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The Specificity Protein Factor Sp1 Mediates Transcriptional Regulation of P2X7 Receptors in the Nervous System*

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Paula García-Huerta[‡§¶1], Miguel Díaz-Hernandez[‡§¶], Esmerilda G. Delicado[‡§¶], María Pimentel-Santillana[‖], Mª Teresa Miras-Portugal[‡§¶], and Rosa Gómez-Villafuertes[‡§¶2]

From the [‡]Departamento de Bioquímica y Biología Molecular and [§]Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense de Madrid, 28040 Madrid, Spain, [¶]Instituto de Investigación Sanitaria del Hospital Clínico San Carlos, 28040 Madrid, Spain, and [‖]Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas-Universidad Autonoma de Madrid, 28029 Madrid, Spain

Background: Purinergic P2X7 receptors regulate proliferation, differentiation, and cell death in both the CNS and non-CNS tissues.

Results: Sp1 factor activates the *P2rx7* promoter. This regulation is abolished by SP1 binding sites mutation, Sp1 knockdown, and mithramycin A treatment.

Conclusion: Sp1 regulates the expression of P2X7 receptor.

Significance: Learning how P2X7 expression is controlled is crucial for understanding P2X7-mediated brain processes in health and disease.

P2X7 receptors are involved not only in physiological functions but also in pathological brain processes. Although an increasing number of findings indicate that altered receptor expression has a causative role in neurodegenerative diseases and cancer, little is known about how expression of *P2rx7* gene is controlled. Here we reported the first molecular and functional evidence that Specificity protein 1 (Sp1) transcription factor plays a pivotal role in the transcriptional regulation of P2X7 receptor. We delimited a minimal region in the murine *P2rx7* promoter containing four SP1 sites, two of them being highly conserved in mammals. The functionality of these SP1 sites was confirmed by site-directed mutagenesis and Sp1 overexpression/down-regulation in neuroblastoma cells. Inhibition of Sp1-mediated transcriptional activation by mithramycin A reduced endogenous P2X7 receptor levels in primary cultures of cortical neurons and astrocytes. Using *P2rx7*-EGFP transgenic mice that express enhanced green fluorescent protein under the control of *P2rx7* promoter, we found a high correlation between reporter expression and Sp1 levels in the brain, demonstrating that Sp1 is a key element in the transcriptional regulation of P2X7 receptor in the nervous system. Finally, we found that Sp1 mediates P2X7 receptor up-regulation in neuroblastoma cells cultured in the absence of serum, a condition that enhances chromatin accessibility and facilitates the exposure of SP1 binding sites.

Purinergic P2X7 receptor belongs to the family of ATP-gated cation channels that mediate a non-selective cation conductance when activated by an appropriate ligand. Currently, seven different P2X subunits (P2X1-P2X7) and various splice variants have been molecularly identified in mammals, with P2X7 receptor being the most divergent member of this family in terms of molecular structure, pharmacology, and function (1). The P2X7 receptor forms trimeric complexes of identical subunits in the plasma membrane and is unable to oligomerize with other P2X receptors (2). Although its structure is basically similar to other P2X receptor subunits, having two transmembrane domains and a large extracellular loop, the intracellular carboxyl terminal domain of P2X7 receptor is much longer (239 amino acids) (3). Within the P2X family, the P2X7 receptor has a distinguished role in the central nervous system (CNS) for its implication in several biological tasks such as learning and memory, circadian rhythms, mood, and motivation (4). In the last years the knowledge about the role of P2X7 receptors in the CNS, in terms of intracellular pathways coupled to its activation, has been noticeably improved. Thus, P2X7 receptors induce calcium/calmodulin-dependent protein kinase II phosphorylation in cerebellar granule neurons (5) and are also coupled to GSK-3 inhibition and neuroprotection in the same neuronal model (6). Moreover, calcium/calmodulin-dependent kinase II signaling cascade mediates P2X7 receptor-dependent inhibition of axonal growth and branching in hippocampal neurons and neuroblastoma cells (7, 8). P2X7 receptors seem to be involved not only in physiological functions but also in a variety of pathological brain processes, where the functional expression level of P2X7 receptor seems to be crucial. This has been highlighted by an increasing number of findings that aberrant or altered receptor expression and function have a causative role in neurodegenerative diseases, mood disorders, and neuropathic pain (4, 9–12). In addition, P2X7 receptor is highly expressed in a variety of human neuroblastoma cells from either primary tumors or cell lines, and its activation increases the proliferation and growth of the tumors (13, 14). Altogether, these evidences point to P2X7 receptor as an appealing target

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[2] Supported by Spanish Ion Channel Initiative. To whom correspondence should be addressed: Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain. Tel.: 34-913943890; Fax: 34-913943909; E-mail: marosa@vet.ucm.es.

for pharmacological intervention; however, little is known about the transcriptional regulation of P2X7 receptor expression in the CNS.

The gene encoding P2X7 subunit was initially cloned from a rat brain cDNA library (15) and afterward was identified in many other mammalian and non-mammalian species including human and mouse (16, 17). There are more than 815 single nucleotide polymorphisms (SNPs)[3] that have been described in the human *P2RX7* gene, but only a few of them have been functionally characterized to cause either loss or gain of receptor function (18–26). Interestingly, five SNPs have been identified upstream of exon 1 of P2RX7 gene, although none of them has been associated with a specific ATP response phenotype at the moment (27). First studies reported P2X7 promoter activity within a 2-kb DNA segment of the 5′ of the gene (28). Afterward, the active promoter of the human *P2RX7* gene was located in the −158/+32-nucleotide region surrounding the transcription start site, although the transcription factors involved in the promoter activity were unknown (29). To further characterize the molecular mechanisms underlying transcriptional regulation of P2X7 receptor, we cloned and functionally identified the active promoter of the murine *P2rx7* gene. We found that *P2rx7* gene promoter region lacks TATA and CAAT boxes and contains seven putative motifs for the Specificity protein 1 (Sp1) family of transcription factors, with at least two of them fully functional and conserved between species. Using interference and overexpression experiments, we demonstrate that Sp1 up-regulates endogenous P2X7 mRNA and protein levels in neuroblastoma Neuro-2a (N2a) cells. Mithramycin A, an inhibitor of Sp1-mediated transcriptional activation, decreases *P2rx7* gene expression in N2a neuroblastoma cells, primary cultures of mouse cortical neurons and astrocytes, and macrophages. Moreover, using *P2rx7*-EGFP transgenic mice that express enhanced green fluorescent protein (EGFP) under the control of *P2rx7* promoter, we observed that most cells expressing P2X7 receptors in the brain of newborn mice also contain high amounts of Sp1 factor. We also described that Sp1 mediates up-regulation of P2X7 receptor expression under serum deprivation, a condition that has been previously reported to enhance open chromatin accessibility, facilitating exposure of SP1 binding sites.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Antibodies used in the study were Sp1 (Merck, catalog #07-645), P2X7 receptor intracellular epitope (Alomone Laboratories, catalog #PR-004), GAPDH (Ambion, catalog #AM4300), NeuN (Chemicon International, catalog #MAB377), GFAP (Santa Cruz Biotechnology, catalog #sc-9065), and Iba1 (Wako, catalog #019-19741). Horseradish peroxidase-conjugated secondary antibodies were from Dako. Cy3™-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch. Penicillin, streptomycin, kanamycin, amphotericin, and Glutamax® were from Invitrogen. All other chemicals were from Sigma.

[3] The abbreviations used are: SNPs, single nucleotide polymorphisms; EGFP, EGF protein; N2a, Neuro-2a cells; Q-PCR, quantitative real time-PCR; Sp1, Specificity protein 1, TSS, transcription start site; ANOVA, analysis of variance.

Genomic Cloning of the Mouse P2rx7 Gene and Construction of Several Deletion Reporter Plasmids—A PCR-based technique was used to clone a 3450-kb fragment of the mouse *P2rx7* gene 5′-flanking region ranging from −3212 bp upstream to +220 bp downstream of the transcription start site (TSS). Sense and antisense oligonucleotides (5′-TGTTACGGCTGCATAGTC-TGTCCT-3′ and 5′-GGATCCGGGTGACTTTGTTTGTCT-3′, respectively) were chosen from genomic sequence of the mouse *P2rx7* gene. The putative TSS designated as +1 was obtained from the mRNA sequences available in GenBank™ corresponding to the transcript variants 1, 2, 3, and 4 (accession nos. NM_011027, NM_001038845, NM_001038839, and NM_001038887, respectively). The 3450-kb genomic fragment was isolated by PCR amplification from mouse genomic DNA using PfuUltra™ Hotstart DNA polymerase (Stratagene). Genomic DNA (extracted from neuroblastoma N2a) was obtained with DNeasy Blood & Tissue (Qiagen) following the manufacturer's instructions. Afterward, genomic fragment was adenylated and cloned in the pGEM-T® Easy vector (Promega) and subjected to double-strand DNA sequencing. A set of truncated constructs was then generated by sequential deletions of the 5′ end by PCR (Fig. 2*A*). Different forward primers bearing a XhoI site and several antisense oligonucleotides bearing a HindIII site at the 5′ end were used for direct cloning into pGL4.23 firefly luciferase reporter vector (Promega). Deletion plasmids were confirmed by sequencing and restriction enzyme digestion. Primer nucleotide sequences are listed in Table 1.

Site-directed Mutagenesis—To assess whether SP1c and SP1d binding sites were responsible for the transcriptional activation of *P2rx7* promoter, mutation of SP1c and SP1d sites was performed by PCR site-directed mutagenesis using pP2X7-F2 and pP2X7-F3 as a template, respectively. PCR was followed by DpnI digestion (QuikChange XL, Stratagene). The designed primers (SP1c-mut forward (fw) 5′-GGAGGCTAGCGGGGC-TTGGTCATCTGCCAGCC-3′, SP1c-mut reverse (rv) 5′-GGCTGGCAGATGACCAAGCCCCGCTAGCCTCC-3′, SP1d-mut fw 5′-GTTGCCAGGACCCGCAACCGCTGCAGTCAC-TGG-3′ and SP1d-mut rv 5′-CCAGTGACTGCAGCGGTTG-CGGGTCCTGGCAAC-3′) replace two nucleotides of each SP1 binding site (mutated nucleotides underlined).

Animals, Cell Culture, and Transfection—All animal procedures were carried out at the Universidad Complutense de Madrid in accordance with European and Spanish regulations (86/609/CEE; RD1201/2005) following the guidelines of the International Council for the Laboratory Animal Science. Male or female C57B1/6J mice were used at different development stages (E18 mouse embryos for neuronal cultures and P0-P1 postnatal day old pups for astrocyte cultures). *P2rx7*-EGFP reporter mice that express EGFP immediately downstream of the *P2rx7* promoter were obtained from United States National Institutes of Health Mutant Mouse Regional Resource Centers (stock 011959-UCD) (30) and were granted by Dr. M. Nedergaard (University of Rochester Medical School, Rochester, NY).

Primary cultures of cortical neurons and astrocytes were prepared as previously described (31). Briefly, the cortex was dissected and dissociated using the Papain Dissociation System (Worthington Biochemical, Lakewood, NJ). For neuronal cultures, cells were plated at a density of 200,000 cells/cm^2 on

TABLE 1
Oligonucleotide primers used to obtain luciferase reporter plasmids
fw, forward; rv, reverse.

Fragment name	Oligonucleotides used to amplified each fragment	Fragment size
		bp
A	fw, 5'-GGTTTGGAAATCAGGCAGCTAG-3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	2332
B	fw, 5'-GTGCTGGCTAGAGAGGGCTACA-3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	1795
C	fw, 5'-GTGGTCCCTCGCAACTATAGTC-3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	1256
D	fw, 5'-GATAATCTCTGTGGGGTGCGGT-3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	670
E	fw, 5'-GATAATCTCTGTGGGGTGCGGT-3' rv, 5'-CATTAAGCTTGTTGCCTGTTTGAGACCTGTTC-3'	150
F	fw, 5'-GCAGCTCGAGCATCTAGGCCCTTGTCTGAG- 3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	468
G	fw, 5'-CGCCCTCGAGGCAGTCACTGGAGGAACTGGAAG-3' rv, 5'-CGATTGAAGCTTGGTGACTTTGTTTGTCT-3'	203
F1	fw, 5'-GCAGCTCGAGCATCTAGGCCCTTGTCTGAG-3' rv, 5'-TTAAAAGCTTCCCTCCCCACCCCCTTTCCTTG-3'	110
F2	fw, 5'-AGGACTCGAGGTGGGGAGGGGGAATTTTAAAAATG-3' rv, 5'-CAAGAAGCTTGTCCTACAGGCCTGGCTGGCAG-3'	107
F3	fw, 5'-GCCACTCGAGCCTGTAGGACTTGGTTCTTG-3' rv, 5'-TTCTAAGCTTGAACGGTTAACTTCCAGTTC-3'	100

6-well plates coated with 10 μg/ml poly-L-lysine (Biochrom AG) and 3 μg/ml laminin. After plating, neurons were cultured for 24 h in Neurobasal medium supplemented with 1% B-27 (both from Invitrogen), 0.5 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. For astrocyte cultures, cells were plated onto culture flasks (~100,000 cells/cm^2) and grown until confluence in DMEM supplemented with 10% fetal bovine serum (FBS) (EuroClone), 50 units/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml kanamycin, and 2.5 μg/ml amphotericin. Afterward, astrocytes were trypsinized and plated on 6-well plates coated with poly-L-lysine at a density of 40,000 cells/cm^2. Microglial contamination of primary cultures of neurons and astrocytes was checked by Western blot using antibodies that recognize marker proteins for neurons, astrocytes, and microglial cells (NeuN, GFAP and Iba1, respectively). Macrophage cell line RAW264.7 and neuroblastoma cell line N2a were grown in DMEM supplemented with 10% FBS, Glutamax®, 100 units/ml penicillin, and 100 μg/ml streptomycin. Elicited peritoneal macrophages were obtained as previously described (32). Briefly, 60 h before the assay, 10-week-old mice were intraperitoneal injected with 2.5 ml of 3% thioglycolate broth. Afterward, mice were killed by cervical dislocation and injected intraperitoneally with 10 ml of sterile RPMI 1640 medium. The peritoneal fluid was carefully aspirated, avoiding hemorrhage, and kept at 4 °C to prevent the adhesion of the macrophages to the plastic. Spleens of the same animals were also harvested for immunohistochemical studies. Cells were washed twice with ice-cold PBS and seeded at 30,000 cells/cm^2 in RPMI 1640 medium supplemented with 10% of heat-inactivated FBS and antibiotics. After incubation for 3 h at 37 °C in a 5% CO_2 atmosphere, non-adherent cells were removed by extensive washing with PBS. All cell cultures were grown at 37 °C in humidified atmosphere containing 5% CO_2.

When appropriate, mithramycin A, a selective inhibitor of Sp1-mediated transcriptional activation, was assayed. Cells were treated 24 h after plating with 300 nM mithramycin A for 24–48 h and then lysed for either RNA or protein extraction. The stock solution of mithramycin A was prepared in methanol (vehicle) to a concentration of 1 mM. Control cells were treated with the same concentration of vehicle solution (final dilution factor higher than 1:3000). For serum withdrawal experiments, N2a cells were seeded on 6-well plates at a density of 75,000 cels/cm^2 for 24 h. Afterward, complete medium was changed to DMEM without serum, and cells remained 24, 48, or 72 h depending on each experiment. Transient transfections of plasmid DNAs were carried out using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions.

Luciferase Reporter Assay—Cell lines were plated on 24-well plates coated with poly-L-lysine the day before transfection (cells reached ~80% confluence the day of transfection). A mixture of 0.64 μg of pGL4.23-based constructs, and 0.16 μg of *Renilla* luciferase vector pGL4.74[hRluc/TK] were cotransfected into cells. The final DNA concentration in all experiments was preserved by the addition of empty expression vector when necessary. Cells were harvested after 24–48 h and assayed for luciferase activity. 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.

Sp1 Overexpression and Small Hairpin (shRNA) Transfection Experiments—The mouse Sp1 cDNA was amplified from the commercial plasmid pENTR223.1 (Source BioScience, Nottingham, UK) by using the oligonucleotides forward (5′-CTAGCTC-GAGATGAGCGACCAAGATCACTC-3′) and reverse (5′-CTA-GGAATTCTTAGAAACCATTGCCACTGA-3′). The amplified fragment was digested with XhoI and EcoRI enzymes and subcloned into the corresponding sites of pIRES-EGFP vector (Clontech Laboratories) for expression in mammalian cells. The ligation product was confirmed by sequencing. N2a cells and astrocytes were transiently transfected with 4 μg of DNA. After 6 h, the medium was removed, and cells were further incubated for the indicated time periods in culture medium. The parallel expression of EGFP from this vector allowed the identification of transfected cells by green fluorescence.

Sp1 knockdown was achieved by RNA interference using a vector-based shRNA approach (pRFP-C-RS vector, OriGene). The shRNA target sequences used were: shSP1.1 (5′-CCTTGCTACCTGTCAACAGCGTTTCTGCA-3′) and shSP1.2 (5′-AGGACAGACTCAGTATGTGACCAATGTAC-3′). To specifically rule out the potential nonspecific effect induced by expression of the shRNAs, control cells were transfected with a scrambled negative control non-effective shRNA (5′-GCACTACCAGAGCTAACTCAGATAGTACT-3′) (OriGene). N2a cells were transiently transfected with 4 μg of DNA. After 6 h the medium was removed, and cells were further incubated for the indicated time periods in culture medium. The concomitant expression of RFP from these vectors allowed transfected cells to be identified by red fluorescence.

Quantitative Real Time-PCR (Q-PCR)—Total RNA was extracted from cultured cells using RNeasy® plus mini kit (Qiagen) following the manufacturer's instructions. After digestion with TURBO DNase (Ambion), total RNA was quantified and reverse-transcribed using M-MLV reverse transcriptase, 6 μg of random primers, and 350 μM dNTPs (Invitrogen). Q-PCRs were performed using gene-specific primers and Taqman MGB probes for mouse P2X7, Sp1, and GAPDH (all from Applied Biosystems). Fast thermal cycling was performed using a StepOnePlus® Real-Time System (Applied Biosystems) as follows: denaturation, 1 cycle of 95 °C for 20 s followed by 40 cycles each of 95 °C for 1 s and 60 °C for 20 s. The results were normalized as indicated by parallel amplification of GAPDH.

Western Blot—Cells were lysed and homogenized for 1 h at 4 °C in lysis buffer containing 50 mM Tris/HCl, 150 mM NaCl, 1% Nonidet P40, and Complete™ Protease Inhibitor Mixture Tablets (Roche Diagnostics), pH 7.4. Separation of the proteins (50 μg of total protein/well) was performed on 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose transfer membrane (PROTRAN®, Whatman GmbH), saturated for 1 h at room temperature with 5% nonfat dried milk in 0.1% TBS/Tween, and incubated overnight at 4 °C with the following commercial primary antibodies: anti-Sp1 (1:1000, 95–105 kDa) (33), anti-P2X7 receptor (1:1000, 75 kDa) (8), anti-GAPDH (1:5000, 37 kDa) (34), anti-NeuN (1:1000, 46, 48, and 66 kDa) (10), anti-GFAP (1:200, 50 kDa) (34), and anti-Iba1 (1:1000, 17 kDa) (10). Proteins were visualized by chemiluminescence using the ECL Chemiluminescence (Amersham Biosciences GE Healthcare), and quantified using ImageJ free software (National Institutes of Health).

Immunocytochemical and Immunohistochemical Studies—Cells cultured on coverslips placed in 35-mm dishes (250,000 cells per well) were washed with PBS and fixed with 4% paraformaldehyde for 15 min. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum and 10% FBS for 1 h at room temperature. After washing with 3% BSA in PBS, cells were incubated for 1 h with primary antibodies against either P2X7 receptor (1:200), Sp1 (1:200), or CD11 (1:200). Afterward, cells were washed with PBS and incubated for 1 h with Cy3™ secondary antibody. Nuclei were counterstained with DAPI (Invitrogen). Coverslips were mounted on glass slides using FluoroSave™ Reagent (Calbiochem). For immunohistochemical studies, brains from P0 postnatal-day-old pups and spleens from 10-week-old mice were harvested and fixed in 4% paraformaldehyde for 48 h at 4 °C and cryoprotected in sucrose before sectioning. Sections were pretreated with Sudan black B, 1% BSA, and 1% Triton X-100 in PBS followed by incubation with the primary antibody anti-Sp1 when indicated. Sections were washed again and incubated with secondary antibody coupled to Cy3™ when necessary. Sections were also treated with DAPI and then coverslipped with FluoroSave™ reagent. Confocal images were acquired with a TCS SPE microscope from Leica Microsystems (Wetzlar, Germany).

Statistical Analysis—Data were analyzed using one way ANOVA with the post hoc Newman-Keuls test or, for two-group comparisons, Student's *t* test (Graph Pad Prism 5, Graph Pad Software Inc., San Diego, CA). Data are expressed as the mean ± S.E. Differences were considered significant at $p \leq 0.05$.

RESULTS

P2rx7 Gene Promoter Lacks TATA/CAAT Boxes and Has Multiple Sp1 Motifs—The murine *P2rx7* gene codifies for at least 5 transcript variants named 1, 2, 3, 4, and *k* (35). To characterize the minimal *P2rx7* promoter region involved in the regulation of *P2rx7* gene expression, we cloned a 2334-bp fragment of the 5′-flanking region of the gene from mouse genomic DNA. This fragment corresponds to positions −2114 to +220 bp relative to the TSS. The putative TSS was designated as +1 and was obtained from the mRNA sequences available in GenBank™ corresponding to the transcript variants 1, 2, 3, and 4 (accession nos. NM_011027, NM_001038845, NM_001038839, and NM_001038887, respectively). A computer-based transcription binding site search using the Genomatix MatInspector software reveals that this 5′-proximal regulatory region lacks TATA and CAAT boxes and contains seven putative motifs for the Sp1 family of transcription factors (Fig. 1*A*). All of these sites have a very high similarity with the theoretical matrix (score >0.85) and are located in both strands: forward strand (sites −1457/−1441, −281/-265, −155/−139, −150/−134, and −79/−63 bp) and reverse strand (−1/+16 and +131/+147 bp) (Table 2). Interestingly, most putative SP1 sites were located close to the TSS, suggesting the implication of Sp1 family of transcription factors as potential key regulators for *P2rx7* gene expression. Methylation degree of CpG islands contained within the SP1 consensus elements may interfere with the binding of Sp1 to DNA, modulating the Sp1-dependent transcription of genes (36). Using CpGPlot software, the presence of CpG islands at the 5′-proximal regulatory region of the murine *P2rx7* gene was analyzed based on two basic parameters: a CG percentage higher than 50% and a ratio of observed-to-expected higher than 0.6 (Fig. 1*B*). Bioinformatics analysis showed that murine *P2rx7* promoter lacks CpG islands, discarding that methylation of SP1 sites located into the promoter region could be interfering Sp1 binding to DNA.

In addition, the 5′-flanking sequence of *P2rx7* promoter contains other putative regulatory elements including four motifs for AP1 (activator protein 1), one CREB (c-AMP-responsive element-binding proteins) binding element, one E-box binding site, one HIF (hypoxia-inducible factor) motif, three STAT (signal transducer and activator of transcription) binding elements,

A

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-2114                                                                                       5'- AGGTTTGGAAATCA
                                            YY1F                 TCF/LEF1
-2100  GGCAGCTAGCCCTCTAGTTCTACCCACTAGACTTTACTGCTCTAATGGAAGCCCTTTGGCCTACTATTTGTTACATTCATCTTTGGTGTGTGTGTGTGTG
-2000  TGTGTGTGTAGCTTGCGTGACCACCTGTGCATATTTGTGCACAGTTTTAATGGAGTCAGTTCTCTCCAAGTATTCGGGTCCTGGGGACCGAACTCAGGTC
                    CREB
-1900  ATCAGGCTTGATGGCACCTTCGTCATCTCACTGGTCCCATTTATTGAAGGTTACTGTACTCCTCCGAGTTTTGAAAGAGTAGCGACAAAGGTCAGGGCAG
-1800  AGTTAAGAGAAAGCGGTAAGAGTGTGCTGGCTAGGGGCTGGCGAGATGGCTCAGTGGTTAAGAGCGCCGTCTGCTTTTCCAAAGGTCCCGAGTTCAAATC
         E-box
-1700  CGAGCAACCACATGGTGGCTCACAACTATCCGTAACGAGATCTGATGCCCTCTTCTGGAGTATCTGAGGACAGCTATAGTGTACTTACATATAATAAATA
-1600  AATAAATGTTCAAAAAAAAAAAGTGTGCTGGCTAGAGAGGGCTACAGAGGGAAGCCCTTACCCCTCCGGGGCCTCCCGAGTAAACAGAGAGGGTTGGGAG
                                                 SP1
-1500  ACGCATCTAGCATCACTGCCTTCTAGTCCCTTGAACTGGAGCCAACAGGGGCAGGGGCCTTGATGTAGATCATAAGGAGGAGAAAGTACAAATTAAGTTA
-1400  GGCTGGAAACATGTAAGTCTGCCTGGCCCATAATGCAGCAGGCAAGACAGCAGTAGGTTGGGATGCTTGCGGGGAGATGGTTCTTTCTCCCTAGGGAGGG
                                                       STAT                      STAT              HIFF
-1300  ATAACGCCTGAGTGACTGTTGCTTCCTTTCTCTAACGTCTAACATCCCACTTGTGCCTGGAAGATTCCAGGTCTCAGAGAAGCAGCAGCCATAGCCACGT
-1200  TAGGACAGAGCCTCAGACACCAGGCTCTGCAGGTGCCTCATTTGAGGACGATCGGGTCTTTATCACCCAGGACCACCGATCCACTGTGTAAACCGTGTGG
-1100  GAAAGGCCTGCTTTATACATCCTGCTTTCCTACAGCCTGTCGGAGCAGGGTTTCAGCCCTGGTGTGGTCCCTCGCAACTATAGTCCCAGAATTTGTGAGC
                                                                       AP1
-1000  TGGAGGTGTGGGAAAGATGAAGGCTTAAGGGTCAGTCTCAGCCTCACAGTAAGTTACAGAGAAGTCTGAGTTACACGAGACCCTGTCTCAAAACAAAACA
                                                                                                  STAT
 -900  AATAGAGAATAAAACAACAAAACCGCACAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATCAACCAAACAAGATTTCCTCTCAACTCCACCTTCCTCA
                                                                                                TCF/LEF1
 -800  GAATTCTACAATGAGGCTACCTCTTCTACTATTTGGAGATAGCATTGGTCCTCTCTCATGAGGTGTCTCCATGGTCAGGAGGGATACTGGATGCAGCTTT
 -700  GTTCTTGGTGGTCTAGGACTGACTGGACCATGAGATGACCTCCCGGGTCCCGAGCAGGATGAAGTGTAGATGGAGGAGAGAGTGAGACAAGCGTGGATAT
                                   YY1F                                    YY1F
 -600  AATCTTCCCATCCCACAATGAATCTGATCTGGCAGCCATCTTTGGCAGACCAGAGTATCCCGTGGAGCAGCGGAGGACAAAGATGCCATTCAGTCATCGG
                      TCF/LEF1         TCF/LEF1
 -500  GTGCACCTGTACATGTGGACTGAACAAAGGGTTGTCCGGAAAACAAAGGGATAATCTCTGTGGGGTGCGGTGGGGCTCACCTATAATCCCAGCACTCGGG
                                       AP1   AP1
 -400  AGGTGGAGGCAGGAGGATCAGAAGTTCATAGCTAGCCTTGAGTGAGTAACAAATCTAAGGTCAGCCTGGCTGCTTGAGAACAGGTCTCAAACAGGCAACA
                      SP1                                                 AP1
 -300  AAAGAAATGGATGGGTCTGAGTGGGGGTGGGGGGGCTTGAGGCAGTGGTCGCATCTAGGCCCTTGTCTGAGTCACACTGGGTCTTACAACATTTGAAGGA
                                                   SP1            SP1       YY1F
 -200  CTGCCCTTTTCTCTCAAATGAAATGAGTTCCAAAAGAGCACAAGGAAAGGGGGTGGGGAGGGGGAATTTTAAAAATGCCCATCTTCTGAACACCATCTTT
                       SP1
 -100  GTGCGGGCGTCTGGGGGAGGCTAGCGGGGCGGGGTCATCTGCCAGCCAGGCCTGTAGGACTTGGTTCTTGTTTATTCCACTCACATTCAGGGCAGTTGCC
        SP1
   +1  AGGACCCGCCCCCGCTGCAGTCACTGGAGGAACTGGAAGTTAACCGTTCCTGCTGAGAAATCGGTGTGTTTCCTTTGGCTGCTCCTAGGTGAGGGTTTGC
                                       SP1
 +100  TGTGGTCTAGCCTGGGAAGTAGGTCTAGCCCAAGTCCAGCCTGAACAGAGCGAGCCTGTCGCCATGCCGGCTTGCTGCAGCTGGAACGATGTCTTGCAGT
          TCF/LEF1
 +200  ATGAGACAAACAAAGTCACC-3'
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B

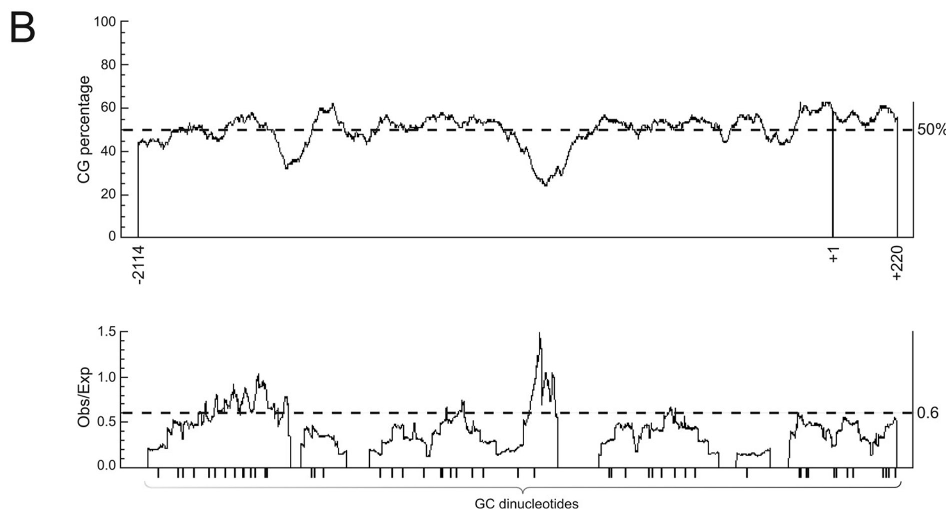

TABLE 2

Main putative transcription factor binding sites found in the *P2rx7* promoter region

Start/End pos., starting/ending position of the consensus binding site in the sequence (relative to +1 TTS); Str., strand sense; Matrix sim., matrix (groups of functionally similar transcription factors) similarity factor (0–1); Core sim., core consensus sequence (4 highest conserved positions) similarity factor (0–1); AP1, activator protein 1; CREB, cAMP-responsive element-binding proteins; E-box, E-box binding factors; HIFF, hypoxia-inducible factor, bHLH/PAS protein family; SP1/GC, stimulating protein 1/GC box elements; STAT, signal transducer and activator of transcription; TCF/LEF1, involved in the Wnt signal transduction pathway; YY1F, activator/repressor binding to transcription initiation site.

Family	Factor	Start pos.	End pos.	Str.	Core sim.	Matrix sim.	Sequence
AP1	AP1	−937	−925	(+)	1.000	0.874	gtctGAGTtacac
	AP1	−365	−353	(+)	1.000	0.878	ccttGAGTgagta
	AP1	−361	−349	(+)	1.000	0.904	gagtGAGTaacaa
	AP1	−236	−224	(+)	1.000	0.968	gtctgAGTCacac
CREB	CREB	−1887	−1867	(−)	1.000	0.918	ccagtgagaTGACgaaggtgc
E-box	c-Myc/Max	−1696	−1684	(+)	0.860	0.931	caaccaCATGgtg
HIFF	HIFF	−1210	−1194	(−)	1.000	0.953	tgtcctaaCGTGgctat
SP1/GC	SP1	−1457	−1441	(+)	1.000	0.919	aacagGGGCaggggcct
	GC box	−281	−265	(+)	0.872	0.912	agtgggGGTGggggggc
	GC box	−155	−139	(+)	0.872	0.904	aaagggGGTGgggaggg
	SP1	−150	−134	(+)	0.877	0.890	gggtgGGGAgggggaat
	SP1	−79	−63	(+)	1.000	0.998	tagcgGGGCggggtcat
	SP1	−1	+16	(−)	1.000	0.973	agcggGGGCgggtcctg
	SP2	+131	+147	(−)	1.000	0.854	tgttcaggctGGACttg
STAT	STAT3	−1253	−1235	(−)	1.000	0.977	aatcTTCCaggcacaagtg
	STAT3	−1240	−1222	(+)	1.000	0.964	aagaTTCCaggtctcagag
	STAT6	−811	−793	(+)	1.000	0.954	caccTTCCtcagaattcta
TCF/LEF1	TCF/LEF1	−2052	−2036	(−)	1.000	0.879	agtaggcCAAAgggctt
	TCF/LEF1	−709	−693	(−)	1.000	0.890	ccaagaaCAAAgctgca
	TCF/LEF1	−483	−467	(+)	1.000	0.908	gactgaaCAAAgggttg
	TCF/LEF1	−464	−448	(+)	1.000	0.962	cggaaaaCAAAgggata
	TCF/LEF1	+204	+220	(+)	1.000	0.873	agacaaaCAAAgtcacc
YY1F	YY2F	−2068	−2048	(−)	1.000	0.872	ggcttCCATtagagcagtaaa
	YY1F	−570	−550	(+)	1000	0.875	ggcagCCATctttggcagacc
	REX1	−520	−500	(+)	1.000	0.874	agatgCCATtcagtcatcggg
	YY1F	−127	−107	(+)	1.000	0.960	aatgcCCATcttctgaacacc

five TCF/LEF1 (involved in Wnt signal transduction pathway) binding sites, and four YY1F (activator/repressor binding to transcription initiation site) motifs (Fig. 1*A* and Table 2).

Functional Analysis of P2rx7 Gene Promoter—To study the transcriptional activity of the *P2rx7* promoter fragment, sequential deletions of the 5′-flanking region from −2114 to +220 bp of the *P2rx7* proximal promoter were amplified by PCR and subcloned into pGL4.23. This reporter vector contains a minimal promoter and a multiple cloning site upstream of the luciferase reporter gene exhibiting very low basal luciferase expression. A schematic representation of the promoter fragments cloned into pGL4.23 with the location of TSS is shown in Fig. 2*A*. The *P2rx7* promoter constructs were cotransfected into N2a cells with pGL4.74[hRluc/TK] plasmid (a transfection efficiency control), and their basal transcription activities were assessed 24 h after transfection.

Compared with empty vector, pP2X7-A exhibited a significant luciferase activity in N2a cells (1.53 ± 0.12) (Fig. 2*B*, *bar A*). Plasmid pP2X7-B containing the region from −1577 to +220 bp displayed higher promoter activity than pP2X7-A (2.04 ± 0.33) (Fig. 2*B*, *bar B*). Plasmid pP2X7-C, containing 1258 bp from −1038 to +220 bp had a strong effect on the promoter activity, reflected by a significant increase in luciferase activity to 3.45 ± 0.26 (Fig. 2*B*, *bar C*). Plasmids pP2X7-D (−451 to +220 bp) and pP2X7-F (−249 to +220 bp) displayed a luciferase activity similar to pP2X7-C (2.97 ± 0.19 and 2.98 ± 0.24, respectively) (Fig. 2*B*, *bars D* and *F*). Deletion of 3′-522 bp or 5′-468 bp from the D fragment to generate pP2X7-E (−451 to −302 bp) or pP2X7-G (+17 to +220 bp), respectively, drastically reduced the luciferase activity to empty vector values (0.87 ± 0.09 and 0.97 ± 0.09, respectively) (Fig. 2*B*, *bars E* and *G*). Taken together, these data suggest that the sequence from −249 to +17 bp, containing the TSS, includes the sequence of nucleotides necessary for basal transcription of the *P2rx7* gene.

As previously shown, a transcription factor binding site search revealed that the 2334-bp 5′-flanking region of the *P2rx7* gene contains seven putative SP1 binding sites, four of which are included into the −249/+17 bp sequence (Fig. 3*A*, Table 2). To examine functional SP1 regulatory elements in the promoter region of the *P2rx7* gene, three more deletion plasmids were constructed, and luciferase assays were performed. The first plasmid pP2X7-F1 (−249 to −139 bp) encloses the first two putative SP1 binding sites (SP1a and SP1b), the second plasmid pP2X7-F2 (-148 to −41 bp) includes the second and third putative SP1 motifs (SP1b and SP1c), and the plasmid pP2X7-F3 (−50 to +49 bp) contains the TSS and the fourth putative SP1 binding site (SP1d) (Fig. 3*B*). The pP2X7-F1 construct behaved as the empty vector (2.13 ± 0.27), whereas pP2X7-F3 plasmid displayed similar luciferase activity compared with whole F fragment (3.55 ± 0.39 and 2.98 ± 0.24, respectively) (Fig. 3*C*, *bars F*, *F1*, and *F3*). Interestingly, pP2X7-F2 containing SP1b and SP1c exerted a significant up-

FIGURE 1. **Sequence features of the 5′-flanking region of the mouse *P2rx7* gene promoter.** *A*, shown is *in silico* analysis of the putative transcription factor binding sites. A 2334-bp fragment of the 5′-flanking region of the *P2rx7* gene was isolated from mouse genomic DNA. Nucleotide numbering is relative to the first nucleotide (adenine +1) of the TSS, which is indicated in a *gray background*. The sequence lacks TATA and CAAT boxes. The positions of putative transcription factor binding motives identified using the Genomatix MatInspector software tool are *boxed*. *B*, shown is a schematic representation of the *P2rx7* promoter comprising the region −2114 to +220 bp. CG sites are depicted by *black bars*. CG content is shown as percentage of the total number of G+C (*top*) and by methylation-susceptible CG pairs, represented by the observed *versus* expected index (*Obs/Exp*; *bottom*).

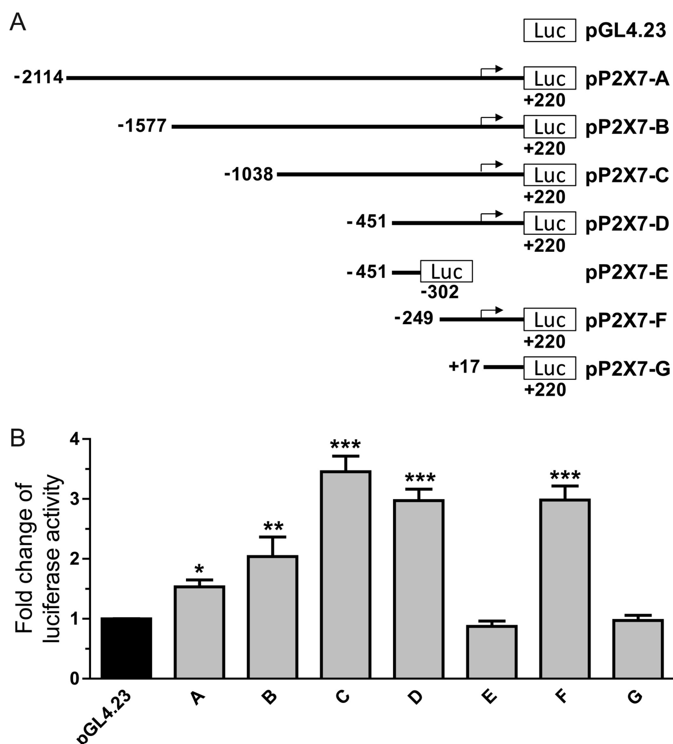

FIGURE 2. **Deletion analysis of the mouse *P2rx7* gene promoter.** *A*, shown is a schematic diagram of the P2X7 promoter constructs consisting of a 5′-flanking region with serial deletions cloned into the pGL4.23 luciferase reporter vector. The *arrow* shows the direction of transcription. *Numbers* represent the end points of each construct. *B*, plasmid constructs were cotransfected with *Renilla* luciferase vector pGL4.74[hRluc/TK] into N2a cells. 24 h after transfection, cells were harvested, and luciferase activity was measured. *Renilla* luciferase activity was used to normalize the transfection efficiency. The values represent the mean ± S.E. of at least three independent experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA with the post hoc Newman-Keuls test).

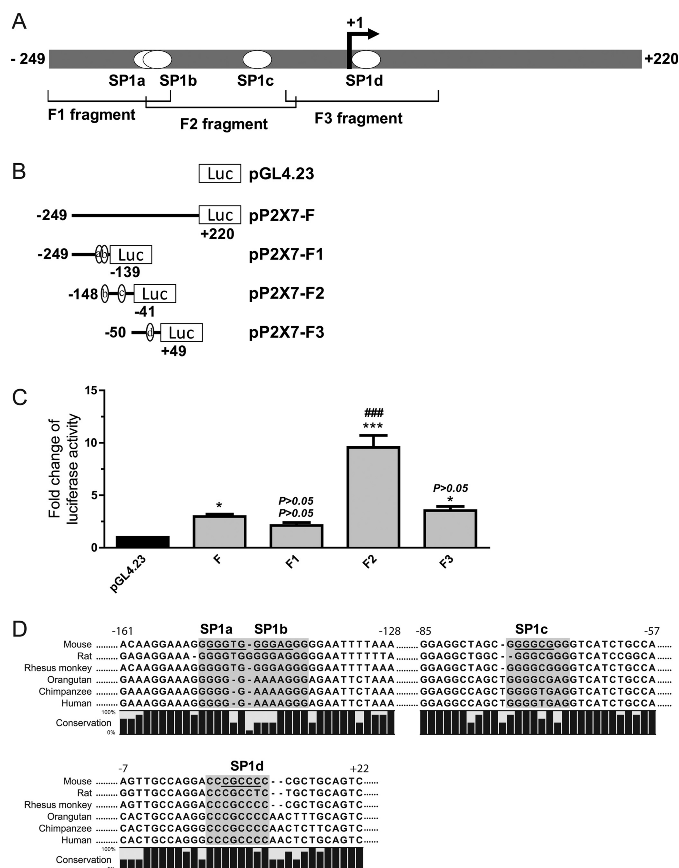

FIGURE 3. **Functional analyses of putative SP1 elements in the mouse *P2rx7* gene promoter.** *A*, shown is a schematic representation of the F fragment (−249 to +220) cloned into the pGL4.23 reporter vector containing the four SP1 binding sites located close to the TSS (+1). F fragment was divided in three subfragments named F1 (−249 to −139), F2 (−148 to −41), and F3 (−50 to +49) as indicated. *B*, deletion constructs of the whole F fragment were obtained by PCR and cloned into pGL4.23. *Numbers* represent the end points of each construct. *White ellipses* represent SP1 sites location. *C*, plasmid constructs were cotransfected with pGL4.74[hRluc/TK] vector into N2a cells. 24 h after transfection, luciferase activity was measured. *Renilla* luciferase activity was used to normalize the transfection efficiency. The values represent the mean ± S.E. of at least four independent experiments in triplicate. *, $p < 0.05$; ***, $p < 0.001$ *versus* empty vector; ###, $p < 0.001$ *versus* F fragment (ANOVA with the *post hoc* Newman-Keuls test). *D*, shown is sequence alignment of the SP1 binding sites located along the mouse *P2rx7* gene promoter across different mammalian species. Sequences were obtained from NCBI-GenBank™ and Ensembl databases. Numbers refer to the mouse sequence and ranges from −161 to +22 bp. Alignments of the putative regulatory sites are shown in a *gray background*. *Black bars* indicate the percentage of conservation with the mouse sequence. Putative core sequences for the binding of Sp1 are *underlined*.

regulation in the *P2rx7* promoter activity, inducing an increase in luciferase activity of 9.56 ± 1.16 (Fig. 3*C*, *bar F2*). This result suggests that SP1c could be the most potent motif in the regulation of *P2rx7* gene transcription.

SP1 Binding Sites Are Functional and Highly Conserved among Different Species—To evaluate the importance of each putative SP1 regulatory site contained into the −249/+17 bp sequence, the upstream sequence of P2X7 receptor genes from several mammalian species were compared. To perform the alignments, the same SP1-containing regions than those we were studying in mouse were selected. As shown in Fig. 3*D*, SP1a and SP1b putative sites are well conserved in mouse, rat, and macaque (rhesus monkey) but disappeared in human and other non-human hominids such as orangutan and chimpanzee. On the contrary, SP1c and SP1d putative sites show a high degree of homology between species. SP1c site is perfectly conserved between mouse, rat, macaque, and orangutan. We notice that the putative SP1c site shows a C/T substitution in chimpanzee and human; however, this nucleotide modification produces a GT-box that also behaves as a putative SP1 binding site (37, 38). The SP1d putative site, located in the region surrounding TSS (+1), is highly conserved between species. These results showed that SP1c and SP1d binding sites and their relative distances are conserved, suggesting an important regulatory role of Sp1 factors in the expression of P2X7 receptor in mammals.

To confirm the involvement of SP1c and SP1d binding sites in the regulation of *P2rx7* promoter, we performed reporter luciferase experiments in N2a cells with pP2X7-F2 and pP2X7-F3 promoter constructs bearing a double point mutation at each of the two SP1 cores (Fig. 4*A*). The mutations completely blocked the promoter activity observed in non-mutated constructs, indicating that both SP1 sites are actively regulating *P2rx7* gene expression (Fig. 4*B*). To explore whether the regulation of *P2rx7* gene expression by Sp1 is limited to neuronal cells, we performed analogous experiments in immune cells that express high levels of P2X7 receptors (39). Thus the macrophage RAW264.7 cell line was transfected with pP2X7-F2 or

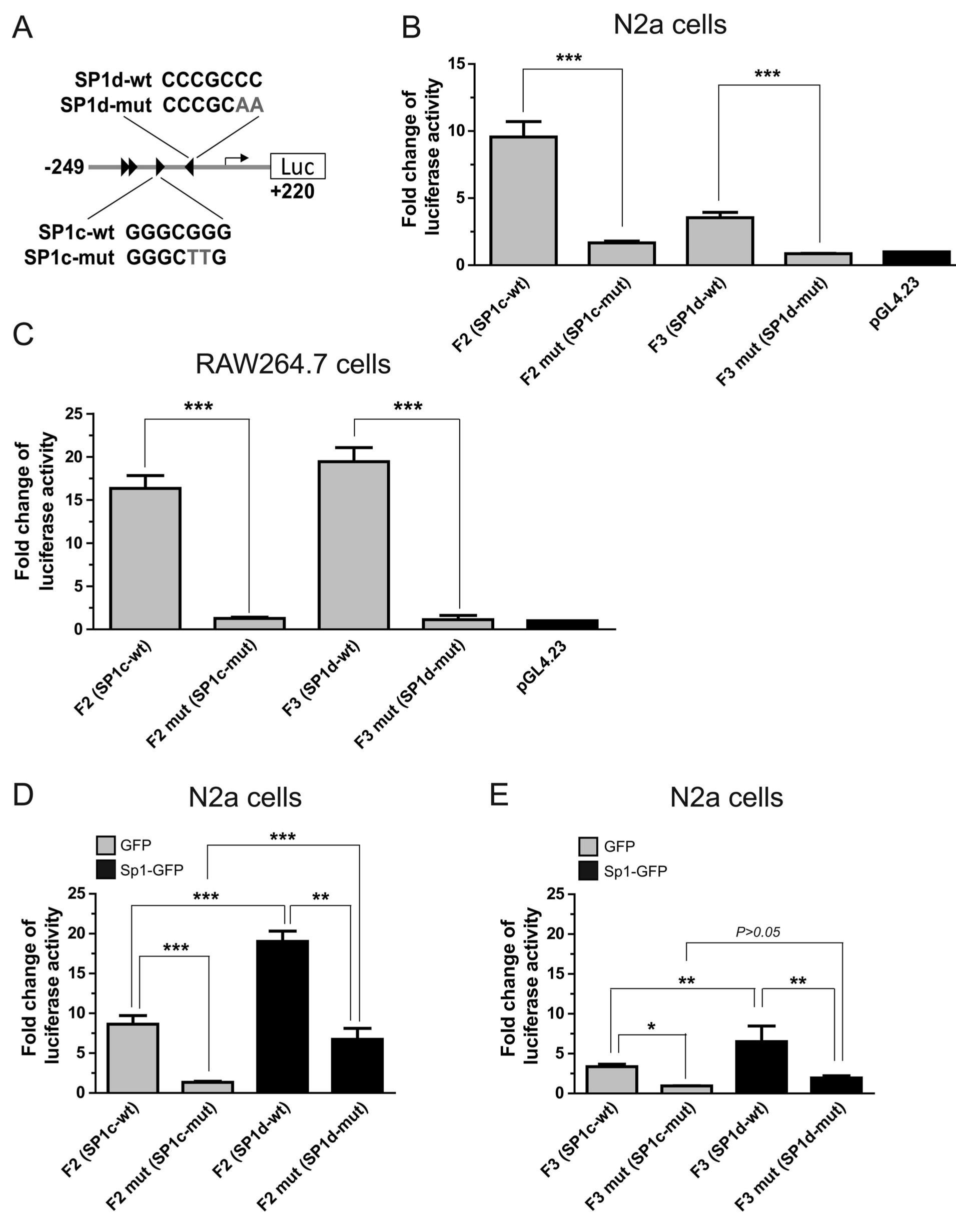

FIGURE 4. **SP1c and SP1d binding sites are directly involved in the transcriptional activation of *P2rx7* promoter by Sp1 protein.** *A*, shown is a schematic representation of the F fragment (−249 to +220) cloned into the pGL4.23 reporter vector containing four SP1 binding sites located close to the TSS (+1). The position and orientation of SP1 sites are indicated by *arrowheads*. The sequences of SP1c and SP1d sites are depicted as well as the double point mutations (in *gray*) performed to eliminate Sp1-dependent regulation. In N2a cells (*B*) and RAW264.7 cells (*C*) mutation of SP1c and SP1d sites inhibits activation of basal transcription exerted by pP2X7-F2 and pP2X7-F3 constructs, respectively. Reporter activity is shown for pP2X7-F2 (*F2*), mutated pP2X7-F2 containing the mutation of SP1c site (*F2 mut*), pP2X7-F3 (*F3*), mutated pP2X7-F3 containing the mutation of SP1d site (*F3 mut*), and empty vector (*pGL4.23*). *D*, overexpression of Sp1 protein increases promoter activity induced by pP2X7-F2 construct. The pP2X7-F2 plasmid (*F2*) containing SP1c site was cotransfected with Sp1 expression plasmid (*Sp1-GFP*) or empty vector (GFP) into N2a cells. Mutation of SP1c binding site (*F2 mut*) reduces promoter activity even in presence when Sp1 is overexpressed. *E*, shown is overexpression of Sp1 protein increases promoter activity induced by pP2X7-F3 construct. The pP2X7-F3 plasmid (*F3*)-containing SP1d site was cotransfected with Sp1 plasmid (*Sp1-GFP*) or empty vector (*GFP*) into N2a cells. Mutation of SP1d binding site (*F3 mut*) inhibits promoter activity. The values represent the mean ± S.E. of at least three independent experiments in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (ANOVA with the *post hoc* Newman-Keuls test).

pP2X7-F3 promoter constructs, and luciferase assays were performed. Interestingly, both pP2X7-F2 and pP2X7-F3 promoter constructs behaved equally potent, inducing an increase in luciferase activity of 16.34 ± 1.50 and 19.45 ± 1.63, respectively. Moreover, mutation of Sp1 cores completely blocked the promoter activity observed in non-mutated constructs (Fig. 4*C*). Altogether these results demonstrate that the regulation of P2X7 expression by Sp1 is not tissue-restricted.

To verify that both SP1c and SP1d are functional binding sites, the *P2rx7* promoter activity was studied in cells overexpressing Sp1 protein. Either Sp1 expression plasmid (Sp1-GFP) or empty vector (GFP) was cotransfected with pP2X7-F2, pP2X7-F3, or empty pGL4.23 into N2a cells. Cells were harvested 48 h after transfection, and luciferase activity was measured. As shown in Fig. 4*D*, pP2X7-F2 luciferase activity was noticeably enhanced in cells transfected with Sp1 vector by 2-fold. This increase was significantly reduced when SP1c site was mutated. We notice that SP1c mutation did not completely reverse the transcriptional activity of F2 construct when Sp1 protein was overexpressed, probably due to Sp1 binding to the

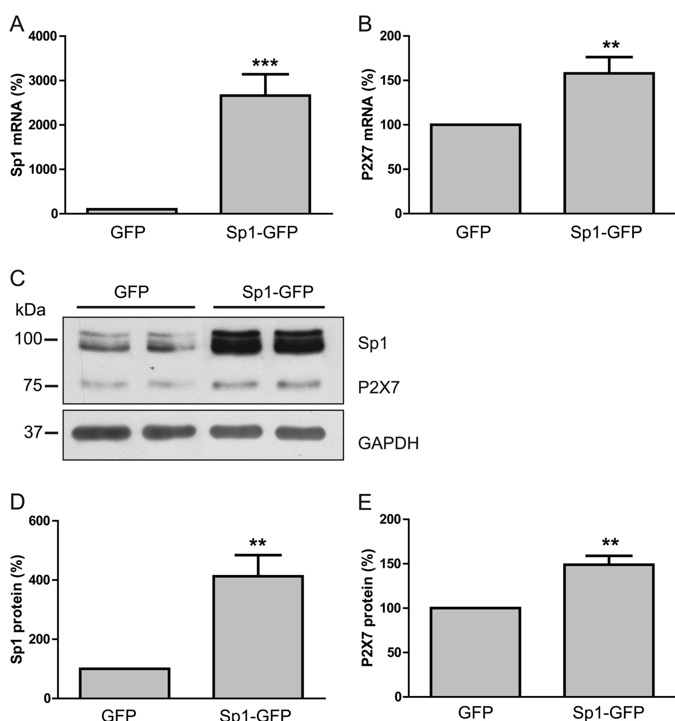

FIGURE 5. **Facilitation of endogenous P2X7 receptor expression by Sp1 overexpression.** N2a cells were transfected with Sp1 expression plasmid (*Sp1-GFP*) or empty vector (*GFP*) and analyzed 48 h later for Sp1 (*A*) and P2X7 (*B*) mRNA levels by Q-PCR. *C*, SDS-PAGE was performed to analyze cell lysates from Sp1-transfected N2a cells. Both Sp1 and P2X7 proteins were detected at a size of around 95–105 and 75 kDa, respectively. GAPDH was used as internal loading control. Densitometric analysis was performed using Image J software (*D* and *E*). The values represent the mean ± S.E. of three independent experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's *t* test).

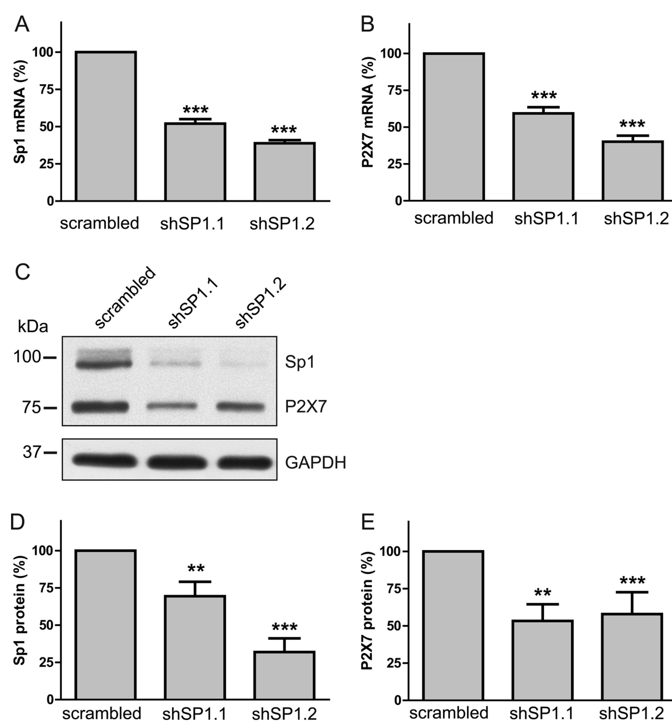

FIGURE 6. **Down-regulation of endogenous P2X7 receptor expression by Sp1 interference.** Endogenous Sp1 expression was knocked down in N2a cells using two specific shRNA (*shSP1.1* and *shSP1.2*) and analyzed 48 h later for Sp1 (*A*) and P2X7 (*B*) mRNA level by Q-PCR. Scrambled shRNA was used as negative control. *C*, silencing of Sp1 reduces both Sp1 and P2X7 proteins expression. SDS-PAGE was performed to analyze cell lysates from N2a cells transfected with shSP1.1, shSP1.2, or scrambled for 72 h. GAPDH was used as internal loading control. Densitometric analysis was performed using Image J software (*D* and *E*). The values represent the mean ± S.E. of three independent experiments in duplicate or triplicate. **, $p < 0.01$; ***, $p < 0.001$ (Student's *t* test).

SP1b motif, which is also included in F2 fragment (Fig. 3*B*). Sp1 overexpression was also able to increase 2-fold the transcriptional activity of pP2X7-F3 (Fig. 4*E*), although in this case the increment in luciferase activity was completely abolished by SP1d site mutation even in the presence of an excess of Sp1 protein. These data clearly demonstrate that overexpression of Sp1 significantly up-regulates *P2rx7* gene promoter activity by its binding to SP1 sites.

Sp1 Is Crucial for the Basal Transcription of Endogenous P2X7 Receptor—In transient transfection experiments using luciferase reporter plasmids, the proximal promoter region of *P2rx7* gene is not packaged into a chromatin-like structure, leading to high accessibility to different nuclear factors. Therefore, it is essential to study the promoter activity in its native chromatin context. To investigate whether Sp1 plays a relevant role in the transcriptional regulation of endogenous *P2rx7* gene, N2a cells were transiently transfected with Sp1 expression plasmid (Sp1-GFP) or empty vector (GFP), and both mRNA and protein levels were quantified 48 h and 72 h after transfection, respectively. Q-PCR studies revealed that the endogenous levels of both Sp1 and P2X7 transcripts were significantly increased in Sp1 over-expressing cells (Fig. 5, *A* and *B*). Moreover, Western blot analysis also demonstrated that Sp1 overexpression markedly enhanced the protein levels of both Sp1 (Fig. 5, *C* and *D*) and P2X7 receptor (Fig. 5, *C* and *E*) in N2a cells.

To further assess the relevance of Sp1 in *P2rx7* transcription, endogenous Sp1 expression was knocked down in murine N2a cells using two specific shRNA (shSP1.1 and shSP1.2). N2a cells were transiently transfected with shRNAs or scrambled vector, and both mRNA and protein levels were quantified 48 and 72 h after transfection, respectively. As expected, Sp1 interference significantly reduced both the transcript and the protein levels of Sp1 (Fig. 6*A*, *C*, and *D*) and P2X7 receptor (Fig. 6, *B*, *C*, and *E*) in N2a cells. Taken together, these data are consistent with the results obtained from the luciferase assays, corroborating that Sp1 factor plays an important role in the transcriptional regulation of endogenous P2X7 receptor.

Sp1 Up-regulates P2rx7 Gene Expression in Primary Cultures of Mouse Cortical Neurons and Astrocytes—To determine whether SP1 binding elements contained in the *P2rx7* promoter functionally regulate P2X7 receptor expression not only in a cell line but also in primary cultures from mouse brain, cortical neurons and astrocytes were treated with mithramycin A, an antibiotic reported to block the binding of Sp1 to GC-rich regions in the DNA (40). First, we confirmed that the level of contaminant microglia in cultures of neurons and astrocytes was almost undetectable by Western blot techniques (data not shown). The treatment with 300 nM mithramycin A for 24 h markedly reduced the endogenous levels of P2X7 mRNA in both astrocytes (Fig. 7*A*) and neurons (Fig. 7*B*). P2X7 protein levels were also significantly reduced in astrocytes and neurons

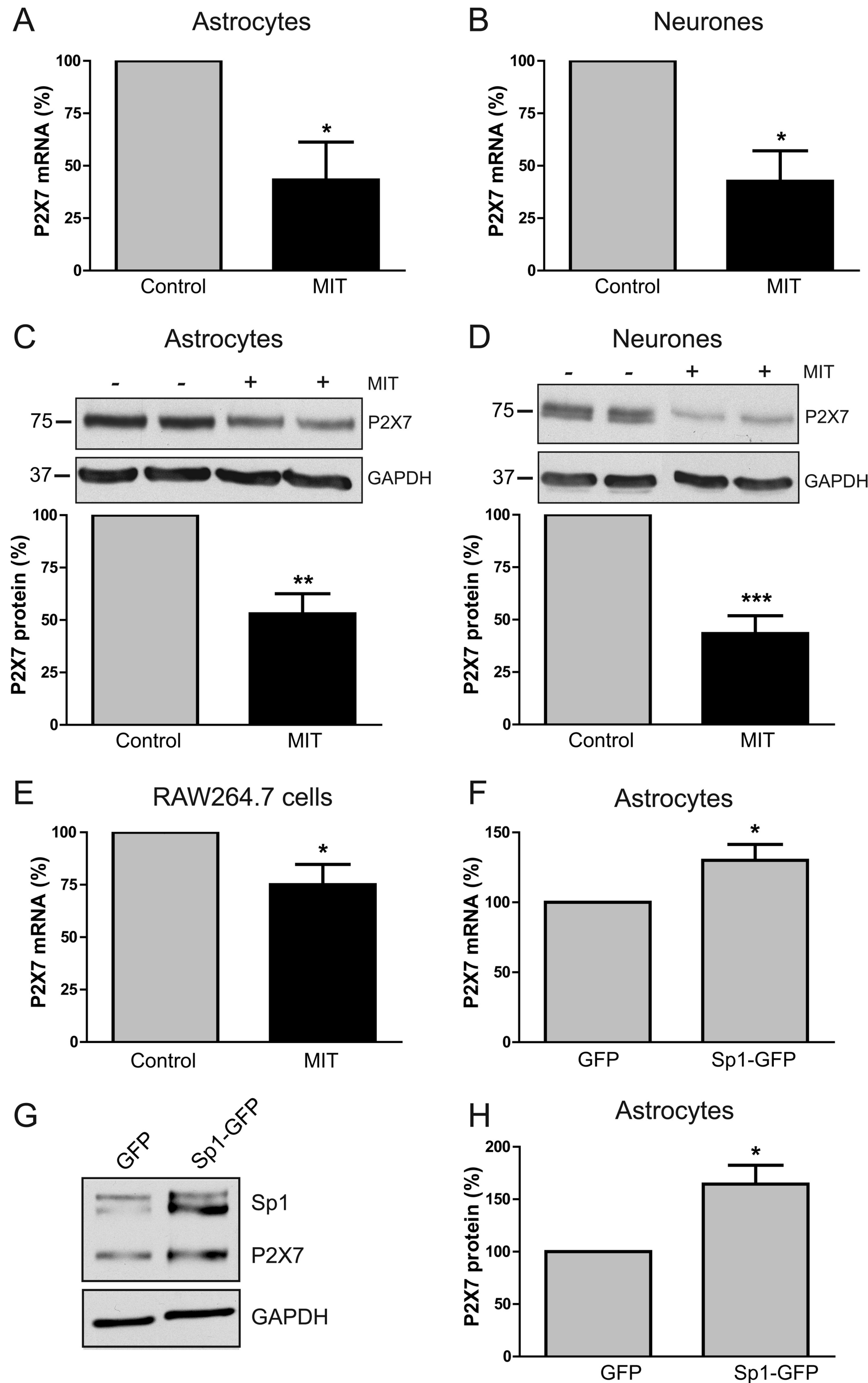

FIGURE 7. **Sp1-dependent regulation of P2X7 expression in primary cultures of cortical astrocytes and neurons.** Primary cultures of cortical astrocytes (*A*) and neurons (*B*) were treated with 300 nM mithramycin A (*MIT*) or vehicle (control) for 24 h. Total RNA was extracted and analyzed for P2X7 mRNA levels by Q-PCR. SDS-PAGE was performed to analyze cell lysates from primary cultures of cortical astrocytes (*C*) and neurons (*D*) treated with 300 nM mithramycin A or vehicle for 48 h. GAPDH was used as internal loading control. *E*, after 24 h of treatment with mithramycin A or vehicle, total RNA from RAW264.7 cells was extracted and analyzed for P2X7 mRNA expression by Q-PCR. *F*, cortical astrocytes were transfected with Sp1 expression plasmid (*Sp1-GFP*) or empty vector (*GFP*) and analyzed 48 h later for P2X7 mRNA level by Q-PCR. *G*, SDS-PAGE was performed to analyze cell lysates from Sp1-transfected astrocytes using anti-P2X7 antibodies. The ratio of P2X7 to GAPDH protein level was calculated by densitometric analysis (*H*). The values represent the mean ± S.E. of three independent experiments in duplicate or triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's *t* test).

treated with mithramycin A for 48 h (Fig. 7, *C* and *D*). When analogous assay was performed in the macrophage RAW264.7 cell line, mithramycin A was also able to reduce the endogenous levels of P2X7 mRNA, corroborating that the regulation of *P2rx7* gene expression by Sp1 is not limited to neural cells (Fig. 7*E*). Next, cortical astrocytes were transiently transfected with Sp1 expression plasmid (Sp1-GFP) or empty vector (GFP), and both mRNA and protein levels were quantified 48 and 72 h after transfection, respectively. Overexpression of Sp1 enhanced transcript (Fig. 7*F*) and protein levels (Fig. 7, *G* and *H*) of endogenous P2X7 receptor, indicating that Sp1 is also able to regulate endogenous P2X7 receptor expression in primary cultures from mouse brain.

Distribution of P2X7 Receptor and Sp1 Factor in the Postnatal Murine Brain—To determine whether Sp1 is also able to regulate P2rx7 gene expression *in vivo*, we sought to analyze the distribution of Sp1 and P2X7 receptor in the brain of newborn (P0) mice. To perform this study we used *P2rx7*-EGFP transgenic mice that express EGFP under the control of *P2rx7* promoter (30). First, to validate the reliability of the *P2rx7*-EGFP reporter mice, we analyzed the expression of EGFP in immune tissues/cells, which natively express high levels of P2X7 receptors (39). As expected, both peritoneal macrophages (Fig. 8*A*) and spleen (Fig. 8, *B* and *C*) express high levels of EGFP in 10-week-old mice, indicating the consistency of the reporter mice model. CD11 was used as protein marker of macrophages. Regarding the distribution of Sp1 and P2X7 receptor in the mouse brain, we found the EGFP fluorescence signal in a small population of cortical cells, mostly located in the upper layers of the cerebral cortex (Fig. 9, *A* and *E*). Immunohistochemical studies using antibodies against Sp1 protein showed that basal levels of Sp1 were widely expressed in the cortex, although some cells showed a higher Sp1 expression compared with the neighboring ones (Fig. 9, *B* and *F*). We notice that 76.09 ± 6.74% ($n = 68$ cells) EGFP-positive cells also showed a strong Sp1-positive immunostaining (Fig. 9, *A–H*). To confirm this observation, other brain regions enriched in EGFP-positive cells were analyzed, and we found a strong EGFP fluorescence signal at the pons of newborn mice (Fig. 9*J*). Interestingly, 62.81 ± 9.45% ($n = 143$ cells) of EGFP-positive cells colocalized with Sp1-positive cells in this brain region (Fig. 9, *J–L*). Thus we confirmed that cells expressing P2X7 receptor also contain a high amount of Sp1 factor.

Sp1 Mediates Up-regulation of P2X7 Receptor Expression under Serum Deprivation—Previous studies reported that serum deprivation of a human hepatocarcinoma cell line enhances open chromatin accessibility and helps to expose SP1 binding sites (41). Moreover, it is well known that Sp1 is an autoregulated gene (42–44). To examine the effects of serum deprivation in Sp1 and P2X7 expression, N2a cells were cultured in the absence of FBS, and both mRNA and protein levels were quantified. After 24 h serum starvation a significant up-regulation of both Sp1 and P2X7 transcripts was observed, being even more evident after 48 h (Fig. 10, *A* and *B*). Treatment of N2a cells with 300 nM mithramycin for 48 h resulted in the reduction of both Sp1 and P2X7 mRNA levels in control conditions (cells cultured in 10% FBS) and also in cells cultured in the absence of serum (Fig. 10, *A* and *B*). When protein levels

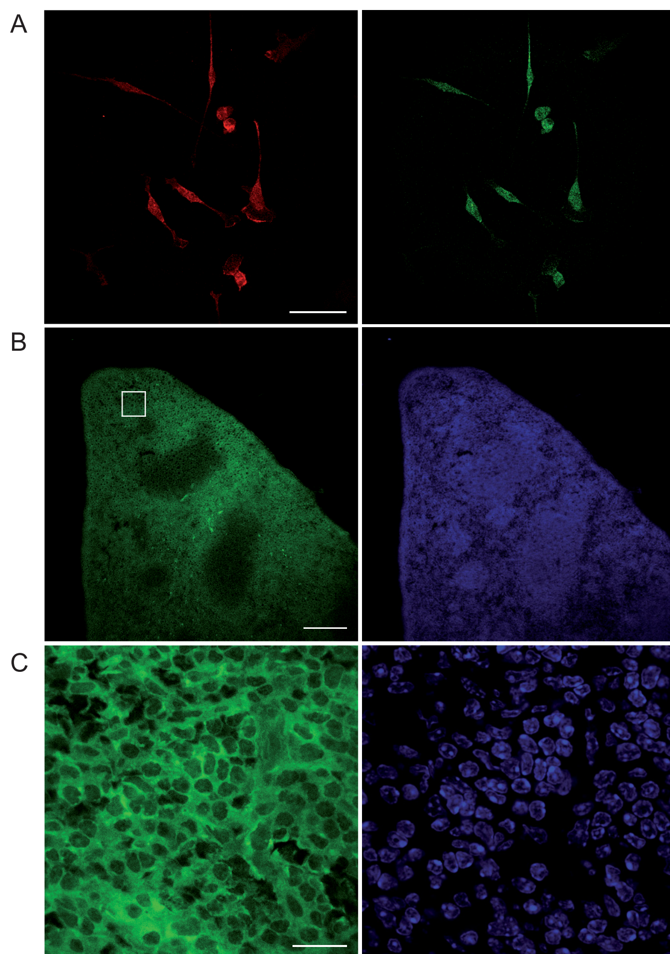

FIGURE 8. **Expression of P2X7 receptors in macrophages and spleen from adult *P2rx7*-EGFP mice.** *A*, representative photomicrographs show the macrophage marker CD11 (*red*) and native EGFP immunofluorescence (*green*) in elicited peritoneal macrophages from 10-week-old P2rx7-EGFP mice. *Scale bar* = 50 μm. *B*, photomicrographs show native EGFP immunofluorescence (*green*) in spleen from 10-week-old *P2rx7*-EGFP mice. Nuclei are counterstained with DAPI (*blue*). *Scale bar* = 300 μm. The *square* in *panel B* is shown at higher magnification in *C*. *Scale bar* = 15 μm.

were quantified, both Western blot and immunocytochemical experiments demonstrated that serum withdrawal produced a rapid and transient increase in Sp1 protein after 24 h serum removal (Fig. 10, *C*, *E*, and *F*), whereas the expression P2X7 receptor was gradually increased during 48–72 h of serum withdrawal (Fig. 10, *D*, *G*, and *H*). These results indicate that serum deprivation facilitates the up-regulation of *Sp1* gene exerted by Sp1 factor and, consequently, an increase in the endogenous levels of P2X7 receptor takes place.

DISCUSSION

Purinergic receptors have been shown to be widely distributed throughout the nervous system, being present in both neurons and glial cells (45). It is well known that activation of P2X receptors by ATP is implicated in fast excitatory neurotransmission, presynaptic regulation of neurotransmitter release, cell proliferation, axonal growth and development, and also in disease and cytotoxicity (6, 7, 46–50). Within the P2X family, the P2X7 receptor has a distinguished role in the central nervous system for its implication in both normal behavior and

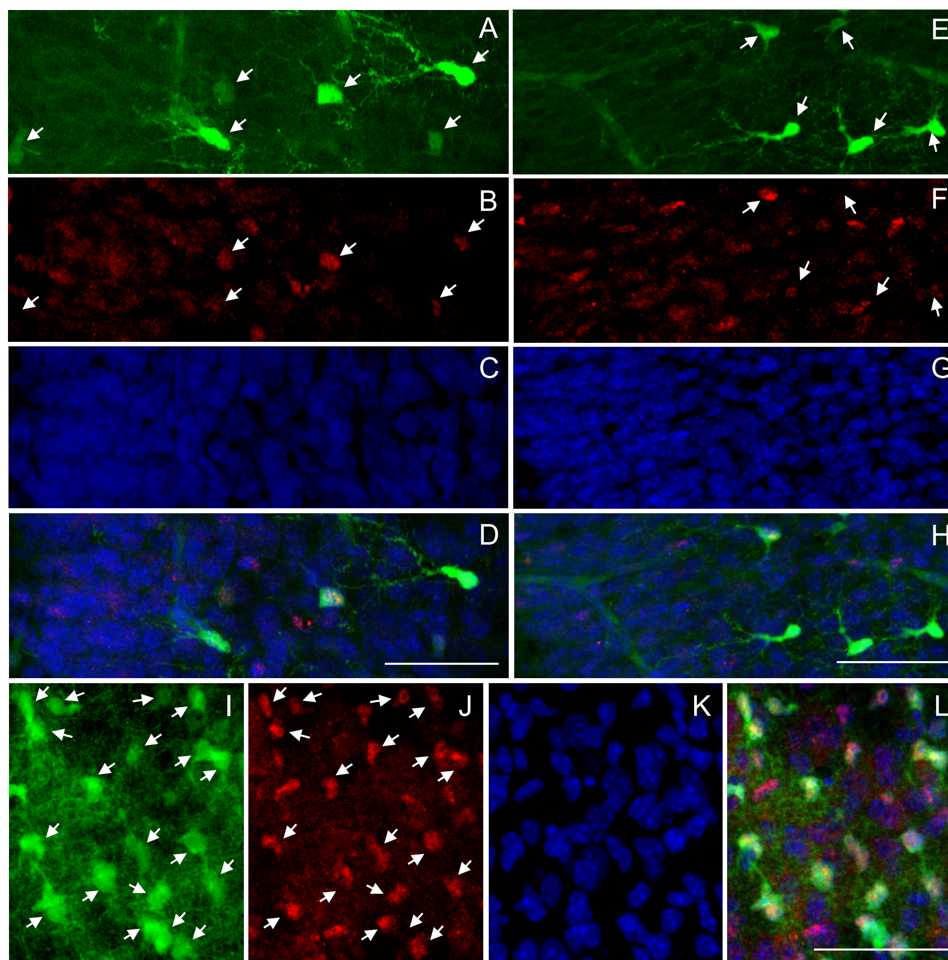

FIGURE 9. ***In vivo* colocalization of P2X7 receptor and Sp1 factor in the brain from neonatal *P2rx7*-EGFP mice.** Representative photomicrographs show native EGFP immunofluorescence (*A* and *E*, *green*) and Sp1 immunostaining (*B* and *F*, *red*) in different cortical subfields from P2rx7-EGFP mice. *Arrows* mark the position of EGFP-positive cells. Nuclei are counterstained with DAPI (*C* and *G*, *blue*). Merge images are shown (*D* and *H*). Photomicrographs show native EGFP immunofluorescence (*I*, *green*) and Sp1 immunostaining (*J*, *red*) in pons, a brain region with a high P2X7 receptor expression in neonatal *P2rx7*-EGFP mice. *Arrows* indicate EGFP-positive cells. Nuclei are counterstained with DAPI (*K*, *blue*). A merge image is shown (*L*). *Scale bars* = 50 μm.

pathological brain functions including neurodegenerative diseases and neuropsychiatric disorders (4). Moreover, high P2X7 expression in most human neuroblastoma so far investigated seems to be related with malignant cell growth and progression (13, 14, 51). All these evidences point to P2X7 receptor as a relevant pharmacological target for the treatment of both neurodegenerative disorders and cancer, thus it seems necessary to understand the mechanisms involved in the regulation of P2X7 receptor expression.

This study reports for the first time that Sp1 is a key factor necessary for the basal expression of P2X7 receptor in the nervous system. Previous studies located the active promoter of the human *P2RX7* gene in the −158/+32-nucleotide region surrounding the transcription start site, although the transcription factors involved in the promoter activity were unknown (28, 29). To characterize the molecular mechanisms that control P2X7 expression, we cloned and characterized 2334 bp of the 5′-flanking region of the murine *P2rx7* gene (from −2114 to +220). Bioinformatics analysis showed that the *P2rx7* gene promoter, unlike most type II eukaryotic gene promoters, does not contain TATA or CAAT boxes. It is well established that genes lacking a typical TATA box in their promoter sequence depend on multiple upstream regulator sequences for their activation (52). Software analysis of putative transcription factor binding indicated that the 5′-proximal regulatory region of the murine *P2rx7* gene contains several putative regulatory elements including AP1 (activator protein 1), CREB (c-AMP-responsive element-binding protein), E-box, HIF (hypoxia-inducible factor), SP1, STAT (signal transducer and activator of transcription), TCF/LEF1, and YY1F (activator/repressor binding to transcription initiation site), suggesting that P2X7 expression is tightly regulated at the transcriptional level. The most striking feature revealed by the *in silico* analysis of *P2rx7* promoter is the presence of seven putative SP1 motifs, most of them located close to the TSS. The Sp1 family of transcription factors recognizes GC and GT boxes at the DNA and has been widely described as general transcription factors frequently involved in transcriptional regulation of gene promoters that lack consensus TATA and CAAT boxes (38). Transfection experiments using different 5′-flanking sequences linked to the luciferase gene showed that 266 bp of the mouse *P2rx7* gene (from −249 to +17) contain the minimal promoter region. This fragment comprises the TSS and four putative SP1 sites (named SP1a, -b, -c, and -d, respectively). By deletion analysis, this

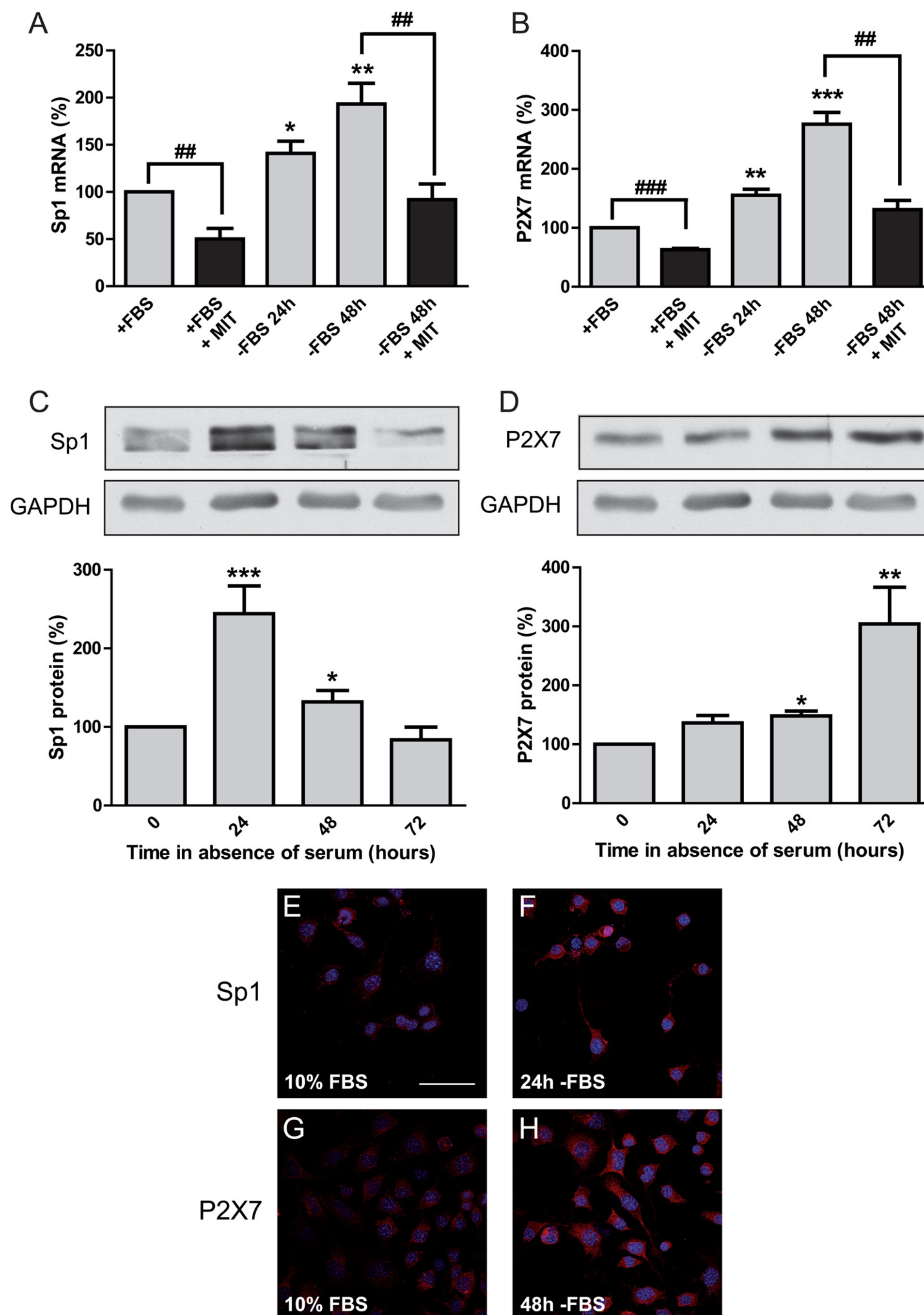

FIGURE 10. **Up-regulation of P2X7 receptor expression under serum deprivation.** Changes in Sp1 (*A*) and P2X7 (*B*) transcript levels in N2a cells cultured under serum deprivation for 24 or 48 h are shown. In some cases 300 nM mithramycin A (*MIT*) treatment was performed. Total RNA was extracted and analyzed for Sp1 and P2X7 mRNA by Q-PCR. The values represent the mean ± S.E. of three independent experiments in duplicate or triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* control (ANOVA with the post hoc Newman-Keuls test); ##, $p < 0.01$; ###, $p < 0.001$ (Student's *t* test). Changes in Sp1 (*C*) and P2X7 (*D*) protein levels under serum deprivation during the whole detection period are shown. SDS-PAGE was performed to analyze Sp1 and P2X7 receptor expression in cell lysates from N2a cells cultured for 0, 24, 48, or 72 h in the absence of serum (FBS). GAPDH was used as internal loading control. The values represent the mean ± S.E. of three independent experiments in duplicate or triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *versus* control (ANOVA with the post hoc Newman-Keuls test). N2a cells were cultured in the presence or absence of serum for 24 or 48 h. Afterward, cells were fixed and immunostained with antibodies against Sp1 (*E* and *F*, *red*) or P2X7 (*G* and *H*, *red*). Nuclei were labeled with DAPI (*blue*). *Scale bar* = 50 μm.

region of maximum promoter activity could be subdivided into two segments, F2 (−148 to −41 bp), which increased promoter efficiency 3-fold compared with complete F fragment, and a proximal segment F3 (−50 to +49 bp), which displayed a significant promoter activity similar to that exerted by complete F construct. These findings indicate that the major elements responsible for transcriptional activation must be confined to these regions. Interestingly, both F2 and F3 constructs contains putative SP1 sites (SP1c and SP1d, respectively) that are structurally identical to the consensus site (G/T)GGGCGG(G/A)(G/A)(C/T) (53), although SP1d is located in the reverse strand. It is well established that DNA methylation of CpG islands is an important mechanism for transcriptional regulation of multiple genes in mammals (54–56). Methylation degree of CpG

islands contained within the SP1 consensus elements may interfere with the binding of Sp1 to DNA, modulating the Sp1-dependent transcription of genes (36). Bioinformatics analysis revealed that the 5′-proximal regulatory region of the murine *P2rx7* gene lacks CpG islands, so we ruled out that methylation of SP1 sites located into the promoter region could be interfering Sp1 binding to DNA. However, we cannot discard an epigenetic control of *P2rx7* gene as frequently CpG regions outside the active promoter can regulate transcription by modulation of DNA methylation. Indeed, the presence of a CpG-rich 547-bp region downstream of the active promoter of the human *P2RX7* gene that regulates its transcription has been previously reported (29).

Our data from site-directed mutation showed that replacement of two nucleotides in both SP1c and SP1d was enough to completely block the reporter activity. Therefore, we investigated whether SP1 binding sites identified in the murine promoter were also present in the upstream sequence of P2X7 genes from other mammals, as relevant transcriptional regulatory elements often show conservation between species (57). The species analyzed included rat, macaque, chimpanzee, orangutan, and human. Sequence alignment showed conserved SP1c and SP1d sites in identical positions across all species examined, suggesting that the involvement of Sp factors in the regulation of P2X7 transcription has been conserved during evolution. We notice that P2X7 receptor gene is highly polymorphic, with 40 coding variants reported in humans and more than 12 non-synonymous SNPs characterized for their effect on function (58). At least eight SNPs in *P2RX7* confer loss-of-function in the P2X7 signaling pathway, giving rise to severe functional defects (20, 59). Only two variants have been shown to confer gain of function (26). Currently, five SNPs have also been identified in the *P2RX7* promoter, although none of them appears to be associated with an altered ATP response phenotype (27). However, support for a regulatory role for the promoter SNPs comes from the fact that a positive association between a *P2RX7* promoter polymorphism (−762, T/C) and a major susceptibility to tuberculosis has been reported (60). Based on this precedent, we speculate that the presence of promoter SNPs in the region containing SP1c and/or SP1d could alter binding of Sp1 transcription factor, resulting in a decrease in P2X7 receptor gene transcription. Analysis of new promoter polymorphisms on P2X7 receptor function could be relevant to identify the regulatory mechanisms underlying the heterogeneity in ATP responsiveness observed within human populations.

Currently the Sp1 family of transcription factors consists of nine proteins (Sp1–9), with Sp1 being the first member indentified and cloned (38). Often, for several cell types and promoters, Sp1 and Sp3 have been reported as the major GC/GT box binding activities, being broadly expressed in most cells and tissues (61). It is well known that Sp1 can be regulated by glycosylation (62) and phosphorylation (53) and can directly interact with the basal transcription machinery to induce Sp1-dependent transcription of target genes (63). However, although Sp3 was found to be highly homologous to Sp1 with similar affinities for GC/GT boxes, there are some striking functional differences. Sp3 has been shown to act as a transcriptional activator in some cellular contexts, whereas in other experimental conditions Sp3 remains inactive or represses transcription driven by Sp1 or other transcription factors (61). Given the dual nature of Sp3, our experiments were focused on Sp1 protein as the classical transcriptional activator of GC box-containing genes. Overexpression of Sp1 protein increased gene promoter activity in N2a cells, resulting in a significant increase in luciferase activity and endogenous P2X7 mRNA and protein levels. Similar results were obtained in the macrophage cell line RAW264.7 indicating that the regulation of P2X7 expression by Sp1 is not restricted to neuronal cells. As expected, SP1c and SP1d mutations were able to disrupt Sp1-enhanced promoter activity in both N2a and RAW264.7 cells, suggesting that Sp1 binds to SP1c and SP1d sites to positively regulate *P2rx7* gene expression. The role of Sp1 as a key factor necessary for the basal activation of *P2rx7* gene transcription has been also corroborated by either Sp1 overexpression or interference of endogenous Sp1 protein in neuroblastoma cells.

Mithramycin A is an antibiotic isolated from various strains of the bacterium *Streptomyces* that has been used in the past in the treatment of several types of cancer including testicular carcinoma, chronic myeloid leukemia, and acute myeloid leukemia (64). More recently, mithramycin has been used in combination with other drugs such as apoptosis-inducing agents, chemotherapeutic drugs, or antiangiogenic compounds as a novel therapy for pancreatic cancer and other tumors (44). Here we show that neuroblastoma cells, macrophages, and primary cultures of cortical neurons and astrocytes treated with mithramycin A underwent a reduction in endogenous P2X7 receptor levels. The mechanism of action of mithramycin involves a non-covalently binding to GC-rich DNA sequences, preventing Sp1 from binding to a variety of promoters of genes involved in cell death, cell migration, and immune recognition of tumor cells (65). Noteworthy, high levels of P2X7 receptor expression have been found in various cancer cells, including human primary neuroblastoma tumors and neuroblastoma cell lines where P2X7 receptors support tumor growth (13, 51, 66). Although the source of extracellular ATP required to keep a tonic P2X7 receptor activation remains unclear, the exocytotic release of ATP coupled to P2X7 receptor stimulation has been demonstrated in neuroblastoma cells (67). In addition, a growing number of studies suggest a noticeable convergence between genes involved in transformation and those necessary for some types of neurodegeneration. In this line, mithramycin has been reported to prolong survival in mice models of Huntington disease (68–72) and to protect against dopaminergic neurotoxicity after methamphetamine administration (73). In addition, mutant huntingtin has been reported to inhibit Sp1-medianted gene transcription (74, 75), and human *huntingtin* gene expression is transcriptionally regulated by Sp1 (76). P2X7 receptors are also implicated in the development of several neurodegenerative pathologies such as Huntington and Alzheimer disease (9, 77, 78). Moreover, P2X7 receptors regulate both axonal development in hippocampal neurons and differentiation of neuroblastoma cells through a Ca^{2+}/calmodulin-dependent kinase II-related mechanism (7, 8). Our analysis of the expression of the P2X7 receptor in newborn *P2rx7*-EGFP mice, where the brain cytoarchitecture is still in progress, showed a good correlation between the presence of P2X7 receptors and

the cellular content in Sp1 factor. These data are in agreement with previous studies reporting that Sp1 is essential for early embryonic development (79) and that the level of Sp1 rises during development in the neural tissue of early fetuses (80). These evidences point to Sp1 as an important regulator of cellular processes during brain development and differentiation.

Serum deprivation has been described to enhance open chromatin accessibility, facilitating exposure of SP1 binding sites. Furthermore, Sp1 binding to its regulatory SP1 sites acts as a docking partner for recruiting RNA polymerase II to the promoter, which in turn results in up-regulation of gene expression (41). According to this premise, an increase in P2X7 expression after serum withdrawal could be correlated with an increase in Sp1 binding to SP1 sites at the *P2rx7* promoter. To confirm this hypothesis, we analyzed both P2X7 and Sp1 levels in differentiated *versus* proliferated N2a cells. Serum deprivation resulted in a significant increase in Sp1 mRNA followed by an expected increase in the transcript levels of P2X7 receptor. Both increases were completely abolished by mithramycin A treatment, indicating that both *Sp1* and *P2rx7* genes are being transcriptionally up-regulated by Sp1 binding to their promoters. We notice that the Sp1 and P2X7 proteins are enhanced under serum deprivation, but the increments observed were not synchronized. This evidence could be relevant in explaining why in newborn *P2rx7*-EGFP mice brains some EGFP-positive cells do not simultaneously express high levels of Sp1 protein.

In conclusion, regulation of P2X7 receptor expression may play a very important role in the development of different pathologies, including cancer and neurodegenerative diseases. By cloning and functionally characterizing the *P2rx7* gene promoter, our experiments provide the first molecular evidence that Sp1 plays an important role in the control of *P2rx7* gene expression in the nervous system. Therefore, we suggest that the gene encoding P2X7 subunit could be just one of the thousands of genes implicated in the control of cell growth, differentiation, and cell death whose expression is transcriptionally regulated by Sp1 (81, 82). Future studies will be focused on clarifying the role of Sp1 in the control of P2X7 receptor expression during brain development and disease.

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