Erythropoietin regulates POMC expression via STAT3 and potentiates leptin response

Soumyadeep Dey*, Xiaoxia Li[†], Ruifeng Teng, Mawadda Alnaeeli, ZhiYong Chen, Heather Rogers and Constance Tom Noguchi

Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 10, Room 9N319, 10 Center Drive MSC-1822, Bethesda, Maryland 20892-1822, USA

*S Dey and X Li contributed equally to this work)

[†]X Li is now at Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100191, China

Correspondence should be addressed to C T Noguchi
Email
connien@helix.nih.gov

Abstract

The arcuate nucleus of the hypothalamus is essential for metabolic homeostasis and responds to leptin by producing several neuropeptides including proopiomelanocortin (POMC). We previously reported that high-dose erythropoietin (Epo) treatment in mice while increasing hematocrit reduced body weight, fat mass, and food intake and increased energy expenditure. Moreover, we showed that mice with Epo receptor (EpoR) restricted to erythroid cells (ΔEpoR_E) became obese and exhibited decreased energy expenditure. Epo/EpoR signaling was found to promote hypothalamus POMC expression independently from leptin. Herein we used WT and ΔEpoR_E mice and hypothalamus-derived neural culture system to study the signaling pathways activated by Epo in POMC neurons. We show that Epo stimulation activated STAT3 signaling and upregulated POMC expression in WT neural cultures. ΔEpoR_E mice hypothalamus showed reduced POMC levels and lower STAT3 phosphorylation, with and without leptin treatment, compared to *in vivo* and *ex vivo* WT controls. Collectively, these data show that Epo regulates hypothalamus POMC expression via STAT3 activation, and provide a previously unrecognized link between Epo and leptin response.

Key Words

- ▶ erythropoietin
- ▶ POMC
- ▶ hypothalamus and neuroendocrinology
- ▶ gene expression

Journal of Molecular Endocrinology
(2016) 56, 55–67

Introduction

The hematopoietic cytokine erythropoietin (Epo), the primary regulator of erythrocyte production, is mainly produced in the kidneys in a hypoxia-dependent manner. Epo receptor (EpoR) expression is highest in erythroid progenitors, and its deletion in mice ($\text{EpoR}^{-/-}$) is embryonic lethal due to severe anemia (Wu *et al.* 1995). In addition, EpoR signaling in non-hematopoietic cells has pleiotropic effects beyond erythropoiesis (Noguchi

et al. 2008, Alnaeeli *et al.* 2012). In the brain, Epo has been associated with neurogenesis, neuroprotection, neural progenitor cell (NPC) migration, and memory retrieval (Shingo *et al.* 2001, Tsai *et al.* 2006, Wang *et al.* 2006, Chen *et al.* 2007, Miskowiak *et al.* 2007, Wang *et al.* 2008, Mengozzi *et al.* 2012). Epo treatment in mice increases hematocrit and can also increase energy expenditure, reduce blood glucose levels, and reduce appetite and body

fat mass (Hojman *et al.* 2009, Katz *et al.* 2010, Foskett *et al.* 2011, Teng *et al.* 2011). Expression of an erythroid tissue-specific *EpoR* transgene rescued the embryonic lethal phenotype in *EpoR*^{-/-} mice (Suzuki *et al.* 2002). This mouse model has *EpoR* expression restricted to erythroid tissue (Δ EpoR_E) (Suzuki *et al.* 2002). Previously, we reported that Δ EpoR_E mice exhibit increased body weight from the first postnatal week, show decreased energy expenditure, and develop metabolic syndromes with age via effects on white adipose tissue and hypothalamus (Teng *et al.* 2011).

The hypothalamus is a major central neuroendocrine regulation center involved in the control of energy homeostasis via orexigenic agouti-related peptide (AgRP)/neuropeptideY (NPY)-producing neurons and anorexigenic proopiomelanocortin (POMC)-producing neurons (Bouyer & Simerly 2013). Ablation of POMC neurons and loss of POMC-derived transmitters lead to obesity (Yaswen *et al.* 1999), further underscoring the importance of POMC neurons in regulation of energy homeostasis. Considering our reported findings that show high *EpoR* expression in hypothalamus, regulation of appetite and energy expenditure by exogenous Epo treatment in mice, and POMC induction in response to Epo treatment (Foskett *et al.* 2011, Teng *et al.* 2011), here we examine the regulation of hypothalamus POMC neuron function/response to *EpoR* signaling.

Leptin, a hormone secreted by adipose tissue into the circulation, binds its long-form receptor (LepRb) in the arcuate nucleus of the hypothalamus and activates signal transducer and activator of transcription 3 (STAT3), which positively regulates POMC expression (Bates *et al.* 2003). The largest population of LepRb-expressing neurons is in the hypothalamus (Zuure *et al.* 2013). Mice lacking *leptin*, *LepRb*, or *STAT3* genes become obese with a concomitant decrease in POMC expression (Gao *et al.* 2004, Diano *et al.* 2011, Harlan *et al.* 2011). Conversely, leptin treatment in mice reduced food intake and increased oxygen consumption (Coppari *et al.* 2005). Interestingly, Epo and leptin are both members of the hematopoietic cytokine superfamily and have similar protein structure (Ouyang & He 2003, Fruhbeck 2006), receptor structure (Constantinescu *et al.* 2001, Mancour *et al.* 2012), and signaling mechanism (Banks *et al.* 2000, Zhang *et al.* 2014), and stimulate anorexic response after administration (Balthasar *et al.* 2004, Teng *et al.* 2011). Therefore, here we investigate potential links between Epo and leptin action, regulation of hypothalamus POMC neuron function/response to *EpoR* signaling, and potential cross talk between Epo and leptin signaling in POMC neurons.

Materials and methods

Animal studies

Δ EpoR_E mice were generated using the *TgEpoR* transgene consisting of *EpoR* cDNA driven by the GATA-1 locus erythroid regulatory domain (Suzuki *et al.* 2002) bred onto an *EpoR* null (*EpoR*^{-/-}) background. This rescues the *EpoR*^{-/-} phenotype from severe anemia and death *in utero* (Suzuki *et al.* 2002). These Δ EpoR_E mice can survive through adulthood and their hematocrits are normal. The Δ EpoR_E mice are on of C57BL/6 background and age-matched C57BL/6 WT mice were used as controls. For Epo treatment (3000 U/kg body weight; Epoetin alpha, Amgen, Thousand Oaks, CA, USA), the mice received s.c. injections three times at 0930 h per week. For leptin treatment (5 mg/kg body weight; Peprtech, Rocky Hill, NJ, USA), mice received i.p. injection twice daily (0930 h and 1730 h) for three consecutive days. Mice were housed in individual cages for body weight and food intake studies, kept on 12 h light/darkness cycle, and fed normal chow diet. Studies were conducted following National Institutes of Health guidelines under institution-approved animal protocol.

NPC cultures

Embryos at 15.5 days (E15.5) were harvested from pregnant mice. The developing hypothalamus and cortex were dissected and transferred separately to serum-free media containing DMEM (with L-glutamine)/F12 (1:1) (Invitrogen) supplemented with B27 (Invitrogen), EGF (PeproTech, 20 ng/ml), FGF-2 (PeproTech, 20 ng/ml), and 0.5 units/ml Pen-Strep (Invitrogen). Hypothalamus and cortex tissues were mechanically dissociated into single-cell suspension with a pipette. Cultures were also established from hypothalamus of newborn WT and Δ EpoR_E pups. Cell viability was assessed using trypan blue exclusion. Dissociated cells were cultured as an adherent monolayer and seeded on tissue culture plates pre-coated with 15 μ g/ml poly-L-ornithine (PLO) (Sigma) and 1 μ g/ml laminin (Sigma) with a seeding density of 1×10^5 cells/cm². After 7 days, cells reached around 90% confluency and were passaged at 1:4 to new plates and designated as passage 1. Expansion cultures were passaged every 5 days regularly from first passage onward at 1:4 ratio and recorded as passage 2 through passage 10.

Quantitative RT-PCR

Total RNA was extracted using RNeasy kit (Qiagen) and reverse transcribed using Molony Murine Leukemia virus

(MuLV) reverse transcriptase and the reagents from GeneAmp RNA PCR (Applied Biosystems). The SYBR green-I (Life Technologies)-based relative quantitative real-time PCR was performed on a 7900HT Thermocycler (Applied Biosystems). All primers were designed to span exon junctions in order to prevent the amplification of contaminating genomic DNA and β -Actin mRNA expression was used as a reference. The primer sequences are as follows: *mEpoR*-forward, ACGCTTGGAAGAC-TTGGTGTG; *mEpoR*-reverse, TGTGGCAGTGAACACCA-GAA; *POMC*-forward, TTTCCTGGCAACGGAGA TGA; *POMC*-reverse, CCACCGTAACGCTTGTCCTT; *NPY*-forward, TCGTGTGTTTGGGCATTCTG; *NPY*-reverse, TCTG-GTGATGAGATTGATGTAGTG; *AgRP*-forward, TGACTGC AATGTTGCTGAGTTGTG; *AgRP*-reverse, CTAGGTGCG ACTACAGAGGTTTCG; β -Actin-forward, CAACGGCTCCGG-CATGTGCAAAG, β -Actin-reverse, GGTGTGATGGTGGGAA TGGGTCAAG; *nestin*-forward, AGAGATTGGAAGGCCG CTGG; *nestin*-reverse, CACCTTCCAGGATCTGAGCGA; β -tubulin-forward, GGGCCAAGTTCTGGGAGGTCATC; β -tubulin-reverse, GCACATACTTGTGAGAGGAGGCC.

Western blotting

For western blot analysis, NPC cultures were treated with Epo for 30 min to detect phosphorylated-STAT3 (p-STAT3), phosphorylated-JAK2 (p-JAK2), and phosphorylated-Akt (p-Akt) and for 24 h to detect POMC expression. Cells were lysed in RIPA buffer and protein was resolved on the basis of size in an NuPAGE electrophoresis system (Life Technologies). The primary antibodies used were anti-p-STAT3-Tyr705 (9131), anti-STAT3 (4904), anti-p-JAK2-Tyr1007 (4406), anti-JAK2 (3230), anti- β -Actin (4967) (from Cell Signaling, Danvers, MA, USA), anti-POMC (ab14064), and anti-GAPDH (ab9485) (from Abcam). All antibodies were used at 1:1000 dilution. Either β -Actin or GAPDH protein expression was used to confirm equal protein loading between lanes.

Immunofluorescence analysis

After overnight fasting, mice were treated either with leptin, Epo, or saline at 0930 h. After 90 min, animals were anesthetized with isoflurane and perfused with ice-cold PBS via the heart and then fixed with 100 ml of 4% paraformaldehyde (PFA) solution (Sigma). Brain was removed, post-fixed overnight in 4% PFA, followed by overnight cryoprotection in 30% sucrose, and cut in 10- μ m coronal sections, collected in five series. Immunostaining was done on serial sections with α -MSH antibody

(20074, Immunostar, Hudson, WI, USA) at 1:4000 dilution and counterstained with DAPI for nuclear staining. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Abcam) was used as a secondary antibody. The saline-treated group served as a negative control and tested antibody specificity. For staining NPC cultures, passage 3 cells were grown in cover slips coated with PLO and laminin as described above for NPC culture, and fixed with 4% PFA for 20 min. Due to variability in commercial lots of EpoR polyclonal antibody, we used the MAB developed at Genetics Institute against the EpoR extracellular domain (mh2er.16.5.1) that previously provided consistent results for immunohistochemistry and has been characterized for specificity (D'Andrea *et al.* 1993, Anagnostou *et al.* 1994, Teng *et al.* 2011). Anti-EpoR antibody was used at 1:25 dilution. Passage 1 NPC cells were also used as a negative control for EpoR staining. To detect POMC expression, cells were stained using POMC antibody (H-029-30, Phoenix Pharmaceuticals, Burlingame, CA, USA) at 1:1000 dilution. For fluorescent detection of POMC and EpoR, Alexa488-conjugated goat anti-rabbit IgG and Alexa594-conjugated goat anti-mouse IgG respectively (Abcam) were used.

Statistical analysis

Differences among groups were analyzed by ANOVA with *post-hoc* comparison by Student's *t* test for continuous variables and χ^2 or Fisher's exact test for nominal variables, as appropriate, followed by Bonferroni correction for multiple comparisons. All results are reported as mean $\pm$ s.d. from at least three independent experiments. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

Primary hypothalamus NPC cultures express POMC and EpoR

We previously reported that EpoR protein in mouse hypothalamus localizes to POMC-expressing neurons, and Epo directly regulates POMC expression in these neurons (Teng *et al.* 2011). To study Epo/EpoR-mediated regulation of POMC in mouse hypothalamus, we established NPC cultures from E15.5 mouse hypothalamus. For comparison, primary NPCs derived from E15.5 hippocampus and cortex were used because these brain regions also express EpoR (Yu *et al.* 2002, Chen *et al.* 2007). POMC expression in the respective cultures, determined by quantitative RT-PCR, revealed that *POMC* mRNA was mainly expressed in hypothalamus-derived NPC cultures

and ten times higher than levels detected in cultures derived from cortex or hippocampus (Fig. 1A). In contrast, *EpoR* mRNA expression was readily detected in NPC cultures from each of these three regions with expression highest in the hippocampus. The levels in the hypothalamus were one-half to one-third the levels in the hippocampus or cortex (Fig. 1B).

NPC cultures as an adherent monolayer are prone to density-dependent differentiation. Adherent NPC cultures were passaged when confluence was >90% to promote density-dependent differentiation. With increasing passage, *nestin* mRNA expression decreased by more than an order of magnitude (Fig. 1C) while β -tubulin mRNA expression increased between passage 1 and passage 3

Figure 1

Gene expression in primary neural progenitor cell (NPC) cultures from WT hypothalamus. (A and B) *POMC* (A) and *EpoR* (B) mRNA levels in NPCs derived from embryonic day E15.5 cortex (cor), hippocampus (hip), and hypothalamus (hyp) from WT mice as measured by quantitative real-time (RT) PCR and normalized to β -Actin mRNA levels. (C, D, E and F) Quantification of *nestin* (C), β -tubulin (D), *POMC* (E), and *EpoR* (F) mRNA levels in hypothalamus NPC culture at passages 1, 3, and 10 relative to β -Actin mRNA levels as determined by RT-PCR. Reactions were run in triplicate and results are representative of three independent experiments (G) western blotting for *POMC* (top panel) and β -Actin (middle panel)

protein expression, and quantitative densitometry analysis (lower panel) at passages 1, 3, 5, and 8 of hypothalamus NPC cultures are shown. Densitometry data is representative of three independent experiments. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-way ANOVA followed by Bonferroni correction). (H) Double fluorescent immunostaining for *POMC* (green) and *EpoR* (red) on hypothalamus NPC cultures at passage 3 and stained with DAPI staining (blue) for nuclei and merged for triple stained image. Scale bar represents 100 μ m.

(Fig. 1D), indicating a shift from NPCs to immature neurons concomitant with an increase in POMC and *EpoR* mRNA expression (Fig. 1E and F). POMC protein production examined by western blotting increased between passage 1 and passage 3, was maximal between passages 3 and 5, and then decreased by passage 8 (Fig. 1G). These data suggest that the increase in POMC expression and protein production in these cultures correlates with a shift in nestin-expressing NPCs toward β -tubulin- and POMC-expressing immature neurons with a maximal shift occurring between passages 3 and 5. At passage 10, *EpoR* mRNA continued to be expressed, but β -tubulin and POMC mRNA levels declined (Fig. 1D and E). Immunostaining of passage 3 cells also showed colocalization of POMC and *EpoR* protein expression and the presence of cells that did not stain for either, suggesting *EpoR* expression is specific for POMC neurons in this population of cells (Fig. 1H). These POMC- and *EpoR*-expressing cells comprised ~50% of total cell population in culture at this stage. Moreover, there were no cells that expressed *EpoR* only, and not POMC. Immunostaining of passage 1 cells with anti-POMC and anti-*EpoR* antibodies served as negative controls and confirmed the specificity of these antibodies. The apparent patterns of cytoplasmic anti-*EpoR* staining in POMC neurons may relate to the processing and trafficking of *EpoR* and the reported observations that only a minor fraction of the Golgi-processed receptor is on the cell surface and a few high affinity *Epo* surface binding receptors in neuronal cells are sufficient for *Epo*-activated cell signaling (Hilton *et al.* 1995, Um *et al.* 2007). In all subsequent experiments, cells from passages 3 to 5 which showed optimum POMC and *EpoR* expression were used. In cortex-derived NPC cultures, the low level of POMC expression did not increase with time in passage indicating that cortex-derived NPCs did not exhibit the ability to self-differentiate into POMC-expressing neurons (data not shown).

Epo increases POMC expression in hypothalamus NPC cultures via JAK2/STAT3 pathway

Epo treatment of hypothalamus NPC cultures increased POMC mRNA expression in a dose-dependent manner as determined by quantitative RT-PCR (Fig. 2A). Similar increase in POMC protein expression was also observed as determined by western blotting (Fig. 2B). In addition to regulating POMC, *Epo* treatment increased JAK2- and STAT3-phosphorylation in NPC cultures (Fig. 2C). Pretreatment with STAT3 inhibitor WP1066 (3 μ M) for

30 min was sufficient to abrogate *Epo* induction of POMC (Fig. 2D). While β -tubulin expression did not change with *Epo* treatment, there was a modest decrease (10% or less) in β -tubulin with WP1066 treatment with and without *Epo* (Fig. 2F). These results suggest that STAT3 phosphorylation is required for *Epo* induction of POMC and that inhibition of STAT3 phosphorylation by WP1066 decreases POMC expression by reducing the amount of POMC expression with minimal change in β -tubulin and the proportion of cells with the potential to express POMC. *Epo* treatment of hypothalamus NPC cultures at 10 and 20 U/ml also resulted in a small but significant increase in cell number in a dose-dependent manner at both 32 h and 48 h of treatment (Fig. 2F).

Loss of *EpoR* signaling in hypothalamus NPC cultures affects their leptin response

We previously reported that leptin is not required for the effects of exogenous *Epo* treatment on body weight and fat mass loss (Teng *et al.* 2011). However, *Epo* signaling in NPC is mediated via STAT3 pathway (Fig. 2), which is also activated in the arcuate nucleus after leptin treatment. Whether *Epo*/*EpoR* signaling affects leptin response in hypothalamus was unknown. To investigate this question, hypothalamus-derived NPC cultures were generated from neonate WT and Δ *EpoR_E* mice. With leptin and *Epo* treatment, WT cultures showed an increase in STAT3 phosphorylation (Fig. 3A). In contrast, NPC cultures from Δ *EpoR_E* mice did not show the increase in STAT3 phosphorylations expected with *Epo* treatment (Fig. 3A). Interestingly, Δ *EpoR_E* hypothalamus cultures also did not exhibit the expected increase in STAT3 phosphorylation with leptin treatment (Fig. 3A). Of note, baseline levels of STAT3 phosphorylation were lower in Δ *EpoR_E* hypothalamus NPC cultures compared to those from WT hypothalamus (comparing saline treatment lanes, Fig. 3A). Moreover, *Epo* and leptin combination treatments were not more effective than *Epo* or leptin treatment alone in inducing STAT3 phosphorylation (Fig. 3A). Consistent with STAT3 activation, 24 h treatment with either leptin or *Epo* increased POMC mRNA expression in WT hypothalamus cultures by two- to 2.5-fold, with combination treatment no better than individual treatment conditions (Fig. 3B). In Δ *EpoR_E* hypothalamus cultures, baseline POMC mRNA expression appeared to be fourfold lower than WT cultures, but was induced approximately twofold by leptin or the combination of leptin plus *Epo* treatment, but not by *Epo* alone (Fig. 3B). Note that even with leptin treatment,

Figure 2

Epo treatment induces proliferation, POMC expression, and JAK2 and STAT3 signaling in WT hypothalamus NPC culture. (A and B) Dose-dependent increase in *POMC* mRNA levels relative to untreated cells and adjusted to β -Actin mRNA levels (A), and *POMC* protein normalized to β -Actin after 24 h of Epo-treatment (B) were determined. Densitometry data is representative of three independent experiments. (C) Western blotting for phosphorylated-JAK2 (p-JAK2), total JAK2 (t-JAK2), phosphorylated-STAT3 (p-STAT3), total STAT3 (t-STAT3), and β -Actin protein in control- and Epo-treated (20 U/ml) NPC cultures, and quantitative densitometry analysis of band intensity ratio of p-STAT3 and t-STAT3 are shown. Densitometry data

is representative of three independent experiments. (D and E) Level of *POMC* (D) and β -tubulin (E) mRNA as measured by RT-PCR analysis of NPCs after saline treatment (Cont), Epo treatment, Epo with STAT3 inhibitor WP1066 and WP1066 only. The mRNA expression levels are relative to saline-treated (Cont) cells and adjusted to β -Actin mRNA levels, and represents mean $\pm$ s.d. from triplicate reactions. (F) Proliferation of NPC cultures after 32 h and 48 h of Epo treatment at 0-, 10-, and 20-U/ml doses. Relative cell numbers are representative of triplicate treatments for each dose. Statistical significance is indicated by * P < 0.05 and ** P < 0.01 (one-way ANOVA followed by Bonferroni correction).

POMC expression in Δ EpoR_E cultures was only half the level of control-treated WT cultures (Fig. 3B), and no significant induction in STAT3 phosphorylation was detected (Fig. 3A). Conversely, *NPY* and *AgRP* mRNA expression did not show any difference between WT and Δ EpoR_E hypothalamus NPC cultures, and was reduced similarly by leptin treatment only (Fig. 3C and D). Combination treatment or Epo alone did not affect either *NPY* or *AgRP* mRNA expression (Fig. 3C and D). These data support the previous finding that EpoR expression is specific for POMC neurons (Teng *et al.* 2011) and lack of EpoR neither affect *NPY* and *AgRP* expression nor its response after leptin treatment. These observations collectively point to an important and previously unrecognized

role for EpoR signaling to contribute to leptin response in hypothalamus NPC *ex vivo*. Interestingly, *LepRb* mRNA levels showed a small but significant reduction in Δ EpoR_E cultures compared to the WT (Fig. 3E), but cell proliferation during the NPC culture is not affected by loss of EpoR (Fig. 3F).

Absence of hypothalamus EpoR signaling reduces *in vivo* STAT3 phosphorylation and POMC production, blunts leptin-induced STAT3 activation but not the leptin-induced relative increase of POMC

Ex vivo hypothalamus NPC cultures provided evidence for Epo-stimulated STAT3-activation (Figs 2 and 3). Loss

Figure 3

EpoR signaling is required for optimum leptin response in NPC cultures. (A) Western blotting and quantitative densitometry analysis were used to determine levels of phosphorylated-STAT3, total-STAT3, and β -Actin protein levels (A), in WT and Δ EpoR_E NPC cultures after control saline treatment, Epo treatment, Epo+leptin treatment, and leptin treatment. Epo and leptin was used at 20 U/ml and 1000 U/ml respectively and densitometry analysis is representative of three independent experiments (B, C and D) *POMC* (B), *NPY* (C), and *AgRP* (D) mRNA expression in WT and Δ EpoR_E NPC cultures were determined after Epo, Epo+leptin, and leptin

treatments, relative to control saline-treated WT cells. (E) *LepRb* mRNA expression in WT and Δ EpoR_E NPC cultures. Gene expressions were adjusted to β -Actin mRNA levels and RT-PCR reactions were run in triplicate. (F) Fold change in numbers of NPC from WT and Δ EpoR_E E15.5 embryos. P0 represents cell proliferation from day 1 to day 7 of culture, and P1–P5 represents passages 1–5 after every 5 days. Fold change is representative of three independent experiments. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA followed by Bonferroni correction).

of endogenous Epo activity in Δ EpoR_E hypothalamus should also reduce STAT3 phosphorylation *in vivo*. Δ EpoR_E mice show age-dependent obesity, and at 8 months are 30% or greater in body mass vs WT due to increased fat mass with a corresponding increase in circulating leptin levels (Teng *et al.* 2011). To avoid this apparent obesity related increase in leptin level and the expected change in STAT3 phosphorylation related to increased leptin signaling, we examined hypothalamus STAT3 phosphorylation in young mice prior to development of fat tissue. At postnatal day 2–3, STAT3 phosphorylation in the hypothalamus of Δ EpoR_E mice was reduced by threefold compared with WT mice while levels of total STAT3 were comparable (Fig. 4A). The difference in STAT3 phosphorylation was also evident in Δ EpoR_E mice at postnatal day 4–5 (Fig. 4B).

Regulation of body weight involves a balance between energy intake and energy expenditure. Leptin has the ability to decrease food intake, increase energy expenditure and promote body weight loss. We investigated whether the changed energy balance of Δ EpoR_E mice involved alterations in leptin-mediated signaling in the hypothalamus (Fig. 4C and F). Three-week old mice were used to eliminate possible leptin resistance associated with

older, obese Δ EpoR_E mice, and also to ensure complete development of neurons of the arcuate nucleus. Δ EpoR_E hypothalamus showed a lower level of STAT3 phosphorylation compared to WT hypothalamus (Fig. 4C). However, the difference is reduced compared with mice at postnatal days 2–3 (Fig. 4A) and days 4–5 old (Fig. 4B). This suggests that Δ EpoR_E mice by 3 weeks develop a compensatory mechanism that restores STAT3 phosphorylation closer to normal WT levels. In contrast to *ex vivo* Δ EpoR_E hypothalamus cultures that show no leptin-induced STAT3 phosphorylation (Fig. 3A), Δ EpoR_E mice exhibited a blunted *in vivo* leptin-induced STAT3 phosphorylation in hypothalamus compared with WT mice (Fig. 4C). This suggests that, *in vivo*, other mechanisms attempt to compensate for the apparent loss of direct leptin induction of STAT3 phosphorylation in hypothalamus NPC derived cells.

In vivo, baseline POMC protein levels were lower in Δ EpoR_E mice, analogous to the reduced *POMC* mRNA in Δ EpoR_E hypothalamus cultures (Fig. 3B), and leptin could induce POMC expression only up to baseline WT levels (Fig. 4D). These data suggest that *in vivo* leptin response in Δ EpoR_E hypothalamus is blunted with respect to STAT3 phosphorylation, and the reduction in baseline STAT3

Figure 4

ΔEpoR_E mice show blunted STAT3 activation but equivalent POMC induction in the hypothalamus after leptin treatment compared to WT mice. (A and B) Western blotting and quantitative densitometry analysis (from three pups each of WT and ΔEpoR_E) were used to compare phosphorylated-STAT3 (p-STAT3), total-STAT3 (t-STAT3), and GAPDH protein levels in WT and ΔEpoR_E pups at postnatal day 2-3 (A) and postnatal day 4-5 (B). (C and D) Western blotting and quantitative densitometry analysis demonstrating

phosphorylated-STAT3 (p-STAT3) and total-STAT3 (t-STAT3) (C), POMC and GAPDH (D) in a 3-week-old female WT and ΔEpoR_E mice 45 min after treated with leptin (5 mg/kg body weight) or saline (n=3 for each treatment group). (E and F) Body weight (E), and food intake (F) were determined in WT and ΔEpoR_E mice after saline- or leptin-treatment twice daily for 3 days (n=4 for each treatment group). Statistical significance is indicated by * $P < 0.05$, and ** $P < 0.01$ (one-way ANOVA followed by Bonferroni correction).

activation results in a lower level of POMC in ΔEpoR_E mice, compared with WT mice without and with leptin treatment. Leptin induction of POMC in both WT and ΔEpoR_E mice was also reflected in a small but significant

decrease in body weight after leptin treatment in WT mice (6%) and in an apparent smaller decrease in ΔEpoR_E mice (2.5%) (Fig. 4E). Food intake for 3 days was significantly lower in leptin-treated WT mice, and also showed a

reduced trend in ΔEpoR_E mice (Fig. 4F). In summary, these data suggest that loss of EpoR in young mice reduces baseline hypothalamus STAT3 activation and lowers POMC expression in the hypothalamus. Moreover, leptin sensitivity with respect to body weight loss and reduced food intake are still evident in ΔEpoR_E mice.

Finally, immunohistochemical staining of brain sections displaying the arcuate nucleus region of the hypothalamus also showed lower staining for $\alpha\text{-MSH}$, a cleavage product of POMC, in ΔEpoR_E mice compared to WT mice (Fig. 5A and B). Both Epo and leptin treatment of WT mice induced $\alpha\text{-MSH}$ expression (Fig. 5C and E).

Figure 5

Induction of $\alpha\text{-MSH}$, a POMC product, in the arcuate nucleus region of the mouse brain after either saline, Epo, or leptin treatment. Representative fluorescence photomicrographs for $\alpha\text{-MSH}$ immunoreactivity in brain sections prepared from the mouse mediobasal hypothalamus consisting of the arcuate nucleus region are shown. Three-week old female WT

(A, C and E) or ΔEpoR_E (B, D and F) mice were treated with either saline (A and B), Epo (3000 U/kg body weight) (C and D), or Leptin (5 mg/kg body weight) (E and F). Scale bar represents 100 μm . 3V, third ventricle; ARC, arcuate nucleus; ME, median eminence.

As expected, the ΔEpoR_E mice brain sections showed higher immunoreactivity to $\alpha\text{-MSH}$ only after leptin treatment and not Epo treatment (Fig. 5D and F).

Discussion

EpoR is expressed in the POMC neurons of the hypothalamus, a master regulatory site of appetite and energy expenditure, and the increased POMC expression in Epo-treated WT mice suggested that Epo could directly stimulate these POMC neurons (Teng *et al.* 2011), although the detailed mechanism of POMC neuron response to Epo was uncertain. We previously showed that Epo treatment in WT mice decreases body weight and fat mass by reducing food intake and increasing energy expenditure (Foskett *et al.* 2011, Teng *et al.* 2011). While EpoR is expressed in several brain regions including hypothalamus, hippocampus and cortex, NPC cultures from the hypothalamus show unique induction of POMC expression with Epo treatment. Using hypothalamus-derived NPC cultures, we studied the signaling pathways activated by Epo in POMC neurons and investigated the crosstalk between LepRb and EpoR signaling.

Epo and leptin are both members of the class-I cytokine superfamily (Ouyang & He 2003, Fruhbeck 2006) and act through the JAK/STAT signaling pathway (Constantinescu *et al.* 2001, Mancour *et al.* 2012). Leptin stimulation of POMC expression in the hypothalamus is mediated via STAT3 activation (Bates *et al.* 2003) and we found that Epo-stimulated increase of POMC is also dependent on STAT3 activation. The requirement for EpoR for normal hypothalamus response is further confirmed by analysis of hypothalamus from ΔEpoR_E mice with EpoR expression restricted to erythroid tissue. Neonatal ΔEpoR_E mice show reduced STAT3 phosphorylation and lower level of POMC expression in the hypothalamus compared with WT mice. In addition, corresponding hypothalamus NPC cultures from ΔEpoR_E mice lack activation of STAT3 or induction of POMC with Epo treatment. Evidence that loss of EpoR may blunt the leptin response is further suggested by *ex vivo* findings from ΔEpoR_E hypothalamus cultures that also exhibit loss of leptin-induced increase in STAT3 phosphorylation. These data provide evidence that endogenous Epo signaling in WT hypothalamus contributes to STAT3 phosphorylation while loss of Epo signaling reduces endogenous baseline level of STAT3 activation and POMC production. The reduction in endogenous STAT3 activity in ΔEpoR_E mice is consistent with the severe obese phenotype observed in mice with neural-specific

disruption of STAT3 that underscore its importance in the CNS in regulating energy homeostasis (Gao *et al.* 2004). Interestingly, with leptin treatment in the ΔEpoR_E mice, induction of STAT3 phosphorylation in the hypothalamus is reduced to half that in treated WT mice. While the low level of POMC expression in ΔEpoR_E hypothalamus is increased with leptin treatment and fold induction of POMC in ΔEpoR_E and WT hypothalamus are similar, the level of POMC remains 50% or less than treated WT hypothalamus. The lack of leptin induced STAT3 activation observed in ΔEpoR_E hypothalamus cultures suggests that other Epo-independent signaling pathways compensate in part to provide leptin induced POMC expression in ΔEpoR_E hypothalamus. Epo influence on leptin-responsive weight loss is also suggested by the lower percentage weight decrease in ΔEpoR_E mice.

The finding that leptin requires functional EpoR for normal levels of STAT3 activation and POMC induction in hypothalamus provides an explanation for the low POMC levels in hypothalamus of ΔEpoR_E mice despite elevated circulating leptin levels and for the resultant altered energy homeostasis (Teng *et al.* 2011), and suggests disruption of critical leptin regulation of energy efficiency in the hypothalamus. However, a combination of Epo/leptin treatment did not induce higher POMC expression, which raises the possibility that Epo may prime POMC neurons in the hypothalamus for subsequent leptin action, and the kinetics of treatment is critical to see a combined Epo/leptin effect on POMC expression. Although lack of EpoR does not affect NPC proliferation in culture, LepRb expression is modestly reduced in ΔEpoR_E NPC cultures. This raises the possibility that EpoR signaling is required for the complete maturation of POMC neurons without affecting its numbers.

These data suggest that direct response by the hypothalamus to Epo treatment contributes, in part, to regulation of body weight and fat mass via STAT3 activation and POMC induction. The ΔEpoR_E mice provide further evidence that increased metabolic load of Epo-stimulated erythropoiesis cannot explain the body weight reduction after Epo treatment in WT mice. Specifically, ΔEpoR_E mice treated with Epo exhibit the expected increase in hematocrit, but without the significant reduction in body mass observed in WT mice (Teng *et al.* 2011). Finally, transgene-driven expressions of *EpoR* in ΔEpoR_E mice hematopoietic cells and spleen are comparable to endogenous *EpoR* levels and also ΔEpoR_E mice do not show any difference in hematocrit levels (Suzuki *et al.* 2002, Teng *et al.* 2011).

Leptin receptor is distributed in the arcuate nucleus and other regions of the hypothalamus and in extra-hypothalamic autonomic control sites as well as cortex and hippocampus (Caron *et al.* 2010), indicating that leptin response associated with energy balance involves multiple regions of the brain. EpoR is also expressed in various sites in the brain, such as cortex and hippocampus that are also linked to food intake (Morton *et al.* 2006, Volkow *et al.* 2011), raising the possibility that Epo activity in the brain beyond POMC neurons may modulate metabolic activity. Although AgRP/NPY neurons in the arcuate nucleus of the hypothalamus also contribute to leptin regulation of POMC, EpoR expression was not detected in AgRP/NPY neurons (Teng *et al.* 2011). Moreover, we did not see any difference in AgRP and NPY expression between WT and Δ EpoR_E hypothalamus NPC cultures, and NPY expression was reduced after leptin treatment only. This also suggests that the effect we see in Δ EpoR_E mice is specifically due to Epo-mediated regulation of POMC neurons and not due to secondary effects on AgRP and NPY neurons.

Similar to acute leptin administration (Hill *et al.* 2008), exogenous Epo treatment in WT mice also results in a reduction in food intake and body weight, and an increase in physical activity with activation of POMC neurons (Teng *et al.* 2011). The uncoupling of food intake and energy expenditure has been observed in POMC neurons, and selective deletion of leptin receptor in POMC neurons fails to affect food intake (Balthasar *et al.* 2004). It has been argued that neural control over feeding behavior has several layers of redundancy (Schwartz *et al.* 2003). It should be noted that although the knockout of anorexigenic neuropeptide-expressing POMC gene causes increased food intake (Yaswen *et al.* 1999), the knockout of orexigenic NPY gene does not affect daily food intake (Erickson *et al.* 1996). In this regard, we observed that the Δ EpoR_E mice that show a 50% reduction in POMC expression in the hypothalamus develop obesity, with reduced physical activity, but no detectable increase in food intake (Teng *et al.* 2011).

Endogenous Epo is present in brain and cerebral spinal fluid (Chavez *et al.* 2006), in addition to the adult kidney, Epo can be produced in an oxygen-dependent manner by astrocytes and neurons (Chavez *et al.* 2006) and provide a local source for Epo production to stimulate the EpoR-expressing neural cells in the hypothalamus. Interestingly, hypothalamic astrocytes have also been suggested to influence metabolic status and obesity (Fuente-Martin *et al.* 2012). High fat diet feeding in mice results in inflammatory response and increase in pro-inflammatory

markers in astrocytes, together with a decrease in POMC neurons in the arcuate nucleus (Thaler *et al.* 2012).

Overall, in this study we identified a previously unknown mechanism of EpoR-dependent regulation of POMC expression via STAT3 activation and LepRb signaling in the hypothalamus. We further explored the molecular links underlying the contribution of loss of EpoR in non-hematopoietic tissue to the obese phenotype in Δ EpoR_E mice. Our study also identified the importance of endogenous Epo signaling in regulation of energy homeostasis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, USA.

Author contribution statement

S D and X L designed and performed experiments; S D wrote the paper; R T, M A, Z C, H R, contributed by technical assistance, advice, and manuscript revision; C T N developed the concept, supervised the study, and wrote the paper.

References

- Alnaeeli M, Wang L, Piknova B, Rogers H, Li X & Noguchi CT 2012 Erythropoietin in brain development and beyond. *Anatomy Research International* **2012** 953264. (doi:10.1155/2012/953264)
- Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N & Noguchi CT 1994 Erythropoietin receptor mRNA expression in human endothelial cells. *PNAS* **91** 3974–3978. (doi:10.1073/pnas.91.9.3974)
- Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC Jr, Elmquist JK *et al.* 2004 Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* **42** 983–991. (doi:10.1016/j.neuron.2004.06.004)
- Banks AS, Davis SM, Bates SH & Myers MG Jr 2000 Activation of downstream signals by the long form of the leptin receptor. *Journal of Biological Chemistry* **275** 14563–14572. (doi:10.1074/jbc.275.19.14563)
- Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E *et al.* 2003 STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* **421** 856–859. (doi:10.1038/nature01388)
- Bouyer K & Simerly RB 2013 Neonatal leptin exposure specifies innervation of presympathetic hypothalamic neurons and improves the metabolic status of leptin-deficient mice. *Journal of Neuroscience* **33** 840–851. (doi:10.1523/JNEUROSCI.3215-12.2013)
- Caron E, Sachot C, Prevot V & Bouret SG 2010 Distribution of leptin-sensitive cells in the postnatal and adult mouse brain. *Journal of Comparative Neurology* **518** 459–476. (doi:10.1002/cne.22219)
- Chavez JC, Baranova O, Lin J & Pichiule P 2006 The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the

- oxygen-dependent expression of erythropoietin in cortical astrocytes. *Journal of Neuroscience* **26** 9471–9481. (doi:10.1523/JNEUROSCI.2838-06.2006)
- Chen ZY, Asavaritikrai P, Prchal JT & Noguchi CT 2007 Endogenous erythropoietin signaling is required for normal neural progenitor cell proliferation. *Journal of Biological Chemistry* **282** 25875–25883. (doi:10.1074/jbc.M701988200)
- Constantinescu SN, Keren T, Socolovsky M, Nam H, Henis YI & Lodish HF 2001 Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *PNAS* **98** 4379–4384. (doi:10.1073/pnas.081069198)
- Coppari R, Ichinose M, Lee CE, Pullen AE, Kenny CD, McGovern RA, Tang V, Liu SM, Ludwig T, Chua SC Jr *et al.* 2005 The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity. *Cell Metabolism* **1** 63–72. (doi:10.1016/j.cmet.2004.12.004)
- D'Andrea AD, Rup BJ, Fisher MJ & Jones S 1993 Anti-erythropoietin receptor (EPO-R) monoclonal antibodies inhibit erythropoietin binding and neutralize bioactivity. *Blood* **82** 46–52.
- Diano S, Liu ZW, Jeong JK, Dietrich MO, Ruan HB, Kim E, Suyama S, Kelly K, Gyengesi E, Arbisser JL *et al.* 2011 Peroxisome proliferation-associated control of reactive oxygen species sets melanocortin tone and feeding in diet-induced obesity. *Nature Medicine* **17** 1121–1127. (doi:10.1038/nm.2421)
- Erickson JC, Clegg KE & Palmiter RD 1996 Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* **381** 415–421. (doi:10.1038/381415a0)
- Foskett A, Alnaeeli M, Wang L, Teng R & Noguchi CT 2011 The effects of erythropoietin dose titration during high-fat diet-induced obesity. *Journal of Biomedicine & Biotechnology* **2011** 373781. (doi:10.1155/2011/373781)
- Fruhbeck G 2006 Intracellular signalling pathways activated by leptin. *Biochemical Journal* **393** 7–20. (doi:10.1042/BJ20051578)
- Fuente-Martín E, García-Caceres C, Granado M, de Ceballos ML, Sanchez-Garrido MA, Sarman B, Liu ZW, Dietrich MO, Tena-Sempere M, Argente-Arizon P *et al.* 2012 Leptin regulates glutamate and glucose transporters in hypothalamic astrocytes. *Journal of Clinical Investigation* **122** 3900–3913. (doi:10.1172/JCI64102)
- Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI & Fu XY 2004 Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *PNAS* **101** 4661–4666. (doi:10.1073/pnas.0303992101)
- Harlan SM, Morgan DA, Agassandian K, Guo DF, Cassell MD, Sigmund CD, Mark AL & Rahmouni K 2011 Ablation of the leptin receptor in the hypothalamic arcuate nucleus abrogates leptin-induced sympathetic activation. *Circulation Research* **108** 808–812. (doi:10.1161/CIRCRESAHA.111.240226)
- Hill JW, Williams KW, Ye C, Luo J, Balthasar N, Coppari R, Cowley MA, Cantley LC, Lowell BB & Elmquist JK 2008 Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. *Journal of Clinical Investigation* **118** 1796–1805. (doi:10.1172/JCI32964)
- Hilton DJ, Watowich SS, Murray PJ & Lodish HF 1995 Increased cell surface expression and enhanced folding in the endoplasmic reticulum of a mutant erythropoietin receptor. *PNAS* **92** 190–194. (doi:10.1073/pnas.92.1.190)
- Hojman P, Brolin C, Gissel H, Brandt C, Zerahn B, Pedersen BK & Gehl J 2009 Erythropoietin over-expression protects against diet-induced obesity in mice through increased fat oxidation in muscles. *PLoS ONE* **4** e5894. (doi:10.1371/journal.pone.0005894)
- Katz O, Stuble M, Golishevski N, Lifshitz L, Tremblay ML, Gassmann M, Mittelman M & Neumann D 2010 Erythropoietin treatment leads to reduced blood glucose levels and body mass: insights from murine models. *Journal of Endocrinology* **205** 87–95. (doi:10.1677/JOE-09-0425)
- Mancour LV, Daghestani HN, Dutta S, Westfield GH, Schilling J, Oleskie AN, Herbstman JF, Chou SZ & Skiniotis G 2012 Ligand-induced architecture of the leptin receptor signaling complex. *Molecular Cell* **48** 655–661. (doi:10.1016/j.molcel.2012.09.003)
- Mengozzi M, Cervellini I, Villa P, Erbayraktar Z, Gokmen N, Yilmaz O, Erbayraktar S, Manohasandra M, Van Hummelen P, Vandenabeele P *et al.* 2012 Erythropoietin-induced changes in brain gene expression reveal induction of synaptic plasticity genes in experimental stroke. *PNAS* **109** 9617–9622. (doi:10.1073/pnas.1200554109)
- Miskowiak K, O'Sullivan U & Harmer CJ 2007 Erythropoietin enhances hippocampal response during memory retrieval in humans. *Journal of Neuroscience* **27** 2788–2792. (doi:10.1523/JNEUROSCI.5013-06.2007)
- Morton GJ, Cummings DE, Baskin DG, Barsh GS & Schwartz MW 2006 Central nervous system control of food intake and body weight. *Nature* **443** 289–295. (doi:10.1038/nature05026)
- Noguchi CT, Wang L, Rogers HM, Teng R & Jia Y 2008 Survival and proliferative roles of erythropoietin beyond the erythroid lineage. *Expert Reviews in Molecular Medicine* **10** e36. (doi:10.1017/S1462399408000860)
- Ouyang S & He F 2003 Phylogeny of a growth hormone-like cytokine superfamily based upon 3D structure. *Journal of Molecular Evolution* **56** 131–136. (doi:10.1007/s00239-002-2385-2)
- Schwartz MW, Woods SC, Seeley RJ, Barsh GS, Baskin DG & Leibel RL 2003 Is the energy homeostasis system inherently biased toward weight gain? *Diabetes* **52** 232–238. (doi:10.2337/diabetes.52.2.232)
- Shingo T, Sorokan ST, Shimazaki T & Weiss S 2001 Erythropoietin regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells. *Journal of Neuroscience* **21** 9733–9743.
- Suzuki N, Ohneda O, Takahashi S, Higuchi M, Mukai HY, Nakahata T, Imagawa S & Yamamoto M 2002 Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. *Blood* **100** 2279–2288. (doi:10.1182/blood-2002-01-0124)
- Teng R, Gavrilova O, Suzuki N, Chanturiya T, Schimel D, Hugendubler L, Mammen S, Yver DR, Cushman SW, Mueller E *et al.* 2011 Disrupted erythropoietin signalling promotes obesity and alters hypothalamus proopiomelanocortin production. *Nature Communications* **2** 520. (doi:10.1038/ncomms1526)
- Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, Zhao X, Sarruf DA, Izgur V, Maravilla KR *et al.* 2012 Obesity is associated with hypothalamic injury in rodents and humans. *Journal of Clinical Investigation* **122** 153–162. (doi:10.1172/JCI59660)
- Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, Liu X, Wu H & Carmichael ST 2006 A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. *Journal of Neuroscience* **26** 1269–1274. (doi:10.1523/JNEUROSCI.4480-05.2006)
- Um M, Gross AW & Lodish HF 2007 A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells. *Cellular Signalling* **19** 634–645. (doi:10.1016/j.cellsig.2006.08.014)
- Volkow ND, Wang GJ & Baler RD 2011 Reward, dopamine and the control of food intake: implications for obesity. *Trends in Cognitive Sciences* **15** 37–46. (doi:10.1016/j.tics.2010.11.001)
- Wang L, Zhang ZG, Zhang RL, Gregg SR, Hozeska-Solgot A, LeTourneau Y, Wang Y & Chopp M 2006 Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. *Journal of Neuroscience* **26** 5996–6003. (doi:10.1523/JNEUROSCI.5380-05.2006)
- Wang L, Chopp M, Gregg SR, Zhang RL, Teng H, Jiang A, Feng Y & Zhang ZG 2008 Neural progenitor cells treated with EPO induce angiogenesis through the production of VEGF. *Journal of Cerebral Blood Flow and Metabolism* **28** 1361–1368. (doi:10.1038/jcbfm.2008.32)
- Wu H, Liu X, Jaenisch R & Lodish HF 1995 Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* **83** 59–67. (doi:10.1016/0092-8674(95)90234-1)

- Yaswen L, Diehl N, Brennan MB & Hochgeschwender U 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nature Medicine* **5** 1066–1070. (doi:10.1038/12506)
- Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, Lin CS, Nikodem VM, Hempstead B, Flanders KC *et al.* 2002 Erythropoietin receptor signalling is required for normal brain development. *Development* **129** 505–516.
- Zhang Y, Wang L, Dey S, Alnaeeli M, Suresh S, Rogers H, Teng R & Noguchi CT 2014 Erythropoietin action in stress response, tissue maintenance and metabolism. *International Journal of Molecular Sciences* **15** 10296–10333. (doi:10.3390/ijms150610296)
- Zuure WA, Roberts AL, Quennell JH & Anderson GM 2013 Leptin signaling in GABA neurons, but not glutamate neurons, is required for reproductive function. *Journal of Neuroscience* **33** 17874–17883. (doi:10.1523/JNEUROSCI.2278-13.2013)

Received in final form 10 November 2015

Accepted 12 November 2015

Accepted Preprint published online 12 November 2015