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Transcriptional Regulation of The Alzheimer's Disease-related Gene, *Nicastrin**

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Abstract The gamma-secretase complex mediates the final cleavage of APP to generate the principal component of amyloid plaques in the brains of Alzheimer's disease patients. Four integral membrane proteins (PS, NCT, PEN-2 and APH-1) are essential and sufficient for gamma-secretase activity. To identify the promoter of human nicastrin gene (*NCT*), its 5′-flanking region has been characterized and a 270 bp fragment containing the TSS(transcription start site) for the promoter activity has been identified. EMSA assays confirmed that all four AP-1 binding sites and two NFAT sites in the *NCT* promoter region were able to bind relative transcription factors *in vitro*. Mutations, as well as treatment with PDTC, which adjust the regulatory effect of AP-1 and NFAT, altered *NCT* promoter activity in both HeLa cells and rat cortical neurons. The results demonstrated that AP-1 and NFAT are involved in the regulation of h*NCT* transcription and suggest that balanced activation of AP-1 and NFAT ensures a strict temporal and tissue-specific control of NCT transcription.

Key words Alzheimer's disease, *nicastrin*, promoter, transcriptional regulation, AP-1, NFAT
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Alzheimer's disease (AD), the most common form of senile dementia, is characterized neuropathologically by extensive neuronal loss and the presence of neurofibrillary tangles and senile plaques in the brain. Gamma-secretase has been implicated in the development of AD because of its role in the cleavage of amyloid precursor protein (APP) and the production of Aβ peptide, the main constituent of amyloid plaques. Besides APP, an increasing number of γ-secretase substrates have been identified, including Notch, E-cadherin, ErbB4 , nectin-1, CD44, lipoprotein receptor related protein (LRP), N-cadherin, sterol regulatory element-binding protein (SREBP), interferon response element (IRE1), and activated transcription factor 6 (ATF-6), thus implicating γ-secretase in many normal cellular processing events. Gamma-secretase is a high-molecular-weight complex. The following four different integral membrane proteins are essential and sufficient for γ-secretase activity: presenilin (PS, including PS1 and PS2), nicastrin, anterior pharynx defective (APH)-1, and presenilin enhancer (PEN)-2. Overexpression and expression knockdown studies have provided strong evidence that these proteins synergistically regulate each other in several different ways, including the level of protein, maturation, transportation, degradation, conformational alteration, and endoproteolysis.

Nicastrin (NCT) is a type-1 transmembrane protein that undergoes glycosylation and sialylation within the secretory pathway to yield a mature ~150 ku protein, the largest and only glycosylated component of the γ-secretase complex[1~4]. NCT has been shown to play a critical role in γ-secretase activity, as inhibition of *NCT* function *in vitro* and *in vivo* results in a complete loss of APP and Notch cleavage[4~6]. Further analysis has confirmed that NCT may function as the substrate acceptor of γ-secretase[7]. In addition, the identified endogenous negative regulators of γ-secretase activity, ERK1/2 and Rer1p, have been confirmed to bind to and work through NCT[8, 9]. These data demonstrate that NCT is essential for the

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γ-secretase complex and that NCT has both positive and negative functions in the regulation of γ-secretase activity. Notably, knocking out *NCT* in mice results in patterning defects that resemble, but are not identical to those of PS [10] or APH-1 [11] null embryos, implying that *NCT* may possess some unique physiologic functions, in addition to γ-secretase activity [12, 13]. To further our understanding of *NCT*, we identified and characterized the promoter region of *NCT* and demonstrated that transcription factor (TF) AP-1 and NFAT may play a role in regulating *NCT* transcription.

1 Materials and methods

1.1 Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Primary rat neuronal cultures were derived from the cerebral cortices of embryonic day 17～19 Sprague-Dawley rat embryos and cultured in serum-free Neurobasal TM-A Medium (Invitrogen, Carlsbad, CA, USA) with B-27 supplement (Invitrogen) [14]. All cells were maintained at 37℃ in an incubator containing 5% CO_2. For induction, 32 h after transfection, rat cortical neurons and HeLa cells were treated with 100 or 150 μmol/L of pyrrolidine dithiocarbamate (PDTC; Sigma Chemical Co., St. Louis, MO, USA), respectively, for 4 h.

1.2 5′-Rapid amplification of cDNA ends(RACE) and primer extension analysis

Total RNA was extracted from fresh healthy human fetal brain with Trizol reagent (Gibco). 5′-RACE was performed using the 5′-Full RACE Core Set kit (TaKaRa) with 5 μg total RNA as the starting material. Primer RT (5′ pATCTTCCTCTCC 3′) was used for synthesis of 5′-RACE cDNA. Primer sequences for 5′-RACE reactions were: S (*NCT*)1 (5′ ACGGCAGGGGGTGGCTCGGGGCTG 3′), S(*NCT*)2(5′ TCTCCTTCGCCTTCTGTCTTTCTGC 3′), A (*NCT*)1 (5′ ACCCCCTGCCGTAGCCATCTTG 3′) and A (*NCT*)2(5′ CCTCTCTGCTGAGCGGAAGCCC-CC 3′). They were located in the known sharing 5′ flanking sequence and the first 2 codon region of all h*NCT* gene splice forms, respectively. All primer-binding sites are shown schematically in Figure 1a. The PCR products were cloned into pGEM-T vector (Promega) and sequenced with primer T7 and SP6. Primer Extension analysis was done using a Primer Extension System——AMV Reverse Transcriptase kit (Promega). 10 pmol of the 30-mer PE primer, 5′ ACC-CCCTGCCGTAGCCATCTTG 3′, complementary to nt -4 to 18 with respect to ATG was end-labeled with 1 μl of [γ-^{32}P] ATP (1.11×10^{17} Bq/mol, 3.7×10^{11} Bq/L, Amersham Pharmacia Biotech) using T4 polynucleotide kinase. Then, 5 μg of total RNA from fresh human fetal brain was hybridized with 30 000 cpm of the labeled primer in a total volume of 11 μl, according to the manual.

1.3 Cloning of the *NCT* promoter, construction of luciferase reporter plasmids, and site-directed mutagenesis

PCR was performed to amplify the 5′-flanking regions of the *NCT* gene by using human peripheral blood lymphocyte genomic DNA as a template. The primers used to generate different promoter deletion plasmids were as follows (Table 1). *Mlu* Ⅰ and *Bgl* Ⅱ restriction sites were contained in the primers so that the resulting PCR-amplified fragments could be cloned into the multicloning sites upstream of the luciferase reporter gene in the pGL3-enhancer expression vector (Promega).

Table 1 Primers used for promoter-reporter plasmids construction

Vector	Upper primer	Lower primer
pGL3-E1768	5′ cgacgcgtCCCATTTTGCACACAGGCTGCTATC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E1343	5′ cgacgcgtATTCACACATTGATAACAACCTCC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E1226	5′ cgacgcgtAGATGAGGAAATACAGACTTAGAG 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E1025	5′ cgacgcgtTCTAACGTTCTGAGGCTAATACTG 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E786	5′ cgacgcgtTTATAAGCCTCAAGTACTATTTCC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E749	5′ cgacgcgtTTATAAGCCTCAAGTACTATTTCC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E597	5′ cgacgcgtCCCATTTTGCACACAGGCTGCTATC 3′	5′ gaagatctACAAGCTGTGACTTGGATGACTTAC 3′
pGL3-E420	5′ gcgacgcgtACCGCTAGCCAGGCCCAACCTCTC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E355	5′ cgacgcgtATTTCGACCAACACCTCTACC 3′	5′ gaagatctCCTCTCTGCTGAGCGGAAGCCCCC 3′
pGL3-E270	5′ cgacgcgtATTTCGACCAACACCTCTACC 3′	5′ gggagatctAGCCTAAGAGACCGGAAGTTCGTG 3′
pGL3-E237	5′ cgacgcgtATTTCGACCAACACCTCTACC 3′	5′ gcgagatctACCGCGGGAGAGGGTCGCTGACG 3′
pGL3-E196	5′ cgacgcgtATTTCGACCAACACCTCTACC 3′	5′ gaagatctTCCGAAGGGCAGCAGGGTG 3′

The catalytic sites of the restriction enzyme are underlined. The additional arbitrary sequences are in lowercase.

Site-directed mutations were integrated into the *NCT* promoter fragments E420 by overlapping PCR and the mutated fragments were then cloned into the pGL3-basic expression vector (Promega). To generate these mutants, two end primers were used in combination with various primers with mutations (Table 2).

Table 2 Primers used for site-directed mutagenesis

Luc vector	Name	Primer
pGL3-Bm(ATF6+HIF)	ATF+HIF-fmut	5′ TGCCCTTCGGACGCCTC**A**AC**A**TCAGCGACCCTCTCCCGC
pGL3-Bm(AP1-1)	Ap1- rmut1	5′ TGGATGTCAGGAGCGGCGC**GT**ACACCGGGGAAGAGAAAATG
pGL3-Bm(AP1-2)	Ap1-rmut2	5′ AACATGGAGGTTGGATGT**AC**GGAGCGGCGCTGACACCG
pGL3-Bm(AP1-3)	Ap1-fmut3	5′ TGCCCTTCGGA GCCTCCACGT**AC**GCGACCCTCTCCCG
pGL3-Bm(AP1-4)	Ap1-fmut4	5′ TCCGGGCCACAGAGACGGTGT**AC**GTGGTAGCCTAGAGAGG
pGL3-Bm(AP4-1)	Ap4-fmut1	5′ ATGTCTTAATCCGAGCCCCGACACACCCT**A**CT**A**CCC
pGL3-Bm(AP4-2)	Ap4-fmut2	5′ AACGGGGGCTTCCGCT**T**AG**T**AGAGAGGCAAGATGGC
pGL3-Bm(NFAT-1)	NFAT- fmut1	5′ TCTCCCGCGGTT**GAG**GGAAGCGCCTGGAAACACGAAC
pGL3-Bm(NFAT-2)	NFAT- fmut2	5′ AGCGCCT**CTC**AACACGAACTTCCGGTCTCTTAG
pGL3-Bm(TCF/LEF)	TCF/LEF-mu1	5′ AGACCAAGAGCGCGCGGG**CTG**AAGGTAGTGGAGAATAAAAG
pGL3-Bm(AP1-3+AP1-4)	Ap1-fmut3	5′ TGCCCTTCGGACGCCTCCACGT**AC**GCGACCCTCTCCCG
	Ap1-fmut4	5′ TCCGGGCCACAGAGACGGTGT**AC**GTGGTAGCCTAGAGAGG
pGL3-Bm(AP1-3+NFAT-1)	Ap1-fmut3	5′ TGCCCTTCGGA GCCTCCACGT**AC**GCGACCCTCTCCCG
	NFAT- fmut1	5′ TCTCCCGCGGTT**GAG**GGAAGCGCCTGGAAACACGAAC
pGL3-Bm(AP1-4+NFAT-2)	Ap1-fmut4	5′ TCCGGGCCACAGAGACGGTGT**AC**GTGGTAGCCTAGAGAGG
	NFAT- fmut2	5′ AGCGCCT**CTC**AACACGAACTTCCGGTCTCTTAG
pGL3-Bm(NFAT-1+NFAT-2)	NFAT- fmut1	5′ TCTCCCGCGGTT**GAG**GGAAGCGCCTGGAAACACGAAC
	NFAT- fmut2	5′ AGCGCCT**CTC**AACACGAACTTCCGGTCTCTTAG
pGL3-Bm(AP4-1+AP4-2)	Ap4-fmut1	5′ ATGTCTTAATCCGAGCCCCGACACACCCT**A**CT**A**CCC
	Ap4-fmut2	5′ AACGGGGGCTTCCGCT**T**AG**T**AGAGAGGCAAGATGGC

Letters in shadows are mutated.

1.4 Transient transfection and reporter gene assays

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. To normalize the different transfection efficiencies of various luciferase constructs, the phRL-SV40 plasmid containing the Renilla Luciferase gene was co-transfected into the cells in a molar ratio of 1∶50 (phRL-SV40∶pGL3). Luciferase activity was assayed 24 h after transfection of the constructs. Cell extracts were prepared by lysing the cells with freshly diluted 1× passive lysis buffer (Promega) and assayed for firefly luciferase activity and Renilla luciferase activity with the Dual-Luciferase® Reporter (DLR™) Assay System (Promega), according to the manufacturer's recommendations. The enzymatic activity of the individual samples was measured twice.

1.5 Statistical analysis

Student's *t*-test was used. The results are expressed as the $\bar{x} \pm s$. For all tests, $P < 0.05$ was considered significant (*).

1.6 Electrophoretic mobility shift assay (EMSA)

Fresh, healthy human fetal brain nuclear extracts were prepared using a nuclear extract kit (Activemotif). Synthetic sense and antisense oligonucleotides (Table 3) were used as probes in gel-shift and super-shift assays. Oligonucleotides were annealed with their respective reverse complements to generate double-stranded oligonucleotides, then were labeled at the 5′ end by T4 polynucleotide kinase with [γ-^{32}P] ATP and purified on Microspin G-25 columns (Amersham Biosciences). 15 μg of fresh, healthy human fetal brain nuclear extracts in a 9 μl final volume of 1× EMSA binding buffer (Promega), with or without competitor oligonucleotides, were incubated at room temperature for 20 min, after which the probes ($2\times10^4 \sim 3\times10^4$ cpm) were added and the reactions were allowed to incubate at room temperature for a further 30 min. Competition binding assays were performed by the addition of excessive non-radioactive specific or non-specific competitor oligonucleotides. The samples were resolved by 4% non-denaturing polyacrylamide gel electrophoresis (PAGE) in 0.5×TBE (44.5 mmol/L Tris, 44.5 mmol/L boric acid, and 1 mmol/L EDTA pH 8.3) buffer and visualized by autoradiography by exposure to X-ray film at –70℃.

Table 3 Nucleotide sequences of oligonucleotide probes used in EMSA

Oligonucleotides	Nucleotide sequences
AP1	5′ CGCTTGATGAGTCAGCCGGAA 3′
AP1-W12	5′ CTTCCCCGGTGTCAGCGCCGCTCCTGACATCCAACCTC 3′
AP1-M1	5′ CTTCCCCGGTGTacGCGCCGCTCCTGACATCCAACCTC 3′
AP1-M2	5′ CTTCCCCGGTGTCAGCGCCGCTCCgtACATCCAACCTC 3′
AP1-M12	5′ CTTCCCCGGTGTacGCGCCGCTCCgtACATCCAACCTC 3′
AP1-W3	5′ CCCTTCGGACGCCTCCACGTCAGCGACCCTCTCCCGCG 3
AP1-M3	5′ CCCTTCGGACGCCTCCACGTacGCGACCCTCTCCCGCG 3′
AP1-W4	5′ CCGGGCCACAGAGACGGTGTCAGTGGTAGCCTAGAGAG 3′
AP1-M4	5′ CCGGGCCACAGAGACGGTGTacGTGGTAGCCTAGAGAG 3′
NFAT	5′ TAGCTATGGAAACTCTATA 3′
NFAT-W12	5′ CTCCCGCGGTTTCCGGAAGCGCCTGGAAACACGAACTT 3′
NFAT-M1	5′ CTCCCGCGGTTgagGGAAGCGCCTGGAAACACGAACTT 3′
NFAT-M2	5′ CTCCCGCGGTTTCCGGAAGCGCCTctcAACACGAACTT 3′
NFAT-M12	5′ CTCCCGCGGTTgagGGAAGCGCCTctcAACACGAACTT 3′
NFAT+AP1	5′ TCGACAAAAGGCGGAAAGAAACAGTCATTTC 3′
TCF/LEF	5′ GATCTAGGGCACCCTTTGAAGCTCT 3′
ATF6	5′ CTCGAGACAGGTGCTGACGTGGCATTC 3′

W: Wildtype; M: Mutant; Sequence without W and M are the classic binding oligonucleotide probes. Letters in shadows are core nucleotide bases for the binding sites; letters in lower case are mutated.

2 Results

2.1 Identification of the transcriptional start site by 5′-RACE and primer extension analysis

Sequence analysis of the 5′-RACE products mapped the transcriptional start site at positions nt-14 relative to the initiating ATG of NM-015331(Figure 1b). The result was confirmed by primer extension analysis (Figure 1c). Taken together, these experimental approaches identified a NM-015331-specific transcriptional start site at nt-14 as the main transcriptional start site of *NCT* in human fetal brain.

2.2 Promoter analysis of the *NCT* gene

To study the transcriptional regulation of *NCT*, we analyzed the genomic DNA sequence of *NCT* by using multiple promoter prediction software. The prediction results suggested that two potential promoter regions are located within 1 800 bp upstream of ATG. To investigate the functional relevance of the *in silico* identified promoter sequences, we performed functional experiments by cloning a series of DNA fragments containing 5′ upstream sequences of the *NCT* gene and inserting them into a luciferase reporter gene construct (Figure 2b). Considering that *NCT* shows an extensive expression pattern and the main pathologic changes of AD occur in the brain, we examined *NCT* promoter-reporter constructs in primary rat neurons, which are likely to provide the most similar circumstances to the human brain. In addition, we used HeLa cells as a control for convenience (Figure 2b). Compared with the empty vector, the basal promoter of *NCT* is located at

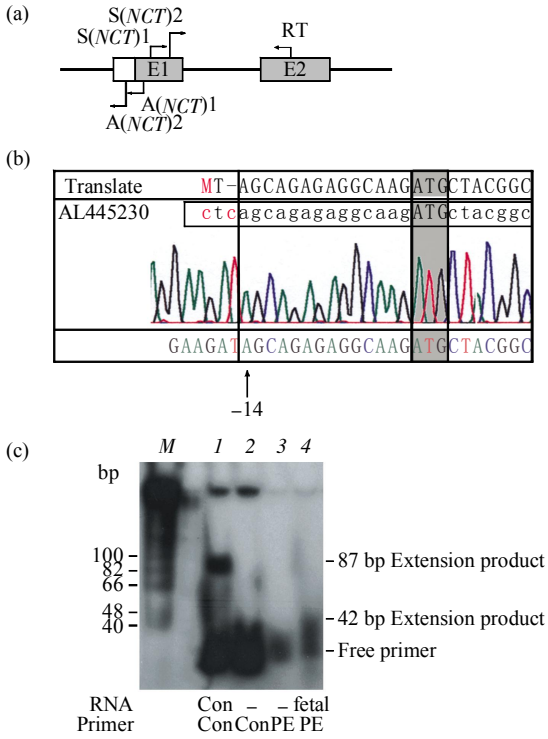

Fig. 1 Identification of the 5′ end sequence of *NCT* mRNA in human fetal brain

(a) Schematic diagram of 5′-RACE primer binding sites and the orientation of the respective primers. The line denotes genomic DNA of the human *NCT* gene displayed in the 5′～3′ direction and the first two exons of NM-015331 are shown as gray boxes and primers as arrows. 5′-RACE (b) and primer extension (c) was performed using mRNA from fresh human fetal brain as a template.

–359/–90 region that drives the transcription of reporter gene in both neuronal and non-neuronal cells. A 420 bp fragment in –424/–4 shows significantly enhanced activity, suggesting the presence of positive *cis*-acting element within region –424/–359 and the presence of negative one within region –752/–424. We also find a 355 bp fragment in –359/–4 showing differential promoter activity in neuronal and non-neuronal cells. The result is consistent with the fact that *NCT* is widely expressed while the expression level between tissues is not the same. To understand the mechanism behind it needs further research.

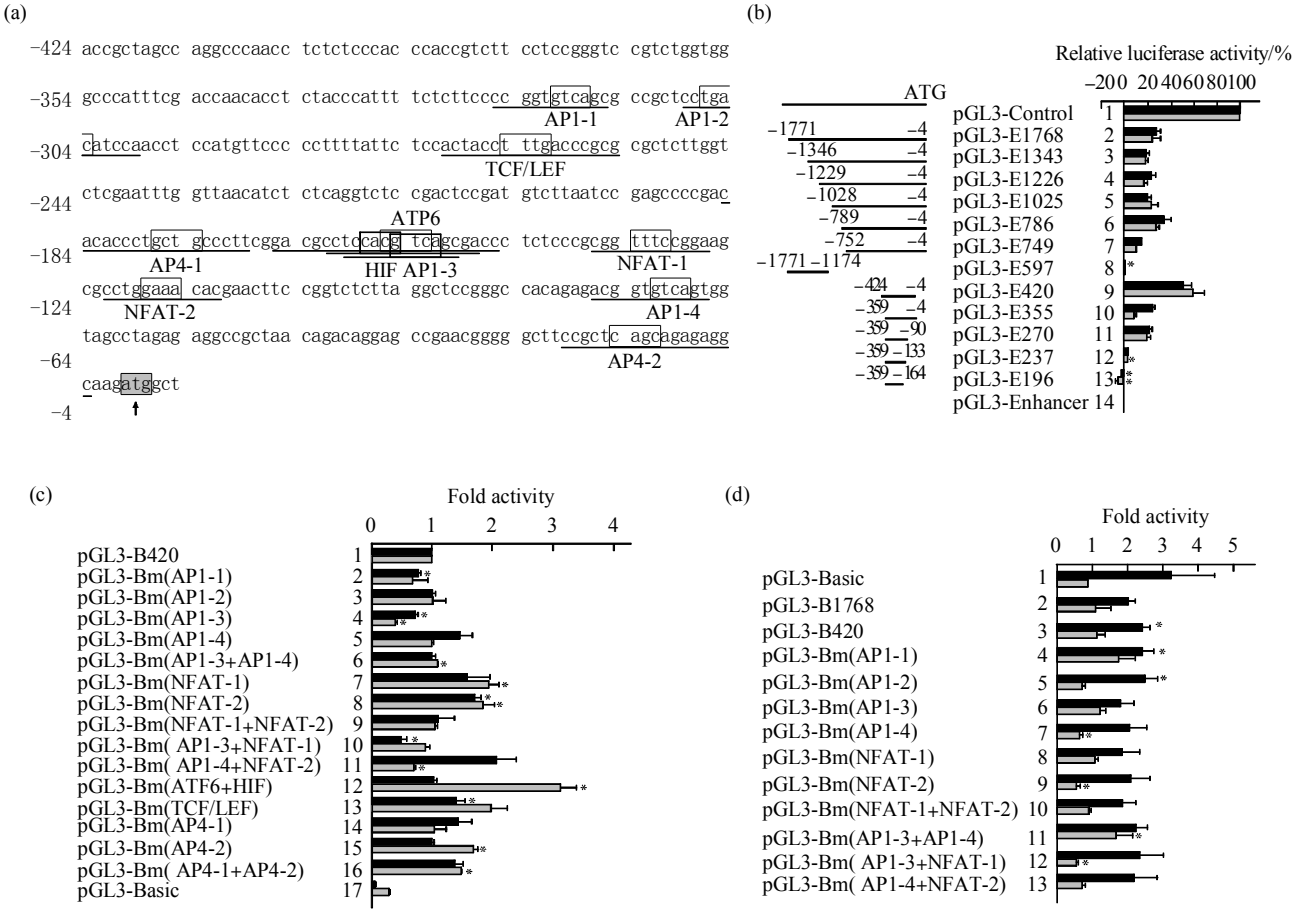

Fig. 2 Sequence features and activity analysis of the human *NCT* gene promoter

(a) The 420 bp fragment of the 5′-flanking region of the human *NCT* gene. The numbering was based on the translation start site, defined as +1. The putative transcription factor binding sites are underlined and its core nucleotides are boxed. (b) Schematic diagram of the *NCT* promoter deletion constructs with their relative luciferase activity (RLA) in HeLa cells and rat cortical neurons. The numbers represent the end-points of each construct. The values represent $\bar{x} \pm s$ *vs*. pGL3-control. (c) Mutation analysis of the potential transcription factor binding sites. Data represent $\bar{x} \pm s$ *vs*. WT by Student's *t*-test. (d) Induction of PDTC altered the *NCT* promoter activity in both HeLa cells and rat cortical neurons. Data represent the $\bar{x} \pm s$ *vs*. relative untreated by Student's *t*-test. ■: HeLa cell; □: Rat cortical neuron.

2.3 AP-1 combined with NFAT regulates *NCT* expression

To identify potential TF binding sites on the *NCT* promoter region, we analyzed the 420 bp sequence with the highest promoter activity using MatInspector 2.2 software (Genomatrix, Munich, Germany). Prediction analysis revealed that this region possessed multiple potential TF binding sites (Figure 2a). To define the potential TFs, we mutated these potential TF binding sites individually or collectively, followed by promoter activity analysis. The mutations' relative luciferase activities were compared with that of the wildtype. Although major mutations of the potential transcriptional binding sites could cause a significant change in the promoter activity, either in HeLa cells or rat cortical neurons, or both, compared with that of the wild-type 420 bp promoter fragment (Figure 2c), none of them resulted in complete abolishment of promoter activity. Notably, mutations in the AP-1 and NFAT binding sites showed a significant change in promoter

activity, suggesting that they are potential TFs regulating *NCT* transcription, which may function either independently or cooperatively.

To confirm the role of AP-1 and NFAT on transcription expression of *NCT in vivo*, we treated both HeLa cells and rat cortical neurons with PDTC (Figure 2d). The fold activity was gotten by comparing the relative luciferase activity from treated cells with that from untreated cells. It is reported that PDTC prevents NFAT DNA-binding activity and simultaneously activates the binding of AP-1 [15]. In HeLa cells, endogenous NFAT levels are not sufficient to achieve activation[16, 17]. Therefore, the main function of PDTC in HeLa cells is to activate the AP-1 pathway. Consistent with the activation function, HeLa cells treated with PDTC showed increased luciferase expression in wild type 420 bp *NCT* promoter(Lane *2*), and the single mutation in the first two AP-1 binding sites did not abolish the activation (Lane *3*, *4*). On the contrary, both the single and combined mutations in the last two AP-1 binding sites, as well as the two NFAT binding sites, resulted in no reaction to the PDTC treatment (Lane *5*～*12*). In rat cortical neurons, the situation is a little complicated, where both NFAT and AP-1 play a role in the regulation of transcription and PDTC has both a positive and negative effect. We can see that PDTC treatment led to either an increase (Lane *10*) or a decrease (Lane *6*, *8*, and *11*) in the luciferase activity driven by mutated *NCT* promoter, while the wild type showed no change (Lane *2*). These suggest that the combined function between NFAT and AP-1 play a role in the impalpable regulation of *NCT* transcription that allows the generation of specific, tightly-controlled responses to various stimuli.

2.4 The *NCT* gene promoter contains AP-1 and NFAT binding sites

To further investigate the binding of AP-1 and NFAT to the promoter region, we performed gel shift assays. For NFAT, a double-stranded oligonucleotide sequence containing both of the NFAT sites was synthesized and labeled as the probe. As shown in Figure 3a (Lane *2*), a protein-DNA complex was

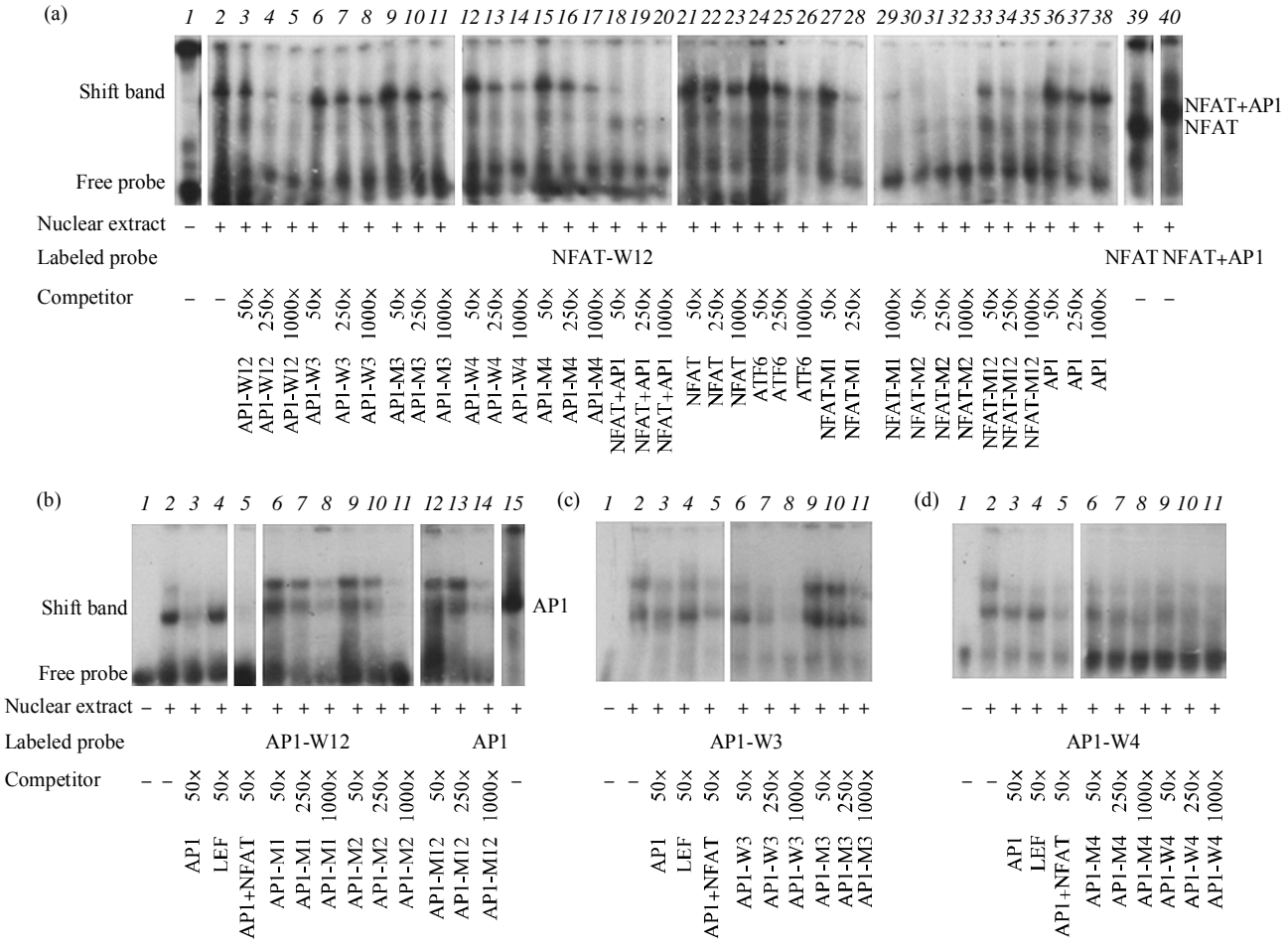

Fig. 3 EMSA for the *NCT* promoter

(a) NFAT binding. (b) AP-1 binding in the first two sites. (c) AP-1 binding in the 3rd site. (d) AP-1 binding in the last site.

detected which was larger than those formed from the classic binding oligonucleotide probe of NFAT (Lane *39*) or NFAT/AP-1 (NFAT +AP1; Lane *40*). Competition assays indicated that the formation of the complex could be affected by the NFAT/AP-1 consensus (Lane *18*～*20*), a *NCT* AP1 probe (Lane *3*～*5*, *6*～*11*, and *12*～*17*) and a *NCT*-mutated NFAT probe (Lane *27*～*35*), as well as the ATF6 consensus (Lane *24*～*26*), indicating that the NFAT binding sites in the *NCT* promoter region may function through binding with multiple different kinds of TFs, which may involve NFAT, AP-1, and ATF6 proteins.

For AP-1 (Figure 3b～d), three double-stranded oligonucleotide sequences corresponding to the four predicted binding sites in the *NCT* promoter region were synthesized and labeled as the probe, among which the AP1-w12 probe contained the first two predicted sites. All of the three probes were able to form two protein-DNA complexes (Lane *2*), the smaller of which is consistent with that formed by the classic AP1 consensus probe (Figure 3b; Lane *15*). In competition assays, the NFAT/AP-1 consensus, as well as their relative probe, showed the strongest competitive effects, suggesting that all four predicted AP-1 binding sites, especially the last two, were most likely to function cooperatively with the proximal NFAT binding sites.

3 Discussion

The experiments presented herein represent the first functional characterization of the human *NCT* promoter, in which we isolated and identified the 1 768 bp of the 5′-flanking region of the human *NCT* gene (Figure 2b). Analysis of this region revealed that the sequence lacks both CAAT and TATA boxes. There is also no initiator element, which in the absence of a TATA box, commonly drives transcriptional initiation [18]. In contrast, this region is GC-rich and contains overlapping binding sites for different TFs (Figure 2a). These features revealed its similarity with those of housekeeping genes and might result in initiation of transcription at multiple locations[19, 20], the former of which is inconsistent with the ubiquitous expression of *NCT* and its critical function. The *NCT* TSS identified in our experiments is within a region which contains the majority of the reported TSSs, although few *NCT* TSSs are upstream of this area. This could result from the different materials and methods adopted, or the h*NCT* gene has more than one TSS, and the one located at nt –14 is the major TSS in the human fetal brain.

The HeLa cells is an immortal cell line widely used in scientific research, which is created by horizontal gene transfer from human papillomavirus 18 (HPV18) to human cervical cells. It is different from either parent cells in many ways, including the number of chromosomes in genome. The rat cortical neurons are miscellaneous cultures from rat embryos. Though we tried our best to minimize the proportion of the neuroglias in the process of culture, they still comprise both neurons and neuroglias. Nowadays, the primary rat neuronal cultures are used as a desirable cell model for neurodegenerative diseases. Deriving from different species, composing of different kinds of cell types, residing in different stages of cell cycle and so on lead to various differences between these two cultures. These differences, especially the different signal transduction pathways, resulted in the disparate promoter activities of the –359/–4 fragment and they may also be the main reason, parting from the background effect of the vector backbone, for the inconsistency of the mutations and PDTC treatment data from HeLa cells and rat cortical neurons. On the other hand, the inconsistency of the data suggests the complicated transcriptional regulation mechanism of *NCT* gene, which palpably reflects the differences between HeLa cells and rat cortical neurons. To elucidate the inconsistency, we need to further our understanding on the differences between the two cultures and identify the responding *cis*-acting elements in *NCT* promoter.

Preliminary research has shown that AP-1 is involved in distinct processes in the brain, specifically, neurodegeneration and neuroregeneration and exhibits both neuroprotective and pathologic-degenerative functions. Most notably, apoptosis mediated by activated AP-1 affects hippocampal and nigral neurons, which are most involved in the pathogenesis of AD [21]. The NFAT family of TFs, as integrators of multiple signaling pathways, can bind to chromatin in combination with other TFs and collectively regulate the expression of different genes, and genes related with nervous system development in particular [22]. Additionally, APP [23] and Notch ligands [24] have been reported to be capable of regulating AP-1 expression or activation, independent of γ-secretase-mediated cleavage. In our experiments, we showed that mutations in the predicted AP-1 and NFAT binding

sites (Figure 2c), as well as the induction of PDTC treatment(Figure 2d), resulted in significant fluctuation of the luciferase expression level driven by the *NCT* promoter in both HeLa cells and rat cortical neurons. The physical bond between AP-1/NFAT proteins and the *NCT* promoter region was further confirmed by EMSA *in vitro* (Figure 3). Of course, the two TFs' effects on *NCT* expression are still to be tested on the protein level before validation can be assumed. The combined regulation of TFs AP-1 and NFAT as a powerful mechanism that enables tight control of gene expression, has been well-demonstrated in the immune system[25]. Considering their co-existence, it is probably possible that the cooperation between NFAT and AP-1 exists in the nervous system, too. If the NCT protein level is indeed regulated by their synergistic function, we may figure out a pattern that the activity of the cleavage enzyme is regulated by its substrates. The mutation in the second AP-4 site significantly increased the luciferase activity (Figure 2c; Lane *15*, *16*), suggesting that TF AP-4 might play a role in regulating *NCT* transcription. Mutations in the last AP-4 sites in the APH-1A promoter region exhibits similar effects[26]. Two components of the γ-secretase complex, PEN-2[27] and PS1[28], have been identified as cAMP-response element-binding protein (CREB)-regulated genes. Additionally, APP and BACE1, the first cleavage enzyme following which the proteolysis of APP is catalyzed by γ-secretase, have been shown to be putative CREB binding sites in their promoter region[29]. These data strongly suggest that a common mechanism may exist to synchronously regulate the expression of genes involved in the AD pathomechanism, and TF AP-4 might be a member of it.

As stated above, *NCT* may possess some unique physiologic functions independent of γ-secretase activity. Transcriptional regulation, as an important mechanism of regulating its function, is definitely involved. An in depth understanding of the regulatory mechanism of *NCT* transcription could not only facilitate our understanding of its physiologic functions, but also be conducive to identifying the targets that can potentially be used in therapeutic intervention of AD.

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老年痴呆症相关基因 *Nicastrin* 的转录调控 *

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摘要 APP 蛋白经过降解，形成老年痴呆症患者脑内老年斑的主要成分．由 PS(早老素)，NCT， PEN-2 和 APH-1 4 种膜蛋白组成的 γ 分泌酶催化该降解过程．为了了解人类 *nicastrin*(*NCT*)基因的转录调控机制，确定了其在人脑中的转录起始位点以及其编码区上游大小不等片段的转录起始活性．EMSA 分析证实 *NCT* 启动子区的 4 个 AP-1 结合位点和 2 个 NFAT 结合位点能够与相应的转录因子结合，能够改变转录因子调控能力的定点突变和 PDTC 诱导使得 *NCT* 启动子在 HeLa 细胞和大鼠皮质神经元中的启动活性都有所改变．以上结果说明：AP-1 和 NFAT 确实参与了人类 *NCT* 基因的转录调控．

关键词 老年痴呆症，*Nicastrin*，启动子，转录调控，AP-1，NFAT
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