

# Neuropeptide Y receptor-specifically modulates human neutrophil function

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## Abstract

Despite a continuously growing body of evidence highlighting the role of NPY in the immune system, surprisingly little is known about its ability to alter human leukocyte function. We therefore set out to examine NPY receptor expression and functional effects of NPY in freshly isolated human neutrophils. Our results not only demonstrate for the first time the presence of specific NPY receptors on human neutrophils, but also unveil of how these receptors differentially modulate critical functions of neutrophils such as phagocytosis of bacteria as well as the release of reactive oxygen species.

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## 1. Introduction

Research in the last two decades has provided compelling evidence that the immune system not only functions as a self-regulatory and autonomous entity, but is subject to significant modulation by other supersystems, such as the nervous or the endocrine system (Bedoui et al., 2003a; Elenkov et al., 2000; Kohm and Sanders, 2001). Importantly, such interactions have been implicated in a growing number of diseases, e.g. autoimmunity (Bedoui et al., 2004), chronic inflammation (Marshall, 2004) and sepsis (Aldridge, 2002; Elenkov et al., 2000).

A major pathway for the bidirectional interaction between the nervous and the immune system is provided by the sympathetic

nervous system (Elenkov et al., 2000; Kohm and Sanders, 2001). Though earlier studies focused mostly on the catecholamines, more recent work has established that other sympathetic transmitters, such as neuropeptide Y (NPY), also play a significant role within the neuroimmune crosstalk (Bedoui et al., 2003a). NPY, a 36 amino acid peptide, is released from sympathetic nerves innervating primary and secondary lymphoid organs (Lundberg et al., 1985) and modulates a variety of immunological functions, including chemotaxis (Straub et al., 2000), T lymphocyte differentiation (Kawamura et al., 1998; Levite and Chowers, 2001) and leukocyte migration (Bedoui et al., 2001). Notably, Hauser et al. (1993) demonstrated that NPY treatment prolongs the survival of endotoxemic rats, implying a protective role for NPY in sepsis. Though these initial studies attributed the protective-like action of NPY entirely to its complex cardiovascular function (Qureshi et al., 1998), we recently demonstrated that the reduced lethality is also associated with significantly decreased tissue infiltration of neutrophils and T lymphocytes due

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to increased adhesiveness to the marginal pool (Nave et al., 2004), suggesting that the immunomodulatory action of NPY is also relevant to sepsis.

Neutrophils are an essential component of the body's first line of defense. With their ability to eliminate invading microbes e.g. by means of the secretion of highly reactive oxygen species and phagocytosis, neutrophils play a key role in the pathophysiology of sepsis (Aldridge, 2002). The production of oxygen species, often referred to as respiratory burst, is a tightly regulated mechanism, involving NADPH-oxidase and myeloperoxidase as key enzymes. Bacterial products such as fMLP and cytokines are potent activators of the respiratory burst. These factors initiate a cascade of intracellular events that particularly involve G-protein coupled receptors and protein kinase C activation (Quinn and Gauss, 2004).

NPY also appears to be a modulator of the respiratory burst, as the fMLP-evoked respiratory burst is modulated by NPY in a dose-dependent manner both *in vitro* and *in vivo* (Hafström et al., 1993; von Hörsten et al., 1998). However, much of the interaction of NPY with neutrophils remains unknown. For example, it is unclear whether NPY also interferes with other critical neutrophil functions, such as phagocytosis, and what NPY receptors mediate such effects. Based on the demonstration of protective effects of NPY in experimental sepsis that also target neutrophils (Nave et al., 2004), and promising findings regarding the interplay between human neutrophils and NPY (Hafström et al., 1993), we set out to further characterize the effect of NPY on human neutrophils function. To this end, we examined freshly isolated human neutrophils for the expression of functional NPY receptors and assessed whether NPY modulates the phagocytotic abilities of the neutrophils.

## 2. Materials and methods

### 2.1. Samples and peptides

For the *in vitro* experiments with human neutrophils, heparinized blood was collected from healthy volunteers. The institutional ethics committee approved the study and informed consent was obtained from the donors. NPY (Michel et al., 1998; Tatemoto et al., 1982; Tatemoto et al., 1985), pancreatic polypeptide (Michel et al., 1998), Leu<sup>31</sup>Pro<sup>34</sup>-NPY (Fuhlendorff et al., 1990), NPY<sub>13–36</sub> (Doods et al., 1999; Michel et al., 1998; Schwartz et al., 1987) and D-Trp<sup>32</sup> (Hwa et al., 1999) were obtained from PolyPeptide Laboratories (Wolfenbüttel, Germany). BIBO 3304 (Wieland et al., 1998) and BIIE 0246 (Doods et al., 1999) were kindly provided by Boehringer Ingelheim (Biberach, Germany).

### 2.2. Isolation of neutrophil granulocytes

Venous blood samples (10 ml), anticoagulated with Lithium-heparin, were drawn from healthy blood donors in the morning. To obtain neutrophils, blood samples were underlaid with Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 30 min to separate neutrophils from peripheral blood mononuclear cells (PBMC). The granulocyte layer usually

contained >98% granulocytes with <1% contamination with PBMC and we routinely retrieved 1–3 million granulocytes from an individual preparation that were then used for the assays described below.

### 2.3. PCR

For RT-PCR neutrophils were separated from peripheral blood (40 ml) of healthy volunteers using a cell sorter gating on granulocytes according to the FSC versus SSC properties of the cells (FACStar plus, Becton Dickinson, Heidelberg, Germany). Since NPY receptors are abundantly expressed in the central nervous system, we used commercially available human brain suspensions as positive controls (BD Biosciences, Palo Alto, CA). RNA was extracted using the RNeasy spin column purification kit (Qiagen, Hilden, Germany), and concentrations were measured using a photometer (OD 260 nm). M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) was used to perform reverse transcription. Identical amounts of RNA were used. PCR reactions were performed in a 20 µl volume with 0.5 µM primers: Y1R sense 5'GTAGGTATTGCTGTGATTGGG-3' and antisense 5'CTCTGGAAGTTTTTGTTCAGGA-3'; Y2R sense 5'CCTACTGCTCCATCATCTTGC-3' and antisense 5'GTAGTTGCTGTTCATCCAGCC-3'; Y4R sense 5'GTGTTTCAC AAGGGCACCTA-3' and antisense 5'TGCCACTTAGCCTCAGGGA-3'; Y5R (sense) 5'-AGC-CATGTGCCATATCATGC-3' and (anti-sense) 5'-GAGGCAGGA-TATACTGCACT-3'; NPY sense 5'AGCCATGTGCCATATTAGC-3' and antisense 5'GGCAGAATA TACTGAACTAGC-3'; β-tubulin rRNA sense 5'TTCCCTGG CCAGCTSAANGCN-GAGCTNCGCAAG-3' and antisense 5'-CATGCCCTCG CCNGTGTACCAGTGNANGAAGGC-3', 1.5 mM MgCl<sub>2</sub> and 10 µl Qiagen Mastermix. After 10 min denaturation at 95 °C, 32–38 cycles with 95 °C for 30 s, 54 °C (β-tubulin)/58 °C (Y1R, Y5R)/59 °C (Y4R)/61 °C (NPY, CD26) for 30 s, and 72 °C 60 s were performed. After PCR amplification, the reactions were analyzed by gel electrophoresis. Samples were diluted 1:2 with loading dye and loaded onto 2% agarose gels containing ethidium bromide. Gels were run for 45 min at 80 V. Expression of 18S served as positive control.

Quantitative analysis of NPY receptor RNA was performed using a smart cycler (Cepheid, France) taking advantage of the QuantiTect SYBR Green™ technology (Qiagen). RNA from sorted neutrophils was isolated as described above. Each PCR cycle comprised melting at 95 °C for 15 s, annealing at a temperature specific for each gene (hCD26: 56 °C, hNPY: 54 °C, hNPYY1: 57.5 °C, hNPYY2: 58 °C, hNPYY4: 55 °C, hNPYY5: 56 °C, hGAPDH: 55 °C) for 30 s, and an extension at 72 °C for 30 s. Each PCR amplification was performed in triplicates. The optimal parameters for the PCR reactions were defined empirically. The purity of the amplified PCR products was verified by melting curves. hGAPDH was used as housekeeping gene. The specificity of the oligonucleotides (see above) used to amplify the NPY receptors was determined using cell lines transfected with specific cDNAs (SK-N-MC cells — Y1R; stably transfected HEC-1B — Y5; transiently transfected COS-7-cells — Y2R and Y4R).

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