Published in final edited form as:

J Mol Neurosci. 2013 March ; 49(3): 523–530. doi:10.1007/s12031-012-9924-0.

Protective role of astrocytic leptin signaling against excitotoxicity

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Abstract

Both pro- and anti-convulsive roles of leptin have been reported, suggesting cell-specific actions of leptin in different models of seizure and epilepsy. The goal of our study was to determine the regulation and function of astrocytic leptin receptors in a mouse model of epilepsy and glutamate-induced cytotoxicity. We show that in pilocarpine-challenged mice developing epilepsy with recurrent seizures after a latent period of 2 weeks, hippocampal leptin receptor (ObR) immunofluorescence was increased at 6 weeks. This was more pronounced in astrocytes than in neurons. In cultured astrocytes, glutamate increased ObRa and ObRb expression, whereas leptin pretreatment attenuated glial cytotoxicity by excess glutamate, reflected by better preserved ATP production. The protective role of astrocytic leptin signaling is further supported by the higher lethality of the astrocyte specific leptin receptor knockout mice in the initial phase of seizure production. Thus, leptin signaling in astrocytes plays a protective role against seizure, and the effects are at least partially mediated by attenuation of glutamate toxicity. Astrocytic leptin signaling, therefore, may be a novel therapeutic target.

Keywords

Astrocyte; Leptin; ObR; Epilepsy; Glutamate; Cytotoxicity

Introduction

Leptin is a 16 kDa protein mainly produced by fat tissue in the periphery. It crosses the blood-brain barrier (BBB) by a saturable transport system (Banks et al., 1996; Maresh et al., 2001) to produce neurotrophic, neuromodulatory, and proinflammatory actions. Receptors for leptin (ObR) are present in the hippocampus (Shioda et al., 1998; Wu et al., 2012), and leptin can activate potassium channels to regulate the excitation of hippocampal neurons (Shanley et al., 2002).

Leptin has been reported to exert both anti-epileptic and pro-convulsive roles. It shows anticonvulsant properties in two rodent models of seizure, after either direct injection into the cortex or through intranasal administration (Xu et al., 2008). Similarly, leptin has neuroprotective effect in rats with kainite acid-induced seizures (Obeid et al., 2010). By contrast, it has also been shown to have pro-convulsive effects (Lynch, III et al., 2010). The different effects of leptin in epilepsy may be related to the use of different models or cell-

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specific effects of leptin administered at different disease stages. In other disease models, we have shown that leptin receptors in astrocytes and neurons may show opposite directions of change. These include mouse models of adult-onset obesity either with the agouti viable yellow mutation (Pan et al., 2008) or diet-induced obesity (Hsueh et al., 2009a), and experimental autoimmune encephalomyelitis (Wu et al., 2012). We also showed that astrocytes may compete with neurons for the availability of leptin to induce STAT3 signaling (Pan et al., 2011). Based on the analogous evidence, we propose that astrocytic leptin signaling may underlie the dual effects of leptin in seizures and epilepsy.

During seizure, persistently high levels of extracellular glutamate are a leading cause of excitotoxicity. Seizure involves an impaired clearance of glutamate. This contrasts with normal conditions in which glutamate is a key transmitter of bidirectional communication between astrocytes and neurons (Haydon and Carmignoto, 2006; Nedergaard et al., 2003). Epilepsy, or recurrent unprovoked seizures, is associated with reactive gliosis that encompasses anatomical and biochemical changes in astrocytes (Rothstein et al., 1996; Tashiro et al., 2002). Excess glutamate is not only a neurotoxin, but it can also impair astroglial function by glial swelling. This occurs at concentrations similar to those required to induce neuronal cell death and is seen in C6 glioma cells (Schneider et al., 1992) as well as primary astrocyte cultures (Chan et al., 1990; Han et al., 2004). Since glutamate buffering is a major function of astrocytes, we studied C6 astrocytoma cells in response to glutamate treatment in parallel with the mouse model of pilocarpine-induced epilepsy. C6 cells constitute a widely accepted model to study astrocytic response to excitotoxicity. These cells express both leptin receptors and leptin itself as an autocrine factor (Morash et al., 2000). In response to the endotoxin lipopolysaccharide (LPS), C6 cells show upregulation of ObR (Hsueh et al., 2009b). Thus, C6 cells are well suited to test the possible protective effects of leptin in glutamate-induced excitotoxicity. The results from these in-vitro and in-vivo approaches are consistent in supporting a beneficial role of astrocytic leptin signaling to attenuate seizure-induced damage.

Materials and Methods

Mouse model of pilocarpine-induced seizure and subsequent development of epilepsy

C57BL/6J male mice (B6, Jackson Laboratories, Bar Harbor, ME) were used to induce epilepsy at the age of eight weeks following a protocol approved by the Institutional Animal Care and Use Committee (IACUC). The mice were group-housed in a 12 h light-dark cycle and had free access to food and water. To induce seizure, pilocarpine (350 mg/kg) was injected intraperitoneally (ip) following an established method (Turski et al., 1983; Turski et al., 1989). Sustained seizure was observed within 10–30 min. Seizures were allowed to continue for 30 min and were then stopped by diazepam (1 mg/kg ip). The control mice received phosphate-buffered saline (PBS) instead of pilocarpine, but the rest of the treatment was the same. After the cessation of initial seizures by treatment with diazepam, the mice underwent a latent period of 12 d. Seizures recurred later, resulting in epilepsy, i.e., recurrent unprovoked seizures. The severity of epilepsy was graded according to the Racine scale: Stage 0 = no convulsion; Stage 1 = facial automatism; Stage 2 = head nodding; Stage 3 = unilateral forelimb clonus; Stage 4 = bilateral forelimb clonus; and Stage 5 = rearing, falling, and generalized convulsions (Racine et al., 1972). The epileptic mice were in Racine stages 3–5, when they were processed for immunohistochemistry (IHC) at about 6 weeks after the initial seizure induction.

IHC

Groups of mice (n = 3 /group) with epilepsy (pilocarpine treated) or without (PBS-treated) were anesthetized and perfused intracardially with 4 % paraformaldehyde (PFA). IHC was

performed on free-floating coronal sections of 20 μm thickness as previously described (Pan et al., 2008). After permeabilization and blocking of nonspecific binding, the sections were incubated with primary antibodies. Two ObR antibodies were used in different sections: the M18 antibody for ObR recognizes the membrane juxtapositional cytoplasmic domain aa877–894 (sc1834, 1:100 Santa Cruz Biotechnology, Santa Cruz, CA) and the K20 antibody (sc-1835) recognizes the N-terminus of all ObR isoforms. The marker used for astrocytes and astrogliosis was glial fibrillary acidic protein (GFAP) antibody (AB5804, 1:500, Chemicon, Temecula, CA). After overnight incubation at 4 °C and thorough washes, the sections were incubated with Alexa488 or Alexa594-labeled secondary antibodies (Invitrogen, Eugene, OR) at room temperature for 1 h. The specificity of the staining was shown by the lack of signal in negative controls, including preadsorption of the primary antibody with a specific blocking peptide and omission of primary antibody. For double-labeling immunofluorescence studies with a second primary antibody against GFAP, single staining experiments with each primary antibody were performed and compared to rule out the cross-reactivity of the irrelevant antibodies. Confocal images were acquired on an Olympus FV1000 microscope in the laboratory, with Argon laser excitation/filter for emission at 488/505–525 and 543/560–660 (nm) for Alexa488 and Alexa594, respectively.

Western blotting (WB) to determine ObR and GFAP expression in C6 cells after glutamate treatment

The C6 astrocytoma cells (American Type Culture Collection, ATCC, Manassas, VA) were seeded on 6-well plates and cultured in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. When the cells reached 90% confluency, they were treated with glutamate (0, 0.05, 0.1, 0.5 and 1 mM) (Sigma, St. Louis, MO) in DMEM and 10% FBS with the 0 group receiving medium only. Cells were divided into groups with glutamate (100 μM), leptin (100 ng/ml) or a combination of both glutamate and leptin. After 24 h, the cells were collected by scraping and lysed in Radioimmunoprecipitation Assay (RIPA) buffer in the presence of a complete protease inhibitor cocktail (Pierce, Rockford, IL) at 4 °C. Protein concentration was determined with a Micro BCA™ Protein Assay Kit (Pierce). Forty μg of protein was electrophoresed on 10% SDS-PAGE, transferred to cellulose membrane, blocked, and probed with anti-ObR antibody (M18, Santa Cruz) and GFAP (Sigma, St. Louis, MO). The expression of β -actin was also determined as a reference loading control. After further incubation with horseradish peroxidase-conjugated secondary antibodies, the signals were developed with enhanced chemiluminescence agent (Pierce). Signal intensity was determined by the NIH Image J program.

Effects of ObRa or ObRb overexpression on the cellular response to glutamate

C6 cells grown at 90% confluency were suspended in electroporation buffer provided in the Nucleofector Kit V (Amaxa, Gaithersburg, MD) to a final concentration of $2 \times 10^6/100 \mu\text{L}$ after detachment by trypsin. A mixture of 100 μL cell suspension and 1 μg plasmids (ObRa or ObRb) was electronically transfected by use of the Nucleofector Device (Amaxa) with program G-030. The ObRa and ObRb plasmids in pcDNA3.1(–) originated from the Bjorbaek lab (Bjorbaek et al., 1997) and has been used to test leptin signaling in a variety of cells in our lab (Tu et al., 2007; Tu et al., 2010). The cells were aliquoted into 24-well plates and cultured in 5% CO_2 at 37°C for another 24 h. The cells were treated with glutamate (100 μM) or PBS vehicle for 24 h, and ATP production was measured.

ATP assay

A ViaLight Cell Proliferation and Cytotoxicity Bioassay Kit (LONZA) was used to determine the release of ATP from C6 cells and primary astrocytes. The kit measures cell viability in terms of cellular ATP, as described previously (Yu et al., 2007). The cells were lysed by incubation with lysis buffer for 10 min. The lysate was mixed with 1:1 AK reagent

incubated for 2 min, and the luminescence was determined in a 96-well plate by use of a microplate reader (Molecular Devices). C6 cells or primary astrocytes were treated with the following conditions: glutamate (100 μ M; 48 h), leptin (100 ng/ml; 24 h), or a combination of both glutamate and leptin for 24 h and pre-exposure to glutamate (100 μ M) for the first 24 h followed by a combination of both glutamate and leptin for the next 24 h.

Response of astrocyte-specific leptin receptor knockout (ALKO) mice to seizure induction

ALKO and littermate control male and female FVB mice were used at age 8–16 weeks following a protocol approved by IACUC. The ALKO mice were generated in our laboratory by crossing GFAP-cre mice (Jackson Laboratories) and ObR-floxed mice from the Chua Laboratory, as described and characterized in detail elsewhere (Hsuchou et al., 2011). Unlike neuronal knockout mice, the ALKO mice are not obese. A dose of 300 mg/kg of pilocarpine was used to reduce mortality. The censor time was 30 min.

Statistical analyses

Means are presented with their standard errors. For single measures, analysis of variance was performed to compare the difference among groups, followed by Bonferroni's and Dunnett's multiple comparison post-hoc tests where appropriate.

Results

1. Behavioral characteristics of pilocarpine-induced seizures

After ip injection of pilocarpine (350 mg/Kg bodyweight), 13 out of 20 C57 male mice showed continuous, recurrent seizures in the first hour. The induction rate was 65%. During status epilepticus, 4 mice died within 30 min, showing 31% mortality. For the remaining 9 mice that survived until 1 h after pilocarpine induction, diazepam treatment at 1 h effectively stopped the seizure. Video monitoring showed that the mice had recurrent seizures 14 ± 4 days after pilocarpine treatment, after an initial seizure-free period of about 2 weeks. The Racine score, reflecting the severity of recurrent unprovoked seizures (i.e., epilepsy), was at Stages 3–5. At about 6 weeks, the epileptic mice and their PBS controls were processed for terminal studies, including IHC or snap freezing of hippocampal and other brain regions for future WB and qPCR analyses.

2. Upregulation of astrocytic ObR in the hippocampus of epileptic mice

To determine whether ObR levels are altered in reactive astrocytes, we examined ObR immunoreactivity in GFAP-positive cells in the hippocampi of mice with epilepsy. Three epileptic mice at Racine scale of 5 were studied with three matched controls at age 3 months, 40 d after ip pilocarpine. In the hippocampus of the control mice, ObR immunoreactivity was mainly seen in GFAP (+) cells, shown by confocal microscopic analysis of images after double-labeling with GFAP and ObR. Both M18 and K20 antibodies of ObR showed substantial colocalization with GFAP, although a smaller number of neurons also showed ObR immunoreactivity that tended to be at a lower level of fluorescent intensity. This indicates a higher level of astrocytic than neuronal expression of ObR protein. In the hippocampus of mice with epilepsy, GFAP immunofluorescent intensity was increased, and the cells had more engorged processes and enlarged cell bodies, indicative of reactive astrogliosis. In these GFAP (+) cells, ObR immunofluorescent intensity was also increased. Besides astrocytes, neurons also showed increased levels of ObR immunoreactivity, although not as abundant as that seen in astrocytes (Fig.1A and 1B).

3. Effect of glutamate treatment on GFAP and ObR expression in C6 glioma cells

Glutamate toxicity is a major cause of CNS damage in seizure. To further determine how astroglial leptin receptors participate, we used C6 glioma cells and first tested how glutamate regulates leptin receptor expression. In C6 cells treated with four different concentrations of glutamate (0.05 – 1 mM) for 24 h, there was an increase of both ObRa and ObRb immunoreactivity in comparison with the non-treated control. In high concentrations (0.5 and 1 mM), there was also an increase of GFAP, a marker of reactive astrogliosis (Fig. 2A). Cell density was reduced. Thus, there was dose-dependent cytotoxicity. In addition, glutamate induced upregulation of ObRa at all doses while astrogliosis was seen only at high doses.

To determine whether leptin and glutamate interact with each other in inducing ObR expression, we incubated C6 cells with glutamate (100 μ M), leptin (100 ng/ml), or a combination of both glutamate and leptin for 24 h ($n = 2$ /group). While ObRa protein expression was increased by glutamate and to a lesser extent by leptin, co-treatment with leptin reduced the level of ObRa in comparison with the cells treated with glutamate alone (Fig. 2B and 2C).

4. Consequence of increased expression of ObR subtypes on glutamate toxicity

The effect of glutamate on ATP synthesis was measured by the ViaLight cytotoxicity assay. In the resting state, there was no significant difference in cytotoxicity among groups of cells over-expressing the pcDNA empty vector, the short isoform ObRa, or the STAT3-activating long isoform ObRb ($n = 6$ /group). Thus, the introduction of any of the plasmids did not increase cell damage as a result of electroporation. In the presence of glutamate, however, there was a significant reduction of ATP production only in the group of cells over-expressing ObRa ($p < 0.005$, $n = 6$ /group). Thus, overexpression of ObRa appeared to correlate with greater cytotoxicity (Fig. 3).

5. Leptin rescues astrocytes from glutamate gliotoxicity

Although astrocytes are responsible for recycling glutamate and are generally resistant to gliotoxicity, their vulnerability to a glutamate insult varies according to the gliotoxic mechanism incurred. It has been reported that prolonged exposure to 100 μ M glutamate injures astrocytes via oxidative stress (Chen et al., 2000). As shown in figure 4, there was a loss in cell viability (reduction in ATP levels), when C6 cells were exposed to 100 μ M glutamate for 48 h.

To determine whether leptin has a rescue effect on cells that already experienced excitatory gliotoxicity, C6 cells were exposed to 100 μ M glutamate for 24 h, followed by treatment with leptin in the continued presence of glutamate. While positive control C6 cells receiving glutamate for 48 h had a significant reduction of ATP levels, co-treatment of the cells with leptin in the last 24 h attenuated the reduction of ATP and helped to maintain the number of surviving astrocytes (Fig. 4). Thus, leptin co-treatment reduced glutamate toxicity.

6. Astrocytes from ALKO mice lacking leptin signaling show increased glutamate gliotoxicity

As shown above, leptin treatment increased both ObRa and ObRb, and lessened glutamate toxicity in general. However, overexpression of ObRa had an opposite effect and reduced ATP production in C6 cells. This suggests receptor subtype-specific, as well as cell type-specific, actions of leptin. The ALKO mice have a mutant ObR in astrocytes lacking signaling functions (Hsueh et al., 2011), enabling us to use primary astrocyte culture to determine the role of leptin signaling. Similar to C6 cells, wildtype (WT) astrocytes had a reduction of ATP levels after prolonged exposure to glutamate, and this was reversed after

the addition of leptin (Fig. 5). By contrast, ALKO astrocytes did not show a reversal of the reduced ATP production. It appears that the presence of normal leptin signaling in astrocytes helped to reduce glutamate-induced gliotoxicity, though we have not been able to determine the differential effects of ObRa and ObRb, or a role of the soluble leptin receptor.

7. Effect of ALKO on pilocarpine-induced seizures

Since cellular studies pointed to a protective role of leptin in astrocytes after exposure to glutamate-induced gliotoxicity, we hypothesized that the ALKO mutation would worsen epilepsy. We used a lower dose of pilocarpine to induce seizures in the ALKO and WT mice on their FVB background (300 mg/kg) than in the C57 mice (350 mg/kg), but the susceptibility of the FVB mice to seizure-related mortality was higher than we expected, with most mice dying before, or shortly after, stopping seizures by diazepam injection at 30 min. All 6 WT mice died between 9 and 24 min with a mean survival time of 18 min, except for one mouse which crossed the censor time for survival. Among the 15 ALKO mice, 2 mice survived past the censored time of 30 min; the rest of mice in this group had a mean survival time of 15 min. At each time point the ALKO mice had lower survival (Fig. 6).

Discussion

We selected the pilocarpine model of epilepsy for our experiments because it consistently represents major features of human limbic seizures: similar histopathologic changes, recurrent spontaneous seizures that do not remit, and sprouting in the supragranular layer of the dentate gyrus. The current study clearly shows an upregulation of ObR in the hippocampal astrocytes of pilocarpine-induced epileptic mice. Glutamate also increased GFAP and ObRa in cultured astrocytes. Upregulation of ObR has been shown in astrocytes of the hippocampal and hypothalamic regions in a mouse model of experimental autoimmune encephalomyelitis (Wu et al., 2012), in agouti viable yellow mice with spontaneous adult-onset obesity (Pan et al., 2008), and in mice with diet-induced obesity (Hsueh et al., 2009a). In normal animals, a neurotrophic effect of leptin is established (Bouret et al., 2004). In seizure and epilepsy models, both protective and proconvulsive actions of leptin have been reported. For example, in rats with focal seizures induced by neocortical injections of 4-aminopyridine, co-injection of leptin shortens the duration and frequency of seizures (Xu et al., 2008). In mice with pentylenetetrazole-induced generalized convulsive seizures, intranasal leptin delays the onset of seizure, and leptin also reduces neuronal spiking in an in-vitro seizure model (Xu et al., 2008). But in another study, leptin showed a dose-dependent convulsive effect when administered along with NMDA or kainite (Lynch, III et al., 2010). Thus it is possible that leptin shows biphasic effects that are determined by cell-specific actions of the adipokine.

Our results show that astrocytic leptin receptors were increased in the epileptic mice along with astrogliosis. Reactive astrogliosis is a complex regulatory change and affects neuronal viability and axonal regeneration or retraction. In higher vertebrates, reactive gliosis resulting from injury to the CNS is characterized by an increase of GFAP immunoreactivity (Lee et al., 2000). Astrocytes can secrete extracellular matrix molecules and growth factors that influence neuronal survival; they can promote synapse formation, maintain synaptic function by taking up synaptically released glutamate, and actively modulate synaptic function (Pfrieger and Barres, 1996). Glutamate-stimulated astrocytes in culture can release D-serine, an agonist at the NMDA receptor glycine site (Schell et al., 1995). Shortly after seizures, astrocytes release several cytokines and growth factors such as the neuroprotective basic fibroblast growth factor and leukocyte inhibitory factor, which seem to be involved in upregulation of GFAP mRNA after seizures (Jankowsky and Patterson, 2001). These functions may underlie a protective role of astrocytes in epilepsy, leading to the questions of whether leptin signaling through the upregulated astrocytic ObR has beneficial effects, and

whether different ObR isoforms have differential effects related to their distinctive signaling properties.

Glutamate increased ObRa expression in C6 cells doses-dependently. A dose range from 0.05 to 1 mM of glutamate for 24 h enlarged cell volume, and all cells attained the typical morphology of astrocytic swelling. High concentrations of glutamate are neurotoxic, and they may also cause swelling in astrocytes, eventually leading to cell death. There is a dose- and time-dependent transition from astrocytic swelling to cell death when exposed to glutamate (Chen et al., 2000). This indicates that excessive levels of glutamate are not only toxic to neurons but also to astrocytes (Bridges et al., 1992). In our cellular studies, leptin was able to rescue astrocytes from glutamate-induced gliotoxicity. Astrocytes release several gliotransmitters (Araque and Navarrete, 2010) that affect neuronal activity, among which ATP is a primary astrocytic gliotransmitter (Halassa et al., 2009). The cytotoxicity kit incorporating detection of cellular ATP therefore provides a convenient measure of cell viability.

There may be several mechanism by which leptin protects astrocytes from glutamate gliotoxicity. Leptin can provide mitochondrial stabilization and reduction of oxidative stress, thereby protecting hippocampal neurons against glutamate excitotoxicity (Guo et al., 2008). Leptin's action on astrocytes might counteract oxidative stress induced by glutamate, or it may enhance the activity of glutamine synthetase to accelerate the conversion of glutamate to the less toxic glutamine. Regardless, leptin signaling appears to recruit cytoprotective mechanisms, probably through ObRb, whereas excess ObRa in the transfected C6 cells shows the opposite effect of reduced ATP production.

It should be noted that we did not use scopolamine methyl bromide pretreatment to reduce the peripheral cholinergic effects of pilocarpine. This might have contributed to a high mortality, particularly in the FVB strains, and opposite results from that mentioned in a review (Pan et al., 2012) in regard to a worsening of survival of the ALKO mice lacking astrocytic leptin signaling. We used a longer time course of leptin treatment and higher concentration than that used in earlier studies with different models from our lab (He et al., 2009; Hsueh et al., 2009a; Tu et al., 2010). Though the concentration of leptin is pharmacological rather than physiological, it at least shows a proof of principle that leptin signaling may provide a cytoprotective effect against excitotoxicity.

To summarize, epilepsy resulted in upregulation of ObR in the hippocampus. In glial cell culture, glutamate induced the upregulation of ObRa, and leptin treatment attenuated it. Leptin also increased cellular ATP production and protected glial cells from a decrease of ATP production indicative of cell viability. ALKO mice lacking astrocytic leptin signaling showed higher mortality in response to seizure induction than the WT FVB mice. These novel findings of a protective role of astrocytic leptin signaling point to the therapeutic potential of enhancing astrocytic-specific leptin signaling against epilepsy.

Acknowledgments

Funding support was provided by NIH (DK54880 and DK92245 to AJK, and NS62291 to WP). The ObR-floxed mice used to generate ALKO mice originated from Dr. Streamson Chua, Jr. (Department of Pediatrics, Albert Einstein Medical College, New York). The ObRa and ObRb plasmids were kindly provided by Dr. Christian Bjorbaek (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston). We thank Dr. Damir Janigro and Dr. Kirsten Stone for helpful discussions, Dr. Barry Robert for assistance in initiating seizure recording and video monitoring, and Ms. Yuping Wang for technical support.

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Figure 1. Expression of astrocytic ObR in hippocampi of epileptic mice. Astrocytes were immunostained with (A) M18 antibody that recognizes the cytoplasmic domain of all isoforms of ObR (green) and GFAP (red), or (B) K20 antibody against the N-terminus of mouse ObR (green) and GFAP (red). Colocalization of ObR with GFAP was visualized as orange color.

Figure 2.

Exposure to gliotoxic concentrations of glutamate induces expression of ObRa and GFAP, shown by WB in C6 cells. (A) Glutamate showed a dose-dependent effect in a range of 0.05 –1 mM for 24 h. (B) Though both glutamate (100 μ M) and leptin (100 ng/ml) increased ObRa, leptin co-treatment attenuated glutamate-induced ObRa expression at 24 h. (C) Densitometric analysis of WB signals in Fig 2B. The reference gene β -actin served as a

loading control and was unchanged by treatment. Data are mean $\pm$ SEM from $n = 2$ /group.
* $p < 0.05$; ** $p < 0.01$.

Figure 3.

Effect of overexpression of ObRa or ObRb in C6 cells on ATP production in response to PBS vehicle or glutamate (100 μ m) for 24 h (n = 6 /group). The measured ATP is expressed as relative luminescence units per mg of protein. *** p < 0.005 vs control.

Figure 4.

Effect of leptin co-treatment on concurrent or pre-existing glutamate cytotoxicity, determined by the Vialight cell viability assay for ATP production. C6 cells were treated with glutamate (100 μ m) for 24 or 48 h, with or without leptin (100 ng/ml) in the last 24 h, as shown in detail in the figure (n = 6 /group). Quantification of the measured ATP is expressed as relative luminescence units. *** $p < 0.005$ vs control.

Figure 5. Comparison of WT and ALKO astrocytes in response to glutamate gliotoxicity. ATP production was measured in cells treated with glutamate (100 μ M for 24 or 48 h), with or without leptin (100 ng/ml in the last 24 h). ATP levels were measured by the Vialight cell viability assay in comparison with ATP standards [nM] (n = 4 /group). ** p < 0.01; *** p < 0.005 vs WT control.

Figure 6.

The effect of pilocarpine on mouse survival in the acute phase after seizure induction by pilocarpine in the ALKO (n = 15) and WT FVB (n = 6) groups. The ALKO mice had consistently shorter survival times than the WT mice. The survival rate at 25 min censor time after pilocarpine injection was 13% in ALKO mice and 17% in the WT mice.