THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 36, Issue of September 4, pp. 23004–23011, 1998

Printed in U.S.A.

Serine Proteases in Rodent Hippocampus*

(Received for publication, April 23, 1998)

Ben J. Davies, Benjamin S. Pickard, Muriel Steel, Richard G. M. Morris‡, and Richard Lathe‡§

From the Center for Genome Research and the ‡Center for Neuroscience, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, United Kingdom

Brain serine proteases are implicated in developmental processes, synaptic plasticity, and in disorders including Alzheimer's disease. The spectrum of the major enzymes expressed in brain has not been established previously. We now present a systematic study of the serine proteases expressed in adult rat and mouse hippocampus. Using a combination of techniques including polymerase chain reaction amplification and Northern blotting we show that tissue-type plasminogen activator (t-PA) is the major species represented. Unexpectedly, the next most abundant species were RNK-Met-1, a lymphocyte protease not reported previously in brain, and two new family members, BSP1 (brain serine protease 1) and BSP2. We report full-length sequences of the two new proteases; homologies indicate that these are of tryptic specificity. Although BSP2 is expressed in several brain regions, BSP1 expression is strikingly restricted to hippocampus. Other enzymes represented, but at lower levels, included elastase IV, proteinase 3, complement C2, chymotrypsin B, chymotrypsin-like protein, and Hageman factor. Although thrombin and urokinase-type plasminogen activator were not detected in the primary screen, low level expression was confirmed using specific polymerase chain reaction primers. In contrast, and despite robust expression of t-PA, the usual t-PA substrate plasminogen was not expressed at detectable levels.

Cell migration and synapse formation during development appear to involve the ordered remodeling of the extracellular matrix (1–3). In brain, major extracellular matrix components include the collagens, laminin, fibronectin, thrombospondin, and diverse proteoglycans that play a central role in regulating the cellular microenvironment (4–6). Metalloproteases are generally held to play a central role in cleavage of extracellular matrix components during neuronal outgrowth, plasticity, and in disease states (7). These are expressed as inactive zymogens that require proteolytic cleavage, predominantly by the serine protease plasmin, to gain full enzymatic activity. In turn, plasmin is activated by cleavage of its precursor, plasminogen, through the action of two further serine proteases, the plasminogen activators (PAs)[1] u-PA and t-PA (8); the paradigm for PA action is thus in converting plasminogen to active plasmin, initiating tissue remodeling.

High levels of PA activity are associated with the developing brain where cell migration and proliferation are active processes (9–11). PA activity is also documented in the adult hippocampus and the cerebellum (12), brain regions exhibiting activity-dependent structural plasticity (13). Furthermore, t-PA expression is up-regulated by seizure, kindling, the induction of long term potentiation in the dentate gyrus (14), and by motor learning in the cerebellum (15). Although t-PA null mice showed no gross morphological abnormalities in the brain (16), there were deficits in the maintenance of long lasting hippocampal long term potentiation (17, 18); they were also resistant to excitotoxic insults (19, 20).

Another well characterized serine protease, thrombin, may also play a role in brain function. This trypsin-like protease is reported to be widely expressed in adult brain and has been shown to reverse neuroblastoma differentiation (21), astrocyte stellation (22), and to modulate neuronal response to injury (23, 24). A potent thrombin inhibitor that is widely expressed in brain, protease nexin I, has also been implicated in the regulation of synaptic plasticity (25).

Serine proteases are implicated additionally in the pathophysiology of Alzheimer's disease, a progressive neurodegenerative disease in which early dysfunction of the hippocampus is a prominent feature. The characteristic deposition of amyloid in brain is thought to result from abnormal proteolytic processing of the amyloid precursor protein (APP) (26). Furthermore, APP itself contains a serine protease inhibitor domain (nexin II) of unknown function (27).

The recent reports of a number of other distinct serine proteases and their inhibitors within the nervous system (28–32) suggest a requirement for well regulated proteolysis in normal brain function. Nonetheless, the identities and abundances of the major brain serine proteases have not been established. In the present report we address the diversity of serine proteases present in adult brain. Our attention has focused on the hippocampus, a region wherein synaptic plasticity is well documented (33) and which is held to play a central role in the encoding of certain types of memory (34–36). Notably, synaptic plasticity in the hippocampus is accompanied by pronounced structural changes in spine morphology (37), implying proteolytic remodeling, whereas early dysfunction of the hippocampal formation is a feature of Alzheimer's disease.

We have sought to determine the range of serine proteases expressed in this brain region and have focused on the principal group or "clan" of enzymes, encompassing chymotrypsin, in which the three invariant catalytic residues are present in the

* This work was supported by grants from the Medical Research Council (to R. L. and R. G. M. M.), a charitable foundation, by Biotechnology and Biological Sciences Research Council funding to the Center for Genome Research, and by a Medical Research Council studentship (to B. D.). This work was performed in partial fulfillment of the requirements for a doctoral degree (to B. D.) by the University of Edinburgh. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ005641 and AJ005642.

§ To whom correspondence should be addressed. Tel.: 44-131-650-8590; Fax: 44-131-667-0164; E-mail: rlathe@ed.ac.uk.

[1] The abbreviations used are: PA(s), plasminogen activator(s); t-PA, tissue-type PA; u-PA, urokinase-type PA; APP, amyloid precursor protein; PCR, polymerase chain reaction; BSP, brain serine protease; CTRL, chymotrypsin-like protein.

order NH_2-His-Asp-Ser-COOH (38). We present a systematic study on the different species expressed in rodent hippocampal formation. We report that at least 10 members of this clan, including two new family members, are expressed in this one brain region.

MATERIALS AND METHODS

cDNA Preparation—After terminal anesthesia (CO_2) of adult rats (Fisher) the hippocampal formation was dissected, including areas CA1–3 and dentate gyrus, subiculum, alvear and fimbrial fibers but excluding fornix and afferent structures such as septum and entorhinal cortex. Total RNAs were prepared by a standard acid phenol procedure (39) and poly(A)$^+$ mRNA selected by affinity chromatography on oligo(dT)-cellulose. cDNA synthesis used oligo(dT)$_{12}$ and Superscript II reverse transcriptase (Life Technologies, Inc.).

PCR and cDNA Cloning—Hippocampus cDNA was amplified using degenerate primers (see Fig. 1; extended (5′) with octanucleotides containing *Eco*RI (upstream primer) or *Xba*I (downstream primer) restriction sites) and *Taq* polymerase (Promega) for 30 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min). Products were cloned into plasmids using a TA cloning kit (Invitrogen) or by conventional restriction enzyme cleavage (*Eco*RI plus *Xba*I) and religation. For hybridization screening, colonies were transferred to Hybond-N membranes (Amersham Pharmacia Biotech), denatured (0.5 M NaOH, 1.5 M NaCl, 2 min), renatured (1 M Tris·HCl, pH 7.4, 1.5 M NaCl, 5 min), rinsed, dried, and baked (2 h, 80 °C). Hybridization as described (40) used probes radiolabeled by random primed DNA polymerization. Full-length cDNA cloning employed a rat hippocampal cDNA library (41) and/or PCR cloning of cDNA extremities by a previously described method.[2] For PCR analysis of known sequences, 1 μg of poly(A)$^+$ RNA from rat brain, liver, or kidney was reverse transcribed and PCR amplified using primers for hypoxanthine phosphoribosyltransferase (5′-dGGGGGCTATAAGTTC-TTTGCTGAC-3′ and 5′-dCTTTTCCACTTTCGCTGATGACAC-3′), plasminogen (5′-dGGAGTACTGTGAGATTCCGTCC-3′ and 5′-dGTG-GTGAAGCACCAAGGG-3′), prothrombin (5′-dCAGGCCTGACAT-CAACTCCACCAC-3′ and 5′-dCACCACGCGCCCTCCTCATCC-3′), or u-PA (5′-dTCGTTGGGGGAGAATTCACTGTCG-3′ and 5′-dT-GGGGCTGCTTGCACTGTTCG-3′). PCR was for 20, 25, or 30 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min). Products were visualized by electrophoresis (1.5% agarose) and blotted (Hybond-N membrane, Amersham Pharmacia Biotech) before hybridization.

DNA Sequence Characterization—Dideoxy sequencing of cDNAs in plasmid vectors used the Sequenase 2.0 kit (Amersham Pharmacia Biotech); to sequence larger cDNAs restriction enzyme subfragments were cloned into pBluescript. Sequence data, obtained from both strands, were analyzed and aligned using the University of Wisconsin Genetics Computer Group package (UWGCG version 8, 1994).

Northern Analysis—Total RNAs (20 μg) were electrophoresed on 1% agarose in the presence of 18% w/v HCHO, blotted (Hybond-N, Amersham), and baked (2 h, 80 °C). Probes were prepared by random priming. After hybridization (40) membranes were exposed for autoradiography. The loading control probe was a 0.5-kilobase cDNA encoding the ubiquitously expressed rat ribosomal protein S26 (43).

In Situ Hybridization—Frozen horizontal 10-μm sections of brain were fixed (4% paraformaldehyde, 10 min), rinsed, treated with proteinase K (20 μg/ml in 50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 5 min), and refixed. After acetylation (0.25% acetic anhydride in 100 mM triethanolamine, 0.8% w/v NaCl, pH 8.0, 10 min) sections were dehydrated by passing though increasing v/v ethanol concentrations (30–100% over 10 min). After chloroform treatment (5 min) and rinsing in ethanol, sections were dried. Hybridization (16 h, 50 °C) with plasmid-generated riboprobes was in 4 × standard saline citrate (1 × SSC = 0.15 M NaCl, 0.015 M Na_3citrate), 50% v/v formamide, 10% w/v dextran sulfate, 1 × Denhardt's solution, 0.1% SDS, 50 μg/ml denatured salmon sperm DNA, 25 μg/ml yeast tRNA. Slides were washed (4 × 15 min, 1 × SSC, 60 °C; 2 × 30 min, 1 × SSC, 20 °C), dipped into photographic liquid emulsion (NTB-2, Kodak), exposed, and developed according to the manufacturer's specifications.

RESULTS

Multiple Serine Proteases in the Rat Hippocampus; Two New Enzymes—To address the spectrum of serine proteases expressed in rat hippocampus, PCR based on conserved motifs in the major (chymotrypsin-type) clan of serine proteases (Fig. 1*A*) was used to amplify and clone products from rat hippocampus cDNA. The most frequent products (t-PA and RNK-Met-1, below) were used to screen a further 1,000 clones. Clones hybridizing at high stringency were excluded, and additional family members were sequenced.

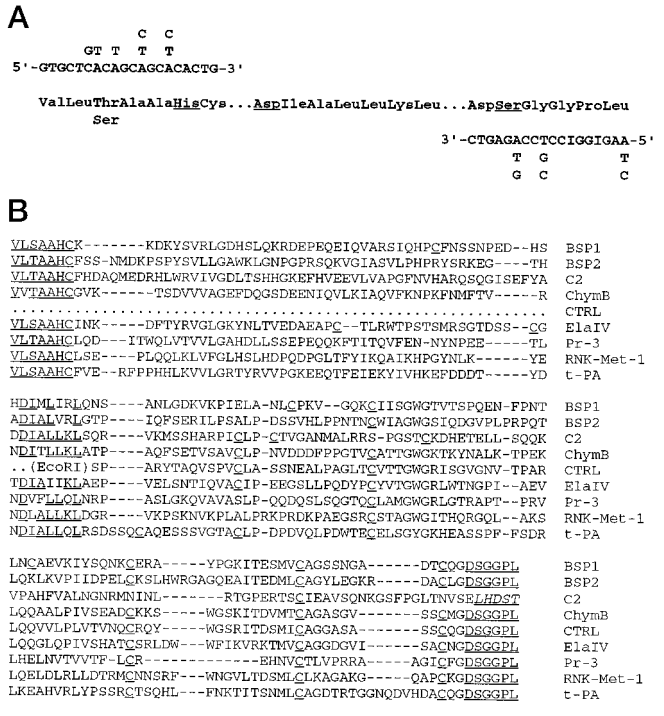

FIG. 1. **PCR amplification of chymotryptic clan serine proteases from rodent hippocampus using degenerate deoxynucleotide primer pairs.** *Panel A*, the catalytic triad and oligonucleotide primer sequences selected (I, deoxyinosine). *Panel B*, alignment of nine amino acid sequences encoded by PCR products; regions corresponding to the primers, the catalytic triad, and all cysteine residues are *underlined*. These sequences derive from rat hippocampus; a final sequence (Hageman factor, not presented) was derived from mouse hippocampus (see Table I) and was found to be identical (with the exception of primer homology regions) to the existing data base entry. Abbreviated names are: *C2*, complement factor C2; *ChymB*, chymotrypsin B; *ElaIV*, elastase IV; *Pr-3*, proteinase 3; as well as RNK-Met-1 and t-PA. For chymotrypsin B the Leu-Val change in the region corresponding to the 5′-primer is presumed to be an artifact of primer synthesis; for CTRL the single cloned PCR product analyzed contained a 5′-truncation caused by internal cleavage of the PCR product (*Eco*RI); for complement C2 the 3′-sequence terminated in a sequence complementary to the 5′-primer (translation product in *italics*).

Nine different serine protease sequences were initially detected; seven were characterized previously, and two corresponded to novel sequences, dubbed BSP1 (brain serine protease-1) and BSP2 (Table I). All clones characterized contained sequence features and homologies characteristic of this class of enzymes (Fig. 1*B*).

t-PA and RNK-Met-1 Are Expressed Abundantly in Brain—The major products obtained corresponded to t-PA and RNK-Met-1. t-PA was reported previously in brain, but RNK-Met-1 was unexpected. Two experiments were performed to reinforce the conclusion that t-PA and RNK-Met-1 are the most abundant species expressed in hippocampus. First we performed amplification from mouse rather than rat hippocampus. In a small scale screen (30 clones sequenced) >50% of cDNAs from mouse brain again corresponded to t-PA plus RNK-Met-1, whereas chymotrypsin B and elastase IV were also identified (not presented). A single clone encoding Hageman factor, a sequence not detected in the rat hippocampal PCR product, was also obtained from mouse hippocampus (Table I).

[2] Steel, M., Moss, J., Clark, K. A., Kearns, I. R., Davies, C. H., Morris, R. G. M., Skarnes, W., and Lathe, R. (1998) *Hippocampus*, in press.

TABLE I
Serine protease sequences in rodent brain

PCR products were generated from rat hippocampal RNA using degenerate primers corresponding to conserved regions of serine proteases of the chymotrypsin clan (Fig. 1), cloned, and sequenced as described under "Materials and Methods."

Enzyme	Expression: frequency in cloned PCR products	Detected by Northern analysis
	%	
t-PA	63	+
RNK-Met-1	26	+
BSP2	3.9	+
BSP1	0.8	+
Elastase IV	0.9	−
Proteinase 3	0.9	−
Complement C2	0.4	−
Chymotrypsin B	0.2	−
CTRL	0.1	−
Nonspecific products[a]	3.8	
Hageman factor[b]	NA	−
Prothrombin	Low level[c]	−
u-PA	Low level[c]	−
Plasminogen	Absent[c]	−

[a] Nonspecific products were sequences that failed to contain any features indicative of members of the serine protease family.
[b] The single clone encoding Hageman factor was obtained in a small scale screen of PCR products from mouse hippocampus. NA, not applicable.
[c] The presence of prothrombin and u-PA sequences, but absence of plasminogen, was demonstrated by specific PCR amplification (see Fig. 6).

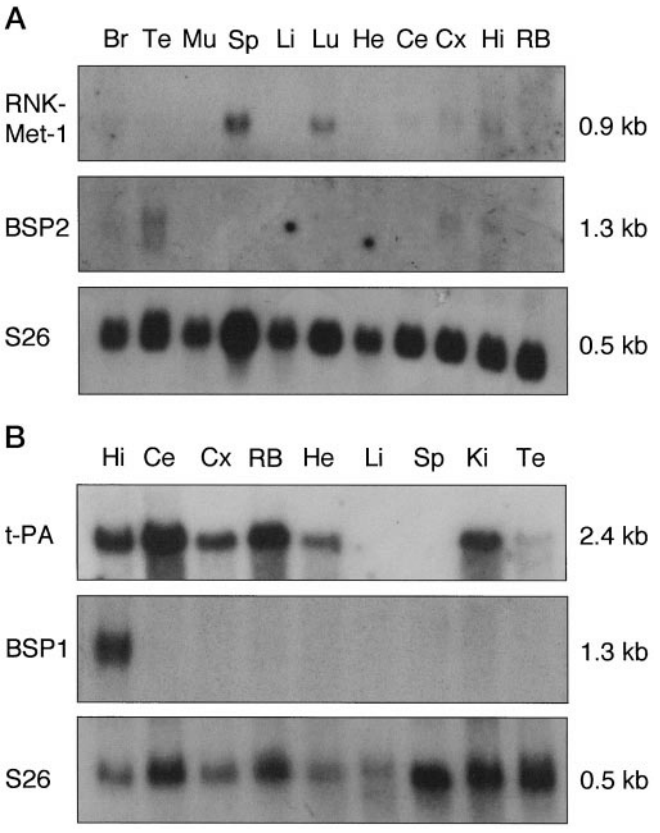

FIG. 2. **Tissue distribution of RNK-Met-1, t-PA, BSP1, and BSP2 mRNAs analyzed by Northern blotting.** Tissue samples were: *Br*, brain; *Te*, testis; *Mu*, muscle; *Sp*, spleen; *Li*, liver; *Lu*, lung; *He*, heart; *Ce*, cerebellum; *Cx*, cortex; *Hi*, hippocampus; *RB*, remainder of brain once hippocampus, cerebellum, and cortex had been removed. Northern blots were hybridized with probes corresponding to RNK-Met-1 and, after stripping, sequentially with probes corresponding to BSP-2 and the loading control S26 (*panel A*) and with probes for t-PA and subsequently with BSP1 and S26 as before (*panel B*).

Second, brain expression of t-PA and RNK-Met-1 was examined by Northern blotting (Fig. 2). Expression of RNK-Met-1 was detected readily in lung and spleen as recorded earlier (44), but in addition, brain expression was detected at comparable levels (Fig. 2*A*). t-PA mRNA was represented abundantly, as expected, in a number of tissues and brain regions (Fig. 2*B*). We then performed *in situ* hybridization to sections of adult rat brain using an RNK-Met-1 probe (the *in situ* distribution of t-PA mRNA being reported elsewhere; 12, 45). mRNA was detected in many brain regions; expression was particularly abundant in dentate gyrus, and lower levels were seen in hippocampal CA fields and in deep and superficial layers of the cerebral cortex (see Fig. 4, *A–C*).

Sequences of BSP1 and BSP2—To characterize further the two new serine protease species obtained in this screen, full-length cDNA cloning was performed. For BSP1, PCR amplification between a known sequence and oligo(dT) or a 5′-tail obtained the complete sequence. Full-length BSP2 cDNA was obtained from a rat hippocampal cDNA library. The sequences of the BSP1 and BSP2 cDNAs and the encoded polypeptides are presented in Fig. 3, *A* and *B*.

The deduced BSP1 translation product contains the features of a typical serine protease and is most closely similar to trypsin (40% sequence similarity) (Fig. 3*A*). The predicted polypeptide (260 amino acids, ~28.6 kDa) contains a hydrophobic signal sequence followed by a sequence motif (Lys ↓ Ile-Leu-Glu-Gly), suggestive of activation of the precursor polypeptide by cleavage by a trypsin-like protease (46). Cleavage at this site is predicted to liberate the active protease (~25.1 kDa).

For the second new enzyme, BSP2, the deduced mature polypeptide is most similar to mast cell tryptase (32% homology) and also contains the triad of catalytic residues characteristic of this family of serine proteases (Fig. 3*B*). However, we were unable to establish the start of the open reading frame unambiguously. The most likely initiation codon (ATG at position 89 in the cDNA) is out of phase with the remainder of the sequence and is followed by a chain termination codon (Fig. 3*B*). In contrast, the open reading frame from position 121 (ACC, Thr) prefixes a long hydrophobic coding sequence (nucleotides 121–162), clearly indicative of a signal sequence, shortly followed by a likely cleavage sequence for a trypsin-like activating enzyme (Arg ↓ Val-Val-Gly-Gly); the remainder of the polypeptide ensues. This predicts that the size of the mature BSP2 enzyme is 258 amino acids (~28.4 kDa). To validate the potential frameshift in the vicinity of the ATG, genomic clones were obtained from two inbred laboratory strains of rat; both confirmed the sequence as depicted in the figure (not presented), ruling out the possibility that the apparent frameshift is a cloning artifact.

Substrate specificity is associated with the presence of particular amino acids within the protease backbone (47). In BSP1 as well as in BSP2, the Asp, Gly, and Gly residues at positions 189, 216, and 226, respectively (numbering according to chymotrypsin; Ref. 48), indicates that both enzymes have tryptic specificity.

BSP1 Expression Is Restricted to the Hippocampus, whereas BSP2 Is Expressed More Widely in Brain—Northern analysis was performed to confirm brain expression of BSP1 and BSP2. The BSP1 probe yielded a signal at 1.3 kilobases in the hippocampal sample but not in other tissues or brain regions examined (Fig. 2*B*). The BSP2 probe also identified a transcript at about 1.3 kilobases in cortex and hippocampus; a similar sized transcript was observed in testis but not in other tissues examined (Fig. 2*A*).

In situ hybridization to brain sections was then performed. BSP1 transcripts were found in the hippocampal CA subfields,

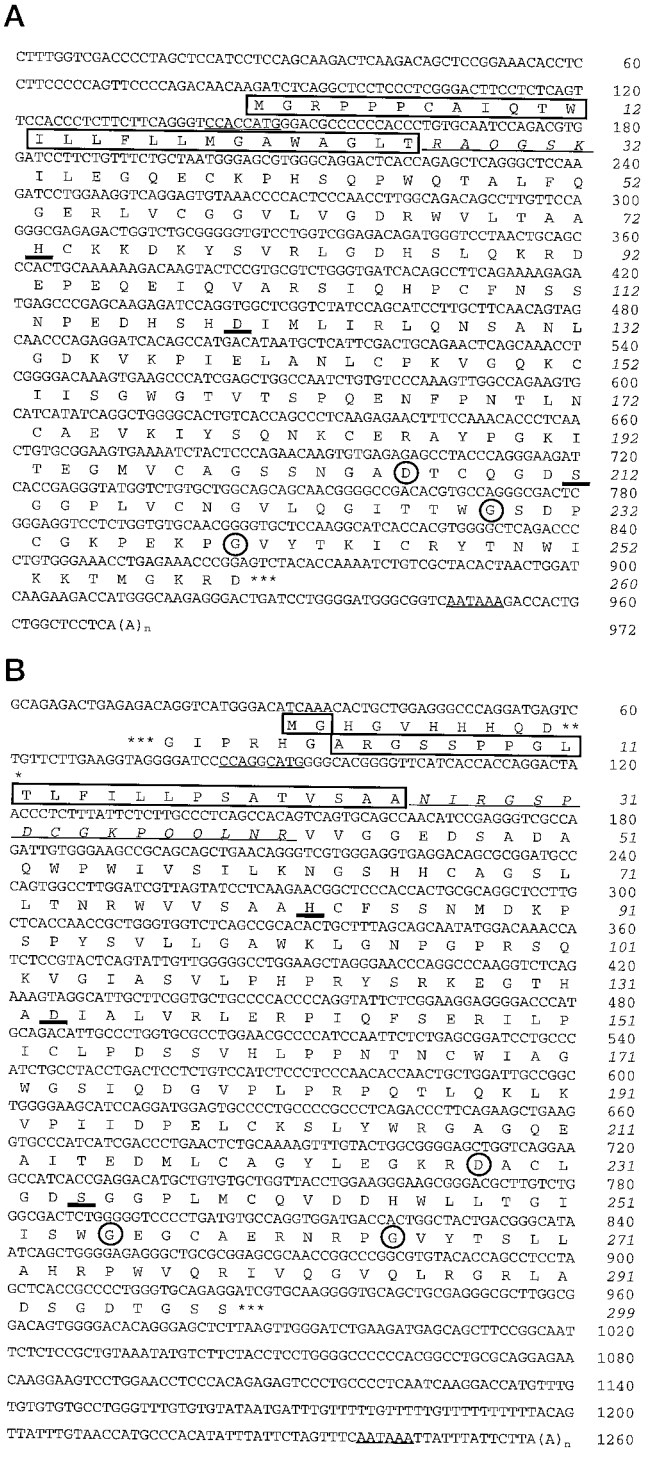

FIG. 3. **Sequences of cDNAs of BSP1 and BSP2 and the encoded polypeptides.** *Panel A*, sequence of BSP1 cDNA and the encoded polypeptide. *Panel B*, sequence of BSP2 and the encoded polypeptide. In the nucleotide sequence possible initiation codons, accompanying Kozak consensus sequences, and potential polyadenylation sequences are *underlined*. For uncertainties concerning the initiation site for BSP2 translation, see "Results." In the deduced polypeptide sequences the putative signal sequences are *boxed*, and the inferred proenzyme activation domain is in *italics*. The catalytic triad residues are *underlined*, and the three residues contributing to substrate specificity (and corresponding to residues 189, 216, and 226 of chymotrypsin) are *circled*.

with expression in CA1 and CA3 but only low level expression in CA2. Expression was also detected in the polymorphic layer of the dentate gyrus and within deep layers of the entorhinal cortex (Fig. 4, *D–F*). No significant expression was detected in other regions of the adult rat brain; overall, expression was substantially restricted to the hippocampal formation.

Robust BSP2 expression was detected throughout the CA fields of the hippocampus and at a lower level in the dentate gyrus. Expression was also recorded in the superficial layers of the cerebral cortex and the motor trigeminal, medullary, and reticular nuclei (Fig. 4, *G–I*, see also Fig. 5*A*).

Other Known Serine Proteases Are Expressed at Low Levels in Brain—Proteases elastase IV, proteinase 3, complement C2, chymotrypsin B, chymotrypsin-like protein (CTRL), and Hageman factor were also represented in hippocampal cDNA. However, in no case did Northern blotting of rat or mouse brain RNA reveal a detectable signal (not shown), indicating that expression levels are below the sensitivity threshold for this technique and correspondingly lower than those of t-PA, RNK-Met-1, BSP1, and BSP2.

In situ hybridization revealed CTRL mRNA within the cerebellum and the hippocampal formation with diffuse expression throughout other brain regions (Fig. 5*B*). Diffuse complement C2 mRNA was present throughout the brain, particularly in cerebral vasculature (Fig. 5, *C* and *D*). Elastase IV mRNA was present in cerebellum, deep and superficial regions of the cerebral cortex, and the CA1 field of the hippocampus (Fig. 5, *E* and *F*). A very low level of proteinase 3 and chymotrypsin B expression was also confirmed in brain by *in situ* hybridization (not shown).

Plasminogen mRNA Is Not Expressed in Brain, whereas Prothrombin and u-PA mRNAs Are Expressed Only at Low Levels—Although t-PA mRNA is represented abundantly in brain, clones corresponding to its usual target, plasminogen, were not obtained. Our analysis also failed to reveal sequences encoding (pro)thrombin and u-PA, two additional enzymes reported in rat brain (49, 50). Because Northern analysis of brain RNA failed to generate detectable signals using u-PA, prothrombin, or plasminogen probes (not shown) we used a more sensitive PCR-based assay. cDNA prepared from brain and positive control tissue (liver or kidney) was amplified using specific primers. An internal control employed coamplification of sequences encoding the ubiquitously expressed enzyme hypoxanthine phosphoribosyltransferase. Genomic DNA contamination was not a complicating factor because the primers amplify across substantial introns. Southern hybridization was used to confirm the identities of the PCR products.

Low level expression of prothrombin and u-PA was confirmed by specific PCR (Fig. 6, *A* and *B*). In contrast, specific PCR amplification of plasminogen sequences from total brain cDNA failed to generate a detectable product, whereas a strong product was obtained from the control tissue, liver. Both brain and liver yielded an easily detectable product corresponding to hypoxanthine phosphoribosyltransferase (Fig. 6*C*), confirming the integrity of the brain and liver cDNA preparations. We conclude that the level of plasminogen expression in rat brain is below the detection limit of the very sensitive PCR procedure employed.

DISCUSSION

We have explored the identities of the major serine proteases expressed in the central nervous system. We focused on the rodent hippocampus, a brain region in which synaptic plasticity is well characterized and which is thought to contribute to the formation of certain types of memory. Using a combination of techniques, including the use of PCR amplification based on conserved regions in the major (chymotryptic) clan of serine proteases, we demonstrate the presence of mRNAs corresponding to 12 serine proteases, including two new family members (BSP1 and BSP2, for brain serine protease).

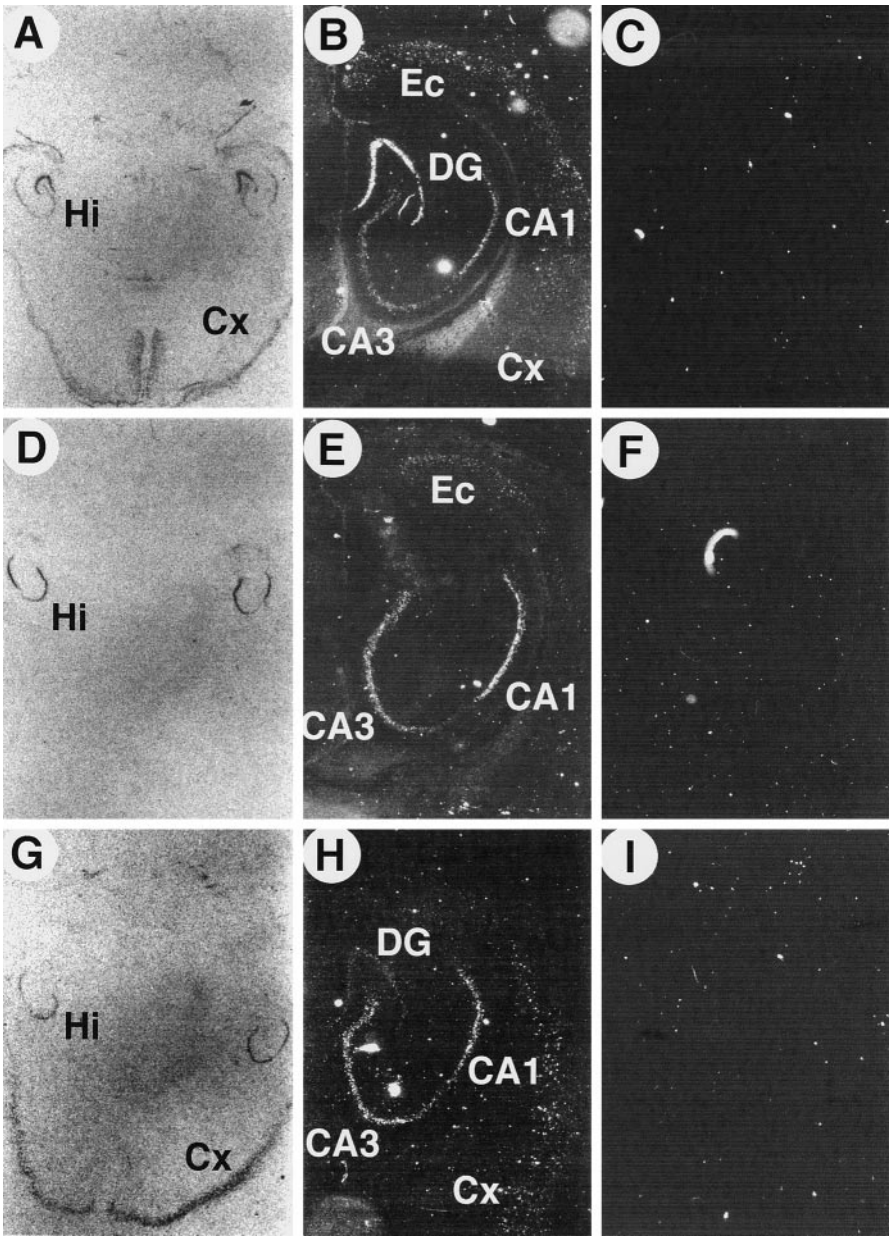

FIG. 4. ***In situ*** **hybridization of serine proteases to sections of rat brain.** Probes were: RNK-Met-1 (*panels A–C*), BSP1 (*panels D–F),* BSP2 (*panels G–I*). *Left panels* (*A*, *D*, and *G*) are autoradiograms of hybridized sections; *center panels* (*B*, *E*, and *H*), dark-field images of representative higher power images obtained after emulsion dipping of the sections; *right panels* (*C*, *F*, and *I*), images corresponding to the *center panels* but in which control sense rather than antisense probes were employed. *CA1* and *CA3*, subregions 1 and 3 of the *cornu ammonis* (hippocampus); *Hi*, hippocampus; *Cx*, cortex; *DG*, dentate gyrus; *Ec*, entorhinal cortex.

The most abundant species, confirmed by Northern blotting, corresponded to t-PA (63% of cloned rat hippocampus PCR products), RNK-Met-1 (26%), BSP2 (3.9%), and BSP1 (0.8%). Expression of the remaining eight species was not detected by Northern blotting (Table I); these are probably expressed at much lower levels.

We surmise that this distribution is a reliable estimate of the major serine proteases present in brain. Although the inherent bias of the PCR approach leaves open the possibility that our analysis might have failed to identify one or more major serine proteases expressed in hippocampus, we think this unlikely. First, although there was some bias, the abundance of the individual PCR products detected is grossly paralleled by their mRNA levels as determined by Northern blotting. Second, the known brain serine proteases not detected in our study all contain sequence motifs matching the primers employed; their absence is more consistent with low level expression than with PCR bias. Our one caveat is that the secondary screen removed sequences hybridizing at high stringency with t-PA and RNK-Met-1 probes; we would therefore fail to detect low abundance products that are extremely similar to these two species.

t-PA and RNK-Met-1—t-PA was recorded previously in mouse brain (12) with expression detected in microglia and some hippocampal neurons (19, 51). In rat, brain expression was restricted to the ventricular ependyma and olfactory nerve layer (52) with additional expression in subcommissural organ and cerebellar Purkinje neurons (45). However, widespread expression in rat brain revealed by our Northern analyses indicates that the rat and mouse patterns of expression are likely to be similar.

The second most abundantly expressed enzyme, RNK-Met-1, was unexpected. This enzyme was originally described in cytolytic T lymphocytes, wherein it was proposed to play a role in target cell death (44). The initial characterization recorded no RNK-Met-1 expression in brain, in clear contrast to our results. However, we have confirmed strong brain expression by Northern blotting and *in situ* hybridization. The role of RNK-Met-1 in brain is unknown, although its substrate specificity (favoring cleavage after a Met residue) suggests that the enzyme might contribute to processing of the Alzheimer's APP by the β-secretase pathway, where cleavage after Met has been described (26).

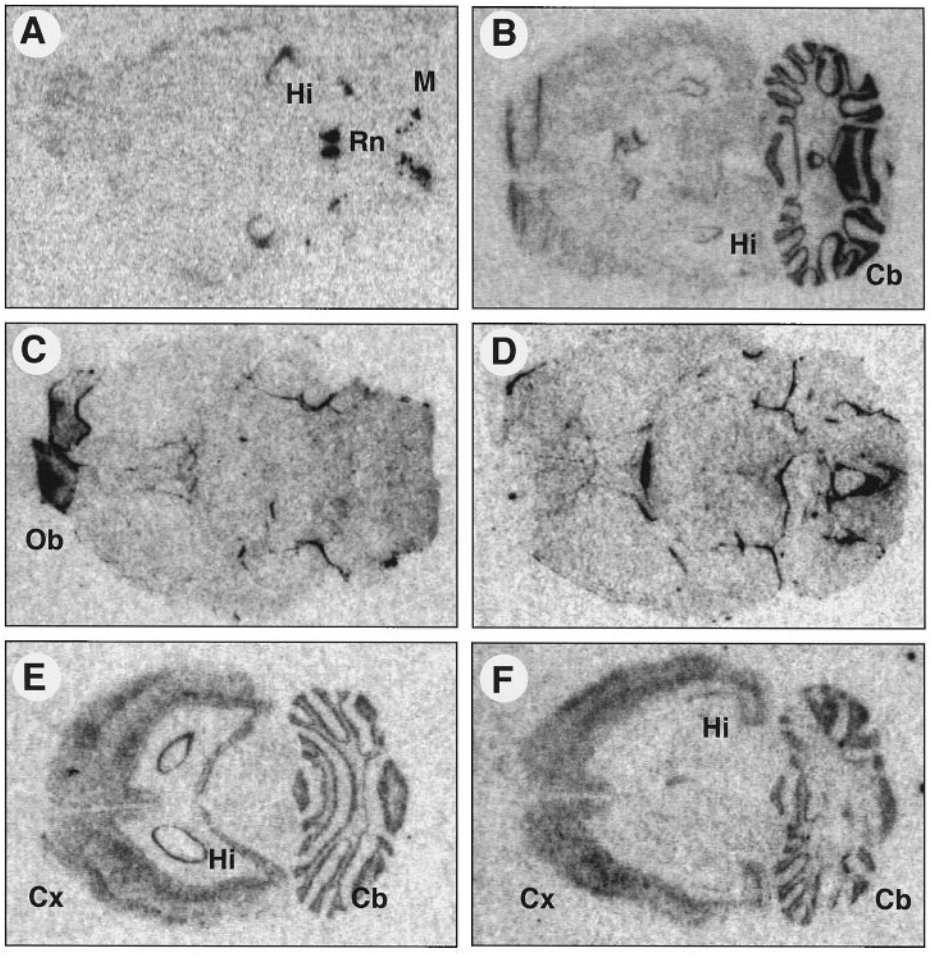

FIG. 5. ***In situ* hybridization analysis of serine protease expression.** Horizontal sections of rat brain were hybridized with probes specific for RNK-Met-1 (*panel A*), CTRL (*panel B*), complement C2 (*panels C* and *D*), and elastase IV (*panels E* and *F*). For RNK-Met-1 expression see also Fig. 4. *Hi*, hippocampus; *Rn*, reticular nucleus; *M*, medullary nucleus; *Ob*, olfactory bulb; *Cx*, cortex; *Cb*, cerebellum.

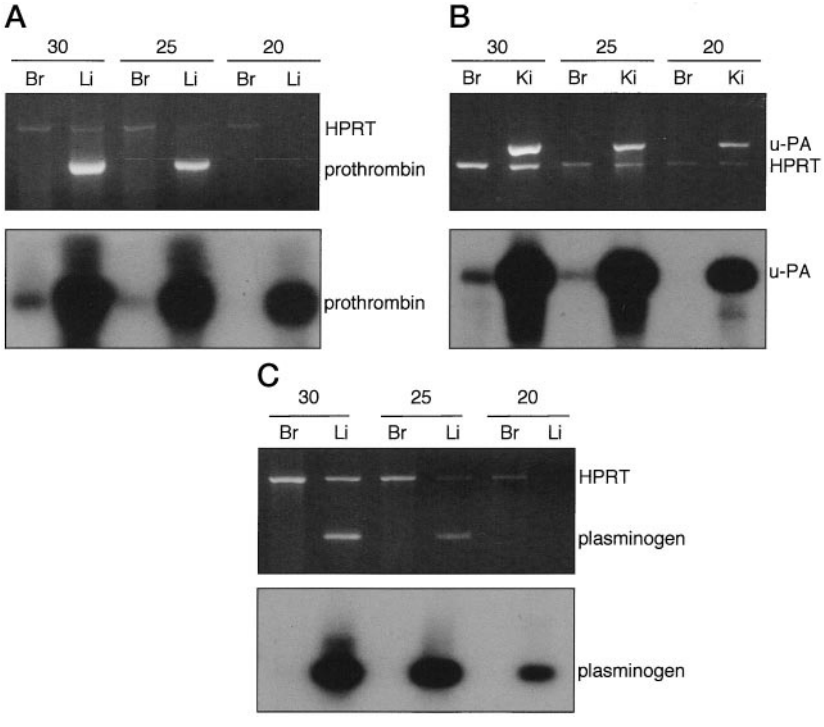

FIG. 6. **PCR analysis of prothrombin, plasminogen, and u-PA expression in rat brain.** Total brain (*Br*), liver (*Li*), or kidney (*Ki*) RNA samples were reverse transcribed and PCR amplified for 20, 25, or 30 cycles as indicated using primer pairs specific for thrombin and the internal control hypoxanthine phosphoribosyltransferase (*HPRT*, *panel A*), u-PA and hypoxanthine phosphoribosyltransferase (*panel B*), plasminogen and hypoxanthine phosphoribosyltransferase (*panel C*). PCR products were resolved by agarose gel electrophoresis (*upper panels*) and analyzed by Southern blotting and hybridization to gene-specific probes (*lower panels*).

Brain Serine Proteases BSP1 and BSP2—Both mRNAs are strongly expressed in brain. BSP2 has not been reported elsewhere, whereas we find BSP1 to be similar to a mouse serine protease "neuropsin" (28) that was reported after our characterization of BSP1. Sequence homology (92% at the amino acid level) and the concurrence between the expression patterns described in rat (this work) and in mouse (28) suggest that BSP1 may be the rat ortholog of this mouse enzyme.

Both BSP1 and BSP2 are most closely related to the tryptic subfamily of enzymes, whereas trypsin itself was not detected

in hippocampus in our study. BSP1 mRNA is highly enriched in hippocampal formation, with expression restricted to the CA1 and CA3 fields and entorhinal cortex but with only low level expression in CA2. Expression was not detected in dentate granule cells. BSP2 is expressed more widely in brain, throughout the CA fields of the hippocampus, the dentate gyrus, and in the superficial layers of the cerebral cortex.

The BSP2 cDNA sequence was found to contain an unusual feature in that the most likely ATG for translation initiation was out of phase with the remainder of the open reading frame encoding the BSP2 polypeptide; this was confirmed by genomic analysis. Open reading frame conservation and robust expression argue against the product of a transcribed pseudogene. Although ribosomal frameshifting could be invoked as an explanation, we suspect an intractable sequencing artifact in the vicinity of the proposed initiation codon. Considerable secondary structure in the region of the ATG may support this interpretation.

Enzymes Elastase IV, Proteinase 3, Complement C2, Hageman Factor, and CTRL—In situ hybridization revealed these species at low to moderate levels in rat brain. Expression was not detectable by Northern analysis, indicating that their levels are significantly below those of t-PA, RNK-Met-1, BSP1, and BSP2.

Elastase activity associated with leukocytes and microglia (53) can be attributed to neutrophil elastase; brain expression of elastase IV, a pancreatic enzyme, has not been reported previously. In addition to a possible role in matrix degradation, elastase can cleave both protease nexin I (54) and APP, this latter at the COOH terminus of the β-amyloid domain, and could constitute a potential γ-secretase (55).

Proteinase 3 enzyme is one of three neutral proteases characterized in polymorphonuclear leukocytes (56), thus brain expression within microglia may be anticipated. Complement protein 2 (C2) is one of a group of proteins responsible for the inactivation of invading pathogens; Hageman factor (factor XII), an enzyme involved in blood coagulation and complement activation, was also detected. Notably, a role for complement proteins in the brain is suggested by the fact that mice lacking active C5 exhibit altered glutamate receptor responses and accelerated excitotoxic damage after kainate infusion (57). Hageman factor can activate latent growth and neuroprotective factors such as hepatocyte growth factor (58) and was reported in Alzheimer's disease plaques (59).

Other proteases detected include chymotrypsin and the closely related protein CTRL; their specific function in this tissue is not known, although chymotryptic enzymes can cleave APP *in vitro* at the NH_2 terminus of the β-amyloid domain (60).

Prothrombin and u-PA—We report low level expression of mRNAs encoding these two enzymes, confirming previous reports of their expression in rat brain (49, 50). The low level expression of u-PA contrasts with the abundant expression of t-PA in rat brain, arguing that t-PA is the predominant PA in brain.

Plasminogen—The paradigm for t-PA action is in plasminogen activation; however, our analysis failed to provide evidence for local synthesis of plasminogen in rat brain. Sensitive gene-specific reverse transcription and PCR were unable to detect plasminogen transcripts in whole rat brain RNA. This same result was obtained using an alternative set of PCR primers or using mouse instead of rat brain (not presented). Despite reports of plasminogen mRNA and protein in mouse hippocampus (51), our results argue that plasminogen production in brain is at least 10^3-fold lower than in liver and is unlikely to be of major biological significance.

Brain t-PA is therefore unlikely to initiate a proteolytic cascade through the sequential activation of plasminogen and metalloproteases. We suggest that t-PA might play a different role. In support, knockout mice lacking either t-PA or plasminogen display different phenotypes. Microglial activation is attenuated in t-PA null mice but not in animals lacking plasminogen (51). Possible alternative substrates for t-PA include hepatocyte growth/scatter factor and macrophage-stimulating protein. These growth factors, although proteolytically inactive, share sequence similarity with plasminogen and require proenzyme cleavage for activation. Hepatocyte growth factor, a neurotrophic factor, has been shown to be activated by t-PA mediated cleavage (61, 62).

The diversity of serine proteases expressed in brain suggests that this family may regulate neuronal function in a variety of different ways. In addition to extracellular matrix remodeling, growth factor activation, and receptor binding (as shown for *e.g.* thrombin and u-PA), the reported modulation of ion channel function by extracellular proteases (*e.g.* Ref. 63) adds a further dimension to their potential roles. The discovery of a serine protease, BSP1, expressed specifically in subfields of the hippocampal formation is of some interest; this implies that the hippocampus has a requirement for selective proteolysis which is unique to this brain region. Transcription regulatory elements derived from the BSP1 gene may lend themselves to selective exploration of hippocampal function in transgenic animal systems (64–66).

It is not known whether the primary roles of the BSP1 and BSP2 are, as we infer for t-PA, in the activation of latent growth factors or whether they instead participate directly or indirectly in structural remodeling. Notably, serine proteases with tryptic activity have been implicated in the processing of APP (67), whereas the protease inhibitor domain (nexin II) of APP is active against trypsin (42). Both BSP1 and BSP2 are trypsin-like, and interactions with APP/nexin II are likely. Further studies on these enzymes, including the elucidation of their substrate specificity and preparation of mutant mice, will be required to cast light on their roles in brain function.

Acknowledgments—We acknowledge the essential contribution of the staff who assisted with the procurement of brain samples.

REFERENCES

1. Krystosek, A., and Seeds, N. W. (1981) *Science* **213,** 1532–1533
2. Moonen, G., Gray-Wagemann, M. P., and Selak, I. (1982) *Nature* **298,** 753–755
3. McGuire, P. G., and Seeds, N. W. (1990) *Neuron* **4,** 633–642
4. Rutka, J. T., Apodaca, G., Stern, R., and Rosenblum, M. (1988) *J. Neurosurg.* **69,** 155–170
5. Venstrom, K. A., and Reichardt, L. (1993) *FASEB J.* **7,** 995–1003
6. Werb, Z. (1997) *Cell* **91,** 439–442
7. Yong, V. W., Krekoski, C. A., Forsyth, P. A., Bell, R., and Edwards, D. R. (1998) *Trends Neurosci.* **21,** 75–80
8. Saksela, O., and Rifkin, D. B. (1988) *Annu. Rev. Cell Biol.* **4,** 93–126
9. Valinsky, J. E., and LeDourain, N. M. (1985) *EMBO J.* **4,** 1403–1046
10. Krystosek, A., and Seeds, N. W. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7810–7814
11. Friedman, G. C., and Seeds N. W. (1994) *Dev. Brain Res.* **81,** 41–49
12. Sappino, A.-P., Madani, R., Huarte, J., Belin, D., Kiss, J.-Z., Wohlwend, A., and Vassalli, J.-D. (1993) *J. Clin. Invest.* **92,** 679–685
13. Bailey, C. H., and Kandel, E. R. (1993) *Annu. Rev. Physiol.* **55,** 397–426
14. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel E. R., and Kuhl, D. (1993) *Nature* **361,** 453–457
15. Seeds, N. W., Williams, B. L., and Bickford, P. C. (1995) *Science* **270,** 1992–1994
16. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J. J., Collen, D., and Mulligan, R. C. (1994) *Nature* **368,** 419–424
17. Frey, U., Muller, M., and Kuhl, D. (1996) *J. Neurosci.* **16,** 2057–2063
18. Huang, Y.-Y., Bach, M. E., Lipp, H.-P., Zhuo, M., Wolfer, D. P., Hawkins, R. D., Schoonjans, L., Kandel, E. R., Godfraind, J.-M., Mulligan, R., Collen, D., and Carmeliet, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8699–8704
19. Tsirka, S. E., Gualandris, A., Amaral, D. G., and Strickland, S. (1995) *Nature* **377,** 340–344
20. Tsirka, S. E., Rogove, A. D., and Strickland, S. (1996) *Nature* **384,** 123–124
21. Gurwitz, D., and Cunningham, D. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3440–3444
22. Cavanaugh, K. P., Gurwitz, D., Cunningham, D. D., and Bradshaw, R. A. (1990) *J. Neurochem.* **54,** 1735–1743
23. Smith-Swintosky, V. L., Zimmer, S., Fenton, J. W., and Mattson, M. P. (1995)

J. Neurochem. **65,** 1415–1418
24. Smith-Swintosky, V. L., Zimmer, S., Fenton, J. W., II, and Mattson, M. P. (1995) *J. Neurosci.* **15,** 5840–5850
25. Lüthi, A., van der Putten, H., Boteri, F. M., Mansuy, I. M., Meins, M., Frey, U., Sansig, G., Portet, C., Schmutz, M., Schroder, M., Nitsch, C., Laurent, J.-P., and Monard, D. (1997) *J. Neurosci.* **17,** 4688–4699
26. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) *Nature* **325,** 733–736
27. Ponte, P., Gonzalez-deWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) *Nature* **331,** 525–527
28. Chen, Z.-L., Yoshida, S., Kato, K., Momota, Y., Suzuki, J., Tanaka, T., Ito, J., Nishino, S., Aimoto, S., Kiyama, H., and Shiosaka, S. J. (1995) *Neuroscience* **15,** 5088–5097
29. Gschwend, T. P., Krueger, S. R., Kozlov, S. V., Wolfer, D. P., and Sonderegger, P. (1997) *Mol. Cell. Neurosci.* **9,** 207–219
30. Yamashiro, K., Tsuruoka, N., Kodama, S., Tsujimoto, M., Tamamura, Y., Tanaka, T., Nakazoto, H., and Yamaguchi, N. (1997) *Biochim. Biophys. Acta* **1350,** 11–14
31. Scarisbrick, I. A., Towner, M. D., and Isackson, P. J. (1997) *J. Neurosci.* **17,** 8156–8168
32. Krueger, S. R., Ghisu, G. P., Cinelli, P., Gschwend, T. P., Osterwalder, T., Wolfer, D. P., and Sonderegger, P. (1997) *J. Neurosci.* **17,** 8984–8996
33. Bliss, T. V. P., and Collingridge, G. L. (1993) *Nature* **361,** 31–39
34. Scoville, W. B., and Milner, B. (1957) *J. Neurol. Neurosurg. Psychiatry* **20,** 11–12
35. O'Keefe, J., and Nadel, L. (1978) *The Hippocampus as a Cognitive Map,* Clarendon Press, Oxford
36. Squire, L. R. (1992) *Psychol. Rev.* **99,** 195–231
37. Geinisman, Y., de Toledo-Morrell, L., and Morrell, F. (1991) *Brain Res.* **566,** 77–88
38. Rawlings, N. D., and Barrett, A. J. (1994) *Methods Enzymol.* **244,** 19–61
39. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
40. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1991–1995
41. Stapleton, G., Steel, M., Richardson, M., Mason, J. O., Rose, K. A., Morris, R. G. M., and Lathe, R. (1995) *J. Biol. Chem.* **270,** 29739–29745
42. Kitaguchi, N., Takahashi, Y., Oishi, K., Shiojiri, S., Tokushima, Y., Utsunomiya, T., and Ito, H. (1990) *Biochim. Biophys. Acta* **1038,** 105–113
43. Vincent, S., Marty, L., and Fort, P. (1993) *Nucleic Acids Res.* **21,** 1498
44. Smyth, M. J., Wiltrout, T., Trapani, J. A., Ottaway, K. S., Sowder, R., Henderson, L. E., Kam, C.-M., Powers, J. C., Young, H. A., and Sayers, T. J. (1992) *J. Biol. Chem.* **267,** 24418–24425
45. Ware, J. H., Dibenedetto, A. J., and Pittman, R. N. (1995) *Brain Res. Bull.* **37,** 275–281
46. Polgár, L. (1989) *Mechanisms of Protease Action,* CRC Press, Boca Raton, FL
47. Perona, J. J., Heldstrom, L., Rutter, W. J., and Fletterick, R. J. (1995) *Biochemistry* **34,** 1489–1499
48. Hartley, B. S. (1964) *Nature* **201,** 1284–1287
49. Dihanich, M., Kaser, M., Reinhard, E., Cunningham, D., and Monard, D. (1991) *Neuron* **6,** 575–581
50. Masos, T., and Miskin, R. (1996) *Mol. Brain Res.* **35,** 139–148
51. Tsirka, S. E., Rogove, A. D., Bugge, T. H., Degen, J. L., and Strickland, S. (1997) *J. Neurosci.* **17,** 543–552
52. Dent, M. A. R., Sumi, Y., Morris, R. J., and Seeley, P. J. (1993) *Eur. J. Neurosci.* **5,** 633–647
53. Nakajima, K., Shimojo, M., Hamanoue, M., Ishiura, S., Sugita, H., and Kohsaka, S. (1992) *J. Neurochem.* **58,** 1401–1408
54. Nick, H., Hofsteenge, J., Shaw, E., Rovelli, G., and Monard, D. (1990) *Biochemistry* **29,** 2417–2421
55. Evin, G., Cappai, R., Li, Q. X., Culvenor, J. G., Small, D. H., Beyreuther, K., and Masters, C. L. (1995) *Biochemistry* **34,** 14185–14192
56. Rao, N. V., Wehner, N. G., Marshall, B. C., Sturrock, A. B., Huecksteadt, T. P., Rao, G. V., Gray, B. H., and Hoidal, J. R. (1991) *Ann. N. Y. Acad. Sci.* **624,** 60–68
57. Pasinetti, G. M., Tocco, G., Sakhi, S., Musleh, D. W., DeSimoni, M. G., Mascarucci, P., Schreiber, S., Baudry, M., and Finch, C. E. (1996) *Neurobiol. Dis.* **3,** 197–204
58. Shimomura, T., Miyazawa, K., Komitama, Y., Hiraoka, H., Naka, D., Morimoto, Y., and Kitamura, N. (1995) *Eur. J. Biochem.* **229,** 257–261
59. Yasuhara, O., Walker, D. G., and McGeer, P. L. (1994) *Brain Res.* **654,** 234–240
60. Nelson, R. B., Siman, R., Iqbal, M. A., and Potter, H. (1993) *J. Neurochem.* **61,** 567–577
61. Mars, W. M., Zarnegar, R., and Michalopoulos, G. K. (1993) *Am. J. Pathol.* **143,** 949–958
62. Thewke, D. P., and Seeds, N. W. (1996) *J. Neurosci.* **16,** 6933–6944
63. Vallet, V., Chraibi, A., Gaeggeler, H. P., Horisberger, J. D., and Rossier, B. C. (1997) *Nature* **389,** 607–610
64. Lathe, R., and Morris, R. G. M. (1994) *Neuropathol. Appl. Neurobiol.* **20,** 350–358
65. Mayford, M. A. T., and Kandel, E. R. (1995) *Curr. Opin. Neurobiol.* **5,** 141–148
66. Chen, C., and Tonegawa, S. (1997) *Annu. Rev. Neurosci.* **20,** 157–184
67. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) *Science* **248,** 1122–1124