Special protein 4 (Sp4) regulates the transcription of AMPA receptor subunit GluA2 (Gria2)

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Abstract

The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are important glutamatergic receptors mediating fast excitatory synaptic transmission in the brain. The regulation of the four subunits of AMPA receptors, GluA1-4, is poorly understood. Excitatory synaptic transmission is highly energy-demanding, and this energy is derived mainly from the oxidative pathway. Recently, we found that specificity factor regulates all subunits of cytochrome c oxidase (COX), a critical energy-generating enzyme. COX is also regulated by nuclear respiratory factor 1 (NRF-1), which transcriptionally controls the Gria2 (GluA2) gene of AMPA receptors. The goal of the present study was to test our hypothesis that Sp-factors (Sp1, Sp3, and/or Sp4) also regulate AMPA subunit genes. If so, we wish to determine if Sp-factors and NRF-1 function via a complementary, concurrent and parallel, or a combination of complementary and concurrent/parallel mechanism. By means of multiple approaches, including electrophoretic mobility shift and supershift assays, chromatin immunoprecipitation, promoter mutations, real-time quantitative PCR, and western blot analysis, we found that Sp4, but not Sp1 or Sp3, regulates the Gria2, but not Gria1, 3, or 4, subunit gene of the AMPA receptor in a concurrent and parallel manner with NRF-1. Thus, Sp4 and NRF-1 both mediate the tight coupling between neuronal activity and energy metabolism at the transcriptional level.

1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain, and the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are a major class of glutamatergic receptors (for review see [1]). AMPA receptors are heterotetramers composed of various combinations of the GluA1, GluA2, GluA3, and GluA4 subunits [2]. They are widely expressed in the brain, where they mediate the majority of ionotropic, ligand-gated, fast excitatory synaptic transmission [1]. Such, AMPA receptors play crucial roles in normal neuronal activity, including synaptic plasticity, synaptic scaling, homeostatic synaptic plasticity, learning and memory, as well as in synaptogenesis and the formation of neuronal circuitry [3-6]. Most AMPA receptors in the cerebral neocortex and hippocampus contain the GluA2 subunit, usually in combination with the GluA1 subunit [7,8]. The GluA2 subunit is unique, as its inclusion in the heterotetramer renders AMPA receptors significantly less permeable to Ca2+ [9]. In the adult, GluA2 expression is much lower than those of GluA1 or GluA2 [7,10]. The GluA4 subunit is present mainly during development [11].

We have shown previously that the expression of GluA2 is governed by neuronal activity [12,13]. Excitatory glutamatergic neuronal activity is highly energy-demanding, with most of this energy utilized to extrude excess cations that enter through glutamatergic receptors (for reviews see [14]). Thus, at the cellular level, an intimate link exists between excitatory activity and the energy requirements of neurons. Recently, we found that this link extends to the molecular level, in that the same transcription factor, nuclear respiratory factor 1 (NRF-1), co-regulates genes that mediate energy metabolism and neuronal activity. Specifically, NRF-1 regulates the expression of all 13 subunits of the energy-generating enzyme, cytochrome c oxidase (COX), as well as the expression of the Gria2 subunit of the AMPA receptor [15,16].

Specificity protein (Sp) is a family of zinc-finger transcription factors that binds to GC-rich DNA motifs (for review see [17]). Sp1, Sp3, and Sp4 are three members of the Sp family that compete for the same cis-motifs: the GC box ‘GGGCGG’ with high affinity, or the GT (‘GGGTGC’) and CT (‘CCTCC’) boxes with significantly lower affinities [18,19]. While the expressions of Sp1 and Sp3 are ubiquitous, that of Sp4 is restricted mainly to neurons and testicular cells [19]. Recently, we found that, like NRF-1, Sp factors also regulate all 13 subunits of COX [20]. The question arises as to whether Sp factors also couple energy metabolism to neuronal activity by regulating specific subunits of the AMPA receptor. If so, do they operate with NRF-1 via a complementary

Keywords: AMPA receptor, Gene regulation, GluA2, Sp4, Specificity protein 4, Transcription factor

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Abbreviations: Sp, specificity protein transcription factor; Gria, gene name for AMPA receptor subunit

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(regulating different subunits), concurrent and parallel (regulating the same subunit in the same direction), or a combination of complementary and concurrent/parallel mechanism? The goal of the present study was to test our hypothesis that Sp1, Sp3, and Sp4 also mediate the transcriptional coupling of synaptic transmission and energy metabolism.

2. Material and methods

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996), and all protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee (approval can be provided upon request). All efforts were made to minimize the number of animals used and their suffering.

2.1. Cell culture

The mouse neuroblastoma (N2a) cell line was acquired from ATCC (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (100 μg/mL) at 37 °C with 5% CO2.

 Cultures of murine or rat primary visual cortical neurons were carried out as described previously [21]. Briefly, neonatal mouse or rat pups (1-day-old) were killed by decapitation. Visual cortical tissue was removed, trypsinized, and triturated. Individual neurons were then plated in poly-L-lysine-coated, 35 mm dishes at a density of 200,000 cells/dish and maintained in Neurobasal-A media supplemented with B27. To suppress the proliferation of glial cells, Ara-C (Sigma, St Louis, MO, USA) was added.

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

DNA sequences surrounding the transcription start points (TSPs) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit genes (Gria1–4) were derived from the NCBI mouse genome database (Gria1 GenBank ID: NC_000077.6, Gria2 GenBank ID: NC_000069.6, Gria3 GenBank ID: NC_000086.7, and Gria4 GenBank ID: NC_000075.6). Computer-assisted search for the typical Sp1 binding motif (‘GGGCGG’), the atypical Sp1 binding motifs (‘GGGTGG’ and ‘CCCTCC’), or their complements, was conducted on sequences encompassing 1 kb upstream and 1 kb downstream of the TSP of each gene. These motifs were applicable to Sp1, Sp3, or Sp4.

To determine the degree of conservation of the Sp binding motif among species, NCBI's Ensembl interface was used to align promoters of AMPA receptor subunits from mice, rats, and humans.

2.3. Electrophoretic mobility shift and supershift assays

To determine if Sp1, Sp3, or Sp4 bound in vivto to putative Sp binding elements in the promoter regions of AMPA receptor subunit genes, electrophoretic mobility shift assays (EMSA) were carried out as described previously with a few modifications [22]. Briefly, oligonucleotide probes containing putative Sp binding sequences on each AMPA receptor subunit promoter (identified from in silico analysis), were synthesized (Table 1), annealed, and labeled with [α-32P]dATP (50 μCi/200 ng; Perkin-Elmer, Shelton, CT, USA) using Klenow fragment (Invitrogen).

 Nuclear extract from mouse primary visual cortical tissue and HeLa cells were isolated as described previously [23,24]. 10 μg of either mouse cortical and/or HeLa nuclear extract was incubated with 2 μg of calf thymus DNA and with each labeled EMSA probe. Supershift assays were performed with 1 μg of Sp4, 3, or 1 specific antibody (Sp4, V-20, SC645; Sp3, H-225; SC13018; Sp1, H-225, SC14027; all from Santa Cruz Biotechnology (SCBT), Santa Cruz, CA, USA) and incubated with the probe/nuclear extract mixture for 20 min.

2.4. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed on murine visual cortical tissue as described previously [24]. Briefly, murine visual cortical tissue was quickly removed, finely chopped, fixed in 2% formaldehyde, and resuspended in swelling buffer (85 mM KCl, 5 mM PIPES, pH 8.0, 1% Nonidet P-40, and protease inhibitors). The tissue was homogenized in a Dounce tissue homogenizer and centrifuged to isolate the nuclei. The nuclei were resuspended in 2% formaldehyde, and resuspended in 2% agarose gels in 2% formaldehyde, and resuspended in 2% agarose gels in 0.25× Tris-borate-EDTA buffer. Results were visualized on a phosphorimager and exposed on film. The positive control probe, murine GM3 synthase gene, is known to bind Sp1 and contains two Sp1 binding sites in a tandem repeat [25]. The negative controls were AMPA receptor subunit probes with mutated Sp binding sequences (Table 1).

2.5. Construction of luciferase reporter vectors and transfection in N2a cells

The Gria2 luciferase reporter construct was created by PCR amplification of the mouse Gria2 proximal promoter (primers listed in Table 3). The PCR product was digested with restriction enzymes (Mlu and BglIII), and ligated into Promega’s pG3 luciferase vectors (E1751, Promega, Madison, WI, USA). The QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) was used to mutate putative Sp binding sites. Primers used to create Sp mutations are listed in Table 3. Sequencing was done to verify all constructs.

N2a cells, plated at 60% confluency in 24-well plates, were transfected with either 0.6 μg of the Gria2 reporter construct or 0.6 μg of the Gria2 reporter construct with mutated Sp binding site. pRL-TK (0.06 μg), a vector with a thymidine kinase promoter that constitutively expresses renilla luciferase, was added to each well and served as an internal control for

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**Table 1**

<table>
<thead>
<tr>
<th>Gene promoter</th>
<th>Position</th>
<th>EMSA sequence</th>
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</thead>
<tbody>
<tr>
<td>Gria1</td>
<td>−189/−172</td>
<td>F: 5′ TTTTGGAGAGCGGAGGGATGAT 3′</td>
</tr>
<tr>
<td>Gria2</td>
<td>−89/−64</td>
<td>F: 5′ TTTTGGCGTGGCCTAGGTGATCC 3′</td>
</tr>
<tr>
<td>Gria3</td>
<td>−87/−69</td>
<td>F: 5′ TTTTGGCGTGGCCTAGGTGATCC 3′</td>
</tr>
<tr>
<td>Gria4</td>
<td>+507/+527</td>
<td>R: 5′ TTTTGGCGTGGCCTAGGTGATCC 3′</td>
</tr>
<tr>
<td>GM3 synthase</td>
<td>−58/−38</td>
<td>F: 5′ TTTTGGCGTGGCCTAGGTGATCC 3′</td>
</tr>
<tr>
<td>Mutant Sp Gria2</td>
<td>−89/−64</td>
<td>R: 5′ TTTTGGAGAGCGGAGGGATGAT 3′</td>
</tr>
</tbody>
</table>

The Sp4, Sp3, and Sp1 antibodies were tested for specificity using western blot analysis and showed two adjacent bands at the appropriate molecular weights. These bands corresponded to the phosphorylated and non-phosphorylated forms of Sp factors. For the cold competition experiment, nuclear extracts were incubated with 100-fold excess of unlabeled oligonucleotides. All reactions were loaded onto 4.5% polyacrylamide gel (58:1, Acrylamide: Bisacrylamide) and run for 3 h at 200 V in 0.25× Tris-borate-EDTA buffer. Results were visualized on a phosphorimager and exposed on film. The positive control probe, murine GM3 synthase gene, is known to bind Sp1 and contains two Sp1 binding sites in a tandem repeat [25]. The negative controls were AMPA receptor subunit probes with mutated Sp binding sequences (Table 1).
the assay, KCl stimulation (at a final concentration of 20 mM) was performed for 5 h as described previously [27]. Cells were harvested 48 h after transfection and the lysates were measured for luciferase activity. The average from six independent transfections for each promoter construct is reported.

2.6. Plasmid construction of Sp1, Sp3, and Sp4 shRNA, transfection, and KCl treatment

Sp1 silencing was carried out using a combination of three to five Sp1-specific hairpin RNA (shRNA) (sc-29488, SCBT). Specific shRNA sequences for silencing murine Sp3 or Sp4 were chosen from the RNAi Consortium’s Public TRC Cloning Database at the Broad Institute (Table 4). The sequences were cloned into Addgene’s pLKO.1 TRC cloning vector (Plasmid 10878, Addgene, Cambridge, MA, USA). The pLKO.1 non-mammalian shRNA control vector (SHC002, Sigma), which contains a scrambled shRNA sequence that targets no known mammalian genes, was used as the control.

N2a cells at 60% confluence in 6-well dishes were transfected the day after plating with either the pLKO.1 non-mammalian control vector (3 μg), or the Sp1, Sp3, or Sp4 shRNA vector constructs (3 μg). TurboGFP (1 μg) vector was added to all wells to monitor transfection and selection efficiency. Transfection was performed using 5 μL of JetPrime transfection reagent (PolyPlus Transfection, Illkirch, France) per well. To select for purely transfected cells, 5 μg/mL of puromycin was added 1.5 days after transfection. Transfection efficiency was around 75% but the addition of puromycin effectively yielded 100% transfected cells. KCl stimulation was performed on rat visual cortical neurons as described for N2a cells above.

2.7. Sp1, Sp3, and Sp4 over-expression and TTX treatment

cDNA clones for human Sp1, Sp3, and Sp4 (Open Biosystems, Lafayette, CO, USA) were cloned into pcDNA Dest40 vectors (Gateway Multisite Cloning kit, Invitrogen) according to the manufacturer’s instructions and as described previously [20].

Transfection procedure for N2a cells and primary neuronal culture was similar to that described above. Either 1.5 μg of Sp1, Sp3, or Sp4 over-expression vector, or 1 μg of the pcDNA3.1 empty vector and 0.5 μg of turboGFP vector was used for both N2a cells and primary neuronal cultures. Three days of TTX treatment (0.4 μM) commenced the day after plating as previously described [27]. Primary neuronal cultures and N2a cells with TTX were harvested 4 days after transfection, whereas those without TTX were harvested 2 days after transfection.

2.8. RNA isolation and cDNA synthesis

TRIZOL (Invitrogen) was used to isolate total RNA according to the manufacturer’s instructions. DNase I treatment was done on 1 μg of total RNA, and cDNA was synthesized with iScript cDNA synthesis kit (Open Biosystems, Lafayette, CA).
2.1. Western blot analysis

Controls for N2a cells and primary neurons, respectively. GC

2.11. Statistical analysis

USA) and ECL reagent was used to visualize protein position and intensi-

Secondary antibodies were from Vector Laboratories (Burlingame, CA,

Davis, CA, USA), GluA3 (1:50; sc-7613, Santa Cruz) and GluA4 (1:50;

(1:1000; Santa Cruz), Sp3 (1:1000; Santa

(1:1000; Santa Cruz), GluA3 (1:1000; Santa Cruz), GluA1 (1:500; Ab1504, Millipore Chemicon, Billerica, MA, USA),

GluA2 (1:200; 75-002 clone 121/32, UC Davis, NIH NeuroMab Facility,

Davies, CA, USA), GluA3 (1:50; sc-7613, Santa Cruz) and GluA4 (1:50;

sc-7614, Santa Cruz), The loading control was β-actin (1:5000; Sigma).

Secondary antibodies were from Vector Laboratories (Burlingame, CA,

USA) and ECL reagent was used to visualize protein position and intensi-

Gel Doc from BioRad (Hercules, CA, USA) was used to perform quanti-

tative analyses of relative changes.

2.11. Statistical analysis

Analysis of variance (ANOVA) was applied to determine significance
among group means. The Student’s t-test was then applied to determine significance between two groups. P-values of 0.05 or less were considered
significant.

3. Results

3.1. In silico promoter analysis of AMPA receptor subunit genes

Computer assisted search for the typical Sp binding motif, the
GC-box (GGCGGCG), in the DNA sequence 1 kb upstream and 1 kb
downstream of the transcription start site (TSP) of the AMPA receptor
subunit genes revealed the motif on the Gria3 and Gria4, but not the
Gria1 or Gria2 promoter region. The Gria2 promoter contained the
atypical Sp binding motif, the GT-box (GGTG). The Gria1 promoter

contained GC rich regions, in which sequences resembling the Sp bind-
ing motif were present (see Table 1 for binding motifs).

3.2. In vitro binding of Sp proteins to AMPA receptor subunit promoters

The ability of Sp1, Sp3, and Sp4 to bind candidate sites in vitro was
determined by electrophoretic mobility shift (EMSA) and supershift as-
says. The positive control for Sp binding was the GM3 synthase promoter
[25], which contained two Sp1 binding sites (2 GC-boxes) in tandem.
When incubated with HeLa nuclear extract, GM3 synthase formed
specific DNA/Sp1, DNA/Sp3, and DNA/Sp4 shift and super shift com-
plexes (Fig. 1, lane 6 for Sp1/Sp3/Sp4 shift, lanes 8, 9, and 10 for Sp1,
Sp3, and Sp4 supershifts, respectively). As Sp1 is capable of homotypic
interactions that lead to multimeric complexes [17], the Sp1 supershift
reaction may be detecting two Sp1 proteins bound to the tandem
GC-boxes (Fig. 1, lane 8, Sp1 Supershift*). When incubated with
mouse visual cortical tissue nuclear extract, GM3 synthase formed spe-
cific DNA/Sp3 and DNA/Sp4 shift and supershift complexes (Fig. 1,
lane 1 for Sp3/Sp4 shift, lanes 4 and 5 for Sp3 and Sp4 supershift, respec-
tively) but did not form a DNA/Sp1 shift or supershift complex (Fig. 1,
lanes 1 and 3 respectively). The lack of a strong Sp4 supershift with
HeLa nuclear extract as compared to mouse visual cortical nuclear ex-
tact is consistent with our previous observations of different levels of
Sp4 abundance in the two nuclear extracts and with its reported neu-
onal distribution [19,28]. Similarly, the lack of Sp1 shift or supershift with
mouse cortical nuclear extract as compared to HeLa nuclear extract was
due to the lower abundance of Sp1 in mouse cortical nuclear extract as
compared to HeLa nuclear extract [28]. The shift bands for visual cortical and
HeLa nuclear extract were competed out with the addition of cold
competitors (Fig. 1, lanes 2 and 7, respectively).

When mouse visual cortical nuclear extract was incubated with
AMPA receptor subunit probes containing putative Sp sites, Gria1 gave
positive shift and supershift bands against Sp factors. Specifically, a
shift band was observed (Fig. 1, lane 11) that was competed out with
the addition of unlabeled competitors (Fig. 1, lane 12). A supershift band
against Sp4 was present (Fig. 1, lane 15) but there were no
supershift bands against Sp1 or Sp3 (Fig. 1, lanes 13 and 14, respectively).
The addition of unlabeled probe with mutated Sp binding site did not
compete out the Sp4 shift band (Fig. 1, lane 16) and shift reactions with
mutated Sp site did not reveal Sp binding (Fig. 1, lane 17). Gria1, Gria3, and
Gria4 did not give positive shift (Fig. 1, lanes 18, 20, and 22, respec-
tively) nor Sp4 supershift bands (Fig. 1, lanes 19, 21, and 23, respectively).

3.3. In vivo interaction of Sp factors with AMPA receptor subunit genes in murine visual cortex

Interactions of Sp factors with AMPA receptor subunit genes were
verified in vivo by chromatin immunoprecipitation (ChIP) assays of mouse visual cortical nuclear extract. As seen in Fig. 2,
PCR products from all 0.5% and 0.1% input DNA reactions revealed
specific bands. There were enriched bands for GM3 synthase
and neurotrophin 3 positive controls in the Sp4 immunoprecipitated
samples. An enriched band was present for Gria2, but not for the β-actin
negative control nor for the Gria1, Gria3, and Gria4 promoters. No enrichment was found for samples immunoprecipitated with NGFR nor
in the “no antibody” negative controls. There was also no enrichment of
DNA in the Sp1 or Sp3 immunoprecipitated samples for any of the tested
regions (data not shown).

3.4. Effect of mutated Sp binding site on Gria2 promoter

The wild type Gria2 promoter as well as the Gria2 promoter with
mutated Sp binding site were transfected separately into N2a cells. Analysis revealed a significant 56% decrease in the activity of Gria2
promoter containing the mutated Sp motif as compared to the wild type
control (P < 0.001, Fig. 3).

Table 5

Real-time qPCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gria1</td>
<td>F: 5′ GAGCAGACCAGGACCGCTCTGA 3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′ CTCTTGGGCTGCGCGGATT 3′</td>
</tr>
<tr>
<td>Gria2</td>
<td>F: 5′ AAGAAATCTGCGGACCGACAC 3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′ CCACACACGCTCTGCTCAGTGC 3′</td>
</tr>
<tr>
<td>Gria3</td>
<td>F: 5′ TTGAAAGGCGCCAGGAAGAAT 3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′ CACCTCTTCTTCGTCGTCAGG 3′</td>
</tr>
<tr>
<td>Gria4</td>
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<tr>
<td></td>
<td>R: 5′ AGTAGATGTACGACCGACGAC 3′</td>
</tr>
<tr>
<td>Sp1</td>
<td>F: 5′ GTTCGGACGATTAACTCAGTGG 3′</td>
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<tr>
<td></td>
<td>R: 5′ GCGGACTGACGTGTACGTCG 3′</td>
</tr>
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<td>Sp3</td>
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<tr>
<td></td>
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<tr>
<td>Sp4</td>
<td>F: 5′ TTGCAACGACCAGGAGCACG 3′</td>
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<tr>
<td></td>
<td>R: 5′ GCTCTCTTCCTCCTGTCGTCCT 3′</td>
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<td>Actb</td>
<td>F: 5′ GTGACATGCATGTCATGAAGA 3′</td>
</tr>
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<td></td>
<td>R: 5′ GCGGACTGACGTGTACGTCG 3′</td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: 5′ AGCTGCGTGAAAGGATTGTCG 3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′ GGCGGTTGTGCGCGCAGAC 3′</td>
</tr>
</tbody>
</table>
3.5. Effect of mutated Sp binding sites on the response of the Gria2 promoter to KCl stimulation

We have shown previously that the KCl-mediated increase in neuronal activity up-regulates the Gria2 promoter. To determine if Sp factor binding is necessary for this activity-mediated up-regulation, Gria2 control promoters or Gria2 promoters with mutated Sp site were transfected into N2a cells and subjected to depolarizing KCl (20 mM) treatment. As shown in Fig. 3, N2a cells transfected with the control Gria2 promoter and subjected to KCl stimulation showed a 47% increase in promoter binding necessary for this activity-mediated up-regulation, Gria2 control promoters or Gria2 promoters with mutated Sp site were transfected into N2a cells and subjected to depolarizing KCl (20 mM) treatment. As shown in Fig. 3, N2a cells transfected with the control Gria2 promoter and subjected to KCl stimulation showed a 47% increase in promoter binding.
3.7. Effect of silencing Sp1, Sp3, and Sp4 by RNA interference on AMPA receptor subunits in N2a cells

To determine whether a knockdown of Sp1, Sp3, or Sp4 had an effect on the expression of AMPA receptor subunits, respective Sp proteins were silenced in N2a cells by means of 2–5 specific plasmid shRNA vectors. Quantitative real-time PCR indicated that silencing Sp4 decreased Sp4 mRNA levels by 68% (P < 0.001, Fig. 4A) and Sp4 protein levels by 56% (P < 0.01, Fig. 4C–D). Sp4 silencing also decreased Gria2 mRNA levels by 62% (P < 0.001; Fig. 4B) and protein levels by 41% (P < 0.05, Fig. 4E–F), mRNA levels of Gria1, Gria3, and Gria4, however, did not change significantly with Sp4 silencing (Fig. 4B), nor did the protein level of GluA1 (Fig. 4E–F), GluA3 or GluA4 (Supplementary Fig. 1A–B).

Silencing of Sp1 resulted in a 71% decrease in its mRNA levels (P < 0.001, Fig. 4A) and a 63% decrease in its protein levels (P < 0.01, Fig. 4C–D). Silencing of Sp3 resulted in a 66% decrease in its mRNA levels (P < 0.001, Fig. 4A) and a 52% decrease in its protein levels (P < 0.01, Fig. 4C–D). However, mRNA levels of Gria1, Gria3, and Gria4 (Fig. 4B), or protein levels of GluA1 and GluA2 (Fig. 4E–F), GluA3 and GluA4 (Supplementary Fig. 1A–B) were not changed significantly with Sp1 or Sp3 silencing.

3.8. Effect of over-expressing Sp1, Sp3, and Sp4 on AMPA receptor subunits in N2a cells

To determine whether over-expressing Sp1, Sp3, or Sp4 had an effect on the expression of the AMPA receptor subunits, plasmids expressing Sp1, Sp3, or Sp4 were transfected into N2a cells. β-actin served as the internal control. Sp4 over-expression increased its mRNA levels approximately 30-fold (P < 0.001, Fig. 5A) and its protein levels by 132% (P < 0.01, Fig. 5C–D). The mRNA and protein levels of GluA2 were also increased by 135% and 47%, respectively (P < 0.001, Fig. 5B and P < 0.05, Fig. 5E–F, respectively). On the other hand, mRNA levels of Gria1, Gria3, and Gria4 did not change significantly with Sp4 over-expression (Fig. 5B), and neither did the protein level of GluA1 (Fig. 5E–F), nor of GluA3 and GluA4 (Supplementary Fig. 1C–D).

Sp1 over-expression led to a significant 27-fold increase in its mRNA levels (P < 0.001, Fig. 5A) and a significant 63% increase in its protein levels (P < 0.01, Fig. 5C–D). Sp3 over-expression led to a significant 39-fold increase in its mRNA levels (P < 0.001, Fig. 5A) and a significant 146% increase in its protein levels (P < 0.05, Fig. 5C–D). However, mRNA levels of Gria1–4 (Fig. 5B) or protein levels of GluA1 and GluA2 (Fig. 5E–F), or of GluA3 and GluA4 (Supplementary Fig. 1C–D) did not change significantly with over-expression of Sp1 or Sp3.

3.9. Effect of over-expressing Sp1, Sp3, and Sp4 on AMPA receptor subunits in primary neurons

Cultured mouse cortical neurons were transfected with Sp1, Sp3, or Sp4 expression vectors to determine whether the lack of an effect seen with over-expression of Sp1 and Sp3 was restricted to N2a cells. Gapdh served as the internal control. Sp4 over-expression resulted in a 29-fold increase in its mRNA levels (P < 0.01, Fig. 5G). Gria2 mRNA levels also increased significantly by 131% with Sp4 over-expression (P < 0.01; Fig. 5H). However, Gria1, Gria3, and Gria4 mRNA levels were not changed significantly (Fig. 5H).

Over-expression of Sp1 resulted in a significant 63-fold increase in its mRNA levels (P < 0.01, Fig. 5G) and over-expression of Sp3 resulted in significant 31-fold increase in its mRNA levels (P < 0.01, Fig. 5G). Gria2 mRNA levels increased significantly with Sp1 and Sp3 over-expression (P < 0.05 and P < 0.01, respectively; Fig. 5H), but those of Gria1, Gria3, or Gria4 did not (Fig. 5H).

3.10. Silencing Sp4 abolished KCl-induced transcript up-regulation of Gria2 in N2a cells

Gria2 mRNA levels are up-regulated in response to neuronal activity induced by physiological concentrations of KCl [15]. To determine whether Sp1, Sp3, or Sp4 was necessary for this up-regulation, control N2a cells and cells transfected with the respective shRNAs against these proteins were subjected to 20 mM KCl for 5 h. While Gria2 transcript levels were significantly increased by 110% (P < 0.001) with KCl-induced depolarization, silencing of only Sp4 abolished this increase (Fig. 6A). Silencing of Sp1 or Sp3 did not prevent the KCl-induced increase of Gria2 transcript (Fig. 6A). KCl treatment increased transcript levels of Gria1, Gria3, and Gria4 significantly (P < 0.001; Fig. 6A), but levels of these subunits were not significantly changed with silencing Sp1, Sp3, or Sp4 (Fig. 6A).

3.11. Over-expression of Sp4 rescued tetrodotoxin-induced transcript reduction of Gria2 in N2a cells

Gria2 mRNA levels are known to be down-regulated in response to a decrease in neuronal activity induced by the action potential blocker TTX [15,29]. To determine if Sp factors could rescue the decrease in AMPA transcript levels induced by the TTX-mediated decrease in neuronal activity, control N2a cells and cells transfected with Sp1, Sp3, or Sp4 over-expression vectors were subjected to 0.4 μM TTX-treatment for 3 days. TTX significantly decreased Gria2 transcript levels by 44% (P < 0.01; Fig. 6B) but the decrease was...
abolished by Sp4 over-expression \( P < 0.001 \) as compared to TTX alone; Fig. 6B). Gria1, Gria3, and Gria4 transcript levels decreased significantly with TTX treatment \( P < 0.01, P < 0.001, \) respectively; Fig. 6B), but they were not rescued by Sp4 over-expression (Fig. 6B). Over-expressing Sp1 or Sp3 could not rescue the TTX-mediated decrease in Gria1–4 transcript levels (Fig. 6B).
Fig. 5. Effect of over-expressing Sp1, Sp3, or Sp4 on the transcript levels of AMPA receptor subunit genes. (A) N2a cells transfected with Sp1, Sp3, and Sp4 expression vectors revealed an up-regulation of Sp1, Sp3, and Sp4 transcripts, respectively. N = 6. (B) Sp4 over-expression, but not Sp1 or Sp3 over-expression, increased Gria2 transcript levels. N = 6. (C–D) In N2a cells, protein levels of Sp1, Sp3, and Sp4 were increased significantly with over-expression of Sp1, Sp3, and Sp4, respectively. A representative blot of the loading control, β-actin, is shown. N = 3. (E–F) In N2a cells, the protein level of GluA2, but not that of GluA1, was increased significantly with over-expression of Sp4. Protein levels of GluA1 and GluA2 did not change significantly with over-expressing Sp1 or Sp3. The loading control was β-actin. N = 3. (G) Over-expression of Sp1, Sp3, or Sp4 in primary neurons increased transcript levels of Sp1, Sp3, and Sp4, respectively. N = 3. (H) Sp4, Sp3, and Sp1 over-expression increased Gria2 transcript levels, but not Gria1, Gria3 and Gria4 transcript levels, in primary neurons. N = 3. P<0.05, P<0.01, and P<0.001 when compared to controls.
3.12. Silencing Sp4 abolished KCl-induced transcript up-regulation of Gria2 in primary neurons

Transcript levels of Sp4, Gria1, Gria2, Gria3, and Gria4 in rat visual cortical neurons were all significantly up-regulated by 20 mM KCl for 5 h \((P < 0.05-0.01; \text{Fig. 7A})\). When neurons were transfected with Sp4 shRNA, transcript levels of Sp4 and Gria2 were significantly down-regulated to 44% and 45%, respectively \((P < 0.01\) and \(P < 0.05\), respectively\), whereas those of Gria1, Gria3, and Gria4 were not affected \((\text{Fig. 7A})\). Likewise, silencing Sp4 abolished the increase \((\text{Fig. 7A})\) in transcript levels of Sp4 and Gria2 induced by KCl depolarization \((P < 0.01)\), but did not affect those of Gria1, Gria3, and Gria4 \((\text{Fig. 7A})\).

3.13. Over-expression of Sp4 rescued tetrodotoxin-induced transcript reduction of Gria2 in primary neurons

Transcript levels of Sp4, Gria1, Gria2, Gria3, and Gria4 in rat visual cortical neurons were all significantly down-regulated by 0.4 mM TTX-treatment for 3 days \((P < 0.05; \text{Fig. 7B})\). Over-expressing Sp4 significantly up-regulated Sp4 and Gria2 transcripts by 163% and 237%, respectively \((P < 0.01\) and \(P < 0.001\), respectively\), but did not affect those of Gria1, Gria3, and Gria4 \((\text{Fig. 7B})\). Sp4 over-expression also rescued Sp4 and Gria2 transcripts from being down-regulated by TTX \((P < 0.05\) and \(P < 0.001\), respectively, as compared to TTX alone\), but had no effect on those of Gria1, Gria3, and Gria4 \((\text{Fig. 7B})\).

3.14. Homology of the Sp binding site

The functional Gria2 Sp binding site is conserved among mice, rats, and humans \((\text{Fig. 8})\).

4. Discussion

Using multiple molecular and biochemical approaches, including in silico analysis, electrophoretic mobility shift and supershift assays,
ChIP, RNA interference and over-expression assays in both N2a cells and rodent primary cortical neurons, we document for the first time that it is Sp4, and not Sp1 or Sp3, transcription factor that regulates the GluA2 (Gria2), and not GluA1 (Gria1), GluA3 (Gria3), or GluA4 (Gria4), subunit of the AMPA receptor in neurons. Furthermore, a knockdown of Sp4 did not allow for the activity-mediated increase of Gria2, whereas over-expression of Sp4 rescued the TTX-mediated down-regulations of Gria2. The Sp binding motif on the Gria2 promoter is conserved among mice, rats, and humans.

AMPA receptors are excitatory glutamatergic neurotransmitter receptors that mediate most of the excitatory neurotransmission in the central nervous system (for review see [11]). These receptors are heterotetrameric proteins composed of GluA1–4 subunits. The majority of AMPA receptors in the adult cortex and hippocampus contain the Gria2 subunit in GluA1/Glia2 heterotetramers [2,7,8]. The expression of GluA3 is lower than that of GluA1, and the Gria4 subunit is developmentally expressed [7,8,11]. Our current study indicates that Sp4 regulates only the Gria2 subunit of AMPA receptors.

Sp4 is in the specificity protein/Krüppel-like (SP/KLF) family of zinc finger transcription factors that bind to GC-rich sequences of DNA (for review see [17]). Of the 25 members in the SP/KLF family, Sp4 shares critical amino acid homology in its DNA binding domain with specificity proteins 1 (Sp1) and 3 (Sp3), with all three factors competing for the same cis-regulatory elements [17,18]. However, unlike Sp1 and Sp3, whose expression is ubiquitous, Sp4 is found primarily in the brain and testes [19,30]. The murine brain has a greater abundance of Sp4 than either HeLa or N2a cells, whereas the opposite is true for Sp1 and Sp3 [28]. Furthermore, in primary neurons, mRNA and protein levels of Sp4 are greater than those of Sp1 [31]. In glial cells, however, Sp1 and Sp3 are the major Sp factors present, with negligible expression of Sp4 [31]. Neurological expression of Sp4 increases with development and Sp4-null male mice do not breed despite having a normal reproductive system, suggesting that the deficit lies in the nervous system [19]. Homozygous splice site mutations in the Gria2 promoter impede the ability of Gria2 protein to activate transcription in both brain and testis [20,21]. Sp4 mice have vacuolization in the hippocampal gray matter and deficits in both sensorimotor gating and contextual memory. Restoration of Sp4 rescues all observable molecular, histological, and behavioral abnormalities in these mice [32].

The Sp cis-motif was previously identified on the Gria2 promoter [33], but consistent with the importance of Sp4 in neurons is our finding that it is Sp4, not Sp1 or Sp3, that regulates the expression of Gria2 in neurons through this motif. Our results of a positive regulation of Gria2 after over-expression of Sp1 and Sp3 in primary neurons may be due to the absence of smaller isoforms of Sp1 and Sp3 in primary neurons [28]. The smaller Sp3 isoforms are known to act as transcriptional repressors and the same may be true for Sp1 [31]. Their absence in primary neurons may allow over-expression of the exogenous full-length Sp3 or Sp1 to activate expression of target genes. N2a cells, on the other hand, possess the smaller isoforms of Sp3 and Sp1 [28], and so they did not exhibit positive regulation of Gria2 with an over-expression of exogenous full-length Sp3 or Sp1.

Of the four AMPA subunits, GluA1 to 4, it is remarkable that Sp4 regulates only GluA2. This subunit is quite unique, in that it undergoes hydrolytic editing of its pre-mRNA in its transmembrane segment 2 from glutamine to arginine [9], thereby rendering GluA2-containing AMPA receptors less permeable to Ca\(^{2+}\); and GluA2 homotetrameric AMPA receptors are completely impermeable to Ca\(^{2+}\) [9,34,35]. Thus, GluA2 subunit determines the functional properties of AMPA receptors, including the I–V behavior of the channel and permeability to Ca\(^{2+}\) [36,37]. GluA2-containing AMPA receptors also exhibit low single channel conductance as compared to AMPA receptors that do not contain GluA2 [37–40].

The special properties of the GluA2 subunit render it important for normal neuronal activities, including synaptic plasticity, learning, and memory (for reviews see [35]). GluA2 is involved in bidirectional plasticity in the synapse as well as in AMPA receptor maintenance, internalization, and distance-dependent scaling [41–44]. Knocking down GluA2 blocks synaptic scaling [45]. GluA2 subunits are able to stimulate synaptic function and promote the growth and formation of dendritic spines in cultured cortical neurons [43]. This is thought to be through a direct interaction of GluA2’s N-terminal domain with the cell-adhesion molecule, N-cadherin [43]. A mechanism for the maintenance of long-term potentiation (LTP), thought to underlie experience-dependent plasticity, learning, and memory, can be through an increase in GluA2-containing receptors in hippocampal synapses [46–48]. Knocking out Gria2 results in multiple behavioral abnormalities, including deficits in object exploration, grooming, eye-closure reflex, and spatial and non-spatial learning [49]. Thus, by regulating GluA2, it is likely that Sp4 plays an important role in mediating many of these neuronal and synaptic processes.

The Gria2 subunit responds to changes in neuronal activity. Specifically, physiological concentrations of KCl, a depolarizing agent that activates neuronal activity, increases both transcript and protein expression of Gria2 in cultured visual cortical neurons [13]. On the other hand, decreasing neuronal activity through TTX-induced impulse blockade down-regulates GluA2 mRNA and protein levels in these neurons [13]. Parallel to these neuronal activity-mediated changes is a change in transcript and protein levels of cytochrome c oxidase (COX) [13], a critical enzyme for energy generation in neurons [50]. In the supragranular and infragranular layers of the macaque visual cortex, Gria2 expression and COX activity levels are governed by visual input and neuronal activity [12]. Specifically, monocular impulse blockade induces a down-regulation of both GluA2 expression and COX activity in deprived ocular dominance columns [12]. Thus, both GluA2 and COX are tightly regulated at the cellular level by neuronal activity.

We have previously shown that Sp factors modulate COX mRNA and protein levels in neurons [20]. Sp4, by regulating GluA2 expression, couples neuronal activity and energy metabolism at the transcriptional level. GluA2 expression is also controlled by nuclear respiratory factor 1 (NRF-1) [15], a transcription factor that regulates all 13 COX subunits [16,22,29]. The present study indicates that Sp4 and NRF-1 employ the concurrent and parallel mechanism to regulate the GluA2, but not GluA1, GluA3, or GluA4 subunits.

A possible benefit of the concurrent and parallel mechanism is that the GluA2 subunit, important for AMPA receptor physiology and function, is regulated by multiple transcription factors. However, it is unlikely that these factors function redundantly. While both Sp4 and NRF-1 are positively regulated by neuronal activity [27,28,51], NRF-1 can interact directly with an upstream coactivator, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), to induce target gene expression [52,53], whereas Sp factors are not known to interact with PGC-1α directly. However, Sp factors can interact with host cell factor 1 (HCF-1) [54], a co-activator that reportedly interacts with PGC-1α [55], resulting in a possible indirect interaction between Sp factors and PGC-1α in the induction of target gene expression. Sp factors are also known to interact with a multitude of cellular proteins and undergo numerous post-translational modifications [30,54]. Other contributing factors, such as the cellular environment, cell signaling, and varying amounts of neuronal activity may differentially recruit NRF-1 or Sp4 to regulate their common target genes, such as Gria2.

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Conflict of interest

The authors declare no competing financial interest.

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References


[28] A. Priya, K. Johar, M.T. Wong-Riley, 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, Biochim. Biophys. Acta 1833 (2013) 48–58.