# Early life stress experience may blunt hypothalamic leptin signalling

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The aim of this study was to investigate whether neonatal maternal separation (MS) – chronic stress experience in early life – affects the anorectic efficacy of leptin in the offspring at adolescence. Sprague–Dawley pups were separated from the dam daily for 3 h during postnatal day 1–14 or left undisturbed as non-handled controls (NH). NH and MS male pups received an intraperitoneal leptin (100 μg/kg) or saline on postnatal day (PND) 28, and then food intake and body weight gain were recorded. The hypothalamic levels of leptin-signalling-related genes, phosphorylated signal transducer and activator of transcription-3 (pSTAT3) and protein-tyrosine phosphatase 1B (PTP1B) were examined at 40 min after a single injection of leptin on PND 39 by immunohistochemistry and Western blot analysis. Leptin-induced suppressions in food intake and weight gain was observed in NH pups, but not in MS. Leptin increased pSTAT3 in the hypothalamic arcuate nucleus of NH pups, but not of MS. Interestingly, basal levels of the hypothalamic PTP1B and pSTAT3 were increased in MS pups compared with NH controls. The results suggest that neonatal MS experience may blunt the anorectic efficacy of leptin later in life, possibly in relation with increased expressions of PTP1B and/or pSTAT3 in the hypothalamus.

[Lee JH, Yoo SB, Kim JY, Lee JY, Kim BT, Park K and Jahng JW 2017 Early life stress experience may blunt hypothalamic leptin signalling. *J. Biosci.* **42** 131–138]

## 1. Introduction

Neonatal maternal separation is considered an animal model of stressful experience early in life. Many of studies have demonstrated that neonatal maternal separation may lead to permanent alterations in the characteristics of the hypothalamic–pituitary–adrenal (HPA) axis responding to stress and the development of depression- and anxiety-like behaviours later in life (see Jahng 2011 for review). We have previously demonstrated that rats that experienced 3 h of daily maternal separation during the first 2 weeks of birth (MS) exhibited depression- and anxiety-like behaviours (Lee *et al*. 2007; Ryu *et al.* 2009; Yoo *et al*. 2013). Interestingly, MS rats showed binge-like eating with increased HPA axis activity when they were challenged with repeated fasting/refeeding cycles during the adolescent period (Ryu *et al*. 2008). Not only the corticosterone increase but also the hypothalamic feeding peptides expressions responding to food deprivation was exaggerated by MS experience (Ryu *et al*. 2008; Yoo *et al*. 2011). Also, stress-induced weight gain was observed in MS rats (Yoo *et al*. 2013). These findings led us to hypothesize that MS experience may blunt the hypothalamic anorexic signalling system responding to physiologic stresses.

**Keywords.** Food intake; hypothalamus; neonatal maternal separation; stress

Abbreviations used: ARC, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; HPA, hypothalamic–pituitary–adrenal; JAK2, Janus kinase 2; MS, maternal separation; NH, non-handled controls; PND, postnatal day; POMC, proopiomelanocortin; pSTAT3, phosphorylated signal transducer and activator of transcription-3; PTP1B, protein-tyrosine phosphatase 1B


**DOI:** 10.1007/s12038-016-9656-3

Published online: 21 December 2016

Adipose hormone leptin is known to suppress feeding and increase energy expenditure resulting in body weight loss in rodents (Pelleymounter *et al*. 1995; Schwartz *et al*. 1996). Blood leptin levels are proportional to the body fat mass and are normally elevated in human obesity (Considine *et al*. 1996). Leptin is synthesized in adipocytes and released into the blood stream, and acts on its receptors in the hypothalamic arcuate nucleus (ARC) to regulate energy expenditure and weight gain. Anorexic efficacy of leptin; i.e. reducing food intake and weight gain, is known to be mediated by the effector neurons in the ARC, such as the neurons expressing either the orexic peptides, increasing food intake and weight gain, or the anorexic peptides, suppressing food intake and weight gain. It has been reported that leptin inhibits gene expression of the orexic peptide neuropeptide Y and induces gene expression of the anorexic peptides proopiomelanocortin (POMC) or cocaine- and amphetamine-regulated transcript (CART) in the ARC (Schwartz *et al.* 2000; Sahu 2004).

Leptin binding to its receptor causes the autophosphorylation and activation of Janus kinase 2 (JAK2), and in turn phosphorylation of downstream signalling molecules signal transducer and activator of transcription-3 (STAT3) and STAT5. And then pSTAT3/pSTAT5 dimers are translocated into the nucleus to induce the transcription of target genes (Ghilardi and Skoda 1997; Sahu 2011). The JAK2-STAT3 pathway in the hypothalamus is crucial in maintaining energy homeostasis, evidenced by the morbid obesity of mice lacking STAT3 in brain (Gao *et al*. 2004). The growing interest in obesity control has brought into focus the role of protein-tyrosine phosphatase 1B (PTP1B) as a deregulator of leptin signalling (Morris and Rui 2009). Studies have reported that chronic stress or postnatal caloric restriction increases PTP1B level in brain regions (Shin *et al*. 2012; Qin *et al*. 2015).

In this study, we examined first the anorectic efficacy of leptin injection on food intake and weight gain of MS rats at adolescence, and then the hypothalamic levels of leptin signalling molecules pSTAT3 and PTP1B with immunohistochemistry and Western blot analysis.

## 2. Materials and methods

### 2.1 *Animals*

Sprague–Dawley rats were purchased (Samtako Bio, Osan,Republic of Korea), and cared for in a pathogen-free barrier area with constant control of temperature (22±1°C), humidity (55%) and a 12/12 h light/dark cycle (lights-on at 07:00 h). Standard laboratory food (Purina Rodent Chow; Purina Co., Seoul, Republic of Korea) and membrane-filtered purified water were available *ad libitum*. Animals were cared for according to the Guideline for Animal Experiments 2000, edited by the Korean Academy of Medical Sciences, which is consistent with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, revised 1996. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Seoul National University.

Nulliparous females and proven breeder males were used for breeding in the laboratory of the animal facility, and the pups were reared in a controlled manner to minimize and standardize unwanted environmental stimulation from *in utero* life. On the next morning of delivery [postnatal day (PND) 1], pups were culled to 5 males and 5 females per litter. Each litter was assigned either to the MS group or the non-handled (NH) group. MS was performed as previously described (Lee *et al*. 2007; Ryu *et al*. 2009; Yoo *et al*. 2013). In brief, MS pups were removed from their dams and home cage and placed closely together in a new cage bedded with woodchips (Aspen shaving, Animal JS Bedding, Cheongyang, Korea) for 180 min, and then returned to their home cage and dams. No additional treatment to keep the pups warm during the separation period other than placing them closely together, was offered, and thus, pup cooling during MS was expected. MS was performed during 09:00 h –12:00 h daily from PND 1–14, and then the pups were left with their dams undisturbed until weaning on PND 22. The NH group remained undisturbed until weaning except for routine cage cleaning. For cage cleaning, all rats were moved to a clean cage twice a week. On the weaning day, male pups in each litter were randomly selected and placed in each cage in a group of 2 or 3, and female pups were excluded from this study.

### 2.2 *Drug treatments*

Rats in each cage were assigned together either for leptin (NH/leptin or MS/leptin) or for vehicle (NH/saline or MS/saline) randomly (n=9–10 in each group, total 38 rats from 8 different litters), and received an intraperitoneal injection of leptin (Peprotech, Rocky Hill, NJ, USA) at a dose of 100 μg/2 mL saline/kg or the same injection volume of saline at 30 min before lights-off on PND 28, at early adolescence (Lukkes *et al*. 2009). Food intake and weight gain during 24 h after the leptin or saline injections were recorded. Total amounts of food consumed by the pups in each cage were divided by the number of pups in each cage and each calculated value was considered as n=1 for the evaluation of 24 h food intake of each rat.

For the analyses of immunostaining and Western blot, rats in each group received an intraperitoneal injection of leptin (100 μg/2 mL saline/kg) or the same injection volume of saline again at 30 min before lights-off on PND 39, and were sacrificed at 40 min after the injections. The injection

schedule was decided on in order to minimize any effects of the previous injections done for behavioural assessment, and rats were at mid-adolescence (Lukkes *et al*. 2009). The hypothalamic brain samples were processed for pSTAT3 and STAT3 immunohistochemistry or PTP1B Western blot analysis.

### 2.3 *Immunohistochemistry*

Forty minutes after the leptin or saline injections, rats were anesthetized with overdoses of sodium pentobarbital (Hallym Pharmaceutical Co., Seoul, Korea) and transcardially perfused first with heparinized isotonic saline and then with 4% paraformaldehyde (Merck Co., Damstadt, Germany) in 0.1 M sodium phosphate buffer. Brains were rapidly dissected out, blocked, post-fixed for 2 h, and then transferred into 30% sucrose (Sigma Co., MO, USA) overnight for cryoprotection. Forty-micron coronal sections were cut on a freezing, sliding microtome (HM440E, Microm Co., Germany). Alternate sections were collected throughout the rostro-caudal extent of the hypothalamic arcuate nucleus (ARC). Immunohistochemistry was performed with standard DAB reaction using commercial ABC kit (Vectastain Elite Kit, Vector Laboratories, CA, USA) as previously described (Jahng *et al*. 1998). Monoclonal mouse anti-pSTAT3 antibodies (1:500 dilution, Cell Signaling Tech Inc., Danvers, MA, USA) were used as primary antibodies, and biotinylated anti-mouse IgG (1:200 dilution, Vector Laboratories, CA, USA) as secondary. The remaining alternate sections were processed for STAT3 immunohistochemistry [Polyclonal rabbit anti-STAT3 antibodies (1:1000 dilution, Calbiochem, Darmstadt, Germany) as primary antibodies, and biotinylated anti-rabbit IgG (1:200 dilution, Vector Laboratories, CA, USA) as secondary]. Immunostained sections were mounted in an anatomical order onto gelatin-coated slides from 0.05 M phosphate buffer, air-dried, dehydrated through a graded ethanol to xylene, and cover-slipped with Permount.

The number of pSTAT3 immunopositive cells in each section was blind-counted by hand, and STAT3 auto-counted, after digitizing the immunostained sections using an Olympus BX-51 microscope (Olympus Co., Tokyo, Japan) attached to a Leica image analysis system (DFC290, Leica Microsystems Gmbh, Wetzlar, Germany). STAT3 immunopositive cells in 720×540 micron images were quantified with Multi Gauge V3.0 software (FUJIFILM, Japan) and the mean relative optical density of pixels with densities of at least 2 S.D. above the mean density of the image background was analysed. pSTAT3 manual count was done and double-confirmed. The number of cells in three sections from the ARC region (closest sections to bregma – 1.88 mm; Paxions and Watson 1986) from each brain was averaged per section, and the individual mean counts were averaged across rats within experimental groups.

### 2.4 *Western blot analysis*

Rats were briefly anesthetized in a carbon dioxide chamber at 40 min after the leptin or saline injections, and once unresponsive, brains were removed immediately after decapitation, and the hypothalamic tissues were rapidly dissected on ice, transferred to liquid nitrogen and stored at –80°C. The tissues were homogenized in a single detergent lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton X-100; protease and phosphatase inhibitor cocktail 0.5%) and then centrifuged at 13000*g* for 20 min at 4°C. The supernatants transferred into new tubes were measured for protein contents using a protein assay kit (Biorad DC, Biorad, Inc., Hercules, CA), aliquoted at a 40 μg/20 μL concentration in lysis buffer. The aliquoted samples were then mixed with loading buffer for the next step or stored at –80°C.

The samples were mixed with loading buffer (100 mM Tris, pH 6.8; 200 mM dithiothreitol; 4% SDS; 20% glycerol; 0.2% bromophenol blue) at 1:1 dilution, boiled for 5 min, quickly chilled on ice, and then electrophoresed on 12% SDS-polyacrylamide Tris-glycine gels. The proteins transferred onto nitrocellulose membranes (Hybond-C, Amersham, Bucks, UK) were treated with 5% nonfat dry milk in 1X phosphate buffered saline-Tween (1.46 mM $NaH_2PO_4H_2O$; 8.05 mM $Na_2HPO_4$; 144.72 mM NaCl; 5% Tween 20) overnight at 4°C. The membranes were reacted with monoclonal mouse anti-PTP1B antibodies (Calbiochem, Darmstadt, Germany) at 1:1000 dilution for 1 h, and then reacted with HRP-conjugated goat anti-mouse antibodies (Vector laboratories, CA, USA) at 1:1000 dilution for 1 h at room temperature. The bound antibodies were detected with chemiluminescence according to the manufacturer's instructions (SUPEX, Neuronex, Korea), and quantified using a digital image analysis system (LAS-1000, Fujifilm, Japan).

### 2.5 *Statistical analysis*

Data were analysed by one- and two-way analysis of variance (ANOVA) and preplanned comparisons with the control performed by *post hoc* Fisher's PLSD test using StatView software (Abacus, Berkeley, CA). The level of significance was set at $P<0.05$, and all values were presented as means ± S.E.M.

## 3. Results

Food intake was measured for 24 h following an intraperitoneal injection of leptin or saline (figure 1A). Leptin administration at a dose of 100 μg/kg suppressed food intake of NH pups ($P=0.0499$, NH/saline vs. NH/leptin), but not of MS pups. Analysis of food intake with two-way ANOVA revealed no significant effect of MS or leptin and no

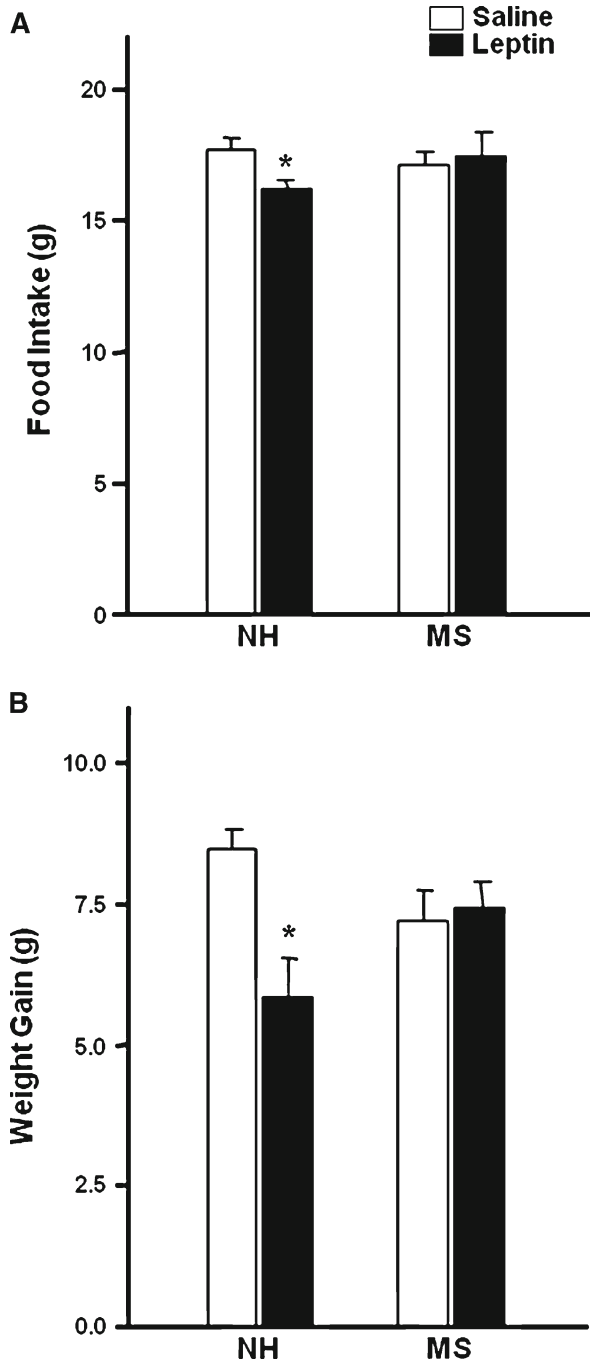


**Figure 1.** Food intake (**A**) and body weight gain (**B**) during 24 h after leptin or saline injection. NH and MS pups received an intraperitoneal injection of leptin at a dose of 100 μg/2 mL saline/kg or the same injection volume of saline at 30 min before lights-off on PND 28. NH, non-handled; MS, maternal separation; *$P$<0.05 vs. NH/saline. Data are presented by means ± S.E.M.

interaction between separation and drug. Weight gain of leptin-injected NH pups during the 24 h of period following the injection was significantly reduced ($P$=0.0016) compared with saline-injected controls; however, leptin-induced weight loss was not observed in the MS group (figure 1B). Analysis of weight gain with two-way ANOVA revealed no significant effect of MS, but a main effect of leptin [$F$(1,33)=5.036, $P$=0.0317] and interaction between MS and leptin [$F$(1,33)=7.354, $P$=0.0105].

Tissue sections of the hypothalamic ARC were prepared at 40 min after the injections and processed for pSTAT3 or STAT3 immunohistochemistry (figure 2A). Leptin significantly increased the number of pSTAT3 immunopositive cells in the ARC of NH pups ($P$=0.0046, NH/saline vs. NH/leptin), but this increase was not observed in MS pups (figure 2B). Interestingly, the number of pSTAT3 immunopositive cells in the ARC of saline-injected MS pups were increased ($P$=0.0199) compared with saline-injected NH pups. Analysis of pSTAT3 levels in the ARC with two-way ANOVA revealed significant effects of MS [$F$(1,15)=6.331, $P$=0.0237] and leptin [$F$(1,15)=5.299, $P$=0.0361], and interaction between MS and leptin [$F$(1,15)=5.393, $P$=0.0347]. Neither leptin nor MS experience altered STAT3 levels in the ARC.

In the Western blot analysis of the hypothalamic PTP1B, leptin did not affect PTP1B levels in both groups. However, the hypothalamic PTP1B levels of saline-injected MS pups was markedly increased ($P$=0.0493) compared with saline-injected NH pups (figure 3).

## 4. Discussion

Exogenic leptin as a potent anorectic molecule has been reported to suppress food intake and weight gain in rodent models (Pelleymounter *et al*. 1995; Schwartz *et al*. 1996). In the present study, intraperitoneal leptin at a dose of 100 μg/kg suppressed food intake and weight gain of NH control rats at early adolescence, proving its anorectic efficacy. The effect of leptin administration in NH rats seemed to be more obvious in weight gain than in food intake. It has been reported that leptin not only suppresses food intake but also increases locomotor activity and energy expenditure (Pelleymounter *et al*. 1995; Morton *et al*. 2011; Ribeiro *et al*. 2011). Although we did not measure the leptin-induced increases in locomotor activity and energy expenditure in the present study, it is plausible that leptin-induced weight loss in NH rats might have been contributed by increased locomotor activity and energy expenditure, in addition to reduced food intake. Interestingly, leptin-induced anorexia was not observed in rats that experienced neonatal maternal separation in the present study, suggesting that neonatal MS experience may blunt the hypothalamic leptin signalling system. To the extent of our knowledge, this is the first report demonstrating that early life stressful experiences may affect the development of the leptin signalling system in

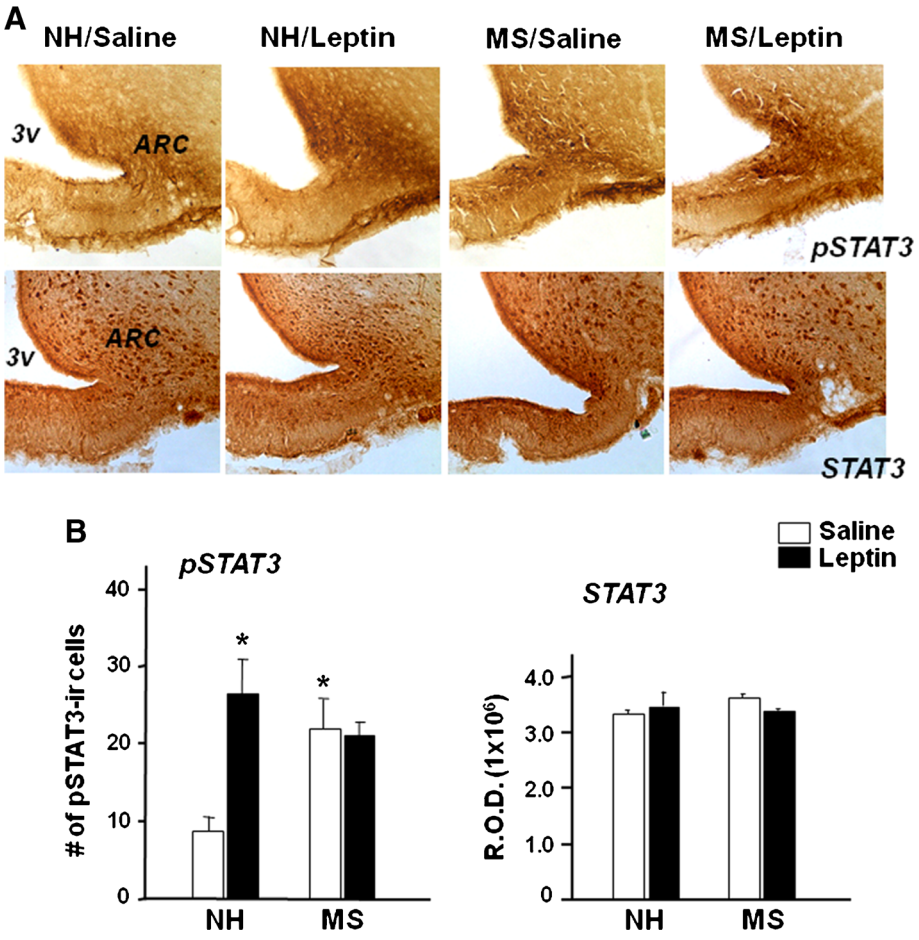


**Figure 2.** Representative microscopic photos (**A**) and quantification (**B**) of pSTAT3/STAT3 immuno-histochemistry in the arcuate nucleus. NH and MS pups received an intraperitoneal injection of leptin at a dose of 100 μg/2 mL saline/kg or the same injection volume of saline at 30 min before lights-off on PND 39, and were sacrificed at 40 min after the injections. NH, non-handled; MS, maternal separation; 3v, third ventricle; ARC, arcuate nucleus; R.O.D., relative optical density; **P*<0.05 vs. NH/saline. Data are presented by means ± S.E.M.

brain. Many studies have reported that leptin is a stress-response hormone and its action may be related with the HPA axis activity (Heiman *et al*. 1997; Konishi *et al*. 2006; Tasker 2006; Malendowicz *et al*. 2007; Jahng *et al*. 2008). In accordance with many other studies done with various types of MS models, our MS model showed a permanent alteration in the HPA axis activity later in life (Jahng 2011). Thus, it is suggested that alterations in the HPA axis activity by neonatal MS experience may affect, at least partly, the development of the leptin signalling system in MS brain.

Leptin binding to its receptor activates the JAK-STAT3 pathway (Ghilardi and Skoda 1997; Sahu 2011), and the leptin signalling via the JAK-STAT3 pathway in the hypothalamus appeared to be crucial in maintaining energy homeostasis and preventing extra weight gain (Gao *et al*. 2004). In this study, intraperitoneal leptin markedly increased the pSTAT3 level, but not STAT3, in the hypothalamic arcuate nucleus of NH control rats, showing the activation of leptin signalling system in the arcuate nucleus where neurons containing the anorectic neuropeptides, such as POMC or CART, are located. However, leptin-induced phosphorylation of STAT3 was not observed in the adolescent MS rats, suggesting that leptin signalling in the hypothalamic arcuate nucleus is blunted by neonatal MS experience. Circulating leptin level, which was decreased during food deprivation (Ahima *et al*. 1996; Schwartz *et al*. 1995; Makimura *et al*. 2003), is increased with refeeding (Wronska *et al*. 2014) and exerts its anorectic efficacy partly by increased expression of POMC and CART in the arcuate nucleus (Korner *et al*. 1999; Schwartz *et al*. 2000). We have previously reported that the adolescent MS pups, but not NH controls, show sustained hyperphagia during repeated

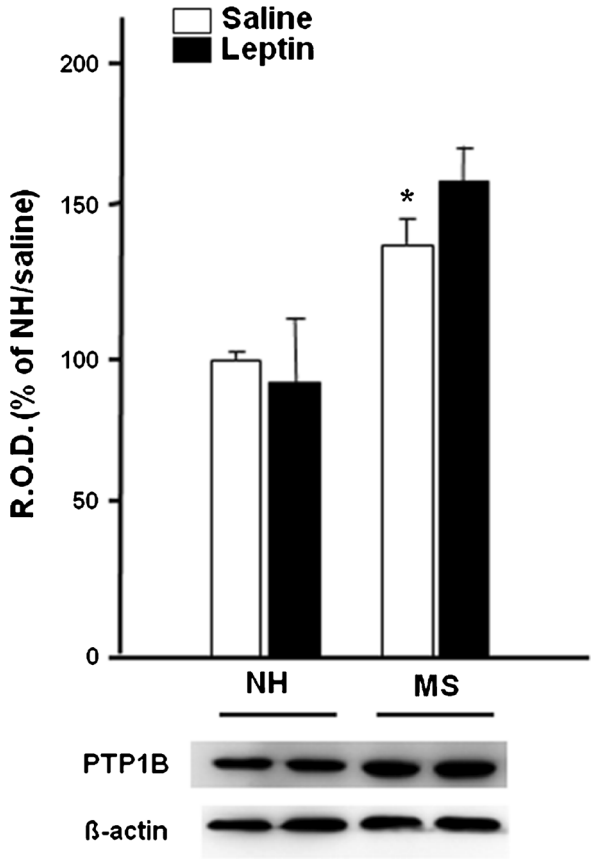


**Figure 3.** Western blot analysis of PTP1B. NH and MS pups received an intraperitoneal injection of leptin at a dose of 100 μg/2 mL saline/kg or the same injection volume of saline at 30 min before lights-off on PND 39, and were sacrificed at 40 min after the injections. NH, non-handled; MS, maternal separation; R.O.D., relative optical density; *$P$<0.05 vs. NH/saline. Data are presented as means ± S.E.M.

fasting/refeeding cycles (Ryu *et al*. 2008). Taken together, it is concluded that a blunted leptin action in the arcuate nucleus of MS rats during the fasting/refeeding cycles might have contributed, at least partly, to the development of sustained hyperphagia.

In this study, the hypothalamic PTP1B level was markedly increased in MS rats compared with NH controls, suggesting a tonic increase of the hypothalamic PTP1B by neonatal MS experience. PTP1B induces dephosphorylation of JAK2, terminating leptin signal transduction (Kaszubska *et al*. 2002; Zabolotny *et al*. 2002). Thus, blunted leptin signalling in MS rats was plausibly due to the increased PTP1B level in the hypothalamus. A high level of PTP1B protein in the hypothalamus is associated with leptin resistance, hyperphagia and obesity (Picardi *et al*. 2008; Chiarreotto-Ropelle *et al*. 2013). Deregulation of the leptin signalling by PTP1B is related to diabetes and obesity (Cheng *et al*. 2002; Zabolotny *et al*. 2002; Asante-Appiah and Kennedy 2003). PTP1B ablation in POMC neurons resulted in leptin hypersensitivity and increased the energy expenditure in mice (Banno *et al*. 2010). Together with our previous report (Ryu *et al*. 2008), it is concluded that adolescent MS rats showed the sustained hyperphagia during fasting/refeeding cycles, at least partly, due to an increased PTP1B level in the hypothalamus, possibly blunting the leptin action. In most studies, increased PTP1B expression in obese states correlates with increased PTP1B activity (Dadke *et al*. 2000; Taghibiglou *et al*. 2002; Wu *et al*. 2005), implicating regulation of PTP1B protein expression as a major mechanism mediating increased PTP1B activity. However, it has not been clear how PTP1B expression is regulated *in vivo*. Chronic stress increased PTP1B activity in the amygdala and induced anxiety in mice, and the behavioural adversity was restored by PTP1B inhibition or by glucocorticoid receptor antagonism (Qin *et al*. 2015). Also, postnatal caloric restriction increased the hypothalamic PTP1B level (Shin *et al*. 2012). Our MS rats were separated from dams for 3 h daily during the first 2 weeks of birth; i.e. the MS pups experienced not only maternal deprivation but also food deprivation for 3 h daily. Although we did not examine the amygdala PTP1B level in this study, and Qin *et al*. (2015) did not report the hypothalamic PTP1B level in their stress model, it is likely that the increased PTP1B level in the hypothalamus of our MS rats is a consequence of repeated (chronic) separation and metabolic stresses in their early lives.

Lastly, pSTAT3 immunopositive cells were increased in the arcuate nucleus of MS rats compared with NH controls in this study. When STAT3 is phosphorylated as a part of leptin signalling cascade, pSTAT3 dimers are translocated into the nucleus and initiate the leptin-induced genes expression, such as POMC or CART (Sahu 2004). However, basal expression levels of POMC and CART in the arcuate nucleus of MS females did not differ from their NH controls (Yoo *et al*. 2011), and the effect of postnatal caloric restriction on the leptin signalling did not show gender differences (Shin *et al*. 2012). Thus, it is not likely that the increased pSTAT3 in the arcuate nucleus of MS rats is associated with increased expression of the anorectic genes POMC and CART. In fact, basal food intake and weight gain of MS pups during adolescent period did not differ from the age-matched NH controls (Ryu *et al*. 2009). Constitutive activation of STAT3 was reported in a cancer cell line, and the STAT3 target genes included ones that were involved in stress response (Xiong *et al*. 2012). Thus, it is likely that neonatal maternal separation, i.e. early life stressful experiences per se, is implicated in the regulatory mechanism underlying a tonic increase of the arcuate pSTAT3 level in MS rats. Further studies are warranted to define the molecular mechanisms involved in the MS-induced increases of pSTAT3 and PTP1B.

**Acknowledgements**

This study was supported by a grant from the National Research Foundation of Korea through the Oromaxillofacial Dysfunction Research Center for the Elderly (2015048003) at Seoul National University in Korea.

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*MS received 05 August 2016; accepted 02 November 2016*

Corresponding editor: Neeraj Jain