

Nerve Injury Enhances Rat Neuronal Glutamate Transporter Expression: Identification by Differential Display PCR

Sumiko Kiryu, Gui Lan Yao, Naonori Morita, Hidemasa Kato, and Hiroshi Kiyama

Department of Neuroanatomy, Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan

An increase in neuronal glutamate transporter expression after nerve injury was demonstrated by means of differential display PCR (DD-PCR) coupled with *in situ* hybridization. DD-PCR was carried out to compare differences in expression of mRNAs between axotomized and normal hypoglossal motoneurons in the rat. The expression of several gene fragments were found to be increased following nerve injury; the full length cDNA corresponding to one fragment was cloned by subsequent rat cDNA library screening. The close homology of glutamate transporters with our rat cDNA led us to conclude that this clone corresponds to the rat neuronal glutamate transporter (rat EAAC1). We speculate that the upregulation of this glutamate uptake system may increase the resistance of these cells against neurotoxic glutamate accumulation during the process of nerve regeneration.

[Key words: nerve regeneration, hypoglossal nerve, neuronal high affinity glutamate transporter, glutamate toxicity, cell death]

Nerve regeneration is a complex process involving many metabolic and catabolic pathway. Previous reports have demonstrated that molecules such as growth factors (Heumann et al., 1987a,b; Eckenstein et al., 1991; Kobayashi et al., 1993), some peptides (Saika et al., 1991) and cytoskeletal proteins (Tetzlaff et al., 1991; Tsui et al., 1991) are upregulated during peripheral nerve regeneration. However, the molecular events associated with axotomy are still not well understood.

Recently, we have focused our attention on several molecules that are involved in the signal transduction pathways during hypoglossal nerve regeneration (Ohno et al., 1994; Saika et al., 1994; Kiryu et al., 1995; Morita et al., 1995). These studies implicate some signal transduction pathways such as that involving Ras pathway in regeneration associated signaling pathway. By examining individual intracellular signaling molecules associated with regeneration, we hope to gain insight into the phenomena underlying of successful nerve regeneration. However, it is likely that a number of other so far unknown molecules and genes will play an important role in nerve regeneration.

Therefore, an alternative method to quickly isolate those genes which may be involved in the nerve regeneration event, has been sought. The development of a new technique termed differential display PCR (DD-PCR) has considerably simplified the identification of genes upregulated after axotomy or lesion, replacing earlier subtractive strategies (Liang et al., 1992; Liang and Pardee, 1992). The method is based on directly comparing the mRNAs expressed in two or more cell populations, separating their reverse transcription-PCR products and comparing band patterns. We have used this method to identify a gene not previously implicated in peripheral nerve regeneration. Using this approach, we identified the rat homolog of the neuronal high affinity glutamate transporter (rabbit EAAC1 or human EAAT3) (Kanai and Hediger, 1992; Meister et al., 1993; Arriza et al., 1994) and showed that this glutamate transporter is upregulated in injured motoneurons. High affinity glutamate transporter have recently been defined by molecular cloning (Kanai and Hediger et al., 1992; Pines et al., 1992; Storck et al., 1992; Arriza et al., 1994) and represent a new family of Na⁺ and K⁺ coupled electrogenic transporters, which have no significant primary structural homology to the superfamily of Na⁺ and Cl⁻ coupled transporters (Amara, 1993). These transporters mediate the cellular uptake of acidic and neutral amino acids from the synaptic cleft into glial cells (GLAST, GLT1) or nerve endings (EAAC1). Since glutamate is neurotoxic (Benveniste et al., 1984; McBean et al., 1985; Choi et al., 1988) as well as being the principal excitatory amino acid neurotransmitter (Fonnum et al., 1984), transporter-mediated uptake systems may serve to maintain extracellular glutamate concentrations below toxic levels (Nicholls et al., 1990; Eliasof et al., 1993). It has also been suggested that several neurodegenerative disorders are due to dysfunction of glutamate transporters (Rothstein et al., 1992). In this study, we show that rat EAAC1, cloned by DD-PCR, is associated with axotomy-induced nerve regeneration. In addition, the distribution of rat EAAC1 mRNA was demonstrated in the adult rat brain.

Materials and Methods

Animals. Seventy male Wistar rats weighing about 100 gm were anesthetized with pentobarbital (45 mg/kg), positioned supine and their right hypoglossal nerve cut with scissors, the hypoglossal nuclei were then dissected from the operated- and normal-sides (Fig. 1A). Seventy hypoglossal nuclei (operated and normal) were collected and frozen in liquid nitrogen. For *in situ* hybridization, rats were sacrificed 1, 3, 5, 7, 14, 21, 28, and 35 d after the operation (three rats each point).

Differential display PCR (DD-PCR). DD-PCR was carried out (Fig. 1A) as previously described (Liang et al., 1992; Liang and Pardee, 1992; Bauer et al., 1993). Total RNA (approximately 70 µg) was obtained from either the operated or the normal hypoglossal nuclei 7 d after surgery. Total RNA (each 0.2 µg) was converted to cDNA with superscript reverse transcriptase (GIBCO/BRL) and nucleotide oligo dT₁₈.

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Correspondence should be addressed to Dr. Hiroshi Kiyama, Department of Neuroanatomy, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan.

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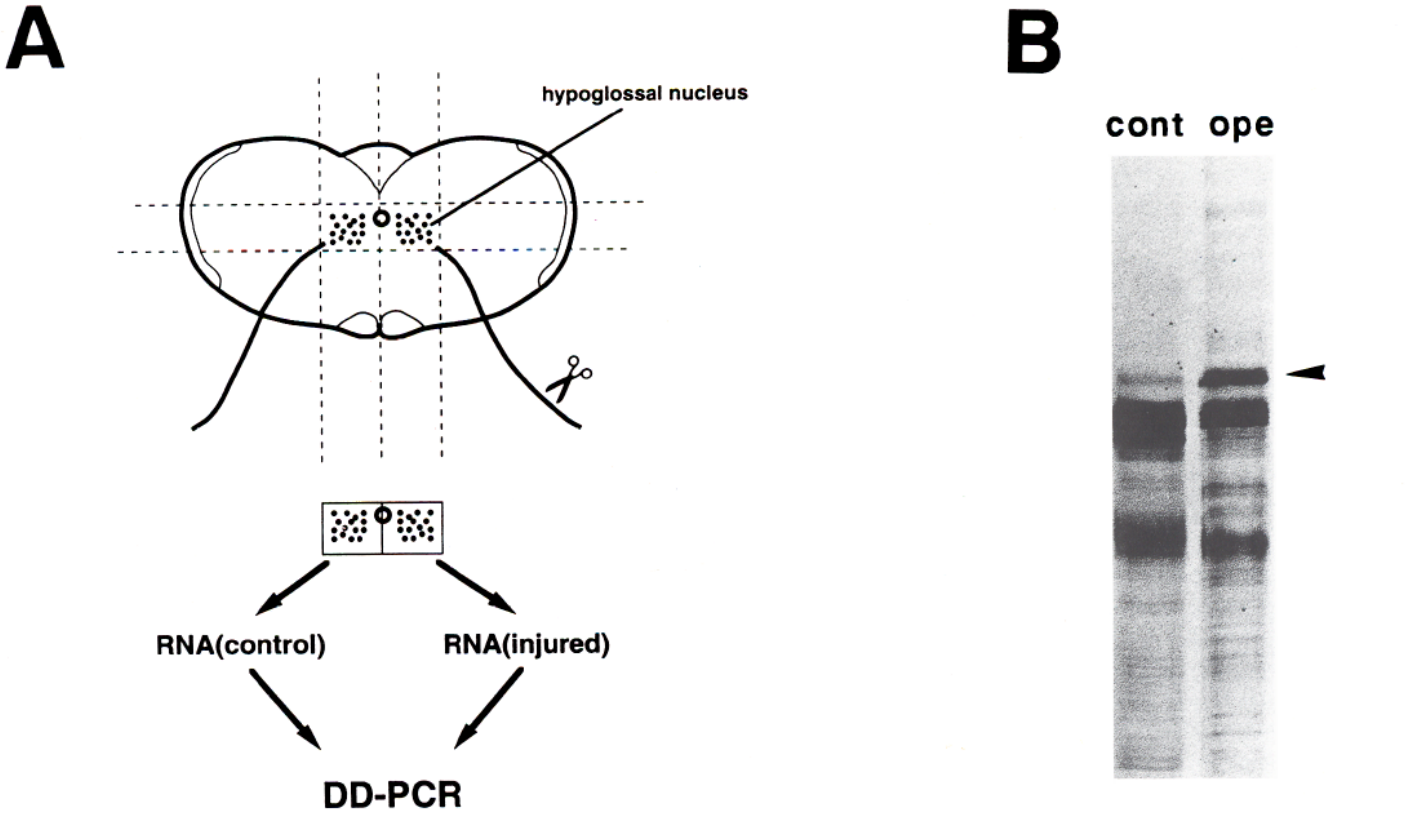


Figure 1. *A*, Preparation of tissue for DD-PCR. Differential display comparing mRNAs from both the normal (*left*) and injured (*right*) hypoglossal nuclei; tissue was carefully dissected and total RNA was prepared from each hypoglossal nucleus. *B* shows autoradiography of amplified ³⁵S-labeled PCR products (after electrophoresis in a 5% polyacrylamide gel) using two arbitrary primers. The *left lane* corresponds to PCR products derived from the normal hypoglossal nucleus and the *right lane* is from the operated side. *Arrowhead* indicates a differentially expressed band which is located at about 200 bp in size.

Subsequently, 1/10 volume of the each pool of cDNA was amplified by PCR in the presence of α -³⁵S dATP (New England Nuclear-Du Pont, Natick, MA) using two arbitrary primers, 5'-AGGGGAAGTCTGGGTCGTC-3' and 5'-GGCCTTCATGTTAATGATGCAATTAAGTCT-3', which were selected among 87 arbitrary primers by chance. The cycling parameters are as follows: denaturation at 94°C for 5 min, 40 cycles with denaturation at 94°C for 30 sec, annealing at 42°C for 1 min and extension at 72°C for 1.5 min and an additional extension period at 72°C for 5 min. Radiolabeled PCR products were analyzed by electrophoresis on a 5% sequencing gel, and visualized by autoradiography. Differentially upregulated bands were

recovered from dried denaturing polyacrylamide gels and reamplified in a 40 cycles PCR using the same primers. Reamplified cDNA products were cloned into PCRTMII vectors using the TA cloning kit (Invitrogen, San Diego, CA).

In situ hybridization. Animals were decapitated 1, 2, 3, 5, 7, 14, and 21 d after surgery. Their brains were removed quickly and frozen in powdered dry ice; 20 μ m thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane coated slides, and stored at -80°C until used. Just before use, the sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min, washed in PB, treated with 10 μ g/ml proteinase-K in 50 mM Tris-HCL and 5 mM

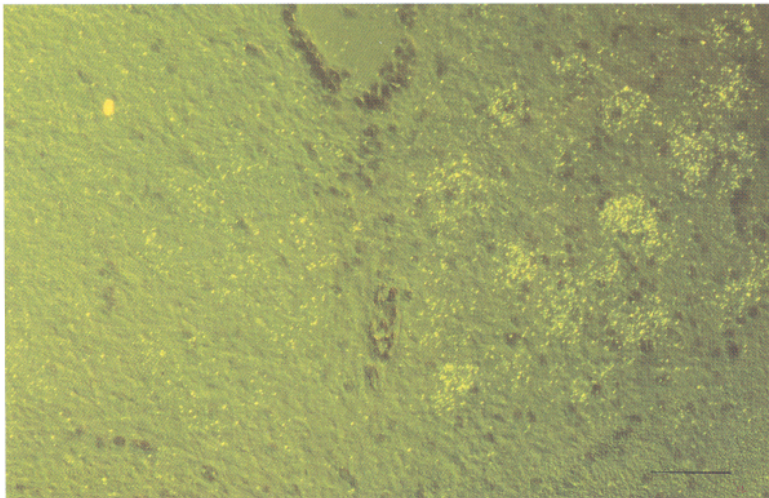


Figure 2. Histological display by *in situ* hybridization using the cRNA probe derived from cDNA fragment shown in Figure 1*B* (*arrowhead*). The section was obtained from an animal whose unilateral hypoglossal nerve was axotomized 7 d before (*right side* is the operated side). The counterstained emulsion autoradiography is observed under semi-darkfield observation. Note the accumulation of silver grain is observed only on the injured motoneurons. Scale bar, 40 μ m.

GGT CCC CAG CCG CAG TGG CAG CTC TGA CAG CCG CCG CTC GCG TGT GTC TCC ACC GTG CCC 60
 ATG ATC CCT CAT CCC ACA TCC GCG CCG CTC CCG TCC TCA CAA ACG CCG GTG ACC ATC 120
 ATG GGG AAG CCC ACC AGC TGG CAG TGC GCG CCG TGC CCG CTC GTG CCG AAT CAG TGG CTT 180
 M S K P L S G C D M R R F L R H W L S 240

CTG CTC TCC ACC GTG GCG GCG GTC GTA CTG GCG ATC GTG GTA GGA GTC TTT CGA GGA 300
 L E F A D V V A T V L S G L R A R I T M S F L V F S 360

CAG AGT GAG CTC TCC AAT CTG GAT AAA TGC TAC TTT GGG TTT CCT GGA GAA ATT CTG ATG 390
 H S E L S N L D K F V F G F E R A V F I L M 420

CGG ATG CTG AAG CTC GTA ATT ATG CCA CTG ATT ATA TCC ACG ATG ATC ACA GGT GTC GCT 450
 R H L K L L V I M P L S S H I T G V A 480

GCA CTG GAT TCC AAC GTA TCT GGG AAA ATT GGT TCT TGT GCT GTA GTA TAT TAT TTC TCC 490
 A L D S N V S L G K I L G T V L V G V A V V Y Y F S 520

ACC ACC GTC ATT GCT GTA ATC CTA GGT ATG GTG GTA GTG AGC ATC AAC CCT GGT GTC 540
 T T V I A V I L G I T V L V G V S I K P G V V 580

ACT CAG AAA GTG AAT GAA ATC AAC AGC ACA GGC AAA CCT CTT GAA GTC AGC ACT GTG GAC 590
 T Q K V N E I R N C R F P E V G A C T P E V S T V D 600

GCC ATG TGG GAT CTG ATC AGG AAC ATG TCC CTT GAG AAC CTG GTC CAA GCG TGT TTT CAG 630
 A M L D L I R N M T F C F E N L G I I I F A C F Q 660

CAG TAC AAA ACC AAG CCG GAA GAA GTG AAG CCG GGT AGT CCT GGG GCG AAC CAG ACA 690
 Q Y K T K R R E E G A P A S D P E G G N Q T 720

GAG GTG TCT GCT ACC ACG GCG ATG ACA ACA TGT GCT GAG A A K A A G A A G A A T A C A A A 750
 E V S V T T A M T M S E N K A A K A A G A A T A C A A A 800

ATC GTG CCG CTG TAC TCA GCG ATC ACA GTC CTG CCG GTC ATT ATC TTC TGC CTC GTC 810
 I V G L V I G S D G I N V L R I M S F L V D 840

TTT GGA GTC CTT ATT GGA AAA ATG GGA GAA AAA GGG CAG ATT CTG GTC GAT TTC TTT AAT 870
 F G L V I G G A A A G A A A G G C A T T C T G G T A T T T C L V F F N 900

CGC TGG AGT GAC CCG ACC ATT AAA ATT GTC CAG ATC ATC TGT TAC ATG CCG ATC GGT 930
 L S D A T M K A T H K I F I M C I T M S F L V F 960

ATT TTG TCT CTA ATC GCT GGG AAG ATC ATA GAA GGT GAA CAG TGG GAA ATA TTC GCG AAG 990
 I E L F A N L V R A T A L V D W E I F R R K 1020

TTG GCG CTT TAT ATG GCG ACT CTC GTG AGT GGG CTT GCA ATC CAC TCC CTC GTA GTG CTG 1050
 L V L F M A T V L S G L R I A H S L V L F 1080

CCA CTG ATC TAT TTA ATA GTC CTC GGG AAG AAC CTT CTC CCG TTT GCT TGG GCG ATG GCG 1090
 F L I V R A T E F V R L G M H A V A 1120

CAG CTT CCG CTG ACA GCT CTC ATG ATC TCA TGT ACG TCG GCA ACC CTT CCA GTT ACA TTC 1140
 Q A L L L T A L M I S E R L P V V T F 1160

CGC TCT GCG GAA GAA AAG AAC CAG CTG GAC AAG AGT ATC ACA AGA TTT GTC CTG CCG GTC 1200
 R C A E F A N L V R A T A L V D W E I F R R K 1260

GCC GCG ACC ATC AAC ATG GAC GCG GCT GCG CTC TAT GAA CCG GTG GCA GCT GTC TTC ATT 1290
 G A T I N M H D G T A L V G T A L V D W E I F R R K 1350

CGC CAA GTG AAC GGC ATG CAG CTG AGC ATT GGG CAG ATC ATC AGC ATC AGC ATC ACA GCC 1320
 A A V N G M D L S I T I S I T A 1380

ACC GCT GCT AGC ATT GGA GCT CCG GGT GTC CCG CAG GCT GCG CTG GTC ACC ATG GTG ATC 1400
 T A A S I G A A G A V G V P A G L V T M V I 1420

GTG TTG AGT GCG GCG GGG CCG CTT CAG GAG CAG ACC CTT ATC ATT GCG GCG GAC TGG 1440
 V L S A V G L L P A E D V T L I I A V D W 440

CTC CTG GAC CCG TTC AGG ACC ATG GTG AAC GTT CCG GGT GAT GCG TTT GGG ACG GGC ATC 1500
 L L D R F R T M V M V L G G T A F G T I 460

GTG GAG AAG CTG TCC AAG AAG GAG CTG GAG CAG GTG GAT GTC TCC TCT GAA GTC AAC ATT 1560
 V E K L F A S K K E L E G V D S E V M I 480

GTG AAC CCG TTT GCG TGG GAG CCG ACA ATC CTG GAT AAT GAA GAC TCA GAT ACG AAG AAG 1620
 V H F A N L V R A T A L V D W E I F R R K 1680

TCT TAC GTC AAT GGG GCG TCG TCG GTA GAC AAA TCT GAC ACC ATC TGG TTT ACT CAG ACC 1690
 S V A N D K S D T I L P T G 1720

GTG CAG TTC TAG ATG CCT GGT TCT AGA TCG CCG GGA CTG TGA AGG GCA TCT CCG AAG GAG 1740
 Q F P 1760

CCA TCT CTT AGC CAA CTC CCG GGT TAA GCA CCG AAG AGG ACA AGA ATC AAC TGT ACA 1800
 TTT AGT TTT ATG ATA AAG CCT CCA GAT TAT TTT GTA TAT TTG GCT CTA TAC GCT TTG TCT 1860

TCT GGG TTT GGG AAC GTG GGG TGG GAT GAA CCG AAG GAA ATT AAG AAA GCT GTC TTA TCT 1920
 GSA CTT TCT AAT TCA GCA GTA TAT GAC GAA CCG GCA CCG GCA CCG GGT CTA GTC GTC 2160

AGG TCA TGG GTC TGG GAG AAA ATT TCT TTT CTC AMG CAA ACA ATA TTT GGG GTT TTT 2040
 AAA CAA TAT TAT TGG CTA CAA ATT TTT CAG GCT TTC TAT TGG CCG GGC TTC CTT TCT 2100

TTT CTC ACT TTT TCA TTA TTT TCT AGC TCA AAA GCG CCA CCG AGT CCA GTC GTD CCG 2160
 AAT TTT CCA TTT TGC CTT ACT CCA GCA CTC TCA ACT AGT AGT GCG AAA TTT TCC ATT TTG 2220

ACC TCA TCT CCA GCA ACT CTC AAA CTA CTT TGG GGA AAA AAA AAG CAA AAA GGA TCA 2280
 GCA TAG TCT GCA ATA ACA GTT CCA AGA TAT TGT GGG CTT GGG AAG GGA AGG GTT TTT 2340

TAT TCA ATG TAC TGT ATT GGG ACC CCG GAT GTC AGT GGT CCG TAT AGA ACT ATA TGT ATA 2400
 TGT GTG TAC AAT TAT TAT TTT CAT GTA ATT TCT AAG ACA GAG ATC AGT AAT GAA CTA TCA 2460

ATG TCA ACA AAT CTT TAT TTT AGT TCA ATC AGG GCT TTA ACT AGG GCT TTA ACT AGG GCT 2520
 TTC AGA GCT CAG GAG GAC CTT CTC AGG CCG TCT GGG GTG AAG AAT CAA GTT GCT GGG TTA 2580

CTG TGC TCC AAG CCC TAA AGG AAG TCT AGT GTG GCG CAG CAA TCC ATT GAG AAC ATC 2640
 TAA GCT CAC GCT ACC TGG AGG GGA AAC CTT TGT GCG GCA TGG AAT GAG TAC ATT TAC 2700

AAA GGG ACC GTA GAG GGA AGA TGT GCT CCG ATA GCA ACA GGA GGC TTC ATT CTT CAA GTA 2760
 CAC TTC AAG GCG CAC TTC ACA AGG CTG TCA CCG CTT TTC TGC CTT TCT CTA ACC ACC 2820

AGG GCG AAA CCA GCA GTC GCG ACC CAT CAC CTA AGC TGT TCC AAA ATG GGC TGC ATA AAT 2880

TGA ATA AGC TTC ATC GGA AGG TGA CCA AGC TAT TCT AAT ACT GTA GTC TTT TTA TAT TAG 2940
 CTT TTC TTA TTT TAT TTT AGT TTT TTA AGA CAG AGT TGT TCT AON TAG CCG ATG 3000

GCC TGG AAC TCA CTA TGT AGA CTA CAG TGG CCT CAA ACT TGT AGA GAT CTT CCG AGT 3060
 TCC TCG TTA GTG CCG TGA AAG GCA AGT GCG ACC ATG CCG ACT TAT ATG CTT AAT TCT 3120

TAC AGA ATA AAA ACC GCA AAT GCG CCA CAG CTT ACT GAT ACC AGT AGT TGT TGT GGG 3180
 APT CTT CAG TCT GTC TCT GGA GAA CAG TTT TCT GAA AAT CCT GGC CTT GTG GCT TAG AAT 3240

CTG CTT GCT GTC TTT CTC CTO CTA ATT TCT GAA GAT GAA CTA TTA AAA TTC TAC ACT TCT 3300
 CCG ACC CTT GTC CTA ATC ACA ACC AAC CCA AGT TTT TTA ATG TGG AGG TAT CCG TAG 3360

CAG CAG TCT TGT TGT AGT GCG CTT AAC TCA TCT TAA ACC CCG GCT TTT ATA TTT GAG AAG 3420
 CCA GAA ATC GTG CCA AAG ATA GCA AAG TAA ACG AGT GTT GAT GAT CAG CAT GGC CAG 3480

CAT CAG CTT GAC CTT GAT AGT GCA CCA CCG ACT TTT AGT TAT AAA TAT CCG TAG 3540
 TCT TTA TAG GAA CCA AAT CTA AAT CTA AAT GGT GGA TGA ATA CTT TAT GCA GGA ATG 3600

GCT GGT TCT GTC CTT GAT GGT GCT GAT CAG TGG CAC TGA ATT AAT TGG CAG AGC 3660
 TCT TCA CCA GTT CAG TGG GGT AAT CTT TGT AAT CAG CTG CTT AAC CTA CTA TTT TAT 3720

CTC AAG AGC ACC CTT CTT ATT TTT CTC AAT AAA CAT CTC CCA CAC TTT CTT CTA AAA 3780
 AAA AAA AAA AAA AAA 3795

EAAC1 rat 1:MGKPTSSGC-DWRRFLRNHMLLSTVAAVLGVVGLVRHSELNLDKFFAFPGELL
 EAAC1 rabbit 1:....ARK..DS-K...K.N.V.....V.....I.....EY.N.T.
 EAAT3 human 1:....ARK...PS.K...K.N.V.....TT.....E..N.T.E.....

EAAC1 rat 61:MRMLKLVIMPLIISSMITGVAALDSNVSGKIGLCAVVVYFTTIVAVILGIVLVSIKPG
 EAAC1 rabbit 61:.....L..V.....R..L..C.I.....TASD.T.K.G
 EAAT3 human 61:.....I.L.....R.....C.L.....

EAAC1 rat 121:VTQKVNENRTGKTPVSTVDAMLDLIRNMPENLVQACFQYKTKREEVKPASDPGNG
 EAAC1 rabbit 121:.....D..D..S.....R.....L..C.I.....TASD.T.K.G
 EAAT3 human 121:.....G..A...S.....P...EM.M

EAAC1 rat 181:TEVSVTAMTT-MSENKTKYKIVGLYSDGINVLGLIIFCLVPLVIGKMEKGGQILVDF
 EAAC1 rabbit 181:..E...AV...R...RV.....V.....V.....
 EAAT3 human 181:..E.F.AV...AI.K.....M.....V.....

EAAC1 rat 241:FNALSDATMKIVQIIMCYPGIGLFLLAGKIIIEVDWEIFRKLGLYMATVLSGLAIHSLV
 EAAC1 rabbit 241:.....L.....V.....I.....
 EAAT3 human 241:.....L.....T.....I.....

EAAC1 rat 301:VLPLIYFIVVRKNPFRFALGMAQALLTALMISSSSATLPVTFRAEAKNHDKRITRFVL
 EAAC1 rabbit 301:I.....M..T.....R.....
 EAAT3 human 301:I.....M.....N.Q.....

EAAC1 rat 361:PVGATINMDGTALYEAVVFLAQVNGMDLSIGQIITISITATAASIGAGVQAGLVTM
 EAAC1 rabbit 361:.....L.D.....V.....
 EAAT3 human 361:.....L.DL..G.....S.....

EAAC1 rat 421:VIVLSAVGLPAEDVTLIIAIVDMLDRFRMTVNVLDGAFGTGIVKLSKKELECVDSVSEV
 EAAC1 rabbit 421:.....V.....M.....
 EAAT3 human 421:.....M.....

EAAC1 rat 481:NIVNPFALPEPTILDNEDSDTKKSYVNGGFSVDSKSDTISFTQTSQF
 EAAC1 rabbit 481:.....SAT.....I...A.....
 EAAT3 human 481:.....S.....A.....

Figure 4. Alignment of neuronal high affinity glutamate transporter sequence. Regions of serine rich motif (SSSS) and conserved amino acids (AAXFIAQ) are underlined. Sequences are taken from the following references: EAAC1 (Kanai and Hediger, 1992), EAAT3 (Arriza et al., 1994).

500 µg/ml yeast transfer RNA, and 200 µg/ml salmon sperm DNA) were denatured for 2 min at 80°C, quenched on ice and placed on the these sections. Hybridization was performed in a humid chamber overnight at 55°C. Hybridized sections were rinsed briefly in 5 × SSC-1 % 2-mercaptoethanol at 55°C, washed in 50% deionized formamide, 2 × SSC and 10% 2-mercaptoethanol (high stringency buffer) for 30 min at 60°C. After rinsing the sections in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA), they were treated with 1 µg/ml RNase-A in RNase buffer for 30 min at 37°C and washed in RNase buffer. Sections were then incubated in buffer as described above, rinsed with 2 and 0.1 × SSC for 10 min each at room temperature, dehydrated in an ascending ethanol series, and air dried. Sections were then exposed to x-ray film for 2 weeks, whereafter they were dipped in Ilford K-5 photoemulsion (Ilford, UK) diluted 6:4 in water. Sections were then exposed for 3–4 weeks at 4°C, developed in Kodak D19 developer, counterstained with thionin, dehydrated in a graded series of ethanol to xylene, and coverslipped before microscopic observation.

Relative quantification of mRNA. The grain intensity on the x-ray film was measured. The relative area occupied by autoradiographic grains in the hypoglossal nuclei was measured bilaterally on the x-ray film using a computerized image analysis system (MCID: Image Res. Inc., Ontario, Canada). In the same sections, we calculated the difference in the optical density between the right (ipsilateral side) and the left hypoglossal nuclei (contralateral side). For statistical analysis, at least eight sections from three rats were studied.

cDNA library screening. A rat brain cDNA library(Uni-ZAP XR Library, Stratagene, La Jolla, CA) was screened with a subcloned PCR-derived DNA fragment to obtain a larger cDNA clone. The probe was labeled with α-³²P dCTP (Amersham, UK) by multiprime DNA labeling system (Amersham, UK). Approximately 1 × 10⁶ plaques were plated at a density of 1 × 10⁴ pfu/150 mm dish and screened following conditions for hybridization: 1 × 10⁶ cpm/ml in 6 × SSC, 5 × Denhardt's and 0.5% SDS for hybridization at 65°C, and washed at 65°C in 2 × SSC and 0.5% SDS. Eleven positive plaques were obtained and purified by *in vivo* excision for plasmids. Both strands of these cDNA inserts were sequenced automatically by the dyeprimer cycle sequence kit using an Applied Biosystems model 373A DNA sequencer (Applied Biosystems, Inc., CA).

Results

Differential display PCR

The expression of mRNAs 7 d after nerve cut were analyzed using DD-PCR. We performed PCR amplifications with two

Figure 3. Nucleotide and deduced amino-acid sequence of the rat EAAC1. Eleven positive plaques were isolated. The location of the cDNA fragment obtained by DD-PCR relative to the original cDNA is underlined. Putative protein kinase C-dependent phosphorylation sites (S/T-X-K/R) are located at residues 87, 115, 121, 164, 247, 340, 466, 500. Potential N-linked glycosylation sites are located at 85, 178, 195. Regions of serine-rich motif and the conserved seven amino acids, AAXFIAQ are underlined in bold. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with following accession number: D63772.

EDTA for 10 min and then fixed again. After washing in distilled water, sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, rinsed with PB, dehydrated in ascending ethanol series (70 %, 95% and 100%), defatted in chloroform, rinsed in ethanol and air dried. ³⁵S-labeled RNA probes were prepared by *in vitro* transcription of the EAAC1 cDNA (2586–2837) in pCRTMII by using SP6 or T7 RNA polymerase and α-³⁵S-UTP (New England Nuclear-Du Pont, Natick, MA). The labeled probes (5 × 10⁶ cpm/ml) in hybridization buffer (50 % deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM PB, 10% dextran sulfate, 1 × Denhardt's solution, 0.2% sarcosyl,

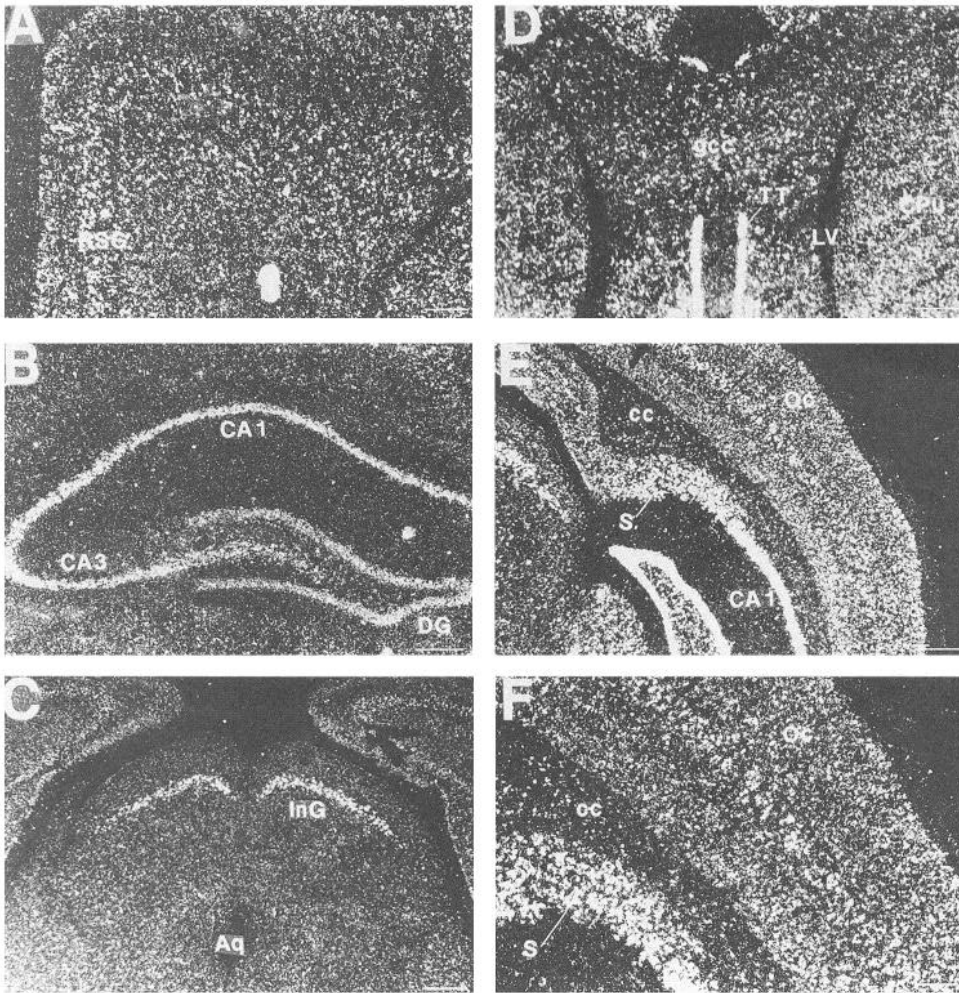


Figure 5. Expression of the rat EAAC1 mRNA in rat brain. *A–C* show a strong expression of the rat EAAC1 mRNA in neurons of the retrosplenial granular cortex (*A*), the hippocampus (*B*), and the superior colliculus (*C*). *D–F* indicate the rat EAAC1 mRNA expression in the corpus callosum (*gcc*, *cc*) at three different levels of the brain. In addition, intense hybridization signal is also observed in various regions such as the CPu (*D*), cerebral cortex (*E*, *F*) and *S* (*E*, *F*). Abbreviations: *RSg*, retrosplenial granular cortex; *CA1* and *3*, fields of CA1 and 3 of Ammon's horn; *DG*, dentate gyrus; *InG*, intermediate gray layer superior colliculus; *Aq*, aqueduct; *gcc*, genu corpus callosum; *TT*, tenia tecta; *LV*, lateral ventricle; *CPu*, caudate putamen; *cc*, corpus callosum; *Oc*, occipital cortex; *S*, subiculum. Scale bars: *A*, *D*, *E*, 300 μ m; *B*, 140 μ m; *C*, 165 μ m; *F*, 120 μ m.

primer combinations. The pattern of amplified cDNA fragments is indicated in Figure 1*B*. A cDNA fragment located at about 200 bp (arrowhead) was amplified to a greater degree on the operated side. This 200 bp band was excised and used for further analysis. In order to eliminate possible false positive signal, the observed increase in mRNA expression was confirmed on tissue sections by *in situ* hybridization. The cDNA fragment recovered from the differential display analysis was reamplified and used as a probe for *in situ* hybridization. This histological survey revealed a significant increase in mRNA expression in cells in the injured hypoglossal nucleus (Fig. 2).

Molecular cloning

We screened a rat brain cDNA library to isolate a full length cDNA clone using radio-labeled cDNA fragments recovered by DD-PCR. Among the 11 positive plaques identified, 4 plaques contained a full length clone. Searching of the nucleotide database, DDBJ, revealed that this clone had high homology with a neuronal high affinity glutamate transporter isolated from rabbit small intestine EAAC1 (Kanai et al., 1992) and human brain EAAT3 (Arriza et al., 1994). The nucleotide sequence of this clone is shown in Figure 3. cDNA sequencing revealed an open reading frame from nucleotides 121–1689 which encoded a 523 amino acid protein. Protein homology revealed a 89% sequence identity with rabbit EAAC1 and a 90% identity with the human EAAT3. Alignment of the amino acid sequences illustrates the

extensive conservation (Fig. 4). Thus, we conclude that this clone is a rat homolog of the rabbit EAAC1 and human EAAT3 clones.

Distribution of rat EAAC1 in the brain

To identify if the cloned glutamate transporter is of the neuronal type or not, *in situ* hybridization histochemistry was performed on fresh frozen rat brain sections. The expression of the rat clone EAAC1 was observed primarily in most neurons with various levels of expression. In particular, neurons in the cerebral cortex, hippocampus, and restricted layers of the superior colliculus (the inter mediate gray layer) expressed substantial amounts of the rat EAAC1 mRNA (Fig. 5). The distribution of rat EAAC1 mRNA in the brain was similar to that of EAAC1 immunoreactivity (Rothstein et al., 1994). In addition, flat glial cells in the corpus callosum, presumed to be oligodendrocyte, also expressed mRNA for the high affinity glutamate transporter (Fig. 5*D–F*).

Expression profile of the rat EAAC1 during nerve regeneration

We carried out *in situ* hybridization to evaluate changes in expression of the rat EAAC1 clone over time during nerve regeneration (Fig. 6, 7). A slight increase in the rat EAAC1 hybridization signal was detected initially in the ipsilateral hypoglossal nucleus 1 d after the nerve cut, however the intensity of the hybridization signal markedly increased to peak levels during

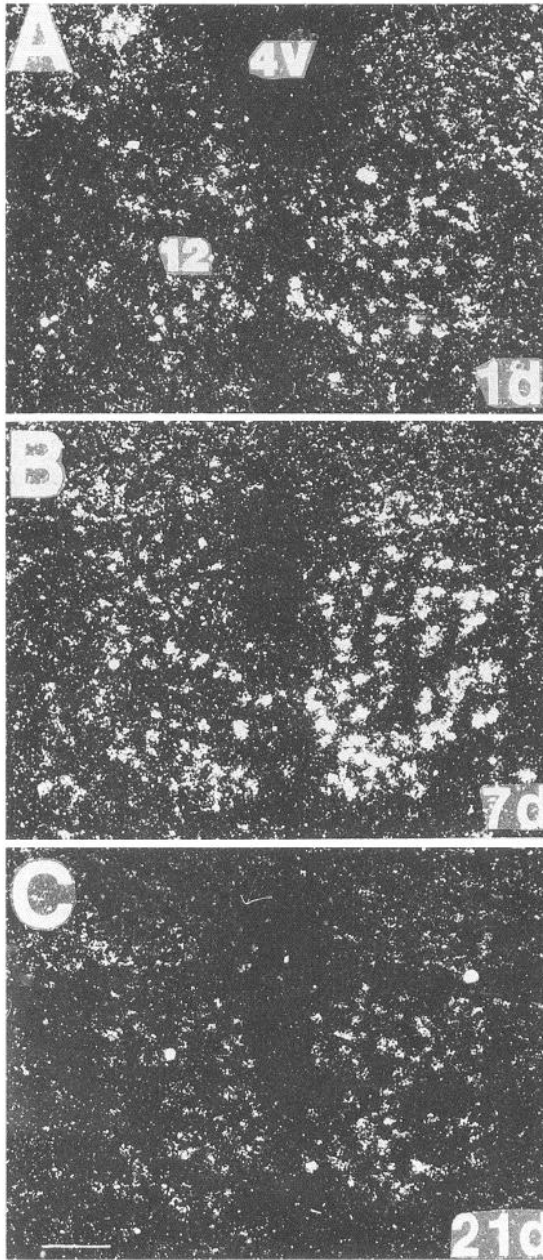


Figure 6. Expression of the rat EAAC1 mRNA in the hypoglossal nucleus 1 d (A), 7 d (B), and 21 d (C) after unilateral hypoglossal nerve transection (right side). 4V, Fourth ventricle; 12, hypoglossal nucleus. Scale bar, 75 μ m.

the following 3 d (Fig. 7) and persisted at this level for about 7 d. Thereafter, the hybridization signal gradually decreased to control level over the following 3 weeks.

Discussion

In the present study, the technique of DD-PCR was used to identify genes associated with hypoglossal nerve regeneration. In particular, one candidate cDNA fragment (200 bp) was isolated and sequenced (Fig. 1). Although this method can be used successfully to isolate candidate cDNAs, it has some technical disadvantages over traditional differential expression cloning methods; one disadvantage is the high incidence of false positives (Nishio et al., 1994). To eliminate these false positives, *in*

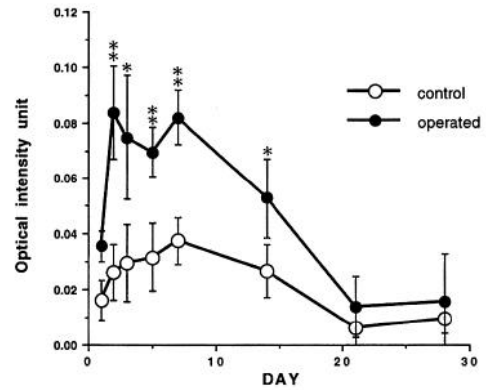


Figure 7. Expression profile of rat EAAC1 mRNA in both resection side (solid circle) and control side (open circle). Each point shows the average intensity of the positive signals and its SD. Asterisks denote statistically significant differences (Student's *t* test) from control: *, $p < 0.05$; **, $p < 0.01$.

situ hybridization histochemistry was used as a secondary screening method. This histological approach, *in situ differential display*, allows false positive clones to be effectively eliminated. About 60–70% of bands initially displayed on the gel were false positives when assessed using *in situ* hybridization.

Subsequent screening of a cDNA library showed that the clone isolated here corresponded to the rat homolog of a neuronal high affinity glutamate transporter. Comparing the amino acid sequences of the rat clone with the rabbit and human sequences revealed that the rat homolog also conserves an AAX-FIAQ structural motif and serine-rich sequences (SSSS) (Figs. 3, 4). *In situ* hybridization further suggested that the present clone was expressed primarily in neurons although some glial expression was observed. Taking all this data into consideration, we suggest that the clone derived by DD-PCR is the rat homolog of the rabbit EAAC1/human EAAT3 sequences. Further, expression of this neuronal type of glutamate transporter is upregulated following peripheral nerve injury.

Glutamate is the major excitatory neurotransmitter in the nervous system as well as a neurotoxin. Under normal conditions, glutamate signal transmission is terminated by the rapid uptake of glutamate into presynaptic nerve terminals and into surrounding glia (Nicholls and Attwell, 1990). It is conceivable that abnormal glutamate uptake may be closely related to an increase in extracellular glutamate concentrations as observed in certain neurodegenerative diseases (Drejer et al., 1985; Silverstein et al., 1986; Ikeda et al., 1989). However, the kinetics of the glutamate transport system in such conditions is still controversial. Several studies have demonstrated that an excess of glutamate in the synaptic cleft results from a reduction in the density of glutamate transporter in spinal cord and brain tissues from patients with amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1992). Further, in ischemia or anoxia, rundown of Na^+ , K^+ and voltage gradients is expected to initiate reversal of the Na^+ -dependent and electrogenic glutamate uptake carrier, releasing excess glutamate into the extracellular space (Szatkowski et al., 1990; Lysko et al., 1994a,b). This abnormal Ca^{2+} -independent nonvesicular release of glutamate results in a reduction of glutamate clearance at the synaptic cleft, activation of NMDA receptors and subsequent Ca^{2+} influx leading to neural death (Andreeva et al., 1991; Waxman et al., 1991). After traumatic brain injury, an increase in extracellular concentrations of glutamate is also

observed (Nilsson et al., 1990, 1994). In addition, Takata (1993) has reported that following hypoglossal nerve axotomy the inhibitory input to the hypoglossal motoneurons decreased whereas the excitatory component remained. This may also contribute the increase of extracellular glutamate. Thus, the present study indicates that axotomized motoneurons produce substantial amounts of glutamate transporter, possibly in an attempt to remove extracellular glutamate and reduce glutamate concentrations to within the physiological range. Thus, at least in the present case the neuronal glutamate transporter isolated may be functioning to reduce local glutamate concentrations and thus increase neuronal survival. Enhanced expression of the rat EAAC1 was not detected immediately after the nerve injury; the maximum expression was seen from day 4 to day 11, and thereafter the expression of rat EAAC1 returned to control levels during the following 3 weeks (Fig. 7). This period corresponds to only 1/2 the period required for complete reinnervation of injured axons (Kobayashi et al., 1994). During this earlier phase of nerve regeneration, injured motoneurons lose their synaptic contacts, suggesting that the newly synthesized glutamate transporter may be functioning not to terminate glutamate signaling, but for some other function. It is possible that these newly synthesized glutamate transporters may function to reduce glutamate levels and therefore protect damaged motor neurons from glutamate mediated cell death. Alternatively it may be that the cells require increased glutamate for protein synthesis.

The family of glutamate transporters is generally divided into two subsets, neuronal (e.g., EAAC1) (Kanai and Hediger, 1992; Arriza et al., 1994; Kanai et al., 1994) and glial (e.g., GLAST, GLT1) (Pines et al., 1992; Storck et al., 1992; Kanner, 1993). Analysis of the expression pattern of rat EAAC1 mRNA in brain, by *in situ* hybridization histochemistry indicates that the rat EAAC1 is localized widely in neurons as described previously (Kanai and Hediger, 1992; Rothstein et al., 1994). However, the intensity of the rat EAAC1 mRNA signal varied among different populations of neurons. Some neurons express little or almost no EAAC1 mRNA. Since many populations of neurons use glutamate for neurotransmission, there may be additional subtypes of neuronal glutamate transporters which are enriched in regions where the rat EAAC1 is not widely expressed. The neuronal glutamate transporters EAAC1 and EAAT3 have been shown to be expressed in neurons; however, we report here that mRNA for the cloned rat EAAC1 was additionally expressed in flat-shaped cells in the white matter, for example the corpus cullosum (Fig. 5). Thus, although the rat EAAC1 is a major neuronal glutamate transporter, it is also expressed in some glial components such as the oligodendrocytes.

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