

Long-Lasting Effects of Elevated Neonatal Leptin on Rat Hippocampal Function, Synaptic Proteins and NMDA Receptor Subunits

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The high circulating levels of leptin in neonatal rodents do not seem to be regulating energy balance at this age, but rather may play an important role for brain development. We tested the hypothesis that high neonatal leptin levels modify hippocampal function and production of synaptic proteins with possible long-term consequences on long-term potentiation (LTP) in adulthood. We first showed that in postnatal day (PND) 10 neonates, acute leptin treatment functionally activated leptin receptors (ObR) in the CA1 and DG regions of the hippocampus through the induction of phosphoERK1/2, but not phosphoSTAT3 protein although both phospho-proteins were induced in the arcuate nucleus. We next examined whether chronic leptin administration (3 mg/kg BW, intraperitoneally) during the first 2 weeks of life (postnatal day, PND 2–14) produces a functional signal in the hippocampus that alters the expression of NMDA receptor subunits (NR1, NR2A, NR2B), synaptic proteins and LTP in the short and long-term. In PND 10 as in adults (PND 70) rats, chronic leptin treatment increased NR1 expression in the hippocampus while reducing NR2B protein levels. Elevated hippocampal concentrations of synapsin2A and synaptophysin were detected during leptin treatment on PND 10 suggesting increased neurotransmitter release. In adults, only SNAP-25 expression was increased after neonatal leptin treatment. LTP was reduced dramatically by leptin treatment in preweaning rats although the changes did not persist until adulthood. Elevated exposure to leptin during a critical period of neonatal hippocampal development might serve to enhance NMDA-dependent functions other than LTP and have important effects on synaptogenesis and neurotransmitter release. © 2007 Wiley-Liss, Inc.

Key words: neonatal hippocampus; leptin receptor; development; LTP; NMDA receptor subunits; synapsin IIA; synaptophysin; SNAP25

The neonatal period in the rat represents a critical window for brain development and in particular for the hippocampal formation (Avishai-Eliner et al., 2002). This period is very sensitive to environmental influences

including maternal regulation (Liu et al., 2000). An important aspect of maternal regulation is mediated through milk composition, and we have shown previously that fat increase in the maternal diet induces significant increases in plasma levels of leptin in pups (Trottier et al., 1998). Leptin, the protein product of the *ob* gene, is primarily involved in the control of energy balance, whereby high levels of this protein in adult animals are generally associated with reduced food intake and fat accumulation and increased thermogenesis (Ahima et al., 2000). A strikingly different scenario is seen in developing animals, however. Both in humans and rodents circulating leptin concentrations are elevated during infancy, (Ahima et al., 1997; Gomez et al., 1999) yet administration of leptin in developing rodents does not reduce food intake (Mistry et al., 1999; Proulx et al., 2002), although it still affects thermogenesis and fat accumulation (Hamman and Matthaei, 1996; Proulx et al., 2002). The high levels of leptin observed during infancy and the diminution of its ability to affect food intake suggest that leptin may have particular functions during this critical phase of development (Steppan and Swick, 1999) such as angiogenesis, cellular proliferation or apoptosis (Kim et al., 2003; Russo et al., 2004) and possibly also hippocampal excitability (Shanley et al., 2001) and production of synaptic proteins participating in synaptogenesis (Ahima et al., 1999).

Many structural neuronal abnormalities are found in the hippocampus of leptin deficient rodent models (*ob/ob* mouse, *db/db* mouse, or *fa/fa* rat) (Bereiter and

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Jeanrenaud 1979, 1980) that are associated with impaired LTP in the CA1 and poor performance in the water maze (Li et al., 2002). In the *ob/ob* mouse, leptin replacement increased brain growth and DNA content (Steppan and Swick, 1999) as well as synaptic proteins such as syntaxin-1 and synaptosomal associated protein (SNAP-25) (Ahima et al., 1999). Recently, Bouret et al. (2004a) showed that neonatal leptin treatment of *ob/ob* mice could rescue the development of projections from the arcuate nucleus to other feeding-related areas and this effect might be mediated by increased neurite outgrowth (Bouret et al., 2004b). Leptin receptors are found in several CNS areas including the hippocampus (Hakansson et al., 1998) where uptake of serum leptin is high (Banks, 2004) and direct effects of leptin have been documented in adult rats. Acute leptin treatment enhances NMDA receptor function and increases intracellular Ca^{2+} levels in hippocampal cultures (Shanley et al., 2001) although a reduction in neuronal excitability by high doses of leptin has also been shown and might specifically involve PI3-kinase activation (Shanley et al., 2002a,b). In anesthetized adult rats, leptin either enhanced or inhibited LTP according to the dose injected in the dentate gyrus (Wayner et al., 2004).

We develop the hypothesis that the high leptin levels seen in developing pups play a critical role to modify hippocampal function and synaptic proteins with possible long-term consequences on memory in adulthood. We examined whether acute neonatal leptin could elicit a functional signal in the hippocampus and whether chronic leptin administration during the first 2 weeks of neonatal life could modify the expression of NMDA receptor subunits, synaptic proteins and LTP in the short- and long-term. We report that NR1 subunit and synaptic protein expression is increased by neonatal leptin until adulthood, even though we did not observe long-lasting changes in LTP after neonatal leptin treatment.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley females (Charles River, St. Constant, Canada) were received on Gestation Day (GD) 17–18 and housed individually in plastic cages with food and water available ad lib. All animals were housed under constant environmental conditions of temperature (22–25°C), humidity (70–80%) and light (12-hr light-dark cycle with lights on at 08:00). The day of birth was considered Day 0 and litters were culled to 10 pups/mother on Day 2 of life. Other than daily disturbances due to the administration of the treatment (5 min maximum) for the chronic group, pups were kept undisturbed with their mother until tested. Litter weight was recorded every morning before the injections. Only males were used in the experiments. All protocols were approved by the Animal Care Committee at McGill University and followed ethical guidelines from the CCAC.

Chronic and Acute Leptin Treatments

Murine leptin was obtained lyophilized from Preprotech Inc. (Rocky Hill, NJ) and reconstituted in 10 mM Tris buffer at

a pH of 9.5. After dissolution, the pH was readjusted to 7.4 by addition of HCl 1 N. For the chronic treatment, each litter was subdivided into two groups receiving daily intraperitoneal (i.p.) injections of 50 μ l of either leptin (L) (3 mg/kg BW) or vehicle (V) (10 mM Tris-HCl, pH 7.4) between postnatal days (PND) 2–14 of life. All injections were given in the morning between 08:30–10:00. Acute vehicle or leptin (3 mg/kg BW, L) treatment was administered on PND 10 for a subset of naive animals who were returned to their mothers in a quiet room before sacrifice 1 hr after the injection. Control pups were sacrificed without any injection for determination of hippocampal leptin receptor (ObRb) expression. Trunk blood was collected in chilled Eppendorf tubes containing 10 μ l of EDTA (60 mg/ml) and plasma was kept frozen at –20°C until assayed for leptin concentrations (to verify for injection).

Tissue Collection

Brains were collected rapidly and either dissected for hippocampal collection (Western blots) and frozen or post-fixed in a chilled solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, 4°C) for 2 days followed by immersion in a solution of 10% sucrose in phosphate buffer (0.1 M, pH 7.4) for 2 days at 4°C. Brains were then frozen at –80°C until processed for in situ hybridization. Retroperitoneal fat pads and adrenal glands were dissected and weighted. In a subset of animals, rats were anesthetized and sacrificed by transcardiac perfusion with 0.9% saline, followed by 4% paraformaldehyde. Brains were postfixed as described above and prepared for immunohistochemistry.

In Situ Hybridization

Twenty μ m coronal brain sections were collected onto poly-L-lysine coated slides, allowed to desiccate overnight under vacuum at 4°C and kept at –80°C until processed for hybridization. For determination of SOCS-3 and Ob-R, we used specific cDNA fragments as described previously (Richard et al., 1996; Huang et al., 1996; Lebel et al., 2000). For the ObR probe, total cDNA was obtained from rat cerebellum and PCR amplified with specific sense (5-ATGAAGTGGCTTAGA-ATCCCTTCG-3) and antisense (5-TACTTCAAAGAGTGT-CCGCTC-3) primers. Ob-R cDNA (349 bp) was then cloned in pGEM-T vector (Promega, Madison, WI) and sequenced. Sense and antisense RNA probes used in in situ hybridization experiments were obtained by in vitro transcription of cDNAs using SP6 and T7 polymerase (Promega) as described by Simmons et al. (1989). The probes were radiolabeled by incorporation of 35 S-dUTP (Amersham-Pharmacia Biotech, Piscataway, NJ). Before hybridization, the radiolabeled probes were purified with Qiagen's RNEasy kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's recommendations. Sections were fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37°C with proteinase K (20 mg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8), acetylated 10 min with acetic anhydride (0.25% in 0.1 M triethylamine [TEA], pH 8) and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. For control RNase treatment, slides were incubated with RNase (10 mg/ml) in RNase buffer for 1 hr at 37°C. After three washes in KPBS buffer, slides were

dehydrated as described above and treated as for the normal sense and antisense hybridization. After vacuum drying for at least 2 hr, 90 μ l of hybridization solution mixture were spotted on each slide. Hybridization buffer contained 500 μ l of formamide, 60 μ l of 5 M NaCl, 10 μ l of 1 M Tris, pH 8.0, 2 μ l of 0.5 M EDTA, pH 8.0, 50 μ l of 20 \times Denhart's solution, 200 μ l of 50% dextran sulfate, 50 μ l of 10 mg/ml tRNA, 10 μ l of 1 M DTT, 118 μ l DEPC water, and 1×10^7 cpm/ml of the 35 S labeled RNA SOCS-3 or Ob-R probe. The slides were sealed with coverslips and incubated overnight at 60°C. The next day, slides were rinsed 1×20 min and 4×5 min in $4 \times$ SSC (0.6 M NaCl, 60 mM sodium citrate buffer, pH 7), digested 30 min at 37°C with RNase-A (10 mg/ml in 10 mM Tris-500 mM NaCl containing 1 mM EDTA), washed in descending concentrations of SSC (2 \times , 10 min; 1 \times , 5 min; 0.5 \times , 10 min; 0.1 \times , 30 min at 60°C), and dehydrated through graded concentrations (50, 70, 95, 100%) of alcohol. After 2 hr of vacuum drying, the slides were exposed to X-ray film (Eastman Kodak, Rochester, NY) for 3 days (SOCS-3) and 4 days (ObR). Radioactive standards prepared from brain paste with 14 C were exposed simultaneously. Hybridization signal was quantified from brain sections (grain counting) selected in the middle hippocampal area using a computerized densitometry by means of an MCID image analyzer system (Imaging Research Inc., St. Catherine, Canada). For each experimental group, 4–6 animals were analyzed with an average of 6–9 sections/animal. Once removed from the autoradiography cassettes, the slides were defatted in toluene and dipped in NTB2 nuclear emulsion (Eastman Kodak). Exposure time to emulsion varied for each transcript. After developing, slides were rinsed in running tap water for 1 hr, counterstained with thionin (0.25%), and coverslipped with DPX.

For determination of NR1, NR2A, and NR2B mRNA, we used oligonucleotides probes that were described previously (Monyer et al., 1994; Liu et al., 2000) with some variations in the hybridization protocol. Sequences were 5'-TTC CTC CTC CTC CTC ACT GTT CAC CTT GAA TCG GCC AAA GGG ACT-3' for NR1, 5'-AGA AGG CCC GTG GGA GCT TTC CCT TTG GCT AAG TTT C-3' for NR2A and 5'-GGG CCT CCT GGC TCT CTG CCA TCG GCT AGG CAC CTG TTG TAA CCC-3' for NR2B, respectively. Scrambled oligonucleotides of the same size were used as controls for the hybridization protocol. The brain sections were post fixed for 10 min in paraformaldehyde (4%), 5 min in 2 \times SSC, and 10 min in 0.1 M TEA pH 8.0 with 0.25% acetic anhydride, washed 5 min in 2 \times SSC, defatted consecutively through graded concentrations (50, 70, 95, and 100%) of alcohol each for 3 min, then placed in chloroform 10 min, and rehydrated through graded concentrations (100, 95%) of alcohol each for 3 min. After air-drying for at least 2 hr, 150 μ l of hybridization solution mixture were spotted on each slide. Hybridization buffer contained 50% deionized formamide, 600 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% Denhardt's solution 100 \times , 100 μ g/ml of denatured salmon sperm DNA, 100 μ g/ml of yeast tRNA, 10% dextran sulfate, 5 mM dithiothreitol, 1×10^7 cpm/ml of the 35 S labeled NR1, NR2A, or NR2B probe and volume was completed to 1 ml with sterile water. The slides were cover slip-sealed and incubated overnight at 42°C. The next

day, the cover slips were removed and the slides were rinsed 1 min in 2 \times SSC, 3 dips in 2 \times SSC at RT, followed by a wash for 60 min in 100 ml of 1 \times SSC at 60°C under constant stirring. The sections were quickly rinsed in tap water. After at least 2 hr of air-drying, slides were exposed to Kodak Biomax MR scientific imaging film (Amersham Biosciences Inc.) together with radioactive 14 C standards for 5 days (NR1), 7 days (NR2A), and 7 days (NR2B). Hybridization signal on the autoradiograms was quantified from brain sections selected in the middle hippocampal area and for each experimental group, 4–6 animals were analyzed with an average of 3–9 sections/animal. Hybridization of sections with scrambled oligonucleotides did not result in specific signal above the background signal of the film.

Immunohistochemistry for Phospho-STAT3 and Phospho-ERK42/44

Fifty micrograms floating sections throughout the hippocampus of vehicle or leptin-injected pups ($n = 3$ –6/group) were incubated in 1% H₂O₂ and 1% NaOH in KPBS buffer to quench endogenous peroxidase activity. Sections were rinsed for 10 min in 0.3% glycine, followed by incubation for 10 min in 0.03% SDS in dH₂O. After short rinses (2 \times 5 min) in KPBS, sections were incubated in 50% ETOH for 1 hr, washed and air dried for 5–10 min. Sections were then incubated for 20 min with 4% normal goat serum (NGS) in 0.4% Triton X-100 and 1% BSA to block non-specific sites. The sections were then incubated in KPBS-0.1% Triton X-100 (0.1 M, pH 7.4, containing 1% BSA and 1% NGS) with the primary antibody either directed against phospho-STAT3 (New England Biolabs, dilution 1:1,000) or against phospho-ERK42/44 (New England Biolabs, dilution 1:500) overnight at 4°C and for 1–2 hr at room temperature. The next day, sections are briefly washed in KPBS buffer and incubated with the biotinylated secondary antibodies (Goat anti-rabbit, Vectastain, Vector Laboratories, Burlingame, CA, dilution 1:200) for 2 hr at room temperature. Sections were then washed and incubated with Vectastain Elite ABC reagent followed by detection using the DAB reaction. Sections are then mounted on slides, dried and coverslipped with Permount. Immunolabeling was observed using a digital camera (SPOT RT 741 KE Slider, Diagnostic Instruments Inc.) mounted on a microscope and connected to a PC computer. Acquired images were processed by Adobe Photoshop 4.0. For quantification of the specific immunoreactive pERK and pSTAT3 signal, the number of immunoreactive cells were counted over 4–18 sections per animal and for each region. Counting was carried out by two investigators blind to the experimental group.

Western Blotting

Hippocampi collected at the time of sacrifice were used for determination of NR1, NR2A, NR2B proteins as well as synaptic proteins such as synapsin IIA, synaptophysin, and SNAP25 concentrations after chronic neonatal vehicle or leptin injection. One hippocampus per animal ($n = 6$ /age group) was used for determination of proteins at both ages (PND 10 and 68–70). Antibodies for NR1, NR2A, NR2B were purchased from Chemicon International (Temecula, CA). Anti-

synapsin IIA was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), anti-synaptophysin was purchased from Sigma (St. Louis, MO) and anti-SNAP25 was purchased from Alomone Laboratories (ANR-001, Jerusalem, Israel). Hippocampi were homogenized in protein extracting buffer (1× PBS pH7.4 with 1 mM EDTA, 0.2 TIU/ml aprotinin, 2 μM leupeptin, 60 μM benzamidine, and 1 mM PMSF), heated at 100°C for 5 min and centrifuged for 15 min at 15,000 rpm. Supernatants were assayed for protein concentrations using the BioRad reagent (BioRad Laboratories) and samples from equivalent amounts of proteins were compared in each experiment. Homogenates were separated on 12–15% SDS-polyacrylamide gels and proteins were transferred electrophoretically to nitrocellulose membranes overnight at RT. Membranes were blocked with 5% fat-free Carnation powdered milk, 0.1% Tween 20 in 0.05 M Tris-buffer saline (TBS) (pH 7.4) and incubated with the primary antibodies overnight at 4°C and at a concentration of 0.5 μg/ml (NR1, 1:200), 0.2 μg/ml (NR2A, 1:500), 0.1 μg/ml (NR2B, 1:500), 1 μg/ml (SNAP-25, 1:1,000), 0.2 μg/ml (Synapsin IIA; 1:1,000), and 5.36 μg/ml (Synaptophysin, 1:2,500). After several washes in TBS-Tween, membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit antibody (1:2,000, Amersham, Oakville, Canada) for NR1, NR2A, NR2B, SNAP25, or HPR-conjugated anti-goat antibody (1:2,000, Santa Cruz Biotech) for synapsin IIA and HPR-conjugated anti-mouse antibody (1:2,000, Amersham) for synaptophysin. Detection was carried out by an enhanced chemiluminescence (ECL) detection system from Roche Laboratories (Montreal, Canada) on Kodak BioMax XAR films. Optical density was measured on films using computerized densitometry by means of an MCID image analyzer system (Imaging Research Inc., St. Catherine, Canada). Membranes were then washed with TBS-Tween (2 × 10 min) and stripped with 0.05 M Tris-HCl containing 0.7% β-mercaptoethanol and 2% SDS for 2 hr at 70°C. After washing and blocking as described above for the primary antibodies against specific proteins, membranes were incubated with anti-actin antibodies (1:1,000, Santa Cruz Biotech) and processed as described above. Data were normalized against optical density values obtained on the same sample and membrane for actin concentrations.

Electrophysiological Recordings

Experimental procedures were according to the protocol of Yang et al. (1999). Hippocampal slices were prepared from vehicle or leptin-treated male neonates on PND 18–20 (vehicle $n = 13$; Leptin $n = 9$) and on PND 73–80 (vehicle $n = 9$; leptin $n = 7$). Rats were killed by decapitation and the brain was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (aCSF), pH 7.4, equilibrated with 95% O₂–5% CO₂, and containing (mM): 126 NaCl, 24 NaHCO₃, 10 glucose, 3 KCl, 2 MgSO₄, 1.25 NaH₂PO₄ and 2 CaCl₂. Transverse slices (400 μm) were cut from the hippocampus using a Vibroslice (Campden Instruments Ltd, UK). Slices were placed on a nylon net in a conventional gas–fluid interface chamber in which oxygenated aCSF was perfused at a rate of 1.0 ml/min. Slices were maintained at 28°C with a controlled heat source, and their upper surface was exposed to

a warm, humidified 95% O₂, 5% CO₂ atmosphere. In all slices, a surgical cut was made between CA3 and CA1 regions to eliminate the propagation of burst discharges generated in the CA3 region during perfusion with bicuculline (Miles and Wong, 1983). There was a recovery period of 1 hr before electrophysiological recordings. For field potential recordings of the population EPSP (fEPSP), glass micropipettes were filled with aCSF (resistance 2–4 M) and positioned with the aid of a dissecting microscope in stratum radiatum below the surface of the slice. For stimulation, a monopolar insulated tungsten microelectrode was placed in stratum radiatum near the CA3 region at least 1.0 mm lateral from the recording electrode. Constant current pulses (50 μsec, 10–275 μA) were delivered using a stimulus isolator (WPI, A360, Sarasota, FL). Evoked field potentials were recorded with a modified model 3100 amplifier (A–M Systems Carlsborg, WA). Responses were digitized and stored on a microcomputer and analyzed using the software package pClamp 9.0 (Axon Instruments). Responses to test-pulses were monitored every 30 sec using an intensity adjusted to evoke fEPSPs with an amplitude of 50% of maximum. This intermediate intensity level was determined in each slice by delivering pulses from 25–350 μA in 25 μA increments. There was a 15-min baseline period in all experiments. The LTP induction paradigm used was a single train of pulses (50 μsec duration at 100 Hz for 1 sec). All fEPSPs were evoked during bath application of 10 μM bicuculline methiodide (Sigma Chemical). The slope and peak amplitude of fEPSPs were analyzed. The slope of the fEPSP was measured at half-amplitude at a constant latency. Data were standardized to the mean of baseline responses. Long-term potentiation effects were assessed by comparing the means for the 10–15 min baseline period and the period 40–45 min post-tetanus with matched samples.

Statistical Analysis

The data are presented as means ± SEM. Statistical analyses of the data were carried out using two-way or three-way analysis of variance (ANOVA), followed by a test of simple effects when appropriate. Homogeneity of variance was assessed by Bartlett's test and *P*-values were obtained by Tukey HSD (honestly significant differences) or Student's Neuman-Keul's post-hoc tests as appropriate. For simple comparisons between two groups, Student's *t*-test was carried out. The number of animals per group (*n*) is indicated.

RESULTS

Effects of Acute Leptin Injection on the Neonatal Hippocampus

We first determined whether leptin receptors are present in the neonatal hippocampus and whether acute peripheral leptin injection could have functional effects on the hippocampus of PND 10 pups. As shown in Figure 1, we found expression of ObR mRNA in several regions of the neonatal hippocampus and in the arcuate nucleus as a comparison. The expression of ObR was greater in the dentate gyrus compared to other hippocampal regions ($P < 0.01$) and compared to the arcuate nucleus ($P < 0.01$), a known target area of leptin action

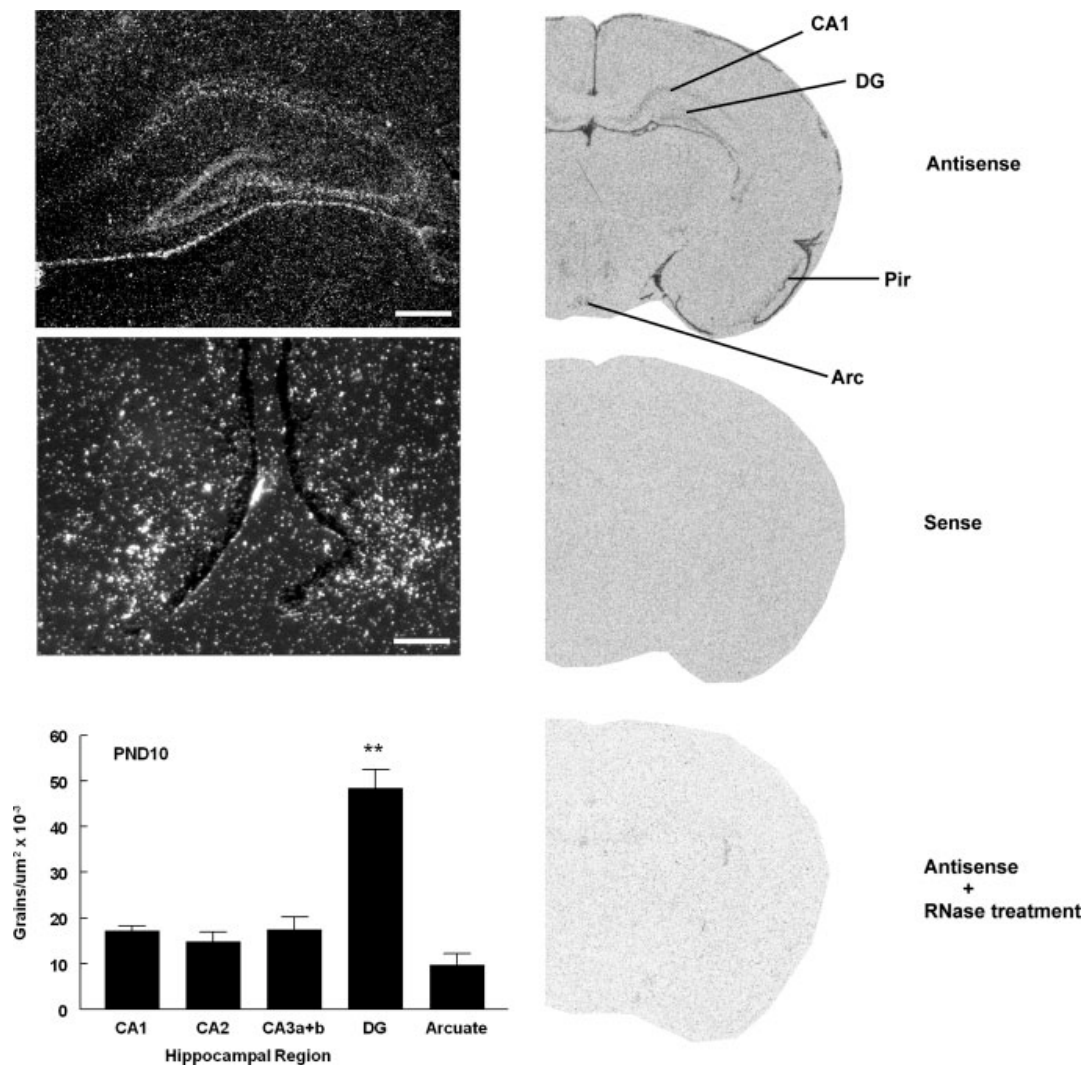


Fig. 1. **Left:** Representative dark field photographs of ObR (including ObRa and ObRb) mRNA expression in the hippocampus (**top**) and arcuate nucleus (**bottom**) of naive PND 10 neonates. High silver grains concentration indicate specific hybridization with cDNA probes in the CA1, CA2, CA3a+b, and dentate gyrus (DG) regions of the hippocampus and arcuate nucleus. Scale bar = 500 μm for low (5×, top); 250 μm for high (10×, bottom) magnification. Quantification of the hybridized signal (grain counting) for ObR mRNA in the CA1, CA2, CA3a+b, and DG regions of the hippocampus in PND 10 pups. Quantification of the signal in the arcuate

nucleus was used as a comparison. Quantification was carried out on an average of six to nine sections per animal and at least four animals were used. Leptin receptor mRNA expression was significantly higher ($P < 0.01$) in the DG compared to other regions of the hippocampus or the arcuate nucleus. **Right:** Specific hybridization for leptin receptor mRNA in PND 10 pups using the antisense (top) and sense (middle) RNA probes as well as the antisense probe with prior RNase treatment (bottom). Note that specific signal was only observed with the antisense probe in the hippocampus, choroid plexus, piriform cortex, and arcuate nucleus.

(Elias, 2000). Analysis of the CA3 region of the hippocampus included both the CA3a and CA3b subdivisions of this pyramidal field (Brunson et al., 2001). To test whether leptin administration could induce functional intracellular messengers in the hippocampus, we identified, counted, and compared cells immunoreactive for the expression of phospho-STAT3 (pSTAT3) and phospho-ERK42/44 (pERK) in the arcuate nucleus (Fig. 2) and hippocampus (Fig. 3) between vehicle and leptin-injected PND 10 pups. In agreement with previous

studies showing a maximal production of leptin-induced signaling molecules (STAT3) at 60 min after i.p. leptin treatment (Mutze et al., 2006), we found a significant induction of pSTAT3 and to a lesser extent, pERK immunoreactivity in the neonatal arcuate nucleus (Fig. 2, Table I). Some pSTAT3 staining was also observed in the DMH and in the VMH (not shown). In contrast, in the hippocampus, leptin induced no detectable pSTAT3 staining, but increased pERK immunoreactivity in PND 10 neonates (Fig. 3, Table I). Immunohistochemical

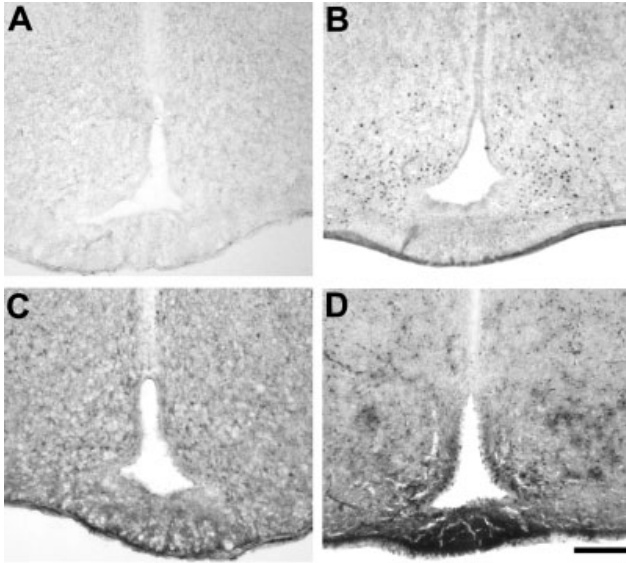


Fig. 2. Immunostaining for phospho-STAT3 (A, B) and phospho-ERK44/42 (C, D) in the arcuate nucleus brain of PND 10 rat pups 1 hr after acute leptin (3 mg/kg b.w.) injection. Arcuate nucleus sections from vehicle- (A,C) or leptin- (B,D) treated pups show a clear expression of phospho-STAT3 and phospho-ERK44/42 proteins in arcuate neurons after acute injection of leptin. Scale bar = 100 μ m for all pictures taken at a 10 \times magnification.

staining for pERK in the hippocampus was confined to the molecular layer of the dentate gyrus and to the stratum oriens of the CA1 region (Swanson, 1998). Although we did not carry out double staining, the shape and location of pERK-IR positive cells suggest that these are GABAergic interneurons rather than glial cells. A small significant induction of SOCS-3 mRNA, a specific inhibitor of leptin action produced on functional stimulation of ObRb receptors and pSTAT3 signaling was observed in the arcuate nucleus (vehicle = 0.948 ± 0.05 optical density unit (OD), leptin = 1.08 ± 0.05 , $P < 0.05$, one-tail t -test), but not in any region of the hippocampus.

Effects of Chronic Neonatal Leptin on the Expression of Hippocampal NMDA Receptor Subunits and Synaptic Proteins

Chronic leptin administration tended to reduce body weight (vehicle, 20.09 ± 0.25 g; leptin, 19.67 ± 0.49) and fat pad weight (vehicle, 0.72 ± 0.16 mg; leptin, 0.54 ± 0.07) in PND 10 pups, but these differences, although documented earlier (Oates et al., 2000; Proulx et al., 2001, 2002), did not reach significance in the present experiments. Plasma leptin levels reached after i.p. injection of leptin usually peak (20-fold increase over basal) at 3 hr after injection and return to normal levels by 12 hr after treatment (Proulx et al., 2002). In previous experiments, we found that chronic leptin treatment significantly increased circulating levels of leptin in pups (vehicle = 3.27 ± 0.7 ng/ml; leptin 3mg/kg = 13.6 ± 2.2) (Oates et al., 2000). We measured leptin-

induced changes in hippocampal NR1, NR2A, NR2B protein expression and mRNA levels during neonatal treatment (PND 10) and in adulthood on male rats (PND 68–70). On PND 10, chronic leptin treatment increased both the mRNA levels ($P < 0.05$ in the CA3) and protein expression ($P < 0.01$) of the NR1 subunit (Fig. 4), whereas there was little effect on NR2A subunit expression. In contrast to NR1, leptin induced a small but significant decrease in NR2B protein expression and a trend toward reduced NR2B mRNA levels in pyramidal neuron populations. Around PND 70 (Fig. 5), the increase in NR1 subunit expression was still maintained although changes in NR1 mRNA levels did not reach statistical significance. NR1 protein levels were still elevated ($P < 0.05$) in adult rats that received neonatal leptin treatment. The decline in NR2B subunit expression induced by leptin at early ages was not maintained in adulthood. In the CA1, CA2, and DG region of the hippocampus, neonatal leptin treatment significantly increased expression of the NR2A subunit mRNA levels ($P < 0.01$ in CA1 and $P < 0.05$ in CA2 and DG) although these changes in mRNA levels did not translate into increases in protein expression in the whole hippocampus. For protein expression, age effects were significant for NR1 ($P = 0.001$), NR2A ($P = 0.001$) and NR2B ($P = 0.0263$), but treatment effects were found only for NR1 ($P = 0.0001$) and NR2B ($P = 0.0692$). Age by treatment interaction was only close to significant for NR1 expression ($P = 0.0962$).

As described previously (Ahima et al., 1999), leptin replacement in *ob/ob* mice increased the expression of several synaptic proteins including SNAP-25 and synaptophysin. We tested whether repeated neonatal leptin treatment could induce the expression of these proteins in pups and maintain the induction until adulthood. As shown in Figure 6, both synapsin IIA and synaptophysin concentrations were increased by leptin in PND 10 pups ($P < 0.01$), in agreement with a participation of these proteins to increased neurotransmitter release during leptin treatment. Although these proteins were not significantly altered in adult rats, concentrations of SNAP25, a synaptic protein mostly associated with presynaptic terminals was increased by neonatal leptin treatment in adults ($P < 0.05$) (Fig. 7). When both age groups (PND 10 and 70) were combined in the statistical analysis, we found a significant age \times treatment effect as well as a significant age \times treatment interaction for synapsin IIA (age: $P = 0.004$; treatment: $P = 0.0128$; age \times treatment: $P = 0.0184$). The age and treatment effects were maintained for synaptophysin (age: $P = 0.009$, treatment: $P = 0.052$) and SNAP25 (age: $P = 0.001$, treatment: $P = 0.0478$), but there was no age \times treatment interaction for these proteins.

Changes in Hippocampal CA1 Long-Term Potentiation With Leptin Treatment

We investigated whether LTP induced by a 100 Hz 1sec tetanus was altered by leptin-treatment. Four to

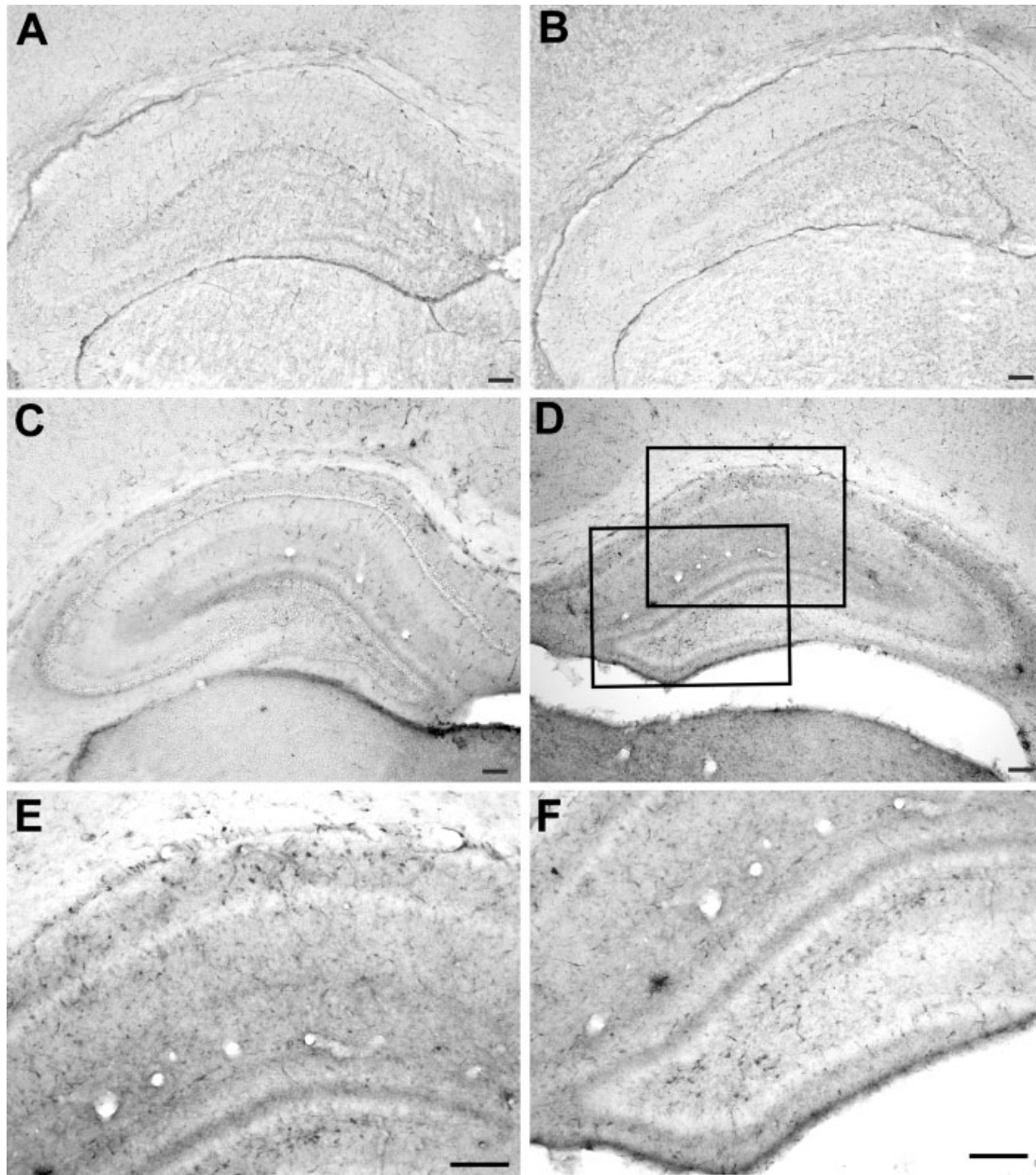


Fig. 3. Immunohistochemical detection of phospho-STAT3 (A, B) and phospho-ERK44/42 (C–F) in the hippocampus of PND 10 rat pups treated acutely with either vehicle (A, C) or leptin (3mg/kg b.w., i.p.) (B, D–F). Compared to vehicle injection (A, C), leptin induced pERK44/42 (D), but not phospho-STAT3 (B) expression in the CA1 and DG region of the hippocampus 1 hr after treatment. At

a higher magnification, we observed a specific activation of phospho-ERK42/44 in the stratum oriens of the CA1 region of the hippocampus (E) and in the molecular layer of the dentate gyrus (F). Squares in (D) indicate the regions being magnified in (E) (CA1) and (F) (DG), respectively. Scale bar = 250 μ m for high (E, F = 10 \times) and low (A–C = 4 \times) magnification.

seven days after the end of neonatal leptin treatment, we observed a dramatic effect on LTP in preweaning male rats (PND 18–21) (Fig. 8, left panel). Indeed, LTP of fEPSPs was $121 \pm 6\%$ ($n = 9$) in controls but only $84 \pm 13\%$ ($n = 8$) in the leptin treated group ($P < 0.05$). No significant differences, however, were noted for LTP in older male rats at PND 32–35 (data not shown) and

PND 70 between controls ($206 \pm 31\%$, $n = 9$) and leptin-treatment ($239 \pm 39\%$, $n = 7$) (Fig. 8, right panel).

DISCUSSION

Our studies show that in addition to the classical hypothalamic targets for leptin in the CNS, this protein

TABLE I. Quantification of Phospho-STAT3 and Phospho-ERK42/44 Immunoreactive Cells 1 Hr After Intraperitoneal Leptin Injection in PND10 Neonates

Region	Treatment	Phospho-STAT3 (n)	Phospho-ERK42/44 (n)
CA1	Vehicle	ND	12.8 ± 2.1 (3)
	Leptin	ND	20.8 ± 3.2 (3)*
DG	Vehicle	ND	8.5 ± 1.5 (4)
	Leptin	ND	17.7 ± 4.6 (4)*
ARC	Vehicle	20.0 ± 6 (6)	43.2 ± 5.9 (4)
	Leptin	104.0 ± 14 (6)**	57.1 ± 7.4 (4)

All values are means ± SEM. For each animal, the number of immunoreactive cells were counted over 4–18 sections for each region. ARC, arcuate nucleus of the hypothalamus; CA1, pyramidal cell field of the hippocampus; DG, dentate gyrus of the hippocampus; ND, not detected.

* $P < 0.05$ compared to vehicle-injected animals (Student's *t*-test).

** $P < 0.01$ compared to vehicle-injected animals (Student's *t*-test).

can also affect hippocampal function, in particular during the neonatal period. Using chronic neonatal treatment with leptin to exemplify the normal physiologic effect of leptin during this critical window of hippocampal development and mimic levels found during exposure to a high fat milk (leptin levels: control milk = 5.3 ± 0.4 ng/ml; high fat milk = 13.7 ± 1.2) (Trottier et al., 1998; Oates et al., 2000), we found a persistent effect of this protein to increase expression of NMDAR subunits, in particular NR1, and expression of synaptic proteins. Functional activation of hippocampal discharge during LTP was abolished in preweaning rats after leptin treatment, but this effect did not persist until adulthood.

In agreement with previous immunohistochemical detection of leptin receptors (ObR) in the hippocampus of adult rodents (Hakansson et al., 1998), we detected a significant expression of leptin receptors (including ObRb and ObRa forms) in all four subregions of the neonatal hippocampus. There has been mounting evidence that the hippocampus represents a target for leptin in adults as both acute electrophysiologic and pharmacologic studies have shown a role for leptin in modulating LTP in vitro (Shanley et al., 2001; Li et al., 2002). In contrast, however, to the arcuate nucleus and other hypothalamic structures where the production of phospho-STAT3 can be used as an index of functional activation of leptin receptors (Bates and Myers, 2004; Bjorbaek, 2004; Myers, 2004), we did not observe any production of phospho-STAT3 in the neonatal hippocampus 1 hr after acute leptin injection. Instead, leptin injection increased the expression of phospho-ERK42/44 (pERK1/2) in the molecular layer of the dentate gyrus and in the stratum oriens of the CA1 area, where many types of interneurons are located (Maccaferri, 2005). One of the main signaling pathways used by leptin on binding to its functional receptor ObRb, is the activation of JAK-1 and JAK-2 proteins, which induces phosphorylation of STAT proteins, including STAT3, STAT5, and STAT6 (Bjorbaek et al., 1997; Cattaneo et al., 1999; Fruhbeck, 2006) and the production of SOCS-3 (suppressor of cytokine signaling-3), an endoge-

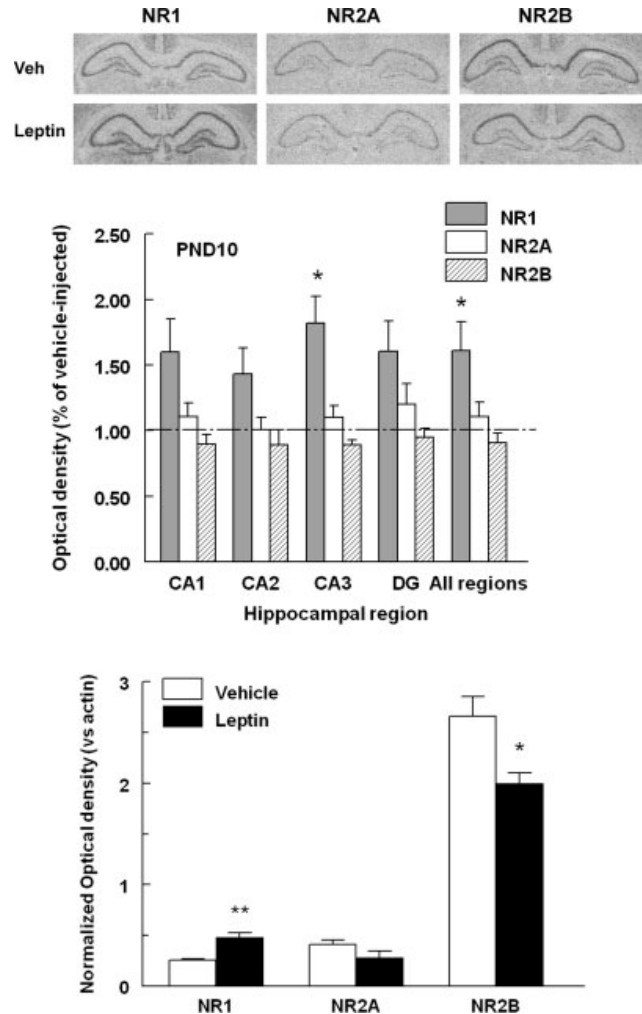


Fig. 4. Leptin-induced changes in NMDA receptor subunits in 10-day-old pups treated daily with either vehicle (open bars) or leptin (3 mg/kg b.w., i.p., closed bars) starting on PND 2. Animals were sacrificed 24 hr after the last vehicle or leptin injection. **Top:** In situ hybridization signal for NR1, NR2A, NR2B subunit mRNA levels in the different regions of the hippocampus. All optical density measurements were taken in the middle portion of the hippocampus between 3.14–4.16 mm posterior to bregma (Paxinos and Swanson, 1982). Values are expressed as percentage of optical density measured in vehicle-injected pups in two experiments and represent the mean ± SEM of 3–4 animals/group. * $P < 0.05$ compared to vehicle-injected group. **Bottom:** Quantification of NR1, NR2A and NR2B protein expression in the hippocampus of PND 10 as measured by Western blots. Values represent the mean ± SEM of six determinations per group. * $P < 0.05$; ** $P < 0.01$ compared to vehicle group. There was no significant differences in leptin-induced expression of NR1 mRNA among different hippocampal regions.

nous inhibitor of leptin action (Dunn et al., 2005). In our experiments and those of others (Mutze et al., 2006), we failed to detect the production of either phospho-STAT3 protein or SOCS-3 mRNA after acute leptin administration in the hippocampus although we can-

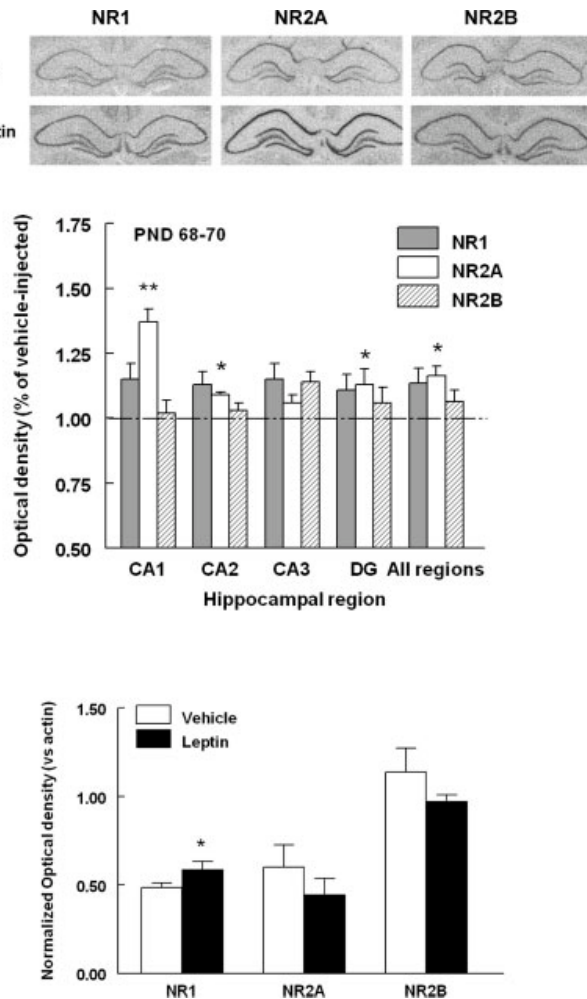


Fig. 5. Long lasting effects of leptin on NMDA receptor subunits in 68–70 day-old male rats treated neonatally (PND 2–14) with either vehicle (open bars) or leptin (3 mg/kg b.w., i.p., closed bars). **Top:** In situ hybridization signal for NR1, NR2A, NR2B subunit mRNA levels in the different regions of the hippocampus. All optical density measurements were taken in the middle portion of the hippocampus between 3.14–4.16 mm posterior to bregma (Paxinos and Swanson, 1982). Values are expressed as percentage of optical density measured in vehicle-injected rats and represent the mean \pm SEM of four to seven animals/group. * $P < 0.05$; ** $P < 0.01$ compared to vehicle-injected group. **Bottom:** Quantification of NR1, NR2A and NR2B protein expression in the hippocampus of adult male rats as measured by Western blots. Values represent the mean \pm SEM of six determinations per group. * $P < 0.05$ compared to vehicle-injected group.

not rule out the possibility that leptin treatment might have activated other STAT proteins. In addition to this pathway, the phosphorylation of JAK2 after leptin activation of both the short (ObRa) and the long (ObRb) forms of the receptor (Bjorbaek et al., 2001; Bjorbaek and Kahn, 2004) also leads to the induction of pERK42/44 via the recruitment of SHP-2, one of the initial steps in the p21ras-ERK signaling pathway (Bates and Myers, 2004). Although our experiments do not

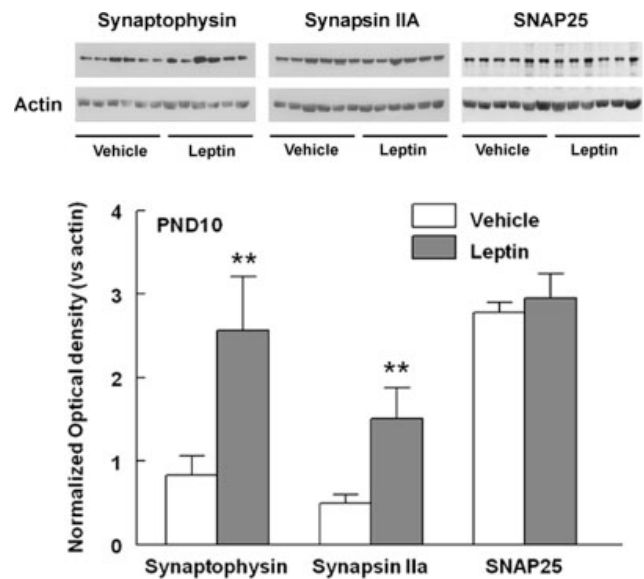


Fig. 6. **Top:** Representative Western blotting of the effects of chronic leptin treatment (PND 2–9) on synaptophysin, synapsin IIA and SNAP25 concentrations in the hippocampus of PND 10 pups. Protein expression was normalized against actin concentrations. **Bottom:** Semi-quantitative determination of synaptic proteins (Synapsin IIA, SNAP25 and synaptophysin) concentrations by Western blotting in the hippocampus of PND 10 pups after neonatal vehicle (open bars) or leptin treatment (dark bars). All values were normalized against actin concentrations. Values represent the mean \pm SEM of six determinations per group. ** $P < 0.01$ compared to vehicle-injected pups.

allow us to determine whether the activation of pERK is direct via leptin receptor activation of hippocampal cells or transsynaptic due to the relatively long time interval (60 min) used to detect pERK, the presence of leptin receptors in this area of the hippocampus would suggest that we have a direct activation. Furthermore, Mutze et al. (2006) have shown that 60 min represents an optimal time point for leptin-induced STAT-3 activation in several regions of the adult rat brain expressing leptin receptors and for which a direct effect of leptin is reported after intraperitoneal injection. Thus, the signaling pathways used by leptin in the neonatal hippocampus might be distinct from those primarily activated in hypothalamic targets and involve at least activation of ERK42/44 proteins.

Interestingly, leptin-induced activation of ERK has been shown to mediate apoptosis in bone marrow stromal cells (Kim et al., 2003), but not proliferation of breast cancer cells, which is dependent on STAT-3 production (Yin et al., 2004). Similarly, in neonatal hippocampal neurons, leptin might be important for cellular proliferation or apoptotic processes or control some aspect of synapse formation or neurite extension as described recently for hypothalamic arcuate neurons (Bouret et al., 2004; Pinto et al., 2004). Indeed, we found that chronic neonatal leptin treatment induced the

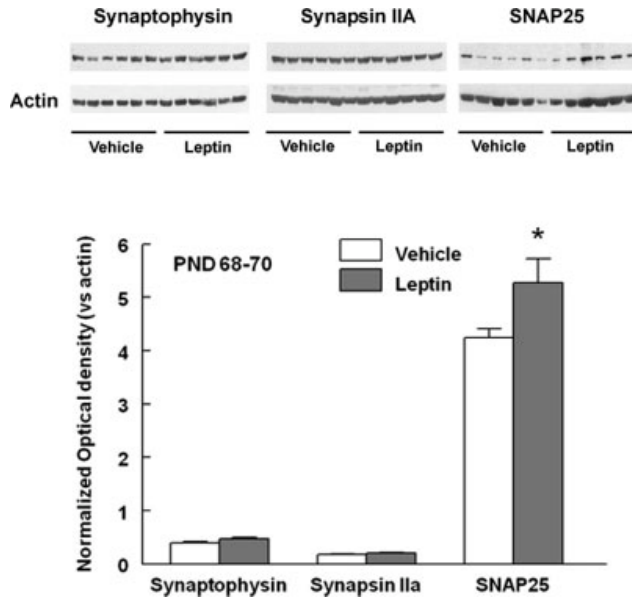


Fig. 7. **Top:** Representative Western blotting of the long-lasting effects of chronic neonatal leptin treatment (PND 2–14) on synaptophysin, synapsin IIA, and SNAP25 concentrations in the hippocampus of adult (PND 68–70) rats. Protein expression was normalized against actin concentrations. **Bottom:** Semi-quantitative determination of synaptic proteins (Synapsin IIA, SNAP25, and synaptophysin) concentrations by Western blotting in the hippocampus of adult rats after neonatal vehicle (open bars) or leptin treatment (dark bars). All values were normalized against actin concentrations. Values represent the mean \pm SEM of six determinations per group. * $P < 0.05$, compared to vehicle-injected pups.

expression of specific synaptic proteins such as synaptophysin and synapsin IIA, which are presynaptic, vesicle-associated proteins implicated in neurotransmitter release and synapse formation (Becher et al., 1999; Chin et al., 1999). The increase in these presynaptic proteins did not persist in adults, in line with the higher state of plasticity typically observed in the early neonatal period. Leptin-induced increase in synaptophysin and synapsin IIA in neonates might indicate a greater number of vesicles in presynaptic terminals and a higher probability for neurotransmitter release at this time. Conversely, the membrane bound increase in SNAP-25 protein in adults treated with leptin neonatally might show enhanced maturation of presynaptic terminals and efficacy of vesicular fusion with presynaptic terminals. Our results are in agreement with earlier studies showing a deficit in synaptic proteins in the hippocampus of rodent models deficient in leptin production or signaling (Ahima et al., 1999).

One of our main findings is that neonatal leptin treatment increased the expression of NR1 subunit of NMDA receptors in all regions of the hippocampus and that the increase in protein expression lasted until adulthood. A concomitant reduction in NR2B was observed at the protein level, but only during neonatal treatment with leptin. NMDA receptor subunits are known to change dramatically during postnatal development

(Monyer et al., 1994; Aamodt and Constantine-Paton, 1999) with a large increase in the NR1 subunit and a switch between high expression of the NR2B form to that of the NR2A form in the second postnatal week. These changes contribute to high synaptic plasticity during this period of hippocampal development. Investigation into the promoter region of the NR1 subunit (Myers et al., 1999) and several in vivo and in vitro (Liu et al., 2001, 2004; Okamoto et al., 2002) studies have pointed to several regulators of NR1 expression, in particular those related to the production of transcription factors binding the AP-1 site such as Fos and Jun (Hiroi et al., 1998), and the GSG site such as Sp1 proteins. In the case of leptin signaling through pERK42/44, a number of transcriptional factors (i.e., CREB, Sp1 and c-Fos) might be produced in activated neurons to enhance transcription of the NR1 promoter (Liu et al., 2001). In the neonatal hippocampus, we detected activation of pERK in the stratum oriens of CA1 and the molecular layer of the dentate gyrus, but not in pyramidal or granule cells of these regions. Interestingly, activation of stratum oriens interneurons has been suggested to provide important feedback information to pyramidal CA1 cells. Although our study did not identify the phenotype of neurons expressing pERK after leptin administration, it is possible that these interneurons are GABAergic because this is the nature of a large portion of horizontal neurons in the stratum oriens. In the molecular zone of the dentate gyrus, activation of precursor neurons might be important for the final make-up of NMDA receptor subunits in the mature granule neurons.

In addition to a direct effect of neonatal leptin via pERK activation, leptin administration might also increase NR1 subunit expression through a reduction of circulating glucocorticoid levels (Mangat et al., 1998) because leptin inhibits endogenous adrenal production of corticosterone in neonates as well as in adults (Salzmann et al., 2004) and causes upregulation of hippocampal glucocorticoid receptors (GR) (Proulx et al., 2001). Others have shown that removal of glucocorticoids in prepubertal rats was associated with an increase in NR1 mRNA levels comparable to that observed in our study (Lee et al., 2003). Neonatal changes in NR2B expression might also be linked to reduced glucocorticoid secretion after leptin treatment (Lee et al., 2003) or to increased synaptic activity as suggested by increased expression of synaptic proteins. Indeed, depolarization and the resulting increased synaptic activity has been shown to reduce NR2B expression in cerebellar neurons (Myers et al., 1999). The reduced expression of NR2B in our young pups treated with leptin might also signal that this treatment modified the timing or amplitude of the shift in NR2B versus NR2A subunits that normally occurs in this period of hippocampal development (Monyer et al., 1994).

Because neonatal leptin treatment modified NR1 and NR2B subunit expression in neonates and adults, we expected to see functional consequences of these changes to enhance hippocampal long-term potentiation (LTP) at both ages. Acute leptin treatment in vivo

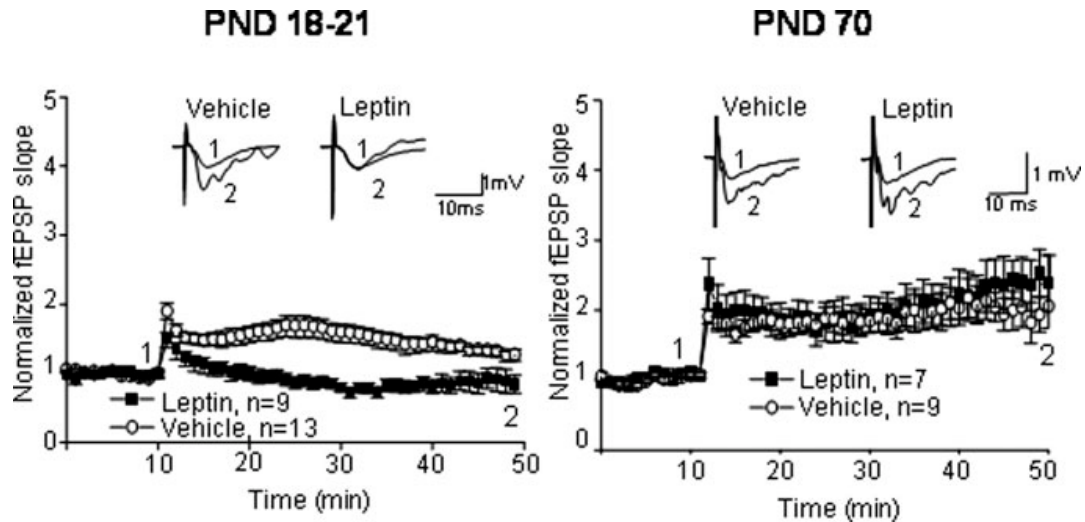


Fig. 8. LTP is abolished in PND 18–21 but not in PND 70 male rats treated neonatally with leptin (PND 2–14). Induction of LTP was done in rat hippocampal slices obtained from age-matched vehicle-treated rats or after neonatal leptin-treatment. Representative fEPSPs were taken before and after LTP induction at the time points

(1 and 2) indicated in the graphs. **Left:** LTP could not be induced at PND 18–21 after neonatal leptin treatment (vehicle, $n = 13$; leptin, $n = 9$). **Right:** in contrast, LTP elicited in the older PND 70 leptin-treated rats or after neonatal leptin-treatment. Representative fEPSPs were taken before and after LTP induction at the time points (vehicle, $n = 9$; leptin, $n = 7$).

(Wayner et al., 2004) and in adult hippocampal slices in vitro (Shanley et al., 2001; Li et al., 2002) have shown that low doses of leptin consistently enhance LTP. Alternatively, impaired LTP and spatial memory have been observed in rodent models of leptin receptor deficiency (Li et al., 2002), suggesting that in adults, leptin displays beneficial effects on hippocampus-mediated functions. Surprisingly, neonatal leptin treatment produced a dramatic suppression of LTP in the CA1 region of the hippocampus 1 week after termination of the treatment in preweaning pups. This effect could not be attributed to increases in GABAergic interneuron neurotransmission because the LTP experiments were carried out in the presence of a GABA_A receptor antagonist, bicuculline. Although we have not determined the mechanism underlying the suppression of LTP, it could be linked potentially to the dose of leptin that was used, as high leptin concentrations reduced LTP in previous experimental paradigms (Li et al., 2002; Wayner et al., 2004). Because we used a repeated chronic leptin treatment that would likely favor higher leptin concentrations, our protocol could promote leptin inhibition of LTP. Another plausible explanation for the suppression of LTP after leptin treatment is the ability of this protein to stimulate IL-1 β levels in the hippocampus (Hosoi, 2002), a cytokine known to induce long-lasting LTP suppression both in vitro and in vivo (Cunningham et al., 1996; Kelly et al., 2003). The fact that early neonatal treatment with leptin suppressed LTP during a critical phase of hippocampal development and that the effect persisted at least 1 week after the end of treatment suggested possible long-term consequences on adult LTP. However, despite the increased expression of NR1 subunits in adults, this was not the case. Although the

role of NR1 subunits on LTP seems to be relatively clear, there remains controversy about the specific roles of NR2A and NR2B on LTP and LTD processes. A reduction in NR2B could counteract the effect of leptin-induced NR1 expression on LTP (Kohr et al., 2003) and recent reports show that for both hippocampal (Liu et al., 2004a) and perirhinal (Barker et al., 2006) plasticity, an increase in NR2A expression would enhance the expression of LTP, whereas increased NR2B is more critical for the induction of LTD. In adult rats, we observed an increased expression of NR2A mRNA levels in the CA1 region, which together with increased NR1 expression should have favored the expression of LTP. Although it is plausible that the magnitude of changes observed in NMDAR subunits expression are too small to affect LTP in our adult offspring, additional experiments are required to clarify the exact mechanisms involved.

In summary, our study shows that increased exposure to leptin during the first 2 weeks of neonatal development, similar to what might occur on exposure to high fat maternal milk can have significant effects on hippocampal function in the long-term. Our results suggest that leptin signaling in the neonatal hippocampus might occur through ERK1/2 (pERK42/44) activation, in contrast to the classical JAK/STAT activation in other CNS targets for leptin. Through this novel mechanism of action, leptin dramatically alters the expression of NMDA receptor subunits and synaptic proteins, suggesting enhanced neurotransmitter release during neonatal leptin treatment. The persistent changes in hippocampal NR1 subunits and SNAP25 expression induced by high neonatal leptin might affect non-LTP dependent hippocampal function or neurotransmitter release in this structure.

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