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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF GPCR23/LPA4 AS A CANDIDATE G PROTEIN-COUPLED RECEPTOR FOR GUANOSINE.

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SUMMARY

Several studies have shown that guanine-based purines exert biological effects on the central nervous system (CNS), possibly through membrane receptors, but at the present there are not reports related to the identification of such specific receptor(s).

According to the results shown in this thesis, we have identified the first guanosine G protein-coupled receptor GPCR23, also known as LPA4 receptor. [^{3}H]-Guanosine radioligand binding assay reveals that [^{3}H]-Guanosine binding to membrane fractions is greatly enhanced by GPCR23 overexpression and reduced by GPCR23 silencing. Furthermore, in [^{35}S] GTPγS binding assay experiments, Guanosine causes a functional G-protein coupled receptor activation in U87-GPCR23 overexpressing cells with an EC50= 8,067 nM. The binding site for [^{3}H]-guanosine is highly specific as well as both lysophosphatidic acid (LPA) and guanine agonists are 10 times less effective than guanosine in displacing 50 nM [^{3}H]-guanosine binding.

In order to correlate the effects of guanosine in the CNS to a putative interaction with specific binding sites and in particular to GPCR activation, we performed, in different brain areas [^{3}H]-Guanosine radioligand binding assay and [^{35}S]-GTPγS binding assay. Among the examined brain tissues, the cerebral cortex showed the highest maximal number of binding sites for Guanosine as compared to other brain regions. In each tested brain area, the saturation curves indicates the presence of a single high affinity binding site since it is resolved by non-linear regression analysis with a one-site model. In cortical membranes KD value is 143,8 nM and Bmax 3713 fmol/mg protein. The other considered areas show lower Bmax values for [^{3}H]-Guanosine, with the following rank order: cerebral cortex>hippocampus>striatum>spinal cord. The existence of a specific receptor coupled to a G protein for guanosine in cortical membranes, thus compatible with GPCR23, is also validated by [^{35}S] GTPγS binding assay experiments that demonstrate the activation of a G protein-coupled receptor in response to guanosine both in autoradiography sagittal sections and in cerebral cortex membranes.

With the purpose of evaluate downstream signaling activated by guanosine interaction with its binding sites; we conducted in vivo and in vitro experiments. According to our

results, Guanosine effects in cerebral cortex may be mediated by ERK1/2 and/or PLC pathways activation. In particular, i.p. administration of 7,5 mg/kg in rats induced ERK enhanced phosphorylation in cortical tissue, with a peak effect at 30 minutes after injection . On the other hand, treatment of cortical neurons with guanosine causes at 7,5 minutes both PLCγ and ERK1/2 pathways activation.

Taken together, our findings demonstrate that GPCR23 is the first Receptor for Guanosine and suggest an involvement of GPCR23 in the functional response of cerebral cortex to Guanosine. Even if these observations do not exclude a possible involvement of other unidentified receptors, our study lays the foundation for identification of receptors responsive to Guanine-based purines (GBPs), both in nervous system and in other peripheral tissues and may provide new targets for neuroprotection and neuromodulation.

INTRODUCTION

Purinergic system

Purine bases (adenine and guanine) and their pyrimidine counterparts (thymine, cytosine, and uracil) are the building blocks of DNA and RNA. However purines bases, their corresponding nucleosides, such as adenosine and guanosine and their metabolic products like the nucleoside inosine and the bases hypoxanthine and xanthine, as well as purine nucleotides, adenosine 5' triphosphate (ATP), adenosine 5' diphosphate (ADP), adenosine 5' monophoshate (AMP), guanosine 5' triphosphate (GTP), guanosine 5' diphosphate (GDP) and guanosine 5' monophosphate (GMP), are ubiquitous molecules found within and outside the cells (Rathbone et al., 1999b).

Purine nucleotides, mainly ATP, are involved in biochemical pathways and energy transfer within the cell. Moreover, the cyclic nucleotides adenosine3',5' -cylic monophosphate (cAMP) and guanosine3',5' -cylic monophosphate (cGMP) act as intracellular second messenger molecules during signal transduction. Despite their intracellular properties, purines exert also many effects extracellularly.

The first evidence for purines acting as extracellular mediators was given in 1929 by Drury & Szent-Györgyi, who discovered that extracellular adenosine is released by the heart during ischemia, triggers negative chronotropic effect on the heart, mediates dilatation of coronary vessels, and inhibits intestinal smooth muscle (Drury and Szent-Gyorgyi, 1929). It was also demonstrated that ATP is responsible for many purine-mediated physiological reactions (Drury, 1936) and that ATP could be released from nerves upon stimulation; indeed, through a firefly luminescence method for ATP detection, electrical stimulation of the rabbit great auricular nerve resulted in a transient elevation of extracellular ATP, establishing a foundation for the theory of purinergic neurotransmission (HOLTON, 1959).

Signaling by extracellular purines has been implicated in the control of a wide variety of physiological processes: vascular smooth muscle tone (Drury and Szent-Gyorgyi, 1929), cardiac muscle, platelet aggregation (Erlinge and Burnstock, 2008),

gastrointestinal mobility (Burnstock, 2008a), respiratory and renal function (Arulkumaran et al., 2013;Burnstock et al., 2013;Taylor et al., 2009).

Despite all these findings, during the last years particular attention has been given to purinergic regulation in central and peripheral nervous system.

Potential cellular sources of extracellular purines in the nervous system include neurons, glia, microglia, endothelial cells and blood.

Studies in vitro and in vivo have demonstrated that astrocytes are an important source of purines both in physiological and in pathological conditions. Like neurons, astrocytes release both purine nucleotides and nucleosides (Ballerini et al., 1996;Ballerini et al., 1995;Caciagli et al., 1988;Caciagli et al., 1989;Meghji et al., 1989).

Astrocytes are involved in multiple brain functions in physiological conditions, participating in neuronal development, synaptic activity, homeostatic control of the extracellular environment, and also in processes related to brain injuries, by arresting and repairing further brain damage (Chen and Swanson, 2003).

Astrocytes, as well as neurons, are also responsible for both nucleoside metabolism and uptake of adenosine and guanosine (Parkinson et al., 2005). Uptake of purine and pyrimidine nucleosides by astrocytes is also important for nucleic acid synthesis and synthesis of AMP, ADP, and ATP from adenosine and GTP from guanosine (Rathbone et al., 1999b)(see Fig.**1**).

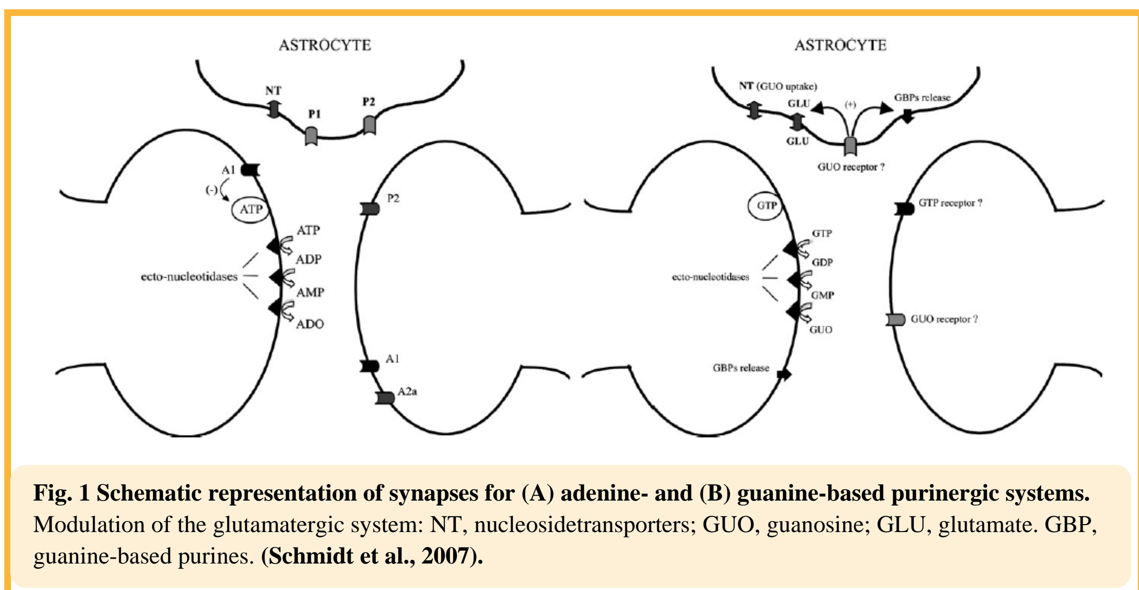


Fig. 1 Schematic representation of synapses for (A) adenine- and (B) guanine-based purinergic systems. Modulation of the glutamatergic system: NT, nucleosidetransporters; GUO, guanosine; GLU, glutamate. GBP, guanine-based purines. **(Schmidt et al., 2007).**

A recent study (Peng et al., 2005) identified 2 equilibrative nucleoside transporters in astrocytes (ENT1 and ENT2), together with the concentrative nucleoside transporter (CNT2) responsible for nucleoside uptake (see Fig.**2**).

The enzymes involved in extracellular nucleotide hydrolysis include membrane-bound ectonucleotidases, ectonucleotidases released from membranes, and the naturally occurring soluble nucleotidases. These enzymes, in association with ecto-5-nucleotidase, hydrolyze extracellular nucleotides in a stepwise fashion down to nucleosides and are crucial for physiological modulation of CNS functions, as well as for the purine-dependent neuroprotective activities against brain insults (Sebastiao et al., 1999). These enzymes can be released to the extracellular space (CSF) from choroid plexus, endothelial cells or even microglia (Heine et al., 2001;Zimmermann, 2006a;Zimmermann, 2006b) and play an important regulatory role of the purinergic system under physiological and pathological conditions. Confirming this issue, a tonic release of ATP from neurons, its hydrolysis by ectonucleotidases and subsequent re-uptake by axons appears crucial for normal axonal growth.

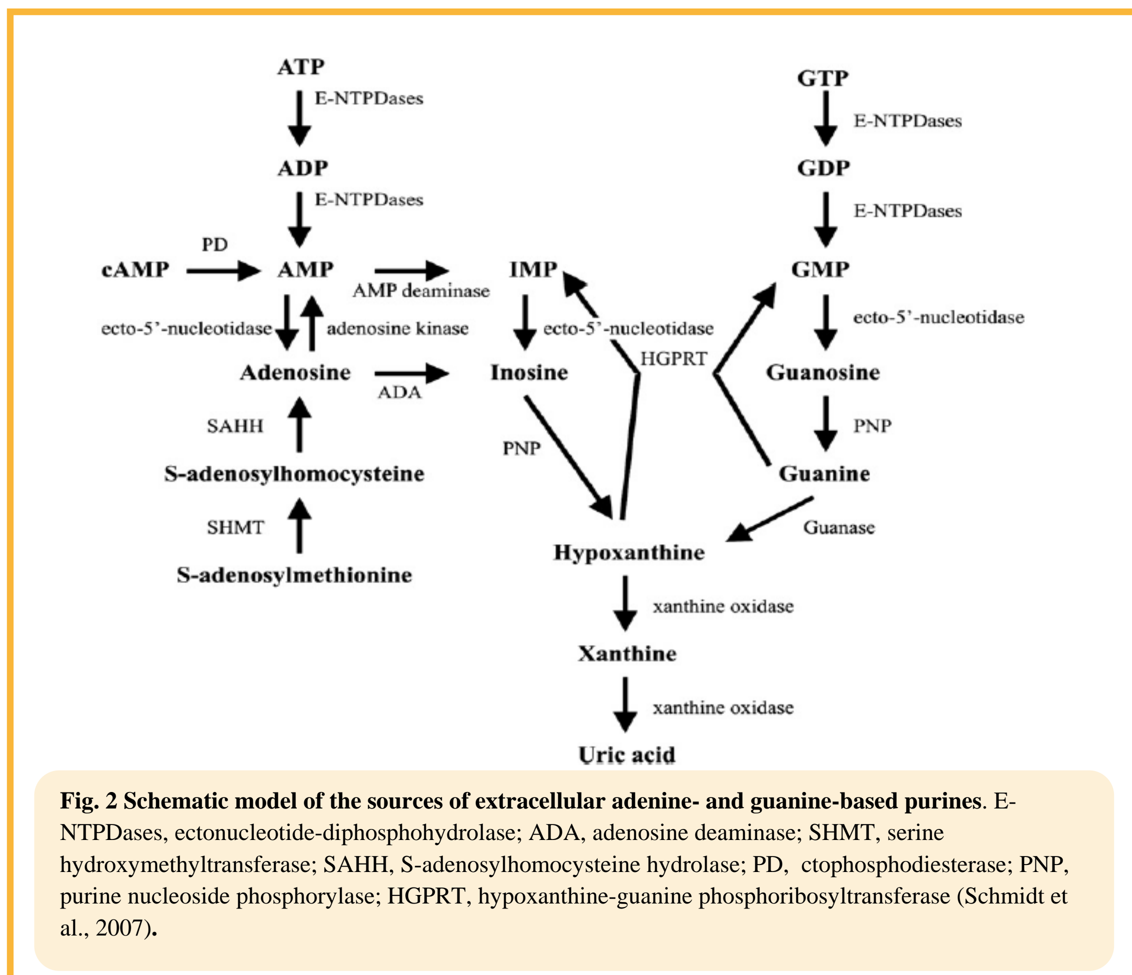


Fig. 2 Schematic model of the sources of extracellular adenine- and guanine-based purines. E-NTPDases, ectonucleotide-diphosphohydrolase; ADA, adenosine deaminase; SHMT, serine hydroxymethyltransferase; SAHH, S-adenosylhomocysteine hydrolase; PD, ctophosphodiesterase; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase (Schmidt et al., 2007).

Several studies have indicated a direct trophic role for extracellular purines in the development and maintenance of the nervous system and its response to disease or injury (Neary et al., 1996). For example, purine nucleotides may regulate neurite

outgrowth (Gysbers and Rathbone, 1996a;Gysbers and Rathbone, 1992), the proliferation of glial cells (Abbracchio et al., 1994;Christjanson et al., 1993;Ciccarelli et al., 2000;Kim et al., 1991;Neary et al., 1996;Rathbone et al., 1991;Rathbone et al., 1992d) and of brain capillary endothelial cells (Rathbone et al., 1992d;Rathbone et al., 1992a;Rathbone et al., 1992c;Rathbone et al., 1992b). Purinergic system has also been shown to regulate neurotrophin and pleiotrophin synthesis and release (Ciccarelli et al., 1997;Di et al., 2001;Gysbers and Rathbone, 1996c;Middlemiss et al., 1995). Moreover they play a role in the outgrowth of neuritic processes (Gysbers and Rathbone, 1996a;Gysbers and Rathbone, 1992) and in the activation of microglia and in glial scarring (Neary et al., 1996), in addition they are able to confer neuroprotection (Di et al., 2001;Di et al., 2004).

Purine nucleosides and nucleotides, especially guanosine, ATP and GTP stimulate incorporation of [3H] thymidine into DNA of astrocytes and microglia and concomitant mitosis in vitro. Extracellular purines also stimulate the synthesis and release of protein trophic factors by astrocytes, including bFGF (basic fibroblast growth factor), nerve growth factor (NGF), neurotrophin-3 and ciliary neurotrophic factor.

There are both short-term purinergic signaling in neurotransmission, neuromodulation and secretion and longterm (trophic) purinergic signaling of cell proliferation, differentiation and death in development and regeneration. While early studies were largely focused on short-term purinergic signaling in such events as neurotransmission, neuromodulation, secretion, chemoattraction and acute inflammation, there has been increasing interest in long-term (trophic) signaling involving cell proliferation, differentiation, motility and death in development, regeneration, wound healing, restenosis, epithelial cell turnover, cancer and ageing (Abbracchio and Burnstock, 1998) (Burnstock and Verkhratsky, 2010). For example in blood vessels, there is dual short-term control of vascular tone by ATP (Burnstock, 2002). In addition, there is long-term control of cell proliferation and differentiation, migration and death involved in neovascularization, restenosis following angioplasty and atherosclerosis (Erlinge and Burnstock, 2008). Furtheremore, involvement of purinergic signaling in development, ageing and regeneration has been described (Burnstock, 2007a).

Indeed, there is much current interest in neuron-glial cell interactions in the CNS (Burnstock et al., 2011;Fields and Burnstock, 2006) and there is increasing attention to the potential roles of purinergic signalling in trauma and ischemia, neurodegenerative conditions including Alzheimer’s, Parkinson’s and Huntington’s diseases, multiple

sclerosis and amyotrophic lateral sclerosis (Burnstock, 2007a), epilepsy, neuropsychiatric diseases and mood disorders (Burnstock, 2008b). In addition, large quantities of purines, particularly guanosine and, to a lesser extent adenosine, are released extracellularly following ischemia or trauma.

In these effects it could be supposed a cooperative interaction between adenenine- and guanine- purinergic systems. Indeed, the extracellular purine nucleotide GTP enhances the tonic release of adenine nucleotides, whereas the nucleoside guanosine stimulates tonic release of adenosine and its metabolic products. The trophic effects of guanosine and GTP may depend, at least in part, on this process.

Recent works enhance the role of purinergic signaling in stem cell differentiation and tissue regeneration (Glaser et al., 2012). Stem cells of diverse origins, such as from adipose, cardiac, and neural tissues, can restore and regenerate damaged tissues by secreting paracrine factors including purines and pyrimidines.

Nucleotides promote proliferation and differentiation of transplanted and endogenous stem cells by providing adequate stem cell niches and reduce the risk of transplant rejection and cell death. Therapeutic applications based on activation of purinergic signaling are foreseen for kidney and heart muscle regeneration.(**Fig 3**) (Coppi et al., 2007;Millart et al., 2009).

Purinergic therapeutic strategies are being developed for treatment of gut, kidney, bladder, lung, skeletal and reproductive system disorders, pain and cancer.

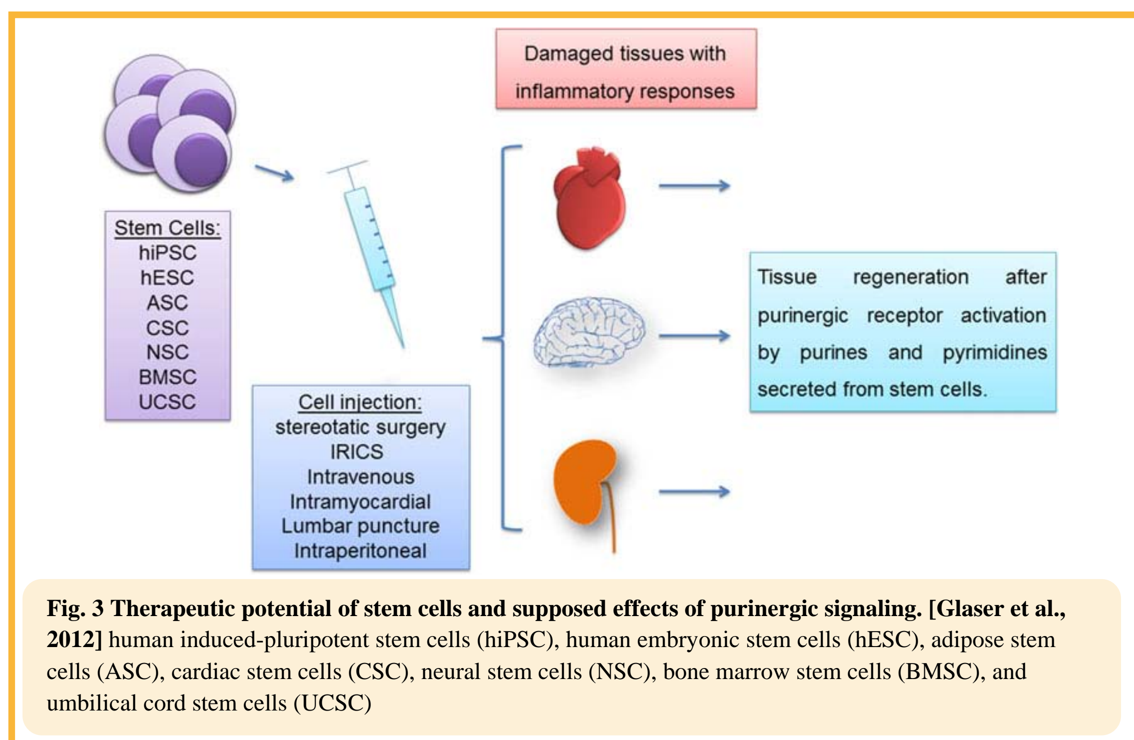


Fig. 3 Therapeutic potential of stem cells and supposed effects of purinergic signaling. [Glaser et al., 2012] human induced-pluripotent stem cells (hiPSC), human embryonic stem cells (hESC), adipose stem cells (ASC), cardiac stem cells (CSC), neural stem cells (NSC), bone marrow stem cells (BMSC), and umbilical cord stem cells (UCSC)

At present, several lines of pre-clinical evidence support the possibility of encouraging beneficial effects resulting from the pharmacological modulation of purinergic pathways also in several digestive pathological conditions, such as visceral pain, diarrhea, bowel inflammation, ischemia and functional disorders. (Antonioli et al., 2013).

It appears that the purinergic system plays a significant role in the regulation of gastric and enteric motility (Gallego et al., 2006;Grbic et al., 2008;Longhurst et al., 1996;Nylund et al., 2007;O' Donnell and Puri, 2008;Wunderlich et al., 2008;Yiangou et al., 2001), affetting both inhibitory effects and tone increments (Giaroni et al., 2002;Mule et al., 2005;Zizzo et al., 2006;Zizzo et al., 2007;Zizzo et al., 2008).

Increasing evidence has demonstrated that various enteric cells (such as neuronal, glial, epithelial and immune cells) are endowed with complex metabolic pathways, which are essential to shape the purinergic signaling in accordance with variations of tissue health conditions (Burnstock and Verkhratsky, 2009;Kukulski et al., 2011;Volonte and D'Ambrosi, 2009). A great deal of evidence has shown a widespread and heterogeneous distribution of purinergic receptors throughout the gut, as well as their relevant contribution to the regulation of intestinal motility, both through a modulation of neurotransmitter release from myenteric nerves and a direct control of smooth muscle contractility (Antonioli et al., 2010;Antonioli et al., 2011b;Antonioli et al., 2006;Burnstock, 2008c;Burnstock, 2011;Burnstock and Novak, 2012;Chandrasekharan et al., 2009;Fornai et al., 2009;Fozard, 2003;Gallego et al., 2011;Gallego et al., 2006;Vieira et al., 2011).

The presence of such an intricate network makes it difficult to determine with accuracy the functional relevance in the regulation of duration, magnitude and direction of purinergic signaling in the gut (Amadio et al., 2011;Antonioli et al., 2011a).

The current body of knowledge strongly corroborates the concept that purinergic pathways play a pivotal role in the integration of physiological functions, providing an ideal bridge among different structures (neurons, glia, neuromuscular compartment, mucosal layer and immune cells).

Adenosine - purinergic system

The nucleotide ATP and the nucleoside adenosine are usually considered the main effectors of the purinergic system (Ralevic and Burnstock, 1998).

Adenosine 5'-triphosphate (ATP) was identified in 1970 as the transmitter responsible for non-adrenergic, noncholinergic neurotransmission in the gut and bladder and the term 'purinergic' was coined. (Burnstock, 1972). Burnstock developed the concept of purinergic transmission in the peripheral nervous system, demonstrating that ATP fully conforms to the criteria for the definition of a neurotransmitter:

(**i**) ATP is synthesized and stored in presynaptic terminals;

(**ii**) ATP is released upon nerve stimulation;

(**iii**) extracellular ATP can be rapidly degraded by coenzymes;

(**iv**)pharmacological agents that inhibited the effects of endogenous ATP also suppressed the effects of nerve stimulation.

In 1976 purinergic cotransmission was proposed and ATP is now recognized as a cotransmitter in all nerves in the peripheral and central nervous systems. Important landmark papers in the early 1990s, described ATP mediation of fast purinergic synaptic transmission in both peripheral ganglia (Evans et al., 1992;Silinsky et al., 1992) and in the CNS (Edwards et al., 1992). Purinergic cotransmission is now well established, in sympathetic and parasympathetic nerves, sensory-motor and enteric nerves. More recently, ATP has been shown to be co-released with glutamate, GABA, dopamine, NA, 5-hydroxytryptamine and acetylcholine (ACh) in different populations of nerve fibres in the central nervous system (CNS) (Burnstock, 2007a). The neuromodulatory effects and sources of adenosine have been well characterized. Extracellular adenosine is enzymatically formed from extracellular nucleotides or comes from the release of intracellular adenosine (Brundege and Dunwiddie, 1997).

Brain extracellular adenosine and ATP act not only as neurotransmitters and neuromodulators, but also as trophic factors involved in plastic processes, such as memory and learning, collateral sprouting of nerve processes, neuroprotection against noxious stimuli, and regulation of cell number through induction of apoptosis (programmed cell death); (Ciccarelli et al., 1999;Ciccarelli et al., 2001).

Moreover, many properties of the Central Nervous System can be brought again in the enteric nervous system and also the number of the transmitters is comparable. Among the plethora of chemical agents modulating gastrointestinal functions a central role is

played by adenine-based purines (Burnstock, 2009). There is abundant evidence that extracellular ATP and adenine-based nucleotides have a significant impact on the physiology of enteric neurotransmission as non-adrenergic, non-cholinergic (NANC) modulators of gastrointestinal motility inducing contractile or relaxing responses of GI smooth muscle (Christofi et al., 1992;Coupar, 1999;Lee et al., 2001;Lee and Parsons, 2000;Moody and Burnstock, 1982;Vizi and Knoll, 1976).

Receptors for adenine based purines

Implicit in purinergic transmission is the existence of specific receptors. A basis for distinguishing two types of purinergic receptors was proposed in 1978, one selective for adenosine (called P1), which was antagonized by methylxanthines, and the other selective for ATP/ ADP (called P2) (Burnstock, 1980). There are both postjunctional receptors as P2, and prejunctional P1 receptors mediating neuromodulatory negative-feedback responses or autoregulation of transmitter release. [**Table.1**]

Concerning ATP receptors, on the basis of molecular structure and transduction mechanisms, it was proposed that P2 should belong to 2 major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors (Abbracchio and Burnstock, 1994). This nomenclature has been widely accepted, and currently 7 P2X subtypes and 8 P2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as purines (Burnstock, 2007b).

P2X receptors mediate the flow of Ca2+, Na+, and K+, whereas P2Y metabotropic receptors, via G proteins, activate second messenger systems, such as phospholipase C (PLC) and phospholipase A2 (PLA2). ADP and pyrimidine nucleotides (UTP and UDP) also activate some subtypes of P2 receptors. Two further P2 receptor subtypes were proposed, P2T on platelets and P2Z on macrophages, and receptors that responded to pyrimidines and to purines were named P2U receptors (Gordon, 1986).

Concerning adenosine receptors, to date 4 different adenosine P1 receptor subtypes, which are classified as A1, A2A, A2B and A3 have been cloned and characterized (Brundege and Dunwiddie, 1997;Cunha, 2005;Fredholm et al., 2001;Fredholm et al., 2005;Ralevic and Burnstock, 1998). A1 and A3 receptors inhibit, whereas A2a and A2b receptors stimulate, adenylate cyclase. Both A1 and A3 receptors also increase inositol-3-phosphate (IP3) formation. The human A2B receptor has also been found to regulate PLC activity (Burnstock, 2007b). Adenosine A1 receptors are widely distributed in the central nervous system (CNS) and have been shown to decrease neuronal excitability

and synaptic activity and to inhibit the release of several neurotransmitters, such as glutamate, dopamine, serotonin, noradrenaline, and acetylcholine. A2A receptors are concentrated in dopamine-rich areas, modulating dopaminergic activity, but are also present in the hippocampus and cerebral cortex. A2B receptors are less well characterized and have been suggested to interact with inflammatory mediators. Similarly, A3 receptors have also been related to inflammation, especially in lungs (Burnstock, 2007a).

A number of P1 subtype-selective agonists and antagonists have been identified. All of the known P1 receptor agonists are closely related to adenosine in structure. Methylxanthines, such as caffeine and theophylline, are the classical nonselective A1–A2 adenosine antagonists.

Many non-neural as well as neuronal cells express multiple receptors (Burnstock and Knight, 2004) and this poses problems about how they interact to mediate physiological events. For example some receptors mediate fast signalling, others slow signalling. While the mRNA is present, the receptor protein may only be expressed under pathological conditions; also different concentrations of endogenous agonists may trigger different receptors. For example, Considering the multiple receptors on microglia, P2Y12 receptors mediate cell migration, an unidentified P2 receptor subtype mediates phenotype changes during migration, P2X4 and P2X7 receptors are up-regulated and mediate neuropathic pain and P2Y6 receptors mediate phagocytotic activity (Inoue, 2008). Selective agonists and antagonists to many of the P1, P2X and P2Y receptor subtypes are now recognized. A breakthrough paper describing the crystal structure of the ATP-gated P2X4 receptor set the stage for comparable studies of other P2X receptor subtypes (Kawate et al., 2009). This has given invaluable information to the medicinal chemists aiming to design selective agonists and antagonists, which for many years have posed difficult challenges (Gunosewoyo and Kassiou, 2010;Jacobson and Boeynaems, 2010;Press and Fozard, 2010). Polymorphic variations of the human P2X7 receptor (Bradley et al., 2011;Gu et al., 2001) raise serious problems about the development of P2X7 receptor antagonists, which are much needed for the treatment of inflammatory disorders (Burnstock and Kennedy, 2011).

It is becoming clear that the purinergic signalling system has an early evolutionary basis (Burnstock and Verkhratsky, 2009;Fountain and Burnstock, 2009;Galimov, 2009;Hoyle, 2011) with fascinating recent studies showing cloned receptors that resemble mammalian P2X receptors in two primitive invertebrates, Dictyostelium and

Schistosoma and in green algae (Agboh et al., 2004;Fountain et al., 2007;Fountain et al., 2008). It was concluded that purinergic signalling was already present in early eukaryotes before the divergence of the plant lineage and the lineage leading to metazoans (Sreedharan et al., 2010). P2X3 receptors were cloned in 1995 and shown to be largely located in small nociceptive sensory nerves that label with isolectin B4 (Bradbury et al., 1995;Chen et al., 1995). Central projections are located in the inner lamina 2 of the dorsal horn of the spinal cord and peripheral extensions in skin, tongue and visceral organs.

RECEPTOR	MAIN DISTRIBUTION
P1 ADENOSINE	
A1	Brain, spinal cord, testis, heart, autonomic nerve terminals.
A2A	Brain, heart, lungs, spleen.
A2B	Large intestine, bladder.
A3	Lung, liver, brain, testis, heart.
P2X	
P2X1	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons.
P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic, sensory ganglia, pancreas.
P2X3	Sensory neurones, NTS, some sympathetic Neurons.
P2X4	CNS, testis, colon, endothelial cells and microglia.
P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord, heart, adrenal medulla.
P2X6	CNS and motor neurons in spinal cord.
P2X7	Immune cells (mast cells, macrophages), pancreas, skin, microglia.
P2Y	
P2Y1	Epithelial and endothelial cells, platelets, immune cells, osteoclasts, brain.
P2Y2	Immune cells, epithelial and endothelial cells, kidney tubules and osteoblasts.
P2Y4	Endothelial cells, placenta, spleen, thymus.
P2Y6	Airway and intestinal epithelial cells, placenta, T cells, thymus, microglia (activated).
P2Y11	Spleen, intestine, granulocytes.
P2Y12	Platelets, glial cells
P2Y13	Spleen, brain, lymph nodes, bone marrow , erythrocytes.
P2Y14	Placenta, adipose tissue, stomach, intestine, discrete brain regions, mast cells.

Table 1. Characteristics of purine-regulated receptors (Cheung et al., 2003)

Guanosine - purinergic system

Similarly to the well-characterized adenine-based purinergic system, a similar story has been taking place with the extracellular roles of guanine-based purines. Traditionally, guanine-based purines have been studied as modulators of intracellular processes, especially regarding the activity of G proteins for signal transduction.

Guanine-based purines have been further investigated as a neurotransmission/ neuromodulator system in terms of physiological, pharmacological, biochemical, and genetic parameters. In a recent past many studies hypothesize that a guanine-based purinergic system plays significant roles in the nervous system, providing new targets for neuroprotection and neuromodulation. The nucleotides GTP, GDP, GMP, and the nucleoside guanosine have been shown to exert extracellular effects, such as modulation of the glutamatergic activity in physiological and pathological conditions, effects on memory and behavior (Roesler et al., 2000;Vinade et al., 2005), and trophic effects on neural cells (Ciccarelli et al., 2001), not related to their direct modulation of G proteins, both in vitro (Baron et al., 1989;Burgos et al., 1998;Burgos et al., 2000a;Burgos et al., 2000b;Burgos et al., 2000c;Paz et al., 1994;Ramos et al., 1997;Souza and Ramirez, 1991;Tasca et al., 2004) and in vivo (Lara et al., 2001;Schmidt et al., 2000).

Guanine-based purines, in the nervous system, mediate both immediate effects, such as neurotransmission, and trophic effects which induce changes in cell metabolism, structure and function.

GTP may be stored in synaptic vesicles (Santos et al., 2006;Zimmermann and Braun, 1996) and indirect evidence indicated that guanosine could be released from synaptosomes (Fredholm and Vernet, 1979). Cultured astrocytes may release guanine-based purines (Ciccarelli et al., 1999), a process that increased after hypoxia/hypoglycemia. Of note, the release of guanine-based purines was much greater than that of their adenine-based counterparts. In cultured astrocytes, inhibition of ecto-5'-nucleotidase activity significantly reduced accumulation of extracellular guanosine, indicating that, like extracellular adenosine, it is to some extent derived from the extracellular metabolism of guanine nucleotides (Caciagli et al., 2000). Interestingly, at high concentrations, GDP hydrolysis rate is greater than that of ADP, perhaps favoring the accumulation of GMP and consequently guanosine. The enzymes involved in GBPs metabolism, can be released to the extracellular space (CSF) from choroid plexus, endothelial cells or even microglia (Zimmermann, 1996;Zimmermann,

2006a;Zimmermann, 2006b) and play an important regulatory role of the purinergic system under physiological and pathological conditions.

It has been classically demonstrated that by acting via G proteins, GTP is able to simultaneously inhibit binding of neurotransmitters (and their agonists) to metabotropic receptors and modulate adenylate cyclase activity (Gudermann et al., 1997). However, the effects of guanine nucleotides on kainic acid (a glutamatergic ligand to receptors not coupled to G proteins) binding site and on adenylate cyclase activity could be dissociated (Souza and Ramirez, 1991). The inhibition of kainic acid binding by guanine nucleotides was not dependent on a G protein-mediated system. This result corroborated studies which had previously shown that the inhibitory effects of guanine nucleotides on the binding of glutamate or ionotropic glutamatergic ligands presented several inconsistencies, when compared with studies on receptors known to be coupled to their second messengers through a G protein (Baron et al., 1989;Butcher et al., 1986;Hood et al., 1990;Monahan et al., 1988;Paas et al., 1996;Sharif and Roberts, 1981).

In all these studies, the nucleoside guanosine had no effect on the binding of glutamate and analogs to glutamate receptors (Porciuncula et al., 2002;Souza and Ramirez, 1991). It was observed that guanine nucleotides inhibited glutamate-stimulated GFAP (astrocytic protein) phosphorylation (Tasca et al., 1995), glutamate (and analogs)-induced increase in intracellular cAMP levels (Tasca et al., 1998), glutamate-induced luminescence (Regner et al., 1998), kainate-stimulated LDH release (Burgos et al., 1998), kainate-activated currents (Aleu et al., 1999;Burgos et al., 2003), and kainate-stimulated increase in Ca^{2+} influx (Burgos et al., 2000a;Burgos et al., 2000b;Burgos et al., 2000c). Guanine nucleotides administered intracerebroventricularly had long been shown (Baron et al., 1989) to prevent seizures induced by quinolinic acid, a toxin that overstimulates the glutamatergic neurotransmission (Stone, 2001). This effect was compatible with the antagonistic properties of guanine nucleotides on glutamate receptors. Additional studies also provided evidence that guanosine and GMP administered intracerebroventricularly (i.c.v.), intraperitonially or orally protected against seizures induced by the glutamatergic agents quinolinic acid, kainate and α-dendrotoxin in adult and young rodents (de Oliveira et al., 2004;Schmidt et al., 2005;Soares et al., 2004;Vinade et al., 2005;Vinade et al., 2003). Guanine-based purines, mainly GMP and guanosine, have usually presented similar neuroprotective profile in several in vivo and in vitro protocols (Lara et al., 2001;Schmidt et al.,

2000;Schmidt et al., 2005;Vinade et al., 2005;Vinade et al., 2003). However, most effects of nucleotides (mainly GMP) seemed to be due to their conversion to guanosine.

Anticonvulsant effects of i.c.v. GTP and GDP seemed to be mediated by their conversion to guanosine, since their poorly hydrolysable analogs GTPγS, GppNHp, and GDPβS were not capable of preventing seizures induced by quinolinic acid in mice (Schmidt et al., 2005). Guanosine is able to increase the glutamate uptake by cultured astrocytes and brain slices (Frizzo et al., 2001;Frizzo et al., 2002;Frizzo et al., 2003). In basal or physiological conditions, the effects of guanosine on glutamate uptake in brain slices seemed to be age (more in young animals)- and structure (more in cortex)-dependent but, in excitotoxic conditions, guanosine was more broadly involved in modulating glutamate uptake (Frizzo et al., 2005;Gottfried et al., 2002;Thomazi et al., 2004). Guanosine stimulatory effect on astrocytic uptake of glutamate is exerted from the extracellular side and is independent of adenosine and its receptors (Frizzo et al., 2001). Guanosine seems to be mediator of the stimulatory effect of guanine-based purines on the astrocytic uptake of glutamate, and this process was independent of adenosine and relatively specific for glutamate (Frizzo et al., 2003). As astrocytic uptake of glutamate is the most important mechanism for terminating its actions within the synapse, the stimulation of uptake by guanosine may be a relevant process in regulating glutamatergic neurotransmission, especially under excitotoxic conditions (Chen and Swanson, 2003;Duan et al., 1999;Matute et al., 2006;Schousboe and Waagepetersen, 2005). Quinolinic acid stimulates glutamate uptake by synaptic vesicles, an effect prevented by glutamate antagonists and the guanine-based purines guanosine and GMP. GTP, GDP, GMP, and guanosine are able to inhibit glutamate uptake by synaptic vesicles in vitro (Tasca et al., 2004), pointing to an intracellular interaction between guanine-based purines and the glutamatergic neurotransmission.

Several studies have indicated that guanosine may be a neuroprotective endogenous compound released under excitotoxic conditions, preventing further toxicity to neurons. Both neuronal and astrocytic cell cultures are able to release guanosine and adenosine under basal or toxic (ischemic) conditions (Ciccarelli et al., 1999;Ciccarelli et al., 2001;Dobolyi et al., 2000) and kainate stimulates the release of guanosine (Dobolyi et al., 2000). Interestingly, guanosine protected brain slices exposed to hypoxia/hypoglycemia (Frizzo et al., 2002) and medium from astrocytes treated with guanosine prevented NMDA-induced toxicity in neurons (Caciagli et al., 2000).

GMP and guanosine are capable to modulate memory processes since pretraining administration of both guanine-based purines impaired retention of inhibitory avoidance responses in rats (Roesler et al., 2000). The guanine-based purine effects on memory were reproduced with anticonvulsant doses after acute/chronic intraperitonial/oral administration and adenosine-receptor antagonists failed to prevent these effects (Vinade et al., 2003;Vinade et al., 2004). Furthermore, the amnesic effect related to the pretreatment with GMP also depended on its conversion to guanosine (Saute et al., 2006). These findings suggest an amnesic effect of guanosine on inhibitory avoidance in rodents, in a pattern compatible with inhibition of glutamatergic activity and independent of adenosine A1 and A2A receptors.

In addition to their effects on neurotransmission, guanine based purines also have important trophic functions, affecting the development, structure or maintenance of neural cells, as observed by Rathbone's group (Rathbone et al., 1999a).

Both extracellular guanosine and GTP, apparently through different mechanisms:

(i) have mitogenic effects promoting astroblast growth (Kim et al., 1991);

(ii) are potent stimulators of in vitro axonal growth and proliferation of a wide range of cell types (Rathbone et al., 1992c;Rathbone et al., 1992b);

(iii) can exert trophic effects on the nervous system (Rathbone et al., 1998;Rathbone et al., 1999a), including stimulation of astrocyte proliferation (Ciccarelli et al., 2000;Kim et al., 1991), synthesis and release of trophic factors such as immunoreactive nerve growth factor from astrocyte cultures (Caciagli et al., 2000;Middlemiss et al., 1995);

(iv) can enhance the differentiation of PC12 cells and hippocampal neurons in vitro (Gysbers and Rathbone, 1992;Gysbers and Rathbone, 1996b).

The role of GTP as a trophic mediator received strong support from data confirming that specific binding sites for GTP are present on the plasma membrane of neuronal-like PC12 cells (Guarnieri et al., 2004;Gysbers et al., 2000;Gysbers and Rathbone, 1996c) and C2C12 mouse skeletal muscle cells (Mancinelli et al., 2012;Pietrangelo et al., 2002) and that GTP is stored in synaptic vesicles (Santos et al., 2006;Zimmermann, 1996). Extracellular GTP enhances the neuritogenic effects of nerve growth factor on PC12 cells, significantly increasing the proportion of cells that have neuritis (Guarnieri et al., 2004;Gysbers et al., 2000;Gysbers and Rathbone, 1996a). Although some extracellular effects of GTP might be related to its conversion to guanosine, other findings indicate that a different mechanism of action between them may be present, as in the case of neurite outgrowth stimulation.

Guanosine has also been shown to stimulate the output of adenine-based purines from astrocytes and triggered these cells to proliferate and to produce large amounts of neuroprotective factors (Ciccarelli et al., 2001).

In the U87 glioma cell line and in several different human tumoral cell lines, Guanine, Guanosine and GMP exerted a marked inhibition of proliferation that was not seen with other tested nucleotides, nucleosides and nucleobases. Indeed, weak effects were detected by treatment with Adenosine and AMP, thus suggesting that GUO induced effects were not mediated by P1 receptors (Garozzo et al., 2010).

The effects of GBPs probably enroll intracellular metabolism. It was demonstrated by experiments in cell lines bearing an inactive form of HGPRT (Hypoxanthine-guanine phosphoribosyltransferase), a transferase that catalyzes conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. The lack of activity of Guanosine in the cell line with mutated HGPRT (C32TG cell line) or its reduced potency in U87 cells silenced for the HGPRT transcript, confirmed the central role of such enzymatic conversion in growth-inhibitory effects of Guanosine.

Supporting this issue, Guanine-based purines have recently been enrolled in the pathogenesis of the Lesch-Nyhan syndrome, a rare inherited disorder caused by a deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), produced by mutations in the HGPRT gene located on X chromosome (Deutsch et al., 2005). Investigation of this disorder and the neurobiological consequences of the hypoxanthine phosphoribosyltransferase (HGPRT) deficiency demonstrated the potential roles that guanine-based purines play in neurodevelopment and as neuromodulators and neurotransmitters. Conceivably, diminished reutilization of free guanine bases due to absent or negligent HGPRT activity and relatively high guanase activity in the brain could lead to deficient pools of guanosine associated with glutamatergic synapses in Lesch-Nyhan syndrome. Nonetheless, if a guanosine deficiency were to exist in Lesch-Nyhan syndrome, the administration of guanosine itself or its analogs that could promote glutamate uptake might be a useful pharmacological strategy to be considered in the treatment of this disorder.

Guanine-based purines seem to have a role in avoiding apoptotic cell degeneration implicated in the pathophysiology of Alzheimer's disease. Recently, it was shown that extracellular guanosine inhibited staurosporine-induced apoptosis in astrocytes (Di et al., 2004). Guanosine has also been shown to protect SH-SY5Y cells against beta-amyloid-induced apoptosis (Pettifer et al., 2004). More recently, guanosine was shown

to dose-dependently inhibit the CD40-induced expression in mouse microglia cells, as well as functional CD40 signaling by suppressing IL-6 production (D'Alimonte et al., 2007).

Additional evidence suggests that guanosine increases cholesterol efflux from astrocytes and rat glioma cells and increases the expression of apolipoprotein E (ApoE) in astrocytes (Ballerini et al., 2006). Considering the fact that cell cholesterol depletion has recently been linked to a reduction in beta-amyloid formation, and cholesterol balance is essential in neuronal plasticity and in stabilization of synaptic transmission, these findings point to a role of guanosine as a potential pharmacological tool in the modulation of cholesterol homeostasis in the brain.

Recent findings suggest that guanine-based purines, especially guanosine, may also be a new target for trauma rehabilitation or CNS diseases related to demyelination. Administration of guanosine for 7 consecutive days improved locomotor function and spinal cord remyelination in rats submitted to a spinal cord injury model (Jiang et al., 2003). Guanosine-induced remyelination seemed to result from activation of endogenous oligodendrocyte lineage cells. These findings may have significant implications for chronic demyelinating diseases. Additionally, the trophic effects of GTP and guanosine may have physiological and therapeutic implications in sprouting and functional recovery after neuronal injury in the CNS, due to the high levels of nucleosides and nucleotides released from dead or injured cells (Ciccarelli et al., 2001).

The potential ability of exogenously administered guanine-based purines to provide an alternative source of energy to ATP has been suggested as an explanatory hypothesis for their neuroprotective effects in the context of oxidative stress and cell damage (Ciccarelli et al., 2001;Litsky et al., 1999).

Close to the CNS effects, Guanine-based purines can be considered a modulatory agent for intestinal and gastric contractility (Zizzo et al., 2011;Zizzo et al., 2013). According to Zizzo et al., guanine-based purines do not play a modulatory role on the spontaneous contractile activity of murine distal colonic circular muscle, but guanosine and guanine were able to interfere with the enteric neurotransmission, negatively modulating, in a concentration-dependent manner, the excitatory cholinergic neurotransmission. Moreover, the effects of guanine-based purines persisted in the presence of P1 and P2 purinoceptor antagonists, indicating that guanosine and guanine did not interact with adenine-based purine receptors. In addition in a recent work Zizzo and cooperators showed that intragastric gavage of guanosine in vivo delays gastric emptying and that

exogenous guanosine in vitro relaxes murine gastric preparations. Those effects probably depend by guanosine cellular uptake and involve adenylyl cyclase activation, which leads to an increase in the cAMP intracellular levels (Zizzo et al., 2013).
All together, these studies on the extracellular effects of guanine-based purines have strengthened the proposal for a specific guanine based purinergic system in addition to the well-known adenine based purinergic system, although specific receptors for guanine based purines have not still been identified.

Guanine-based purines receptors and second messenger hypothesys

Evidences for putative receptors or specific binding sites for either guanosine or GTP in astrocytes (Ciccarelli et al., 2001), in PC12 cells (Gysbers et al., 2000) and in other cell types (Vuorinen and Laustiola, 1992) have been reported during the last years, although specific receptors for guanine based purines haven't been already identified.
Neither guanosine nor GTP binds with high affinity to adenine-based purine receptors (Muller and Scior, 1993), suggesting that guanine-based purines have distinct cellular targets from adenine-based purines. A possibility is that some actions of guanine-based purines could be mediated intracellularly after their uptake or may be indirect, occurring as a result of stimulating the synthesis and release of trophic factors and/or enhancing the effects of these specific trophic factors. However, with respect to a specific neurotrophic role for guanosine, its extracellular levels remained elevated for up to a week after focal brain injury (Uemura et al., 1991). Additionally, many trophic effects of guanine-based purines were not affected by the nucleoside uptake inhibitors, such as NBTI or dipyridamole (Gysbers and Rathbone, 1992), indicating that they are triggered extracellularly. The ability of guanine based purines to stimulate proliferation of rat brain microglia in a concentration-dependent manner appears to be mediated by specific purinergic receptors that recognize adenine-based purines (Ciccarelli et al., 2000). But this explanation is also incomplete, since many of the effects of guanine-based purines persist in the presence of P1 and/or P2 purine receptor antagonists (Frizzo et al., 2001;Gysbers and Rathbone, 1992;Tasca and Souza, 2000), suggesting the presence of different receptors and mechanism of actions for guanine-based purines, maybe depending on the cell type involeved or on the specific effect activated. Moreover, several of the effects of guanosine may be mediated through G protein-dependent signaling pathways involving cyclic nucleotides or MAP kinase pathway (Caciagli et

al., 2000;Gysbers and Rathbone, 1996c), raising the possibility that some of the effects of guanine-based purines, particularly guanosine, involve activation of cell surface receptors coupled to G proteins.

Signal transduction mechanisms linked to guanosine receptor are not fully understood; they may be linked via G proteins to the MAPK cascade since the ability of guanosine to enhance synthesis of trophic factors in astrocytes is associated with an increase in phosphorylation of ERK1 and ERK2 and is blocked by pretreatment with pertussis toxin (Caciagli et al., 2000). Also the antiapoptotic effects of guanosine seemed to be mediated by activation of the PI3K/Akt/PKB and MAPK (ERK1/2 and p38) pathways (D'Alimonte et al., 2007).

Several lines of evidence have raised the possibility that specific cell surface receptors for guanosine may exist (Rathbone et al., 1999b). A key prediction of this hypothesis is that the plasma membranes contain specific high affinity binding sites for guanosine. The first direct evidence for the presence of high-affinity binding sites for [^{3}H]-guanosine in rat brain membrane preparations was given in 2002 by Traversa and collegues (Traversa et al., 2002), who demonstrated the presence of specific guanosine binding sites distinct from the well characterized adenosine and purine nucleotide binding sites. Neither adenosine, ATP, nor the non-specific adenosine receptor antagonists caffeine and theophylline, reduced [^{3}H]-guanosine binding. These observations led scientists to suggest the presence of a putative cell surface receptor for guanosine.

In 2003, Traversa et al. (Traversa et al., 2003) reported experiments showing that guanosine binding sites in rat brain membranes belong to the class of G-protein coupled receptors (GPCRs), since pretreatment with PTX decreased [^{3}H]-guanosine specific binding, and involves cAMP as a possible intracellular signaling pathway. PTX catalyzes the ADP-ribosylation of the α_i subunits of the heterotrimeric G protein. This prevents the G proteins from interacting with G protein-coupled receptors on the cell membrane, thus interfering with intracellular communication(Burns, 1988). The Gi subunits remain locked in their GDP-bound, inactive state, thus unable to inhibit adenyl cyclase activity, leading to increased cellular concentrations of cAMP.

In the reported experiments, the cAMP increase is specific for guanosine, since neither the pretreatment with adenosine deaminase (which converts adenosine to inosine) nor the A(1) and A(2) adenosine receptor antagonists were able to modify the guanosine-induced cAMP accumulation.

Later, Volpini and collaborators (Volpini et al., 2011) using the innovative DELFIA Eu-GTP binding assay, proved that guanosine and 6-thioguanosine and their derivatives activate a putative GPCR that is different from the well-characterized purinergic adenosine receptors.

Together, all these data suggest the existence of unknown and specific G-protein coupled receptors for guanosine and its derivates.

GPCR 23 Hypothesis

In collaboration with the group of Prof. Condorelli from University of Catania, we have hypothesized that GPCR23 may represent a receptor for Guanine based purines. Our unpublished data have indicated that guanine based purines caused antiproliferative effects in glioma U87 cell line. Candidate receptors for GBPs were then seleceted on the basis of their homology to purinoreceptors P1-P2. After checking for the expression of selected receptors in U87 cell line (Garozzo et al., 2010), specific siRNAs were designed against GPCR3, GPCR21, GPCR22, GPCR23, P2Y5; these receptors were silenced by siRNA lipotransfection and cell proliferation in response to GBPs was evaluated. Interestingly, GPCR23-silencing dramatically reduced (Guanosine) GUO and (Guanine) GUA antiproliferative effects in U87 cell lines. Moreover, GPCR23-overexpressing clones, stably transfected with recombinant expression vectors, displayed an enhanced sensitivity to GUA and GUO that was reverted by GPCR23 siRNA-mediated silencing, thus confirming a possible role of GPCR23 in GBPs responses (Garozzo R et al, unpublished data). The GPCR23 gene (Accession Number NM_005296) (also called P2Y9 and P2Y5-like because it shares a high homology with human P2Y5) is located on chromosome Xq13-q21.1, and contains an intronless open reading frame of 1113 bp encoding 370 amino acids (O'Dowd et al., 1997). The protein sequence shows 33% identities and 56% conserved amino acid residues vs P2Y1 ADP receptor. Moreover specific sites, involved in ligand interaction and conserved in P2Y protein family, are present in GPCR23 protein. The consensus sequence SILFLTCIS, found in almost all functionally defined P2Y receptors (von, I and Wetter, 2000), is conserved in GPCR23-protein sequence with a single substitution (SMLFLTCIS). The P2Y1 residue Ser314, that, by mutagenesis studies, has been shown to be involved in H bond formation with N1 of purine (Jiang et al., 1997;Moro et al., 1998) is also

conserved (+T301), while no residues involved in interaction with 5’-diphosphate groups are conserved (Abbracchio et al., 2003;Costanzi et al., 2004).

With regards to specific ligands and biological functions of this receptor, previous functional studies reported GPCR23 as a receptor activated by Lysophosphatidic acid (LPA) (Lee et al., 2007;Noguchi et al., 2003;Yanagida et al., 2007). P2y9/GPCR23 specifically binds to LPA and mediates LPA-induced adenylyl cyclase stimulation and intracellular Ca^{2+} mobilization in p2y9/GPR23-expressing CHO cells (Noguchi et al., 2003). According to Noguchi's results, LPA induced an increase in cAMP levels in p2y9/GPCR23-expressing CHO and pretreatment of the cells with pertussis toxin further increased the cAMP. In mock-transfected CHO cells, LPA induced no change or a decrease in cAMP levels and pretreatment of the cells with pertussis toxin attenuated an LPA-induced decrease in cAMP levels.

LPA is a bioactive lipid mediator with diverse physiological and pathological actions on many cell types. At least six G protein–coupled receptors (GPCRs) have been identified to mediate a wide range of biological functions of LPA (An et al., 1998;Bandoh et al., 1999;Hecht et al., 1996;Im et al., 2000;Lee et al., 2006;Noguchi et al., 2003;Pasternack et al., 2008). The LPA1/Edg2, LPA2/Edg4 and LPA3/Edg7 receptors are members of the endothelial cell differentiation gene (Edg) family, sharing 50–57% homology in their amino acid sequences (An et al., 1998;Bandoh et al., 1999;Hecht et al., 1996;Im et al., 2000).

LPA4/GPCR23/p2y9, a member of the purinergic receptor family, and the related LPA5/GPR92 and p2y5 are structurally distant from the Edg LPA1–3 receptors (Lee et al., 2006;Noguchi et al., 2003;Pasternack et al., 2008).

Interestingly, p2y9/GPCR23 shares only 20–24% amino acid identities with Edg-2/LPA1, Edg-4/LPA2, and Edg-7/LPA3, and the phylogenetic analysis shows that p2y9/GPCR23 is distant from the Edg family and most closely related to the orphan receptor p2y5 and relatively close to the functional receptors for nucleotides (P2Y1, P2Y4, and P2Y6) (Boeynaems et al., 2000). (**Fig. 4**)

The mRNA levels of p2y9/GPCR23 are significantly high in ovary. A prominent expression of Edg-4/LPA2 has been shown in primary cultures and established lines of ovarian cancer cells (Goetzl et al., 1999;Oyesanya et al., 2008). The EST cDNA encoding p2y9/GPCR23 was originally isolated from human brain (Janssens et al., 1997;O'Dowd et al., 1997). Although, in Noguchi study high expression of

p2y9/GPCR23 was not detected in brain, the authors suppose that specific types of cells in restricted brain areas express p2y9/GPCR23.

To determine biological functions of p2y9/GPCR23/LPA4, Lee (Lee et al., 2008) and cooperators have characterized LPA4- deficient mice. The chimeric male mice were bred with C57BL/6 females to generate heterozygous female founder mice ($X^{+}X^{-}$) and WT males ($X^{+}Y$), which were further intercrossed to generate hemizygous males ($X^{-}Y$). The lpa4 KO mice were grossly indistinguishable from their WT or heterozygous littermates in appearance, size, and behavior. They did not show any defects in mating, pregnancy, or litter sizes. There were no gross abnormalities in the internal organs of LPA4-deficient adults.

Although p2y9/GPCR23/LPA4-deficient mice displayed no apparent abnormalities, p2y9/GPCR23/LPA4-deficient mouse embryonic fibroblasts (MEFs) were hypersensitive to LPA-induced cell migration. Consistent with negative modulation of the phosphatidylinositol 3 kinase pathway by p2y9/GPCR23/LPA4, p2y9/GPCR23/LPA4-deficiency potentiated Akt and Rac but decreased Rho activation induced by LPA. Ectopic expression of p2y9/GPCR23/LPA4 strongly inhibited migration and invasion of human cancer cells. When coexpressed with LPA1-receptor in B103 neuroblastoma cells devoid of endogenous LPA receptors, p2y9/GPCR23/LPA4 attenuated LPA1-receptor derived migration and invasion, indicating functional antagonism between the two subtypes of LPA receptors. These results provide genetic and biochemical evidence that p2y9/GPCR23/LPA4 is distant for genetical origin and activity from the other Edg LPA receptors.

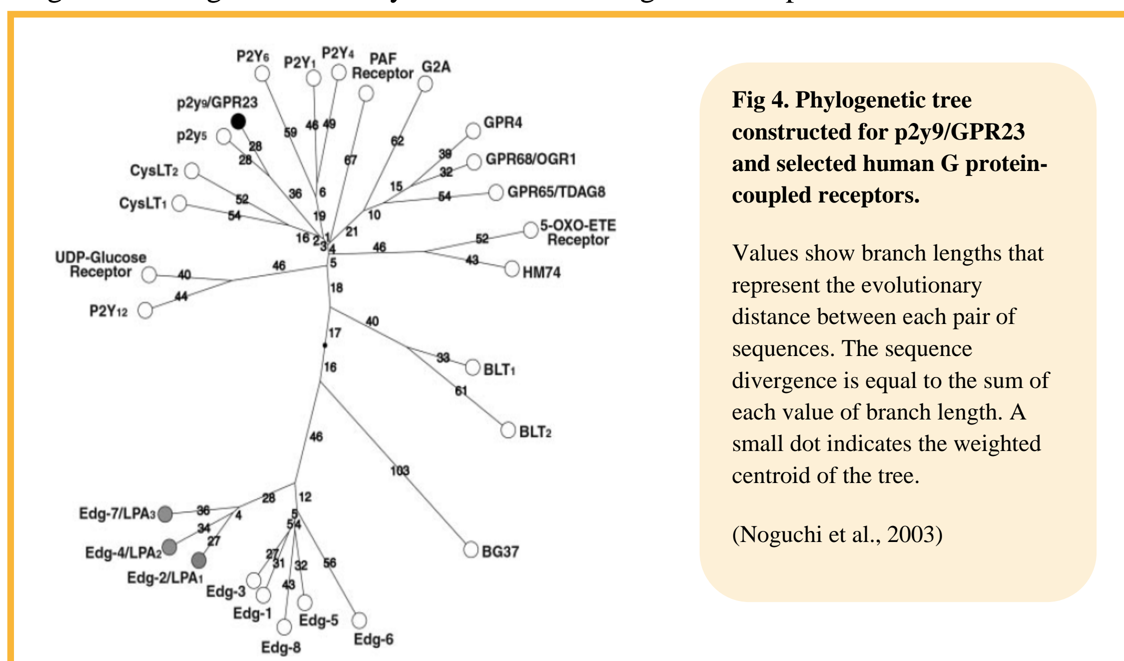


Fig 4. Phylogenetic tree constructed for p2y9/GPR23 and selected human G protein-coupled receptors.

Values show branch lengths that represent the evolutionary distance between each pair of sequences. The sequence divergence is equal to the sum of each value of branch length. A small dot indicates the weighted centroid of the tree.

(Noguchi et al., 2003)

GENERAL AIMS

Current evidences suggest the existence of a specific guanine-based purinergic system on the brain in addition to the well-characterized adenine-based purinergic system. Guanosine and related purines (guanine, GMP. GDP, GTP) modulate glutamatergic parameters, such as glutamate uptake by astrocytes and synaptic vesicles, seizures induced by glutamatergic agents, response to ischemia and excitotoxicity, and are able to affect learning, memory and anxiety. Additionally, guanine-based purines exert important trophic functions, playing an important role in the development, structure, or maintenance of neural cells and astrocytes.

Several studies have shown that guanine-based purines exert biological effects especially on the Central Nervous System (CNS), possibly through membrane receptors, but at the present there are no reports related to the identification of such specific receptor(s)

Recently our unpublished data have shown that guanosine exert antiproliferative effects in a human glioma cell line (U87), and that those effects seems to be modulated by G protein coupled receptor GPCR 23, also known as LPA4 receptor. Indeed, the silencing of this receptor reduces significantly the antiproliferative effects of guanosine, while stably transfected cell clones over-expressing GPCR23 increase sensitivity to guanosine.

With the present study we aimed to:

- Analyze and characterize the specific interaction between guanosine and GPCR23, both in U87 cell lines and in brain tissues;
- Evaluate GPCR23 activation by guanosine, both in U87 cell lines and in cerebral cortex;
- Evaluate downstream signaling activated by guanosine in cerebral cortex.

MATERIALS AND METHODS

U87 Cell cultures

The human tumor cell lines were obtained from the American Type Culture Collection (ATCC, Teddington, UK). Human glioma cell lines, (U87-MG ATCC number: HTB-14 and U373-MG ATCC number: HBT-17) were cultured in RPMI 1640 (Cat. No. 61870-010, GIBCO, Invitrogen, Scotland, UK). The growing medium was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Cat. No. 10270-106, Gibco, Invitrogen, Scotland) and penicillin-streptomycin (50 units-50 μg for ml). Due to the supplementation with serum, a final concentration of 0.5 μM GUA and 0.1 μM GUO was present in each growing media and these concentration can be considered negligible for experiments herein reported. The cell cultures were incubated at 37° C in a humidified 5% CO2 incubator and the culture medium was changed twice a week.

siRNA transfection

U87 cells were transfected with siRNAs at 50% confluency using Oligofectamine (Invitrogen). The day before transfection, the cells were trypsinized, counted, plated at 4x103 cells/well in 96 well plates "Nunclon TM Microwell TM" (Nunc) in the medium containing 10% FBS, and were incubated at 37°C. After 24 h, cells were transfected according to the manufacturer's procedure in the absence (mock transfected), in the presence of the ctrl RNA, or in the presence of specific siRNAs (final concentration 50 nM). siRNAs were chemically synthesized by MWG Biotech AG (Germany) using the following primers: GPR23-siRNA: sense sequence: 5'-GAUCUCUGGAACUGCAUUC-dTT-3', antisense: 5'-GAAUGCAGUUCCAGAGAUC- dTT-3').

Human GPR23: subcloning in expression vectors and generation of stably transfected cell lines

The PCR product containing the entire coding sequence of the human GPCR23 mRNA was cloned into the expression vector pcDNATM 3.1D/V5-His-TOPO (Invitrogen, life technologies, UK). The GPCR23 ORF fragment was amplified with the following primers: Fw 5'-CACCATGGGTGACAGAAGAT-3' and Rv 5'-TGCTAGAATC CACCTTTTAG-3'. To confirm the orientation of the GPCR23 insert, the construct, pcDNA 3.1 D/V5-His-TOPO/GPR23 was subjected to restriction enzyme digestion in the appropriate analysis buffer with BamH I (Promega, Madison, USA) and, to double digestion with XbaI/HindIII (Promega, Madison, USA). The digestion products were analyzed by electrophoresis in 0.8% agarose 1 x TAE gel and ethidium bromide staining. Moreover, to confirm the identity of the GPCR23 entire open reading frame, DNA sequencing was performed by standard fluorescent dideoxy chain-termination procedure with the Abi Prism 377 automatic sequencer (MWG). MACAW alignment allowed us to compare the sequence of the insert with the GPCR23 gene. U87 cell lines were stably transfected with the pcDNA 3.1D/V5-His-TOPO /GPCR23, or with the control vector, pcDNA 3.1D/V5-His-TOPO/lacZ, using lipofectamine reagent (Invitrogen, life technologies, Carlsbad, CA) according to the manufacturer's procedure. Cells were cultured in the growth medium supplemented with geneticin (G418, 600 ug/ml). After 1-2 week, individual clones were isolated with the purpose of selecting clones with the highest expression of the receptors. The different clones were maintained in their medium containing 500 ug/ml G418.

Animals

In vivo / ex vivo studies were performed in Wistar rats (200–250 g) obtained from our animal facility. The rats were housed under alternating 12 h periods of light and darkness in a temperature (24±2 °C) and humidity-controlled room. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12,1987; NIH Guide for the Care and Use

of Laboratory Animals, NIH Publication no. 80-23, 1985 and Guidelines for the Use of Animals in Biomedical Research, Thromb. Haemost. 58, 1078–1084,1987). All efforts were made to minimize the number of animals used and all experiments were approved by the local ethical committee.

In vivo treatments

Rats were treated with guanosine (Sigma-Aldrich Chemie Steinheim, Germany, G-6264) dissolved in saline and administered systemically with a single intraperitoneal (i.p.) injection of 7.5 mg/kg. Control rats received the same amount of saline. Rats were sacrificed at different time points from treatment. Rats were killed under deep anesthesia, brains were rapidly removed and cortex dissected out, quickly frozen in cooled isopentane and stored at -70°C. Three independent experiments have been made and each experimental group consisted of at least four rats. Dissected cortexes, were homogenized at 4 °C in cold RIPA buffer (50mM TrisHCl pH7.4, 150mM NaCl, 1% Triton X-100, 0.1%SDS and H2O), protease inhibitor cocktail (Sigma-Aldrich, cat. num P8340), phosphatase inhibitor cocktail 2 (Sigma-Aldrich, cat. num P5726) and incubated on ice for 30 min.

Primary cortical neuron cultures

Primary cultures of rat cortical neurons were prepared from the cortex of E18 old Wistar rat embryos. After treatment with trypsin 2.5% for 25 minutes at 37° C in PBS-BSA- Glucose solution, cortexes were washed in PBS-BSA glucose with 50% Horse serum in order to stop trypsin activity, and disrupted with a Pasteur and 22 GA syringe. The cell suspensiones were seeded in 12-well plates coated with poly-L-lysine (0.5mg/ml) at the density of 8×105/well (for in vitro studies) or in 100 mm well with poly.L-lysine (0.5mg/ml) at the density of 12x106 (for binbing studies). After the cells were attached to the substrate, were incubated in serum-free Neurobasal medium with 2% B27 ingredient (Invitrogen, 21103-049), 0.5mM L-glutamine, 100U/ml penicillin/100U/ml streptomycin), in a humidified atmosphere of 5% CO2 at 37°C, with half of the medium being changed every 3 days. To suppress the glial growth of

dividing cells, after one day cytosine β-D-arabinofuranoside 1mM (c-Ara 1768Sigma-Aldrich) was supplemented in the medium, and left for 48h. Cortical neurons were maintained in culture and on day 12 were exposed to treatment.

In vitro treatments

Cortical neurons on day 12 were exposed to treatment with guanosine (Sigma-Aldrich Chemie Steinheim, Germany, G-6264) according to dose-effect and related time points shown in results section. At the end of treatment and after washing with ice cold PBS, cells were scraped and incubated for 30 min on ice with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, SDS 0.1%, H20), protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Samples were processed for evaluation of pERK1/2 and pPLCγ levels as described below in Western blotting section.

Western blot for p-Erk1/2 and p-PLCγ analysis in animal tissues and cell preparation

Cortex homogenates and embryonic cortical neurons homogenates were centrifuged at 1,000g for 30 min at 4°C. The supernatant was stored at 20°C, aliquots were taken for protein determination by the method of Lowry et al. 1951 (LOWRY et al., 1951) and 50 ug of proteins were used for p-Erk1/2 and p-PLCγ western blots. The samples and mol. wt. markers (161-0375, Bio-Rad Laboratories S.r.l.,Segrate (MI), Italy), were run on 10% polyacrylamide gel for and p-Erk1/2, and on 6% polyacrylamide gel for p-PLCγ at 100 V and electrophoretically transferred onto nitrocellulose membrane (Hybond-C-extra, GE Healthcare, formerly Amersham, Europe GmbH e Filiale Italiana, Milan, Italy). The membranes were incubated for 1 h in blocking buffer: 1 TBS, 0.1% Tween-20, 5% w/v nonfat dry milk. Following three washing for 7 min. with TBS/T, the membranes were incubated with gentle shaking overnight at 4°C with specific antibody in primary antibody dilution buffer: 1x TBS, 0.1% Tween-20. The following antibodies were used: anti-phosphorylated Erk1/2 antibody (Rabbit phospho-p44/42 MAPK, Thr202/Tyr204 antibody 1:2000; 9101 Cell Signaling) or anti-phosphorylated

PLCγ (rabbit phospho-PLCg1 (Tyr783) antibody, 1:1000; 2821 Cell Signaling). Following three washing for 7 min with TBS/T, the membranes were incubated for 1 h at room temperature with antirabbit IgG horseradish peroxidase-conjugated diluted 1:5000 (Sc, 2004, Santa Cruz Biotechnology) and relative bands were visualized with chemiluminescence reagent (ECL, GE Healthcare, formerly Amersham, Europe GmbH e Filiale Italiana, Milan, Italy) according to the manufacturer's instructions. The blot is exposed to autoradiography film (Amersham Hyperfilm ECL; 28-9068-36), developed in Kodak D19 developer and fixer (Eastman-Kodak, Rochester, NY, USA), and the densitometric evaluation of bands was performed by measuring the optical density (O.D.) using NIH ImageJ software(Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-20011).

Rat tissues membranes

Rats were killed under deep anesthesia and different tissues (hippocampus, cortex, spinal cord, striatum) were dissected, rapidly frozen in cooled isopentane and stored at -70°C until use. Tissues were homogenized in ice-cold Tris-EGTA buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl2, 100 mM NaCl, pH 7.4) by four strokes, using an Ultra-turrax TP 18/10 instrument (Janke & Kunkel, IKA Werk Staufen, Germany) for 5 s at maximum setting level. The homogenate was centrifuged at 600 g, for 5 min at 4°C; the pellet was resuspended and centrifuged again at the same conditions. The supernatant was centrifuged at 16170 g for 20 min at 4°C in a Centra MP4R refrigerated centrifuge with a 851(651) rotor, two times. After each centrifugation the supernatant was discarded and the pellet was resuspended in a minimum volume of ice-cold buffer plus protease inhibitor cocktail (P8340, SigmaeAldrich S.r.l., Milan, Italy). During the quantification step the membranes were kept on ice. Protein concentration was measured by the Lowry assay and the membrane homogenates were stored at -70°C until use.

Cortical neuron - U87 cell membranes preparation

Cortical neurons on day 12, WT - GPCR23 overexpressing - GPCR23 silenced cells, after washing with ice cold PBS, were scraped, incubated in ice-cold Tris-EGTA buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl2, 100 mM NaCl, pH 7.4) with 5µl /mL protease inhibitor cocktail (P8340, SigmaeAldrich S.r.l., Milan, Italy) and sonicated for a minute with a 1 ml insulin syringe. The homogenate was centrifuged at 1000 g for 10 min at 4°C in a Centra MP4R refrigerated centrifuge with a 851(651) rotor. After this centrifugation the pellet was discarded and the supernatant was centrifugated again at 16170 g for 45 min at 4°C. The obtained membranes pellet was resuspended in a minimum volume of ice-cold Tris-EGTA buffer. During the quantification step the membranes were kept on ice. Protein concentration was measured by the Lowry assay (LOWRY et al., 1951) and the membrane homogenates were stored at -70°C until use.

[^{3}H]-Guo-Radioligand Binding Assay

Membranes (50 µg per sample) were incubated for 60 min at 30 °C in a total volume of 0.5 ml 50 mM Tris-HCl pH 7.4 with [^{3}H]-guanosine (Hartmann Analytic). For saturation experiments in U87 cells a concentration range of 5 - 200 nM of [^{3}H]-guanosine was used. For experiments in rat brain areas membranes a concentration range of 5 - 500 nM of [^{3}H]-guanosine was used. Non-specific binding was determined preparing each sample corrisponding to a specific [^{3}H]-guanosine concentration with the addition of 1 mM unlabeled guanosine. Specific binding was calculated by subtracting non-specific from total binding. In competition experiments, displacing agents and 50 nM of [^{3}H]-Guanosine were added and the reaction was started by adding the membranes. After incubation tubes were immediately put on ice and added of 3ml of ice-cold Tris HCl 50 mM pH 7.4 to stop the reaction. Samples were filtrated through Whatman GF/B glass fiber filters (presoaked in tris HCl 50 mM pH 7.4), followed by rapid washing with 5×2 ml volume of ice-cold 50 mM Tris (pH7.4). Filters were dried for 1 hour at 30 °C and immersed in 5 mL of Ready Safe scintillation cocktail (Beckman) in 6 mL scintillation vials. Bound radioactivity was determined after 18

hours by liquid scintillation counting in a Beckman Coulter LS6500 Multipurpose Scintillation Counter.

[^{35}S]-GTPγS Binding Assay

Membranes (50 µg) were pre-incubated for 15 min at 30 °C in GTPγS assay buffer (50 mM Tris-HCl , 0.2 mM EGTA, 3 mM MgCl2, 100 mM NaCl, pH 7.4) plus 50 µM GDP. The reaction was started by the addition of a mixture composed by the tested agonist at the chosen concentrations and 0.6 nM [^{35}S] GTPγS for treated samples and just the addition of 0.6 nM [^{35}S] GTPγS for the control samples, in a final volume of 500 µl. The samples were incubated for 1 h at 30°C. Total binding was defined as the amount of radioactivity observed when 0.6 nM [^{35}S]GTPγS was incubated in the absence of any agonist. Non-specific binding was determined by the addition of 100 µM unlabelled GTPγS. Specific binding was calculated by subtracting nonspecific from total binding. After incubation samples were quickly put on ice and added of 3ml of ice-cold Tris HCl 50 mM pH 7.4. The reaction was stopped by filtration through Whatman GF/B glass fiber filters (presoaked in tris HCl 50 mM pH 7.4), followed by rapid washing with 5×2 ml volume of ice-cold 50 mM Tris (pH7.4). Filters were dried for 1 hour at 30 °C and immersed in 5 mL of Ready Safe scintillation cocktail (Beckman) in 6 mL scintillation vials. Bound radioactivity was determined after 18 hours by liquid scintillation counting in a Beckman Coulter LS6500 Multipurpose Scintillation Counter.

[^{35}S]-GTPγS Autoradiografy

Rats were killed under deep anesthesia by cervical dislocation; brains were removed and immediately immersed in isopentane and stored at -70°C until use. Sagittal sections (14 µm) were cut on a cryostat (Leica CM3050 S) and stored at -70°C until processed as described below. Sections were rinsed in assay buffer (50 mM Tris-HCl , 0.2 mM EGTA, 3 mM MgCl2, 100 mM NaCl, pH 7.4) containing 2 mM GDP for 15 min at 25°C. The sections were then incubated for 2 hr at 25°C in assay buffer with 0.6 nM [^{35}S]GTPγS and 2 mM GDP, with and without different concentrations of the agonist

guanosine. Sections were then rinsed twice (2 minutes each) in ice-cold 50 mM Tris-HCl buffer, pH 7.4 and once quickly in distilled H2O. Sections were dried at room temperature for 1 hour and exposed to autoradiography film (Kodak), developed in Kodak D19 developer and fixer (Eastman-Kodak, Rochester, NY, USA), and the densitometric evaluation of cerebral areas was performed by measuring the optical density using NIH ImageJ software.

RESULTS

U87 CELL MEMBRANES EXPERIMENTS

Binding of [^{3}H]-guanosine to U87 cell membranes

To characterize the binding of Guanosine to GPCR23, we conducted membrane radioligand binding assays using [^{3}H]-Guanosine on U87 wt cells, U87 GPCR23 overexpressing cells and U87 GPCR23-silenced cells. [^{3}H]-Guanosine binding to membrane fractions was greatly enhanced by GPCR23 overexpression and inhibited by GPCR23 silencing (**Fig.5**). In each case, the saturation isotherm studies showed that the binding became saturable at about 70-100 nM of [^{3}H]-guanosine concentrations. Values are the means of five experiments, each point performed in triplicate. The pooled data were fitted by a computerized non-linear regression analysis and resolved for the presence of a single high affinity binding site with an apparent KD=59.5 nM and Bmax=809.5 fmol mg−1 protein in U87 GPR23-overexpressing cells, a KD=43.8 nM and Bmax=499.5 fmol mg−1 protein in U87 wt cells and a KD=54.1 nM and Bmax= 336.7 fmol mg−1 protein in U87 GPR-23 silenced cells.

The Scatchard plot yields a straight line of slope Ka, where Ka is the affinity constant for ligand binding. The affinity constant is the inverse of the dissociation constant. The intercept on the X axis is Bmax. A Scatchard plot is a plot of the ratio of concentrations of bound ligand to unbound ligand versus the bound ligand concentration. It is a method for analyzing data for freely reversible ligand/receptor binding interactions (Voet and Donald, 1995).

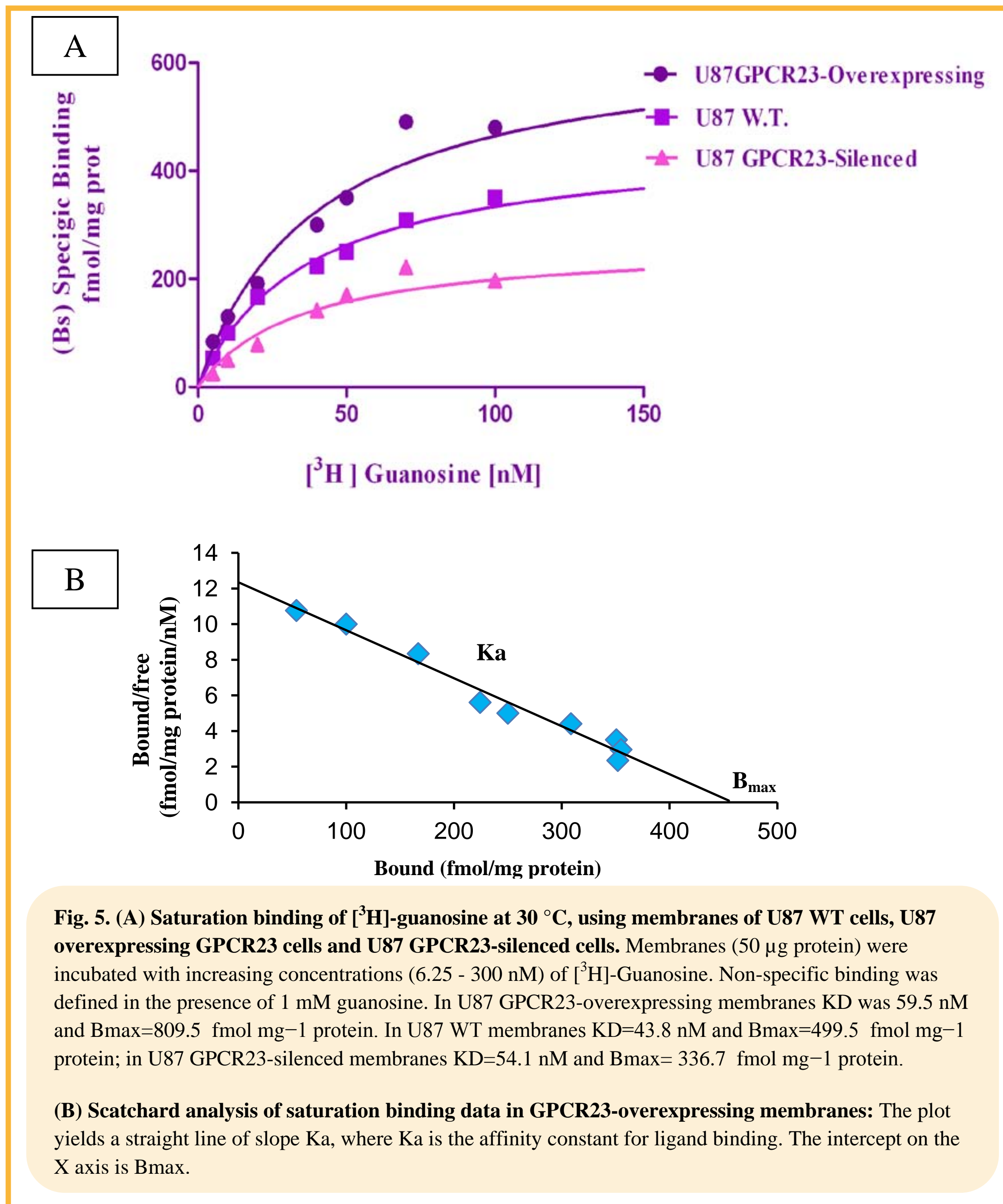


Fig. 5. (A) Saturation binding of [^{3}H]-guanosine at 30 °C, using membranes of U87 WT cells, U87 overexpressing GPCR23 cells and U87 GPCR23-silenced cells. Membranes (50 µg protein) were incubated with increasing concentrations (6.25 - 300 nM) of [^{3}H]-Guanosine. Non-specific binding was defined in the presence of 1 mM guanosine. In U87 GPCR23-overexpressing membranes KD was 59.5 nM and Bmax=809.5 fmol mg−1 protein. In U87 WT membranes KD=43.8 nM and Bmax=499.5 fmol mg−1 protein; in U87 GPCR23-silenced membranes KD=54.1 nM and Bmax= 336.7 fmol mg−1 protein.

(B) Scatchard analysis of saturation binding data in GPCR23-overexpressing membranes: The plot yields a straight line of slope Ka, where Ka is the affinity constant for ligand binding. The intercept on the X axis is Bmax.

Association kinetics

The time course of 5 nM [^{3}H]-Guanosine binding to U87 GPCR-23 overexpressing cells showed that steady state was reached within10 min and was maintained for up to 30 min. (**Fig. 6**). Membranes were incubated with [^{3}H]-guanosine 5 nM under standard assay conditions for the different time intervals (1 min; 5min; 10 min; 20 min; 30 min). 1 mM of cold guanosine was used to detect non-specific binding. The values are the mean of six experiments, each point in triplicate. The association kinetic is very rapid, confirming the specific interaction of Guanosine with its binding site.

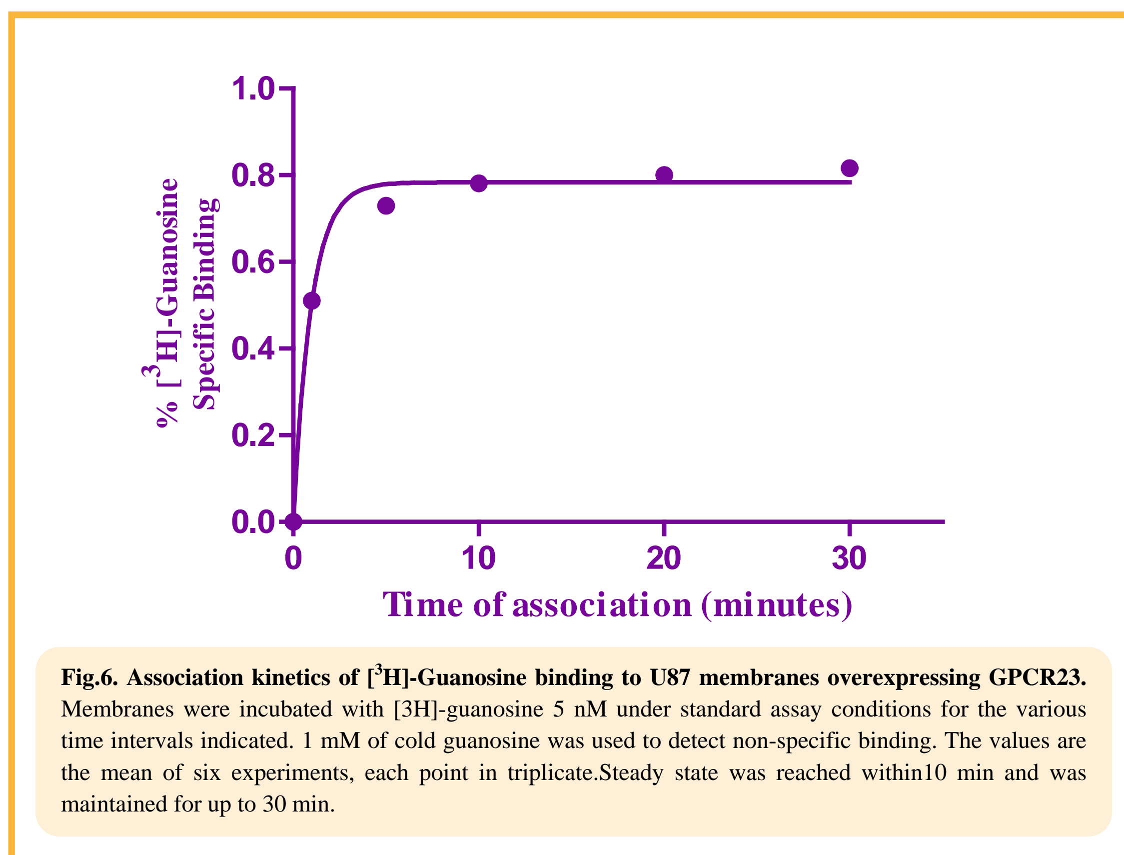


Fig.6. Association kinetics of [^{3}H]-Guanosine binding to U87 membranes overexpressing GPCR23. Membranes were incubated with [3H]-guanosine 5 nM under standard assay conditions for the various time intervals indicated. 1 mM of cold guanosine was used to detect non-specific binding. The values are the mean of six experiments, each point in triplicate.Steady state was reached within10 min and was maintained for up to 30 min.

Displacement studies

To determine the specificity of guanosine binding for its receptor site we tested the ability of other purines (Guanine) and LPA (Lysophosphatidic acid), a previously identified ligand for GPCR23 (Lee et al., 2008;Noguchi et al., 2003), to displace specifically-bound [^{3}H]-guanosine to U87 GPR23-overexpressing membranes. None of

the substances tested were potent displacers of [^{3}H]-guanosine. Displacement curves were resolved by non-linear regression analysis with a one-site model. The addition of LPA caused a weak concentration-dependent reduction of the specific binding of [^{3}H]-guanosine in a concentration range of 1nM – 10µM, and reached about 30% reduction at concentration of 100µM. Only the highest concentration of LPA 1mM was able to significantly displace most of the [^{3}H]-guanosine binding. In contrast, Guanine was unable to displace [^{3}H]-guanosine from its binding sites even at high concentrations, displaying a maximum 25% reduction of the specific binding of [^{3}H]-guanosine at 1 mM concentration. The values are the mean of three experiments, each point in triplicate. Data were fitted by a computerized non-linear regression analysis and resolved with a one site model. (**Fig.7**)

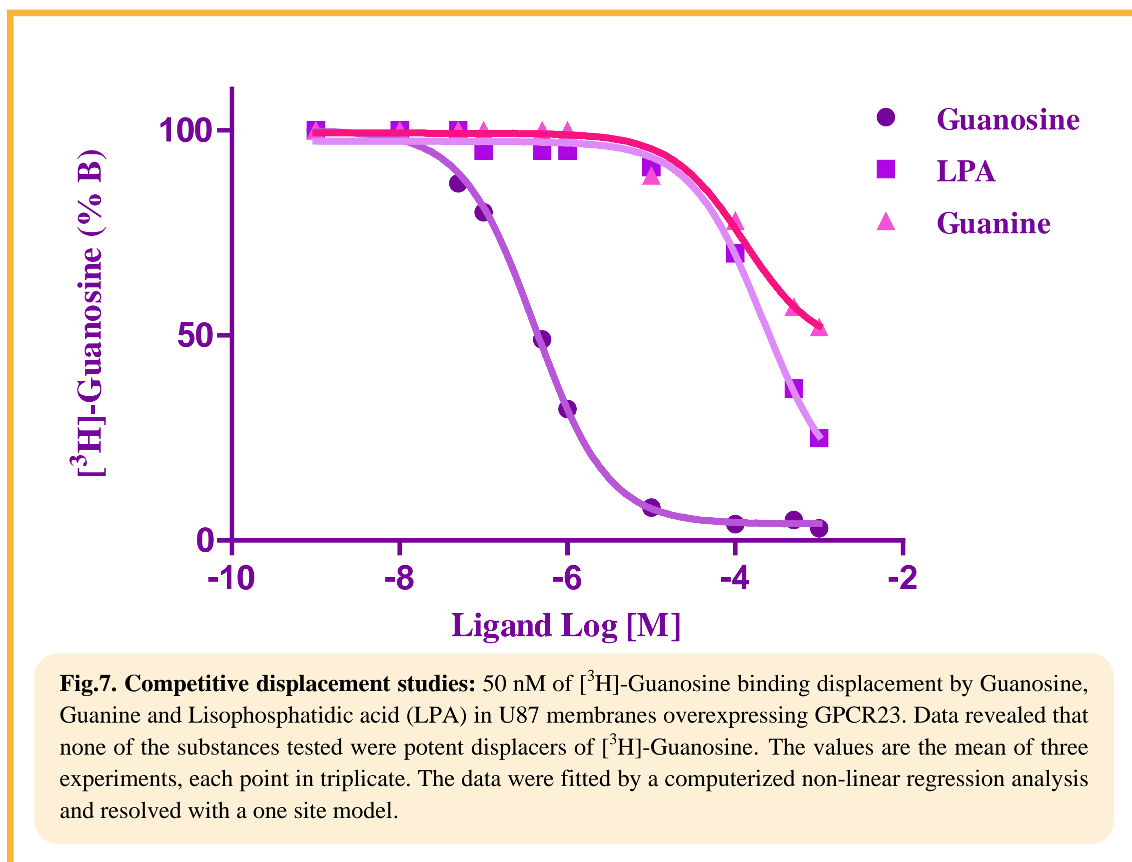


Fig.7. Competitive displacement studies: 50 nM of [^{3}H]-Guanosine binding displacement by Guanosine, Guanine and Lisophosphatidic acid (LPA) in U87 membranes overexpressing GPCR23. Data revealed that none of the substances tested were potent displacers of [^{3}H]-Guanosine. The values are the mean of three experiments, each point in triplicate. The data were fitted by a computerized non-linear regression analysis and resolved with a one site model.

[^{35}S] GTPγS Binding Assay

Because GPCRs stimulation by agonists results in increased binding of GTP to G proteins (which can, in turn, be quantified by measuring [^{35}S]GTPγS binding to purified membranes), activation of GPCR23 was determined by testing the increase of [^{35}S]GTPγS binding following incubation of membranes from U87 wt and U87

GPCR23 overexpressing cells with Guanosine. Optimal [^{35}S]GTPγS binding conditions were determined in preliminary experiments. Concentration–response curves were resolved by non-linear regression analysis using the program Prism 5.00. We found that Guanosine increased [^{35}S]GTPγS binding in a dose-dependent manner both in U87 wt and U87 GPCR23 over-expressing cells. U87 GPCR23 overexpressing cells show higher receptor activation compared to U87 wt cells, with an apparent EC50= 8,067 nM in U87 GPCR23-overexpressing cells and EC50= 51nM in U87 wt. (**Fig. 8**)

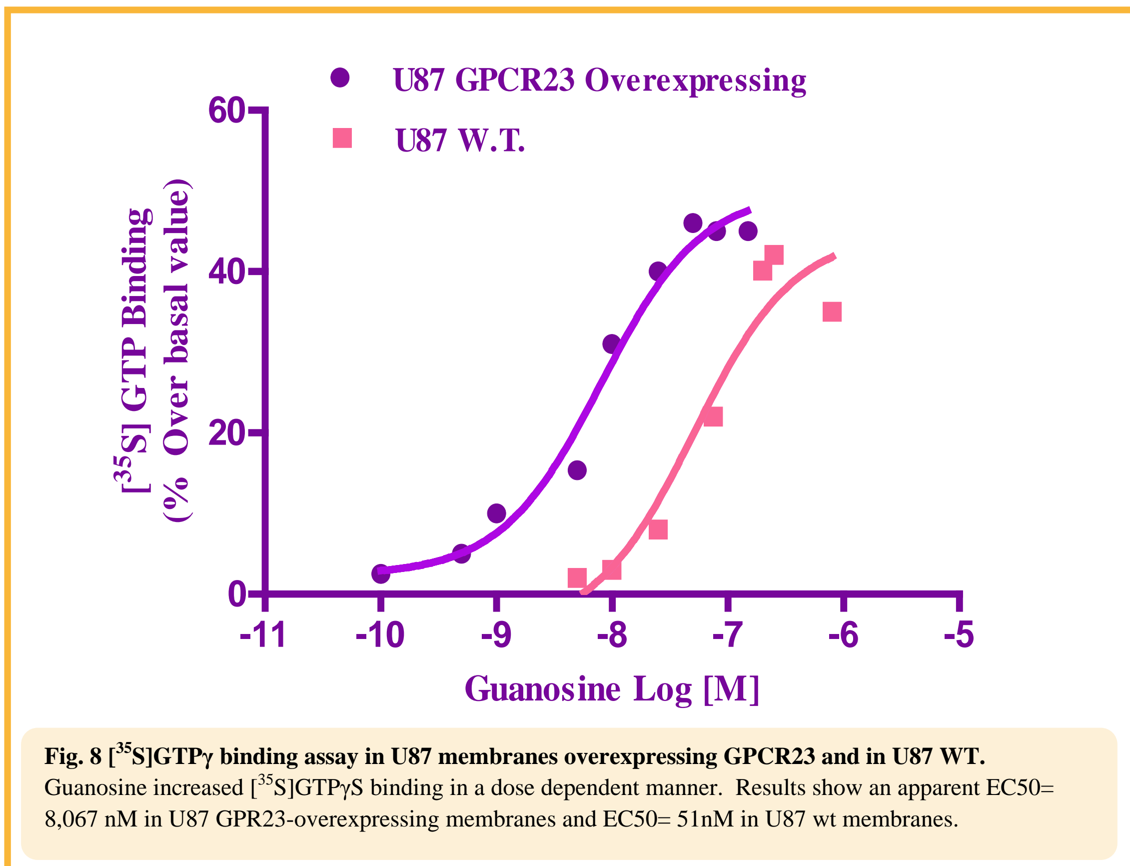


Fig. 8 [^{35}S]GTPγ binding assay in U87 membranes overexpressing GPCR23 and in U87 WT. Guanosine increased [^{35}S]GTPγS binding in a dose dependent manner. Results show an apparent EC50= 8,067 nM in U87 GPR23-overexpressing membranes and EC50= 51nM in U87 wt membranes.

Weaker partial GPCR activation was observed incubating membranes from U87 GPCR23 overexpressing cells with Guanine. In this case we found an EC50= 203 nM and a lower maximal efficacy compared with guanosine, confirming that guanosine is a more potent and more effective GPCR23 ligand. (**Fig.9**)

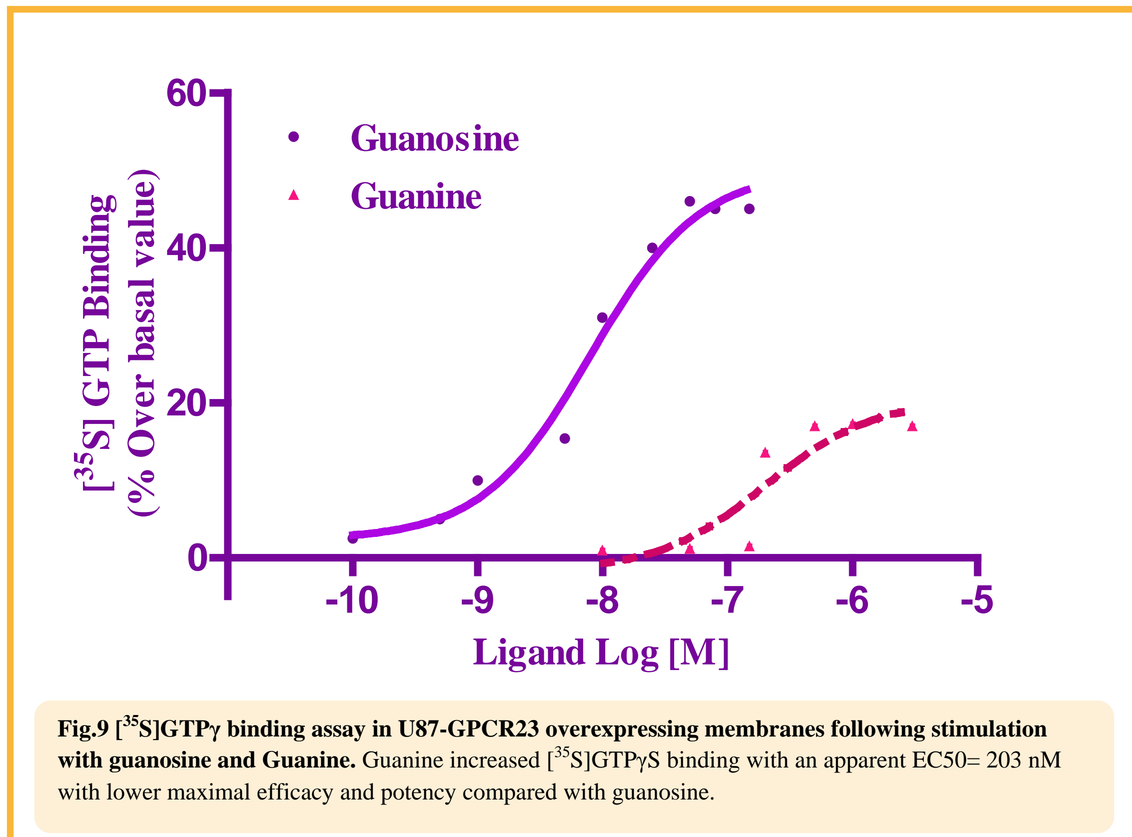


Fig.9 [^{35}S]GTPγ binding assay in U87-GPCR23 overexpressing membranes following stimulation with guanosine and Guanine. Guanine increased [^{35}S]GTPγS binding with an apparent EC50= 203 nM with lower maximal efficacy and potency compared with guanosine.

RAT CEREBRAL CELL MEMBRANES EXPERIMENTS

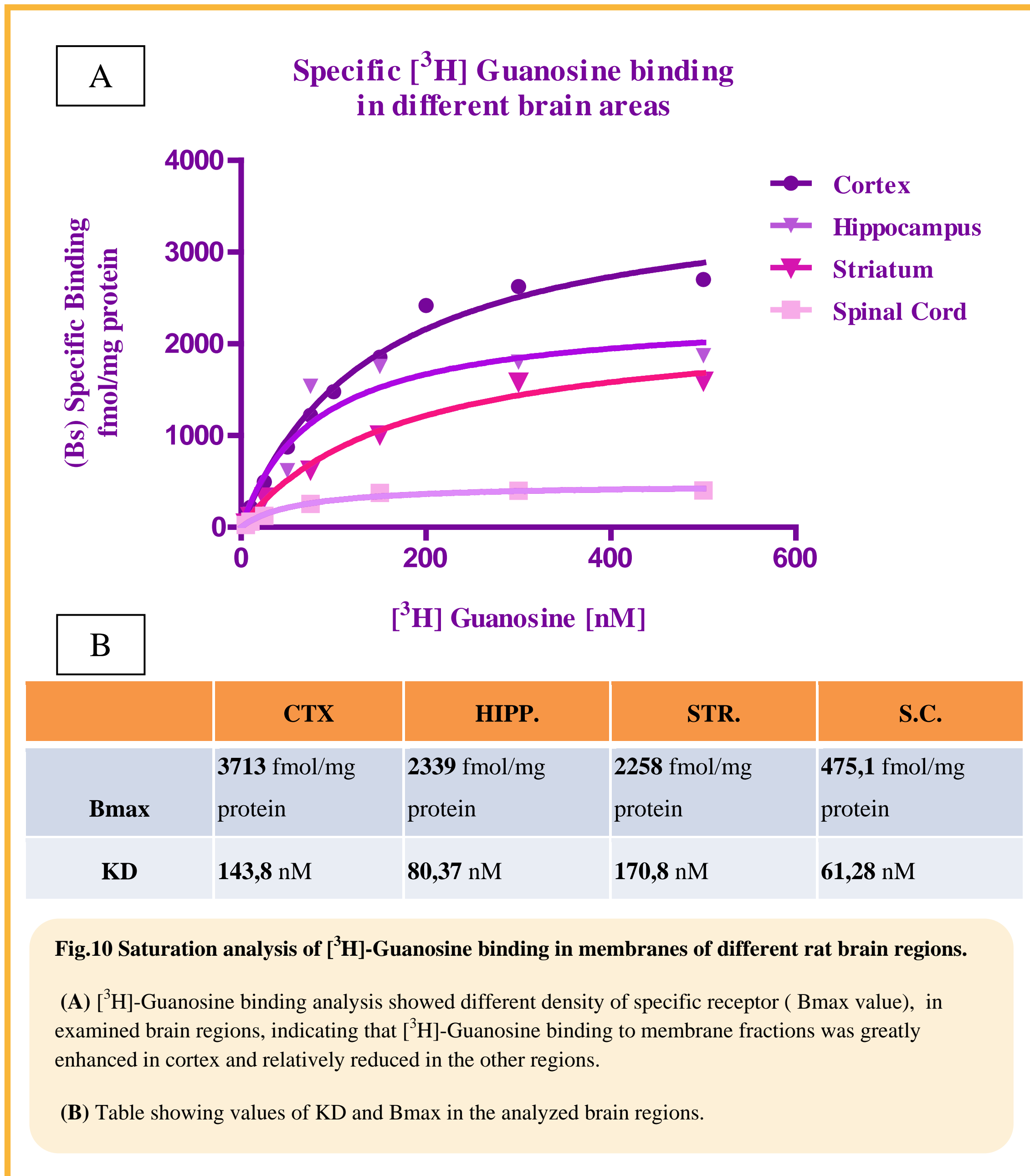
Binding of [^{3}H]-guanosine to different brain region membranes

To characterize the binding of Guanosine to different rat brain areas, we conducted membrane radioligand binding assays using [^{3}H]-Guanosine on cell membranes from cortex, hippocampus, spinal cord, striatum. [^{3}H]-Guanosine binding to membrane fractions was higher in cortex and relatively reduced in the other regions (**Fig. 10**). In each case, the saturation isotherm studies showed that the binding became saturable at about 300 - 400 nM of [^{3}H]-guanosine concentrations. The pooled data were fitted by a computerized non-linear regression analysis and resolved for the presence of a single high affinity binding site with an apparent KD=143,8 nM and Bmax= 3713fmol mg−1 protein in cortex membranes, a KD=80,37 nM and Bmax= 2339 fmol mg−1 protein in hippocampus, a KD= 170,8 nM and Bmax= 2258 fmol mg−1 protein in striatum membranes and KD= 61,28 nM and Bmax= 475,1 fmol mg−1 protein in membranes derived from spinal cord cells (**table B**).

Since the cortex showed the highest binding capacity for guanosine we used cortical membranes to conduct association and displacement studies.

Association and dissociation kinetics

In order to evaluate the association kinetics in brain tissues we conducted association and dissociation experiments in cortical membrane fractions. The time course of [^{3}H]-guanosine binding to cortex membranes showed that the steady state was reached within 60 min and was maintained for up to 3 hours. The dissociation of 50 nM [^{3}H]-guanosine from its binding site was measured by adding an excess of unlabeled guanosine after an equilibration time of 60 min. The addition of 1 mM guanosine reduced [^{3}H]-guanosine binding by about 60% at 5 min, and reached a plateau within 10 min. (**Fig.11**). No further reduction of binding was observed over longer periods of incubation.



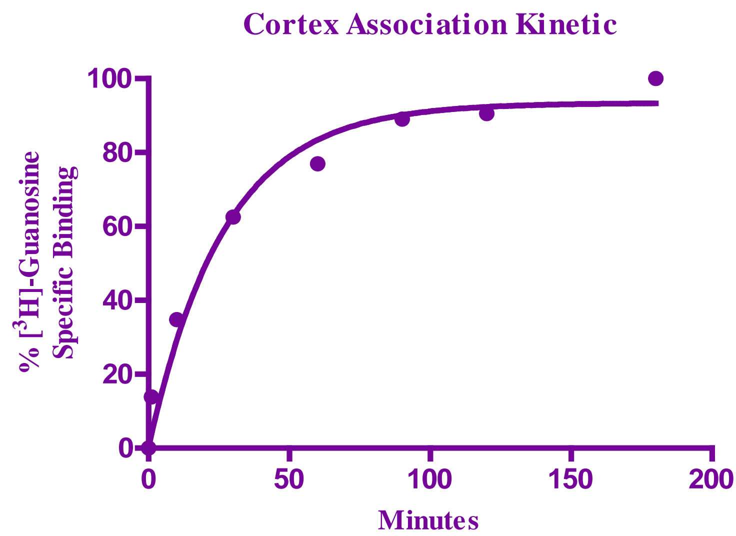
	CTX	HIPP.	STR.	S.C.
Bmax	**3713** fmol/mg protein	**2339** fmol/mg protein	**2258** fmol/mg protein	**475,1** fmol/mg protein
KD	**143,8** nM	**80,37** nM	**170,8** nM	**61,28** nM

Fig.10 Saturation analysis of [^{3}H]-Guanosine binding in membranes of different rat brain regions.

(A) [^{3}H]-Guanosine binding analysis showed different density of specific receptor (Bmax value), in examined brain regions, indicating that [^{3}H]-Guanosine binding to membrane fractions was greatly enhanced in cortex and relatively reduced in the other regions.

(B) Table showing values of KD and Bmax in the analyzed brain regions.

A



B

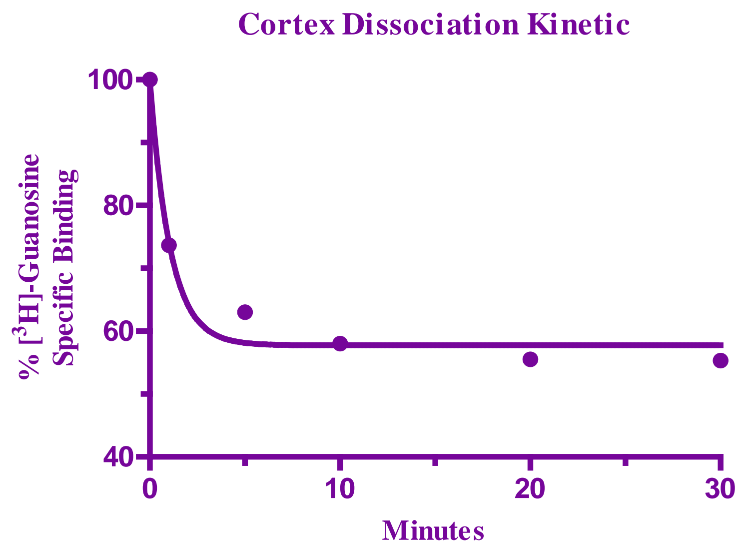


Fig.11 Association and dissociation kinetics of [^{3}H]-Guanosine binding in cerebral cortex membranes.

(A) Time-course of [^{3}H]-Guanosine binding: steady state was reached within 60 min and was maintained for up to 180 min.

(B) Time-course of [^{3}H]-Guanosine dissociation: 1 mM of cold guanosine reduced [^{3}H]-Guanosine binding at about 60% in 5 min and reached a plateau within 10 min.

Competitive displacement studies

To determine the specificity of guanosine binding for its interaction sites in cortex we check the ability of Guanine and LPA to displace specifically-bound [^{3}H]-guanosine to membranes. Displacement curves were resolved by non-linear regression analysis with a one-site model. The addition of Guanine caused a weak concentration-dependent reduction of the specific binding of [^{3}H]-Guanosine in a concentration range of 1nM – 10 µM, and reached about 30% reduction at concentration of 50 µM. Only the highest concentration of LPA 1mM was able to significantly displace most of the [^{3}H]-guanosine binding. (**Fig.12**). These data suggest the specific interaction between guanosine and its binding sites, not displaceable by GPCR 23 ligand LPA.

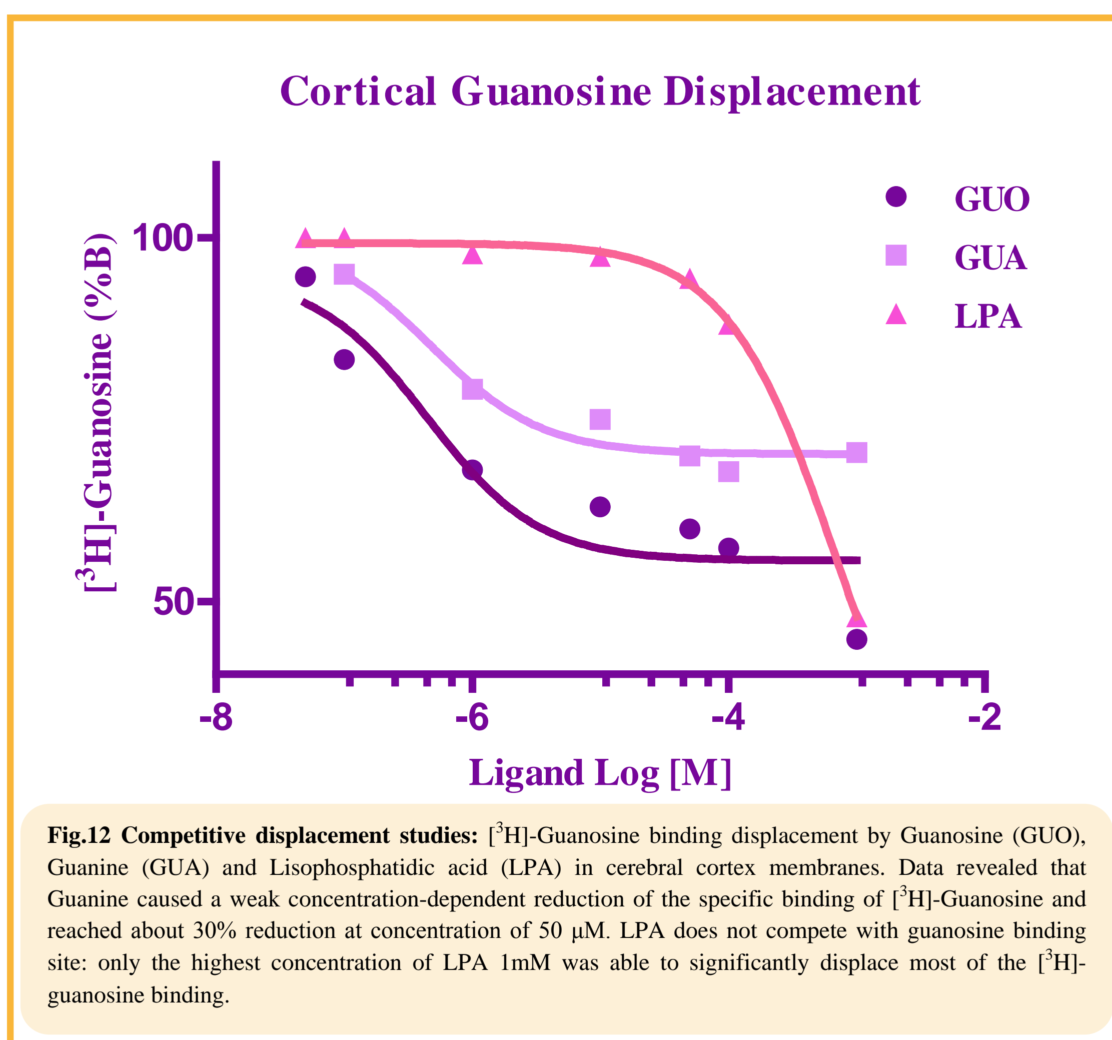


Fig.12 Competitive displacement studies: [^{3}H]-Guanosine binding displacement by Guanosine (GUO), Guanine (GUA) and Lisophosphatidic acid (LPA) in cerebral cortex membranes. Data revealed that Guanine caused a weak concentration-dependent reduction of the specific binding of [^{3}H]-Guanosine and reached about 30% reduction at concentration of 50 µM. LPA does not compete with guanosine binding site: only the highest concentration of LPA 1mM was able to significantly displace most of the [^{3}H]-guanosine binding.

CORTICAL G-PROTEIN COUPLED RECEPTOR FUNCTIONAL ACTIVATION

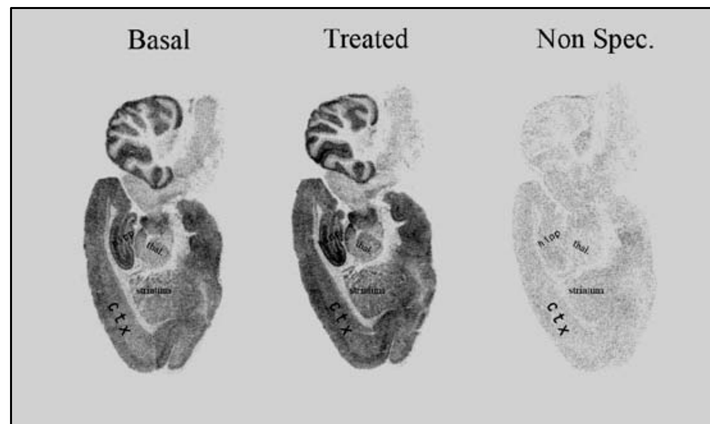
Ligand activity was also evaluated using [^{35}S] GTPγS autoradiography and [^{35}S] GTPγS binding assay, which are a direct measurement of receptor activation in response to agonist occupancy.

Guanosine-stimulated [^{35}S] GTPγS autoradiography

Representative sagittal rat brain sections were processed to examine basal and guanosine-stimulated [^{35}S] GTPγS binding. Brain sections were incubated with different Guanosine concentration (from 500 nM to 1,5 uM) to evaluate GPCR activation. The addition of 800 nM (or more) of guanosine induced stimulation of [^{35}S] GTPγS binding over basal levels. Basal [^{35}S] GTPγS binding was defined in the absence of guanosine and was reduced to minimal levels by adding high concentrations (2 mM) of GDP. Nonspecific binding was evaluated using 1mM of cold GTP. Brain sections were analyzed densitometrically and mean levels of basal and guanosine-stimulated [^{35}S] GTPγS binding are showed in **Fig. 13.**

Guanosine-stimulated [^{35}S]GTPγS binding was distributed heterogeneously throughout the brain. In general, low levels of basal [^{35}S]GTPγS binding were found throughout the brain, although higher basal [^{35}S] GTPγS binding was detected in specific regions, such as hippocampus and cerebellum, probably as the result of the presence of endogenous neurotransmitters in the brain (Moore et al., 2000).

Interestingly, the cerebral cortex showed the highest receptor activation following guanosine stimulation.



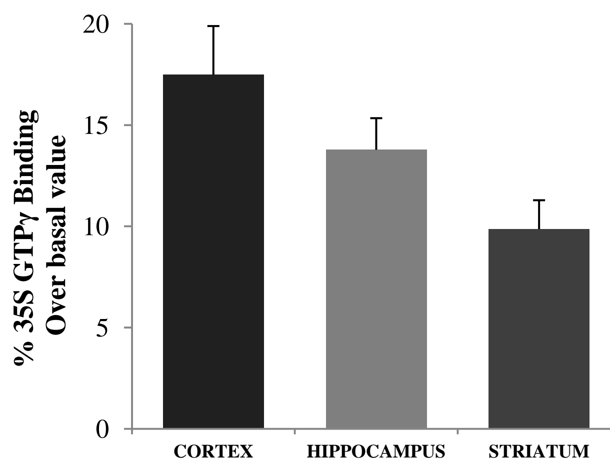


Fig. 13 [^{35}S]GTPγ binding autoradiography Representative autoradiograms of control (basal) and guanosine-treated sagittal brain sections showing [^{35}S]GTPγ binding levels in different brain regions. [^{35}S]GTPγ binding revealed different levels of receptor(s) activation by guanosine in various brain regions. Cortex is the brain structure whit the highest receptor activation after guanosine stimulation. Brain sections were analyzed densitometrically and mean levels of basal and guanosine-stimulated [^{35}S] GTPγS binding are presented.

Guanosine- stimulated [^{35}S] GTPγS Binding Assay in membranes

Rat cortical membranes were incubated with increasing concentration of guanosine to assess total G-protein coupled receptor activation in cortical tissue. Concentration–effect curves were generated in membranes from the frontal pole of the brain. Data were analyzed using GraphPad Prism 5.0.1 (GraphPad, San Diego, CA). Concentration–response curves were fitted using sigmoidal nonlinear regression analysis and a 4-

parameter fit. EC50 (pEC50=log10 •EC50) and intrinsic activity (IA) values were derived from the curve fit. The Intrinsic Activity (IA) is the maximal response of test compound as defined by the top of the asymptote maximum. Experiments were repeated more than 3 times in triplicate and data are shown as mean±S.E.M. Responses were normalized as percentage of [^{35}S]GTPγS binding over basal counts (0%). Guanosine increased [^{35}S]GTPγS binding in a dose dependent manner with an apparent EC50= 100nM. Maximal stimulation of [^{35}S]GTPγS binding was obtained with Guanosine 250 nM, which determined an increase (about 21%) in [^{35}S]GTPγS binding to cortical membranes. (**Fig.14**)

These results confirmed the presence of functional G-protein coupled receptor(s) responsive to guanosine

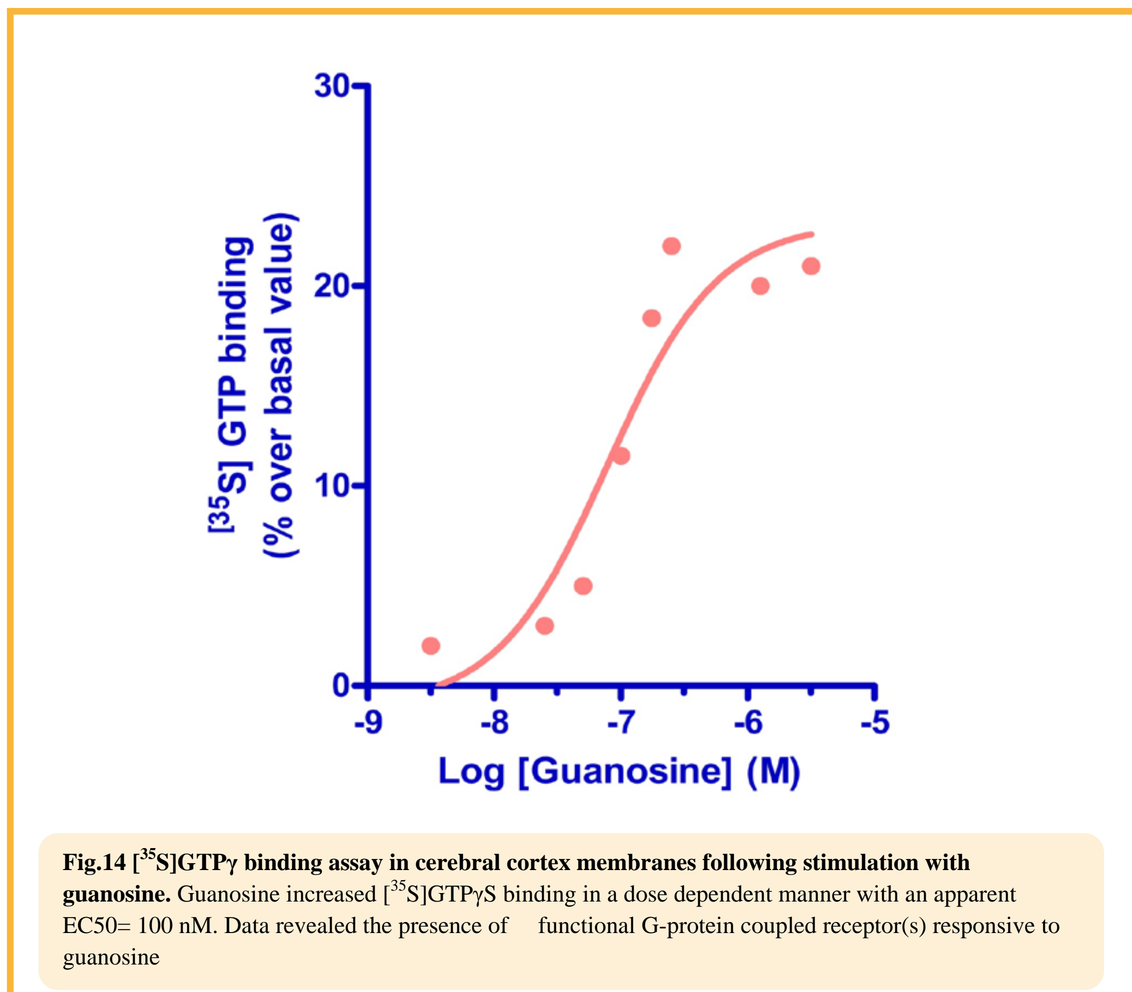


Fig.14 [^{35}S]GTPγ binding assay in cerebral cortex membranes following stimulation with guanosine. Guanosine increased [^{35}S]GTPγS binding in a dose dependent manner with an apparent EC50= 100 nM. Data revealed the presence of functional G-protein coupled receptor(s) responsive to guanosine

EVALUATION OF DOWNSTREAM PATHWAYS ACTIVATED BY GUANOSINE

Since cortical tissue showed functional binding sites for guanosine, further studies have been carried out to evaluate downstream signaling activated by guanosine interaction with its receptors, both in vitro and in vivo.

In view of the fact that many works underline guanosine effects in rat brain after i.p. administration of 7,5 mg/Kg of Guanosine, rats were treated with this dose of the purine for different time points: 7 minutes, 30 minutes, 1 hour, 4 hours, 8 hours and 24 hours. Cortex of both controls and treated rats was dissecetd to value pathways activation. (**Fig. 15**) In vivo experiments showed p-ERK1/2 enhanced phosporylation 7 minutes after administration with a pick-effect after 30 minutes, probably due to a receptor activation. In one hour this effect seems to decrease and then rising again getting the maximum effect 8 hours after treatment. This rebound could be ascribed to a more complex cellular rearrangement.

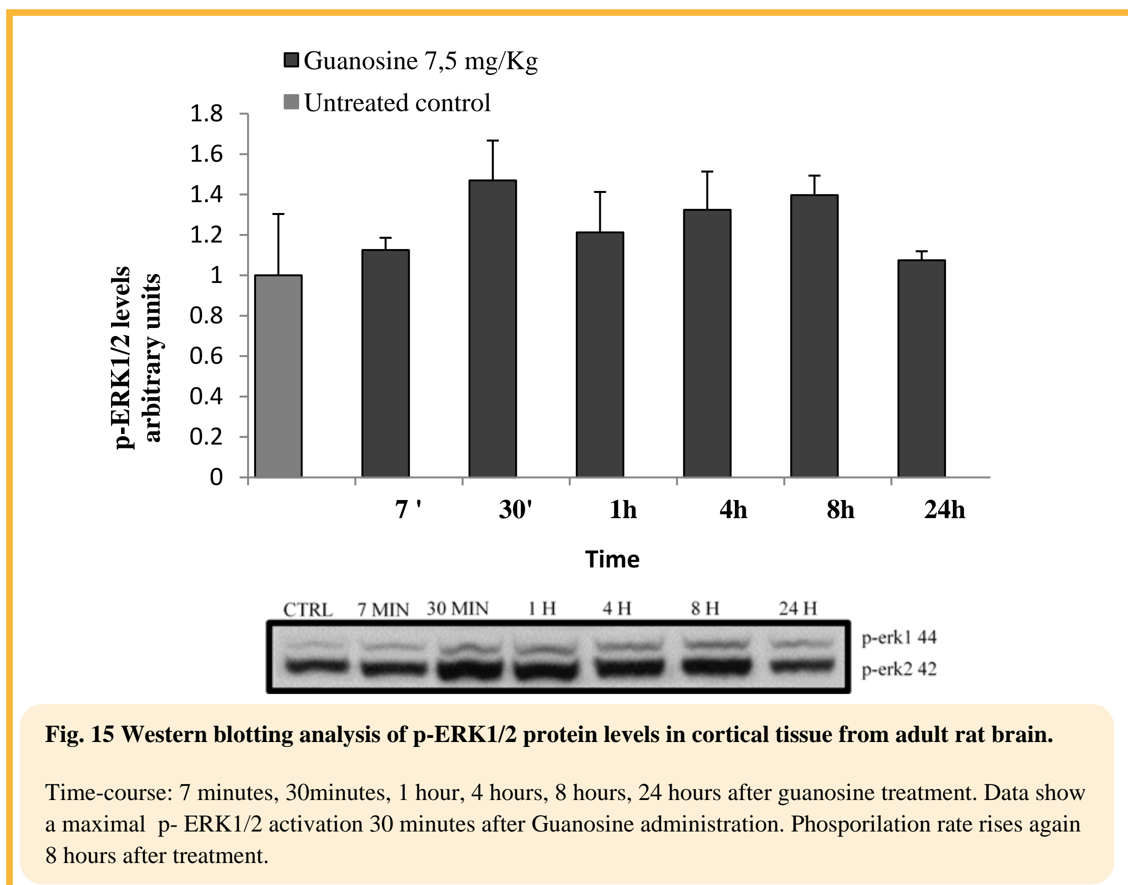


Fig. 15 Western blotting analysis of p-ERK1/2 protein levels in cortical tissue from adult rat brain.

Time-course: 7 minutes, 30minutes, 1 hour, 4 hours, 8 hours, 24 hours after guanosine treatment. Data show a maximal p- ERK1/2 activation 30 minutes after Guanosine administration. Phosporilation rate rises again 8 hours after treatment.

We also treated embryonic cortical cells with many concentrations of guanosine for different time points of treatment. In particular, we analyzed phosphorylation of PLCγ and ERK1/2 using immunoblotting, with PLCγ and ERK phospo specific antibodies. Guanosine treatment resulted in enhancement of both PLCγ and ERK phosphorylation (**Fig. 16-17**). Dose-effect esperiments showed a dose dependent phosphorylation of ERK1/2 and PLCγ, with a maximal activation with 100-200 μM of Guanosine. In each case time course evaluation underline the maximal effect after 7,5 minutes of guanosine administration.

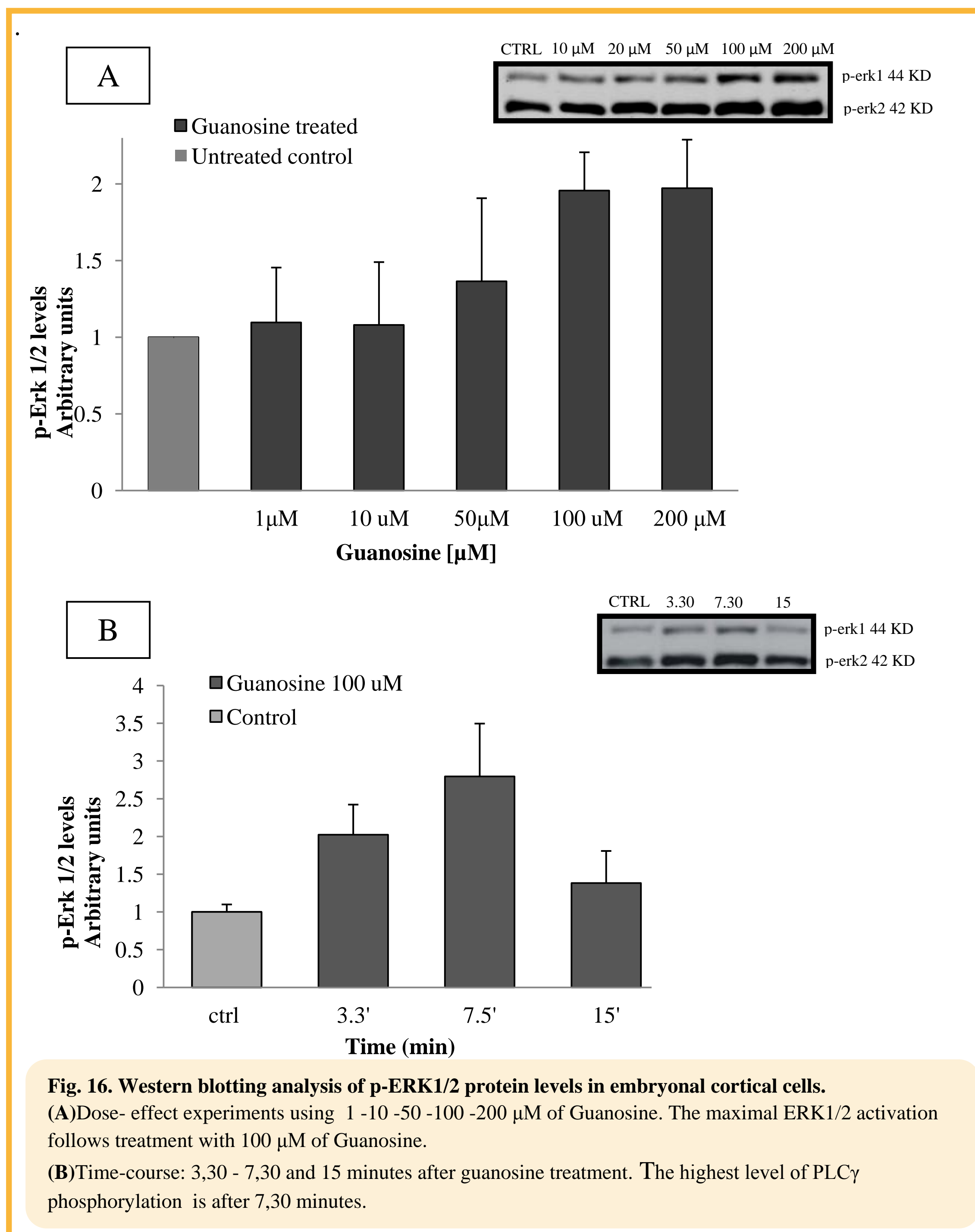


Fig. 16. Western blotting analysis of p-ERK1/2 protein levels in embryonal cortical cells.
(**A**)Dose- effect experiments using 1 -10 -50 -100 -200 μM of Guanosine. The maximal ERK1/2 activation follows treatment with 100 μM of Guanosine.
(**B**)Time-course: 3,30 - 7,30 and 15 minutes after guanosine treatment. The highest level of PLCγ phosphorylation is after 7,30 minutes.

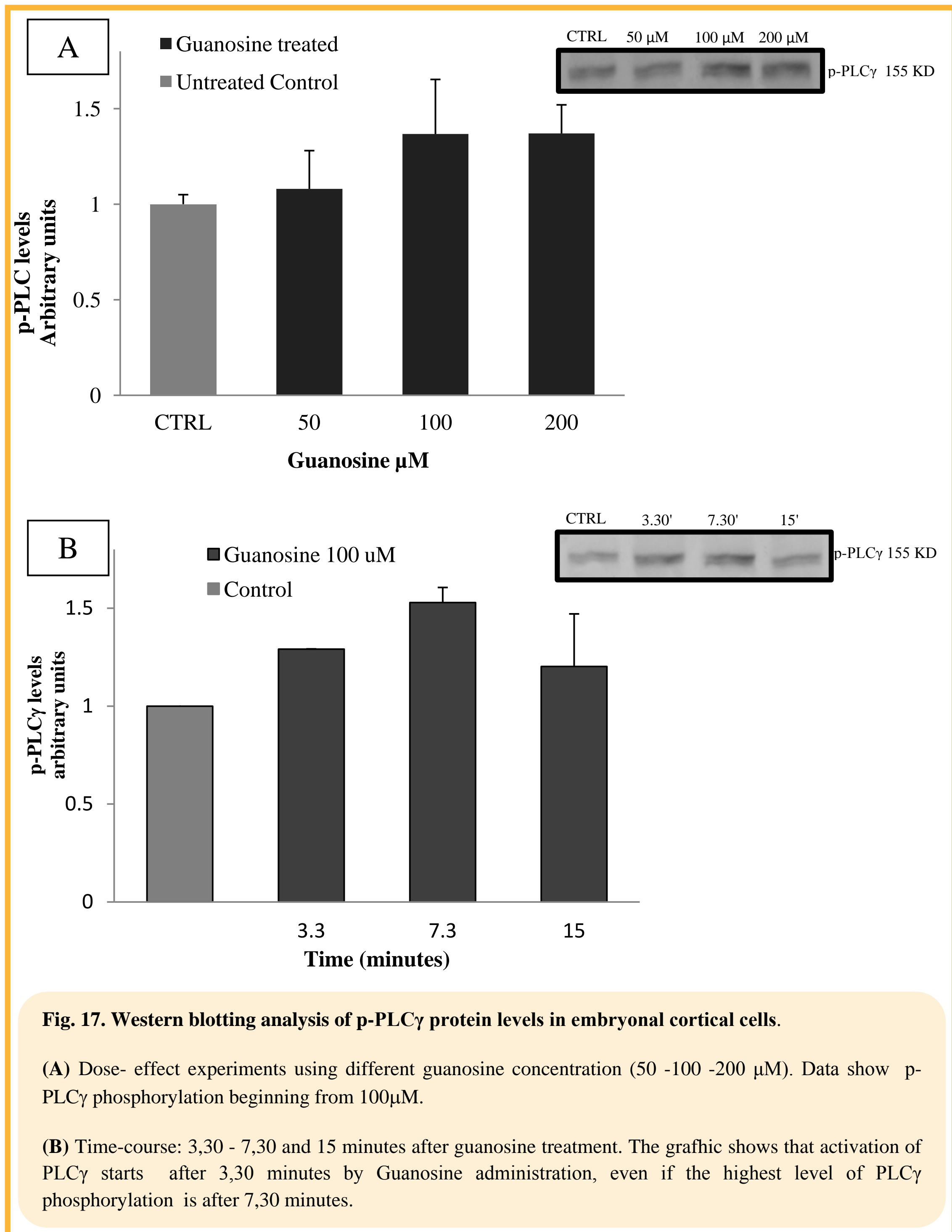


Fig. 17. Western blotting analysis of p-PLCγ protein levels in embryonal cortical cells.

(A) Dose- effect experiments using different guanosine concentration (50 -100 -200 µM). Data show p-PLCγ phosphorylation beginning from 100µM.

(B) Time-course: 3,30 - 7,30 and 15 minutes after guanosine treatment. The grafhic shows that activation of PLCγ starts after 3,30 minutes by Guanosine administration, even if the highest level of PLCγ phosphorylation is after 7,30 minutes.

DISCUSSION

Guanine-based purinergic system plays significant roles in the nervous system, providing new targets for neuroprotection and neuromodulation. Indeed, Guanosine and Guanine nucleotides exert plenty of extracellular brain effects both in in vitro and in vivo studies: neuroprotective modulation of the glutamatergic system; synthesis and release of trophic factors; protection against hypoxia and hypoglycemia; reduction of hyperlocomotion both in schizophrenia models and in experimental spinal cord injury models; prevention of beta-amyloid-induced apoptosis; amnesia and antidepressant effect.

Guanine-based purines have been relatively neglected when compared to adenine-based purines even if this system represents a very interesting target for new pharmacological options to treat neuronal diseases.

In the recent past a modest advance on guanosine receptor characterization and elucidation of its mechanism of action has been emerged, since data suggesting G-protein coupled receptors for guanosine in rat brain membranes have been shown (Traversa et al., 2002;Traversa et al., 2003;Volpini et al., 2011); furthermore, it has been demonstrated that several effects of Guanosine may be mediated through signalling pathways dependent on G-proteins, which involve cyclic nucleotides or MAP kinases (Di et al., 2004;Gysbers and Rathbone, 1992;Rathbone et al., 1991).

Together all these data raise the hypothesis that activation of unidentified cell-surface receptors is involved in mediating Guanine-based purines (GBPs) effects.

Assuming a possible receptor-mediated mechanism for (GBPs), our group, in collaboration with Condorelli's team from University of Catania, speculated about a specific G-protein coupled receptor implication. According to our hypothesis, GPCR 23 may represent the first identified specific receptor for Guanosine. Indeed, in our experiments, GPCR23 silencing reduced Guanosine antiproliferative effects in U87 glioma cell line; moreover GPCR23-overespressing clones, stably trasfected with recombinant expression vectors, displayed an enhanced sensitivity to Guanosine. The GPCR23 gene (Accession Number NM_005296) (also called P2Y9 and P2Y5-like because it shares a high homology with human P2Y5) is located on chromosome Xq13-q21.1. The protein sequence shows 33% identities and 56% conserved amino acid

residues vs P2Y1 ADP receptor. Moreover specific sites, involved in ligand interaction and conserved in P2Y protein family, are present in GPCR23 protein. The high homology with purinergic receptor, together with GPCR23 involvement in antiproliferative effect of guanosine in U87 glioma cell line, let us speculate a direct interaction between Guanosine and the above-mentioned receptor.

Thus, the main aim of the present thesis was to evaluate and characterize the interaction of Guanosine with GPCR 23, and to assess the functional activation of GPCR23 and the downstream signaling in response to Guanosine,

The results of the present study identify GPCR23 as the first G protein-coupled receptor, for guanosine. Our data report the presence of a high-affinity binding site for [^{3}H]-guanosine in U87 human glioma cell line membrane preparations.

[^{3}H]-Guanosine radioligand binding assay reveals that [^{3}H]-Guanosine binding to U87 membrane fractions is greatly enhanced by GPCR23 overexpression and inhibited by GPCR23 silencing. The saturation curve indicates the presence of a single high affinity binding site since it is resolved by non-linear regression analysis. Membrane fractions of U87 glioma cells stably transfected with p2y9/GPCR23 show a specific binding activity for [^{3}H]-Guanosine, characterized by a dissociation constant (KD) of 59.5 nM and a maximum binding capacity (Bmax) of 809.5 fmol mg^{-1} protein. Instead our results indicates a KD=43.8 nM and Bmax=499.5 fmol mg^{-1} protein in U87 wt cells and a KD=54.1 nM and Bmax= 336.7 fmol mg^{-1} protein in U87 GPCR23-silenced cells (**Fig.5.**). Bmax value estimates the total density of receptors for guanosine; thus, the higher binding ability of [^{3}H]-Guanosine in GPCR23 overexpressing membranes despite WT and GPCR23 silenced membranes correlates with GPCR23 expression and underlies GPCR23 involvement in guanosine binding. Moreover a KD value in order of nanomolar concentration points out a good affinity interaction between Guanosine and GPCR23. Furthermore, association kinetics of [^{3}H]-Guanosine in GPCR23-overexpressing U87 membranes is very rapid, which is characteristic of the binding of several natural compounds to their receptors (**Fig.6**). All together, these results indicates a specific high-affinity binding between [^{3}H]-Guanosine and GPCR23.

The competition curves of guanosine and related compounds, at concentrations ranging over several orders of magnitude, demonstrate the specificity of [^{3}H]-guanosine binding site.

Given that GPCR23 is known to be the fourth receptor for LPA (LPA4), structurally different from EDG receptors, we tested the ability of LPA and the purine Guanine to

displace specifically-bound [^{3}H]-guanosine to U87 GPR23-overexpressing membranes. The binding site for [^{3}H]-guanosine results highly specific and both LPA and guanine are 10 times less effective than guanosine in displacing 50 nM [^{3}H]-guanosine binding (**Fig. 7**). Guanine is a purine structurally analogue of guanosine, except for the presence of a furanosic ribose ring in guanosine. The finding that Guanine is not able to significantly displace the binding of [^{3}H]-guanosine indicates that the two molecules do not compete for the same receptor site, as also reported by Traversa and colleagues (Traversa et al., 2002), and the lower effectiveness of guanine suggests the importance of ribose for the success of ligand-receptor binding.
The fact that LPA, previously identified as a GPCR23 ligand, is less effective in displacing [^{3}H]-guanosine binding and that it is able to compete with Guanosine only at very high concentrations (starting at 100 uM) suggests that probably GPCR23 binds both ligands , but shows higher affinity for Guanosine. However, we can also speculate that the two ligands bind to different sites of the same receptor, working through an allosteric relationship. The hypothesis that GPCR23 may bind both ligands, although structurally very different, is in agreement with data reported by Noguchi (Noguchi et al., 2003) who, on the basis of the Phylogenetic analysis, assumed that the P2Y9/GPCR23 could interact with both lipids or nucleotides . Moreover, the notion that LPA represents the main agonist of GPCR23 has been recently challenged by Yin and co-workers (Yin and Yu, 2009) who, using a newly developed GPCR assay that measures beta-arrestin binding to GPCRs, did not observe any response of GPCR23 to LPA up to 100 μM. Surely, the relationships between LPA, guanosine and GPCR23 require further investigations.

To assess the fnctional activation of GPCR23 in response to guanosine, we performed [^{35}S] GTPγS binding assay experiments both in GPCR23-overexpressing and in wt U87 cell membranes (**Fig.8**). Guanosine induces a functional G protein coupled receptor activation in U87 cells, characterized by an EC50= 8,067 nM in GPCR23 overexpressing cells and an EC50= 51nM in WT cells. Even if these data do not exclude the addional involvement of other GPCRs, they indicate that GPCR23 is activated in response to guanosine. In fact, the lower Ec50 value of GPCR23 overespressing cells, as compared to wt cells, suggests that the presence of GPCR23 contributes to delevelop stronger receptor activation in response to guanosine. The specificity of GPCR23 activation by Guanosine was also assessed by comparing

Guanine and Guanosine GPCR activation ability in U87 GPCR-23 overexpressing cell membranes. We found a weaker partial GPCR activation (Ec50=203nM) in response to Guanine, as compared to guanosine (**Fig.9**), confirming that Guanosine is a more potent and more effective GPCR23 ligand.

Our data showing activation of GPCR by Guanosine are corroborated by several studies. Recently, data from independent groups (Traversa et al., 2002;Traversa et al., 2003;Volpini et al., 2011) have suggested the hypothesis of specific G-protein coupled receptors activated by guanosine in primary cultured rat astrocytes and in rat brain membranes. In particular Volpini et al (Volpini et al., 2011) confirmed the hypothesis of GPCR activation mediated by Guanosine in rat brain membrans using a time-resolved fluorometric (TRF) assay that allows the examination of GPCR activation by monitoring the binding of a non-hydrolysable europium-labeled GTP (Eu-GTP) (Frang et al., 2003). Additionally, according with our data, they demonstrated that guanine is unable to activate the receptor, confirming that the ribose is important for receptor activation.

Since numerous evidences have suggested that guanine based purines modulate many functions on the nervous system and the existence of a specific guanine-based purinergic system has been proposed, in order to correlate the effects of guanosine in the CNS to a putative interaction with specific binding sites and in particular with GPCR activation, we performed, in different brain areas, [^{3}H]-Guanosine radioligand binding assay and [^{35}S] GTPγS binding assay. Among the examined brain tissues, the cerebral cortex showed the highest maximal number of binding sites for Guanosine as compared to other brain regions. The saturation curves indicates in each tested brain area the presence of a single high affinity binding site since it is resolved by non-linear regression analysis with a one-site model. In cortical membranes KD value is 143,8 nM and Bmax 3713 fmol/mg protein. The other considered areas show lower Bmax values for [^{3}H]-Guanosine, with the following rank order: cerebral cortex > hippocampus > striatum > spinal cord (**Fig.10**).

Since cortex shows the highest binding capacity for guanosine, we used cortical membranes to characterize [^{3}H]-Guanosine binding in brain tissue. Specific binding of guanosine is rapid, reversible and saturable, thus fulfils important criteria for the characterization of hormone and drug receptors.

Both association and dissociation kinetics let us to suppose a high-affinity interaction between guanosine and cortical membranes, confirming the presence of specific binding sites (**Fig.11**). Competitive binding experiments with LPA and Guanine, using cortical membranes, confirmed that LPA is not able to significantly displace most of the [^{3}H]-guanosine binding, except for the highest concentration used (1mM). On the other hand, the addition of Guanine causes only a weak concentration-dependent reduction of the specific binding of [^{3}H]-Guanosine, that reaches about 30% at the concentration of 50 μM (**Fig.12**).

Thus, our data indicates the presence of [^{3}H]-guanosine binding sites in the rat brain, highly concentrated in the cerebral cortex, compatible with GPCR23 that, in previous studies, by PCR and in situ hybridization, has been shown to be expressed mainly in the cerebral cortex (data not shown). However, our data do not demonstrate that guanosine binds specifically to GPCR23 and, in addition, other possible membrane binding sites could also exist in cortical tissue, such as unidentified receptors and purine nucleoside transporters (Flanagan and Meckling-Gill, 1997).

The existence of a specific G protein coupled receptor for guanosine in cortical membranes, thus compatible with GPCR23, is also validated by [^{35}S] GTPγS binding assay experiments that demonstrate an activation of a G protein-coupled receptor in response to guanosine both in autoradiography sagittal sections and in cerebral cortex membranes. .

In rat brain sagittal sections guanosine induces stimulation of [^{35}S] GTPγS binding over basal levels. Guanosine-stimulated [^{35}S]GTPγS binding is distributed heterogeneously throughout the brain, and also in this case, cortex is the brain region whit the highest receptor activation in response to guanosine (**Fig.13**). To authenticate this result, we performed [^{35}S] GTPγS binding assay experiments on cortical membranes preparation, confirming the activation of a G protein-coupled receptor in response to Guanosine characterized by an EC50= 100nM (**Fig.14**). According to our results, suggesting the cerebral cortex as the brain area with the highest number of binding sites for guanosine, it has been demonstrated that in basal or physiological conditions, the effects of guanosine on glutamate uptake in brain slices seems to be age (more in young animals)- and structure (more in cortex)-dependent but, in excitotoxic conditions, guanosine is more broadly involved in modulating glutamate uptake (Frizzo et al., 2005;Gottfried et al., 2002;Thomazi et al., 2004).

In order to evaluate downstream signaling activated by guanosine interaction with its binding sites, we conducted in vivo and in vitro experiments.

According to the results shown in this thesis, Guanosine effects in cerebral cortex may be mediated by ERK1/2 and//or PLCγ pathways activation.

I.P. administration of 7,5 mg/kg in rats induced ERK enhanced phosphorylation in cortical tissue already at 7 minutes after injection; this effect is maintained for up to 8 hours, it shows a peak-effect at 30 minutes and disappears at 24 hours after injection (**Fig.15**). The 30 minutes rapid effect could be explained speculating membrane receptor activation by guanosine that quickly stimulates downstream signals. On the other hand, the long-term effect on Erk phosporylation could be ascribed to a more complex biochemical mechanism, involving the stimulation of the synthesis and release of trophic factors and adenine-based purines from astrocytes and other cells, which may, in turn, be responsible for some other effects of guanosine (Ciccarelli et al., 2000).

We have also investigated the effects, in terms of intracellular signaling activation, of guanosine treatment in vitro, in cortical primay neurons. Treatment of cortical neurons with guanosine causes both PLCγ and ERK1/2 pathways activation. In both cases, the phosphorylation starts to be increased at 3.5 minutes, becomes maximal at 7,5 minutes and decreases to basal levels already at 15 minutes following guanosine treatment (**Fig.16-Fig.17**). The earliness and the short duration of the effect suggest that the two explored signaling are direct downstreams of guanosine receptor/s and exclude a mechanism enrolling synthesis or release of new factor or other purines from astrocytes or other cells. Furthermore, the potential involvement in PLC/Erk activation of trophic factors released by astrocytes, which are the main source of such factors following guanosine stmulation (Ciccarelli et al., 2000;Di et al., 2002;Traversa et al., 2002), is excluded in our *in vitro* model, since astrocytes growth and survival are blocked during cortical neurons culturing.

The hypothesis that several effects of guanine based purines, guanosine in particular, may be mediated through G protein-dependent signaling pathways involving cyclic nucleotides or MAP kinases is well supported by several in vivo and in vitro evidences (D'Alimonte et al., 2007;Gysbers and Rathbone, 1996c;Traversa et al., 2002). For instance, according to Traversa and collegues (Traversa et al., 2003), Guanosine caused a dose-dependent increase in intracellular cAMP accumulation in slices of rat cerebral

cortex; furthermore, the ability of guanosine to enhance synthesis of trophic factors in astrocytes is associated with an increase in phosphorylation of ERK1 and ERK2 and is blocked by pretreatment with pertussis toxin (Caciagli et al., 2000); moreover, the antiapoptotic effects of guanosine seem to be mediated by activation of the PI3K/Akt/PKB and MAPK (ERK1/2 and p38) pathways (D'Alimonte et al., 2007).

In conclusion, several studies have shown that guanosine plays significant roles in the central nervous system possibly through membrane receptors, but at the present there are not reports related to the identification of such specific receptor(s). Taken together, our findings demonstrate that GPCR23 is the first Receptor for Guanosine even if further investigation is necessary in terms of physiological, pharmacological and biochemical parameters. Our study lays the foundation for identification of receptors responsive to Guanosine-based purines, both in nervous system and in other peripheral tissues and may provide new targets for neuroprotection and neuromodulation.

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