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Immunohistochemical evidence of functional leptin receptor expression in neuronal and endothelial cells of the rat brain

Jörg Mütze [a], Joachim Roth [a], Rüdiger Gerstberger [a], Kiyoshi Matsumura [b], Thomas Hübschle [a,*]

[a] *Veterinary-Physiology, Justus-Liebig-University Giessen, Frankfurter Strasse 100, D-35392 Giessen, Germany*
[b] *Osaka Institute of Technology, 1-79-1 Kitayama, Hirakata-City, Osaka 573-0196, Japan*

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Abstract

Leptin binding to its functional receptor leads to activation of the JAK-STAT-signaling pathway and especially to the activation of the signal transducer and activator of transcription factor 3 (STAT3). The immunohistochemical detection of nuclear STAT3 translocation is used as a neuroanatomical mapping tool to determine leptin-responsive cells in the rat brain. This study neuroanatomically identifies those brain cell phenotypes showing STAT3 activation after intraperitoneal leptin treatment (5 mg/kg) using immunohistochemical colocalization with neuronal and endothelial cell marker proteins. Leptin treatment induced nuclear STAT3 signals with the strongest response observed 90 min after the treatment. The caudobasal hypothalamus showed a particularly pronounced STAT3 response. Leptin-induced nuclear STAT3 signals were additionally determined in the solitary tract nucleus, the choroid plexus and in the brain endothelium. The vast majority of STAT3-responsive cells proved to be neurons located in the caudobasal hypothalamus, however, a marked number of brain endothelial cells distributed throughout the entire brain got activated as well. In conclusion, neurons and non-neuronal brain cells, e.g., endothelial or choroid plexus cells, seem to express functional leptin receptors and might thereby mediate leptin-dependent functions in the rat brain.

Keywords: Leptin-target cells; Functional leptin receptor (Ob-Rb); Brain endothelium; Signal transducer and activator of transcription 3 (STAT3); Hypothalamus

Circulating leptin exerts its regulatory actions by the activation of functional receptors (Ob-Rb) that are primarily expressed in the hypothalamus. Leptin binding to this long splice variant leads to the activation of the JAK-STAT-signaling pathway and especially to the activation of the signal transducer and activator of transcription factor 3 (STAT3). Consequently, putative involvement of Ob-Rb receptors in any particular leptin-dependent function can be investigated via the analysis of STAT3 activation, e.g., STAT3 phosphorylation and further nuclear translocation of this transcription factor. We, as also others, have used the immunohistochemical detection of nuclear STAT3 translocation as a neuroanatomical mapping tool to determine leptin-responsive cells in the rodent brain [14,16,17,22,23,28].

Only limited information is available on the putative brain cell phenotypes that show leptin-induced STAT3 activation after in vivo leptin challenge. We showed that nuclear STAT3 signals in the rat brain induced by intracerebroventricular (i.c.v.) leptin treatment did not colocalize with astrocytic or oligodendrocytic cell markers, while a subset of nuclear STAT3 signals clearly colocalized with Fos-activated neurons [16]. In line with these findings, leptin-dependent neuronal STAT3 activation has been reported in a subpopulation of TRH-expressing neurons located in the hypothalamic paraventricular nucleus and POMC-expressing neurons in the arcuate nucleus (ARC) [17,23]. In contrast, it has been clearly shown that besides STAT3 activation in neurons, several other brain cell phenotypes do also respond with nuclear STAT3 signals to various leptin-independent (patho-)physiological stimuli. It would thus be of interest to determine other non-neuronal leptin-sensitive brain cell phenotypes, which functionally respond to leptin stimulation in vivo. First, but not conclusive, evidence indicated the expression of the long leptin receptor isoform in the rodent brain. Prominent Ob-Rb expression was reported for hypothalamic [3–9,20,21] and extrahypothalamic [5,8,21] parenchymal cells and neurons. In contrast, brain endothelial cells seem to express the functional receptor at low level [6].

* Corresponding author. Tel.: +49 641 9938 155; fax: +49 641 9938 159.
E-mail address: Thomas.Huebschle@vetmed.uni-giessen.de (T. Hübschle).

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So far, nuclear STAT3 activation after in vivo stimulation with leptin has been shown – in colocalization experiments and therefore without any doubt – exclusively for neurons. It seems to be likely that those brain cells that express the functional leptin receptor are the same cells that would respond with a nuclear STAT3 translocation after leptin stimulation. To test this assumption we attempted to neuroanatomically map those brain cells that show STAT3 activation after intraperitoneal (i.p.) leptin treatment with 5 mg/kg, a dose commonly used for systemic leptin stimulations in rodents and strong enough to provide a presence of high amounts of this hormone in the blood and possibly in the brain. The major purpose of this study is the immunohistochemical phenotype analysis of the leptin-dependent STAT3-activated brain cells.

Twenty male Wistar rats with body weights (BW) of 237 ± 12 g (mean ± S.E.M.) were used for this neuroanatomical study. Animals were kept individually under a 12:12 h day–night cycle with lights on from 7:00 a.m. to 7:00 p.m. with a room temperature adjustment to $23 \pm 1\,^{\circ}$C. All experiments were carried out in accordance with the local Ethics Committee (approval number GI 18/2 – Nr. 42/00). Thirteen animals received an i.p. injection of recombinant murine leptin (gift from Sanofi-Aventis Pharma, Frankfurt, Germany) at a dose of 5 mg/kg BW using four different stimulation periods (30 min: $N = 2$; 60 and 90 min: $N = 4$; 120 min: $N = 3$). Seven control animals were injected i.p. with an equivalent volume of pyrogen-free 0.9% saline using three stimulation periods (60 min: $N = 2$; 90 min: $N = 4$; 120 min: $N = 1$).

STAT3 immunohistochemistry was performed on fresh-frozen immersion-fixed 20 μm rat brain sections as previously described [26]. To detect STAT3 signals, a rabbit anti-STAT3 antibody (sc-482, Santa Cruz Biotechnology, Heidelberg, Germany) was used at a 1:4000 dilution in PB. The use of this antibody in the analysis of both the non-phosphorylated and phosphorylated STAT3 protein as an index of constitutive versus functionally activated STAT3 expression has been clearly demonstrated in the rat brain [16]. Omission of the primary STAT3 antibody as well as preabsorption control experiments in the rat confirmed the specificity of the STAT3 immunohistochemistry [15]. The STAT3 antibody was targeted with a secondary biotinylated goat anti-rabbit antibody (1:200, Vector, Linaris Biologische Produkte, Wertheim-Bettingen, Germany) and finally visualized with Cy3-conjugated streptavidin (1:800, Jackson Immuno-Research, Dianova, Hamburg, Germany).

Colocalization experiments were performed to investigate the nuclear location of the STAT3 immunoreactivity and to analyze the phenotype of those cells responding with a leptin-induced nuclear STAT3 translocation. STAT3 analysis was combined with both the nuclear DAPI stain (Molecular Probes, MoBiTec GmbH, Göttingen, Germany) to verify nuclear localization of the STAT3 signals [12,16,26] and with the immunohistochemical detection of cell-specific neuronal and endothelial marker proteins. Putative leptin-target neurons were assessed with a mouse monoclonal anti-neuron-specific nuclear protein antibody (1:100; anti-NeuN, Calbiochem, VWR Deutschland GmbH, Darmstadt, Germany). Putative leptin-responsive STAT3-activated brain endothelial cells were analyzed using a polyclonal sheep anti-rat von Willebrand factor antibody (1:3000; anti-vW; SARTW-HRP, Affinity Biologicals, Essen, Germany). All sections were coverslipped with a glycerol/PB solution and stored at 4 °C until microscopical analysis was performed.

Sections were analyzed and documented with a conventional light/fluorescent Olympus BX50 microscope (Olympus Optical Co., Hamburg, Germany) as previously described [12,26]. Digital brain maps were arranged with the digital rat brain map atlas of Paxinos and Watson [25]. To judge for specific leptin-induced nuclear translocation of the transcription factor STAT3 within distinct brain nuclei, a semi-quantitative analysis was performed estimating the densities of nuclear STAT3 translocation in a five-point rating scale.

Plasma leptin concentrations were analyzed with a commercial rat leptin radioimmunoassay kit (Biotrend Chemicals GmbH, Köln, Germany) in the Rowett Research Institute, Aberdeen, Scotland. Bioactive plasma IL-6 was measured by a bioassay as previously described [12,26]. Leptin and IL-6 plasma concentrations are presented as means ± S.E.M.

Leptin-target cells were mapped in those brain structures showing a leptin-induced nuclear STAT3 translocation after systemic challenge with 5 mg/kg leptin and compared to the controls. Four different stimulation periods were used ranging from 30 to 120 min (Table 1). The caudobasal hypothalamus seems to be the location at which the most pronounced leptin-induced nuclear STAT3 activation occurs in the brain. The strongest STAT3 response with moderate to high density of nuclear STAT3 expression was observed 90 min after i.p. leptin treatment. This can be depicted from the overview of the caudobasal hypothalamus (Fig. 1). While 90 min after the treatment, the control showed a low nuclear STAT3 expression in the basolateral ARC and some potentially weak cytoplasmatic STAT3 signals throughout that area (Fig. 1A); in contrast, the leptin-treated animal showed a strong nuclear STAT3 expression (Fig. 1B). This proved to be true for many caudobasal hypothalamic nuclei such as the ARC, dorsomedial hypothalamic nuclei (DMH), periarcuate area (PAA), ventral premammillary nucleus (PMV), retrochiasmatic area (RCH) and the ventromedial hypothalamic nucleus (VMH). The intensity of the STAT3 immunoreactivity was strongest at this 90 min time point. Some additional moderate nuclear STAT3 expression was detected in the brainstem solitary tract nucleus. Thirty and sixty minutes after leptin treatment, a tendency of increasing nuclear STAT3 expression was seen. One hundred twenty minutes after leptin treatment nuclear STAT3 signals slowly disappeared.

The direct proof of leptin-induced STAT3 accumulation within cell nuclei is given in colocalization experiments with the nuclear DAPI stain. Systemic treatment of rats with leptin induces, 60 min (Fig. 2B) and 90 min (Fig. 2A and C–H) after its injection, a nuclear translocation of STAT3 immunoreactivity (pink color) in various brain nuclei of the caudobasal hypothalamus, the ARC (Fig. 2A and B), the VMH (Fig. 2A and C–E), the PAA (Fig. 2A) and the RCH (Fig. 2F–H). STAT3 immunoreactivity is shown in red, all DAPI-stained cell nuclei are given in blue and consequently intense pink signals indicate nuclear