

# Neuropeptide AF and FF Modulation of Adipocyte Metabolism

PRIMARY INSIGHTS FROM FUNCTIONAL GENOMICS AND EFFECTS ON  $\beta$ -ADRENERGIC RESPONSIVENESS\*

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The presence of a neuropeptide AF and FF receptor (NPFF-R2) mRNA in human adipose tissue (Elshourbagy, N. A., Ames, R. S., Fitzgerald, L. R., Foley, J. J., Chambers, J. K., Szekeres, P. G., Evans, N. A., Schmidt, D. B., Buckley, P. T., Dytko, G. M., Murdock, P. R., Tan, K. B., Shabon, U., Nuthulaganti, P., Wang, D. Y., Wilson, S., Bergsma, D. J., and Sarau, H. M. (2000) *J. Biol. Chem.* 275, 25965–25971) suggested these peptides, principally recognized for their pain modulating effects, may also impact on adipocyte metabolism, an aspect that has not been explored previously. Our aim was thus to obtain more insights into the actions of these peptides on adipocytes, an approach initially undertaken with a functional genomic assay. First we showed that 3T3-L1 adipocytes express both NPFF-R1 and NPFF-R2 transcripts, and that NPAF binds adipocyte membranes with a nanomolar affinity as assessed by surface plasmon resonance technology. Then, and following a 24-h treatment with NPFF or NPAF (1  $\mu$ M), we have measured using real-time quantitative reverse transcriptase-PCR the mRNA steady state levels of already well characterized genes involved in key pathways of adipose metabolism. Among the 45 genes tested, few were modulated by NPFF (~10%) and a larger number by NPAF (~27%). Interestingly, NPAF increased the mRNA levels of  $\beta$ 2- and  $\beta$ 3-adrenergic receptors (AR), and to a lesser extent those of  $\beta$ 1-ARs. These variations in catecholamine receptor mRNAs correlated with a clear induction in the density of  $\beta$ 2- and  $\beta$ 3-AR proteins, and in the potency of  $\beta$ -AR subtype-selective agonists to stimulate adenylyl cyclase activity. Altogether, these data show that NPFF-R1 and NPFF-R2 are functionally present in adipocytes and suggest that besides their well described pain modulation effects, NPAF and to a lesser extent NPFF, may have a global impact on body energy storage and utilization.

The amidated neuropeptide FF (NPFF)<sup>1</sup> and neuropeptide AF (NPAF), often referred as to mammalian FMRFamide-like

peptide or morphine-modulating peptides, have long been recognized for their pain modulation effects as well as for their fundamental role in opioid analgesia and tolerance development (for review, see Refs. 1 and 2).

However, in line with original identification of the molluscan neuropeptide FMRFamide and analogues as cardioexcitatory agents (3, 4), these peptides have been shown to produce several other peripheral effects. In particular, NPFF and/or NPAF modulate cardiac and vascular function (5, 6), insulin and somatostatin secretion (7, 8), food intake (9), adrenal aldosterone production (10), or body temperature (11).

The gene encoding the precursor for these peptides has been recently described in human, rat, mouse, and bovine species (12, 13); the two peptides are encoded as a single copy by the same precursor. In the rat, expression of the NPAF and NPFF precursor is limited to discrete regions of the central nervous system including the hypothalamus, medulla, and dorsal horn of the spinal cord (13). In human, the NPAF and NPFF precursor genes are expressed in various peripheral organs as well as in nervous central regions with the highest levels in the cerebellum (14). Detection of pulsatile secretion of NPFF in human plasma suggests that the peptides could act as hormones (15) and is relevant to the various peripheral actions reported for these peptides.

Two human receptors for NPFF and NPAF have very recently been identified (14, 16, 17). The first reported human receptor for NPFF and NPAF, initially called HLWAR77, is a previous orphan 7-transmembrane domain G protein-coupled receptor, which when stably expressed in HEK293 exhibit similar nanomolar affinity for both NPAF and NPFF (14). Bonini *et al.* (16) as well as Hinuma *et al.* (17) identified another receptor, sharing 78% amino acid identity with the precedent, with nanomolar affinity for NPAF and NPFF, called NPFF1. Bonini *et al.* (16) also identified the receptor characterized by Elshourbagy and collaborators (14) and named it NPFF2. These NPAF/NPFF receptors will be called hereafter NPFF-R1 for NPFF1, and NPFF-R2 for HLWAR77 or NPFF2. Tissue distribution analysis of NPFF-R mRNAs in human revealed a wide expression in various brain areas and little or no expression in other tested peripheral organs (14, 16). In the central and peripheral

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<sup>1</sup> The abbreviations used are: NPFF, neuropeptide FF;  $\beta$ -AR,  $\beta$ -adrenergic receptor; BRL37344, sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl]phenoxyacetate sesquihydrate (*RR,SS*-diastereoisomer); CGP20712A, ( $\pm$ )-(2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-(4-(1-methyl-4-trifluoromethyl-2-imidazolylphenoxy)-2-

propanolmethanesulfonate; [<sup>125</sup>I]CYP, (-)-[<sup>125</sup>I]iodocyanopindolol; G3PDH, glycerol-3-phosphate dehydrogenase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTP $\gamma$ S, guanosine 5'-O-(3-thio-triphosphate); ICI118551, erythro-( $\pm$ )-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ISO, (-)-isoproterenol; NPAF, neuropeptide AF; NPFF-R(s), NPFF or NPAF receptor(s); RT, reverse transcription; RU, resonance unit; HPA, hydrophobic association; full names of gene abbreviations are given in Table I.

nervous systems, *NPFF-R1* mRNA levels are the highest in spinal chord, hippocampus, and amygdala (16), whereas *NPFF-R2* mRNA are the highest in cingulate gyrus and the lowest in cerebellum (14). In human peripheral organs, *NPFF-R1* transcripts are found at low levels in lung and small intestine (16), whereas *NPFF-R2* expression is virtually restricted to adipose tissue with the exception of placenta. Although the restricted and limited expression in peripheral organs do not reconcile multiple reported peripheral effects of NPAF or NPFF and may suggest the existence of an additional receptor subtype(s) (1), it highlights possible modulatory metabolic effects of these neuropeptides on adipose tissue, an aspect that has never been reported or explored.

For such a purpose we have designed an assay aimed at providing insights into the biological effects of NPAF or NPFF on adipocytes. We used as a model the mouse 3T3-L1 preadipose cell line that mimics the morphological, metabolic, and hormonal features of adipose tissue development (18). After having verified that 3T3-L1-differentiated fat cells express *NPFF-R1* and *NPFF-R2*, mature 3T3-L1 adipocytes were exposed to NPAF or NPFF stimulation and mRNA steady state levels of about 50 genes, which products are involved in key pathways of adipose metabolism, were measured by quantitative real-time RT-PCR. The well characterized tested genes are implicated in the six following pathways: insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation, and secretion of factors. With such an approach, one could evidence that NPAF, and to a lesser extent NPFF, modulate in adipocytes a finite number of genes among those tested. The products of these target genes are involved in several facets of adipocyte biology. Interestingly, we observed that NPAF induces the mRNA levels of the three  $\beta$ -adrenergic receptor ( $\beta$ -AR) subtypes. To evaluate the consequences of modulation of  $\beta$ -AR transcripts, we focused the last part of our work on the characterization of NPAF effects on  $\beta$ -AR protein expression and functional responsiveness. In agreement with variations in mRNA levels, the neuropeptide increases  $\beta$ 2- and  $\beta$ 3-AR populations. This was paralleled by a higher potency of  $\beta$ 2- or  $\beta$ 3-AR-selective agonists and of (-)-epinephrine to stimulate adenylyl cyclase activity. This fine regulation may represent a physiological mechanism by which in fat cells, the neuropeptide could promote the vascular over the neuronal adrenergic control of cAMP-dependent biological events.

#### MATERIALS AND METHODS

**Cell Culture**—3T3-L1 cells (ATCC number CL-173) were grown and differentiated at 37 °C in an atmosphere of air/CO<sub>2</sub> (90:10, v/v) in Dulbecco's modified Eagle's medium (Invitrogen) with 4.5 g/liter of D-glucose, 10% fetal calf serum, penicillin/streptomycin (50 units of penicillin/50  $\mu$ g of streptomycin/ml of medium). Two days after reaching confluence, cells were induced into differentiation with a 2-day incubation in Dulbecco's modified Eagle's medium, 10% fetal calf serum containing insulin (1  $\mu$ g/ml), dexamethasone (0.25  $\mu$ M), and isobutylmethylxanthine (0.1 mM) (all from Sigma). Then preadipocytes were shifted in Dulbecco's modified Eagle's medium, 10% fetal calf serum supplemented with insulin (1  $\mu$ g/ml). After 10 days, when adipocytes have accumulated numerous lipid droplets as judged by Oil Red O staining (19), cells were placed for 16–18 h in a defined medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 (1:1, v/v), 4.5 g/liter of D-glucose, L-glutamine, penicillin/streptomycin, 5% bovine serum albumin, then treated for 24 or 48 h with the indicated concentrations of human NPAF (AGEGLNSQFWSLAAPQRFa) or human NPFF (SQAFLFQPQRFa) (Bachem). Each experiment was performed three to five times.

**Interaction of NPAF with Membranes from Adipocytes Assessed by Surface Plasmon Resonance**—Membranes from mature 3T3-L1 adipocytes were prepared by hypotonic lysis in 20 mM HEPES, pH 7.5, 1 mM EDTA, in the presence of protease inhibitors, followed by a 500-g supernatant centrifugation at 48,000  $\times$  g. Proteins were measured

using the Bradford Coomassie dye method using bovine serum albumin as standard (Pierce). Adipocyte membranes were immobilized on hydrophobic surfaces to allow subsequent measurement by surface plasmon resonance (Biacore) of NPAF binding. For such a purpose, the HPA (hydrophobic association) sensor chip surface was cleaned with 50  $\mu$ l of 40 mM *N*-octylglucoside in water at a flow rate of 10  $\mu$ l/min. After 10–30 s, 10  $\mu$ l of adipocyte membranes (100  $\mu$ g of protein/ml) diluted in phosphate-buffered saline were injected onto the surface in the sample flow cell at a flow rate of 3  $\mu$ l/min. Another flow cell also activated with *N*-octylglucoside but without membrane served as control. The process of spontaneous fusion of adipocyte membranes to the HPA surface was monitored as the sensorgram reading begins to level out after reaching a plateau. To allow formation of an homogenous surface, *i.e.* lipid monolayer, 10  $\mu$ l of 10 mM NaOH was injected at a flow rate of 10  $\mu$ l/min according to the manufacturers recommendation (Biacore). Then bovine serum albumin (10  $\mu$ l at 0.1 mg/ml) was injected at a flow rate of 10  $\mu$ l/min to fill out putative gaps that could have remained on the surface. All experiments were done at 25 °C in a continuous flow of phosphate-buffered saline, pH 7.4. HPA surface regeneration was achieved with repeated washes with 10 mM glycine at pH 2.0. NPAF was used at the indicated concentrations to monitor interaction with adipocyte membranes. Binding curves obtained using two concentrations of NPAF (10 and 1000 nM) were analyzed (BiaEvaluation software), and the data sets were best fitted with a 1:1 Langmuir binding interaction. Nonspecific binding to membranes was determined using an irrelevant peptide of the same size as NPAF: COOH-GSKGSKGSKGSKGSKGSK-NH<sub>2</sub>. Experiments were reproduced at least 3 times.

**RNA Preparation and Real-time Quantitative RT-PCR**—Total RNA was prepared as described (20). cDNA was synthesized from 5  $\mu$ g of total RNA in 20  $\mu$ l using random hexamers and murine Moloney leukemia virus reverse transcriptase (Invitrogen). Design of primers was done using either the Primer Express (Applied Biosystems) or Oligo (MedProbe, Norway) software. Real-time quantitative RT-PCR analyses for the genes described in Table I were performed starting with 50 ng of reverse transcribed total RNA (diluted in 5  $\mu$ l of 1 $\times$  Sybr Green buffer), with 200 nM of both sense and antisense primers (Genset) in a final volume of 25  $\mu$ l using the Sybr Green PCR core reagents in a ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). Fluorescence was generated after laser excitation by bound Sybr Green to double strand DNA. Because we used Sybr Green in measurements of amplification-associated fluorescence for real-time quantitative RT-PCR, it was important to verify that generated fluorescence was not overestimated by contaminations resulting from residual genomic DNA amplification (using controls without reverse transcriptase) and/or from primer dimers formation (controls with no DNA template nor reverse transcriptase). RT-PCR products were also analyzed on ethidium bromide-stained agarose to ensure that a single amplicon of the expected size was indeed obtained. To measure PCR efficiency, serial dilutions of reverse transcribed RNA (0.1 pg to 200 ng) were amplified, and a line was obtained by plotting cycle threshold ( $C_T$ ) values as a function of starting reverse transcribed RNA, the slope of which was used for efficiency calculation using the formula  $E = 10^{(1/\text{slope})} - 1$  (21). Ribosomal 18 S RNA and *GAPDH* (which are not modified by treatment with the neuropeptides) amplifications were used to account for variability in the initial quantities of cDNA. Relative quantitation for any given gene, expressed as -fold variation over control (untreated cells), was calculated after determination of the difference between  $C_T$  of the given gene A and that of the calibrator gene B (*GAPDH*) in treated cells ( $\Delta C_{T1} = C_{TA} - C_{TB}$ ) and untreated cells ( $\Delta C_{T0} = C_{T0A} - C_{T0B}$ ) using the  $2^{-\Delta\Delta C_T(1-0)}$  formula (21). *GAPDH* expression of a control RNA was used as interplate calibrator.  $C_T$  values are means of triplicate measurements. Experiments were repeated 3 to 5 times. For quantitation of murine *NPFF-Rs*, standard curves were determined after amplification of  $5 \times 10^2$  to  $5 \times 10^6$  copies of purified amplicons generated from 3T3-L1 cDNA using the sense and antisense primers. All primer sequences are presented in Table I.

**Enzyme and Binding Assays**—3T3-L1 adipocytes were rinsed two times with phosphate-buffered saline, then harvested and homogenized at 4 °C using a Dounce B pestle (20 strokes) in 1 mM EDTA, 25 mM Tris-HCl, pH 7.5. Homogenates were centrifuged at 100,000  $\times$  g for 30 min at 4 °C, and membrane pellets were resuspended in the homogenization buffer and stored at -80 °C until use in adenylyl cyclase or binding assays. Protein was assayed (22) using bovine serum albumin as a standard. Cell triglyceride content was determined with the Infinity<sup>TM</sup> triglyceride kit (Sigma).

Adenylyl cyclase (EC 4.6.1.1) activity was measured for 10 min at 35 °C in a 50- $\mu$ l standard assay consisting of 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (ICN Pharmaceuticals), 1 mM cAMP, 10 mM phosphocreatine, 0.5 unit of

creatine phosphokinase, 100  $\mu\text{M}$  GTP (except when GTP $\gamma\text{S}$ , NaF, or forskolin were used), 10 mM MgCl<sub>2</sub>, 0.2 mM EDTA, and 50 mM Tris-HCl, pH 7.5, with or without a  $\beta$ -adrenergic effector. The reaction was initiated by the addition of crude membranes (20  $\mu\text{g}$  of protein) and terminated as described (23).

Glycerol-3-phosphate dehydrogenase (G3PDH) activity was measured in the cytosolic fraction by recording the initial rate of oxidation of NADH at 340 nm at 25 °C (24). The standard mixture contained 50 mM triethanolamine, 25 mM HCl buffer, pH 7.5, 1 mM EDTA, 0.13 mM  $\beta$ -NADH, 1 mM dihydroxyacetone phosphate, 1 mM 2-mercaptoethanol, and variable amounts of 100,000  $\times$  g supernatants.

For [<sup>125</sup>I]CYP binding experiments, membrane aliquots (40–60  $\mu\text{g}$  of protein) were incubated for 30 min at 37 °C with [<sup>125</sup>I]CYP (Amersham Biosciences) with or without competing ligand in a final volume of 100  $\mu\text{l}$  consisting of 10 mM MgCl<sub>2</sub>, 100  $\mu\text{M}$  GTP, 50 mM Tris-HCl, pH 7.5. After dilution in 2 ml of ice-cold 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.5, separation of bound from free radioligand was achieved by vacuum filtration over Whatman GF/C glass fiber disks presoaked in 0.3% polyethyleneimine, followed by three washes with the same buffer. Saturation experiments were performed with [<sup>125</sup>I]CYP concentrations ranging from 5 to 4000 pM. Competition experiments were carried out at 30 pM [<sup>125</sup>I]CYP. Nonspecific binding was determined in the presence of 100  $\mu\text{M}$  ( $\pm$ )-propranolol and represented  $9.4 \pm 0.5\%$  of total binding at 30 pM [<sup>125</sup>I]CYP. CGP20712A was a gift from Novartis (Basel, Switzerland), and ICI118551 was provided by ICI Pharma (France Division, Cergy-Pontoise). All other  $\beta$ -AR ligands were from Sigma. Data from saturation and competition experiments were analyzed with the EBDA and LIGAND programs (Biosoft Elsevier, Cambridge, United Kingdom).

**Statistical Analysis**—Results are presented as mean  $\pm$  S.E. of at least three independent experiments. Statistical significance was assessed by ANOVA followed by Newman-Keuls comparison tests (Statistica, StatSoft Inc.). A  $p < 0.05$  was considered as the threshold of statistical significance.

## RESULTS

The presence of a neuropeptide AF and FF receptor expression in human adipose tissue suggested that NPAF or NPFF, besides their well documented pain modulating effects, may impact on adipocyte metabolism. Using a well characterized murine preadipose cell line, we first ensured that mature 3T3-L1 adipocytes expressed NPFF-R1 and/or NPFF-R2 and that NPAF binds to adipocyte membranes. Then, to gather initial information as regards to the actions of these peptides on adipocytes, we have treated differentiated 3T3-L1 murine adipocytes with the two neuropeptides and measured by real-time quantitative RT-PCR the expression of various genes which products are known to be involved in key pathways of adipose metabolism, including insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation, and secretion of factors (Table I).

**NPAF Binds to Adipocyte Membranes**—We have analyzed the NPAF binding capability on adipocyte membranes using plasmon resonance technology. For such a purpose, adipocyte membranes were immobilized as a monolayer on an hydrophobic sensor chip (see “Materials and Methods”), thus giving a substantial increase in resonance units (1677 RU) reflecting effective immobilization (Fig. 1A). Whereas phosphate-buffered saline control led only to a 19.9 RU response, NPAF increased resonance response to 43.8 and 138 RU at concentrations of 10 nM and 1  $\mu\text{M}$ , respectively (Fig. 1B). On the contrary, an irrelevant control peptide of the same size as NPAF gave very low signals (1.2 and  $-0.7$  RU at 1 and 10  $\mu\text{M}$ , respectively), whereas in the same experiment NPAF at 1  $\mu\text{M}$  produces a 150 RU response (Fig. 1C). Binding curves were fitted using a 1:1 Langmuir binding interaction model giving a  $K_D$  value of immobilized adipocytes membranes for NPAF of 7.7 nM. These data show that NPAF is able to bind specifically to adipocyte membranes, supposedly to NPFF-R1 and/or NPFF-R2, with a nanomolar range affinity.

**NPAF and NPFF Modulation of Gene Expression in 3T3-L1 Adipocytes**—For most of tested genes, PCR efficiencies were close to 1 ( $1.1 \pm 0.07$ , mean  $\pm$  S.E.,  $n = 21$ ) indicating a doubling of DNA at each PCR cycle, as theoretically expected. It was thus possible to sort the genes by their relative expression levels compared with a chosen reference, e.g. ribosomal 18 S RNA, on the basis of their respective cycle threshold. Data obtained in nonstimulated control adipocytes are presented (Fig. 2). The expression levels of the genes could be scaled between  $10^{-1}$  and  $10^{-8}$  times that of reverse transcribed r18 S RNA, then divided into 3 arbitrary expression levels: high (above  $10^{-3.3}$ ), medium (between  $10^{-3.3}$  and  $10^{-5.7}$ ), and low (below  $10^{-5.7}$ ). These corresponded to PCR cycle thresholds ranging from 14 to 34, from GAPDH to ACE, with a cycle threshold of 10 for 18 S. The highest levels were obtained for SCD-1, LPL, aP2, G3PDH, or FAS; medium levels for, e.g. perilipin, caveolin-1, SR-BI, GLUT1, CD36, HSL,  $\beta$ 3-AR, PPAR $\gamma$ , UCP2, ABC1, or leptin, and lowest levels for insulin receptor, GLUT4, hexokinase-II, SREBP-1c, or PI3K. Both mNPFF-R1 and mNPFF-R2 were expressed in 3T3-L1 adipocytes at low levels (Fig. 2). Expression levels of mNPFF-R1 and mNPFF-R2 were of similar magnitude in preadipocytes and adipocytes (not shown).

When 3T3-L1 cells were exposed to 1  $\mu\text{M}$  NPFF for 24 h, the expression of only four genes was significantly affected: C/EBP $\alpha$ , PI3K, GLUT1, and ACE expression were increased by a factor of  $\sim 2$ –3 (Fig. 3A). By contrast, NPAF significantly modulated the expression of 12 genes among those tested (Fig. 3B). These are mainly genes coding for transcription factors (1.5–5-fold induction in C/EBP $\alpha$ , C/EBP $\beta$ , Id3, SREBP-1c, and SREBP-2 mRNA levels), or for proteins involved in glucose transport (GLUT1 and GLUT4) and insulin signaling (insulin receptor and PI3K) or for a secreted factor (cardiotrophin-1). Interestingly, NPAF also increased the mRNA steady state levels of all the three  $\beta$ -ARs subtypes.

To verify that the inducing effect of NPFF or NPAF on the expression of several adipocyte genes did not result from a general promoting action of the peptides on the level of adipocyte differentiation, we also tested two classical biochemical markers of terminal adipose maturation, i.e. cell triglyceride content and G3PDH specific activity (25). As shown in Table II, neither NPAF nor NPFF exerted any significant effect on 3T3-L1 adipocyte triglyceride content or G3PDH activity. This observation, in addition with the absence of NPAF or NPFF effect on the levels of a large panel of adipocyte mRNAs (Fig. 3), support the view that the induction by the peptides of a finite number of transcripts rather corresponds to a specific modulating effect on these targets than to a more general action on adipocyte maturation level.

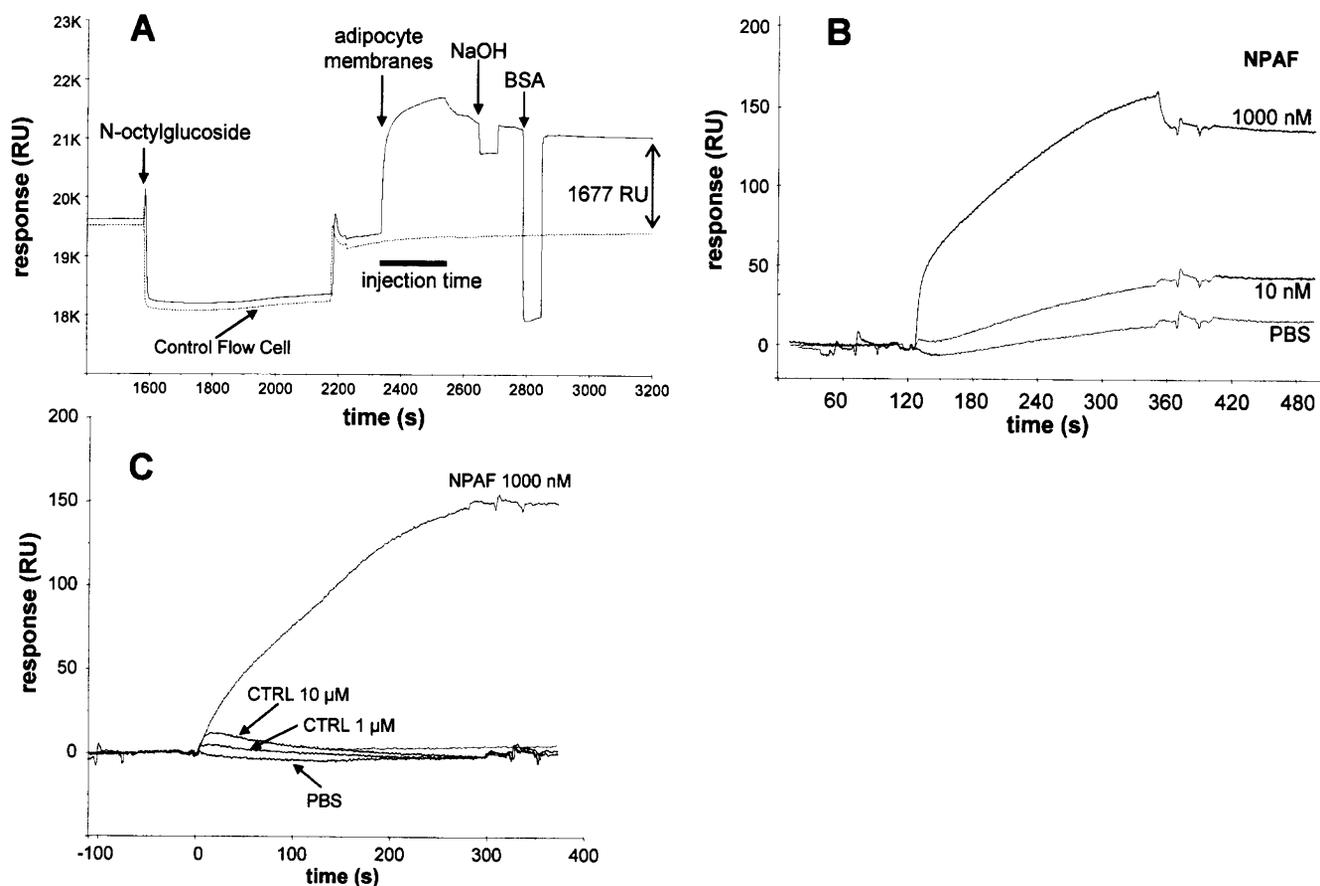
Thus, whereas NPFF only modulated the expression of a limited number of genes, NPAF was able to increase the abundance of a larger variety of transcripts. To further document the effect of NPAF on adipocytes, we decided to focus our study onto a more limited number of proteins that ensure key roles in adipocytes. We chose to more extensively characterize the NPAF effect on the  $\beta$ -adrenoreceptor subtypes expression and function, which are chiefly governed by mRNA abundance. Indeed, several studies in 3T3 preadipose cell lines have shown that variations in  $\beta$ 1,  $\beta$ 2-, or  $\beta$ 3-adrenoreceptor gene expression in response to various hormones, cytokines, and nutrients are accompanied by the corresponding changes in protein binding capacity and function (20, 26–31). Moreover, the major physiological importance of the  $\beta$ -adrenergic system in adipocyte is well established: in this cell type,  $\beta$ -adrenoreceptors not only mediate lipolysis, but are also involved in the negative and

TABLE I  
 Primer sequences for genes involved in key pathways of adipose metabolism

The abbreviation of the genes, full name, accession number or locus, and corresponding primer numbers, and 5' to 3' nucleotide sequences of the sense and antisense primers are presented.

Genes	Full name	Accession number/locus	Primer number (S/AS)	Sense primer	Antisense primer
ACE	Angiotensin-I converting enzyme	MUSACEA	875/876	CTCCCTGGGGCTGTTACCT	ACTGCATGAAATCTGGATGTGG
Akt/PKB	Protein kinase B	NM_009652	1077/1078	AGGTGCTGGAGGACAACGAC	CCCACCGAGCCTCTGTGTAG
Apelin	Pre-pro apelin	AB023494	899/900	CTGTGCTCTGGCTCTCCTT	TGTTCTGCGAAATTTCCCTCTG
Angiotensinogen	Angiotensinogen	AH005858	798/799	GCCAGACACCCCTGCTA	GTTCCTGGGGCTCACTCC
aP2	Fatty-acid binding protein	MUSLBP	733/734	AACACCGAATTTCTCTT	ACACATTCACACACCAG
$\beta$ 1-AR	$\beta$ 1-Adrenoceptor	MUSADRR	893/894	GCTGCAGACTCTACCA	GCGAGGTAGCGGGTCCAG
$\beta$ 2-AR	$\beta$ 2-Adrenoceptor	MMB2ARG	895/896	CACAGCCATTGCCAAGTTCG	GCGGCCTATTCTTGGTCAGC
$\beta$ 3-AR	$\beta$ 3-Adrenoceptor	MMB3A	1159/1160	CCACTCCGGAAACACCG	GGCAGTAGATGACCGGGTTG
$\beta$ -Actin	$\beta$ -Actin	MMACTBR2	45/46	GAGACTTCAACACCCC	GTGGTGTGAAGCTGTAGCC
CAAT/enhancer binding protein alpha	CAAT/enhancer binding protein alpha	M62362	718/719	CCGGGAACTCTAACTC	GATGTAGGGCTGATGT
C/EBP $\beta$	CAAT/enhancer binding protein beta	X62600	737/738	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
CT-1	Cardiotrophin-1	MMU18366	927/928	CCAGCATGAGCCAGAGGA	GCCGGGTGGTGAGAAAG
Caveolin-1	Caveolin-1	MMU07645	1022/1023	AACATCTACAAGCCCAACAAGG	GGTTCTGCAATCACAATCTCAAAGTC
Caveolin-2	Caveolin-2	AF141322	1026/1027	TGACGCCTACAGCCACCACA	CAAAACAGGATACCCGCAATGAAG
CD36/FAT	CD36 antigen/fatty acid transporter	MUSCDANTI	909/910	GATGTGGAACCCTACTGGATTAC	GGTCCAGTCTCATTTAGCCACAGTA
FAS	Fatty acid synthase	MMFASC	825/826	TGCTCCAGCTGCAAGC	GCCCGTAGCTCTGGGTGA
G3PDH	Glycerol-3-phosphate dehydrogenase-1	MUSGPDY	747/748	TGATGGGGGCCAACAT	TGCCCGTCCGCCAGTC
GAPDH	Glyceraldehyde-phosphate dehydrogenase	MUSGAPDH	546/547	GGCCATCACAGTCTTCTGG	ACCACAGTCCATGCCATCACTGCCA
GLUT1	Glucose transporter type 1	MUSGLUTRN	1068/1069	CCATCCACACACTCACCAC	GCCCAGGATCAGCATCTCAA
GLUT4	Glucose transporter type 4	MUSGT2A	1058/1059	CTTCTTTGAGATTGGCCCTGG	AGGTGAAGATGAAGAAGCCAAAGC
Hexokinase II	Hexokinase type II	NM_013820	1101/1102	ACGGAGCTCAACCAAAACA	CCATCCGGATTGACCTCA
HMG-CoA Rase	HMG-coenzymeA reductase	MUSHMGOA	1040/1041	GATCTGGCAGTCAGTGGAA	GTTTAGCCGCTATGCTCC
HSL	Hormone-sensitive lipase	MMU08188	808/809	CCTCATGGCTCAACTCC	GGTTCCTGACTATGGGTGA
Id1	Inhibitor of differentiation-1	MMU43884	991/992	GCTGCTACTCACGCTCAAG	GCCGTTACAGGGTGTG
Id2	Inhibitor of differentiation-2	NM_010496	995/996	GAAAAACAGCTGTGGACCA	CCAGGCGATCTGCAAGT
Id3	Inhibitor of differentiation-3	NM_008321	999/1000	CTGTACAGCGGGTGTG	CACCTGGCTAAGCTGAGTGC
Ins-Recep	Insulin receptor	MUSINSR	885/886	CCGAAGATTTCCGAGACCTCAG	GGATACGGACCAAGTCGATAGTG
LDL-R	Low density lipoprotein receptor	NM_01070	1044/1045	GAGGAACGGCCGCTGAA	GTGCTGGATGGGGAGGTCT
Leptin	Leptin	MMU18812	753/754	GACACAAACCCCTCAT	CAGTGTCTGGTCCATCT
LPL	Lipoprotein lipase	MUSLPL01	831/832	AGGACCCCTGGAAGAC	GGCACCCAACTCTCAT
NPFF-R1	NPFF-FF receptor 1	this paper	1291/1292	CCCCGAGTCTGAACGAGA	TAGTAGGAAGAGAAGGTGAGGCTGG
NPFF-R2	NPFF-FF receptor 2	this paper	972/974	GGACTCAAACTCTCAGAAAGCTGGAA	CCATGCAACAGACAAGATCAGGA
PAI-1	Plasminogen activator inhibitor-1	MUSPAI1	1062/1063	TCAGCCCTTGCTTGCCTCAT	GCATAGCCAGCACCCAGGA
Perilipin	Perilipin-A	NA <sup>a</sup>	731/732	TGCTGGATGGAGACCTC	ACCGCTCCATGCTCCA
PLTP	Phospholipid transfer protein	U28960	1139/1140	GGAAGCCGCTCTCAGTGTCTA	GCCACGAAGTGTATCCCTCA
PI3K	Phosphatidylinositol-3-phosphate regulatory subunit	U50413	1052/1053	GCCCTCTGTAGTGGCC	GCGAGATAGCGTTTGAAGAACA
PPAR $\gamma$ 1+2	Peroxisome proliferator-activated receptor $\gamma$	U01664	855/856	AGGCCGAGAAGAGAAGCTGTTG	TGGCCACCTTTTGGCTCTGCTC
RPL19	Ribosomal protein large subunit 19 kDa	NM_000981	1089/1090	GACGGAAGGGCAGCATATG	TGTGGATGTGCTCATGAGG
SCD-1	Stearoyl CoA desaturase	AAA40103	763/764	TGGTGTGGCTGCTTGTG	GCGTGGCCAGGATGAAG
SR-BI	Scavenger receptor class B type I	U37799	1081/1082	TTGGCTGTTTGTGGGATG	GGATTCGGGTGTCAATGAAGG
SREBP-1a	Sterol regulatory element binding protein-1a	AB017337	1007/1008	GCGCCATGGACGAGCTG	TTGGCACCTGGGCTCT
SREBP-1c	Sterol regulatory element binding protein-1c	From Shimomura 97	1121/1122	GGAGCCATGGATTGCACAT	GCTTCCAGAGAGAGGCCAG
SREBP-2	Sterol regulatory element binding protein-2	NA	1036/1037	CCCTTGACTCTCTGTGCA	GCTTGAGTGGGGCGAATC
UCP2	Uncoupling protein type-2	AF111999	790/791	CCGGGGCTCTGGAAAG	CCCAAGCGGAGAAAGGA
UCP3	Uncoupling protein type-3	AF053352	796/797	CMAATGGCTCTACAG	TGTAGGCATCATAGTCCC
VLDL-R	Very low density lipoprotein receptor	L33417	1007/1008	GCGCCATGGACGAGCTG	TTGGCACCTGGGCTGCT
18 S	18 S Ribosomal RNA	MMRNA18	813/814	GGGAGCCCTGAGAAACGGC	GGGTCCGGAGTGGGTAAATTT

<sup>a</sup> NA, not applicable.



**FIG. 1. NPAF binds to adipocyte membranes in a biosensor assay.** Adipocyte membranes were immobilized on HPA hydrophobic surfaces to allow subsequent measurement of NPAF binding by surface plasmon resonance (Biacore). The process of spontaneous fusion of adipocyte membranes to the HPA surface was monitored as the sensorgram reading begins to level out to the reaching a plateau (*panel A*). NPAF was used at the indicated concentrations to monitor interaction with adipocyte membranes (*panel B*). The figure displays a single representative experiment. Nonspecific binding to membranes was determined using the 18-amino acid peptide COOH-GSKGSKGSKGSKGSKGSK-NH<sub>2</sub> (*panel C*). Experiments were reproduced at least 3 times. For more details, see “Materials and Methods” and “Results.”

positive control of lipogenesis and thermogenesis, respectively (review in Ref. 32).

**Regulation by NPAF of  $\beta$ -ARs Density and Functional Expression**—Saturation and competition binding experiments were carried out on membrane fractions to determine the levels of  $\beta$ -AR subtype density in 3T3-L1 adipocytes cultured in the absence or presence of 1  $\mu$ M NPAF for 48 h. In agreement with the results of real-time RT-PCR analysis, NPAF up-regulated the  $\beta$ 3-AR (Table III). The density of  $\beta$ 3-ARs, corresponding to the  $B_{\max}$  of the low affinity component for [<sup>125</sup>I]CYP (33), was increased by about 60% after NPAF exposure ( $p < 0.02$ ). The amount of high affinity sites that represented the sum of  $\beta$ 1- and  $\beta$ 2-ARs was slightly and not significantly induced in the presence of NPAF. No difference in the  $K_D$  values of the two binding classes for the radioligand could be detected between control and NPAF-treated cells.

[<sup>125</sup>I]CYP competition binding experiments against  $\beta$ -AR subtype-selective ligands were also performed to determine the relative proportions of  $\beta$ 1- and  $\beta$ 2-ARs. These competition studies were carried out at a low (30 pM) [<sup>125</sup>I]CYP concentration, at which no significant occupancy of the  $\beta$ 3-AR by [<sup>125</sup>I]CYP occurs given the poor affinity of the  $\beta$ 3-AR for this radioligand. Under these conditions, competition of [<sup>125</sup>I]CYP with  $\beta$ 1- or  $\beta$ 2-AR subtype-selective antagonists allowed estimation of the relative proportions of each of the two  $\beta$ -AR subtypes, with no significant interference of the  $\beta$ 3-AR population. Analysis of the displacement curves of [<sup>125</sup>I]CYP by the  $\beta$ 1-AR-selective antagonist CGP20712A or by the  $\beta$ 2-AR-selective antagonist

ICI118551 gave concordant results (Table IV). In the presence of NPAF there was a 4.5–7-fold induction in  $\beta$ 2-AR density. By contrast NPAF only caused a very weak but significant 8–13% increase in  $\beta$ 1-AR population.

To determine the functional consequences of  $\beta$ -AR subtype regulation by NPAF, adenylyl cyclase activity was measured in response to various  $\beta$ -AR agonists on membranes from control or NPAF-treated adipocytes. We first compared the relative potencies of the catecholamines (–)-isoproterenol, (–)-norepinephrine, and (–)-epinephrine to activate adenylyl cyclase between control 3T3-L1 adipocytes and cells exposed for 48 h to 1  $\mu$ M NPAF (Table V). There was no significant difference in maximal catecholamine-induced adenylyl cyclase activity between control and NPAF-treated cells. Likewise, NPAF did not modify the potencies of (–)-isoproterenol and of (–)-norepinephrine to activate adenylyl cyclase (similar  $EC_{50}$  values in control and NPAF-exposed cells). By contrast, (–)-epinephrine, known to preferentially stimulate the  $\beta$ 2-AR subtype, has a higher potency (*i.e.* a decreased  $EC_{50}$  value) in NPAF-treated cells as compared with control cells. Further investigations were also performed with  $\beta$ 1-,  $\beta$ 2-, and  $\beta$ 3-AR-selective agonists (Table V). The potency of the  $\beta$ 1-AR-selective agonist ( $\pm$ )-dobutamine remained unaffected after exposure to NPAF. On the contrary, the  $\beta$ 2-AR-selective agonist ( $\pm$ )-fenoterol and the  $\beta$ 3-AR-selective agonist ( $\pm$ )-BRL37344 were significantly more potent in NPAF-exposed 3T3-L1 adipocytes than in control fat cells.

To ascertain the physiological relevance of the NPAF-in-

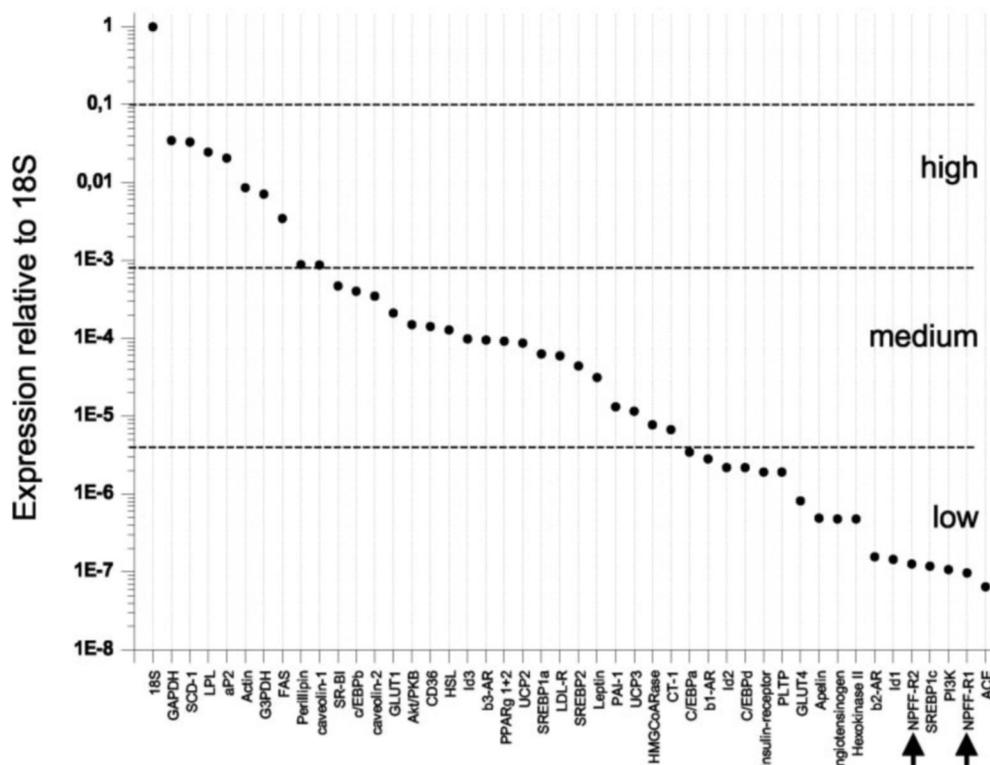


FIG. 2. Snapshot of gene expression levels in 3T3-L1 adipocytes relative to r18 S. The relative expression levels of the indicated genes have been compared with that of ribosomal 18 S RNA. Mean data obtained in unstimulated control adipocytes are presented ( $n = 3-5$ ). The expression levels of the genes have been scaled between  $10^{-1}$  and  $10^{-8}$  times that of reverse transcribed r18 S RNA, then divided into 3 arbitrary expression levels each of  $10^{-2.33}$  wide: high, medium, and low. *NPFF-R1* and *NPFF-R2* are indicated with arrows.

duced changes in the potency of ( $-$ )-epinephrine, ( $\pm$ )-fenoterol, and ( $\pm$ )-BRL37344 to stimulate adenylyl cyclase, 3T3-L1 adipocytes were exposed for 48 h to various concentrations of NPAF ranging from 1 nM to 1  $\mu$ M. Then, adenylyl cyclase activity was tested in response to increasing concentrations of each of the three  $\beta$ -AR agonists (Table VI). NPAF clearly provoked a dose-dependent increase in the potency of ( $-$ )-epinephrine, ( $\pm$ )-fenoterol, and ( $\pm$ )-BRL37344 to activate adenylyl cyclase. This effect was statistically significant from 10 nM NPAF for ( $-$ )-epinephrine and ( $\pm$ )-BRL37344, and from 1 nM NPAF for ( $\pm$ )-fenoterol. The maximal effect was obtained at 100 nM of the neuropeptide. The concentration of NPAF giving a half-maximal effect on  $\beta$ -adrenergic potency was in the 2–3 nM range for the three  $\beta$ -AR agonists. This concentration is in agreement with the estimated  $K_D$  value drawn from plasmon resonance technology binding experiments on adipocyte membranes (Fig. 1). These observations strongly support the view that the NPAF-induced modulation in catecholamine responsiveness is of physiological relevance.

In control and NPAF-treated adipocytes, we also measured adenylyl cyclase activity in response to maximal concentrations of G protein or adenylyl cyclase effectors. As shown in Table VII, NPAF exposure did not modulate adenylyl cyclase activity in response to the G protein effectors GTP $\gamma$ S and NaF, or to the adenylyl cyclase activator forskolin. Taken together, our [ $^{125}$ I]CYP binding studies and adenylyl cyclase experiments support the view that NPAF modulates  $\beta$ -AR subtype expression and  $\beta$ -AR subtype responsiveness in 3T3-L1 adipocytes.

#### DISCUSSION

The presence of a substantial expression of a neuropeptide AF and FF receptor mRNA in human adipose tissue (14) suggested that these two neuropeptides, principally recognized for their pain modulating effects (reviewed in Refs. 1 and 2), may also impact on adipocyte metabolism. The goal of the present

study was thus to investigate the effects of these peptides on adipocyte function using the murine preadipose 3T3-L1 cell line as a model.

We have first demonstrated that NPAF binds to 3T3-L1 adipocyte membranes with a nanomolar affinity (7 nM) (Fig. 1), similar to that reported for the human- or rat-cloned NPFF-R2 (14, 16, 34, 35), and that both *NPFF-R1* and *NPFF-R2* mRNAs were expressed in 3T3-L1 adipocytes (Fig. 2). Thus, 3T3-L1 adipocytes appear to be a good model to study the effects of these neuropeptides on adipocyte function. For such a purpose, we have designed a functional genomics assay aimed at obtaining a rather wide picture of NPAF or NPFF putative actions on adipose metabolism using 3T3-L1 adipocytes. This assay relies on the quantitation after stimulation by both neuropeptides of mRNA steady state levels of already known and well characterized genes implicated in key pathways of adipose metabolism. With such an approach we have obtained relevant primary information as regards to the action of these peptides on adipocyte metabolism. Based on these results we have further investigated in more detail the inducing effect of NPAF on  $\beta$ -adrenergic receptor subtype expression and function.

Our findings underline that while NPFF has discrete action on adipocyte gene expression, NPAF exerts much wider effects. Indeed, NPFF affected the expression of four genes only: *GLUT1*, *PI3K*, *C/EBP $\alpha$* , and *ACE*, all of which were increased. By contrast, NPAF modulates that of 12 genes, corresponding to  $\sim 27\%$  of tested genes. Among these are transcription factors involved in the regulation of maintenance of adipocyte phenotype and/or differentiation that all are increased: *C/EBP $\alpha$* , *C/EBP $\beta$* , and *Id3* (36, 37). At this point it should be noted that the effects of NPAF or NPFF cannot be ascribed to an action on adipocyte differentiation as some known gene markers of adipocyte differentiation are not modified (e.g. *aP2*, *FAS*, *LPL*, and *G3PDH*), whereas others are (e.g. *C/EBP $\alpha$* , *GLUT4*, and  $\beta$ 3-

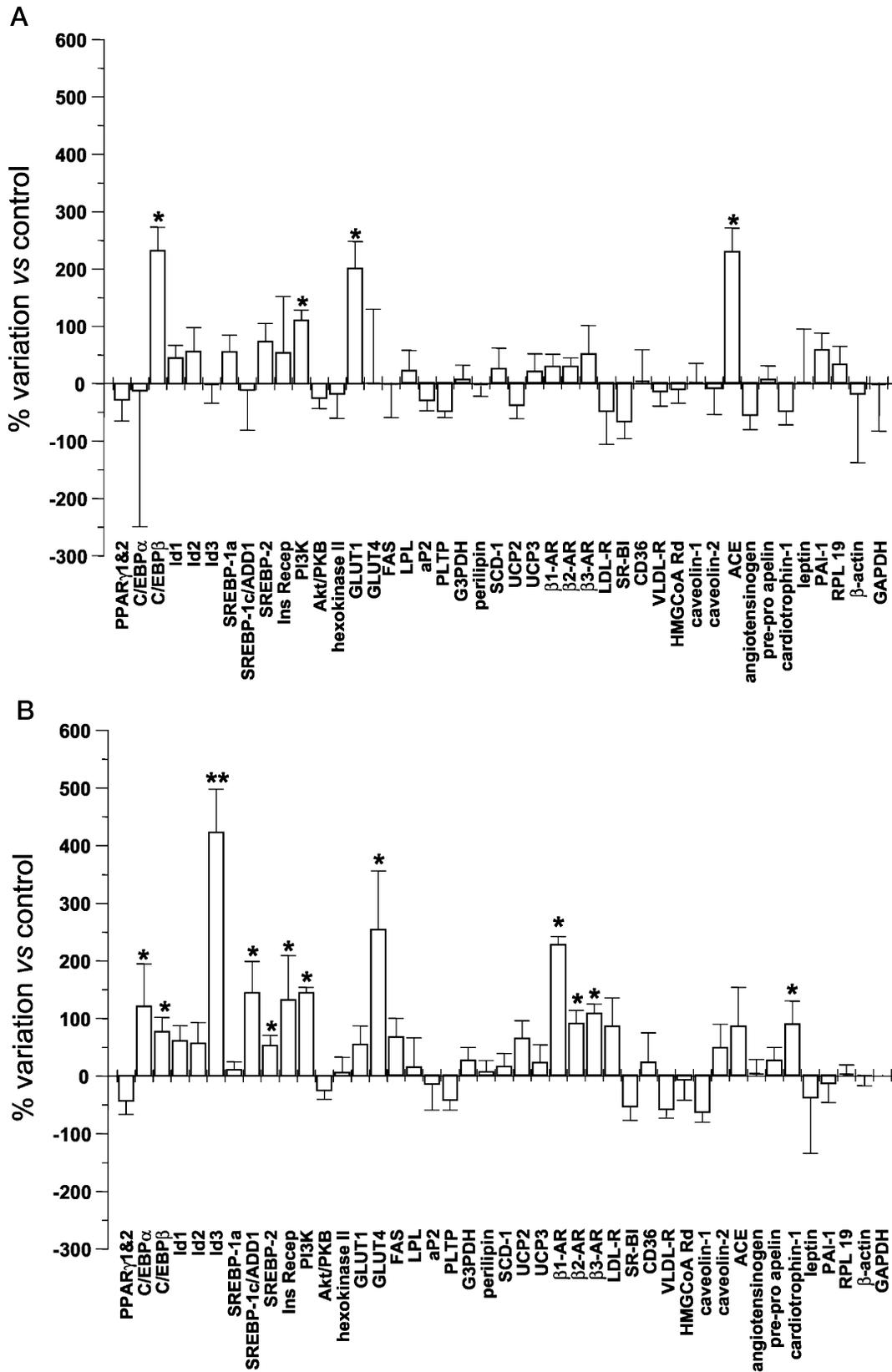


FIG. 3. Variation in gene expression in 3T3-L1 adipocytes upon treatment with NPAF and NPFF. 3T3-L1 mature adipocytes were treated with 1  $\mu$ M NPFF (panel A) or NPAF (panel B) for 24 h. Total RNA was prepared and mRNA steady state levels of well characterized genes implicated in the six following pathways of adipocyte metabolism were measured by quantitative RT-PCR: insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation, and secretion of factors. Results are presented as -fold increase over control values using the  $2^{-\Delta\Delta CT}$  formula. Data presented are mean  $\pm$  S.E. of three to five independent experiments. For additional details see "Materials and Methods." Significant variation over control assessed by one-way ANOVA are labeled as: \* for  $p < 0.05$  and \*\* for  $p < 0.01$ .

TABLE II

Absence of NPAF or NPFF effect on 3T3-L1 adipocyte triglyceride content and on glycerol-3-phosphate dehydrogenase activity

Cells extracts (homogenates and  $100,000 \times g$  supernatants) were prepared from control and NPAF-or NPFF-treated ( $1 \mu\text{M}$  for 48 h) 3T3-L1 adipocytes, and were tested for triglyceride content (in nmol/dish) and G3PDH activity nanomole (in nmole of NADH/min/mg of protein). Data were also normalized to the corresponding control values. Results are expressed as mean  $\pm$  S.E. of eight independent experiments.

Culture conditions	Triglyceride content		G3PDH activity	
	nmol/dish	% of control	nmol NADH/min/mg	% of control
Control	548.7 $\pm$ 40.6	100	505.0 $\pm$ 17.1	100
NPAF	570.3 $\pm$ 50.2	104	484.1 $\pm$ 12.8	96
NPFF	568.6 $\pm$ 50.6	103.5	481.8 $\pm$ 13.3	95.5

TABLE III

Characterization of [ $^{125}\text{I}$ ]CYP-binding sites in membranes from control and NPAF-exposed 3T3-L1 adipocytes

Membranes from control and NPAF-treated ( $1 \mu\text{M}$  for 48 h) 3T3-L1 adipocytes were tested in [ $^{125}\text{I}$ ]CYP saturation binding experiments using a wide range of concentrations (5–4000 pM) of the radioligand. Scatchard analysis of the data with the EBDA/LIGAND program was used to calculate the  $K_D$  and the  $B_{\text{max}}$  values of the high- ( $\beta_1$ - and  $\beta_2$ -ARs) and the low ( $\beta_3$ -AR) affinity sites for [ $^{125}\text{I}$ ]CYP. Results are expressed as mean  $\pm$  S.E. of six separate experiments. The percentage of each affinity binding class is indicated in parentheses after the  $B_{\text{max}}$  values.

Culture conditions	[ $^{125}\text{I}$ ]CYP-binding sites			
	High affinity ( $\beta_1$ - and $\beta_2$ -ARs)		Low affinity ( $\beta_3$ -AR)	
	$K_D$	$B_{\text{max}}$	$K_D$	$B_{\text{max}}$
	pM	fMol/mg	pM	fMol/mg
Control	35.4 $\pm$ 5.6	11.4 $\pm$ 2.1 (26.5%)	770 $\pm$ 136	31.6 $\pm$ 2.5 (73.5%)
NPAF	39.6 $\pm$ 4.9	14.2 $\pm$ 1.9 (22.2%)	876 $\pm$ 160	49.7 $\pm$ 5.6 (77.8%) <sup>a</sup>

<sup>a</sup>  $P < 0.02$ , NPAF-treated versus control adipocytes.

TABLE IV

Competition of [ $^{125}\text{I}$ ]CYP against  $\beta_1$ - and  $\beta_2$ -AR selective antagonists in membrane from control and NPAF-exposed cells

Membranes were prepared from control and NPAF-treated ( $1 \mu\text{M}$  for 48 h) 3T3-L1 adipocytes. Competition binding experiments were performed at 30 pM [ $^{125}\text{I}$ ]CYP in the absence or presence of various concentrations of CGP20712A and ICI118551,  $\beta_1$ - and  $\beta_2$ -AR-selective antagonists, respectively. Data from displacement of [ $^{125}\text{I}$ ]CYP binding by these subtype-selective ligands were used to calculate the  $K_i$  values for each affinity component. The corresponding  $B_{\text{max}}$  values were derived from total  $\beta$ -AR density ( $\beta_1$ - plus  $\beta_2$ -AR) drawn from [ $^{125}\text{I}$ ]CYP saturation experiments (see Table III), and taking into account the percentage of each affinity component (indicated in parentheses after the  $B_{\text{max}}$  values) obtained from competition experiments. Results are expressed as mean  $\pm$  S.E. of six separate experiments.

Ligand	Selectivity	Binding affinity (related $\beta$ -AR subtype)	Culture conditions			
			Control		NPAF	
			$K_i$	$B_{\text{max}}$	$K_i$	$B_{\text{max}}$
			nM	fMol/mg	nM	fMol/mg
CGP20712A	$\beta_1$	High ( $\beta_1$ -AR)	9.2 $\pm$ 1.2	11.0 $\pm$ 0.2 (96%)	10.1 $\pm$ 1.0	12.4 $\pm$ 0.6 (87%) <sup>a</sup>
	$\beta_2$	Low ( $\beta_2$ -AR)	919 $\pm$ 320	0.4 $\pm$ 0.2 (4%)	704 $\pm$ 178	1.8 $\pm$ 0.6 (13%) <sup>a</sup>
ICI118551	$\beta_2$	High ( $\beta_2$ -AR)	1.6 $\pm$ 0.7	0.3 $\pm$ 0.2 (3%)	1.3 $\pm$ 0.5	2.2 $\pm$ 0.2 (15%) <sup>b</sup>
	$\beta_1$	Low ( $\beta_1$ -AR)	118 $\pm$ 15	11.1 $\pm$ 0.2 (97%)	172 $\pm$ 25	12.0 $\pm$ 0.2 (85%) <sup>c</sup>

<sup>a</sup>  $p < 0.05$ ,  $p < 0.01$ .

<sup>b</sup>  $p < 0.001$ , NPAF-treated versus control adipocytes.

<sup>c</sup>  $p < 0.01$ , NPAF-treated versus control adipocytes.

TABLE V

Relative potencies of various  $\beta$ -AR agonists for stimulating adenylyl cyclase activity in control and NPAF-treated 3T3-L1 adipocytes

Adenylyl cyclase activity was measured in membranes from control and NPAF-exposed ( $1 \mu\text{M}$  for 48 h) 3T3-L1 adipocytes and in response to increasing concentrations of the indicated  $\beta$ -AR agonists.  $\text{EC}_{50}$  values (in  $\mu\text{M}$ ) are the concentrations of each ligand required for a half-maximal stimulation of adenylyl cyclase.  $V_{\text{max}}$  values (in pmol of cAMP/min/mg of protein) are expressed as agonist-stimulated over basal adenylyl cyclase activity. Results represent the mean  $\pm$  S.E. of 5–12 independent experiments. Basal adenylyl cyclase activity was  $6.7 \pm 1.0$  and  $6.8 \pm 1.1$  pmol of cAMP/min/mg of protein in control and NPAF-exposed cells, respectively.

$\beta$ -AR agonist	Culture conditions			
	Control		NPAF	
	$\text{EC}_{50}$	$V_{\text{max}}$	$\text{EC}_{50}$	$V_{\text{max}}$
	$\mu\text{M}$	pmol cAMP/min/mg	$\mu\text{M}$	pmol cAMP/min/mg
(-)-Isoproterenol	0.71 $\pm$ 0.08	35.9 $\pm$ 3.9	0.60 $\pm$ 0.05	39.5 $\pm$ 3.7
(-)-Norepinephrine	4.26 $\pm$ 0.32	38.1 $\pm$ 4.7	4.64 $\pm$ 0.48	40.8 $\pm$ 6.2
(-)-Epinephrine	4.07 $\pm$ 0.62	31.3 $\pm$ 5.4	2.17 $\pm$ 0.63 <sup>a</sup>	31.6 $\pm$ 5.0
( $\pm$ )-Dobutamine	2.18 $\pm$ 0.27	8.3 $\pm$ 0.9	3.13 $\pm$ 0.93	9.6 $\pm$ 1.5
( $\pm$ )-Fenoterol	6.94 $\pm$ 0.31	13.8 $\pm$ 1.5	4.02 $\pm$ 0.89 <sup>b</sup>	14.8 $\pm$ 1.7
( $\pm$ )-BRL37344	0.98 $\pm$ 0.17	20.0 $\pm$ 1.5	0.57 $\pm$ 0.06 <sup>a</sup>	22.4 $\pm$ 1.0

<sup>a</sup>  $p < 0.05$ .

<sup>b</sup>  $p < 0.01$ , NPAF-treated versus control adipocytes.

AR). Furthermore, two classical biochemical markers of adipocyte maturation level, cell triglyceride content and G3PDH specific activity, remain unaltered in the presence of NPAF or NPFF (Table II). Other genes altered by NPAF treatment en-

code products involved in insulin-mediated regulation of gene expression, *SREBP-1c* (reviewed by Ref. 38), or cholesterol metabolism, *SREBP-2* (39). Although *SREBP-1c* and *SREBP-2* are increased by NPAF treatment, it is interesting to note that

TABLE VI  
Dose-dependent effect of NPAF on the potency of several  $\beta$ -AR agonists to stimulate adenylyl cyclase

Cell extracts were prepared from control 3T3-L1 adipocytes and from cells exposed for 48 h to various concentrations (1–1000 nM) of NPAF. Adenylyl cyclase activity was then measured in response to increasing concentrations of (–)-epinephrine, (±)-fenoterol, and (±)-BRL37344. EC<sub>50</sub> values (in  $\mu$ M) is the concentration of each  $\beta$ -AR agonist required for a half-maximal stimulation of adenylyl cyclase. V<sub>max</sub> values (in pmol of cAMP/min/mg of protein) are expressed as agonist-stimulated over basal adenylyl cyclase activity. Results represent the mean  $\pm$  S.E. of six independent experiments. Basal adenylyl cyclase activity was 8.6  $\pm$  0.4, 8.7  $\pm$  0.7, 8.0  $\pm$  0.5, 8.6  $\pm$  0.6, and 8.6  $\pm$  0.5 pmol of cAMP/min/mg in control cells and in cells treated with 1, 10, 100, and 1000 nM NPAF, respectively.

NPAF concentration (nM)	$\beta$ -AR agonist					
	(–)-Epinephrine		(±)-Fenoterol		(±)-BRL37344	
	EC <sub>50</sub>	V <sub>max</sub>	EC <sub>50</sub>	V <sub>max</sub>	EC <sub>50</sub>	V <sub>max</sub>
nM	$\mu$ M	pmol cAMP/min/mg	$\mu$ M	pmol cAMP/min/mg	$\mu$ M	pmol cAMP/min/mg
0 (control)	4.56 $\pm$ 0.54	39.4 $\pm$ 3.5	9.93 $\pm$ 0.70	26.0 $\pm$ 2.0	0.58 $\pm$ 0.05	25.7 $\pm$ 2.9
1	3.37 $\pm$ 0.44	41.5 $\pm$ 5.0	7.79 $\pm$ 0.35 <sup>a</sup>	25.2 $\pm$ 2.0	0.39 $\pm$ 0.06	24.9 $\pm$ 2.4
10	3.40 $\pm$ 0.31 <sup>a</sup>	41.0 $\pm$ 4.1	7.24 $\pm$ 0.23 <sup>b</sup>	25.3 $\pm$ 2.8	0.36 $\pm$ 0.03 <sup>a</sup>	22.0 $\pm$ 1.7
100	2.62 $\pm$ 0.25 <sup>a</sup>	43.3 $\pm$ 5.8	5.84 $\pm$ 0.18 <sup>b</sup>	27.5 $\pm$ 3.7	0.29 $\pm$ 0.02 <sup>b</sup>	26.0 $\pm$ 3.9
1000	2.88 $\pm$ 0.27 <sup>a</sup>	46.1 $\pm$ 5.1	5.84 $\pm$ 0.54 <sup>b</sup>	27.4 $\pm$ 3.1	0.29 $\pm$ 0.02 <sup>b</sup>	28.6 $\pm$ 3.5

<sup>a</sup>  $p < 0.05$ .

<sup>b</sup>  $p < 0.01$ , NPAF-treated versus control adipocytes.

TABLE VII  
Absence of NPAF effect on adenylyl cyclase stimulated by G-protein or adenylyl cyclase effectors

Membranes were prepared from control adipocytes or from adipocytes exposed for 48 h to 1  $\mu$ M NPAF. Adenylyl cyclase in response to an optimal concentration of each indicated effector was determined. Results are expressed as effector-stimulated over basal adenylyl cyclase activity, and represent the mean  $\pm$  S.E. of six independent experiments. Basal adenylyl cyclase activity was similar in control and NPAF-treated cells (see Table V).

Effector	Adenylyl cyclase activity (over basal)	
	Control	NPAF
	pmol cAMP/min/mg protein	
GTP $\gamma$ S (100 $\mu$ M)	52.6 $\pm$ 2.9	50.8 $\pm$ 1.7
NaF (10 mM)	105.4 $\pm$ 21.8	94.2 $\pm$ 23.3
Forskolin (100 $\mu$ M)	378.9 $\pm$ 51.2	344.0 $\pm$ 54.8

their known gene targets are not modulated, such as *FAS* (40), *SCD-1* (41), *LDL-R* (42), *LPL* (43), or *HMG-CoA reductase* (44). It can be suggested that site-1 and site-2 proteases, which in conditions of sterol depletion are instrumental regulator of SREBPs release from endoplasmic reticulum (reviewed in Ref. 45), are not modulated by NPAF. By inference one can speculate that NPAF induction of *SREBPs* might synergize with states of cholesterol depletion, in the turning on activation of cholesterol and lipogenic genes. Of interest too, NPAF or NPFF treatment of adipocytes lead to an increase in mRNAs from secreted products such as ACE and cardiotrophin-1, both involved in the control of blood pressure and cardiac remodeling or hypertrophy (46, 47). In line with the well recognized status of adipose as a secretory organs (reviewed in Ref. 48 and 49), this study shows that 3T3-L1 adipocytes expressed two additional secreted products: cardiotrophin-1 and apelin, a ligand for APJ receptor (50).

Messenger RNA levels of the three  $\beta$ -AR subtypes were also increased, but at various levels, by NPAF exposure. Whereas there was about a 2-fold induction in  $\beta$ 2- and  $\beta$ 3-AR mRNA abundance,  $\beta$ 1-AR transcripts were much more weakly enhanced by the neuropeptide treatment. Two major arguments prompted us to further investigate the potential relationship between the variations in  $\beta$ -AR subtype transcripts and those of the corresponding proteins and functions. (i) First in adipocytes,  $\beta$ -ARs play a pivotal physiological role and mediate pleiotropic functions of catecholamines. For instance, adipocyte  $\beta$ -ARs not only activate lipolysis and thermogenesis, but also exert a negative control on lipogenesis and glucose transport (32). Thus, modulation of  $\beta$ -AR expression could have consequences on different metabolic pathways of fat cells. (ii) Second, several studies have previously reported a good correla-

tion between the levels and  $\beta$ -AR mRNAs,  $\beta$ -AR proteins, and  $\beta$ -AR sensitivity (20, 26–31).

In agreement with the results on  $\beta$ -AR subtype transcripts, we observed a preferential increase in  $\beta$ 2- and  $\beta$ 3-AR populations, whereas the induction in  $\beta$ 1-AR density appeared very weak (Tables III and IV). Overall, the regulation of  $\beta$ 2- and  $\beta$ 3-AR number is accompanied by an increased potency (decreased EC<sub>50</sub> values) of the  $\beta$ 2-AR-selective (fenoterol) and the  $\beta$ 3-AR-selective (BRL37344) agonists to stimulate adenylyl cyclase (Table V). Likewise, among the three tested catecholamines, (–)-isoproterenol, (–)-norepinephrine, and (–)-epinephrine, only (–)-epinephrine had an increased potency to activate adenylyl cyclase in NPAF-exposed as compared with control cells, in agreement with its higher ability to stimulate the  $\beta$ 2-AR subtype. Following NPAF exposure, the increased potency of (–)-epinephrine, (±)-fenoterol, and (±)-BRL37344 to stimulate adenylyl cyclase was detectable at low concentrations of the neuropeptide (Table VI). The half-maximal effect of NPAF was observed at nanomolar concentrations that are in close agreement with the affinity of the receptor for its endogenous ligand, thus suggesting that the modulation of adipocyte catecholamine responsiveness may have physiological implications. Furthermore, adenylyl cyclase activity in response to optimal concentrations of G-protein or adenylyl cyclase effectors was not affected by NPAF exposure (Table VII). Whereas we cannot exclude an effect of the neuropeptide distal to the  $\beta$ -ARs, both data converge to suggest that NPAF can modulate  $\beta$ -AR responsiveness through a differential regulation of  $\beta$ -AR subtype expression. It is also noticeable that despite the accessory expression of  $\beta$ 2-ARs in mature 3T3-L1 adipocytes, even after NPAF exposure, the up-regulation of this  $\beta$ -AR subtype caused by the neuropeptide is sufficient to induce an increased  $\beta$ 2-adrenergic responsiveness. This observation is in line with studies showing that as compared with the  $\beta$ 1-AR, the  $\beta$ 2-AR has a higher intrinsic efficacy to stimulate adenylyl cyclase (51) and a greater degree of both physical and functional agonist-promoted coupling with Gs (52).

The main roles ensured by NPAF and NPFF concern their antioxioid properties in the central nervous system. Peripheral effects of these peptides are poorly documented, particularly their potential interaction with catecholamine responsiveness. However, it has been previously reported that NPFF/AF receptors are present in the rat heart, and that peripheral administration of NPFF modulates blood pressure and heart rate (5). Overall, the authors have suggested that these peripheral cardiovascular responses could be mediated by functional interactions between adrenergic and NPFF/AF systems. In adipose tissue, it is thus conceivable that NPAF, through its own re-

ceptors, could mediate a modulation in  $\beta 2$ - and  $\beta 3$ -AR expression. Especially, the induction in  $\beta 2$ -AR density caused by NPAF will increase the potency of epinephrine as compared with norepinephrine for stimulating the adenylyl cyclase system, and could have consequences on several cAMP-dependent processes in adipocytes, including lipolysis, thermogenesis, and anti-lipogenesis. As regards to the dual innervation and vascularization of white and brown adipose tissues, this switch in  $\beta$ -AR expression may privilege the vascular (*i.e.* by epinephrine) over the sympathetic control (*i.e.* by norepinephrine) of energy expenditure in situations that could increase NPAF levels. It has been reported that neuropeptide FF, and possibly NPAF, which is encoded by the same gene precursor (12, 13), is secreted in the plasma in a pulsatile manner suggesting that the peptides could act as hormones (15). Under conditions of chronic increase in plasma NPAF, it can be anticipated that modifications of adipocyte catecholamine responsiveness could occur.

Gene profiling of a limited number of informative genes has revealed a fruitful approach to achieve initial gain of knowledge concerning NPAF and NPAFF action on adipocytes. The dynamics of quantitative RT-PCR, which is of about one million between the highest and the lowest tested genes, is far above other current gene expression measurement techniques. The separation of gene expression into three arbitrary high, medium, and low expression levels also stresses that genes encoding enzyme involved in the key functions of adipocytes, *i.e.* storage and synthesis of lipids and sterols, are predominantly expressed.

In conclusion, based on the fact that a neuropeptide FF and AF receptors are substantially expressed in adipose tissue, we have explored NPAF and NPAFF effects on adipocytes primary starting with a functional genomics approach. This relied on the analysis of a wide panel of well characterized genes involved in key pathways of adipocyte metabolism. Gene expression and functional data converged and showed that these peptides affect several adipocyte metabolic pathways, including catecholamine responsiveness. Thus, besides their well described pain modulation effects, NPAF and to a lesser extent NPAFF may have global impact on body energy storage and utilization.

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