In NTera2/D1 cells, antibodies directed to the Gram negative bacterium Neisseria gonorrhoeae (NG) cross-react with the heat shock protein Hsp60 and lead to impaired neurite outgrowth

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Introduction

First trimester maternal infections with the Gram-negative bacterium Neisseria gonorrhoeae (NG) lead to an increased risk for the offspring to suffer from psychotic symptoms in later life *. We hypothesize here an antibody mediated mechanism for this, and therefore investigate interactions and effects of a commercial rabbit antiserum directed to NG (α-NG) with and in human NTera2/D1 cells, a model for human neuronal differentiation *in vitro*.

* Sørensen et al., 2009, Schizophrenia Bull. 35, 631-637

Materials&Methods

Antibodies and proteins:
Anti-Neisseria gonorrhoeae (**α-NG**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN285584.
Anti-Neisseria meningitidis (**α-NM**): rabbit polyclonal, Antikoerper-online.de, Cat. Nr. ABIN285585
Anti-β-actin (**α-β-actin**) mouse monoclonal, Sigma-Aldrich, Cat. Nr. A5441.
Recombinant human heat shock protein **Hsp60**, Antikoerper-online.de, Cat. Nr. ABIN621577.

Cell culture:
Human NTera2/D1 cells (DSMZ, Braunschweig, Germany) were maintained in DMEM, supplemented with 10% FCS, 10% αMEM, 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, 10% αMEM, and PS. For neuronal differentiation, cells were treated for 6 weeks with 10µmol/l retinoic acid. To test actions of α-NG on neurite-outgrowth, differentiating NTera2/D1 cells were treated with 10µg/ml of α-NG, sodium-azide of which has been removed by microdialysis using Amicon-Ultra filter units (Millipore). Cells were imaged with a digital camera mounted on an inverted microscope (Nikon). For every treatment and timepoint, values were determined at 10 randomly chosen fields in 8 independent experiments.

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 Axiophot microscope equipped with epifluorescence (Zeiss).

Western blot analysis:
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 β-actin. Blots were densitometrically evaluated using the IMAL image analysis program. Significance was analysed by Students two-tailed t-test

2D-Gel electrophoresis:
Cells were harvested in 2x ampholyte sample buffer, and protein concentration was determined by a densitometric method. For the 1st dimension, 30µg of total cellular protein was loaded on Polyacrylamide gel slices, containing ampholytes (pH3,5-10) for pH-dependent isoelectric focusing. Gel slices were then equilibrated in Laemmli sample buffer and located on top of a standard SDS polyacrylamide gel. For the 2nd dimension, separation occurred due to the molecular weight, and a partial Western transfer onto PVDF membrane was performed by shortening transfer time. Whereas the PVDF membrane was stained by immune incubation, the remaining proteins on the polyacrylamide gel were stained with Coomassie blue. By overlay of the immunoblot with the stained gel, the α-NG immunreactive protein spot could be identified and excised.

Digestion and LC-Q-TOF MS/MS analysis:
The excised protein spot was digested as described previously*. Obtained peptides were reconstituted in an aqueous solution and introduced onto two consecutive nano-C18-reversed-phase chromatography columns using an auto CapLC sampler (Waters). The separated peptides were analyzed in a Q-TOF Ultima Global mass spectrometer (Waters) equipped with a nanoflow ESI Z-spray source in the positive ion mode. Data were acquired with the MassLynx (v4.0) software on a Windows NT PC and further processed using ProteinLynx Global Server (PLGS; v 2.2; Micromass). Obtained peak lists were searched online (http://www.matrixscience.com) by the MASCOT algorithm against the NCBI and SwissProt database.
*Asif et al., 2010, Electrophoresis 31, 1947-1958.

α-NG immunoreactivity in NTera2/D1 cells corresponds to a 60-70 kDa protein band

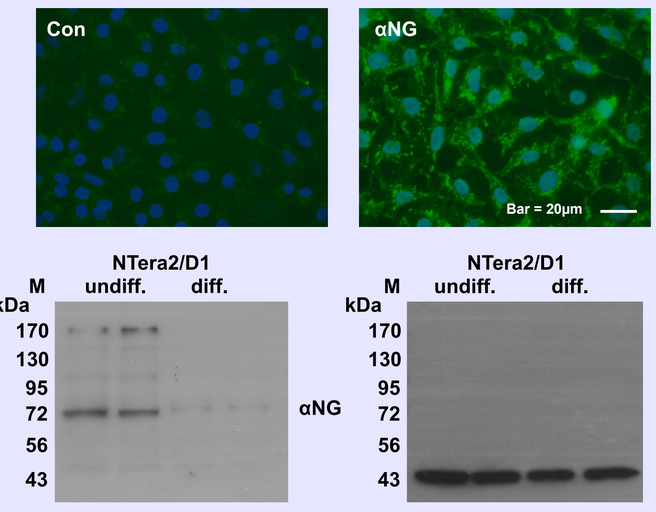


Figure 01

α-NG-specific immunoreactivity in NTera2/D1 cells is not identical to that of α-NM

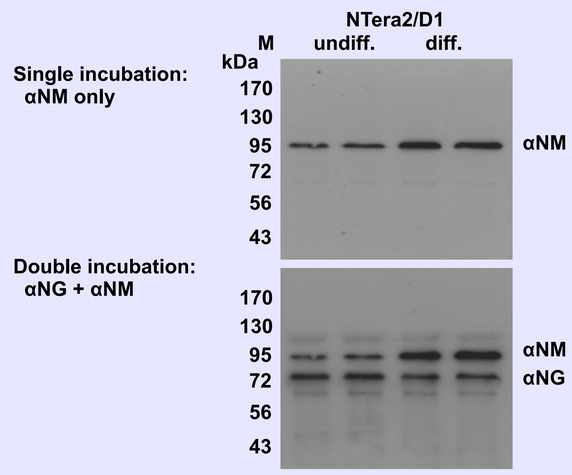


Figure 02

A Coomassie stained spot on a 2D protein gel can be identified as α-NG-specific by immunoblot after partial Western transfer

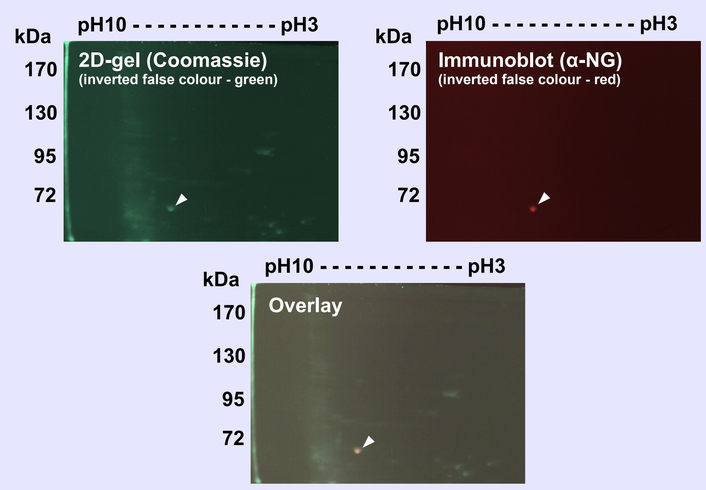


Figure 03

LC-Q-TOF MS/MS analysis identifies the α-NG immunoreactive protein spot as the mitochondrial heat shock protein Hsp60

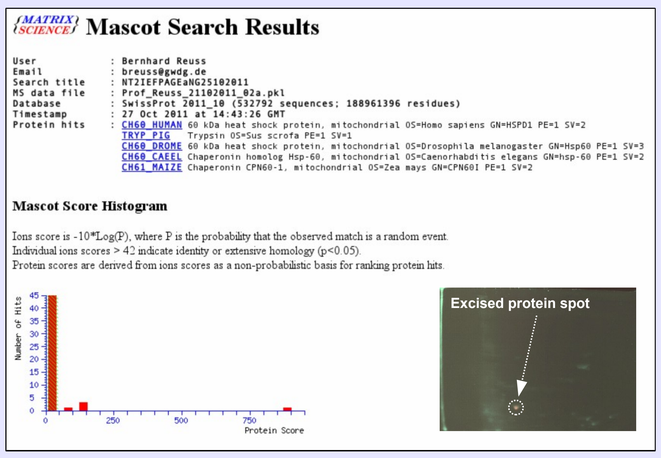
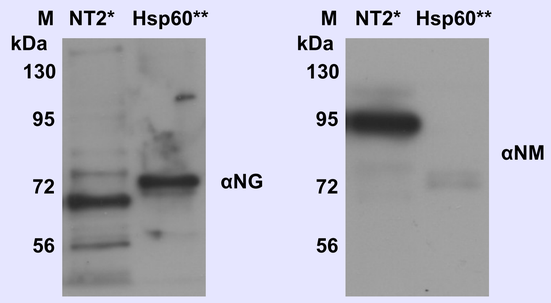


Figure 04

Crossreactivity of α-NG with a commercial Hsp60 protein confirms the result obtained by mass-spectroscopy



*NT2 = NTera2/D1 whole cell protein extract
**Hsp60 = recombinant Hsp60 protein (n.b.: due to an added His-tag, molecular weight of this protein is slightly incrased compared to the size of the natural variant)

Figure 05

α-NG but not α-NM reduces neurite outgrowth in NTera2/D1 cells during RA-dependent neuronal differentiation

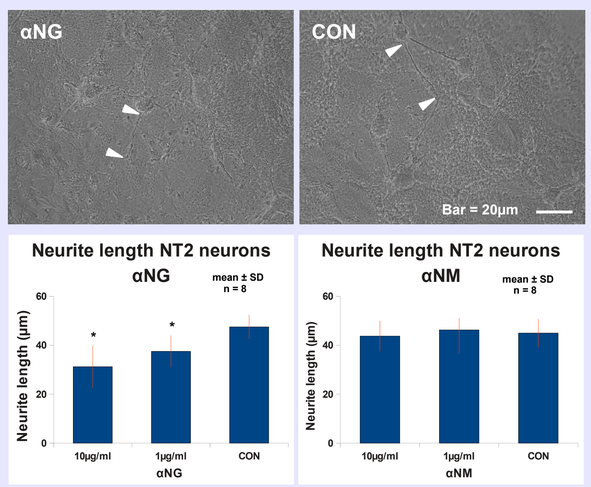


Figure 06

Results

Fluorescent immunocytochemistry revealed α-NG to label in undifferentiated NTera2/D1 cells, antigens located in an intracellular organelle, which by subsequent Western blot analysis revealed a molecular weight between 56 and 72 kDa (Fig. 01). In contrast, an antiserum directed to Neisseria meningitidis (α-NM) reacts with an antigen of a higher molecular weight of between 72 and 95 kDa, revealing the observed interactions to be antibody specific (Fig. 02). By 2D-Gel-electrophoresis combined with partial Western transfer, a protein spot with strong α-NG-specific immunoreactivity could be localized (Fig. 03). After isolation, the resulting protein could be identified by LC-Q-TOF MS/MS analysis as the human mitochondrial heat-shock protein Hsp60 (Fig. 04). This could be confirmed by Western blot analysis for interaction of α-NG with a commercially available recombinant Hsp60 protein, with which α-NM failed to interact (Fig. 05). Finally, effects of α-NG on neurite outgrowth in retinoic acid stimulated NTera2/D1 cells was analysed, demonstrating that 10µg/ml α-NG leads to decreased neurite length, whereas 10µg/ml α-NM has no such effect (Fig. 06).

Conclusions

These results demonstrate for the first time that antibodies directed to Neisseria gonorrhoeae are able to interact with the human mitochondrial heat-shock protein Hsp60. This result seems indeed to be of some interest, since autoantibodies to Hsp60 have already been previously reported in schizophrenic patients*. Nevertheless, how these interactions could lead to diminished neurite outgrowth in NTera2/D1 cells, and whether this is in fact of pathogenetic relevance for psychotic symptomatology remains to be clarified in the future.

* Wang et al., 2003, Biol Psychiatry 53:361-375.

Acknowledgements

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