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**Effect of alpha lipoic acid and choline-containing compounds on the expression of some astroglial biomarkers during proliferation and differentiation of astrocytes and neuroblastoma cultures**

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# CHAPTER 1

## INTRODUCTION

The central nervous system of vertebrates (CNS) is formed by neurons and glia, two heterogeneous classes of cells playing main and complex functions. During the neurogenesis, the proliferation and differentiation processes and the consequent formation in different types of neural cells play a key role in the development of the nervous system.

The interactions between neurons and astrocytes play a crucial role during the process of development and in the adult brain. During development, in fact, glial cells promote the growth of the precursors.

Astrocytes (star-shaped cells) are involved in the physical structuring of the brain and they are the most abundant glial cells in the brain that are closely associated with neuronal synapses and regulate the transmission of electrical impulses within the brain.

Astrocytes function to maintain the homeostatic environment of the CNS and also play an important role in immune regulation, acting as a source of chemokines, cytokines, and effector molecules. The idea that astrocytes have active roles in the modulation of neuronal activity and synaptic neurotransmission is now widely accepted.

In addition, it is well known that glial cells are also involved in providing neurotrophic signals to neurons required for their survival, proliferation and differentiation.

Glial cells contribute to control neuronal activities and are influenced by nerve cells through cell-to-cell interactions or through the release of soluble molecules such as neurotransmitters.

In the interactive dialogue between neurons and glia, the growth factors, coordinate some complex processes, such as the development and maturation of astrocytes and neurons. Growth factors also influence the activity of the target cells by interaction with specific receptors located on the plasma membrane.

Furthermore, it is known in the literature that the astroglia proliferation is closely related to cell growth (Wang et al. 1995).

In addition, steroid hormones play a key role on the function and development of the brain in mammals. In particular, it has been observed that estrogens act as protective factors in neurodegenerative diseases, such as Parkinson's disease and Alzheimer's diseases (Xu et al. 1998). In contrast to this regulatory function and protective, estrogens play a different role during neuronal development. Alterations in the levels of estrogen in the central nervous system (CNS) in development, affect critical aspects of cell differentiation including the extension of neurites, synapse formation, myelination, the expression of neurotransmitters and neuropeptides, death and survival cell (Miranda et al. 1994). Estrogens are crucial hormones for differentiation of the CNS. In addition, estrogens affect the astroglial compartment and can promote the effects of neurotrophins at the level of the genome through interactions with the regulatory pathways of neurotrophic factors (Toran-Allerand 1996).

## **GROWTH FACTORS**

The growth factors (GFs) are protein molecules with low molecular weight that play an important role in cellular communication, through interaction with specific receptors located in the plasma membrane of target cells. The growth factor interaction greatly resembles that of some hormones.

At the level of the S.N.C. astrocytes express receptors for various GFs, neurotransmitters and neuromodulators. Nerve cells can respond to growth factors of astrocytic origin and can control the functions astrocyte through different signaling molecules and pathways of intracellular signal transduction. The growth factors, neurotransmitters, and peptides represent the principal agents of cell signaling. The GFs include both the components that stimulate proliferation, as mitogens, and the components that depress it (for example, TGF  $\beta$  and interferons).

However, these types of response are attributable to distinct GFs, as a single factor may stimulate or inhibit the proliferation in different cell populations, or in the same cell type, but in different conditions (concentration and time of application of the factor growth, functional status of the target cells, etc.). The stimulation of the cells with GFs induce a proliferative effect after about 15-20 hours after the receipt of the stimulus. Mitogenic activity exercised by growth factors on quiescent cells induces G<sub>0</sub>-G<sub>1</sub> transition phase of the cell cycle, resulting a progression versus the S phase of DNA replication.

This process is characterized at least by two subphases: an initial "competence" phase, which in the case of quiescent cells coincides with their transition from G<sub>0</sub> to G<sub>1</sub> phase, followed by a "progression" phase in which the cell synthesizes components necessary for DNA synthesis, such as DNA polymerase, the "Proliferating cell nuclear antigen" (PCNA), ribonucleotide reductase, thymidine kinase.

In some cases, the stimulation of proliferation requires the presence of the growth factor during all the time that elapses between the receipt of the stimulus and the stage of progression. In other cases, instead, it is sufficient that the growth factor is present only during the phase of competence.

The epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), the insulin-like growth factor I (IGF-I), and insulin (INS) are neurotrophic agents inducing neuronal and astroglial cells proliferation and differentiation in culture (Avola et al. 1988a; Avola et al. 1988b).

Stiles C.D. and coll. (Stiles et al. 1979) have classified the growth factors such as factors of "*competence*" or "*progression*". These two classes of growth factors cooperate for a full mitogenic response of the cell.

The factors of "competence" are not able to initiate DNA synthesis, but are able to induce the "competence" to respond to other growth factors ("progression" factors) that stimulate the "progression" through the cell cycle. The competent cells do not respond to the factors of "progression" and stop growth. The factors of "competence" include: the platelet-derived growth



factor (PDGF) and fibroblast growth factor (FGF), while the factors of "progression" include the epidermal growth factor (EGF), and the insulin growth factor family (IGFs).

In addition to growth factors mentioned above, there is a wide range of molecules that participate in various development processes and neuronal growth. Among the most important are the class of neurotrophins that includes the "nerve growth factor" (NGF), the "brain derived neurotrophic factor" (BDNF) and neurotrophin 3 and 5.

The biological action of NGF is mediated by its binding to two classes of receptors localized on the plasma membrane: p75, known as low-affinity receptor, which binds also to other neurotrophins, and Trk-A, a glycoprotein of 140 kDa with activity of tyrosine kinases. NGF has multiple functions and in the near future could be used in the treatment of certain diseases such as neurotrophic corneal ulcer, Parkinson's disease, Alzheimer's disease, multiple sclerosis and some peripheral neuropathies.

### ***Fibroblast growth factors (FGFs)***

The family of fibroblast growth factors (FGFs) includes the aFGF and bFGF, two mitogenic proteins that were originally purified basing on their ability to bind to heparin, the FGF-5, FGF-6, the factor growth of keratinocytes, and the products of oncogenes int-2 and hst (Hefti and Knusel 1988).

The fibroblast growth factors are able to elicit strong morphological effects on the astroblasto, characterized by a narrowing of its cell body, and by an increase of the extension of cellular processes.

The FGFs are present in many peripheral tissues and are potent mitogens agents for various types of cells (Baird et al. 1986). The brain and the pituitary gland are particularly rich in bFGF. The basic fibroblast growth factor (bFGF) is a protein from the PM of about 17,000 D, can stimulate the proliferation of endothelial, mesenchymal and neuronal cells.

The bFGF promotes the survival and differentiation of cholinergic neurons in rat mesencephalic culture, of mesencephalic dopaminergic neurons (Mayer et al. 1993) new-striatal GABAergic neurons (Zhou and DiFiglia 1993) and immortalized hypothalamic neurons (Gallo et al. 1996).

### ***Epidermal growth factor (EGF)***

The epidermal growth factor (EGF) is an acidic protein, that belongs to a large family of proteins which includes some viral proteins.

It is a "*progression*" polypeptide factor (molecular weight of about 6000 Da), and it is known in the literature its stimulatory effect on the proliferation of the epidermis, its inhibitory effect on gastric secretion, as well as the proliferation and differentiation of various cell types (Avola et al. 1988a).

A lot of studies concerning EGF were addressed both to the role as a mitogen, both to its ability to affect the differentiation of not-neuronal cells (Carpenter G. and Cohen S. 1979), such as astroglial cells where the EGF stimulates both the synthesis of DNA and RNA, that the activity of fosfoinositol 3-kinase (Avola R. et al. 1988a and 1988b). The EGF is able, moreover, to modify the morphology and to induce astrocyte "up-regulation" in the levels of glutamine synthetase and S-100 (Avola R. et al. 1988a and 1988b).

In recent years it has been demonstrated that EGF induces numerous biological responses:

- increased or decreased cell adhesion
- morphological changes
- induction of ornithine decarboxylase
- phosphorylation reactions.

### ***Insulin***

Insulin and insulin like growth factors of type I and II are a family of proteins involved in the regulation of metabolism and cell growth of various tissues.

Insulin is a peptide hormone secreted by the cells of the pancreatic island of Langerhans  $\beta$ , has a PM of 5807 Da and consists of two polypeptide chains of 21 (A-chain) and 30 (B-chain) amino acids.

Insulin is able to promote some metabolic pathways, and in particular it induces some key enzymes of glycolysis and causes a lowering of the levels of activity of fructose 1,6-bisphosphatase and pyruvic carboxykinase in the liver, causing a slowdown in gluconeogenesis.

The central nervous system has been regarded, for a long time, as a court Insulin-dependent, because the insulin produced by the pancreas crosses the blood-brain barrier to a limited extent.

Currently there is increasing evidence that insulin found in the brain is synthesized on site probably by astrocytes and that insulin can function in nerve cells, more like that neurotransmitter neuromodulator (Wei et al. 1990).

Insulin, synthesized and released by nerve cells, has also been associated with the growth of the brain. In fact, it is well known that the extent of protein synthesis in the brain is positively correlated with the number of insulin receptors since it promotes an active uptake of amino acids through specific transporters and that insulin stimulates the synthesis of macromolecules in mixed cultures brain and in cultured astrocytes from neonatal rat (Avola et al. 1988a; Avola et al. 1988b).

This action may be specific for astrocytes, which regulate the local availability of glucose in the central nervous system. In this way the Insulin could affect the availability of extra glucose in case of ischemic events, due to its ability to promote the "*uptake*" of glucose through its specific carrier (GLUT4).

### ***Insulin like growth factors (IGF-I and IGF-II)***

Insulin like growth factors (IGFs) (IGF-I and IGF-II) are two peptides (MW about 7500) that promote cell growth and are structurally related to insulin.

The IGF-I is a potent mitogen and acts through interaction with its specific receptor type I, and it belongs to the family of tyrosine kinase receptors. This receptor for IGF-I has a higher affinity than IGF-II and insulin. The receptors for IGF-I are widely distributed in the central nervous system of mammals. It is likely that, through the link with its high affinity receptor, the IGF-I is able to increase the synthesis of RNA in neurons and to stimulate DNA synthesis in embryonic brain cells in vitro.

In the brain, IGF-I and IGF-II expression is remarkable during early development, but their expression decreases in the adult brain.

The IGF-I stimulates glial and neuronal growth and in the CNS increases the production of myelin stimulates DNA synthesis and induces a phenotype catecholaminergic in neural cells of chicken crest (Nataf and Monier 1992). In addition, in astrocytes, IGF-I stimulates cell proliferation and glucose "uptake".

Insulin, IGF-I and IGF-II promotes the survival and stimulate neurite outgrowth in central neurons and peripheral culture, including mesencephalic dopaminergic neurons (Engele and Bohn 1991).

The fact that IGF-I is expressed in neurons during synaptogenesis in the projection, made me think for a functional role of IGF-I in the synaptic formation or stabilization (Hefti and Knusel 1988).

IGF-I has also an important role in programmed cell death mechanisms by exerting an antiapoptotic role (LeRoith et al. 1995).

### ***Cholinergic precursors***

Phospholipids are important components of mammalian cells, including neurons and glial cells, and exert different biological functions. They form lipid bilayers that provide structural integrity necessary for protein function, act as energy reservoirs, and serve as precursors for various second messengers. A better knowledge of various aspects of phospholipid metabolism will contribute to understand the role of lipids in maintaining cell physiology and how alterations in their metabolism can contribute to various nervous system disorders.

Choline is a quaternary amine obtained primarily from the diet and synthesized in brain and liver as well. Choline is also a precursor in the biosynthesis of the neurotransmitter acetylcholine (ACh) and of phosphatidylcholine (PC), a major membrane constituent (Wurtman 1992). Choline plays a crucial role as a key structural and functional component of cell membranes (Wurtman 1992).

The neurotransmitter acetylcholine may have an important role in controlling glial activation. Glial cells release the acetylcholine degrading enzyme acetylcholinesterase (AChE) (Anderson et al. 2008; Bond et al. 2006) and express cholinergic receptors (Xiu et al. 2006). Glial response to cholinergic activation results from the balance between the direct hyperpolarizing action of acetylcholine and the depolarizing modulation of glutamate from the neighbouring neurons (Amenta and Tayebati 2008; Seigneur et al. 2006).

Degeneration of basal forebrain cholinergic system is thought to play an important role in the pathophysiology of Alzheimer's disease and dementia with Lewy bodies, which represent common forms of dementia in the elderly.

The neuropathology of Alzheimer's disease and dementia with Lewy bodies is related to cholinergic dysfunctions and the main therapeutic strategies to counter these disorders are represented by enhancement of cholinergic neurotransmission primarily by slowing-down AChE/cholinesterase degradation with cholinesterase inhibitors (for a review see (Amenta and Tayebati 2008; Parnetti et al. 2007)). Cholinergic precursor loading therapy was the first approach tried to relief cognitive impairment in dementia disorders, but controlled clinical trials failed to show significant improvements with choline or phosphatidylcholine (lecithin), a choline-containing phospholipids, alone or in association with cholinesterase inhibitors (tacrine plus choline, or physostigmine plus choline) (Parnetti et al. 2007). The reasons for the lack of effect of this precursor strategy are unclear, but some recent studies are suggesting that negative effects with choline or phosphatidylcholine cannot be generalized for all cholinergic precursors (Parnetti et al.

2007). This is true for the cholinergic precursors cytidine 50-diphosphocholine (CDP-choline) and choline alphoscerate (alpha-glyceryl-phosphorylcholine), which increase acetylcholine content and release. Among these two precursors, choline alphoscerate is more effective than CDP-choline in rising plasma choline levels (Amenta and Tayebati 2008).

Astroglial cells during proliferation and differentiation in primary cultures represent a valuable tool to study biochemical mechanisms involved during development and maturation of these cultured nerve cells. Neuronal nicotinic acetylcholine receptors are expressed by hippocampal astrocytes and their activation produces rapid currents and calcium transients (Sharma and Vijayaraghavan 2001).

### ***STEROID HORMONES***

The organization of neural circuits is controlled by a broad spectrum of neuroendocrine responses. In particular, the cognitive and behavioral functions in adult mammals are constantly affected by specific sex hormones and the different exposure of the central nervous system (CNS) to steroid hormones produced by the gonads, especially estrogens and androgens.

The biosynthesis of steroid hormones is made from cholesterol, which is the precursor of the five most important classes of steroids, hormones, such as progestins and in particular progesterone, androgens, including testosterone, estrogens, which include estrone and estradiol (E<sub>2</sub>), glucocorticoids, such as cortisol and mineralocorticoids such as aldosterone. The most important sites of synthesis of these classes of hormones are: progestins in the corpus luteum, estrogen in the ovaries, androgens in the testes, glucocorticoids and mineralocorticoids in the adrenal cortex.

Steroid hormones once in the bloodstream, bind in large part to carrier proteins albumin and globulins.

As part of the nervous system estrogen, together with glucocorticoids and androgen hormones are key to many brain activity.

The presence of estrogen appears to be crucial during brain development for perinatal sexual differentiation, masculinization and development of structures and functions of the CNS (AP

Arnold and RA Gorski 1984). Numerous studies have confirmed that perinatal exposure to estrogen in the CNS changes permanently glial and neuronal morphology.

Estrogens also modulate functional and neuromorphological properties such as cell size, the formation of synapses, axonal growth and dendritic arborization and are involved in the control of neuronal development and, in particular, in the formation of brain sex-specific circuits. The formation of brain estrogens is catalyzed by the enzyme aromatase (also known by the name of estrogen synthase or P450arom). The enzyme aromatase is known for its ability to catalyze the conversion of androgens such as testosterone and androstenedione into estrogens such as  $\beta$  17- E<sub>2</sub> and estrone.

Many studies, in which were used monolayer cell cultures of rodent brain, have shown that the expression of aromatase and its activity are mainly localized in neurons with very low levels in glial cells (Beyer and Hutchison 1997). Some researchers (Zwain et al. 1997), found that the production of E<sub>2</sub> and the expression of aromatase in cultured astrocytes derived from the cerebral cortex of neonatal rats, suggesting that under specific culture conditions, aromatase can be expressed by astrocytes. Astrocytes are potentially able to express the aromatase in response to injury and the distribution of aromatase immunoreactive astrocytes after a lesion and the distribution of reactive astrocytes after brain damage. Estrogens that are formed in astrocytes would be issued and would act as a trophic factor for damaged neurons in the processes of cell growth (Garcia-Segura et al. 2001). Glial cells do not express the aromatase under normal conditions, the induction of the enzyme may be part of the program of glial activation and may cooperate with the new conditions in which it comes to finding the nerve tissue after damage.

The brain aromatase activity appears to be mainly regulated by the steroid hormones.

The receptors for steroid hormones are also phosphoproteins and their functions are regulated by phosphorylation. This post-translational modification may play a role in the nuclear translocation, in DNA binding, and in interactions with other proteins in trans-activation. The primary site of phosphorylation is induced dall'E<sub>2</sub> serine 118 (Ser118).

The action of estrogens on neurite outgrowth includes the stimulation of the release of  $\text{Ca}^{++}$  from intracellular stores and the consequent activation of the transductional cascade cAMP/PKA/pCREB (Beyer 1999). The mobilization of cytosolic calcium and the subsequent activation of intracellular  $\text{Ca}^{++}$  cascades seems to be a prerequisite for neuronal survival and dendritic growth.

It has been shown that estrogen inducing an increase of  $\text{Ca}^{++}$  a adenylate cyclase stimulate, through the activation of a kinase  $\text{Ca}^{++}$ /Calmodulin dependent (CaMK) (DMF Cooper et al. 1995). The rapid increase of  $\text{Ca}^{2+}$  ions is specific for  $17\text{-}\beta\text{E}_2$ , in fact, testosterone and  $\beta\text{E}_2$  at high concentrations do not seem to affect the levels of intracellular  $\text{Ca}^{2+}$ .

In the nerve cells have been described interactions between the signaling pathways dell'E2 and growth factors (Singh et al. 1999; Toran-Allerand et al. 1988). In particular, has been demonstrated co-localization of estrogen receptor with the ligand of neurotrophins and their receptor systems (p75 and the receptor tyrosine kinase) in neurons of the CNS in development (Toran-Allerand et al. 1992). The IGF-I is a growth factor with prominent neurotrophic effects, which stimulates the differentiation and survival of specific neuronal populations.

Estrogens may have a neuroprotective effect by preventing programmed cell death, the effect of many stress agents, reducing the risk and improving the symptoms of neurodegenerative diseases.

One of the mechanisms by which estrogen may exert their neuroprotective effects, involving molecules important to apoptosis. The family of proteins related to the Bcl-2 is involved in the regulation of cell death of many types of cells, including neurons. Some members of this family such as Bcl-2 and Bcl-XL are negative regulators of apoptosis, other as Bax, Bad and Bid, act as positive regulators of apoptosis (Martinou et al. 1994).

Depletion of estrogen after menopause is thought to increase the susceptibility of women to Alzheimer's. In fact, in women there is an increased prevalence of Alzheimer's disease (Bachman et al. 1992) than men. The estrogen replacement therapy may have therapeutic effects against Alzheimer's disease and improve cognitive function and stopping the progression of the disease. However, the mechanism by which estrogens induce neuroprotection is unclear. As well as in



neurodegeneration is apoptosis involved, in neuroprotection by estrogen seems involved the modulation of apoptosis.

### ***ALPHA LIPOIC ACID***

Lipoic acid (1,2-dithiolane-3-pentanoic acid; LA) as well as its reduced form dihydrolipoic acid (DHLA) are compounds having a chiral center (Fig. 1). LA contains two thiol groups, which may be oxidized or reduced. It is part of a redox pair, being the oxidized member of the reduced form dihydrolipoic acid (DHLA) and both the oxidized and reduced forms of LA are antioxidants. In addition, the asymmetrical carbon atom, provides special optical properties.

LA has two enantiomers: the R-enantiomer [R-LA or (+)LA] and the S-enantiomer [S-LA or (-)LA]. LA is present in nature as R-enantiomer (Fig. 1), but synthetic LA is a racemic mixture of (+)LA and (-)LA [(+/-)LA]. Furthermore, both forms have different functions. LA is also an pivotal component of mitochondrial complex of four important proteins participating on the synthesis and degradation of glycine molecule.

In view of its important role in biochemical processes, LA was enclosed into vitamin B complex, although, now researchers do not consider it a vitamin. The chemical activity of LA and DHLA is mainly based in its dithiolane ring (Fig. 1), in addition, the position of the two sulfur atoms in the ring creates an exceptionally high electron density, which confers special properties to LA. These structural features provide to LA a potential reactive under physiological conditions. It is well known that in biological systems only the NAD(P)H/NAD(P)<sup>+</sup> redox couple has a higher reduction potential. DHLA, the reduced form of LA, exercises an antioxidant effect directly by donating electrons to a pro-oxidant or an oxidized molecule. It can regenerate reduced vitamin C from dehydroascorbic acid and it can indirectly regenerate vitamin E back from its oxidized state. Moreover, LA metabolites have been shown to have antiinflammatory and antioxidant effects (Kwiecien et al. 2013). However, it has been shown that LA is able to exert a significant antioxidant effect through a scavenger activity on free radicals (Packer et al. 2001), as well as its capability of

LA to chelate metals (Ghibu et al. 2009). Thus, the chemical nature of LA and DHLA make them capable of taking part to a variety of biochemical reactions where redox state is meaningful.

A significant activity of LA is the capability to chelate toxic metals and also to increase glutathione levels inside the cells. Glutathione plays important role in the capability of the system to chelate and discharge a wide variety of toxins and toxic metals. Several metals known to form these complexes are manganese, zinc, cadmium, lead, cobalt, nickel, iron, copper, cadmium, arsenic and mercury.

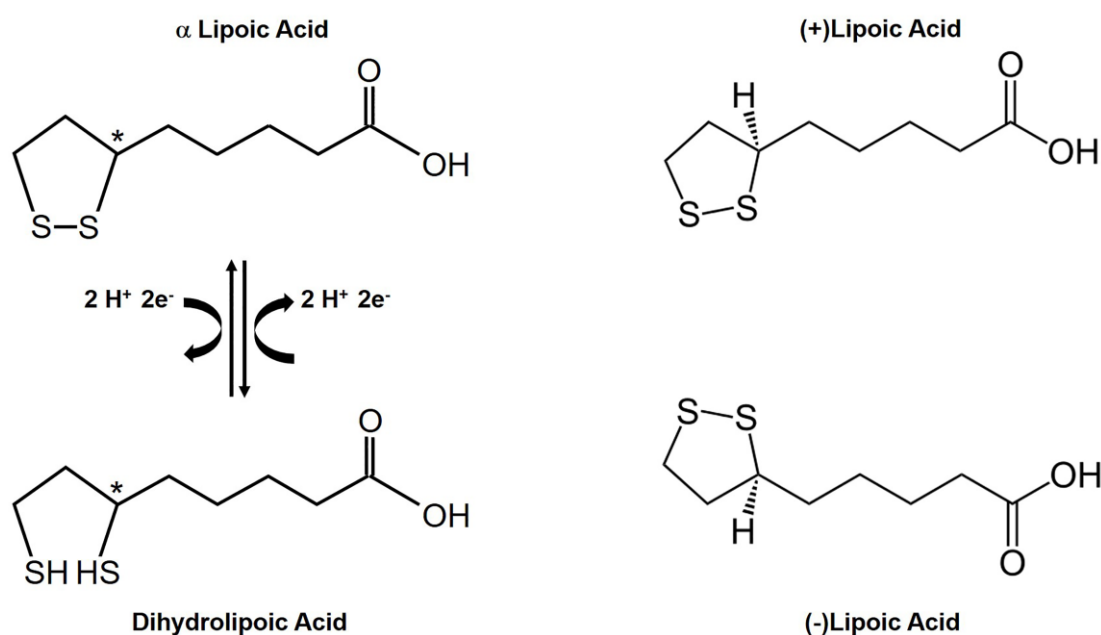


Fig. 1

LA is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans (Smith et al. 2004). Endogenously synthesized LA is covalently bound to specific proteins, which function as cofactors for mitochondrial dehydrogenase enzyme complexes.

In addition, to the physiological functions of protein-bound LA, there is an increasing scientific and medical interest in potential therapeutic uses of pharmacological doses of free LA. Considering its role in biochemical processes, lipoic acid was initially included in the vitamin B complex.

LA is unique among natural antioxidants in its ability to fulfil all of these requirements, making it a potentially highly effective therapeutic agent for a number of conditions in which oxidative damage has been implicated.

Furthermore, since oxidative stress cooperates to develop the disease pathogenesis, it has been investigated to use LA as a treatment alternative for Alzheimer's disease and diabetic polyneuropathy. There is evidence that LA performs therapeutic activity in diabetic condition because it is able to induce a lowering glucose levels. In fact, LA is administered to the patients suffering of diabetic polyneuropathy, a pathologic condition associated with increased oxidative stress. (Golbidi et al. 2011; Vallianou et al. 2009; Ziegler et al. 2004).

LA supplementation has multiple beneficial effects on the regression of the mitochondrial function and on oxidative stress associated with several diseases and aging. However, appropriate plasma levels need to be obtained in order to warrant maximum therapeutic benefit. The use of the LA as drug or food supplement is interfered by its rapid metabolism (man plasma half live of 30 min and bioavailability after oral administration of 30%) and its stability problems since it is known that LA can polymerize. Its degradation in the presence of light was characterized by a physical change in the compound and a shift in the ultraviolet spectrum.

## **Lipoic acid and cancer**

Oxidative stress possesses also a main role in tumorigenesis (Durand and Mach 2013). LA has been administered as an anticancer agent mainly in experimental studies of different tumorigenesis cells type with encouraging results (Al Abdan 2012; Feuerecker et al. 2012; Guais et al. 2012; Kim et al. 2012; Mantovani et al. 2003; Michikoshi et al. 2013). Until now the molecular mechanisms implicated in this process are still unknown. In addition to its antioxidant power, another possibility could be its capability to provoke cellular apoptosis as recently demonstrated in lung cells (Michikoshi et al. 2013). This effects may originated by activation of caspase proteins induced by endoplasmic reticulum stress (Mantovani et al. 2003).

Another possible hypothesis could be linked to the cancer cells metabolism, which converts and transforms preferentially the glucose to lactate, a mechanism known as the Warburg effect (Kim et al. 2012). In fact, LA is the cofactor of pyruvate dehydrogenase which converts and transforms the pyruvate molecules to acetyl CoA, resulting in a decrement in the formation of lactate molecules (Feuerrecker et al. 2012). The clear and immediate consequence of this process is the block of the glycolytic way. In addition, the block of mTOR (target of rapamycin), a well known signaling pathway involved on cell growth and correlated to insulin receptor phosphorylation- PI3K-AKT activation, has been showed in several researches using insulinoma cells (Targonsky et al. 2006). This action provoked an inhibition of insulin secretion as well as a decrement beta-cells growth (Targonsky et al. 2006).

Combinations of LA with well-known drugs as well as the synthesis of LA conjugates with other bioactive scaffolds are two strategies toward the development of effective agents for the prevention or treatment of various disorders and diseases. In agreement to the reviewed patents, LA can be used in combination therapy, with drugs (anticancer, antidiabetic, antimicrobial) having synergistic effects and reduced toxicity (Kates et al. 2014). In fact, in some research studies, LA was administered associated with other antioxidant agents or with other anticancer drugs (Diesel et al. 2007).

### **Lipoic acid in the mechanisms of resistance to bortezomib in SH-SY5Y neuroblastoma cells**

Neuroblastoma (NB), the well known extracranial solid cancer in childhood, is the most common cancer in infancy (Park et al. 2010). It is a neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system (SNS). Neuroblastoma is one of the few human malignancies able to vary from the highly aggressive chemoresistant disease to the spontaneous regression (Ohira and Nakagawara 2010). Although the standard treatment for neuroblastoma is based on combination chemotherapy with drugs such as doxorubicin, vincristine, cyclophosphamide

and cisplatin, the disease becomes chemoresistant over time and is eventually fatal. Therefore, novel therapeutic approaches to treat this disease are needed.

Bortezomib (BTZ), a reversible inhibitor of the 26S proteasome, is used as first line drug for treating multiple myeloma (MM) (Romano et al. 2013). Recent experimental evidence has shown that BTZ treatment is able to overcome cancer cell resistance in different solid tumors, including NB (Mujtaba and Dou 2011). A synergistic effect of its combination with doxorubicin in vitro has been reported (Du et al. 2012). BTZ treatment in NB cell lines induces over-expression of heme oxygenase 1 (HO-1) which characterizes the increase of resistance to proteasome-inhibition (Furfaro et al. 2014). Recently, some researches showed that BTZ-induced HO-1 is involved in resistance to proteasome inhibitor and is involved in genomic instability of MM (Tibullo et al. 2016). HO-1, also known as heat shock protein 32, is an enzyme that catalyze the heme degradation to generate biliverdin, free heme iron and carbon monoxide. The classical physiological functions of HO-1 is to decrease oxidative stress and inflammatory responses and to protect against apoptosis by the removal of heme, a potent pro-oxidant and pro-inflammatory mediator. In neoplastic cells HO-1 is considered to play a major role as an essential survival factor, protecting against chemotherapy-induced increase in ROS (Abe 2011; Goswami et al. 2008; Meister et al. 2007; Richardson et al. 2014; Teicher et al. 1999).

In addition, it is well known that bortezomib induces endoplasmic reticulum (ER) stress and its adaptive response pathway, known as the unfolded protein response (UPR). The UPR enables the cell to survive reversible environmental stresses.

However, if the stress is severe or prolonged, UPR activation eventually leads to cell-cycle arrest (Brewer and Diehl 2000; Brewer et al. 1999) and the induction of apoptosis (Zinszner et al. 1998). Emerging data indicate that ER stress is also a potent inducer of macroautophagy, a process whereby eukaryotic cells recycle their macromolecules and organelles. Depending on the context, autophagy counterbalances ER stress-induced ER expansion, enhances cell survival or leads the cell to non-apoptotic death. Recent studies have been revealed that autophagy plays a critical role in cell

death decisions, and autophagy induction is correlated to apoptosis resistance. Effective autophagy may reduce cell death by inhibiting apoptosis (Degenhardt et al. 2006; Zinszner et al. 1998), whereas inhibition of autophagy may promote cell death by potentiating apoptosis (Liu et al. 2011; Zhou et al. 2014). Autophagy refers to an evolutionarily conserved process in which cytoplasmic components are sequestered in double-membraned autophagosomes. Auto-phagosomes are eventually fused with lysosomes in order to form autolysosomes for the purpose of recycling cellular components to maintain cytoplasmic homeostasis (Lum et al. 2005). Concerning the cell death/survival decisions, the role of autophagy is highly contextual, autophagy can act as an alternative cell death pathway or it can play a cytoprotective role (Mani et al. 2015).

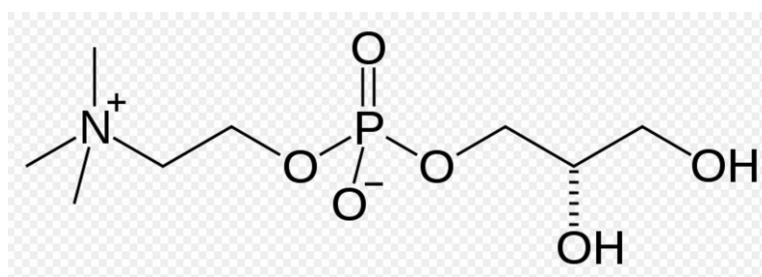
The antioxidant role played by LA and its particular ability in restoring glutathione content may be correlated to proliferative and differentiative state of astrocyte cells (Bramanti et al. 2010b), as demonstrated by up and down modulation of several astroglial biomarkers expression.

In neurological fields, accumulating evidence suggests that LA has neuroprotective effects in the models of both brain ischemia (Clark et al. 2001) and neurodegeneration such as Alzheimer's disease (AD) (Siedlak et al. 2009) and Parkinson's disease (PD) (Karunakaran et al. 2007). Recently, it has been demonstrated that LA prevented cell death induced by drugs which deplete glutathione in NB cells (Yamada et al. 2011).

### **Alpha glycerylphosphorylcholine (Choline alphoscerate, $\alpha$ GPC)**

Changes in cholinergic function are implicated in the pathogenesis of learning and memory alterations occurring in adult-onset cholinergic dysfunction including dementia disorders (Gottfries et al., 1994). The cholinergic system is not the only neurotransmitter system affected in cognitive dysfunction common of Alzheimer's disease or vascular dementia, but analysis of its involvement in cognitive functions has shown that central cholinergic receptors might be involved in learning and memory through complex mechanisms (Gottfries et al., 1994).

Cholinergic strategies were therefore developed for restoring deficient cholinergic neurotransmission which occurs primarily in basal forebrain (Terry et al., 2003). Cholinergic precursors have represented an old approach to treat cholinergic dysfunction and cognitive decline in adult-onset dementia disorders (Amenta et al., 2001; Parnetti et al., 2007). Many of these precursors were early leaved because their efficacy was not clearly demonstrated. This is not true for choline alfoscerate, a cholinergic precursor available in the pharmaceutical market of several countries, which has been studied both in preclinical paradigms and in clinical trials.

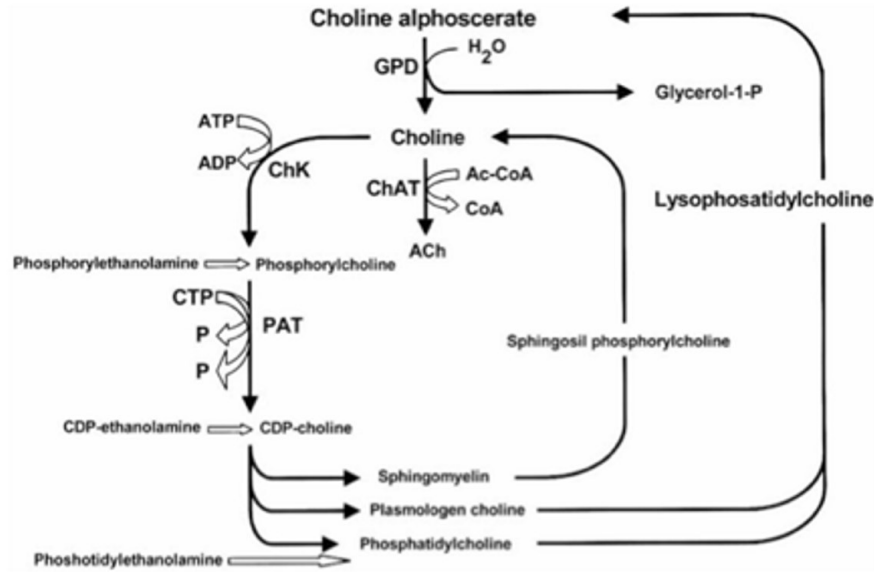


**Figure A: Chemical structure of choline alfoscerate**

Choline alfoscerate or alpha-glycerylphosphorylcholine (ATC code N07AX02) (GPC) (Figure A) is a semi-synthetic derivative of lecithin. Following oral administration, it is converted to phosphorylcholine, a metabolically active form of choline able to reach cholinergic nerve terminals where it increases acetylcholine synthesis, levels and release (Amenta et al., 2008).

Figure B summarizes acetylcholine anabolic pathways (Amenta et al., 2008).

As shown, the enzyme glyceryl phosphorylcholine diesterase transforms alpha glyceryl-phosphorylcholine into a molecule of choline and another of glycerol-1-phosphate.



**Figure B:** Acetylcholine synthetic pathways . Interference of choline-containing compounds.

This figure shows the steps in which choline alphoscerate can influence neurotransmitter biosynthesis. Cytidin diphosphate (CDP); Cytidin triphosphate (CTP); Glycerol-phosphorylcholine diesterase (GPD); Choline acetyltransferase (ChAT); Choline kinase (ChK); Phosphocholine cytidyl transferase (PCT)

Mechanisms of action of choline alphoscerate are mainly two. In fact the compound interferes with brain phospholipid metabolism and increases brain choline and acetylcholine levels and release (Amenta et al., 2008; Amenta et al., 2001).

Pharmacodynamic studies on choline alphoscerate during phases of development of the compound were focused primarily on its role in potentiating brain cholinergic neurotransmission and in interfering with brain phospholipid metabolism. Pre-clinical studies have demonstrated that choline alphoscerate increases the release of acetylcholine in rat hippocampus, facilitates learning and memory in experimental animals (Sigala et al., 1992), improves brain transduction mechanisms (Lopez et al., 1991; Schettini et al., 1990) and decreases the age-dependent structural changes occurring in the rat frontal cortex and hippocampus (Amenta et al., 1993). Moreover, the compound contributes to anabolic processes responsible for membrane phospholipid and glycerolipid synthesis, positively influencing membrane fluidity (Aleppo et al., 1994). In several animal



paradigms of impaired cognitive function, choline alfoscerate was demonstrated to improve cognitive deficit in experimental models of aging brain (Canonica et al., 1990; Drago et al., 1990) and to reverse mnemonic deficits induced by scopolamine administration (Parnetti et al., 2007; Sigala et al., 1992). Based on the above evidence, the central parasymphomimetic activity of choline alfoscerate was defined, suggesting its clinical use in patients affected by cognitive decline. Consistently with the activity profile, choline alfoscerate was classified as a centrally acting parasymphomimetic drugs both in international pharmacopoeias (Reynolds et al., 1996) and in the Chemical Therapeutical Anatomical Classification.

A restorative role of choline alfoscerate on central cholinergic system was also documented by studies performed in old rodents. In these investigations the compound was able to counter age-related changes in brain acetylcholine synthesizing (choline acetyltransferase) and degrading (acetylcholinesterase) enzymes (Amenta et al., 1994) and some subtypes of muscarinic cholinergic receptors (Amenta et al., 1994; Muccioli et al., 1996).

Neuroprotective effects of choline alfoscerate were also documented in a rodent model of altered cholinergic neurotransmission caused by lesioning of the Nucleus Basalis Magnocellularis which represents the main source of cholinergic innervation of cerebral neocortex (Amenta et al., 1995; Bronzetti et al., 1993). A neuroprotective effect of treatment with choline alfoscerate on hippocampus microanatomy and glial reaction which represents an early sign of brain damage was documented in spontaneously hypertensive rats, used as an animal model of brain vascular injury (Tomassoni et al., 2006). A model used to mimic to some extent neuropathological changes occurring in vascular dementia (Tomassoni et al., 2006). Among cholinergic precursors tested, choline alfoscerate elicited the most relevant stimulation on vesicular acetylcholine transporter, and choline transporter in the same model of brain vascular injury suggesting that it represents a strong enhancer of central cholinergic neurotransmission (Tayebati et al., 2011).

Effects of choline alfoscerate were limited not only in rodent models of aging or lesioning of brain cholinergic nuclei, but also in Rhesus monkeys. In this species the compound revealed general

facilitatory properties on retinal neurotransmission as well as specific spatial frequency tuning effects on retinal information processing (Antal et al., 1999).

Another series of more recent studies has shown that association of choline alphoscerate with (acetyl)cholinesterase inhibitors potentiates effects on cholinergic neurotransmission. In fact, administration of choline alphoscerate plus the acetylcholinesterase inhibitor rivastigmine induced an increase of brain acetylcholine levels and of high affinity choline uptake binding sites more pronounced than single drugs (Amenta et al., 2006). This investigation has suggested that combination of a suitable precursor of brain acetylcholine such as choline alphoscerate and of an acetylcholinesterase inhibitor may represent an association worthwhile of being further investigated as a cholinergic replacement therapy in pathologies characterized by impaired cholinergic neurotransmission (Amenta et al., 2006). This working hypothesis was supported by the demonstration of a more sustained neuroprotective action by choline alphoscerate plus the acetylcholinesterase inhibitor galantamine than the two drugs administered alone (Tayebati et al., 2009).

### **Intermediate filaments cytoskeletal proteins**

One of the main hallmarks of developmental neurobiology is to understand the molecular mechanisms by which such cellular diversity is generated. Such diversification occurs at an early stage of development, especially by activation of sets of cell type-specific genes, which gives cells distinctive functions and morphological characteristics. Some of these cell-specific genes are the intermediate filaments (IF) protein genes, which are regulated during cell development. The two major IF proteins of astrocytes are vimentin and GFAP. In the course of astrocyte development, a transition in the expression of IF protein genes is observed. Early during development, radial glia and immature astrocytes express mainly vimentin. Towards the end of gestation, a switch occurs whereby vimentin is progressively replaced by GFAP in differentiated astroglial cells. At present, there is no consensus on the functional role of these IF proteins. The application of molecular

genetic approaches to IF function has been providing some significant insights as well as raising new questions about the functional role of individual IF proteins.

## **Vimentin**

Vimentin IFs are the only IF type found in a variety of cells including astrocytes, fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes (Evans 1998). Functional analysis of the vimentin gene promoter has already been carried out and several negative and positive elements were identified within this region (Gomes et al. 1999). Data obtained from vimentin knockout mice (-/-) demonstrated that those animals developed and reproduced without presenting an obvious new phenotype, thus heavily calling into question the biological function of vimentin (Colucci-Guyon et al. 1994). Several data, however, argue in favor of a relevant function for vimentin. Using the same vimentin (-/-) lineage as Colucci-Guyon and collaborators found that GFAP filaments were also absent in certain glial cells that normally co-express vimentin and GFAP such as the Bergmann glia and an astrocyte subpopulation of the corpus callosum (Colucci-Guyon et al. 1994). This was not due to the inability to express GFAP. Transfection of cultured vimentin-/- astrocytes with a vimentin cDNA restores the vimentin-GFAP filament network, suggesting that in these cells vimentin might be required for coassembly with GFAP filaments (Galou et al. 1996).

Reactive gliosis is a prominent result of many types of insult to the central nervous system (CNS) and leads to the formation of glial scar that impedes the regeneration of axons. The intermediate filament protein vimentin is found in pathology of the CNS, mainly in the vicinity of injuries to the CNS. In the present study some authors investigated the role of vimentin in the formation of glial scars in vitro and in vivo by using immunohistochemistry, Western blot analysis, and in situ hybridization. In vitro experiments showed that the intensity of immunofluorescent labeling for vimentin and glial fibrillary acidic protein (GFAP) was consistently decreased in astrocytes after transfection with a retrovirus carrying antisense complementary DNA (cDNA) for vimentin. Transfection also inhibited the growth of astrocytes and decreased the expression of vimentin

mRNA. In vivo studies demonstrated that transfection with the retrovirus carrying the antisense cDNA vimentin inhibited the upregulation of vimentin and GFAP in stab wounds in rat cerebrum. These results suggest that vimentin may play a key role in the formation of glial scars in the CNS. Moreover, vimentin appears to accompany the formation of glial scars. Vimentin may stabilize the formation of GFAP-type IF in some reactive astrocytes, and its expression may be required for the formation of GFAP in these cells (Galou et al. 1996). In normal adult CNS, vimentin is not expressed in astrocytes, but only in some specialized glial cells such as those of Bergmann glia and radial glia, and ependymal cells. These findings suggest that vimentin may take part in the formation of glial scar, and that there may be a relationship between the expression of GFAP and that of vimentin. During the formation of GFAP networks in some reactive astrocytes, vimentin may act as a cytoskeleton associated protein (Fuchs and Cleveland 1998). At early stages of CNS development, IF in radial glia and immature astrocytes are composed of vimentin. Subsequently, at about the time of birth, a transition from vimentin to GFAP takes place; vimentin disappears and is progressively replaced by GFAP in differentiated astroglial cells, which transiently coexpress these two proteins (Galou et al. 1996). The transient expression of vimentin observed in the present study has also been observed immunocytochemically in most models of gliosis (Stringer 1996). In the normal adult rodent brain, vimentin expression is restricted to specialized glia such as ependymal cells, Bergmann glia of the cerebellum, and Schwann cells, which has led to the suggestion that vimentin may be a more specific marker of gliosis than is GFAP (Lenz et al. 1997). Galou founds that the astrocytes in the immediate vicinity of stab wounds expressed considerable GFAP (Galou et al. 1996). However, these cells did not express GFAP after the vimentin gene had been knocked out, whereas in wild-type mice the cells not only expressed vimentin but also expressed GFAP. These results suggest that the expression of GFAP in these astrocytes depends on the expression of vimentin, and that changes in the expression of vimentin affect the expression of GFAP. In summary, the authors Lin and Kai found that the expression of vimentin and GFAP increased markedly after injury to CNS, and that restricting vimentin decreased the expression both of

vimentin and GFAP, as well as formation of glial scar. In addition, the authors therefore believe that vimentin may play an important role in reactive gliosis and the formation of glial scar. Accordingly, we suggest that manipulating the expression of vimentin may control reactive gliosis and provide an environment that favours the regeneration of injured axons.

### **Glial fibrillary acidic protein (GFAP)**

Glial fibrillary acidic protein is an intermediate filament (IF) protein that is expressed by numerous cell types of the central nervous system (CNS) including astrocytes and ependymal cells. GFAP is a type III IF protein that maps, in humans, to 17q21. It is closely related to its non-epithelial family members, vimentin, desmin, and peripherin, which are all involved in the structure and function of the cell's cytoskeleton. GFAP is thought to help to maintain astrocyte mechanical strength, as well as the shape of cells but its exact function remains poorly understood, despite the number of studies using it as a cell marker.

GFAP is expressed in the central nervous system in astrocyte cells. It is involved in many important CNS processes, including cell communication and the functioning of the blood brain barrier. GFAP is proposed to play a role in astrocyte-neuron interactions as well as cell-cell communication. In vitro, using antisense RNA, astrocytes lacking GFAP do not form the extensions usually present with neurons.

In addition, GFAP has been widely recognized as an astrocyte differentiation marker, constituting the major intermediate filament (IF) protein of mature astrocyte (Bramanti et al. 2010a). GFAP synthesis is considered an important element of the developmental program of astrocyte differentiation and is part of the reactive response to almost any CNS injury (Bramanti et al. 2010a). It is involved in many important CNS processes, including cell communication and functioning of the blood-brain barrier (Grasso et al. 2014). During GFAP network formation in some reactive astrocytes, vimentin may act as a cytoskeleton associated protein. At early stages of CNS development, IF in radial glia and immature astrocytes are composed of vimentin (Bramanti et al.

2010a). The gene GFAP encodes an intermediate filament protein (50kDa) of mature astrocytes, which may be used as a marker for distinguishing astrocytes from other glial cells during development of the central nervous system. Defects in this gene causes Alexander disease. It is a rare disorder of astrocytes in the CNS

### **Tissue transglutaminase (TG-2)**

Tissue transglutaminase (TG-2) is an important calcium dependent protein, which represents a normal constituent of central and peripheral nervous systems during fetal stages of development. This enzyme is a member of transglutaminases family that catalyzes the formation of isopeptide bridges by calcium  $\text{Ca}^{2+}$ -dependent-cross linking of the carboxamide moiety of a peptide-bound glutamine either to the  $\epsilon$ -amino group of a peptide-bound lysine, or to polyamines, with liberation of ammonia. Type-2 transglutaminase (TG-2), which is the most ubiquitous TG isoform, is a multifunctional enzyme involved in the regulation of cell differentiation and survival (Milakovic et al. 2004). TG-2 is the only member of TGs playing a role in cell signaling transduction, differentiation and apoptosis. TG-2 is induced during apoptotic death and is implicated in a variety of human disorders including central nervous system (CNS) disorders (Fesus and Piacentini 2002; Mastroberardino et al. 2002).  $\text{Ca}^{2+}$  ions are key regulators of TG-2 activity. When intracellular  $\text{Ca}^{2+}$  is low, TG-2 behaves like a G protein, coupling different receptors to phospholipase C (Nakaoka et al. 1994). The multiplicity of TG-2 function also depends on its intracellular location.

### **Cyclin D**

Cyclin D is a member of the cyclin protein family that is involved in regulating cell cycle progression. The synthesis of cyclin D is initiated during  $G_1$  and drives the  $G_1/S$  phase transition. Once the cells reach a critical cell size (and if no mating partner is present in yeast) and if growth factors and mitogens (for multicellular organism) or nutrients (for unicellular organism) are present, cells enter the cell cycle. In general, all stages of the cell cycle are chronologically separated in

humans and are triggered by cyclin-Cdk complexes which are periodically expressed and partially redundant in function. Cyclins are eukaryotic proteins that form holoenzymes with cyclin-dependent protein kinases (Cdk), which they activate. The abundance of cyclins is generally regulated by protein synthesis and degradation through an APC/c dependent pathway.

Cyclin D is one of the major cyclins produced in terms of its functional importance. It interacts with four Cdks: Cdk2, 4, 5, and 6. In proliferating cells, cyclin D-Cdk4/6 complex accumulation is of great importance for cell cycle progression. Namely, cyclin D-Cdk4/6 complex partially phosphorylates Rb, which is able to induce expression of some genes (for example: cyclin E) important for S phase progression.

Growth factors stimulate the Ras/Raf/ERK that induce cyclin D production. One of the members of the pathways, MAPK activates a transcription factor Myc, which alters transcription of genes important in cell cycle, among which is cyclin D. In this way, cyclin D is synthesized as long as the growth factor is present.

Even though cyclin D levels in proliferating cells are sustained as long as the growth factors are present, a key player for G<sub>1</sub>/S transition is active cyclin D-Cdk4/6 complexes. Despite this, cyclin D has no effect on G<sub>1</sub>/S transition unless it forms a complex with Cdk 4 or 6.

One of the best known substrates of cyclin D/Cdk4 and -6 is the retinoblastoma tumor suppressor protein (Rb). Rb is an important regulator of genes responsible for progression through the cell cycle, in particular through G<sub>1</sub>/S phase.

In its un-phosphorylated form, Rb binds a member of E<sub>2</sub>F family of transcription factors which controls expression of several genes involved in cell cycle progression (example, cyclin E). Rb acts as a repressor, so in complex with E<sub>2</sub>F it prevents expression of E<sub>2</sub>F regulated genes, and this inhibits cells from progressing through G<sub>1</sub>. Active cyclin D/Cdk4 and 6 inhibit Rb by partial phosphorylation, reducing its binding to E<sub>2</sub>F and thereby allowing E<sub>2</sub>F-mediated activation of the transcription of the cyclin E gene and the cell progresses towards S-phase. Subsequently, cyclin E fully phosphorylates Rb and completes its inactivation.

Cyclin D is regulated by the downstream pathway of mitogen receptors via the Ras/MAP kinase and the  $\beta$ -catenin-Tcf/LEF pathways and PI3K. The MAP kinase ERK activates the downstream transcription factors Myc and AP-1 which in turn activate the transcription of the Cdk4, Cdk6 and Cyclin D genes, and increase ribosome biogenesis. Rho family GTPases and Focal Adhesion Kinase (FAK) activate Cyclin D gene in response to integrin.

## **Ornithine decarboxylase**

The Ornithine decarboxylase (ODC) is an enzyme involved in polyamines metabolism: by decarboxylation of ornithine, reaction catalyzed by the enzyme ornithine decarboxylase (ODC), is formed putrescine.

The expression of this enzyme is regulated by different stages, by transcriptional and post-transductional levels.

The first reaction consists in the production of putrescine by the ODC enzyme. It requires pyridoxal phosphate as a cofactor and also reducing agents containing thiol groups.

The polyamines are divalent regulators of cell function, promote the growth or cell death depending on the environmental signals and recently it has been shown that the polyamines are also involved in cell cycle regulation.

Recent "in vivo" studies shown that during the cell cycle also occur changes in the activity of ODC and in the concentration of polyamines.

Supporting the correlation between polyamines and cell growth, some reports indicate that high levels of polyamines, resulting in an increase of their synthesis, are present in the cells that make up many solid tumors, where the ODC results to be overexpressed, as well as in different precancerous manifestations and consequently exposure to chemical carcinogens.

## **Mitogen-activated protein (MAP) kinases**



Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. The closest relatives of MAPKs are the cyclin-dependent kinases (CDKs). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis-among many others. The first mitogen-activated protein kinase to be discovered was ERK1 (MAPK3) in mammals. Since ERK1 and its close relative ERK2 (MAPK1) are both involved in growth factor signaling, the family was termed "mitogen-activated".

MAP kinases are found in eukaryotes only, but they are fairly diverse and encountered in all animals, fungi and plants, and even in an array of unicellular eukaryotes. Most MAPKs have a number of shared characteristics, such as the activation dependent on two phosphorylation events, a three-tiered pathway architecture and similar substrate recognition sites. These are the "classical" MAP kinases. But there are also some ancient outliers from the group as sketched above, that do not have dual phosphorylation sites, only form two-tiered pathways, and lack the features required by other MAPKs for substrate binding. These are usually referred to as "atypical" MAPKs. It is yet unclear if the atypical MAPKs form a single group as opposed to the classical ones.

Mitogen-activated protein kinases are catalytically inactive in their base form. In order to become active, they require (potentially multiple) phosphorylation events in their activation loops. This is conducted by specialized enzymes of the STE protein kinase group.

In the case of classical MAP kinases, the activation loop contains a characteristic TxY (threonine-x-tyrosine) motif (TEY in mammalian ERK1 and ERK2, TDY in ERK5, TPY in JNKs, TGY in p38 kinases) that needs to be phosphorylated on both the threonine and the tyrosine residues in order to lock the kinase domain in a catalytically competent conformation. In vivo and in vitro, phosphorylation of tyrosine precedes phosphorylation of threonine, although phosphorylation of either residue can occur in the absence of the other.

The ERK1/2 pathway of mammals is probably the best characterized MAPK system. The most important upstream activators of this pathway are the Raf proteins (A-Raf, B-Raf or c-Raf), the key mediators of response to growth factors (EGF, FGF, PDGF, etc.); but other MAP3 kinases such as c-Mos and Tpl2/Cot can also play the same role. All these enzymes phosphorylate and thus activate MKK1 and/or MKK2 kinases, that are highly specific for ERK1 and ERK2. The latter phosphorylate a number of substrates important for cell proliferation and cell cycle progression (RSK kinases, Elk-1 transcription factor, etc.)

### **Extracellular Regulated Kinases (ERKs)**

Extracellular-signal-regulated kinases (ERKs), a well known member of the MAP kinase family, act as an integration point for multiple biochemical signals, and in addition, they are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. The activation of this kinase requires its phosphorylation by upstream kinases. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens, activate the ERK pathway. Some studies demonstrated an alternative estrogen signaling pathway that displayed similarities to the one used by growth factor and specifically the MAP-kinase cascade (Singh et al. 1999).

## CHAPTER 2

### AIMS OF INVESTIGATION

In view of the possible interest of selected cholinergic precursors to counter cholinergic deficits typical of several neurological diseases, the first session of present study we will design to evaluate the effects of treatment for 24h with acetylcholine and the cholinergic precursors choline, CDP-choline and choline alfoscerate on TG-2 expression and pattern in primary cultures of rat astrocytes at 14, 21 and 35 days in vitro (DIV).

In addition, as mentioned before, because  $\alpha$ -lipoic acid plays a pivotal role as antioxidant and metabolic component of some enzymatic complexes involved in glucose metabolism of different cell types, the second session of our research we will focus to evaluate the effect of (+)lipoic acid or (+/-)lipoic acid and /or 10 mM  $\alpha$ -GPC for 24h treatment on astroglial cell cultures.

In particular, we will evaluate the expression of some proliferation and differentiation markers in 15 or 21 DIV astrocyte cultures treated with 50  $\mu$ M (+) lipoic acid or (+/-)lipoic acid and /or 10 mM  $\alpha$ -GPC for 24h. In particular, we will evaluate by Western blot analysis the expression of GFAP, vimentin, cyclin D1 Ornithine decarboxylase and MAP-kinase, a signalling transduction pathway biomarker, in 15 or 21 DIV astrocyte cultures. In addition, it will interesting to evaluate the possible genoprotective effect by analysis of DNA status detected by Alkaline Comet assay.

Furthermore, in the third session of the investigation, we will study the estrogens and growth factors activities as mitogens promoting cellular proliferation. Although it was considered originally that these agents manifested their mitogenic action through separate pathways, more recent data suggest that EGF and estrogen-mediated signaling pathways are intertwined. For this reason, the aim of the third session of our investigation will be particularly devoted to evidence the interactions between the “competence” growth factor bFGF and/or estrogen 17- $\beta$ -estradiol and the “progression” growth

factors (EGF or IGF-I or INS) on DNA labeling and on proliferation and differentiation activity of primary astroglial cell cultures under different experimental conditions.

In particular, our attention will be focused to study first the labeling of [methyl-3H]thymidine in the DNA of primary astrocyte cultures at 24 days in vitro (DIV), pretreated with estradiol (17- $\beta$ -E<sub>2</sub>) and/or “competence” growth factor bFGF and subsequent treated with “progression” GFs by two or by three in the last 12h or 24h.

Subsequently, we will study Cyclin D1, ERK1/2, GFAP and vimentin expression in our astroglial cultures at 24 DIV pretreated for 36h with estradiol and treated with “competence and progression” growth factors by two or by three for 24h.

In addition, we will hypothesize that the joint pretreatment with “competence” growth factor bFGF and DEX and subsequent treatment with “progression” GFs can stimulated an upward modulation of cellular proliferation and differentiation.

This last evidence seeks to evaluate the eventual synergistic effect played by trophic factors added in the presence of glucocorticoids and to elucidate the different behaviors of competence and progression GFs interacting with DEX under our experimental conditions. Additionally, this last one seeks to elucidate the relationships among these classes of neuroactive molecules, steroids, and GFs in the regulation of astrocyte proliferation and differentiation in culture that might have significant implications for future therapeutic approaches to neurologic disorders associated with astrogliosis.

Finally, the last session of the research project will be focused in order to evaluate the antioxidant effects of LA combined with BTZ on NB cell lines. In particular, we will focus our attention on HO-1 modulation, on gene HMOX-1, as well as on the study of several of ER-stress proteins expression markers, such as the chaperons Binding Immunoglobulin Protein (BiP1) and Inositol-requiring enzyme 1 (IRE1 $\alpha$ ), ER oxidoreductin 1 (ERO1 $\alpha$ ) and Protein disulfide isomerase (PDI), activated by cell in order to counteract ER stress response.

Finally, we will study some protein related to the autophagy such as the protein of Autophagy protein 5 (ATG5), Microtubule-associated protein 1 (MAP1) and Beclin-1 protein (BECN1).

## CHAPTER 3

### MATERIAL AND METHODS

#### Astroglial cell cultures

Primary cultures of astrocytes were prepared from new-born albino rat brains (1–2-day-old Wistar strain) as previously described (Bramanti et al. 2008b; Prezzavento et al. 2007). In particular, cerebral tissues, after dissection and careful removal of the meninges, were mechanically dissociated through sterile meshes of 82- $\mu\text{m}$  pore size (Nitex). Isolated cells were suspended in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (50 mg/ml), and penicillin (50 U/ml) and plated into T-75  $\text{cm}^2$  flask with a density of  $3 \times 10^6$  cells (Campisi et al. 2008). The low initial plating density of dissociated cells was meant to favor the growth of astrocytes with only slight oligodendroglial and microglial contamination. Cells were maintained at 37°C in a 5%  $\text{CO}_2$ /95%  $\text{O}_2$  air humidified atmosphere for 2 weeks, and the medium was removed every 3 days. Astroglial cells were characterized by immunofluorescent staining with the glial marker GFAP, (Bramanti et al. 2007b), and were subject to treatment.

#### Treatment with Cholinergic Precursors

To synchronize cell cycle, at 12 or 19 or 33 DIV the culture medium containing the serum was removed and replaced with DMEM containing BSA (1 mg/1 ml). After 24 h, cell cultures were divided in five groups; four groups were exposed for 24h at different concentration (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$  and 10  $\mu\text{M}$ ) of choline chlorure, CDPC or  $\alpha\text{GPC}$ . One group of cells, used as a control, was maintained in medium containing Albumin from bovine serum (BSA) for the same time of drug treated-cultures. Four replicate experiments were performed for each sample. The time of cultures exposure to drugs were chosen, when TGs activity reacting the maximum levels at 24h (data not

shown). In previous experiments, on the basis of MTT assay, microscopic observation and GFAP immunolabeling we established that the no-toxic drug concentrations was up to 10  $\mu$ M.

### **Monodansyl-Cadaverine Labelling of Astrocytes**

TG-mediated-monodansyl-cadaverine (DC) uptake into living cells after drug treatments at concentrations of 0.1 $\mu$ M, 1 $\mu$ M and 10  $\mu$ M was assessed by confocal laser scanning microscopy (CLSM) cytofluorescence according to Ientile and collaborators [14]. Cultures were first observed using a 403/1.0 NA oil immersion objective using a Leica DM IRB inverted fluorescence microscope (Leica Microsystems, Heidelberg, Mannheim, Germany) and then viewed at a Leica TCS SP2 CLS microscope excitation at 488 nm, using He/Neon and Ar/Kr as laser sources. Fluorescence emitted from specimens was sent through a Leica AOTF mechanism (Acoustic Optical Turnable Filter) and pinhole, to two different photomultiplier tubes (PMTs). Image outputs electronically generated by the same parameter settings (frame scanning, pinhole aperture, gain voltage, pixels and spatial resolution) were measured and compared for intensity (treated vs. control), after non-specific fluorescence subtraction. Under these conditions TG-mediated-DC appeared as a green fluorophore (for further details see [8, 15, 18]). Sequential sections obtained by the CLS microscope Z-axis stepping capability, were combined to form an extended depth of a focus image, and standard image processing was performed to enhance brightness and contrast, using Leica Confocal Software (version 2.0 build 0585). The sequence of these pictures was then analyzed by an image analyzer (IAAS Delta System, Roma). The optical density of fluorescence per cell area was then calculated by the image analyzer and the value were normalized for the intensity of histofluorescence of control cells considered to correspond to “one”.

### **Drug Treatment for lipolic acid on astroglial cell cultures**

Astrocyte cultures at 15 DIV and 21 DIV were maintained under following experimental conditions:

lipoic acid was dissolved in 0.5M DMSO and then diluted in culture medium at the concentration 1mM. Catalase (1000 U/ml) was added and the solution was incubated at 37°C for 30 minutes. This solution was diluted 1:10 in the culture medium in order to obtain 50 µM lipoic acid final concentration. As reported below in dose-response figure, the best concentration of (+/-)lipoic acid or (+) lipoic acid was 50mM

#### **Lipoic acid and αGPC treatments on astroglial cell cultures:**

1. Control untreated astrocyte cultures
2. 50 µM (+/-)lipoic acid or (+)lipoic acid-treated astrocyte cultures
3. 10 mM α-GPC-treated astrocyte cultures
4. 50 µM (+/-)lipoic acid + 10 mM α-GPC or (+)lipoic acid + 10 mM α-GPC treated astrocyte cultures

#### **Immunocytochemical Analysis**

Concerning immunocytochemical analysis, the cells were fixed with 4% paraformaldehyde in 0.1 M tris saline buffer (TBS) for 20 min. Non specific antibody reactions were blocked with 5% normal goat serum for 1 h at room temperature. Fixed cells were incubated overnight at 4°C with primary antibodies directed against GFAP (1:200; Chemicon). After three washes, cells were incubated with secondary antibody conjugate with Fluorescein-5-isothiocyanate (FITC) for 1 h at room temperature (1:200; Chemicon).

#### **Comet assay**

The Comet Assay, a gel electrophoretic technique particularly advantageous to assess the presence of DNA fragmentation in every single cell was performed on astroglial cells treated as previously reported according to a modification of the protocol reported by Singh and collaborators.



In 10  $\mu$ l cell suspension are added 65  $\mu$ l of Low melting agarose (LMA) to 0.7%. The incorporated cells are arranged on a slide previously coated with a agarose thin layer in normal melting point (NMA) at 1%. The sandwich structure is completed by overlaying a further layer of LMA to 0.7% (85  $\mu$ l).

Lysis of cells: the slides are immersed in lysis solution at pH 10 (N-lauryl-sarcosine 1%, NaCl 2.5 M, 100 mM Na<sub>2</sub>EDTA, Triton X-100 1%, DMSO 10%) for 1h at 4°C. The strongly alkaline conditions of the lysis induce proteins denaturation, including histone, and the hydrolysis of RNA. The presence of the hydroxyl group in position 2' on the ribose causes the formation of unstable intermediates that are cyclic further hydrolysed with formation of a mixture of nucleoside monophosphates 2' and 3'. The absence of this group in the DNA molecule protects the latter from hydrolysis, allowing only the denaturation. Moreover, the presence of DMSO, a destabilizing the double helix, allows to obtain the DNA separated into two filaments.

Electrophoresis: finished lysis slides are positioned in the electrophoretic tank containing, for the alkaline Comet, running buffer at pH > 13 (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA) and left for 20 minutes in the dark, in order to facilitate unwinding of the DNA. After this step starts the electrophoretic running for 30 minutes at constant voltage (0.7V/cm). At the end of the stroke the slides are washed twice with the neutralization solution to pH 7.5 (0.4 M Tris-HCl) and were scored using a Leica fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Dedicated software (Leica-QWIN) allowed the analysis and the quantification of DNA damage by measuring the level of DNA damage as: (1) percentage of the fragmented DNA (TDNA), since it is considered to be the most comprehensive and meaningful Comet parameter.

#### **Drug treatment in astroglial cell cultures at 24 DIV for DNA labeling assay:**

Astroglial cells were maintained in serum-supplemented medium (SSM) as previously described (Bramanti et al. 2008a). Astroglial cells were washed in DMEM without serum and were incubated in the same medium supplemented with fatty acid-free bovine serum albumin (BSA, 1 mg/ml,

chemically defined medium) at each time interval during growth, as indicated (DIV), 48h after the last medium change. Cells were deprived of serum for 24h (starvation period) and then incubated for different time periods (24h or 36h) with estradiol or/and growth factors as described successively. Figure 2 drug treatment: bFGF (5 ng/ml) and 17- $\beta$ -E<sub>2</sub> (5 nM) were added for 24h or 36h respectively in 22 DIV astrocyte cultures. After the above mentioned pretreatment, the astroglial cell cultures were treated in the last 12h with “progression” growth factors EGF (10 ng/ml), IGF-I (10 ng/ml), INS (10  $\mu$ g/ml) (fig. 2), as follow:

1. Control;
2. E<sub>2</sub> (5 nM) 36h
3. bFGF (5 ng/ml) 24h
4. bFGF (5 ng/ml) 24h / E<sub>2</sub> (5 nM) 36h
5. bFGF (5 ng/ml) 24h / E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml) (last 12h)
6. bFGF (5 ng/ml) 24h / E<sub>2</sub> (5 nM) 36h + IGF-I (10 ng/ml) (last 12h)
7. bFGF (5 ng/ml) 24h / E<sub>2</sub> (5 nM) 36h + INS (10  $\mu$ g/ml) (last 12h)

### **Drug treatment in astroglial cell cultures at 22 DIV for DNA labeling assay and protein expression detection**

17- $\beta$ -E<sub>2</sub> (5 nM) was added for 36h in 22 DIV astrocyte cultures. After this last pretreatment, the astroglial cell cultures were treated in the last 24h with the following “competence and progression“ growth factors, as follow:

1. Control;
2. E<sub>2</sub> (5 nM) 36h
3. E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml)+bFGF (5 ng/ml) (last 24h)
4. E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml)+IGF-I (10 ng/ml) (last 24h)
5. E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml)+INS (10  $\mu$ g/ml) (last 24h)
6. E<sub>2</sub> (5 nM) 36h + bFGF (5 ng/ml)+IGF-I (10 ng/ml) (last 24h)

7. E<sub>2</sub> (5 nM) 36h + bFGF (5 ng/ml)+INS (10 µg/ml) (last 24h)
8. E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml)+bFGF (5 ng/ml)+IGF-I (10 ng/ml) (last 24h)
9. E<sub>2</sub> (5 nM) 36h + EGF (10 ng/ml)+bFGF (5 ng/ml)+INS (10 µg/ml) (last 24h)

**For DNA labeling assay:** at each time [methyl-3H]thymidine (2 µCi/ml of culture medium) was added and incubation was continued for 2h at 37°C. At the end of the incubation, DNA labeling was determined as described below.

### **Determination of Astroglial cell Viability**

Cell viability was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)2,5-diphenyl] tetrazolium bromide (MTT) reduction assay. This cell proliferation assay was used as a quantitative colorimetric method for measurements of cellular cytotoxicity (Bramanti et al. 2015). Briefly, MTT was added to each well with a final concentration of 1.0 mg/ml and incubated for 1 hr in a CO<sub>2</sub> incubator. The dark blue formazan crystals formed in intact cells were extracted with 250 µl of dimethyl sulfoxide, and the absorbance was read at 595 nm with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT). Results were expressed as the percentage MTT reduction of control cells.

### **[Methyl-3H]Thymidine incorporation into DNA**

T-75 cm<sup>2</sup> flasks were rinsed three times with ice-cold 0.9% NaCl-solution, pH 7.4, and cells were extracted with 1 N perchloric acid for 30 min at 4°C. Acid insoluble material was washed three times with 0.5 N perchloric acid, once with ethanol, and solubilized in 0.3 N NaOH at 37°C for 30 min. Aliquots were taken for protein and radioactivity measurements. Proteins were determined by the Lowry's method using BSA as the standard. Further methodological details are reported elsewhere (Avola et al. 1988a; Avola et al. 1988b).

### **NB cell cultures and treatments**

NB cell lines were maintained in adhesion with DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin at 37°C and 5% CO<sub>2</sub>. LA was added 24h before the addition of BTZ for LA/BTZ combination. BTZ was used at 50 nM. For estimation of the effect of BTZ on ER stress markers and HO-1 expression, NB cells were seeded in 6-well culture plate at density 5×10<sup>5</sup> cell per well (about 60% confluency), and treated with BTZ alone and in combination with 5 mM 4-Sodium phenylbutyrate (4PBA, Sigma-Aldrich, Milan, Italy) for 6 and 24h; and with 10 μM thapsigargin (Santa Cruz Biotechnology) alone and in combination with 5 mM 4-PBA for 24h. For viability assay, NB cells were seeded on 96 well black culture plate (Eppendorf, Milan, Italy) at density 1×10<sup>4</sup> cell per well, and subsequently treated with 100 μM of LA. After 24 hours, 50nM BTZ alone and in combination with LA was added to cell cultures for 24 hours. All agents were diluted directly in cell culture medium.

### **NB Cell viability assay**

Cell viability was assessed using ATPlite 1step assay (PerkinElmer, Milan, Italy) according to the manufacturers' protocol. Briefly, the 96-well black culture plate was taken from the incubator and equilibrated at room temperature for 30 minutes. Subsequently, to each well containing 100 μl of the cell suspension (5×10<sup>5</sup> cells/ml), 100 μl of reconstituted reagent was added and the plate was shaken for 20 minutes at 700 rpm using orbital shaker (Stuart Scientific, Staffordshire, UK). The luminescence was measured using Victor3 (PerkinElmer, Milan, Italy). Viability of the cells was expressed as percentage of vitality of untreated cells.

### **HO-1 gene expression analysis by real-time PCR (qRT-PCR) in NB cells**

RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent [61]. HO-1 mRNA expression was assessed by TaqMan Gene Expression, Applied Biosystem and quantified using a fluorescencebased real-time detection method by 7900HT Fast Real Time PCR

System (Life technologies, Carlsbad, CA, USA). For each sample, the relative expression level of HO-1 (Hs01110250\_m1) mRNA was normalized using GAPDH (Hs02758991\_g1) as an invariant control.

### **Western blot analysis**

Briefly, for western blot analysis 30 µg of protein was loaded onto a 12% polyacrylamide gel MiniPROTEAN® TGX™ (BIO-RAD, Milan, Italy) followed by electrotransfer to nitrocellulose membrane TransBlot® Turbo™ (BIO-RAD, Milan, Italy) using TransBlot® SE Semi-Dry Transfer Cell (BIO-RAD, Milan, Italy). Subsequently, membrane was blocked in Odyssey Blocking Buffer (Licor, Milan, Italy) for 1h at room temperature. After blocking, membrane was three times washed in PBS for 5 minutes and incubated with primary antibodies against HO-1 (1:1000) (anti-rabbit, Cat. No. BML-HC3001-0025, Life Sciences, Milan, Italy), BiP (1:1000) (anti-rabbit, Cat. No. 3177S, Cell Signaling Technology, Milan, Italy), Iron Responsive Element1α (IRE1α) (1:1000) (anti-rabbit, Cat. No. 3294S, Cell Signaling Technology, Milan, Italy), PDI (1:1000) (anti-rabbit, Cat. No. 5683S, Cell Signaling Technology, Milan, Italy), ERO (1:1000), ATG5, BECN1, MAP1 and β-actin (1:1000) (anti-mouse, Cat. No. 4967S, Cell Signaling Technology, Milan, Italy), overnight at 4°C. Next day, membranes were three times washed in PBS for 5 minutes and incubated with Infrared anti-mouse IRDye800CW (1:5000) and anti-rabbit IRDye700CW secondary antibodies (1:5000) in PBS/0.5% Tween-20 for 1h at room temperature. All antibodies were diluted in Odyssey Blocking Buffer. The blots were visualized using Odyssey Infrared Imaging Scanner (Licor, Milan, Italy) and protein levels were quantified by densitometric analysis of antibody responses. Data were normalized to protein levels of β-actin for cytoplasmic fraction. For extraction of cytoplasmic proteins, M-PER™ Cytoplasmic Extraction Reagents (Life technologies, Milan, Italy) was used following manufacturer's instructions.

### **Immunofluorescence**

Cells were grown directly on coverslips before immunofluorescence. After washing with phosphatebuffered saline (PBS), cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 20 minutes at room temperature. After fixation, cells were three times washed in PBS for 5 minutes and blocked in Odyssey Blocking Buffer for 1h at room temperature. Subsequently, the cells were incubated with primary antibody against HO-1 (anti-rabbit, Cat. No. BMLHC3001-0025, Life Sciences, Milan, Italy) at dilution 1:200 and against  $\beta$ -actin (anti-mouse, Cat. No. 4967S, Cell Signaling Technology, Milan, Italy) at dilution 1:200, overnight at 4°C. Next day, cells were three times washed in PBS for 5 minutes and incubated with secondary antibodies: TRITC (anti-mouse, Cat. No. sc-3796, Santa Cruz Biotechnology, ) at dilution 1:200, and FITC (anti-rabbit, Cat. No. sc-2012, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilution 1:200 for 1h at room temperature. All antibodies were diluted in Odyssey Blocking Buffer. The slides were mounted with medium containing DAPI (4', 6-diamidino-2phenylindole, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to visualize nuclei. The fluorescent images were obtained using a Confocal Laser Scanning Microscopy (CLSM, Zeiss LSM700, Milan, Italy).

### **Statistical Analysis**

Statistical analyses were made by Prism Software (Graphpad Software Inc., La Jolla, CA, USA), (Graphpad Prism, data analysis software, RRID: rid\_000081). The data are expressed as mean  $\pm$  SEM. Statistical analysis was carried out by ANOVA test: it is used to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\*  $p < 0.05$ ) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\*  $p < 0.01$ ) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*)  $p < 0.001$ ) were considered highly statistically significant.

## CHAPTER 4

### RESULTS

#### *Treatment with Choline containing compounds on astrocyte cultures*

Primary astroglial cell cultures were characterized by Glial Fibrillary Acidic Protein (GFAP) and glutamine synthetase activity as previously reported [3, 5, 8]. This analysis revealed that approximately 90% of cells were GFAP positive, indicating that cultures were enriched in astrocytes (data not shown).

DC cytofluorescence revealed no effect on TGs activity after treatment for 24 h with different concentrations of ACh compared to untreated cells (Figs. 1a, 1b and 2). An intense DC-fluorescence was observed in choline chloride treated cells at 0.1  $\mu\text{M}$  (Figs. 1c and 2) compared to control untreated cultures at 14 DIV (Figs. 1a).

Cytofluorescence intensity at 1  $\mu\text{M}$  (Figs. 1c and 2) was similar to control and slightly decreased at 10  $\mu\text{M}$  (Fig. 2). Fluorescence was located mainly in perinuclear areas both in untreated and in 0.1  $\mu\text{M}$  choline chloride treated cells. Using the 0.1  $\mu\text{M}$  choline concentration, fluorescence was also observed in the nuclear compartment (Fig. 1C, D).

CLSM analysis showed a dose-dependent increase of DC cytofluorescence in CDP-choline treated astrocytes (Figs. 1e, f and 2) in comparison to untreated cells at 14 DIV (Figs. 1a and 2).

Astrocytes exposed to the highest CDP-choline analysis appeared wrinkled (Fig. 1). An increased fluorescence, localized mainly along cytosolic prolongaments was observed in 1  $\mu\text{M}$  CDP-choline exposed 14 DIV astroglial cells (Fig. 2E, F) respect to untreated astrocytes (Fig. 2E, F). Treatment for 24h with  $\alpha$ -GPC at 1  $\mu\text{M}$  (Fig. 1h) increased DC cytofluorescence compared to control cells (Figs. 1 and 2). In 0.1  $\mu\text{M}$   $\alpha$ -GPC-treated astrocytes at 14 DIV fluorescence was found both in the cytosol and in the nuclear compartment (Fig. 1g). A decreased fluorescence was observed in astroglial cell cultures treated with 10  $\mu\text{M}$   $\alpha$ -GPC (Fig. 2).

Data of immunochemical (Western blot) analysis of the effect of different treatments on TG-2 expression, which are consistent with those of cytofluorescence are shown in Figs. 3–5. As can be seen, in astroglial cell cultures at 14 DIV, treatment for 24 h with 1  $\mu$ M choline or  $\alpha$ -GPC increased TG-2 expression in comparison with untreated control cultures, whereas treatment for the same time and for the same concentration of ACh or CDP-choline (1  $\mu$ M) and ACh (1  $\mu$ M) had no effect. Exposure of 21 or 35 DIV astroglial cell cultures to a 1  $\mu$ M concentration of choline, CDP-choline or  $\alpha$ -GPC induced a light decrease of TG-2 expression (Figs. 4 and 5). Treatment of 21 DIV cultures for 24 h with 1  $\mu$ M ACh remarkably decreased TG-2 expression (Fig. 4). A comparison of data of immunochemical analysis of TG-2 expression in astrocytes cultures at 14 DIV (Fig. 5, left hand panel) or at 35 DIV (Fig. 5, right hand panel) for 24 h shows a remarkable decrease of TG-2 immunoreactivity at 35 DIV (Fig. 5). Addition of 20% FCS to cell cultures at 14 or 35 DIV induced a remarkable increase of TG-2 expression, in comparison with BSA (1 mg/ml) addition, whose results represent the untreated control cultures (Fig. 5). Data on the influence of treatment with cholinergic precursors investigated on cyclin D1 expression in astroglial cell cultures at 14 DIV are shown in Fig. 6. A decrease of cyclin D1 immunoreactivity was noticeable after exposure of cultures to 1  $\mu$ M acetylcholine for 24h but not for 48h, to 1  $\mu$ M choline for both 24h and 48h and to 1  $\mu$ M CDP-choline for 24h but not for 48h. The effect elicited by treatment of cultures with 1  $\mu$ M  $\alpha$ -GPC consisted in a remarkable reduction of cyclin D1 expression after 24h exposure and no effect after 48h exposure (Fig. 6).

A significant increase on cyclin D1 expression was found in astroglial cell cultures at 35 DIV after exposure for 24 h to 0.1  $\mu$ M or 1  $\mu$ M, ACh, whereas treatment of cultures at 35 DIV with precursors tested at the concentration of 0.1  $\mu$ M and 1  $\mu$ M induced a decrease of Cyclin D1 expression (Fig. 7).

### **$\alpha$ Lipoic Acid (LA) - Alpha glycerylphosphorylcholine ( $\alpha$ GPG)**



Immunocytochemical analysis showed GFAP positivity of astroglial cells cultures treated with (+)thioctic acid or (+/-) thioctic acid and/or  $\alpha$ -GPC (fig.8).

Western blotting analysis showed that in 15 DIV astrocyte cultures, the treatment with (+)lipoic acid alone induced an highly significant enhancement in the expression of well known proliferation marker cyclin D1 respect to the values of untreated control ones (fig.9). Conversely not significant modification of cyclin D1 expression in  $\alpha$ -GPC-treated astrocyte cultures was observed; instead, the treatment with (+)lipoic acid and  $\alpha$ -GPC both together significant decreased it (fig.9). In 21 DIV astrocyte cultures the treatment with (+/-)lipoic acid alone or in combination with  $\alpha$ -GPC showed no significant modification of cyclin D1 expression. A slight enhancement of cyclin D1 expression was observed in  $\alpha$ GPC treated ones (fig.9).

On the other hand, in 15 DIV astrocyte cultures the treatment with (+)lipoic acid alone induced an highly significant enhancement of ornithine decarboxilase expression, a well known marker associated with increased cell growth (fig.10). The treatment  $\alpha$ -GPC alone or in combination with (+)lipoic acid showed a marked decrement of ornithine decarboxilase expression (fig.10). In 21 DIV astrocyte cultures, the treatment with (+/-)lipoic acid in combination with  $\alpha$ GPC significantly increased ODC expression; instead, no significant modification was observed after addition of (+/-)lipoic acid or  $\alpha$ -GPC alone (fig.10).

The MAP-kinase expression enhanced after treatment with thioctic acid and  $\alpha$ -GPC alone at 15 and 21 DIV, instead, the treatment with  $\alpha$ -GPC and (+)lipoic acid both together significant decreased it (fig.11).

The analysis of DNA status by Alkaline Comet assay showed any significant modifications induced by the treatment with (+)lipoic acid resembling rather a possible genoprotective effect (fig.12). ex. 5

## **Pretreatment bFGF-E<sub>2</sub>**

To assess the viability of astroglial cells in cultures at 24 DIV, pretreated for 24h with bFGF or/and E<sub>2</sub> for 36h and subsequently treated in the last 12/24h with “progression” growth factors, was performed MTT analysis with tetrazolium salts (Fig. 13). We observed that all the treatments used did not induce any change on the normal cellular viability respect to control ones (Fig. 13), therefore all the treatments used had no harmful effects on cellular metabolic activity.

In fig. 14 are reported the results concerning astrocyte cultures at 24 DIV, previously induced to 24h starvation period, and than pretreated for 24h with bFGF and for 36h with E<sub>2</sub>, and treated in the last 12h with EGF, or IGF-I or INS. In particular, pretreatment for 24h with bFGF and subsequent exposure for 36h to E<sub>2</sub> and EGF or IGF-I or INS in the last 12h stimulated DNA labeling especially when the cultures were treated with “progression” growth factors. In these conditions a significant increase of [methyl-<sup>3</sup>H]-thymidine incorporation was found compared to untreated controls, as well as to control cultures pretreated with bFGF and subsequently with E<sub>2</sub> (Fig. 14). Basic FGF pretreatment and subsequent treatment with E<sub>2</sub> for 36h induced a stimulation of [methyl-<sup>3</sup>H]-thymidine incorporation into DNA also, whereas a 36h E<sub>2</sub> treatment alone not significantly decreased it (Fig. 14).

Figure 15 showed the DNA labeling in astrocyte cultures at 24 DIV pretreated for 36h with E<sub>2</sub> and than treated with “competence and progression” growth factors. The 36h E<sub>2</sub> treatment alone decreased not significantly DNA labeling, but contemporary addition of E<sub>2</sub> with two or three growth factors induced a remarkable stimulation of methyl-[<sup>3</sup>H]thymidine incorporation into DNA (Fig. 15). When E<sub>2</sub> was co-added with EGF and INS or with EGF, bFGF and INS, the increase of [<sup>3</sup>H]thymidine incorporation reached the maximum value, demonstrating an astroglial synergistic mitogenic effect evoked by contemporary treatment with growth factors in the presence of estrogens (Fig. 15).

The Cyclin D1 expression data, evaluated by western blot analysis, remind the trend of DNA labeling values (Fig.16). In particular, when astrocyte cultures were pretreated for 36h with E<sub>2</sub> and

subsequently treated with “competence and progression” growth factors by two or by three, cyclin D1 expression was markedly increased (Fig.16).

In figure 17 are reported data concerning ERK 1/2 expression by western blot analysis in astrocyte cultures at 24 DIV in the above mentioned experimental conditions. All the treatments (EGF, bFGF, INS, IGF-I alone or in combination by two or by three) induced an high significant increase of the ERK 1/2 expression comparing this result to the untreated control and also to the E<sub>2</sub> 36h treated ones (Fig. 17).

Figure 18 showed GFAP expression in astrocyte cultures at 24 DIV pretreated for 36h with estradiol alone or plus the above mentioned “competence and progression” growth factors by two or by three. The data obtained indicated that GFAP expression was markedly increased when the cultures were treated with the neurotrophic factors, especially with the following treatments EGF+bFGF, or EGF+INS or bFGF+INS, and with the following treatments also EGF+bFGF+IGF-I or EGF+bFGF+INS comparing results to untreated control and also to the E<sub>2</sub> 36h treated ones (Fig.18).

Data concerning vimentin expression (Fig. 19) evaluated by western blot analysis showed an increment of this cytoskeletal marker in all the treatments used in comparison to untreated control and also to the E<sub>2</sub> 36h treated ones. The enhancement on vimentin expression was particularly evident when the cultures were treated with “competence and progression” growth factors by two or by three (Fig. 19).

### **The pretreatment with competence GF and DEX and subsequent treatment with progression GF**

To assess the viability of astroglial cells in cultures pretreated for 24 hr with competence growth factor bFGF and subsequently treated with DEX 10<sup>-9</sup> M for 48 hr with progression GFs (EGF, IGF-1, or INS) alone or two of them added in the last 12 hr, MTT analysis was performed with

tetrazolium salts (Fig. 20). We observed that the astroglial cell viability was not significantly modified for all the treatments used compared with untreated controls at 25 DIV (Fig. 20).

Data with respect to immunocytochemical analysis evaluated for GFAP, the well-known astrocyte differentiation marker, showed that all of the treatments were positive for GFAP (Fig. 21A). Intracellular localization of GFAP and relative morphological modifications induced by our treatments were evaluated (Fig. 21A). In particular, protein localization was found in the cytoplasm and in subcellular compartments, and a typical polygonal flat shape of astrocyte was also ascertained (Fig. 21A).

In Figure 21B, results are displayed with respect to GFAP expression in astrocyte cultures at 25 DIV pretreated for 24 hr with competence growth factor bFGF and subsequently treated with DEX  $10^{-9}$  M for 48hr and then with the previously mentioned progression GFs.

The pretreatment with bFGF alone or in combination with the subsequent treatment with DEX  $10^{-9}$  M for 48 hr induced an enhancement of GFAP expression with respect to untreated controls (Fig. 21B). The treatment with progression GFs EGF, INS, and IGF-1 alone in the last 12 hr significantly increased GFAP expression with respect to untreated controls and also with respect to SSM cultures (Fig. 21B). No significant modification in GFAP expression with respect to untreated controls was observed in the other treatments in which the GFs were coadded. In addition, GFAP expression was significantly decreased after coaddition of EGF+INS and EGF+IGF-1 respect to bFGF 24-hr/DEX 48-hr pretreated cultures (Fig. 21B).

Figure 22 shows vimentin expression in astrocyte cultures at 25 DIV under the same experimental conditions. It is easy to observe a marked enhancement of vimentin expression for all the treatments with progression GFs added alone or in combination compared with untreated controls. This enhancement was particularly evident in the treatment with INS alone in the last 12 hr (Fig. 22).

In Figure 23, data are displayed with respect to ERK2 expression in astrocyte cultures at 25 DIV under the above-mentioned experimental conditions. All the treatments (EGF, INS, and IGF-1 alone

or in combination) induced a highly significant increase of the ERK2 expression in comparison with the untreated controls and also with SSM cultures (Fig. 23).

### **Effects of LA on BTZ induced-cytotoxicity in neuroblastoma cell lines**

To study of BTZ effects on cell proliferation, we performed the cytotoxicity assay by ATPLite one step kit. We observed that the BTZ 50nM was able to inhibit the proliferation in neuroblastoma cell lines SK-N-BE and SH-SY5Y ( $p < 0.001$ ) (Fig. 24). Moreover, we tested the LA effects on neuroblastoma cell lines proliferation alone or in combination with BTZ. The survival in LA alone condition not showed any significant change, while, in combination with BTZ, LA was capable to revert the cytotoxicity effect of BTZ in neuroblastoma cells ( $p < 0.001$ ) (Fig.24).

### **LA reduces HMOX1 expression induced by BTZ and increase its nuclear traslocation**

In order to evaluate the cell response after BTZ treatment alone or in combination with LA, we analyzed the hemeoxygenase-1 (HMOX1) expression in NB cells .We observed a significant increase of HMOX1 mRNA expression in SK-N-BE and SH-SY5Y cells after treatment with BTZ 50 nM. BTZ induced up-regulation of HMOX1 of about 100 folds ( $p < 0.0001$ ) (Fig.25A). The evaluation of HMOX1 protein expression by western blot assay, confirmed the increase induced by BTZ treatment in neuroblastoma cells (Fig. 25B). In our recent studies, we demonstrated that HMOX1 nuclear translocation regulates BTZ-induced cytotoxicity in cancer cells (Tibullo et al. 2016). To study the nuclear localization of HMOX1 in neuroblastoma cells, we performed immunofluorescence assay in NB cell lines. Then cells were stained with immunofluorescent antibody. We observed by confocal microscope an increase of protein expression and nuclear localization signal of HMOX1 in neuroblastoma cells after treatment with BTZ (Fig. 26) compared

with untreated. Cytoplasmic localization of HMOX-1 was disrupted when bortezomib and LA were used in combination (Fig. 26). Moreover, LA did not induce HMOX1 expression, but in combination with BTZ, LA was able to revert the increase of HMOX1 induced-BTZ alone and promote its nuclear localization (Fig. 26).

### **LA modulates both ER-stress and autophagy signaling**

It has been shown previously that bortezomib-induced apoptosis is associated with increased endoplasmic reticulum (ER) stress, activating the terminal unfolded protein response (UPR) (Davenport et al. 2007; Meister et al. 2010). In order to check the activation of ER-stress response after proteasome inhibition, we performed western blot assay in SH-SY5Y cell line treated with BTZ. Our data shown that BTZ was able to induce protein expression of ER-stress markers, in particular the Binding Immunoglobulin Protein (BiP1) and Inositol-requiring enzyme 1 (IRE1 $\alpha$ ), ER oxidoreductin 1 (ERO1 $\alpha$ ) and Protein disulfide isomerase (PDI) ( $p < 0,0001$ ) compared to control ones. When NB cell lines were exposed to BTZ in combination with LA 100  $\mu$ M, we observed a concomitant reduction of ER-stress protein levels ( $p < 0,001$ ) respect to BTZ alone treatment (Fig. 27).

Regarding autophagy, we evaluated the protein expression of Autophagy protein 5 (ATG5), Microtubule-associated protein 1 (MAP1) and Beclin-1 protein (BECN1). Figure 27 shows that treatment with BTZ alone did not induce autophagy-related proteins expression compared to controls. Interestingly, NB cells co-treated with LA shown higher ATG5, MAP1 and BECN1 protein levels ( $p < 0,001$ ) than BTZ alone. This set of experiments showed that LA prevented the overexpression of ER-stress related proteins and promote autophagy signaling.

## CHAPTER 5

### 5. DISCUSSION

#### **Treatment with Choline containing compound on astrocyte cultures**

The purpose of this first session of the investigation was to assess the influence in primary astrocyte cultures at different stages of growth, development and maturation (14, 21 and 35 DIV) of different cholinergic precursors (choline chlorure, CDP-choline and  $\alpha$ -GPC) and of ACh used as a reference compound, on TG-2 expression, as differentiation marker.

In cholinergic neurons choline is generated from the hydrolysis of phosphatidylcholine by a phospholipase D (PLD)-type enzyme and is used for the synthesis of the neurotransmitter Ach [1]. CDP-choline and its hydrolysis products (cytidine and choline) play important roles in generating phospholipids involved in membrane formation and repair. Moreover, the compound has beneficial physiological actions on cellular functions, that have been extensively studied [12]. Exogenously administered CDPcholine prevents, reduces, or reverses effects of ischemia and/or hypoxia in most animal and cellular models investigated, and acts in head trauma models to decrease and limit nerve cell membrane damage, restore intracellular regulatory enzyme sensitivity and function, and limit edema [26, 27]. Beneficial effects of exogenous citicoline also have been postulated and/or reported in experimental models for dyskinesia, Parkinson's disease, cardiovascular disease, aging, Alzheimer's disease, learning and memory impairment, and cholinergic stimulation [27].

The neurochemical profile of  $\alpha$ -GPC consists in an initial short stimulatory effect on ACh release paralleled by a significant and long lasting increase of ACh synthesis, resulting in an increase storage of ACh and a raised secretion upon stimulation [24]. Administration of  $\alpha$ -GPC before a behavioural test prevented learning impairment induced by scopolamine. It has been suggested that these behavioural effects evoked by this drug may be related to its property to increase hippocampal ACh synthesis and release [7, 24].  $\alpha$ -GPC is a cholinergic precursor, which has been shown to be

effective in countering cognitive symptoms in forms of dementia disorders of degenerative, vascular or combined origin [25]. The observation that treatment with  $\alpha$ -GPC attenuates the extent of glial reaction in the hippocampus of SHR suggests also that the compound may afford neuroprotection in this animal model of vascular brain damage and has an influence on astroglial dynamics [25].

In the present study we have observed that DC-fluorescent induced by exposure of cultures to 0.1  $\mu$ M choline was located predominantly at perinuclear level, whereas fluorescence induced by a higher concentration of the compound (1  $\mu$ M) involved also the nuclear compartment.

This suggests an involvement of TGs activity not only during cell differentiation, but also in proliferating astroglial cells maintained in the presence of cholinergic modulators. An increased fluorescence localized prevalently along cytosolic processes in 1  $\mu$ M CDP-choline treated astrocyte cultures compared to untreated ones was observed. This suggests an intense TGs activity during different biochemical pathways and physiological processes consequent to CDP-choline activity (for a review see [1]). Treatment for 24h with 1  $\mu$ M  $\alpha$ -GPC raised TGs activity to compare to control astrocytes at 14 DIV and induced an increase of cytofluorescence in both cytosol and nuclear compartments of cultured astrocytes. These findings suggest a role of the compound during astrocyte differentiation processes probably related to nucleus-cytosol cross-talking in proliferating astroglial cells in a particular stage of development in vitro.

ACh at the different concentrations used had no effect on TG-2 activity compared to untreated control cells. This suggests that effects observed in our model with cholinergic precursors is not related with effects of the neurotransmitter itself. Possible explanations for these phenomenon is that cholinergic precursors are not transformed into ACh in this model or that amounts of ACh they can generate is not enough for activating muscarinic (G protein-coupled) or nicotinic (ionotropic) receptors [13, 21] to elicit biological actions of ACh.

Western-blot analysis of TG-2 expression in astrocytes cultures at 14, 21 and 35 DIV showed that treatment for 24 h with 1  $\mu$ M ACh and 1  $\mu$ M CDP-choline for 24h had no effect on TG-2



expression whereas  $\alpha$ -GPC and choline at the same concentration enhanced TG-2 expression in astrocyte cultures at 14 DIV compared to untreated ones.

The TG-2 expression unaffected by CDP-choline indicates a reduced protein biosynthesis, even though a marked DC fluorescence distribution evidences the enzymatic TGs activity independently on protein expression. In astroglial cell culture at 21 DIV 1  $\mu$ M choline, CDP-choline and  $\alpha$ -GPC addition slightly decreased TG-2 expression as well as  $\alpha$ -GPC addition in 35 DIV cultures; but this reduction is more pronounced after 1  $\mu$ M ACh treatment. These findings suggest a down-regulation of TG-2 expression during different stages of maturation and differentiation of our cell culture model and the lack of involvement of a cholinergic transmission mechanisms in the astroglial cells, in the absence of neuron-glia cross-talking which is relevant for neurotransmitter modulatory activity.

Moreover, glial fibrillary acid protein (GFAP) and vimentin western blot analysis assessed a particular cytoskeletal network involvement in order to better clarify the proliferative and differentiative status of our astroglial cell cultures, respectively [6].

In conclusion, our findings suggest that our in vitro model may represent an excellent tool not only to evaluate astroglial cell proliferation and differentiation in culture, but also to study the effects of ischemia and/or hypoxia induced in some model cellular systems, in order to better precede, reduce and counteract it after cholinergic precursor exposure.

In addition, Ach, cholin, CDP-choline and  $\alpha$ -GPC beneficial administration may be also considered therapeutically very useful and particularly effective for some important neurodegenerative diseases, such cerebral aging, stroke, cerebral ischemia, Parkinson's and Alzheimer's disease.

### **Lipoic acid- $\alpha$ GPC treatment**

It is well known that glial cells are involved in providing neurotrophic signals required for survival, proliferation and differentiation neurons (Bramanti et al., 2010).

Besides their physiological involvement, astrocytes play an important role in pathological conditions of the nervous system.

Several lines of evidence indicate that glia influences the growth, migration and differentiation of neurons, but the effect of neuronal cells on astrocytes is far from being well understood. Increasing evidence has been accumulated indicating that neurons are modulators of astrocyte gene expression and differentiation (Bramanti et al., 2010).

It is well known that LA is a neuroprotective antioxidant agent able to act by scavenging reactive oxygen species and stimulation of glutathione synthesis.

In the literature are reported data concerning novel therapeutic approaches for different neurodegenerative diseases associated with oxidative stress. In particular, the raceme LA is used in clinical practice, even if the R(+)-LA is the preferred isomer in biological systems.

Other studies indicate that peak plasma levels of the R(+)-LA are significantly higher than S-LA even though both isomers are rapidly metabolized into bisnorlipoate, tetranorlipoate and b-hydroxy-bisnorlipoate, which are all readily secreted (Teichert et al., 2003).

In order to better clarify the antioxidant role of R(+)-LA in astroglial compartment, we investigated the effect of both R(+)-LA and raceme LA on astroglial cell proliferation and differentiation in primary cultures.

In addition, particular attention has been devoted to study the involvement of both compounds on the expression of some different biomarkers related to the astroglial cytoskeletal network, cell cycle and signalling transduction pathways, in our in vitro model.

Concerning the results obtained in our astroglial cell cultures treated with  $\alpha$ -lipoic acid or  $\alpha$ GPC alone or both in combination, is particularly important to underline that the addition of single drugs ( $\alpha$ -lipoic acid or  $\alpha$ GPC) induced an "up" modulation of the expression of biomarkers used in our study. On the contrary, the co-treatment with both  $\alpha$ -lipoic acid+ $\alpha$ GPC showed not significant modification or a down regulation of the above mentioned biomarkers (GFAP, vimentin, cyclin D1, O.D.C. and MAP-kinases). This last finding demonstrated no additional effect after the co-treatment

with both  $\alpha$ -lipoic acid +  $\alpha$ GPC respect to the drugs addition alone. It necessary a further investigation to better clarify the particular mechanism evoked by the treatment of these neuroprotective agents in our in vitro models.

It is necessary further study to clarify the specific mechanism evoked by the processing of these neuroprotective agents in our in vitro models. A possible explanation of this unexpected results may depend on the fact that,  $\alpha$ -lipoic acid, by acting as “insulin mimetic agent” and stimulating the receptors, would elicit a mechanism of action that could interfere on common signal transduction pathways causing a desensitization of the same ones and thereby lead to a “down regulation” of the expression of biomarkers studied.

In conclusion, these preliminary findings may represent a very good “tool” in order to better clarify the antioxidant and metabolic role played by  $\alpha$ -lipoic acid in proliferating and differentiating astroglial cell cultures, during an interactive cross-talk between glial and neuronal cells, after brain lesions or damages, that may occur in some neurodegenerative diseases, as Alzheimer’s and Parkinson’s diseases, Huntington's Còrea , Stroke, Ictus correlated to oxidative stress.

## **Pretreatment with competence growth factors and E<sub>2</sub> and treatment with progression GFs**

The astrocytes proliferation and differentiation represents a valuable tool to study biochemical mechanisms involved in their development and maturation. Glial cells are involved in providing neurotrophic signals to neurons and they are required for their survival, proliferation, and differentiation (Bramanti et al. 2010a).

In addition, to their physiological involvement, astrocytes play an important role in pathological conditions of the nervous system. Several lines of evidence indicate that glia influence the growth, migration, and differentiation of neurons, but the effect of neuronal cells on astrocytes is far from

being well understood. Increasing evidence indicates that neurons are modulators of astrocyte gene expression and differentiation (Bramanti et al. 2010b).

The neurotrophic and neuroprotective effects of estrogens have been widely studied in various experimental models, both *in vivo* and *in vitro*.

In addition, serum deprivation is one exogenous stimulus capable to enhance spontaneous or growth factor-induced astroglial differentiation (Bramanti et al. 2007a).

It is well known that growth factors contained in serum are crucially required for the growth of cell cultured *in vitro*. Serum deprived cultures cease to growth and serum restoring causes the cells to enter S phase. Although each serum factor may have a differential effect on DNA, RNA, protein synthesis, of particular cell types, the combined action of them is required for the optimal cell growth. Competence growth factors act on cells that are either in G<sub>0</sub> or early G<sub>1</sub> phase of the cycle, rendering them competent to initiate DNA replication.

The progression growth factors would allow the progression of cells through the prereplicative phase of the cycle, inducing cells to traverse into S, G<sub>2</sub> and M phases.

The crucial aim of present research was to study the interesting and interactive role exerted by estrogen and growth factors added both together, as well as to clarify the different activity exercised by “competence” and “progression” growth factors respectively. Data results of our *in vitro* model demonstrated that growth factor–estrogen interaction induced a marked enhancement of astrocytes proliferation and maturation processes.

The treatment with estradiol alone induced a weak but not significant reduction of DNA synthesis, whereas the co-addition of estradiol plus growth factors markedly increased it (Fig. 14). This suggests that astroglia responds synergistically to the proliferative effect induced by co-addition of growth factors in the presence of E<sub>2</sub>. Moreover, pretreatment with bFGF alone in untreated cultures or treatment with E<sub>2</sub> in bFGF-pretreated cultures, markedly stimulated DNA synthesis, whereas the 24h E<sub>2</sub> treatment reduced it (Fig. 14). These findings demonstrate a synergistic effect evoked by the interaction between E<sub>2</sub> and growth factors during astroglial cell proliferation and differentiation in

bFGF pretreated cultures. When “progression” growth factors were added in the presence of a differentiative factor such as E<sub>2</sub>, GFAP and vimentin expression were markedly enhanced comparing results to untreated control ones.

The co-addition of estradiol plus “progression” growth factors induced a marked DNA macromolecular synthesis, specially when estradiol was co-added with EGF+bFGF+INS (Fig. 15). Strangely, estradiol added alone had not peculiar effect on DNA labeling, while bFGF treatment alone or in combination with estradiol markedly stimulated it (Fig. 15). This last finding suggests the hypothesis that estradiol acts like “competence factor” when it is added alone in astrocyte cultures, whereas in the presence of bFGF competence growth factors it shows a synergic effect with them and promotes progression through the cell cycle.

Each of the well known four phases of the cell cycle: G<sub>1</sub>, S, G<sub>2</sub> and M, is regulated by the coordinated action of kinases and proteases (King et al. 1996). When deprived of serum, the cells continue to cycle until they complete mitosis, whereupon they exit into the G<sub>0</sub> state. These cells can be reintroduced into the cell cycle by the re-addition of serum or purified growth factor. The mitogen must be present in G<sub>1</sub> phase until to phosphorylation of Rb (R point), which is several hours prior to the transition between G<sub>1</sub> and S (Planas-Silva and Weinberg 1997). Thus in serum deprived cells, all of the growth factor-stimulated events that are necessary for completion of one round of the cell cycle occur before the R point.

Our results on cyclin D1 expression indicated that E<sub>2</sub> as well as “competence and progression” growth factors stimulated cyclin D1 expression (Fig. 4), therefore neurotrophic factors addition enhanced proliferation processes. In fact, cyclin D1 is a transcriptional co-regulator in astroglial cells and it may be considered, as an important marker to evaluate the involvement of different compounds on growth, development and maturation of our *in vitro* model (Bramanti et al. 2010a).

These data on Cyclin D1 expression (Fig. 16) are well correlated to those ones obtained by DNA labeling (Fig.15) in astroglial cells maintained under different culture conditions. In particular, it is markedly evident the synergistic mytogenic role played by “competence and progression” growth

factors added in the last 24h of the 36h E<sub>2</sub>-treatment. This in order to better regulate DNA synthesis during astroglial proliferation in different phases of cell cycle.

In addition, growth factors promote phosphorylation of Rb by regulating the activity of the G1 Cdk. This involves promoting the synthesis and stability of cyclin subunits, as well as decreasing the levels of Cdk inhibitors (Jones and Kazlauskas 2000).

According to these results "competence and progression" growth factors would act sequentially and synergistically during cell cycle. The similar action of some growth factors in the prereplicative phase suggest the operation of certain common intracellular pathways.

Concerning ERK 1/2 expression in astrocyte cultures at 24 DIV (fig. 17) our findings showed a marked up regulation of protein in all the treatment performed. ERK 1/2, a well known member of the MAP kinase family, acts as an integration point for multiple biochemical signals, and it is involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. The activation of this kinase requires its phosphorylation by upstream kinases.

Other studies demonstrated an alternative estrogen signaling pathway that displayed similarities to the one used by growth factor and specifically the MAP-kinase cascade (Singh et al. 1999).

It is also evident that the response of cells to extracellular signalling agents such as hormones and growth factors, may result in either stimulation or inhibition of specific functions associated with structural components of the membrane, the cytoplasm, the mitochondria, the nucleus or other cellular compartments. Frequently, the cellular response includes alteration in the regulation of gene expression, and certain cells may show a mitogenic response.

Concerning cytoskeletal proteins expression, when "progression" growth factors were added only in the last 24h in the presence of a differentiating agent, such as 17- $\beta$ -estradiol, GFAP and vimentin expression were markedly enhanced. It have been widely demonstrated and accepted in literature the evidence that GFAP synthesis is an important element of the developmental program of astrocyte differentiation. During the formation of GFAP networks in some reactive astrocytes,

vimentin may act as a cytoskeleton associated protein (Pixley and de Vellis 1984). Subsequently, at about the time of birth, a transition from vimentin to GFAP takes place; vimentin disappears and is progressively replaced by GFAP in differentiated astroglial cells, which transiently co express these two proteins. Furthermore, cytoskeletal proteins GFAP and vimentin represent intermediate filaments of astrocyte used like differentiation parameters (Bramanti et al. 2010b).

Regarding astroglial cell differentiation processes may be hypothesized an involvement of both PI-3K and NFkB pathways.

These two pathways may operate in association (Martin et al. 2001) and GFAP promoter possess a NFkB-like recognizing sequence.

Therefore, we can not exclude that this mechanism may be responsible for the reduction of DNA synthesis and enhanced GFAP expression in our *in vitro* model when estradiol was added only.

Moreover, it has been widely demonstrated that GFAP expression in astroglial cell cultures is regulated by various hormones and growth factors, indicating a differentiating role played by numerous trophic proteins present in serum, that are able to stimulate cytoskeletal protein expression (Morrison et al. 1985).

Estradiol may act directly upon astrocytes, and that the direction of the transcriptional response can be influenced by astrocyte–neuron interactions. Estrogen receptor may also cross-talk directly with neurotrophin receptor signaling cascades (Toran-Allerand 1996). By acting via its own receptor, E<sub>2</sub> not only can elicit direct classical transcriptional effects, but also could indirectly regulate gene transcription by activating ERKs (Font de Mora and Brown 2000). This may account for its ability to regulate genes devoid of an apparent estrogen responsive element (Sukovich et al. 1994).

Our findings confirmed the complex and crucial role played by estradiol and “competence” and “progression” growth factor, during astroglial cell proliferation and differentiation in cultures, maintained under different experimental conditions. The estradiol added alone didn’t have a significative effect on astrocytes proliferation and differentiation, but the contemporary addition of

this estrogen with “progression” growth factors induced a marked increment of proliferative and differentiative astroglial markers used.

An increasing number of evidence suggests that estradiol exerts significant neuroprotective effects against a variety of neurodegenerative pathologies; however, the molecular mechanisms underlying the beneficial actions of these hormones in the brain are not completely understood. Further studies are needed to better characterize the modes of action of estrogens in the brain: from their cellular targets to the downstream molecular effectors, in order to provide a rationale for their pharmacological exploitation as effective neuroprotective agents.

Finally, our findings suggest an intertwined dialogue between these two class of neuroactive molecules and confirm the complex role played by estradiol and both “competence” and “progression” growth factors, in regulating specific proteins expression during astroglial cell proliferation and differentiation. That could have an important involvement in future therapeutic strategies correlated to neurological and neurodegenerative disorders.

### **Pretreatment with competence GFs and DEX and subsequent treatment with progression GFs**

Cellular proliferation and differentiation are important activities in nervous tissue, and in this field glial cells have several prominent functions, such as providing neurotrophic activity, maintaining homeostasis, and giving support and protection for neurons in the central and peripheral nervous systems (Bramanti et al. 2010a). Consequently, some important functions carried out by astrocytes include reserving nutrients toward the nervous tissue, supporting ion balance and removing excess ions, and assisting neurons to form synaptic connections among one another other as well as playing an interesting role in the repair and scarring processes of the brain following some injuries. Therefore, the study of astroglial cell proliferation and differentiation represents an interesting way to observe and understand the mechanisms involved in glial development. In fact, some studies



report that glia influence the growth and differentiation of neurons, but the effect of neuronal cells on astrocytes is not well understood. Growing evidence indicates that neurons are modulators of astroglial gene expression (Bramanti et al. 2010b).

The present study evaluates the main role of some GFs added simultaneously in the presence of the DEX steroid hormone. In particular, we assessed the different behaviors of competence and progression GFs examined under different experimental conditions in cultures. The findings indicate that glucocorticoids may cooperate with GFs or may abrogate their effects depending on the experimental culture conditions used as well as the exposure time and the type of GFs added. DEX upregulated astroglial intermediate filament protein expression depending on its addition and on the time of treatment (after competence factor bFGF and before progression GFs [EGF, INS, IGF-1] alone or both together). This suggests the occurrence of an interaction between GFs and glucocorticoids regulating astroglial cell maturation and differentiation through a synergistic action on the cytoskeletal protein network.

On the other hand, the relationships between glucocorticoid receptor and transcription factors are well known, including the ubiquitous transcription factor nuclear factor-kB (NF-kB).

It has been well documented that NF-kB regulates not only cell survival but also proliferation through upregulation of cell-cycle-regulated proteins such as cyclin D1 (Joyce et al. 1999). It is probable that the growth-arresting and differentiation-promoting properties of DEX that we observed in our experiments depends on these mechanisms.

With respect to GFAP expression (Fig. 21B), the addition of EGF, IGF-1, or INS alone induced an upregulation of cytoskeletal protein; in contrast, the coaddition of EGF+IGF-1 or EGF+INS induced a downregulation of GFAP expression when the results were compared with cultures pretreated with bFGF 24 hr/DEX 48 hr. These data confirm the modulation of the expression of this cytoskeletal protein involved in the astroglial differentiation under different experimental conditions. Moreover, these findings highlight a possible competitive rather than cumulative effect

evoked by the coaddition of EGF+INS or EGF+IGF-1 with respect to the single GF treatments used.

With respect to the marked enhancement of vimentin, expression for all the treatments used confirms the main activity of DEX and progression GFs. Glucocorticoids can upregulate the high-mobility group (HMG)-1 and -2 proteins involved in chromatin remodeling during cell differentiation; DEX stimulates astrocytes to release the HMG-1 protein that promotes membrane-mediated differentiation processes (Passalacqua et al. 1998). These observations could explain the effects of DEX on vimentin expression. In fact, all progression factors significantly increased vimentin levels.

Data with respect to ERK2 expression in astrocyte cultures at 25 DIV (Fig. 23) show a marked upregulation of protein in all treatments performed. ERK2 is a wellknown member of the MAPK family. It acts as an integration point for multiple biochemical signals and is involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation, and development. It is known that the activation of this kinase requires its phosphorylation by upstream kinases. Upon activation, this kinase translocates to the nucleus of the stimulated cells, where it phosphorylates nuclear targets.

In the present study, the increase of the expression of ERK2 is an indication of increased cell proliferation and differentiation. In particular, these results demonstrate that pretreatment with the competence growth factor bFGF+DEX and the subsequent treatment with progression GFs (EGF, INS, IGF-1) stimulates biochemical events related to the MAPK/ERK pathway that oversees the subsequent downstream molecular mechanisms. The MAPK/ERK pathway is a well-known chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. It is hypothesized that the cellular response to extracellular signaling agents, such as hormones and GFs, may induce stimulation or inhibition of specific functions associated with some cellular compartments or with the nucleus. Often, the response is a modification of gene expression.

In conclusion, our results indicate that the addition of GFs modulates GFAP, vimentin, and ERK2 expression, depending on the pretreatment used. In particular, the activity of progression GFs induces a significant enhancement of the above-mentioned biomarkers. These findings support the hypothesis of the presence of meaningful interconnections among these categories of neuroactive molecules that validate the main role performed by DEX and GFs during the developmental signals, such as cell proliferation, differentiation processes, and neural survival. This underscores the importance of basic biomedical research for clarifying the molecular mechanisms involved in biological processes of some neurologic disorders associated with astrocytosis to help in future clinical approaches for these pathologies.

### **Effect of lipoic acid in the mechanisms of resistance to bortezomib in SH-SY5Y neuroblastoma cells**

In the present last research session, we shown the crucial role played by LA in the development of chemoresistance in NB cells treated with BTZ. Our data indicate that in combination with BTZ, LA acts as chemical chaperone reducing the stress response induced by proteasome inhibition. Firstly, we showed that BTZ significantly reduces cell viability. These data are consistent with previous observations showing that BTZ, a boronic acid dipeptide inhibitor of the 26S proteasome (Teicher et al. 1999) down regulates the expression of several antiapoptotic factors and induces caspase-dependent apoptosis (Almond and Cohen 2002; Hideshima et al. 2002). It is well known that proteasome inhibitors induces ER stress, resulting in a unfolded protein response (UPR) and escalation of reactive oxygen species (ROS) that leads to cell death. In cancer cells, HO-1 is considered to play a major role as an essential survival factor, protecting against chemotherapy-induced increase in ROS (Goswami et al. 2008; Meister et al. 2007; Salerno et al. 2013). These observations were confirmed in our study. Under our experimental conditions, HO-1 upregulation was observed following BTZ treatment on all tested NB cell lines, suggesting a protective role

against BTZ-induced ROS. Concomitantly to HO-1 upregulation we showed a significant induction of ER-stress. Other papers demonstrated that BTZ enhances ER-stress (Chien et al. 2014; Obeng et al. 2006) and that HO-1 upregulation is dependent on ER-stress (Tibullo et al. 2016). However, autophagy, another cell death program, occurs widely in cancer cells under unfavorable conditions. Whether the autophagy that occurs in response to various stimuli leads to cell survival or cell death remains controversial. The consensus is that the outcome of autophagy activation is partly dependent on the tumor type and treatment characteristics. Additionally, the specific signal transduction pathways involved in the autophagy process are not exactly the same under different conditions (He and Klionsky 2009; Klionsky 2005; Mizushima et al. 1999). Several lines of evidences substantiate these hypothesis. In lung cancer and multiple myeloma autophagy flux protects cells from apoptotic death (Wu et al. 2016). In our experimental model, we did not observe activation of autophagy-related proteins in NB cells treated with BTZ, thus we supposed that in this contest autophagy is activated to promote cell survival. Cell ability to adapt to oxidative stress by detoxifying reactive oxygen species (ROS) and toxic molecules has been demonstrated to play a role in the failure of different anticancer therapies (Diehn et al. 2009; Gorrini et al. 2013). Indeed, the imbalance of redox state, induced by different antitumour drugs, can be counteracted by the up-regulation of antioxidants, leading to tumour cell survival. LA is a neuroprotective antioxidant agent able to act as scavenger of ROS. Moreover, it has been demonstrated that LA prevented cell death induced by drugs which deplete glutathione in NB cells (Yamada et al. 2011). In order to demonstrate the implications of LA in cell resistance to BTZ cytotoxic effect, we co-treated NB cells with LA 100  $\mu$ M. Our data shown an increase of viability in all NB cell lines treated with both BTZ and LA. Compared to NB cells treated with BTZ alone, the co-treatment with LA induced a down regulation of HO-1 and ER-stress. In addition, we observed a significantly increase of autophagy following treatment both with combination of BTZ and LA. Furthermore, LA is able to increase autophagy in NB cells alone compared to BTZ treatment. Previous researches demonstrated that HO-1 is overexpressed in NB cells BTZ-resistant (Furfaro et al. 2016), although

we shown both a significantly down regulation of HO-1 and cell survival induced by LA. Concomitantly, in the same condition, we observed a nuclear localization of HO-1 using immunofluorescence assay compared to NB cells treated with BTZ alone. Nuclear localization of HO-1 has been demonstrated in different situations (Ghobrial et al. 2011; Li Volti et al. 2004) and may serve to upregulate cytoprotective genes against oxidative stress (Lin et al. 2007). In this regard, it has previously shown that nuclear HO-1 is able to protect leukemic cells from drug-induced toxicity and that could be implicated as a regulator of DNA repair activities (Gandini et al. 2012; Tibullo et al. 2013). Thus we supposed that LA induces cytoprotection in NB cell lines promoting intracellular HO-1 compartmentalization rather enzymatic activity. Some researchers (Biswas et al. 2014) showed that nuclear HO-1 modulates the activation of Nrf2, leading to an adaptive reprogramming that enhances antioxidant defenses. To confirm the pivotal role of LA as chemical chaperone and its antioxidant properties, we treated NB cells with Thapsigargin that was able to induce all ER-stress proteins and HO-1 and this effect was reversed by addition of LA (data not shown). Recent studies have been revealed that autophagy plays a critical role in cell death decisions, and autophagy induction is correlated to apoptosis resistance (Maiuri et al. 2007; Wu et al. 2015). Effective autophagy may reduce cell death by inhibiting apoptosis (Degenhardt et al. 2006; O'Donovan et al. 2011), whereas inhibition of autophagy may promote cell death by potentiating apoptosis (Liu et al. 2011; Zhou et al. 2014). Our results demonstrated that LA-induced autophagy of NB cells mediates the cell death resistance to BTZ.

In conclusion, the mechanisms of cytoprotection of LA against NB cells treated with BTZ seem to be complex. Our hypothesis is that antioxidant properties of LA under our experimental conditions is not due to upregulation or enzymatic activity of HO-1 in response to stress induction by BTZ rather its nuclear localization. Recently, some interesting researches (Tibullo et al. 2016) demonstrated that protective effect of HO-1 on drug-induced cytotoxicity in leukemic and myeloma cells does not involve its enzymatic byproducts, but rather its nuclear translocation following proteolytic cleavage. Furthermore, they shown that HO-1 upregulation is dependent on ER-stress

suggesting a link between oxidative stress and UPR. Whether nuclear HO-1 can regulate the transcription of genes implicated in drug-resistance awaits further investigations. All these data demonstrated that LA protects NB cells by stress and damage induced by BTZ since reduces ER-stress and activates autophagy as mechanism of cell survival. This work confirms the neuroprotective effects of LA in neurological field and suggests that it should not use in treatment of neuroblastoma disease, since reduces both redox escalation and cellular damage induced by bortezomib.

## CHAPTER 6

### CONCLUDING REMARKS

Redox reactions belong to the major metabolic pathways of the cells. The shift of the balance between pro- and antioxidants can account for mechanisms implicated in pathogenesis and/or progression of many, apparently unconnected pathological states, like tumors, neurodegenerative diseases, diabetes, etc. This underlines the importance of antioxidants in the therapy of these diseases.

In addition, as mentioned before, LA acts as antioxidant to directly scavenge ROS and RNOS, chelate transition and heavy metal ions and mediate the recycling of other endogenous antioxidants as well as glutathione. LA also modulates various signaling cascades either by receptor-mediated or non-receptor mediated processes.

Therefore, the beneficial properties of LA should not be doubted. However, further studies in the specific disease model systems are required to set proper dosage regimen as well as its involvement in cell growth and differentiation processes.

### FUTURE PERSPECTIVES

Because LA performs a pleiotropic action on different pathways linked to the above mentioned diseases, its use as a potential therapeutic agent is very interesting and hopeful.

The activity of LA as a therapeutic agent is corroborated not only for its actions as scavenger of ROS/RNS, but also for its capability to influence signaling cascades. The stimulation of signal transduction molecules containing critical cysteine residues may thus prove to be clinically useful and should be examined in detail in the next future.

One more significant remark for future researches is the differential outcome of LA administered in vitro and in vivo. There is still much to learn regarding to the metabolic destiny of LA and the

effects of the various metabolites on the cells. In fact, it is obvious that LA is quickly removed from plasma and tissues, whereas (DH)LA may persist within cell culture media for long time. This underlines the importance of using in vivo models in order to confirm the data obtained in vitro.

Many preclinical efforts on LA should be conducted keeping in mind these considerations. Finally, these studies had the objective to evaluate the effects induced by treatments with cholinergic precursors and Lipoic acid on proliferation and differentiation mechanisms, as well as the antioxidant and protective effect, in our experimental models, in order to better clarify their complex role played in several biochemical processes.

Finally, we still need more knowledges to better characterize in deep about these interesting compounds in preclinical phase, before to offer and extend its use in routine clinical practice.



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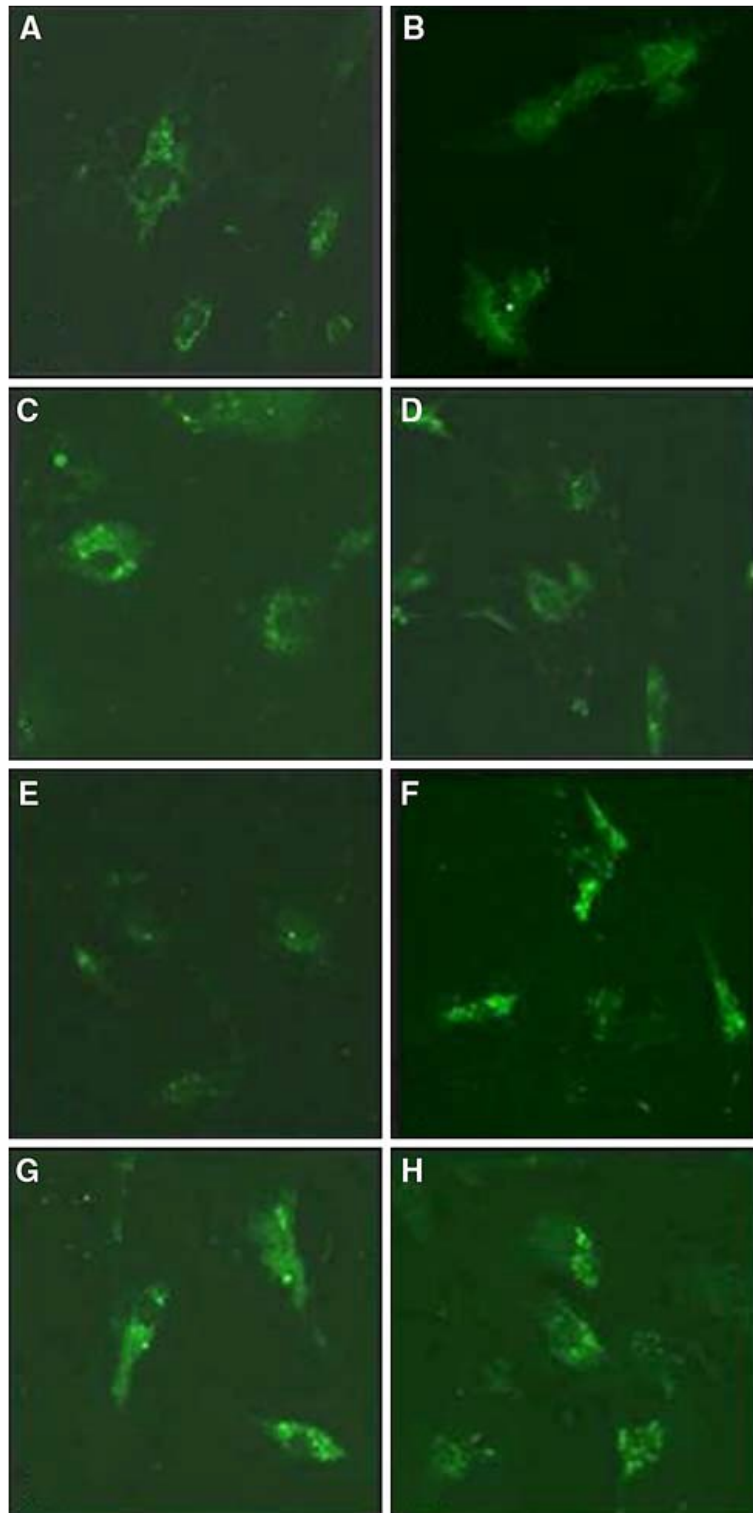
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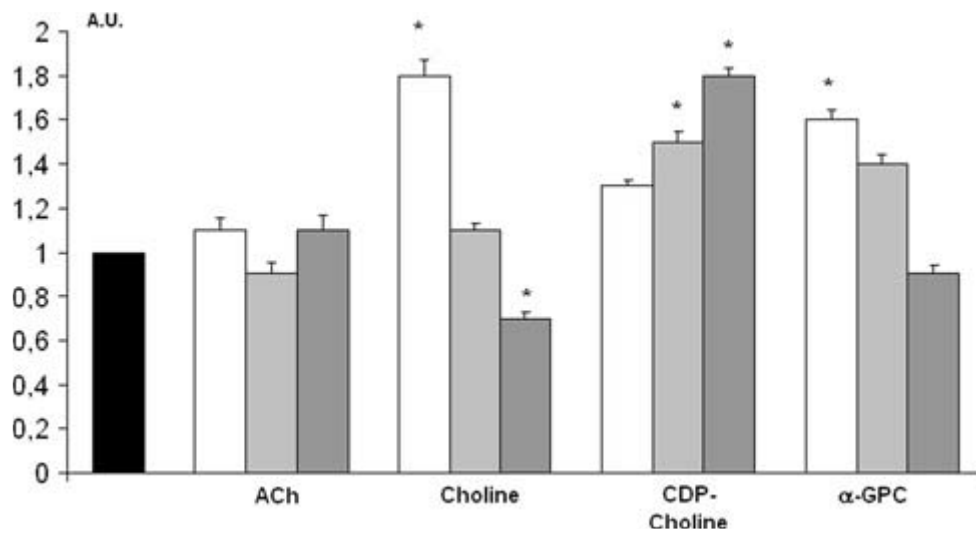
Zwain IH, Yen SS, Cheng CY. 1997. Astrocytes cultured in vitro produce estradiol-17beta and express aromatase cytochrome P-450 (P-450 AROM) mRNA. *Biochim Biophys Acta* 1334(2-3):338-348.



**Fig. 1**

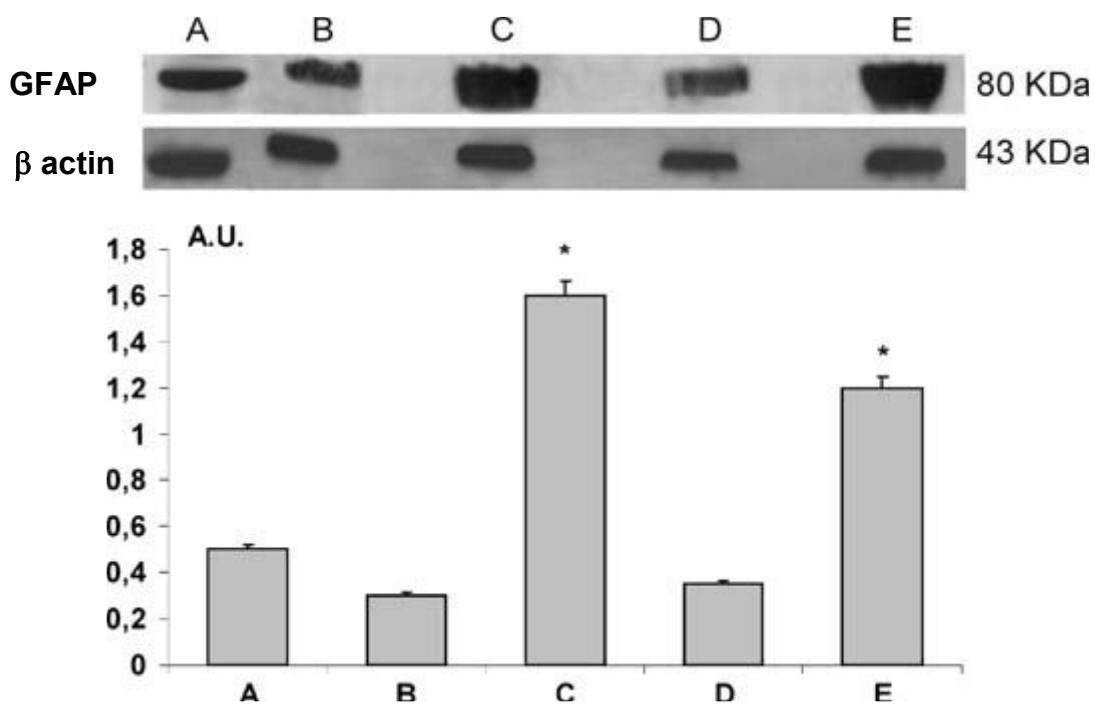
Confocal laser scanning microscopy (CLSM) analysis by DC cytofluorescence incorporated into living astrocytes cultures at 14 DIV untreated (A) or treated for 24 h with ACh (B); choline chloride (C, D), CDP-choline (e, f),  $\alpha$ -GPC (G, H) and at concentration of 0.1  $\mu$ M (C, E, G) and 1  $\mu$ M (B, D, F, H)





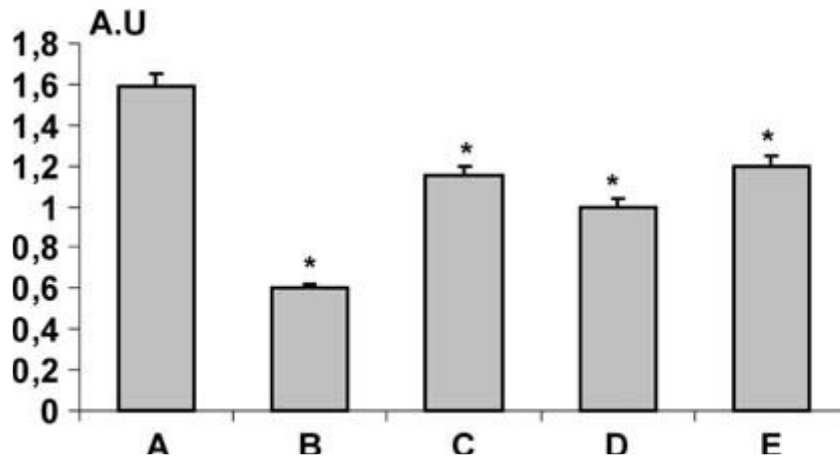
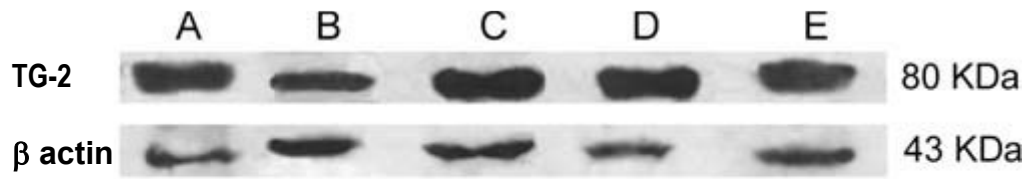
**Fig. 2**

Quantitative analysis of confocal laser scanning microscopy (CLSM) of living astrocytes cultures at 14 DIV. The data represent the optical density of fluorescence per cell area normalized for the intensity of cytofluorescence of control cells considered to correspond to “one”. Black bar: Control; White bar: concentration of 0.1 μM; light gray: concentration of 1 μM; dark gray: concentration of 10 μM. \*p < 0.05 vs. control cultures



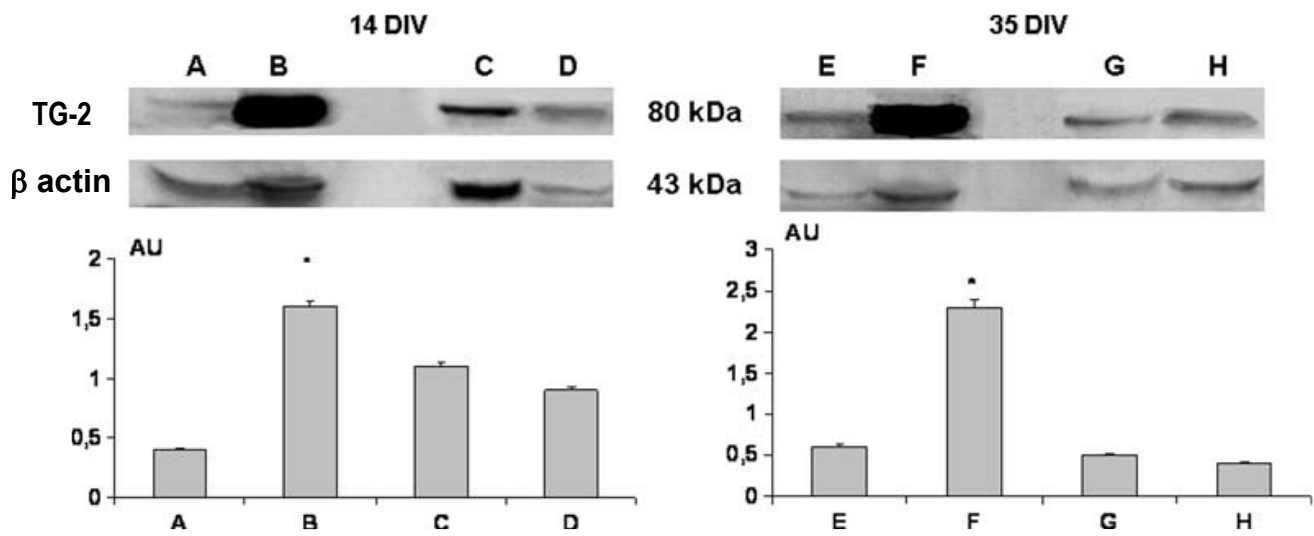
**Fig. 3**

Western Blotting analysis of TG-2 expression in astrocytes control cultures at 14 DIV (A), treated for 24 h with acetylcholine (B) and cholinergic precursors (C: choline; D: CDP-choline; E:  $\alpha$ -GPC) at the concentration of 1  $\mu$ M. Each value of densitometric analysis is in A.U. (arbitrary units) and represents the means  $\pm$  standard error of the mean (SEM) of values obtained from five different dishes. \*  $p < 0.05$  vs. control cultures



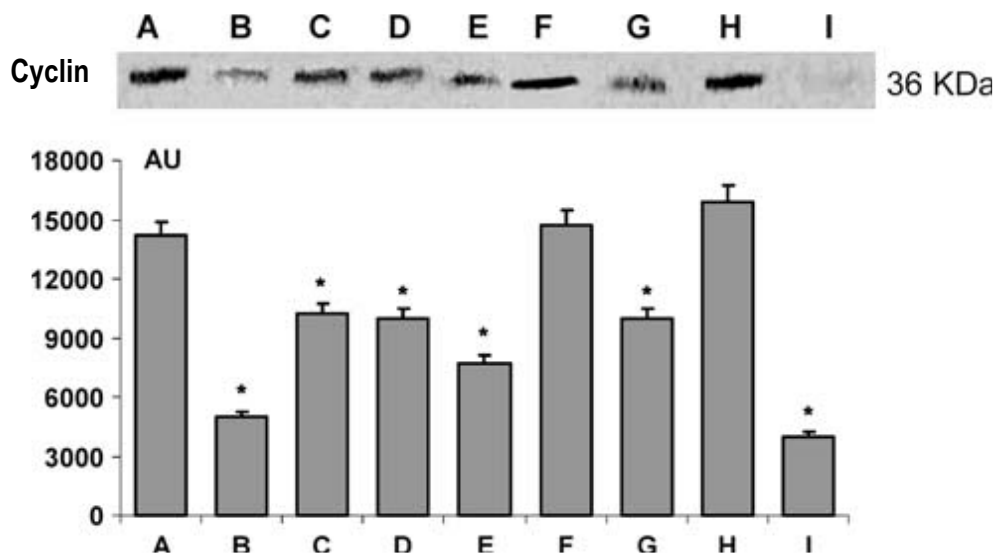
**Fig. 4**

Western Blotting analysis of TG-2 expression in astrocytes at 21 DIV (A) treated for 24 h with acetylcholine (B) and choline precursors (C: choline; D: CDP-choline; E:  $\alpha$ -GPC) at the concentration of 1  $\mu$ M. Each value expressed as A.U. (arbitrary units) is the average  $\pm$  standard error of the mean (SEM) of values from five different dishes. \* $p < 0.05$  vs. control cultures



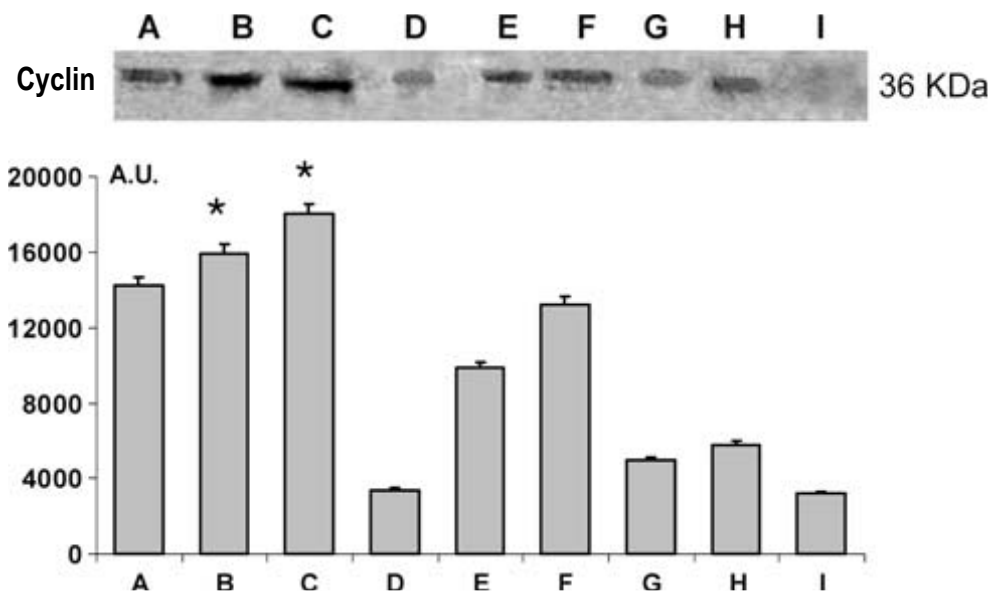
**Fig. 5**

Western Blotting analysis of TG-2 expression in astrocytes cultures at 14 DIV (left panel) treated for 24 h with acetylcholine (C) and  $\alpha$ -GPC (D) compared to 35 DIV cell cultures (right panel) treated for 24 h with acetylcholine (G) and  $\alpha$ -GPC (H). Astrocytes cultures maintained in the presence of BSA at 14 DIV (A) or 35 DIV (E) were considered as untreated controls cultures in contrast with culture maintained in the presence of 20% of fetal calf serum at 14 DIV (B) or 35 DIV (F). Each value expressed as A.U. (arbitrary units) is the average  $\pm$  standard error of the mean (SEM) of values from five different dishes. \* $p < 0.05$  vs. control cultures



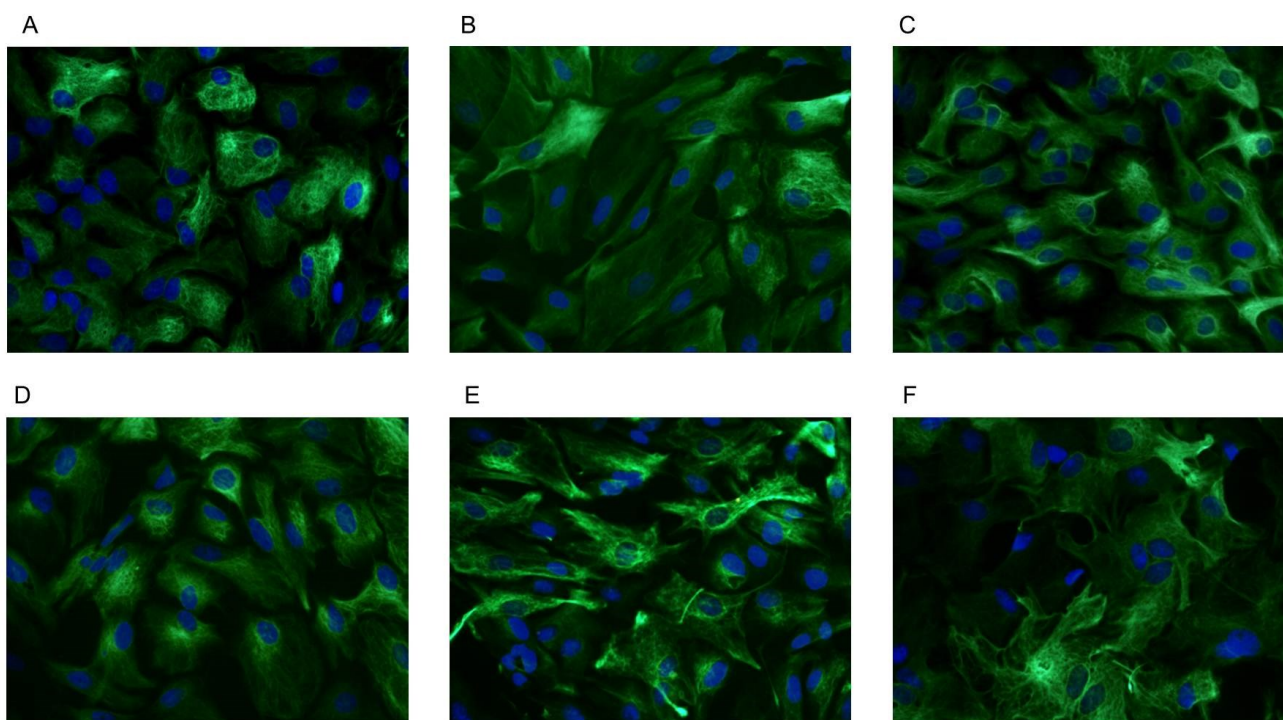
**Fig. 6**

Western Blotting analysis of cyclin D1 expression in astrocytes control cultures at 14 DIV (A) or treated for 24h or 48h with choline precursors at the concentration of 1  $\mu$ M. B: 24h treatment with ACh; C: 24h treatment with choline chloride; D: 24h treatment with CDP-choline; E: 24h treatment with  $\alpha$ -GPC; F: 48 h treatment with ACh; G: 48h treatment with choline chloride; H: 48h treatment with CDP-choline; I: 48h treatment with  $\alpha$ -GPC. Each value expressed as A.U. (arbitrary units) is the average  $\pm$  standard error of the mean (SEM) of values from five different dishes



**Fig. 7**

Western Blotting analysis of cyclin D1 expression in astrocytes control cultures at 35 DIV (A) or treated for 24 h with acetylcholine and choline precursors at the concentration of 0.1 and 1 μM. B: Treatment with ACh at concentration of 0.1 μM; C: treatment with ACh at concentration of 1 μM; D: treatment with choline chloride at concentration of 0.1 μM; E: treatment with choline chloride at concentration of 1 μM; F: treatment with CDPcholine at concentration of 0.1 μM; G: treatment with CDP-choline at concentration of 1 μM; H: treatment with αGPC at concentration of 0.1 μM; I: treatment with αGPC at concentration of 1 μM. Each value expressed as A.U. (arbitrary units) is the average ± standard error of the mean (SEM) of values from five different dishes



40X

**FIGURES LEGEND**

**A : Control**

**B : (+)lipoic acid**

**C : (+/-)lipoic acid**

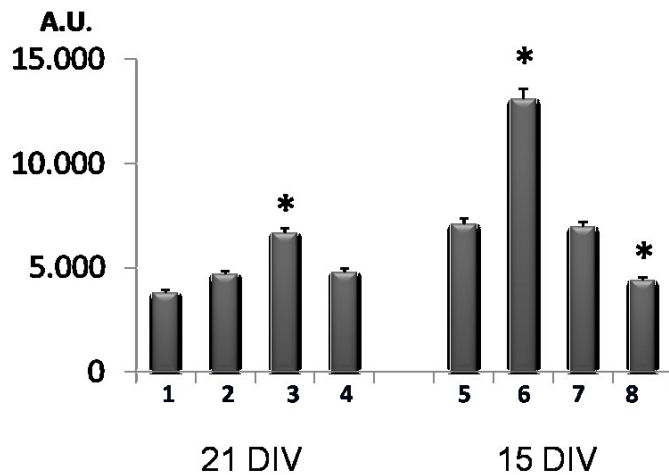
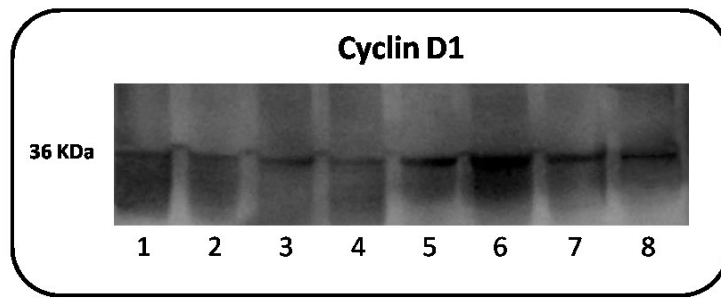
**D :  $\alpha$ -GPC**

**E : (+)lipoic acid +  $\alpha$ -GPC**

**F : (+/-)lipoic acid +  $\alpha$ -GPC**

**Fig. 8**

**Figure 8:** Immunocytochemical analysis for GFAP after (+)lipoic acid treatment (C),  $\alpha$ -GPC treatment (D), (+)lipoic acid+ $\alpha$ -GPC (E) and  $\alpha$ GPC treatment (F) in astrocyte cultures. Data show that all of the treatments above mentioned astroglial marker.



#### FIGURES LEGEND

1. CTRL
2. (+)lipoic acid
3.  $\alpha$ -GPC
4. (+)lipoic acid +  $\alpha$ -GPC

**Fig. 9**

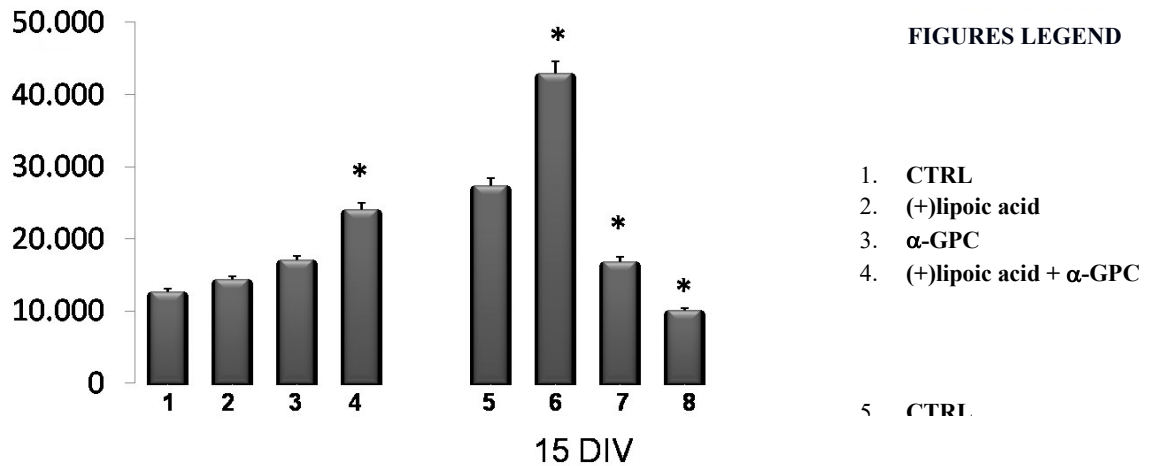
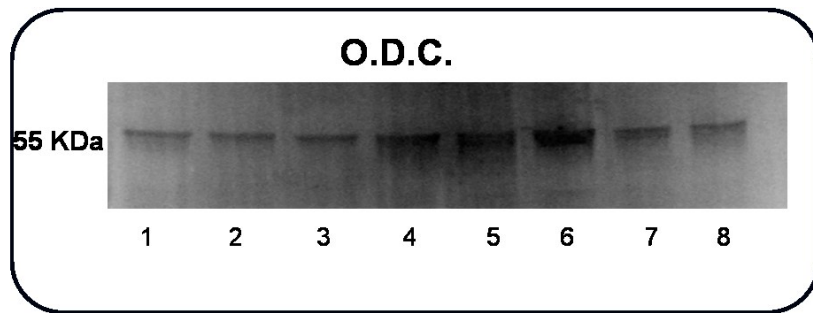
**Figure 9:** Western blotting analysis data for Cyclin D1 expression:

Treatment in astrocyte cultures at 21 DIV: Control (1), after (+/-)lipoic acid treatment (2),  $\alpha$ GPC treatment (3), (+/-)lipoic acid +  $\alpha$ GPC treatment (4);

Treatment in astrocyte cultures at 15 DIV: Control (5), after (+)lipoic acid treatment (6),  $\alpha$ GPC treatment (7), (+)lipoic acid+ $\alpha$ GPC (8)

Each value expressed as A.U. (arbitrary units) is the average  $\pm$  standard error of the mean (S.E.M.) of values from five different dishes. Statistical significance was expressed at p values of  $<0.05$





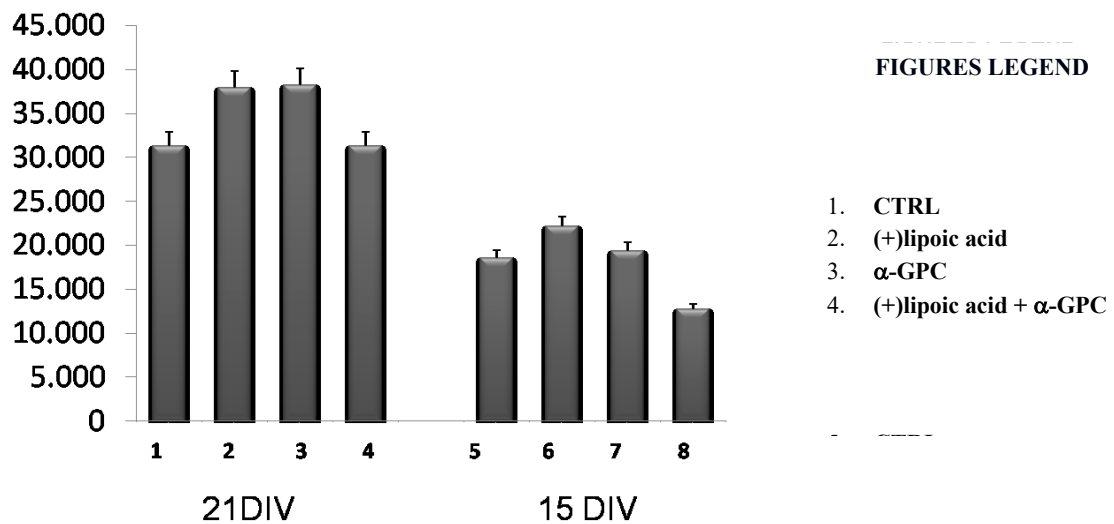
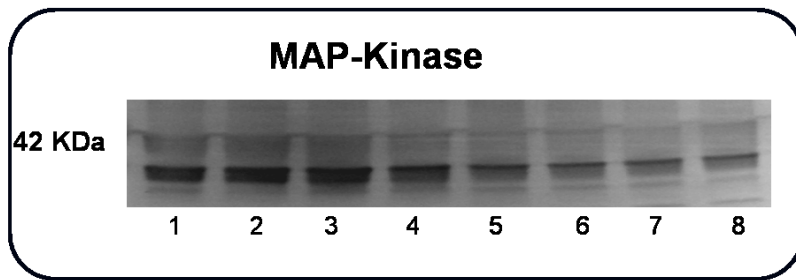
**Fig. 10**

**Figure 10:** Western blotting analysis data for ornithine decarboxylase (O.D.C.) expression:

Treatment in astrocyte cultures at 21 DIV: Control (1), after (+/-)lipoic acid treatment (2), αGPC treatment (3), (+/-)lipoic acid + αGPC treatment (4);

Treatment in astrocyte cultures at 15 DIV: Control (5), after (+)lipoic acid treatment (6), αGPC treatment (7), (+)lipoic acid + αGPC (8)

Each value expressed as A.U. (arbitrary units) is the average ± standard error of the mean (S.E.M.) of values from five different dishes. Statistical significance was expressed at p values of <0.05



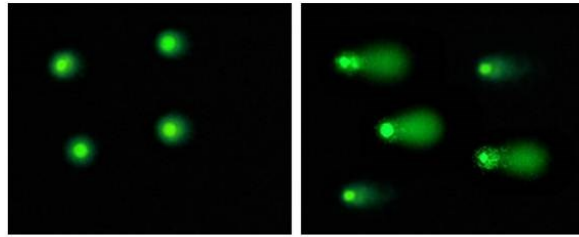
**Fig. 11**

**Figure 11:** Western blotting analysis data for MAP-kinase expression:

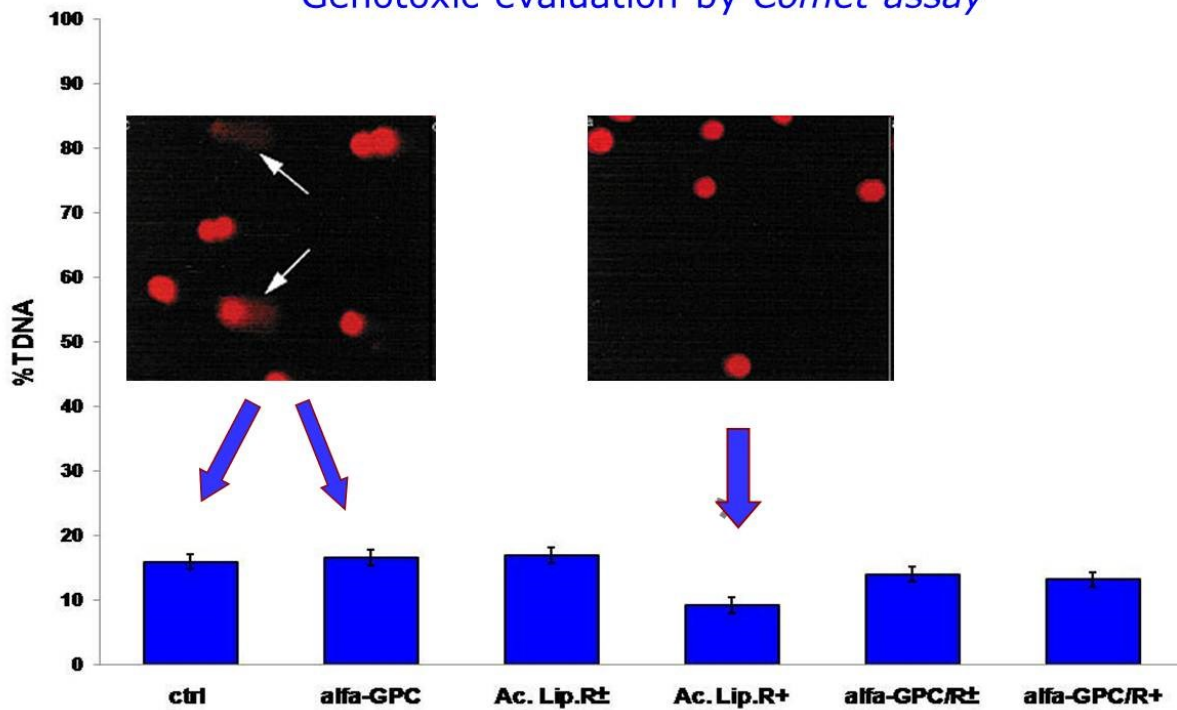
Treatment in astrocyte cultures at 21 DIV: Control (1), after (+/-)lipoic acid treatment (2),  $\alpha$ GPC treatment (3), (+/-)lipoic acid +  $\alpha$ GPC treatment (4);

Treatment in astrocyte cultures at 15 DIV: Control (5), after (+)lipoic acid treatment (6),  $\alpha$ GPC treatment (7), (+)lipoic acid +  $\alpha$ GPC (8)

Each value expressed as A.U. (arbitrary units) is the average  $\pm$  standard error of the mean (S.E.M.) of values from five different dishes. Statistical significance was expressed at p values of  $<0.05$

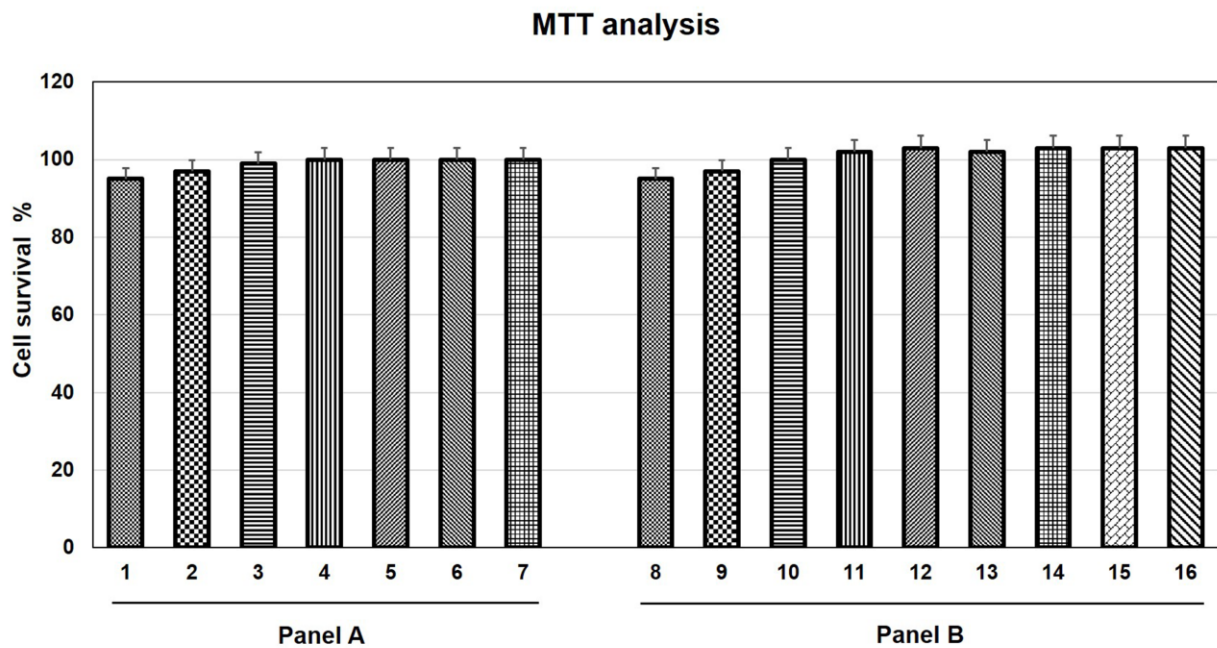


Genotoxic evaluation by *Comet assay*



**Fig. 12**

**Figure 12:** Genotoxic evaluation by Comet Assay: The analysis of DNA status by Alkaline Comet assay showed any significant modifications induced by the treatment with (+)lipoic acid resembling rather a possible genoprotective effect.



**Fig. 13**

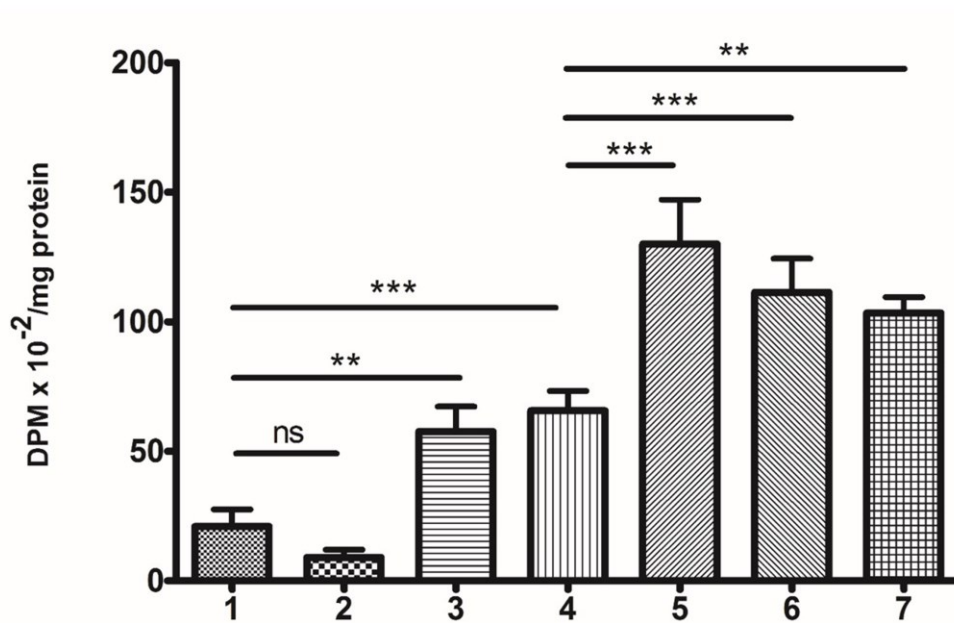
**Fig. 13: Determination of Cell Viability of 24 DIV astrocyte cultures**

MTT analysis for cell viability evaluation of astroglial cells in culture at 24 DIV, pretreated with bFGF 24h or/and E<sub>2</sub> 36h and subsequently treated in the last 12/24h with “progression” growth factors. Treatments Panel A: 1. Control; 2. E<sub>2</sub> 36h; 3. bFGF 24h; 4. bFGF 24h / E<sub>2</sub> 36h; 5. bFGF 24h / E<sub>2</sub> 36h + EGF (last 12h); 6. bFGF 24h / E<sub>2</sub> 36h + IGF-I (last 12h); 7. bFGF 24h / E<sub>2</sub> 36h + INS (last 12h). Treatments Panel B: 8. Control; 9. E<sub>2</sub> 36h; 10. E<sub>2</sub> 36h + EGF+bFGF (last 24h); 11. E<sub>2</sub> 36h + EGF+IGF-I (last 24h); 12. E<sub>2</sub> 36h + EGF+INS (last 24h); 13. E<sub>2</sub> 36h + bFGF+IGF-I (last 24h); 14. E<sub>2</sub> 36h + bFGF+INS (last 24h); 15. E<sub>2</sub> 36h + EGF+bFGF+IGF-I (last 24h); 16. E<sub>2</sub> 36h + EGF+bFGF+INS (last 24h).

Statistical analysis were performed with Graphpad Prism, data analysis software, La Jolla, CA, USA; RRID: rid\_000081). The data are expressed as mean ± SEM .

## DNA labeling in astrocyte cultures at 24 DIV

pretreated with E<sub>2</sub> or/and bFGF and than treated with “progression” growth factors in the last 12h

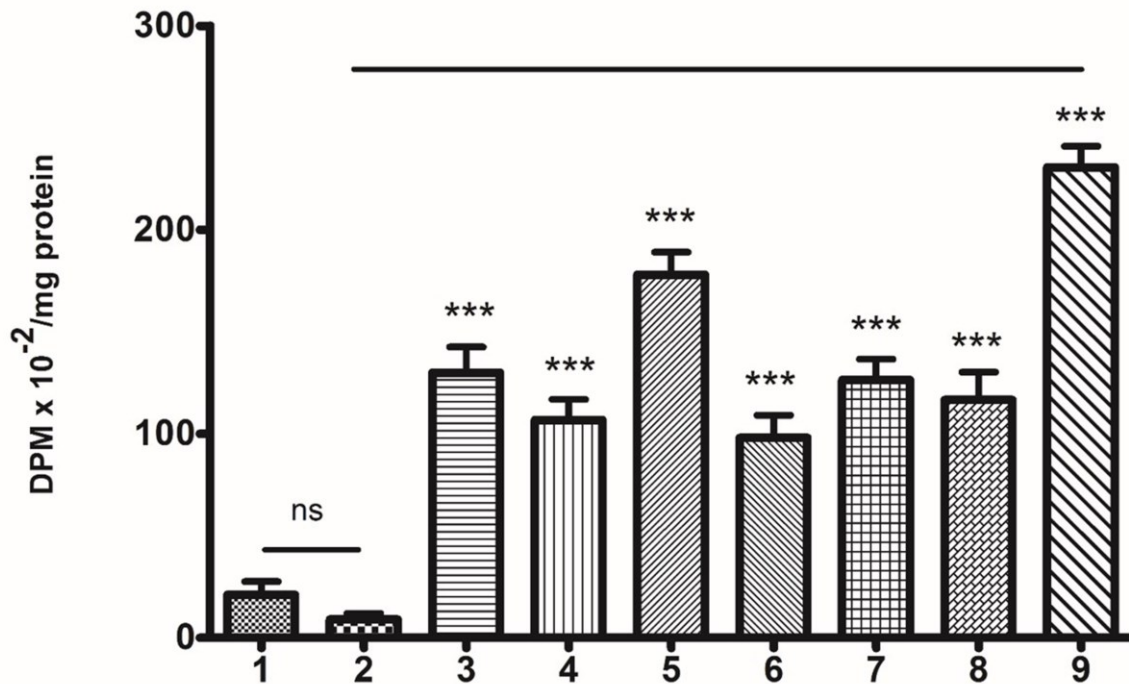


**Fig. 14**

**Fig. 14: DNA labeling analysis in astrocyte cultures at 24 DIV** pretreated with bFGF 24h or/and E<sub>2</sub> 36h and than treated in with “progression” growth factors, as follow: 1. Control; 2. E<sub>2</sub> 36h; 3. bFGF 24h; 4. bFGF 24h / E<sub>2</sub> 36h; 5. bFGF 24h / E<sub>2</sub> 36h + EGF (last 12h); 6. bFGF 24h / E<sub>2</sub> 36h + IGF-I (last 12h); 7. bFGF 24h / E<sub>2</sub> 36h + INS (last 12h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.

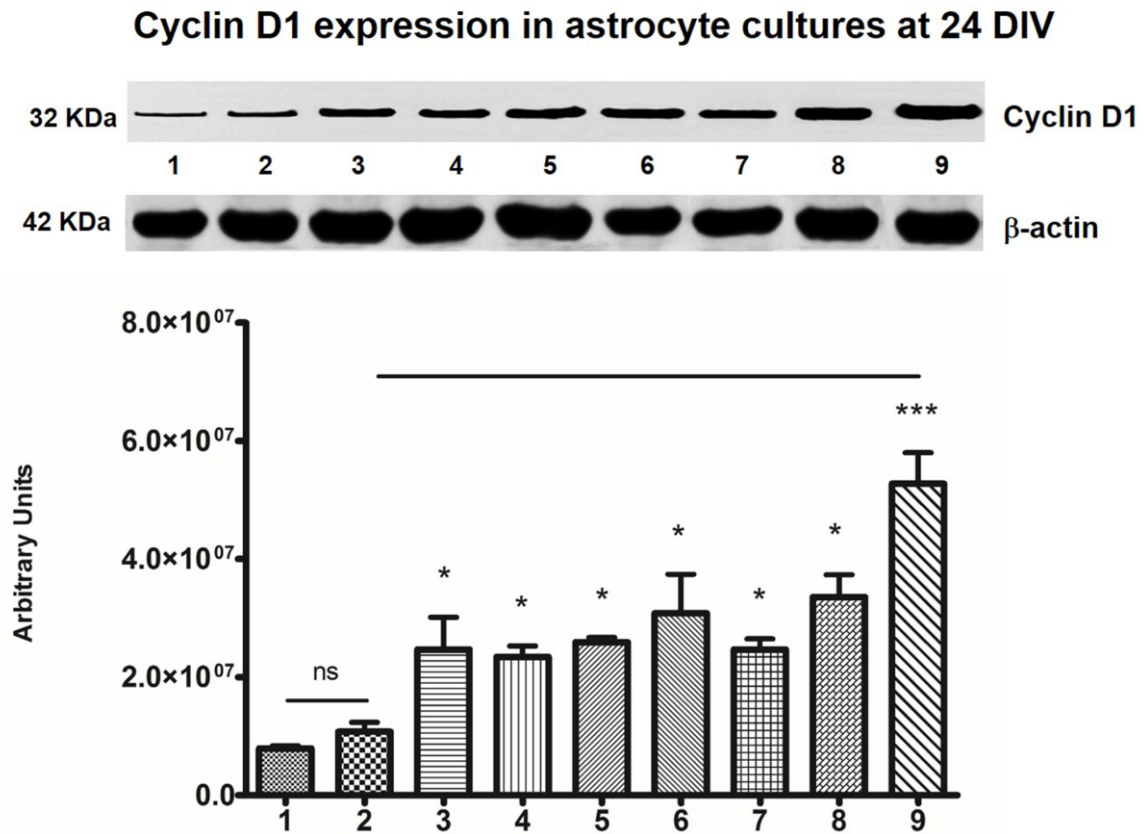
## DNA labeling in astrocyte cultures at 24 DIV

pretreated with E<sub>2</sub> and then treated with “progression” growth factors in the last 24h



**Fig. 15**

**Fig. 15: DNA labeling analysis in astrocyte cultures at 24 DIV** pretreated with estradiol 36h and then treated with “competence and progression” growth factors, as follow: 1. Control; 2. E<sub>2</sub> 36h; 3. E<sub>2</sub> 36h + EGF+bFGF (last 24h); 4. E<sub>2</sub> 36h + EGF+IGF-I (last 24h); 5. E<sub>2</sub> 36h + EGF+INS (last 24h); 6. E<sub>2</sub> 36h + bFGF+IGF-I (last 24h); 7. E<sub>2</sub> 36h + bFGF+INS (last 24h); 8. E<sub>2</sub> 36h + EGF+bFGF+IGF-I (last 24h); 9. E<sub>2</sub> 36h + EGF+bFGF+INS (last 24h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.



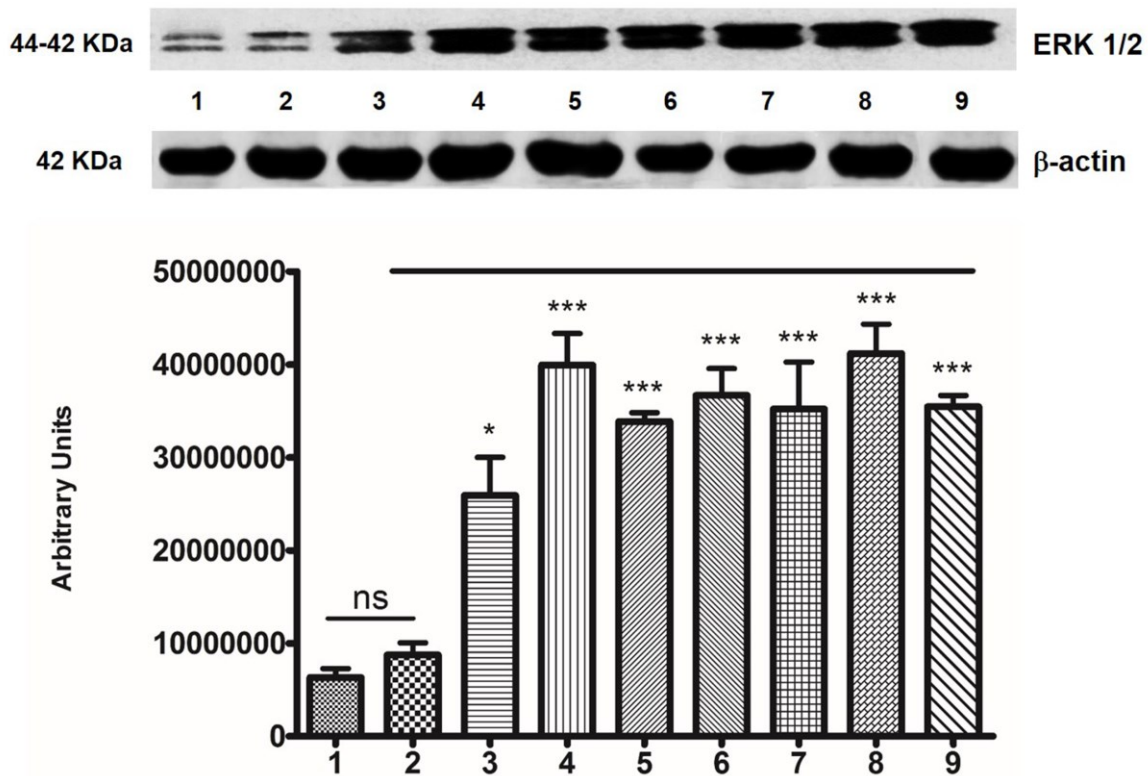
**Fig. 16**

**Fig. 16: Western blotting analysis for Cyclin D1 expression in astrocyte cultures at 24 DIV**

Effect of pretreatment of estradiol alone or added with two or three growth factors on Cyclin D1 expression. Astrocyte cultures at 22 DIV, after 24h starvation period, were treated with estradiol alone 36h or plus “competence and progression” growth factors by two or by three, as follow: 1. Control; 2. E<sub>2</sub> for 36h; 3. E<sub>2</sub> for 36h + EGF+bFGF (last 24h); 4. E<sub>2</sub> for 36h + EGF+IGF-I (last 24h); 5. E<sub>2</sub> for 36h + EGF+INS (last 24h); 6. E<sub>2</sub> for 36h + bFGF+IGF-I (last 24h); 7. E<sub>2</sub> for 36h + bFGF+INS (last 24h); 8. E<sub>2</sub> for 36h + EGF+bFGF+IGF-I (last 24h); 9. E<sub>2</sub> for 36h + EGF+bFGF+INS (last 24h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.



## ERK1/2 expression in astrocyte cultures at 24 DIV



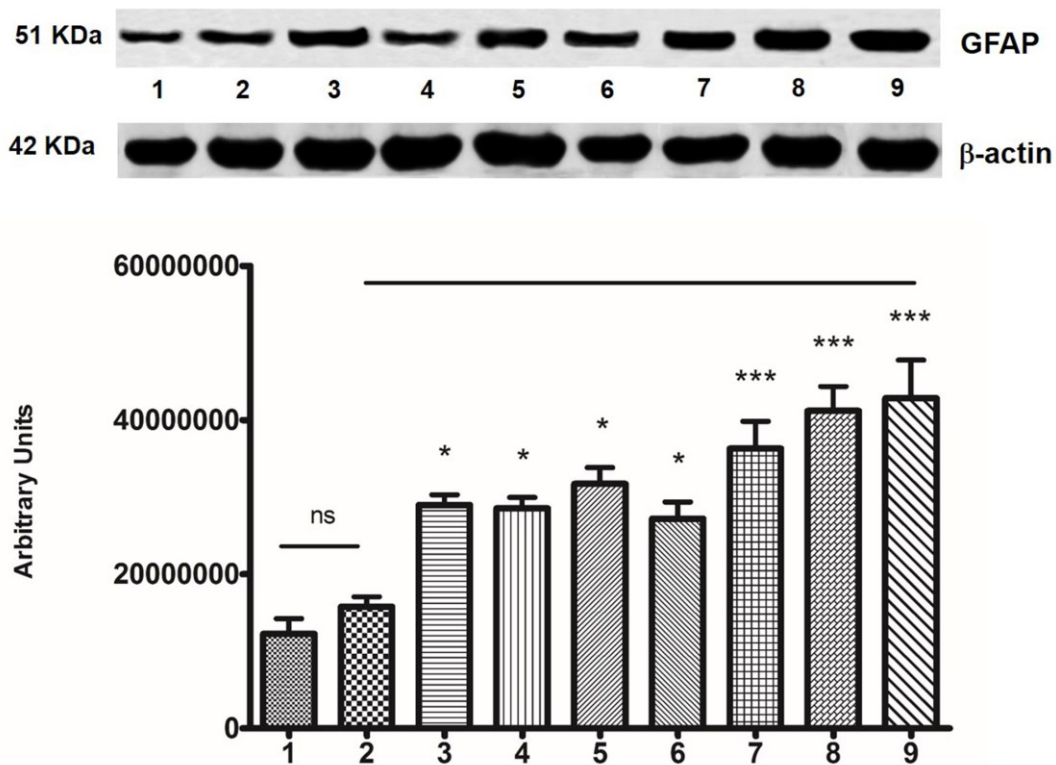
**Fig. 17**

### Fig. 17: Western blotting analysis for ERK 1/2 expression in astrocyte cultures at 24 DIV

Effect of pretreatment of estradiol alone or added with two or three growth factors on ERK1/2 expression. Astrocyte cultures at 22 DIV, after 24h starvation period, were treated with estradiol alone 36h or plus “competence and progression” growth factors by two or by three, as follow: 1. Control; 2. E<sub>2</sub> for 36h; 3. E<sub>2</sub> for 36h + EGF+bFGF (last 24h); 4. E<sub>2</sub> for 36h + EGF+IGF-I (last 24h); 5. E<sub>2</sub> for 36h + EGF+INS (last 24h); 6. E<sub>2</sub> for 36h + bFGF+IGF-I (last 24h); 7. E<sub>2</sub> for 36h + bFGF+INS (last 24h); 8. E<sub>2</sub> for 36h + EGF+bFGF+IGF-I (last 24h); 9. E<sub>2</sub> for 36h + EGF+bFGF+INS (last 24h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.



## GFAP expression in astrocyte cultures at 24 DIV

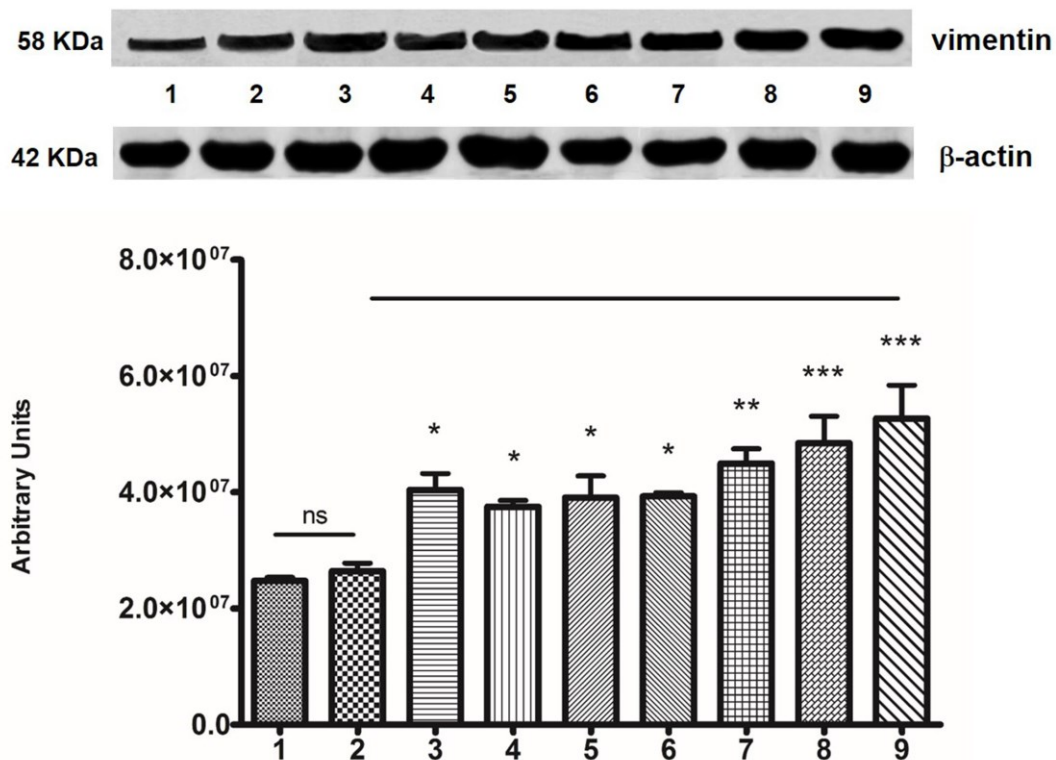


**Fig. 18**

### Fig. 18: Western blotting analysis for GFAP expression in astrocyte cultures at 24 DIV

Effect of pretreatment of estradiol alone or added with two or three growth factors on GFAP expression. Astrocyte cultures at 22 DIV, after 24h starvation period, were treated with estradiol alone 36h or plus “competence and progression” growth factors by two or by three, as follow: 1. Control; 2. E<sub>2</sub> for 36h; 3. E<sub>2</sub> for 36h + EGF+bFGF (last 24h); 4. E<sub>2</sub> for 36h + EGF+IGF-I (last 24h); 5. E<sub>2</sub> for 36h + EGF+INS (last 24h); 6. E<sub>2</sub> for 36h + bFGF+IGF-I (last 24h); 7. E<sub>2</sub> for 36h + bFGF+INS (last 24h); 8. E<sub>2</sub> for 36h + EGF+bFGF+IGF-I (last 24h); 9. E<sub>2</sub> for 36h + EGF+bFGF+INS (last 24h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.

## Vimentin expression in astrocyte cultures at 24 DIV



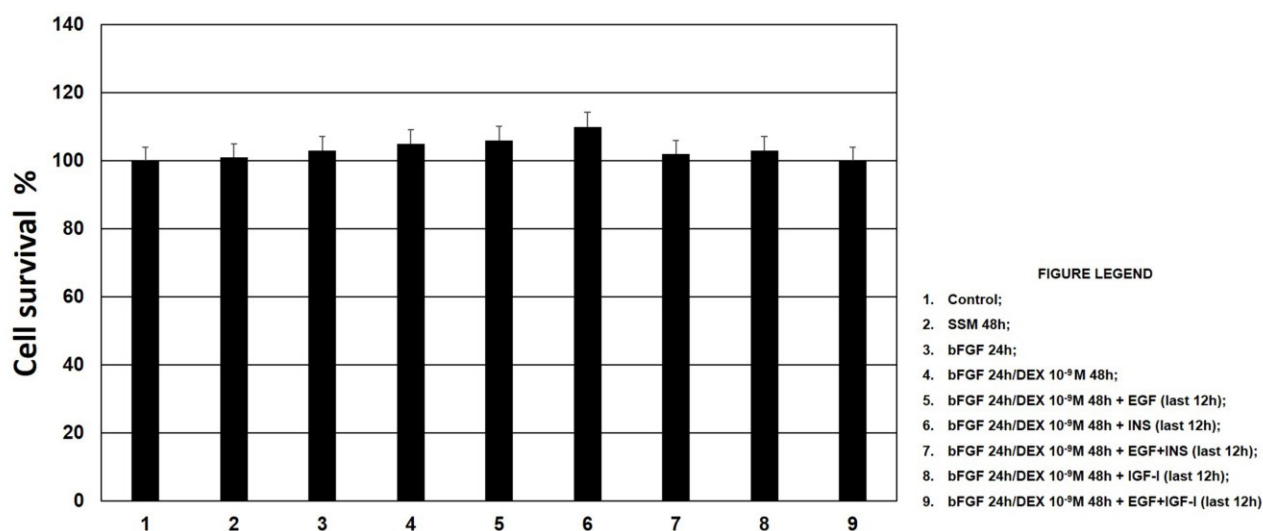
**Fig. 19**

### Fig. 19: Western blotting analysis for vimentin expression in astrocyte cultures at 24 DIV

Effect of pretreatment of estradiol alone or added with two or three growth factors on vimentin expression. Astrocyte cultures at 22 DIV, after 24h starvation period, were treated with estradiol alone 36h or plus “competence and progression” growth factors by two or by three, as follow: 1. Control; 2. E<sub>2</sub> for 36h; 3. E<sub>2</sub> for 36h + EGF+bFGF (last 24h); 4. E<sub>2</sub> for 36h + EGF+IGF-I (last 24h); 5. E<sub>2</sub> for 36h + EGF+INS (last 24h); 6. E<sub>2</sub> for 36h + bFGF+IGF-I (last 24h); 7. E<sub>2</sub> for 36h + bFGF+INS (last 24h); 8. E<sub>2</sub> for 36h + EGF+bFGF+IGF-I (last 24h); 9. E<sub>2</sub> for 36h + EGF+bFGF+INS (last 24h). Statistical analysis was carried out by Graphpad Prism data analysis software, La Jolla, CA, USA; (RRID: rid\_000081). The data are expressed as mean ± SEM of at least five independent experiments. Statistical analysis was carried out by ANOVA test to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 (\* p<0.05) was accepted as statistically significant between experimental and control groups; p-values of less than 0.01 (\*\* p<0.01) were considered much more statistically significant; p-values of less than 0.001 (\*\*\*) p<0.001) were considered highly statistically significant.



### MTT analysis of astrocyte cultures at 25 DIV

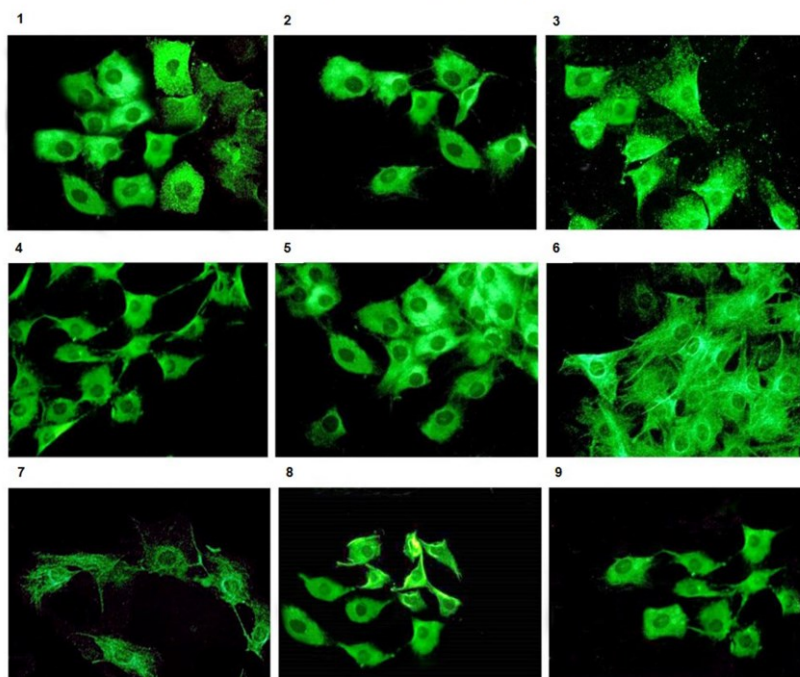


**Fig. 20**

**Fig. 20: Determination of cell viability of 25 DIV astrocyte cultures.**

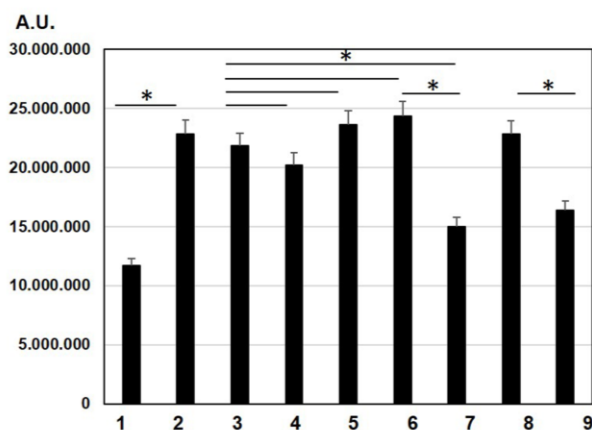
MTT analysis with tetrazolium salts for cell viability evaluation of 25 DIV astrocyte cultures pretreated with competence factor bFGF for 24 hr and subsequent treatment with DEX 10<sup>-9</sup> M for 48 hr and in the last 12 hr with progression GFs (EGF, INS, or IGF-1) alone or two of them together. 1, Control; 2, SSM 48 hr; 3, bFGF 24 hr; 4, bFGF 24 hr/DEX 48 hr; 5, bFGF 24 hr/DEX 48 hr + EGF (last 12 hr); 6, bFGF 24 hr/DEX 48 hr + INS (last 12 hr); 7, bFGF 24 hr/ DEX 48 hr + EGF+INS (last 12 hr); 8, bFGF 24 hr/DEX 48 hr + IGF-1 (last 12 hr); 9, bFGF 24 hr/DEX 48 hr + EGF+IGF-1 (last 12 hr).

### GFAP Immunocytochemical analysis at 25 DIV



Panel a

### GFAP expression in astrocyte cultures at 25 DIV

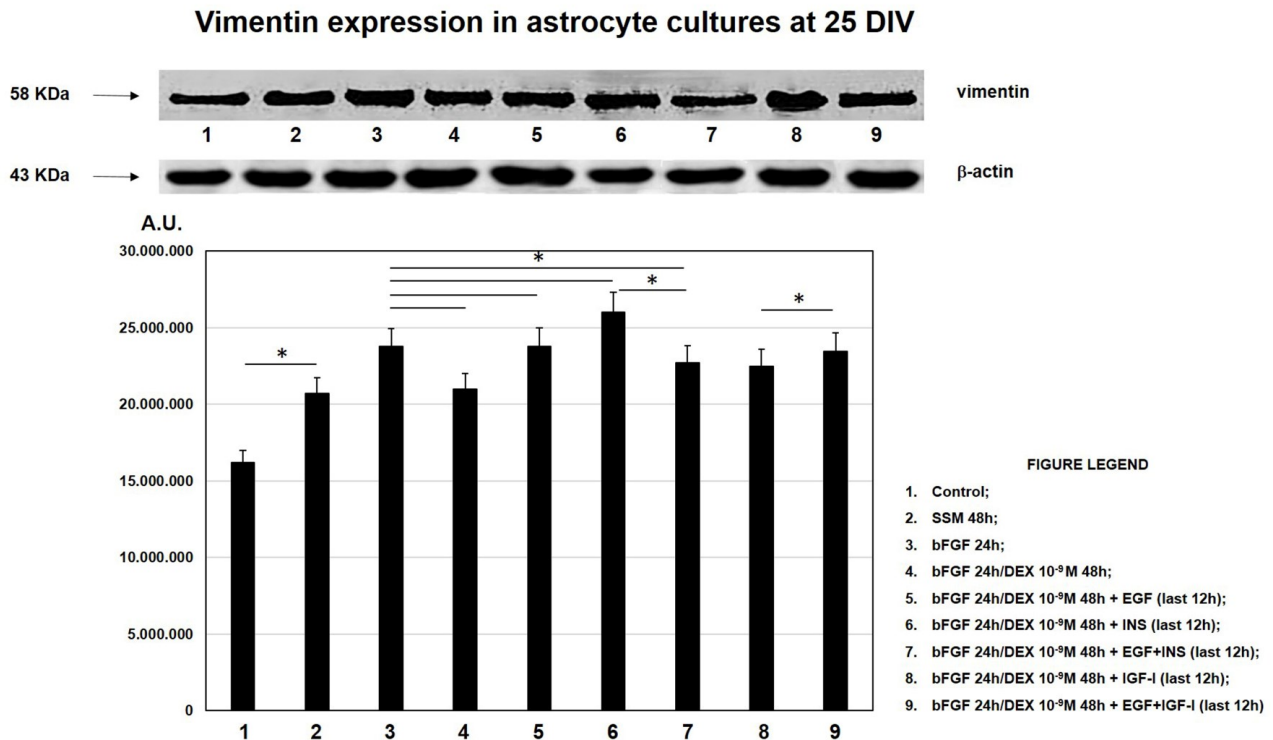


Panel b

**Fig. 21**

**A:** Immunocytochemical analysis for GFAP in astrocyte cultures at 25 DIV. Data show that all of the treatments are positive for the astroglial markers that have been discussed. **B:** Western blotting analysis for GFAP expression in astrocyte cultures at 25 DIV. Western blotting data for GFAP expression in astrocyte cultures at 25 DIV pretreated with

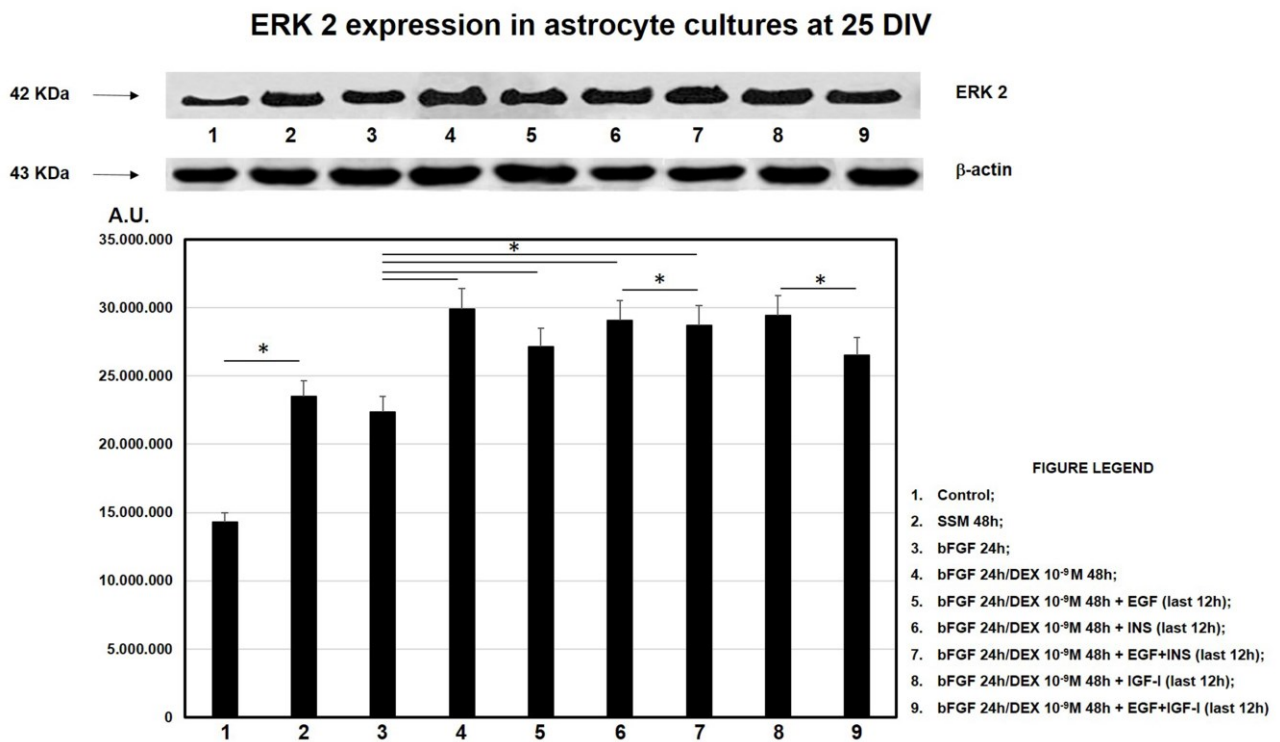
competence factor bFGF for 24 hr and subsequent treatment with DEX  $10^{-9}$  M for 48 hr and in the last 12 hr with progression GFs (EGF, INS, or IGF-1) alone or two of them together. 1, Control; 2, SSM 48 hr; 3, bFGF 24 hr; 4, bFGF 24 hr/DEX 48 hr; 5, bFGF 24 hr/DEX 48 hr + EGF (last 12 hr); 6, bFGF 24 hr/DEX 48 hr + INS (last 12 hr); 7, bFGF 24 hr/DEX 48 hr + EGF + INS (last 12 hr); 8, bFGF 24 hr/DEX 48 hr + IGF-1 (last 12 hr); 9, bFGF 24 hr/DEX 48 hr + EGF+IGF-1 (last 12 hr). Data are mean  $\pm$  SEM. Statistical analysis was carried out by ANOVA.  $P < 0.05$  was accepted as statistically significant between experimental and control groups.



**Fig. 22**

**Fig. 22: Western blotting analysis for vimentin expression in astrocyte cultures at 25 DIV.**

Western blotting data for vimentin expression in astrocyte cultures at 25 DIV pretreated with competence factor bFGF for 24 hr and subsequent treatment with DEX  $10^{-9}$  M for 48 hr and in the last 12 hr with progression GFs (EGF, INS, or IGF-1) alone or two of them together. 1, Control; 2, SSM 48 hr; 3, bFGF 24 hr; 4, bFGF 24 hr/DEX 48 hr; 5, bFGF 24 hr/DEX 48 hr + EGF (last 12 hr); 6, bFGF 24 hr/DEX 48 hr + INS (last 12 hr); 7, bFGF 24 hr/DEX 48 hr + EGF + INS (last 12 hr); 8, bFGF 24 hr/DEX 48 hr + IGF-1 (last 12 hr); 9, bFGF 24 hr/DEX 48 hr + EGF + IGF-1 (last 12 hr). Data are mean  $\pm$  SEM. Statistical analysis was carried out by ANOVA.  $P < 0.05$  was accepted as statistically significant between experimental and control groups.



**Fig. 23**

**Fig. 23: Western blotting analysis for ERK2 expression in astrocyte cultures at 25 DIV.**

Western blotting data for ERK2 expression in astrocyte cultures at 25 DIV pretreated with competence factor bFGF for 24 hr and subsequent treatment with DEX 10<sup>-9</sup> M for 48 hr and in the last 12 hr with progression GFs (EGF, INS, or IGF-1) alone or two of them together. 1, Control; 2, SSM 48 hr; 3, bFGF 24 hr; 4, bFGF 24 hr/DEX 48 hr; 5, bFGF 24 hr/DEX 48 hr + EGF (last 12 hr); 6, bFGF 24 hr/DEX 48 hr + INS (last 12 hr); 7, bFGF 24 hr/ DEX 48 hr + EGF + INS (last 12 hr); 8, bFGF 24 hr/DEX 48 hr + IGF-1 (last 12 hr); 9, bFGF 24 hr/DEX 48 hr + EGF + IGF-1 (last 12 hr). Data are mean ± SEM. Statistical analysis was carried out by ANOVA. P < 0.05 was accepted as statistically significant between experimental and control groups.

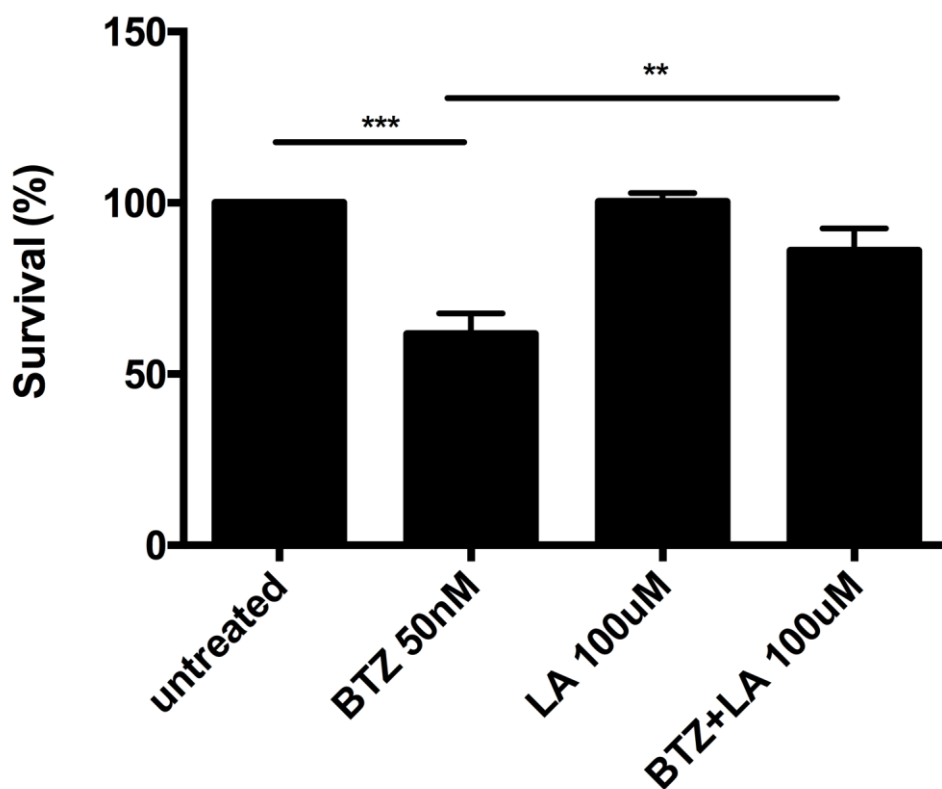
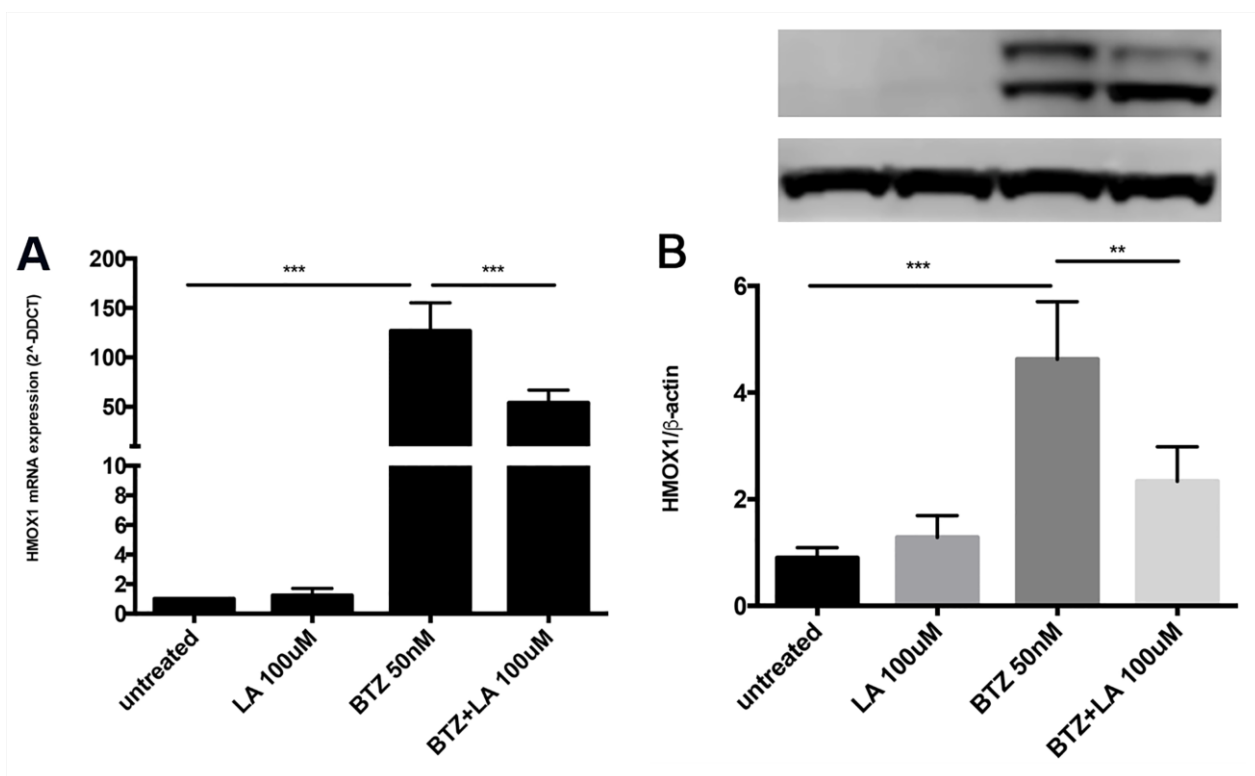


Fig. 24

Fig. 24: Determination of cell viability of NB cell lines

MTT analysis with tetrazolium salts for cell viability evaluation of NB cell lines untreated, treated with BTZ (50nM) alone; treated with LA (100  $\mu$ M) alone and treated with BTZ+LA in combination.

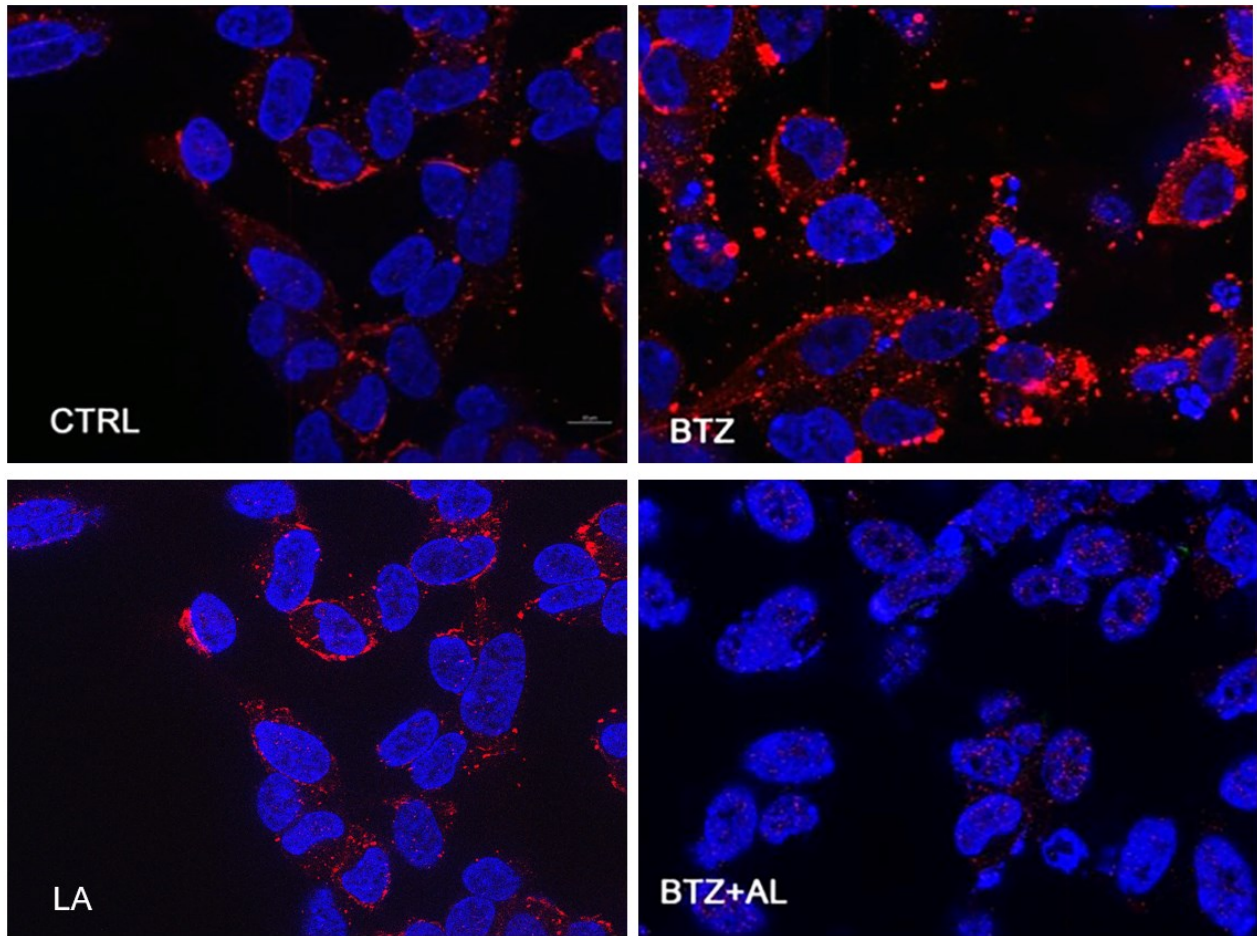




**Fig. 25**

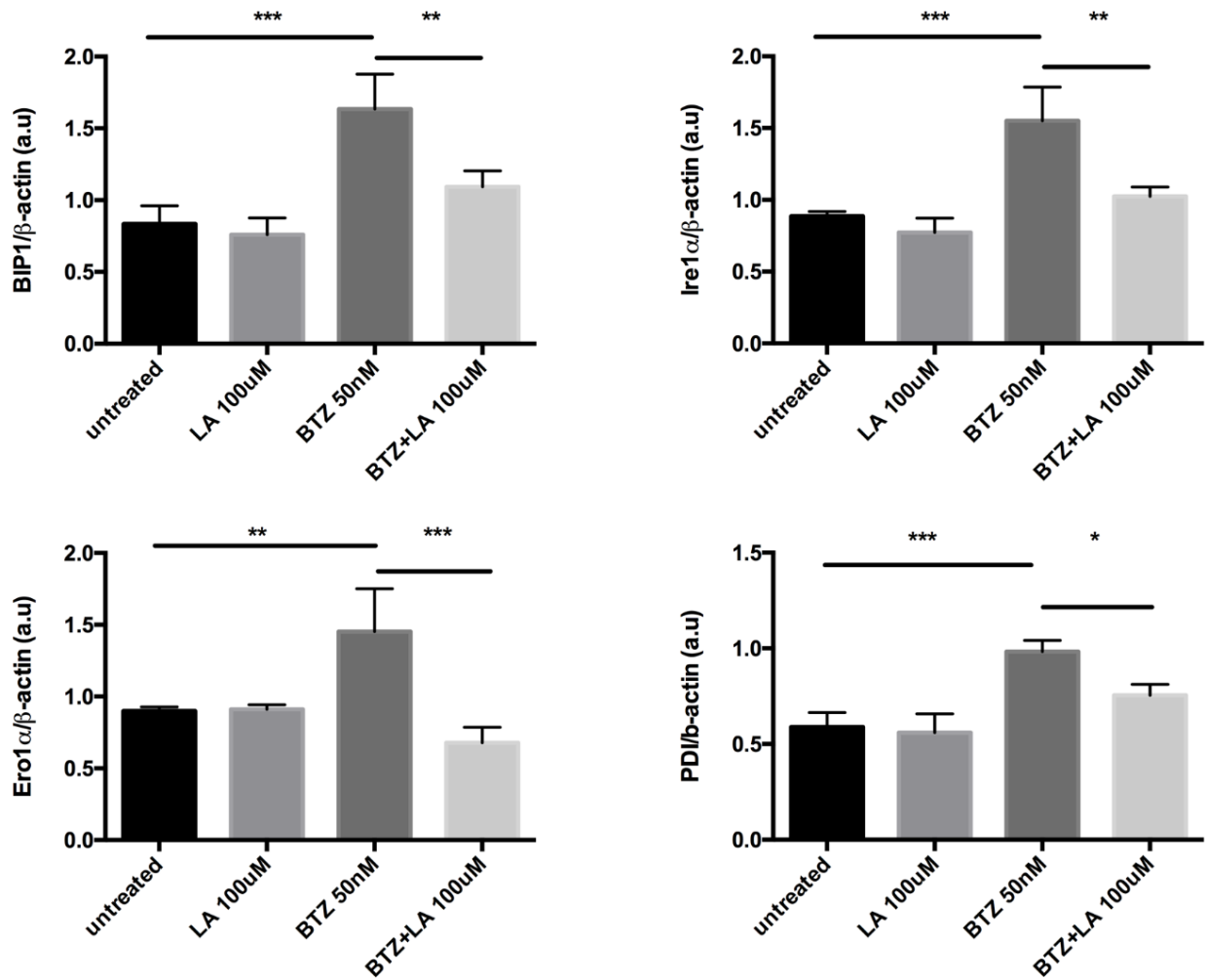
**Fig. 25 A:** Evaluation of HMOX-1 gene expression after treatment with LA (100 uM) alone; with BTZ (50 nM) alone and with BTZ+LA in combination, in NB cell lines.

**Fig. 25 B:** Evaluation of HMOX-1 protein expression in NB cell lines, after treatment with LA (100 uM) alone; with BTZ (50 nM) alone and with BTZ+LA in combination.



**Fig. 26**

**Fig. 26:** Confocal Laser Scanning Microscopy analysis of HO-1 localization in NB cell lines. The detection of HO-1 was performed by incubation with anti-rabbit secondary antibody followed by monoclonal antibody conjugated with TRITC (red). The counter-staining of the cells was performed using the nuclear dye DAPI (blue)



**Fig. 27**

**Fig. 27:**

Comparison of Gene Expression Chaperon levels of Binding Immunoglobulin Protein (BiP1), Inositol-requiring enzyme 1 (IRE1 $\alpha$ ), ER oxidoreductin 1 (ERO1 $\alpha$ ) and Protein disulfide isomerase (PDI) in Neuoblastoma cell lines treated with BTZ (50 nM) alone; with LA (100  $\mu$ M) alone and in combination with LA: BTZ+LA (100  $\mu$ M).

**Table 1.** Table of Primary Antibodies Used

<b>Antigen</b>	<b>Description of Immunogen</b>	<b>Source, Host Species, Cat. #, Clone or Lot#, RRID</b>	<b>Concentration Used</b>
Beta-Actin monoclonal antibody [AC-15]	Synthetic peptide: DDDIAALVIDNGSGL conjugated to KLH, corresponding to amino acids 1-14 of <i>Xenopus laevis</i> Actin (beta).	GeneTex, mouse monoclonal antibody, GTX26276, Clone AC-15, RRID:AB_367161	1:2000 in PBS (WB)
Cyclin D1	Synthetic peptide corresponding to residues near the C terminus of human Cyclin D1	GeneTex, mouse monoclonal antibody, GTX72094, Clone P2D11F11, RRID: AB_383363	1:2000 in PBS (WB)
Anti-MAP Kinase Kinase 1 / 2, (ERK 1/2)	synthetic phosphopeptide Ser <sup>217/221</sup> corresponding to residues around Ser <sup>217/221</sup> of human MEK 1/2.	Sigma-Aldrich, mouse, polyclonal antibody, Cat# M7683, RRID: AB_260653	1:2000 in PBS (WB)
Anti-GFAP	purified GFAP from pig spinal cord.	Sigma-Aldrich, mouse monoclonal antibody, G6171, Clone G-A-5, RRID: AB_1840893	1:2000 in PBS (WB)
Anti-Vimentin	pig eye lens vimentin	Sigma-Aldrich, mouse monoclonal antibody, V6389, V9 monoclonal, RRID: AB_609914	1:2000 in PBS (WB)
Goat Anti-Mouse IgG	Purified mouse IgG, Fc fragment	Abcam, Mouse IgG secondary, ab49724, polyclonal antibody, RRID: AB_954556	1:1000 in PBS (WB)

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