# Antibodies to Neisseria gonorrhoeae reduce NGF-dependent neurite outgrowth, and phosphorylation of transcription factors FoxO3a and Stat3 in PC12-cells *in vitro*

**B. Reuss**

University of Göttingen, Insitute for Neuroanatomy
Kreuzbergring 36, 37075 Göttingen, Germany

## Introduction

Neisseria gonorrhoeae is a Gram-negative bacterium, and a major cause for clinical and subclinical reproductive tract infections. During pregnancy, first trimester prenatal maternal gonococcal infections are linked to an increased risk for the offspring to suffer from psychotic symptoms in later life (Babulas et al., 2006, Am. J. Psychiatry 163, 927-929; Sørensen et al., 2009, Schizophrenia Bull. 35, 631-637). Since psychosis is often associated with impaired neurite formation in the prefrontal cortex, I have tested here the effects of a commercially available polyclonal antiserum from rabbit directed to Neisseria gonorrhoeae (αNG), on Nerve growth factor (NGF)-dependent neurite outgrowth in PC12-cells, a commonly used *in vitro* model for neuronal differentiation.

## Materials&Methods

**Antibodies:**
Anti-Neisseria gonorrhoeae (**α-NG**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN285584.
Anti-activating-transcription-factor-3 (**α-ATF-3**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN883952.
Anti-activating-transcription-factor-5 (**α-ATF-5**): rabbit polyclonal, Abcam, Cat. Nr. ab1370.
Anti-Signal-transducer-and-activator-of-transcription-1 (**α-Stat-1**): mouse monoclonal, Zymed (Invitrogen), Cat. Nr. 33-1400.
Anti-Signal-transducer-and-activator-of-transcription-3-pSer727 (**α-Stat-3-pSer727**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN482587.
Anti-Forkhead-Box-Protein-O3a-pSer253 (**α-FoxO3a-pSer253**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN349221.
Anti-β-actin (**α-β-actin**) mouse monoclonal, Sigma-Aldrich, Cat. Nr. A5441.

**Cell culture:**
Rat PC12-pheochromocytoma-cells (DSMZ, Braunschweig, Germany) were maintained in Dulbeccos modified Eagle Medium (DMEM), supplemented with 10% fetal-calf-serum (FCS), 5% horse-serum (HS), and Penicillin/Streptomycin (PS). Medium was exchanged twice per week. 5000 cells/well were seeded on a 6-well cell-culture-plate and were preincubated for one day with DMEM, supplemented with 0,5% FCS, 0,25% HS and PS. For differentiation, and induction of neurite-outgrowth, 10ng/ml of NGF was added to the cell-culture-medium. To analyze the effects of αNG on neurite-outgrowth, PC12 cells were treated in parallel with 10µg/ml of a polyclonal antiserum from rabbit directed to Neisseria gonorhoeae (αNG), sodium-azide of which has been removed by microdialysis using Amicon-Ultra filter units (Millipore). Some cultures were also treated with antipsychotic drugs Haloperidol (HAL; 0,1µmol/l), Clozapine (CLZ; 0,1µmol/l), Olanzapine (OLA; 10µmol/l) and Risperidone (RIS; 1µmol/l). Cells were imaged with a digital camera system mounted on an inverted microscope (Nikon). For every treatment and timepoint, values were determined at 10 randomly chosen fields in 8 independent experiments.

**Western blot:**
Cells were harvested in 5x Laemmli sample-buffer and protein concentration was determined. 5µg of total cellular protein was separated by polyacrylamide gel electrophoresis (8,5%). After tank-blot Western-transfer onto PVDF-membrane, membranes were blocked with dry milk and incubated with primary antibodies (1:2000, 4°C) over night. After washing, either mouse or rabbit specific peroxidase-coupled secondary antibodies (1:10.000) were applied for 90' at RT, and after washing, ECL-detection was performed. For relative quantification, blots were stripped with NaOH (1mol/l) for 15' and were incubated with an antibody directed to the house-keeping protein β-actin. Blots were densitometrically evaluated using the IMAL image analysis program. Significance of the obtained results were analysed with Students two-tailed t-test

**Immunocytochemistry:**
Cells were washed with PBS and fixed for 10' with 4% paraformaldehyde. After permeabilization with Acetone/Methanol (1:1), followed by three washes with PBS, cells were blocked for 1h with goat serum (1:50) in PBS. Primary antibodies were applied over night at 4°C, followed by three washes with PBS and biotin-coupled secondary antibodies (1:400 at RT). After three washes with PBS, FITC-coupled Streptavidin was used to fluorescently label the antibody tagged proteins. After three final washing steps, microscopic detection was performed using an Axiocam digital camera system mounted on an Axiovert inverted microscope equipped with epifluorescence (Zeiss).

## αNG specific antibodies impair neurite outgrowth in NGF-stimulated PC12 cells

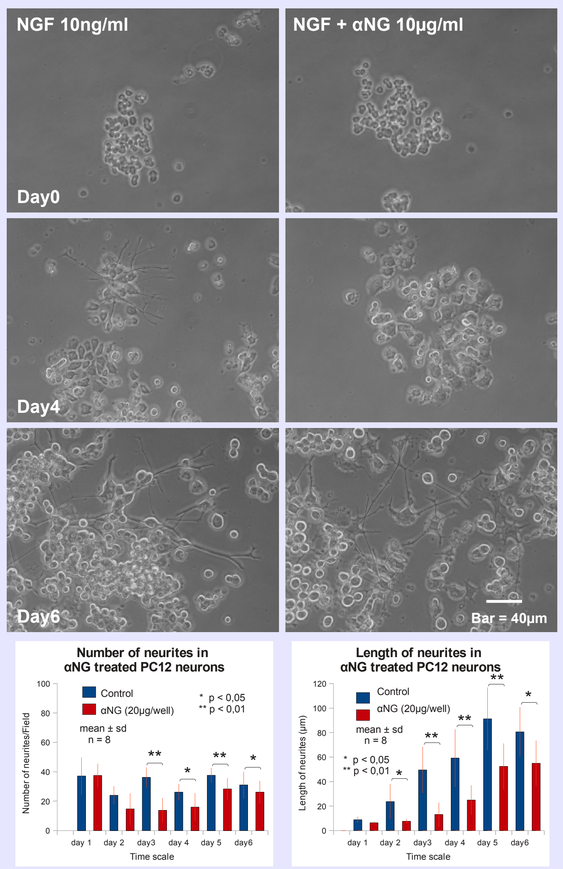



Figure 01

## Neuroleptics revert the αNG dependent decrease in neurite length of PC12 cells

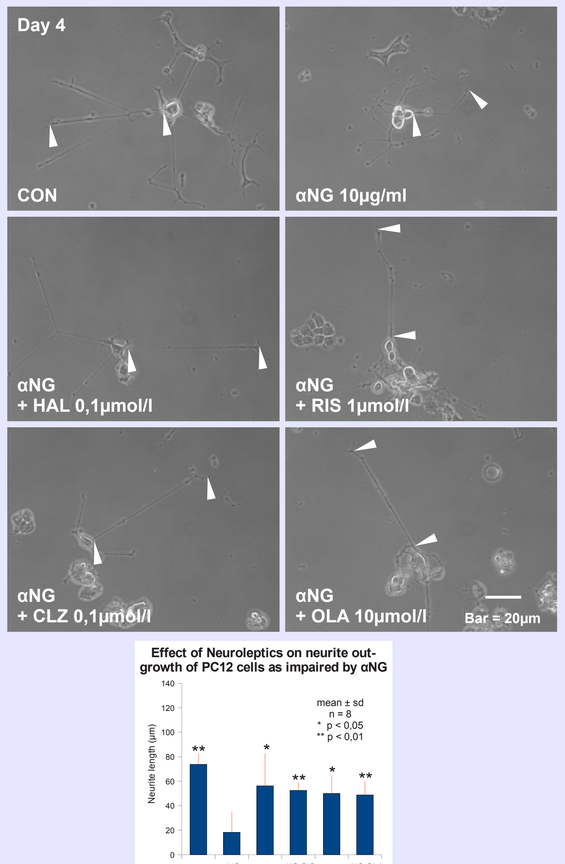
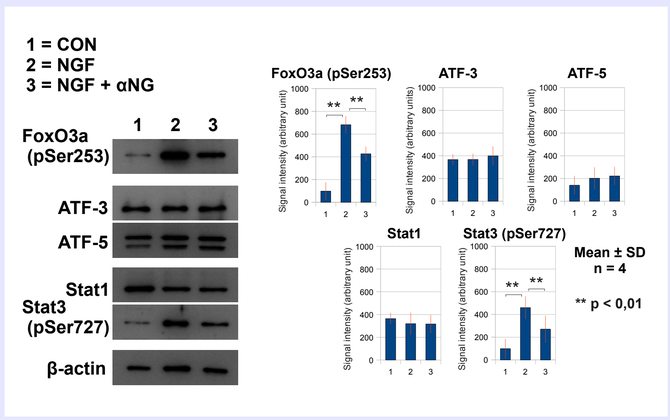



Figure 02

## αNG leads to reduced phosphorylation of the transcription factors FoxO3a and Stat3



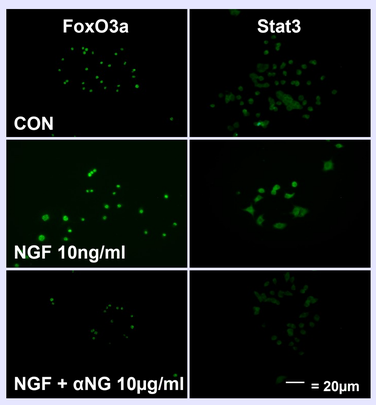




Figure 03

## Decreased phosphorylation of FoxO3a and Stat3 by αNG is not restituted by neuroleptic drug treatment

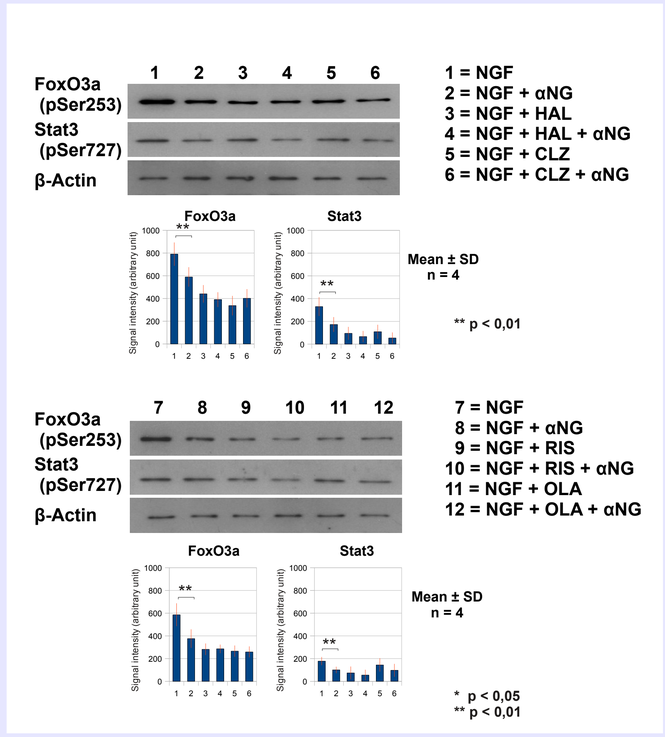



Figure 04

## Results

Incubation of differentiating PC12-cells with 10µg/ml of αNG leads to a significant reduction of NGF-dependent neurite outgrowth as compared to cultures treated with NGF alone (Fig. 01). Both, neurite length, as well as number of neurites per cell are significantly reduced. Interestingly, the reduction in neurite outgrowth caused in PC12-cells by αNG treatment, can be reversed by parallel application of the antipsychotic drugs Haloperidol (HAL; 0,1 µmol/l), Risperidone (RIS; 1µmol/l), Clozapine (CLZ; 0,1µmol/l), and Olanzapine (OLA; 10µmol/l) (Fig. 02). As revealed by Western blot analysis, αNG-treatment of differentiating-PC12 cells leads to a reduction in the phosphorylation of the transcription factors Fox-O3a and Stat3, whereas phosphorylation of Stat1, as well as expression of ATF-3 and ATF-5 are not altered (Fig. 03). In contrast to this, phosphorylation of Fox O3a and Stat3 are not changed upon reversal of neurite outgrowth by the application of neuroleptic drugs Haloperidol and Clozapine, or Risperidone and Olanzapine (Fig. 04).

## Conclusions

These results suggest that antibodies directed primarily to bacteria-specific antigens are able to crossreact with neuronal antigens, leading to impaired neurite outgrowth in PC12-cells *in vitro*, and that this impairment can be overcome by parallel treatment of the cells with neuroleptic drugs. In addition, they demonstrate that the effects of bacteria-specific antibodies may be mediated by the regulation of neuritogenic transcription factors such as FoxO3a and Stat3. Although this mechanism might be relevant for a better understanding of the pathogenesis of psychotic symptoms, its *in vivo* relevance, as well as the identity of the affected target molecule(s) still remain to be clarified in the future.

## Acknowledgements

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