Neuropilin-1 Is a Direct Target of the Transcription Factor E2F1 during Cerebral Ischemia-Induced Neuronal Death In Vivo[▽]

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The nuclear transcription factor E2F1 plays an important role in modulating neuronal death in response to excitotoxicity and cerebral ischemia. Here, by comparing gene expression in brain cortices from E2F1^{+/+} and E2F1^{-/-} mice using a custom high-density DNA microarray, we identified a group of putative E2F1 target genes that might be responsible for ischemia-induced E2F1-dependent neuronal death. Neuropilin 1 (NRP-1), a receptor for semaphorin 3A-mediated axon growth cone collapse and retraction, was confirmed to be a direct target of E2F1 based on (i) the fact that the NRP-1 promoter sequence contains an E2F1 binding site, (ii) reactivation of NRP-1 expression in E2F1^{-/-} neurons when the E2F1 gene was replaced, (iii) activation of the NRP-1 promoter by E2F1 in a luciferase reporter assay, (iv) electrophoretic mobility gel shift analysis confirmation of the presence of an E2F binding sequence in the NRP-1 promoter, and (v) the fact that a chromatin immunoprecipitation assay showed that E2F1 binds directly to the endogenous NRP-1 promoter. Interestingly, the temporal induction in cerebral ischemia-induced E2F1 binding to the NRP-1 promoter correlated with the temporal-induction profile of NRP-1 mRNA, confirming that E2F1 positively regulates NRP-1 during cerebral ischemia. Functional analysis also showed that NRP-1 receptor expression was extremely low in E2F1^{-/-} neurons, which led to the diminished response to semaphorin 3A-induced axonal shortening and neuronal death. An NRP-1 selective peptide inhibitor provided neuroprotection against oxygen-glucose deprivation. Taken together, these findings support a model in which E2F1 targets NRP-1 to modulate axonal damage and neuronal death in response to cerebral ischemia.

Molecular mechanisms of neuronal death caused by cerebral ischemia are still the subjects of intense investigation. Previous studies, including our own, have shown that the transcription factor E2F1 plays an important role in modulating ischemia-induced neuronal death (11, 19, 22, 24, 44). Adenovirus (Ad)-mediated overexpression of E2F1 in cerebellar granule neurons (CGNs) resulted in neuronal apoptosis (18, 20, 35), while E2F1-deficient neurons were resistant to death evoked by K⁺ withdrawal (14, 44), staurosporine (18), dopamine (19, 20), 6-hydroxydopamine (19), and oxygen-glucose deprivation (11). In addition, E2F1 knockout mice were more resistant to ischemia-induced brain damage than their wild-type littermates (32, 33). The transcription factor E2F1 is a regulator of cell cycle progression from G₁/S (8, 45). E2F1 is regulated through interaction with the retinoblastoma proteins (Rb). When released from its binding with Rb, following Rb hyperphosphorylation, E2F1 promotes neuronal death via Bax and caspase activation (14). Phosphorylation of Rb is likely to result from a number of different signaling pathways; two of the possible candidates are the cyclin-dependent kinases (46) and p38 MAPK (24, 47). E2F1-mediated neuronal death involves activation of gene transcription and repression (15, 20, 31). Large-scale DNA microarray screening and chromatin immunopre-

cipitation (ChIP) techniques have been used to identify potential targets of the transcription factor E2F1 (8, 25, 51); however, how E2F1 precisely modulates neuronal death is far from clear.

Ample studies have shown that cerebral ischemia in animal models and human patients is associated with the growth of new blood vessels and with neuronal remodeling (2–4, 22). On the molecular level, these processes may be regulated by common molecules, such as the neuropilin family members (NRPs) (2, 10, 52). NRPs were originally identified as receptors for the semaphorin family of secreted polypeptides (12, 16, 30). The neuropilin family contains two members, NRP-1 and NRP-2. They are non-tyrosine kinase transmembrane proteins. NRP-1 and NRP-2 have short intracellular segments that lack cytoplasmic signal transduction domains. Therefore, NRPs participate in signal transduction as coreceptors with plexins and vascular endothelial growth factor (VEGF) receptors. In addition to being modulators of VEGF during angiogenesis and vasculogenesis, NRPs also function as receptors for axon guidance factors, such as semaphorin 3A, during the process of axonal pathfinding. NRP-1 is a cell surface glycoprotein expressed on axons (9, 26). Overexpression of NRP-1 in transgenic mice results in embryonic lethality associated with excessive defects in neuronal guidance, as well as vascular formation (29). Targeted disruption of the NRP-1 gene in mice is also embryonic lethal due to defects in neuronal patterning and insufficient vascularization (28).

The indication that NRPs may be involved in mediating axonal damage comes from studies showing that the formation of brain lesions induces changes in the expression of chemore-

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pulsive semaphorins, which have been found to be related to the successful regeneration of injured neurons or their failure and death. Mammalian semaphorins and their chick homologues, the collapsins, are a family of transmembrane or secreted glycoproteins present in neuronal tissues that act as mediators of neuronal guidance by inducing growth cone collapse (21, 37). The collapsin/semaphorin-induced growth cone collapse is mediated through NRPs, as demonstrated by the abrogation of these effects by using anti-NRP-1 antibodies (16, 30). In fact, the biological activities of the repulsive axon guidance molecule semaphorin 3A are known to be responsible for the elimination of neurons when axons are still too far away from the target during development (2, 37). The most direct piece of evidence implicating axon guidance molecules in mediating axonal damage during neuronal death is that blocking semaphorin 3A in dopamine-treated cerebellar granule neurons provides protection against neuronal death (42, 43). It is therefore not surprising to find that semaphorins are involved in the pathological processes of several neurodegenerative diseases, including Alzheimer's disease, motor neuron degeneration, and cerebral ischemia, as reviewed by De Winter et al. (7).

In the present study, we identified E2F1 target genes by using custom DNA microarrays and determined that NRP-1 is a bona fide transcriptional target of the transcription factor E2F1 during neuronal death caused by cerebral ischemia.

MATERIALS AND METHODS

Cerebral ischemia produced by MCAO. All procedures using animals were approved by the local Animal Care Committee. C57BL/6 mice (20 to 23 g) were obtained from Charles River (St. Foie, Quebec, Canada). The E2F1^{-/-} (stock 2785) and E2F1^{+/+} (stock 101045) mice were crosses between C57BL/6 and SV129 strains and were initially obtained from Jackson Laboratories (Bar Harbor, ME) but were bred and genotyped in house as previously described (18). Under temporary isoflurane anesthesia, the mice were subjected to middle cerebral artery occlusion (MCAO) using an intraluminal filament, as previously described (32). After 1 h of MCAO, the filament was withdrawn, blood flow was restored to normal by laser Doppler flowmetry, and the wounds were sutured. Sham-operated mice, which were subjected to the same brain surgery but no MCAO, were used as controls. The animals were sacrificed after 24 h of reperfusion. Consistency in brain infarctions was evaluated by cutting the brains into three 2-mm-thick coronal slices through the forebrain, which were stained with 5 ml of 2% 2,3,5-triphenyltetrazolium chloride for 90 min at 37°C. The tissue was rinsed with saline, and the formazan product was solubilized in ethanol-dimethyl sulfoxide (1:1). After a 24-h incubation in the dark, the red solvent extracts were diluted 1:20 with fresh ethanol-dimethyl sulfoxide solvent in three tubes and placed in cuvettes. Absorbance was measured at 485 nm in a spectrophotometer, and the values were averaged. The percentage decrease in brain 2,3,5-triphenyltetrazolium chloride staining in the ischemic side of the brain was compared to that in the contralateral side of the brain of the same animal using the following equation: percent decrease = $[1 - (\text{absorbance of ischemic hemisphere} / \text{absorbance of contralateral hemisphere})] \times 100$.

RNA extraction and labeling. Snap-frozen brains were subjected to RNA isolation. Total RNA was extracted from the ischemic mouse cortices using Trizol reagent (GIBCO-BRL Life Technologies) as previously described (24). DNA contamination was monitored using reverse transcription (RT)-PCR to detect β -actin with a primer pair capable of detecting a 78-bp intron as described previously (23). RNA quality was assessed by gel electrophoresis, as well as by the ratios of optical densities at 260 and 280 nm (above 1.95). A total of 10 μ g RNA was reversed transcribed into single-stranded cDNA in the presence of [α -³²P]dCTP, as previously described (1), and used for hybridization of DNA microarrays.

Microarray analysis of E2F1-regulated genes. The set of brain-relevant DNA microarrays containing 3,456 sequence-verified cDNA fragments on nylon membranes were built as described previously (1). The microarray contained genes representing all of the major cellular types of the brain, including neurons,

astrocytes, microglia, and oligodendrocytes. Twenty micrograms of total RNAs was isolated from the brains of focal ischemic mice and reverse transcribed into single-stranded cDNA. The cDNA was labeled with [α -³²P]dCTP and used to hybridize with the DNA microarray at 50°C overnight. The membrane was rinsed with the first wash solution (2 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate) once at 50°C and then twice at room temperature with the first wash solution. The membrane was scanned and quantified with a Molecular Dynamics STORM densitometer (Sunnyvale, CA). Four repeat screenings were performed, and the average intensity of the hybridization was calculated with a Microsoft Excel spreadsheet. The spot intensity was subtracted from the background signals, and the intensities of the four repeats were averaged. The z ratio was calculated, and changes in the z ratio between ischemia and sham were calculated using the following formula as described previously (17): ratio (ischemic - sham) = z trans average [ischemic] - z trans average [sham] / (standard deviation [ischemic - sham]). A gene expression was regarded as up- or down-regulated if the z ratio was more than +1.5 or less than -1.5, respectively. The z ratio at 1.5 is about a fivefold change from the average intensity. Only genes that changed consistently more than fivefold among the four comparisons were listed and analyzed in the study.

Semiquantitative RT-PCR. The optimal PCR amplification conditions and cycle number were determined experimentally to ensure specific and exponential signal generation. Expression of β -actin mRNA was used as an internal standard to quantify the relative gene expression as described before (23). A negative control (without the addition of cDNA) was included in each PCR run.

ChIP assay. The ChIP analyses were performed using a magnetic-bead-based ChIP-IT Express kit exactly following the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, CGNs were grown on 100-mm tissue culture dishes for 7 days in vitro (DIV). The cells were then cross-linked with 1% formaldehyde for 10 min, harvested, and chromatin immunoprecipitated with 3 to 5 μ g of the indicated antibodies: anti-E2F1 (Active Motif; 39313) and anti-mouse immunoglobulin G (IgG) (Sigma). After sonication, the resulting DNA fragments in the range of 500 to 1,500 bp were analyzed by PCR using a pair of primers (Fwd, 5'-ACCCATCCTCGCCTTTATG-3', and Revs, 5'-CGTGTGTTGATCTTAGAGAACTGT-3') spanning the putative E2F1 binding site in the NRP-1 promoter region. A ChIP assay was also performed without an antibody to serve as a negative control.

EMSA. An electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts essentially as described previously (6). Nuclear protein was extracted from mouse brains following the protocol described previously (41). A double-stranded (ds) E2F-binding oligonucleotide with the sequence 5'-TGCTCTAAGAAAGTCTGCCTATGCTTTACGTGGCAGACTGGG-3' in the NRP-1 promoter region (40) was labeled with [α -³²P]dCTP (Amersham) using T4 polynucleotide kinase (Amersham). A mouse conserved E2F1-specific binding sequence (18) (see Fig. 4C) was made to serve as a positive control (CTL hot probe). Eight micrograms of nuclear extract was incubated with an excess of [α -³²P]dCTP-labeled dsDNA probe (60,000 cpm/0.2 ng of DNA). The binding reaction (20 μ l) was carried out at room temperature for 20 min in binding buffer (20 mM HEPES, pH 7.6, 0.2 mM EDTA, 100 mM KCl, 5% glycerol, and 2 mM dithiothreitol) and 0.1 μ g of nonspecific competitor poly(dI-dC) (Amersham). Each new preparation of poly(dI-dC) was titrated to determine the ideal concentration for EMSA. In an E2F1 supershift experiment, poly(dI-dC) was not added to the reaction mixture to produce an optimal supershift. Monoclonal E2F1 antibody (2 μ l/reaction; KH95 X; Santa Cruz Biotechnology) was used for the antibody supershift assay. Complexes were resolved on a 5% gel and run for 2 h, dried, and visualized by autoradiography.

Cell culture and treatment. Primary cultures of CGNs were prepared from 6- to 8-day-postnatal mice (E2F1^{+/+} and E2F1^{-/-}), taking account of the importance of ensuring the same developmental sensitivity to the excitotoxin between experimental groups, as described previously (44). The cells were plated on 24-well dishes containing polylysine-coated coverslips at a density of 6×10^5 per well. After approximately 18 h, cytosine- β -D-arabino-furanoside was added to a final concentration of 5 μ M to prevent glial cell proliferation. Recombinant semaphorin 3A (R&D Systems) was added to the cultures at either 1 or 3 DIV. Oxygen glucose deprivation (OGD) treatment of CGNs was performed exactly as described previously (11).

Adenovirus infection. The replication-defective adenoviruses encoding full-length E2F1 cDNA or E2F1 mutant cDNA (a point mutant at 138 amino acids, which abolishes DNA binding capacity) in an adenoviral shuttle vector containing a green fluorescent protein (GFP) marker were used from stocks created previously in the laboratory by Hou et al. (20). Primary glial cells were infected at 80% confluence in 10-cm dishes with adenovirus particles at a multiplicity of infection of 10. The infectivity level reached maximum 3 days postinfection. Equal infectivities between cell samples were ensured by manually counting glial

cells expressing GFP. After 3 days of infection, the cells were scraped from the dish, and total protein was extracted and quantified. Western blotting was performed using these protein samples, and levels of NRP-1 expression were compared between E2F1^{-/-} and E2F1^{+/+} glial cells.

Promoter luciferase assay. Cultured primary glial cells from E2F1^{+/+} and E2F1^{-/-} brains were transfected with 10 μ g PLG3-luciferase-NRP-1 promoter construct using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). After 3 days of transfection to allow gene expression, cells were collected and lysed in luciferase buffer. Luciferase activity was determined with the luciferase reporter assay system following the manufacturer's instructions (Promega Corporation).

Measurement of neurite outgrowth and cell death. CGNs were fixed with fresh 4% paraformaldehyde for 20 min and subjected to immunostaining with primary antibody against MAP-2 or β -III-tubulin (Chemicon International). Under a fluorescent microscope, digital images at a resolution of 3,000 dots/in were taken of 10 randomly selected fields that contained more than 300 cells. Axonal lengths on the digitized images were measured using Image J, an NIH image analysis system (<http://rsb.info.nih.gov/ij/>). Cells were also counted using Image J. These analyses were performed in a double-blinded fashion, and the data were averaged and plotted for statistical analysis. Neuronal death was also measured by adding propidium iodide (PI) at 10- μ g/ml concentration to the live cultures. Cellular fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 645 nm in a Cytofluor 2350 fluorescence measurement system (Millipore). Viability was normalized to the control cell readings.

Double immunofluorescent staining. The procedures for fluorescence immunocytochemistry were exactly as described previously (24). The primary antibody against NRP-1 was purchased from Sigma and used at 1:200 dilution.

Western blotting. The procedures for Western blotting were exactly as described previously (18). Nuclear proteins were extracted, and immunoprecipitation was performed following our previously published procedures (21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The intensities of bands were quantified using Molecular Dynamics Image-Quant software (Sunnyvale, CA).

Data analysis. All data were analyzed by one-way analysis of variance (ANOVA) and further post hoc tests for significant groups using Tukey's test.

RESULTS

Identification of NRP-1 as a putative E2F1 target gene using a DNA microarray. The transcription factor E2F1 plays a key role in modulating neuronal death following cerebral ischemia. In order to identify E2F1 target genes which might be directly responsible for cerebral ischemia-induced E2F1-dependent neuronal damage, we first determined the time course changes of E2F1 gene expression in the cortex using immunoprecipitation-Western blotting. E2F1 expression transiently increased following focal ischemia starting after 2 h of reperfusion, and a relatively high E2F1 level was maintained at the 6-h reperfusion time point in comparison with other time points and with the contralateral side of the brain (22, 32). Therefore, ischemic brains at 6 h after reperfusion were selected for RNA extraction and DNA microarray analysis. The gene expression profile from the E2F1^{+/+} mouse cortex was compared with that derived from E2F1^{-/-} littermates (Fig. 1). We identified 374 genes whose expression levels had changed more than fivefold (either up or down) in the cortices of E2F1^{-/-} mice, indicating that these genes were putative E2F1 targets (Fig. 1A to C). Furthermore, after comparison of these genes with those changed following ischemia, 206 of the putative E2F1 target genes appeared to be responsive to MCAO (Fig. 1A). Oligonucleotides specific to the candidate E2F1-targeted ischemia-responsive genes were then commercially synthesized and printed in house to generate a custom E2F1 microarray. This custom microarray was screened with mRNA extracted from the ischemic mouse brain, which allowed us to identify a smaller group of genes potentially targeted by E2F1. In addition,

FIG. 1. Identification of E2F1 target genes using DNA microarrays. (A) High-density DNA microarrays containing 3,456 brain genes were screened with ³²P-labeled cDNA probes prepared from E2F1^{+/+} and E2F1^{-/-} mouse brains either with or without focal cerebral ischemia. We identified 375 differentially expressed genes in the E2F1^{-/-} mouse brains, which represented putative E2F1 target genes. By comparing them with genes having altered expression levels following cerebral ischemia, we identified 206 putative E2F1 target genes that appeared to be responsive to ischemia. (B) Plot showing no differential gene expression between the sham-operated and ischemic animals ($R^2 = 0.98$). (C) Plot showing a drastic alteration in the level of gene expression following ischemia ($R^2 = 0.67$). The arrows in panels B and C indicate the altered genes. (D) A few examples of differentially expressed putative E2F1 target genes, confirmed by RT-PCR.

tion, these genes were also responsive to MCAO-induced neuronal damage. Based on these studies, several genes were selected for confirmation of differential expression in E2F1^{-/-} and E2F1^{+/+} neurons by using semiquantitative RT-PCR (Fig. 1D). For example, NRP-1 and caveolin 1 were down-regulated in E2F1^{-/-} brains, while p38a and GM2A were up-regulated in E2F1^{-/-} brains (Fig. 1D). Other genes, such as those for NRP-2, SNAP23, caspase 8, and β -actin, did not change their levels of expression. It is interesting that, although NRP-1 and NRP-2 belong to the same family, only NRP-1 expression appeared to be regulated by E2F1. Because NRPs are known to play important roles in ischemia-induced new growth of blood vessels and neuronal remodeling (2), we investigated the

FIG. 2. E2F1^{-/-} CGNs have significantly reduced NRP-1 expression. (A) mRNAs and proteins were extracted from E2F1^{+/+} and E2F1^{-/-} mouse brains. Equal amounts of total RNA were used for the first-strand cDNA synthesis. PCRs were performed using the cDNAs to detect NRP-1 and β -actin levels; β -actin was used as an internal control for equal mRNA loading. (B) The intensities of the bands were quantified using densitometry, and the ratio of NRP-1 to actin was calculated and plotted. (C and D) Western blotting was performed to detect NRP-1 protein expression (C), and GAPDH was used as a control for equal protein loading. No detectable NRP-1 protein was found in E2F1^{-/-} brain lysate (C and D). Statistical analysis was performed; **, $P < 0.001$ by ANOVA. The error bars indicate standard deviations. (E) Replication-defective adenoviral constructs expressing GFP only (Ad-GFP), a mutant E2F1 (Ad-Emut), and the full-length E2F1 (Ad-E2F1) were made and used to infect cultured primary glial cells derived from the brains of E2F1^{+/+} and E2F1^{-/-} mice. Noninfected cells were used as the baseline control for NRP-1 expression (CTL). After 3 days of infection, cells were collected for Western blotting to detect NRP-1 expression. GAPDH Western blotting was performed to show equal protein loading.

significance of the differential expression of NRP-1 in ischemic brains.

Lack of NRP-1 expression in E2F1^{-/-} neurons. To confirm the differential expression of NRP-1 in E2F1^{+/+} and E2F1^{-/-} neurons, levels of NRP-1 mRNA and protein were determined in E2F1^{+/+} and E2F1^{-/-} mouse brains by using semiquantitative RT-PCR and Western blotting, respectively. As shown in Fig. 2A to D, the level of NRP-1 mRNA was extremely low in the E2F1^{-/-} mouse cortex. Similarly, NRP-1 protein expres-

FIG. 3. E2F1^{-/-} CGNs are less susceptible to semaphorin 3A-mediated inhibition of axonal outgrowth. Semaphorin 3A at the indicated concentrations was added to CGNs at the time of plating. After 3 days in culture, cells were fixed in 4% paraformaldehyde and immunostained with β -III-tubulin to detect axons. As shown in panels A and C, untreated E2F1^{+/+} and E2F1^{-/-} neurons had developed long axons and neurites and started to form networks of connections at 3 DIV. However, semaphorin 3A treatment caused a significant inhibition of axonal outgrowth in E2F1^{+/+} CGNs (B and E), but not in E2F1^{-/-} CGNs (D and E). Digital images of axons were taken, and axon lengths were measured using Image J software. **, $P < 0.01$ by one-way ANOVA. The error bars indicate standard deviations.

sion was almost absent from the E2F1^{-/-} brain, suggesting that the transcription factor E2F1 positively regulates NRP-1 gene expression.

To demonstrate that E2F1 indeed transcriptionally activates NRP-1, a gene replacement experiment was performed. Replication-defective adenovirus carrying E2F1 was used to reintroduce E2F1 into E2F1^{-/-} CGNs (Fig. 2E). Adenoviral constructs expressing either GFP alone (Ad-GFP), an E2F1 mutant (Ad-Emut, a point mutant of E2F1 at 138 amino acids which abolished promoter binding capability [20]), or the full-length E2F1 (Ad-E2F1) were used to infect 7-day-old CGNs derived from E2F1^{-/-} and E2F1^{+/+} mouse brains. The Ad constructs have been shown in our laboratory and others to be

FIG. 4. Identification of an E2F1 binding site in the NRP-1 promoter. (A) ChIP assays were performed on CGNs. Chromatin was isolated and immunoprecipitated with either an antibody specific to E2F1 or a nonspecific mouse IgG (Neg IgG) or no antibody (Ab) as negative controls. PCR for the NRP-1 promoter sequence was carried out to detect NRP-1 promoter sequence. Input chromatin represents the total chromatin serving as a positive control. (B) The density of each PCR band was quantified and normalized against the input band and plotted. The error bars indicate standard deviations. (C) A mouse E2F1 consensus binding sequence was used to identify homologous sequences in the NRP-1 promoter region by pairwise alignment using a BLAST search. (D) The sequence identified on the NRP-1 promoter was chemically synthesized to make a dsDNA, which was then labeled with radioactive [α - 32 P]dCTP for EMSA (NRP1 hot probe). Nuclear fractions were isolated from E2F1^{+/+} and E2F1^{-/-} brain extracts and mixed with radioactive dsDNA NRP1 hot probes. Several controls

effective in expressing the encoded genes in neurons (20, 35). Uninfected controls were also used to detect basal levels of NRP-1 expression and to ensure that infection alone with viruses that do not carry the E2F1 gene did not alter the levels of NRP-1. The results from this experiment, as shown in Fig. 2E, determined that when the E2F1 gene was replaced in the E2F1^{-/-} cells, the wild-type level of NRP-1 expression was restored. Controls in which cells were infected with GFP alone or with mutant E2F1 did not show induction of NRP-1 expression (Fig. 2E). GAPDH was used as a loading control to show equal protein loading (Fig. 2E).

To show that E2F1^{-/-} neurons indeed do not express functional NRP-1, cultured CGNs were treated with semaphorin 3A, which is known to inhibit axonal outgrowth through NRP-1-mediated signal transduction (26). As shown in Fig. 3, adding 0.1 μ g/ml or 5 μ g/ml of semaphorin 3A to 0-DIV E2F1^{+/+} CGNs significantly inhibited axonal outgrowth after 3 days, but not in E2F1^{-/-} CGNs. Axons were identified by immunostaining for β -III-tubulin (Fig. 3A to D). The lengths of axons were measured on digitized images of axons using Image J software and quantified as shown in Fig. 3E. Indeed, the absence of E2F1 expression significantly ameliorated semaphorin 3A-mediated inhibition of axonal outgrowth. Taken together, these studies demonstrated that E2F1 positively targets NRP-1 and that E2F1^{-/-} CGNs lack the expression of functional NRP-1 receptors.

Neuropilin is a direct target of E2F1. To demonstrate that the transcription factor E2F1 directly binds to the endogenous NRP-1 promoter, a ChIP assay was performed on formalin-fixed CGNs, using an E2F1-specific antibody. As shown in Fig. 4A and B, E2F1 indeed bound to the NRP-1 promoter, while a negative IgG showed very low nonspecific background binding. The intensity of each PCR band was normalized against the ChIP input band to show the relative abundance of E2F1 binding to the NRP-1 promoter (Fig. 4B). To further identify the specific promoter sequence on the NRP-1 promoter that E2F1 targets, a bioinformatics approach was taken to search for the putative E2F1 binding sequences in the promoter region of the NRP-1 gene. Using the consensus mouse E2F1 DNA binding sequence (18), a putative E2F1 DNA binding site was identified in the promoter region of NRP-1 (Fig. 4C).

were used in the study, including CTL hot probe (a conserved mouse E2F1 binding promoter DNA sequence as a positive control), CTL and NRP1 cold probes (100 times the amount of excess CTL and NRP1 cold probes, respectively), and E2F1 antibody supershift. Specific E2F1 binding to the NRP-1 promoter sequence was identified, as indicated by an arrow in panel D. The supershift pattern is characteristic of E2F1, which when mixed with the antibody and in the absence of poly(dI-dC), completely shifted the binding of E2F1 to the dsDNA probe. (E) Activation of NRP-1 promoter by E2F1 was demonstrated by the use of a luciferase reporter assay. The E2F1 binding site sequence from the NRP-1 promoter was subcloned into the PGL3 promoterless expression vector. Several controls were also used, including GLO buffer alone, nontransfected cells, and cells transfected with empty vector. Primary glial cells derived from E2F1^{+/+} and E2F1^{-/-} mouse brains were transfected with the constructs for 3 days. After that, cells were collected, and their lysates were quantified. Equal amounts of proteins were subjected to luciferase assay using a Promega kit following the manufacturer's instructions. A 10-fold increase in luciferase activity was detected in the NRP-1 promoter construct-transfected E2F1^{+/+} cells.

FIG. 5. E2F1 positively regulates NRP-1 expression during cerebral ischemia. (A) Nuclear fractions were isolated from both the ischemic (L) and contralateral (R) sides of MCAO mouse brains. EMSA was performed using these nuclear extracts. Sham-operated brains were used as a control to determine the baseline level of E2F1 binding to the promoter. (B) The E2F1 band was quantified by densitometry measurement and plotted. Temporal changes in E2F1 binding on the ischemic side of the brain were normalized against those on the contralateral side of the brain. (C) E2F1 immunoprecipitation (IP) was performed against nuclear extracts obtained from ischemic mouse brains. (D) Western blotting was then performed on equal amounts of the IP products to show increased E2F1 levels following MCAO by densitometry measurement and normalization against the E2F1 level in the sham-operated brain. (E) RT-PCR was performed using RNA from the ischemic side of the MCAO mouse brains to detect changes in NRP-1. (F) β -Actin was used as an internal control for quantification of NRP-1 levels. * and ** indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively, by one-way ANOVA with a post hoc Tukey's test for significant groups. The error bars indicate standard deviations.

An oligonucleotide sequence corresponding to the E2F1 binding site on NRP-1 was synthesized and used for EMSA (Fig. 4C and D) to determine sequence-specific binding by E2F1. Indeed, as shown in Fig. 4D, E2F1-specific binding was detected in the brain nuclear extract. This binding was specific, as determined against three controls, i.e., supershifting with an E2F1 antibody, abolishing E2F1 binding with cold-probe com-

petition, and specific binding to the conserved E2F1 sequence (Fig. 4D, CTL hot probe). Finally, to assess whether the binding of E2F1 to the NRP-1 promoter sequence was sufficient to activate the promoter and lead to gene transcription, a luciferase reporter assay was designed and constructed to show the activation of the NRP-1 promoter by E2F1. The putative E2F1 binding region on the

NRP-1 promoter (150 bp) was amplified using PCR, and the fragment was subcloned into a promoterless PGL3 expression vector. A deletion mutant (100 bp) and a fragment of DNA from the adjacent region of the putative binding site (150 bp) were also constructed as negative controls. Actively proliferating glial cells from E2F1^{+/+} and E2F1^{-/-} brains were transfected with the constructs. As shown in Fig. 4E, PGL3/NRP-1 promoter-transfected E2F1^{+/+} cells showed a >10-fold increase in luciferase activity compared to those transfected with the vector alone or compared to E2F1^{-/-} cells transfected with the PGL3/NRP-1 promoter construct (Fig. 4E). These experiments confirmed that NRP-1 is a direct transcriptional target of E2F1.

E2F1 targets NRP-1 in vivo during MCAO. To determine whether E2F1 targets NRP-1 during cerebral ischemia, nuclear extracts from ischemic brains were subjected to EMSA using the NRP-1 promoter sequence, which was shown to be specific to E2F1 binding. As shown in Fig. 5A and B, E2F1 binding to the NRP-1 promoter sequence significantly increased after 2 h and 8 h of reperfusion in the ischemic side of the cortex. No significant changes in E2F1 binding occurred on the contralateral side of the brain. This binding profile correlated with the temporal profiles of induction in the levels of E2F1 protein (Fig. 5C and D) and NRP-1 mRNA (Fig. 5E and F) in response to MCAO. Nuclear extracts from ischemic brains were immunoprecipitated with an E2F1-specific antibody followed by Western blotting to detect E2F1. More than threefold increase in E2F1 expression occurred after 8 h of reperfusion in MCAO brains (Fig. 5C and D). This induction in the E2F1 protein level coincided with the induction of NRP-1 mRNA. Using semiquantitative RT-PCR, a clear induction of NRP-1 mRNA occurred after 2 h and 8 h of reperfusion of the ischemic side of the brain (Fig. 5E and F). Double immunostaining was also performed to detect the colocalization of E2F1 with NRP-1 expression (Fig. 6A to H). MCAO after 8 h of reperfusion evoked a dramatic increase in nuclear E2F1 expression and NRP-1 expression in the parenchyma of the E2F1^{+/+} mouse brain (Fig. 6A to D). In contrast, no E2F1 expression was detected in the E2F1^{-/-} mouse brain either before or after MCAO (Fig. 6E and G). Similarly, no obvious changes in NRP-1 expression occurred in E2F1 knockout mouse brains (Fig. 6F and H). Collectively, these studies demonstrated that E2F1 occupied the promoter site of the NRP-1 gene during ischemia to up-regulate the expression of NRP-1 in response to ischemia.

NRP-1 is involved in neuronal death. To determine the role of NRP-1 in adult mature neurons, mature postmitotic CGNs cultured for 3 DIV were treated with semaphorin 3A. These CGNs were shown in our previous studies to have fully expressed receptors for glutamate and could indeed respond to glutamate-mediated excitotoxicity (24, 34). Interestingly, semaphorin 3A added to 3-DIV CGNs for 18 h not only led to significant shortening of the axons of E2F1^{+/+} CGNs (Fig. 7A), but also caused a significant induction in the number of neuronal deaths in comparison with those of E2F1^{-/-} CGNs in a time-dependent manner (Fig. 7B).

Unfortunately an NRP-1^{-/-} mouse was unavailable due to the embryonic lethality of this mutation, and thus, genetic tests to identify the contribution of the NRP1 pathway in postischemic processes became more difficult. To circumvent this difficulty, we used an NRP-1-specific peptide inhibitor to see whether blocking NRP-1 function would have any protective benefits for neurons against ischemia. This peptide, which is a

FIG. 6. Colocalization of E2F1 and NRP-1 expression in the ischemic mouse brain. (A to H) E2F1^{+/+} and E2F1^{-/-} mice were subjected to MCAO and reperfusion as described in Materials and Methods. Formaldehyde-fixed paraffin sections were cut and double immunostained with antibodies to E2F1 (red) and NRP-1 (green). (C and D) Increases in nuclear E2F1 (red) and cytosolic and membrane NRP-1 (green) occurred in the E2F1^{+/+} ischemic brains. The arrows (C and D) indicate positive cells. The arrowheads (B and E to H) indicate E2F1- or NRP-1-negative cells/staining. Scale bar = 100 nm.

known NRP-1 inhibitor specifically blocking NRP-1 interaction with semaphorin 3A (50), was added to cultured CGNs before OGD, which is a well-established in vitro model to simulate ischemic damage to neurons. Indeed, our previous studies have shown that OGD treatment elicits the expression of E2F1 and causes neuronal death (11, 22). Using RT-PCR and Western blotting, increased NRP-1 expression levels were detected starting 2 h after reperfusion in CGNs treated with OGD (Fig. 7C). There was more than 10-fold induction of cell death in OGD-treated CGNs compared with the untreated CGNs (Fig. 7D). Adding NRP-1 inhibitor peptide to the CGN cultures protected neurons from OGD-induced death (Fig. 7D), lending further support to the idea that NRP-1 is important in ischemic neuronal death.

Taken together, these results demonstrated that NRP-1 participated in E2F1-mediated neuronal death, possibly by mod-

FIG. 7. Semaphorin 3A causes axonal damage and neuronal death. Mature postmitotic CGNs at 3 DIV were treated with semaphorin 3A for 18 h. The CGNs were either fixed with 4% paraformaldehyde to immunostain for β -III-tubulin (A) or supplemented with 10 μ g/ml PI for cell viability assays (B). (A) Axon lengths were measured on β -III-tubulin-stained slides, and the data were plotted. E2F1^{-/-} CGN axons were not affected by semaphorin 3A treatment, while E2F1^{+/+} neurons had significantly shortened axons following both 0.1- μ g/ml and 5- μ g/ml semaphorin 3A treatment. The error bars indicate standard deviations. (B) A cell death assay was performed using PI staining, which detected significant neuronal death after 18 and 24 h of semaphorin 3A treatment. **, $P < 0.01$ by ANOVA and post hoc Tukey's test. (C) CGNs were plated in 10-cm dishes and subjected to OGD treatment for 90 min. Individual plates were reperused for either 2, 6, or 10 h with regular culture media. At these time points, the cells were scraped from the plate and pelleted, and total protein extracts were collected for RNA or protein extraction to detect changes in NRP-1 expression. (D) For NRP-1 inhibitor studies, CGNs were preincubated for 30 min with or without 0.5 μ g/ml of synthetic peptide inhibitor. The cells were subsequently subjected to 90 min of OGD and reperused for 24 h, at which point the cells were fixed with 4% fresh paraformaldehyde and double immunostained for DAPI and MAP2. Cell death was quantified by manual counting of condensed DAPI-stained nuclei, and the induction in neuronal death (n -fold) was determined.

FIG. 8. Schematic diagram depicting pathways of axonal damage and neuronal death mediated by E2F1 activation of NRP-1 during cerebral ischemia. Ref, references.

ulating axonal retraction and shortening. A working model is proposed in Fig. 8 in which E2F1 modulates neuronal death not only through Bax/caspase activation, but also through positive regulation of NRP-1 expression, which in turn causes remodeling of distal axons. Whether axonal damage through NRP-1 activation acts as an early death signal for the entire neuron or is just part of the simultaneous neuronal destruction process remains to be investigated.

DISCUSSION

E2F1 is a key regulator of many genes involved in cell cycle progression and death. Accumulating evidence has indicated that E2F1 modulates neuronal death through activation or repression of genes (15, 22, 31). Despite intensive efforts to hunt for E2F1 target genes through large-scale screening using chromatin immunoprecipitation or microarrays (25, 48, 49), very little information is yet available about E2F1 targets in neuronal death. Analysis of 1,400 proximal human promoters revealed E2F binding to a much larger set of cell cycle-regulated promoters, including G₁/S transition, DNA replication, mitosis, and DNA damage and repair checkpoints (39). The present work for the first time demonstrated that NRP-1, an axonal-guidance molecule, is also a direct target of E2F1 in neuronal death caused by cerebral ischemia. Using a high-

density DNA microarray, we compared the expression profiles of ischemia-induced genes between E2F1^{+/+} and E2F1^{-/-} mouse cortices. When we identified more than 200 putative E2F1 target genes that were also responsive to ischemia, it became apparent that the activities of the transcription factor E2F1 impinged on several signaling pathways during neuronal death. This conclusion lends further support to the idea that E2F1 is at the hub of cell death pathways (8, 15, 45). It is most interesting that, based on our microarray data, in addition to NRP-1, which was selectively down-regulated in E2F1^{-/-} brains, several genes associated with axonal collapse and plasticity were also found to have changed, for example, those encoding cofilin and collapsin response mediator proteins (reference 21 and unpublished data). These results prompted us to investigate further whether selective modulation of NRP-1 by E2F1 plays a role in axonal damage, which may serve as an early distal signal for neuronal death in response to cerebral ischemia.

Key findings from the present study demonstrated that E2F1 is a positive regulator of NRP-1. The supporting evidence is as follows: (i) E2F1 binds to the NRP-1 promoter sequence with selectivity both *in vitro* (EMSA data) and *in vivo* (ChIP data), (ii) NRP-1 expression is significantly reduced in E2F1^{-/-} neurons compared with E2F1^{+/+} neurons, (iii) replacing the E2F1 gene using a replication-defective adenoviral vector in E2F1^{-/-} cells restores NRP-1 expression, (iv) E2F1 occupation of the NRP-1 promoter is associated with the up-regulation of NRP-1 mRNA expression, and (v) an NRP-1 promoter-reporter construct can be positively regulated by E2F1. Therefore, it is reasonable to believe that NRP-1 is one of the death receptors selectively augmented by E2F1 during axonal retraction and shortening caused by cerebral ischemia.

It is interesting that NRP-1, but not NRP-2, was selectively targeted by E2F1. Extensive bioinformatics searches failed to reveal a putative E2F1 binding site on the available NRP-2 promoter sequence, which further supports the hypothesis that NRP-2 may play a role different from that of NRP-1 during neuronal death. Indeed, although several reports have shown transient increases in the expression of NRP-1, NRP-2, and semaphorin 3A in ischemic brains, the specific molecular pathways and significance of up-regulation of these genes remain unclear (10, 52). It has been suggested that these molecules are involved in the extensive regrowth of new blood vessels and neuronal modeling in stroke brains (2, 52). For example, up-regulation of NRP-1, in concert with VEGF and its receptors, contributes to neovascular formation in the adult ischemic brain (52). The expression of semaphorin 3A is also increased following cerebral ischemia. For example, semaphorin 3A mRNA levels increased in the noninfarcted parietal cortex on the ischemic side 6 h after MCAO (10, 52). The increased semaphorin 3A expression prevented axons from growing beyond the area where semaphorin 3A was expressed, indicating that semaphorin 3A either elicits neuronal death or prevents neuronal regeneration (13, 36). In fact, dopamine treatment of cultured sympathetic neurons induces the expression of semaphorin 3A, which eventually causes neuronal death (43). Further, adding semaphorin 3A or a semaphorin 3A-derived peptide to cultured cerebellar granule neurons or retinal ganglion cells also induces neuronal death (42). These studies indicate that semaphorin 3A transmits a death signal through NRPs.

The present study provided new evidence to support the idea that the repellent guidance cue semaphorin 3A causes axonal retraction and neuronal death through the NRP-1 receptor. We used cultured pure neuronal cells (95% neurons in our CGN cultures) to show that semaphorin 3A elicited axonal shortening and neuronal death in adult neurons, while in the absence of NRP-1 expression, damage to neurons by semaphorin 3A was alleviated. NRP-1 peptide inhibitor was also effective in neuroprotection against semaphorin 3A toxicity. Since NRP-1 plays a role in the death of adult neurons in response to chemorepellent guidance cues and NRP-1 is a *de novo* target of E2F1 during neuronal death in response to cerebral ischemia, our future studies will be directed toward testing blockers to the NRP-1/semaphorin pathway *in vivo* for development as potential therapeutics for cerebral ischemia-induced brain damage.

Although focal cerebral ischemia, such as the mouse model used in the present study, appears to produce a localized focal lesion on the ipsilateral side of the brain, based on gross pathological parameters, increasing genomic evidence indicates that altered gene expression also occurs on the contralateral side of the brain (17, 27). It is therefore not surprising to see a relatively smaller increase in E2F1 binding to the contralateral side of the ischemic brain on the EMSA (Fig. 5A) in comparison with the sham brain, suggesting a potential up-regulation of NRP-1 expression. The significance of this remains unclear; it may, however, suggest subtle damage to the contralateral side of the brain. Another question, a logical extension of the current study, is to what extent E2F1 regulates other partners of NRP-1 complexes. For example, L1, a cell adhesion molecule of the Ig superfamily (IgCAM), also plays a critical role in the formation of neuronal networks by participating in the signaling of semaphorin 3A (5). Plexins are also the prerequisite partners for NRP-1 intracellular signaling (38). To begin to address these questions, we performed promoter searches for E2F1 binding sequences. However, a search failed to reveal potential E2F1 binding sequences on L1 and plexins. Further studies are definitely required.

In summary, understanding the molecular mechanisms of neuronal death in ischemic brains is essential for developing therapeutics against stroke. Based on our previous studies, the transcription factor E2F1 plays a significant role in modulating neuronal death. The absence of E2F1 expression has been shown to be very neuroprotective against cerebral ischemia, both *in vitro* and *in vivo* (11, 19, 24, 31, 32, 35, 44). The present study for the first time reports the discovery that E2F1 also positively targets NRP-1, a receptor for axonal-growth guidance cues, and that NRP-1 activation leads to axonal retraction and neuronal death. Inhibition of this pathway may therefore have the potential for protection of both axons and neurons.

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