

Neurotrophin and GDNF Expression Increases in Rat Adrenal Glands During Experimental Colitis

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Submitted: November 1, 2001
Accepted: November 18, 2001

Key words: **neurotrophins, adrenal glands, chromaffin cells, experimental colitis**

Neuroendocrinology Letters 2001; 22:461-466 pii: NEL220601A07 Copyright © Neuroendocrinology Letters 2001

Abstract

OBJECTIVES: Neurotrophins and GDNF have been recently recognized as important local regulators of inflammatory processes of the gut.

RESULTS: We now demonstrate that experimental TNBS-colitis is associated with the increased expression of neurotrophins and GDNF in the adrenal glands. In histological sections of the adrenals from untreated control animals, faint immunolabeling for BDNF, NT-3 and NGF was detectable in the adrenal cortex, with some additional labeling for NGF over the adrenal medulla, whereas GDNF immunolabeling was confined to the adrenal medulla. Induction of TNBS-colitis markedly increased NGF, BDNF, and NT-3 expression within the adrenal cortex after 8 h. NGF declined to basal levels after 7 days. In case of BDNF and NT-3 basal expression levels were reached after 14 days. GDNF expression was robustly upregulated in the adrenal medulla 8 h after induction of colitis and stayed elevated for up to 14 days.

CONCLUSION: Together these observations suggest that neurotrophins and GDNF might act as local modulators of components of the HPA-axis during peripheral inflammation.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) as well as the neurotrophins, nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF) are potent survival factors for distinct populations of peripheral and central nervous system neurons [1, 2]. In addition all these growth factors control the development and/or function of hematopoietic cells [3–8]. Recently, we and others provided evidence that neurotrophins are also capable of modulating inflammatory processes. In the inflamed gut of patients with Crohn's disease and ulcerative colitis NGF and NT-3 are strongly upregulated in colonic epithelial cells, inflammatory cells infiltrating the lamina propria, and in sensory neurons of the colon [9, 10]. Moreover, these studies revealed that immunoblockade of NGF and NT-3 severely worsens experimental colitis in rats.

The hypothalamic-pituitary-adrenal (HPA) axis plays a critical role in modulating peripheral inflammation. Activation of the HPA axis results in the increased release of glucocorticoids and the subsequent suppression of the immune and inflammatory responses (for review see [11]). The communication between the immune system and components of the HPA axis occurs via neuronal as well as humoral mechanisms [12]. To date, recognized humoral activators of the HPA axis are several cytokines, including IL-1, IL-6, and tumor necrosis factor α (TNF α) as well as growth factors such as NGF and epidermal growth factor (EGF) [11]. Recent studies revealed that experimental colitis is associated with a stimulation of adrenocortical cell function [13]. In the present study, we demonstrate that experimental colitis results in a robust upregulation of NGF, BDNF, NT-3 and GDNF in the adrenal gland, implying a potential role of these growth factors as local modulators of adrenal function during peripheral inflammation.

Materials and Methods

Induction of TNB-colitis. Adult male Sprague-Dawley rats (250 g, n=3 for each group) were anaesthetized with a mixture of ketamine (50 mg/kg) and xylazine (2 mg/kg) given intramuscularly. The distal colon was cleaned carefully with a small balloon catheter prior to the enema. To induce mild chronic colitis, a total volume of 0.7 ml of trinitrobenzene sulfonic acid (30 mg/kg in 50% ethanol) was then instilled into the colon via a plastic feeding tube (8F) followed by 1.5 ml air. Animals were sacrificed at various time points (0, 8h, 12h, 1d, 7d, 14d, 21d) after induction of colitis by CO₂-asphyxiation. The adrenal glands and the colon were dissected and snap frozen for RNA extraction or fixed in paraformaldehyde for

immunostaining and evaluation of the macroscopic damage score.

Macroscopic Damage Score. The macroscopic damage score was determined as previously described [10]. In short, the frequency of edema, erosions, and ulcerations was evaluated within the distal 4 cm of the colon by two investigators in a blinded fashion using the following scale and a maximal score of 13: Edema: 0 = no edema, 1 = < 50%, 2 \geq 50% and < 75%, 3 = \geq 75%; erosions: 0 = no erosions, 1 = < 25%, 2 = \geq 25% and < 50%, 3 = \geq 50% and < 75%, 4 = \geq 75%; ulceration: 0 = no ulceration, 3 = < 25%, 4 = \geq 25% and < 50%, 5 = \geq 50% and < 75%, 6 = \geq 75%.

Immunostaining. The paraffin embedded tissue was sectioned (4 μ m) and mounted on superfrost plus slides (Menzel, Göttingen, Germany). Endogenous peroxidase activity was blocked with 3% H₂O₂ for 30 minutes. Cells were subsequently permeabilized with 0.3% TritonX 100 in PBS for 10 min, and non-specific binding sites were blocked by incubating sections in 10% normal goat serum. The following primary antibodies were used: anti-NGF (1:250); anti-BDNF (1:100); anti-NT-3 (1:250); anti-GDNF (1:250); all from Prohormone Science CA, USA). Sections were incubated overnight at RT with the primary antibodies followed by incubation with biotinylated secondary antibodies (goat anti rabbit IgG; Dianova Hamburg, Germany; 1:100) for 60 min at RT. Antibody labeling was detected using the Vectastain ABC-Kit (Vector Laboratories) and visualized with the AEC Substrate System (DAKO). Sections were counterstained with hematoxylin, briefly fixed in 0.25% ammonia water, and embedded with glycerol gelatin. Specificity of the staining was controlled by incubating sections with normal rabbit serum or by omitting the primary antibodies.

RT-PCR analysis. Total RNA was isolated from rat adrenal glands with the TriReagent RNA isolation kit (MRC, Cincinnati, OH, USA) following the manufacturer's instructions. Ten μ g of total RNA were annealed with oligo dT primer (Promega) at 65°C for 10 minutes. Reverse transcription was carried out at 42°C for 1 h in first strand buffer (GibcoBRL, Scotland) supplemented with 0,1 M DTT, 10 mM of each dNTP (Boehringer, Mannheim, Germany), and 200 units of Superscript II RNase H Reverse Transcriptase (Gibco BRL) in a total volume of 20 μ l. Obtained cDNA was amplified in a final volume of 50 μ l with 2 μ l cDNA, 5 μ l of 10 x PCR buffer (Perkin-Elmer, Branchburg, NJ, USA), MgCl₂ (final concentration; 1,5 mmol/l), 10 mM of each dNTP, 10 pmol of each sense and antisense primer, and 1 μ l of Amplitaq DNA polymerase (Perkin-Elmer). In all experiments, β -actin served as an internal control. The used primer sequences were NGF SENSE 5'-CCAAGGGAGCAGCTTTCTATCCTGG-3', NGF ANTISENSE

5'-GGCAGTGTCAAGGGAATGCTGAAGT-3', BDNF SENSE
 5'-AGCCTCCTTCTCTTCTGCTGGA-3', BDNF ANTISENSE
 5'-CTTTTGTCTATGCCCTGCAGCCTT-3', NT-3 SENSE
 5'-TTTCTCGCTTATCTCCGTGGCATCC-3', NT-3 ANTISENSE
 5'-GGCAGGGTGCTCTGGTAATTTCT-3', GDNF SENSE
 5'-GAAGTTATGGGATGTCGTGGC-3', GDNF ANTISENSE
 5'-CGTAGCCCAACCCAAGTCAG-3'.

Amplification was carried out with 35 cycles at the following conditions: 30 s denaturation at 95°C, 30 s annealing at specific temperatures for each set of primers (55–61°C), and 1 min elongation at 72°C. PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide.

Results

In accordance with our previous findings [10], TNBS-induced colitis in rats resulted in a macroscopic damage of the colon, as evidenced by the appearance of edema, erosions, and ulcerations within 8 h after treatment (Fig. 1). Maximal tissue damage occurred after 7 days with signs of recovery thereafter. In histological sections of adrenals obtained from control animals, faint immunostaining for NGF was present in both the adrenal medulla and cortex, whereas BDNF and NT-3 immunoreactivity seemed restricted to adrenal cortex (Fig. 2A-C). Staining with GDNF antibodies resulted in a faint, but specific labeling of the adrenal medulla (Fig. 2D). In animals suffering from experimental colitis, NGF expression markedly increased in the adrenal cortex 8 h after treatment with TNBS (Fig. 2 (A2)). The NGF expression was not uniform, but predominantly occurred in a patch-like manner within the zona fasciculata. NGF immunostaining decreased 1 day after TNBS treatment and reached basal expression levels after 7 days (data not shown). Similar to NGF, TNBS-induced colitis resulted in a patchy increase in BDNF and NT-3 expression within the adrenal cortex 8 h after TNBS-infusion (Fig. 2 (B2,C2)). Whereas BDNF immunostaining was predominantly localized in the external part of the zona fasciculata, NT-3 expression was predominantly present in the internal part of the zona fasciculata and the adjacent zona reticularis. Both BDNF and NT-3 immunostaining gradually decreased reaching basal expression levels 14 days after induction of experimental colitis. In apparent contrast to neurotrophins, TNBS-induced colitis resulted in a robust increase in GDNF expression within the adrenal medulla (Fig. 2 (D2)). This increase in GDNF expression was present 8 h after TNBS infusion and was still detectable up to 14 days. Since the observed increases in adrenal growth factor levels could either result from enhanced synthesis within the organ itself or the stimulated import via the blood stream and/or nerve terminals, we further

determined whether mRNA's encoding the various growth factors are present within the adrenals. RT-PCR allowed the amplification of BDNF, NT-3, NGF and GDNF mRNA from adrenal tissue, demonstrating that all these growth factors are synthesized by adrenal cells (Fig. 3).

Discussion

Neurotrophic factors have been recently recognized as potent modulators of the immune system. In the present study, we sought to determine whether peripheral inflammatory processes would affect the expression of neurotrophic factors in the adrenal gland, a component of the HPA-axis recently found to be activated during colitis [13]. We observed that adrenal BDNF, NT-3, NGF, and GDNF expression robustly increased upon induction of experimental colitis, thus pointing to a yet unrecognized role of these growth factors in the coordination of the neuro-immune-endocrine response during peripheral inflammatory processes.

In both control animals and animals suffering from mild colitis neurotrophin and GDNF immunoreactivity was detectable in discrete areas of the adrenal gland. In accordance with a previous observation [14], GDNF immunolabeling was confined to the adrenal medulla. Immunoreaction for BDNF and NT-3 was present in the adrenal cortex, whereas NGF immunolabeling occurred within both the adrenal cortex and medulla. This is in further accordance with findings from recent in-situ hybridization studies which showed a restricted expression of mRNA encoding BDNF and NT-3 within the adrenal cortex of adult rats [15]. Similar information on the localization of NGF mRNA within the adrenal gland is currently not available. Notable differences in the

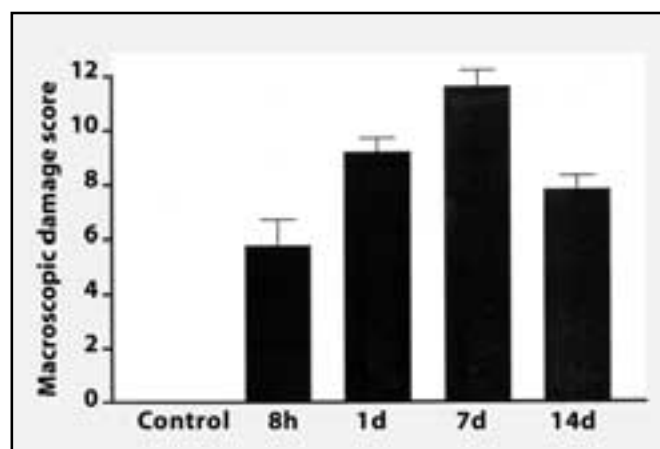


Fig. 1. The macroscopic damage score during the time course of TNBS-induced experimental colitis was calculated by evaluation of edema, erosions and ulcerations in the inflamed colon (mean ± SEM).

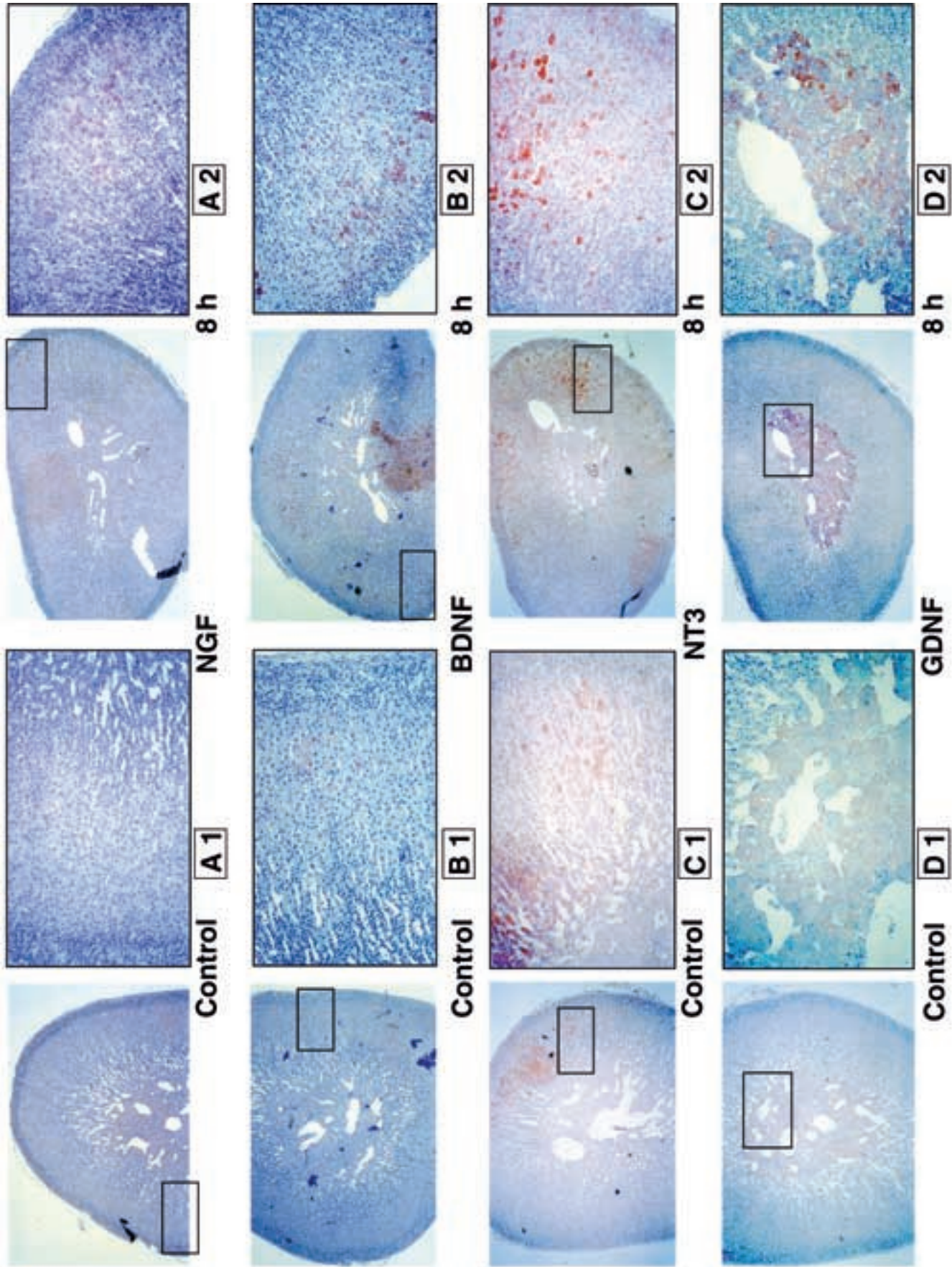


Fig. 2. Histological sections of adrenals were immunostained (red) with specific antibodies to (A) NGF, (B) BDNF, (C) NT3 and (D) GDNF. Expression of the neurotrophins is shown in control animals without colitis (A1 – D1) and 8h after induction of TNBS-colitis (A2 – D2). Sections are stained using the ABC method. Cells are counterstained with hematoxylin (blue).

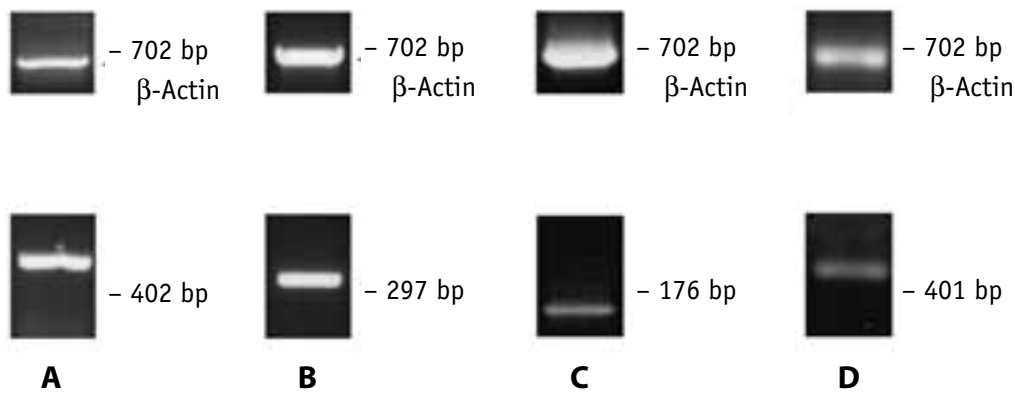


Fig. 3. RT-PCR shows the expression of mRNA's encoding for (A) NGF, (B) BDNF, (C) NT3 and (D) GDNF in rat adrenals. In all experiments β -actin mRNA expression served as an internal control.

reported distribution of BDNF and NT-3 mRNAs and protein expression, as observed in our present studies, concerned the localization within the different layers of the adrenal cortex. Whereas Schober *et al.* [15] found that both BDNF and NT-3 mRNA are expressed within the innermost layer of the zona reticularis of the adult adrenal cortex, we observed the most intense immunostaining for BDNF and NT-3 within the zona fasciculata, which in case of NT-3 further extended to the outer part of the zona reticularis. The reason for this discrepancy is presently unknown and could reflect strain-specific differences in neurotrophins expression within the adrenal cortex.

The signal(s) by which peripheral inflammatory processes induce growth factor expression in the adrenal gland remain(s) to be identified. Potential candidates are pro-inflammatory cytokines (e.g. IL-1, IL-6, or TNF α) that are known to trigger the hypothalamic-adrenocortical (HPA) axis by inducing ACTH secretion or by direct stimulation of the cytokine receptors in the adrenals [11]. The possible involvement of cytokines in these inductive effects is further underlined by the recent observation that adrenal IL-6 levels increase during colitis [13]. In addition it has been demonstrated that IL-6 promotes neurotrophin expression in CNS glia [16]. However, both medulla and adrenocortical cells are densely innervated by sensory and/or preganglionic sympathetic fibers [17, 18] and there is evidence that adrenal steroid secretion not only occurs in response to hormonal stimuli, but also in response to neuronal activity [12]. Consequently, the regulation of growth factor expression in adrenals by a humoral or a neuronal mechanism seems equally possible. It is further of note that neurotrophin expression seen in TNBS-treated animals was not uniform, but occurred in a patch-like manner. Presently the easiest explanation for this expression pattern is the existence of functional domains within the zona fasciculata and reticularis of the adrenal cortex.

The function of neurotrophins and GDNF in the adrenals is currently not well characterized. It has been suggested that adrenal neurotrophins and GDNF represent target-derived survival factors for sensory and preganglionic sympathetic neurons [15]. In context of our present observations, it is has to be stressed that adrenal neurotrophins might also function as autocrine/paracrine growth factors. Although only few adrenal cells seem to express the high-affinity receptor for BDNF and NT-3 *trkB* and *trkC*, respectively [15, 19], a major population of chromaffin cells was found to express the high-affinity NGF receptor, *trkA* [20, 21]. Moreover, basically all chromaffin cells show expression of the low-affinity neurotrophin receptor, p75 [20, 21], which binds all neurotrophins with equal affinities. Functional neurotrophin and/or GDNF receptors are also present on various cells of the immune system [3–7]. Thus, it seems further conceivable that adrenal neurotrophins and GDNF could modulate local immune pathways. One important future issue will be to define the changes associated with the observed increases in adrenal growth factor expression upon colitis. In addition, future studies have to establish whether stimulation of growth factor expression in the adrenals represents a general response to peripheral inflammatory processes.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft DFG Re 789/2–3

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