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## Neuregulin-1, the Fetal Endothelium, and Brain Damage in Preterm Newborns

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### Abstract

**Objective:** To assess the potential role for Neuregulin-1 (NRG1) as a systemic endogenous protector in the setting of perinatal inflammatory brain damage.

**Methods:** We measured *NRG1*- protein and mRNA levels in human umbilical venous endothelial cells (HUVECs) of different gestational ages at various durations of exposure to lipopolysaccharide (LPS). In parallel, we genotyped the donor individuals for SNP8NRG221533, a disease-related single nucleotide polymorphism in the 5' region upstream of the *NRG1* sequence. Intracellular NRG1 localization was visualized by confocal microscopy. Furthermore we analyzed the relationship between SNP8NRG221533 genotype and neurodevelopmental outcome in children born preterm.

**Results:** We observed a positive dose-response-relationship between *NRG1*-mRNA and intracellular protein levels with both advancing gestational age and duration of LPS exposure in HUVECs. The presence of allele C at the SNP8NRG221533 locus was associated with an increased cellular production of NRG1 in HUVECs, and with a significantly reduced risk for cerebral palsy and developmental delay in children born preterm.

**Interpretation:** In conclusion, our data indicate that gestational age, duration of LPS exposure, and the SNP8NRG221533 genotype affect NRG1 levels. Our results support the hypothesis that NRG1 may qualify as an endogenous protector during fetal development.

### Keywords

Neuregulin-1; HUVECs; inflammation; SNP8NRG221533; neuroprotector; brain damage

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## Introduction

Preterm birth is associated with an increased risk of neonatal brain damage (Ferriero 2004) and long-term developmental delay (Stephens and Vohr 2009). Intrauterine infection leads to inflammatory responses (Dudley 1997; Dammann, Allred et al. 2004; Romero, Espinoza et al. 2006; Romero, Espinoza et al. 2007) that contribute to prematurity and neonatal morbidity. The umbilical vessel endothelium is one of the first fetal interfaces exposed to inflammatory challenges, e.g. lipopolysaccharides (LPS). Indeed, endothelial cells have a remarkable capacity to respond to stressors (Dauphinee and Karsan 2006; Magder, Neculcea et al. 2006).

Neuregulins (NRGs) play important roles during fetal brain (Bernstein, Lendeckel et al. 2006), heart (Gassmann, Casagrande et al. 1995) and lung development (Dammann, Nielsen et al. 2003), and are involved in inflammatory processes (Xu, Jiang et al. 2004; Xu, Ford et al. 2005). Several NRG isoforms (Meyer, Yamaai et al. 1997) are produced by alternative splicing (Wen, Suggs et al. 1994; Falls 2003). One of these, NRG1, appears to help signal the onset of surfactant synthesis in the fetal lung (Dammann, Nielsen et al. 2003) and might qualify as a brain protector in experimental ischemia (Xu, Jiang et al. 2004; Xu, Ford et al. 2005). We have recently suggested that NRG1 might play a role not only in adult (Deadwyler, Pouly et al. 2000; Gerecke, Wyss et al. 2004), but also in neonatal brain disorders (Dammann, Bueter et al. 2007).

The role of single nucleotide polymorphisms (SNPs) in inflammation-associated genes, e. g. interleukin (IL)-10, as risk-modulators for neonatal disorders has previously been investigated in a pilot study (Dordelmann, Kerk et al. 2006; Dammann, Brinkhaus et al. 2009). Now we wanted to expand this scenario to include *NRG1* and one of its SNPs (SNP8NRG221533) that has been associated with schizophrenia (Stefansson, Sigurdsson et al. 2002; Stefansson, Sarginson et al. 2003).

We hypothesized, that

1. NRG1 is released by HUVECs in response to LPS-exposure
2. *NRG1* gene expression and NRG1 protein production differ with both gestational age and LPS treatment duration
3. SNP8NRG221533 alters *NRG1* gene expression; and
4. There is a reduced risk for neonatal brain damage and developmental delay among children with a high producer allele of SNP8NRG221533.

## Methods and Patients

### Cell culture

Umbilical cords were collected from babies born either before 30 weeks gestation (immature group) or after completion of 37<sup>th</sup> week of gestation (mature group). To reduce the likelihood of obtaining biased results all umbilical cords showing histological evidence for inflammation (funisitis) were excluded from our study. Endothelial cells were harvested from human umbilical cord vessels within 1 – 5 days of delivery (Bueter, Dammann et al. 2006). Briefly, umbilical veins and arteries were cannulated with blunt needles, rinsed with sterile PBS buffer, and treated with type I collagenase (0.04 %, GIBCO, Invitrogen, Karlsruhe) for 25 minutes at 37°C. Cell-collagenase suspension was collected and centrifuged at 250 × g for 5 minutes at 4°C. Cells were resuspended, and plated in endothelial cell growth medium containing 10 % fetal calf serum (FCS). Cells were washed after 2-3 hours of adherence and grown until confluence. We used cells up to the 5<sup>th</sup> passage for the experimental assays. Before treatment, cells were starved for two hours in Dulbecco's Modified Eagle's Medium (DMEM) containing

0.1 % FCS. Treatment was performed with 100 ng/ml LPS for 1.5, 3, 6, 12, and 24 hours. Untreated controls were kept in culture in DMEM for the same time.

### NRG1 localization by confocal microscopy

HUVECs were grown on glass slides for 24 hours and starved in serum-free DMEM for 2 hours. Cells were either treated with LPS (100ng/ml) for 30 minutes, 24 hours or left in DMEM as a control for the same time at 37°C. Immunofluorescence staining was performed as previously described (Padmakumar, Abraham et al. 2004). Briefly, cells were fixed with 3% paraformaldehyde for 20 minutes followed by permeabilisation with 0.2% Triton X-100 for 2 minutes. After 1 hour blocking in 10% normal goat serum, fixed cells were incubated with the specific primary NRG1 antibody (rabbit polyclonal IgG Neuregulin-Ab 2; Neo Markers, Fremont, CA) at room temperature for 1-2 hours. Cells were washed with PBS and incubated with the appropriate secondary antibody conjugated with Alexa568. Subsequently cells were incubated in 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, Heidelberg, Germany) for 10 minutes. Cells were mounted in Gelvatol/DABCO and analyzed using a Leica TCS-SP2 confocal laser scanning microscope.

### Genotyping for SNP8NRG221533

We genotyped both HUVECs obtained from umbilical cords and lymphocyte DNA obtained from whole blood samples (Dordelmann, Kerk et al. 2006). Genomic DNA was isolated using standard phenol-chloroform extraction. Polymerase chain reactions (PCR) were performed on 100 ng of genomic DNA using primers 5'-ACCTAAGATGTCCAAGAGACAG-3' (forward) and 5'-GACTGGAAGCCATGTATCTTTATTGT -3' (reverse) and HotStart *Taq* DNA polymerase (5 U/μl, Qiagen, Hilden, Germany). Thirty-six cycles were performed with 15 minutes denaturation at 95°C, 1 minute annealing at 62° and 1 minute extension at 72°C. The artificially introduced mismatch in the reverse primer allowed for a subsequent allelic discrimination by the restriction enzyme *RsaI*. PCR products were incubated with *RsaI* (New England BioLabs, Frankfurt, Germany) at 37°C overnight and restriction fragment length analysis (RFLP) was performed by 3 % agarose gel electrophoresis.

### ELISA

All sample-measurements were carried out in quadruplicates. Cells were scraped, lysed by sonication (3 × 30 seconds), lyophilized, and resuspended in 200μl PBS. The amount of total protein was measured by DC Protein Assay (Bio-Rad, Munich, Germany). ELISA was performed using the Human NRG1-beta 1/HRG1-beta 1 DuoSet (DY 377 R&D Systems, Wiesbaden, Germany). 96-well-plates were coated overnight with 100 μl capture antibody (4 μg/ml Heregulin Ab-1 Clone 7D5, Neo Markers, Fremont, CA) at 4°C. After overnight blocking with 1 % protease-free BSA and 5 % sucrose, 100 μl of sample or standard were incubated overnight at 4°C. 100 μl detection antibody (200 ng/ml Heregulin/Neuregulin Ab-2, biotin-labeled, Neomarkers), solved in PBS with 1 % protease-free BSA and 2 % normal goat serum was added for two hours. Cells were incubated in the dark in 50 μl Streptavidin HRP for 20 minutes followed by 100 μl substrate solution for 20 minutes. Substrate reaction was stopped by adding 50 μl 2N H<sub>2</sub>SO<sub>4</sub>. Photometric extinction was measured at 450 nm vs 570 nm. NRG1 concentration was calculated based on 1mg of total protein and presented as % of controls.

### RT-Real-time-PCR

Total RNA was isolated using a guanidinium-phenol based extraction procedure (Abgene, Hamburg, Germany). 1 μg of total RNA was subjected to reverse transcription using a First-Strand cDNA Synthesis Kit (GE Healthcare). Real-time PCR was performed using a commercially available primer assay for *NRG1* and a SYBR Green Master Mix (Qiagen,

Hilden, Germany) following the manufacturer's protocol with 40 cycles and an annealing at 60°C on a Sequence Detection System 7000 (Applied Biosystems).  $\beta$ -actin served as the housekeeping gene and was amplified on the same plate using primers 5'-AGATGACCCAGATCATGTTTGAG-3' and 5'-GAGTCCATCACGATGCCAGTG-3'. We calculated means and DCt values using the ABI PRISM 7000 SDS software (Applied Biosystems, Darmstadt, Germany) for relative quantification. Corresponding control values were subtracted from treated samples to calculate DDCt values. A negative result indicates a higher *NRG1* gene expression in the treated sample compared to the respective untreated control.

### Statistics

P-values were calculated using the two-tailed t-test for equal variances. Equality of variances was tested by Levene Test. Potential associations between genotypes, antenatal characteristics, and clinical outcome were calculated using logistic regression and the one-sided Cochran-Armitage test for trend (publicly available syntax was obtained from <http://listserv.uga.edu/>). All analyses were performed with SPSS 15.0 (SPSS Inc., 2006).

### Study population

We evaluated 54 children < 32 weeks gestation from the Developmental Follow Up Program at Hannover Medical School (Hannover, Germany). Children were at least two years of age. A comprehensive physical examination and the Denver developmental screening (DDST) II test were administered by one paediatrician. Clinical data were abstracted from medical charts. Peripheral lymphocytes were obtained from whole blood samples for SNP8NRG221533 genotyping as described above.

The study was approved by the institutional review board at Hannover Medical School. The parents of all study participants gave their written informed consent. Clinical data were extracted from mothers' and infants' medical records.

### Outcome measures and potential confounders (Dordelmann, Kerk et al. 2006)

Cystic periventricular leukomalacia (cPVL) was defined by echolucent areas in the white matter at any time, location, and of any extent. Cerebral palsy (CP) was defined as abnormal control of movement or posture and not as a result of progressive disease (Kuban and Leviton 1994). Developmental delay (DD) was defined as  $\geq 2$  years of age corrected for prematurity as a delay  $\geq 12$  months on at least one of the 4 Denver Developmental Screening Test (DDST) II subscales (Frankenburg, Dodds et al. 1992). Examinations were performed by the same pediatrician.

Gestational age was recorded as completed weeks based on the last menstrual period and on early ultrasound examinations during pregnancy. Children whose birth weight had been below the 10<sup>th</sup> percentile compared to gestation-, sex-, singleton-/multiple-specific German reference data (Voigt, Schneider et al. 1996) were defined as being small for gestational age (SGA). Prenatal glucocorticoid exposure was defined as complete when two doses of betamethasone were administered at a 24-hour interval, with the first dose >48 h prior to delivery. We reported whether the mother had ruptures of membranes (ROM) >12 hours, preterm labour (PTL) unresponsive to tocolytic therapy, or histological or clinical chorioamnionitis (CAM: white blood cells  $\geq 15 \times 10^3/\mu\text{L}$ , C-reactive protein  $\geq 20$  mg/dL, pyrexia  $\geq 38^\circ\text{C}$ , uterine pain or tenderness). One infant was randomly chosen out of each multiple pregnancy.

## Data Analysis

We report univariable and multivariable relationships between clinical variables, SNPs and outcomes in terms of odds ratios (ORs) and their 95% confidence intervals (CIs). In cases where we found an association with an OR whose CI excluded the null (i.e., 1.0) we still considered it worthwhile reporting this result. However, in constellations where the CI included the null, we do not believe that our study bears the potential of proving the absence of an association.

As the multifactorial pathogenesis of our clinical outcomes is widely known, we first performed univariable analyses to identify potential associations between antenatal characteristics and both the SNP and clinical outcomes. In a second step we utilized logistic regression analyses adjusting for *NRG1* genotype and one potential confounding factor at a time.

## Results

### NRG1 protein levels after LPS exposure (Figure 1)

Mature and immature HUVECs responded significantly different to 24 hours of LPS-treatment. Treated mature cells seemed to increase their intracellular NRG1 protein expression about two-fold, to a mean of  $233 \pm 23\%$  (mean $\pm$ SEM, N=2,  $p=0.11$ ) of control cells, while NRG1 protein expression was slightly reduced in immature cells to  $79 \pm 8\%$  (mean $\pm$ SEM, N= 3,  $p=0.13$ ) of control cells. The difference between mature and immature cells was statistically significant ( $p=0.005$ ). Despite different basal expression levels both immature ( $144 \pm 5\%$  of control, mean  $\pm$ SEM, N= 3,  $p=0.01$ ) and mature HUVECs ( $167 \pm 50\%$ , mean $\pm$ SEM, N= 2,  $p=0.41$ ) seemed to increase NRG1 secretion into their supernatants in response to 24 hours of LPS exposure.

### Both maturity and genotype affect NRG1-mRNA-levels (Figure 2)

We confirmed that HUVECs do indeed produce *NRG1* mRNA transcripts. Similar to protein levels, *NRG1* expression was significantly higher in mature cells than in cells from immature infants after 24 hours of LPS exposure (Fig. 2.1). The difference in *NRG1* gene expression between mature and immature cells became more prominent with increasing duration of LPS exposure and reached nominal statistical significance (N=12,  $p=0.007$ ) after 24 hours. At that time point, LPS-treated mature cells surpassed their controls by  $0.5 \pm 0.6$  cycles (mean DDCT  $\pm$ SEM, N=6,  $p=0.36$ ) whereas LPS-treated immature cells exceeded the threshold almost two PCR cycles later (mean DDCT $\pm$ SEM =  $1.8 \pm 0.3$ , N=6,  $p=0.006$ ) than untreated controls which is consistent with an about three-fold lower mRNA abundance. Stratification by SNP8NRG221533 genotype revealed that cells with at least one C-allele seemed to express more *NRG1* mRNA than TT homozygous cells in both immature and mature cells (Fig.2.2).

### NRG1-localization in HUVECs in response to LPS-treatment (Figure 3)

Neuregulin-1 was diffusely expressed in the cytoplasm of untreated serum-starved mature and immature HUVECs (Fig. 3.1a and 3.2a). Mature cells also showed remarkable membrane NRG1 enrichment after serum starvation (Fig. 3.1a) and LPS-treatment (Fig. 3.1b). Over time, NRG1 seemed to accumulate in the nucleus (Fig. 3.1d) with a loss of membrane staining in LPS-treated cells. In immature HUVECs, NRG1 localization was more diffuse in and around the nuclear region with only very little membrane staining in the untreated cells (Fig. 3.2a and c). The localization in the cell membrane disappeared completely after LPS-treatment (Fig. 3.2d). With increasing duration of LPS-exposure, nuclear NRG1 localization increased in immature cells (Fig. 3.2c). LPS-treatment appeared to amplify this effect with special enhancement in the nucleoli in both gestational ages (Fig. 3.1d and 3.2d).



### Human study population characteristics (Table 1)

Among the 54 children born before 32 weeks of gestation, forty-two (77%) infants had at least one C-allele at the SNP8NRG221533 gene locus (CC: N=6; CT: N=36; TT: N=12) (Table 1). There were no significant associations between perinatal risk factors and SNP8NRG221533 genotype, although infants carrying at least one C-allele tended to be less likely to be born after preterm labor (60%) than those with two T-alleles (83%), and were also less likely to be male (45% vs. 67%).

Ten infants (19%) had cystic periventricular leukomalacia (cPVL), 5 (9%) had cerebral palsy (CP), and 9 (17%) had developmental delay  $\geq 12$  months (DD). Low gestational age and birth weight were the strongest risk factors for all three outcomes. Only male gender, preterm labor and chorioamnionitis appeared to be potential confounders of the observed relationship between SNP8NRG221533 genotype and outcomes by virtue of being associated with both.

### SNP8NRG221533 and reduced risk for neurodisability (Table 2)

The 42 children who were homo- or heterozygous for the C-allele had lower percentages of adverse clinical outcomes compared to those with a TT genotype. Among those homozygous for the C-allele, all outcomes were absent. A one-sided Cochran-Armitage test revealed a significant trend with  $p=0.02$  for CP and  $p=0.03$  for DD (Table 2) suggesting a linear relationship between the number of C-alleles and neurological outcome. Adjustment for single confounders did not result in a reduction of the effect of the SNP8NRG221533 genotype on outcome observed in univariable analyses (Table 3). We considered our dataset too small to adjust for more than one variable at a time.

## Discussion

The goal of our study was to explore a potentially protective role for NRG1 in neonatal brain damage (Dammann, Bueter et al. 2007) based on experimental evidence from other groups obtained in several damage models (Xu, Jiang et al. 2004; Xu, Ford et al. 2005; Li, Xu et al. 2007; Lok, Wang et al. 2007). Our results suggest that NRG1 is expressed and might even be systemically released by human umbilical venous endothelial cells in response to LPS challenge. We found support for a dose-response-relationship between the amount of NRG1 expression and (1) maturity level of cells and (2) the presence of the C-allele of SNP8NRG221533. In preterm infants, the presence of at least one C-allele was associated with a reduced risk for adverse neurologic outcome.

Among candidates for endogenous protectors are growth factors that are “developmentally regulated”, i.e., they are more abundant in term than in preterm newborns (Dammann and Leviton 1999). This concept helps explain the prominently increased risk for brain damage among preterm infants by postulating a decreased availability of protectors.

To qualify as a perinatal endogenous protector, NRG1 first of all needs to be expressed at the human systemic level. Our present and previous (Bueter, Dammann et al. 2006) results suggest that NRG1 is expressed by human endothelial cells as early as mid-gestation.

Second, we would expect a developmentally-regulated endogenous protector to increase with (a) advanced gestational age and (b) after exposure to potential adverse stimuli. Indeed, we found support for both these hypotheses (Figures 1 and 2).

NRG1 plays major roles in lung (Dammann, Nielsen et al. 2003) and brain (Esper, Pankonin et al. 2006) development. We therefore wanted to begin exploring the possibility that NRG1 is involved in inflammation-related processes in preterm and term settings. Although studies on LPS-induced genes in HUVECs are available (Ahn, Choe et al. 2003; Dauphinee and Karsan

2006), our study is the first to show that LPS-exposure appears to influence NRG1 expression in HUVECs. Our failure to detect a prominent response to LPS of immature cells might be part of the reason why preterm infants are at increased risk for inflammation-associated organ damage (Dammann, Leviton et al. 2005). Certainly, we have to take into account that these results might be affected by independent events besides the maturity level of the cells. Indeed we did not have access to the medical history of umbilical cord donors and the etiology of preterm delivery. Nevertheless we eliminated the possibility of acute inflammation as the cause for preterm delivery, by excluding umbilical cords with histological signs of funisitis to prevent result biasing by activated endothelial cells. Taken together, our findings further support the contention that the concept of developmental regulation observed for a multitude of proteins (Dammann and Leviton 1999) might also apply to NRG1.

While no clear trends were present during the first hours of LPS-exposure (Fig. 2.1), the difference between immature and mature cells regarding mRNA production started increasing after 3 hours, reaching nominal statistical significance at 24 hours. Although immature cells might have a similar capacity of withstanding inflammatory challenge during the first hours, they seemed to “burn out” after 5-6 hours, while mature cells continued producing NRG1. Thus, although immature cells seem to be able to upregulate *NRG1* mRNA production, they do not fully respond to demand by sustaining protein production.

Perinatal infection and inflammation appear to play a role in both, neonatal brain damage (Dammann and Leviton 2004) and in the etiology of adult neurological diseases such as schizophrenia (Brown 2006) or Parkinson's disease (Ling, Zhu et al. 2006; Snyder-Keller and Stark 2008). Others have identified potential interactions between pro-inflammatory and *NRG1* SNPs in schizophrenia (Hanninen, Katila et al. 2007). We hypothesized that, if genetic polymorphisms of *NRG1* were associated with differential gene expression and systemic availability of NRG1 protein, they might also be associated with an altered risk for neurodevelopmental outcome in preterm infants. Our results indicate that both immature and mature cells with at least one SNP8NRG221533 C-allele seem to be capable of producing higher quantities of *NRG1* mRNA than cells with the homozygous TT-genotype (Fig. 2.2). Therefore, we explored in a small clinical population of children born before 32 weeks of gestation the relationship between SNP8NRG221533, one of the multiple SNPs in the *NRG1* gene associated with schizophrenia (Li, Collier et al. 2006; Norton, Williams et al. 2006), and clinical outcome (Table 1). We found that, while the schizophrenia literature indicates a risk increase with the C-allele in the SNP8NRG221533 locus, our results suggest a decrease in neonatal brain damage and developmental long-term problems.

What might explain this “development-disease-paradox”? While we speculate that increased NRG1 levels (and the SNP8NRG221533 allele) protect the preterm newborn's brain by compensating for maturation deficits, increased NRG1 levels in the prefrontal cortex are associated with schizophrenia (Chong, Thompson et al. 2008). We propose that the function of growth factors such as NRG1 might change over time, just like cell proliferation is of critical importance during normal fetal development and also contributes to disease processes in aging cells (e.g., tumor growth). Two major parts in cell biology, namely chronic ER-stress induced changes in unfolding protein response (Naidoo, RevNeuro Sci 2009) and aging induced changes in autophagy (Salminen et Kaarmiranta 2008, Trends Mol Med 2009) might be major players in these events. Another explanation of this paradox is that it is a chance finding. However, the possibility that the finding is real deserves further investigation.

Interestingly, the C allele that appeared neuroprotective in our case-only study of preterm newborns, has recently been associated with an increased frontal brain activation in a working memory task of healthy individuals (Krug, Markov et al. 2008). If NRG1 was indeed neuroprotective, further studies are warranted to elucidate how exactly the protective

mechanism might look like. For example, experiments are needed that incorporate the effects of inflammation on the developing NRG-system in concert with, e.g., the developing oligodendrocyte (Sussman, Vartanian et al. 2005), one of the key cellular components of the developing brain white matter that is likely to be a target for inflammatory challenge (Pang, Zheng et al. 2007).

It is well-established that neuregulins act locally and in a paracrine manner (Meyer and Birchmeier 1995). The overall hypothesis of our study, however, was that NRG1 might play a role as a responder to inflammation on the systemic level. In earlier studies, NRG1 expression had been identified in cardiac endothelium (Carraway, Weber et al. 1997; Zhang, Sliwkowski et al. 1997; Zhao, Sawyer et al. 1998), brain white matter (Winterer, Konrad et al. 2008), and mesenchymal stem cells (Gui, Wang et al. 2007). More recently, Neuregulin-1 mRNA, protein expression, and activity towards the receptors erbB2 and erbB3 were detected in brain microvascular endothelial cells (BMECs), and evidence for a cytoprotective effect of NRG1 in this cell type has been presented (Lok, Sardi et al. 2009).

We previously found that expression of erbB receptors for NRG in HUVECs was altered by stimulation with NRG1 (Bueter, Dammann et al. 2006). We therefore hypothesized that HUVECs themselves should be capable of expressing the NRG1 protein. Indeed, we found support for this using both ELISA techniques (Fig. 1) and confocal microscopy (Fig. 3). Our previous (Bueter, Dammann et al. 2006) findings also indicate that nuclear shifting of erbB4, another one of the NRG1 receptors, after NRG1 stimulation is amplified by LPS-treatment. We speculate that NRG1 uses the erbB4 receptor for nuclear transport, since erbB4 contains a nuclear localization signal. During systemic inflammation the NRG1-erbB4 complex might play a central role in the cellular immune response, just as erbB2, an orphan receptor of the erbB receptor family, appears to play a role in interleukin-6 signalling (Qiu, Ravi et al. 1998).

In summary, our data suggest that carrying at least one C-allele of the SNP8NRG221533 might lead to an increase in *NRG1* gene expression in human umbilical venous endothelial cells, that exposure to pro-inflammatory stimuli further increases this effect, and that NRG1 might be a systemic endogenous neuroprotector in preterm newborns. Future research should include studies that correlate systemic NRG1 level in fetuses/newborns with both, fetal and maternal genotypes and neurologic outcomes.

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## Abbreviations

BPD	Bronchopulmonary dysplasia
CAM	Chorioamnionitis
CI	Confidence interval
CLD	Chronic lung disease
CP	Cerebral palsy
cPVL	Cystic periventricular leukomalacia
DD	Developmental delay
NRG	Neuregulin



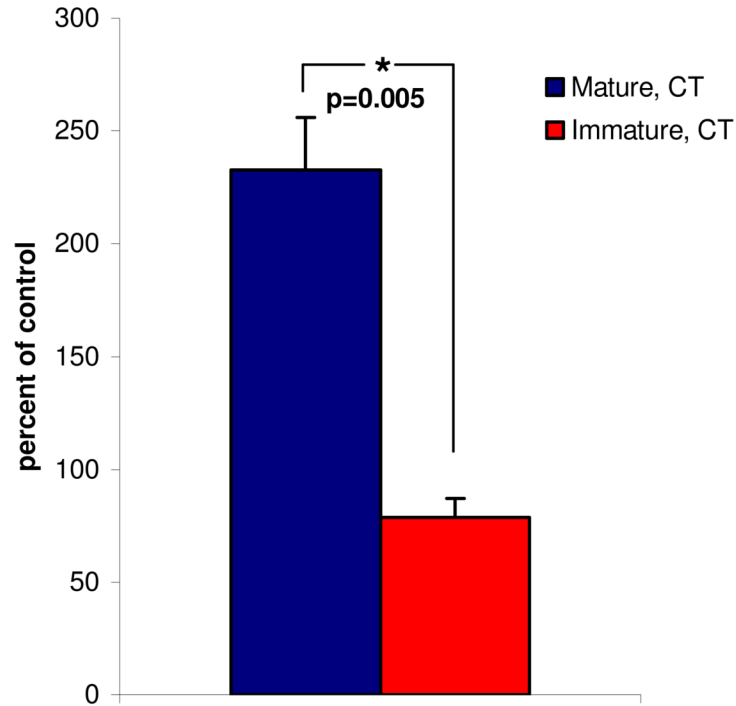
OR	Odds ratio
PTL	Preterm labor
PVE	Periventricular echodensity
ROM	Rupture of membranes
SGA	Small for gestational age

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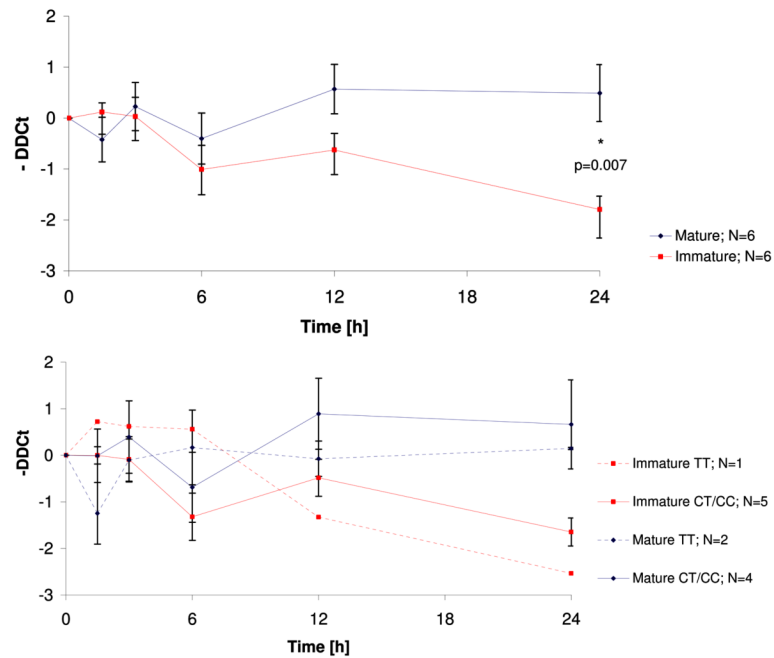
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**Figure 1. NRG1 protein concentration in human umbilical vein endothelial cells (HUVECs) from mature compared with premature newborns**

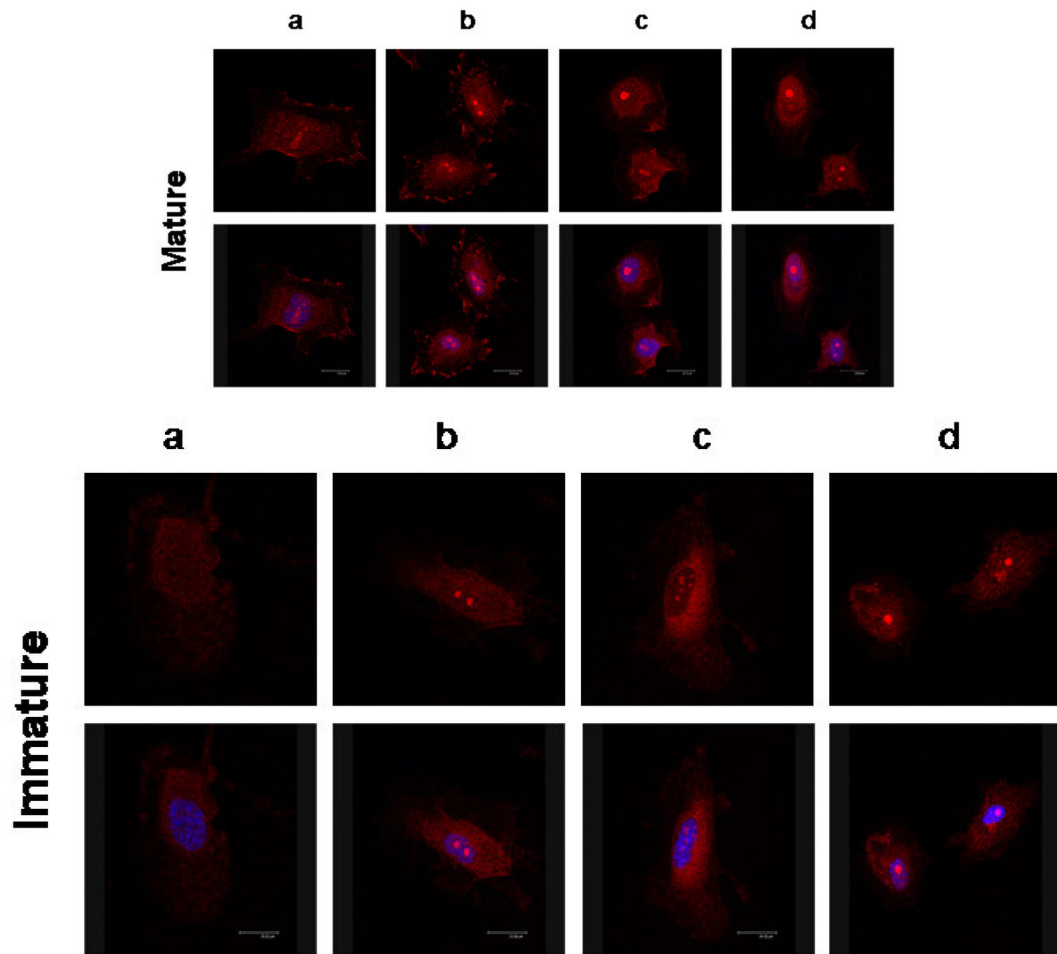
Mean intracellular concentration of NRG1 protein in HUVECs from term (mature) (N=2) vs. preterm (immature) newborns (N=3). All HUVECs carried the same genotype (CT) at the SNP8NRG221533 locus. NRG1 concentration was measured by ELISA after 24 hours of LPS-treatment (100ng/ml) and is presented as percent of the experiment specific non-treated control cells. Error bars indicate standard errors of the mean (SEM).



**Figure 2. Relative NRG1 mRNA concentrations in HUVECs dependent on gestational age (2.1) and stratified by NRG1 genotype (2.2)**

NRG1 mRNA concentration was measured by real time RT-PCR. Data are presented as mean  $DDCt_{\pm SEM}$ . Cells were treated with LPS (100ng/ml) for 1.5, 3, 6, 12 and 24 hours. DDCts were calculated as the difference between treated sample and their non-treated experiment-specific control cells with a negative result in case of a lead in crossing the threshold line thereby indicating more NRG1 mRNA within the raw material.





**Figure 3. Nuclear localization of NRG1 in human umbilical venous endothelial cells (HUVECs) in response to LPS-treatment**

Cellular localization of NRG1 (red) in mature (3.1) and immature (3.2) HUVECs after 30 minutes (a) and 24 hours (c) serum starvation and equivalent durations of LPS-treatment (b, d). The nucleus marked by DAPI is indicated by blue color in the bottom rows.

**Table 1**

Characteristics of the study population. Data are given for all children and for groups defined by NRG1 genotype CC/CT vs. TT, and the presence or absence of PVL, CP and DD >= 12 months. If not indicated otherwise, numbers are column percents. Variables tested as potential confounding factors are marked in grey.

	All children N=54		SNP8NRG221533 genotype		Cystic Periventricular Leukomalacia			Cerebral Palsy			Developmental Delay ≥ 12 months		
	CC, CT N=42	TT N=12	OR (CI 95%)	OR (CI 95%)	Yes N=10	No N=44	OR (CI 95%)	Yes N=5	No N=49	OR (CI 95%)	Yes N=9	No N=45	OR (CI 95%)
NRG1 CC/CT	%	%	-	-	%	%	%	%	%	%	%	%	%
Gestation < 30 wks	63	67	0.8 (0.2-3.1)		90	57	6.8 (0.8-59)	80	61	2.5 (0.3-24)	89	58	5.9 (0.7-51)
Birth weight < 1000g	44	33	1.8 (0.5-7.0)		70	39	3.7 (0.8-16)	80	41	5.8 (0.6-56)	67	40	3.0 (0.7-14)
Small for gestational age	22	17	1.6 (0.3-8.4)		20	23	0.9 (0.2-4.7)	40	20	2.6 (0.4-18)	33	20	2.0 (0.4-9.6)
Male	50	45	0.4 (0.1-1.6)		60	48	1.6 (0.4-6.6)	60	49	1.6 (0.2-10)	67	47	2.3 (0.5-10)
Mother German	72	83	0.5 (0.1-2.3)		60	75	0.5 (0.1-2.1)	100	69	-	78	71	1.4 (0.3-7.8)
Multiple pregnancy	17	14	0.5 (0.1-2.4)		10	18	0.5 (0.1-4.5)	20	16	1.3 (0.1-13)	11	18	0.6 (0.1-5.3)
Prenatal glucocorticoid, complete	81	76	100		70	84	0.4 (0.1-2.1)	80	82	0.9 (0.1-9.1)	78	82	0.8 (0.1-4.3)
Pregnancy induced hypertension	19	17	1.2 (0.2-6.5)		20	18	1.1 (0.2-6.3)	20	18	1.1 (0.1-11)	11	20	0.5 (0.1-4.5)
Preterm labor	65	83	0.3 (0.1-1.5)		80	61	2.5 (0.5-13)	80	63	2.3 (0.2-22)	89	60	5.3 (0.6-46)
Rupture of membranes > 12hrs	22	17	1.6 (0.3-8.4)		30	20	1.7 (0.4-7.8)	20	22	0.9 (0.1-8.5)	22	22	1.0 (0.2-5.6)
Chorioamnionitis	30	29	0.8 (0.2-3.2)		60	23	5.1 (1.2-22)	20	31	0.6 (0.1-5.5)	44	27	2.2 (0.5-9.6)

**Table 2**

Associations between SNP8NRG221533 genotypes and neurologic outcome.

		SNP8NRG221533 genotype					
		CC	CT	TT	Total		
		n	n	n	N	<i>p</i>	
<b>Cystic Periventricular Leukomalacia</b>	yes	0	7	3	10	0.121	
	no	6	29	9	44		
<b>Cerebral Palsy</b>	yes	0	2	3	5	<b>0.021</b>	
	no	6	34	9	49		
<b>Developmental Delay ≥ 12 months</b>	yes	0	5	4	9	<b>0.027</b>	
	no	6	31	8	45		

Table 3

Risk analyses for development of neurodevelopmental outcome using SNP8NRG221533 (CC/CT vs TT) and one confounder at a time

	Cystic Periventricular Leukomalacia (n=10)	Cerebral Palsy (n=5)	Developmental Delay ≥ 12 months (n=9)
	OR (CI 95 %)	OR (CI 95 %)	OR (CI 95 %)
SNP8NRG221533 (CC/CT vs TT)	0.6 (0.1-3.1)	0.2 (0.02-1.1)	0.3 (0.1-1.3)
Gestational age < 30 wks	6.8 (0.8-58)	2.5 (0.2-26)	6.0 (0.7-54)
SNP8NRG221533 (CC/CT vs TT)	0.5 (0.1-2.4)	<b>0.1 (0.01-0.8)</b>	<b>0.2 (0.04-1.0)</b>
Birth weight < 1000g	4.1 (0.9-19)	11 (0.9-140)	4.1 (0.8-22)
SNP8NRG221533 (CC/CT vs TT)	0.7 (0.1-3.1)	0.2 (0.02-1.1)	0.3 (0.1-1.4)
Male	1.5 (0.4-6.4)	1.1 (0.2-8.1)	1.9 (0.4-9.0)
SNP8NRG221533 (CC/CT vs TT)	0.7 (0.2-3.5)	0.2 (0.02-1.2)	0.4 (0.1-1.6)
Preterm labor	2.4 (0.4-13)	1.5 (0.1-17)	4.4 (0.5-39)
SNP8NRG221533 (CC/CT vs TT)	0.6 (0.1-3.2)	<b>0.1 (0.02-1.0)</b>	0.3 (0.1-1.3)
Chorioamnionitis	<b>5.1 (1.2-22)</b>	0.5 (0.1-5.2)	2.2 (0.5-10)