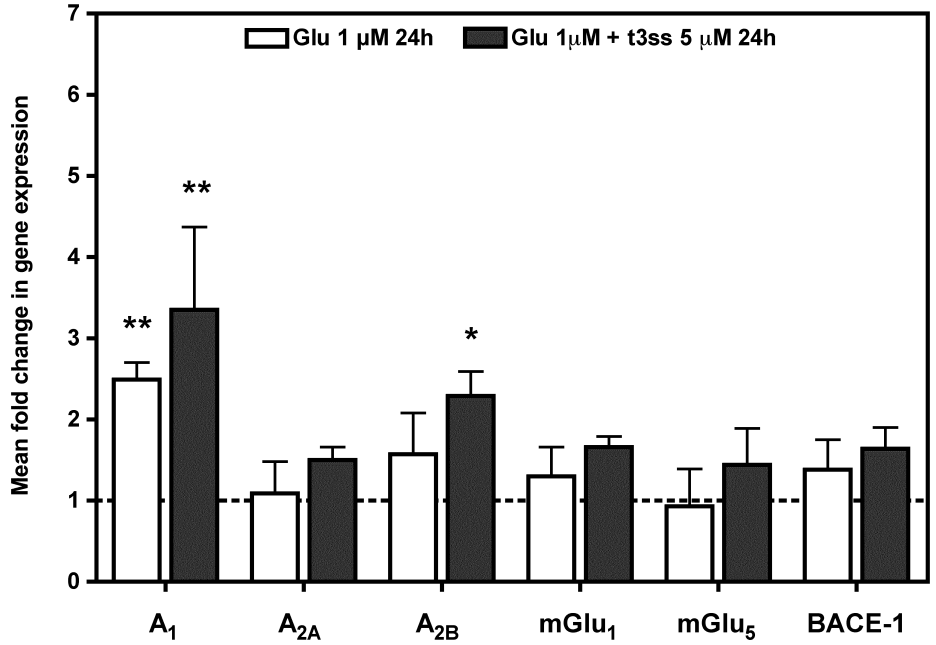


Figure 36. **Relative gene expression in SH-SY5Y exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y treated with 100 μM L-Glutamate (L-Glu) and together with [60]fullerene hydrosoluble derivative (t3ss) for 6 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. *$p<0,05$ significantly different from control. Control corresponding to the dotted line.

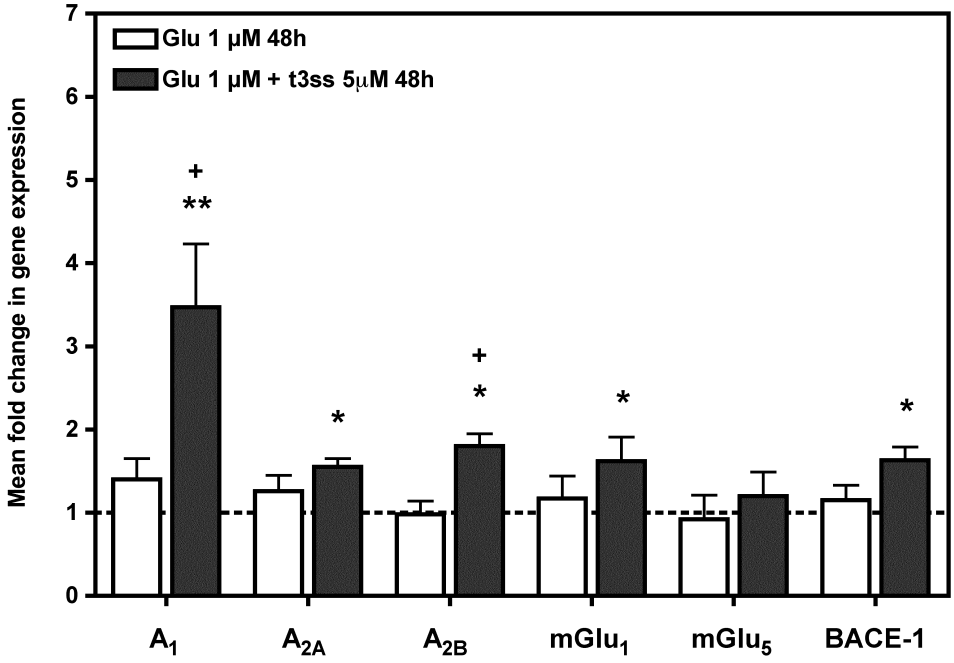
Results from real time PCR assay, indicate that only $mGlu_1$ gene expression was significantly decreased after considered treatment with 100 μM L-Glu and 5 μM t3ss for 6 h. However, although not significant, a decrease was also observed in A_1 and BACE-1 genes in all treatment assayed, suggesting different and independent regulatory mechanisms elicited by glutamate and t3ss.

Subsequently we evaluated the activity in gene expression of t3ss derivative, at the same concentration, in combination with 1-10 μM L-Glu for long-time exposure to SH-SY5Y cells. We first considered the treatment of cells with 1 μM L-Glu in combination with 5 μM t3ss at 24 h (fig. 37 panel A); the results showed an increase in the gene expression of adenosine A_1 receptors by the combination of both treatment, that maintained a significant value of increase respect to the control, and a tendency, although not significant, to increase the gene expression of the receptors respect to the treatment with L-Glutamate alone. The combination of the two treatment significantly increased the gene expression of adenosine A_{2B} receptors, and more in general seem to produce a tendency in increase the gene expression of all considered target, although not significantly. By using the same treatment in cells but for 48h, we confirmed that tendency to increase in the most part of considered gene targets the effect of glutamate treatment (fig. 37 panel B). If we consider the next treatment at 72h of SH-SY5Y cells exposure to 1 μM L-Glutamate and in combination with 5 μM we observed a normalization in levels of the most part of gene expression (fig. 37 panel C), with the only exception for gene expression of adenosine A_1 receptors, in which the contemporary use of L-Glutamate and t3ss maintained the significant increase of gene expression observed in the previous time of exposure.

A


Glu 1 µM 24h
Glu 1µM + t3ss 5 µM 24h
Mean fold change in gene expression
0
1
2
3
4
5
6
7
**
**
*
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

B


Glu 1 µM 48h
Glu 1 µM + t3ss 5µM 48h
Mean fold change in gene expression
0
1
2
3
4
5
6
7
+
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*
*
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

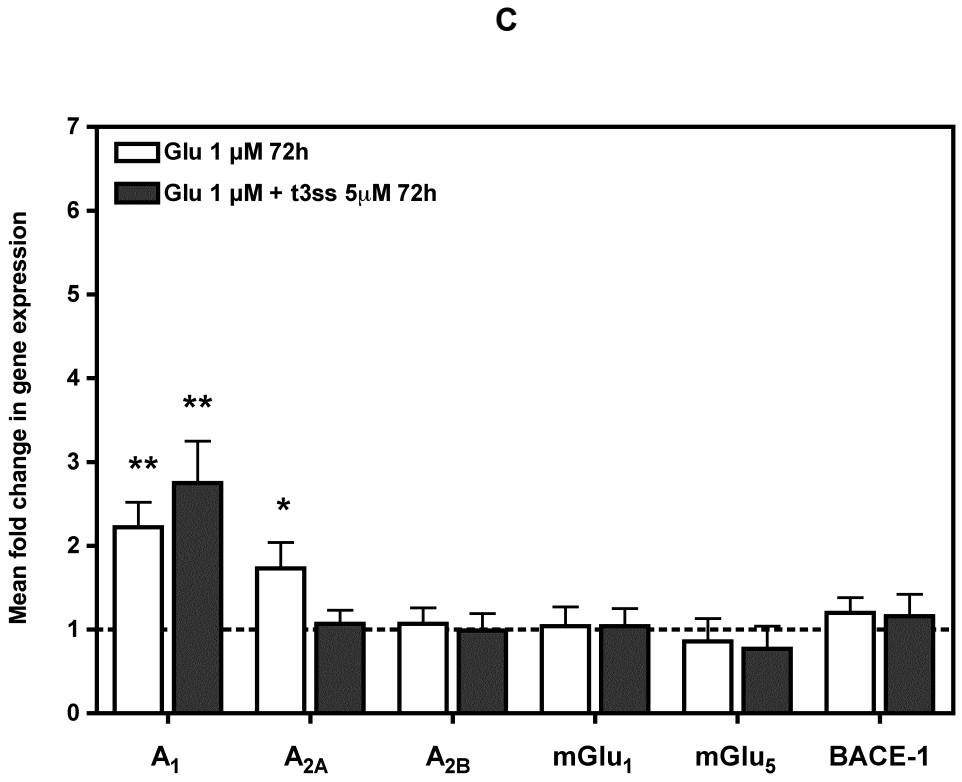
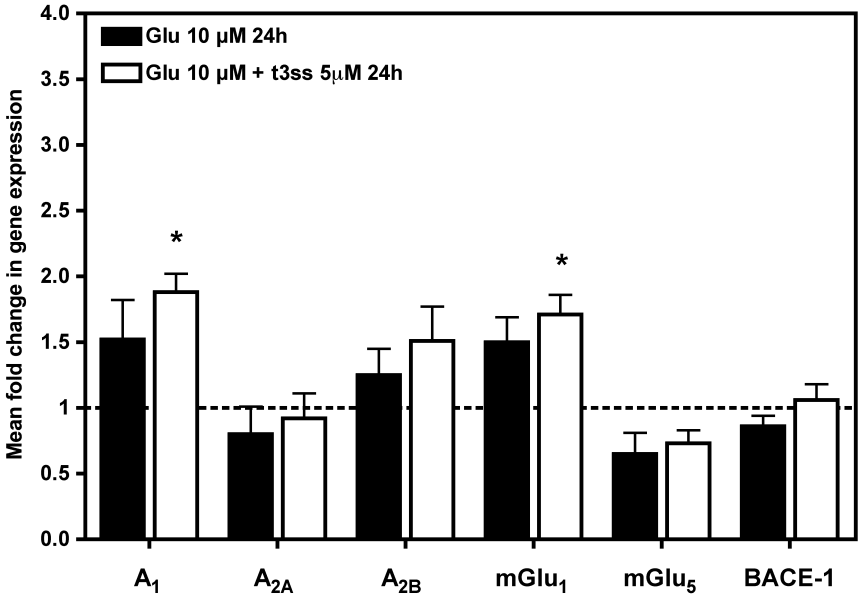


Figure 37. **Relative gene expression in SH-SY5Y exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 1 μM L-Glutamate (L-Glu) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h (A), 48 h (B) and 72 h (C). The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. *$p<0,05$ significantly different from control; **$p<0,01$ significantly different from control. +$p<0,05$ significantly different from treatment with L-Gutamate alone. Control corresponding to the dotted line.

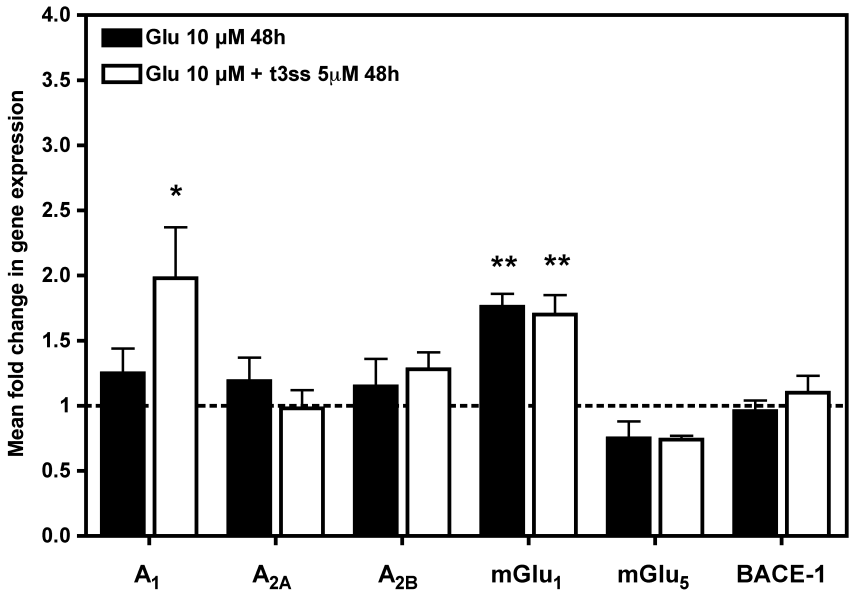
Next step was to analyze the gene expression of the same target as previously discussed in SH-SY5Y cells at different long-time exposure to 10 μM L-Glutamate alone and in combination with 5 μM t3ss. After 24 h of treatment with L-Glutamate and t3ss derivative at reported concentration (fig. 38 panel A), we observed a significant increase in adenosine A_1 receptors in cells subjected to combined treatment; a similar effect was observed for the gene expression of $mGlu_1$ receptors. Furthermore, as previously commented for the treatment with 1 μM L-Glu, it was observed a certain tendency to increase, although not significantly, the gene expression in the combined treatment with respect to glutamate treatment. This tendency disappear at 48

and 72 h, with the exception of adenosine A_1 receptors which were highly expressed after glutamate plus t3ss treatment as compared with glutamate alone treatment (fig. 38 B and C). Therefore a possible effect of [60]fullerene t3ss derivative in modulating the activity of adenosine A_1 receptors is observed, what could be related with its protective role during long-time exposure of cells to L-Glutamate.

A



B



C

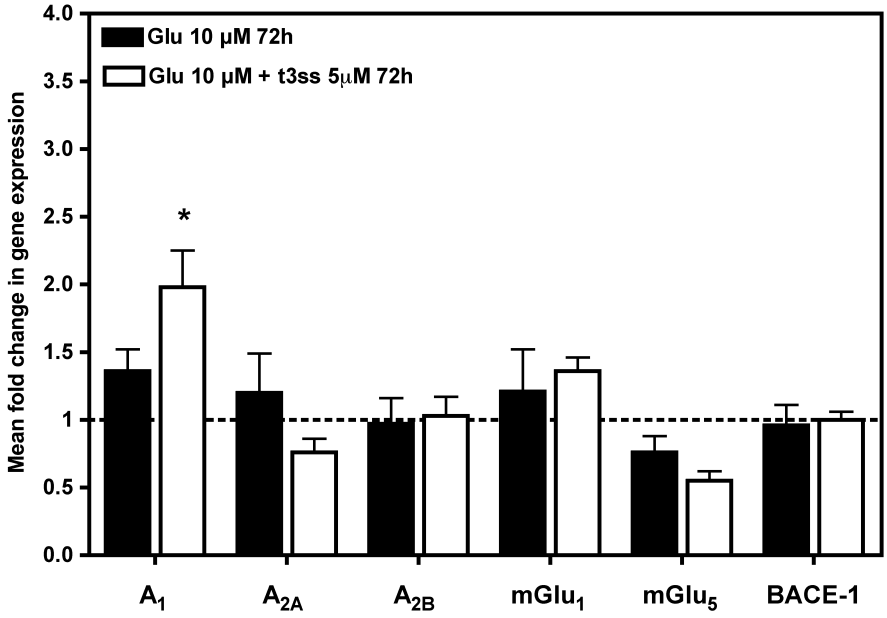


Figure 38. **Relative gene expression in SH-SY5Y exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 10 µM L-Glutamate (L-Glu) and together with 5 µM [60]fullerene hydrosoluble derivative (t3ss) for 24 h (A), 48 h (B) and 72 h (C). The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. *p<0,05 significantly different from control; **p<0,01 significantly different from control. Control corresponding to the dotted line.

3.1 Study of the activity of [60]fullerene hydrosoluble derivative t3ss and L-Glutamate on differentiated SH-SY5Y cells.

In the following section we will consider the results obtained by treating differentiated SH-SY5Y human neuroblastoma cells with t3ss [60]fullerene derivative and L-Glutamate. The purpose of the next experimental section dedicated to differentiated SH-SY5Y cells, was to study the toxicity of L-Glutamate and others neurotoxic compound in a cellular model mimicking human neurons, in order to create experimental conditions closer to real processes occurring in human brain during neurodegenerative diseases. In that sense by using a fully differentiated human neurons proceeding from SH-SY5Y cells, we investigated mainly the activity of t3ss derivative on viability of

cells and on gene receptors expression for the same target as we described before. At the same time we tried to mimic what is occurring in human brain, by exposing cells to moderate-high neurotoxic conditions by using L-Glutamate 100nM/1-10μM for long time exposure (24 to 72h). We first investigated the viability of neuronal cells after exposure to 5μM t3ss for 6-24-48 and 72h (fig. 39);

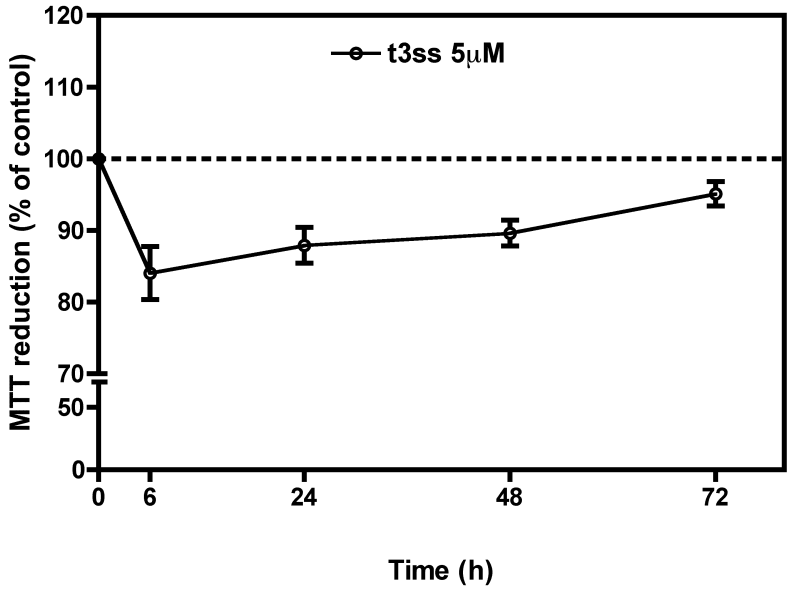


Figure 39. **Profile of viability of differentiated SH-SY5Y cells after exposure to t3ss**. Differentiated cells SH-Sy5y were exposed to 5 μM [60]fullerene hydrosoluble derivative (t3ss), for 6, 24, 48 and 72h, as showed in figure. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and conducted as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, by using GraphPad ver. 5.0 as the *software*.

Results showed a tendency in loss of viability, after 6h of treatment with t3ss derivative, whereas this toxic effect was lower for longer time of treatment. Anyway the treatment with t3ss 5μM was demonstrated to be not very toxic in neuronal cells maintaining the viability around 90% of the control

during 72h. Furthermore, this low toxic effect was lower than that detected in undifferentiated SH-SY5Y cells (fig. 28).

The next step was to investigate the gene expression in differentiated SH-SY5Y during treatment with t3ss derivative; for that purpose we considered the same gene target as before for undifferentiated SH-SY5Y cells, and the same long-time exposure of neuronal cells to 5μM t3ss like used for the study of viability. The summary of gene expression evaluated by real time RT-PCR technique in differentiated SH-SY5Y cells after t3ss treatment is reported (fig. 40).

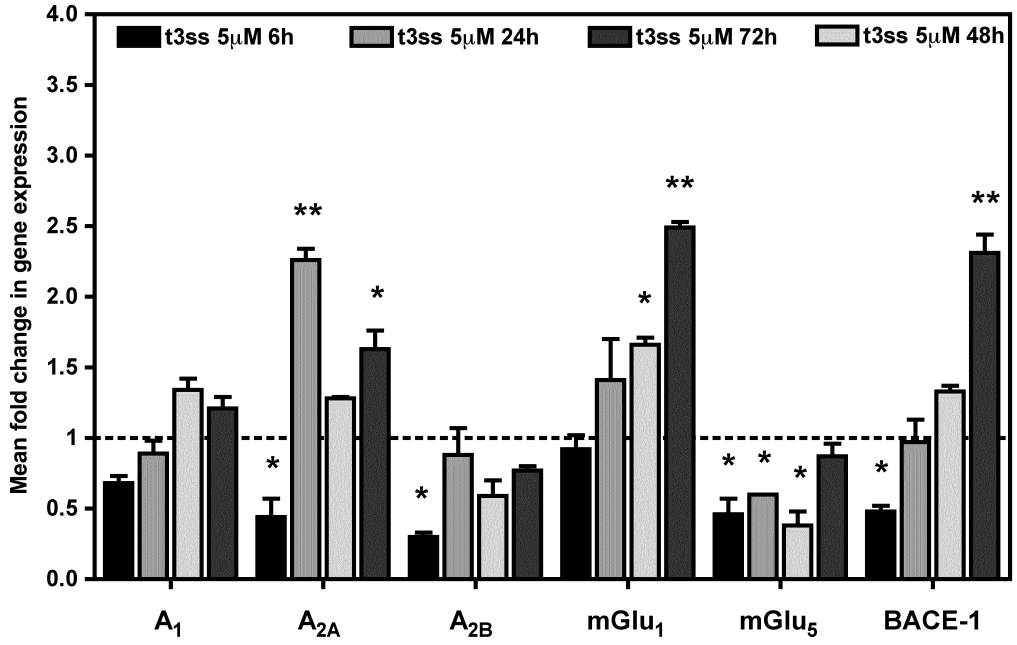


Figure 40.**Relative gene expression in differentiated SH-SY5Y cells exposed to t3ss derivative, evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on differentiated SH-SY5Y cells treated with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 6, 24, 48, 72 h. The obtained extracted RNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. *p<0,05, **p<0,01 significantly respect to the control. Control is representing by dotted line.

Results from real time RT-PCR showed significant increase in gene coding for adenosine A_{2A} receptors with a tendency to modulate with time. Gene expression of $mGlu_1$ receptors and BACE-1 were also increased at 24/48 and 72 h. Interestingly, almost all genes assayed had significant lower

expression at 6 h of treatment, respect control level, in the case of A_1, A_{2B} and $mGlu_5$ or higher levels than controls in the case of A_{2A}, $mGlu_1$ and BACE-1 at 24 h and longer time of treatments. The next step was to investigate the activity of sub-toxic exposure of differentiated SH-SY5Y cells to L-Glutamate; to do that we considered three concentrations of L-Glutamate as 100 nM, 1 μM, 10 μM, those were used because the long-time of exposure we planned to perform in differentiated SH-SY5Y cells, in order to re-create the typical conditions to which human neurons are subjected when in pathological and/or stressed situations. We first investigated the viability of differentiated cells exposed to 100nM and 1-10μM L-Glutamate for at least 72 h of treatment.

We continued by analyzing the viability of cells in the presence of L-Glutamate at different concentrations and for different time of exposure (fig. 41). Differentiated SH-SY5Y cells suffer a moderate toxicity to glutamate, decreasing viability a 30% at 100 μM glutamate as soon as 6 h and maintaining this effect even for 72 h. A similar constant effect was observed considering lower glutamate concentrations. Thus, at 1 and 10 μM glutamate 80 % of cells were viable at all times assayed and at 100 μM L-Glu viable cells were 85%. Comparison of viability values obtained in differentiated and non differentiated SH-SY5Y cells, clearly shows that toxic effect of glutamate i significantly higher in undifferentiated cells at 6 hours of treatement, while at longer time of exposure there are not differences.

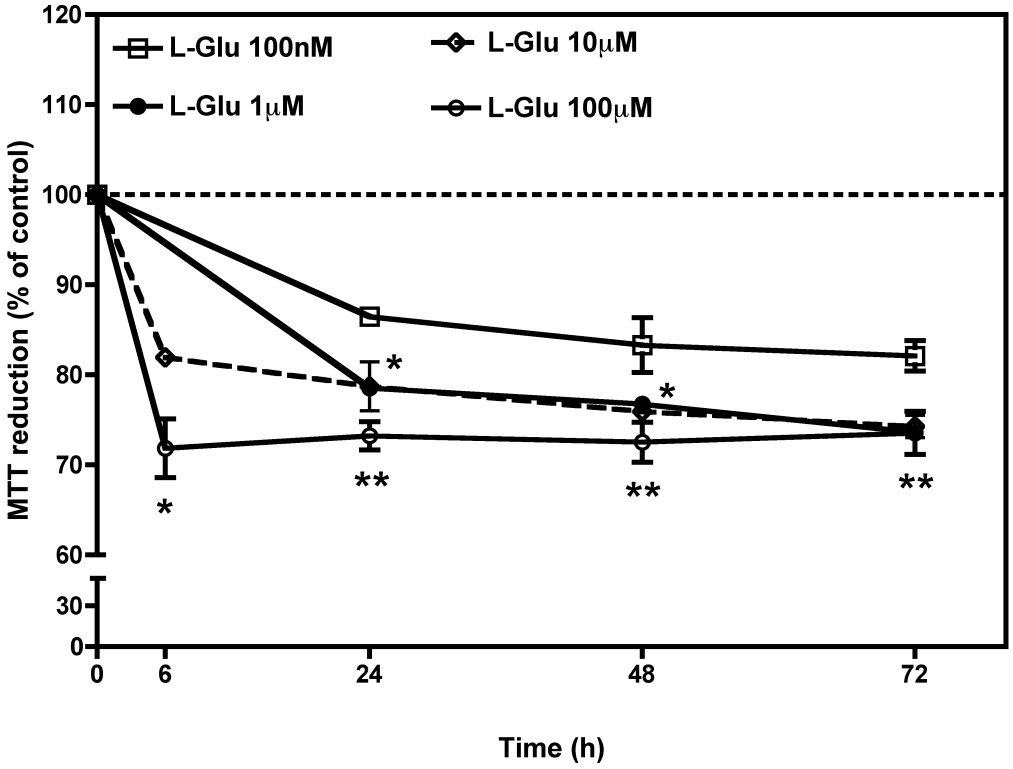


Figure 41. **Profile of viability of differentiated SH-SY5Y cells after exposure to L-Glutamate, evaluated by MTT reduction assay.** Differentiated cells SH-Sy5y were exposed to 100 nM and 1-10-100 μM L-Glutamate (L-Glu), for 6, 24, 48 and 72 h, as showed in figure. Values of cell survival, are from the average of three independent treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, by using GraphPad 5.0 as the *software*. *p<0,05, **p<0,01 significantly different from control.

In any case, we demonstrated that the tendency of t3ss derivative to protect undifferentiated SH-SY5Y cells against toxic effect of L-Glutamate is maintained although less evident, in differentiated cells (fig.42).

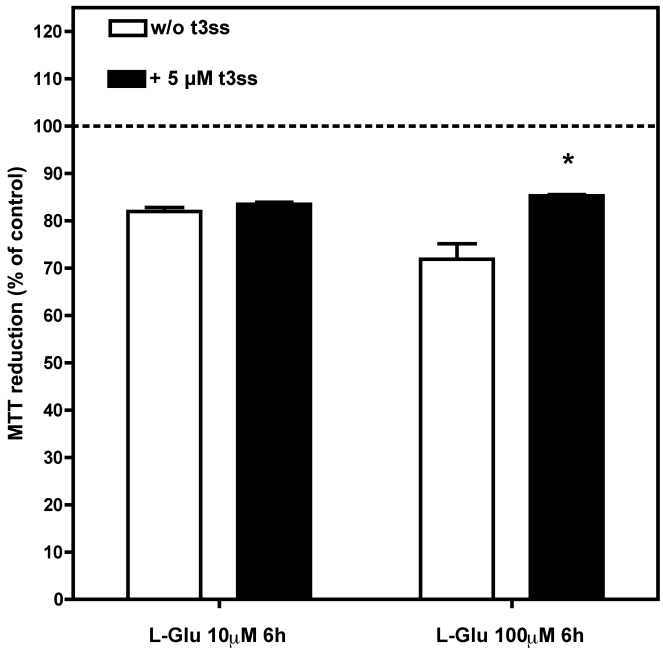
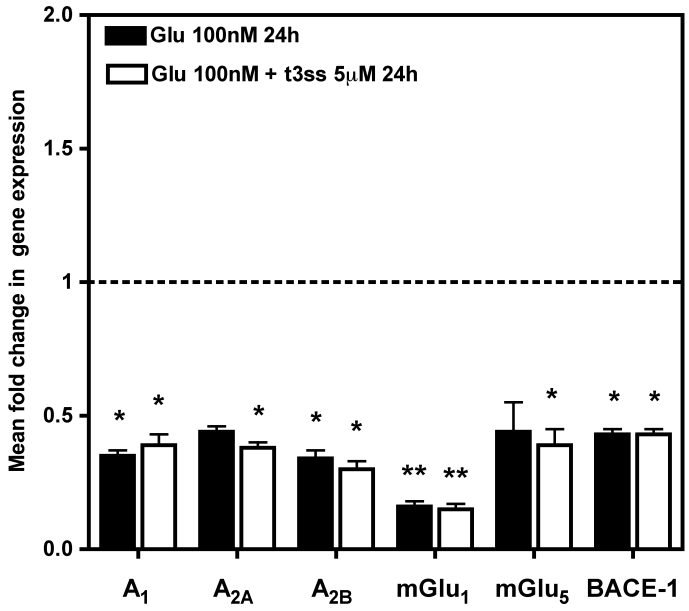


Figure 42. **Viability of differentiated SH-SY5Y cells after exposure to L-Glutamate alone and together with t3ss derivative, evaluated by MTT reduction assay.** Differentiated cells SH-SY5Y were exposed to 10 - 100 μM L-Glutamate (L-Glu) and together with 5 μM t3ss derivative (t3ss), for 6 h as showed in figure. Cell survival was obtained by MTT assay reduction as described in *Methods*, about three independent days treatment, corresponding each one to at least 8-10 values of MTT absorbance for each considered treatment, calculated as reported in *Methods*. Statistical analysis, and relative conversion, from MTT absorbance to percent of cell survival, were performed by using GraphPad 5.0 as *software*. *$p<0,05$ significantly different from control. Control corresponding to the dotted line as 100% of cell survival.

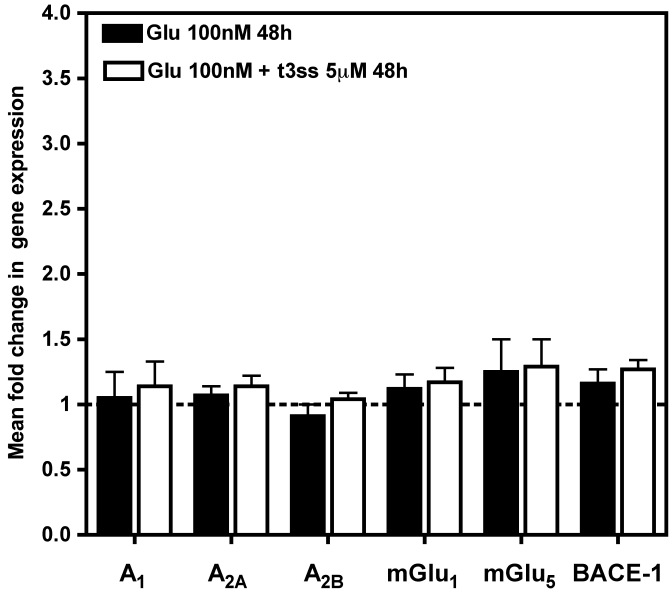
In particular we observed a more significant recovery of cells by the contemporary use of 5 μM t3ss in the presence of 100μM L-Glutamate, like observed in undifferentiated cells. In order to analyze the effect of t3ss derivative on the gene expression of differentiated SH-SY5Y cells treated with L-Glutamate at the same concentrations and time considered for the viability study. We first considered the concentration of 100 nM L-Glutamate and cells exposed for 24, 48 and 72 h in the presence or the absence of 5 μM t3ss, and we evaluated the gene expression as in undifferentiated cells (fig. 43). If we consider the treatment with 100 nM L-Glutamate at 24h all considered gene expression target are significantly decreased (fig. 43a) with not apparent difference respect to the contemporary presence of 5 μM t3ss. However, at 48

and 72 h of treatment 100 nM Glutamate did not modify gene expression. Moreover, presence of 5 μM t3ss has no effect over glutamate treatment.

A



B



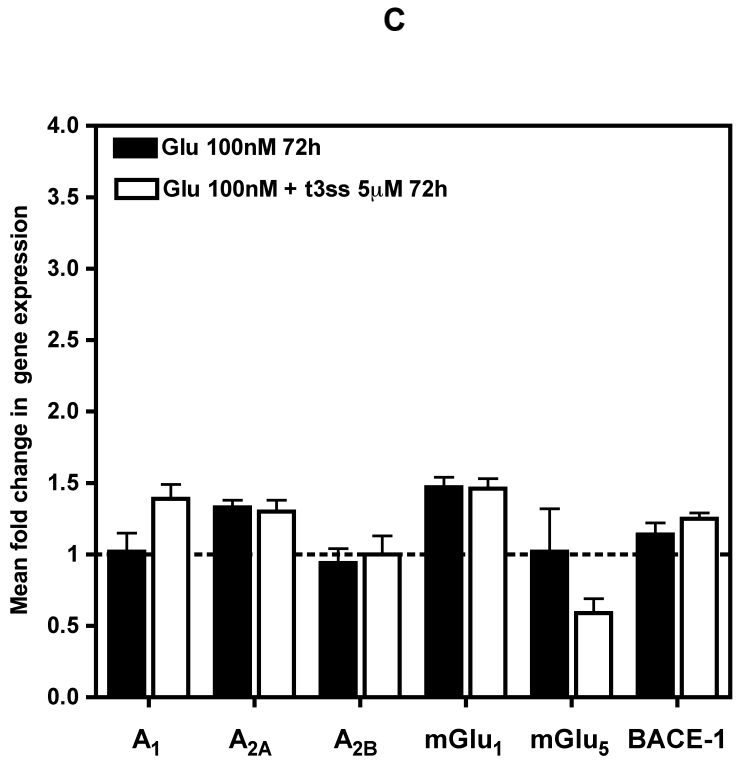
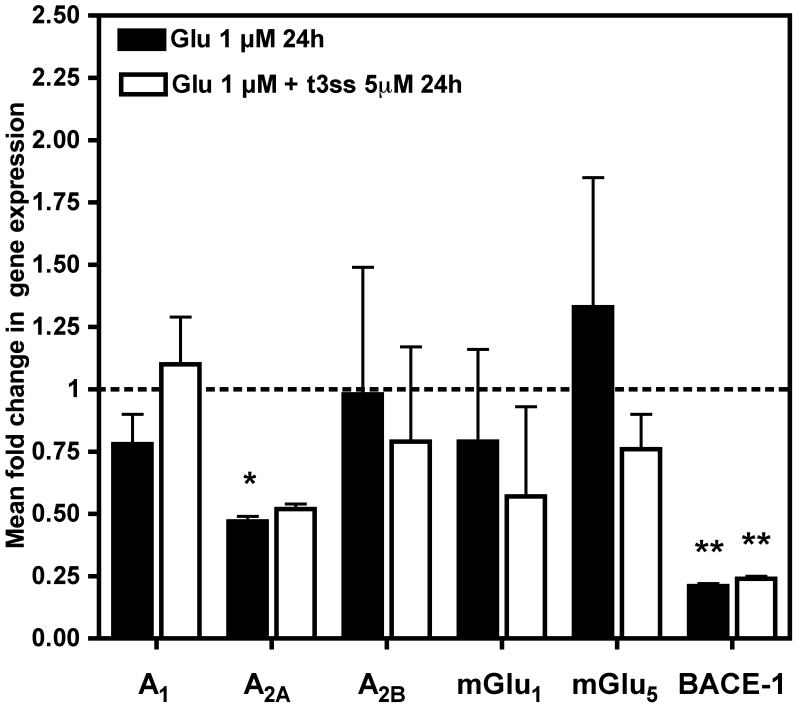


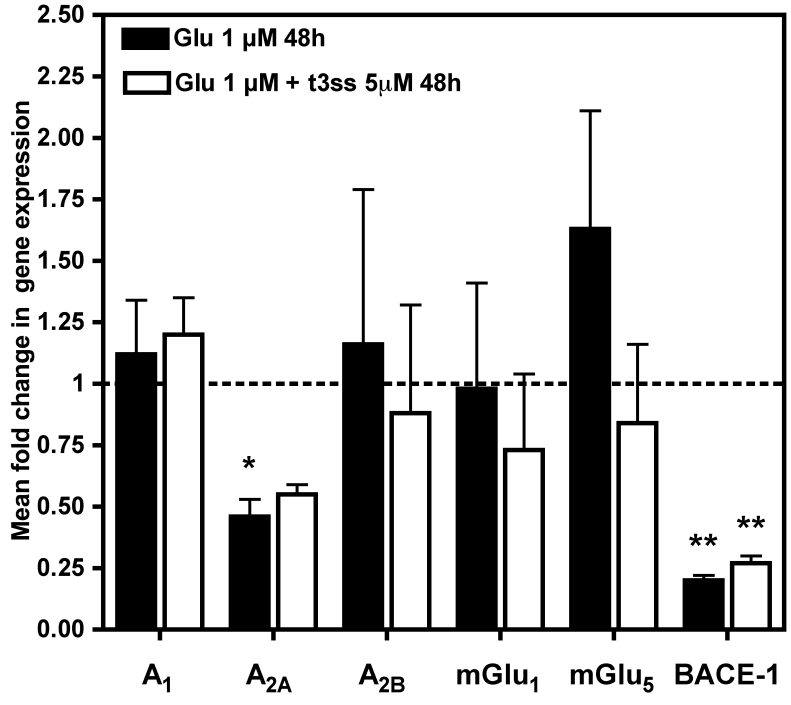
Figure 43. **Relative gene expression in differentiated SH-SY5Y cells exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 100 nM L-Glutamate (L-Glu) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h (A), 48 h (B) and 72 h (C). The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad . 5.0 *software* and as described in *Methods*. *p<0,05 significantly different from control; **p<0,01 significantly different from control. Control, corresponding to the dotted line.

We increased glutamate concentration to 1 μM and the same analysis of gene expression was performed (fig. 44). These results corroborated the absence of effect by t3ss presence during treatment we detected at 100 nM L-Glu. Interestingly, at 24 h reduction in gene expression was lower in A_{2A} and BACE-1 genes and disappear in A_1, A_{2B}, $mGlu_1$ and $mGlu_5$ genes at 1μM glutamate treatment (fig. 44 A), if compared to 100 nM glutamate (fig. 43 A).

A


Glu 1 µM 24h
Glu 1 µM + t3ss 5µM 24h
Mean fold change in gene expression
2.50
2.25
2.00
1.75
1.50
1.25
1
0.75
0.50
0.25
0.00
*
**
**
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

B


Glu 1 µM 48h
Glu 1 µM + t3ss 5µM 48h
Mean fold change in gene expression
2.50
2.25
2.00
1.75
1.50
1.25
1
0.75
0.50
0.25
0.00
*
**
**
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

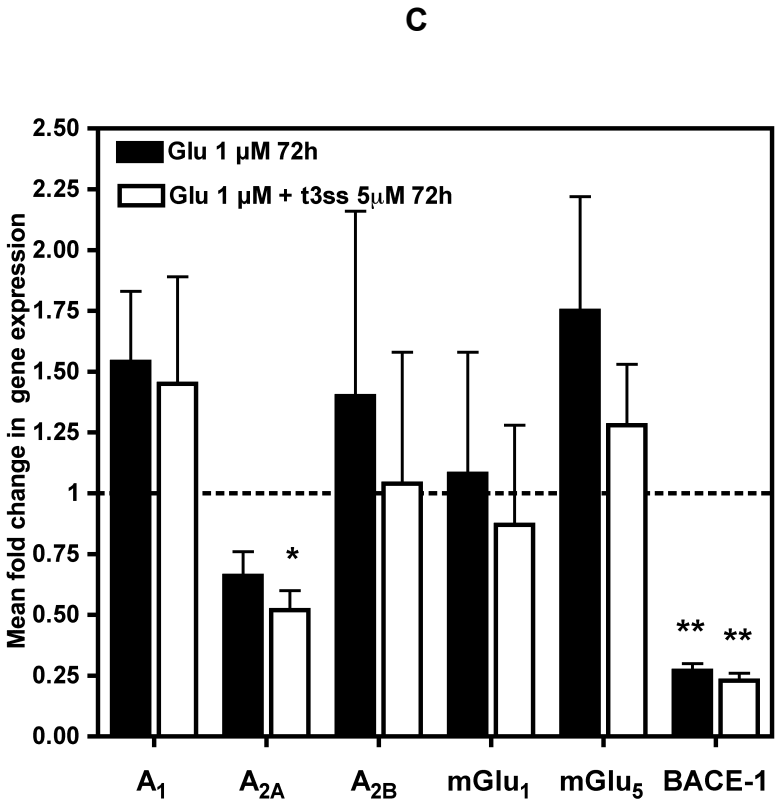


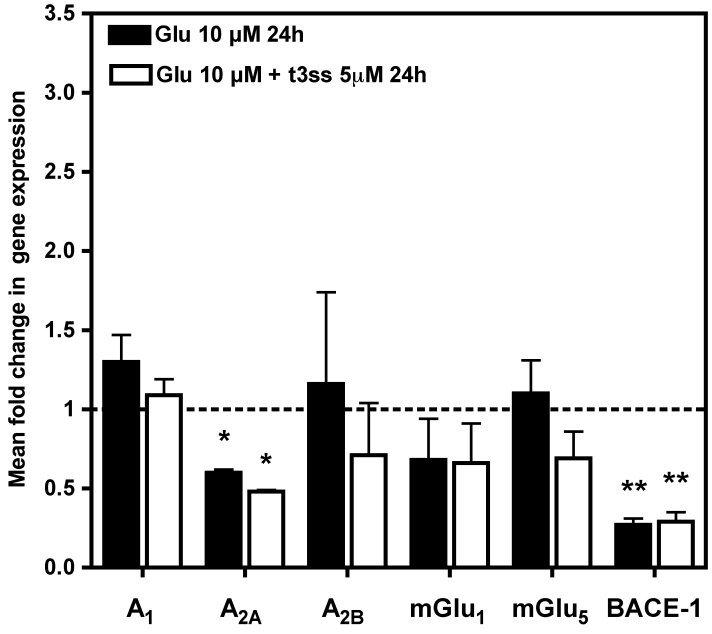
Figure 44.**Relative gene expression in differentiated SH-SY5Y cells exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 1 μM L-Glutamate (L-Glu) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h (A), 48 h (B) and 72 h (C). The obtained extracted RNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad . 5.0 *software* and as described in *Methods*. *p<0,05 significantly different from control; **p<0,01 significantly different from control. Control, corresponding to the dotted line.

.

Similarly to that previously observed for the treatment with 1 μM L-Glutamate, the gene expression of differentiated SH-SY5Y cells did not suffer any relevant change in gene expression after exposure to 10 μM L-Glutamate together with 5 μM t3ss derivative, except to a small but significant decrease in gene expression of A_{2A} and BACE-1 (fig. 45 A). Furthermore, as observed for L-Glutamate 1 μM, t3ss derivative does not contribute in any case to change the gene expression, in the sense that all the observed effect is carried out by L-Glutamate alone. Furthermore, in all remaining cases, the little difference observed between treatment with L-Glutamate and together with t3ss derivative, and some tendency observed in affect the gene expression of different targets, is mainly due to the assay standard deviation

error, not to a real effect of the treatment. This is the case of gene expression of A_{2B} receptors after treatment with 10 μM L-Glutamate alone at 72 h, showing to significantly increased the gene expression, even the value is considered with an high assay deviation (fig. 45 C). As well as by consider the treatment with 10 μM L-Glutamate combined with t3ss derivative we observed a significant increase in gene expression of A_1 receptors, thus indicating once more, a relationship between exposure of cell to toxic dose of L-Glutamate, and increase of A_1 receptors as response to the activity of t3ss derivative. Nevertheless, the effect of L-Glutamate may due to the long time differentiated SH-SY5Y cells were exposed, most than to the concentration used (fig. 45 C).

A



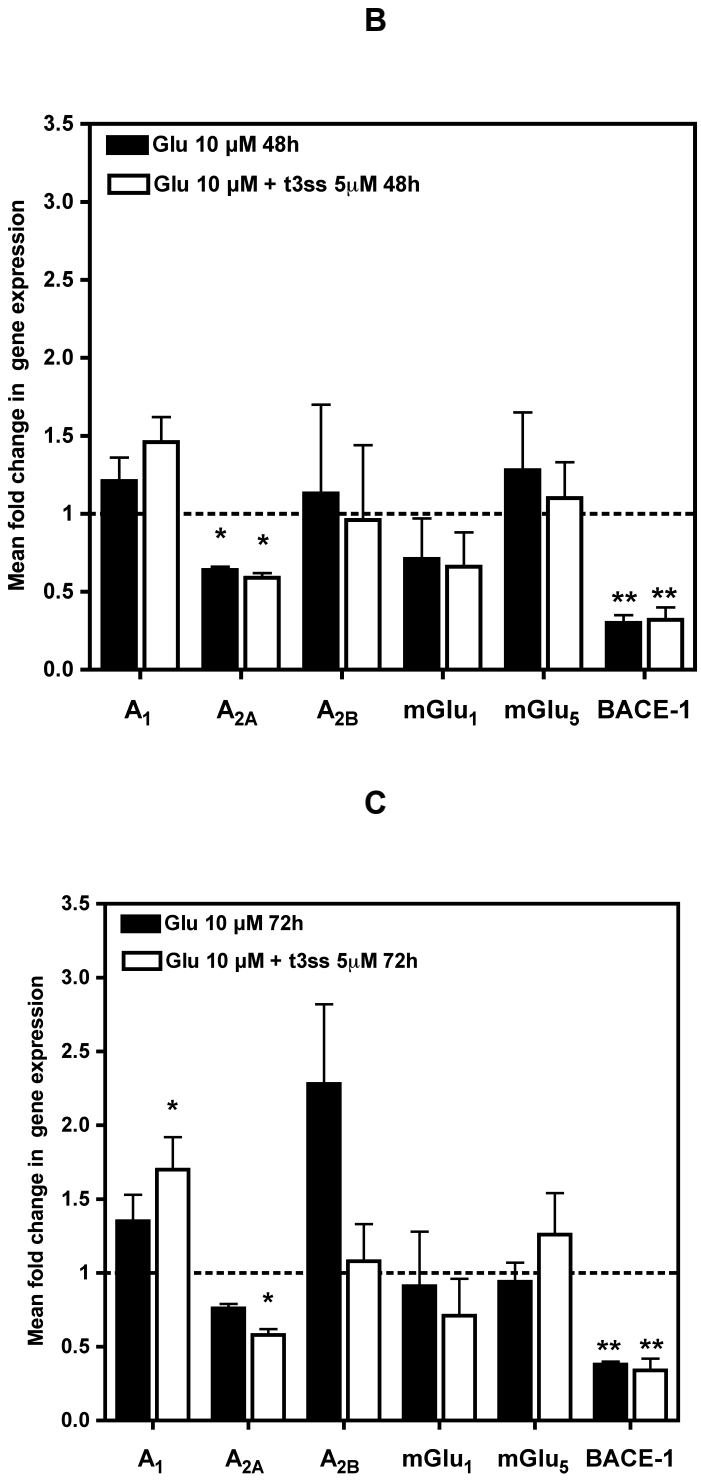


Figure 45. **Relative gene expression in differentiated SH-SY5Y cells exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 10 µM L-Glutamate (L-Glu) and together with 5 µM [60]fullerene hydrosoluble derivative (t3ss) for 24 h (A), 48 h (B) and 72 h (C). The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad ver. 5.0 *software* and following what described in *Methods*. *$p<0,05$ significantly different from control; **$p<0,01$ significantly different from control. Control, corresponding to the dotted line.

We also evaluated the gene expression by RT-PCR of differentiated SH-SY5Y after exposure to 100 μM L-Glu 6h and together with 5 μM t3ss derivative. The significant effect observed was the one by t3ss derivative treatment alone, able to decrease the gene expression of all considered target, except for $mGlu_1$. The treatment with L-Glu did not seem to affect significantly the gene expression of considered targets, while the contemporary presence of t3ss derivative, noticed a slight change in gene expression, respect to the treatment with L-Glutamate alone, even not significant. Thus the main effect on gene expression, after considered treatments, appear clearly to be evoked by 5 μM t3ss derivative alone, like for example in the most patent case observed, the decrease in gene expression of A_{2A}, A_{2B} and $mGlu_5$. Nevertheless, by considering BACE-1 expression after treatment with 100 μM L-Glu 6 h alone, a little not significant increase was observed, while considering the contemporary presence of 5 μM t3ss derivative, and the treatment with t3ss derivative alone, a decrease in the gene expression of BACE-1 enzyme was observed; thus suggesting a possible modulation on BACE-1 gene expression elicited by t3ss derivative (fig. 46).

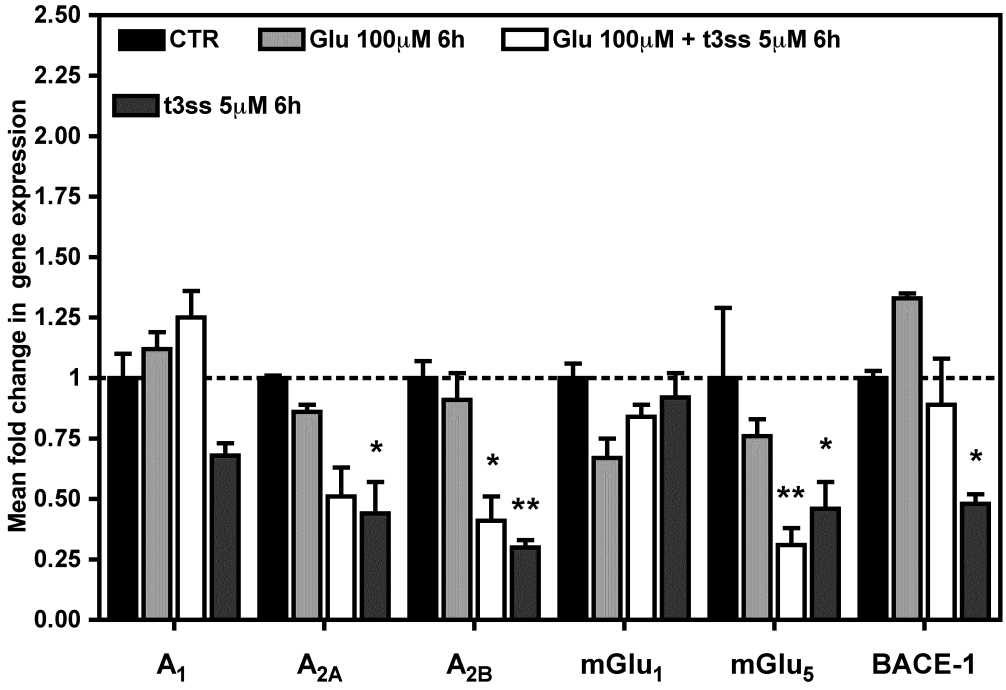


Figure 46.**Relative gene expression in differentiated SH-SY5Y cells, exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 100 μM L-Glutamate (L-Glu) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 6 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. *p<0,05, **p<0,01 significantly different from control. Control, corresponding to the dotted line.

The following treatment considered was 100 μM L-Glutamate and together with 5 μM t3ss derivative for 24 h of exposure to differentiated SH-SY5Y cells. The most interesting effect was observed on gene expression of A_{2A} receptors, increased by treatment with t3ss derivative alone (fig. 47), while during the treatment at 6 h we observed a decrease in gene expression of A_{2A} receptors, by considering the same treatment with t3ss derivative. No further significant effects on gene expression, were observed by considering both the considered treatment with L-Glu and t3ss derivative at 24 h, except for a significant decrease in gene expression of $mGlu_5$, apparently due only to the activity of t3ss derivative, as confirmed also by consider the same treatment at 6 h, like previously discussed (fig. 46). These results finally suggest a certain modulation by t3ss derivative of gene expression of A_{2A} and

$mGlu_5$ receptors, as reported by both considered times of treatment at 6 and 24 h.

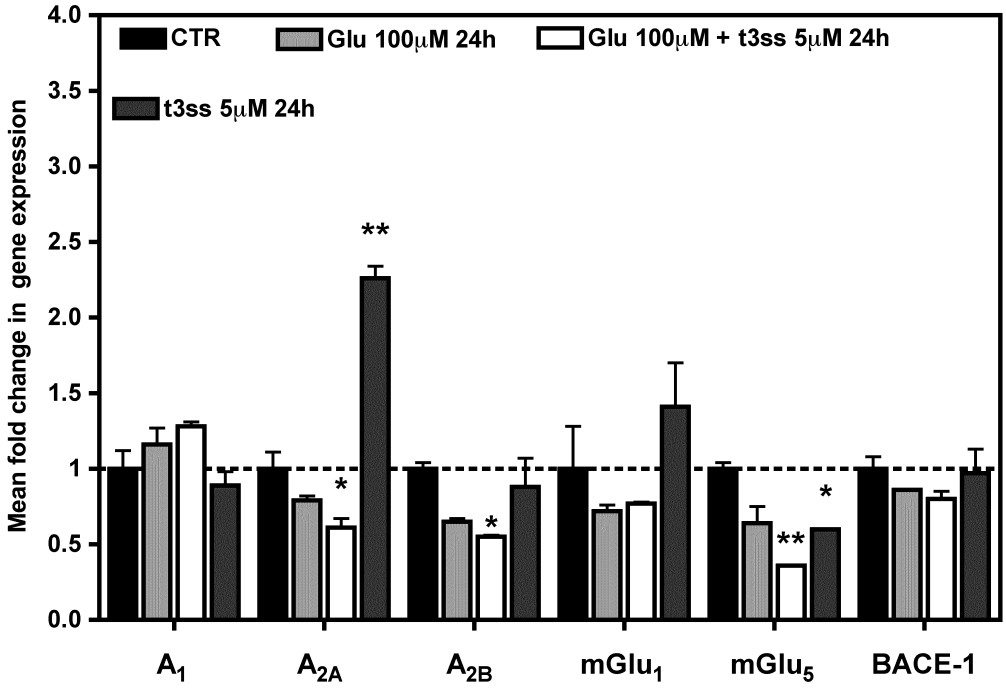


Figure 47. **Relative gene expression in differentiated SH-SY5Y cells, exposed to L-Glutamate and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 100 μM L-Glutamate (L-Glu) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h. The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and as described in *Methods*. *$p<0,05$, **$p<0,01$ significantly different from control. Control, corresponding to the dotted line.

4.1 Study on the effect of amyloid-beta peptide fragment 25-35 on undifferentiated SH-SY5Y cells and in combination with t3ss derivative.

In the following section we investigated the effect of amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) on undifferentiated SH-SY5Y cells. Like discussed in the *Introduction* of the present work, amyloid-beta peptide is the major responsible for a drastic worsening in conditions of human neuronal cells in many neurodegenerative diseases. Therefore we used that treatment alone and in combination with [60]fullerene t3ss derivative to explore the effects on our cells model. We first focused on viability of cells after exposure to 10 and 25 μM $A\beta_{25-35}$ for 6h and in combination with 5μM t3ss. During the treatment with $A\beta_{25-35}$ 10μM we observed a high and time dependent toxicity towards cells. Interestingly the toxicity of 10 μM $A\beta_{25-35}$ is partially reverted by the contemporary presence of 5 μM t3ss, in all times assayed (fig. 48).

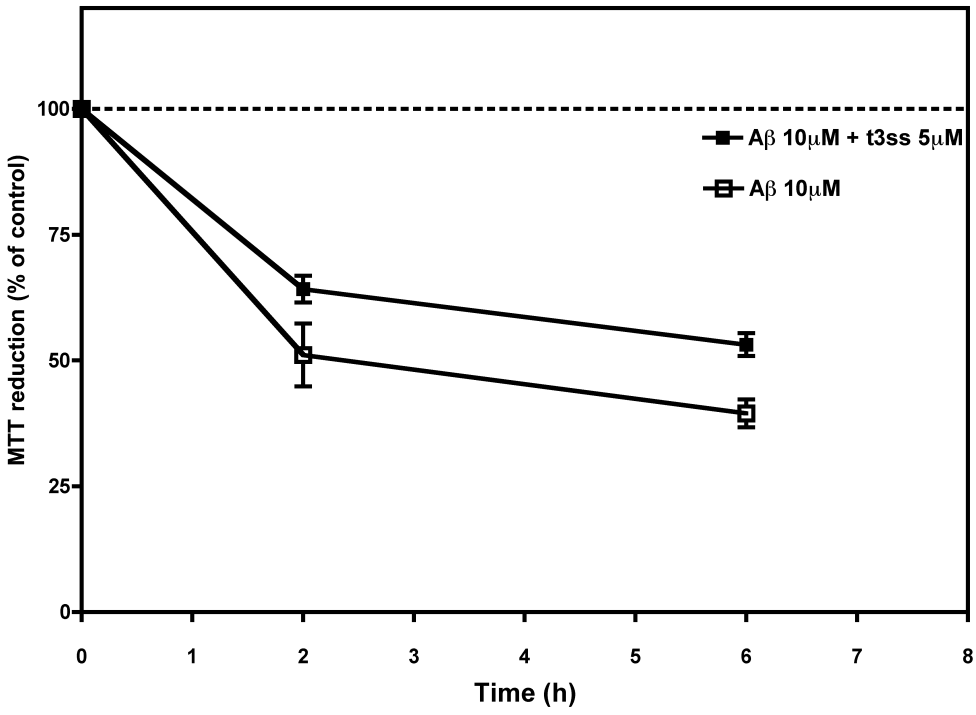


Figure 48. **Viability of SH-SY5Y after exposure to $A\beta_{25-35}$, and together with t3ss derivative, evaluated by MTT reduction method.** Undifferentiated SH-SY5Y cells were exposed to 10 μM amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 2 and 6 h, as showed in figure. Obtained

values of cells survival, are the average from three independent experiments, and analyzed by MTT reduction assay as described in *Methods*. Data were further analyzed by using Graph Pad 5.0 *software*, and treatment compared with 100% of cells survival, represented by dotted line in figure, as control. Not statistically significant differences have been observed by consider the viability of cells in presence of amyloid alone and together with t3ss derivative.

A similar effect but more evident and significant was observed by considering the treatment with 25 μM $A\beta_{25-35}$ and in combination with 5 μM t3ss (fig. 49). These results showed a more toxic effect of the used concentration of $A\beta_{25-35}$ indicating a dose and time dependent toxicity of amyloid-beta peptide towards SH-SY5Y cells. At the same time, we observed a more evident effect of recovery from Aβ toxicity when were treated at the same time with 5 μM t3ss, indicating in this case, like observed before for treatment with L-Glutamate, a protective effect of t3ss derivative in undifferentiated cells.

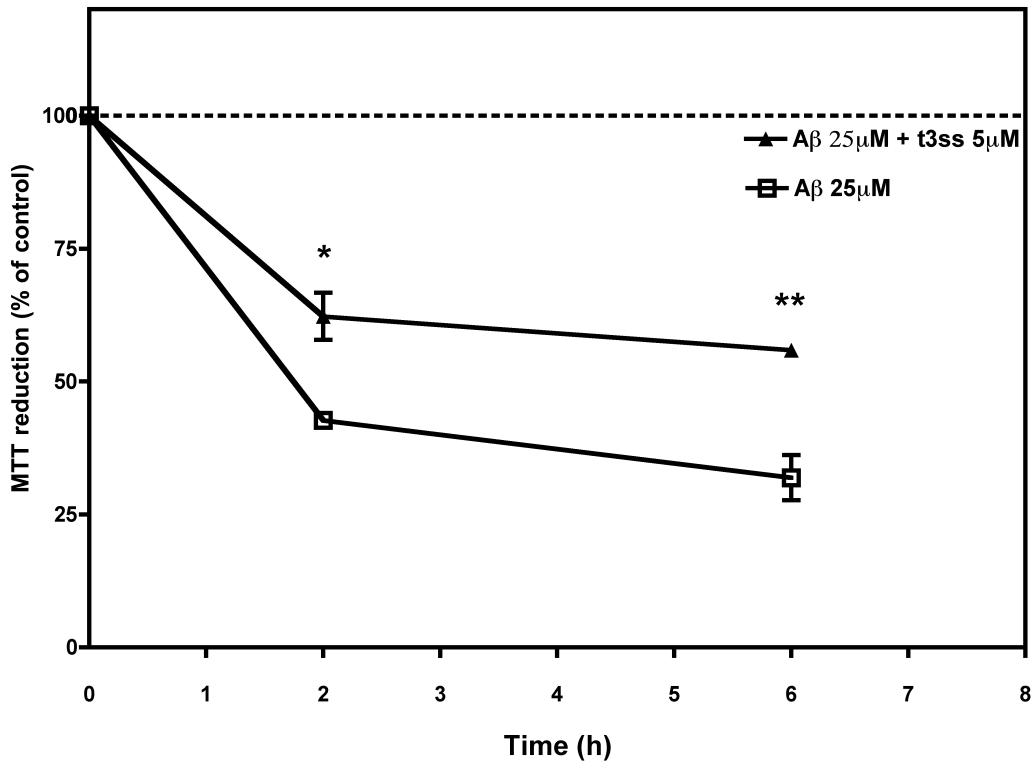


Figure 49. **Viability of SH-SY5Y after exposure to $A\beta_{25-35}$,and together with t3ss derivative, evaluated by MTT reduction method.** Undifferentiated SH-SY5Y cells were exposed to 25 μM amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 2 and 6 h, as showed in figure. Obtained values of cells survival, are the average from three days independent experiments, and analyzed by MTT reduction assay as described in *Methods*. Data were further analyzed by

using Graph Pad 5.0 *software*, and treatment compared with 100% of cells survival, represented by dotted line in figure, as control. *p<0,05 significantly different from the correspondent treatment with Aβ alone ; **p<0,01 significantly different from the correspondent treatment with Aβ alone.

Furthermore, t3ss derivative appear to have an high and interesting efficacy if we consider the treatment with 25 μM $A\beta_{25-35}$ alone at 6h in which we a observed a loss of viability until 30% of control. The combination with 5 μM t3ss at the same time of exposure, allow that viability of cells is maintaining around 70% of control. That means a recovery of 45% of cellular death elicited by Aβ treatement. That could be promising in future tests for the activity of [60]fullerene derivative. That protective effect can also be observed, although not significantly, during the treatment of differentiated SH-SY5Y cells with $A\beta_{25-35}$ and its combination with t3ss derivative, at the same concentration as before (fig. 50). Thus, by considering the viability of differentiated cells exposed to amyloid-beta peptide 10-25μM for 6h, and in the presence of 5 μM t3ss, we observed a tendency to protect cells, even the loss of viability in the case of cells treated with amyloid alone (fig. 50), is less evident respect to the same treatment in undifferentiated cells (fig. 49-50). Like observed after treating differentiated cells with L-Glu, a same highly toxic treatment is more tolerated in differentiated cells, than Aβ in this case in undifferentiated. In that sense we observed a certain resistance of differentiated cells to toxic treatment, due most probably to the differentiation processes. Anyway, it is maintained the tendency of protective effect by t3ss derivative (fig. 50). The next step was to evaluate the gene expression of target involved in neurodegeneration and discussed before, by considering the same time of exposure, the same concentration of $A\beta_{25-35}$ and t3ss derivative using to perform the viability in undifferentiated cells. We focused only in the gene expression of undifferentiated treated cells, mainly because the amyloid peptide in differentiated cells is resulted to be less toxic than the undifferentiated and consequently the protective effect of t3ss derivative was less evident.

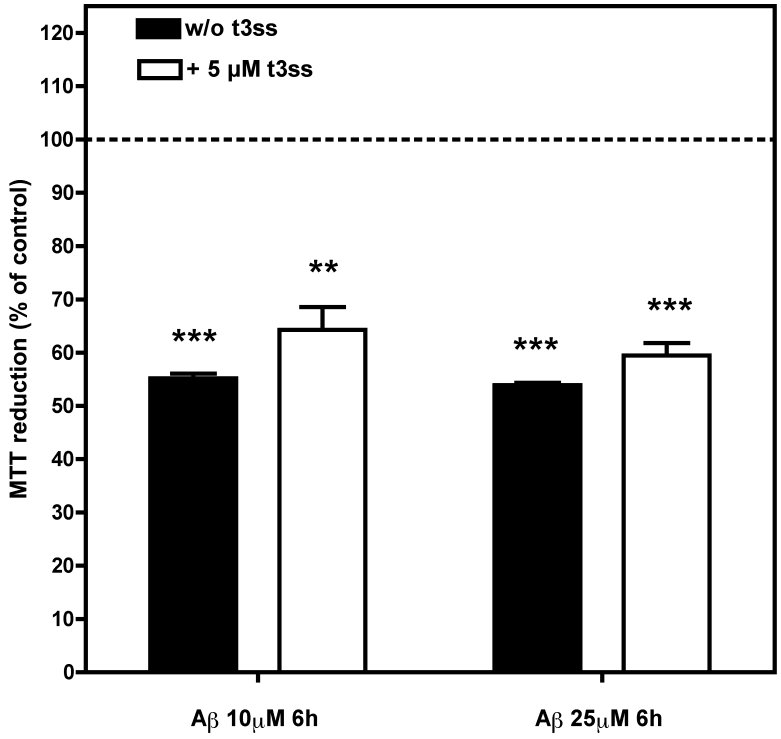


Figure 50. **Viability of differentiated SH-SY5Y after exposure to $A\beta_{25-35}$,and together with t3ss derivative, evaluated by MTT reduction method.** Undifferentiated SH-SY5Y cells were exposed to 10 -25 µM amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) and together with 5 µM [60]fullerene hydrosoluble derivative (t3ss) for 2 and 6 h, as showed in figure. Obtained values of cells survival, are the average from three days independent experiments, and analyzed by MTT reduction assay as described in *Methods*. Data were further analyzed by using Graph Pad 5.0 *software*, and treatment compared with 100% of cells survival, represented by dotted line in figure, as control. **p<0,01 significantly different from the control ; ***p<0,001 significantly different from the control.

The results by real time RT-PCR obtained by treating undifferentiated SH-SY5Y cells with $A\beta_{25-35}$ 10µM showed not significant differences with respect to the controls, mainly if we consider the combined treatment with t3ss 5µM. Anyway we observed a tendency to decrease the gene expression of the targets (fig. 51) after Aβ treatment. The treatment with 10 µM $A\beta_{25-35}$ combined with 5µ M t3ss seem to increase the gene expression of the target mainly respect to the treatment with amyloid alone. That tendency is evident for every considered gene target except for BACE-1.

The results from real time RT-PCR did not show any significant variations in gene expression of SH-SY5Y cells treated respect to their controls, except for some kind of tendency exerted by t3ss derivative in reversing the effect of 25 μM $A\beta_{25-35}$ at 6 h on the gene expression of certain targets. That is for example the case of gene expression of adenosine A_1 receptors, where a tendency to increase the gene expression by adding t3ss derivative to the treatment is observed. A similar tendency was also observed about gene expression of adenosine A_{2A} and A_{2B} receptors. Furthermore, we noticed a certain effect, although not significant, on gene expression of $mGlu_5$ and BACE-1, which were reduced probably by the $A\beta_{25-35}$ presence, if we consider the value respect to the treatment with t3ss derivative alone (fig. 52).

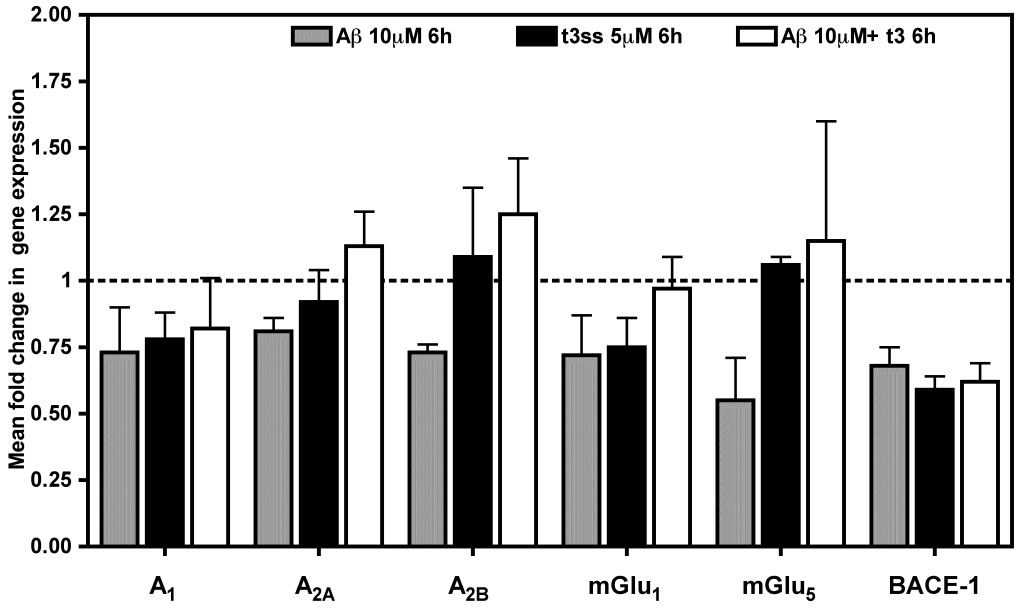


Figure 51. **Relative gene expression in undifferentiated SH-SY5Y cells, exposed to amyloid-beta peptide and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 10 μM amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h. The obtained extracted RNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. Control, corresponding to the dotted line.

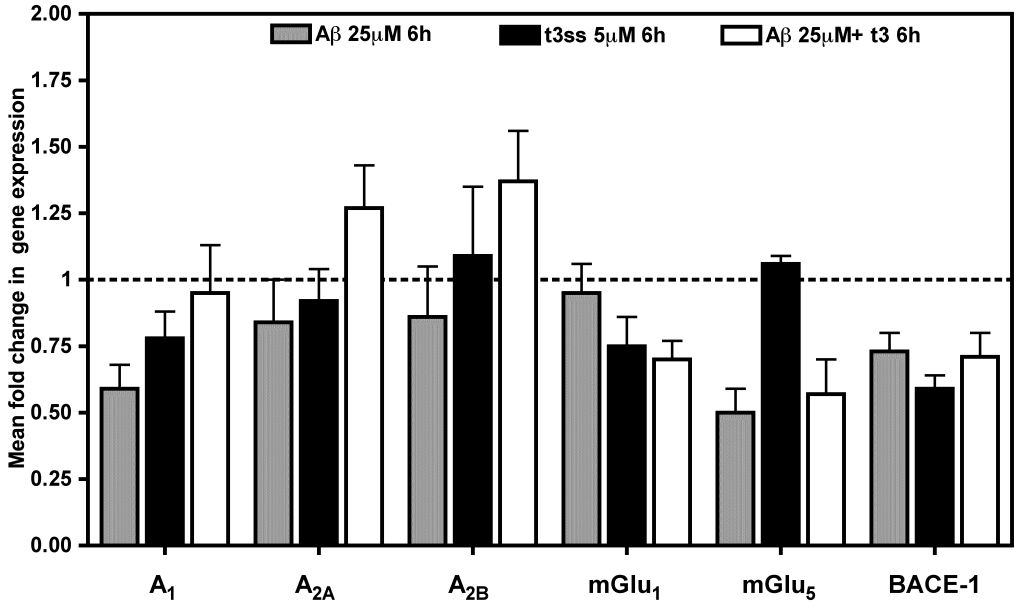


Figure 52. **Relative gene expression in undifferentiated SH-SY5Y cells, exposed to amyloid-beta peptide and together with t3ss derivative evaluated by using real time RT-PCR.** The relative gene expression analysis was performed on SH-SY5Y exposed to 25 μM amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) and together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) for 24 h. The obtained extracted RNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figures. Data obtained were analyzed on GraphPad 5.0 *software* and following what described in *Methods*. Control corresponding to the dotted line.

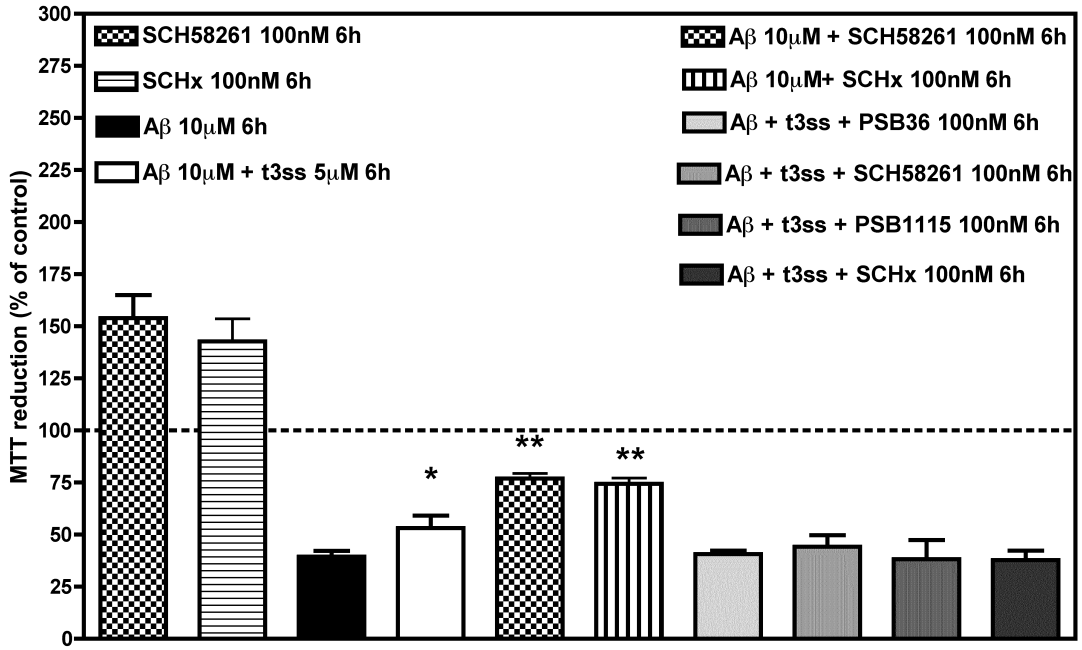
Excepting some cases, the observed tendency of the two considered treatment with amyloid-beta peptide in undifferentiated SH-SY5Y cells, is to decrease the gene expression of the most part of targets, while the treatment with t3ss alone seem to not affect the gene expression. At the same time, the contemporary presence of 5 μM t3ss during $A\beta_{25-35}$ treatment, revert the effect of amyloid alone on gene expression, what was observed for both concentrations of amyloid considered. Thus, these results make us suggest that the presence of t3ss derivative in toxic exposure of cells to amyloid-beta peptide could be related with a certain modulation of adenosine receptors mainly A_1, A_{2A} and A_{2B}, and BACE-1 gene. This modulation could have a role in the protection exerted by t3ss against Aβ toxicity.

5.1 Adenosine and metabotropic glutamate receptors antagonists on undifferentiated SH-SY5Y cells: study of the effect alone and in combination with L-Glutamate, amyloid-beta peptide and t3ss [60]fullerene derivative.

In this section we analyzed the effect of adenosine receptors antagonist type A_{2A}(SCH58261) and A_3(SCHx), previously synthesized by prof. Piero Spalluto from University of Trieste (Italy), beeing the second one as newest compound not yet fully tested. We focused first on the effect of the antagonist alone and subsequently the contemporary treatment with L-Glutamate, amyloid-beta peptide and t3ss [60]fullerene derivative on undifferentiated SH-SY5Y cells. Furthermore, in the case of contemporary use of antagonist with toxic and t3ss treatments, we considered to use more antagonist like ones for A_1(PSB36) and A_{2B}(PSB1115) adenosine receptors, and metabotropic glutamate receptors $mGlu_1$(JNJ16259) and $mGlu_5$(MPEP), in order to see if there were some relationship between the observed protective effect of t3ss on cells, and its action on some of these and previously considered receptors. We first considered the activity on viability of undifferentiated SH-SY5Y cells of the adenosine receptors antagonist A_1 (PSB36), A_{2A} (SCH58261), A_{2B} (PSB) and A_3(SCHx) in combination with amyloid-beta peptide (Aβ) and t3ss derivative. Results from MTT reduction assay, indicated that these A_{2A} and A_3 antagonists induced a positive effect on cells growth, in the sense that they seem to stimulate cell growth in a consistent way even after only 6h of treatment (fig. 53 panel A). The profile of viability for undifferentiated SH-SY5Y cells after 6h treatment with 10 μM Aβ and in combination with 5 μM t3ss and adenosine receptors antagonist (all at 100nM), showed a loss of efficacy in the protective effect of t3ss against toxicity evoked by amyloid-beta peptide. In particular, like previously observed, the contemporary use of t3ss derivative during the toxic treatment with amyloid-beta peptide, partially and significantly recovered cells from death. However the contemporary use of antagonist of adenosine receptors and t3ss derivative during a toxic exposure of cells to 10μM Aβ for 6h, seems to avoid the observed protective effect of t3ss derivative (fig. 53 panel A). At

the same time by treating SH-SY5Y cells with amyloid-beta peptide and t3ss derivative for 6h and at the same concentration as above, and by using instead of adenosine antagonist receptors, the metabotropic glutamate antagonist receptors of $mGlu_1$ (JNJ1625) and $mGlu_5$(MPEP) , we observed a similar effect on viability of cells, with the loss of the protective effect of t3ss derivative against toxicity evoked by 10 μM Aβ, when these antagonists are contemporary used (fig. 53 panel B). When cells have been exposed for 6h to amyloid-beta peptide 10μM in presence of antagonist of A_{2A} and A_3, SCH58261 and SCHx, there was a great recovery of cells from amyloid toxicity, with an induced growth of cells (fig. 53 panel A).

A



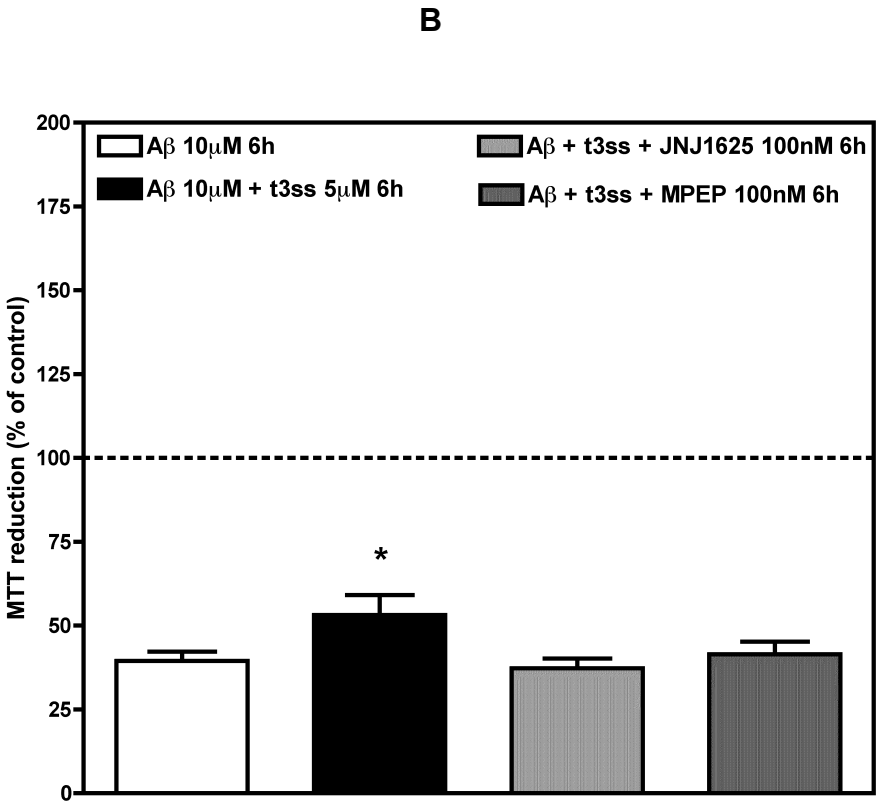


Figure 53. **MTT reduction viability assay of undifferentiated SH-SY5Y cells treated with amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) alone and in combination with t3ss, and both in combination with adenosine and metabotropic glutamate receptors antagonists**. (**A**) Undifferentiated cells were 6 h treated with 100 nM of adenosine A_{2A} (SCH58261) and A_3 (SCHx) receptors antagonist, with 10 μM amyloid-beta peptide (Aβ) alone and together with 5 μM [60]fullerene hydrosoluble derivative, with 10 μM amyloid-beta peptide (Aβ) together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) and in combination with 100 nM adenosine A_1(PSB36), A_{2A}(SCH58261), A_{2B}(1115), A_3(SCHx) receptors antagonist as showed in figure. (**B**). Undifferentiated SH-SY5Y cells 6h treated with 10 μM amyloid-beta peptide (Aβ) alone and together with 5 μM t3ss and both in combination with 100 nM $mGlu_1$(JNJ1625), $mGlu_5$(MPEP) receptors antagonist as showed in figure.

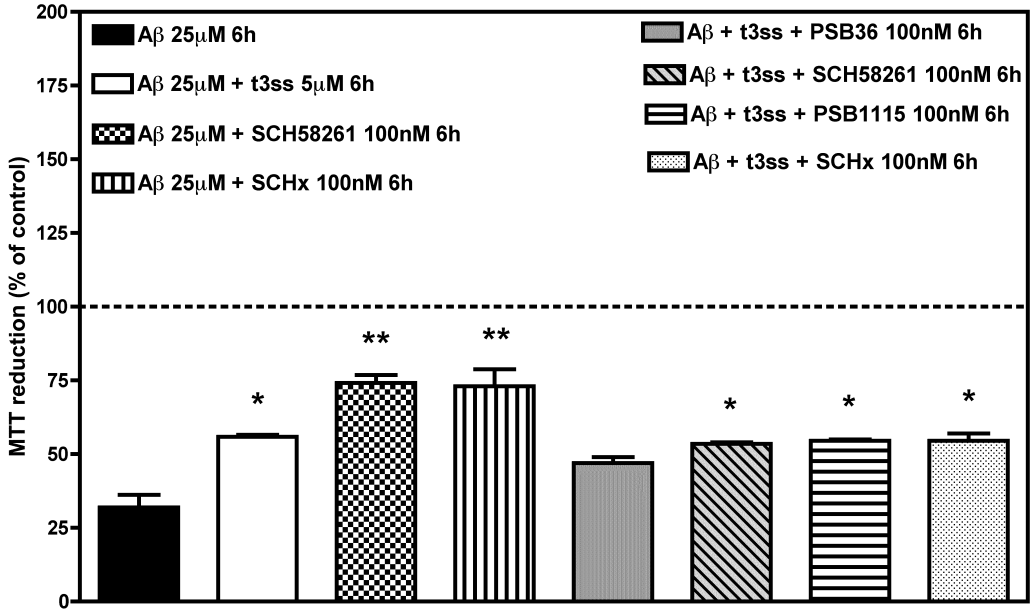
Values of cells survival were found by MTT reduction assay as reported in *Methods*, and further analyzed by using GraphPad ver. 5.0 as *software* for statistical analysis. Results herein reported were collected from three independent days assays, and each value of treatment correspond to the analysis of at least 8-10 values of MTT absorbance. Control is considered as 100% of cell survival, and represented in figure as a dotted line. *$p<0,05$ significantly different from the treatment with amyloid-beta peptide alone. **$p<0,01$ significantly different from the treatment with amyloid-beta peptide alone.

These results were followed by the observation that the SH-SY5Y cell survival by treating with 25 μM amyloid-beta peptide for 6h, in the presence or the absence of t3ss derivative and the contemporary use of adenosine and metabotropic glutamate receptors antagonist, in the same conditions as

previously described, had a different behaviour. In this case we observed that the contemporary use of adenosine receptors antagonist, without any significant difference between values, both with 5μM t3ss treatment, maintained the observed protective effect of t3ss derivative against amyloid toxicity (fig. 54 panel A). On the other hand, by considering the treatment of SH-SY5Y cells with 25 μM amyloid for 6h, alone and in combination with antagonist of adenosine receptors A_{2A}(SCH58261) and A_3(SCHx), we observed, as previously for the treatment with 10 μM Aβ, a great and significant recovery of cells from amyloid toxicity, even greater than by using t3ss 5μM during the same treatment (fig. 54 panel A).

Furthermore, by using the same treatment as previously with 25 μM amyloid-beta peptide and 5 μM t3ss but in the presence or the absence of $mGlu_1$(JNJ1625) and $mGlu_5$(MPEP) metabotropic glutamate receptors antagonist we also observed the same survival profile as before, in the sense that the protective effect of t3ss against amyloid toxicity by their contemporary use, is maintained (fig. 54 panel B).

A



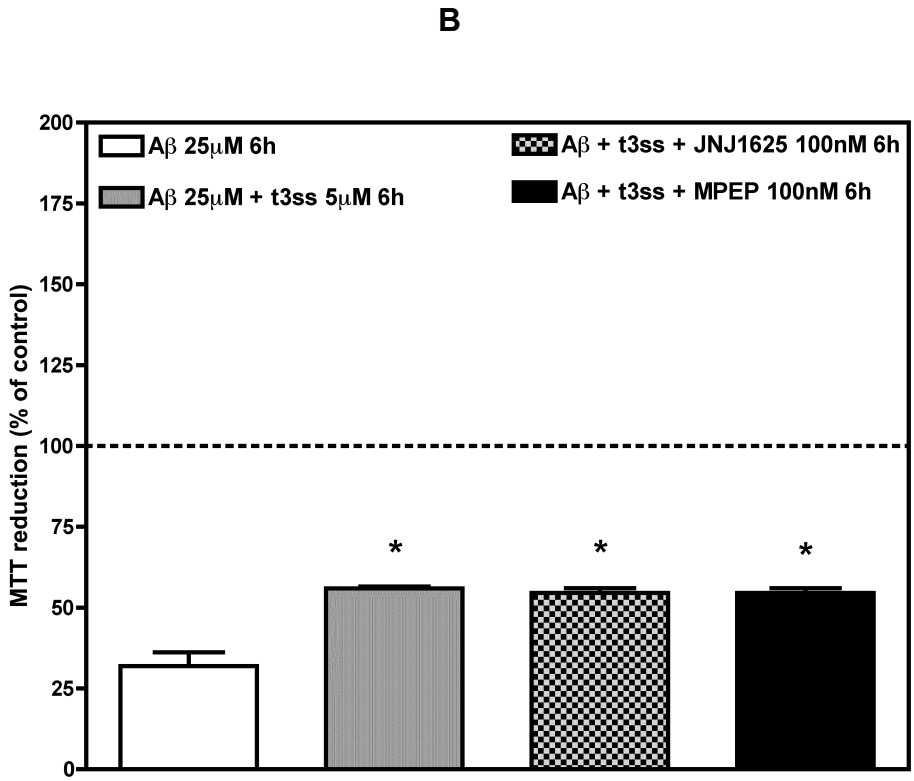


Figure 54. **MTT reduction viability assay of undifferentiated SH-SY5Y cells treated with amyloid-beta peptide fragment 25-35 ($A\beta_{25-35}$) alone and in combination with t3ss, and both in combination with adenosine receptors antagonists**. (**A**) Undifferentiated cells were 6 h treated with 25 μM amyloid-beta peptide (Aβ) alone and together with 5 μM [60]fullerene hydrosoluble derivative, with 25 μM amyloid-beta peptide (Aβ) together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) and in combination with 100 nM adenosine A_1(PSB36), A_{2A}(SCH58261), A_{2B}(1115), A_3(SCHx) receptors antagonist as showed in figure. (**B**). Undifferentiated SH-SY5Y cells 6h treated with 25 μM amyloid-beta peptide (Aβ) alone and together with 5 μM t3ss and both in combination with 100 nM $mGlu_1$(JNJ1625), $mGlu_5$(MPEP) receptors antagonist as showed in figure.

Values of cells survival were found by MTT reduction assay as reported in *Methods*, and further analyzed by using GraphPad ver. 5.0 as *software* for statistical analysis. Results herein reported were collected from three independent days assays, and each value of treatment correspond to the analysis of at least 8-10 values of MTT absorbance. Control is considered as 100% of cell survival, and represented in figure as a dotted line. *$p<0,05$ significantly different from the treatment with amyloid-beta peptide alone. **$p<0,01$ significantly different from the treatment with amyloid-beta peptide alone.

As we previously observed about SH-SY5Y cells survival data, after treating 6 h with amyloid-beta peptide, t3ss, and described antagonists, is not applicable to what we observed by treating cells with 100μM L-Glutamate in

the presence of t3ss and in the absence or the presence of adenosine/glutamate metabotropic receptors antagonists. Survival data by using L-Glutamate instead of amyloid and t3ss in combinations with different receptors antagonist, revealed an increase in the protective effect against L-Glutamate toxicity by using t3ss and those antagonist at the same time. In particular we observed that by using a combination of 5μM t3ss and adenosine receptors antagonist, the protective role of t3ss was enhanced when blocking A_1 receptors (PSB36), and (to higher extend) when blocking A_{2A} (SCH58261), A_{2B} (PSB1115) and A_3 (SCHx) receptors (fig. 55 panel A). Furthermore, an increase in the protective effect of t3s derivative was also observed by the contemporary use of metabotropic $mGlu_1$ and $mGlu_5$ receptors antagonist, JNJ 1625 and MPEP respectively, the second one being little more effective (fig. 55 panel B). These described results may be explained by a more consistent influence of L-Glutamate treatment on adenosine and metabotropic glutamate receptors with respect to what we previously observed for amyloid-beta peptide. Nevertheless, if we consider, as previously for amyloid-beta peptide, results of viability obtained by treating cells with 100 μM L-Glutamate at 6 h alone and in presence of adenosine A_{2A} (SCH58261) and A_3 (SCHx) receptors antagonist, we observe that the protective effect of these compounds against L-Glutamate toxicity, is more than simply significant, it is surprising. In fact, a great protective effect is provided by these two antagonists, by inducing cells growth, like what observed in the case of cells survival in presence of those antagonist alone, (fig. 55 panel A). The most surprising, it's that the recovery effect against L-Glutamate toxicity is greater than the corresponding observed for t3ss derivative alone, suggesting that by antagonizing these two receptors, like observed for amyloid-beta peptide, it could be possible to limiting the toxic effect of neurotoxic compounds. Therefore A_{2A} and A_3 adenosine receptors, seem to be involved in the mechanism of cells degeneration.

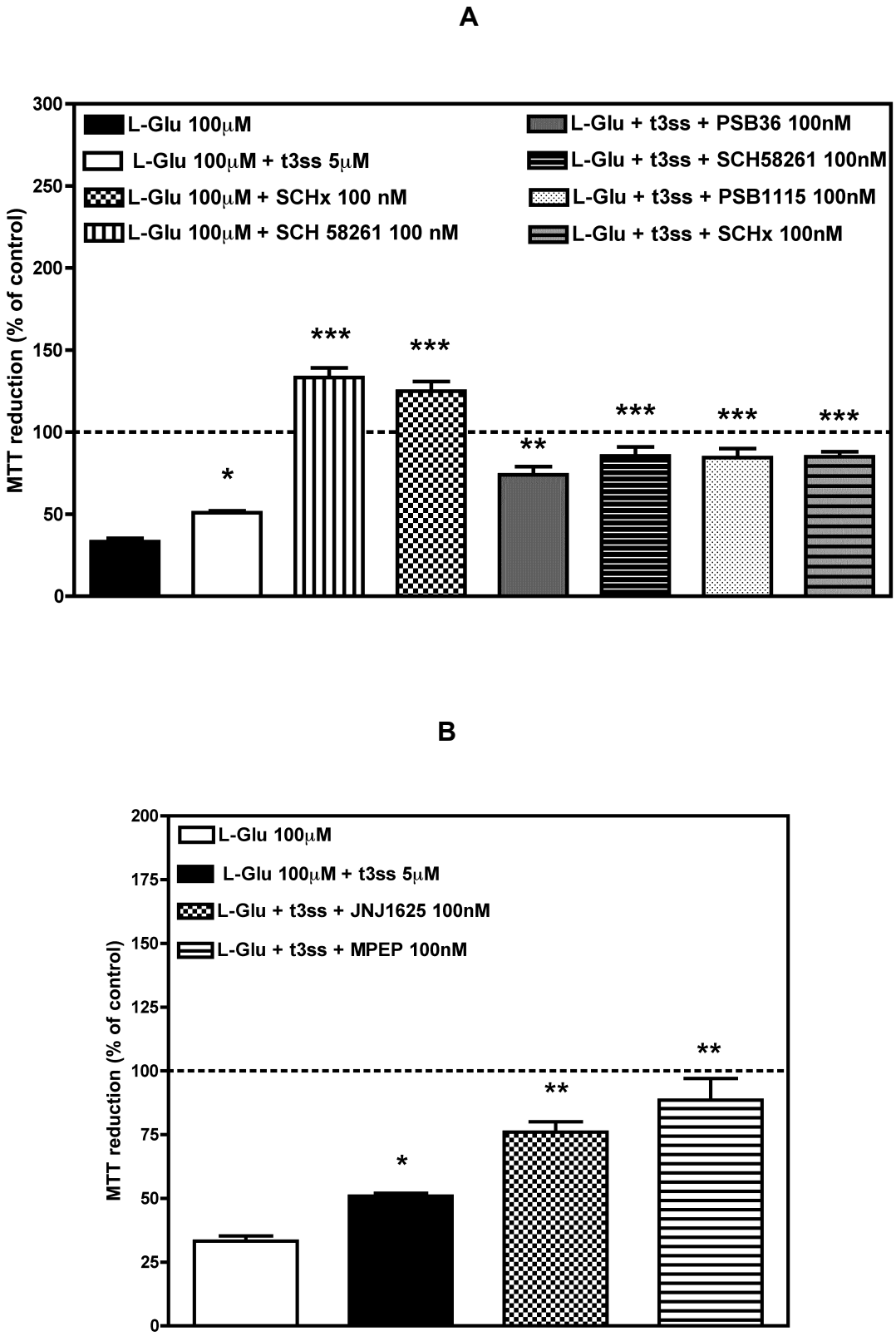


Figure 55. . **MTT reduction viability assay of undifferentiated SH-SY5Y cells treated with L-Glutamate and in combination with t3ss, and both in combination with adenosine and metabotropic glutamate receptors antagonists**. (**A**) Undifferentiated cells were 6 h treated with 100 μM L-Glutamate (L-Glu) alone and together with 5 μM [60]fullerene hydrosoluble derivative, and 100 nM of adenosine A_{2A} (SCH58261) and A_3 (SCHx) receptors

antagonist, with 100 μM L-Glutamate (L-Glu) together with 5 μM [60]fullerene hydrosoluble derivative (t3ss) and both in combination with 100 nM adenosine A_1(PSB36), A_{2A}(SCH58261), A_{2B}(1115), A_3(SCHx) receptors antagonist as showed in figure. (**B**) Undifferentiated SH-SY5Y cells 6h treated with 100 μM L-Glutamate (L-Glu) alone and together with 5 μM t3ss and both in combination with 100 nM $mGlu_1$(JNJ1625), $mGlu_5$(MPEP) receptors antagonist as showed in figure.

Values of cells survival were found by MTT reduction assay as reported in *Methods*, and further analyzed by using GraphPad ver. 5.0 as *software* for statistical analysis. Results herein reported were collected from three independent days assays, and each value of treatment correspond to the analysis of at least 8-10 values of MTT absorbance. Control is considered as 100% of cell survival, and represented in figure as a dotted line. *$p<0,05$ significantly different from the treatment with L-Glutamate alone; **$p<0,01$ significantly different from the treatment with L-Glutamate alone; ***$p<0,001$ significantly different from the treatment with L-Glutamate alone.

In the following section we describe results obtained by treating undifferentiated SH-SY5Y cells with adenosine A_{2A}(SCH58261) and A_3(SCHx) receptors antagonist for 6h of exposure at 100 nM, in order to evaluate if they are able to produce any effect on gene expression. Using RT-PCR technique and considering the same pattern of gene target receptors used and fully described in previous sections, we evaluated the effect of such antagonist on gene expression. From RT-PCR data we observed a clear and significant effect on the gene expression of receptors $mGlu_1$ and $mGlu_5$ (fig. 56). Furthermore the considered treatment act in opposite way on gene receptors expression, in the sense that we observed how adenosine receptors antagonist decrease the gene expression of $mGlu_1$ receptors and increase the gene expression of $mGlu_5$. The observed modulation by adenosine A_{2A} (SCH58261) and A_3 (SCHx) receptors antagonist, on gene expression of $mGlu_1$ and $mGlu_5$, could be related to the protective effect exhibited by these, on undifferentiated SH-SY5Y cell survival, during treatment with 25 μM amyloid-beta peptide, as well as in the case of treatment with 100 μM L-Glutamate, as previously discussed (figures 53, 54 and 55).

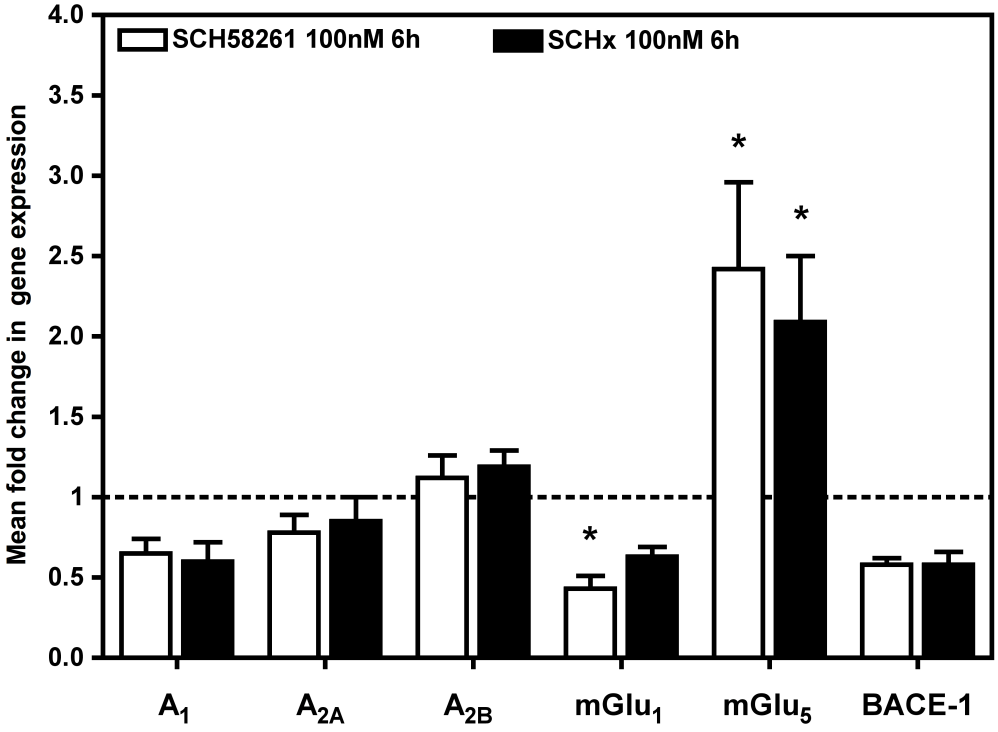


Figure 56. **Relative gene expression evaluated by RT-PCR technique on undifferentiated SH-SY5Y cells exposed to 6 h adenosine receptors antagonist.** Undifferentiated SH-SY5Y cells were exposed for 6 h to 100 nM of adenosine A_{2A}(SCH58261) and A_3 (SCHx) receptors antagonist, thus the extracted mRNA were processed as reported in *Methods*, analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Results of relative gene expression presented in figure were analyzed on GraphPad ver. 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent days of experiments. Control is showed in figure as a dotted line. *p<0,05 significantly different from the control.

6.1 Effect of [60]fullerene hydrosoluble derivative t3ss on undifferentiated SH-SY5Y cells during hypoxic conditions.

In the following section we report results obtained by studying the effect of [60]fullerene hydrosoluble derivative on undifferentiated SH-SY5Y cells exposed to low concentrations (5%) of O_2, and for that reason under conditions of oxidative stress, in order to have an *in vitro* model of what usually occur *in vivo* neuronal cells during ischemic damage. We first evaluated the survival of cells, by MTT reduction assay, in the absence and/or in the presence of t3ss derivative at increasing concentrations. We've chosen

to expose undifferentiated SH-SY5Y cells to 6 and 24 h of hypoxic conditions in the presence of t3ss derivative. We observed that cells suffered a relevant toxicity during hypoxic conditions with a 50% of cell survival in the case of 6 h exposure and only 40% of survival after 24 h of exposure (fig. 57). On the other hand by considering 6h exposure of cells to hypoxia, t3ss derivative exhibited a protective effect that is concentration-dependent, reaching values of total recovery at the highest concentration used. We also noticed that by adding t3ss derivative to cells during exposition to hypoxia for 24 h, viable cells reach a plateau stage in the case of the 24 h hypoxia, meaning that t3ss, even at high concentrations is unable to completely recover cells from hypoxia, probably because of the hard stress conditions, the cells are subjected. That is understandable because the duration of the treatment is longer (24 h) and cells are in time to activate compensation and protective mechanism against free radical damage, helped by t3ss derivative acting like radical scavenger as probably happens at 6h of hypoxia.

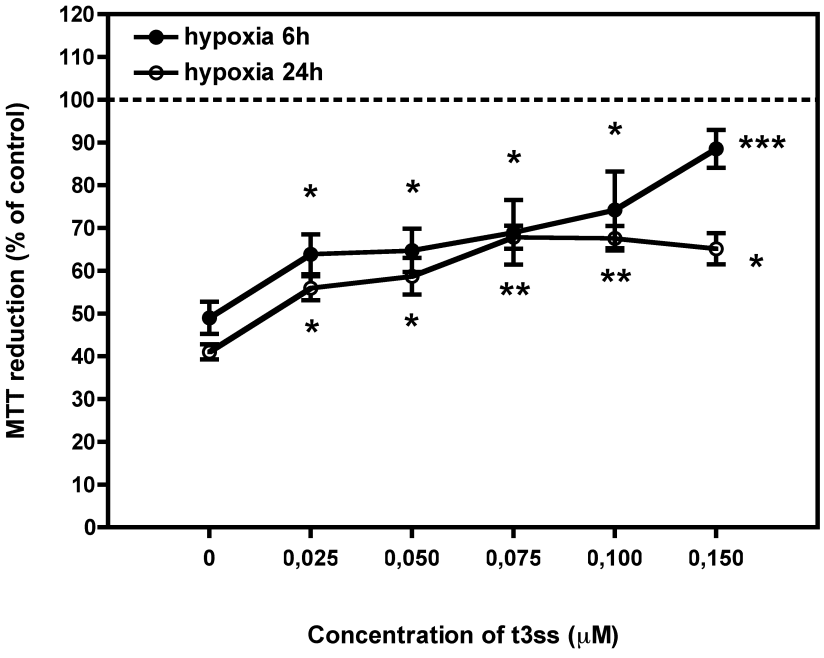
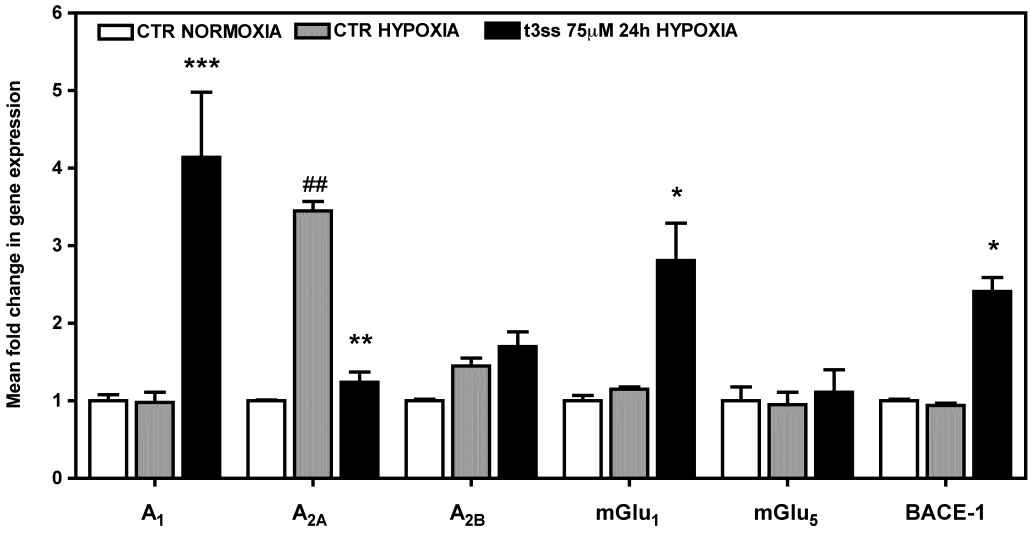


Figure 57. **MTT reduction viability assay on undifferentiated SH-SY5Y cells submitted to hypoxic conditions (5% of O_2 and 95% of N_2) in the absence or the presence of t3ss derivative**. Undifferentiated SH-SY5Y cells were subjected to 6 and 24 h of hypoxic conditions, as indicated in title, in presence or absence of different concentrations of [60]fullerene hydrosoluble t3ss derivative (t3ss), as showed in figure. The obtained values are the average from three independent experiments, collected following MTT reduction assay protocol as described in *Methods*. Data were thus analyzed to statistically compared them by

using GraphPad 5.0 *software*. The dotted line indicates the 100% of cells survival intended as cells growing in normal oxygen conditions (25% of O_2). *p<0,05 significantly different from cells survival in absence of t3ss derivative; **p<0,01 significantly different from cells survival in absence of t3ss derivative;***p<0,001significantly different from cells survival in absence of t3ss derivative .

We thus proceed to investigate the receptors involved in hypoxia by treating undifferentiated SH-SY5Y cells with 75μM t3ss for 24, 48 and 72 h in hypoxic conditions. We evaluated the expression for the same gene targets as described and considered until now, in order to quantify any interesting change that could be related to the protective effect of t3ss previously describe. We considered only one concentration of t3ss for that purpose, the one that demonstrated to be effective for both time of exposure to hypoxia and with the same values of cell recovery. We also decided to not investigate the acute exposure to hypoxic conditions (6h) by the point of view of gene expression, concentrating our study more on long term exposure (for more explanations and details see discussion). The RT-PCR values revealed a significant effect of t3ss derivative on the most part of considered gene targets. Thus we have for example that at 24h of hypoxic conditions t3ss derivative significantly increase, respect to the hypoxic controls, the gene expression of A_1 adenosine receptors, decreased the gene expression of A_{2A} adenosine $mGlu_1$ receptors and BACE-1 (fig. 58 panel A).

A



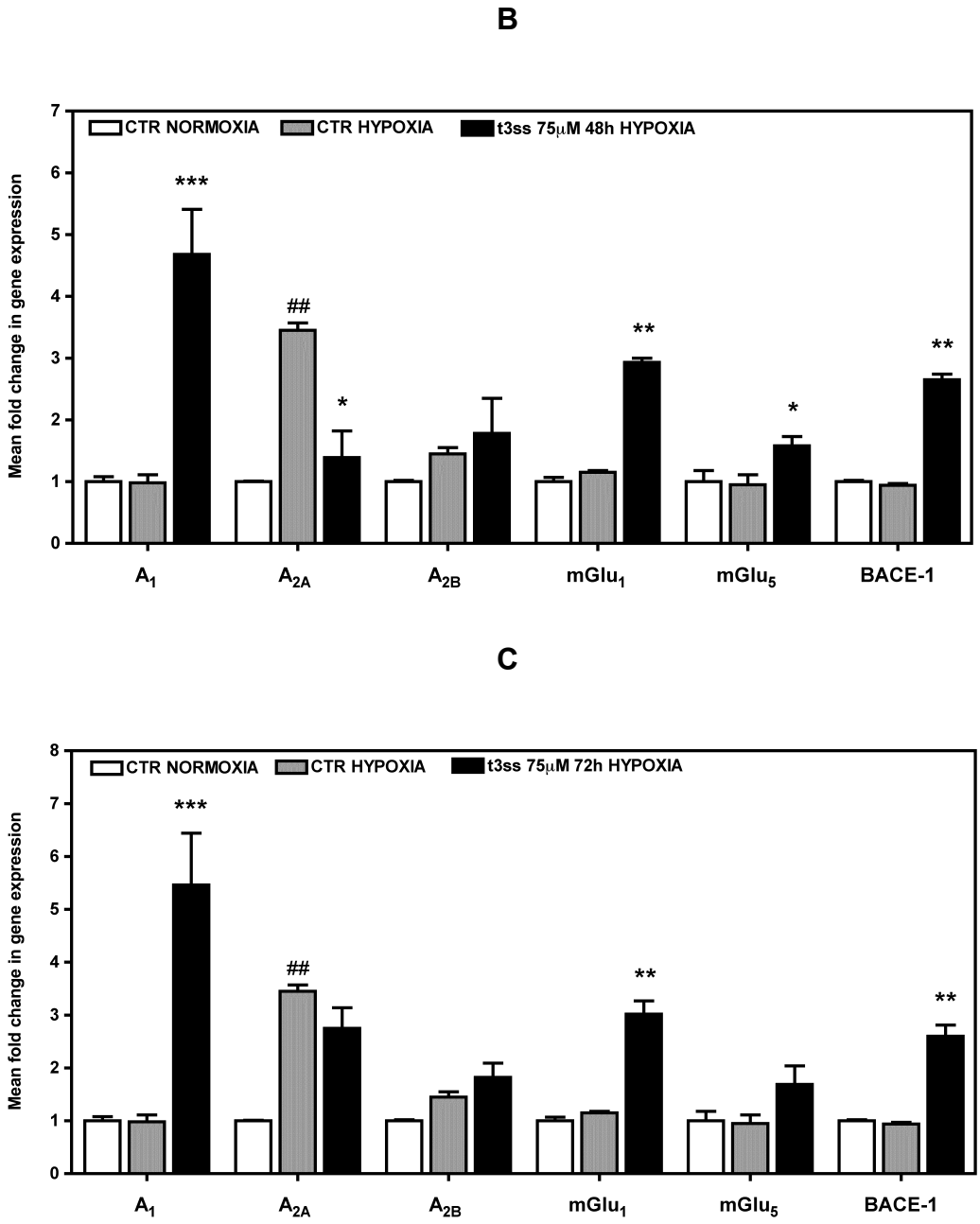


Figure 58. **Gene expression evaluated by RT-PCR technique in SH-SY5Y cells exposed to hypoxic conditions**. Undifferentiated SH-SY5Y cells were subjected to hypoxic conditions (5 % of O_2 and 95% of N_2) in the absence (CTR HYPOXIA) and presence of 75 μM t3ss derivative for 24 h (**A**), 48 h (**B**) and 72 h (**C**). The mRNA extracted from cells after exposure to these conditions were analyzed following the RT-PCR protocol, as described in *Methods*, and compared to mRNA extracted from undifferentiated SH-SY5Y cells not subjected to hypoxic conditions (CTR NORMOXIA). The obtained extracted mRNA was analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Values obtained are the average of three independent days exposition of cells to hypoxic or normoxia conditions, and compared by using GraphPad ver. 5.0 as *software* for statistical analysis. ## $p<0,01$ significantly different from CTR NORMOXIA values; * $p<0,05$ significantly different from CTR HYPOXIA values; **$p<0,01$ significantly different from CTR HYPOXIA values.

Like observed for the following exposure-time of cells to hypoxic conditions, the profile of gene expression at 48 and 72 h is maintained quite similar than the initial exposure for 24h. In particular, we have that t3ss derivative is inducing a great increase of gene expression for A_1 adenosine receptors, which is higher for longer time of treatment (fig. 59 panel B and C). Furthermore while the profile of gene expression for BACE-1, or $mGlu_1$ and $mGlu_5$ is not suffering relevant difference also considering the consequently times of exposure to hypoxic conditions, we noticed that gene expression of A_{2A} adenosine receptors is growing about the treatment with t3ss derivative, from the first time of exposure to the last (fig. 59 panel A-B-C). It is clear from results of gene expression that during hypoxia conditions, no relevant changes were observed in gene expression of hypoxic controls respect to the normoxic controls, except by considering the gene expression of A_{2A} adenosine receptors, in which we notice an increase of gene values of these respect to normoxia controls and respect to those of t3ss derivative treated. In particular, we assisted to a constant and significant effect of t3ss derivative on all the considered gene target and time of exposition to hypoxic conditions. Nevertheless if we consider that t3ss derivative induced a significant increase in the most part of considered gene target, we also have noticed that t3ss derivative caused a decrease of A_{2A} adenosine receptors gene expression with respect to the hypoxic control, mainly for 24 h and 48 h of exposure to hypoxic conditions. After 72 h of exposure to low concentrations of O_2 we observed that t3ss derivative is loosing its effect of counterbalancing the effect of hypoxia in the gene expression of A_{2A} adenosine receptors. Only in this case in fact, we assisted to an opposite effect respect of all the considered targets, in which the tendency of t3ss derivative is to increase the gene expression, respect to hypoxia controls. In the described case for adenosine A_{2A} receptors, is clear that the effect of t3ss derivative is to maintain low the value of gene expression, against the hypoxia tendency to increase that expression.

7.1 Effect of [60]fullerene hydrosoluble t3ss derivative on rat cortical neurons exposed to toxic concentrations of L-Glutamate and amyloid-beta peptide.

We now consider the results obtained by treating neuronal cells from brain of foetuses rat (for specifications see *Material* and *Methods*), with toxic concentrations of L-Glutamate and amyloid-beta peptide in the presence or the absence of t3ss derivative, in order to describe a possible effect of [60]fullerene derivative on this cellular model (different from human cells model). First, we evaluated the profile of gene expression, for the same target gene previously described, during at least two weeks of culture of rat's neurons, and by extracting mRNA from cells every two day. As figure 61 shows, the expression of gene we were interested on, changed along the culture time (Scheme of figure 59).

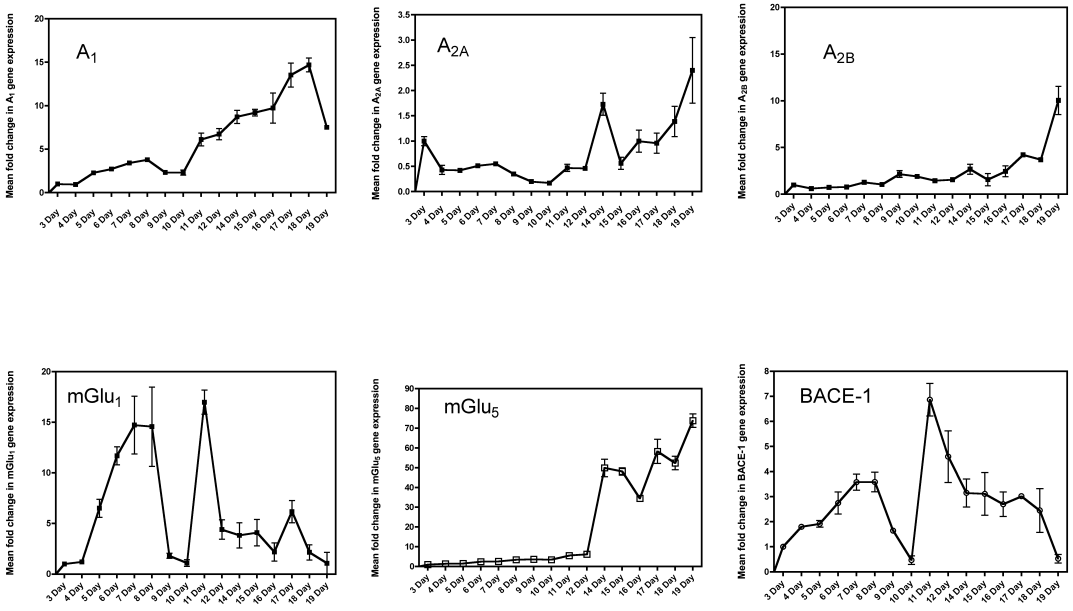


Figure 59. **Profile of gene expression, evaluated by RT-PCR technique, during 19 days of normal incubation and maintenance of rat's neuronal cells.** Rat's cortical neurons were normally incubated as described in *Methods*, and mRNA extracted at designed day, from day 3 consecutively to day 19. The following gene targets have been evaluated (from up left to bottom right): A_1, A_{2A}, A_{2B}, $mGlu_1$, $mGlu_5$ and BACE-1. Values of gene expression, were obtained according to RT-PCR assay protocol as described in *Methods*, by RNA extracted

from neuronal cells derived from cortical region of brain of three days independent sacrificed rats. Data of relative gene expression were analyzed on GraphPad 5.0.

We noticed that except for $mGlu_1$ and BACE-1 gene targets, the remaining seem to increase the expression by passing time. Furthermore, we assisted to a dramatic decrease during the last days of cells life; instead the other targets tended to maintain a more regular profile by increasing gene expression during the last period. We decided to continue the study with t3ss derivative and toxic treatments in neuronal cells from rat's brain, by using cells between the day 9 and day 14 in culture. Then we evaluated the survival of neuronal cells in the presence of toxic concentrations of L-Glutamate and amyloid-beta peptide and with the contemporary presence of t3ss derivative, by using MTT reduction assay. The results obtained by treating cells with 100 μM L-Glutamate showed a moderate high toxicity after 2h of exposure, and a very high toxicity after 6h of treatment. At the same time by contemporary use of t3ss derivative, we assisted to a partial but significant recovery of cells (fig. 60 panel A), as observed in other cell model previously used, for the same treatment. In the case of treatment with amyloid-beta peptide, we observed a moderate toxicity by using 10 μM of amyloid, and a toxicity from moderate to high when 25 μM amyloid was used. Both treatments were also depending on the duration of exposure. At the same time, by using t3ss derivative during amyloid treatment, a general tendency to recover cells was observed, although significant only in the case of treatment with amyloid 25μM 2h (fig. 60 panel B). Nevertheless, the already observed protective effect of t3ss derivative, appear to be confirmed even in the case of rat's neurons.

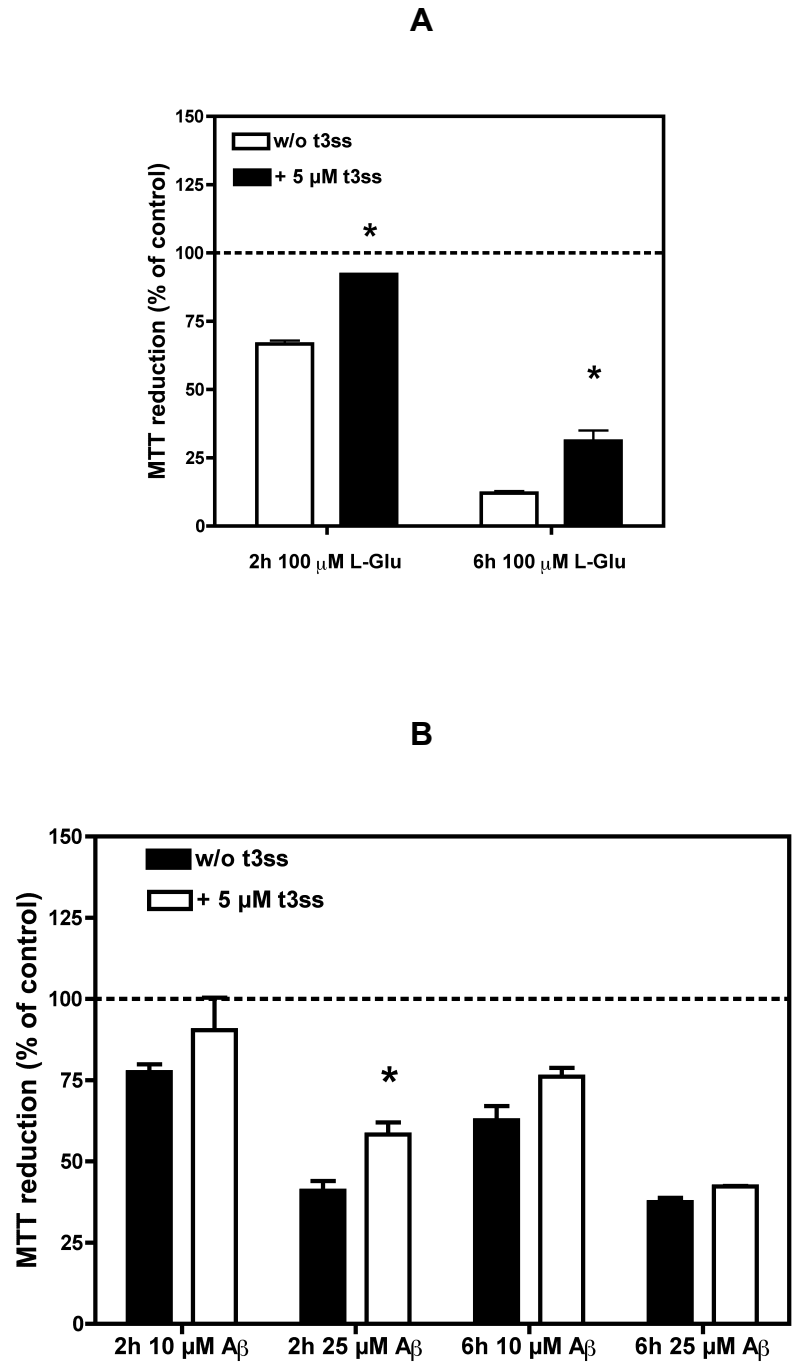


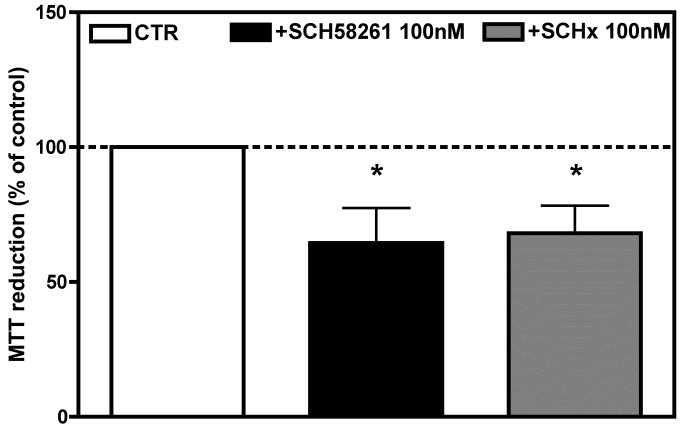
Figure 60. **MTT reduction assay to evaluate survival of rat's neuronal cells treated with L-Glutamate and amyloid-beta peptide and in presence of t3ss derivative**. Rat's neuronal cells growth between the day 9 and day 14 and maintained as described in *Methods*, were exposed for 2 and 6 h to 100 µM L-Glutamate and together with 5 µM [60]fullerene hydrosoluble t3ss derivative (t3ss) as showed in figure (**A**). Rat's neurons in the same growth conditions were also exposed for 2 and 6 h to 10 and 25 µM of amyloid-beta peptide (Aβ) and together with 5 µM t3ss derivative (t3ss) as showed in figure (**B**). Values obtain following the protocol of MTT reduction assay, as reported in *Methods*, were analyzed and compared from the average of three days independent rat's cells culture, deriving from three different sacrificed rat's brain, as reported in *Methods*. Data were thus statistically analyzed and compared by using GraphPad 5.0 *software*. *$p<0,05$ significantly different from control. Control as intended as 100% of cells survival, and indicated in figure as a dotted line.

7.2 Study of the effect of adenosine and metabotropic glutamate receptors antagonists on rat cortical neurons survival.

Similarly to that performed on SH-SY5Y cells, we evaluated neuronal cells survival in presence of antagonist of adenosine and metabotropic glutamate receptors. In particular we first evaluated the profile of survival for rat's neuronal cells in the presence of the two new antagonist of adenosine receptors A_{2A}(SCH58261) and A_3(SCHx), while the next step was to study the activity of a toxic treatment like L-Glutamate and in the presence of t3ss derivative, by contemporary using a specific receptor antagonists. Like explained before, the goal was to understand if there will be any variation, by using a specific antagonist, on the observed protective effect of t3ss derivative. However it must be underlined that the used receptor antagonists SCH58261 and SCHx are mainly active on human receptor. Nevertheless it was demonstrated to exist a partial similarity between the receptors of these two species, so the same compound could be effective on both receptors from species. Thus, by the treatment of rat's neurons with antagonist of adenosine receptors A_{2A} and A_3, we observed a moderate toxicity on cells survival (fig. 61 panel A), both at 100nM and for 6h treatment. On the other hand some interesting results were obtained from viability assay by treating cells with 100 μM L-Glutamate and in the presence of 5 μM t3ss both with adenosine receptors and metabotropic glutamate receptors antagonist, the same used for SH-SY5Y cells. It was interesting to notice that in the most part of considered treatments with glutamate antagonist and t3ss, no difference was observed between the contemporary presence of a specific antagonist, glutamate and t3ss and effect of the combination of t3ss and glutamate. However by using antagonist of A_1 and $mGlu_5$ we observed a difference with respect to the treatment with glutamate and t3ss derivative. In particular, by using A_1 antagonist PSB36 we assisted to a tendency to decrease the protective effect of t3ss (fig. 61 panel B), by using the $mGlu_5$ MPEP antagonist, the activity of t3ss derivative seems to be enhanced against L-Glutamate toxicity (fig 61 panel C). Thus, it was demonstrated that these antagonist, although more active on humans in the case of SCH58261 and

SCHx, may have some kind of activity on rat's receptors, and modulate the t3ss activity.

A



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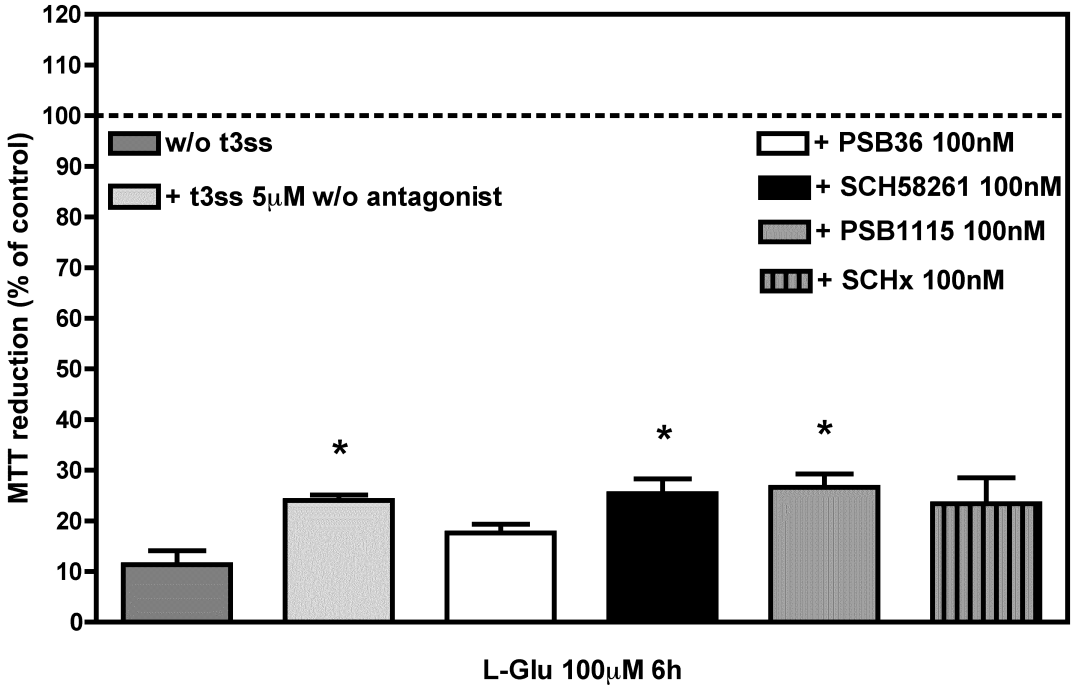


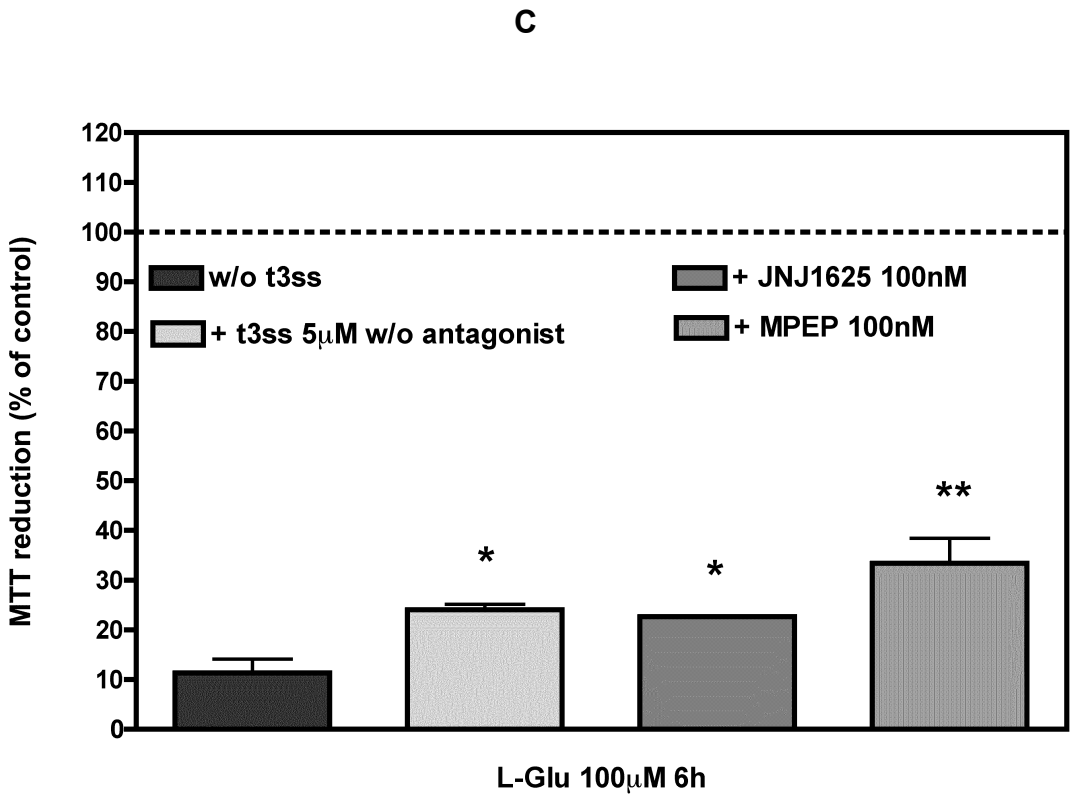


Figure 61. **MTT reduction viability assay of neuronal cells from rat's brain exposed to adenosine antagonist, and combination of L-Glutamate together with t3ss derivative and adenosine and metabotropic glutamate receptors antagonist**. (**A**) Rat's neurons were exposed for 6 h to 100 nM A_{2A} (SCH58261) and A_3 (SCHx) adenosine receptor antagonists.*p<0,05 significantly different from control bar, and also represented as a dotted line of 100% of cell survival. (**B**) Rat's neurons were exposed for 6 h to 100μM L-Glutamate (L-Glu) in the absence and in the presence of 5 μM [60]fullerene hydrosoluble derivative (t3ss) and 5 μM t3ss together with 100 nM A_1(PSB36), A_{2A}(SCH58261), A_{2B}(PSB1115), A_3(SCHx) adenosine receptor antagonist, as showed in figure. (**C**) Rat's neurons were exposed for 6 h to 100μM L-Glutamate (L-Glu) in the absence and in the presence of 5 μM [60]fullerene hydrosoluble derivative (t3ss) and 5 μM t3ss together with 100 nM and A_3(SCHx), $mGlu_1$(JNJ1625), $mGlu_5$(MPEP) receptor antagonists. Values obtained from MTT reduction assay are the result of the average of three independent days of experiments, each one from at least 8-10 MTT reduction values, following the protocol described in *Methods*. indicated time and concentrations. Data obtained from MTT reduction assay, were then analyzed by using GraphPad ver. 5.0 as *software* for statistical analysis. *p<0,05 significantly different from cell survival in presence of 100 μM L-Glutamate alone;**p<0,01 significantly different from cell survival in presence of 100 μM L-Glutamate alone. In all panels the Control values are represented by dotted line and as 100% of cells survival.

7.3 Study of the gene expression in rat cortical neurons during treatment with [60]fullerene derivative t3ss and L-Glutamate: effect of long-term exposure.

In order to complete the study on the activity of [60]fullerene derivative t3ss on rat's neuronal cells, we analyzed the receptors involved during the exposure to t3ss derivative, from the point of view of gene expression, evaluated by RT-PCR technique. In this case we considered two different treatment with t3ss derivative, at 1μM and 5μM, by considering the chance to obtain a similar protective effect as the previously reported in other models, by decreasing the concentration of t3ss derivative used, and also to avert from a possible toxic range dose in rat's neuronal cells. We also considered long-time exposure of cells to t3ss derivative for 24, 48 and 72 h, to obtain comparable results to those in SH-SY5Y cells, by treating with 1 μM t3ss. We noticed some significant effect on many gene targets, more relevant in the case of gene expression of A_{2B}, which was greatly increased in all considered time of exposure. At the same time we observed the same gene expression profile for A_1 and BACE-1 starting with a significant increase of gene expression at 24 h of t3ss treatment, and no changes at 48 h and 72 h. On the other hand, t3ss derivative seems to exert an opposite effect on gene expression of A_{2A} and $mGlu_1$, with a great decrease of gene expression, maintained on all considered time of exposure, suggesting that the observed effect of t3ss derivative on that cells could be related to the suppression of these receptors activity (fig. 62 panel A).

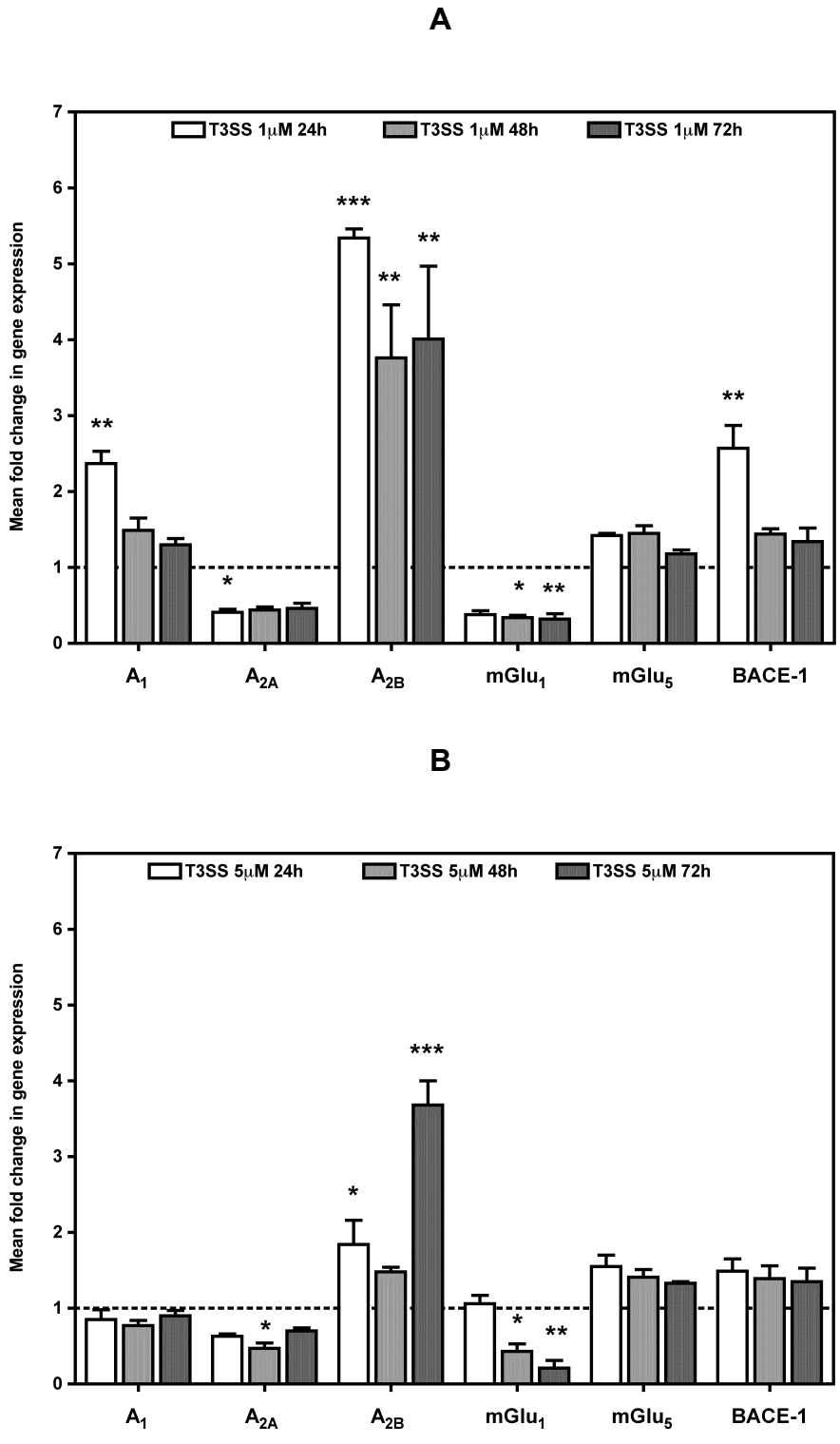
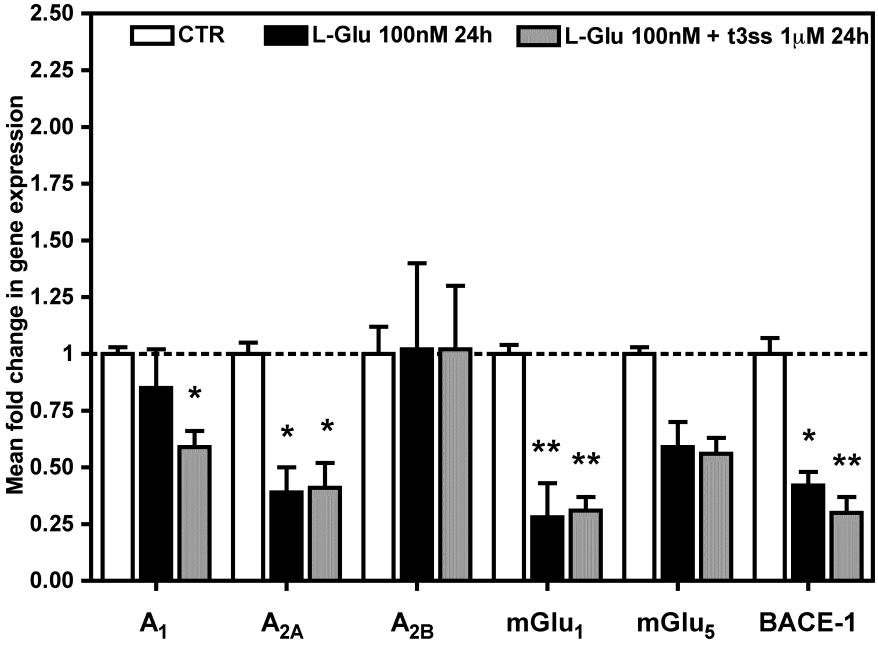


Figure 62. **Relative gene expression evaluated by RT-PCR technique, in rat's neuronal cells after treatment with t3ss derivative.** (**A**) Rat's neurons were exposed for 24, 48 and 72 h to 1μM [60]fullerene hydrosoluble derivative (t3ss). (**B**) Rat's neurons were exposed for 24, 48 and 72 h to 5μM [60]fullerene hydrosoluble derivative (t3ss). For the treatment with t3ss derivative were used rat's neuronal cells from foetuses rat's brain, following the protocol indicated in *Methods*, about growth and maintenance. The mRNA proceeding from cells were processed as reported in *Methods* and analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure.

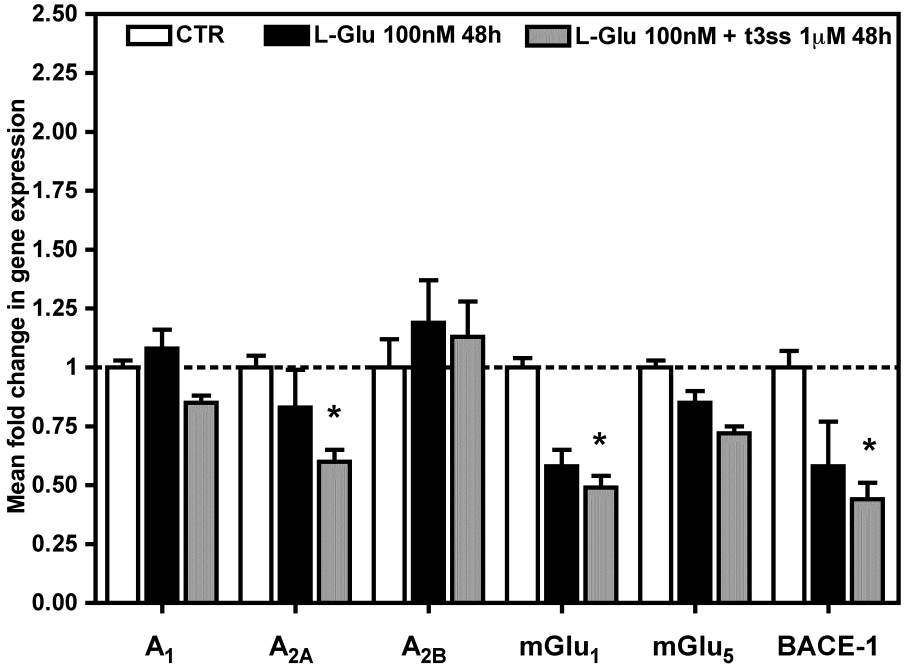
Results of relative gene expression presented in figures were analyzed on GraphPad 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent experiments. Control is showed in figures as a dotted line. *p<0,05 significantly different from the control; **p<0,01 significantly different from the control; ***p<0,001 significantly different from the control.

In the case of treatment with 5μM t3ss we observed a similar gene expression profile, mainly in the case of A_{2B}, A_{2A} and $mGlu_1$ receptors gene targets, with a great and significantly increase of gene expression for A_{2B} and a tendency to decrease gene expression in the case of A_{2A}, most significant for $mGlu_1$ (fig. 62 B). Thus, it was confirmed the previous hypothesis of that t3ss derivative activity on neuronal cells may be due to an influence on A_{2A}, A_{2B} and $mGlu_1$ receptors turn over. We then provided a study on this neuronal model, by considering how long-term exposure to L-Glutamate may affect the expression of certain gene targets, mainly the same analyzed previously. To this end we used two moderate toxic concentrations of L-Glutamate as 100 nM and 1 μM, with contemporary presence of t3ss derivative at 1 and 5 μM, and for different exposure time: 24-48 and 72h. At the same time it must be considered that even moderate toxic, those L-Glu concentrations may be considered as the typical conditions occurring in brain during an excitotoxic injury, and for that the following results may be suggestive. First, by considering the treatment with 100 nM L-Glutamate and in presence of 1 μM t3ss, we noticed a great influence of these treatment on gene target, mainly at 24h exposure in which it could be observed a decrease in the gene expression of all considered target. This effect of 100 nM Glutamate was not modulated by the presence of 1 μM t3ss. Moreover, at 48 h the decrease of gene expression was only detected for A_{2A}, $mGlu_1$, and BACE-1 while at 72 h neither glutamate nor its combination with t3ss modified gene expression (fig. 63).

A


CTR
L-Glu 100nM 24h
L-Glu 100nM + t3ss 1μM 24h
Mean fold change in gene expression
2.50
2.25
2.00
1.75
1.50
1.25
1
0.75
0.50
0.25
0.00
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

B


CTR
L-Glu 100nM 48h
L-Glu 100nM + t3ss 1μM 48h
Mean fold change in gene expression
2.50
2.25
2.00
1.75
1.50
1.25
1
0.75
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0.00
A1
A2A
A2B
mGlu1
mGlu5
BACE-1

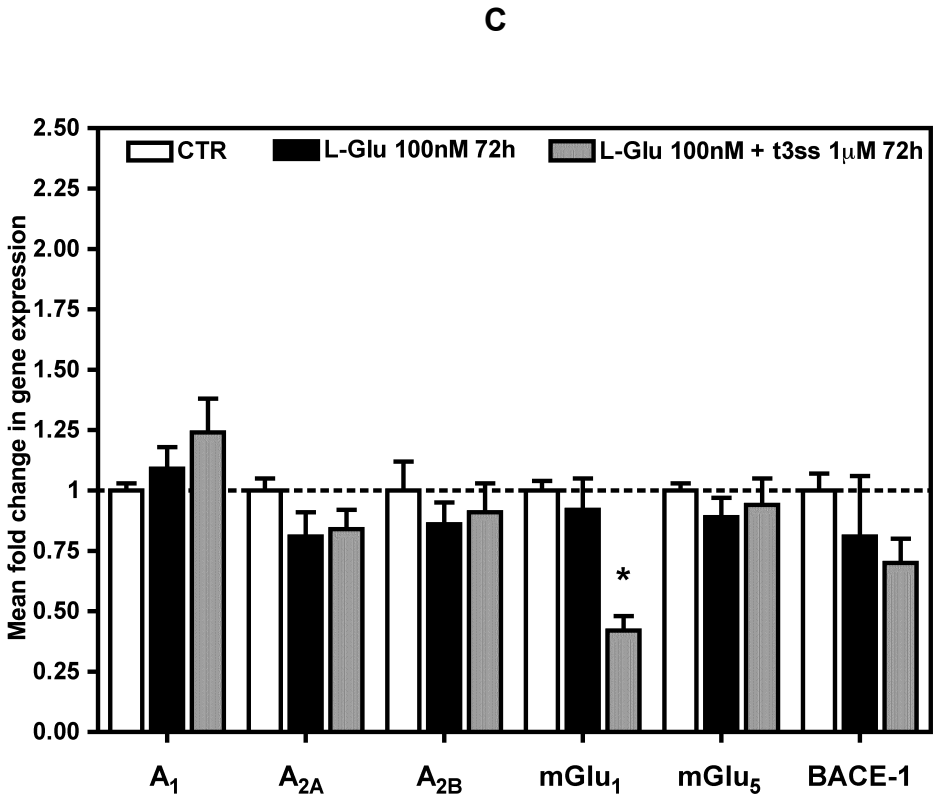
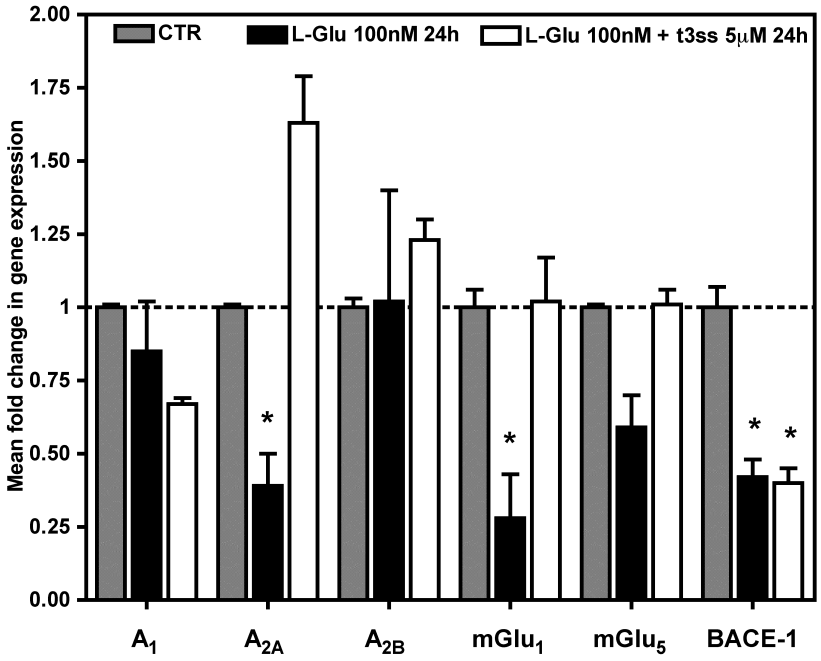


Figure 63. **Relative gene expression evaluated by RT-PCR technique, in rat's neuronal cells after treatment with L-Glutamate together with t3ss derivative.** (**A**) Rat's neurons were exposed for 24 h to 100nM L-Glutamate (L-Glu) and together with 1μM [60]fullerene hydrosoluble derivative (t3ss). (**B**) Rat's neurons were exposed for 48 h to 100 nM L-Glutamate (L-Glu) and together with 1μM [60]fullerene hydrosoluble derivative (t3ss).(**C**) Rat's neurons were exposed for 72 h to 100 nM L-Glutamate (L-Glu) and together with 1μM [60]fullerene hydrosoluble derivative (t3ss).For the treatment with L-Glutamate and t3ss derivative we used rat's neuronal cells from foetuses rat's brain, following the protocol indicated in *Methods*, about growth and maintenance. The mRNA proceeding from cells were processed as reported in *Methods* and analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Results of relative gene expression presented in figures were analyzed on GraphPad 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent days of experiments. Control is showed in figures as a dotted line. *$p<0,05$ significantly different from the control; **$p<0,01$ significantly different from the control.

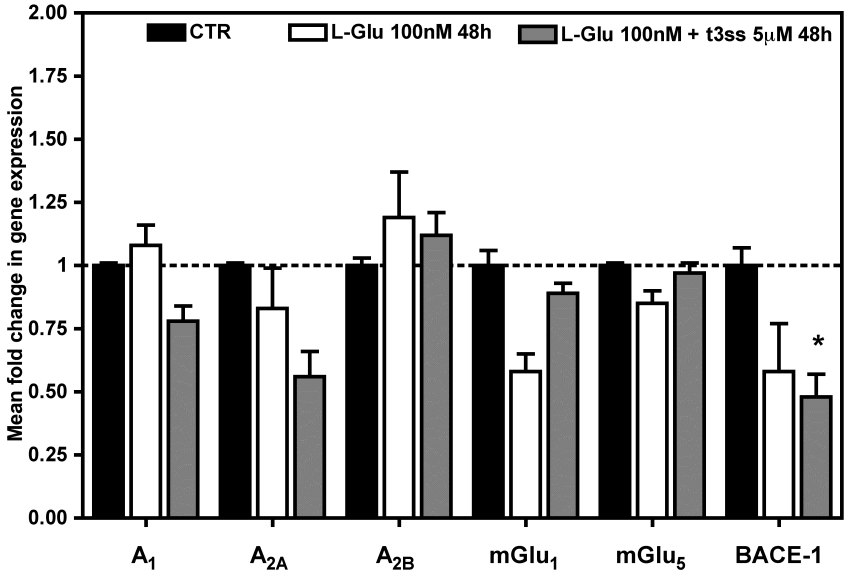
Thus the results from gene expression on rat's neurons exposed to 100 nM L-Glutamate and 5 μM t3ss derivative, did not show any difference from what previously observed by using 1 μM of t3ss derivative. The profile of gene expression by using 100 nM L-Glu and 5 μM t3ss derivative, indicated that no changes in genes expression occurrs at 48 and 72 h of treatment, respect to what previously observed, at the same time of exposure, by using 1 μM t3ss derivative. Surprisingly, the 24 h exposure of rat's cells to 100 nM L-

Glutamate and 5 μM t3ss derivative, did not show a decrease of gene expression of A_{2A}, $mGlu_1$ and $mGlu_5$, like instead observed by using 1 μM t3ss derivative. Thus indicating a different concentration-dependent modulation on gene expression exerted by t3ss derivative on rat's cells (fig. 64).

A



B



C

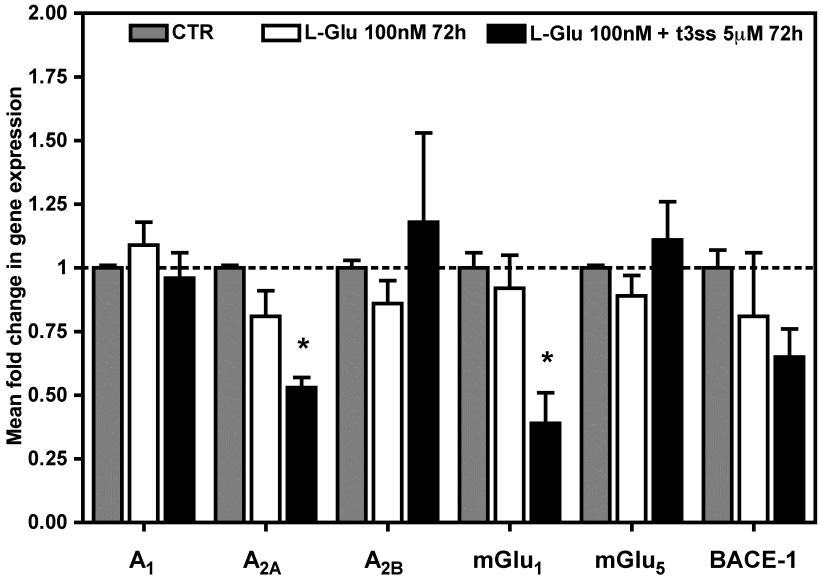
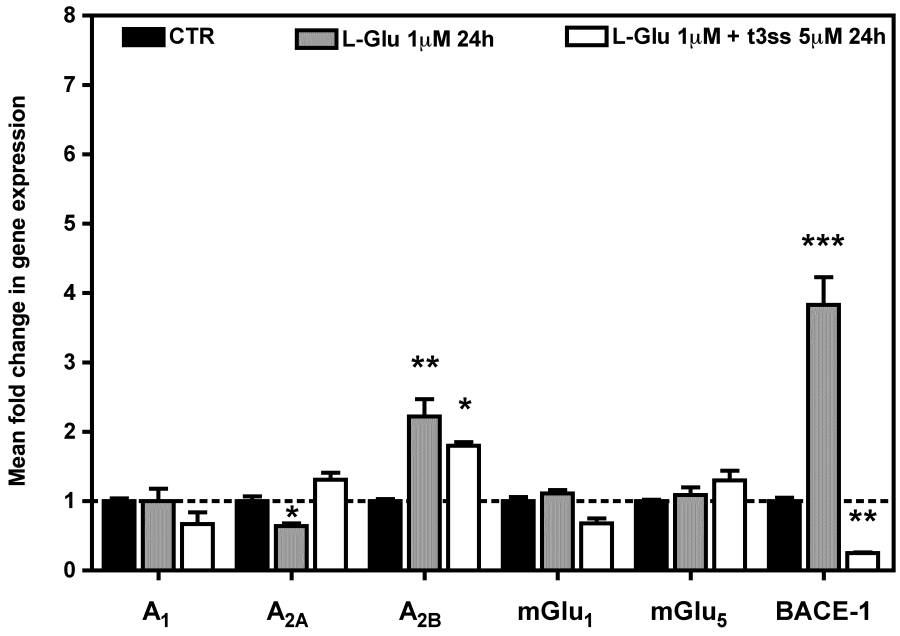


Figure 64. **Relative gene expression evaluated by RT-PCR technique, in rat's neuronal cells after treatment with L-Glutamate together with t3ss derivative.** (**A**) Rat's neurons were exposed for 24 h to 100nM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss). (**B**) Rat's neurons were exposed for 48 h to 100 nM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss).(**C**) Rat's neurons were exposed for 72 h to 100 nM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss).For the treatment with L-Glutamate and t3ss derivative we used rat's neuronal cells from foetuses rat's brain, following the protocol indicated in *Methods*, about growth and maintenance. The RNA proceeding from cells were processed as reported in *Methods* and analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Results of relative gene expression presented in figures were analyzed on GraphPad 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent days of experiments. Control is showed in figures as a dotted line. *$p<0,05$ significantly different from the control.

We next analyzed gene expression in rat cortical neurons by increasing glutamate concentration to 1 μM and using 5 μM t3ss derivative, thus we obtained probably the most interesting results on gene expression, after treating rat's neuronal cells for 24, 48 and 72h. For example, after 24h exposure to L-Glutamate 1 μM we noticed a great increase of BACE-1 expression, completely counterbalance by the contemporary presence of t3ss derivative. In the case of A_{2A} and A_{2B} receptors, the effect by L-Glutamate is clearly counterbalance by t3ss derivative producing an opposite change in gene expression (fig. 65 panel A). Furthermore these effects are confirmed at

48 h exposure to L-Glutamate and t3ss derivative; in particular, the effect of 1 μM L-Glutamate alone cause a great increase in gene expression of BACE-1 which is again counterbalance by the contemporary presence of t3ss derivative (fig. 65 panel B). The activity on gene expression of A_{2B} is confirmed by a significant increase of values with respect to the control caused by both considered treatments; while A_{2A} and $mGlu_1$ gene expression is forced to dramatically decrease by the combination of t3ss and L-Glutamate (fig. 65 panel B). Finally, what we obtained by considering the values of gene expression for 72h of exposure to L-Glutamate and t3ss derivative is a great increase of the expression in gene of A_{2B} receptor. On the other hand a dramatically decrease in gene expression of $mGlu_1$ was observed, mainly caused by the presence of t3ss derivative. At the same time, it was confirmed the effect of L-Glutamate 1μM to greatly increase the gene expression of BACE-1, while an opposite effect is observed when t3ss derivative was used both in combination wth L-Glutamate, similarly to detected at 24 and 48 h (fig. 65 panel C).

A



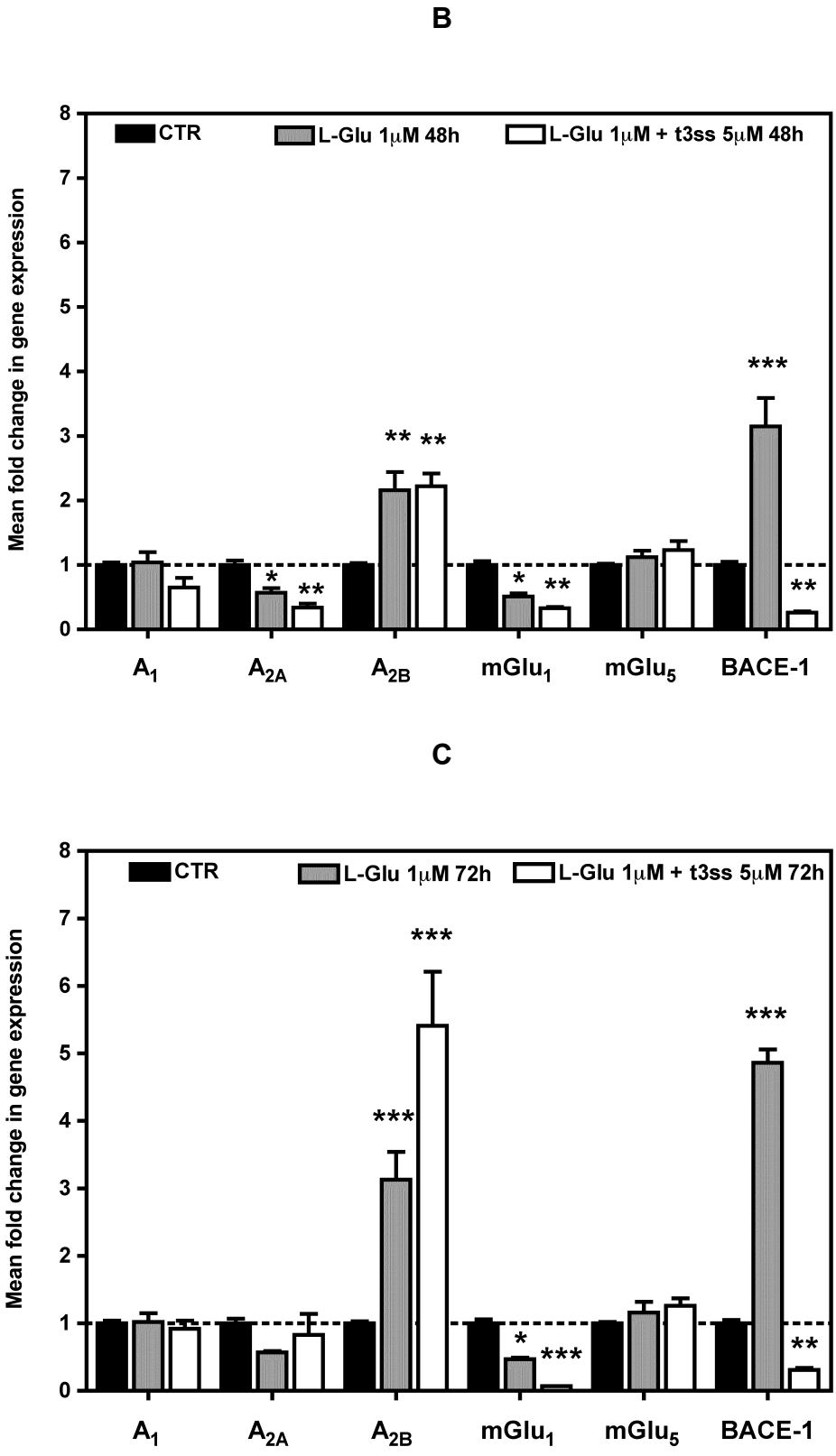


Figure 65. **Relative gene expression evaluated by RT-PCR technique, in rat's neuronal cells after treatment with L-Glutamate together with t3ss derivative.** (**A**) Rat's neurons were exposed for 24 h to 1 μM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss). (**B**) Rat's neurons were exposed for 48 h to 1 μM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss).(**C**) Rat's neurons

were exposed for 72 h to 1 μM L-Glutamate (L-Glu) and together with 5μM [60]fullerene hydrosoluble derivative (t3ss).For the treatment with L-Glutamate and t3ss derivative we used rat's neuronal cells from foetuses rat's brain, following the protocol indicated in *Methods*, about growth and maintenance. The RNA proceeding from cells were processed as reported in *Methods* and analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Results of relative gene expression presented in figures were analyzed on GraphPad 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent days of experiments. Control is showed in figures as a dotted line. *p<0,05 **p<0,01, ***p<0,001 significantly different from the control.

8.1 Study of the effect of functionalized partially hydrosoluble Nanohorns and hydrosoluble gold-nanoparticle on undifferentiated SH-SY5Y cells and rat cortical neurons vaibility and gene expression.

From data previously showed, it seems that t3ss derivative could portect cells against some kind of toxic stimuli. In order to study the activity of different carbon-made compounds and asses whether they exhibit also a protective role, we investigated the activity of partially hydrosoluble and functionalized nanohorns (NH) (fig. 66), also to compare with the activity of [60]fullerene derivative previously discussed.

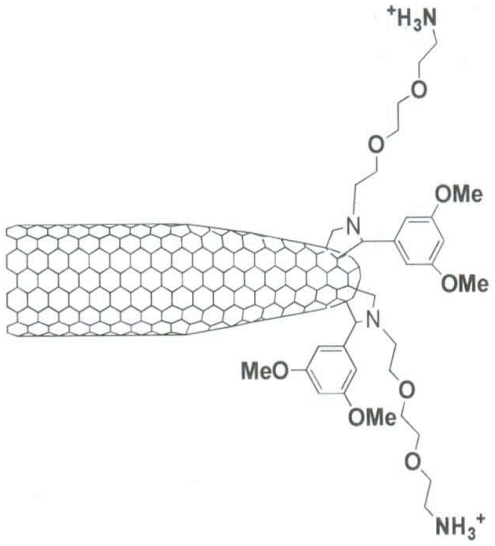


Figure 66. Structure of partially hydrosoluble nanohorns used in the present work. For more details and synthetic route see Rubio *et al.,* 2009. For more details on its use during treatment and general management, see *Methods*.

As well as for the nanohorns in the present section we provided a preliminary study on the activity of hydrosoluble gold-nanaoparticle, in order to compare as well its activity on our neurodegenerative models. For this purpose we used gold nanoparticles (GNP) functionalized with an alkyl S terminated chain (fig. 68), purchased from Sigma Aldrich.

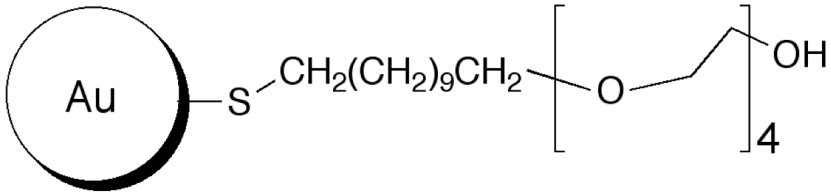


Figure 67. Structure of gold nano particle used in the present study, and purchased from Sigma. For more details see material and *Methods*.

Then we first evaluated the cells survival in the presence of nanohorns NH (fig. 68) and GNP (fig. 69) on undifferentiated SH-SY5Y cells. From viability results we observed toxicity from moderate to high after 6h exposure of cells to NH, with a toxicity profile doses-dependent. A moderate toxicity, was also observed by cells exposed to GNP, presenting a similar dose-dependent profile.

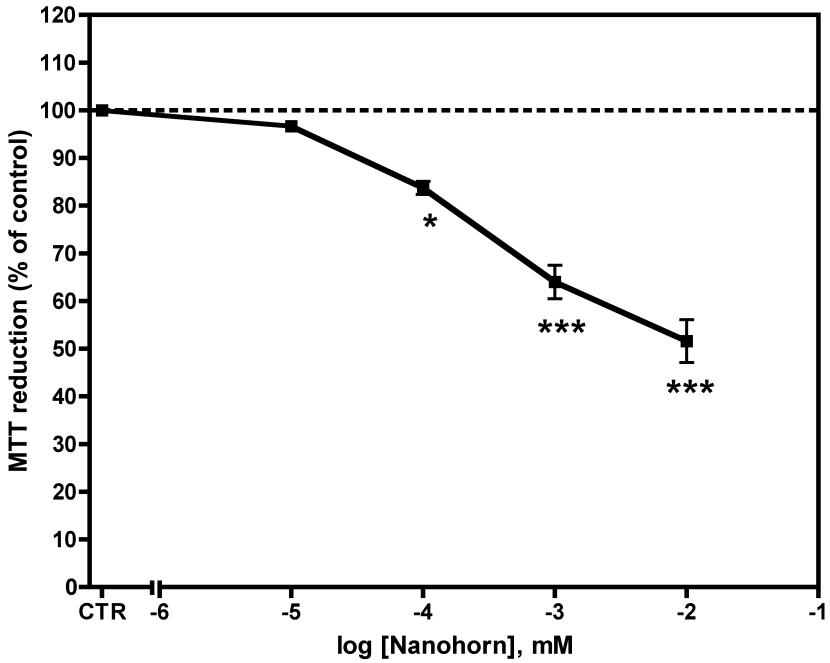


Figure 68. **MTT reduction viability assay evaluated in undifferentiated SH-SY5Y cells exposed to crescent concentrations of nanohorns**. Cells SH-Sy5y have been exposed to different concentration gold nano-particle for 6 h, as indicated in figure. Treatments were

prepared by taking known volumes from stock solution of nanohorn (100 μM), and by diluting directly into cells culture medium, to obtain final concentration of nanhorns, for treatment on cells, as showed in figure and described in *Methods*. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad ver. 5.0 as the *software*. *p<0,05 significantly different from control; ***p<0,001 significantly different from control.

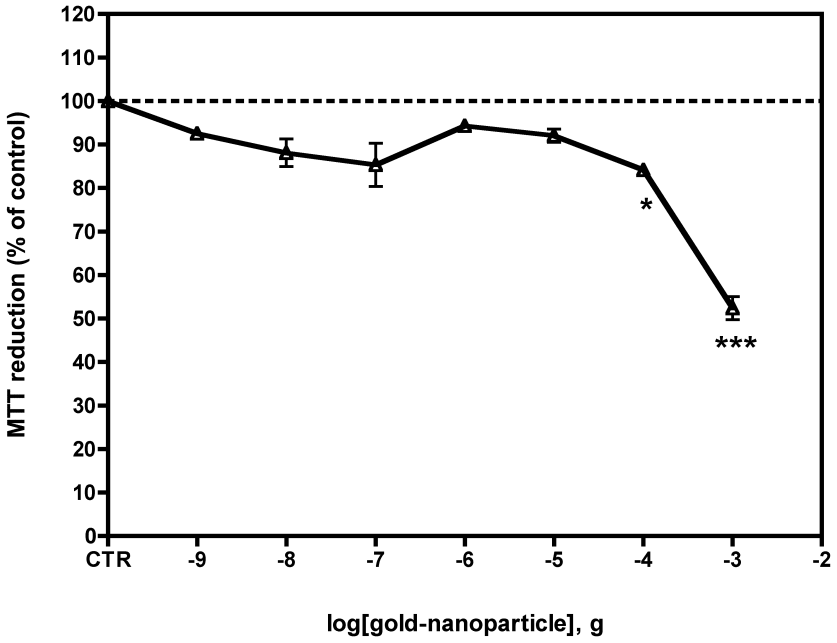


Figure 69. **MTT reduction viability assay on undifferentiated SH-SY5Y cells exposed to crescent concentrations of gold nano-particle.** Cells SH-Sy5y have been exposed to different concentration gold nano-particle for 6 h, as showed in figure. Treatments were prepared by taking known volumes from stock solution of gold nano-particle (2% w/v), and by diluting directly into cells culture medium, to obtain final concentration of gold nano-particle, for treatment on cells, as showed in figure and described in *Methods*. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad 5.0 as the *software*. *p<0,05, ***p<0,001 significantly different from control.

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In the same way we obtained a profile for survival of rat's neuronal cells in the presence of nanohorns (fig. 70). The results provided a moderate to high toxicity of nanohorns on neuronal cells, showing a 45% of cells survival by using 1 μM of NH, and suggesting rat cortical neurons as more vulnerable to NH as compared to SH-SY5Y.

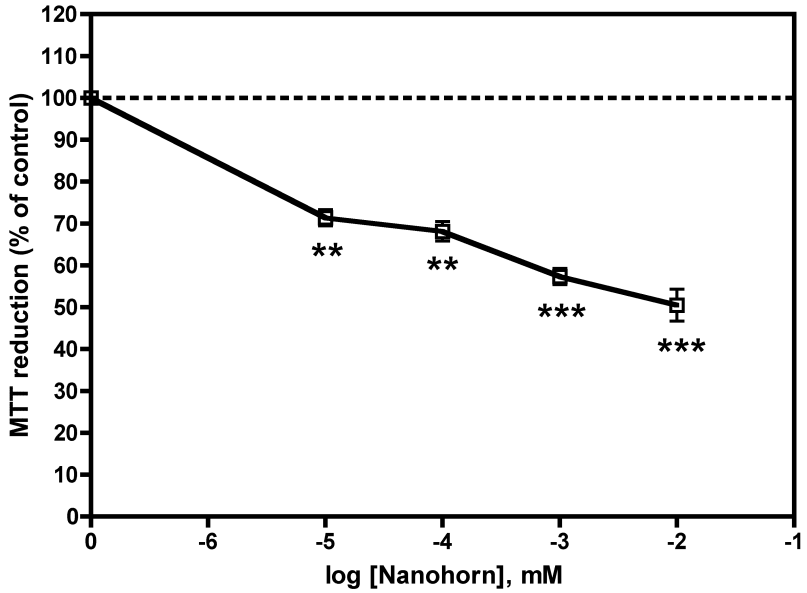


Figure 70. **MTT reduction viability assay performed in rat' neuronal cells after exposure to nanohorns.** Rat cortical neurons have been exposed to different concentration gold nano-particle for 6 h, as showed in figure. Treatments were prepared by taking known volumes from stock solution of nanohorn (100 μM), and by diluting directly into cells culture medium, to obtain final concentration of nanhorns, for treatment on cells, as showed in figure and described in *Methods*. Values of cell survival, are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad 5.0 as the *software*. *p<0,05 significantly different from control; ***p<0,001 significantly different from control.

We subsequently provided a study on SH-SY5Y cells survival in the presence of toxic concentrations of L-Glutamate and amyloid-beta peptide as treatment alone, and in the presence of not highly toxic concentrations of NH (100 nM) and GNP (10 μg). Thus by treating cells with 10-100μM L-Glutamate and in the presence of NH and GNP (fig. 71), we obtained surprising results, in the sense that we observed a recovery of cells from both L-Glutamate toxicity conditions, which was even more significant by considering the higher treatment with L-Glutamate 100 μM (fig. 71 B). Furthermore, in both conditions, the most efficient recovery effect from L-Glu toxicity, was elicited by GNP (fig. 71 panel A and B).

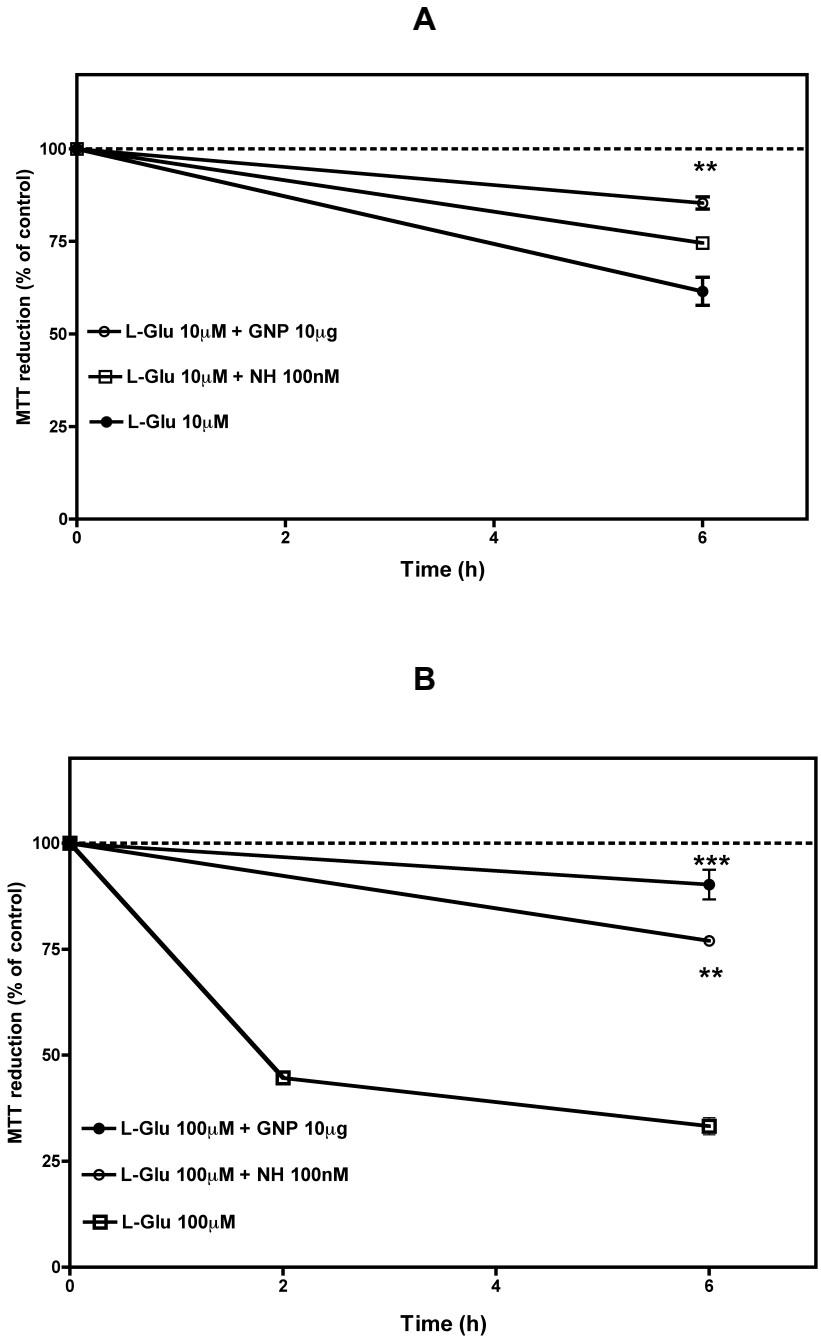
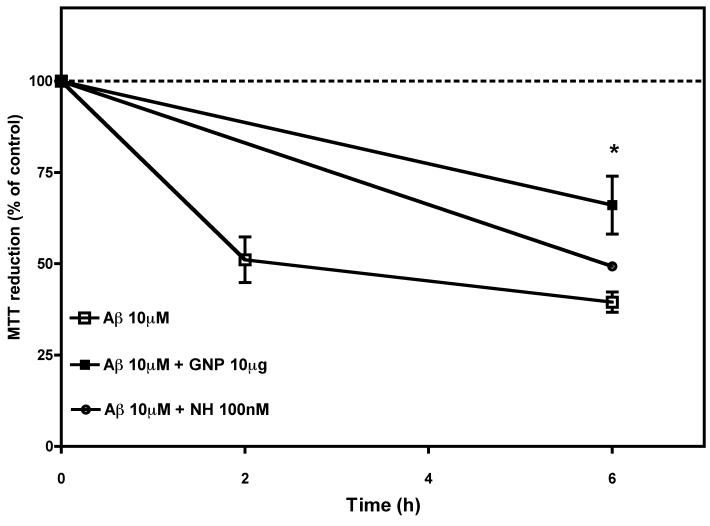


Figure 71. **MTT reduction viability assay, on undifferentiated SH-SY5Y cells after exposure to L-Glutamate, and together with Nanohorns and gold nanoparticle**. (**A**) Undifferentiated SH-SY5Y cells were exposed to 10 μM L-Glutamate (L-Glu) and together with 100 nM of nanohorns (NH) and 10 μg of gold nanoparticle (GNP), for 6 h exposure, as showed in figure. (**B**) Undifferentiated SH-SY5Y cells were exposed to 100 μM L-Glutamate (L-Glu) and together with 100 nM of nanohorns (NH) and 10 μg of gold nanoparticle (GNP), for 6 h exposure, as showed in figure. Values of cell survival are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad 5.0 *software*. *p<0,05 significantly different from cells treated with L-Glutamate alone; ***p<0,001 significantly different from cells treated with L-Glutamate alone.

We next considered the same type of study on SH-SY5Y cells, treated with amyloid-beta peptide instead of L-Glutamate, and maintaining the same concentrations previously used for nanohorns and gold nanoparticles. Results indicated also in this case a recovery effect of NH and GNP, by considering both the treatment with 10 and 25 μM amyloid-beta peptide, evaluated only at 6h, like in the case of L-Glutamate. A significant effect on cells survival was observed by using GNP, more efficient than that exerted by NH in the same conditions (fig 72 panel A and B). Furthermore we observed that the protective effect exerted by NH during toxic treatment, is quite similar to that observed by using t3ss derivative in presence of the same degree of toxicity in undifferentiated SH-SY5Y cells. Thus respect to the protective effect exhibited by t3ss derivative in the same cells model, and conditions, nanohorns demonstrated to be less effective. At the same time nanohorns, demonstrated to own higher toxicity profile on cells, respect to t3ss derivative, probably involving the limited solubility of nanohorns in water compared to t3ss derivative. Nevertheless, from these studies we demonstrated that t3ss derivative, it is not the only compound able to have a protective effect on cells during toxic stimuli, but exhibiting the best profile of high dose-toxicity related on cell viability, among the considered class of hydrosoluble nanocompound.

A



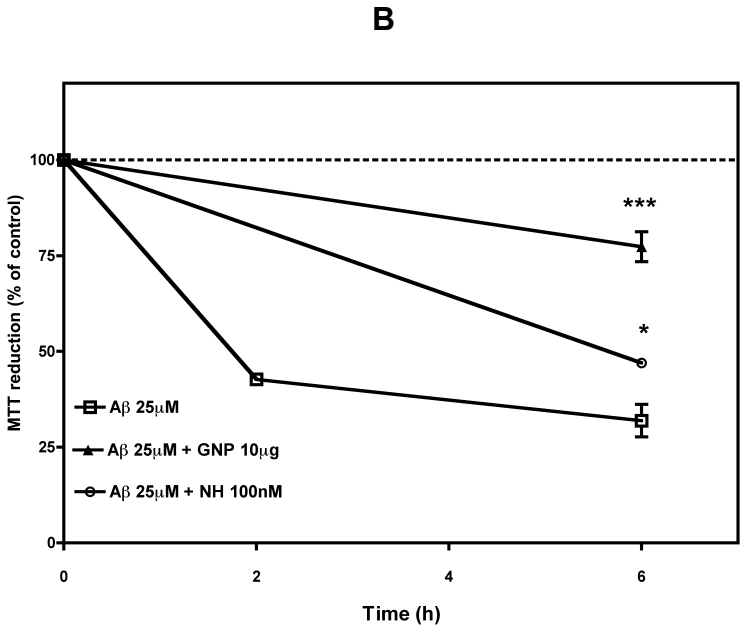


Figure 72. **MTT reduction viability assay, on undifferentiated SH-SY5Y cells after exposure to amyloid-beta peptide, and together with Nanohorns and gold nanoparticle**. (**A**) Undifferentiated SH-SY5Y cells were exposed to 10 μM amyloid-beta peptide (Aβ) and together with 100 nM of nanohorns (NH) and 10 μg of gold nanoparticle (GNP), for 6 h exposure, as showed in figure. (**B**) Undifferentiated SH-SY5Y cells were exposed to 25 μM amyloid-beta peptide (Aβ) and together with 100 nM of nanohorns (NH) and 10 μg of gold nanoparticle (GNP), for 6 h exposure, as showed in figure. Values of cell survival are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad ver. 5.0 as the *software*. *$p<0,05$ significantly different from cells treated with amyloid alone; ***$p<0,001$ significantly different from cells treated with amyloid alone.

A profile of rat's neuronal cells survival in the presence of nanohorns and adenosine A_{2A}(SCH58261) and A_3(SCHx) receptor antagonists was also provided. By exposing cells to 6h treatment with 1 and 10 μM NH and in presence of those antagonist, we obtained a partially but significant protective effect of both adenosine antagonist against nanohorns toxicity, although less evident when using adenosine A_3 receptor antagonist (fig. 73). Thus a certain activity of these two adenosine receptor antagonist in toxicity mechanism, has been observed.

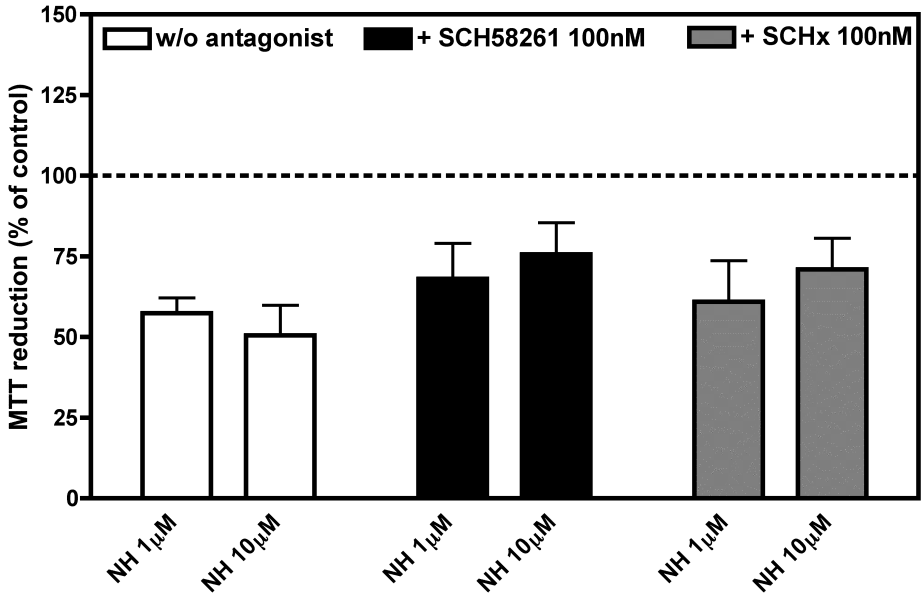


Figure 73. **MTT reduction viability assay of rat's neuronal cells exposed to nanohorns (NH) and in presence of adenosine receptor antagonists.** Undifferentiated SH-SY5Y cells were exposed for 6 h to 1-10μM Nanohorns (NH) and together with 100 nM adenosine A_{2A} (SCH58261) and A_3 (SCHx) receptor antagonists at showed in figure. Values of cell survival are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of survival as assay control, and indicated in figure as a dotted line, by using GraphPad 5.0 *software*.

To conclude this section dedicated to this preliminary study with GNP and NH, we to evaluated the gene expression for the receptors we were interested on, during exposure of undifferentiated SH-SY5Y to non-toxic concentrations of nanohorns. Thus, after 6h exposure to 10 and 100 nM NH, we evaluated the gene expression by considering the same gene targets as previously described (fig. 74). From RT-PCR values we observed some kind of tendency by nanohorns in affecting the gene expression of different targets, however the main closer to a significant effect is the modulation of gene expression of adenosine A_1 receptors. Nevertheless, these are preliminary results, needing further experiments, mainly by considering higher concentrations of nanohorns, and in combination with toxic treatment, like performed for t3ss derivative and as well as for gold nanoparticles.

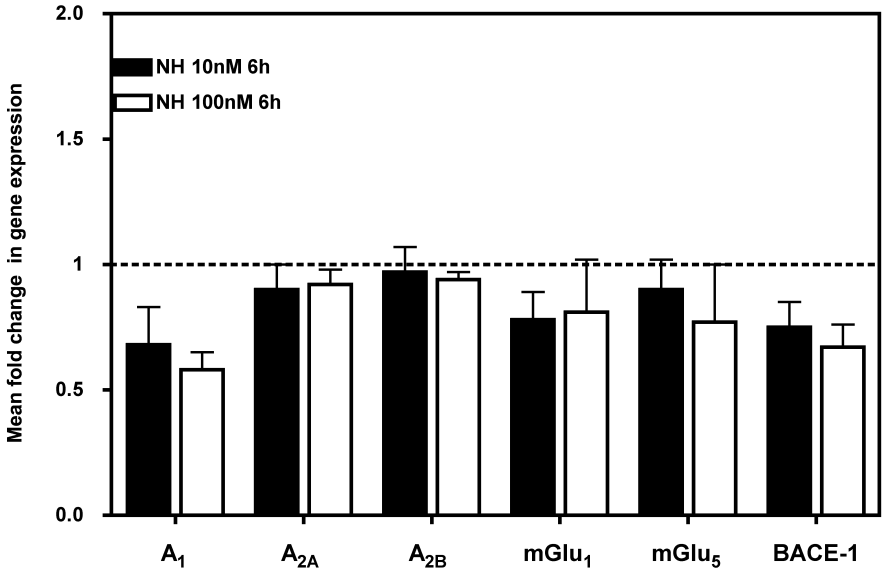
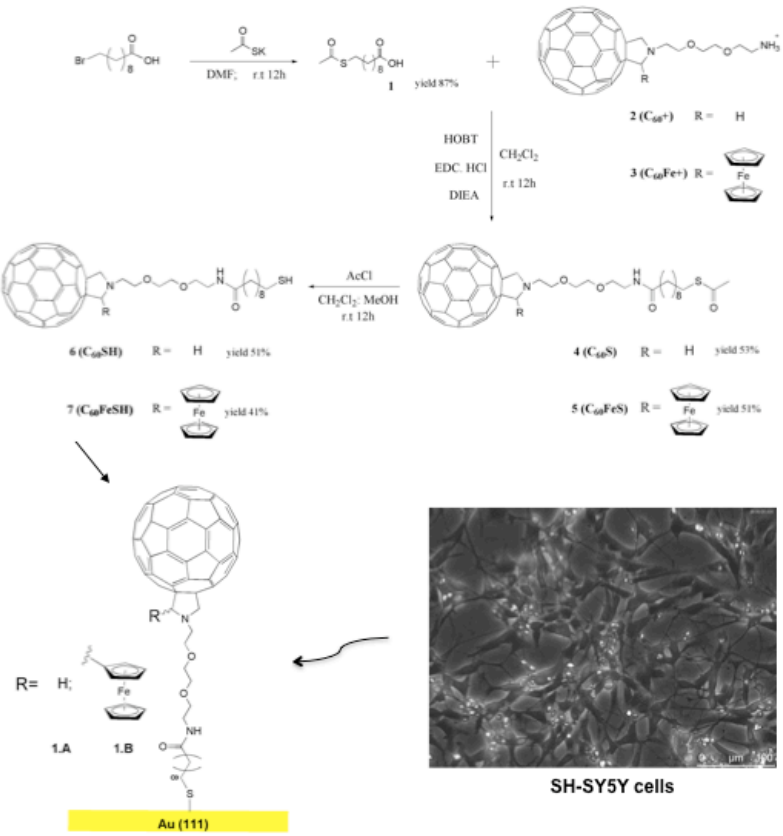


Figure 74. **Gene expression evaluated by RT-PCR technique, in undifferentiated SH-SY5Y cells exposed to nanohorn.** Undifferentiated SH-SY5Y cells were exposed for 6 h to 10-100 nM of Nanohorns (NH) as showed in figure. The mRNA proceeding from cells were processed as reported in *Methods* and analyzed for the following gene target: adenosine A_1, A_{2A}, A_{2B} receptors, $mGlu_1$ and $mGlu_5$ receptors and BACE-1 enzyme, as showed in figure. Results of relative gene expression presented in figures were analyzed on GraphPad 5.0 *sofware*, also for statistical analysis and to compare values. Values obtained are the average from the values of three independent days of experiments. Control assay is showed in figure as a dotted line.

9.1 Study on [60]fullerene based gold substrates, by using undifferentiated and differentiated SH-SY5Y cells.

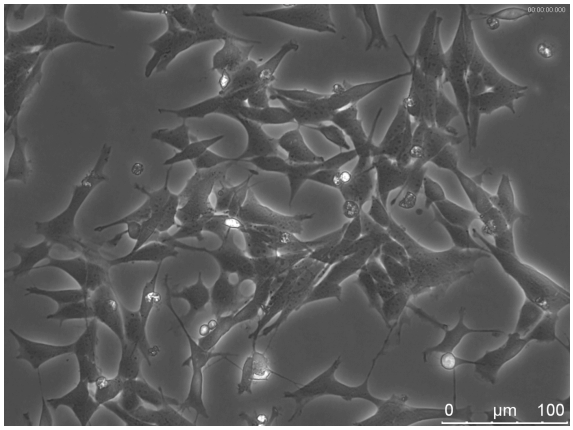
In the following section we aimed to study the biocompatibility of [60]fullerenes based surfaces, SAM's (Self-Assembled Monolayers), as described in *Introduction*. The first step was to choice a possible biocompatible substrate, in that sense a good approach was found by Au covered mica substrate, as one of the main reported metal useful in engineering fullerene modified surfaces. The [60]fullerene were synthesized as mono-substituted pyrrolidine N-substituted as 10 carbon terms alkyl chains, free thiol terminated, necessary to link the structure on Au surface (scheme above, **1.A**). Moreover, as extensively reported in literature, [60]fullerene may take part in structure acting as electron storage and donor, in combination for example with electron-dono-acceptor compounds, and porphyrins and ferrocene-fullero substituted are examples (*see* Introduction). In that sense we engineered two type of [60]fullerene mono substituted structure, the one as previously described, as well as with a ferrocene moiety, in order to influence the electronic properties of [60]fullerene derivative on surfaces (scheme above, compound **1.B**). In the present study we provided to evaluated the biocompatibility of [60]fullerene modified materials by appreciating the morphological changes occurring in undifferentiated SH-SY5Y cells growth on surfaces, as well as after the differentiation into human neuronal cells. We first evaluated SEM images of cells on surfaces, and subsequently, we tested surfaces as possible therapeutics, by treating cells maintained on surfaces with a toxic concentration of L-Glutamate.



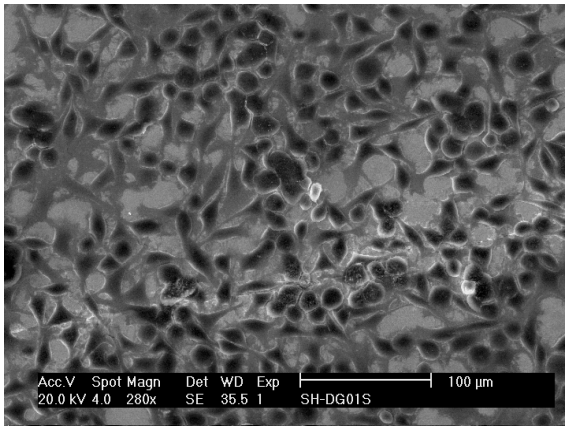
In the scheme above a description of the synthetic route to obtain [60]fullerene gold based surfaces. Undifferentiated SH-SY5Y cells were growth on [60]fullerene modified Au surfaces, and successfully differentiated following the protocols as reported in *Materials and Methods*, and also for more details.

So we first evaluated the biocompatibility of [60]fullerene based gold surfaces, by SEM images we compared undifferentiated cells 24h growth on a normal Petri plaque, and those growth on modified surfaces. From SEM images, not great differences were observed between cells growth on normal Petri plaque(fig. 75 A) and cells growth on modified surfaces, in this case with [60]fullerene-ferrocene monosubstituted (fig. 75 B and C). Furthermore, also no difference in cells behavoiur were observed between the two [60]fullerene derived gold surfaces 1.A and 1.B.

A



B



C

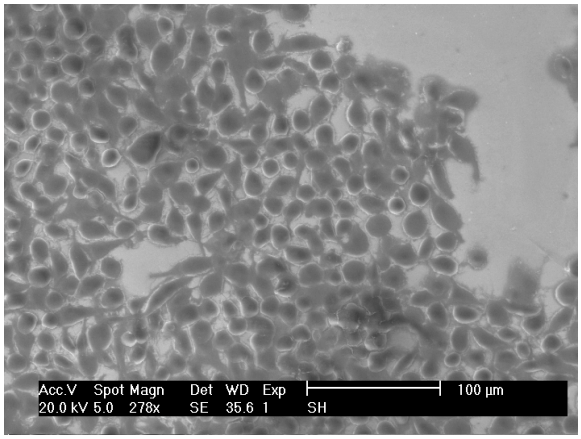
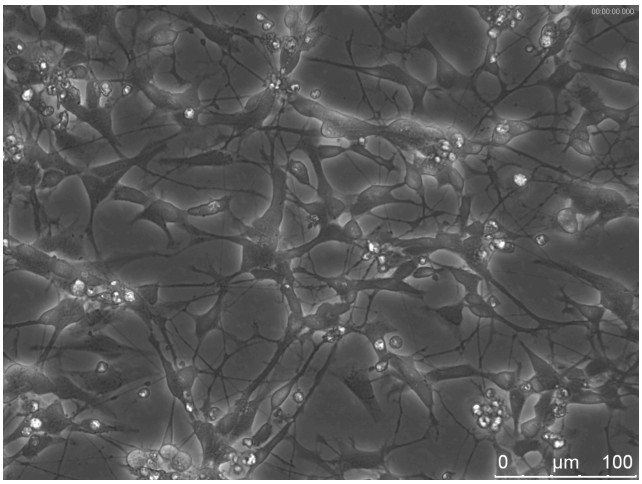


Figure 75. **Images of undifferentiated SH-SY5Y cells 24h growth on different substrates**. Figure 75 **A**. Undifferentiated SH-SY5Y 24 h cells growth on a normal Petri plaque, and image taken by confocal microscopy instrument. Figure 75 **B**. Undifferentiated SH-SY5Y cells 24 h growth on [60]fullerene modified gold substrate (**1.A**). Figure 75 **C.** Undifferentiated SH-

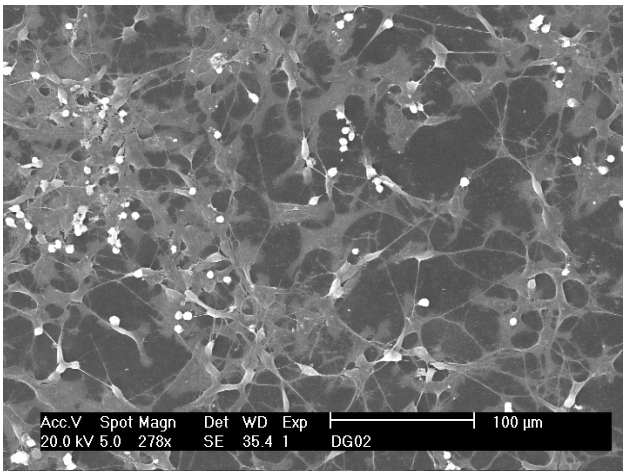
SY5Y cells 24h growth on [60]fullerene modified gold substrate (**1.B**). Images of figures 76 B and C has been taken by SEM instrument, after cells fixation procedure, as reported in *Methods*. SH-SYY cells were 24 h growth and maintained as reported in *Methods*. Image by confocal microscopy have been performed by photography a normal Petri culture plaque, using the instrument reported in *Methods*.

In order to proof the total biocompatibility of synthesized modified substrates, we differentiated SH-SY5Y cells on these surfaces, as well as in normal Petri surfaces. It must be notice that differentiation is a particularly selective process for SH-SY5Y cells, derived from neuroblastoma, that take totally 12 days of cells incubation with two different treatments, *all trans* retinoic acid (ATRA) and Brain Derived Neurotrophic Factor (BDNF). First 7 days treatment with ATRA are necessary to essentially convert the phenotype of cancer cells into a differentiated neuronal-like one. The second part 5 days of treatment with BDNF, is necessary to form neuritis networks between cells, if already differentiated. We finally obtained fully differentiated human neuronal cells on [60]fullerene based gold surfaces, without essentially no difference between the cells differentiated on normal Petri plaque and gold modified surfaces (fig. 76).

A



B



C

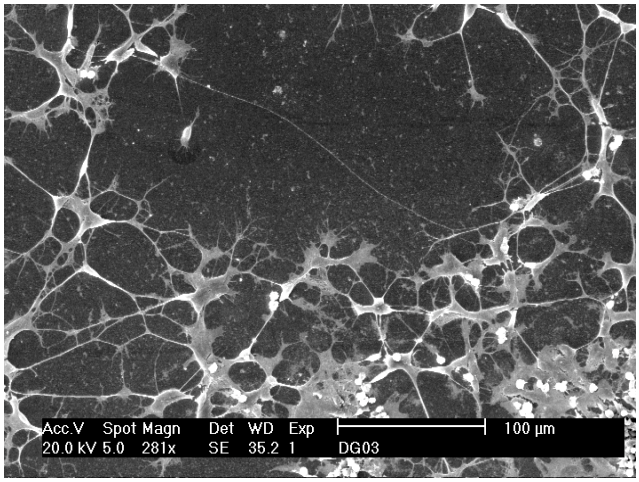


Figure 76. **Images of 12 days differentiated SH-SY5Y growing on different substrates.** Figure 76 **A**. Image of 12 days differentiated SH-SY5Y cells seeded on normal Petri plaque, photographed by confocal microscopy. Figure 76 **B**. SEM image of 12 days differentiated SH-SY5Y cells growing on [60]fullerene modified gold substrates (1. A). Figure 76 **C**. SEM image of 12 days differentiated SH-SY5Y cells growing on [60]fullerene modified gold substrates (1. B). SEM images of differentiated SH-SY5Y cells have been obtained following fixation protocol as described in *Methods*. Image by confocal microscopy have been performed by photography a normal Petri culture plaque, using the instrument reported in *Methods*.

The last step of the present study with [60]fullerenes on surfaces have been to evaluate a possible therapeutic effect of these modified surfaces, looking of any cooperative positive interaction between surfaces and cells exposed to toxic compounds. To this purpose we evaluated the undifferentiated SH-SY5Y cells survival, growth on [60]fullerene modified surfaces, after exposure to 100μM L-Glutamate for 6h, and the results are reported below in figure 77.

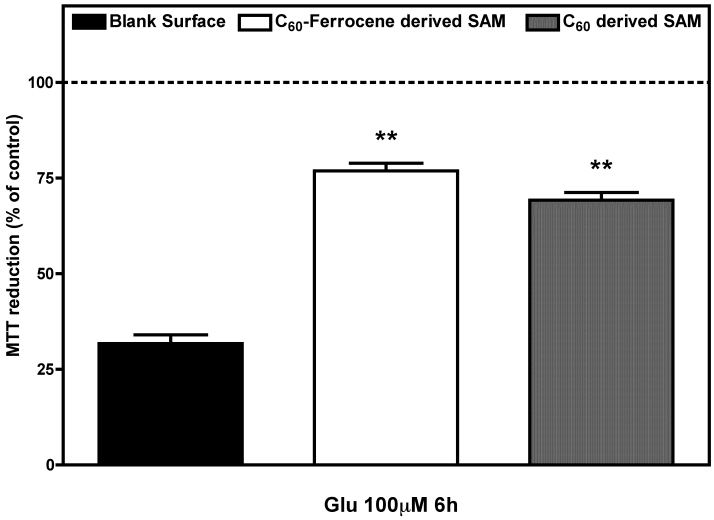


Figure 77. **MTT reduction viability assay on undifferentiated SH-SY5Y cells growth on different surfaces**. Undifferentiated SH-SY5Y cells were growth 48h and maintained on non-modified gold surface (Blank Surface), on [60]fullerene-ferrocene (**1.B**) modified gold surface (C_{60}-Ferrocene derived SAM), on [60]fullerene (**1.A**) modified gold surface (C_{60}-derived SAM), and thus exposed to 100 μM L-Glutamate (Glu) for 6 h. Values of cell survival are from the average of three independent days of treatment, and from MTT reduction assay as at least 8-10 values of absorbance, and as reported in *Methods*. Data were thus analyzed, by comparing with 100% of cells survival as SH-SY5Y growth on a non-modified gold surface and not exposed to 100 μM L-Glutamate, and as assay control, indicated in figure as a dotted line, by using GraphPad ver. 5.0 as the *software*. **p<0,01 significantly different from survival of cells exposed to L-Glutamate on non-modified gold surface.

From viability assay we noticed a protective effect of [60]fullerene modified gold surfaces against L-Glutamate toxicity on cells, mainly if compare with cells growth on a non-modified gold surface. On blank gold surface cells were subjected to all the toxic effect of L-Glutamate with a percentage of survival comparable to those growth and treated on a normal Petri plaque. Furthermore, a small difference in the protective effect of substrate, even not significant, was observed between the considered substrates. In fact the substrate modified with [60]fullerene-ferrocene seems to be a little more active than the other, thus probably confirming that to a modification in the electronic property of [60]fullerene may correspond a modification on its biological properties. Finally, that observed activity of [60]fullerene based surfaces, may open new perspectives for the study of newest modified materials, adapted to interfacing with biological systems.

DISCUSSION

Discussion

1. Endogenous expression of adenosine and metabotropic glutamate receptors in SK-N-MC cells from human neuroepithelioma.

In this work it has been demonstrated that SK-N-MC cells from human neuroepithelioma endogenously express adenosine receptors type A_1, A_{2A}, A_{2B} and metabotropic glutamate receptors type $mGlu_1$, $mGlu_2$, $mGlu_3$ and $mGlu_5$. The expression of these receptors was evaluated by using different techniques, based on the use of specific antibodies (Western Blot), on the expression of specific genes (real time PCR) and quantification of receptors by using radio labelled compounds (radioligand binding assay). These receptors are naturally expressed in this type of cancer cells, suggesting that it could be useful as a study model to understand the regulation and modulation of these receptors and their involvement in processes of excitotoxicity and cell death. Even though it was reported the presence of various receptors endogenously expressed by SK-N-MC cells, we focused specifically on the study of adenosine receptors type A_1, A_{2A}, A_{2B} and metabotropic glutamate type $mGlu_1$ and $mGlu_5$ during specific toxic treatment because they are implicated on phenomena of neurodegeneration and neuroprotection (Skeberdis *et al*., 2001; Pisani *et al*, 2001; Sheldon and Robinson, 2007; Cunha, 2005). Furthermore adenosine A_1 and A_2 receptors modulate neuronal and synaptic function in a range of ways that may make them relevant to the occurrence, development and treatment of brain ischemic damage and degenerative disorders (Stone et al., 2009). These receptors are endogenously expressed in SK-N-MC cells, as here reported, and other proliferating cell lines as C6 glioma (Castillo et al., 2007), 28A (Spielman et al., 1992) and DDT1 MF-2 (Gerwins et al., 1990). SK-N-MC cells present RNA transcripts of genes coding for adenosine A_{2A} and A_{2B} receptors subtypes. However, the corresponding mRNA for A_1 was undetectable in our conditions

2. Study of L-Glutamate related toxicity

The toxicity in cells related with glutamate and called excitotoxicity have been investigated since its first description (Olney, 1969). That type of excitotoxicity is based on the mechanism of action of glutamate that act on NMDA receptors, provoking cellular death by changing the calcium homeostasis (Villmann and Becker, 2007). At the same time the role of metabotropic glutamate receptors have been extensively studied in different cellular model for their implication in neurodegeneration or/and neuroprotection processes during the excitotoxic damage, and in particular the participation of $mGlu_5$ on both the processes (Olive, 2005).

2.1 On SK-N-MC human neuroepithelioma cells

The results obtained from MTT reduction viability assay in SK-N-MC cells exposed to L-Glutamate, showed the high toxicity of that compound and confirmed the high sensibility of brain derived cells, to high concentration of L-Glutamate. The increase in intracellular Calcium levels elicited by glutamate, mainly acting on NMDA and AMPA receptors, is related to neuronal death (Menev *et al*., 1989), and demonstrated to play a decisive role in the ischemic cascade relating to Alzheimer's disease (Hynd *et al*, 2004). On the other hand results about the gene expression evaluated by RT-PCR technique in SK-N-MC cells exposed to L-Glutamate, showed an increase in gene expression of metabotropic glutamate receptors belonging to group I. The most part of the authors reported that the activation of receptors belonging to group I of glutamate is related to an increase of the damage initiated by NMDA receptors (Skeberdis *et al*., 2001), while many other authors report that the activation of receptors belonging to group I during an excitotoxic insult, may cause a cascade of events including the inhibition of endocytosis, inhibition of inflammation and cell death (Baskys *et al.,* 2005). Thus, the activation of receptors of group I seem to facilitate the endocytosis of NMDA receptors, which reduce the excitotoxic damage (Snyder *et al*., 2001). At the same time excitotoxicity mediated by glutamate also involves adenosine receptors, which modulate the release of that aminoacid. In the present work it has been showed how the treatment of cells with L-Glutamate is

effective on the modulation of the expression of adenosine receptors type A_{2A} and A_{2B}, and, even more effective on the expression of metabotropic glutamate receptors of group I, although it was confirmed only by gene expression. Data showed that treatment with L-Glutamate at 10 μM and 100 μM affected the expression of adenosine A_1 receptors. The fact that these receptors increase their expression after excitotoxic exposure to glutamate suggests that adenosine A_1 receptors may have a neuroprotective role. This role has been largely investigated in different model of toxicity (De Mendonça *et al.,* 2000) and has been related to its ability to inhibit the excitability in neuronal cells and synaptic transmission (Cunha, 2005). The neuroprotective role of adenosine A_1 receptors against different excitotoxic stimuli has been extensively demonstrated in *in vivo* and *in vitro* model; as, for example, the model of toxicity induced by kainate and quinolinic acid in hippocampus (MacGregor *et al*., 1993; MacGregor *et al*., 1997). Different *in vivo* models relate adenosine A_1 receptors neuroprotection with neurodegenerative disease. It has been demonstrated for example that by using a model of Huntington disease the injection of A_1 receptors agonist (ADAC) reduced the brain damage (Blum *et al*., 2002), or in sclerosis model the activation of those receptors reduced the inflammation (Tsutsui *et al*., 2004). The neuroprotective role of A_1 could be noticed at different level: by inhibiting the release of excitatory neurotransmitters like glutamate, at presynaptic level, by decreasing all events related with these neurotransmitters, and, at postsynaptic level A_1 receptors could activate "rectification" K^+ channels that produce an hyperpolarisation in neurons (Dunwiddie and Masino, 2001). The activation of these receptors inhibit the activity of NMDA receptors, and consequently reduce the calcium imbalance into cells and all excitotoxic events related (De Mendoça *et al*., 1995). Due to the low not detectable mRNA level, or another unknown reason, it was not possible to directly relate eventually changes in gene expression of adenosine A_1 receptors by RT-PCR technique to exposure of SK-N-MC cells to L-Glutamate. Nevertheless, it was observed by binding radiolabelled assay. An increase in total adenosine A_1 receptors, after exposing cells to L-Glutamate 10 and 100 μM, thus confirming a possible neuroprotective role of these receptors. This hypothetically protective role for adenosine A_1 receptors, during L-Glutamate cells exposure, and related to the increase of these receptors, could not be well applied to adenosine A_{2A}

and A_{2B} receptors. Considering results obtained by evaluating both adenosine A_{2A} and A_{2B} receptors, an increase in gene expression as well as in total number of both receptors has been observed in SK-N-MC cells exposed to L-Glutamate. The few works published about these receptors indicated a neuroprotective role in excitotoxic rat model (Jones *et al*., 1998) or in hemorrhagic *in vivo* brain model (Mayne *et al*., 2001). It was also demonstrated that in pathological conditions there is an increase in adenosine A_{2A} receptors, that was observed in epilepsy model (Rebola *et al*., 2005a), in Parkinson model (Pinna *et al*., 2002) as well as in patient (Calon *et al*., 2004). By our group, where this work was carry out, was demonstrated that in patient with Pick dementia there is an increase in total number of the adenosine receptors A_{2A} (Albasanz *et al*., 2006), as well as in patient with Alzheimer's disease, was observed an increase of A_1 and A_{2A} receptors (Albasanz *et al.,* 2008). It was so confirmed a tendency in the increase of these receptors during excitotoxic events. At the same time the similar behaviour observed for the expression of both adenosine A_{2A} and A_{2B} receptors after L-Glutamate treatment in SK-N-MC cells, could be explained by the fact that these receptors are very similar in terms of structure and for that reason adenosine receptors A_{2B} is usually considered like the same as A_{2A} but with less affinity for its agonist. On the other hand adenosine A_{2B} receptors have been showed to posses their own intracellular signalling pathway, with only a small part shared with the A_{2A} ones. This has focused the attention of many authors, after discover that adenosine A_{2B} receptors may have its own physiological role and could represent a possible therapeutic target in many diseases (for a complete review about it see Feoktistov and Biaggioni, 1997; Stone *et al.,* 2009).

2.2 On undifferentiated SH-SY5Y human neuroblastoma cells

The effect of L-Glutamate on undifferentiated SH-SY5Y cells from human neuroblastoma, seems to complete a part of what previously discussed for SK-N-MC cells. In fact the main observed effect of L-Glutamate is a moderate to high toxicity on cells survival, like in the case of SK-N-MC, even by using this model we decided to focus mainly on the long-term exposure of cells to not high toxic concentrations of L-Glutamate, in order to obtain a profile of gene expression for some of the targets naturally involved in neurodegenerative diseases. In fact, because after treating SH-SY5Y with 100 μM L-Glutamate for 6h, we did not observe any change in gene expression of considered target, we focus mainly on long-term exposure to moderate toxic concentrations of L-Glutamate in order to simulate a hypothetic long excitotoxic effect, as naturally occurring in human brain during neurodegenerative diseases (Hynd et al. 2004). What we were able to confirm, as previously observed in SK-N-MC cells, by protein expression, is that by treating undifferentiated cells with L-Glutamate, and considering the evaluation of gene expression of adenosine A_1 receptors after 24-72h exposure to 1 μM L-Glutamate, we confirmed that adenosine A_1 receptors may have a neuroprotective role against the excitotoxicity of L-Glutamate. Nevertheless by considering the treatment of undifferentiated SH-SY5Y cells with 10 μM L-Glutamate, the previously observed effect on adenosine A_1 receptors disappears, showing only a tendency, but a significant increase in gene expression of mGlu1 receptors was observed, thus confirming by RT-PCR what observed for SK-N-MC and already discussed. Furthermore it should be noticed that only by using 10 μM L-Glutamate we observed an effect (increase) on gene expression of $mGlu_1$ receptors and only a tendency in modulating the expression of A_1, on the other hand by using 1 μM L-Glutamate we observed an effect (increase) on the expression of A_1 receptors and only a tendency to increase the $mGlu_1$ ones. It could be explain by the use of different concentrations of L-Glutamate able to act in a different manner on these two types of receptors. At high (100 μM) concentrations, L-Glutamate could be more effective on the activation of NMDA and consequently modulating the expression of metabotropic receptors $mGlu_1$ as a response to this activation. In fact it has been proposed a relationship between the activation of

NMDA receptors and the expression of receptors belonging to the group I of metabotropic glutamate receptors, by studying neurons from cortical region of rats (Hedinger et al. 2002). On the other hand by using long-exposure to 1 μM L-Glutamate, a moderate toxic concentration, which probably that considered concentration is unable to massive activate NMDA receptors and modulate matabotropic receptors of group I, but able to change the Ca^{2+} imbalance produce as protective response an increased gene expression of adenosine A_1 receptors; it's well known the relationship between the pathways of NMDA receptors and adenosine receptors, and the antagonistic role of those systems (Sweeney, 1997). Unfortunately, we missed to confirm our hypothesis by the evaluation of the protein expression of those targets, after long exposure to L-Glutamate, but it's anyway a promising confirmation of what previously observed for SK-N-MC cells.

2.3 On differentiated SH-SY5Y cells

The choice to differentiate SH-SY5Y cells, have been a convenient method, not really time consuming, to study the effect of neurotoxic treatments in a fully differentiated human neuronal cells, closer to human neurons, proceeding for example from patients. The opportunity to have a further *in vitro* model to study neurodegenerative process and closer to that naturally occurring in human brain, has been previously discussed (Xie et al. 2010). Differentiated SH-SY5Y cells (from this point called neuronal cells), seem to be more resistant to the effect of L-Glutamate, respect to the undifferentiated one; it has been surprising to observe that even for long term exposure to 100 μM L-Glutamate, that is considered a very toxic concentration, during 24, 48 and 72h, no high toxicity was observed, and the survival has never been under 70% of controls. This is one of the most evident differences between undifferentiated and differentiated SH-SY5Y cells. For example by considering the gene expression after 6h treatment with 100 μM L-Glutamate, we observed not significant difference between controls and gene targets, it means that gene expression of considered adenosine and metabotropic receptors are not influenced by the exposure to 100 μM L-Glutamate. Even by considering exposure to

lower concentrations of L-Glutamate, we observed only in the case of 100 nM L-Glutamate 24h, a decrease in gene expression of all targets, but reverted at 48h and at 72h only the gene expression of $mGlu_1$ receptors had significantly increased. On other hand after treating neuronal cells with 10 μM L-Glutamate, it seems to have effect more on gene expression of adenosine A_{2A} and A_{2B} receptors. All the related effect of L-Glutamate on neuronal cells, and the low profile of toxicity, could mean a lower expression of NMDA receptors in these cells, respect to the undifferentiated cells, making them more resistant to L-Glutamate (Pizzi et al., 2002; Singh et al., 2005). The observed effect on gene expression of described target could be related to a different activation of NMDA receptors. It should be noticed for example that, like previously discussed, to an high toxicity by L-Glutamate, corresponds an high activation of NMDA receptors and consequently an increase of expression of A_1 adenosine receptors, which has not been observed in the case of differentiated cells. Nevertheless the main activity of L-Glutamate on gene expression, has been observed on BACE-1 gene target; in fact by passing through exposure of neuronal cells from low to high concentrations of L-Glutamate, we noticed a gradually but significant reverting from the initial decrease in gene expression, by considering 100 nM L-Glutamate, to a tendency in increase the gene expression of BACE-1 by using 100 μM L-Glutamate. This has been the main effect found, in neuronal cells, and in this case, related to long-time exposure and to increasing concentrations of L-Glutamate. It has been demonstrated that the enzyme processing amyloid beta-peptide and related to many neurodegenerative diseases (BACE-1) increase its expression after an increase of intracellular Ca^{2+} level (Cho et al. 2008), this is the case of exposure to high concentration of L-Glutamate. Nevertheless it has been demonstrated how physiologically concentrations of NMDA, may have neuroprotective effect, by preventing Ca^{2+} imbalance into neuronal cells, as well as acting on naturally neurons plasticity. The same could be well applied to non-toxic concentrations of L-Glutamate, acting as agonist on the modulation of NMDA and AMPA receptors (Singh et al. 2005).

2.4 On neuronal cells from rat's brain cortex.

About neuronal cells from cortex region of rat's brain, in this section we only analyzed the survival of cells in the presence of L-Glutamate by MTT reduction assay, without focusing on changes occurring on gene expression of receptors after short-time exposure to L-Glutamate because it has been extensively studied (Castillo et al., 2009). Nevertheless the observed toxic effect on cells survival after exposure to 100 μM L-Glutamate for 2 and 6 h was in agreement to what previously described by others authors (Murphy at al., 1990). In the present we were mainly focused on the effect of L-Glutamate, after long-time of exposure, on gene expression of adenosine and metabotropic glutamate receptors, and BACE-1 enzyme. Similarly to what we observed about differentiated SH-SY5Y cells exposed for large period to L-Glutamate, we have to consider two separate effects based on the doses of L-Glutamate used. In particular from RT-PCR analysis of the gene expression during L-Glutamate treatment we obtained different, and apparently discordant results, on the basis of concentrations of L-Glutamate. After treating cells with 100 nM L-Glutamate we obtained similar results in gene expression as those observed and discussed for SH-SY5Y differentiated cells after treating them with sub-toxic concentrations of L-Glutamate. After treating cells with 100 nM L-Glutamate, the most observed has been a tendency, not significant, to regulate the gene expression of some receptors, like for example A_1, or A_{2A} and A_{2B}. Concerning BACE-1 we observed a significant decrease in the gene expression in all the considered period of exposure to L-Glutamate. Therefore what previously discussed about the regulation made by L-Glutamate, at non-toxic doses, on the gene expression of differentiated SH-SY5Y cells, could be also well applied to neuronal cells from rat's brain. At the same time if we consider the treatment with 1 μM L-Glutamate for long time period, we noticed some significant variations in the gene expression, mainly in A_{2B} adenosine receptors, $mGlu_1$ receptors and BACE-1. It should be also highlight how the observed effect of gene expression is enhanced after 72h of L-Glutamate treatment. The modulation of 1 μM L-Glutamate of gene expression may be due to the fact that this concentration is near to the toxic one. Thus we observed a modulation on gene expression of A_{2B} and $mGlu_1$ as a possible response to a more

effective activation by 1 μM L-Glutamate of NMDA receptors, and a related increase in intracellular Ca^{2+} levels. For example it is known that adenosine $A_{2A/2B}$ receptors and metabotropic glutamate receptors of group I could functionally interact, and regulate each other (Tebano et al. 2005). Furthermore the activation of NMDA by L-Glutamate or other agonist could influence the activity and regulation of metabotropic glutamate receptor belonging to group I (Lea et al. 2002). In addition the increase of intracellular levels of Ca^{2+} deriving from an activation of NMDA receptor by L-Glutamate could induce an increase of the expression of BACE-1, as observed in our case (Cho et al. 2007). This increase of BACE-1 gene expression has been demonstrated in rat's cells induced by a toxic damage (Blasko et al., 2004). Thus a relationship between using of high concentrations of L-Glutamate for long time exposure, and the increase of BACE-1 expression has been here demonstrated, proposing a direct relationship between the formation of toxic amyloid-β peptide and the presence of toxic concentrations of L-Glutamate. As previously reported, BACE-1 enzyme is fully involved in the formation of amylod-β peptide by following the amyloidogenic pathway, as well as its inhibition was demonstrated to be a potential therapeutic approach in neurodisease. Furthermore it has been demonstrated that mGluRs and non amyloidogenic processing pathway have common steps, indicating a further relationship between the role of glutamate and the generation of amyloid-β peptide (*see Introduction*).

3. Study of Amyloid-β peptide induced toxicity

3.1 On SK-N-MC cells from human neuroepithelioma.

Results herein presented clearly demonstrated that SK-N-MC cells suffer toxicity induced by treatment with 25-35 fragment of amyloid-β peptide ($A\beta_{25-35}$). This fragment have been employed in various experimental works focused on demonstrating its toxicity as well as the fragment $A\beta_{1-42}$ (Pike *et al*., 1993; Iversen *et al*., 1995). Toxicity of $A\beta_{25-35}$ have been reported by using MTT reduction method in rat's neurons from cortical region *in vitro*, where the activation of caspase 3 is

responsible for cells death after exposure to $A\beta_{25-35}$ (Harada and Sugimoto, 1999). Furthermore $A\beta_{25-35}$ toxicity is related with concentrations used; in the present work the used concentration was toxic even though there are some authors who confirmed that $A\beta_{25-35}$ at physiological concentrations could act on cells with trophic, antioxidant, antiapoptotic or neuroprotective effects on neuronal cells (Schaeffer *et al*., 2008). Our results showed that $A\beta_{25-35}$ treatment influence in a significant way the gene expression of metabotropic glutamate receptors of group I. Like explained before these receptors could be related with toxicity, neuronal death but also with neuroprotection. More in detail we observed that this treatment in SK-N-MC cells affected $mGlu_5$ gene expression and showed a tendency to change the expression of $mGlu_1$ receptors. For several years there have been controversial data about the neurotoxic role exerted by amyloid-β peptide. Although there are many experimental works indicating that the activity of metabotropic glutamate receptors of group I is excitatory, some data seem to confirm that tonic activation of these receptors change the excitatory neuronal activity into an inhibitory activity, and for that reason into a neuroprotective activity (Herrero *et al*., 1998; Bruno *et al*., 2001). Furthermore results obtained by our group in humans, seem to confirm this hypothesis. Thus it was reported that metabotropic glutamate receptors of group I are decreased in frontal cortex brain of Alzheimer patients (Albasanz *et al*., 2005) in a progressive way as the disease evolve, probably due to an increase in glutamate concentrations in brain, as the disease worst. At the same time the increase of gene expression of these receptors after $A\beta_{25-35}$ treatment in our cells, could represent an attempt of cellular response to decrease toxicity caused by that peptide. The present work demonstrates that amyloid-β peptide treatment influence the expression of A_1 receptors although not significantly. While the tendency in growing the expression of adenosine receptors A_1 after amyloid treatment observed in radiolabelled binding assay could be explained like an intent of cells to protect themselves form toxicity induced by amyloid, the effect on A_{2B} is more complicated to explain. This variation observed in gene expression of adenosine receptors A_{2B}, could be related to the opposite tendency observed in A_{2A} in decrease the expression after amyloid treatment at the same concentration; like explained before in the present work

adenosine A_{2A} and A_{2B} receptors are very similar and could be mutually modulated. Nevertheless, in agreement, the use of antagonist of these receptors has been demonstrated to protect from neurotoxicity induced by $A\beta_{25-35}$ fragments, in granular neurons (Dall'Igna *et al*., 2003). In the present work, on the section dedicated to the study of the activity of adenosine receptors antagonist on undifferentiated SH-SY5Y cells, we demonstrated how A_{2A} antagonist (SCH58261), in cells exposed to toxic stimuli with $A\beta_{25-35}$, exerted a protective effect.

3.2 On undifferentiated and differentiated SH-SY5Y cells from human neuroblastoma and neuronal cells from rat's brain cortex.

In this section we focused on the effect of the treatment with $A\beta_{25-35}$ on the survival of undifferentiated and differentiated SH-SY5Y cells from human neuroblastoma, and on the gene expression of some targets related with neurodegenerative diseases. First, by considering cells survival we observed how $A\beta_{25-35}$ acts differently on undifferentiated and differentiated cells. The differentiated SH-SY5Y cells appear to be more resistant to $A\beta_{25-35}$ toxicity, as previously observed for L-Glutamate, with a cells survival not less than 50% for both considered concentrations. On the other hand undifferentiated cells suffer in a more evident manner the toxicity of $A\beta_{25-35}$ reaching the 30% of survival after 6h exposure to 25 μM $A\beta_{25-35}$. Differentiated SH-SY5Y cells have been demonstrated to have a more efficient antioxidant mechanism, involving a more efficient Ca^{2+}-ATPase machinery and by increasing the total membrane cholesterol component which reduce the vulnerability to bind toxic amyloid aggregates to the membrane (Cecchi et al. 2008). Concerning the gene expression evaluated by RT-PCR on undifferentiated SH-SY5Y cells after 6h treatment with 10-25 μM $A\beta_{25-35}$ 10-25μM, we did not observe any significant changes in gene expression of selected targets, except some tendency in modify the gene expression for example of adenosine A_1 receptors or metabotropic

glutamate $mGlu_1$ and $mGlu_5$ receptors, probably as a consequence of the oxidative damage evoked by $A\beta_{25-35}$ (Sagara and Schubert, 1998), more than a directly activity of $A\beta_{25-35}$ on these receptors (Busciglio et al.,1993). At the same time the tendency observed for both considered treatment with $A\beta_{25-35}$ to decrease the expression of BACE-1 may be related to the forced reduction of the activity of that enzyme by the presence of one of its cleavage products. Furthermore, no differences in the total gene expression of each investigated targets have been observed between not differentiated and differentiated control SH-SY5Y cells, thus indicating, that the increased resistance observed by survival assay to amyloid-β peptide as well as L-Glutamate treatment, exhibited by differentiated SH-SY5Y cells compared to undifferentiated, involve different mechanism than those involving the investigated gene targets (*data not shown*). About neuronal cells from rat's brain cortex, it could be noticed that cells are susceptible in a concentration and time -dependent way to $A\beta_{25-35}$ exposure; by using the higher concentration a 30% of survival was observed. The mechanism of Aβ toxicity is well known and applicable to both human and rat's model; it involves radical formation, oxidative stress, intercalation of Aβ fragments into cell membrane with ions imbalance and alteration of membrane potential, even with accumulation of amyloid aggregates outside cells blocking cell networks (for a complete review on Aβ toxicity *see* Atwood et al., 2003).

4. Effect of hydrosoluble [60]fullerene derivative (t3ss)

The [60]fullerene derivatives have been extensively investigated because their own chemical and interesting biological properties. Due to their physical and chemical aromatic properties which limit the solubility of fullerenes in many organic solvents, the study of their biological properties has been difficult to achieve. In that sense some modification on the main structure of fullerene by adding an proper substituent, allowed to obtain fullerene derivative with a more hydrosoluble character with more chances to be tested on biological assay. In the last decade many authors

demonstrated the promising biological properties of fullerene, instead others demonstrated its toxicity in human cells. The main studied biological property of fullerene has been the antioxidant and radical scavenger one. To this main biological property of fullerene have been related the most of effects observed in biological assays (for a complete review see Satoh *et al*., 2006). It has been observed a protective effect of a [60]fullerene derivative in rat's cortical neurons treated with excitotoxic compound like NMDA (N-methyl D-aspartic acid), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and KA (kainic acid), that have been related to an anti free-radicals effect of fullerene during the apoptotic process (Dugan *et al*., 1996).

4.1 On SK-N-MC cells from human neuroepithelioma

In this section we demonstrated how cells, as well as no suffer toxicity exposed to the treatment with hydrosoluble [60]fullerene derivative t3ss, showed a significant recovery from the toxic effect of L-Glutamate and amyloid beta-peptide, with the contemporary use of t3ss. If the protective effect of [60]fullerene derivative t3ss could be well related to its antioxidant activity during the treatment with the fragment 25-35 of amyloid-β peptide, because amyloid toxicity have been demonstrated to be related to an oxidative damage in cells (Sultana *et al*., 2008). However that effect of t3ss could not explain the protective effect during L-Glutamate treatment. In that sense during L-Glutamate treatment, t3ss modified the expression of adenosine A_1 receptors like confirmed also by radioligand binding assay. However the treatment with amyloid-β peptide in combination with t3ss derivative had no significant effect on the expression of adenosine A_1 receptors. What we observed when cells were treated with L-Glutamate and t3ss have been a significant increase of adenosine A_1 receptors with a Bmax value more similar to that observed for the treatment with t3ss alone than for the treatment with L-Glutamate; this was observed for both the concentrations of L-Glutamate considered for the assay. Therefore in the case of treatment of cells with amyloid-β peptide the observed protective effect of t3ss could be explained by considering the antioxidant and radical scavenger

properties of [60]fullerene derivative against the oxidative stress caused by Aβ, while the observed protective effect of t3ss observed at all times and concentrations of L-Glutamate used, could be related to t3ss effect on modulating adenosine A_1 receptors. At the same time the tendency of t3ss to modulate the metabotropic glutamate receptors group I ($mGlu_1$ and $mGlu_5$) could be considered part of that observed protective effect (see toxicity results for L-Glu), in addition to the effect on adenosine receptors. More in general by evaluating with radioligand binding assay these receptors in intact cells and membrane we observed that t3ss increase their expression, in particular, a significant increase of subtype A_{2B} respect to subtype A_{2A} of adenosine receptors. That result is very interesting because it seems that t3ss is able to modulate the adenosine receptors in particular the subtype A_1 and A_{2B}, by acting directly on their pathways, although more experiments are needed to confirm it. In this sense while adenosine A_1 receptor has been largely investigated related to its protective role, the A_{2B} subtype role has been less understood with respect to the A_{2A} subtype, even if the blockade of those receptors with teophylline has been related to an anti-asthma effect (Weinberger and Hendeles, 1996). In that sense the [60]fullerene derivative t3ss, has been demonstrated to act different as only radical scavenger, involving adenosine and metabotropic glutamate receptors pathways modulation, making a relevant role of the exhibited protective effect.

4.2 On undifferentiated SH-SY5Y cells from human neuroblastoma

As observed in SK-N-MC cells, [60]fullerene hydrosoluble derivative t3ss has been demonstrated to be a valid treatment to revert toxicity derived by treatment with L-Glutamate and Amyloid-β peptide also in SH-SY5Y cells. MTT reduction viability assay, confirmed that the contemporary presence of t3ss derivative during exposure of cells to L-Glutamate and Amyloid-β peptide, is related to a partial or total recovery of cells from induced death. From the previous section we obtained results that correlate this protective effect of t3ss derivative mainly with a modulation of adenosine A_1 receptors, which total expression appeared to increase after treatment with t3ss derivative. Nevertheless data obtained from radiolabel binding assay, (i.e

as number of total A_1 receptors increased), were not supported by gene expression due to problems related to the use of specific gene sequences as previously commented. Considering SH-SY5Y treatment with t3ss derivative, L-Glutamate and amyloid-β peptide, we decided to focus mainly on changes occurring in gene expression, also in order to complete what previously observed in SK-N-MC cells by an exhaustive study by RT-PCR technique. First of all no evident toxicity has been observed after different times of exposure of cells to t3ss derivative, in terms of survival. At the same time no significant effect on the gene expression after long time exposure to t3ss derivative has been observed, except a tendency to increase A_1 gene expression by increasing time of exposure. Nevertheless if we consider the recovery effect on cells viability exerted by t3ss derivative during exposure to L-Glutamate and amyloid-β peptide, we observed some interesting effect on gene expression. In particular by considering 6h treatment with 100 μM L-Glutamate, the only significant effect observed by the combination of t3ss derivative and L-Glutamate on gene expression, has been a decrease in $mGlu_1$ expression. It is known that [60]fullerene derivatives as well as to be a radical scavenger and antioxidant agent, could act on the function of NMDA receptors, by limiting the related toxicity of agonist activation (Dugan et al., 1994); this could involve $mGlu_1$ receptors expression, because it is known that the signalling pathway of mGluR of group I could be related to NMDA receptors (Guo et al., 2004). On the other hand by considering the contemporary treatment for long time exposure (24-72h) to lower concentrations of L-Glutamate, t3ss derivative seems to exert its effect mainly on changing adenosine A_1 receptors gene expression, by increasing it. Furthermore a smaller increase is exerted in A_{2A}, A_{2B} and $mGlu_1$ gene expression by the contemporary treatment with L-Glutamate and t3ss derivative, respect to the treatment with L-Glutamate alone, thus confirming these receptors are sharing some signalling pathways (Ferré et al., 1999). Anyway the only significant effect on gene expression maintained, after 72h exposure to both L-Glutamate (1-10 μM) and t3ss derivative, is the increase adenosine A_1 receptors expression; finally this result may confirm a good contribution of adenosine A_1 receptors on the protective effect observed for t3ss derivative. About the treatment with $A\beta_{25-35}$ we observed a protective effect by t3ss derivative on cells survival more or less evident if the

intensity of Aβ treatment and related toxicity is considered. Nevertheless no evident relationship between the gene expression and protective effect exerted by t3ss derivative has been detected, thus suggesting an effect of t3ss derivative mainly as radicals scavenger, following the oxidative damage evoked by Aβ. In this sense many antioxidant compounds have been demonstrated to revert the oxidative damage caused by $A\beta_{1-42}$ and $A\beta_{25-35}$ fragments (for a review *see* Abramov et al., 2005).

4.3 On differentiated SH-SY5Y cells

The main effect observed by treating differentiated SH-SY5Y cells with t3ss derivative alone for 6, 24, 48 and 72h, was a significant increase in gene expression of A_{2A}, A_{2B}, $mGlu_1$ and BACE-1 targets by considering up to 6h exposure to t3ss derivative. At 6h exposure to t3ss derivative, in fact, the effect on gene expression is the opposite to that observed at 24h of exposure; at the same time no effect on gene expression of A_1 receptors was observed, like for undifferentiated cells. This could be explained by the effect of t3ss derivative on intracellular radicals species, on membrane lipids peroxidation, that could affect Ca^{2+} imbalance, with lower values at 6h and higher for long time of exposure, but not enough to induce a massive cells death, but to influence the expression of the target gene previously described. Another contributing mechanism could be a partial action of [60]fullerenes on NMDA related pathway, like previously discussed. Considering the treatment with sub toxic L-Glutamate concentrations and t3ss derivative, no interesting effect was observed on gene expression, except a general decrease for all the considered gene targets by using 100 nM L-Glutamate, that t3ss derivative was unable to revert, even this has been observed only for 24h treatment. The most interesting effect after cells exposure to non-toxic concentrations of L-Glutamate, has been noticed in the gene expression of BACE-1, that is constantly decreasing for all the considered

treatments, except by using 100 μM L-Glutamate for 6h, when a little increase in BACE-1 expression was observed, counterbalanced by the contemporary presence of t3ss derivative. The magnitude of NMDA receptors activation may play a crucial role in maintaining the Ca^{2+} balance into cells. In this sense, to an increased activation of NMDA receptors may correspond an intracellular Ca^{2+} imbalance, and activation of related pathway, involving cell toxicity as previously discussed. This could be related to what observed by treating cells with increasing concentration of L-Glutamate. The modulation exerted by L-Glutamate on NMDA receptors and consequently on Ca^{2+} homeostasis, may play a differential role on gene expression and on effect exerted by t3ss derivative on cells. In particular, as observed, t3ss derivative resulted to be more efficient as neuroprotective agent, if cells are exposed to high concentrations of L-Glutamate, supposing a more effective effect on NMDA receptors and Ca^{2+} homeostasis. Furthermore, and as previously discussed, intracellular levels of Ca^{2+} are crucial for the regulation of BACE-1 expression (Cho et al., 2008).

4.4 On neuronal cells from rat's brain cortex.

The protective effect of t3ss derivative against toxicity of L-Glutamate and $A\beta_{25-35}$ has been confirmed, as well as discussed in human cells model, in neuronal cells from rat's brain. Significant recovery from cells death by t3ss derivative has been observed after the majority of toxic exposure to L-Glutamate and $A\beta_{25-35}$. Nevertheless also in this study we mainly focused on the long term exposure to [60]fullerene derivative and in combination with a range of concentrations of L-Glutamate from non-toxic to highly toxic, and mainly on gene expression of receptors possibly involved in degeneration evoked by L-Glutamate. First, the effect of [60]fullerenes on cells from rats brain, have been demonstrated that to be non toxic and, furthermore, to protect cells from toxic treatment and induced degeneration (Tikhomirov et al., 2009). It is quite difficult to explain [60]fullerenes protection against L-Glutamate toxicity, without referring to the most studied activity of these compound on AMPA and NMDA receptors, or its effect on Ca^{2+} imbalance. And even

more difficult is to explain results obtained from RT-PCR analysis on gene target, without making hypothesis involving the relationship between apparently different pathways, and even more in rat's neurons, because there are not many example in literature about it. So from now, mainly we attempted to justify some effect of t3ss derivative on gene expression of target involved in neurodegenerative diseases. For example the main observed effect, after expose rat's neuronal cells to t3ss derivative (1-5μM) for long period, was a great increase the adenosine A_{2B} receptors gene expression. At the same time also gene expression of A_{2A} and $mGlu_1$ has been resulted to be affected by t3ss treatment, with a decrease in the total gene expression. The explanation could be related, as previously discussed for the other studied cells model, to the influence that t3ss derivative may have on Ca^{2+} homeostasis, starting from this, is well known how receptors belonging to a same family and with similar structure and common pathways, are mutually regulated. This is the case of gene for A_{2A} and A_{2B} adenosine receptors, and $mGlu_1$ and $mGlu_5$, which after exposure to t3ss derivative appeared to be mutually regulated. Thus it has been demonstrated that mouse knock out for A_{2A} receptors up-regulated A_{2B} receptors (Teng et al., 2008), as well as the alternative roles and regulation of mGluRs belonging to group I (Volk et al., 2006). Furthermore t3ss derivative has been demonstrated to be effective in counterbalance the effect of long exposure to 100 nM L-Glutamate on gene expression, but only by using 5 μM t3ss, no reverting effect by using 1 μM t3ss was observed. In particular after 24h exposure to 100 nM L-Glutamate the contemporary presence of 5 μM t3ss counterbalanced the decrease on gene expression of A_{2A}, $mGlu_1$ and $mGlu_5$ caused by L-Glutamate, inducing an increase in the expression of these targets. As well as it is known that an activation of A_{2A} receptors may be related to a protective effect in rat's subjected to inflammation process (Chen et al., 2009), it is also known the controversial role of metabotropic glutamate receptors of group I, that some authors associate to excitotoxicity and neurodegeneration (Tsai et al., 2009), and others to neuroprotective effect (Kalda et al., 2000). In our case the observed reverting effect on $mGlu_1$ and $mGlu_5$ receptors gene expression, exerted by t3ss derivative against L-Glutamate could be related with a protective effect, as well as the increase on A_{2A} receptors gene expression. Probably the most interesting result by gene expression

was obtained after chronic exposure of neuronal rat's cells to 1 μM L-Glutamate with the contemporary presence of 5 μM t3ss, by considering the expression of BACE-1. In fact we assisted to a dramatically increase of BACE-1 gene expression by L-Glutamate and that effect was counterbalanced by the presence of t3s derivative, suggesting the role of chronic high concentrations of L-Glutamate in amyloidogenic evoked neurodegeneration, and at the same time the possible role of t3ss derivative in limiting it. Furthermore we assisted to a great increase in gene expression of A_{2B} receptors after 72h exposure to the combination of L-Glutamate and t3ss derivative, greater than the treatment with L-Glutamate alone. It has been demonstrated how the concert work of A_{2A} and A_{2B} receptors may induce a strong protection in rat's cells against reperfusion injury (Xi et al., 2009). At the same time the observed gradual decrease in gene expression of $mGlu_1$ receptors, may suggest a direct protective effect of t3ss derivative (Pellegrini, 2003) as well as an interfacing between the adenosine and metabotropic glutamate receptors pathways (Conn and Pin, 1997; Nicoletti et al., 1999).

4.5 Under hypoxic conditions on undifferentiated SH-SY5Y cells

Data from MTT reduction viability assay after exposing undifferentiated SH-SY5Y cells from 6h to 24h of hypoxia conditions, suggest high toxicity suffered by cells. Nevertheless, also in this case, the contemporary presence of t3ss derivative, protected cells from oxidative stress deriving from hypoxic conditions, although higher concentrations than previously used, have been needed. Finally we obtained a viability profile of cells under hypoxic conditions by using increasing concentrations of t3ss derivative, which demonstrates a t3ss dose dependent recovery. Furthermore previously study on toxicity of t3ss derivative on SH-SY5Y cells survival provided similar results as obtained for SK-N-MC cells, with no significant toxicity observed (data not shown). Like extensively discussed, under these conditions of oxidative stress derived from hypoxia, [60]fullerenes are able to exert the maximum of their activity on radical species as radical scavengers, helping the natural protective

mechanism in cells (Beuerle et al. 2008). At the same time our investigation on gene expression during hypoxia, provided some interesting results, involving some targets. Therefore by treating SH-SY5Y cells, exposed to 24, 48 and 72h of hypoxic conditions, in presence of t3ss derivative at 75μM (medium effective dose resulted from survival assay), we observed an interesting changes in gene expression after the early 24h hypoxia and contemporary use of t3ss derivative, that revealed an increase in gene expression of A_1 and BACE-1 and a decrease in gene expression of A_{2A} and $mGlu_1$ receptors. The observed increase in gene expression of A_1 adenosine receptors may be related to the described protective effect exerted by these receptors during hypoxic conditions (Arrigoni et al., 2005), and the ability of t3ss derivative to increase the expression of these receptors has been extensively discussed. Furthermore it has been demonstrated that during oxidative stress and related toxicity, the use of antagonists of adenosine A_{2A} receptors may be considered a valid therapeutic approach (Cieślak et al., 2008), thus suggesting that t3ss derivative by counterbalancing the gene expression of these receptors may contribute to the observed protective effect during hypoxic conditions. About the observed significant increase in gene expression of $mGlu_1$ receptors by using t3ss derivative, respect to the controls of hypoxia, it could be explained by the previously discussed modulation of t3ss derivative on NMDA and Ca^{2+} homeostasis, involving metabotropic glutamate receptors of group I because and their regulation on voltage dependent calcium channels (Endoh, 2004). Furthermore the t3ss derivative could promote antagonist effect on $mGlu_1$ receptors, consequently inducing an increase in gene expression, in that sense has been reported how antagonist of this receptors could reduce the damage in post ischemic conditions (Moroni et al., 2002). About the increase exerted by t3ss derivative on gene expression of BACE-1 during hypoxic conditions, it may be due to a high depletion of BACE-1 active enzyme caused by t3ss derivative, which as a consequence could induce the activation of relative gene expression. In this case, in fact, on the contrary respect to what observed in the case of increase in gene expression of BACE-1 exerted by 1 μM L-Glutamate in rat's cells, values of the increase in gene expression are considerable lower, suggesting us most a modulation in BACE-1 gene expression, and not a forced gene expression directed to the synthesis of new functional BACE-1 enzymes, as in the case of the

treatment with L-Glutamate 1μM observed in rat's cells. To confirm that, further assays, including studies on enzyme activity and total quantification of protein, will be necessary

5. About the activity of the antagonist of adenosine A_1, A_{2A}, A_{2B} and A_3 and $mGlu_1$ and $mGlu_5$ receptors.

Selective antagonist are useful to study changes on a determinated molecular pathway involved in the partial or total reduction of the activation of a specific receptor by the main agonist. Therefore, in our case, we proposed to study how a specific antagonist for the receptors investigated until now, and in many cases implicated in toxic and/or protective effects, may interfere with the observed effect, mainly during the toxic treatment with L-Glutamate and in combination with t3ss derivative. The final goal has been to investigate how a partial modification of the activation of these receptors, may change the activity of t3ss derivative. For this study we considered to use specific antagonists of human adenosine A_1, A_{2A}, A_{2B}, A_3, $mGlu_1$, $mGlu_5$ receptors, and to test them on undifferentiated SH-SY5Y cells and on neuronal cells from rat's brain at the same time as cells were exposed to L-Glutamate or Amyoid-β peptide and in combination with t3ss derivative.

5.1 On SH-SY5Y cells from human neuroblastoma and on neuronal rat's brain derived cells

The exceptional effect obtained by MTT viability assay after exposing SH-SY5Y cells to novel specific antagonist of A_{2A} (SCH58261) and A_3 (SCHx) adenosine receptors has still to be fully investigated. The observed increase in cells growth may have more than one possible explanation. It's known that an activation of adenosine A_3 receptors exhibit an anti-apoptotic and anti-proliferative effect on many class of cancer cells (Brambilla et al, 2000; Gao et al, 2001), thus the use of an antagonist

limit or cancel that effect (Merighi et al. 2002). Nevertheless the role of activation or blockade of adenosine A_{2A} receptors is still controversial, for example Alfinito and co-workers suggest that a blockade of A_{2A} receptors by use of antagonist may contribute to a protective effect preventing cell death (Alfinito et al., 2003) and others authors suggest that an activation of these receptors may have an antimitotic effect and thus an antiproliferative effect (Sun et al., 2006). Results indicated that when cells are treated with 25 μM amyloid-β peptide for 6h and with the contemporary presence of these two antagonist, a great recovery from cells death exerted by Aβ alone was also observed. On the contrary with the contemporary presence of t3ss derivative and adenosine antagonist during exposure to 25 μM Aβ, not cumulative effect on recovery from toxicity was observed. In the same conditions, but changing the toxic treatment with 100 μM L-Glutamate 6h, an increase of the protective effect of t3ss derivative was observed by the contemporary use of all the considered antagonists. This has been unexpected and no apparently explanation is possible, without performing further analysis. Anyway, by considering that the protective effect exhibited by t3ss derivative against Aβ, is not increased by the contemporary presence of these antagonists, but enhanced in the case of L-Glutamate, it is suggested that during Aβ toxicity and protective effect of t3ss derivative the receptors involved are different from those involved in L-Glutamate toxicity and related t3ss protection. At now, without more and completed analysis on that provided effect on cells survival, further hypothesis will be vain. For example we should expect by using an antagonist of adenosine A_1 receptors a decrease of protective effect of t3ss derivative, but opposite results were found. Nevertheless some authors reported a reduction of inflammatory process by using antagonist of A_1 receptors (Nadeem et al. 2006); furthermore antagonists of A_{2A} receptors exhibited neuroprotective effects (Kalda et al., 2006) and today are considered as possible novel therapeutic treatment for Parkinson's disease (Muller and Ferré, 2007). Furthermore it has been also demonstrated how antagonists of A_{2B} receptors attenuate pulmonary inflammation (Sun et al. 2006; Mustafa et al. 2007), and at the same time antagonist of A_3 receptors exhibited moderate but significant antinflammatory properties (Bilkey-Gorzo et al., 2008). In turn, antagonists of mGluRs of group I demonstrated to be neuroprotective in rat's cortical cells after

exposure to NMDA (Bruno et al., 1999). Furthermore the observed significant increase in gene expression of $mGlu_5$ after exposing cells to antagonist of A_{2A} and A_3 receptors for 6 h may be related to a possible functional interaction between these two class of receptors as described by Tebano and co-workers (Tebano et al., 2005). We have also to underline that it's very difficult to compare the herein observed effect for these antagonists to the high number of different models, different conditions and different type of toxicity where these were tested. A great influence is also given by using different concentration of antagonist, which at high concentrations could act as a partial agonist, so finally activating a specific receptor instead to blocking. In neuronal cells from rat's brain, by using the same receptors antagonist, and the same conditions as before, we only noticed the toxicity of the A_{2A} and A_3 adenosine receptors antagonist on cells survival. No difference between the protective effect of t3ss derivative alone or in combination with the receptors antagonist used, were observed after exposure of cells to 100 μM L-Glutamate 6h, except by using $mGlu_5$ receptor antagonist MPEP, able to give a little increase in the protective effect of t3ss derivative. In this sense the protective effect of MPEP has been demonstrated by many authors in Parkinson's rat models (Bao et al., 2001; Battaglia et al., 2004; Baskys et al., 2005). For reason of time we couldn't perform the same study with antagonists more selective for rat's receptors, but it has been planned.

6. Nanohorns and Gold nanoparticles

During the recent years nanotechnologies have been improved in the number of studies and investigations carried out, mainly interfacing news materials with biologic systems. Nanohorns and goldnanoparticles belong to a class of nanomaterials opened to a variety of biological applications, and constantly growing (Roco, 2003). In this study, we provided to interfacing these nanomaterials to our *in vitro* models, in order to investigate the effects and also to compare with those observed for [60]fullerenes t3ss deivative.

6.1 On undifferentiated SH-SY5Y cells from human neuroblastoma and neuronal cells from rat's brain.

Nanohorns (NH) and gold nanoparticles (GNP) used in this study exhibited a moderate to high toxicity in undifferentiated SH-SY5Y cells, also by considering the potential use of gold nanoparticles as anti-proliferative agents (Brown et al., 2010). A similar profile from MTT reduction viability assay was obtained on rat's cells treated with nanohorns. Thus, aimed to investigate a possible effect of NH and GNP during exposure of cells to L-Glutamate and amyloid-β peptide, we detected surprising protective effect from both the used compounds. At the same time we noticed a protective effect of the two antagonist of adenosine receptors A_{2A} (SCH58261) and A_3 (SCHx), by exposing SH-SY5Y cells to toxic concentrations of NH. Like previously discussed, adenosine antagonist may exert protective effect on cells, while the most surprising has been the protective effect of non-toxic concentrations of GNP and NH against L-Glu and Aβ toxicitiy. Nanohorns belonging to the family of carbon nanotubes are extensively studied and exhibit a large number of biological properties and application (for a review see Liu et al., 2009), but also a toxic effect due to the accumulation in cells when not functionalised (Cui et al., 2005; Bottini et al., 2006). In our case by using news partially hydrosoluble functionalised nanohorns we provided a low toxic profile to cells and at the same time a protective effect toward L-Glu and Aβ toxicity, probably because of a radical scavengers mechanism, opening new perspectives for the study of this class of compounds. On the other hand is known that the engineering of modify GNPs is an open field mainly for *in vivo* delivery of therapeuticals (Ghosh et al., 2008) and avoid the toxicity of nanoparticles of gold due to their accumulation in many tissues and organs, with genotoxic, oxidative and cytotoxic effects (Johnston et al., 2010). Herein we want to report one of the first healthy effects on cells ever observed by using GNPs, a protective effect.

6.2 Time-depending gene expression of adenosine, metabotropic glutamate receptors and BACE-1 evaluated in neuronal cells from rat's brain cortex.

The needing to know changes occurring in neuronal cells during physiological not induced ageing, is essential to understand mechanisms involved in neurodegeneration and many neurodiseases. From the extraction of neuronal cells from cortex region of rat's brain and seeding (see *materials and methods*) to obtain a fully population of neuronal cells, 3 days (3 DIV) after seeding no glial cells are present which may interfering with normal neuronal cells. From day third (3 DIV) to day nineteenth (19 DIV) of seeding, we have a normal time course of a rat's neurons culture; it's kindly requested to perform the experiment during this period, to avoid cells degeneration, observable also in morphological changes occurring in cells (Savettieri et al., 1984). We decided to collect RNA from cells in culture every day starting form day 3 to day 19, finally obtaining profiles of gene expression targets showed in figures. As expected, we observed some profile in the gene expression, with a gradually increase, this is the case of adenosine A_{2A}, A_{2B} and metabotropic $mGlu_5$ receptors, and an alternating in gene expression during cells culture with a finally decrease, as in the case of BACE-1 enzyme, A_1 and $mGlu_1$ receptors. As reported by Castillo and co-workers, gene expression of A_1 and A_{2A} receptors is involved in ageing, and mainly due to a decrease of A_1 adenosine, and an increase of A_{2A} in model of *SAM* (Senescence-accelerated mouse), and this modulation is in agreement with our observations in rat's neuronal cells culture (Castillo et al., 2009). Furthermore also the observed increasing in gene expression of A_{2B} receptors in our model of rat's neurons could be explained by relating to what observed in human brain from ageing Pick's disease patients, which demonstrated the up-regulation of A_{2A} and A_{2B} receptors (Albasanz et al., 2006). Nevertheless many studies are in contrast to what observed in our case, neuronal cells from hippocampus region of rats brain, exhibited an increase of $mGlu_1$ expression and a decrease in the expression of $mGlu_5$ receptors along time of culture (Scheiwe et al., 2002; Simonyi et al., 2005). On the other hand an up-regulation of $mGlu_5$ receptors has been also observed in Down's syndrome brains, as ageing models (Oka and Takashima,

1999), and a downregulation of $mGlu_1$ receptors was also observed in neuronal model of Alzheimer's disease (Lee et al., 2002). Thus, depending on the model used, and particular region of brain observed, different results in gene expression of these receptors could be obtained. About the observed decrease in gene expression of BACE-1 enzyme, could be related to a normal decay of physiological processes in cells during aging; it may be noticed in fact, that BACE-1, during physiological conditions, exert its own function on non-APP substrates, and its total abolishment in knock-out mice, have been demonstrated to not have negative behavioural and psychological consequences (Roberds *et al.,* 2001). Therefore, in absence of an initiating factor, maybe including excitotoxicity, pathological activities of BACE-1 are silencing (Cole and Vassar 2007). Thus our results provided an interesting view on gene expression of adenosine and metabotropic receptors involved in normal aging of neuronal cells from rat's brain cortex.

7. [60]fullerenes based surfaces

During the realization of the present work of study about activity of [60]fullerene hydrosoluble derivative on models of neurodegeneration, we decided to verify the biocompatibility of [60]fullerenes linked on a surface. There are many example of substrates coated by different compounds to enhancing growth of certain cells model, like for example in our case, the poly-lysine coated surfaces essential for rat's neuronal cells growth (see *Materials and Methods*). Thus, far away from creating newest modified surfaces for biological purposes, we had simply the idea to proof the cell growth on a gold [60]fullerene modified surface. Once gold modified surfaces with [60]fullerene, were demonstrated to be compatible with undifferentiated SH-SY5Y cells growth, and even more, with the differentiation into fully and healthy human neuronal cells, we studied a possible effect of the own modified gold surface, during 6 h exposure to 100 μM L-Glutamate toxic treatment, by undifferentiated SH-SY5Y cells 24h seeded on that modified surface. Finally, [60]fullerenes modified gold surfaces obtained have been resulted to be effective, even more than the same [60]fullerene hydrosoluble t3ss derivative, in protecting cells from L-Glutamate

toxicity. There are no many examples in literature about [60]fullerene modified surfaces as biologically active, so we can make only some hypothesis about the way to interact with our cell models. As is known that L-Glutamate may induce in cells death by changing membrane potential, causing by intracellular Ca^{2+} imbalance, and also with the depletion of oxydative and redox systems and generation of radical species, have been also investigated [60]fullerenes SAMs with redox properties, as in the case of reduction of glucose by glucose oxidase and mediated by [60]fullerene SAM (Patolsky et al., 1998). In that sense [60]fullerene on surfaces may act as electron acceptors and radical scavengers at the same time of potential imbalanced cells, under L-Glutamate exposure. Finally, [60]fullerene beside exerting a low toxicity in our in vitro models and protective effective as compound in solution against L-Glutamate and amyloid-β peptide toxicity, is also effective as coater to manufacturing biocompatible substrates, even with certain therapeutically properties. Thus, these results, even further and accurate studies are needed, have been opened a promising perspective and, once at all confirming nano-modifed materials as a new challenge also for biology interfacing. Furthermore we attempted to evaluated the cell absorption of hydro soluble derivative of [60]fullerene by electroic microscopy, in order to well understand the process involved in the activity of fullerene into cells, but without success. In this sense, it would be interesting to broad the study of the interaction between [60]fullerene modified surfaces and cells, although we may hypothesize a not direct interaction between cells and [60]fullerene cage, with almost intercalation into lipids bi-layer of cell membrane, to exert the observed protective effect. Probably, that effect is exerted as external electron acceptor system, of radical species generated during L-Glutamate evoked oxidative stress. A similar system has been reported, and provided as [60]fullerene on gold surfaces, increasing the enzymatic activity of Glucose oxidase, by accepting electron proceeding from the enzyme accelerating the conversion of glucose into gluconic acid (Patolsky *et al*, 1998). On the other hand, the hypothetic interaction between [60]fullerene and cell membrane among the permanent intercalation of carbon cage, may generate a toxic effect on cells, relating to the imbalance of ions species. Thus, it is unlikely [60]fullerene modified surfaces act on cells, on that way.

CONCLUSIONS

CONCLUSIONS

1. SK-N-MC cells endogenously express all types of adenosine receptors and groups I and II of metabotropic glutamate receptors.
2. The toxicity of t3ss fullerene derivative is very low in all *in vitro* model assayed.
3. t3ss modulates gene expression of adenosine and metabotropic glutamate receptors in cells.
4. The excitotoxic effect of glutamate is confirmed in these cell models.
5. The neurotoxic effect exhibited by β-amyloid is also confirmed in these cells.
6. Differentiated SH-SY5Y cells are more resistant to these toxic insults probably due to modulation of adenosine receptors.
7. t3ss seems to protect cells against glutamate and β-amyloid toxicity by different molecular mechanisms involving its radical scavenger role and the modulation of adenosine receptor, respectively.
8. Other nanoparticles as nanohorns and gold nanoparticles have a moderate toxicity depending on the concentration and time of exposure and the *in vitro* model analyzed.
9. Gold nanoparticles and nanohorns exhibit a slight protective role against glutamate and β-amyloid toxicity.
10. Linkage of [60]fullerenes to mica gold surfaces could be use as a growing surface for cell culture as SH-SY5Y cells can be fully differentiated and the protective effect of [60]fullerene, exhibited in solution, is preserved.

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