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Specificity protein 4 (Sp4) transcriptionally regulates inhibitory GABAergic receptors in neurons

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Abstract

Previous studies in our laboratory have shown that the neuron-specific specificity protein 4 (Sp4) transcriptionally regulates many excitatory neurotransmitter receptor subunit genes, such as those for *GluN1*, *GluN2A*, and *GluN2B* of N-methyl-D-aspartate (NMDA) receptors and *Gria2* of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. It also regulates *Atp1a1* and *Atp1b1* subunit genes of Na^+/K^+-ATPase, a major energy-consuming enzyme, as well as all 13 subunits of cytochrome c oxidase (COX), an important energy-generating enzyme. Thus, there is a tight coupling between energy consumption, energy production, and excitatory neuronal activity at the transcriptional level in neurons. The question is whether inhibitory neurotransmitter receptors are also regulated by Sp4. In the present study, we tested our hypothesis that Sp4 regulates receptor subunit genes of a major inhibitory neurotransmitter, GABA, specifically $GABA_A$ receptors. By means of multiple approaches, including *in silico* analysis, electrophoretic mobility shift and supershift assays, real-time quantitative PCR, chromatin immunoprecipitation, promoter mutational analysis, over-expression and shRNA of Sp4, functional assays, and western blots, we found that Sp4 functionally regulates the transcription of *Gabra1* ($GABA_A$-α1) and *Gabra2* ($GABA_A$-α2), but not *Gabra3* ($GABA_A$-α3) subunit genes. The binding sites of Sp4 are conserved among rats, humans, and mice. Thus, our results substantiate our hypothesis that Sp4 plays a key role in regulating the transcription of $GABA_A$ receptor subunit genes. They also indicate that Sp4 is in a position to transcriptionally regulate the balance between excitatory and inhibitory neurochemical expressions in neurons.

Keywords

Gene regulation; Sp4; $GABA_A$ receptors; *Gabra1*; *Gabra2*; *Gabra3*

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1. Introduction

Neuronal activity and energy metabolism are tightly coupled processes [1]. Previously, we have shown that the neuron-specific specificity protein 4 (Sp4) transcriptionally regulates many excitatory neurotransmitter receptor subunit genes, such as *Grin1* (GluN1), *Grin2a* (GluN2A), and *Grin2b (*GluN2B) of N-methyl-D-aspartate (NMDA) receptors [2] and *Gria2* of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [3]. It also regulates *Atp1a1* and *Atp1b1* subunit genes of Na^+/K^+-ATPase, a major energy-consuming enzyme [4], as well as all 13 subunits of cytochrome c oxidase (COX), an important energy-generating enzyme [5] in neurons. As neuronal activity involves both excitation and inhibition, the question naturally arises as to how the inhibitory neurotransmitter receptors are transcriptionally regulated, and if Sp4 plays a role in this regulation.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system [6,7] and the fast-acting ionotropic type A receptors ($GABA_AR$) are prevalent among neurons [8,9]. Functional alterations of $GABA_A$ receptors are often associated with a variety of disorders, such as epilepsy, anxiety, insomnia, and schizophrenia [10], linking frequently to an excitation/inhibition imbalance in specific populations of neurons [11–13]. Understanding the genetic mechanism underlying the synaptic balance at the cellular and molecular levels will lead to a better insight into normal and abnormal functioning of neurons, and will lay a foundation for new therapeutic tools for the prevention of a variety of neurological disorders. We have uncovered the transcriptional regulation of a number of excitatory neurotransmitter receptor genes [2,3,14–16]. Deciphering the transcriptional regulation of different inhibitory $GABA_A$ receptor subunit genes will be the next step towards such an understanding. The goal of the present study was to determine if the three major $GABA_A$ receptor subunit genes, *Gabra1* ($GABA_A$ α1), *Gabra2* ($GABA_A$ α2), and *Gabra3* ($GABA_A$ α3) are transcriptionally regulated by the same transcription factor, Sp4, as some of the key excitatory neurotransmitter receptor subunit genes. Our hypothesis is that they are.

By means of multiple approaches, including *in silico* analysis, electrophoretic mobility shift and supershift assays, real-time quantitative PCR, chromatin immunoprecipitation, promoter mutational analysis, over-expression, shRNA, functional assays, and western blots, we found that Sp4 functionally regulates the transcription of *Gabra1* and *Gabra2*, but not *Gabra3* subunit genes in neurons.

2. Materials and Methods

All experiments involving rats were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin (Milwaukee, WI). All efforts were made to minimize the number of animals used and their suffering.

2.1 Primary neuronal cultures

Rat or mouse primary visual cortical neurons were cultured as described previously [17]. In brief, neonatal one-to-two day old pups were euthanized by decapitation. The brains were detached from the skull, meninges were removed, and the visual cortex was dissected. Pieces of the visual cortex were treated with trypsin and suspended by pipetting. Neurons were then dissociated by trituration, and cells were seeded in a six-well plate (35 mm; pre-coated with poly-L-lysine) at a density of 1×10^6 cells/well. Cells were allowed to grow in Neurobasal-A media containing L-glutamine and B27 supplement (Life Technologies, Carlsbad, CA, USA) and maintained in a humidified incubator with 5% CO_2 at 37°C. Cytosine arabinoside (Ara-C) (Sigma, St Louis, MO, USA) was added to the culture media to suppress the division of glial/non-neuronal cells.

2.2 In silico analysis of $GABA_A$ receptor subunit promoters

Using DNAStar Lasergene 8 Suite – Sequence Builder and Genequest software, sequences surrounding I kb upstream and 1 kb downstream of the transcription start points (TSPs) of mouse, rat, and human $GABA_A$ receptor α1, α2, and α3 subunit genes (*Gabra1, Gabra2, and Gabra3*) were derived from the genome database in GenBank and were aligned using Megalign. Computer-assisted search for the typical Sp binding motif (‘GGGCGG’), the atypical Sp binding motifs (‘GGGTGG’ and ‘CCCTCC), or their complements, was conducted to uncover the putative Sp binding sites.

2.3. Preparation of nuclear extracts and electrophoretic mobility shift and supershift assays

Nuclear extraction protocol was as reported previously [18]. In brief, the frozen brain tissue was rinsed in the PBS buffer, spun and transferred to a Dounce tissue homogenizer. Buffer A was added at 2.5 times per g of the tissue weight with a minimum volume of 2 ml. Five strokes of pestle were used to homogenize the tissue to a liquid mass. After induction with NP-40 (0.5%), five additional strokes were applied and the tissue was incubated for 10 min to lyse the cells with the detergent. The homogenate was transferred equally into fresh tubes and centrifuged at 13,000 rpm for 30 sec. The supernatant containing the cytoplasmic constituents was removed, and buffer C was added to the nuclear pellet. Tubes were mixed thoroughly, placed on a rotary shaker for 15 min, and centrifuged. The supernatant containing the protein from the nuclear extract was removed carefully and transferred to a fresh tube. The protein was measured, aliquotted, and stored at −80°C until use.

Electrophoretic mobility shift (EMSA) and supershift assays’ protocols were as described previously [3]. Based on the *in silico* analysis, oligonucleotide probes with putative Sp4 binding site on each promoter were synthesized (Table 1), annealed, and labeled by a fill-in reaction with Klenow fragment and [^{32}P]dATP (50 μCi/200 ng; Perkin-Elmer, Shelton, CT, USA). Each labeled probe was incubated with 2 g of calf thymus DNA and 5 g of brain nuclear extract. For supershift experiments, the reactions were incubated with 2 μg of Sp4 polyclonal antibodies (Sp4, V-20, SC-645, Santa Cruz Biotechnology [SCBT], Santa Cruz, CA, USA) for 20 min at room temperature (RT). For competition, 100-fold excess of unlabeled oligonucleotides were incubated with brain nuclear extract before adding labeled or nonspecific oligonucleotides. Shift reactions were loaded onto 4% polyacrylamide gel

and run at 200 V for 3 h in 0.25X TBE buffer. Gels were dried and exposed for 4–24 h at −80°C. Mouse *GM3 synthase* with a known Sp4 binding site was designed as previously described [5] and was used as a positive control. Sp4 mutants with mutated sequences were used as negative controls.

2.4. Chromatin immunoprecipitation assays

The protocol for *in vivo* chromatin immunoprecipitation (ChIP) was as described previously [2] with a few modifications. Murine visual cortical tissue was kept on ice at all times to avoid protein degradation. Tissue was cut in a petri dish resting on a block of dry ice and then chopped into tiny pieces, fixed in 2% formaldehyde, and resuspended in the swelling buffer (85 mM KCl, 5 mM PIPES, pH 8.0, 1% Nonidet P-40, and protease inhibitors). The tissue was transferred to a Dounce homogenizer, homogenized 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 anti-Sp4 polyclonal antibodies (2 μg). Two μg of anti-nerve growth factor receptor (NGFR) antibodies (sc-6188, SCBT) or 'no antibody' blanks were used as negative controls. Based on our *in silico* analysis, putative Sp binding sites were identified and semi-quantitative PCR was performed by utilizing primers surrounding the Sp binding sites (Table 2). *GM3 synthase* was the positive control. *Neurotrophin 3* [19] was also used (data not shown), and *β-actin* promoter (*Actb*) was the negative control. PCR additives and cycling parameters were used to improve the reproducibility and quality of ChIP. PCR products were visualized in 2% agarose gels.

2.5. Luciferase reporter assay

Luciferase reporter constructs of the three $GABA_A$ receptor subunit promoters (*Gabra1, Gabra2*, and *Gabra3)* were made by PCR cloning the proximal promoter sequences, using genomic DNA prepared from mouse N2a cells [15] as template. Digestion with restriction enzymes was performed, followed by ligation of the product directionally into pGL3 basic vector (Promega, Madison, WI). Sequences of primers used for PCR cloning are provided in Table 3. Site-directed mutations of putative Sp factor binding sites in each promoter were generated using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Primers used for mutagenesis are also listed in Table 3. All constructs were verified by sequencing.

Primary neurons were cultured and plated into 24-well plates before transfection. Each promoter construct was transfected into the primary neurons using Neurofect (Genlantis, San Diego, CA). Each well received 0.6 μg of reporter construct and 0.06 μg of pRL-TK *Renilla* luciferase vector [20]. Firefly luciferase and *Renilla* luciferase activities were measured sequentially using the Dual Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized according to *Renilla* and expressed as relative luciferase units to reflect the promoter activity.

2.6. Sp4 knockdown and KCl treatment

To knockdown Sp4 expression, a vector-based shRNA approach was used, and two target sequences were chosen from the RNAi Consortium's Public TRC Cloning Database at the

Broad Institute (Table 4) and each cloned into the pLKO.1 TRC cloning vector (Plasmid 10878; Addgene, Cambridge, MA, USA) as reported previously [5]. To rule out the non-specific consequences of shRNA, the pLKO.1 non-mammalian shRNA control vector SHC002 (Sigma), a scrambled negative control, was also used.

Transfection of primary neurons was carried out 4 days post-plating with both *Sp4* shRNA constructs (2 μg of each construct) or the pLKO.1 non-mammalian control (2 μg), using Neurofect transfection reagent per 6-well plate according to the manufacturer's instructions (Genlantis). TurboGFP (0.5 μg) vector was added to each well for transfection visualization and selection efficiency. Transfection efficiency was around 50–60% before selection. Puromycin selection, however, effectively yielded 100% transfected cells. Transfection efficiency was observed using green fluorescence. Primary neurons transfected with shRNA against Sp4 were further stimulated with KCl. A final concentration of 20 mM KCl was added to the culture medium for 5 h according to our published method [2]. After this period, cells were harvested for RNA and protein isolation.

2.7. Sp4 over-expression and TTX treatment

Using Gateway Multisite Cloning kit (Invitrogen), human Sp4 clones of cDNA (Open Biosystems Lafayette, CO, USA) were cloned into pcDNA Dest40 vectors according to the manufacturer's instructions and as described previously [21].

As mentioned above, the transfection protocol for over-expressing Sp4 in primary cultures was similar to that described for shRNA. Either 2 μg of *Sp4* over-expression vector, or 1.5 μg of the pcDNA3.1 empty vector and 0.5 μg of turboGFP vector for green fluorescence were used for primary neuronal cultures. Transfected primary neurons were blocked for 3 days with tetrodotoxin (TTX) at a final concentration of 0.4 μM and starting on the day after transfection as previously described [20]. Two to four days after transfection, neurons were harvested for RNA and protein isolation.

2.8. Knockdown of Sp4 followed by over-expression of Sp4 with or without KCl treatment

Two sets of experiments were performed. In the first set, primary neurons in culture were transfected with Sp4 shRNA as described in section *2.6* above for one day, then they were transfected with Sp4 over-expression vectors as described in *2.7* above for another day. In the second set of experiments, primary neurons were also transfected with Sp4 shRNA followed by transfection with Sp4 over-expression vectors; however, in between the two procedures, they were treated with KCl for 5 h as described in section *2.6* above.

2.9. RNA isolation and cDNA synthesis

Cultured and transfected neurons were washed with PBS, followed by the extraction of total RNA by the TRIzol (Life Technologies, Carlsbad, CA, USA) method according to the manufacturer's instructions. Any residual genomic DNA was digested with DNase I on total RNA, and cDNA synthesis was carried out using iScript cDNA synthesis kit (170-8891, BioRad, Hercules, CA, USA).

2.10. Quantification of gene expression by real-time PCR analysis

mRNA levels of various genes were determined in a BioRad iCycler using IQ SYBR Green Supermix and/or Cepheid Smart Cycler Detection system (Cepheid, Sunnyvale, CA, USA) following manufacturer's protocols. Primers for real-time are shown in Table 5. PCR runs: hot start 2 min at 95 C, denaturation 10 s at 95 C, annealing 15 s according to the *T*m of each primer, and extension 10 s at 72 C for 15–30 cycles. Melt curve analyses verify the formation of single desired PCR product. Mouse/rat compatible *Actb* (β-actin) and *Gapdh* were used as internal controls, and the $2^{-\Delta\,\Delta CT}$ method [22] was performed for the relative amount of transcripts.

2.11. Western blots

Control, Sp4 shRNA, and Sp4 over-expression protein samples were harvested using the sample buffer (12.5 mM EDTA, 50 mM Tris–HCl pH 6.8, 1% SDS, 10% glycerol). Equal protein amounts of each sample were electrophoresed in SDS gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in TBS-T (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, and 0.05% Tween 20) at RT for 1h, then incubated with primary antibodies against Sp4 (1:1000; SCBT), $GABA_A$ α1, $GABA_A$ α2 , or $GABA_A$ α3 (all at 1:1000; all from Phosphosolutions, Aurora, CO, USA). The loading control was β-actin (1:5000; Sigma) followed by an incubation with horseradish peroxidase-conjugated secondary antibodies from Vector Laboratories (Burlingame, CA, USA). ECL reagent was used to visualize protein position and intensity on blots, which were exposed to autoradiographic films (RPI, Mount Prospect, IL, USA). Gel Doc system (BioRad) was used to perform quantitative analyses of relative changes.

2.12. Statistical Analysis

Data were analyzed using ANOVA. Significance between two groups was analyzed by student's *t* test. *P* values of 0.05 or less were considered significant.

3. Results

3.1. In silico promoter analysis of $GABA_A$ receptor subunit genes

Proximal promoters of mouse and rat $GABA_A$ subunit genes *Gabra1, Gabra2, and Gabra3* with DNA sequence 1 kb 5' upstream and 1 kb 3' beyond TSP were analyzed using *in silico* analysis. The α1-subunit gene is strongly expressed in all brain regions [23], and we found a GC-rich promoter with many typical Sp binding motifs, the GC-box (GGGCGG), as well as a TATA box upstream of the TSP. The *Gabra2* promoter also contained more than one typical binding motif, 'CCCGCC' as well as atypical binding sites, whereas the *Gabra3* promoter showed atypical Sp binding sites, such as 'GGGTGG' or 'CCCTCC' and were conserved only between mouse and rat, but not in humans.

3.2. In vitro Sp4 bound to GC-box region of $GABA_A$ subunits α1 and α2

We performed EMSA to determine if Sp4 would bind to the GC boxes of *Gabra 1*, *2*, and *3* subunit promoters. Based on our previous finding that *GM3 synthase* formed specific DNA/Sp4 shift and supershift complexes with mouse visual cortical nuclear extract but

almost fade or absent with HeLa cell nuclear extract [3], we used *GM3 synthase* with murine visual cortical nuclear extract as our positive control. Indeed, when incubated with mouse visual cortical tissue nuclear extract, *GM3 synthase* formed specific DNA/ Sp4 shift and supershift complexes (Fig. 1, lanes 1 and 3 for Sp4 shift and supershift, respectively).

When murine visual cortical nuclear extract was incubated with $GABA_A$ receptor subunit probes containing putative Sp4 sites, *Gabra 1* and *Gabra2* gave positive shift bands (Fig.1, lanes 4 and 9, respectively) that were competed out with the addition of unlabeled competitors (Fig. 1, lanes 5 and 10, respectively). A supershift band against Sp4 was present when Sp4 antibody was added to either *Gabra1* or *Gabra2* (Fig.1, lanes 6 and 11). The addition of unlabeled probes with mutated Sp4 binding sites did not compete out the Sp4 shift bands (Fig. 1, lanes 7 and 12). Labeled *Gabra1* and *Gabra2* with Sp4 antibody without any nuclear extract was also run to rule out antibody-oligo interaction, and they did not show any shift or supershift bands (Fig. 1, lanes 8 and 13). *Gabra3* did not give a positive shift (Fig. 1, lane 14) or a Sp4 supershift band (Fig.1, lane 16). Shift, competitor, and supershift reactions with mutated *Gabra1* and *Gabra2* did not show any binding to the Sp4 sites (Fig. 1, lanes 17–22).

3.3. In vivo interaction verification through ChIP assay

To provide further corroboration of the physical association of Sp4 with its binding sites on the $GABA_A$ subunit promoters, we performed a ChIP assay using mouse visual cortical tissue (Fig. 2). A 0.5% dilution of input chromatin was used as a standard to indicate the efficiency of the PCR. As Sp factors are known to regulate *GM3 Synthase* [24], the promoter of these genes were used as positive controls, whereas β-actin exon 5 (*Actb*) served as a negative control.

When the sonicated nuclear lysates were immunoprecipitated with Sp4 polyclonal antibodies and the resultant DNA was subjected to PCR analysis, the anti-Sp4 antibody specifically co-precipitated the *Gabra1* and *Gabra2* promoter fragments, but failed to do so with the *Gabra3* promoter. The appearance of a distinct band with Sp4 antibody verified the *in vivo* interaction of $GABA_A$ receptor subunits *Gabra1* and *Gabra2* with Sp4. Nerve growth factor receptor (NGFR) antibody was used as a control for the immunoprecipitation reaction and it did not yield any PCR product. To rule out the possibility of a bead-DNA interaction, an additional “no antibody” or blank control was used, which did not yield any PCR product.

3.4. Effect of mutated Sp4 binding sites on the Gabra1 and Gabra2 promoters

Gabra1 and *Gabra2* promoters that bound to Sp4 *in vitro* and *in vivo* were each cloned into pGL3 basic luciferase vectors. Site-directed mutations of the putative Sp4 binding sites were constructed. Transfection of control or mutated Sp promoter regions into cultured primary neurons resulted in a significant fall of 53% and 61% in the activity of *Gabra1 and Gabra2* promoters, respectively, containing the mutated Sp4 motif ($P < 0.001$ for all, Fig. 3).

3.5. Knock down of Sp4 decreased mRNA levels of $GABA_A$ receptors and the effect of KCl depolarization in primary neurons

To provide further confirmation to our findings, we employed shRNA constructs targeted to Sp4 mRNA in cultured primary neurons. β-*Actin* was used as an internal control. Sp4 protein showed a significant decline of 76% ($P < 0.001$). $GABA_A$ α1 and $GABA_A$ α2 were decreased by 36.77% and 26.65%, respectively ($P < 0.01$, Fig. 4, A–B), whereas $GABA_A$ α3 did not show any remarkable change (Fig. 4, A–B). With silencing, mRNA levels were also quantified, and β-*Actin* and *Gapdh* were used as internal PCR controls. For transfection controls, neurons received scrambled shRNA, which failed to bind to any known mRNA. Silencing of Sp4 resulted in a significant decrease of 44% in Sp4 mRNA ($P < 0.001$), and significant decreases of 56% and 54% in *Gabra1* and *Gabra2* mRNA levels, respectively ($P < 0.01$ for both, Fig. 4C). On the other hand, mRNA levels of *$GABA_A$ α3* did not show any significant change with silencing (Fig. 4C).

Primary cultured neurons (control and Sp4 transfected) were then subjected to 20 mM KCl for 5 h. As shown in Figure 4C, KCl depolarization significantly increased *Sp4* itself, *Gabra1*, and *Gabra2* transcript levels to 158%, 164%, and 152%, respectively ($P < 0.05$ for all). In the presence of Sp4 shRNA, KCl could no longer increase transcripts of *Sp4, Gabra1 and Gabra2* to levels of control-plus-KCl ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively, when compared to control-plus-KCl). The values of shRNA-plus-KCl were also not significantly different from those of shRNA alone ($P = 0.218$, $P = 0.120$, and $P = 0.202$, respectively, when compared to shRNA alone). The transcript level of *Gabra3* was also significantly increased with KCl treatment ($P < 0.05$), but was not changed in the presence of *Sp4* shRNA.

3.6. Sp4 silencing followed by over-expression rescued the effects of the knockdown

Cultured primary neurons were transfected first with Sp4 shRNA for one day and then with Sp4 over-expression vectors for another day. Over-expression of Sp4 was able to increase *Sp4*, *Gabra1*, and *Gabra*2 transcript levels significantly above those with Sp4 shRNA alone ($P < 0.05$ for all), and these values were not significantly different from those of controls (Fig. 4C). This indicates that the shRNA-induced down-regulation of transcripts was rescued by Sp4 over-expression. However, this combination had no effect on *Gabra3* transcripts (Fig. 4C). Sp4-silenced primary neurons treated with KCl for 5 hours and then transfected with Sp4 over-expression vectors showed increases in their mRNA levels of *Sp4*, *Gabra1*, and *Gabra2* that were significantly above those treated with shRNA and KCl but without over-expression ($P < 0.001$, $P < 0.01$, and $P < 0.01$, respectively, when compared with shRNA-plus-KCl). Again, Sp4 over-expression was able to counter the effect of shRNA and allowed the neurons to be activated by KCl. Thus, the values of transcripts for *Sp4*, *Gabra1*, and *Gabra2* were not significantly different between shRNA-plus-KCl-plus-over-expression and control-plus-KCl. Again, this combination had no effect on *Gabra3* (Fig. 4C).

3.7. Over-expressing Sp4 increased mRNA levels of $GABA_A$ receptors and the effect of TTX blockade in primary neurons

To determine the effect of over-expressing Sp4 on transcript levels of $GABA_A$ receptor subunits *Gabra1* and *Gabra2*, *Sp4* over-expression plasmids were transfected into primary

cultured neurons. β-actin was used as the internal control. Sp4 over-expression increased the protein levels of Sp4 to 196% ($P < 0.001$, Fig. 5A–B), and those of $GABA_A$ α1 and $GABA_A$ α2 to 182% and 144%, respectively ($P < 0.001$ for both, Fig. 5A–B). Likewise, mRNA levels showed a similar pattern of change. Over-expressing Sp4 significantly increased the mRNA levels of Sp4 to 369%, and those of *Gabra1 and Gabra2* to 210% and 293%, respectively ($P < 0.001$ for all, Fig. 5C). On the other hand, neither $GABA_A$ α3 protein nor *Gabra3* mRNA exhibited any significant change with Sp4 over-expression (Fig. 5A–C).

To determine if Sp4 over-expression could rescue $GABA_A$ transcript levels suppressed by TTX-induced impulse blockade, primary cultured neurons (controls and Sp4 transfected) were subjected to 0.4 μM TTX treatments for 3 days. Control neurons transfected with empty vectors were exposed to TTX showed a significant decrease of 60%, 63%, and 62% in transcript levels of *Sp4*, *Gabra1*, and *Gabra2*, respectively ($P < 0.01$, $P < 0.001$, and $P < 0.05$, respectively; Fig. 5C). However, neurons that were transfected with *Sp4* over-expression vectors did not exhibit any down-regulation of their transcript levels of *Sp4*, *Gabra1*, and *Gabra2* with TTX. Rather, these levels were 346%, 179% and 309% respectively, as compared to controls ($P < 0.001$ for *Sp4* and *Gabra2*, and $P < 0.01$ for *Gabra1* as compared to TTX alone; Fig. 5C). *Gabra3* transcript level decreased significantly with TTX treatment ($P < 0.05$) but was not rescued by *Sp4* over-expression (Fig. 5C).

3.8. Homology

The functional Sp4 binding sites are conserved among mice, rats, and humans for *Gabra1* and *Gabra2* (Fig. 6).

4. Discussion

The present study analyzed the proximal promoter regions of $GABA_A$ receptor subunit genes *Gabra1, Gabra2, and Gabra3* by means of multiple molecular and biochemical approaches, including *in silico* analysis, electrophoretic mobility shift and supershift assays, ChIP, RNA interference, over-expression, functional assays, and western blots in rodent primary cortical neurons. We found that *Gabra1* and *Gabra2*, but not *Gabra3*, subunit genes are regulated by the neuron-specific transcription factor Sp4. The typical Sp binding motifs on the *Gabra1 and Gabra2* promoters are conserved among mice, rats, and humans.

Specificity protein (Sp) is a family of zinc finger transcription factors that bind to GC-rich DNA motifs and regulate the expression of a large number of genes implicated in various biological functions [25]. The Sp binding site, also known as the GC box, contains an asymmetric hexanucleotide core ‘GGGCGG’ sequence with high affinity, or the GT (‘GGGTGG’) or CT (‘CCCTCC’) boxes with significantly lower affinities, and is recognized by members of the Sp/KLF family of transcription factors with zinc finger motifs [25,26]. Increasing evidence indicate that Sp4 is amply expressed in the brain, with high levels in neurons and low levels in astrocytes [5,27,28]. *Sp4* reportedly regulates developmental cerebellar dendritic patterning, postnatal development of hippocampal dentate granule cells, postnatal hippocampal cell proliferation, hippocampal long-term potentiation, and animal behaviors including contextual and spatial memory, sensorimotor

gating, and prepulse inhibition, some of which have been associated with psychiatric disorders [29–31]. Although Sp-*cis* motif has been reported in the promoter regions of *Gabra1* and *Gabra2* [32], it has not been characterized previously. The present study provides experimental evidence from multiple approaches that Sp4 functionally regulates the expression of *Gabra1* and *Gabra2* in neurons.

$GABA_A$ receptors are the dominant inhibitory neurotransmitter receptors in the brain, and their kinetics is known to be faster than that of $GABA_B$ by roughly ten-fold [33]. Binding of GABA to ionotropic $GABA_A$ receptors opens the intrinsic ion channels, enabling the influx of chloride through the channels and subsequent hyperpolarization of neurons [34]. At least 19 $GABA_A$ receptor subunit isoforms (α1–6, β1–3, γ1–3, ρ1–3, π, θ, δ ε) have been characterized in mammals [35,36]. The most typical heteropentameric $GABA_A$ receptors contain two α, two β, and one γ subunits [37,38].

Our current study indicates that Sp4 regulates the *Gabra1* and *Gabra2* but not *Gabra3* subunit genes of $GABA_A$ receptors. We have focused on these three subunits because they are developmentally regulated [39–43], vary in receptor kinetics [44,45], and the α subunit provides the interface for binding GABA [46] and benzodiazepine [47].

$GABA_A$ α1 expression increases with age, reaching a stable level in the adult [44,48] as well as in the aging brain [49]. In the immature cortex, the kinetics of the ionotropic $GABA_A$ receptors is relatively slow [45], but it accelerates three-fold during development until $GABA_A$ α1 dominates the receptor complex [50]. $GABA_A$ α1 subunit is responsible for sedative, amnestic, and anticonvulsant actions [51] and is also known to drive ocular dominance plasticity in the visual cortex [52]. The α1 subunit also has a high-affinity for binding GABA and benzodiazepine receptor ligands [53]. It is now clear that this important subunit is transcriptionally regulated by Sp4 (the present study).

The distribution and expression of the $GABA_A$ α2 subunit are reportedly strong in the superficial layers of neocortex and become abundant and widespread within the whole cortex during development; they then show a stable expression only in the superficial layers during adulthood and aging [49]. In the rat brain stem, this subunit is maintained at a steady level during the first three postnatal weeks [42,43]. It is thought to mediate anxiolytic actions [51,54] and is involved in modulating neuronal firing [52]. The persistence of α2 into adulthood underscores the importance of Sp4 in its regulation.

In contrast, the $GABA_A$ α3 subunit is abundantly expressed only during the early postnatal weeks and is decreased or absent in the adult and aging brain [49]. The inclusion of $GABA_A$ α3 in the receptor is reportedly the reason for the slow kinetics during early postnatal development, and a developmental switch from α3 to α1 is essential for a faster kinetics [44]. This developmental switch in $GABA_A$ subunits occurs in rats [41–42,43], cats [40], and macaque monkeys [55]. Such apparent subunit switches have been suggested to underlie the transition from depolarizing/excitatory to a hyperpolarizing/inhibitory mode of GABA action during development [56]. Our present data indicate that Sp4 does not play an important role in regulating this neonatal subunit of $GABA_A$ receptors.

The ratio of the different subunits is a more accurate predictor of $GABA_A$ receptor function than the expression of a single subunit [57,58] . Yu and colleagues [49] have revealed a continuously low α3 subunit and a relatively stable and high α1 and α2 subunit expression in the cortex during normal aging, and suggested that subunit composition may modulate the binding of GABA and benzodiazepine to the receptors. The present study documents that the two key $GABA_A$ receptor subunit genes, *Gabra1* and *Gabra2*, are transcriptionally regulated by Sp4 in neurons, indicating that Sp4's control over $GABA_A$ receptor expression is sustained throughout the postnatal life of the animal.

The fact that Sp4 plays an important role in regulating mediators of inhibitory transmission ($GABA_A$ receptors; the present study) is significant, as we have found that it also regulates many excitatory neurotransmitter receptor subunit genes, such as *Grin1*, *Grin2a*, and *Grin2b* of NMDA receptors [2] and *Gria2* of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [3]. Thus, Sp4 is in an ideal position to regulate the balance between excitation and inhibition to achieve a homeostatic state of neuronal functioning. Normal neuronal functioning is dependent on a proper balance between excitation and inhibition, both at the single cell and neuronal network levels. Neuronal firing rate is thought to be maintained around a homeostatic set point, and the maintenance of that stability is dependent on a number of factors, including synaptic scaling and the balancing of excitation and inhibition [reviewed in 59]. To date, the mechanism underlying such a homeostatic balance has not be fully elucidated. We propose that one such mechanism is via transcriptional regulation, and that Sp4 is capable of regulating mediators of both excitatory glutamatergic [2, 3] and inhibitory GABAergic [present study] neurotransmission. In fact, we have shown that over-expressing Sp4 up-regulates the mRNA and protein levels of both glutamatergic receptors [2,3] and GABAergic receptors (the present study), and knocking down Sp4 down-regulates both. Transcriptional regulation is a viable mechanism that allows for an exquisite orchestration of the delicate balance between excitation and inhibition.

In addition to regulating mediators of neuronal activity, Sp4 also transcriptionally regulates mediators of energy generation (cytochrome c oxidase or COX) as well as energy consumption (Na^+/K^+-ATPase) in neurons [4,5]. COX is the terminal enzyme of the mitochondrial electron transport chain critical in generating ATP, and it has proven to be a sensitive and reliable indicator of neuronal activity [1]. Its13 subunits are derived either from the mitochondrial genome or the nuclear DNA across 9 different chromosomes, and we found that Sp4 regulates all 13 subunits from the two genomes [5]. Na^+/K^+-ATPase is a plasma membrane protein that maintains the ionic imbalance across the membrane by actively extruding Na^+ in exchange for the influx of K^+ [60]. It is a major energy consumer in neurons because of the constant need for membrane repolarization after depolarizing activities, and its distribution is highly correlated with that of COX, a key energy generator [1]. Thus, Sp4 is a master regulator that tightly couples energy metabolism (both generation and consumption) with neuronal activity (both excitation and inhibition) at the transcriptional level. This ensures that the energy requirement of neuronal activity is met with an adequate production of energy. The present study also highlights Sp4's role in mediating a homeostatic balance between excitation and inhibition in the nervous system.

Acknowledgments

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Abbreviations

COX	cytochrome c oxidase
Gabra	gene name for $GABA_A$ receptor subunit
Sp4	specificity protein 4

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Highlights

- Sp4 regulates $GABA_A$ receptor subunits *Gabra1* and *Gabra2*, but not *Gabra3*.
- Over-expression of Sp4 enhances *Gabra1* and *Gabra2* mRNA and protein levels.
- Knocking down Sp4 reduces *Gabra1* and *Gabra2* mRNA and protein levels.
- Sp4 regulates inhibitory and the previously reported excitatory receptor genes.

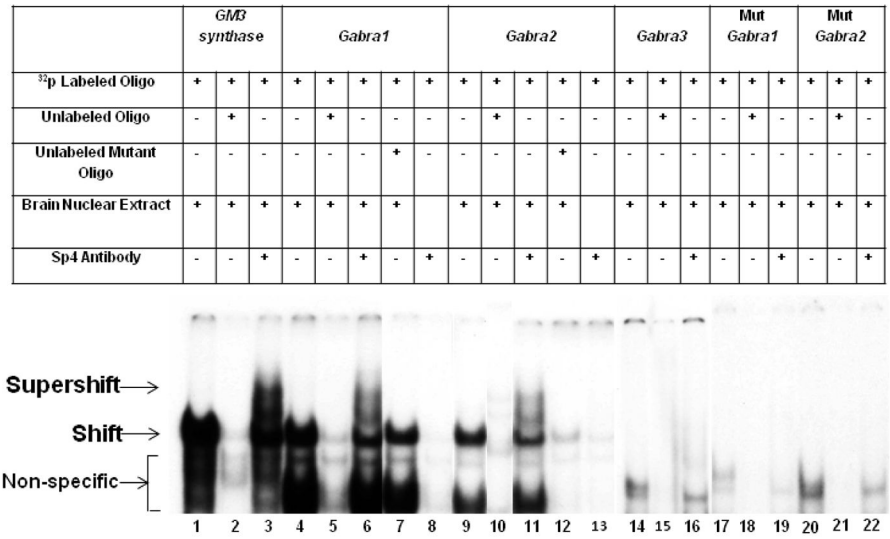

Fig. 1.

In vitro binding of Sp4 transcription factor to putative binding sites on $GABA_A$ receptor subunit promoters using EMSA and supershift assays. All lanes contain ^{32}P-labeled oligonucleotides and are indicated by a "+" or a "−" sign depending on whether they also contain mouse brain nuclear extract, excess unlabeled oligos, unlabeled mutant oligos, or Sp4 antibody. Sp4 shift, supershift, and non-specific complexes are indicated by arrows. The positive control for Sp4 factor binding was *GM3 Synthase*. Specific Sp4 shift bands were revealed upon incubation with cortical nuclear extract (lane 1). Excess unlabeled competitor competed out the shift band (lane 2). The addition of Sp4 antibody yielded a strong supershift band (lane 3) corresponding to the presence of tandem Sp binding. Incubation of cortical nuclear extract with the *Gabra1 and Gabra2* probes yielded specific Sp4 shift bands (lane 4 and 9, respectively) that were competed out with the addition of excess cold probes (lanes 5 and 10, respectively). The addition of Sp4 antibody yielded fainter but specific supershift bands (lanes 6 and 11, respectively). The addition of excess unlabeled mutant *Gabra1* and *Gabra2* probes did not compete out the shift reaction (lanes 7 and 12, respectively). Labeled *Gabra1* and *Gabra2* with Sp4 antibody without any nuclear extract was used as a positive control, to check for any antibody-oligo interaction, and they did not show any shift or supershift bands (lanes 8 and 13). Labeled *Gabra3* probes did not yield specific Sp4 shift (lane 14) or supershift bands (lane 16), and cold competitor was also blank (lane 15). Labeled *Gabra1 and Gabra2* probes with mutant Sp sites did not yield specific Sp4 shift or supershift bands (lanes 17–19 and 20–22, respectively).

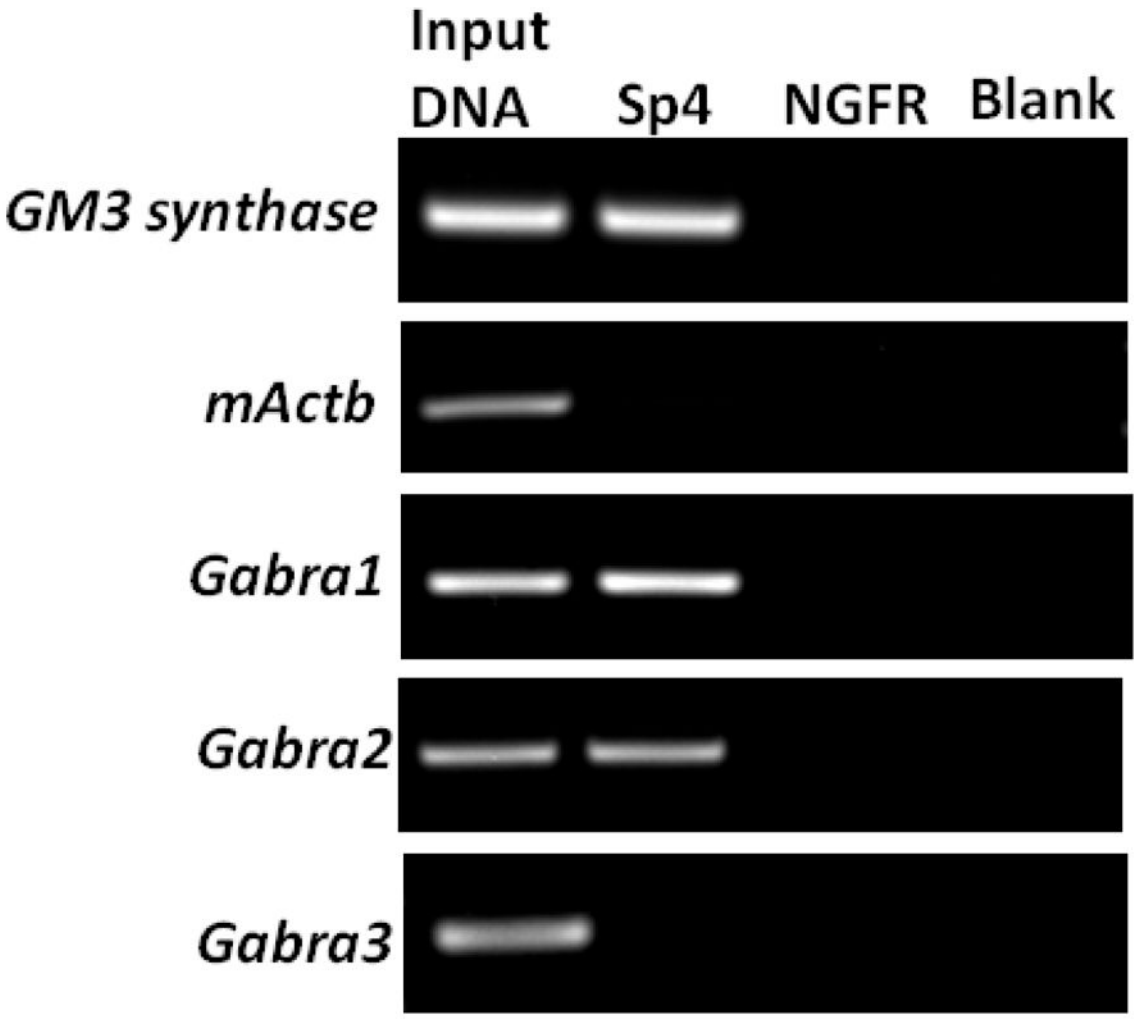

Fig. 2.

In vivo chromatin immunoprecipitation showing Sp4's interactions with $GABA_A$ receptor subunit promoters in mouse visual cortical nuclear extract. Immunoprecipitation was carried out with anti-Sp4 antibodies (Sp4 lane), anti-nerve growth factor receptor p75 antibody (negative control, NGFR lane), or no antibody (negative control, blank lane). 0.5% of input chromatin (Input DNA lane) served as the control for PCR reaction. The positive control for Sp4 binding was *GM3 synthase*, whereas β-Actin was the negative control. Sp4 interacted with *Gabra1* and *Gabra2*, but not with *Gabra3*.

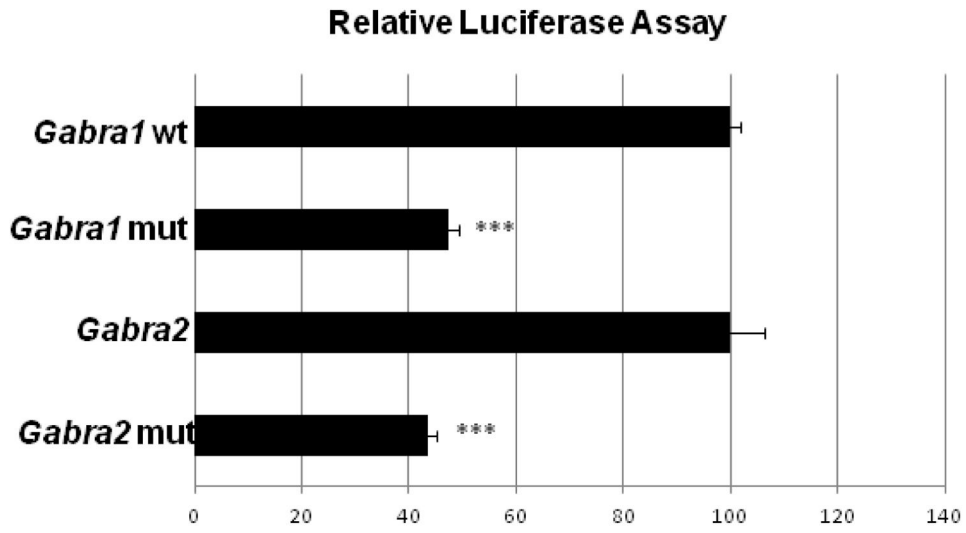

Fig. 3.

Relative luciferase activity of the wild type *Gabra1 and Gabra2* promoters (wt) and the *Gabra1* and *Gabra2* promoters with mutated Sp binding site (mut). Mutating the Sp binding site on *Gabra1 and Gabra2* resulted in a significant decrease in luciferase activity as compared to the wild type controls. N = 3 for each construct. ***= $P < 0.001$ as compared to the wild type.

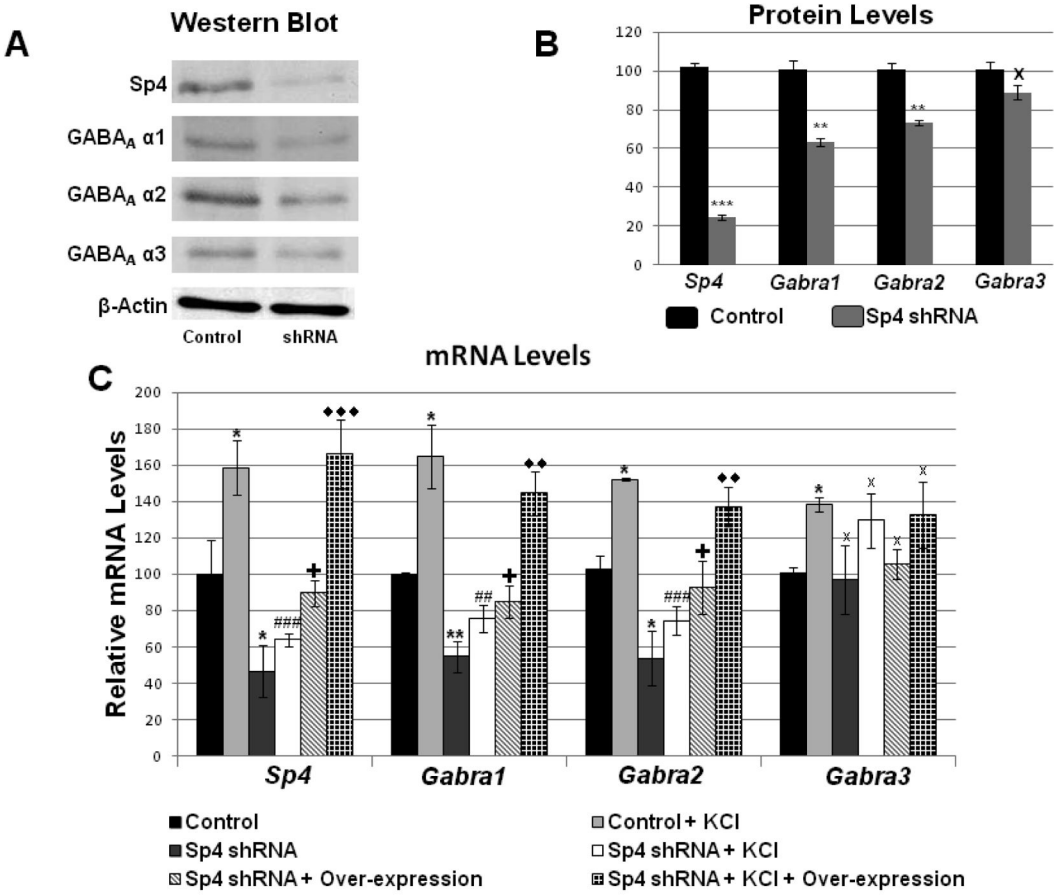

Fig. 4.

Effect of silencing *Sp4*, depolarizing stimulation, and Sp4 overexpressing. (A– B) Western blots revealed a down-regulation of Sp4, GABA$_A$ α1, and GABA$_A$ α2, but not GABA$_A$ α*3* protein levels in *Sp4* shRNA-transfected primary neurons. β-actin served as a loading control (N = 3). Using scrambled vector transfection as a control, primary neurons exposed to KCl up-regulated their mRNA levels for *Sp4*, *Gabra1*, *Gabra*2, and *Gabra3* (* $P < 0.05$ when compared with controls; N = 3). When transfected with Sp4 shRNA, transcript levels of *Sp4*, *Gabra1*, and *Gabra*2 were significantly down-regulated, but not that of *Gabra3* (* $P < 0.05$; ** $P < 0.01$; X = non-significant when compared to controls; N = 3). In the presence of Sp4 shRNA, KCl was no longer able to up-regulate mRNA levels of *Sp4*, *Gabra1*, and *Gabra*2 to that of control + KCl, though it was still able to do so for *Gaba3* (## $P < 0.01$; ### $P < 0.001$; X = non-significant when compared to KCl alone). Transfection of neurons with Sp4 shRNA followed by Sp4 over-expression rescued their *Sp4*, *Gabra1*, and *Gabra*2 transcripts from being down-regulated by Sp4 shRNA, but this treatment had no effect on *Gaba3* ($^+$ $P < 0.05$; X = non-significant when compared to shRNA alone; N = 3). Neurons transfected with Sp4 shRNA followed by Sp4 over-expression as well as KCl treatment showed a level of *Sp4*, *Gabra1*, and *Gabra*2 transcripts that was significantly higher than that of shRNA + KCl, although it had no effect on that of *Gabra3* (◇◇ = $P < 0.01$; ◇◇◇ = $P < 0.001$; X = non-significant when compared to shRNA + KCl; N = 3). All four transcript levels were not significantly different from those of control + KCl.

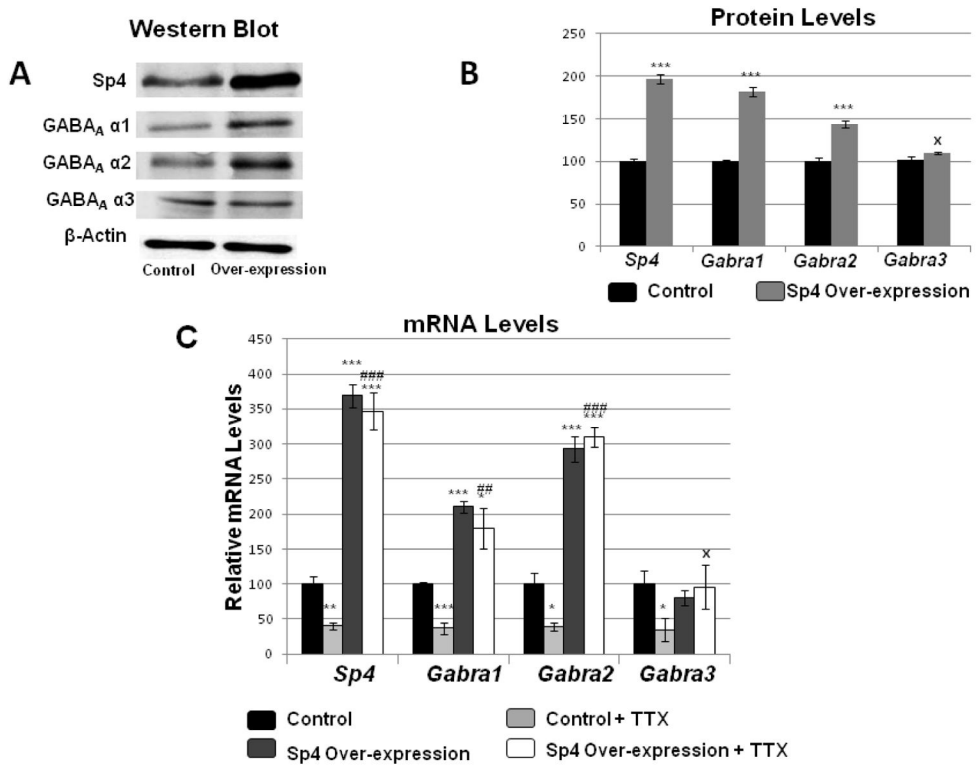

Fig. 5.

Effect of over-expressing *Sp4* on the transcript levels of GABA$_A$ receptor subunit genes. (A-B) Primary neurons transfected with *Sp4* over-expression vectors revealed an up-regulation of Sp4, GABA$_A$ α1, and GABA$_A$ α2 but not GABA$_A$ α3 protein levels using western blots (N = 3). (B) *Sp4* over-expression also increased *Sp4*, *Gabra1 and Gabra2* transcript levels but not that of *Gabra3*. (N = 3). (C) Over-expression of *Sp4* in primary neurons increased mRNA levels of *Sp4*, *Gabra1*, and *Gabra2* mRNA levels in primary neurons, but not of *Gabra3*. N = 3. *= $P < 0.05$, **= $P < 0.01$, and ***= $P < 0.001$ when compared to controls. Transcript levels of Sp4 and all three GABA$_A$ receptor subunit genes were decreased with 3 days of 0.4 μM TTX treatment. *Sp4* over-expression rescued the TTX-mediated down-regulation of *Sp4*, *Gabra1*, and *Gabra2* transcript levels, but not that of *Gabra3*. N =3. *= $P < 0.05$, **= $P < 0.01$, and ***= $P < 0.001$ when compared to controls. ##= $P < 0.01$, ### = $P < 0.001$ and X = non-significant when compared to TTX treatment alone.

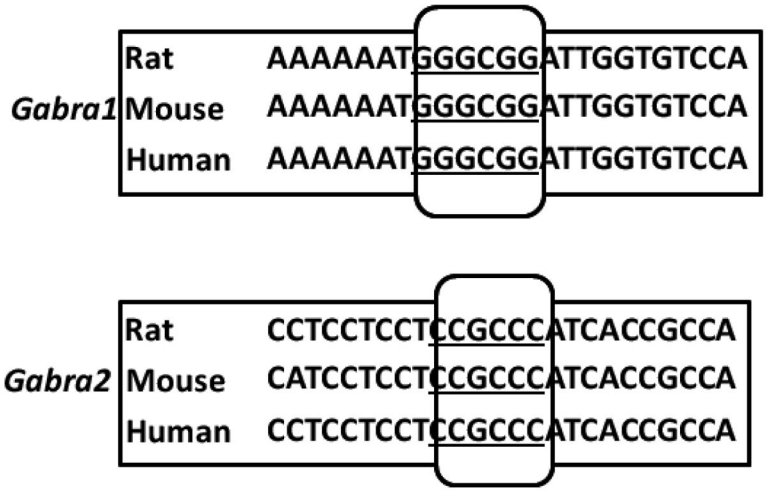

Fig. 6.

Aligned partial sequences of the *Gabra1* and *Gabra2* promoters from mice, rats, and humans showed conserved Sp binding sites (boxed and underlined).

Table 1

EMSA probes with putative Sp4 binding sites underlined.

Gene Promoter	EMSA Sequence
Gabra1	F: 5' TTTTGCATATAAAAAATGGGCGGATTGGTG 3'
	R: 5' TTTTCACCAATCCGCCCATTTTTTATATGC 3'
Gabra2	F: 5' TTTTGGCCAGGATTGGGGTGGGGGATAAGGGG 3'
	R: 5' TTTTCCCCTTATCCCCCACCCCAATCCTGGCC 3'
Gabra3	F: 5' TTTTCTACTTCATTGGGGTGGGGATCAG 3'
	R: 5' TTTTCTGATCCCCACCCCAATGAAGTAG 3'
GM3 Synthase	F: 5' TTTTGCGCGACCCCCGCCCCCGCCTA 3'
	R: 5' TTTTTAGGCGGGGGCGGGGTCGCGC 3'
Mutant Sp *Gabra1*	F: 5' TTTTGCATATAAAAAATGGAAGGATTGGTG 3'
	R: 5' TTTTCACCAATCCTTCCATTTTTTATATGC 3'
Mutant Sp *Gabra2*	F: 5' TTTTGGCCAGGATTGGGAAGGGGGATAAGGGG 3'
	R: 5' TTTTCCCCTTATCCCCCTTCCCAATCCTGGCC 3'

Table 2

Primers for ChIP analysis.

Gene Promoter	Sequence	Amplicon Length
Gabra1	F: 5’ GGAATGCTTGCAGCAGATTG 3’	216 bp
	R: 5’ GTAGTAATACGTCCCAGCGC 3’	
Gabra2	F: 5’ GCAGGGTGTGGGCTCCACTC 3’	290 bp
	R: 5’ TAGCGATCGCAGGAGCCGG 3’	
Gabra3	F: 5’ GACTGTTTTGCCTCCTTTGC 3’	336 bp
	R: 5’ CCTTCCATCTCTATGGGTGTGC 3’	
GM3 Synthase	F: 5’ CACCTACTTCTCGGCTGGAG 3’	198 bp
	R: 5’ AATTCAGCCCCGGACAGT 3’	
β-Actin	F: 5’ GCTCTTTTCCAGCCTTCCTT 3’	187 bp
	R: 5’ CGGATGTCAACGTCACACTT 3’	

Table 3

Primers used for promoter cloning and mutagenesis analysis. Mutated Sp4 binding sites are underlined with mutated nucleotides in bold.

Gene Promoter	Primer
Gabra1	F: 5’ CAGACGCGTTGCTTCCTAGCTTGCGTTCA 3’
	R: 5’ CAGCTCGAGGTGCTCCTGCACTGGAGATT 3’
Mutant Sp *Gabra1*	F: 5’ GCGCTGCATATAAAAAATG**T**G**AT**GATTGGTGTCCAGATCCAGTGGCTG 3’
	R: 5’ CAGCCACTGGATCTGGACACCAATC**AT**C**A**CATTTTTTATATGCAGCGC 3’
Gabra2	F: 5’ CAGGGTACCAAGGCGTGATTAGCCCCTTC 3’
	R: 5’ CAGAAGCTTAAGACCAGGCAGCAAACGAA 3’
Mutant of Gabra2	F: 5’ GGCTTGGCCAGGATTGGG**TTTT**GGGATAAGGGGTCCCC 3’
	R: 5’ GGGGACCCCTTATCCC**AA**A**ACCC**AATCCTGGCCAAGCC 3’

Table 4

Sp4 shRNA sequences cloned into the pLKO.1 vector for silencing Sp4

Gene		Hairpin Sequence
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
Gabra1	F: 5’ ACTGTCTTTGGAGTGACGACCGTT 3’
	R: 5’ AATAAACCAGTCCATGGCCGTTGC 3’
Gabra2	F: 5’ TCAGTGCTCGAAATTCCCTTCCCA 3’
	R: 5’ TACACTCTTTCCATCCCAAGCCCA 3’
Gabra3	F: 5’ AGGAAGCTCACACTTTGGTCCTGA 3’
	R: 5’ TTGGCCAGTGGTTCCAGGTAGAAT 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’