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Nuclear respiratory factor 2 regulates the expression of the same NMDA receptor subunit genes as NRF-1: Both factors act by a concurrent and parallel mechanism to couple energy metabolism and synaptic transmission

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ABSTRACT

Neuronal activity and energy metabolism are tightly coupled processes. Previously, we found that nuclear respiratory factor 1 (NRF-1) transcriptionally co-regulates energy metabolism and neuronal activity by regulating all 13 subunits of the critical energy generating enzyme, cytochrome c oxidase (COX), as well as *N*-methyl-D-aspartate (NMDA) receptor subunits 1 and 2B, GluN1 (*Grin1*) and GluN2B (*Grin2b*). We also found that another transcription factor, nuclear respiratory factor 2 (NRF-2 or GA-binding protein) regulates all subunits of COX as well. The goal of the present study was to test our hypothesis that NRF-2 also regulates specific subunits of NMDA receptors, and that it functions with NRF-1 *via* one of three mechanisms: complementary, concurrent and parallel, or a combination of complementary and concurrent/parallel. By means of multiple approaches, including *in silico* analysis, electrophoretic mobility shift and supershift assays, *in vivo* chromatin immunoprecipitation of mouse neuroblastoma cells and rat visual cortical tissue, promoter mutations, real-time quantitative PCR, and western blot analysis, NRF-2 was found to functionally regulate *Grin1* and *Grin2b genes*, but not any other NMDA subunit genes. *Grin1* and *Grin2b* transcripts were up-regulated by depolarizing KCl, but silencing of NRF-2 prevented this up-regulation. On the other hand, overexpression of NRF-2 rescued the down-regulation of these subunits by the impulse blocker TTX. NRF-2 binding sites on *Grin1* and *Grin2b* are conserved among species. Our data indicate that NRF-2 and NRF-1 operate in a concurrent and parallel manner in mediating the tight coupling between energy metabolism and neuronal activity at the molecular level.

1. Introduction

Glutamate is the main excitatory neurotransmitter in the brain and *N*-methyl-D-aspartate (NMDA) receptors are a major type of glutamatergic receptors. NMDA receptors are ligand-gated, voltage-dependent ionotropic receptors (for a review see [1]). They are heterotetrameric proteins composed of the ubiquitous GluN1 subunit in various combinations with the GluN2A-D and GluN3A-B subunits [2–4]. While the NMDA receptors are crucial for the proper functioning and activity of neurons, the properties of the receptors are dictated by their subunit composition. Most NMDA receptors are composed of two GluN1 subunits with two GluN2A or GluN2B subunits [2,5]. GluN1/GluN2A receptors are fast-acting and widely expressed in the adult brain, while the GluN1/GluN2B receptors are slower acting but widely expressed in the neonatal and the adult brain [3]. The GluN2C, GluN2D, GluN3A, and GluN3B receptor subunits are more developmentally and regionally expressed [4,6].

Our laboratory has recently shown that GluN1 and GluN2B receptor subunits are regulated at the transcriptional level by nuclear respiratory factor 1 (NRF-1) [7], which also regulates all 13 subunits of cytochrome c oxidase (COX) [8], an enzyme critical for energy production in neurons (for a review see [9]). Perturbations of neuronal activity result in concurrent changes in mRNA and protein levels of COX subunits as well as those of GluN1 (*Grin1*) and GluN2B (*Grin2b*) subunits [7,8]. Likewise, altering the expression of NRF-1 leads to parallel changes in COX, GluN1, and GluN2B mRNA and protein expression [7,8]. Thus NRF-1 co-regulates mediators of synaptic transmission and energy generation, thereby coupling neuronal activity to energy metabolism.

Nuclear respiratory factor 2 (NRF-2), or GA binding protein (GAPB), is a transcription factor in the E-26 transformation-specific (ETS) family (for a review see [10]). The functional protein is composed of α and β subunits that form either a heterodimer or a heterotetramer ($\alpha_2\beta_2$) [10]. The α subunit contains the ETS DNA binding domain that binds to the 'GGAA' or 'TTCC' motif, and the β subunit contains the transcriptional activation domain [10]. NRF-2 is involved in the control of basic cellular processes, such as cell cycle progression, protein synthesis,

Abbreviations: NRF-2/GABP, Nuclear respiratory factor 2/GA binding protein; Grin, Gene name for NMDA receptor

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and mitochondrial biogenesis [11–13]. NRF-2 is present in neurons, with neuronal activity regulating transcription and nuclear translocation of both its subunits [14–16]. NRF-2 and COX co-exist at the immunohistochemical level, and physiological perturbations of neuronal activity result in parallel changes in NRF-2 and COX protein expression [17,18]. Our lab has recently discovered that NRF-2, like NRF-1, transcriptionally regulates all subunit genes of the COX enzyme [19–21]. The question naturally arises as to whether NRF-2 also couples energy metabolism to neuronal activity by regulating specific subunits of the NMDA receptors. If so, do the two transcription factors operate *via* complementary, concurrent and parallel, or a combined complementary and concurrent/parallel mechanism? The goal of the present study was to test our hypothesis that NRF-2 also mediates the coupling of synaptic transmission and energy metabolism.

2. Material and methods

All experiments were carried out in accordance with the US National Institutes of Health Guide for the care and use of laboratory animals and the Medical College of Wisconsin regulations. All efforts were made to minimize the number of animals and their suffering.

2.1. Cell culture

Murine neuroblastoma (N2a) cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. In silico analysis of promoters of murine NMDA receptor subunit genes

DNA sequences surrounding the transcription start points (TSPs) of *N*-methyl-D-aspartate (NMDA) receptor subunit genes (*Grin1*, *Grin2a-d*, *Grin3a-b*) were derived from the NCBI mouse genome database (*Grin1* GenBank ID: NC_000068.7, *Grin2a* GenBank ID: NC_000082.6, *Grin2b* GenBank ID: NC_000072.6, *Grin2c* GenBank ID: NC_000077.6, *Grin2d* GenBank ID: NC_000073.6, *Grin3a* GenBank ID: NC_000070.6, and *Grin3b* GenBank ID: NC_000076.6). These promoter sequences encompassed 1 kb upstream and 1 kb downstream of the TSP of each gene analyzed. Computer-assisted search for NRF-2's binding motif 'GGAA', or its complement 'TTCC', separated by up to 24 base pairs (bp) from another such NRF-2 binding motif, was conducted on each promoter.

Alignment of human, mouse, and rat promoter sequences was performed with NCBI's Ensembl interface. Mouse NMDA receptor promoter sequences were compared with those of rat and human genomic sequences for conservation of the NRF-2 binding motif.

2.3. Electrophoretic mobility shift and supershift assays

Electrophoretic mobility shift assays (EMSA) for possible NRF-2 interactions with putative binding elements on all NMDA receptor subunit promoters were carried out with a few modifications from methods previously described [21]. Briefly, based on *in silico* analysis, oligonucleotide probes with a putative NRF-2 binding motif in a tandem repeat on each NMDA receptor subunit promoter were synthesized (Table 1A), annealed, and labeled by a Klenow fragment (Invitrogen, Grand Island, NY, USA) fill-in reaction with [α-^{32}P] dATP (50 μCi/200 ng; Perkin-Elmer, Shelton, CT, USA). N2a nuclear extract was isolated using methods described previously [22]. Each labeled EMSA probe was incubated with 2 μg of calf thymus DNA and 15 μg of N2a nuclear extract. The probe reaction was processed for EMSA. Supershift assays were performed with 0.4 μg of NRF-2 specific antibody (polyclonal rabbit antibody, H-180, sc-22810, Santa Cruz Biotechnology, Santa Cruz, CA, USA) added to the probe/nuclear extract mixture and incubated for 20 min at 24 °C. For competition, 100-fold excess of unlabeled oligonucleotides was incubated with nuclear extract before the addition of labeled oligonucleotides. Shift reactions were loaded onto 4.5% polyacrylamide gel (58:1, Acrylamide:Bisacrylamide) and run at 200 V for 4.2 h in 0.25× Tris-borate-EDTA buffer. Results were visualized by autoradiography and exposed on film. Rat cytochrome c oxidase subunit 6b (*COX6b*) with known NRF-2 binding site was designed as previously described [21] and used as a positive control. NRF-2 mutants with mutated sequences, as shown in Table 1B, were used as negative controls.

Table 1A
EMSA probes. Positions of probes are given relative to TSP. Putative NRF-2 binding sites are underlined.

Gene promoter	Position	EMSA sequence
Grin1	−565/	F: 5′ TTTTCTGGGGGAAGGATATTGGTGATTTCCTTCT 3′
	−545	R: 5′ TTTTAGAAGGAAATCACCAATATCCTTCCCCCAG 3′
Grin2a	−334/	F: 5′ TTTTAAATTTGGGGAATCTTGTGTGGAATTTGGA 3′
	−319	R: 5′ TTTTTCCAAATTCCACACAAGATTCCCCAAATTT 3′
Grin2b	−159/	F: 5′ TTTTAGCTTTTCCCACCCCCTGGCTACCCCACTTCCCCCA 3′
	−133	R: 5′ TTTTTGGGGGAAGTGGGGTAGCCAGGGGGTGGGAAAAGCT 3′
Grin2c	−385/	F: 5′ TTTTTATCTGGAAGCTTGAAAGTGGAAAGGCA 3′
	−368	R: 5′ TTTTTGCCTTTCCACTTTCAAGCTTCCAGATA 3′
Grin2d	−330/	F: 5′ TTTTTGGGTTCCATCTTTCCCTTCCACTC 3′
	−314	R: 5′ TTTTGAGTGGAAGGGAAAGATGGAACCCA 3′
Grin3a	−598/	F: 5′ TTTTGAAAAAGGAAGAGAGGAAGTAGAA 3′
	−587	R: 5′ TTTTTTCTACTTCCTCTCTTCCTTTTTC 3′
Grin3b	−183/	F: 5′ TTTTAGTGTACTTTTCCCCCAACAAA 3′
	−180	R: 5′ TTTTTTTGTTGGGGGAAAAGTACACT 3′
COX6b	−47/	F: 5′ TTTTTCCTCTTGCAGCTTCCGGCCAGTC 3′
	−23	R: 5′ TTTTGACTGGCCGGAAGCTGCAAGAGGA 3′

2.4. Chromatin immunoprecipitation (ChIP) assays in N2a cells

ChIP assays were performed similar to those described previously [7]. Briefly, 1×10^6 N2a cells were used for each immunoprecipitation reaction. Cells were fixed with 1% formaldehyde for 10 min at 24 °C. Following formaldehyde fixation, cells were resuspended in swelling buffer (5 mM PIPES, pH 8.0, 85 mM KCl, and 1% Nonidet P-40 (Sigma, St Louis, MO, USA), with protease inhibitors added right before use) and homogenized 10 times in a small pestle Dounce tissue homogenizer (5 mL). Nuclei were then isolated by centrifugation before being subjected to sonication in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1 (Sigma)). The sonicated lysate was immunoprecipitated with either 1 μg of NRF-2 polyclonal rabbit antibody (H-180, Santa Cruz Biotechnology) or 2 μg of anti-nerve growth factor receptor (NGFR) p75 polyclonal goat antibody (C20, sc-6188, Santa Cruz Biotechnology). Semi-quantitative PCR was performed using 1/20th of precipitated chromatin. Primers encompassing putative NRF-2 tandem repeats near TSPs of NMDA receptor subunit genes (identified in *in silico* analysis) were designed (Table 2) as previously described [8]. *COX6b* promoter with NRF-2 binding site was used as a positive control, and exon 8 of *NRF-1*, a region of DNA that does not contain a NRF-2 binding site, was used as a negative

Table 1B
Mutant EMSA probes. Positions of probes are given relative to TSP. Mutated NRF-2 binding sites are underlined.

Gene promoter	Position	Sequence
Grin1	−565/	F: 5′ TTTTCTGGGTTTTGGATATTGGTGATAAAATTCT 3′
	−545	R: 5′ TTTTAGAATTTTATCACCAATATCCAAAACCCAG 3′
Grin2b	−159/	F: 5′ TTTTAGCTTAAAACACCCCCTGGCTACCCCACAAAACCCA 3′
	−133	R: 5′ TTTTTGGGTTTTGTGGGGTAGCCAGGGGGTGTTTTAAGCT 3′
Grin3a	−598/	F: 5′ TTTTGAAAAATTTTGAGATTTTGTAGAA 3′
	−587	R: 5′ TTTTTTCTACAAAATCTCAAAATTTTTC 3′