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Evidence suggesting phosphodiesterase-3B regulation of NPY/AgRP gene expression in mHypoE-46 hypothalamic neurons

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Abstract

Hypothalamic neurons expressing neuropeptide Y (NPY) and agouti related-protein (AgRP) are critical regulators of feeding behavior and body weight, and transduce the action of many peripheral signals including leptin and insulin. However, intracellular signaling molecules involved in regulating NPY/AgRP neuronal activity are incompletely understood. Since phosphodiesterase-3B (PDE3B) mediates the hypothalamic action of leptin and insulin on feeding, and is expressed in NPY/AgRP neurons, PDE3B could play a significant role in regulating NPY/AgRP neuronal activity. To investigate the direct regulation of NPY/AgRP neuronal activity by PDE3B, we examined the effects of gain-of-function or reduced function of PDE3B on NPY/AgRP gene expression in a clonal hypothalamic neuronal cell line, mHypoE-46, which endogenously express NPY, AgRP and PDE3B. Overexpression of PDE3B in mHypoE-46 cells with transfection of pcDNA-3.1-PDE3B expression plasmid significantly decreased NPY and AgRP mRNA levels and p-CREB levels as compared to the control plasmid. For the PDE3B knockdown study, mHypoE-46 cells transfected with lentiviral PDE3BshRNAmir plasmid or non-silencing lentiviral shRNAmir control plasmid were selected with puromycin, and stably transfected cells were grown in culture for 48 hr. Results showed that PDE3BshRNAmir mediated knockdown of PDE3B mRNA and protein levels (~60-70%) caused an increase in both NPY and AgRP gene expression and in p-CREB levels. Together, these results demonstrate a reciprocal change in NPY and AgRP gene expression following overexpression and knockdown of PDE3B, and suggest a significant role for PDE3B in the regulation of NPY/AgRP gene expression in mHypoE-46 hypothalamic neurons.

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Keywords

PDE3B; p-CREB; NPY; AgRP; hypothalamic neurons

1. Introduction

Hypothalamic neurons that express neuropeptide Y (NPY) and agouti related-protein (AgRP) are critical regulators of feeding behavior and body weight [1-6]. NPY/AgRP neurons transduce the action of many peripheral signals including leptin and insulin [3]. Both leptin and insulin inhibit NPY/AgRP neuronal activity and the knockdown of NPY/AgRP in the arcuate nucleus of adult animals causes starvation and body weight loss [1, 3, 4]. However, intracellular signaling molecules involved in regulating NPY/AgRP neuronal activity are not fully understood. We have previously shown that leptin stimulates phosphodiesterase-3B (PDE3B) activity in the hypothalamus, and PDE3B mediates hypothalamic action of leptin on feeding [7, 8]. Our recent studies have demonstrated that, like leptin, insulin also increases PDE3B activity [9] and PDE3B inhibitor, cilostamide, reverses anorectic and the body weight reducing effects of insulin [10]. More recently, we have shown that PDE3B-cAMP pathway of leptin signaling is impaired during the development of diet-induced obesity [11]. Furthermore, PDE3B-cAMP pathway of leptin signaling is also impaired following chronic central leptin infusion in rats in association with the development of resistance to the satiety action of leptin, and the development of leptin resistance in NPY neurons [12, 13]. However, whether altered PDE3B activity in NPY neurons is responsible for the development of leptin resistance in NPY neurons following chronic leptin infusion is not known. Because hypothalamic NPY neurons express PDE3B [14], it raises the possibility that PDE3B could play a significant role in the regulation of NPY/AgRP neuronal activity. To this end, it is important to demonstrate whether PDE3B manipulation, particularly the gain-of-function or the reduced function of PDE3B, modifies the activity of NPY/AgRP neurons. Thus, to begin to address this issue, in the present study we used a clonal hypothalamic neuronal cell line, mHypoE-46, which endogenously express NPY and AgRP [15], to examine directly the consequences of the gain-of-function or reduced function of PDE3B on NPY and AgRP gene expression. We also assessed if PDE3B manipulation modifies phosphorylation of cAMP response element binding protein (CREB), a mechanism that could alter NPY and AgRP gene expression [16, 17].

2. Materials and methods

2.1. Cell culture

The mHypoE-46 cell line, a clonal hypothalamic neuronal cell line expressing NPY/AgRP, has been described previously [15]. mHypoE-46 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, Life Technologies), 25 mM glucose (Sigma, St Louis, MO, USA), and 1% penicillin/streptomycin (Life Technologies); and maintained at 37°C in a humidified chamber supplemented with 5% CO₂.

2.2. Demonstration of PDE3B expression in mHypoE-46 cells

To demonstrate the expression of PDE3B in mHypoE-46 cells we used RT-PCR and western blot. Cells grown in culture as mentioned above were washed with cold PBS and processed for total RNA and protein extraction using standard methods as described below. As positive controls for PDE3B expression, we also extracted total RNA from epididymal white adipose tissue (WAT) and medial basal hypothalamus (MBH) from adult male C57BL/6J mice, which was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. cDNA was made by RT reaction as described by the manufacturer (Life Technologies) and used to examine the presence of PDE3B by PCR using the following primers (Forward: 5'-agtaccgcggaggaaaaagt-3', reverse: 5'-aagtcctcagtcctcagagat-3'). The PCR product (164 bp) was visualized on 1% agarose gel containing ethidium bromide under UV light. Western blotting for PDE3B protein was done using standard procedure with a specific PDE3B antibody (n-terminal, provided by Dr. V. C. Manganiello, NIH) [18] and appropriate secondary antibody.

2.3. Overexpression of PDE3B in mHypoE-46 cells

Murine PDE3B cDNA tagged with HA (hemagglutinin) was a gift from Dr. W. Ogawa (Kobe University, Japan)[19]. HA-tagged PDE3B (3.41 kb) was subcloned into the HindIII-XhoI sites of the pcDNA3.1 mammalian expression vector (Life Technologies). As a control, we used pcDNA3.1-EGFP plasmid, which was prepared by subcloning EGFP cDNA {obtained by digesting pEGFP-C2 vector (Clontech, Mountain View, CA, USA), with NheI/ApaI} into the XbaI-ApaI sites of the pcDNA3.1 vector. Cells were cultured as described above. For transfection, cells were grown in media without antibiotic. The day before transfection, cells were cultured into a 35mm tissue culture plate grown to 50 to 70% confluency and transfected with purified plasmid DNA at 1 or 2µg/plate using the LipofectAMINE 2000 reagent according to the manufacturer's instructions (Life Technologies). Eight hours later, the media were discarded and replaced with a fresh media. Twenty four to 48 hours after transfection, the cells were processed for protein and RNA extraction for western blotting and RT-qPCR, respectively.

2.4. PDE3BshRNAmir mediated PDE3B knockdown in mHypoE-46 cells

For PDE3B knockdown, pGIPZ lentiviral shRNAmir plasmids targeted toward mouse *Pde3b* and a GIPZ non-silencing control lentiviral shRNAmir plasmid (RHS4346) were purchased from Open Biosystems (Huntsville, AL, www.thermoscientificbio.com). We screened five GIPZ lentiviral PDE3BshRNAmir plasmids (RMM4431-101288515, RMM4431-101292490, RMM4431-98766439, RMM4431-99007861, RMM4431-99214565) and compared with non-silencing lentiviral shRNAmir control plasmid to check the efficacy of each construct in knocking down PDE3B in mHypoE-46 cells. The cells were transfected with PDE3BshRNAmir or control plasmid using Arrest-In™ according to the manufacturer's protocol (Open Biosystems) and stable clones were isolated 72 h post-transfection by selection in growth medium supplemented with 2µg/ml Puromycin (Sigma). These clones were propagated and frozen using standard procedure. To screen the efficacy of each PDE3BshRNAmir, the stably transfected cells were plated on 60 mm plate and grown in culture medium supplemented with puromycin (2µg/ml). After 48

hours, the cells were harvested for protein and RNA extraction. PDE3B mRNA and PDE3B protein levels were determined by RT-qPCR and western blotting, respectively. The most efficient knockdowns were achieved by using the RMM4431-99007861 clone (clone#4) (data not shown). We then used stable cells transfected with clone #4 to examine the effects of PDE3B knockdown on NPY and AgRP gene expression and p-CREB levels at 48 hr of culture at which time the cells were harvested for protein and RNA extraction for western blotting and RT-qPCR, respectively.

2.5. Western Blot

The cells or tissues (WAT, MBH) were lysed with a $1 \times$ lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with phenyl-methylsulfonyl fluoride and a cocktail of protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Total protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Fifty μ g of protein, unless indicated otherwise, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gel for PDE3B, 12% gel for p-CREB) and transferred to polyscreen polyvinylidene difluoride membranes. The membranes were then incubated overnight with anti-rabbit PDE3B antibody (dilution 1:1000, provided by Dr. Manganiello) or p-CREB antibody (dilution 1:5000, #9198, Cell Signaling) at 4°C followed by incubation for 60 min with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature. Immunoreactive bands were visualized by Western Lightning chemoluminescence Reagent Plus-ECL as described by the manufacturer (Perkin Elmer, Boston, MA, USA). The membranes were stripped and then blotted with a monoclonal anti- β -actin antibody (dilution 1:40,000, Sigma) or CREB antibody (dilution 1:5000, #9197, Cell Signaling) as appropriate. Images of the bands were scanned and analyzed using NIH IMAGE software (NIH, Bethesda, MD, USA). PDE3B levels were normalized to β -actin, while p-CREB levels were normalized to total CREB and then expressed as relative to control.

2.6. RNA isolation and RT-qPCR

Total cellular RNA was isolated by Trizol reagent (Life Technologies) and assessed for purity and concentration using spectrophotometric analysis. 5 μ g of total RNA was subjected to DNase treatment using RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's protocol and re-extracted with Trizol. cDNA was synthesized from 2 μ g RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The RT reaction consisted of 10 min incubation at 25 °C, 120 min incubation at 37 °C, followed by a 5-min 85 °C termination step, and the resulting cDNA was stored at -20 °C. qPCR reactions were performed with 100 ng of cDNA in duplicate using a 2X Power SYBR Green PCR master mix (Life Technologies) containing 500 nm each primer pair and run on the Applied Biosystems Prism 7900HT real-time PCR machine at 95 °C for 10 min followed by 40 cycles of 95 °C (15 s) and 60 °C (60 s). The primer sequences are as follows: NPY: sense-5'-cagaaaacgccccagaa-3'; anti-sense-5'-aaaagtcgggagaacaagtttcatt-3'; AgRP: sense-5'-cggaggtgctagatccacaga-3'; anti-sense-5'-aggactcgtgcagccttacac-3'. PDE3B: sense-5'-gatggtggtggtgaaagaa-3'; anti-sense-5'-agtgaggtggtgcattag-3'; cyclophilin: sense-5'-aaggtgaaagaaggcatgaac-3'; anti-sense-5'-agctgtccacagtcggaatg-3'. The relative

quantification of mRNA levels (fold change) normalized to cyclophilin was calculated using the $\Delta\Delta C_T$ method from the C_t (threshold cycle) values obtained from ABI SDS software package.

2.7. Statistical analysis

All values are expressed as means $\pm$ SEM. Statistical significance was determined using ANOVA with Fisher's least significant difference (LSD) multiple-range tests or Student's *t*-test. All statistical analyses were done using GB-Stat software for the Macintosh (Dynamic Microsystems, Silver Spring, MD). $P < 0.05$ were considered to be significant.

3. Results

3.1. PDE3B overexpression decreased NPY and AgRP gene expression in mHypoE-46 neuronal cells

The mHypoE-46 neurons have been described to express NPY and AgRP [15]. In this study we first demonstrated that these hypothalamic neuronal cells also express PDE3B as determined by RT-PCR and western blotting (Fig. 1A and B). To examine the effects of PDE3B overexpression on NPY and AgRP gene expression, the cells were transfected with 1 or 2 μ g of pcDNA-3.1-EGFP or pcDNA-3.1-PDE3B expression vector and 24 or 48 hours later, the cells were harvested for PDE3B mRNA and protein levels. We observed a dose and time-dependent increase in both protein and mRNA levels for PDE3B in the pcDNA3.1-HA-PDE3B DNA transfected cells as compared to pcDNA3.1-EGFP transfected control cells (Fig. 1C and D, *Pde3b* mRNA: 24 hr: control-1 μ g: 1.0468 ± 0.172 , PDE3B-1 μ g: 2.947 ± 0.1059 ; control-2 μ g: 1.0001 ± 0.0468 , PDE3B-2 μ g: 37.4737 ± 8.2455 ; 48 hr: control-1 μ g: 1.000 ± 0.009 , PDE3B-1 μ g: 4.5937 ± 1.3393 ; control-2 μ g: 1.0001 ± 0.1394 , PDE3B-2 μ g: 43.683 ± 11.4045 ; Mean $\pm$ SEM; $N = 3$). We then assessed NPY and AgRP mRNA levels by qPCR and observed a significant decrease in both NPY and AgRP mRNA levels following 24 or 48 hours of PDE3B overexpression (Fig. 2A and B). Because transfection with either 1 or 2 μ g of PDE3B expression plasmid decreased NPY and AgRP gene expression, we examined whether PDE3B overexpression with 2 μ g plasmid DNA have any effect on p-CREB levels at 24 hr of overexpression. p-CREB levels in the protein extracts were determined by western blot, and we observed a significant decrease in p-CREB levels following overexpression of PDE3B as compared to that seen after transfection with control plasmid (Fig. 2C and D).

3.2. PDE3B knockdown increases NPY and AgRP gene expression in mHypoE-46 neuronal cells

To knockdown PDE3B in mHypoE-46 neuronal cells, we first generated stable cell lines expressing one of five different lentiviral PDE3BshRNA mir plasmids and examined the efficacy of PDE3B knockdown by assessing the expression of PDE3B by qPCR and western blot. We observed that stable cells expressing clone#4 shRNA mir had maximum PDE3B knockdown (65-70%) at the mRNA and protein levels as compared to those expressing control non-silencing shRNA mir (Fig. 3A-C; *Pde3b* mRNA: control: 1.000 ± 0.0431 , shRNA: 0.3923 ± 0.1257 , $N = 4$; PDE3B protein: control: 1.0421 ± 0.1047 , shRNA: 0.2599 ± 0.0335 , $N = 5$). Most importantly, PDE3B knockdown was associated with a significant

($p<0.01$) increase in NPY and AgRP gene expression (Fig.3D). In addition, PDE3B knockdown significantly ($p<0.01$) increased p-CREB protein levels (Fig. 3E).

4. Discussion

The main objective of this study was to examine whether PDE3B regulates NPY/AgRP gene expression in mHypoE-46 cell, a clonal hypothalamic neuronal cell line. NPY/AgRP neurons of the hypothalamus play a major role in regulating food intake, body weight and glucose homeostasis [1, 2]. They are also the targets of leptin and insulin action in the regulation of energy homeostasis [3]. Whereas the phosphatidylinositol-3-kinase (PI3K) pathway has been established to be a common signaling branch that mediates both leptin and insulin signaling in the hypothalamus[5], we have shown that PDE3B pathway mediates the hypothalamic action of both leptin and insulin in food intake and body weight regulation [7-10]. Furthermore, PI3K is upstream of the PDE3B pathway of leptin signaling in the rat hypothalamus [20]. Thus, PDE3B could represent a key signaling node that integrates both leptin and insulin action in the hypothalamus and therefore, understanding the role of PDE3B in the regulation of gene expression of those hypothalamic neurons, including NPY/AgRP neurons, that play an obligatory role in energy homeostasis, is very important. In this regard, whereas PDE3B is expressed in hypothalamic NPY/AgRP neurons [14], the possibility that PDE3B could modify the activity of these neurons has not been tested. To test this possibility directly, we assessed the effects of overexpression or knockdown of PDE3B on NPY and AgRP gene expression in mHypoE-46 cells, a clonal hypothalamic neuronal cell line, that expresses NPY/AgRP as well as leptin and insulin receptors [15]. Our demonstration that mHypoE-46 cells also express PDE3B is in line with the possibility that PDE3B could modify NPY/AgRP gene expression in these hypothalamic neurons.

In the first experiment, we overexpressed PDE3B in mHypoE-46 cells by transfecting these cells with pcDNA-3.1-PDE3B expression vector and assessed NPY and AgRP gene expression. Our demonstration that PDE3B overexpression for a period of either 24 or 48 hours resulted in a decreased NPY and AgRP gene expression suggests that PDE3B can modify NPY/AgRP gene expression in these neuronal cells. Interestingly, although there was a dose and time dependent increase in PDE3B expression in over-expressed cells, repression in NPY or AgRP gene expression was at about 50% suggesting saturation of the repression. Whereas the underlying mechanism behind this is currently unknown, it is likely that other signaling molecules including those in the PI3K-Akt pathway could also be involved in regulation of NPY/AgRP gene expression in this cell line (15). To further confirm the role of PDE3B in regulating NPY and AgRP gene expression in mHypoE-46 cells, in the second experiment, we tested the hypothesis that knockdown of PDE3B increases NPY and AgRP gene expression in these cells. Our finding that PDE3B knockdown of 65-70% by a specific PDE3B shRNA mir clone resulted in a significant increase in both NPY and AgRP gene expression in mHypoE-46 hypothalamic neurons strongly suggests that PDE3B plays a significant role in the regulation of NPY and AgRP gene expression in these clonal hypothalamic neurons. While the induction of NPY and AgRP gene expression was modest (about 150%) following shRNA mediated knockdown of PDE3B, since a very small induction of NPY can cause a substantial increase in feeding (21), PDE3B regulation of

NPY/AgRP gene expression seen in this cell model could be physiologically relevant if confirmed *in vivo*.

To gain an insight into the mechanism(s) by which the overexpression or knockdown of PDE3B alter NPY and AgRP gene expression, we measured p-CREB protein levels in the cell extract. One of the mechanisms by which CREB is phosphorylated is via an increase in intracellular cAMP levels. Specifically, cAMP-dependent protein kinase A plays a major role in CRE mediated gene induction [22]. Several agents that increase cAMP levels increase the expression of NPY [16]. In the mouse arcuate nucleus (ARC), fasting-induced NPY gene expression is associated with an increase in p-CREB levels [23]. In addition, intracerebroventricular injection of a cAMP analog increases NPY protein levels in the ARC [24], and CREB antisense oligonucleotides prevent the fasting-induced increase in NPY expression in the rat hypothalamus [17]. Also, both NPY and AgRP genes possess CREB-binding sites [15, 16]. Altogether these evidences support the idea that CREB is one of the main transcription factors that regulate NPY expression. Furthermore, PDE inhibitors significantly enhanced the fasting-induced increases in NPY mRNA levels in the ARC of fasted mice [25]. Thus, although the mechanism by which PDE3B regulates NPY and AgRP gene expression is currently unknown and was not addressed in this study, our findings that overexpression and knockdown of PDE3B resulted in decreased and increased p-CREB levels, respectively, suggest the possibility that altered NPY and AgRP gene expression following PDE3B manipulation observed in the present study could be, at least in part, mediated by changes in p-CREB levels in the mHypoE-46 neurons; which requires further investigation.

Identification of an intracellular signaling molecule that can regulate the activity of NPY/AgRP neurons is of immense significance, because these neurons are the major players in controlling energy homeostasis. Although our study was done *in vitro*, the findings that direct PDE3B manipulation by increasing or decreasing the expression of this enzyme results in reciprocal changes in NPY/AgRP gene expression in mHypoE-46 neurons provide a frame work for the future *in vivo* studies dissecting out the role of PDE3B in NPY/AgRP neurons. Specifically, it will be quite interesting to demonstrate if PDE3B overexpression in NPY/AgRP neurons ameliorates or reverse the metabolic phenotypes seen in diet-induced obese (DIO) animals. Overall, our study has unraveled a previously unknown role of PDE3B in the regulation of NPY and AgRP gene expression in the mHypoE-46 hypothalamic neurons.

In summary, we have demonstrated a reciprocal change in NPY and AgRP gene expression following gain-of-function or knockdown of PDE3B in mHypoE-46 hypothalamic neurons in association with a similar change in p-CREB levels. We suggest that, if our finding is confirmed *in vivo*, then it could be a viable approach to manipulate NPY and AgRP neuronal activity during various metabolic disorders including the DIO.

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Highlights

PDE3B is expressed in hypothalamic NPY/AgRP neurons that play a key role in energy homeostasis.

PDE3B is expressed in mHypoE-46 hypothalamic neuronal cells expressing NPY and AgRP.

PDE3B overexpression decreased NPY/AgRP gene expression in mHypoE-46 cells.

PDE3B knockdown increased NPY and AgRP gene expression in mHypoE-46 cells.

This study shows PDE3B regulation of NPY/AgRP gene expression in mHypoE-46 cells.

Fig. 1.

PDE3B expression in mHypoE-46 cells (N46 cells) at basal condition (A, B) and following transfection of mHypoE-46 cells with 1 or 2 µg of pcDNA-3.1-PDE3B plasmid DNA for 24 or 48 hours (C, D). A. RT-PCR products (164 bp) from RNA isolated from N46 cell line (n = 4 different cultures), epididymal white adipose tissue (WAT) and medial basal hypothalamus (MBH) of mice (n = 3) were electrophoresed in a 1% agarose gel containing ethidium bromide and visualized under UV light. MW = 100 bp DNA Ladder (Invitrogen). B. Western blot analysis of PDE3B protein in mHypoE-46 cells (22.5 µg protein, n = four different cultures) and WAT (4 µg protein). C. RT-qPCR analysis of PDE3B mRNA levels. Data are mean ± SEM of three independent incubations. * $p < 0.05$ vs. respective control. D. Western blot analysis of PDE3B protein in transfected mHypoE-46 cells (12.5 µg protein).

Fig. 2.

Effects of PDE3B overexpression on NPY and AgRP mRNA and p-CREB protein levels in mHypoE-46 cells. The cells were transfected with control pcDNA-3.1-GFP (1 and 2 μg) or pcDNA-3.1-PDE3B (1 and 2 μg) plasmid DNA for 24 or 48 hr. RT-qPCR analysis of NPY and AgRP mRNA levels at 24 hr (A) and 48 hr (B) of transfection. (C) Western blot of phosphorylated CREB (p-CREB), (D) densitometric analysis of the immunoreactive bands of p-CREB normalized to total CREB at 24 hr in the cells transfected with 2 μg of DNA. The results are expressed as relative to the corresponding pcDNA-3.1-GFP control levels (set to 1.0). Values are means ± SEM (n = 3 to 5). * $p < 0.05$, ** $p < 0.01$.

Fig. 3.

Effects of shRNA-mediated PDE3B knockdown on NPY and AgRP mRNA and p-CREB protein levels in mHypoE-46 cells. The cells transfected with either control shRNA or PDE3BshRNA plasmid DNA were selected with Puromycin (2 µg/ml). Stably transfected cells were analyzed for PDE3B knockdown at mRNA and protein level, and for NPY and AgRP mRNA and p-CREB protein levels. (A) RT-qPCR analysis of PDE3B mRNA levels, (B) Western blot for PDE3B, (C) densitometric analysis of the immunoreactive bands of PDE3B normalized to β-actin, (D) RT-qPCR analysis of mRNA levels for PDE3B, NPY and AgRP, (E) Western blot for p-CREB and CREB (upper panel) and densitometric analysis of the immunoreactive bands of p-CREB normalized to total CREB (lower panel). The results are expressed as relative to the shRNA control levels (set to 1.0). Values are means ± SEM (n = 3 to 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.