Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2638–2641, March 1997
Neurobiology

A novel seizure-induced synaptotagmin gene identified by differential display

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Communicated by Ruth Sager, Dana–Farber Cancer Institute, Boston, MA, December 23, 1996 (received for review November 27, 1996)

ABSTRACT **Systemic administration of kainic acid, a cyclic analogue of glutamate, produces many of the clinical features of human temporal lobe epilepsy and status epilepticus in rats, including the induction of motor convulsions and the degeneration of neurons in the hippocampus and piriform cortex. Differential display PCR was used to identify mRNAs that are differentially expressed between degenerating and nondegenerating tissues in the brain after kainic acid-induced seizure activity. A novel cDNA fragment expressed in the degenerating hippocampus and piriform cortex, but not in the nondegenerating parietal cortex, was identified, cloned, and sequenced. This novel cDNA fragment identified a new member of the synaptotagmin gene family that is rapidly and transiently induced in response to seizure activity. Differential expression of this synaptotagmin gene, *syt X*, was confirmed by Northern blot analysis and *in situ* hybridization. This novel, inducible synaptotagmin gene may provide a direct link between seizure-induced neuronal gene expression and subsequent modulation of synaptic structure and function.**

Differential display PCR allows the identification of differentially expressed genes by using PAGE to display PCR-amplified cDNA fragments between two or more mRNA populations (1, 2). Differential display PCR was used to identify changes in gene expression that occur in degenerating areas of the rat brain after kainic acid-induced status epilepticus (3, 4). In this model, kainate-induced excitotoxicity leads to delayed neuronal cell death in the hippocampus and piriform cortex but not in the parietal cortex (5). Seizure activity is characterized by the rapid transcriptional activation of immediate early genes whose gene products are believed to control the expression of downstream genes associated with delayed neuronal degeneration (6). We used a differential display strategy that examined gene expression in three different brain tissues over a 12-h time period after kainic acid administration. This differential display strategy allowed us to compare gene expression in the hippocampus and piriform cortex, two brain regions that undergo excitotoxicity-induced neuronal degeneration, with gene expression in the parietal cortex, a region that does not undergo neuronal degeneration (7). Differential display patterns were examined 3, 6, and 12 h after systemic kainic acid administration. These time points were chosen to identify genes that are rapidly induced in response to kainic acid-induced seizures (8) and potential downstream target genes associated with excitotoxicity-induced neuronal degeneration.

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0027-8424/97/942638-4$2.00/0
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MATERIALS AND METHODS

Animals. Adult male Sprague Dawley rats (250–275 g) were injected i.p. with kainic acid (12 mg/kg; Sigma) or a similar volume of vehicle (100 mM PBS). Animal behavior was then monitored for 1–2 h for characteristic signs of status epilepticus. Animals were killed 3, 6, 12, and 24 h after kainic acid administration. Animals that were injected with kainic acid but did not show signs of status epilepticus were used as controls.

RNA Isolation. Tissues used for RNA isolation were rapidly dissected, snap frozen, and stored in liquid nitrogen until used. Total cellular RNA was isolated from frozen tissue using TRIzol (GIBCO/BRL). Total RNA samples used in differential display experiments were treated with RQ1 RNase-free DNase (Promega) to remove contaminating chromosomal DNA. Poly(A)$^+$ RNA was isolated using the Mini RiboSep mRNA Isolation Kit (Collaborative Research).

Differential Display Analysis. Reverse transcription of total RNA and differential display analysis were as described (2) and were analyzed on a 6% polyacrylamide DNA sequencing gel. Recovery and reamplification of the *syt X* cDNA fragment from the differential display gel was also as described (2).

Isolation of *syt X* cDNA Sequence. The *syt X* differential display cDNA fragment was subcloned into the pCR II vector (Invitrogen) and sequenced using the T7 Sequencing Kit (Pharmacia). Primer sequences derived from the differential display cDNA fragment were used to obtain additional cDNA sequence information via 5′-rapid amplification of cDNA ends using the Marathon cDNA Amplification Kit (CLONTECH).

Preparation of *syt X* Probes. The *syt X* cDNA probe used for Northern blot analysis corresponding to nucleotides 810–1553 was agarose gel purified using the Qiaex purification resin (Qiagen) and ^{32}P-labeled using Ready-To-Go DNA Labeling Beads (Pharmacia) (−dCTP). The oligonucleotide probe used for *in situ* hybridization, 5′-TCAGCGTCTAGTCCAGTTCGACACA-CACCT-3′, corresponding to nucleotides 1524–1553 and was chosen based on the low degree of nucleotide similarity to other synaptotagmin genes. The oligonucleotide probe was ^{33}P-labeled using the 3′-End Labeling Kit (Amersham).

Northern Blot Analysis. Poly(A)$^+$ RNA was isolated using the RiboSep mRNA Isolation Kit (Collaborative Research). Denatured RNA samples (2 μg) were electrophoresed in 1.0% agarose–formaldehyde gels, transferred to ZetaProbe GT membrane (Bio-Rad), fixed by UV irradiation, and hybridized with a ^{32}P-labeled cDNA probe prepared from a gel-purified insert. Hybridization and washing conditions were as described (9). Filters were exposed to film at −70°C with an intensifying screen. Normalization for loading was done using the Bio-Rad Model GS-690 Imaging Densitometer and MOLECULAR ANALYST software.

***In Situ* Hybridization.** *In situ* hybridization was as described (7). The oligonucleotide probe used in this experiment, 5′-

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U85513).
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TCAGCGTCTAGTCCAGTTCGACACACACCT-3′, was chosen based on the low degree of nucleotide similarity to other synaptotagmin genes.

Database Searches and Protein Alignment. Database searches and sequence alignment were done using the FASTA and BLAST search servers at the National Center for Biotechnology Information (10). Protein alignments were assembled using the CLUSTALW program at the Baylor College of Medicine, Houston.

RESULTS AND DISCUSSION

Identification and Cloning of Synaptotagmin X. Differential display analysis was used to identify tissue-specific changes in gene expression in response to kainic acid-induced seizure activity. We identified a 228-bp cDNA fragment that appeared to be differentially expressed in the hippocampus and piriform cortex of animals that displayed status epilepticus (Fig. 1). This cDNA fragment was not detected in the parietal cortex or in control animals that were injected with kainic acids but did not exhibit seizure activity. Sequence analysis of the cDNA fragment revealed a high degree of similarity to members of the synaptotagmin gene family (10–12). Approximately 1×10^6 plaques of a poly(A)$^+$-primed λZAP II rat hippocampus cDNA library (Stratagene) were screened using the differential display cDNA fragment. However, no positive clones were isolated with this method, suggesting a low level of *syt X* gene expression in the absence of seizure activity.

Additional cDNA sequences were obtained using rapid amplification of cDNA ends (Fig. 2). The predicted amino acid sequence and overall structure identify the protein as a novel member of the synaptotagmin gene family, which we designated synaptotagmin X (11, 13–18). Synaptotagmin genes are characterized by an amino acid sequence that contains five distinct regions. These consist of an N-terminal, intravesicular sequence of variable length, a transmembrane domain, a cytoplasmic region of variable length, two highly conserved C2 domains, and a short C-terminal tail. Within the cytoplasmic region located between the transmembrane domain and the conserved C2 domains, the *syt X* gene contains additional highly conserved amino acid sequences found in *syt VI* and another synaptotagmin gene, p65C, isolated from the marine ray (19) (Fig. 3). The high degree of amino acid sequence conservation observed in this region suggests that these sequences are functionally important and may define a functionally distinct class of synaptotagmin proteins.

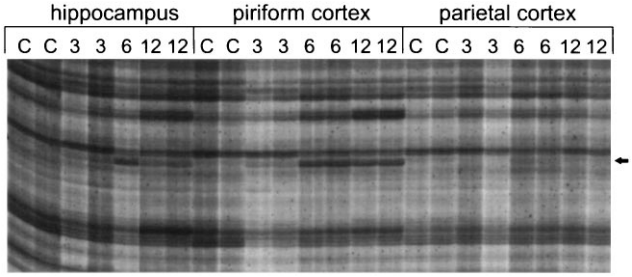

FIG. 1. Differential display of mRNA comparing gene expression among hippocampus, piriform cortex, and parietal cortex after kainic acid administration. Only a portion of the autoradiogram is shown. C, 3, 6, and 12 denote control, 3 h, 6 h, and 12 h, respectively. Differential display reactions were performed in duplicate using 5′-TTTTTTTTTTTCG-3′ as an anchored primer and 5′-TACAACGAGG-3′ as a random arbitrary primer. The arrow indicates a reproducible PCR-amplified cDNA fragment that appears to be induced only in degenerating tissues (hippocampus and piriform cortex) at 6 and 12 h after kainic acid injection. In the hippocampus and piriform cortices, baseline levels of expression are seen in control animals and at the 3-h time point. By comparison, only baseline levels of expression are observed in the parietal cortex, a tissue in which seizure-induced neuronal degeneration is not observed.

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CGCAGACTGGCAGCAGCGTCCGTCGGCGCGCGCCACCCTCCTGCGCCCTTGCCAACCATG 60

GGGCCAACTCGCAACTCTTAAGGCTCAGCCTCGGGCCAAGGGAAATCAGAGAAGCTGCGA 120

GCAGAAGCCAAGATGAGTTTCCGCAAGGAGGACGGGGTGAGCAGCCTGTGCCAGAAGGCG 180
            M  S  F  R  K  E  D  G  V  S  S  L  C  Q  K  A  16

CTGCACATCATCACCGAGCTGTGCTTCGCGGGCCAGGTGGAGTGGGACAAGTGTTCGGGC 240
 L  H  I  I  T  E  L  C  F  A  G  Q  V  E  W  D  K  C  S  G  36

ATCTTTCCAGCCGATAGGAGCGGCCAGGGCGGAGGTGGCACAGACATTTCAGTCAGCCTG 300
 I  F  P  A  D  R  S  G  Q  G  G  G  G  T  D  I [S  V  S] L  56

TTAGCAGTTGTTGTAAGCTTCTGTGGCCTGGCCCTGCTGGTTGTCTCCCTTTTCGTCTTC 360
[L  A  V  V  V  S  F  C  G  L  A  L  L  V  V  S  L] F  V  F  76

TGGAAGTTGTGCTGGCCCTGCTGGAAGAGCAAGCTCGTGGCCCCCAATGTCAGTACCCTT 420
 W  K  L  C  W  P  C  W  K  S  K  L  V  A  P  N  V  S  T  L  96

CCGCAGAGCATCTCAAGTGCTCCAACTGAGGTTTTTGAGACCGAAGAGAAAAAAGAAGTC 480
 P  Q  S  I  S  S  A  P  T  E  V  F  E  T  E  E  K  K  E  V  116

GAAGAAAATGAAAAGCCAGCACCAAAAGCTATTGAGCCTGCAATAAAAATCAGCCACACA 540
 E  E  N  E  K  P  A  P  K  A  I  E  P  A  I  K  I  S  H  T  136

TCCCCCGACATCCCAGCAGAAGTGCAAACTGCTTTAAAGGAGCATTTAATTAAACATGCC 600
 S  P  D  I  P  A  E  V  Q  T  A  L  K  E  H  L  I  K  H  A  156

CGTGTGCAGAGGCAAACGACCGATCCTACATCTTCATCCCGCCACAATTCCTTCAGGAGG 660
 R  V  Q  R  Q  T  T  D  P  T  S  S  S  R  H  N  S  F  R  R  176

CACCTACCAAGGCAAATGAACGTCTCCAGTGTTGACTTTAGCATGGGCACGGAACCTGTC 720
 H  L  P  R  Q  M  N  V  S  S  V  D  F  S  M  G  T  E  P  V  196

TTACAAAGAGGAGAAACCAGAACCAGCATCGGGAGGATAAAACCGGAACTCTATAAGCAG 780
 L  Q  R  G  E  T  R  T  S  I  G  R  I  K  P  E  L  Y  K  Q  216

AAATCAGTTGACTCCGAGGGCAACAGAAAAGACGACGTCAAAACCTGTGGGAAACTTAAC 840
 K  S  V  D  S  E  G  N  R  K  D  D  V  K  T  C  G  K  L  N  236

TTTGCGCTCCAGTACGATTATGAAAATGAACTCCTAGTTGTTAAAATTATCAAAGCTCTA 900
 F  A  L  Q  Y  D  Y  E  N  E  L  L  V  V  K  I  I  K  A  L  256

GATCTCCCAGCTAAAGATTCCACAGGAACTTCTGATCCTTATGTGAAGATCTATCTTCTT 960
 D  L  P  A  K  D  S  T  G  T  S  D  P  Y  V  K  I  Y  L  L  276

CCGGACAGGAAGAAGAAATTTCAGACCCGTGTGCACCGGAAGACTTTAAACCCTCTGTTC 1020
 P  D  R  K  K  K  F  Q  T  R  V  H  R  K  T  L  N  P  L  F  296

GATGAGCTATTTCAGTTCCCGGTGGTTTACGATCAGCTAAGCAACCGCAAATTACACTTT 1080
 D  E  L  F  Q  F  P  V  V  Y  D  Q  L  S  N  R  K  L  H  F  316

AGTATATATGATTTTGACAGATTTTCCAGACACGACATGATTGGGGAAGTGATTCTTGAT 1140
 S  I  Y  D  F  D  R  F  S  R  H  D  M  I  G  E  V  I  L  D  336

AATTTATTTGAAGTCTCTGATCTCTCCAGGGAAGCCACAGTATGGAAAGATATCCACTGT 1200
 N  L  F  E  V  S  D  L  S  R  E  A  T  V  W  K  D  I  H  C  356

GCAACCACAGAAAGCATGGACCTGGGCGAAATCATGTTTTCCCTTTGCTATTTGCCAACT 1260
 A  T  T  E  S  M  D  L  G  E  I  M  F  S  L  C  Y  L  P  T  376

GCTGGTCGCATGACCTTGACTGTGATCAAGTGCAGAAATCTGAAGGCAATGGACATTACC 1320
 A  G  R  M  T  L  T  V  I  K  C  R  N  L  K  A  M  D  I  T  396

GGATCATCAGATCCTTATGTCAAAGTGTCTCTGATGTGTGAGGGCCGAAGACTTAAAAAA 1380
 G  S  S  D  P  Y  V  K  V  S  L  M  C  E  G  R  R  L  K  K  416

AGAAAAACAACTACAAAGAAAAATACTCTCAATCCCGTGTACAATGAAGCCATTATTTTC 1440
 R  K  T  T  T  K  K  N  T  L  N  P  V  Y  N  E  A  I  I  F  436

GATATCCCTCCAGAAAATGTGGACCAGGTCAGCCTGTGCATTGCGGTCATGGATTATGAC 1500
 D  I  P  P  E  N  V  D  Q  V  S  L  C  I  A  V  M  D  Y  D  456

AGGGTGGGACACAATGAGGTCATAGGTGTGTGTCGAACTGGACTAGACGCTGAGGGTCTT 1560
 R  V  G  H  N  E  V  I  G  V  C  R  T  G  L  D  A  E  G  L  476

GGAAGAGACCATTGGAATGAAATGCTGGCCTATCACCGAAAGCCAATCACACACTGGCAC 1620
 G  R  D  H  W  N  E  M  L  A  Y  H  R  K  P  I  T  H  W  H  496

CCCTTG                                                        1626
 P  L                                                         498
```

FIG. 2. Nucleotide and predicted amino acid sequence of synaptotagmin X. Single letter amino acid codes are used, numbering from the first in-phase ATG codon. The putative transmembrane domain is boxed, and the two C2 domains are indicated by shading. Sequence information from a small portion of the C-terminal tail of *syt X* is incomplete.

Expression of *syt X* mRNA in Rat Brain Tissue. The expression of *syt X* mRNA was examined in the hippocampus, piriform cortex, and parietal cortex by Northern blot analysis (Fig. 4*A*).

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Syt VI MSGVWGAGGPRCQAALAVLASLCRARPPPLGLDVETCQSFELQPPEQSPSAADS 54
Syt X      MSFRKEDGVSSLCQKALHIITELCFAGQVEWDKCSGIFPADRSGQGGGGT 50
p65C     MSGDGEDELCRNALALVNELCFSVRGNHNNEKCIEFSYLLRDRDRTRHIET 51

Syt VI GTSVSLLQVVVIVCGVALVAVFFFLFMKLCWMPWRNKEASS---PSSANPASEI 105
Syt X  DISVSLLAVVVSFCGLALLVVSLFVFWKLCWPCWKSKLVAP---NVSTLPQSIS 101
p65C   DISVSLLSVIVTFCGIVLLGVSLFVSWKLCWIPWRDKGLNPQRRDSQHHPHQHL 105

Syt VI LQSPSSRGNMADKLKDPS----------ALGFLEAAVKISHTSPDIPAEVQMSV 149
Syt X  SAPTEVFETEEKKEVEENEKP-------APKAIEPAIKISHISPDIPAEVQTAL 148
p65C   HHHHSHFTDLTVERVDCGPEMPERSYLDLESYPESGIKLSQTSPDIPVDTSSGS 159

Syt VI KEHIMRHTKLQRQTTEDASSTRHTSFKRHLPRQMHVSSVDYGNELPPAA--AEQ 201
Syt X  KEHLIKHARVQRQTTDDTSSSRHNSFRRHLPRQMNVSSVDFSMGTEPVLQRGET 202
p65C   KENNIPNAHSQQQVSAEPPATRFNSLPRPIPQQLSSP--EFGTQADEKV---EQ 208

Syt VI PTSIGRIKPELYKQKSVDGDEAKSEAAKSCGKINFSLRYDYESETLIVRILKAF 255
Syt X  RTSIGRIKPELYKQKSVDSEGNRKDDVKTCGKLNFALQYDYENELLVVKIIKAL 256
p65C   VTSIGQIKPELYKQRSIDT-EAKKHQKVNCGRINFMLRYTYTTEQLVVKILKAL 261

Syt VI DLPAKDFCGSSDPYVKIYLLPDRKCKLQTRVHRKTLNPTFDENFHFPVPYEELA 309
Syt X  DLPAKDSTGTSDPYVKIYLLPDRKKKFQTRVHRKTLNPLFDELFQFPVVYDQLS 310
p65C   DLPAKDANGFSDPYVKIYLLPDRKKKFQTKVHRKTLNPIFNETFQFNVPFNELQ 315

Syt VI DRKLHLSVFDFDRFSRHDMIGEVILDNLFEASDLSRETSIWKDIQYATSESVDL 363
Syt X  NRKLHFSIYDFDRFSRHDMIGEVILDNLFEVSDLSREATVWKDIHCATTESMDL 364
p65C   NRKLHFSVYDFDRFSRHDLIGQVVLDNLLELSDFSEDTTIWRDILEATSEKADL 369

Syt VI GEIMFSLCYLPTAGRLTLTVIKCRNLKAMDITGYSDPYVKVSLLQDGRRLKKKK 417
Syt X  GEIMFSLCYLPTAGRMTLTVIKCRNLKAMDITGSSDPYVKVSLMCEGRRLKKRK 418
p65C   GEINFSLCYLPTAGRLTITIIKATNLKAMDLTGFSDPYVKASLIGDERRLKKRK 423

Syt VI TTIKKNTLNPVYNEAIIFDIPPENMDQVSLLTSVMDYDRVGHNEIIGVCRVGIS 471
Syt X  TTTKKNTLNPVYNEAIIFDIPPENVDQVSLCIAVMDYDRVGHNEVIGVCRTGLD 472
p65C   TSIKKNTLNPVYNEALVFDIPNENMEHVNVIIAVMDYDCIGHNEVIGMCRVGNA 477

Syt VI AEGLGRDHWNEMLAYPRKPIAHWHCLAEVKKSFKEGTPRL-------------- 511
Syt X  AEGLGRDHWNEMLAYHRKPITHWHPL---------------------------- 498
p65C   TDGPGREHWNEMLANPRKPIEQWHQLIEEKVMNSYMTKSFAAGTGATKPVTIVV 531

Syt VI ------------------------------------------------------
Syt X  ------------------------------------------------------
p65C   ESPHSV------------------------------------------------- 537
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FIG. 3. Alignment of rat and marine ray predicted synaptotagmin amino acid sequences. The amino acid sequences of *syt VI* and *syt X* from rat and p65C from marine ray are aligned. Identical amino acid residues for all three genes are highlighted in black. The predicted *syt X* protein is most similar to *syt VI* and p65C, with overall identities of 62% and 52%, respectively. These three synaptotagmin genes share an additional region of amino acid homology that is not found in other synaptotagmin family members. This region is located between the transmembrane domain and the two C2 domains.

This experiment revealed that the *syt X* gene was rapidly and transiently induced in specific brain regions in response to kainic acid-induced status epilepticus. A single 8.0-kb transcript was induced in the hippocampus and piriform cortex but not in the parietal cortex. In the hippocampus, the *syt X* message was elevated at 3 h, strongly induced at 6 h, and returned to basal levels by 12 h after kainic acid administration. In the piriform cortex, a smaller level of *syt X* message induction was observed at 3 and 6 h after kainic acid administration. The piriform cortex also appeared to have a higher level of constitutive *syt X* expression in comparison to the hippocampus and parietal cortex. *In situ* hybridization experiments were performed to verify the tissue-specific pattern of *syt X* gene expression. These experiments confirmed that the *syt X* gene was induced in specific brain regions in response to kainic acid-induced status epilepticus. The *syt X* gene showed tissue-specific expression in layer II of the piriform cortex and the dentate granule cells of the hippocampus (Fig. 4*B*). Most other brain regions showed very low basal level *syt X* gene expression.

The pattern of gene expression observed in both differential display and Northern blot analyses demonstrated an increase in *syt X* gene expression in response to seizure activity. However, the time course of *syt X* gene expression was not completely consistent between these experiments. The difference in these results may be explained by the variability in seizure activity between animals used in differential display and those used in Northern blot analyses (7). *In situ* hybrid-

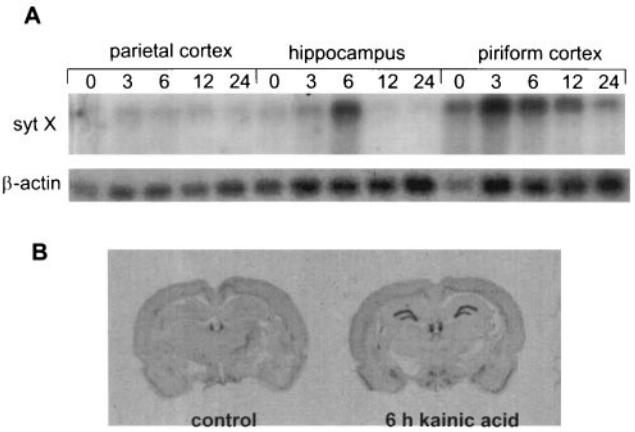

FIG. 4. (*A*) Northern blot analysis of *syt X* expression. Poly(A)$^+$ RNA from parietal cortex, hippocampus, and piriform cortex at 0, 3, 6, 12, and 24 h after kainic acid-induced seizures was probed with *syt X*. The probe identifies a single message that is 8.0 kb in length. The blot was also probed with a β-actin oligonucleotide for the purpose of quantitation. (*B*) *In situ* hybridization of rat brain sections using an oligonucleotide probe specific for the *syt X* gene. Coronal section control and 6-h kainic acid-treated rat brain. Examination of control and kainic acid-treated animals revealed that *syt X* is expressed in the dentate granule cells of the hippocampus and layer II of the piriform cortex.

ization results also showed some variability in the time course of *syt X* gene expression in response to seizure activity (data not shown). It also should be noted that differential display was primarily useful as a screening tool for the identification of differentially expressed genes and not as a quantitative measure of gene expression.

The Ca^{2+}-dependent release of neurotransmitters and neuropeptides from presynaptic nerve terminals is the central event in synaptic neurotransmission (12). Recent genetic and electrophysiological evidence suggests that synaptotagmin I plays an integral role in synaptic vesicle fusion and neurotransmitter release (20–24). However, at least nine other members of the synaptotagmin gene family have been identified, and the role of these genes in synaptic vesicle function is not well understood. The *syt X* gene is unique in that it is structurally distinct from most other synaptotagmins and it demonstrates tissue-specific induction in response to seizure activity. It is particularly interesting that the *syt X* gene is induced in cells that project to neurons that undergo delayed cell death in response to excitotoxic injury (5, 7). The *syt X* gene is induced in the dentate granule cells, a region of the hippocampus that undergoes little or no seizure-induced cell death, so it is unlikely that this gene plays a direct role in the cell death process. However, *syt X* gene expression may play a role in modulating neurotransmitter release in response to excitotoxic neuronal activation. The expression of one other synaptotagmin gene, *syt IV*, has also been reported to be increased after kainic acid-induced seizures (25). It is possible that coordinate expression of specific synaptotagmin genes leads to changes in synaptic structure and function that underlie long term neuronal plasticity.

We thank K. Murphy and M. Peterson for their excellent technical assistance and E. M. Denovan–Wright for careful reading of the manuscript. This work was funded by grants from the Medical Research Council of Canada, Heart and Stroke Foundation of New Brunswick (R.W.C.), the Heart and Stroke Foundation of Nova Scotia (H.A.R.), and the Medical Research Council of Canada/SmithKline Beecham (R.W.C., H.A.R.).

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