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Oxygen restriction of neonate rats elevates neuregulin-1α isoform levels: Possible relationship to schizophrenia

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Abstract

Neuregulin-1 (NRG-1), a replicated gene in schizophrenia-association studies, exhibits six mRNA-types and two types of the EGF-like domain, α and β. The β-isoform was extensivly studied, less is known about the extent and specific localization of adult brain NRG-1α. NRG-1α protein levels were reported reduced in postmortem prefrontal-cortex of schizophrenia patients. NRG-1 type I mRNA levels were found higher in postmortem brain in schizophrenia. In an attempt to decipher between a genetic or environmental involvement in the differences in NRG-1 levels in postmortem brain in schizophrenia, and since obstetric complications were suggested non-genetic risk-factors of schizophrenia, we studied the effect of perinatal hypoxia in rats on brain NRG-1α protein levels. Seven-day-old rats were exposed to hypoxia versus air. Frontal-cortex levels of NRG-1α isoform were quantified at adulthood by Western blotting. Frontal-cortex NRG-1α was 32% elevated in hypoxia-exposed rats. The data support the role of non-genetic factors, e.g. oxygen restriction, in the expression of genes associated with schizophrenia.

Keywords: Neuregulin-1; Schizophrenia; Rat model; Hypoxia; Oxygen restriction; Postmortem brain

1. Introduction

Neuregulin-1 (NRG-1) is an extremley large gene (1.4 MB) encoding over 15 peptides, derived from six alternatively spliced mRNAs (Harrison and Weinberger, 2005; Harrison and Law, 2006; Law et al., 2004) and two types of the EGF-like domain, NRG-1α and β (Falls, 2003). The β-isoform was extensivly studied but less is known about the α-isoform. NRG-1 is involved in neurodevelopmental processes, e.g. neuronal-migration, axon-guidance, synaptogenesis and myelination, neurotransmission and synaptic-plasticity (Lemke, 1996). It is located on chromosome 8p12-21, and is one of highly replicated findings in linkage and association studies of schizophrenia (Li et al., 2006; Munafo et al., 2006). Harrison and Weinberger (2005) suggested that the genetic risk for schizophrenia associated with NRG-1 is mediated by NRG-1 signaling in a complex manner that could be affected by environment as well as genetic factors.

The extent and specific localization of NRG-1α in adult brain has not been elucidated. A recent study reported its expression in cortical neurons in the dorsolateral-prefrontal-cortex (DLPFC), in nerve cells of white-matter, in hippocampal interneurons and in other brain areas (Bernstein et al., 2006). Differently from NRG-1β null mice, NRG-1α$^{-/-}$ mice survive to adulthood, yet they were not phenotyped behaviorally nor studied for CNS morphology and function (Li et al., 2002). NRG-1α protein levels were found reduced in postmortem white and gray matter prefrontal-cortex in schizophrenia (Bertram et al., 2007).

Hashimoto et al. (2004) and Law et al. (2006) reported significantly elevated NRG-1 type I mRNA levels (α and β splice-variants) in postmortem DLPFC and hippocampus, and Hahn et al. (2006) found no difference in NRG-1 protein levels in postmortem prefrontal-cortex slices in schizophrenia. Law et al.'s supplementary discussion (2006) suggested that increased type I NRG-1 expression in schizophrenia may be related to environmental influences on NRG-1 transcript regulation.

Since perinatal oxygen-restriction is implicated as a schizophrenia risk-factor (Cannon et al., 2002) and taken Schmidt-Kastner et al.'s (2006) literature screening to identify

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schizophrenia candidate genes meeting criteria for ischemia-hypoxia regulation pointing at NRG-1, we studied the effect of perinatal oxygen restriction on NRG-1α protein levels.

2. Methods

2.1. Induction of hypoxia to rat pups

All procedures were approved by the Ben-Gurion University Committee for the Ethical Care of Animals in Experiments. Timed pregnant Sprague–Dawley rats were raised until delivery, and pups raised by their dam. At postnatal day (PD) 6 the pups were counted, sex determined and a maximum of 10 pups were left per dam. On PD7 each four pups were subjected to hypoxia conditions or air in a 4-section-chamber. For hypoxia, pups ($n = 25$) were exposed to air mixture of 8% O_2, 0.03% CO_2, balance N_2 for 120 min. For air exposure, pups ($n = 11$) were exposed to air mixture of 20% O_2, 0.03% CO_2, balance N_2 for 120 min. For both conditions flow rate was 0.4–0.5 l/min. The chambers were placed in a 37 °C incubator. Following the exposure to hypoxia the pups were subjected to a 10 min recovery period in air before being returned to their dam. Pups were weaned at PD21 and separated by sex. Only male pups were used for the study of protein levels. Animals were group housed (4–8 animals/cage) in a random treatment combination and maintained on a 12-h light:12-h dark cycle with free access to food and water. On PD56 (post-pubertal age) rats were sacrificed, brains removed, frontal cortex dissected on ice and immediately stored at −70 °C until extraction. Mean rat pups weight did not differ between the two groups at any time point measured.

To confirm the effect of oxygen restriction on the brain, nuclear hypoxia-inducible factor (HIF)-1 protein levels of 6-day-old rat pups exposed to hypoxia conditions were measured. HIF-1 is a transcription factor used as a marker for oxygen deprivation (Carmeliet et al., 1998). A 120 kDa band of HIF-1 protein was detected in the nuclear fraction of only the hypoxic rats [air exposed rats: no HIF-1 immunoreactivity ($n = 3$); hypoxia-exposed rats: 26 ± 13 arbitrary densitometric units/100 μg protein ($n = 6$)].

2.2. Preparation of cytosolic- and nuclear-enriched fractions for HIF-1 Western blotting

Fifty milligrams tissue was homogenized with a hand-held 7 ml Teflon-on-glass grinder (Wheaton, Millville, NJ) in 0.3 ml lysis buffer (15 strokes). Homogenates centrifuged for 10 min at 2500 × g in a refrigerated centrifuge (Eppendorf, Germany). Supernatant and pellet separated for processing to generate cytosolic- and nuclear-enriched extracts, respectively. The supernatant was centrifuged at 14,000 × g and 4 °C for 15 min; the supernatant obtained used as the cytosolic fraction. For nuclear extract the pellet was suspended in 0.3 ml lysis buffer, sonicated 15 s, 4 °C and 50% power capacity, centrifuged at 14,000 × g and 4 °C for 20 min. The supernatant used as the nuclear extract.

2.3. Western-blot analysis

Crude homogenate was prepared as previously described (Kozlovsky et al., 2000). Sodium-dodecyl-sulfate/polyacrylamide gel-electrophoretic separation (SDS-PAGE) and immunoblotting were performed as previously described (Kozlovsky et al., 2000). Briefly, aliquots (25–50 μg, within linear range of quantitative detection) were separated, blotted and probed at room temperature for an hour, then over-night at 4 °C with diluted antibodies [anti-GSK-3β 1:2500, used for normalization among gels as previously described (Kozlovsky et al., 2000) (from BD Transduction-Laboratories, Lexington, KY), anti-NRG-1 1:100 (Santa-Cruz, CA, antibody SC-1791) and anti-HIF-1 1:50 (Santa-Cruz, CA, antibody SC-10790)]. Membranes were probed with secondary antibody anti-mouse 1:10,000 (GSK-3β, Upstate, NY), anti-goat 1:10,000 (NRG-1) or anti-rabbit 1:10,000 (HIF-1) both from Santa-Cruz, CA, for an hour on an orbital shaker at room-temperature. Bands were detected with Western-Blotting Luminol reagent (Santa-Cruz, CA). Densities of immunoreactive bands were quantified using AIDA-2D image-analysis system (Dinco-Israel). The experimenter who carried out the assays (CN) was blind to the identity of the samples.

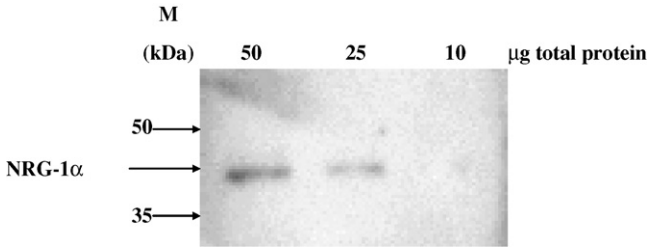

Fig. 1. A representative blot of the linear range of detection of rat frontal cortex NRG-1α 44 kDa protein isoform. M = protein standards marker (kDa).

2.4. Statistical analysis

Mean values were compared by Student's t-test.

3. Results

According to the manufacturer's indication the antibody used recognizes NRG-1α isoforms exhibiting a molecular weight of ∼40 kDa [Heregulin (HRG)-α and HRG-α2B and, to a lesser extent, HRG-α1A and HRG-α3] first purified and identified by Holmes et al. (1992) as a 45 kDa protein. To substantiate the specificity of the manufacturer's antibody, membranes were pre-absorbed with an NRG-1α blocking peptide (SC-1791P) according to the manufacturer's protocol prior to the Western blot analysis. No ∼40 kDa band was detected with the blocking peptide confirming that the band monitored with the antibody represents NRG-1α (data not shown).

Fig. 1 demonstrates a representative blot of the linear range of detection and Fig. 2 demonstrates a representative experiment of NRG-1 44 kDa measurement. Fig. 3 shows that protein levels of the NRG-1α 44 kDa isoform were 32% significantly elevated in the hypoxic rats [119.6 ± 48.9 (S.D.) arbitrary units/μg protein (hypoxia, $n = 25$) versus 90.9 ± 21.8 (air, $n = 11$); $t_{(34)} = 2.43$, $p = 0.02$].

4. Discussion

Bernstein et al. (2006) recently reported widely spread NRG-1α expression in adult brain in neurons of multiple cortical areas. In agreement with Bernstein et al. (2006), using a different manufacturer's antibody, we also found significant abundance of NRG-1α protein in rat frontal cortex. A recent postmortem brain study of Bernstein's group (Bertram et al.,

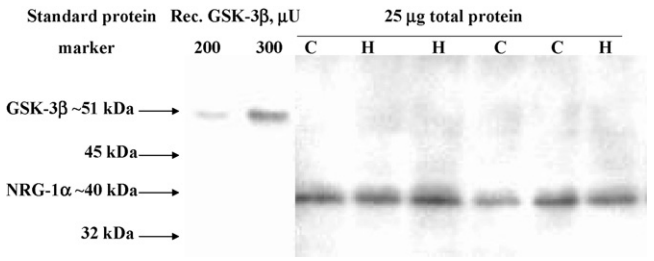

Fig. 2. A representative blot of hypoxic and control rat frontal cortex NRG-1α protein isoform levels. C = control rats, H = hypoxic rats.