Expression of neuromedin B in adipose tissue and its regulation by changes in energy balance

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Abstract

Bombesin is one of the most powerful substances showing anorexic effects in the hypothalamus (Moody TW & Merali Z 2004 Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. *Peptides* **25** 511–520). In mammals, neuromedin B (NMB) is one member of a family of bombesin-like peptides, which have been shown to reduce food intake when administered systemically. Using Taqman real-time PCR with specific primers, we report the expression of NMB mRNA in both human and rodent adipose tissue. Expression of NMB in rodent epididymal adipose tissue was higher than in other tissues studied. Expression of NMB in adipose tissue appears to be regulated by changes in energy balance and leptin. It is decreased fourfold in the epididymal fat depot of *ob/ob* mice when compared with the same fat depot in lean mice. It is further decreased with the intra-peritoneally (i.p.) administration of leptin in both lean and obese *ob/ob* mice. This may relate to its function in food intake regulation or to changes in energy expenditure. We demonstrate that NMB expression in rodent adipose tissue is decreased in cold exposed animals. However, when we investigated the effects of NMB on resting metabolic rate by i.p. injection, there was no effect on oxygen consumption, RQ or physical activity when compared with saline-treated controls. In conclusion, NMB is expressed in both human and rodent adipose tissue and appears to be regulated by changes in energy balance. Given its anorexic effects centrally, it may form part of a new adipose tissue – hypothalamic axis regulating food intake.

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Introduction

Bombesin (BN), originally extracted from frog skin, is also active in mammals (Anastasi *et al.* 1971). It has various physiological effects, for example on food intake, insulin secretion, gastric secretion and contractive effects on the colon or ileum (Erspamer *et al.* 1970). Neuromedin B (NMB) is a member of a family of BN-like peptides, which in mammals also include gastrin-releasing peptide (GRP) and neuromedin (McDonald *et al.* 1979, Minamino *et al.* 1988). These peptides are widely expressed in the pituitary, brain, pancreas, adrenal and gastrointestinal tract, but have not previously been reported in adipose tissue (Ohki-Hamazaki *et al.* 2005). Their receptors are also widely distributed not only in the key areas in the central nervous system (CNS) involved in energy balance regulation (Moody & Merali 2004) but also in the periphery (Merali *et al.* 1999). The BN-like peptides have been shown to bind a range of BN receptors with varying affinity. The BN receptors have been sub-classified into four subtypes, NMB (NMB-preferring receptor), GRP (GRP-preferring receptor), bombesin receptor subtype-3 (BRS-3) (which shows low affinity with both NMB and GRP) and BRS-4 receptors, although the fourth

subtype has been found only in amphibian tissues (Nagalla *et al.* 1995). The amino acid sequences of all the BN receptors are well conserved across species (Ohki-Hamazaki *et al.* 2005).

Given the cross reactivity of NMB for all the BN receptors, it is often difficult to determine which functions are specific to NMB as opposed to the other BN-like peptides (e.g. GRP). An indication of this is provided by knockout studies of the receptors. GRP receptor knockout mice and mice lacking the BRS-3 receptor are obese (Ohki-Hamazaki *et al.* 1997, Ladenheim *et al.* 2002). However, mice lacking the NMB receptor are not obese, but there is an effect on the serotonergic (5-hydroxytryptamine (HT)) system which is linked to food intake (Yamano *et al.* 2002).

In humans, the receptor to NMB has been identified in visceral adipocytes (Yang *et al.* 2003) and Bouchard *et al.* (2004) have shown that NMB is a potential candidate gene for eating disorders (Bouchard *et al.* 2004). They also identified a missense mutation in the NMB gene, which resulted in twice as much body fat gain over a 6-year follow-up period when compared with the wild type population (Bouchard *et al.* 2004). Given that the receptor to NMB is present in human adipose

tissue, we hypothesised that NMB may be secreted by adipose tissue and act in a paracrine or endocrine manner to regulate energy balance.

Here we show that NMB is expressed in mature adipocytes and adipocytes differentiated in primary culture as well as the stromal vascular fraction of adipose tissue. We show that its expression in adipose tissue is regulated by changes in energy balance including obesity and cold exposure, but that peripheral injection of NMB does not affect energy expenditure. We further show that NMB is regulated by leptin *in vivo* and by leptin, triiodothyronine (T_3), thyroxine (T_4), interleukin 6 (IL6) and lipopolysaccharide (LPS) *in vitro* in differentiated mature 3T3-L1 adipocytes.

Materials and methods

Animals

Wild type, obese *ob/ob* and obese *db/db* male mice on the C57BL/6 background (all ~11 weeks of age) were purchased from Harlan UK (Bicester, Oxon, UK). Animals were maintained on a 12:12 h photoperiod with lights on at 0800 h. Room temperature was controlled at 20 °C (± 1 °C) and humidity was regulated to be <70%. Food (Biosure CRM pelleted chow; Special Diets Services, Witham Essex, UK) and water were available *ad libitum* unless stated. The CRM pelleted chow has an estimated metabolisable energy of 12–13 MJ/kg, partitioned as Atwater fuel energy, protein 22%, fat 9% and carbohydrate 69%.

All procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and received ethical approval from the University of Aberdeen or Rowett Research Institute Ethical Review Committee.

Intra-peritoneally (i.p.) leptin administration

To determine the effect of the i.p. administration of leptin on the expression of NMB in adipose tissue, 24 obese (*ob/ob*) mice and 24 lean (+/?) mice were used. Half the mice in each group, with *ad libitum* access to food and water, were injected i.p. once only with leptin (2 mg/kg body weight in saline) in the middle of the light phase. The other half received saline (100 μ l) only. After 1 or 4 h, the mice were killed by cervical dislocation and tissues collected for analysis. Each experimental group consisted of six mice. All murine tissues described were carefully dissected and then frozen immediately in liquid nitrogen and stored at -80 °C until extraction of RNA.

Cold exposure

To examine the effects of cold exposure, C57BL/6 mice were singly housed into M3 shoebox cages with sawdust

but no bedding, at 4 °C over three orders of magnitude in duration, for 6 (control), 60, 600 and 6000 min ($n=5$ per time point). The start time of exposure to cold were staggered so that the time at which exposure ended was always noon. The mice were singly housed to prevent social thermoregulation and had been singly housed in the warm prior to exposure to the cold. Animals were maintained on a 12:12 h photoperiod with lights on at 0800 h. The adipose tissue (epididymal and subcutaneous) was carefully dissected and then frozen immediately in liquid nitrogen and stored at -80 °C until extraction of RNA.

Energy expenditure

Measurements of resting metabolism were made using an open-flow indirect calorimetry apparatus as previously described (Arch *et al.* 2006). Because our systems include an O_2 and a CO_2 analyser uniquely dedicated to each metabolic chamber, these systems permit continuous logging of O_2 consumption and CO_2 production at a user defined interval – in this case every 15 s. The detailed time course of response allowed us to identify periods when the animals had elevated metabolism due to physical activity and thereby quantify not only resting metabolism but also the expenditure of energy on spontaneous physical activity (Hoggard *et al.* 2004).

We measured metabolic rate in lean wild type C57BL/6 mice ($n=16$, 8 per group) prior to (1.5 h) and following (4 h) i.p. administration of either saline or NMB (32 nmol/kg body weight) solubilised in saline (Sigma N3762). Mice were not fed while in the respirometry chamber. Metabolism measurements started between 0900 h and 1000 h. Prior to measurements, all the mice were weighed and their body temperatures were measured using a rectal probe (Model 2751-K and K type thermocouple, Digitron Instrumentation Ltd, Devon, UK). The rectal probe was inserted 2.5–3 cm into the rectum for ~5 s.

Mice were placed in the respirometry chamber at 20 °C (the temperature at which the mice were routinely housed) for 90 min to obtain a pre-injection baseline measurement. They were then removed from the chamber and injected i.p. with either NMB in saline ($n=8$) or saline (200 μ l, $n=8$) alone and within 30 s replaced in the respirometry chamber. Solutions were injected into the mice blind of the solution given to the animal. Metabolism was then monitored for a further 4 h. After 4 h, animals were removed from the chamber and their body weights and body temperatures remeasured. Animals were then killed as described above.

Positive excursions of CO_2 and reductions in O_2 concentration in the traces connected with physical activity were very obvious. To eliminate the effects of activity on metabolism in both the pre- and post-injection phases we measured VO_2 and VCO_2 as the

lowest consecutive 150 s of metabolism. Each animal therefore acted as its own weight-matched control. We defined the treatment effect as the difference between the pre- and post-injection measures of RMR. We used two inclusion zones for the post-injection measures of resting metabolic rate (RMR): first, the entire period between placing animals back into the chamber and their removal 4 h later and second, a restricted period of 6000 s commencing 4500 s after the animals were replaced in the chambers. Respiratory exchange ratio (RQ) was calculated as the minimum CO₂ production divided by the minimum O₂ consumption in pre- and post-injection phases. We estimated the levels of energy expended on physical activity by taking the average metabolic rate throughout the entire pre- and post-injection periods and subtracted from this the resting metabolic rate. In the post-injection period, we performed this analysis both including and excluding the period of elevated activity that mice exhibited for around 15–20 min after being returned to the metabolic chamber. All analyses of metabolism were performed blind of the treatment.

Mature adipocyte isolation

Mature adipocytes were isolated from the murine epididymal fat depot of 11-week-old C57BL/6 male mice. In brief, following dissection the white adipose tissue (WAT) samples were placed in 1% Albumin buffer (1% BSA in HEPES buffer). Following removal of blood vessels, the tissue was diced and transferred to collagenase buffer (1 mg/ml collagenase type II in HEPES buffer, pH 7.4) and placed in a shaking incubator at 37 °C for 60 min, or until digestion was complete. The digested tissue was passed through a 250 µm mesh and centrifuged at 200 *g* for 10 min to pellet the stromal vascular fraction. The mature fat cells floating at the top were then transferred to fresh tubes and treated to three washes with HEPES buffer with centrifugation at 200 *g* for 5 min. Finally, excess buffer was removed and Tri-pure isolation reagent (Roche Diagnostics Ltd) added. The stromal vascular pellet was incubated for 10 min in erythrocyte lysis buffer (154 mM NH₄Cl, 5.7 mM K₂HPO₄ and 0.1 mM EDTA, pH 7.3). The suspension was then centrifuged at 200 *g* for 10 min and the pellet was resuspended in Tri-pure isolation reagent (Roche Diagnostics Ltd).

3T3-L1 Cell culture

3T3-L1 cells were obtained from the LGC Promochem (Teddington, Middlesex, UK) and cultured at 37 °C in a humidified atmosphere of 5% CO₂–95% air. The cells were maintained in culture medium containing the

following: Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 25 mM glucose, 1 mM pyruvate, 4.02 mM L-alanyl-L-glutamine and 10% foetal calf serum (FCS; Sigma). Differentiation was initiated 24 h after confluence by incubation for 2 days in culture medium containing 0.25 µM dexamethasone (Dex), 0.5 mM 3-isobutyl-1-methyl-xanthine and 5 µg/ml insulin (Sigma). This was followed by maintenance in feeding medium (renewed every 2 out of 3 days) consisting of culture medium containing 5 µg/ml insulin. Cells were fully differentiated by day 11 with over 90% of the cells containing lipid droplets. The cells were maintained for up to 11 days after the induction of differentiation and sampled every 2–3 days. In some experiments as described fully differentiated adipocytes at day 11 were incubated for a further 24 h with leptin (1000, 100 and 10 nM), (IL6; 100 ng/ml), (LPS; 75 ng/ml), (T₃; 1000 and 100 nM), (T₄; 1000 and 100 nM) or (Dex; 20 nM). All reagents were purchased from Sigma. Cells were harvested and RNA extracted as described below for PCR analysis.

Human adipose tissue – subjects and protocols

Human subcutaneous adipose tissue was taken from the abdominal subcutaneous wall, during an operation for vertical banded gastroplasty, from two obese female patients. The patients were aged 40 ± 2 years with a BMI of 38.6 ± 1.3. Adipose tissue samples were obtained within 5 min of the tissue being extracted from the patients and frozen immediately in liquid nitrogen. Subjects had fasted overnight prior to surgery. All patients provided informed written consent before inclusion in the study. The study was approved by the Grampian Research Ethics committee.

Real-time quantitative PCR (RTQ-PCR; Taqman system)

RNA was extracted using the Tri-pure isolation reagent (Roche Diagnostics Ltd) for DNA-free RNA, following manufacturer's instructions (AMS Biotechnologies, Abingdon, Oxon, UK). RNA was accurately quantified on the Agilent 2100 Bioanalyzer (Agilent Technologies, South Queensferry, West Lothian, UK). This also shows the quality of the RNA extracted from the tissues. Only RNA showing no degradation was processed further. Reverse transcription was carried out on 3–5 µg RNA using the SuperScript first strand synthesis system (Invitrogen Cat No: 12371-019) according to manufacturer's instructions. The cDNA was amplified by PCR using either rodent specific (Applied Biosystems, Foster City, CA, USA cat number Mm00471534_m1) or human specific (Applied Biosystems cat number Hs00175085_m1) primers for the NMB gene or rodent

specific primers for the leptin gene (Applied Biosystems cat number Mm00434759_m1).

The method for the measurement of gene expression is based upon the 5' exonuclease activity of the Taq polymerase. Briefly, within the amplicon defined by a gene-specific oligonucleotide primer pair an oligonucleotide probe labelled with two fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carboxy-fluorescein, FAM) at the 5'-end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3'-end. During the extension phase of the PCR, the Taq polymerase cleaves the probe releasing the reporter dye. An automated photometric detector combined with special software (ABI Prism 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems) monitors the increasing reporter dye emission. The algorithm normalises the signal to an internal reference (DRn) and calculates the threshold cycle number (CT), when the DRn reaches ten times the s.d. of the baseline. The CT values of the probes are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template versus the corresponding CT values. Commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer) and conditions were employed according to manufacturer's protocol. cDNA (2.5 ml; reverse transcription mixture) and oligonucleotides with a final concentration of 300 nmol/l of primers and 200 nmol/l of TaqMan hybridisation probe were added to 25 ml reaction mix.

PCR was performed on an Applied Biosystems ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems Inc.) under the following conditions: 50 °C (2 min) for 1 cycle, 95 °C (10 min) for 1

cycle, 95 °C (15 s), 60 °C (1 min) for 40 cycles. All samples were run in triplicate and were all within the standard curve.

A serial dilution of known copy numbers of a PCR product served as reference (standard curve) providing a relative quantification of the unknown samples. Target gene expression was related to the housekeeping genes' β -actin (primers obtained from Applied Biosystems cat number rodent Mm00607939_s1 and human 4352935E) and GAPDH (primers obtained from Applied Biosystems cat number Mm99999915_g1), following manufacturer's instructions. All of the primers and probes were purchased from Applied Biosystems as TaqMan Gene Expression Assays which have been designed and validated by Applied Biosystems. Further details of all the primers are available at the Applied Biosystems website under the catalogue numbers provided.

Values were standardised to β -actin and expressed as a percentage of the control. Similar data were obtained when the values were standardised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (data not shown). Controls were carried out to eliminate any potential for spurious amplification from contaminating genomic DNA.

Statistical analysis

Between-group comparisons were made using unpaired Student's *t*-tests. Results were considered statistically significant if the *P* value was ≤ 0.05 . Where appropriate the data were also analysed by two-way ANOVA using Minitab version 14.

Figure 1 Expression of NMB (neuromedin B) mRNA is higher in murine epididymal adipose tissue (Epi) when compared with subcutaneous adipose tissue (sub C), perirenal adipose tissue (Peri), brown adipose tissue (Bat), kidney, liver, heart, spleen, gut, hypothalamus and pituitary (Pit) dissected from lean C57BL/6 mice and human subcutaneous (Human Sub C) adipose tissue (** $P \leq 0.01$). NMB mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB and human NMB respectively. Results are expressed as a percentage of the control that in this case is taken as expression in the lean rodent epididymal fat depot (the highest expression), $n=3$ for each point ($n=2$ for human tissue) $\pm$ s.e.m. related to the expression of the housekeeping gene β -actin. This was replicated in independent amplifications.

Results

Expression of NMB mRNA in murine tissues and in human subcutaneous adipose tissue

To determine the level of expression of NMB mRNA in murine tissues, we carried out real-time PCR with the Taqman system on a range of tissues dissected from lean C57BL/6 mice, which had *ad libitum* access to food and water and compared the level of expression with that for human subcutaneous adipose tissue obtained from the abdominal wall of two obese subjects (Fig. 1).

NMB is highly expressed in murine epididymal adipose tissue when compared with a range of other tissues including other adipose tissue depots studied ($P \leq 0.05$). NMB expression was greater by eightfold when compared with other tissues studied including kidney, liver spleen, gut, hypothalamus and heart. Due to the high level of NMB mRNA expression in the murine epididymal adipose tissue, data are presented for this fat depot in subsequent experiments, but the same trend was also observed in the subcutaneous fat depot. NMB mRNA was also detected in brown adipose tissue in keeping with a possible role for NMB in thermoregulation. NMB was expressed at high levels in the pituitary of these mice in keeping with previous reports (Houben *et al.* 1993). NMB was also detected in human adipose tissue (Fig. 1).

We tried to confirm that the differences in gene expression paralleled differences in protein by western

blotting but a commercial porcine antiserum (Acris Antibodies, Hiddenhausen, Germany) did not cross react sufficiently to murine NMB (data not shown).

Expression of NMB mRNA in the adipose tissue of obese *ob/ob* and *db/db* mice compared with C57BL/6 lean mice. Effect of i.p. administration of leptin when compared with saline-treated controls

To determine whether NMB mRNA in adipose tissue is regulated by changes in energy balance we carried out real-time PCR using the Taqman assay with primers specific to NMB (Fig. 2). NMB is decreased fourfold in the epididymal fat depot of the *ob/ob* mouse when compared with the same fat depot in lean mice on the same C57BL/6 background ($P \leq 0.05$). It is further decreased with the i.p. administration of leptin (2 mg/kg of body weight) in both lean and obese *ob/ob* mice after 4 h ($P \leq 0.05$). Using two-way ANOVA, there is a significant effect of both leptin ($P = 0.02$) and obesity ($P = 0.02$) as well as an interaction of the two ($P = 0.04$) on NMB gene expression. A similar trend was also observed after 1 h i.p. administration of leptin (data not shown).

NMB was also elevated in lean controls when compared with obese *db/db* mice, which have a defect in leptin signalling ($P \leq 0.005$) in agreement with the observed difference between the lean and obese *ob/ob* mice (Fig. 2).

Figure 2 NMB mRNA is higher in the epididymal adipose tissue depot of lean C57BL/6 mice ($P \leq 0.05$) when compared with *ob/ob* and *db/db* mice on the same background. The administration of leptin i.p. at 2 mg/kg of body weight to lean and *ob/ob* mice decreases NMB mRNA in the adipose tissue when compared with saline-treated controls after 4 h ($P \leq 0.05$). NMB mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB. Results are expressed as a percentage of the control that in this case is taken as the expression in the fat depot of the lean saline-treated mice (the highest expression), $n = 6$ for each point $\pm$ S.E.M. related to the expression of the housekeeping gene β -actin. This was replicated in independent amplifications.

Figure 3 First strand cDNA synthesis and subsequent qRT-PCR analysis were performed on RNA isolated from stromal vascular and mature adipocyte fractions separated by collagenase digestion from epididymal fat dissected from C57BL/6 mice. NMB and leptin mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB and leptin respectively. Results are expressed relative to the expression of the housekeeping gene β -actin and as a percentage of the NMB expression in the mature adipocyte fraction. SVF, stromal vascular fraction; Adipocyte, mature adipocytes.

NMB mRNA is expressed in mature adipocytes and the stromal vascular fraction

Adipose tissue is heterogeneous at the cellular level, mature adipocytes comprising no more than 50% of the total cell content. To establish the cellular localisation of NMB in WAT, epididymal fat from C57BL/6 mice was fractionated by collagenase digestion into stromal vascular and mature adipocyte components. NMB mRNA was measured using the Taqman assay and products for NMB mRNA were observed in the

adipocyte and stromal vascular fractions (Fig. 3). As expected, leptin mRNA expression was expressed exclusively in the mature adipocytes.

NMB mRNA is expressed in the murine adipocyte 3T3-L1 cell line

To determine the pattern of expression of NMB mRNA as preadipocytes are differentiated into mature adipocytes, NMB mRNA was measured using the Taqman assay in the 3T3-L1 adipocyte cell line (Fig. 4).

NMB mRNA can be readily detected in preadipocytes and differentiated mature adipocytes. NMB mRNA increased ($P \leq 0.001$) during the first 2 days of culture in medium containing 0.25 μ M Dex, 0.5 mM 3-isobutyl-1-methyl-xanthine and 5 μ g/ml insulin. Thereafter, NMB mRNA levels decreased with increasing differentiation of the preadipocytes into mature adipocytes ($P \leq 0.001$). As expected, leptin mRNA levels increased with increasing differentiation of the preadipocytes into mature adipocytes as previously reported (Rentsch & Chiesi 1996). In addition, differentiation of the 3T3-L1 cells was also evident from the accumulation of intracellular lipid droplets for which the differentiation rate was >90%.

Regulation of NMB expression in differentiated murine adipocyte 3T3-L1 cells

Differentiated murine adipocytes at day 11 were incubated for 24 h with leptin (1000, 100 and 10 nM), (IL6; 100 ng/ml), (LPS; 75 ng/ml), (T_3 ; 1000 nM, 100 nM), (T_4 ; 1000 and 100 nM) and (Dex; 20 nM) and compared with control untreated cells (Fig. 5).

Addition of leptin resulted in a dose-dependent decrease in NMB mRNA expression ($P \leq 0.05$) measured using the Taqman assay (Fig. 5). This is in keeping with the *in vivo* studies showing a decrease in NMB mRNA in adipose tissue in response to i.p. administration of leptin to both lean and obese C57BL/6 mice (Fig. 2).

NMB expression was also decreased with T_3 and T_4 at the higher concentration (1000 nM; $P \leq 0.05$), LPS ($P \leq 0.001$) and IL6 ($P \leq 0.01$), while Dex increased NMB expression ($P \leq 0.01$; Fig. 5).

Expression of NMB mRNA in murine adipose tissue decreased with increasing cold exposure in the C57BL/6 mouse

To determine whether adipose tissue NMB mRNA is altered by cold exposure, we exposed C57BL/6 mice to periods of cold exposure spanning three orders of magnitude in duration from 6 to 6000 min and measured NMB mRNA by the Taqman assay in epididymal (Fig. 6a) and subcutaneous adipose tissue (Fig. 6b). Relative to the 6 min exposure, NMB mRNA expression was decreased

Figure 4 NMB and leptin gene expression in 3T3-L1 cells before and after differentiation into mature adipocytes. Differentiation was initiated on day 0. NMB and leptin mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB and leptin respectively. Results are expressed relative to the expression of the housekeeping gene, β -actin. $n=3$ for each point $\pm$ s.e.m. This was replicated in independent amplifications. *** $P \leq 0.001$.

Figure 5 The addition of leptin (1000, 100 and 10 nM), T₃ (triiodothyronine; 1000 nM), T₄ (thyroxine; 1000 nM), LPS (lipopolysaccharide; 75 ng/ml) and IL6 (interleukin 6; 100 ng/ml) to primary cultures of differentiated adipocytes for 24 h significantly ($P \leq 0.05$) inhibits NMB mRNA expression when compared with untreated control cells, while Dex (dexamethasone; 20 nM) stimulates NMB mRNA expression. No significant effect of T₃ (100 nM) and T₄ (100 nM) was observed at the lower concentrations on NMB expression. NMB mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB. Results are expressed as a percentage of the control untreated differentiated cells. $n=3$ for each point $\pm$ S.E.M. related to the expression of the housekeeping gene β -actin. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. This was replicated in independent amplifications.

with increasing cold exposure. This was significant at 600 min ($P \leq 0.05$). This suggests that peripheral NMB may have a role in thermoregulation or alternatively it may simply be part of the mechanism regulating food intake. In keeping with the role in thermoregulation, previous studies have shown that BN and the BN-like peptides can affect metabolism (Wunder *et al.* 1980, Babcock *et al.* 1992, Itoh *et al.* 1995, Tsushima *et al.* 2003).

Effect of NMB administered acutely to lean C57BL/6 mice on O₂ consumption and CO₂ production

Mice were randomly allocated to the treatment groups. There were no significant differences in body mass or pre-injection RMR between the treatment groups. A typical time course of O₂ consumption for a single individual mouse injected with saline is shown in Fig. 7. This plot illustrates several features that were apparent in the traces of all animals. During the initial phase, after being introduced to the chamber, all the mice exhibited exploratory behaviour and their levels of O₂ consumption remained elevated for around 50–60 min (Fig. 7B). They then settled and during the final 30 min (approximately) were generally observed to be asleep (Fig. 7C). Positive excursions of O₂ consumption for brief periods coinciding with periods of activity were evident on the recordings (Fig. 7D). Following injection, the mice exhibited a second

increase in O₂ consumption coinciding with elevated activity on return to the chambers. Following this period, which lasted 20–30 min, the mice again settled down and positive excursions of the traces due to activity were again evident (Fig. 7H). The pattern of change in the baseline resting O₂ consumption underlying these changes due to activity were very similar between the control and NMB treatment. The mean O₂ consumption averaged across all individuals in each treatment group is illustrated in Fig. 8a (saline) and Fig. 8b (NMB). In these plots, periods when all mice were active or had elevated resting metabolism show as positive excursions, while individual bouts of activity that were not coincident across individuals are damped by the average. In the pre-injection phase, both plots appear identical as would be expected since the groups were matched for body mass and at this point their treatments did not differ. In the saline (Fig. 8a) and NMB injected mice (Fig. 8b), the metabolic rate increased following return to the chamber after injection but then settled to a level where the minimal O₂ consumption had declined slightly relative to that measured pre-injection.

Using the criterion of minimal resting O₂ consumption measured over a continuous period of 150 s over the entire post-injection period, there was no significant effect of NMB when compared with saline, on resting O₂ consumption.

Respiratory exchange ratio (RQ) in the post-injection phase was not significantly ($P > 0.05$) affected by

Figure 6 Expression of NMB mRNA in (a) murine epididymal and (b) subcutaneous adipose tissue is decreased upon cold exposure of the C57BL/6 mouse. The decrease in NMB increases with the increased exposure to cold at 6, 60, 600 ($P \leq 0.05$) and 6000 min compared with the 6-min cold exposure. NMB mRNA was determined by qRT-PCR using the Taqman assay and primers specific for murine NMB. Results are expressed as a percentage of the NMB expression in the 6-min cold exposed fat depot (the highest expression), $n=5$ for each point $\pm$ s.e.m. related to the expression of the housekeeping gene β -actin. This was replicated in independent amplifications.

treatment (data not shown). Levels of energy expended on spontaneous physical activity were also not significantly different either prior to injection ($P > 0.05$) or during the post-injection phase ($P > 0.05$).

In summary, the effect of NMB on O_2 consumption was not significantly different from that of the saline control. This suggests that the NMB changes as a result of cold exposure were precipitated by changes in the cold and not part of the system up-regulating cold-induced thermogenesis.

Discussion

We have shown that NMB mRNA is expressed at high levels in adipose tissue when compared with a range of other tissues including the pituitary, a tissue where expression has previously been reported in the C57BL/6 mouse. Given that BN-like peptides including NMB and GRP administered both centrally and peripherally have previously been shown to inhibit food intake in a variety of species including humans (Merali *et al.* 1999,

Figure 7 Typical time course of oxygen consumption observed in a C57BL/6 mouse during an experimental run to illustrate typical features of the change in metabolism over time. The animal is placed in the chamber at point A. The oxygen consumption increases at point B reflecting activity of the animal in the chamber. The animal eventually falls asleep (point C) although during the decline in metabolism there are still occasional excursions reflecting renewed activity (e.g. point D). The pre-injection RMR is indicated by the level E. At F, the animal is removed from the chamber, rapidly injected i.p. with the treatment compound (in this case saline) and returned immediately into the chamber. As this is performed within a period of about 10 s, during which the chamber is not ventilated, the analyser does not register a decline in the oxygen consumption. There is a second bout of activity (G) following return to the chamber but the animal settled down more quickly in this case. Occasional elevations in metabolism due to activity are still observed in the post-injection phase (H) and the animal is finally removed at point I. The post-injection level of resting metabolism is indicated by the level J. Each measurement is 15 s apart.

Ladenheim & Knipp 2007), we hypothesised that NMB expressed in adipose tissue may play a role in energy balance regulation. This may relate to its function in food intake regulation or to changes in energy expenditure. In support of this, we have shown that NMB expression is decreased in the adipose tissue of both obese *ob/ob* and obese *db/db* mice when compared with lean mice on the same C57BL/6 background. The administration of leptin peripherally to both lean and obese (leptin-deficient) *ob/ob* mice caused a decrease in NMB expression in adipose tissue. This outcome is supported by our *in vitro* findings, which show a dose-dependent inhibition of NMB mRNA by leptin in differentiated 3T3-L1 adipocytes. However, given that NMB is decreased in (leptin-deficient) obese *ob/ob* mice when compared with lean control mice, it suggests that leptin is not the only signal regulating NMB. The possible regulation of NMB by the pituitary–thyroid axis and the pro-inflammatory response associated with

obesity is discussed below. We further demonstrate that NMB expression in rodent adipose tissue is decreased in the subcutaneous fat depot of cold exposed animals. However, when we investigated the effects of NMB on RMR by i.p. injection (32 nmol/kg body weight) there was no effect on oxygen consumption, RQ or physical activity when compared with saline-treated controls.

BN-like peptides have a role in meal termination and are expressed in the gut as well as the brain. Accordingly, it has been proposed that NMB along with GRP may represent one of the physiological mediators of the gut–brain axis (Banks 1980, Gibbs 1985, Panula 1986, Merali *et al.* 1999). It has been proposed that BN-like peptides, including NMB, are released in the GI tract in response to food, and bridge the gut–brain axis to inhibit further food intake (Merali *et al.* 1999). In keeping with this, it has been shown that BN-like peptides are released into the intestinal mucosa in response to nutrients (Schusdziarra *et al.* 1986). Ladenheim *et al.* (1996a,b, 1997) have shown that peripherally injected BN decreased food intake via BN receptors of both GRP and NMB subtypes in the caudal hindbrain. There have been a number of reports where BN/BN-like peptides administered into the hypothalamus induce hypophagia (Stuckey & Gibbs 1982, Kyrkouli *et al.* 1987, Hillebrand *et al.* 2002). The release of BN-like peptides in the paraventricular nucleus (PVN) has been shown to depend on the ingestion of food (Plamondon & Merali 1994). Taken together, this clearly demonstrates the importance of the BN receptor family in the CNS in the regulation of feeding behaviour (Merali *et al.* 1999). NMB expressed in adipose tissue may therefore regulate food intake over the longer term. In keeping with BN-like peptides suppressing food intake, levels of NMB in the adipose tissue of obese (leptin-deficient) *ob/ob* mice are low compared with their lean littermates.

The process through which the BN-like peptides of peripheral origin activate the relevant brain circuits remains to be identified. Although these peptides of peripheral origin may not readily cross the blood brain barrier, they may have some access at sites where the barrier is less restrictive (such as the area postrema; Banks & Kastin 1989, 1990). It has also been suggested that peripheral BN from the gut suppresses food intake via spinal afferent fibres along with vagal afferent mechanisms that ultimately elicit the release of BN-like peptides within the CNS (Merali *et al.* 1999, Michaud *et al.* 1999).

We have shown that the administration of leptin peripherally to both lean and obese (leptin-deficient) *ob/ob* mice causes a decrease in NMB expression in adipose tissue. This is supported by our *in vitro* findings, which show a dose-dependent inhibition of NMB mRNA by leptin in differentiated 3T3-L1 adipocytes. This finds resonance with previous studies, where peripheral

Figure 8 Time courses of oxygen consumption averaged across eight C57BL/6 mice in each group injected with either (a) saline (200 μ l) or (b) NMB (32 nmol/kg body weight) in saline. In saline and NMB injected mice, there was no difference between the pre-injection and post-injection levels of resting metabolism.

administration of leptin to rats (8 μ g/100 g body weight) caused a 30% reduction in NMB concentration in the pituitary when compared with saline-treated controls (Ortiga-Carvalho *et al.* 2002). In the pituitary, the inhibition of NMB by leptin occurs either by a direct action of leptin on the pituitary or indirectly via a stimulation of thyrotropin-releasing hormone (TRH). TRH has also been shown to reduce NMB concentration in the pituitary by up to 45% when administered peripherally to rats compared with saline-treated controls (Ortiga-Carvalho *et al.* 2002). Serum T_3 is also reduced in NMB receptor knockout mice, which taken together suggests that NMB may be important in the pituitary–thyroid axis (Oliveira *et al.* 2006). To investigate this further in adipose tissue, we determined whether T_3 or T_4 had a direct effect on NMB expression in differentiated

3T3-L1 adipocytes. Although only significant at the higher concentrations (1000 nM), we were able to show that both T_3 and T_4 inhibit NMB expression. However, it should be noted that these levels are tenfold higher than the physiological levels of T_3/T_4 (~ 10 nM).

I.c.v. injections of BN and the BN-like peptides have been shown to produce significant dose-dependent hypothermia in rodents (Wunder *et al.* 1980, Babcock *et al.* 1992, Itoh *et al.* 1995, Tsushima *et al.* 2003). However, there is another study that suggests that peripheral administration of BN increased metabolism (Even *et al.* 1991). Because of this conflict and the effect we observed of both T_3 and T_4 on the expression of NMB in adipocytes, which have also been shown to affect metabolic rate, we determined whether peripheral i.p. administration of NMB had a significant effect

on energy expenditure. Support for this hypothesis comes from our demonstration that NMB is decreased in adipose tissue as a result of cold exposure. However, when we studied the acute effect of NMB using open-flow indirect calorimetry apparatus, we observed no effect on RMR at the concentration used (32 nmol/kg body), which we selected because it was a similar dose to that used in previous studies. This suggests that the NMB changes as a result of cold exposure were not part of the system up-regulating cold-induced thermogenesis, but the changes may be part of the mechanism regulating food intake. The decrease we observed in NMB during the cold exposure may be a result of changes in T_3 and T_4 that are activated as a result of cold exposure. A similar decrease in NMB in the pituitary upon cold exposure has been reported previously (Ortiga-Carvalho *et al.* 2003). The changes in NMB expression in adipose tissue as a result of cold exposure may be as a result of changes in energy fluxes stimulating an increase in food intake.

Receptors to NMB have been reported in adipose tissue, which suggests that NMB may have important autocrine effects. In keeping with this, NMB has been shown to induce mitogenesis in adipocyte 3T3 cells (Zachary & Rozengurt 1985). Indeed, using 3T3-L1 cells, the present study indicates that the expression of NMB mRNA is greater in preadipocytes than in differentiated mature adipocytes supporting a role for NMB in preadipocyte mitogenesis. It may be that expression of NMB is more important in preadipocytes; however, significant levels of NMB are detected in the mature adipocytes and adipose tissue itself. In addition, NMB is detected at significant levels in the stromal vascular fraction, which could reflect expression in any component of that fraction, including fibroblasts, vascular endothelial cells and immune cells (including mast cells and macrophages) in addition to immature adipocytes. This suggests that there might be an interaction between the cells of the stromal vascular fraction and the mature adipocytes, which expresses receptors to NMB. It is interesting to note that we also show that NMB expression is decreased by IL6, which is part of the obesity associated inflammatory response and by LPS, which stimulates an inflammatory response (Trayhurn & Wood 2004). This may be one pathway by which NMB expression in the adipose tissue is reduced in the obese *ob/ob* mouse when compared with the lean control mouse. IL6 and other inflammatory cytokines are increased with increasing adiposity in the obese *ob/ob* mouse. As we discussed above, it is unlikely that leptin is responsible for the decrease in NMB in the leptin-deficient *ob/ob* mouse, as we have shown that leptin causes a reduction in NMB expression.

In humans, Bouchard *et al.* (2004) have identified a missense mutation in the NMB gene, which was associated with twice as much body fat gain over a 6-year follow-up period compared with the wild type

population. In keeping with a role for NMB in energy balance in humans, we have also shown that NMB mRNA is present in human adipose tissue at levels comparable with those in rodent adipose tissue.

In conclusion, this is the first report of NMB expression in adipose tissue. We provide evidence to support a possible role of NMB in the regulation of energy balance. Given the anorexic effects of BN-like peptides centrally, NMB expressed by adipose tissue may form part of a new adipose tissue hypothalamic axis regulating food intake.

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References

- Anastasi A, Erspamer V & Bucci M 1971 Isolation and structure of bombesin and alkytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. *Experientia* **27** 166–167.
- Arch JR, Hislop D, Wang SJ & Speakman JR 2006 Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *International Journal of Obesity* **30** 1322–1331.
- Babcock AM, Baker DA & Moody TW 1992 Bombesin-induced hypothermia: a dose–response and receptor antagonist study. *Pharmacology, Biochemistry and Behavior* **43** 957–960.
- Banks WA 1980 Evidence for a cholecystokinin gut–brain axis with modulation by bombesin. *Peptides* **1** 347–351.
- Banks WA & Kastin AJ 1989 Quantifying carrier-mediated transport of peptides from the brain to the blood. *Methods in Enzymology* **168** 652–660.
- Banks WA & Kastin AJ 1990 Exchange of peptides between the circulation and the nervous system: role of the blood–brain barrier. *Advances in Experimental Medicine and Biology* **274** 59–69.
- Bouchard L, Drapeau V, Provencher V, Lemieux S, Chagnon Y, Rice T, Rao DC, Vohl MC, Tremblay A, Bouchard C *et al.* 2004 Neuromedin β : a strong candidate gene linking eating behaviors and susceptibility to obesity. *American Journal of Clinical Nutrition* **80** 1478–1486.
- Erspamer V, Erpamer GF & Inselvini M 1970 Some pharmacological actions of alkytesin and bombesin. *Journal of Pharmacy and Pharmacology* **22** 875–876.
- Even PC, de Saint Hilaire Z & Nicolaidis S 1991 Peripheral administration of bombesin increases metabolism in the rat. *Physiology and Behavior* **49** 439–442.
- Gibbs J 1985 Effect of bombesin on feeding behavior. *Life Sciences* **37** 147–153.
- Hillebrand JJ, de Wied D & Adan RA 2002 Neuropeptides, food intake and body weight regulation: a hypothalamic focus. *Peptides* **23** 2283–2306.
- Hoggard N, Rayner DV, Johnston SL & Speakman JR 2004 Peripherally administered [Nle⁴,D-Phe⁷]- α -melanocyte stimulating hormone increases resting metabolic rate, while peripheral agouti-related protein has no effect, in wild type C57BL/6 and *ob/ob* mice. *Journal of Molecular Endocrinology* **33** 693–703.

- Houben H, Vandenbroucke AT, Verheyden AM & Denef C 1993 Expression of the genes encoding bombesin-related peptides and their receptors in anterior pituitary tissue. *Molecular and Cellular Endocrinology* **97** 159–164.
- Itoh S, Takashima A, Itoh T & Morimoto T 1995 Effects of neuromedins and related peptides on the body temperature of rats. *Japanese Journal of Physiology* **45** 37–45.
- Kyrkouli SE, Stanley BG & Leibowitz SF 1987 Bombesin-induced anorexia: sites of action in the rat brain. *Peptides* **8** 237–241.
- Ladenheim EE & Knipp S 2007 Capsaicin treatment differentially affects feeding suppression by bombesin-like peptides. *Physiology and Behavior* **91** 36–41.
- Ladenheim EE, Taylor JE, Coy DH, Moore KA & Moran TH 1996a Hindbrain GRP receptor blockade antagonizes feeding suppression by peripherally administered GRP. *American Journal of Physiology* **271** R180–R184.
- Ladenheim EE, Wirth KE & Moran TH 1996b Receptor subtype mediation of feeding suppression by bombesin-like peptides. *Pharmacology, Biochemistry and Behavior* **54** 705–711.
- Ladenheim EE, Taylor JE, Coy DH, Carrigan TS, Wohn A & Moran TH 1997 Caudal hindbrain neuromedin B-preferring receptors participate in the control of food intake. *American Journal of Physiology* **272** R433–R437.
- Ladenheim EE, Hampton LL, Whitney AC, White WO, Battey JF & Moran TH 2002 Disruptions in feeding and body weight control in gastrin-releasing peptide receptor deficient mice. *Journal of Endocrinology* **174** 273–281.
- McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR & Mutt V 1979 Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochemical and Biophysical Research Communications* **90** 227–233.
- Merali Z, McIntosh J & Anisman H 1999 Role of bombesin-related peptides in the control of food intake. *Neuropeptides* **33** 376–386.
- Michaud D, Anisman H & Merali Z 1999 Capsaicin-sensitive fibers are required for the anorexic action of systemic but not central bombesin. *American Journal of Physiology* **276** R1617–R1622.
- Minamino N, Kangawa K & Matsuo H 1988 Neuromedin B and neuromedin Ca. Two mammalian bombesin-like peptides identified in porcine spinal cord and brain. *Annals of the New York Academy of Sciences* **547** 373–390.
- Moody TW & Merali Z 2004 Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. *Peptides* **25** 511–520.
- Nagalla SR, Barry BJ, Creswick KC, Eden P, Taylor JT & Spindel ER 1995 Cloning of a receptor for amphibian (Phe13) bombesin distinct from the receptor for gastrin-releasing peptide: identification of a fourth bombesin receptor subtype (BB4). *PNAS* **92** 6205–6209.
- Ohki-Hamazaki H, Watase K, Yamamoto K, Ogura H, Yamano M, Yamada K, Maeno H, Imaki J, Kikuyama S, Wada E *et al.* 1997 Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature* **390** 165–169.
- Ohki-Hamazaki H, Iwabuchi M & Maekawa F 2005 Development and function of bombesin-like peptides and their receptors. *International Journal of Developmental Biology* **49** 293–300.
- Oliveira KJ, Ortiga-Carvalho TM, Cabanelas A, Veiga MA, Aoki K, Ohki-Hamazaki H, Wada K, Wada E & Pazos-Moura CC 2006 Disruption of neuromedin B receptor gene results in dysregulation of the pituitary–thyroid axis. *Journal of Molecular Endocrinology* **36** 73–80.
- Ortiga-Carvalho TM, Oliveira KJ, Soares BA & Pazos-Moura CC 2002 The role of leptin in the regulation of TSH secretion in the fed state: *in vivo* and *in vitro* studies. *Journal of Endocrinology* **174** 121–125.
- Ortiga-Carvalho TM, Oliveira K, de J, Morales MM, Martins VP & Pazos-Moura CC 2003 Thyrotropin secretagogues reduce rat pituitary neuromedin B, a local thyrotropin release inhibitor. *Experimental Biology and Medicine* **228** 1083–1088.
- Panula P 1986 Histochemistry and function of bombesin-like peptides. *Medical Biology* **64** 177–192.
- Plamondon H & Merali Z 1994 Push–pull perfusion reveals meal-dependent changes in the release of bombesin-like peptides in the rat paraventricular nucleus. *Brain Research* **668** 54–61.
- Rentsch J & Chiesi M 1996 Regulation of *Ob* gene messenger-RNA levels in cultured adipocytes. *FEBS Letters* **379** 55–59.
- Schusdziarra V, Schmid R & Classen M 1986 Modulatory glucose effect on bombesin-like immunoreactivity and gastrin secretion from isolated perfused rat stomach. *Diabetes* **35** 791–796.
- Stuckey JA & Gibbs J 1982 Lateral hypothalamic injection of bombesin decreases food intake in rats. *Brain Research Bulletin* **8** 617–621.
- Trayhurn P & Wood IS 2004 Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition* **92** 347–355.
- Tsushima H, Mori M, Fujiwara N & Moriyama A 2003 Pharmacological characteristics of bombesin receptor mediating hypothermia in the central nervous system of rats. *Brain Research* **969** 88–94.
- Wunder BA, Hawkins MF, Avery DD & Swan H 1980 The effects of bombesin injected into the anterior and posterior hypothalamus on body temperature and oxygen consumption. *Neuropharmacology* **19** 1095–1097.
- Yamano M, Ogura H, Okuyama S & Ohki-Hamazaki H 2002 Modulation of 5-HT system in mice with a targeted disruption of neuromedin B receptor. *Journal of Neuroscience Research* **68** 59–64.
- Yang YS, Song HD, Li RY, Zhou LB, Zhu ZD, Hu RM, Han ZG & Chen JL 2003 The gene expression profiling of human visceral adipose tissue and its secretory functions. *Biochemical and Biophysical Research Communications* **300** 839–846.
- Zachary I & Rozengurt E 1985 High-affinity receptors for peptides of the bombesin family in Swiss 3T3 cells. *PNAS* **82** 7616–7620.

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