ORIGINAL ARTICLE

Effect of fluoxetine or adenosine receptor NECA agonist on G-proteins of C6 glioma cells or NK immunocytes

Hana Kovářů [1], František Kovářů [2], Petra Ondráčková [3], Věra Lisá [4], Anna Fišerová [5]

1 1st Faculty of Medicine in Prague, Charles University, Czech Republic
2 University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
3 Veterinary Research Institute, Brno, Czech Republic
4 Institute of Physiology, Academy of Sciences CR, Prague, Czech Republic
5 Institute of Microbiology, Academy of Sciences CR, Prague, Czech Republic

Correspondence to: Prof. RNDr. Hana Kovářů, DSc.
1st Faculty of Medicine in Prague, Charles University
Ke Karlovu 11, 120 00 Prague 2, Czech Republic.
TEL: +420 224965382; FAX: +420 224923077;
E-MAIL: hkovaru@cesnet.cz, hkova@lf1.cuni.cz

Submitted: 2011-04-24 Accepted: 2011-05-20 Published online: 2011-06-29

Key words: **C6 glioma cells; NK cells; 1,4,5 IP3; fluoxetine; astrocyte; trimeric GTP binding protein; G-protein; GPCR; NECA agonist; adenosine receptor; G alpha q/11 signalling; interleukin 2; neuroimmunomodulation**

Neuroendocrinol Lett 2011; **32**(3):274–278 **PMID:** 21712787 NEL320311A10

Abstract

OBJECTIVE: Neurochemical approaches to antidepressant effects and depressive disorder are also focusing on G-protein coupled receptors (GPCR) and subsequent signalling. Trimeric G-proteins play a crucial role in transmembrane signalling, its amplification and processing. It is evident that immune system participates in antidepressant mode of action by neurotransmitter GPCR.

METHODS: We studied the effect of acute administration of fluoxetine or NECA agonist of adenosine receptor (GPCR) on C6 glioma cells and natural killer (NK) cell line, innate immunity. We used immunochemical estimation (ELISA) of the main types of G-protein alpha subunits from isolated membranes of tested cells.

RESULTS: Significant reduction of G alpha q/11 subunits after acute administration of fluoxetine or NECA agonist was found. In contrast, no significant influence of G alpha s or G alpha i1,2 subunit levels of C6 glioma cells were observed. Lowered Gq/11 signalling was in accordance with decreased 2nd messenger 1,4,5 IP3 formation by PLC. Acute effect of fluoxetine or NECA agonist on NK cell line resulted in significantly reduced G alpha q/11 levels without changes in G alpha s and G alpha i1,2. Furthermore, we determined that NECA agonist was able to abolish fluoxetine-evoked G alpha q/11 levels of NK cell line.

CONCLUSIONS: Results show involvement of fluoxetine in the C6 glioma signal transduction and were comparable with NK cells. Similar inhibiton of G alpha q/11 by NECA agonist in both C6 glioma cells and NK cell line was determined. Furthermore NECA induced attenuation of fluoxetine evoked Galpha q/11 signalling can indicate parallel interference between GPCR and final response. Finally, we determined similarity in both interleukin 2, IL2 immunostimulator and fluoxetine evoked G q/11 levels in NK cell line and thus fluoxetine action could be related to signalling aspects of neuroimmunomodulatory activity.

Abbreviations:

AC	- adenylylcyclase
cAMP	- cyclic adenosine monophoshate
G-protein	- trimeric GTP binding protein
GPCR	- G-protein coupled receptor
GTP	- guanosine triphosphate
IP3	- 1,4,5 inositol triphosphate
NA	- noradrenaline
NECA	- 5´-(N-ethylcarboxamido) adenosine
5-HT	- 5-hydroxytryptamine, serotonin
PLC	- phopholipase C
SSRI	- selective serotonin reuptake inhibitor
TCA	- tricyclic antidepressant

INTRODUCTION

Contemporary neurochemical theories suggest that major depression is associated with the imbalance of neurotransmitters serotonin (5-HT), noradrenaline (NA) and dopamine (DA), and antidepressants (AD) reverse this state at presynaptic nerve terminals by mechanisms of reuptake blockade. Moreover, abnormalities are undoubtedly involved. in transmembrane signal transduction via G-protein coupled neurotransmitter receptors and intracellullar signalling cascades modulation are resulting in the activation of multiple pathways (McEwen *et al.* 2010).

Serotonin (5-hydroxytryptamine or 5-HT) plays a pivotal role in the regulation of mood. Although it is generally accepted that 5-HT-mediated processes are fundamental in depressive aetiology, a consensus regarding the specific 5-HT receptor subtypes and 5-HT transporter alterations has not been reached (Millan 2006; Tardito *et al.* 2006).

Serotonergic mechanisms are also active in immunocytes, such as NK cells, lymphocyte subpopulation ($CD56^+$) which play a key role in immunological surveillance against *self-nonself cells*, viral or tumor elements in the organism without prerequisite antigen stimulation NK cells are neurotransmitter receptor rich, highly stress sensitive elements of innate immunity. NK cell activity has been augmented by fluoxetine and paroxetine ex vivo (Frank *et al.* 1999). Impaired NK cytotoxicity (NKCA) was repeatedly demonstrated in major depressive disorder with restoration of activity following antidepressant treatment concurrently with amelioration of depressive symptoms (Frank *et al.* 1999; Evans *et al.* 2008).

Transmembrane signalling system consists of heptahelical receptors (e.g.neurotransmitter) that are coupled with limited repertoire of G proteins and interact with few effector molecules, including adenylyl cyclase (AC), phospholipase C (PLC), cyclic-GMP phosphodiesterase and various ion channels mainly. Functionally important alpha subunits of G proteins are divided into main groups according to their effector effects: G(s), G(i) regulate adenylyl cyclase, and G(q) operates via phospholipase C (Wettschurek & Offermans 2005). Regulation of G protein-coupled receptor (GPCRs) functions includes delayed receptor desensitization and downregulation and induction of adaptive changes in postreceptor signalling cascades and in gene expression, as well as changes in neuroplasticity, cellular resilience, and synaptic plasticity (Tardito *et al.* 2006; Hisaoka *et al.* 2007; Golan *et al.*2010). The basic mechanisms underlying the phenomenon of desensitization and downregulation of GPCRs involve the activities of two families of proteins: G protein-coupled receptor kinases and arrestins (Avissar & Schreiber 2006; Golan *et al.* 2010).

In the family of G protein-coupled receptors, adenosine receptors, AR (P1 purinoceptors) are involved and are divide to A1, A2A, A2B, and A3. In both the immune system and CNS, adenosine participates in regulation of neurotransmission, growth and differentiation events, and immune responses (Lorenzen 1997; Schulte *et al.* 2003). Anxiolytic activity and psychiatric or neurological relationship to modulated adenosine receptors have been described (Lorenzen 1997). It is known that A2b a A3 AR operate via G alpha q/11 (Haskó *et al.* 2008). NECA is suggested as potent A2b agonist of adenosine receptor in immune responses (Jeffe *et al.* 2009).

Rat C6 glioma cells are an established and useful model for studying the phenomena of GPCR desensitization and downregulation (Fishman & Finberg 1987). C6 glioma cells appear to be a suitable model to study the mechanism of action of antidepressants because of no synaptic structures.Thus any change after drug treatment represents a direct "postsynaptic effect" model (Chen & Rasenick 1995; Kovářů *et al.* 2009). The C6 glioma cells are also used to study antidepressant effects on Galpha subunit levels and production of growth factors or cytokines (Hisaoka *et al.* 2007). Furthermore, C6 glioma cells have been shown to respond to antidepressants in a manner similar to primary astroglial cells (Hisaoka *et al.* 2007; Golan *et al.* 2010). The astrocytes are influenced by antidepressant and can represent 5HT receptors only without participation of 5HT transporter (Zhang *et al.* 2010). The absence of own 5HT transporter in C6 glioma cells can be taken in account as not evidenced in available literature data.

Here, we examined the effect of fluoxetine (selective serotonin reuptake inhibitor, SSRI), on G alpha subunit profiles of main G-protein types in C6 glioma cells or rat NK lymphocytes of RNK 16 line, CD45(–). A possible role of adenosine receptor agonist (5´-N-ethylcarboxamido-adenosine, NECA) on G-protein signalling was also analysed.

We also focused our attention on interleukin 2, IL immunostimulator and its effect on G-protein changes NK lymphocytes in respect to neuroimmunomodulatory aspects.

MATERIAL AND METHODS

C6 glioma cells. Cells were cultured in MEM medium, pH 7.3 with 5% fetal calf serum under standard conditions. Confluent cultures were exposed to antidepressant (final 1 μM) for up to 24 h (acute model). Then cells were washed with PBS, harvested by scraping with rubber policeman in PBS, containing proteolytic inhibitors, and cholate membrane extracts were prepared as described previously (Kovářů *et al.* 1998; Fišerová *et al.* 2002; Páv *et al.* 2009).

RNK cells. RNK 16 CD45(–) mutant NK cell line was derived from spontaneous rat leukaemia (F344 strain) and was gift of Dr. Reynolds (NIH, Bethesda). Cells were cultured under standard conditions in supplemented RPMI 1640 medium and 10% fetal calf serum. The modulators were added to cell suspensions (2.10^6/ml) in the following final concentrations: r IL2 - 100 U/ml (Sigma), and 1 μM fluoxetine (Sigma), 0.1 μM NECA (Sigma) for 17 hours. Cholate membrane extracts of cell cultures were prepared as described above. For other details see (Fišerová *et al.* 1997; 2002; Kovářů *et al.* 1998).

G alpha subunit estimation. G alpha chains changes were analysed by ELISA technique with our rabbit antibodies against synthetic C-terminal dekapeptides of alpha chains of Gs, Gi1,2 and Gq/11 (Milligan 1989; 1993). ELISA method of competitive inhibiton was performed (Ransnas & Insel 1989) and modified by use of Maxisorp microtitration plates (NUNC) for noncovalent peptide binding. ELISA method assay estimations was compared with Western immunoblotting (Kovářů *et al.* 1998; 2009). Used monospecific antibodies against individual G alpha dekapeptides were without cross reactivity. Synthetic dekapeptides were prepared in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic,. and primary amino acid sequence of dekapeptides was checked by sequence analysis (Kovářů *et al.* 1998).

Statistical analysis

All results are expressed as the arithmetical mean ±S.E.M. The differences between experimental samples were evaluated by Student´s t- test for unpaired values. P values smaller than $p<0.01$ were marked with (*).

Tab. 1. RNK 16 / CD45 (-) NK lymphocytes.

	% of control		
	G alpha q/11	G alpha s	G alpha i1,2
IL2 (100 U/ml)	60.4 ± 18*	97.3 ± 6	105.7 ± 9
FLUOXETINE (1 μM)	68.2 ± 7*	114.1 ± 9	92.8 ± 7
NECA (0.1 μM)	72.4 ± 9*	88.3 ± 11	117.4 ± 12
+ FLU + NECA	89.3 ± 14	97.1 ± 16	110.1 ± 9

RESULTS AND DISCUSSION

Figure 1 summarizes the acute effect of fluoxetine on C6 glioma cells. Results show highly significant reduction of membrane G alpha α q/11 level confirmed by highly decreased 1,4,5 IP3 concentration (Figure 2), i.e. reduced signalling via G alpha q/11 to effector PLC. Beside this we determined in another study fluoxetine induced translocation of membrane G alpha q/11 subunit into cytosol of C6 glioma cells as a part of desensitization mechanisms (Kovářů *et al.* - in preparation). Two ideas are noteworthy: i) in C6 glioma (astrocytoma) cells with no synaptic structures formed between them, thus alteration observed after fluoxetine treatment represents a direct postsynaptic compartment effect, i.e. fluoxetine effect on postreceptor and postsynaptic level; ii) furthermore, in C6 glioma (astrocytoma) cells, fluoxetine effects can involve probably 5HT 2B receptor linked to Gq/11 pathway probably without 5HT transporter. Cultured primary astrocytes influenced by antidepressant can represent divided functions of 5HT2 receptors only without 5HT transporter (Zhang *et al.* 2010).

Former studies of cultured cells models were focused on tricyclic (TCA)antidepressants mainly and post receptor signalling through G protein coupled adenylyl cyclase pathways were without changes in G alpha subunit levels. (Chen & Rasenick 1995).We know that G alpha q/11 protein is ubiqitous in nervous system and 5HT1 and 5HT2 receptors operate via Gq and PLC. 5HT2 receptors are present in C6 glioma cells and they are coupled via Galpha q/11 protein to the activation of PLC and stimulation of PI hydrolysis (Hubbard and Heppler 2006; Tardito *et al.* 2006). Citalopram (SSRI) antagonizes desensitization of 5HT2A receptor mediated Ca^{2+} mobilization in C6 glioma cells (Kagaya *et al.* 1996).

Another study was based on dissection of the genetic targets of serotonin (5HT), the selective 5HT reuptake inhibitor (SSRI) fluoxetine (Prozac), and the tricyclic antidepressant imipramine. Using the well-established serotonergic response in *Caenorhabditis elegans* egg-laying behavior as a paradigm, they determined that the action of fluoxetine and imipramine at the 5HT reuptake transporter (SERT) and at 5HT receptors are separable mechanisms (Dempsey *et al.* 2005). Furthermore, data on fluoxetine induced 5HT effect are supported by our data – other non-neuronal system – NK cell response corresponds with C6 glioma cells (see Table 1).

The other part of our experiments was focused on GPCR response in respect to adenosine receptor and NECA agonist effect on G protein signalling. We estimated markedly reduced G alpha q/11 level of C6 glioma cells (Figure 1). G alpha q/11 pathway could indicate a role of A2b subtype of AR receptor with low affinity characteristics, but A3R role is not excluded. It is known, that A2b and A3 adenosine receptor subtypes

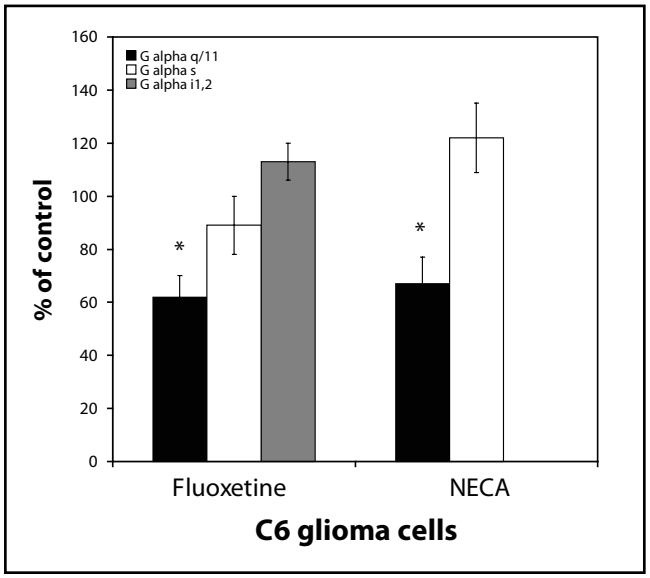

Fig. 1. Acute fluoxetine or NECA effect on G alpha subunit profiles of C6 glioma cells. Results are arithmetical means of three experiments and 6-8 measurements in tetraplets, ± S.E.M.

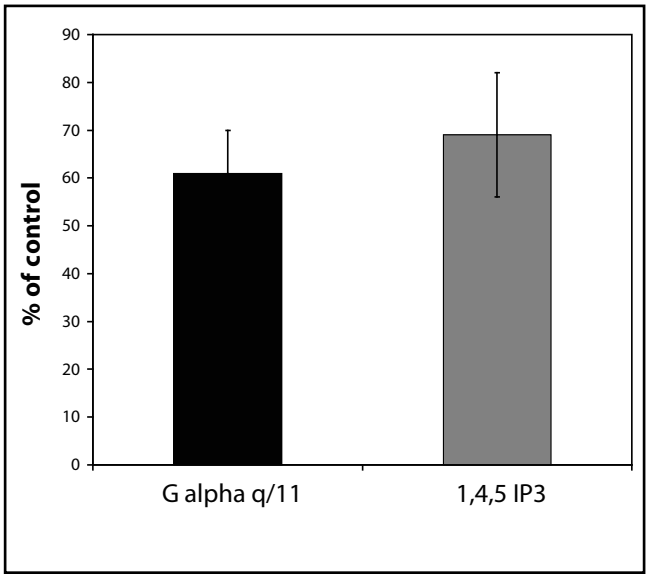

Fig. 2. Acute effct of fluoxetine on Galpha q/11 and 1,4,5 IP3 levels in C6 glioma cells. Results are arithmetical means 3 experiments and 7-8 measurements in tetraplets, ± S.E.M.

operate via G alpha q/11 (Haskó *et al.* 2009; Jeffe *et al.* 2009).

Fluoxetine or NECA agonist (Table 1). Another mechanism of neuroimmunomodulation in relationship to G alpha profiles can be related to adenosine receptor, as analysed with NECA. Our results (Table 1) in NK cell line indicate similar values of fluoxetine or NECA influence on decreased G alpha q/11 subunit compared with C6 glioma cells. Both fluoxetine or NECA agents act via Gq/11 on effector PLC system, indicated by 2nd messenger 1,4,5 IP3 formation, by the enzyme. The NECA as a potent A2b adenosine receptor agonist could participate in the response of NK cells.

The results indicating decreased G alpha q/11 of RNK CD45(–) are in contrast to elevated Gα q/11subunit of another cell line RNK16, CD45(+) cell line. Thus results can indicate that Gα q/11 subunit changes could be related to some membrane influence by CD 45 phenotypic marker, The CD45 marker is phosphatase, regulating protein dephosphorylation (Kovářů *et al.* 2000).

Fluoxetine and NECA effects (Table 1). Fluoxetine induced RNK 16 CD45(–) resulted in similar signaling events, decreased G alpha q/11 which was comparable with NECA alone. Furthermore, data are comparable in both cell systems – NK and C6 glioma cells.

When NECA (in low 0.1 μM) was added to fluoxetine then the abolished Galpha q/11 level of RNK cells was determined. Fluoxetine induced decrease of signalling by NECA is regulated in dose dependent NECA manner. Reduction of G alpha q/11 signalling by fluoxetine by increasing NECA concentration was confirmed by declined levels of 1,4,5 IP3 formation by PLC of C6 glioma cells (Kovářů *et al.* - in preparation). On the other hand, the amplifying NECA effect on fluoxetine evoked apoptosis in C6 glioma cells was observed, indicating distinct pathways in the final cell response (Španová *et al.* 1997; Kovářů *et al.* - in preparation). There are very limited data in literature about adenosine/ adenosine receptor interference with 5-HT receptor signalling .

Interleukin 2, IL2 immunostimulator effects on RNK cells (Table 1) and fluoxetine effects on transmembrane signalling are comparable. If fluoxetine or IL2 effects at postreceptor level of NK cells were similar in the cell signalling, then fluoxetine action could be related to possible neuroimmunomodulatory activity.

We conclude that there is an increasing line of evidence that immune system and brain cells can share common properties of molecular regulations during transduction of neuro-immunomodulator signal(s). It is suggested that future research of neurotransmitter and GPCR systems in neuroimmunomodulation will be fruitfull for both the CNS and immune system and communication between them (Kovářů *et al.* 2009). Undoubtedly, more detailed knowledge remains to be elucidated in this area.

ACKNOWLEDGEMENTS.

This study was supported by grant of Academy of Sciences of Czech Republic, No. IAA 601680801, grants of Ministery of Education of Czech Republic, No 0021620849 and MSMT (CZ.1.05/2.1.00/01.0006, AdmireVet) and grant of MZE (MZE0002716202).

REFERENCES

1 Avissar S, Schreiber G (2006) The involvement of G proteins and regulators of receptor-G protein coupling in the pathophysiology, diagnosis and treatment of mood disorders. Clin Chim Acta. **366**: 37–47.

2 Chen J, Rasenick MM (1995) Chronic treatment of C6 glioma cells with antidepressant drugs increases functional coupling between G protein (Gs) and adenylyl cyclase. J Neurochem. **64**: 724–732.

3 Dempsey CM, Mackenzie SM, Gargus A, Blanco G, Szei JY (2005) Serotonine (5HT), Fluoxetine, Imipramine as dopamine target distinct 5HT receptor signaling to modulate Caenorhabditis elegans egg-laying behavior. Genetics. **169**: 1425–1536.

4 Evans DL, Lunch KG, Bento R, Dubé B, Gettes DR, Nancy B (2008) Serotonin reuptake inhibitor and substance p antagonist enhancement of natural killer cell innate immunity in human immunodeficiency virus/acquired immunodeficiency syndrome. Biol Psychiatry. **63**: 899–905.

5 Fišerová A, Kovářů H, Hajduová Z, Mareš V, Starec M, Křen V, Flieger M, Pospíšil M (1997) Neuroimmunomodulation of natural killer (NK) cells by ergot alkaloid derivates. Physiol Res. **46**: 119–12.

6 Golan M, Schreiber G, Avissar S (2010) Antidepressants increase beta-arrestin2 ubiquitinylation and degradation by the proteasomal pathway in C6 rat glioma cells J. Pharmacol. Exp. Ther. **332**: 970–976.

7 Haskó G, Baláci C, Zoltán H, Vizi ES, Pacher P (2009) A2B adenosine receptors in immunity and inflammation. Trends Immunol. **30**: 263–270.

8 Hisaoka K; Takebayashi M; Tsuchioka M; Maeda TN; Nakata Y; Yamawaki S (2007) Antidepressants increase glial cell line-derived neurotrophic factor production through monoamine-independent activation of protein tyrosine kinase and extracellular signal-regulated kinase in glial cells. J Pharmacol Exp Ther. **121**: 148–157.

9 Hubbard KB, Hepler JR (2006) Cell signalling diversity of theGq alpha family of heterotrimeríc G proteins. Cell Signal. **18**: 135–150.

10 Fishman PH, Finberg JP (1987) Effect of the tricyclic antidepressant desipramine on beta-adrenergic receptors in cultured rat glioma C6 cells. J Neurochem. **49**: 282–289.

11 Frank MG, Hendrieks SE, Johnson DR, Wieseler JL, Burke WJ (1999) Antidepressants augment natural killer cellular activity: in vivo and in vitro. Neuropsychobiology. **39**: 18–24.

12 Jeffe F, Stegmann KA, Broelsch F, Manns MP, Cornberg M, Wedemeyer H (2009) Adenosine and IFN- synergistically increase IFN-production of human NK cells. J Leuk Biol. **85**: 452–461.

13 Kagaya A, Kugaya A, Hayashi T, Okamoto Y, Takebayashi M, Uchitomi Y, Yamawaki S(1996) Effect of citalopram on the desensitization of serotonin-2A receptor-mediated calcium mobilization in rat glioma cells. Progr Neuropsychopharmacol Biol Ppsychiatry. **20**: 157–166.

14 Kovářů H, Kovářů F, Žižkovský V, Velek J (1998) Developmental changes of trimeric GTP-binding proteins in porcine brain and immune system. Acta Vet. Brno. **67**: 15–20.

15 Kovářů H, Fišerová A, Kovářů F, Paclt I, Lisá V (2000) Antidepressant or immunomodulator induced regulation of cell signalling in brain and immune system. Eur Neuropsychopharmacol. **Suppl. 3**: 269–270.

16 Kovaru H, Pav M, Kovaru F, Raboch J, Fiserova A (2009) Cell signalling in CNS and immune system in depression and during antidepressant treatment: focus on glial and natural killer cells. Neuroendocrinol Lett. **30**: 421–428.

17 Lorenzen A (1997) Determination of adenosine receptor-G protein coupling. Signifi cance for psychiatric and neurological disorders. In: G protein methods and protocols.Role of G proteins in psychiatric and neurological disorders. Humana Press, Totowa, N. Jersey: 327–352.

18 McEwen BS, Chattarji S, Diamond DM, Jay (TM), Reagan LP, Svenningsson P, Fuchs E (2010) The neurobiological properties of Tianeptine (Stablon) from monoamine hypothesis to glutamatergic modulation. Mol Psychiatry. **15**: 237–249.

19 Millan MJ (2006) Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. Pharmacol Ther. **110**: 135–370.

20 Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochem. J. **255**: 1–13

21 Milligan G (1993) Regional distribution and quantitative measurement of the phosphoinisitidase C-linked guanine nucleotide binding proteins G11 alpha and Gq alpha in rat brain. J Neurochem. **61**: 845–851.

22 Páv M, Kovářů H, Fišerová A, Havrdová E, Lisá V (2008) Neurobiological aspects of depressive disorder and antidepressant treatment: role of glia. Physiol Res. **57**: 151–64.

23 Pav M, Kovaru H, Kovaru F, Lisa V, P Ondrackova-Zelnickova P , Fiserova A (2009).Acute and chronic effects of antidepressants on the G-protein alpha subunit profiles *in vitro* and *in vivo*. Neuroendocrinol Lett. **30:** 592–598**.**

24 Ransnas LA, Insel PA (1989) Quantification of a guanine nukleotid binding regulatory protein by an enzyme-linked immunosorben competition assay. Anal Biochem. **176**: 185–190.

25 Schulte G, Fredholm BB (2003) Signalling from adenosine receptors to mitogen- activated protein kinases. Cellular Signalling. **15**: 813–827.

26 Španová A, Kovářů H, Lisá V, Lukášová E, Rittich B (1997) Estimation of apoptosis in C6 glioma cells treated with antidepressants. Physiol Res. **46**: 161–164.

27 Tardito D, Perez J, Tiraboshi E, Musazzi L, Racagni G, Popoli M (2006) Signaling Pathways Regulating Gene Expression, Neuroplasticity, and Neurotrophic Mechanisms in the Action of Antidepressants: A Critical Overview. Pharmacol Rev. **58**: 115–34.

28 Wettschureck N, Offermanns S (2005) Mammalian G proteins and their cell type specific functions. Physiol Rev. **85**: 1159–120.

29 Zhang S. Lovatt D Song G. Goldmann S. Nedergaard M. Hertz L. Peng L (2010) 5HT2B receptors are expressed on astrocytes from brain and in culture are a chronic target for all five conventional 'serotonin-specific reuptake inhibitors'. Neuron Glia Biol. **2:** 113–125.