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Bi-phasic regulation of glycogen content in astrocytes via Cav-1/PTEN/AKT/GSK-3 β pathway by fluoxetine

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**Bi-phasic regulation of glycogen content in astrocytes via
Cav-1/PTEN/AKT/GSK-3β pathway by fluoxetine**

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Conflict of interest

The authors declare no conflict of interest.

Abstract

Here we present the data indicating that chronic treatment with fluoxetine regulates Cav-1/PTEN/PI3K/AKT/GSK-3 β signalling pathway and glycogen content in primary cultures of astrocytes with bi-phasic concentration dependence. At lower concentrations fluoxetine down-regulates gene expression of Cav-1, decreases membrane content of PTEN, increases activity of PI3K/AKT, and elevates GSK-3 β phosphorylation thus suppressing its activity. At higher concentrations fluoxetine acts in an inverse fashion. As expected, fluoxetine at lower concentrations increased while at higher concentrations decreased glycogen content in astrocytes. Our findings indicate that bi-phasic regulation of glycogen content via Cav-1/PTEN/AKT/GSK-3 β pathway by fluoxetine may be responsible for both therapeutic and side effects of the drug.

Key words: fluoxetine; Cav-1; PTEN; PI3K/AKT; GSK-3 β ; glycogen

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Introduction

Astrocytes being homeostatic cells of the CNS (Verkhatsky and Butt 2013) are central elements for potassium ion (K^+) buffering, for neurotransmitter homeostasis, for maintenance and regulation of synaptic transmission and for CNS metabolism. Astroglial contribution to major depression (similarly to other psychiatric pathologies - see Verkhatsky et al. 2014) does not involve astrogliosis and hypertrophy; rather the astrodegenerative signs prevail. The number of astrocytes, and expression of astroglial marker GFAP both are significantly decreased in the brains of depressed patients (McNally et al. 2008; Rajkowska et al. 2013). At the same time treatment with antidepressants increases GFAP levels (Barley et al. 2009). Expression of another astroglial marker, the calcium-binding protein S100 β has been reduced in the ventral prefrontal cortex of depressed suicide victims (Klempan et al. 2009). Several other astroglial markers, such as aquaporin 4, astroglial connexins, astroglial plasmalemmal glutamate transporters and glutamine synthetase are all down-regulated in mood disorders (Rajkowska et al. 2013; Barley et al. 2009; Bernard et al. 2011; Sequeira et al. 2009). Reduced number of astrocytes and of their packing density was detected throughout the fronto-limbic areas of the brain, including the orbito-frontal area, and anterior cingulate, prefrontal, entorhinal and subgenual cortices, as well as the amygdala (Rajkowska et al. 2013; Verkhatsky et al. 2014; Popoli et al. 2011; Sanacora et al. 2013).

Previously, we reported that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), acts as an agonist on astroglial 5-HT_{2B} receptors (Kong et al. 2002). Acute treatment with fluoxetine activates 5-HT_{2B} receptors in astroglia, which results in transactivation of epidermal growth factor receptor (EGFR) with subsequent recruitment of its intracellular signal pathways, MAPK/ERK and PI3K/AKT (Li et al. 2008; Ren et al. 2015). The glycogen synthase kinase 3 (GSK-3), the downstream substrate of AKT, is an enzyme, which has been initially discovered as a deactivator of glycogen synthase (GS) that converts glucose to glycogen. In the brain *in vivo*, glycogen is mainly (if not exclusively) localised in astroglia (Ibrahim 1975; Hertz et

al. 2015). The roles of glycogen are not limited to an emergency energy substrate. Glycogen turnover, i.e., interspersed glycogen synthesis and glycogenolysis, is indispensable for learning (Gibbs and Hutchinson 2012; Hertz et al. 2013). The acute memory-enhancing, as well as glycogenolysis-dependent effect of both fluoxetine and paroxetine has been also described (Gibbs and Hertz 2014). Knock-out of brain glycogen synthase abolishes learning of new motor and cognitive skills (Duran et al. 2013). In our laboratory, experiments with primary cultures of astroglial cells have shown that glycogenolysis is essential for many signalling processes, including those enabling K^+ buffering (Xu et al. 2013).

GSK-3 exists in two isoforms, GSK-3 α and GSK-3 β with molecular weight of 51 and 47 kDa respectively (Gould TD et al. 2005). High levels of GSK-3 β are detected in the central nervous system (CNS) (Perez-Costas et al. 2010). AKT phosphorylates GSK-3 β at serine-9 and serine-21, and down-regulates its activity (Fang et al. 2000). The GSK-3 β is an important component of several signalling pathways, such as insulin/insulin-like growth factor (IGF-1) signalling, neurotrophic factor signalling, and the Wnt signalling pathway (Gould and Manji 2005). Alterations in GSK-3 β activity were found in mood disorders, in Alzheimer's disease and in diabetes (Gould and Manji 2005).

Phosphatase and tensin homologue (PTEN) inhibits PI3K/AKT pathway by dephosphorylation of PI3K (Robbins and Hague 2016); whereas caveolae protein, caveolin 1 (Cav-1) that binds directly to PTEN, regulates membrane content and function of the latter (Xia et al. 2010). Caveolae are small lipid rafts of the plasma membrane which contribute to cell signalling (Sowa 2011). Caveolae formation and stabilisation depends on the key structural protein, caveolin (Smart et al. 1999), which occurs in three isoforms of caveolin 1 to 3 (Cav-1, -2, and -3) (Sary et al. 2012). Cav-1 is essential for formation of caveolae in endothelial cells, fibroblasts, and pneumocytes (Razani et al. 2001), whereas the role of Cav-2 remains undefined. Cav-3 is highly expressed as a homodimer in muscle (Chidlow and Sessa 2010). All

three caveolin isoforms have been detected in CNS (Zschocke et al. 2002; Shin et al. 2005).

Previously, we have demonstrated that in astrocytes ammonium decreases GSK-3 β phosphorylation and increases its activity via Cav-1/PTEN/PI3K/AKT signalling pathway. We hypothesised that chronic treatment with fluoxetine may affect glycogen content in astroglial cells via regulation of Cav-1 gene expression. In the present study, we have investigated (i) expression of Cav-1 mRNA and protein in primary astroglial cultures chronically treated with fluoxetine; (ii) effects of chronic treatment of primary astroglial cultures with fluoxetine on membrane content of PTEN and phosphorylation of AKT, and GSK-3 β ; (iii) effects of chronic treatment with fluoxetine on glycogen content in astrocytes *in vitro*.

Materials and methods

Cell cultures

Primary cultures of mouse astrocytes were prepared from the neopallia of the cerebral hemispheres of newborn CD-1 mice as previously described (Hertz et al. 1998; Hertz et al. 1978); cells were sparsely seeded, and grown in Dulbecco’s Minimum Essential Medium (DMEM) with 7.5 mM glucose. After 2 weeks *in vitro*, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. These cultures are highly enriched in astrocytes (>95% purity of glial fibrillary acidic protein- (GFAP-) and glutamine synthetase-expressing astrocytes) (Hertz et al. 1985). Addition of dBcAMP leads to a morphological and functional differentiation as evidenced by the extension of cell processes and increases in several metabolic and functional activities characteristic of astrocytes *in situ* (Meier et al. 1991); in addition this treatment promotes Ca²⁺ flux through voltage-dependent channels (Hertz et al. 1989).

Knock-down of Cav-1

Transfection was performed as previously described (Li et al. 2008). To allow incorporation of siRNAs into astrocytes, 3-week-old astrocytes cultured in Primaria

24-well culture plates were incubated in Dulbecco's medium without serum for 24 h on the day before transfection. Transfection solution containing 2 μ l Oligofectamine, 40 μ l Opti-MEMI, and 2.5 μ l siRNA (666 ng) was added to the culture for 8 h. In siRNA (–) control cultures, transfection solution without siRNA was added. Thereafter, 87.5 μ l DMEM with 37.5 μ l serum was added to the cultures. Effective knockdown of Cav-1 was confirmed by Western blot three days after transfection.

Membrane preparation

A crude membrane preparation from astrocytes or brain tissue was made as previously described by Peng et al (1997). Cells from five 60-mm dishes were collected as one sample, homogenised in SET (0.315 M sucrose, 20 mM TrisCl, and 1 mM EDTA, pH 7.4), and centrifuged for 1 h at 140,000 g. The pellets were collected and resuspended in SET.

Western blotting

Protein content was determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin as the standard. Samples containing 60 μ g protein were applied on slab gels of 10% polyacrylamide and electrophoresed. After transfer to polyvinylidene fluoride (PVDF) membranes, the samples were blocked by 5% skim milk powder in TBS-T (30 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20) for 1 h. The PVDF membranes were incubated with the first antibody, specific to Cav-1, p-AKT/AKT, p-GSK/GSK overnight at 4°C, or PTEN and β -actin for 2 h at room temperature. After washing, the blots were incubated with peroxidase-conjugated affinity-purified goat-anti-mouse or goat-anti-rabbit horseradish antibody for 2 h. Staining was visualised by ECL detection reagents. Digital images obtained using Gel-Imaging System (Tanon 4200, Shanghai, China). Optical density for each band was assessed using the Window Alpha-Ease TM FC 32-bit software.

Determination of glycogen

For determination of glycogen in cultured cells, a TECAN infinite M200 MicroplateAnalyzer (Lausanne, Switzerland) was used to record fluorescence intensity of NADPH, generated from NADP⁺ by the action of glucose-6-phosphate dehydrogenase. This was done in untreated control cultures and in cultures treated with fluoxetine (1 µM, 10 µM) for 2 weeks. After 2 weeks treatment, the astrocytes were washed three times with ice-cold phosphate-buffered saline (PBS) and sonicated in 30 mM HCl (similar solution as the homogenised slices). The suspension was used to measure non-hydrolyzed glycosyl units of glycogen. Briefly, three 50 µl aliquots were sampled. In the first aliquot, 150 µl of acetate buffer (0.1 M, pH 4.65) was added. In the second, 150 µl of a solution containing 1% amyloglucosidase (10 mg/ml) in the acetate buffer was added in order to degrade remaining glycogen to glucose, and the mixture was incubated at room temperature for 30 min. Subsequently the two aliquots were treated identically. Two ml of Tris-HCl buffer (0.1 M, pH 8.1) containing 3.3 mM MgCl₂, 0.2 mM ATP, 25 ug/ml NADP, 4 µg/ml hexokinase, and 2 µg/ml glucose-6-phosphate dehydrogenase was added to each, and the mixture was incubated at room temperature for 30 min. The fluorescence of the NADPH formed in amounts equivalent to glucose metabolised by hexokinase was then read (excitation 340 nm; emission 450 nm). The first aliquot measures the sum of glucose and glucose-6-phosphate in the tissue, whereas the second aliquot in addition to those also measures the glycosyl units from glycogen remaining in the tissue. Determination of the difference between these two aliquots provides a measurement of the amount of the latter. The third aliquot was used to measure the protein content by the Lowry method to normalise the glycogen contents (nmol) per mg protein. A standard curve was made to show fluorescence intensity at different glucose concentrations and glycogen content was calculated, using a conversion factor based on a molecular weight of 180 g/mol for free glucose, but 162 g/mol per glycosyl unit of glycogen.

Materials

Chemicals for preparation of culturing medium were purchased from Sigma (St. Louis, MO, USA) and horse serum from Invitrogen (Carlsbad, CA, USA). Most chemicals,

including dBcAMP and fluoxetine were purchased from Sigma (St. Louis, MO, USA). Antibodies specific to Cav-1 antibody and PTEN were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific to p-AKT, AKT, p-GSK-3 β or GSK-3 β were obtained from Cell Signaling Technology (Cell Signaling, USA). Antibody specific to β -actin was obtained from Sigma-Aldrich (Sigma, USA). The second antibody goat anti-mouse IgG-HRP was purchased from Promega (Madison, USA). The second antibody goat anti-rabbit IgG-HRP and Cav-1 siRNA (catalog no. sc-29942) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Scramble siRNA (5'-ACGACTAGCCTGAACTCAA-3') was synthesised by Sigma (St. Louis, MO, USA). Opti-MEMI and Oligofectamine were purchased from Invitrogen (Carlsbad, CA, USA). Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), hexokinase, glucose-6-phosphate dehydrogenase, and amyloglucosidase were purchased from Sigma (St. Louis, MO, USA).

Statistics

The differences between individual groups were analysed by one-way ANOVA followed by Fisher's LSD test. The level of significance was set at $p < 0.05$.

Results

Acute treatment

In order to differentiate acute and chronic effects of the drug, we obtained the concentration-dependence and monitored the time course of AKT phosphorylation in the context of acute treatment. Acute treatment with fluoxetine for 10 min increased AKT phosphorylation only at 5 μ M ($18.9 \pm 3.4\%$) and at 10 μ M ($21.1 \pm 4.5\%$). Twenty min treatment increased AKT phosphorylation in a concentration-dependent manner with 0.1 μ M to 10 μ M fluoxetine causing $20.9 \pm 6.3\%$ to $62.3 \pm 3.0\%$ increase (Fig. 1). The highest level of phosphorylation was observed at 10 μ M being $162.3 \pm 3.0\%$, of control group. The increase in AKT phosphorylation declined after

40 min with only a small increase at 10 μ M ($17.8 \pm 3.7\%$) and disappeared after 2 hr of treatment (Fig. 1).

Chronic treatment

Treatment of cultured astrocytes with fluoxetine for 2 weeks regulated expression of Cav-1 protein in a concentration-dependent manner with 0.1, 0.5 and 1 μ M fluoxetine causing a significant down-regulation but 5 and 10 μ M a significant up-regulation (Fig. 2A). The lowest level was observed at 1 μ M being $52.6 \pm 1.9\%$ of control, whereas the highest level of expression was determined at 10 μ M being $147.8 \pm 3.1\%$ of control group.

Similar bi-phasic concentration-dependence was also observed in membrane PTEN content with 0.1, 0.5 and 1 μ M fluoxetine causing a significant down-regulation but 5 and 10 μ M a significant up-regulation (Fig. 2B). The lowest level was observed at 1 μ M being $59.7 \pm 7.8\%$ of control, whereas the highest level of expression at 10 μ M being $139.7 \pm 3.9\%$ of control group.

Treatment of cultured astrocytes with fluoxetine for 2 weeks regulated AKT phosphorylation in a concentration-dependent manner with 0.1, 0.5 and 1 μ M fluoxetine causing a significant up-regulation (from $19.2 \pm 0.6\%$ to $33.3 \pm 8.5\%$), whereas 5 and 10 μ M a significant down-regulation (from $26.1 \pm 4.6\%$ to $29.6 \pm 4.2\%$) (Fig. 3A). There is no significant difference between 0.1, 0.5 and 1 μ M and between 5 and 10 μ M.

Similar effect was also observed in GSK-3 β phosphorylation. Treatment of cultured astrocytes with fluoxetine for 2 weeks affected GSK-3 β phosphorylation in a concentration-dependent manner with 0.1, 0.5 and 1 μ M fluoxetine causing a significant up-regulation (from $44.2 \pm 16.3\%$ to $58.2 \pm 10.5\%$) but significant down-regulation (from $39.3 \pm 4.6\%$ to $40.5 \pm 14.2\%$) was observed at 5 and 10 μ M

(Fig. 3B). There is no significant difference between 0.1, 0.5 and 1 μ M and between 5 and 10 μ M.

Effects of Cav-1 siRNA

Cav-1 siRNA reduced protein expression of Cav-1 to $33.9 \pm 6.8\%$ of the control group after 3 days of transfection (Fig. 4A). Cav-1 siRNA had no effect on 0.1, 0.5 and 1 μ M fluoxetine-induced increase of AKT phosphorylation (increased by $23.8 \pm 4.9\%$, $41.3 \pm 6.6\%$ and $36.5 \pm 3.5\%$, respectively), but abolished the decrease of AKT phosphorylation by 5 and 10 μ M fluoxetine ($102.5 \pm 4.8\%$ and $104.1 \pm 2.3\%$ of control group, respectively) (Fig. 4B). Similar results was also obtained in GSK-3 β phosphorylation (respectively increased by $20.2 \pm 6.0\%$, $33.2 \pm 7.5\%$ and $22.3 \pm 1.3\%$ at 0.1, 0.5 and 1 μ M; $102.2 \pm 3.7\%$ and $103.9 \pm 5.5\%$ of control group at 5 and 10 μ M) (Fig. 4C).

Effect of fluoxetine on glycogen content

Treatment with 1 μ M fluoxetine for 2 weeks increased glycogen content to $150.7 \pm 13.6\%$ of the control group. In contrast, treatment with 10 μ M fluoxetine decreased glycogen content to $54.0 \pm 10.2\%$ of the control (Fig. 5).

Discussion

In addition to regulation of glycogen synthesis, GSK-3 β is involved in diverse signalling pathways, such as insulin/insulin-like growth factor (IGF-1) signalling, neurotrophic factor signalling, and the Wnt signalling (Gould and Manji 2005). The signalling roles of GSK-3 β are implicated in several human diseases, such as type 2 diabetes, Alzheimer's disease, cancer, cardiac hypertrophy and hypertension (Phukan et al. 2010). Some biological functions of GSK-3 β may be relevant to mood disorders. It includes neuroprotection, regulation of monoaminergic signalling, brain metabolism and circadian rhythms, and modulating estrogen and glucocorticoid activity (Gould and Manji 2005).

Increased GSK-3 β activity is generally accepted to be one of mechanisms underlying major depression and bipolar disorder. Increase in GSK-3 β activity results from up-regulation of GSK-3 β gene expression or down-regulation of GSK-3 β phosphorylation and hence decrease in the ratio between phosphorylated GSK-3 β and total GSK-3 β . Evidence for the involvement of GSK-3 β in major depression is obtained from both animal models (Szymańska et al. 2009) and depressed patients (Pláteník et al. 2014). Increase in GSK-3 β activity in depressed animals can be reversed by treatment with SSRIs. Mice overexpressing GSK-3 β shows prodepressant-like phenotype and increased sensitivity to chronic mild stress; these behaviour changes can be prevented by chronic, but not by acute treatment with fluoxetine (Zhang et al. 2013). In prenatally stressed rats GSK-3 β levels were significantly elevated in the frontal cortex, but not in the hippocampus (Szymańska et al. 2009), while concentration of phosphorylated GSK-3 β was decreased only in the former structure. Chronic treatment with antidepressant drugs diminished stress-induced alterations in GSK-3 β and phospho-GSK-3 β only in the frontal cortex (Szymańska et al. 2009). In addition, chronic treatment with citalopram alleviated the depression-like behaviors and reversed the decrease of the phosphorylated GSK-3 β in stressed rats (Liu et al. 2012). In postmortem prefrontal cortex of patients with MDD and MDD with suicide, the level of phosphorylated GSK-3 β was decreased (Karege et al. 2012). In elderly depressed patients and Alzheimer's disease patients with depression, a lower level of phosphorylated GSK-3 β in platelets correlated with severity of depression (Diniz et al. 2011; Pláteník et al. 2014).

However, there are data demonstrating increased activity of GSK-3 β following antidepressant treatment. For example, long-term treatment with sertraline increased GSK-3 β activity in platelets of patients with late-life depression (Joaquim et al. 2012). Our present findings suggest that this controversy may result from none-linear effects of SSRIs on regulation of Cav-1 gene expression in astrocytes. Astrocytes express all three isoforms of caveolins (Virgintino et al. 2002), which play complex rolea that are not completely understood. Recently, we have reported that treatment of cultured

astrocytes with ammonium as well as treatment of animals with urease up-regulates astroglial Cav-1 gene expression, which, in turn, increases membrane content of PTEN, inhibits PI3K/AKT signalling, and enhances GSK-3 β activity (Wang et al. 2016).

In the present study, we found that acute treatment with fluoxetine at concentrations of 0.5 to 10 μ M increased AKT phosphorylation in a concentration-dependent manner. This is in agreement with our previously report (Ren et al. 2015) although it contrasts the bi-phasic effect by chronic drug treatment. In addition, this stimulation lasted only for 40 min, and thereafter p-AKT level showed no significant difference from control group from 2 hours to 3 days (data not shown) after drug treatment.

Second, we found that chronic treatment with fluoxetine decreased Cav-1 mRNA and protein expression in cultured astrocytes at lower concentrations, but increased it at higher concentrations. Since Cav-1 contains sequences for PTEN binding (Xia et al. 2010), the up- or down-regulation of Cav-1 expression, in turn, increases or decreased membrane content of PTEN. PTEN is a lipid/protein phosphatase, which induces dephosphorylation and inhibition of PI3K (Robbins and Hague 2016). We also observed that AKT phosphorylation was increased at lower concentrations, but decreased at higher concentrations. The involvement of Cav-1 was further confirmed by the abolishment of fluoxetine-induced inhibition of AKT phosphorylation at higher concentrations by Cav-1 siRNA.

Third, we found that chronic treatment with fluoxetine decreased GSK-3 β phosphorylation in cultured astrocytes at lower concentrations, but increased it at higher concentrations. Down-regulation of GSK-3 β phosphorylation is also related to Cav-1 since Cav-1 siRNA abolished fluoxetine-induced inhibition of AKT phosphorylation at higher concentrations. The regulation of GSK-3 β phosphorylation and activity will change the activity of glycogen synthetase, which, in turn, affects glycogen content in astrocytes. Here, we found that fluoxetine increased glycogen

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content in cultured astrocytes at lower concentrations, but decreased it at higher concentrations. This may be important for behavioural effects of SSRIs. Glycogen turnover, i.e., interspersed glycogen synthesis and glycogenolysis, is indispensable during learning (Gibbs and Hutchinson 2012; Hertz et al. 2013). The acute memory-enhancing, glycogenolysis-dependent effect of both fluoxetine and paroxetine has been mentioned (O'Dowd et al. 1994; 1995; Gibbs and Hertz 2014; Suzuki et al. 2011; Gibbs and Hertz 2014; Gao et al. 2016). Knock-out of brain glycogen synthase abolishes learning of new motor and cognitive skills (Duran et al. 2013).

In conclusion, we demonstrated, for the first time, that chronic treatment with fluoxetine decreases GSK-3 β activity via stimulation of PI3K/AKT pathway by decreasing Cav-1 gene expression and membrane content of PTEN at lower concentrations, but increases GSK-3 β activity via inhibition of PI3K/AKT pathway by increasing Cav-1 gene expression and membrane content of PTEN at higher concentrations. The combined concentration of fluoxetine and its primary metabolite norfluoxetine in plasma of fluoxetine-treated patients is around 1-2 μ M (Bolo et al. 2000; Henry et al. 2005). However, brain concentration of the drug reaches 15-25 μ M (Bolo et al. 2000; Henry et al. 2005). Thus bi-phasic regulation of GSK-3 β activity and glycogen content in astrocytes, as well as in other types of cells in peripheral tissues may contribute to both therapeutic and side effects of the drug.

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Figure legends

Figure 1. The level of AKT phosphorylation induced by acute treatment with fluoxetine.

Cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μ M fluoxetine for 10, 20, 40 min or 2 hrs. A: Immunoblots from representative experiments. Bands of 60 kDa represent p-AKT (Ser473) and total AKT. Similar results were obtained from three independent experiments. B: Phosphorylated AKT and total AKT in drug-treated groups were normalised with control group respectively. Average AKT phosphorylation was quantified as ratio between normalised p-AKT and AKT. SEM values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control groups at the same time period.

Figure 2. Cav-1 protein expression and PTEN membrane content in primary cultures of mouse astrocytes treated with fluoxetine for 2 weeks.

A: Cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μ M fluoxetine for 2 weeks. Immunoblots from representative experiments are shown on the top; bands of 23 kDa and 42 kDa represent Cav-1 and β -actin. Similar results were obtained from three independent experiments. Graph at the bottom shows average Cav-1 protein expression quantified as ratio between Cav-1 and β -actin. Cav-1 to β -actin ratios were normalised to the control. SEM values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, 5 and 10 μ M groups. #Indicates statistically significant ($P<0.05$) difference from control, 0.1, 0.5 and 1 μ M groups.

B: Cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μ M fluoxetine for 2 weeks. Immunoblots from representative experiments are shown on the top; bands of 55 kDa and 42 kDa represent PTEN and β -actin. Similar results were obtained from three independent experiments. Graph at the bottom shows average PTEN membrane

content was quantified as ratios between PTEN and β -actin. Ratios between PTEN and β -actin were normalised to the control. SEM values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, 5 and 10 μ M groups. #Indicates statistically significant ($P<0.05$) difference from control, 0.1, 0.5 and 1 μ M groups.

Figure 3. The level of phosphorylation of AKT and GSK-3 β in primary cultures of mouse astrocytes treated with fluoxetine for 2 weeks.

A: Cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μ M fluoxetine for 2 weeks. Immunoblots from representative experiments are shown on the top; bands of 60 kDa represent p-AKT (Ser473) and total AKT. Similar results were obtained from three independent experiments. Graph at the bottom shows changes in phosphorylated AKT and total AKT in drug-treated groups, which were normalised to the controls. Average AKT phosphorylation was quantified as ratios between normalised p-AKT and AKT. SEM values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, 5 and 10 μ M groups. #Indicates statistically significant ($P<0.05$) difference from control, 0.1, 0.5 and 1 μ M groups.

B: Cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μ M fluoxetine for 2 weeks. Immunoblots from representative experiments are shown on the top; bands of 43 kDa represent p-GSK-3 β and total GSK-3 β . Similar results were obtained from three independent experiments. Graph at the bottom shows changes in phosphorylated GSK-3 β and total GSK-3 β in drug-treated groups normalised to the controls. Average GSK-3 β phosphorylation was quantified as ratios between normalised GSK-3 β and GSK-3 β Average. SEM values are indicated by vertical bars. *Indicates statistically significant ($P<0.05$) difference from control, 5 and 10 μ M groups. #Indicates statistically significant ($P<0.05$) difference from control, 0.1, 0.5 and 1 μ M groups.

Figure 4. The effects of Cav-1 siRNA on phosphorylation of AKT and GSK-3β in primary cultures of mouse astrocytes treated with fluoxetine for 2 weeks.

Cells were treated with either transfection solution without siRNA [siRNA (-)] (Control), with scrambled siRNA or siRNA specific to Cav-1 [siRNA (+)] for three days. Thereafter cells were incubated with 0 (Control), 0.1, 0.5, 1, 5 and 10 μM fluoxetine for 2 weeks.

A: Left: Immunoblots from representative experiments; bands of 23 kDa and 42 kDa represent Cav-1 and β-actin. Similar results were obtained from three independent experiments. Right: average Cav-1 protein expression quantified as ratios between Cav-1 and β-actin. Ratios between Cav-1 and β-actin were normalised to the control. SEM values are indicated by vertical bars. *Indicates statistically significant (P<0.05) difference from control and scrambled siRNA (-) groups.

B: Left: Immunoblots from representative experiments; bands of 60 kDa represent p-AKT (Ser473) and total AKT. Similar results were obtained from three independent experiments. Right: Phosphorylated AKT and total AKT in drug-treated groups were normalised with control group respectively. Average AKT phosphorylation was quantified as ratios between normalised p-AKT and AKT. SEM values are indicated by vertical bars. *Indicates statistically significant (P<0.05) difference from control, 5 and 10 μM groups.

C: Left: Immunoblots from representative experiments; bands of 46 kDa represent p-GSK-3β and total GSK-3β. Similar results were obtained from three independent experiments. Right: Phosphorylated GSK-3β and total GSK-3β in drug-treated groups were normalised with control group respectively. Average GSK-3β phosphorylation was quantified as ratios between normalised GSK-3β and GSK-3β Average. SEM values are indicated by vertical bars. *Indicates statistically significant (P<0.05) difference from control, 5 and 10 μM groups.

Figure 5. Effect of fluoxetine on the glycogen content in cultured astrocytes.

Cultured astrocytes were incubated in untreated control cultures and in cultures treated with fluoxetine (1 μ M or 10 μ M) for 2 weeks. After the experiment glycogen was determined by measuring glucose content fluorometrically before and after breakdown of remaining glycogen in the cells. Average glycogen contents are indicated as percentages of those under control conditions. All values are expressed as mean $\pm$ SEM, indicated by vertical bars and are from 8 to 10 individual cultures.

*Statistically significant ($P < 0.05$) difference from control.

Fig. 1

243x331mm (300 x 300 DPI)

Fig. 2

184x134mm (300 x 300 DPI)

Fig. 3

144x89mm (300 x 300 DPI)

Fig. 4

255x303mm (300 x 300 DPI)

Fig. 5

107x131mm (300 x 300 DPI)