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Identification and Characterization of a Rat Novel Gene *RSEP4* Expressed Specifically in Central Nervous System

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Abstract The low-abundantly expressed genes composed the majorities of the mRNAs expressed in the central nervous system (CNS), and were thought to be important for the normal brain functions. Through differential screening a low-abundance cDNA sublibrary with mRNA from neuropathic pain of chronic constriction injury (CCI) model, we have identified a novel rat gene, rat spinal-cord expression protein 4 gene (*RSEP4*). The total length of RSEP4 cDNA is 2006 bp, with a 501 nucleotide open reading frame (ORF) that encodes a 167 amino acid polypeptide. Northern blot revealed that RSEP4 was expressed specifically in the CNS. *In situ* hybridization showed that the mRNA of RSEP4 was strongly expressed in the CA1, CA2, CA3 and DG regions of hippocampus, the Purkinje cells of cerebellum, and the small sensory neurons of dorsal horn and large motor neurons of ventral horn of spinal cord. Over-expression of RSEP4-EGFP fusion protein in the human embryonic kidney 293T cells showed that RSEP4 protein was mainly localized in the cell cytoplasm. These results suggest that RSEP4 may play some roles in the CNS.

Key words central nervous system (CNS); rat; Northern blot; *in situ* hybridization

The central nervous system (CNS) of vertebrate displays sophisticated functions, such as perception, motion, learning and memory, etc. [1]. These higher-order brain functions are dependent on gene expression alterations [2–5]. The CNS mRNA complexity is rather high in terms of the number of genes that can be expressed in the genome, and more than 50% of mRNAs are made up of thousands of low-abundance transcripts (1–5 copies/cell). Most of these low-abundantly expressed genes are powerful biological effectors, whose physiological consequences, even at barely detectable levels, can be profound and critical for normal CNS functions [6]. Hence, it is a big challenge for neuroscientists to fully characterize these low-abundance transcripts in the CNS.

Injury to peripheral nerves frequently leads to neuropathic pain, which is characterized by spontaneous burning or stabbing pain, exaggerated responses to light touch (tactile allodynia) and painful stimuli (hyperalgesia). The neuropathic pain is extremely difficult to cure, and it is resistant to most pain-killing drugs including morphine. Most evidence indicates that the spinal, supraspinal and brain structures are involved in processing the neuropathic pain [7,8], and many genes are related to neuropathic pain formation [9,10]. However, the molecular mechanisms underlying the neuropathic pain are still poorly understood. The chronic constriction injury (CCI) is one of the most commonly used animal models to study the mechanisms of the neuropathic pain [11].

In this study, using mRNAs from the spinal cord of CCI rat to differentially screen a low abundant expression gene cDNA sublibrary from the rat brain, we isolated and characterized a novel rat gene, rat spinal-cord expression protein 4 gene (*RSEP4*). RSEP4 was specifically expressed

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in the CNS, such as the hippocampus, cerebellum and spinal cord, suggesting that it may play some roles in the CNS.

Materials and Methods

Construction of the low-abundantly expressed gene cDNA sublibrary

The 0.5-day postnatal Sprague-Dawley rats (Shanghai Laboratory Animal Center, SIBS, CAS, China) were sacrificed and brain tissues were collected for RNA extraction using Oligotex reagent (Qiagen). Two micrograms poly(A)$^+$ RNA were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL), and the resulting cDNAs were labeled with [α-^{32}P]dATP as the probe using a random primer strategy (Roche). A brain cDNA library of 8-week male rat (SuperScript™ rat brain cDNA library, Gibco BRL) was screened with the probes as described [12]. Briefly, the blocked membranes were incubated with the probe in 6×SSC, 5×Denhardt, 0.5% SDS, 0.1% BSA, 50% formamide and 100 μg/ml denatured salmon sperm DNA at 42 °C overnight. Then they were washed 10 min at room temperature with 1×SSC and 1% SDS, and 15 min at 55 °C with 0.2×SSC and 0.2% SDS. The air-dried membranes were exposed to X-ray films (Kodak) and the non-hybridizing clones (about 10,000) were selected to construct a low-abundantly expressed gene cDNA sublibrary. This sublibrary was amplified by PCR and made into cDNA microarrays by transferring the PCR products in duplicates to positively charged nylon membranes by Shanghai CASarray Co., Ltd..

CCI model

Thirty-two adult male Sprague-Dawley rats (260–330 g, Shanghai Laboratory Animal Center) were used in this study. Twelve rats underwent CCI, twelve received a sham operation and eight were naive controls. The CCI model was made according to the procedure described previously [11]. Briefly, the rats were anaesthetized with pentobarbital (40 mg/kg), and the sciatic nerves were exposed bilaterally at mid-thigh level by blunt dissection through the biceps femoris muscle. On each side, two loosely constrictive ligatures were made with 4.0 chromic gut sutures surround the nerve at space of about 1 mm. Sham operations were carried out to expose and mobilize the nerve, but there was no ligation. To minimize difference in technique, all operations were done by the same person.

Differential screening of the cDNA array

Two micrograms poly(A)$^+$ RNA were extracted from the L4-5 spinal cord tissues of the CCI model and control rats, and then were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL). The resulting cDNAs were labeled with [α-^{32}P]dATP by random priming. These radioactive probes were used to hybridize cDNA microarrays of the low-abundantly expressed gene cDNA sublibrary, and differentially expressed clones were isolated and subjected to DNA sequence analysis.

Northern blot hybridization

Twenty micrograms of total RNA from different rat tissues were subjected to electrophoresis in 1.0% agarose gel, and transferred onto N$^+$ nylon membrane. Hybridization probes were ^{32}P-labeled RSEP4 cDNA. RNA blots were hybridized at 65 °C in a hybridization solution containing 0.2 M Na_3PO_4 (pH 7.2), 1 mM EDTA (pH 8.0), 1% BSA, 7% SDS, 15% formamide and 100 μg/ml denatured salmon sperm DNA. After overnight hybridization, blots were washed twice in 40 mM Na_3PO_4 (pH 7.2), 1 mM EDTA (pH 8.0) and 1% SDS for 1 h at 65 °C, and were scanned with Typhoon 9410 (Amersham Pharmacia Biotech).

In situ hybridization

The adult Sprague-Dawley rats (Shanghai Laboratory Animal Center) were perfused and their brains and spinal cords were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 4 h at room temperature. Fixed tissues were washed in PBS and then embedded in paraplastic (Sigma). Tissue blocks were sectioned at a thickness of 10 μm. A digoxigenin-labeled cRNA probe was prepared from RSEP4 cDNA fragment by PCR. The resulting fragment was digested with *Eco*RI/*Bam*HI, and inserted into the corresponding sites of pSPT19 vector (Roche). The cloned plasmid DNA was linearized with either *Eco*RI or *Bam*HI, and *in vitro* transcribed with T7 and SP6 RNA polymerases using digoxigenin-labeled UTP to generate sense and antisense riboprobes (SP6/T7 transcription kit, Roche). *In situ* hybridization was performed as described previously [13].

Transfection of EGFP-RSEP4 fusion protein

The full-length open reading frame (ORF, position 280–783 bp) of rat RSEP4 was inserted in-frame into the expression vector (EGFP-C2, Clontech) following the sequence of enhanced green fluorescent protein (EGFP) to create the expression construct, pEGFP-RSEP4. This construct, encoding an EGFP-RSEP4 fusion protein, was

transfected into human embryonic kidney 293T cells. Control cells were transfected with EGFP-C2 which lacked the RSEP4 coding sequence. Three micrograms of EGFP-RSEP4 or EGFP-C2 were transiently transfected into human embryonic kidney 293T cells by calcium phosphate transfection method [12]. Twenty-four hours after transfection, cells were washed with PBS, and fixed 40 min in 4% paraformaldehyde. To visualize nuclei, cells were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Photographs were taken with Leica TCS SP2 laser confocal microscope.

Results

Isolation of the cDNA of a novel rat gene, *RSEP4*

DNA sequence analysis showed that one of the cDNA clones from the low abundant rat brain cDNA sublibrary is a novel gene. The total length of this cDNA is 2006 bp, encompassing a 501 nucleotide ORF that encodes a 167 amino acid residue polypeptide (GenBank accession No. AY540309) (Fig. 1). The cDNA also contained a 279 bp 5′ untranslated region (UTR), as well as a 1223 bp 3′-UTR. There are two polyadenylation signals (AATAAA), one of them is localized at 19 bp upstream of the poly(A)$^+$ tail [14] (Fig. 1). We compared the deduced amino acid sequence of this cDNA with a non-abundant protein database using the BLASTP algorithm program, and found there was no homologous ortholog. We, thus, named this rat novel gene as *RSEP4* and compared its cDNA sequence with the rat genomic sequence at NCBI by the genomic BLAST algorithm (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html). The program identified matching on the rat chromosome Xq34.

Tissue distribution of RSEP4 mRNA

Northern blot analysis was used to analyze the tissue

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   1 ttgggagttcccgtctcagttcagggcttcttttaacttctgcacgatcctttacacagggaggtgcacgcgtag
  76 ggcattaggacccagcgattgttgtagtgagagtggttgaatctcgatctctcaatttttctctccagctccctc
 151 ctctccctttgtcattctctctgctgcctccgcggcgtgcctctagctctccctgtgctaactgcccgcgccttg
 226 acagccgggctgcgcaaagcagagggattagttgggacctgccttggtgacccc
 280 atggcatccccacgaaccataactattgtggccctctctgtggccctgggactcttctttgttttcatggggact
       M  A  S  P  R  T  I  T  I  V  A  L  S  V  A  L  G  L  F  F  V  F  M  G  T   25
 355 atcaagttgacccccaggctcagcaaggatgcctacagtgagatgaaacgtgcttacaagagctatgttcgagcc
       I  K  L  T  P  R  L  S  K  D  A  Y  S  E  M  K  R  A  Y  K  S  Y  V  R  A   50
 430 cttcctctgctgaagaaaatgggcattaattccattctccttcggaaaagcattggtgcgcttgaagtagcctgt
       L  P  L  L  K  K  M  G  I  N  S  I  L  L  R  K  S  I  G  A  L  E  V  A  C   75
 505 ggcattgtcatgaccctggtacccggtcgtcccaaagatgtggccaacttcttcctgctcttgctggtgttggct
       G  I  V  M  T  L  V  P  G  R  P  K  D  V  A  N  F  F  L  L  L  L  V  L  A  100
 580 gtgcttttcttccaccagctggttggcgatcctctcaaacgttatgcccatgctctggtgtttggaatcctgctc
       V  L  F  F  H  Q  L  V  G  D  P  L  K  R  Y  A  H  A  L  V  F  G  I  L  L  125
 655 acctgccgcctgctgattgcccgcaagcctgaagaccggtcttctgagaagaaggctttgcctgagagtgcagaa
       T  C  R  L  L  I  A  R  K  P  E  D  R  S  S  E  K  K  A  L  P  E  S  A     150
 730 gagcaaccgtccttatatgagaaggccccacagggcaaagtgaaggtgtcataa
       E  Q  P  S  L  Y  E  K  A  P  Q  G  K  V  K  V  S  *                       167
 784 aaattggaagagcaaagaatggaccttctaggcagttgcttctttgacacccaagatgacattagtgtgtatgtg
 859 tgtgtgtgttttcctttgatttatttatcttgggagtaaagagaaaaatacaatctgtaagttacttggcttgtg
 934 tacatctataccctaaaatgacctccccacattgacatccgtgtgccacctttaatcactctggagcaacattca
1009 tcttgctgcatgtatatgtgtatgaatataattgaggtgtatttgtgagatggactccagcaagcatgtgactga
1084 gattatgtgtgggggaacgtatataactgccatgtgtgtgggtatacacacacatatagaggaggttcagatctt
1159 gaactaatcctgcacaagtatccttccctctataaattaattccagcaaagacagaataaaagaaccatcccgta
1234 aagagagttgtttctggtggaaaacaaataacctagtttgattcttcaagcaatgcagtaggaggggtctccttg
1309 cctttgtccgagcccaagcaactgagctctttagtggtttcacattcgattcgtgacctttactagacaccttc
1384 aacttttgttgtaagcacaaagctgccccactttaccattatcagcagccaaccaggatctgaagcttccccatc
1459 gtttccattgagggaccagagattggtcctggtacaagtttcaatctccagccctagtggttttctgaaagctgt
1534 aattggatggagaagctatagccactgtttatctgtaggaagagttctctactgctattgattccccactgaact
1609 ttgctaaagttaacaaagaccaaaacacccccctagaaatgtctattttacacctcttcagcattccagagggtgg
1684 agtttagctgggggaggacatttgacatgggttagtcagattgaccagtatggaaatttgcttttgtagttagtt
1759 acacactgctgtttctcttggttaaatgtgctttgatggttttaatattgtgccagggatgggaaacggggtggg
1834 gttgtgtgtggggagagtacttcctgtattttcttcagtgccattgttcttggtaattgatacttgtcttctttc
1909 gctcattctttccaataaagagttttgaaatttggaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
1984 aaaaaaaaaaaaaaaaaaaaaa
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Fig. 1 *RSEP4* cDNA and predicted amino acid sequences

The putative amino acid sequence is indicated in capital, 5′-UTR and 3′-UTR are indicated in lowercase. The number in left side is nucleic acid position and in the right number is amino acid position. The translation initiation codon is boxed, the stop codon is marked with the asterisk and putative polyadenylation signals are underlined.

expression pattern of *RSEP4* in rats. As shown in Fig. 2, RSEP4 mRNA could be detected specifically in the CNS, including the cerebrum, cerebellum, hippocampus, brain stem and spinal cord with the highest expression level in the hippocampus. The RSEP4 expression, however, could not be detected in the non-nerve tissues such as, heart, liver, spleen, lung, kidney, testis and small intestine. There are two bands in the Northern blot of RSEP4, the major transcript is at about 2.0 kb and the minor one is at 4.4 kb.

Expression pattern of RSEP4 gene in the CNS

In situ hybridization was used to investigate the expression pattern of RSEP4 gene in the CNS of rat (Fig. 3). In the cerebral cortex, RSEP4 mRNA was expressed in the posterior part of the prepiriform cortex (PCp), the auditory area of the temporal cortex (Te Aud), and the superior thalamic radiation (Str), with the strongest expression in the granule cells of hippocampal CA1, CA2, CA3 and dentate gyrus (DG) regions [Fig. 3(A,B)]. The transcript of RSEP4 could also be detected throughout the cerebellar cortex especially in the Purkinje cell layers [Fig. 3(C,D)]. In the spinal cord, RSEP4 mRNA was mainly expressed in the gray matter of spinal cord. The expression level is high in laminae I-II and V-VI small sensory neurons in the dorsal horn and even higher in the large neurons of ventral horn [Fig. 3(E,F)]. No signals were detected with sense probes (data not shown).

Subcellular localization of RSEP4 fusion protein

The expression construct of EGFP-RSEP4 fusion protein was transfected into human embryonic kidney 293T cells, and the EGFP fluorescence was analyzed by the confocal microscopy. As shown in Fig. 4, EGFP-RSEP4 fusion protein was localized solely in the cytoplasm, and there was no nucleus localization [Fig. 4(D,F)]. By contrast, the control plasmid (EGFP-C2) showed a uniformed distributed at both cytoplasm and nucleus [Fig. 4(A,C)]. These results suggested that RSEP4 protein was likely localized in the cell cytoplasm.

Discussion

In this study, through differential screening a low-abundance cDNA sublibrary, we identified a novel rat gene, *RSEP4*. Northern blot analysis revealed that *RSEP4* was expressed specifically in the CNS. *In situ* hybridization showed that the high expression of *RSEP4* was in the CA1, CA 2, CA3 and DG regions of hippocampus, the Purkinje cells of cerebellum and the small sensory neurons in the

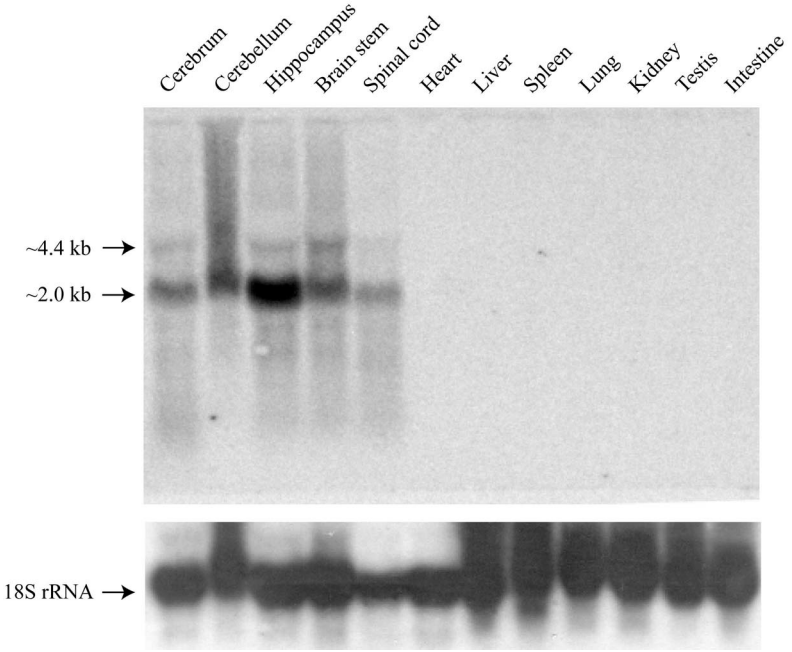

Fig. 2 Northern blots analysis of the expression of rat *RSEP4* gene

Twenty micrograms of total RNA from various tissues of 8-week adult rats were resolved in the agarose gels, and transferred to N^+ nylon membranes and hybridized with a ^{32}P-labeled probe for rat *RSEP4*. The sizes of RSEP4 mRNA were indicated with arrows in the left. The 18S rRNA is used as the loading control.

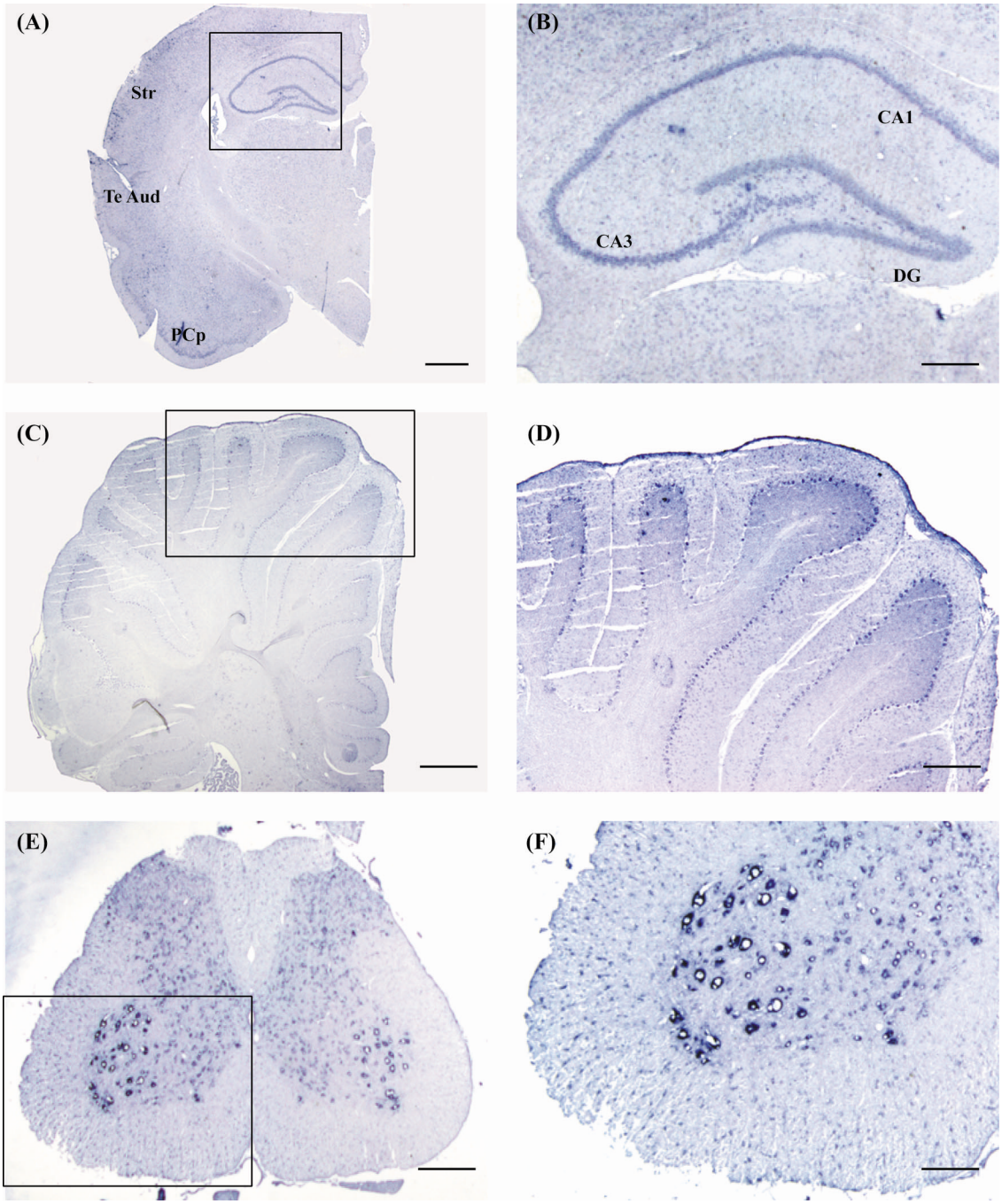

Fig. 3 ***In situ*** **hybridization of rat cerebrum, cerebellum and spinal cord for probing RSEP4 mRNA expression**

Consecutive sections from cerebrum (A), cerebellum (C) and spinal cord (E) of the 8-week rats were hybridized with digoxigenin-labeled cRNA probe from rat RSEP4 cDNA. (B), (D), and (F) The partially magnified images of (A), (C), and (E) respectively. PCp, posterior part of the prepiriform cortex; Str, the superior thalamic radiation; Te Aud, the auditory area of the temporal cortex; DG, dentate gyrus. Bars: 1000 μm in (A) and (C); 500 μm in (D); 250 μm in (B) and (E); 100 μm in (F).

dorsal horn and the large neurons in the ventral horn of spinal cord.

In the dorsal horn of the spinal cord, RSEP4 was expressed in laminae I-II and V-VI, where most neurons had responses to nociceptive stimulation [15], suggesting that this gene was involved in modulation of neuropathic pain.

Numerous studies from human and animal have

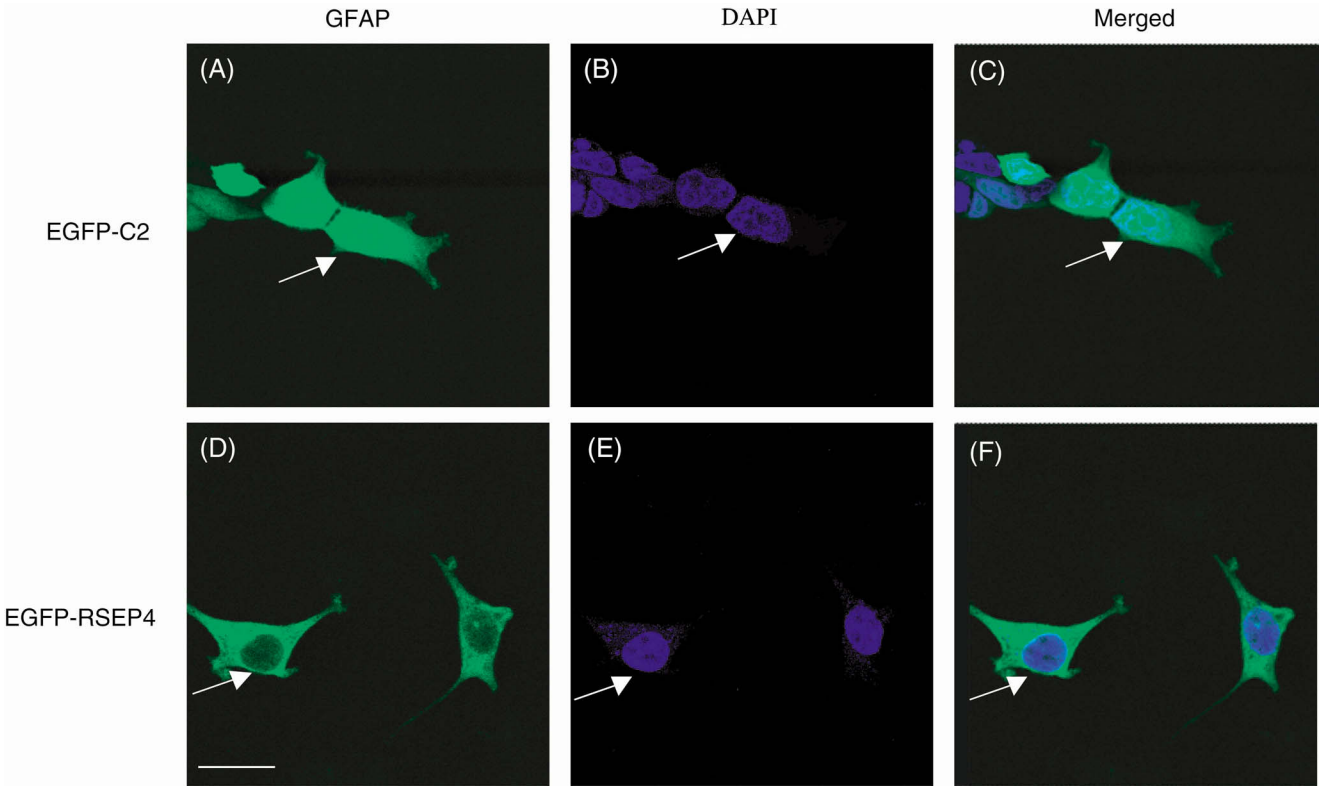

Fig. 4 Subcellular localization of EGFP-RSEP4 fusion protein

The expression plasmid of EGFP-RSEP4 fusion protein (D–F) and control plasmid of EGFP-C2 (A–C) were transfected into human embryonic kidney 293T cells. Twenty-four hours after transfection, the expression of the fusion protein was detected by confocal microscopy (A,D). Nuclei were visualized by DAPI staining (B,E), and the merged images were shown in C and F. The nuclei were indicated by the arrows. Bar, 20 μm.

indicated that the hippocampus including CA1, CA2, CA3 and DG regions is important for the behaviors including learning and memory [16–22]. The cerebellum, which contains the innermost granule cell layer, the Purkinje cell layer and the outmost molecular layer, is crucial for the movement coordination [23–25]. The spinal cord is the unique pathway for information connecting the brain and peripheral nervous system. The large neurons in the ventral horn of spinal cord are the α-motor neurons, which send their axons out via the spinal roots and directly control the muscles [26]. *In situ* hybridization analysis showed that *RSEP4* mRNA was strongly expressed in the CA1, CA2, CA3 and DG regions of hippocampus, the Purkinje cell layer of cerebellar cortex and the α-motor neurons of spinal cord (Fig. 3), suggesting that RSEP4 gene might play some important roles in the learning and memory behavior, the coordination of movements as well as the muscle contraction.

In summary, we have isolated and characterized a novel rat gene, *RSEP4*, from rat spinal cord, and found that this gene expressed specifically in the CNS. Future work will be needed to elucidate the functions of this gene in the CNS.

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