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Sp1 in Astrocyte Is Important for Neurite Outgrowth and Synaptogenesis

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Abstract

In this study, we found that Sp1 was highly expressed in astrocytes, implying that Sp1 might be important for the function of astrocytes. Sp1/GFAP-Cre-ERT2 conditional knockout mice were constructed to study the role of Sp1 in astrocytes. Knockout of Sp1 in astrocytes altered astrocytic morphology and decreased GFAP expression in the cortex and hippocampus but did not affect cell viability. Loss of Sp1 in astrocytes decreased the number of neurons in the cortex and hippocampus. Conditioned medium from primary astrocytes with Sp1 knockout disrupted neuronal dendritic outgrowth and synapse formation, resulting in abnormal learning, memory, and motor behavior. Sp1 knockout in astrocytes altered gene expression, including decreasing the expression of Toll-like receptor 2 and Cfb and increasing the expression of C1q and C4Bp, thereby affecting neurite outgrowth and synapse formation, resulting in disordered neuron function. Studying these gene regulations might be beneficial to understanding neuronal development and brain injury prevention.

Keywords

Sp1; Astrocyte; Neurite outgrowth; Synaptogenesis

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Introduction

Specificity protein 1 (Sp1) belongs to the specificity protein/Krüppel-like factor (SP/KLF) transcription factor family and is a ubiquitously expressed, prototypic C2H2-type zinc finger-containing DNA binding protein that can activate or repress transcription in response to physiologic and pathologic stimuli in mammalian cells [1]. Sp1 is involved in many cellular processes including cell cycle progression, apoptosis, development and differentiation, tumorigenesis, angiogenesis, neuroregeneration, and neuroprotection [2–4]. The expression levels of Sp1 messenger RNA (mRNA) and protein were increased in the brains of APPswe/PS1dE9 transgenic mice, and mithramycin A (MTM) chronic treatment potently inhibited Sp1 activation in APPswe/PS1dE9 mice, resulting in levels of Sp1 that were similar to those in wild-type mice. MTM treatment significantly improved learning and memory deficits [5]. Sp1 is overexpressed that promotes human leucine-rich repeat kinase 2 (LRRK2) gene expression, suggesting that controlling the LRRK2 level regulated by Sp1 signaling may be beneficial to attenuating Parkinson's disease-related neuropathology [6]. Inhibition of Sp1 functions by MTM increased memory defects in AD transgenic mice, but there were no changes in sensorimotor or anxiety tests. Sp1 activation could be a protective response in AD transgenic mice model [7]. Sp1 is also reported to be involved in neuron regeneration and neuroprotection against brain injury such as stroke, spinal cord injury, and brain trauma [8–10]. Our previous studies also showed that Sp1 is upregulated in ischemia stroke and protects neuron survival [11]. However, the functional role of Sp1 in astrocytes is still not clear.

Astrocytes, one type of glial cells in mammals, are important regulators of brain development and physiology function through their interaction with neuronal synapses and provide important metabolic and trophic support to neurons. Astrocytes have been well known for the establishment and maintenance of proper synaptic connectivity and networks [12]. In adult brain, astrocytes are involved in synapse component integrations and protoplasmic astrocytes infiltrate into the neuropil and wrap around synapses. A mouse cortical astrocyte is estimated to contact over 100,000 synapses, but a human astrocyte can contact up to 2,000,000 synapses, indicating that astrocytes have the ability to sense and adhere to synapses [13]. According to previous studies, astrocytic coverage of synaptic contacts is altered during development and in various physiological conditions. Peri-synaptic astrocyte processes can rapidly remove synaptic released neurotransmitters from the interstitial area, avoiding extrasynaptic accumulation. For example, astrocytic processes regulate glutamate transporters, which ensure that glutamate does not accumulate extrasynaptically and prevents excitotoxicity [14]. In addition, astrocytes also control the ionic channel balance at the synapse, which is critical for the sustainability of proper synaptic transmission [15]. Previous studies have indicated that rodent neurons grown in isolation in vitro form few synapses but the addition of astrocytes to the neurons dramatically increases both the number of synapses and the strength of the synapses [16], indicating that astrocytes control synapse development. In this study, we first found that Sp1 is highly expressed in astrocytes, implying that it might be important for the astrocytic function. Therefore, we created a specific functional knockout of Sp1 in astrocytes and studied the role of Sp1 in astrocytes. We found that Sp1 in astrocytes regulates the

expression of many genes that are involved in neuronal developmental processes such as neurite outgrowth and synaptogenesis.

Materials and Methods

Primary Astrocyte Cultures

Primary astrocyte cultures were prepared from mice pups at postnatal days 0–2 as previously described. Briefly, cortices and midbrain were isolated and digested into a single cell suspension. Cells were centrifuged and resuspended in culture maintenance medium (DMEM-F12 (1:1) medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ M non-essential amino acids, 50 U/ml penicillin, and 50 μ g/ml streptomycin) and plated on poly-D-lysine-coated 6- or 24-well plates at 1×10^6 or 1×10^5 cells/well, respectively. Astrocyte culture medium was refreshed every 3 days until treatment 7 days after seeding. Astrocyte cultures were treated with 1 mM of L-leucine methyl ester (LME; Sigma-Aldrich, St. Louis, MO, USA) for 72 h after seeding cells.

Primary Neuron Cultures

Mesencephalic neuron cultures were prepared from mice embryos at gestation day 14 (E14) as described previously. Briefly, midbrain tissues were digested into single cell suspension. Cells were cultured in maintenance medium (MEM medium supplemented with 10% heat-inactivated FBS, 10% heat-inactivated HS, 0.1% (w/v) D-glucose, 1 mM sodium pyruvate, 100 μ M non-essential amino acids, 50 U/ml penicillin, and 50 μ g/ml streptomycin) and seeded on poly-D-lysine-coated 24-well plates at 5×10^5 cells/well. Primary neuron cultures were treated with 10 μ M of cytosine β -D-arabinofuranoside (Ara-C, Sigma) for 3 days after seeding cells for 48 h.

Primary Neuron-Astrocyte Co-cultures

Mesencephalic neuron cultures were prepared from mice embryos at gestation day 14 (E14) as described previously. Cells were cultured and seeded on 24-well plates at 5×10^5 cells/well.

Primary neuron-astrocyte cultures were prepared by plating enriched astrocyte directly on top of the neuron layer prepared into Transwell inserts that were placed above the existing neuron cultures. After 24 h, the two types of cultures were ready for treatment.

Genomic DNA Extraction and Genotyping

The finger tissues were prepared from Sp1/GFAP-Cre-ERT2 mice at postnatal days 5–7. All the tissues were mixed with lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 10% SDS) and protease K (10 mg/ml) that incubated 55 °C on heating block overnight. The tissues were quenched in room temperature and mixed well with 5 M NaCl on ice for 5–10 min. Centrifuge tissue mixture and precipitate genomic DNA with ethanol. Genomic DNA was subjected to PCR with Super Therm tag enzyme (Invitrogen). The primers used are listed as follows: for Sp1, forward: 5'-AACAGGGGCTGGAACAATAG-3' and reverse: 5'-CGTGAGTTCAAGGGAAGACTGG-3', and for Cre, forward: 5'-

CTAAACATGCTTCATCGTCGGGTC-3' and reverse: 5'-
TCTGACCAGAGTCATCCTTAGCG-3'.

Golgi-Cox Staining

Control and Sp1 knockout mice were sacrificed, and whole brain tissues were incubated in impregnation solution at 1–2 weeks. Brain sections (250 µm) were cut using a microtome (Leica Biosystems) and mounted and stained according to the instruction manual of superGolgi kit (Bioenno Tech). After staining, brain sections were dehydrated and cleared in xylene solution and then coverslipped using mounting medium. Sections were photographed under an Olympus BX-51 fluorescent microscope (Olympus) at × 1000 magnification.

Western Blotting

Mouse primary astrocyte and E14 primary neuronal cell lysates were fractionated using SDS-PAGE and transferred onto a PVDF (Millipore, Bedford, MA, USA) membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After incubation with 5% non-fat milk in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) for 1 h, the membranes were incubated with anti-Sp1, anti-NeuN, anti-GFAP (Millipore), anti-MAP2, anti-SNAP25 (Abcam), anti-N-cadherin (GeneTex, CA, USA), and anti-actin (Sigma) antibodies at 4 °C overnight. The membranes were then washed with TBST and incubated with secondary antibodies (Millipore) at room temperature for 1 h blots. After washing with TBST, blots were developed using the ECL system.

Immunofluorescent Staining

Mouse primary astrocytes were seeded onto coverslips. After fixation with 3.7% paraformaldehyde (Sigma) in PBS for 10 min and permeabilization with PBST buffer (containing 0.05% Triton X-100) for 5 min, cells on the coverslip were blocked with 2% bovine serum albumin for 1 h and stained with the antibodies against Sp1, GFAP, and NeuN (1:200; Millipore) at 4 °C overnight. Subsequently, cells on the coverslip were washed with PBS three times and stained with the anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Finally, cells were washed with PBS three times and mounted with 90% glycerol containing DAPI (Invitrogen) and photographed under the Olympus BX-51 fluorescent microscope (Olympus) at × 1000 magnification.

Microarray Analysis

Primary astrocytes cultures were derived from Sp1/GFAP-Cre-ERT2 mice and treated with DMSO or tamoxifen (60 µM) for 24 h. Astrocytes' culture medium with or without knockout of Sp1 that treated primary neuron (E14 embryo) and cell lysate was collected for RNA extraction using TriSure. After reverse transcription, complementary DNA (cDNA) was subjected to microarray analysis performed by Phalanx Biotech Group (Hsinchu, Taiwan). The function group related to proliferation was extracted by Gene Set Enrichment Analysis (GSEA). Microarray data was analyzed by the Rosetta Resolver system (Rosetta Biosoftware), and Pearson's correlation coefficient was used to analyze technical replicates

to assess reproducibility. Gene profiles and proteomic clusters were examined by using the STRING database v11.0.

Frozen Section and Nissl Staining

Sp1/GFAP-Cre-ERT2 mice were knockout Sp1 by using tamoxifen treatment. Sp1 knockout mice were sacrificed, and brain blocks were fixed in 3.7% formaldehyde for 24 h, dehydrated, and embedded in OCT. Sections (40 μ m) were cut and stained with cresyl violet acetate solution (Nissl staining solution) for 4–6 h. After staining, brain sections were dehydrated and cleared in xylene solution. Sections were photographed under the Olympus BX-51 fluorescent microscope (Olympus) at $\times 200$ and $\times 1000$ magnification.

Animal Experiments

The experiments related with animals were approved by the Institutional Animal Care and Use Committee (IACUC) at National Cheng Kung University (NCKU). Conditional knockout Sp1/GFAP-Cre-ERT2 transgenic mice were generated in the National Laboratory Animal Center (NLAC; Taiwan, Tainan). After breeding, Sp1/GFAP-Cre-ERT2 mice were used to study brain development. To knockout of Sp1, 2-month-old transgenic mice were orally administrated with tamoxifen in feed (400 mg/kg) for 1 month. After 1 month, we combined using tamoxifen 5 mg/kg with intraperitoneal (i.p) injection twice/week for 1 month. Sp1/GFAP-Cre-ERT2 mice receiving tamoxifen for 2 months were sacrificed, and brain tissues were analyzed by immunofluorescence staining. All methods involving animals were performed in accordance with the relevant guidelines and regulations.

Rotarod Test

The rotarod test was performed as described previously. Briefly, Sp1 knockout mice were placed in rotating rod and the latency to fall from a rotating rod was scored automatically with infrared sensors in a Rotamex 5 rotarod (Columbus Inst, Columbus, OH, USA).

Object Recognition Test

Object recognition test (ORT) was performed as described previously. Briefly, Sp1 knockout mice were initially habituated to the empty chamber (42 cm $\times$ 42 cm $\times$ 42 cm) by allowing them to freely explore for 10 min each day for 3 days. After 24 h, mice were rehabilitated to the empty chamber for 1 min and then returned to their home cage while two identical objects were placed at two diagonal corners of the chamber. Afterward, mice were placed in the chamber and were allowed to explore two identical objects for 10 min. In the test phase, one familiar object was repositioned, and the other familiar object was placed in the same location during the training trial. One hour after training, mice were placed in the testing chamber for 5 min, the time spent in exploring each object was recorded using a digital video camera, and scoring was performed with the behavioral tracking system EthoVision (Noldus). To analyze cognitive performance, a discrimination index was calculated for each mouse as the following formula: (time spent on the object in novel location) / (time spent on the object in novel location + time spent on the object in familiar location).

Weight-Drop TBI Model

Sham, control, and Sp1 knockout mice weighing 25–30 g were anesthetized lightly by inhalation of isoflurane (3%) in a closed glass chamber for 2 min. The left side of the head, between the eye and ear, was positioned under the guide tube of a weight-drop device and held in place by a sponge. In the device, a cylindrical iron weight (50 g) with a spherical tip was dropped from the full height of the vertical, graduated guide tube (100 cm long). The effect of the injury on the brain was studied at 7 days following the trauma.

2,3,5-Triphenyltetrazolium Chloride Staining

Sham, control, and Sp1 knockout mice were sacrificed after TBI. Fresh brain sections (2 mm) were prepared and incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) solution in a darker container at 37 °C for 20 min. Replacing the TTC solution to 10% formalin solution and storage brain sections at 4 °C was performed overnight. Brain sections were photographed under the Olympus BX-51 microscope (Olympus).

Statistical Analysis

Student's *t* test was used to analyze the difference between two groups in this study. The *p* value of 0.05 was considered statistically significant.

Results

Sp1 Is Highly Expressed in Astrocytes and Involved in Neurite Distribution in the Brain

Our previous study on Sp1 in brain injury indicated that Sp1 increases brain recovery under conditions of stroke [11], implying that Sp1 might be important for the brain in physiological and pathological conditions. In this study, we first studied the level of Sp1 in neurons and astrocytes (Fig. 1A, b). The data indicated that Sp1 was more highly expressed in primary astrocytes compared to its expression in neurons (Fig. 1A). Furthermore, the distribution of Sp1 was studied in the brains of mice (Fig. 1B). We used NeuN and GFAP as markers of neurons and astrocytes in the brain, respectively, to study the distribution of Sp1. The data revealed that Sp1 was more highly expressed in astrocytes than in neurons, suggesting that Sp1 in astrocytes may be crucial for astrocytic function. To study the role of Sp1 in astrocytes in the brain directly, a conditional astrocyte-specific functional knockout of Sp1 was constructed (Fig. 1C). The GFAP2 promoter was used to drive Cre-ERT2 expression, and tamoxifen treatment was used to carry Cre into the nucleus to cut the *loxP* sites, leading to a loss of the DNA binding domain of Sp1 (Fig. 1C). Genotyping demonstrated that Sp1 expression was decreased in the brain but not in other organs, indicating that Sp1 was specifically knocked out in astrocytes (Fig. 1D). Next, we studied the neuronal distribution by using Nissl staining on Sp1^{WT} and Sp1^{f/+} mouse brain sections (Fig. 2). There was no significant difference in brain size between the wild-type and Sp1 knockout mice (Fig. 2A). Interestingly, the neuronal distribution in the cortex and hippocampus areas was very different between Sp1^{WT} and Sp1^{f/+} mouse brain sections (Fig. 2B). In the cortical region, the Nissl body signal was more condense but not hazy in wild-type mice (Fig. 2B(b), left panel) compared to that in Sp1 knockout mice (Fig. 2B(b), right panel), implying that the loss of Sp1 might affect neurite outgrowth. In the hippocampus

area, the Nissl body signal was significantly decreased in the hippocampus in Sp1 knockout mice (Fig. 2B(a, c), right panel) compared to the signal in this region in the wild-type mice (Fig. 2B(a, c), left panel), implying that there may be neuronal loss in Sp1 knockout mice. Based on these results, Sp1 in astrocytes might be important for neurite outgrowth. To further address whether Sp1 is involved in neurite outgrowth, the Sp1 and NeuN signals in wild-type and Sp1 functional knockout mice were quantified by TissueQuest software (Fig. 3). The data indicated that Sp1 expression was decreased in the cortical and hippocampal regions of the knockout mice compared to that in the regions in the wild-type mice (Fig. 3A). Since Sp1 was specifically knocked out in astrocytes, its expression in other cell types in the brain and the incomplete Cre activity meant that the Sp1 signal did not disappear completely. We found that the NeuN signal was also decreased in the cortical and hippocampal regions of Sp1 knockout mice compared to that in wild-type mice (Fig. 3B), suggesting that Sp1 in astrocytes is critical for neurite growth.

Loss of Sp1 Affects Astrocyte Morphology and Function

Next, we studied the role of Sp1 in astrocytes of wild-type and knockout mice (Fig. 4). The data indicated that the Sp1 signal was decreased in various areas of the knockout mice, including the hippocampus, striatum, and corpus callosum, compared to the signal in these regions of the wild-type mice. In conjunction with the Sp1 signal, GFAP was used as an astrocyte marker to study the morphology and viability of astrocytes (Fig. 4A). The data indicated that there was no significant difference in cell number, but the astrocytic morphology was very different between wild-type and Sp1-KO mice, implying that Sp1 is involved in astrocytic function (Fig. 4). In addition, the levels of N-cadherin and SNAP25 were also decreased in these areas in Sp1-KO mice in parallel, implying that Sp1, as a transcriptional factor in astrocytes, might regulate the expression of many genes related to the maintenance of neuron survival (Fig. 4B). Next, primary astrocytes derived from wild-type and Sp1 knockout mice were cultured and treated with tamoxifen, and the cell survival and gene expression profile were then examined (Fig. 5). The data indicated that the morphology of astrocytes in Sp1 knockout mice was altered compared to that in wild-type mice; the intermediate filaments were less abundant in astrocytes of knockout mice compared to those in wild-type mice (Fig. 5A). There was no change in the viability of the astrocytes in the wild-type and Sp1 knockout mice, suggesting that Sp1 might affect the function but not viability of astrocytes (Fig. 5B). Finally, the global gene expression regulated by Sp1 in astrocytes was studied by cDNA array (Fig. 5C). The cDNA array data revealed that 2379 genes were upregulated and 2407 genes were downregulated, including both direct and indirect effects of Sp1 knockout in astrocytes (Fig. 5C). After analysis with bioinformatics software, pathway, Gene Ontology analysis, and proteomic clusters, a large number of genes related to many pathways involved in inflammation and neuron development were regulated by Sp1 (Fig. 5D, supplementary Figs. 2 and 4). In the gene expression profile and proteomic clusters of the Sp1 knockout astrocytes, we found that genes negatively regulated by complement, C4bp, C1q, and C8, were inhibited, but the gene expression of many cytokine genes such as Cxcl1, Cxcl2, Cxcl3, Cxcl5, Cxcl11, Ccl2, Ccl5, Ccl7, Ccl20, Tlr2, and IL-6 was positively regulated, suggesting that Sp1 in astrocytes might repress inflammation through inhibiting the complement system (Fig. 5E, Table 1).

Loss of Sp1 in Astrocytes Affects Neurite Growth

Previous studies have indicated that astrocytes are important for neuron development. Herein, we studied the role of Sp1 in astrocytes in neuron development (Fig. 6). The data indicated that every neuron in the hippocampal area of wild-type mice contained many obvious and clear dendrites. However, in the hippocampus of Sp1 knockout mice, there were fewer and shorter dendrites in every neuron (Fig. 6A). In the cortex region, one major axon and many slim dendrites were found in every neuron of the wild-type mice. However, the axons of the neurons in the cortex region of the Sp1 knockout mice were very different from those in wild-type mice; the axon was shorter, and most of the neurites were lost (Fig. 6A). Based on the neuronal morphology in the cortical and hippocampal regions, we concluded that Sp1 in astrocytes is critical for neuron development. Next, we used the conditioned medium from primary astrocyte culture with or without Sp1 knockout by tamoxifen treatment to treat primary neurons (Fig. 6B). We found that the neurites of primary neurons treated with conditioned medium from wild-type primary astrocytes were crude, but the neurites of those treated with conditioned medium from Sp1 knockout astrocytes were altered to be slim and intermittent, suggesting that the loss of Sp1 in astrocytes might change the repertoire of the conditioned medium, thus affecting neuronal development. We also studied the levels of MAP2 and SNAP25 in the primary neurons treated with conditioned medium with or without tamoxifen treatment (Fig. 6C). Moreover, we used primary neurons co-culture with astrocytes with or without Sp1 knockout by tamoxifen (Fig. 7A). We also found that Sp1 knockout in astrocytes directly caused neurite outgrowth defect (Fig. 7B). The data showed that expression of both MAP2 and SNAP25 was decreased in the Sp1 knockout condition, indicating that Sp1 in astrocytes is important for neuron survival and synapse formation.

Loss of Sp1 in Astrocytes Changes Mouse Behavior

Above, we showed that Sp1 in astrocytes is important for neuron development. Next, the effect of the loss of Sp1 in astrocytes on mouse behavior was examined (Fig. 8). First, we used the rotarod assay to examine the sensorimotor and motor learning abilities of our studied mice (Fig. 8A). The data indicated that mice with the loss of Sp1 in astrocytes remained on the rod for a shorter amount of time than wild-type mice, suggesting that Sp1 might be involved in motor learning, similar to Sp1 expression in the spinal cord. Recognition learning and memory was studied with the ORT (Fig. 7B). First, wild-type and Sp1 knockout mice were trained, and no significant difference was found between wild-type and Sp1 knockout mice in the recognition index (Fig. 8B(a)). In the test trial, the wild-type mice exhibited an increase in the recognition index toward the new object, but there was no change in the Sp1 knockout mice, indicating that Sp1 in astrocytes is involved in recognition learning and memory in mice (Fig. 8B(b)). Next, we studied the role of astrocytic Sp1 in regulating neuronal gene expression. The global gene expression profile was studied in primary neurons treated with conditioned medium from primary astrocytes with or without tamoxifen treatment (Fig. 8A). In neurons treated with conditioned medium from Sp1 knockout astrocytes, 1089 genes involved in various pathways were found to be upregulated and 738 genes downregulated (Fig. 9A, B; Supplementary Fig. 3). Several genes related to learning and memory shown in Table 2 were also regulated by Sp1, suggesting that Sp1 highly expressed in astrocytes regulates the expression of many genes in astrocytes,

subsequently affecting gene expression in neurons. In accordance with the previous studies that have revealed that astrocytes are involved in brain injury conditions [10], herein, we also found that Sp1 in astrocytes regulates the expression of many genes related to inflammation. Therefore, Sp1 in astrocytes might also be involved in the recovery from brain injury. Moreover, we used TBI model and induced brain trauma in Sp1 knockout mice. Results showed that the loss of Sp1 increased brain injury region than that in the control group (Fig. 9C). These results indicated that Sp1 plays a crucial role in neuroprotection and brain functions.

Discussion

In this study, Sp1 was specifically knocked out in astrocytes to study the role of Sp1 in astrocytes and neuronal function and development. The loss of Sp1 in astrocytes activated the innate immune complement system, thus increasing the levels of chemokines and cytokines in astrocytes, thereby inhibiting neurite outgrowth, synaptogenesis, and neuron survival (Fig. 9D).

As a transcriptional factor, Sp1 regulates the expression of various genes involved in physiology and pathology [1]. Bioinformatics analysis revealed that more than 10,000 gene promoters contain Sp1 binding sites, suggesting that Sp1 directly or indirectly regulated the expression of numerous genes. In addition, not only coding genes but also many non-coding genes are regulated by Sp1 [17, 18]. However, Sp1 structure and its knockout mice were still unsolved. In the past, constitutive Sp1 knockout was shown to be embryo lethal by embryo day 11 [19, 20]. Therefore, direct evidence of the function of Sp1 in vivo was still absent. In this study, we first established a conditional astrocyte-specific Sp1 knockout mouse. Based on the experimental results, we demonstrated that Sp1 in astrocytes is involved in neuron survival and development through regulating the expression of many related genes. Most of the previous studies of Sp1 in astrocytes are focused on how Sp1 regulates the expression of individual genes such as cyclin D1, cytochrome P450, and IL-6 [21–24] at the cell level. In neurons, Sp1 has been shown to be involved in neuron development and brain injury. Our previous study indicated that upregulation of Sp1 in neurons under ischemic condition protects neuron survival [11]. In this study, we found that Sp1 is highly expressed in astrocytes, implying that Sp1 is critical for their function. After Sp1 was knocked out in astrocytes, we found that Sp1 not only affected the function and morphology of astrocytes but also decreased neurite outgrowth, synaptogenesis, and neuron survival. Previous studies have known that astrocytes are supporters for maintaining neuron survival [25]. Herein, we provide direct evidence to support that Sp1 in astrocytes is essential for maintaining neuron survival. Sp1 in other cells in the brain such as neurons and microglia might also be important for brain function and development. We will construct conditional Sp1 knockout mice in these specific cells to clarify the role of Sp1 in the brain in the future. In addition, previous studies have also indicated that not only Sp1 but also Sp3 and Sp4 might be important for maintaining neuron function [26, 27]. The ways in which these Sp family members interact to regulate brain function are important and remain to be addressed in the future.

In this study, we found that innate immune systems such as the complement system and chemokine/cytokine system were activated under the loss of Sp1 in astrocytes, suggesting that Sp1 negatively regulates inflammation in astrocytes. Until now, there has been no study to indicate a role of astrocytic Sp1 in inflammation. In neurons, Sp1 increases neuron recovery after brain injury, but there is still no direct evidence to support the relationship between Sp1 and neuroinflammation [7, 28]. We found that Sp1 increases the expression of C4Bp, which is the inhibitor of the C3 convertase, C4bC2b, in the classical pathway, leading to inhibition of complement. In addition, our study also showed that Sp1 decreases the expression of Cfb, which is the activator of the C3 convertase, C3bBb, in the alternative pathway, leading to inhibition of complement. Therefore, in astrocytes, Sp1 might repress the complement system to inhibit the levels of various chemokines and cytokines shown in Table 1, which might be induced in neuroinflammation and act as neurotoxins to damage neurons [29, 30]. Whether Sp1 in other cell types also represses the complement system such as in astrocytes is not yet understood and remains to be clarified in the future. In this study, Sp1 was also shown to decrease the expression of Toll-like receptor type 2, which can be upregulated in degenerative diseases [31]. Previous studies have revealed that neuron and glial cells express Toll-like receptors as well as complement receptors, and the complement components are often expressed in response to brain injury and development [32]. According to previous studies, most C proteins are synthesized in the liver and then transferred to the target organs including the brain through the BBB [33]. However, most C proteins are unlikely to penetrate the brain parenchyma. Therefore, local synthesis of C components by resident cells in the brain is important for the appropriate function of the local defense system. In this study, we found that several C proteins including C4bp, C1Qa, C1Qb, C1Qc, C8b, C8g, and Cfb can be expressed and regulated by Sp1 in astrocytes.

C proteins are thought to have non-immune roles in the brain. C proteins have been found to increase proliferation and regeneration in different tissues and may also perform similar functions in the CNS [34]. Previous studies have indicated that C protein activation products can regulate synapse formation during brain development [35]. Neurons isolated from the developing eye were found to express high levels of C1q mRNA [36]. In a recent study, the loss of C1q, both alone and in conjunction with C3, facilitated the microglial clearance of misfolded proteins, apoptotic neurons, and damaged cells such as neuronal blebs and modulated cytokine profiles to subdue potential neurotoxic inflammatory gene expression [37]. In this study, we found that the loss of Sp1 downregulated C1q expression and disrupted synapse formation, indicating that Sp1 in astrocytes is crucial for neuron synaptogenesis through regulating C1q expression in a non-immune-dependent manner. On the other hand, for immune function, C1q is involved in the initiation stage of complement activation, but two critical downstream key regulators, C4Bp and Cfb, are regulated by Sp1 and repress complement activation, leading to inhibition of chemokine and cytokine expression. In conclusion, Sp1-induced C1q is crucial for neuron development in an inflammation-independent manner, and Sp1 also protects neurons through inhibition of chemokine and cytokine secretion.

In this study, the loss of Sp1 dramatically decreased the learning and memory ability of mice. In the gene expression profile, we also found that many genes related to learning and memory which were regulated by Sp1. For example, Sp1 positively regulated the expression

of synaptic vesicle glycoprotein 2 (Sv2), cholinergic receptor muscarinic 1 (Chrm1), and gamma-aminobutyric acid type A receptor alpha 4 subunit (Gabra4) but negatively regulated the expression of C-C motif chemokine ligand 2 (Ccl2), transmembrane protein 90B (Tmem90b), and crystallin alpha B (Cryab). Sv2 is involved in the regulation of vesicle trafficking and exocytosis in neurons [38, 39]. Chrm1 influences many effects of acetylcholine in the central and peripheral nervous system [40]. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. GABA-A receptors are ligand-gated chloride channels. GABRA4 is involved in the etiology of autism and eventually increases autism risk through its interaction with GABRB1 [41]. In addition, an increase in CCL2 causes neural tube defects [42]. Another name for Tmem90b is synapse differentiation inducing 1, which belongs to the interferon-induced transmembrane family of proteins related to the regulation of excitatory synapse development [43]. Increasing expression of CRYAB occurs in many neurological diseases such as desmin-related myopathy [44]. Sp family members have been studied in the brain, especially in neuronal development, function, and disease [45, 46]. Our previous studies have also indicated that upregulation of Sp1 in damaged neurons increases the recovery ability [11]. Sp3 and Sp4 have also been found to be upregulated in degenerative diseases [47]. Many studies have shown that Sp4 in neurons is critical for neuronal function and development [48, 49]. Here, we found that many genes can be regulated by Sp1 both positively and negatively. When analyzing the Sp1-binding element(s) within these genes, we found that some genes contained Sp1 binding sites, which might indicate that these are Sp1 target genes. In addition, not all target genes were positively regulated by Sp1, which might be related to other factors that interact with Sp1, such as HDACs, p300, and YY1. Different interacting repertoires will affect the effect of Sp1 on its target genes [50–53]. Understanding the role of Sp1 in brain function and development might be beneficial for drug development for the prevention of brain injury and diseases such as neuron degenerative diseases in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Knockout of Sp1 in astrocyte. **A** The expression of Sp1 in primary neuron and astrocytes were analyzed by western blotting and immunofluorescent staining (**B**). **C** The model of Sp1 conditional knockout mice (Sp1/GFAP2-CreERT2) was constructed. **D** Genotyping of Sp1^{WT}, Sp1^{f/+}, and Sp1^{f/f} mice (a). Sp1 levels in the different organs of the knockout mice were studied with western blotting (b). After three independent experiments, the Sp1 level was quantified by statistical analysis with *t* test, **p* < 0.05 (c)

Fig. 2.

Neuron in *Sp1* knockout mice. **A** Nissl staining on *Sp1*^{WT} and *Sp1*^{f/+} mice brain section. **B** The wild-type and *Sp1* knockout mice treated with tamoxifen (TAM, 5 mg/kg). Tissues were stained with Nissl staining on different brain sections ($\times 2$) (a) including the hippocampus ($\times 10$) (b) and the cortex ($\times 10$) (c)

Fig. 3.

Knockout of Sp1 induces neuronal cell death in the cortex and hippocampus. **A, B** (a) Brain sections from TAM-treated wild-type and Sp1 KO mice were used to study Sp1 and NeuN levels by immunofluorescence assay with antibodies against Sp1 (green) (**A**) and NeuN (green) (**B**). Immunofluorescence staining reacted with Sp1 or NeuN antibodies (a) and analyzed by TissueQuest software (b). *hippo* hippocampus

Fig. 4.

Knockout of Sp1 in astrocytes changes the levels of GFAP and related proteins. Brain sections from wild-type and Sp1^{ff} mice were analyzed by immunofluorescence assay with antibodies against GFAP (red) and Sp1 (green) (A). After TAM treatment, wild-type and Sp1^{ff} mice were sacrificed to collect the different regions of brain tissue. Samples were analyzed by Western blotting with antibodies against Sp1, N-cadherin, GFAP, SNAP25, and actin antibodies (B)

Fig. 5. Knockout of Sp1 in primary astrocyte changes cell morphology and gene expression profile. **A** Knockout of Sp1 by tamoxifen (TAM30 and TAM60) treatment in primary astrocyte was used to study the cell morphology by immunofluorescence staining with anti-GFAP antibodies. The cell viability was studied by MTT assay (**B**) and the gene expression profile by cDNA array (**C**). The cDNA array data was analyzed by Gene Ontology analysis (**D**). The proteomic clusters was analyzed by the STRING database (**E**)

Fig. 6.

Loss of Sp1 in astrocyte decreases neuronal dendrites and synapse formation, exercise, and memory function. **A** Golgi-cox staining on control and Sp1^{fl/fl} mice brain sections. Brain sections were photographed under a microscope at $\times 1000$ magnification. **B** The morphology of the E14 primary neurons treated with conditioned medium from Sp1 knockout astrocytes was studied by a DIC microscope. **C** Samples harvested from the E14 primary neurons treated with conditioned medium from Sp1 knockout astrocytes were used to study the levels of MAP2 and SNAP25 by Western blotting with antibodies against indicated proteins (a).

After three independent experiments, the levels of MAP2 and SNAP25 were quantified by statistical assay with t test, $*p < 0.01$ (b)

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Fig. 7.

Loss of Sp1 in astrocyte decreases neurite outgrowth. **A** Astrocytes were seeded into Transwell inserts while neurons growth was confluent in the lower compartment of the 24-well plate as indicated. After seeded for 24 h, neurons and astrocytes were treated with DMSO or tamoxifen (60 ng/ml) and E14 primary neurons were studied by a DIC microscope (**B**). E14 primary neurons co-culture with Sp1 knockout astrocytes were used to study the levels of MAP2 by Western blotting with antibodies against indicated proteins (b). After three independent experiments, the levels of MAP2 were quantified by statistical assay with *t* test, **p* < 0.05, ***p* < 0.01 (c)

Fig. 8.

Loss of Sp1 in astrocytes affects the animal behavior. **A** Rotarod test on control and Sp1^{fl/fl} mice to evaluate exercise abilities. **B** Object recognition test (ORT) on control and Sp1^{fl/fl} mice to evaluate learning and memory abilities. After three independent experiments, the time on the rod (a) and recognition index (b) were quantified by statistical assay with t test, ** $p < 0.01$, *** $p < 0.005$

Fig. 9. Knockout of Sp1 in primary astrocyte changes the complement and inflammatory genes expression. **A** Total RNA was extracted from E14 primary neurons treated with conditioned medium from Sp1 knockout astrocytes for studying the gene expression profile by cDNA array. **(B)** The gene expression repertoire was analyzed by Gene Ontology analysis. **C** Sham, control, and Sp1 knockout mice brain sections were analyzed by TTC staining. **D** Proposed model in this study

Table 1

Sp1 in astrocyte regulates inflammation-related gene expression

Gene symbol	Description	Log2
Sp1-regulated inflammation-related genes		
C4bp	Complement component 4 binding protein	6.6438562
C1qa	Complement component 1, q subcomponent, alpha polypeptide	3.86488994
C1qc	Complement component 1, q subcomponent, C chain	3.80276529
C1qb	Complement component 1, q subcomponent, beta polypeptide	3.53089936
C8b	Complement component 8, beta polypeptide	1.80936423
C8g	Complement component 8, gamma polypeptide	1.636210
Cxcl1	Chemokine (C-X-C motif) ligand 1	- 6.64385619
Ccl20	Chemokine (C-C motif) ligand 20	- 6.64385619
Cxcl2	Chemokine (C-X-C motif) ligand 2	- 6.64385619
Ccl7	Chemokine (C-C motif) ligand 7	- 6.56091223
Ccl2	Chemokine (C-C motif) ligand 2	- 6.18034853
Ccl5	Chemokine (C-C motif) ligand 5	- 5.98764869
Cxcl5	Chemokine (C-X-C motif) ligand 2	- 5.37876455
Tlr2	Toll-like receptor 2	- 4.63239725
Cxcl11	Chemokine (C-X-C motif) ligand 11	- 4.55023762
Cxcl3	Chemokine (C-X-C motif) ligand 3	- 4.38980779
Cfb	Complement factor B	- 4.05449954
Il6	Interleukin 6	- 2.29012501

The mRNA isolated from primary astrocytes with or without tamoxifen treatment was used to do the cDNA array. Genes listed here are altered more than 1.5log2

Primary neuron treated with conditional medium from the primary astrocytes with Sp1 knockout by tamoxifen treatment

Table 2

Gene symbol	Description	Log2
Sp1-regulated learning and memory-related genes		
Sv2b	Synaptic vesicle glycoprotein 2 b	2.42985011
Chrm1	Cholinergic receptor, muscarinic 1, CNS	2.06818201
Gabra4	Gamma-aminobutyric acid (GABA)-A receptor, subunit alpha 4	1.96243945
Lrrtm2	Leucine-rich repeat transmembrane neuronal 2	1.92508294
Lgil1	Leucine-rich repeat LGI family, member 1	1.88894434
Cplx3	Complexin 3	1.82660964
Nrgn	Neurogranin	1.81014654
Grik1	Glutamate receptor, ionotropic, kainate 1	1.78451909
Olfm3	Olfactomedin 3	1.77905819
Arc	Activity-regulated cytoskeletal-associated protein	1.76381458
Chme	Cholinergic receptor, nicotinic, epsilon polypeptide	1.76016538
Syt1	Synaptotagmin 1	1.74676638
Gabrb2	GABA-A receptor, subunit beta 2	1.65852664
Olfm1	Olfactomedin 1	1.63575588
Gabrg2	GABA-A receptor, subunit gamma 2	1.60694544
Nlgn1	Neurexin 1	1.5418095
Calb2	Calbindin 2	1.53260794
Kcnd2	Potassium voltage-gated channel, Shal-related family, member 2	1.53228203
Ccl2	Chemokine (C-C motif) ligand 2	-4.26044417
Tmem90b	Transmembrane protein 90B	-2.03552825
Cryab	Crystallin, alpha B	-2.00286994
Plat	Plasminogen activator, tissue	-1.75176416
Igfb1	Integrin beta 1 (fibronectin receptor beta)	-1.54201186
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1	-1.54154359

The mRNA was isolated from neuron for cDNA array Genes listed here are altered more than 1.5log2