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# Specificity 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 *Gria 1*, *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.

### Keywords

AMPA receptor; gene regulation; GluA2; Sp4; specificity protein 4; transcription factor

## 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



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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]. As 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 $Ca^{2+}$ [9]. In the adult, GluA3 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 GluA2 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 (‘GGGTGG’) and CT (‘CCCTCC’) boxes with significantly lower affinities [17, 18]. 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 (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% $CO_2$.

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 vitro* 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-$^{32}$P] 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]. Ten μg of either mouse cortical and/or HeLa nuclear extract were 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. 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 phosphoimager 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).

### 2.4. 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 and sonicated in SDS lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.1, 10 mM EDTA). Immunoprecipitation was performed with 2 μg of Sp1, Sp3, or Sp4 antibodies. Two μg of anti-nerve growth factor receptor (NGFR) antibodies (sc-6188, SCBT) or 'no antibody' blanks were used as negative controls. Semi-quantitative PCR, utilizing primers (Table 2) surrounding the putative Sp binding sites identified through *in silico* analysis, was performed. Positive controls for Sp factor binding were *GM3 Synthase* (known to bind Sp1) and *neurotrophin 3* (known to bind Sp4) [25, 26]. *β-actin* promoter (*Actb*) was the negative control (Table 2). PCR additives and cycling parameters used to improve the reproducibility and quality of ChIP are listed in Table 2. 2% agarose gels stained with ethidium bromide were used to visualize PCR products.

### 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 (MluI and BgIII), and ligated into Promega's pGL3 luciferase vectors (E1751, Promega, Madison, WI, USA). The QuikChange 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 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 at a final concentration of 20 mM for 5 h as previously described [27].

Transfection of murine or rat primary neuronal cultures was carried out 5 days post-plating with *Sp1*, *Sp3*, or *Sp4* shRNA constructs (2 μg) or the pLKO.1 non-mammalian control (2 μg) using Neurofect transfection reagent per 6-well plate according to the manufacturer's instructions (Genlantis, San Diego, CA). TurboGFP (0.5 μg) vector was added to each well for transfection visualization and selection efficiency. Transfection efficiency was around 40 – 50% before selection. Puromycin selection, however, 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 were 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 (170-8891, BioRad, Hercules, CA, USA).

### 2.9. Real-time quantitative PCR

The Cepheid Smart Cycler Detection system (Cepheid, Sunnyvale, CA, USA) and/or the iCycler System (BioRad) were used with the IQ SYBR Green SuperMix (170-8880, BioRad) to perform real-time quantitative PCR (RTqPCR). The sequences of primers used for analysis are listed in Table 5. Primer sequences were the same for mouse and rat.

RTqPCR conditions were optimized to yield an amplification efficiency of 95% -105%. Products were run on agarose gel to verify the correct amplification length. Melt curve analyses of each PCR reaction was performed to verify the formation of a single desired PCR product. The $2^{-\Delta\Delta CT}$ method was used to quantify the relative amount of transcripts, with β-actin and *Gapdh* acting as the internal controls for N2a cells and primary neurons, respectively.

### 2.10. Western blot analysis

Sample buffer (12.5 mM EDTA, 50 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol) was used to harvest control, shRNA, and over-expression protein samples. Equal protein amounts of each sample was loaded onto 10% SDS-PAGE gel and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk and incubated in primary antibodies against Sp1 (1:1000; Santa Cruz), Sp3 (1:1000; Santa Cruz), Sp4 (1:1000; Santa Cruz), GluA1 (1:500; Ab1504, Millipore Chemicon, Billerica, MA, USA), GluA2 (1:200; 75-002 clone L21/32, UC Davis/NIH NeuroMab Facility, Davis, 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 intensity. Gel Doc from BioRad (Hercules, CA, USA) was used to perform quantitative 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 (GGGCGG), 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 (GGGTGG). The *Gria1* promoter contained GC rich regions, in which sequences resembling the Sp binding 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 assays. 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 complexes (Figure 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

(Figure 1, lane 8, Sp1 Supershift*). When incubated with mouse visual cortical tissue nuclear extract, *GM3 synthase* formed specific DNA/Sp3 and DNA/Sp4 shift and supershift complexes (Figure 1, lane 1 for Sp3/Sp4 shift, lanes 4 and 5 for Sp3 and Sp4 supershift, respectively) but did not form a DNA/Sp1 shift or supershift complex (Figure 1, lanes 1 and 3 respectively). The lack of a strong Sp4 supershift with HeLa nuclear extract as compared to mouse visual cortical nuclear extract is consistent with our previous observations of different levels of Sp4 abundance in the two nuclear extracts and with its reported neuronal 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 (Figure 1, lanes 2 and 7, respectively).

When mouse visual cortical nuclear extract was incubated with AMPA receptor subunit probes containing putative Sp sites, *Gria2* gave positive shift and supershift bands against Sp factors. Specifically, a shift band was observed (Figure 1, lane 11) that was competed out with the addition of unlabeled competitors (Figure 1, lane 12). A supershift band against Sp4 was present (Figure 1, lane 15) but there were no supershift bands against Sp1 or Sp3 (Figure 1, lanes 13 and 14, respectively). The addition of unlabeled probe with mutated Sp binding site did not compete out the Sp4 shift band (Figure 1, lane 16) and shift reactions with mutated Sp site did not reveal Sp binding (Figure 1, lane 17). *Gria1*, *Gria3*, and *Gria4* did not give positive shift (Figure 1, lanes 18, 20, and 22, respectively) nor Sp4 supershift bands (Figure 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 gene promoters were verified *in vivo* by chromatin immunoprecipitation (ChIP) assays of mouse visual cortical nuclear extract. As seen in Figure 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 ($P < 0.001$, Figure 3).

### 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 figure 3, N2a cells transfected with the control *Gria2* promoter and subjected to KCl stimulation showed a 47% increase in promoter activity ($P < 0.001$). This increase was abolished by mutating the Sp binding site (Figure 3), confirming a requirement for Sp binding in the activity-induced up-regulation of the *Gria2* promoter.

### 3.6. 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% ($P < 0.001$, Figure 4A) and Sp4 protein levels by 56% ($P < 0.01$, Figure 4C-D). *Sp4* silencing also decreased *Gria2* mRNA levels by 62% ($P < 0.001$; Figure 4B) and protein levels by 41% ($P < 0.05$, Figure 4E-F). mRNA levels of *Gria1*, *Gria3*, and *Gria4*, however, did not change significantly with *Sp4* silencing (Figure 4B), nor did the protein level of GluA1 (Figure 4E-F), GluA3 or GluA4 (Supplementary Figure 1A-B).

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

### 3.7. Effect of silencing Sp1, Sp3, and Sp4 by RNA interference on AMPA receptor subunits in primary neurons

To determine whether the lack of an effect seen with silencing *Sp1* and *Sp3* was specific to N2a cells, *Sp1*, *Sp3*, and *Sp4* were each silenced in cultured primary mouse cortical neurons. G*apdh* served as the internal control. *Sp4* silencing resulted in a 64% decrease in its mRNA levels ($P < 0.01$, Figure 4G). *Gria2*'s mRNA levels were also decreased significantly by 61% ($P < 0.05$; Figure 4H). However, *Gria1*, *Gria3*, and *Gria4* transcripts were not changed significantly with *Sp4* silencing (Figure 4H).

Silencing of *Sp1* decreased its mRNA levels by 51% ($P < 0.01$, Figure 4G) and silencing of *Sp3* decreased its mRNA levels by 57% ($P < 0.01$, Figure 4G). However, mRNA levels of *Gria1-4* were not changed significantly by the silencing of either *Sp1* or *Sp3* (Figure 4H).

### 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 ($P < 0.001$, Figure 5A) and its protein levels by 132% ($P < 0.01$, Figure 5C-D). The mRNA and protein levels of GluA2 were also increased by 135% and 47%, respectively ($P < 0.001$, Figure 5B and $P < 0.05$, Figure 5E-F, respectively). On the other hand, mRNA levels of *Gria1*, *Gria3*, and *Gria4* did not change significantly with *Sp4* over-expression (Figure 5B), and neither did the protein level of GluA1 (Figure 5E-F), nor of GluA3 and GluA4 (Supplementary Figure 1C-D).

*Sp1* over-expression led to a significant 27-fold increase in its mRNA levels ($P < 0.001$, Figure 5A) and a significant 63% increase in its protein levels ($P < 0.01$, Figure 5C-D). *Sp3* over-expression led to a significant 39-fold increase in its mRNA levels ($P < 0.001$, Figure 5A) and a significant 146% increase in its protein levels ($P < 0.05$, Figure 5C-D). However, mRNA levels of *Gria1-4* (Figure 5B) or protein levels of GluA1 and GluA2 (Figure 5E-F), or of GluA3 and GluA4 (Supplementary Figure 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 ($P < 0.01$, Figure 5G). *Gria2* mRNA levels also increased significantly by 131% with *Sp4* over-expression ($P < 0.01$; Figure 5H). However, *Gria1*, *Gria3*, and *Gria4* mRNA levels were not changed significantly (Figure 5H).

Over-expression of *Sp1* resulted in a significant 63-fold increase in its mRNA levels ($P < 0.01$, Figure 5G) and over-expression of *Sp3* resulted in significant 31-fold increase in its mRNA levels ($P < 0.01$, Figure 5G). *Gria2* mRNA levels increased significantly with *Sp1* and *Sp3* over-expression ($P < 0.05$ and $P < 0.01$, respectively; Figure 5H), but those of *Gria1*, *Gria3*, or *Gria4* did not (Figure 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 (Figure 6A). Silencing of *Sp1* or *Sp3* did not prevent the KCl-induced increase of *Gria2* transcript (Figure 6A). KCl treatment increased transcript levels of *Gria1*, *Gria3*, and *Gria4* significantly ($P < 0.001$; Figure 6A), but levels of these subunits were not significantly changed with silencing *Sp1*, *Sp3*, or *Sp4* (Figure 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% ($P < 0.01$; Figure 6B) but the decrease was abolished by *Sp4* over-expression ($P < 0.001$ as compared to TTX alone; Figure 6B). *Gria1*, *Gria3*, and *Gria4* transcript levels decreased significantly with TTX treatment ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively; Figure 6B), but they were not rescued by *Sp4* over-expression (Figure 6B). Over-expressing *Sp1* or *Sp3* could not rescue the TTX-mediated decrease in *Gria1-4* transcript levels (Figure 6B).

### 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; 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 (Fig. 7A). Likewise, silencing *Sp4* abolished the increase (Figure 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* (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 μM TTX-treatment for 3 days ($P < 0.05$; 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* (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* (Fig. 7B).

### 3.14. Homology of the Sp Binding Site

The functional *Gria2* Sp binding site is conserved among mice, rats, and humans (Figure 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-regulation 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 [1]). These receptors are heterotetrameric proteins composed of GluA1-4 subunits. The majority of AMPA receptors in the adult cortex and hippocampus contain the GluA2 subunit in GluA1/GluA2 heterotetramers [2, 7, 8]. The expression of GluA3 is lower than that of GluA1, and the GluA4 subunit is developmentally expressed [7, 8, 11]. Our current study indicates that Sp4 regulates only the GluA2 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*-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]. Neuronal 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]. Hypomorphic *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 [3, 5]). 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 GluA2 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 GluA2 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, GluA2 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-1 α 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*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **Sp** | Specificity protein transcription factor |
| ***Gria*** | Gene name for AMPA receptor subunit |

## Highlights

- Sp4 regulates the expressions of AMPA receptor subunit GluA2, not GluA1, 3, or 4
- Sp4 silencing prevented the KCl-induced up-regulation of GluA2 (Gria2) transcript
- Sp4 over-expression rescued TTX-mediated down-regulation of Gria2 transcript
- Sp4 regulates Gria2 in a concurrent and parallel manner with NRF-1 and NRF-2
- Sp1 and Sp3 do not regulate the GluA1 -4 AMPA receptor subunits

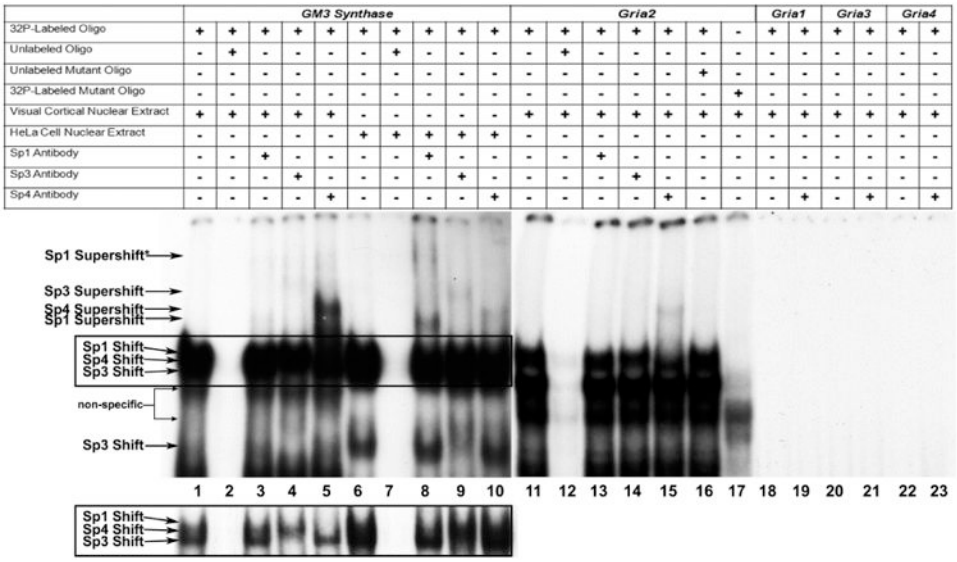


**Figure 1.**

*In vitro* binding of Sp factors to the GM3 Synthase and *Gria2* promoters using EMSA and supershift assays. All lanes contain $^{32}$P-labeled oligonucleotides and are labeled by a "+" or a "−"sign depending on whether they also contain mouse visual cortical or HeLa nuclear extract, excess oligos or mutant oligos (unlabeled), and Sp1, Sp3, or Sp4 antibodies. Sp1, Sp3, or Sp4 shift, supershift, and non-specific complexes are indicated by arrows. The positive control for Sp factor binding was GM3 Synthase. Specific Sp1, 3, or 4 shift bands were revealed upon incubation with cortical (lanes 1-5) or HeLa (lanes 5-10) nuclear extract (lanes 1 and 6, respectively). Excess unlabeled competitor competed the shift bands (lanes 2 and 7). The addition of Sp1 antibody did not yield a supershift band with cortical nuclear extract (lane 3) but gave two specific supershift bands with HeLa nuclear extract (lane 8), corresponding to the presence of tandem Sp binding. The addition of Sp3 antibody yielded a faint specific supershift band with cortical nuclear extract (lane 4) and a stronger supershift band with HeLa nuclear extract (lane 9). The addition of Sp4 antibody yielded a very strong supershift band with cortical nuclear extract (lane 5) and a much fainter supershift band with HeLa nuclear extract (lane 10). The relative levels of the Sp1, Sp3, and Sp4 supershift bands in mouse cortical and HeLa nuclear extract corresponds to the relative levels of these transcription factors in these tissue and cell types.

As Sp1, 3, and 4 all recognize the same *cis* motif, their combined binding yielded a rather thick shift band. However, with shorter gel exposure (boxed lanes 1 to 10 highlighted in the lower left), the individual shift bands can sometimes be dissociated. As shown in lane 1 in the inset, cortical nuclear extract did not reveal any detectable Sp1 shift band, but did show distinct Sp3 and Sp4 bands. The Sp3 shift band disappeared when it was supershifted (lane 4). Likewise, the Sp4 shift band disappeared when it was supershifted (lane 5). With HeLa nuclear extract, the rather abundant presence of both Sp1 and Sp3 caused the bands to merge even with lighter exposure (lane 6). However, the upper portion (Sp1) disappeared when supershifted with Sp1 antibody (lane 8), and the lower portion (Sp3) became lighter when supershifted with Sp3 antibody (lane 9). No change was detectable with anti-Sp4 antibody (lane 10), indicating that HeLa nuclear extract did not contain detectable Sp4.

Incubation of cortical nuclear extract with the *Gria2* probe yielded a specific Sp4 shift band (lane 11) which was competed out with the addition of excess cold probes (lane 12). The addition of Sp4 anitbody yielded a specific supershift band (lane 15), but the addition of Sp1 and Sp3 antibodies did not (lanes 13 and 14). The addition of excess unlabeled mutant *Gria2* probes did not compete out the shift reaction (lane 16). Labeled *Gria2* probes with mutant Sp sites did not yield specific Sp shift bands (lane 17). Labeled *Gria1*, *Gria3*, and *Gria4* probes did not yield specific Sp shift (lanes 18, 20, and 22, respectively) or Sp4 supershift bands (lanes 19, 21, and 23, respectively).

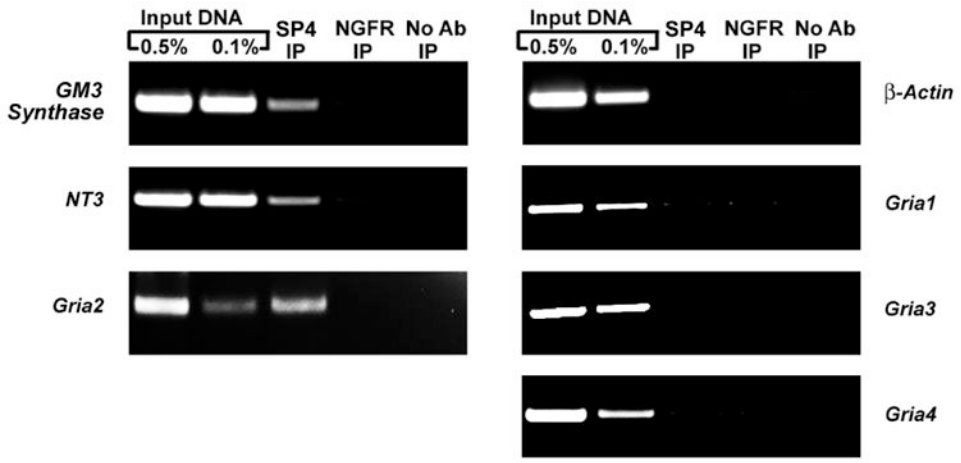


**Figure 2.**

Sp4 interactions with AMPA receptor subunit promoters in mouse visual cortical tissue. Precipitation of chromatin was carried out from mouse visual cortical tissue nuclear extract with anti Sp4 antibodies (Sp4 IP lane), anti-nerve growth factor receptor p75 antibody (negative control, NGFR IP lane), or no antibody (negative control, No Ab lane). 0.5% (input 0.5% IP lane) and 0.1% (input 0.1% IP lane) of input chromatin served as control reactions for PCR. Positive controls for Sp4 binding were *GM3 synthase* and *Neurotrophin 3*, whereas p-Actin was the negative control. Sp4 interacted with *Gria2*, but not with *Gria1*, *Gria3*, or *Gria4*.

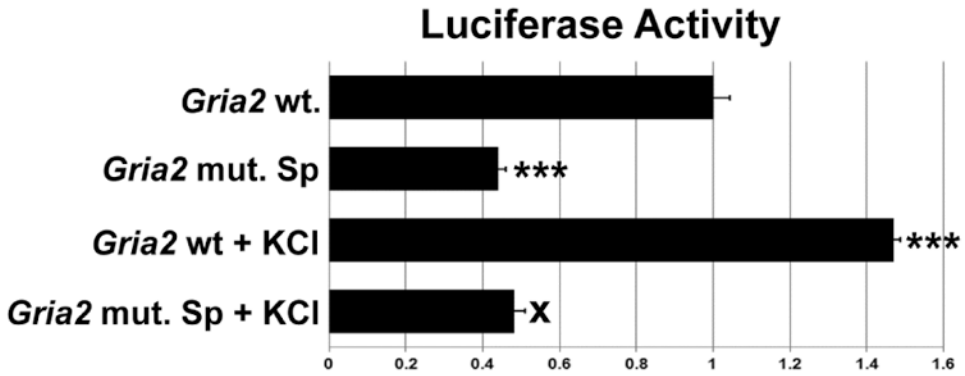


**Figure 3.**

Relative luciferase activity of the wild type *Gria2* promoter (wt) and the *Gria2* promoter with mutated Sp binding site (mut). Mutating the Sp binding site on *Gria2* resulted in a significant decrease in luciferase activity as compared to the wild type promoter. KCl depolarization significantly increased *Gria2* wild type promoter activity, but could not increase activity in the *Gria2* promoter with mutated Sp site. N = 6 for each construct. ***= $P < 0.001$; X = NS. Both mutant and wild type + KCl were compared to the wild type. The mutant + KCl was compared to the mutant.

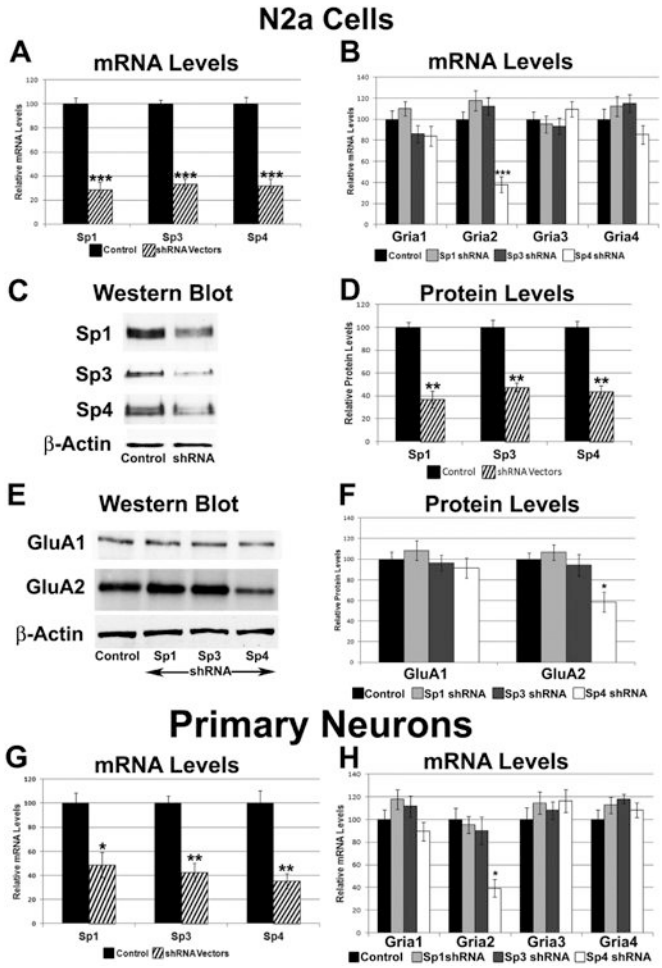


**Figure 4.**

Effect of silencing of *Sp1*, *Sp3*, or *Sp4* on the expression of the AMPA receptor subunit genes using RNA interference. (A) N2a cells transfected with *Sp1*, *Sp3*, or *Sp4* shRNA showed significant down-regulation of *Sp1*, *Sp3*, and *Sp4* transcripts, respectively. N = 6. (B) In N2a cells, *Gria2* transcript levels were decreased with silencing of *Sp4*, but not with silencing of either *Sp3* or *Sp1*. N = 6. (C-D) In N2a cells, protein levels of Sp1, Sp3, and Sp4 decreased significantly with shRNA against *Sp1*, *Sp3*, and *Sp4*, respectively. A representative western blot for β-actin, the loading control, is shown. N = 3. (E-F) *Sp4* silencing reduced the protein levels of GluA2 but not that of GluA1. Protein levels of GluA1 and GluA2 did not change significantly with *Sp1* or *Sp3* silencing. The loading control was β-actin. N = 3. (G) shRNA against *Sp1*, *Sp3*, or *Sp4* showed a significant down-regulation in the transcript levels of *Sp1*, *Sp3*, and *Sp4*, respectively. N = 3. (H) Transcript levels of *Gria2*, but not *Gria1*, *Gria3*, and *Gria4*, decreased with *Sp4* shRNA in primary neurons. Transcript levels of *Gria1-4* did not change significantly with *Sp1* or *Sp3* shRNA in primary neurons. N = 3. *= $P < 0.05$, **= $P < 0.01$, and ***= $P < 0.001$ when compared to controls.

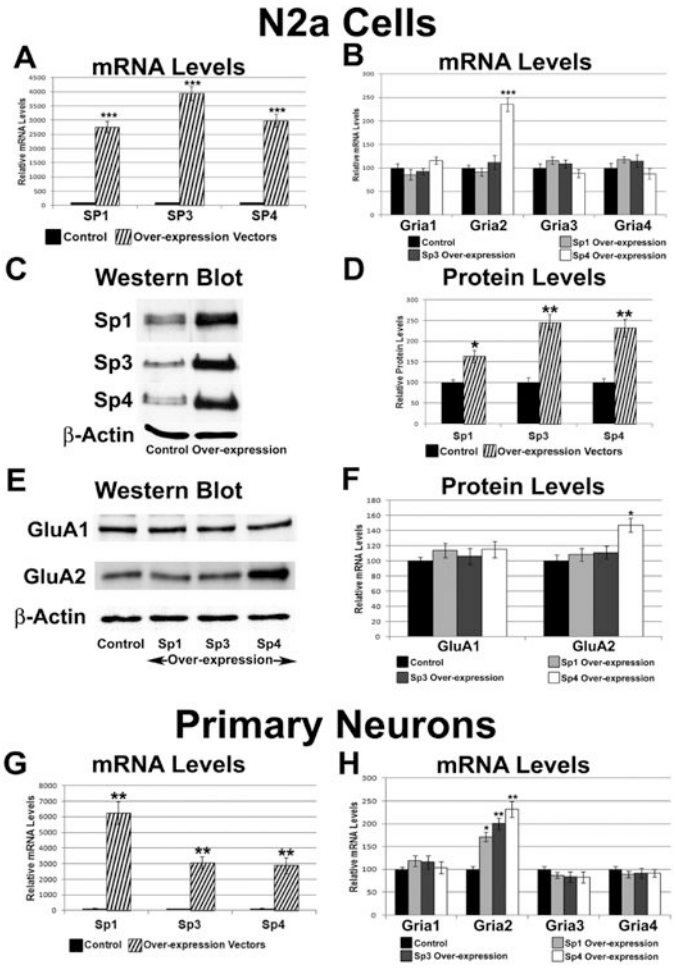


**Figure 5.**

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.

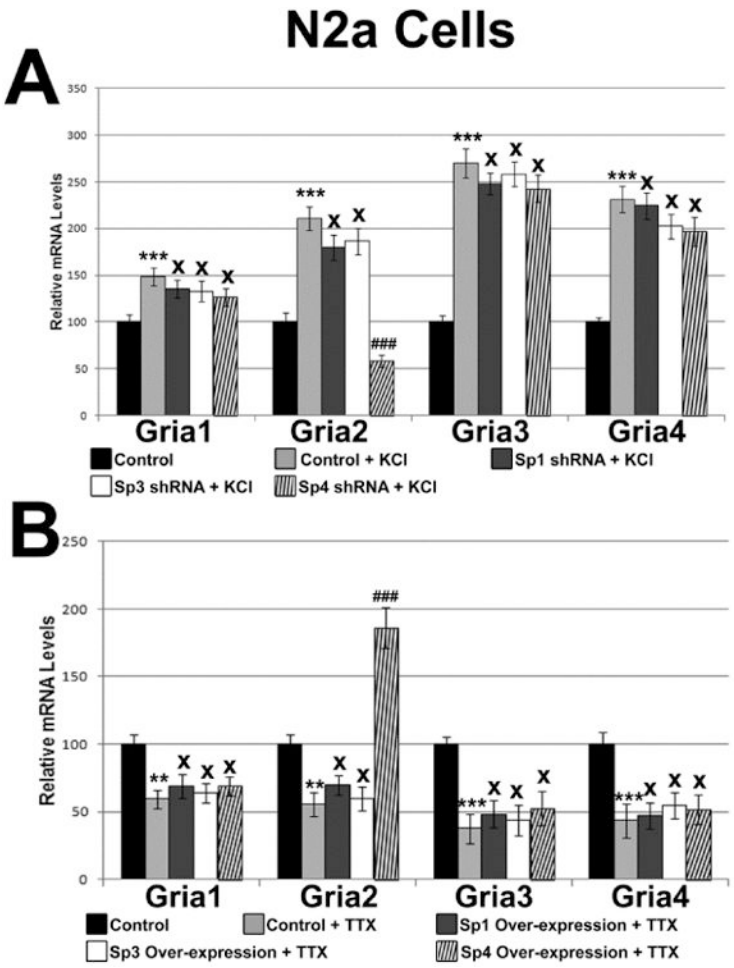


**Figure 6.**

Effect of changes in neuronal activity on *Gria1*, *2*, *3*, and *4* transcript levels in the absence or presence of *Sp1*, *Sp3*, or *Sp4* silencing or over-expression in N2a cells. (A) Transcript levels of all AMPA receptor subunit genes were increased by 5 h of 20 mM KCl treatment. KCl is a depolarizing agent that increases neuronal activity. Silencing *Sp4* did not allow for transcript levels of *Gria2* to increase with KCl, but had no effect on the KCl-induced increase in the transcript levels of *Gria1*, *Gria3*, and *Gria4*. Silencing *Sp1* and *Sp3* did not prevent the KCl-induced up-regulation of *Gria1-4* transcript levels. N = 6. (B) Transcript levels of all AMPA receptor subunit genes were decreased with 3 days of 0.4 μM TTX treatment. TTX decreases neuronal activity by blocking action potentials. *Sp4* over-expression rescued the TTX-mediated down-regulation of *Gria2* transcript levels, but did not rescue the TTX-mediated down-regulation of the *Gria1*, *Gria3*, and *Gria4* transcripts. *Sp1* or *Sp3* over-expression could not rescue the TTX-mediated down-regulation of *Gria1-4* transcript levels. N = 6. *= $P < 0.05$, **= $P < 0.01$, and ***= $P < 0.001$ when compared to controls. ### = $P < 0.001$ and X = non-significant when compared to KCl or TTX treatment alone.

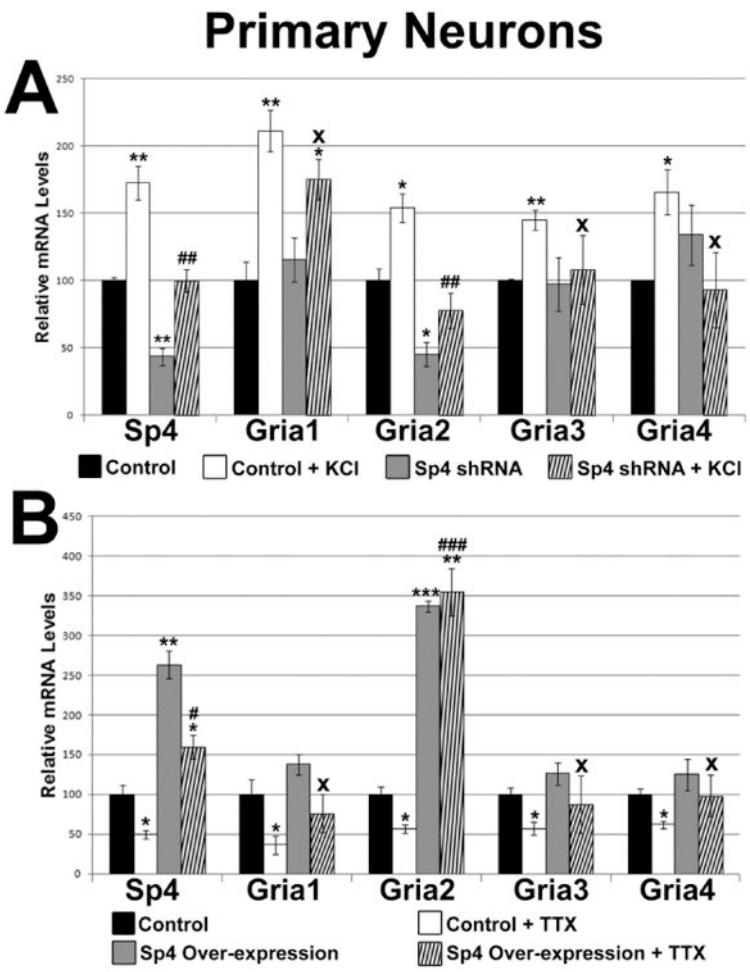


**Figure 7.**

Effect of changes in neuronal activity on *Gria1*, *2*, *3*, and *4* transcript levels in the absence or presence of *Sp4* silencing or over-expression in rat visual cortical neurons. (A) Transcript levels of *Sp4* and all AMPA receptor subunit genes were increased by 5 h of 20 mM KCl treatment. Silencing *Sp4* reduced its own transcript levels as well as that of *Gria2*, but not those of *Gria1*, *3*, or *4*. *Sp4* silencing also did not allow for transcript levels of *Sp4* itself and *Gria2* to increase with KCl, but it had no effect on KCl-induced up-regulation of *Gria1*, *Gria3*, and *Gria4* transcripts. N = 3. (B) Transcript levels of *Sp4* and all AMPA receptor subunit genes were decreased with 3 days of 0.4 μM TTX treatment. *Sp4* over-expression significantly increased its own transcript levels as well as that of *Gria2*, but not those of *Gria1*, *3*, or *4*. *Sp4* over-expression also rescued the TTX-mediated down-regulation of *Gria2* transcript levels, but not those of *Gria1*, *Gria3*, and *Gria4* transcripts. N = 3. *= $P < 0.05$, **= $P < 0.01$, and ***= $P < 0.001$ when compared to controls. # = $P < 0.05$, ## = $P < 0.01$, ### = $P < 0.001$ and X = non-significant when compared to KCl or TTX treatment alone.

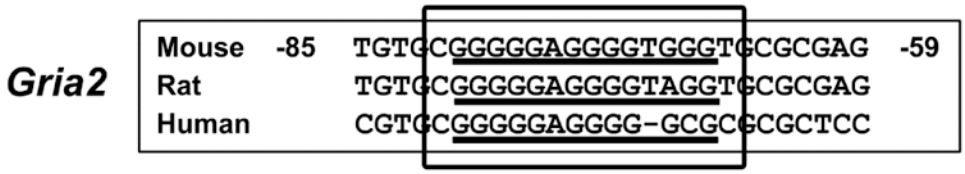


**Figure 8.**

Aligned partial sequences of the *Gria2* promoters from mice, rats, and humans showed conserved Sp binding sites.

## Table 1

EMSA Probes. Positions of probes are given relative to TSP. Putative Sp binding sites are underlined.

| Gene Promoter | Position | EMSA Sequence |
|---|---|---|
| *Gria1* | -189/-172 | F: 5′ TTTTTGGAGAGGCAGGGGTGAT 3′ |
| | | R: 5′ TTTTATCACCCCTGCCTCTCCA 3′ |
| *Gria2* | -89/-64 | F: 5′ TTTTGCGCTGTGCGGGGGAGGGGTGGGTGC 3′ |
| | | R: 5′ TTTTGCACCCACCCCTCCCCCGCACAGCGC 3′ |
| *Gria3* | -87/-69 | F: 5′ TTTTCAATGAACCCGCCTTCCAG 3′ |
| | | R: 5′ TTTTCTGGAAGGCGGGTTCATTG 3′ |
| *Gria4* | +507/+527 | F: 5′ TTTTGTAGCCCGGGCGGGGGTCGGC 3′ |
| | | R: 5′ TTTTGCCGACCCCCGCCCGGGCTAC 3′ |
| *GM3 Synthase* | -58/-38 | F: 5′ TTTTGCGCGACCCCCGCCCCCGCCTA 3′ |
| | | R: 5′ TTTTTAGGCGGGGGCGGGGTCGCGC 3′ |
| Mutant Sp *Gria2* | -89/-64 | F: 5′ TTTTGCGCTGTGTTTTTTTTTTTTGTGTGC 3′ |
| | | R: 5′ TTTTGCACACAAAAAAAAAAAACACAGCGC 3′ |

## Table 2

Primers and conditions used for ChIP analysis.

| Gene Promoter | Sequence | Amplicon Length | Cycling Conditions |
|---|---|---|---|
| *Gria1* | F: 5′ GGGAGGTGAAGGGGACAGTGGG 3′ | 170 bp | 94°: 30s, 57°: 30s, 72°: 45s; 2.5% DMSO |
| | R: 5′ CCCAGGAGCCAGCTCGGAGT 3′ | | |
| *Gria2* | F: 5′ ATCGAATGCGCAAGACTGGA 3′ | 201 bp | 94°: 30s, 60°: 30s, 72°: 45s; 2.5% DMSO |
| | R: 5′ GAAAGTCACAGAGAGGGGCA 3′ | | |
| *Gria3* | F: 5′ CAGAAATCGCTTTGGGGAACC 3′ | 71 bp | 94°: 30s, 60°: 30s, 72°: 45s; 2.5% DMSO |
| | R: 5′ ACTTCTGTCTTCACACCAATCTG 3′ | | |
| *Gria4* | F: 5′ AGCCTCCCTGAAGACCTGGGC 3′ | 231 bp | 94°: 30s, 60°: 30s, 72°: 45s; 2.5% DMSO |
| | R: 5′ GGCTCCCTTGCGCTCCCTTG 3′ | | |
| *GM3 Synthase* | F: 5′ CACCTACTTCTCGGCTGGAG 3′ | 198 bp | 94°: 30s, 57°: 30s, 72°: 45s. |
| | R: 5′ AATTCAGCCCCGGACAGT 3′ | | |
| *NT3* | F: 5′ GAGCAAACTCCAAAATGCCAGG 3′ | 219 bp | 94°: 30s, 57°: 30s, 72°: 45s |
| | R: 5′ AAAGTTGCGCCGGGCTATCTC 3′ | | |
| *β-Actin* | F: 5′ GCTCTTTTCCAGCCTTCCTT 3′ | 187 bp | 94°: 30s, 57°: 30s, 72°: 45s. |
| | R: 5′ CGGATGTCAACGTCACACTT 3′ | | |

## Table 3

Primers used for promoter cloning and mutagenesis analysis. Mutated Sp binding sites are underlined.

| Gene Promoter | Position | Primer |
|---|---|---|
| *Gria2* | -947/+152 | F: 5′ CAGACGCGTCCCAAGCAGGCTCGGTGTAATGA 3′ |
| | | R: 5′ CAGAGATCTGCTGTGGTCCCGGTGTCTGG 3′ |
| Mutant Sp *Gria2* | -90/-52 | F: 5′ GGCGCTGTGCG**T**G**T**GAG**T**G**T**T**T**G**T**GTGCGCGAGCTCGCCG 3′ |
| | | R: 5′ CGGCGAGCTCGCGCAC**A**CAA**A**C**A**CTC**A**C**A**CGCACAGCGCC 3′ |

## Table 4

*Sp3* and *Sp4* shRNA sequences. shRNA sequences cloned into the pLKO.1 vector for silencing of Sp3 and Sp4

| Gene | | Hairpin Sequence |
|---|---|---|
| *Sp3* | 1 | *5′ CCGG*—**ATGAGAAACTGTTGGTATTTA**—*CTCGAG*—**TAAATACCAACAGTTTCTCAT**—*TTTTTG* 3′ |
| | | 5′ AATTCAAAAA—**ATGAGAAACTGTTGGTATTTA**—CTCGAG—**TAAATACCAACAGTTTCTCAT** 3′ |
| | 2 | *5′ CCGG*—**TTACCTTTGTACCAATCAATA**—*CTCGAG*—**TATTGATTGGTACAAAGGTAA**—*TTTTTG* 3′ |
| | | 5′ AATTCAAAAA—**TTACCTTTGTACCAATCAATA**—CTCGAG—**TATTGATTGGTACAAAGGTAA** 3′ |
| *Sp4* | 1 | *5′ CCGG*—**CCAGTAACAATCACTAGTGTT**—*CTCGAG*—**AACACTAGTGATTGTTACTGG**—*TTTTTG* 3′ |
| | | 5′ AATTCAAAAA—**CCAGTAACAATCACTAGTGTT**—CTCGAG—**AACACTAGTGATTGTTACTGG** 3′ |
| | 2 | *5′ CCGG*—**CTGGACAACAGCAGATTATTA**—*CTCGAG*—**TAATAATCTGCTGTTGTCCAG**—*TTTTTG* 3′ |
| | | 5′ AATTCAAAAA—**CTGGACAACAGCAGATTATTA**—CTCGAG—**TAATAATCTGCTGTTGTCCAG** 3′ |

**Table 5**

**Real-time qPCR Primers**

| Gene | Primer |
|---|---|
| *Gria1* | F: 5′ GAGCAACGAAAGCCCTGTGA 3′ |
| | R: 5′ CCCTTGGGTGTCGCAATG 3′ |
| *Gria2* | F: 5′ AAAGAATACCCTGGAGCACAC 3′ |
| | R: 5′ CCAAACAATCTCCTGCATTTCC 3′ |
| *Gria3* | F: 5′ TTCGGAAGTCCAAGGGAAAGT 3′ |
| | R: 5′ CACGGCTTTCTCTGCTCAATG 3′ |
| *Gria4* | F: 5′ GGCTCGTGTCCGCAAGTC 3′ |
| | R: 5′ TTCGCTGCTCAATGTATTCATTC 3′ |
| *Sp1* | F: 5′ CTCCAGACCATTAACCTCAGTG 3′ |
| | R: 5′ ATCATGTATTCCATCACCACCAG 3′ |
| *Sp3* | F: 5′ TCAGGCACAGACAGTGACCCCT 3′ |
| | R: 5′ AGCGTGAGTGTCTGAACAGGCG 3′ |
| *Sp4* | F: 5′ TTGCAGCAAGGCCAGCAGACC 3′ |
| | R: 5′ GCTTCTTCTTTCCTGGTTCACTGCT 3′ |
| *Actb* | F: 5′ GTGACGTTGACATCCGTAAAGA 3′ |
| | R: 5′ GCCGGACTCATCGTACTCC 3′ |
| *Gapdh* | F: 5′ AGGTCGGTGTGAACGGATTTG 3′ |
| | R: 5′ GGGGTCGTTGATGGCAACA 3′ |