Phosphodiesterase-3B is expressed in proopiomelanocortin and neuropeptide Y neurons in the mouse hypothalamus

Maitrayee Sahu, **David G. Litvin**, and **Abhiram Sahu**
Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh, School of Medicine, Magee-Womens Research Institute, Pittsburgh, PA 15213, USA

Abstract

Leptin signaling in the hypothalamus is obligatory for normal food intake and body weight homeostasis. It is now well established that besides the signal transducer and activator of transcription-3 (STAT3) pathway, several non-STAT3 pathways mediate leptin signaling in the hypothalamus. We have previously demonstrated that leptin stimulates phosphodiesterase-3B (PDE3B) activity in the hypothalamus, and PDE3 inhibitor cilostamide reverses anorectic and bodyweight reducing effects of leptin. Recently, we have demonstrated that cilostamide reversed the leptin-induced increase in proopiomelanocortin (POMC) gene expression in the hypothalamus. Because POMC and neuropeptide Y (NPY) neurons are thought to be the major targets of leptin signaling in the hypothalamus, to establish the physiological role of the PDE3B pathway it is important to demonstrate if PDE3B is expressed in these neurons. To this end we examined co-localization of PDE3B with POMC and NPY neurons using immunocytochemistry in POMC-GFP and NPY-GFP mice, respectively. Results showed that PDE3B was highly localized throughout the various hypothalamic sites including the arcuate nucleus (ARC), ventromedial nucleus, dorsomedial nucleus, ventral premammillary nucleus, paraventricular nucleus, and lateral hypothalamus. Importantly, almost all NPY (91.7%) and POMC (97.7%) neurons co-expressed PDE3B. These results suggest a direct role of the PDE3B pathway in mediating leptin signaling in the POMC and NPY neurons –a potential mechanism of leptin signaling in the hypothalamus.

Keywords

phosphodiesterase-3B; POMC; NPY; hypothalamus; leptin

Introduction

Leptin, the product of the obese gene [39], is secreted by the adipocytes and signals nutritional status to key regulatory centers in the hypothalamus and it has emerged as an important signal regulating energy homeostasis [13–15,33]. Central or peripheral leptin administration decreases food intake and body weight in a variety of animals [13, 34]. The deletion of leptin receptor (LEPR) in neurons leads to an obese phenotype [8], and transgenic supplementation of the LEPR in neurons of Lepr[db/db] mice results in an

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amelioration of the obese phenotype [20]. Besides its role in energy homeostasis, leptin also plays important role in reproduction, bone growth and immuno functions [37]. Importantly, most, if not all, of these functions of leptin are mediated at the level of the hypothalamus. Thus, understanding the mechanism of leptin signaling is very important. Several lines of evidence suggest that besides the classical Janus-kinase 2 (JAK2)-signal transducer and activator of transcription-3 (STAT3) pathway [4,14,15,35,36], leptin signaling in the hypothalamus is mediated through various non-STAT3 pathways including including AMP-activated protein kinase (AMPK) [22], mammalian target of rapamycin (mTOR) [10], forkhead protein (FOXO1) [5,17], phosphatidylinositol 3-kinase (PI3K) [23,40], and SHP2-GRB2-Ras-Raf-MAPK (mitogen-activated protein kinase) [2,6,7,38]. We have demonstrated that leptin action is also mediated through an insulin-like signaling pathway involving stimulation of PI3K and phosphodiesterase 3B (PDE3B) activities and reduction in cAMP levels in the hypothalamus [40]. Furthermore, cilostamide, a selective PDE3 inhibitor, reverses the anorectic and body weight reducing effect of leptin [40]. While these results suggest a potential role of the PDE3B pathway in mediating leptin action in the hypothalamus, the physiological role of this pathway of leptin signaling in energy homeostasis remains relatively unknown.

Towards establishing the physiological role of the PDE3B pathway, we have recently demonstrated that PDE3B inhibitor reversed the leptin-induced increase in proopiomelanocortin (POMC) and neurotensin gene expression in the hypothalamus [30]. However, it is still unknown whether leptin directly activates PDE3B in these and other leptin target neurons, a possibility that could be demonstrated if PDE3B is expressed in these neurons. Besides POMC neurons, neuropeptide Y (NPY) neurons in the hypothalamus play a significant role in energy homeostasis, and both POMC and NPY neurons are the major targets of leptin action in the hypothalamus [1,9,12,26,28,33]. Specifically, leptin inhibits NPY gene expression and induces POMC gene expression in the hypothalamus. Thus, to further establish the physiological role of the PDE3B pathway of leptin signaling, we tested the hypothesis that PDE3B is expressed in the POMC and NPY neurons. To this end, we performed dual-label immunocytochemistry (ICC) with a specific PDE3B antibody and GFP (green fluorescence protein) antibody to examine PDE3B co-localization in hypothalamic POMC and NPY neurons in POMC-GFP and NPY–GFP mice, respectively.

Materials and methods

Transgenic mouse lines expressing GFP in either NPY (NPY-GFP) or POMC (POMC-GFP) neurons were kindly provided by Dr Jeffrey Friedman (The Rockefeller University, New York, NY), and were maintained in our animal facility in a light (lights on 0500 h to 1900 h) and temperature (22 °C)-controlled room with food (Pelleted Purina rodent chow) and water available *ad libitum*. The procedures used herein were according to an approved Institutional Animal Care and Use Committee protocol.

Adult male mice (NPY-GFP, n = 4; POMC-GFP, n = 3) were injected stereotaxically with colchicine (1 μl, 20 μg/μl) into the lateral cerebroventricle under pentobarbital anesthesia using an ultra micro-II injection pump (Precision Instruments). Forty-eight hours later, mice were deeply anesthetized with pentobarbital, and perfused transcardially with 0.9% saline (RT) followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The brains were quickly removed from each perfused animal, post-fixed in the same fixative overnight at 4 °C, and cryoprotected in 25% sucrose solution until they sank. The brains were then frozen on dry ice and kept at −80 °C until sectioning. Five series of coronal 25 μm free-floating sections were cut through the mediobasal hypothalamus on a freezing microtome (Leica Sliding Microtome), and stored in cryoprotectant at −20 °C until use.

To demonstrate PDE3B localization in various hypothalamic nuclei, adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) were injected with recombinant murine leptin (5 mg/kg body weight, i.p; A.F. Parlow, NHPP, Torrance, CA) followed 60 minutes later by transcardial perfusion with saline and 4% paraformaldehyde. The brain was processed for sectioning as described above. Free-floating sections were pretreated with 1.8% H_2O_2 in 50 mM KPBS (potassium phosphate buffered saline) for 20 min followed by several washes and incubation in 0.05% glycine for 10 min at RT. Sections were then blocked with 5% normal rabbit serum +1% BSA + 0.4% Triton X-100 and incubated with goat anti-PDE3B (1:500, FabGennix Inc., Frisco, TX) at 4 °C for 48 hr. After washing, sections were incubated with biotinylated rabbit anti-goat secondary antibody (1:1200, 90 min at RT, Vector Laboratories), then washed again and incubated in avidin-biotin complex (Vectastatin, ABC Elite kit, Vector Laboratories) for 90 minutes at RT. Immunoreactive PDE3B was visualized with diaminobenzidine hydrochloride (DAB, Sigma) reaction. Finally, sections were mounted on superfrost slide (Fisher Scientific, Pittsburgh), dried overnight, rehydrated and then dehydrated with increasing concentrations of ethanol, washed in xylene and mounted with DPX. Visualization of immunostaining detected with DAB was performed using a Leica DMRBE microscope (Brodersen Instruments, Co, Valencia, PA), MicroPublisher 5.0 RTV QImaging color digital camera and Bioquant NOVA PRIME Program (BIOQUANT Image Analysis Corporation, Nashville, TN).

For dual-label ICC for PDE3B and GFP (for NPY or POMC), ICC for PDE3B was performed first followed by GFP staining. Free floating tissue sections were pretreated with 1% NaOH and 1% H_2O_2 in H_2O for 20 min. Sections were then blocked for 1 h and incubated with goat anti-PDE3B (1:500, FabGennix Inc.) at 4 °C for 48 h, followed by washing and incubation with Cy3-conjugated donkey anti-goat secondary antibody (1:800, 1 hour RT). Sections were washed and then incubated with Alexa 488-conjugated rabbit anti-GFP (1:1500, Invitrogen). Finally, sections were stained with DRAQ5 (fluorescence DNA dye, 1:2000), mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA) using Fluoromount-G (Southern Biotech), and visualized with an Olympus FluoView Confocal Microscope for green GFP (POMC or NPY), red PDE3B expressing neurons and blue nuclear stain. The specificity of the PDE3B antibody was validated in the following manner. First, pre-absorption of primary antibody with the PDE3B peptide used as immunogen blocked all staining in both single- and dual-label ICC procedures (Fig. 2, preadsorbed). Second, substitution of isotypic serum for primary antibody eliminated all staining (Fig. 1).

Sections were scanned at 1024 × 1024 pixels, 40× objective 0.2micron pixel size, using two or three color image collection (488 nm laser, 543 nm, 633 nm) together with appropriate dichroics and barrier filters. Image planes throughout the depth of the specimen were collected and the neurons expressing both GFP and PDE3B (yellow color in merged images) were counted on at least ten different sections through the entire rostro-caudal extent of the ARC of each brain using the MetaMorph software (Molecular Devices, Sunny Vale, CA). Co-expression values were calculated as percentages of the total number of POMC- or NPY-positive cells expressing PDE3B. All values were expressed as means ± standard error (SE).

Results

As reported previously in rat [28], PDE3B+ve cells were localized in various nuclei in the mouse hypothalamus including the arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), ventral premammillary nucleus (PMv) and lateral hypothalamic areas (LH) (Fig. 1 and data not shown). Among these nuclei, PDE3B was expressed highly in the ARC followed by PVN and PMv. GFP+ve cells identifying the POMC and NPY neurons in POMC-GFP and NPY-GFP mice, respectively, were distributed throughout the ARC as described previously [11,25].

Systematic examination of sections through the rostro-caudal axis of the ARC showed expression of PDE3B (red) in POMC and NPY neurons (green) as shown by the development of yellow color in the merged figures (Fig. 2). Analysis of various sections throughout the ARC in four NPY-GFP and three POMC- GFP mice showed that PDE3B was expressed in almost all POMC and NPY neurons (Fig 3).

Discussion

The present study shows that PDE3B is expressed in POMC and NPY neurons and in those hypothalamic nuclei that have been implicated in energy homeostasis. These results further support a role of PDE3B in energy homeostasis particularly in leptin signaling in the hypothalamus.

Leptin signaling in hypothalamic neurons, particularly in POMC and NPY neurons, is critical for normal energy homeostasis [1,13,28,33]. Understanding the mechanism of leptin signaling in these neurons is important toward identifying any defect in a particular signaling pathway during the development of diet-induced obesity, which could be targeted for therapeutic approaches. Cumulative evidence suggests that besides the STAT3 pathway, several non-STAT3 pathways are integral part of the leptin-signaling network in the hypothalamus that regulates energy homeostasis [28,31]. Our pharmacological studies have identified PDE3B pathway as one of the non-STAT3 pathways of leptin signaling in the hypothalamus in that PDE3 inhibition reverses the anorectic and body weight reducing effects of leptin [40]. In addition, reversal of the leptin-induced STAT3 activation in the hypothalamus by PDE3 inhibition demonstrates a cross talk between the PDE3B and STAT3 pathways of leptin signaling [40]. We have also reported that the PI3K-PDE3B-cAMP pathway but not the STAT3 pathway of leptin signaling in the hypothalamus was impaired during the development of leptin resistance in POMC and NPY neurons following chronic central leptin infusion [27,29,32]. However, the physiological role of hypothalamic PDE3B signaling in energy homeostasis is not clearly understood.

Thus to begin to examine the physiological role of PDE3B signaling in the hypothalamus, in the present study we sought to examine if PDE3B was localized in POMC- and NPY-expressing neurons, two important neuronal subtypes that have been established to play significant role not only in leptin signaling but also in overall energy homeostasis [33]. The finding of PDE3B expression in almost all POMC- and NPY-expressing neurons in the arcuate nucleus together with previous report of leptin receptor expression in these neurons [3] are in favor of a direct role of PDE3B pathway in transducing leptin action in these neurons. Our recent demonstration that PDE3 inhibition by cilostamide reverses the leptin-induced stimulation of hypothalamic POMC and neurotensin (NT) gene expression [30], and the finding that leptin suppresses ghrelin-induced NPY neurons by activation of the PI3K-PDE3B pathway [18] are in line with this possibility.

In addition to PDE3B co-localization in POMC and NPY neurons, we also documented PDE3B expression in those hypothalamic nuclei including the ARC, VMN, DMN, PMv, LH and PVN that are known to express leptin receptor and have been implicated in food intake and body weight regulation. This finding suggests the possibility of PDE3B playing a role in leptin signaling in these nuclei. Whereas the specific role of the PDE3B pathway in mediating leptin signaling in various hypothalamic nuclei is not clearly understood, the role of this pathway in mediating leptin signaling in POMC, NPY and NT neuronal activities is becoming apparent [30]. Also, the demonstration that leptin blocks glucocorticoid-induced endocannabinoid biosynthesis and suppression of excitation in the PVN via a PDE3B-mediated reduction in cAMP levels [21] suggests a role of PVN PDE3B in leptin signaling and energy homeostasis. It is noteworthy that insulin signaling in hypothalamic neurons

including that in POMC and NPY neurons plays a critical role in energy homeostasis [16,19,24]. Thus, PDE3B expression in POMC and NPY neurons and in various hypothalamic nuclei along with our preliminary finding that insulin increases PDE3B activity in the mouse hypothalamus [A. Sahu and M. Sahu, unpublished] may suggest a potential role of PDE3B in mediating insulin action in the hypothalamus.

In summary, we have demonstrated expression of PDE3B in POMC and NPY neurons and in various hypothalamic nuclei that have been implicated in energy homeostasis. This study along with reversal of leptin-induced POMC and NT gene expression by PDE3 inhibition suggest an important role of the PDE3B pathway in mediating action of leptin and other metabolic signals including insulin in these and other hypothalamic neurons.

Acknowledgments

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Highlights

- PDE3B pathway plays an important role in mediating hypothalamic action of leptin.

- This study shows expression of PDE3B in POMC and NPY neurons in the hypothalamus.

- PDE3B is also expressed in the hypothalamic nuclei implicated in energy homeostasis.

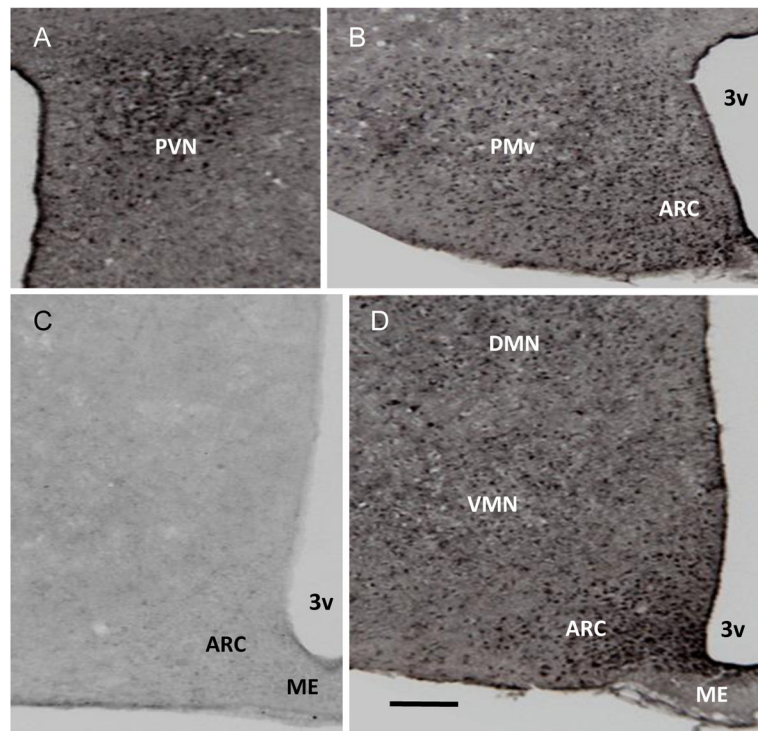- Direct PDE3B signaling appears to mediate leptin action in POMC and NPY neurons.

Fig. 1.
Bright-field photograph showing phosphodiesterase 3B (PDE3B) immunoreactive cells in mouse hypothalamus. (A) PDE3B-ir positive cells in the paraventricular nucleus (PVN). (B) PDE3B-ir positive cells in the ventral premammiliary nucleus (PMv) and arcuate nucleus (ARC) at bregma −2.46 mm. (C) Immunocytochemical reaction with substitution of isotypic serum for PDE3B primary antibody in a section through the median eminence (ME)-ARC area at bregma −1.46 mm. (D) PDE3B-ir positive cells in the ARC, ventromedial nucleus (VMN) and dorsomedial nucleus (DMN) at bregma −1.94 mm. 3v = third ventricle. Scale bar = 100 μm
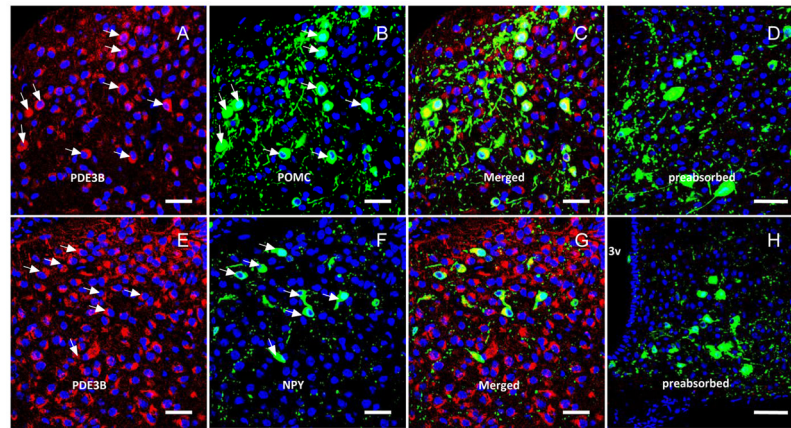
Fig. 2.
Double ICC for PDE3B (red) and GFP-POMC (upper panels, A–C) and GFP-NPY (lower panels, F–G) in the ARC of POMC-GFP or NPY-GFP mice. Blue = nuclear stain. Arrows indicate co-localization. 3v = third ventricle. Note that preabsorption of PDE3B antibody with PDE3B peptide used as immunogen blocked all PDE3B staining as shown in the right panels (D, H). Scale bar = 50 μm
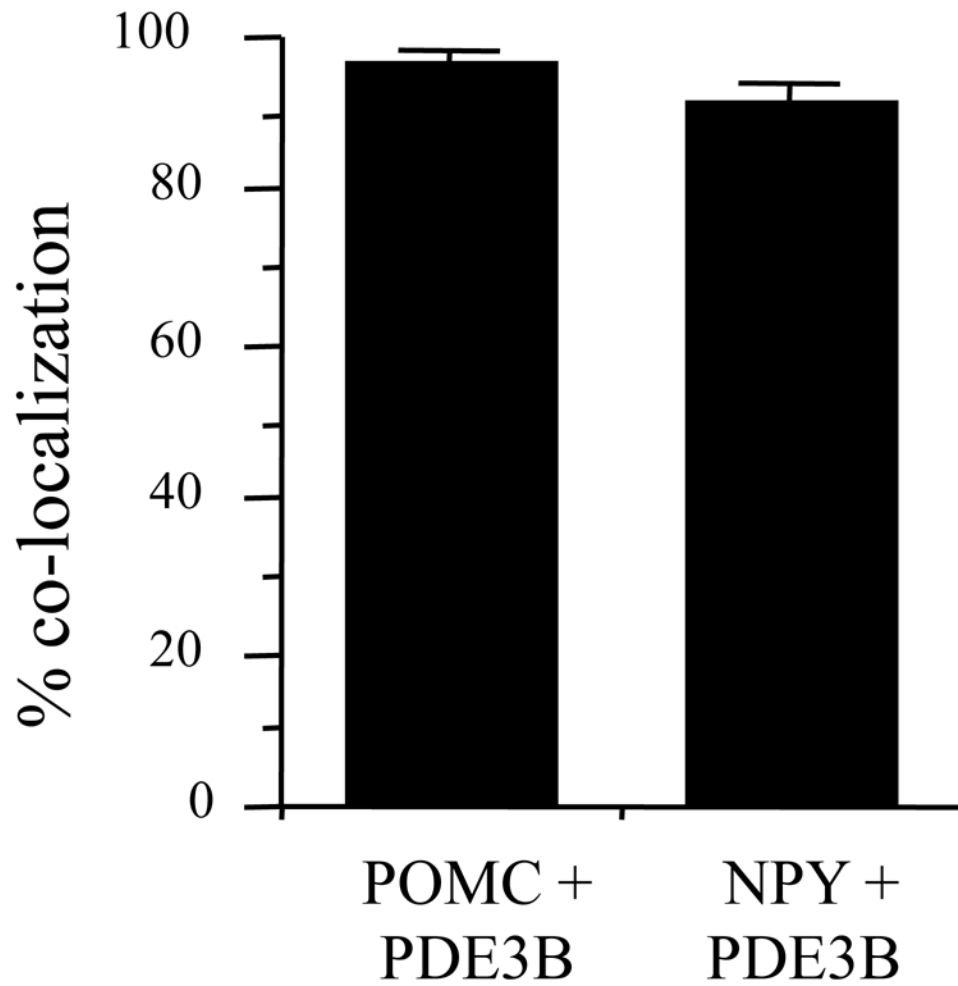
Fig. 3.
Percent of POMC and NPY neurons co-localized with PDE3B in the hypothalamus of POMC-GFP and NPY-GFP mice. Values represent the mean ± SEM for 3 and 4 animals in POMC-GFP and NPY-GFP groups, respectively.