Identification of Brain-derived Neurotrophic Factor Promoter Regions Mediating Tissue-specific, Axotomy-, and Neuronal Activity–induced Expression in Transgenic Mice

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Abstract. The structure of rat brain-derived neurotrophic factor (BDNF) gene is complex; four 5′ exons are linked to separate promoters and one 3′ exon is encoding the BDNF protein. To analyze the relative importance of the regulatory regions in vivo, we have generated transgenic mice with six different promoter constructs of the BDNF gene fused to the chloramphenicol acetyl transferase reporter gene. High level and neuronal expression of the reporter gene, that in many respects recapitulated BDNF gene expression, was achieved by using 9 kb of genomic sequences covering the promoter regions that lie adjacent to each other in the genome (promoters I and II and promoters III and IV, respectively) and by including sequences of BDNF intron-exon splice junctions and 3′ untranslated region in the constructs. The genomic regions responsible for the in vivo upregulation of BDNF expression in the axotomized sciatic nerve and in the brain after kainic acid–induced seizures and KCl-induced spreading depression were mapped. These data show that regulation of the different aspects of BDNF expression is controlled by different regions in vivo, and they suggest that these promoter constructs may be useful for targeted expression of heterologous genes to specific regions of the central and peripheral nervous systems in an inducible manner.

BRAIN-derived neurotrophic factor (BDNF)[1] (Barde et al., 1982; Leibrock et al., 1989) is a member of the neurotrophin family that includes three other structurally related proteins: NGF, neurotrophin-3, and neurotrophin-4 (reviewed in Korsching, 1993; Persson, 1993). In cell culture, BDNF promotes the survival of retinal ganglion cells (Johnson et al., 1986), basal forebrain cholinergic neurons (Alderson et al., 1990), embryonic mesencephalic dopaminergic neurons (Hyman et al., 1991; Knüsel et al., 1991), and embryonic cerebellar granule neurons (Segal et al., 1992). BDNF also promotes neuronal differentiation in neural crest cell cultures (Sieber-Blum, 1991) and enhances early maturational change in dorsal root ganglion cell cultures (Wright et al., 1992). Recently, it was shown that endogenous BDNF is a trophic factor in activity-mediated neuronal survival of cultured embryonic cortical neurons (Ghosh et al., 1994). In vivo BDNF increases the survival of embryonic neurons in the dorsal root ganglia, nodose ganglia (Hofer and Barde, 1988), basal forebrain cholinergic neurons (Knüsel et al., 1992), as well as developing and axotomized motoneurons (Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993). BDNF null-mutated mice develop several sensory deficits, but no obvious defects were seen for motoneurons or central dopaminergic neurons (Ernfors et al., 1994; Jones et al., 1994).

Except for the striatum and septum, BDNF mRNA is expressed in neurons throughout the adult rat brain with the highest level in the hippocampal formation (Ernfors et al., 1990*a*, *b*; Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1990), where also BDNF-like immunoreactivity has been demonstrated (Wetmore et al., 1991). Glutamatergic, GABA-ergic, and cholinergic neurotransmitter systems have been shown to be involved in the regulation of BDNF mRNA expression (Zafra et al., 1990, 1991; Ernfors et al., 1991; Isackson et al., 1991; Dugich-Djordejevic et al., 1992*a*, *b*; Lindefors et al., 1992; Lindvall et al., 1992). Activation of

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1. *Abbreviations used in this paper*: BDNF, brain-derived neurotrophic factor; CAT, chloramphenicol acetyl transferase; KA, kainic acid; NRSE, neural-restrictive silencer element; SD, spreading depression; UTR, untranslated region.

glutamate receptors of both NMDA and non-NMDA subtypes has been implicated in the increase of BDNF mRNA after administration of kainic acid (KA) or after epileptic and ischemic insults (Zafra et al., 1990, 1991; Ernfors et al., 1991; Lindvall et al., 1992). However, the molecular mechanisms governing the increase of BDNF mRNA levels, as well as the mechanisms responsible for the tissue-specific and developmentally regulated expression of BDNF mRNA, are unknown.

It has recently been shown that the organization of BDNF gene is complex (Timmusk et al., 1993). Four short untranslated 5′ exons and one 3′ exon encoding the mature BDNF protein are present within the rat BDNF gene with a separate promoter upstream of each 5′ exon. Alternative promoter usage, differential splicing and the use of two different polyadenylation sites within each of the four transcription units generate altogether eight different BDNF mRNAs (Timmusk et al., 1993). Promoters I–III are used predominantly in the brain, while promoter IV is more active in peripheral tissues. Moreover, the levels of BDNF mRNAs transcribed from promoters I–III are markedly elevated in a promoter-specific subset of hippocampal and neocortical neurons in different models of neuronal activation, while only a modest increase was seen for promoter IV specific mRNA (Falkenberg et al., 1993; Metsis et al., 1993; Timmusk et al., 1993; Kokaia et al., 1994).

In this report, we have analyzed the potential of the promoter regions of the rat BDNF gene to drive expression of a reporter gene in transgenic mice. Tissue-specific and high level expression of the bacterial reporter gene chloramphenicol acetyl transferase (CAT) in correct neuronal populations of transgenic mice brain was achieved by using 9 kb of genomic sequences covering the promoter regions that lie close in the genome (promoters I and II and promoters III and IV, respectively) and by including sequences of BDNF introns with splice donor and acceptor sites and 3′ untranslated region (UTR) in the constructs. We also identified the promoter regions that are involved in the regulation of BDNF induction in different experimental models, i.e., (*a*) in the cerebral cortex after KCl-induced cortical spreading depression; (*b*) in the hippocampus and cerebral cortex after KA-induced seizures; and (*c*) in the axotomized sciatic nerve.

Materials and Methods

General Methods

All molecular biology procedures were performed according to standard practices (Sambrook et al., 1989). Plasmid DNA was purified by alkaline lysis and CsCl centrifugation. Labeling of cRNA and cDNA probes, RNA isolation, CAT assays, RNase protection assay, RNA quantification, and in situ hybridization were performed as shown earlier (Timmusk et al., 1993, 1994*a*). BDNF exon-specific cRNA probes were synthesized from the same fragments of rat BDNF gene as described (Timmusk et al., 1993, 1994*a*). As a probe for CAT, the 0.8-kb coding sequence of it was digested out from pBLCAT2 (Luckow and Schutz, 1987) using XhoII restriction enzyme, subcloned into the Eco RV site of pBSKS vector (Stratagene, La Jolla, CA), and used further to synthesize cRNA or cDNA probes. As a probe for mouse BDNF, a 0.34-kb NarI fragment of mouse BDNF cDNA (Hofer et al., 1990) was used as a template.

Transgenic Mice

Transgenic mice were generated and analyzed for transgene integration as previously described (Nilsson and Lendahl, 1993). Vector sequences were removed from the BDNF promoter constructs and the transgene was injected into the male pronucleus of one-cell mouse embryos obtained from superovulated F1(C57BL6 X CBA). Injected eggs were transferred at the one- or two-cell stage to the oviducts of pseudopregnant foster mothers. Tail biopsy–derived DNA isolated from the resulting offspring (F0) was screened by Southern or dot blot analysis with CAT-specific probe for the presence of the transgene. Different restriction digests were used to identify those animals that contained intact copies of the construct (data not shown). The frequencies for transgenicity were between 15 and 50%. As the integration of transgene in F0 founders may be chimeric, most of the transgenic founders were maintained as lines by breeding them with wild-type mice. The analysis of transgene expression and regulation was performed with 1–2-mo-old F1 animals unless specifically noted.

Animals, Pharmacological Treatments, and Surgery

Adult male and female transgenic mice (body wt = 20–30 g) were used for all experiments. Treatment of animals with kainic acid was performed as described earlier (Zafra et al., 1990) and animals were killed 3–6 h after the injection. Spreading depression produced by repeated topical application of KCl to the cortical surface of mice during 2 h was performed as described in (Kokaia et al., 1993) and mice were killed after another 2 h. Transection of sciatic nerve was performed as previously described (Funakoshi et al., 1993). After all treatments mice were anesthetized with ether and decapitated. The brains, indicated brain regions, and peripheral organs were dissected and immediately frozen on dry ice.

BDNF-CAT Fusion Genes

Promoters of BDNF I, II, III, and IV CAT constructs were all inserted into modified pBLCAT2 vector, replacing the thymidine kinase promoter by cloning into the XbaI and XhoI sites. All these constructs contain SV-40 small t intron and SV-40 polyadenylation signals downstream from CAT gene. The numbering and restriction enzyme sites is corresponding to the published rat BDNF gene sequence (Timmusk et al., 1993).

BDNF I CAT contains a 2.6-kb EcoRI/ClaI fragment of exon I and its 5′ flanking region. The 5′ EcoRI site lies 1.5 kb upstream from the BamHI site located at the first basepair of rat BDNF gene sequence. The 3′ ClaI site is located at 937 bp.

BDNF II CAT contains a 3.7-kb EcoRI/AccI fragment of exon II and its flanking region. This construct has the same 5′ end as BDNF I CAT, but it extends 1.2 kb to the 3′ direction. The 3′ AccI site is located at 2,151 bp inside of exon II.

BDNF III CAT contains a 4.4-kb HindIII/XbaI fragment of exon III and its 5′ flanking region. The 5′ HindIII site lies 3.5 kb upstream from the HincII site located at the first basepair of rat BDNF gene sequence. The 3′ XbaI site is located at 901 bp.

BDNF IV CAT contains a 5.5-kb HindIII/BamHI fragment of exon IV and its 5′ flanking region. This construct has the same 5′ end as BDNF III CAT, but it extends 1.1 kb to the 3′ direction. The 3′ BamHI site is located at 2,051 bp inside of exon IV.

BDNF IV prox CAT contains a 0.9-kb EcoRI/BamHI fragment of exon IV and its 5′ flanking region. This construct is a 5′ deletion of BDNF IV CAT.

BDNF I + II CAT and BDNF III + IV CAT were cloned in pBSKS and they contain the following: (*a*) Promoter region and the 5′ part of the mini-intron including splice donor sites: genomic fragments covering the 5′ and 3′ flanking regions of exons I and II or exons III and IV, respectively. (*b*) 3′ part of the mini-intron including splice acceptor site: a 0.7-kb genomic fragment covering the 5′ flanking region of exon V (coding exon) including 10 bp of exon V (this is the common splice acceptor region for all the BDNF transcripts). This region is common for both minigenes. (*c*) CAT gene (0.7-kb XhoII fragment) without SV-40 small t intron and polyadenylation signals. This region is common for both minigenes. (*d*) 3′ UTR including polyadenylation signals: 3.2-kb genomic fragment covering BDNF 3′ UTR, extending 0.1 kb downstream from the end of the 4.2-kb mRNA. This region is common for both minigenes.

BDNF I + II CAT promoter region contains a 9.5-kb BamHI/SalI genomic fragment including exons I and II and the flanking regions. The 5′ BamHI site lies ~6.5 kb upstream from the BamHI site located at the first bp of rat BDNF gene sequence. The 3′ SalI site lies ~0.7 kb downstream from the end of exon II.

BDNF III + IV CAT promoter region contains a 9-kb BamHI/HindIII genomic fragment including exons III and IV and the flanking regions. The 5′ BamHI site lies ~6 kb upstream from the HincII site located at the first

bp of rat BDNF gene sequence. The 3′ HindIII site lies ~0.7 kb downstream from the end of exon IV.

Results

Expression of BDNF Promoter I, II, III, and IV CAT Constructs in Transgenic Mice

Functional analysis of the proximal promoter regions of the BDNF gene by transient transfections showed that sequences upstream of all four 5′ exons promote expression of the CAT reporter gene in neuronal and nonneuronal cell lines (Timmusk et al., 1993). To analyze the tissue specificity of the rat BDNF gene expression in vivo, transgenic mice were generated that harbored a chimeric gene composed of one of the upstream regions of the 5′ exons of rat BDNF gene fused to sequences encoding the bacterial enzyme CAT. The constructs (designated BDNF I CAT, BDNF II CAT, BDNF III CAT, and BDNF IV CAT) are represented in Fig. 1. All these fusion genes contain SV-40 small t intron and SV-40 polyadenylation signals downstream of the CAT coding region. BDNF I CAT contains a 2.6-kb fragment of exon I and its 5′ flanking region. BDNF II CAT contains a 3.7 kb fragment of exon II and its 5′ flanking region. This construct has the same 5′ end as BDNF I CAT, but it extends 1.2 kb to the 3′ direction and has the 3′ end in exon II. BDNF III CAT contains a 4.4-kb fragment of exon III and its 5′ flanking region. BDNF IV CAT contains a 5.5-kb fragment of exon IV and its 5′ flanking region. This construct has the same 5′ end as BDNF III CAT, but it extends 1.1 kb to the 3′ direction terminating inside exon IV.

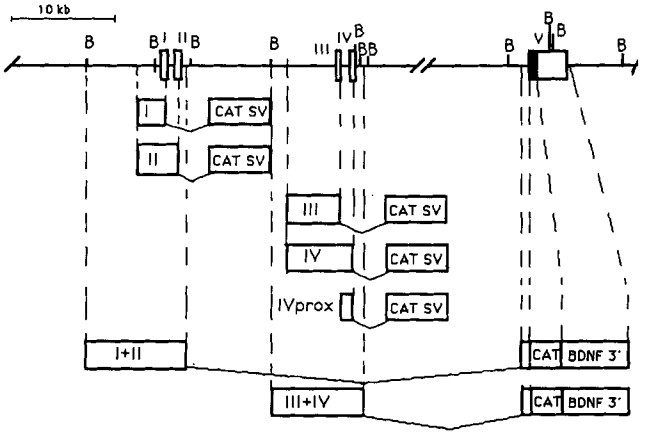


Figure 1. Schematic representation of rat BDNF-CAT fusion genes analyzed in transgenic mice in relation to the rat BDNF gene structure. The gene is shown in top with exons with their corresponding numbering as boxes and introns as lines. UTRs of the exons are indicated by open boxes, and the region corresponding to the prepro-BDNF protein is indicated by the filled box. Restriction sites for BamHI are indicated by B's. The BDNF CAT fusion genes are shown below the schematic map of BDNF gene. Open boxes with I, II, III, IV, IV prox, I + II, and III + IV indicate the upstream regions of BDNF 5′ exons included in BDNF I CAT, BDNF II CAT, BDNF III CAT, BDNF IV CAT, BDNF IV prox CAT, BDNF I + II CAT, and BDNF III + IV CAT, respectively. Broken lines show the regions of rat BDNF gene included in the BDNF-CAT fusion genes. *SV*, SV-40 small t intron and polyadenylation signals. *BDNF 3′*, 3′ UTR of rat BDNF gene.

CAT activity was measured in brain and in 10 nonneural tissues: salivary gland, thymus, heart, lung, spleen, liver, kidney, pancreas, muscle, and testis. As the mouse BDNF gene structure has not been reported, the expression patterns of the BDNF-CAT fusion genes were compared to the levels of rat BDNF mRNAs containing different 5′ exons by RNase protection assay.

2.6 kb of BDNF Promoter I Targets the Expression of Reporter Gene to the Thymus of Transgenic Mice

Eight different transgenic lines expressing CAT under the control of a 2.6-kb 5′ flanking sequence of exon I (BDNF I CAT) were analyzed. CAT activity was detected in the thymus of all of the eight lines tested. In the other organs, including the brain, the levels of CAT activity were close to or below the detection limit (Fig. 2 and Table I). We have recently shown that in the rat this promoter is active in brain and not in lung and heart (Timmusk et al., 1993). RNase

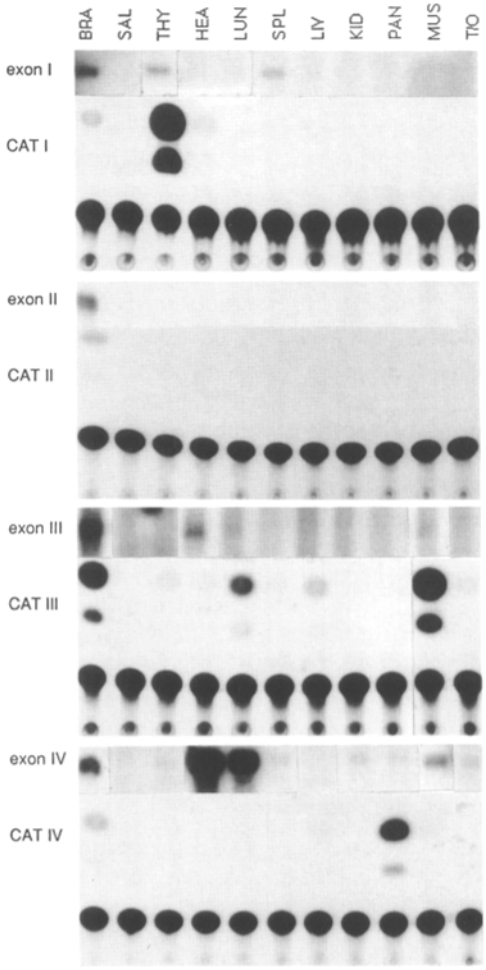


Figure 2. Expression of BDNF I CAT (*CAT I*), BDNF II CAT (*CAT II*), BDNF III CAT (*CAT III*), and BDNF IV CAT (*CAT IV*) in the brain and nonneural tissues of transgenic mice of representative founder lines. 1 mg of protein extract was analyzed in each line by CAT assay. Samples were incubated for 2 h at 37°C. Expression of endogenous rat BDNF exon mRNAs in the same rat tissues analyzed by RNase protection assay is shown in top of the CAT assays of each BDNF-CAT fusion gene.

Table I. Tissue Distribution of CAT Activity in Transgenic Mice with Different BDNF-CAT Fusion Genes

	CAT activity TISSUE										
	BRA	SAL	THY	HEA	LUN	SPL	LIV	KID	PAN	MUS	T/O
I	6	0	8	6	4	6	0	0	0	0	0
n = 8	(+)	−	+	(+)	(+)	(+)	−	−	−	−	−
II	4	0	4	0	0	0	0	0	0	0	0
n = 18	(+)	−	(+)	−	−	−	−	−	−	−	−
III	6	2	4	4	4	4	4	3	3	6	4
n = 11	+	(+)	+	+	+	(+)	(+)	(+)	(+)	+	(+)
IV	11	8	8	8	8	8	8	8	11	8	8
n = 13	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	(+)	(+)
I + II	9	nt	9	1	7	1	0	1	1	1	1
n = 9	+++		++	(+)	+	(+)	−	(+)	(+)	(+)	(+)
III + IV	6	nt	6	2	5	1	1	3	2	2	1
n = 6	+++		++	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)

Average conversion of [^{14}C]chloramphenicol per 1 mg protein during 2 h reaction at 37°C in the expressing founder lines: (+), <1% conversion; +, 1-5%; ++, 5-25%; +++, >25%. I, II, III, IV, I + II, and III + IV, BDNF-CAT fusion genes BDNF I CAT, BDNF II CAT, BDNF III CAT, BDNF IV CAT, BDNF I + II CAT, and BDNF III + IV CAT, repectively; *n*, number of analyzed founder lines; *nt*, not tested; *BRA*, brain; *SAL*, salivary gland; *THY*, thymus; *HEA*, heart; *LUN*, lung; *SPL*, spleen; *LIV*, liver; *KID*, kidney; *PAN*, pancreas; *MUS*, skeletal muscle; *T/O*, testis or ovary.

protection analysis of RNA from 11 different rat tissues showed that, in addition to brain, lower levels of BDNF exon I mRNA are expressed in the thymus and spleen (Fig. 2). These data demonstrate that the 2.6-kb of 5′ flanking BDNF exon I sequence is sufficient to direct appropriate tissue-specific expression of reporter gene in the thymus, but not in the spleen and brain.

3.7 kb of BDNF Promoter II Is Predominantly Silent in Transgenic Mice

18 lines containing CAT under the control of 3.7 kb of the 5′ flanking sequences of BDNF exon II were generated and analyzed. In 14 lines, we were not able to detect any CAT activity. In the remaining four lines, CAT activity was detected only in the brain and thymus, and it was significantly lower than in the thymus of transgenic mice carrying the BDNF I CAT transgene. (Fig. 2 and Table I). RNase protection analysis revealed that the endogenous expression of BDNF exon II mRNA in rat is restricted to the brain (Fig. 2). The 3.7-kb of BDNF exon II 5′ flanking region is apparently lacking positive regulatory sequences that are regulating the endogenous BDNF mRNA expression from this promoter.

4.4 kb of BDNF Promoter III Directs CAT Expression Predominantly in Brain and Muscle

11 different founder lines carrying 4.4 kb of the upstream sequences of BDNF exon III linked to CAT gene were analyzed and in six of them CAT activity was detected (Table I). In two lines, CAT activity was highest in the muscle and brain, and lower levels were present in the lung, thymus, heart, and liver (representative founder line is shown in Fig. 2). In two other lines, CAT activity was detected only in the muscle and brain. In two lines, CAT activity was detected in most of the organs with little differences in the expression levels. In five lines, we did not detect CAT activity in any of the analyzed organs. In the rat, this promoter is active mainly in brain and less also in the heart, as revealed by Northern blotting (Timmusk et al., 1993). RNase protection analysis of 11 rat tissues confirmed that the highest levels of BDNF exon III mRNA are present in the brain and significantly lower levels were seen in heart. Exon III mRNA was also detected in muscle and lung at low (close to detection limit) levels (Fig. 2). Taken together, the results demonstrate that the 4.4-kb of BDNF exon III 5′ flanking region directed the transgenic expression of CAT in tissues that overlap with the endogenous expression pattern of exon III mRNA in the rat.

5.5 kb of BDNF Promoter IV Directs the Expression of Reporter Gene Preferentially in Brain and Pancreas

Promoter IV of the rat BDNF gene is a classical G/C rich promoter that directs the expression of BDNF mRNA mainly outside of the nervous system with the highest levels in lung and heart (Timmusk et al., 1993). Sensitive RNase protection analysis showed that BDNF exon IV mRNA is expressed at low levels in all the rat tissues analyzed, except liver (Fig. 2). 13 different founder lines possessing the reporter gene fused to the 5′ flanking sequences of BDNF exon IV were analyzed (Fig. 2 and Table I). CAT activity was detected in 11 founder lines, showing that the percentage of transgene-expressing mice was higher than for BDNF III CAT. In seven founder lines, the levels were highest in the brain and pancreas, in two lines in the liver and in one line CAT levels were nearly equal in all the tissues analyzed. The expression pattern of BDNF IV CAT in transgenic mice (as shown for one representative founder line in Fig. 2) suggests that the 5.5-kb of rat BDNF promoter IV region includes elements essential for exon IV mRNA regulation in brain but is lacking sequences that are controlling the high level expression of endogenous BDNF in the heart and lung.

Expression of BDNF I + II CAT and III + IV CAT in Transgenic Mice

Partial recapitulation of the tissue specificity of endogenous BDNF mRNA expression and comparatively low transgene expression levels in the transgenic mice possessing the four BDNF promoter constructs described above suggested that these promoter regions of the rat BDNF gene are not

sufficient to account for the full activity of the gene. Promoters I and II and promoters III and IV are adjacent in the genome and display similar regulation patterns during brain development, suggesting the existence of shared regulatory sequences (Timmusk et al., 1994*a*). We therefore decided to generate two new BDNF constructs, designated BDNF I + II CAT and BDNF III + IV CAT (Fig. 1). The promoter region of BDNF I + II CAT consists of a 9.5-kb genomic fragment including exons I and II with their splice donor sites and the flanking regions. The promoter region of BDNF III + IV CAT contains a 9-kb genomic fragment including exons III and IV with the flanking regions. Several studies have shown that transgene constructs that include SV small t intron downstream of coding sequences are not optimal for expression, while inclusion of generic introns in the constructs increases transgene expression in mice (Brinster et al., 1988; Choi et al., 1991; Palmiter et al., 1991). In addition, the requirement of intragenic regulatory elements for tissue-specific and regulated expression has been described for several genes, including neural-specific ones (Vidal et al., 1990; Beaudet et al., 1992; Belecky et al., 1993; Vanselow et al., 1994; Zimmerman et al., 1994). We, therefore, decided to include BDNF mini-introns and 3′ UTR in the constructs (Fig. 1). Except for the promoter regions and splice donor sites, both these constructs also contain 0.7 kb of BDNF coding exon 5′ sequence with splice acceptor site and CAT coding sequence. The SV-40 polyadenylation signals downstream of the CAT gene were replaced by rat BDNF genomic region covering the 3′ UTR of the 4.2-kb mRNA and 0.1 kb of additional downstream sequences. The transgene expression was analyzed in seven different brain regions: cerebral cortex, hippocampus, cerebellum, striatum, thalamus/hypothalamus, midbrain, and brainstem. The analyzed nonneural tissues were the same as for the four transgenic constructs described above, except that expression was not analyzed in salivary gland.

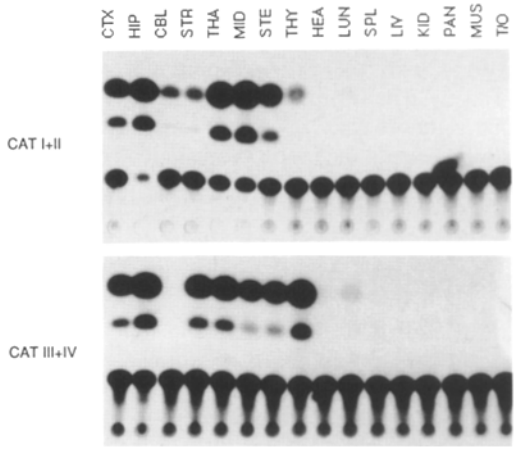


Figure 3. Expression of BDNF I + II CAT (*CAT I* + II) and BDNF III + IV CAT (*CAT III* + IV) in the different brain regions and nonneural tissues of transgenic mice of representative founder lines. 0.1 mg of protein extract was analyzed in each line by CAT assay. Samples were incubated for 2 h at 37°C.

BDNF I + II CAT Directs High Level Expression of the Reporter Gene in the Brain and Thymus of Transgenic Mice

Nine founders possessing the BDNF I + II CAT were obtained and bred to establish F1 transgenic lines to study the expression and regulation of this promoter construct in brain and peripheral tissues (Tables I and II and Fig. 3). All the nine lines expressed high level of CAT activity in the brain. In the thymus, CAT activity was always 5–10-fold lower (Table I and Fig. 3). In seven founder lines, CAT was also expressed in the lung with levels significantly lower than in the thymus. In the brain, the measured CAT activities were

Table II. CAT Activity in Different Brain Regions and the Levels of CAT mRNA in the Hippocampus of Transgenic Mice Possessing BDNF I + II CAT and BDNF III + IV CAT Fusion Genes

	CAT activity BRAIN REGION							CAT mRNA × 100%
Founder line	CTX	HIP	CBL	STR	THA	MID	STE	BDNF mRNA
I + II								
P4	++	+++	+	+	+++	+++	++	200–300%
P11	+	++	+	nt	nt	nt	+	nt
Q2	+	+++	+	+	+++	+++	++	15–40%
Q4	+++	+++	+	++	++	++	++	50–100%
Q6	++	++	+	nt	nt	nt	+	nt
Q9-1	+	++	++	+	++	+++	++	<5%
Q9-2	++	+++	+	++	++	++	+	25–40%
Q11	+	+	(+)	+	(+)	+	(+)	<5%
III + IV								
X7	+	+	−	−	(+)	+	−	<5%
Y3-1	+++	+++	++	+++	+++	+++	+++	15–30%
Y3-2	+++	+++	(+)	+++	+++	+++	++	10–20%
Y7	+++	+++	−	+++	+++	+++	+++	300–400%
Z1	(+)	+++	−	(+)	++	++	+	20–30%
Z8	++	++	++	+++	++	++	+++	20–30%

Average conversion of [^{14}C]chloramphenicol per 0.1 mg protein during 2 h reaction at 37°C in the expressing founder lines: (+), <1% conversion; +, 1–5%; ++, 5–25%; +++, >25%. *I + II* and *III + IV*, BDNF-CAT fusion genes BDNF I + II CAT and BDNF III + IV CAT, repectively; *nt*, not tested; *CTX*, cerebral cortex; *HIP*, hippocampus; *CBL*, cerebellum; *STR*, striatum; *THA*, thalamus and hypothalamus; *MID*, midbrain; *STE*, brainstem.

≤100-fold higher than in the transgenic animals with BDNF I CAT and BDNF II CAT.

CAT activity was analyzed in seven different brain regions (Table II and Fig. 3). The expression patterns varied slightly from one founder line to another, but in most of them, the highest levels were seen in the hippocampus, midbrain, thalamus/hypothalamus, and cerebral cortex. Lower CAT activity was detected in the brainstem and the lowest in the striatum and cerebellum. In the rat brain, the highest levels of endogenous BDNF exon I mRNA, transcribed from promoter I, were seen in the hippocampus and cerebral cortex, and lower levels were present in the other brain regions (Timmusk et al., 1994*a*, *b*). In the cerebellum and striatum, exon I mRNA was not detected. BDNF exon II transcripts, synthesised from promoter II, are expressed throughout the rat brain, with the highest levels in cerebellum, hippocampus, and midbrain RNA (Timmusk et al., 1994*a*, *b*). The transgene expression pattern of BDNF I + II CAT recapitulated that of endogenous rat BDNF mRNA transcribed from promoters I and II of rat BDNF gene with only two minor differences: a lower expression of transgene in the cerebellum and a higher expression in the striatum (Table II). Endogenous mouse BDNF mRNA and transgenic CAT mRNA were quantified in the hippocampus of transgenic animals by RNase protection using known amounts of sense BDNF and CAT mRNA, respectively. The results revealed that in several founder lines the transgene is expressed with levels comparable (founder lines Q4, Q2, and Q9-1) or up to threefold higher (founder line P4) than the endogenous mouse BDNF mRNA (Table II). These results clearly show that the rat BDNF genomic region of BDNF I + II CAT construct directs high level tissue-specific expression of the reporter gene in transgenic mice.

BDNF III + IV CAT Is Expressed at High Levels in the Brain of Transgenic Mice

Six different transgenic mice lines with BDNF III + IV CAT were obtained and the offspring analyzed as shown in Tables I and II and illustrated in Fig. 3. In all these founder lines, transgene expression was highest in brain followed by thymus. Lower levels of CAT activity were seen in the lung of five transgenic lines, in the kidney of three lines, in the heart, pancreas and muscle of two lines, and in the spleen, liver, and testis of one line. In the rat heart and lung, BDNF mRNA is predominantly expressed from promoter IV of the BDNF gene and the total levels are comparable with BDNF mRNA in the brain. The results revealed that BDNF III + IV CAT recapitulated the endogenous BDNF expression in the lung but not in the heart.

Analysis of CAT activity expression patterns in different brain regions of transgenic mice revealed that it was expressed throughout the brain with only small differences in the expression levels. In the cerebellum, CAT activity was absent or significantly lower. Quantification of RNA showed that in the hippocampus, CAT mRNA levels were comparable (founder lines Z1, Z8, Y3-1, and Y3-2) or higher (founder line Y7) as compared to the endogenous mouse BDNF mRNA levels determined in the same animals. BDNF exon III mRNA, that is transcribed from promoter III of the rat BDNF gene, has the most restricted expression pattern in the rat brain having highest levels in the cerebral cortex and hippocampus and 5–10-fold lower levels in the other brain regions. BDNF exon IV mRNA is expressed in all rat brain regions, except striatum, without significant differences in the levels (Timmusk et al., 1994*a*, *b*). Comparison of rat BDNF expression patterns with that of BDNF III + IV CAT in transgenic mice showed that the transgene is expressed in a similar manner to the rat BDNF mRNA transcribed from promoters III and IV. The minor differences from the endogenous pattern were the expression of transgene in the striatum and lower levels of expression in the cerebellum.

5.5 kb of the 5′ Sequence of BDNF Exon IV Is Sufficient to Direct Lesion-induced Expression of CAT in the Sciatic Nerve

BDNF mRNA has been shown to be induced in the distal segment of axotomized sciatic nerve (Meyer et al., 1992) by selective upregulation of exon IV mRNA levels (Funakoshi et al., 1993). To explore the possibility that the induction is caused by differential activation of BDNF promoter IV we studied if axotomy of sciatic nerve induces CAT expression

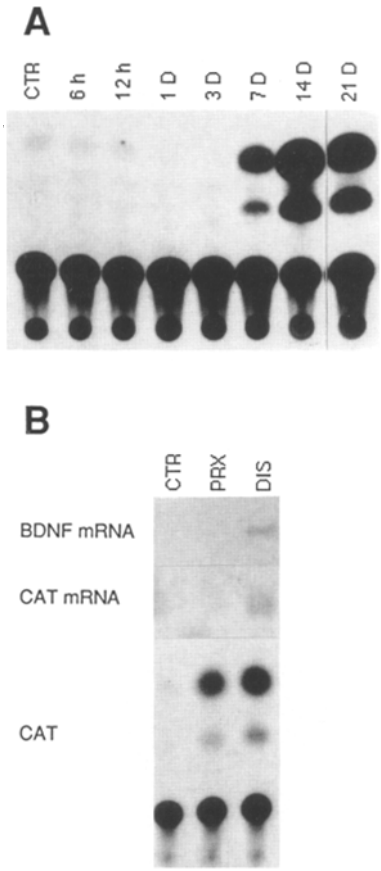


Figure 4. Expression of BDNF IV CAT in axotomized sciatic nerve of A2 founder line. (*A*) Time course of CAT activity changes in the distal segment of transected sciatic nerve. (*B*) CAT activities, CAT mRNA, and mouse BDNF mRNA (coding exon) in different segments of the sciatic nerve 14 d after lesion. 0.1 mg of protein extract was analyzed in each line by CAT assay. Samples were incubated for 2 h at 37°C. (*B*) 10 mg of total RNA was analyzed by RNase protection assay using cRNA probe specific for CAT or mouse BDNF. *CTR*, control sciatic nerve; *PRX*, proximal segment of the transected sciatic nerve, *DIS*, distal segment of the transected sciatic nerve.

in transgenic mice possessing BDNF IV CAT. The sciatic nerves of mice were transected and CAT activity was determined in the distal part of the lesioned nerve at different time points after operation (Fig. 4 *A*). In the intact sciatic nerve, CAT activity was low with levels only marginally above the detection limit. An increase in CAT activity was seen 1 wk after lesion and the levels were fivefold higher 2 and 3 wk after lesion (Fig. 4 *A*). This time course of transgene expression mimics exactly the delayed induction of rat BDNF mRNA after sciatic nerve lesion.

CAT activity and CAT mRNA levels were analyzed in the distal and proximal part of the transected sciatic nerve 2 wk after lesion (Fig. 4 *B*). In the distal part, both CAT activity and CAT mRNA were upregulated. In the proximal segment, only CAT activity was enhanced, indicating that it is not a transcriptional regulation and could be caused by transport and accumulation of CAT protein. Similar results were obtained with mice from two different lines of the same construct. As the regulation of mouse BDNF mRNA in this axotomy model has not been reported, we also determined the mouse BDNF mRNA levels in the same samples (Fig. 4 *B*). Endogenous mouse BDNF mRNA was increased predominantly in the distal segment of axotomized sciatic nerve. These data demonstrate that the 5.5-kb of BDNF exon IV the 5′ sequence is sufficient to direct correctly spatiotemporally regulated expression of reporter gene in the lesioned sciatic nerve.

To study if the proximal region of promoter IV is sufficient to confer the axotomy-induced expression of reporter gene in sciatic nerve, transgenic mice possessing BDNF IV prox CAT (Fig. 1) were generated and the offspring analyzed. This BDNF-CAT fusion gene is a 4.6-kb 5′ deletion of BDNF IV CAT. CAT activity was not induced in the axotomized sciatic nerve of any of the six different founder lines analyzed (data not shown). These results indicate that the regulatory sequences involved in the induction of BDNF expression in the lesioned sciatic nerve are located in the distal part of BDNF promoter IV in the region between 0.9 and 5.5 kb upstream from the 3′ end of exon IV.

BDNF CAT Fusion Genes Are Regulated by Kainate and KCl Depolarization in the Transgenic Mice Brain

KA has been shown to induce BDNF mRNA levels in the adult rat hippocampus and cerebral cortex (Zafra et al., 1990; Ballarin et al., 1991), and the induction is mediated predominantly by promoters I and III of the rat BDNF gene (Timmusk et al., 1993). Therefore, we studied the expression of BDNF-CAT fusion genes in the adult mice brain 3 and 6 h after systemic injection of KA. Kainate did not upregulate CAT activity in the hippocampus and cerebral cortex of any of the transgenic founder lines possessing BDNF I CAT, BDNF II CAT, or BDNF III CAT (Table III). The expression levels of reporter gene in these transgenic mice, detected by CAT assay, were very low (Table I), and transgene mRNA could not be detected by RNase protection analysis. In transgenic mice with BDNF IV CAT, KA did not induce CAT activity, but in the two different founder lines analyzed, marked increases (eight- and fourfold) of CAT mRNA were seen in the hippocampus and cerebral cortex, as analyzed by RNase protection assay (Table III). The promoter region of BDNF IV CAT is a 1-kb 3′ extension of the promoter region

Table III. Induction of CAT Activity and CAT mRNA in Transgenic Mice with Different BDNF-CAT Fusion Genes 3 h after KA Treatment or 4 h after SD Caused by Application of KCl

	Kainate induction				SD induction	
	CAT activity		CAT mRNA		CAT activity	CAT mRNA
Reporter construct	HIP	CTX	HIP	CTX	CTX	CTX
I	0/6	0/6	nd	nd	nt	nt
II	0/5	0/5	nd	nd	nt	nt
III	0/5	0/5	nd	nd	nt	nt
IV	0/6	0/6	2/2	2/2	0/2	1/2
I + II	1/6	0/6	3/3	2/2	0/2	1/2
III + IV	1/4	1/4	4/4	4/4	0/1	1/1

Numeral values (positive/analyzed) present the fraction of animals where the induction of CAT expression was observed. I, II, III, IV, I + II, and III + IV, BDNF-CAT fusion genes BDNF I CAT, BDNF II CAT, BDNF III CAT, BDNF IV CAT, BDNF I + II CAT, and BDNF III + IV CAT, repectively; *nd*, not detected; *nt*, not tested; *HIP*, hippocampus; *CTX*, cerebral cortex.

of BDNF III CAT and the induction was caused by preferential usage of promoter III. In all the analyzed founder lines of BDNF I + II CAT and BDNF III + IV CAT, the levels of CAT mRNA were induced three- to sixfold in the hippocampus and cerebral cortex through preferential usage of

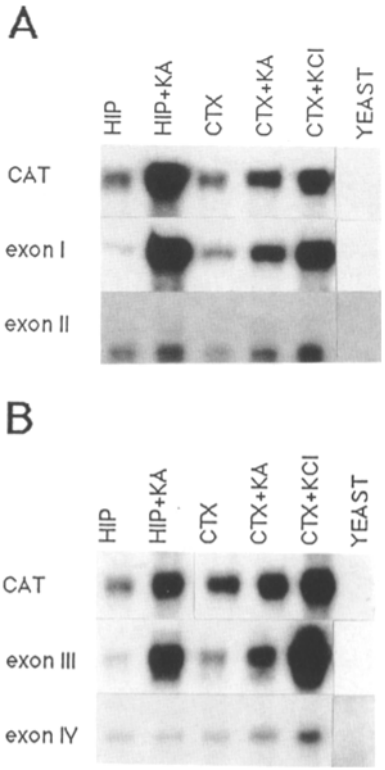


Figure 5. Expression of total CAT mRNA and CAT mRNA containing different transgenic 5′ exons of rat BDNF gene in the hippocampus and cerebral cortex of transgenic mice 3 h after kainic acid treatment or 4 h after repeated episodes of spreading depression by KCl application. (*A*) Q4 founder line of BDNF I + II CAT. (*B*) Y7 founder line of BDNF III + IV CAT. mRNA expression was analyzed by RNase protection assays as described in Materials and Methods. *yeast*, Yeast tRNA.

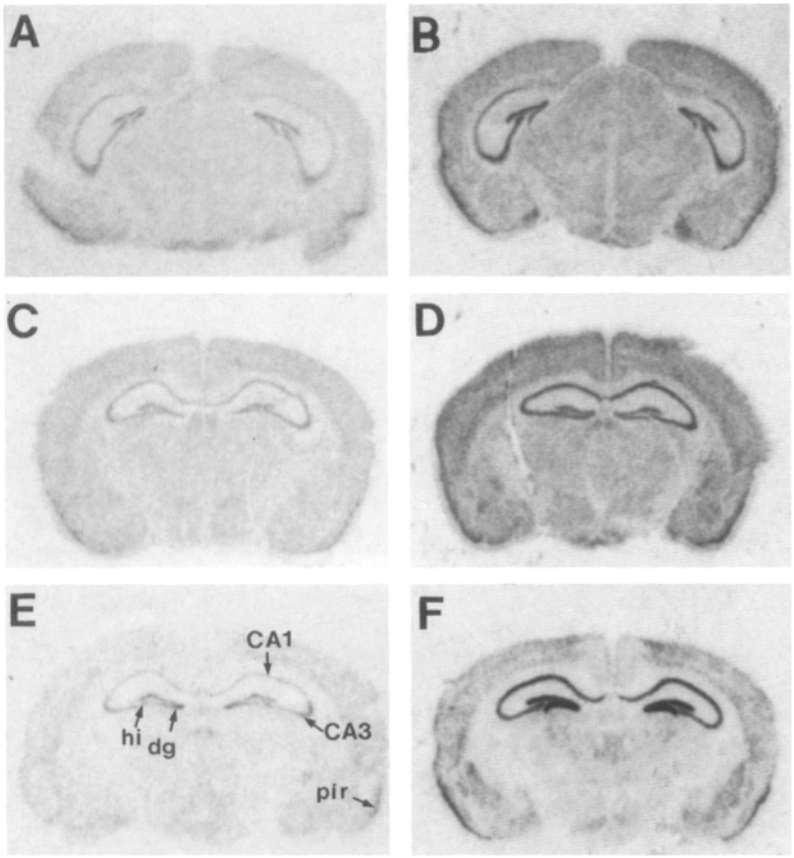


Figure 6. Light-field photomicrographs of autoradiograms of in situ hybridization showing the expression of CAT mRNA and mouse BDNF mRNA in the brain of control (*A*, *C*, and *E*) and kainic acid treated transgenic mice (*B*, *D*, and *F*). Coronal sections were hybridized to cRNA probes specific for CAT (*A–D*) or mouse BDNF coding exon (*E* and *F*). (*A*, *B*, *E*, and *F*) P4 founder line of I + II CAT transgenic mouse. (*C* and *D*) Y7 founder line of III + IV CAT transgenic mouse. *dg*, Dentate gyrus; *CA1* and *CA3*, pyramidal layers CA1 and CA3 of the hippocampus; *hi*, hilar region of the hippocampus; *pir*, piriform cortex.

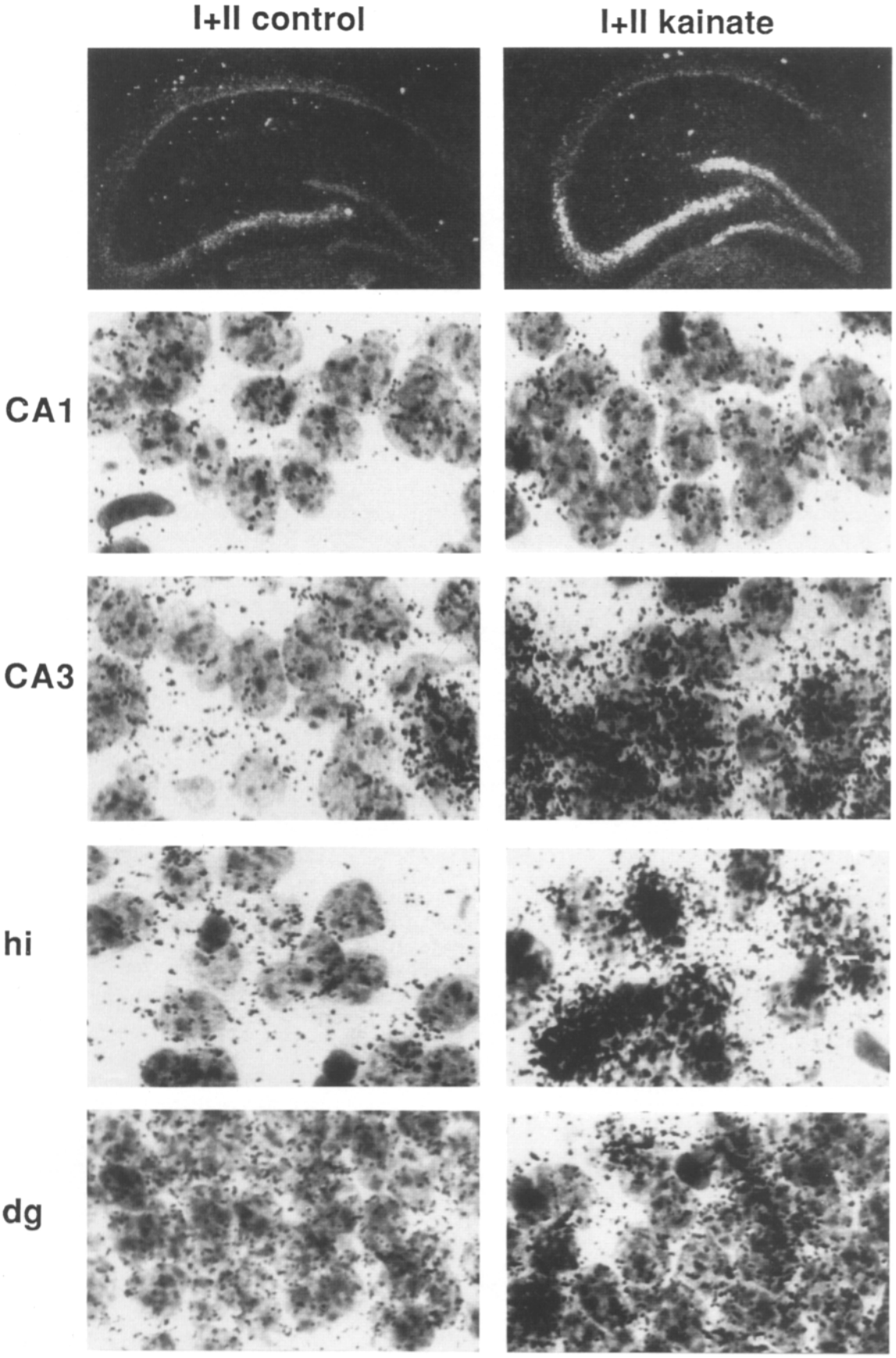
transgenic BDNF exons I and III, respectively (Table III and Fig. 5), that resembles the induction of rat BDNF mRNA. In contrast to this, CAT activity was induced (1.5-fold) only in the hippocampus of one of the analyzed lines with BDNF I + II CAT. In transgenic mice possessing BDNF III + IV CAT, twofold increases of CAT activity were seen only in the hippocampus of Y7 and in the cerebral cortex of Z1 founder line (Table III).

Spreading depression (SD), caused by KCl depolarization of cortical neurons, has been shown to induce BDNF mRNA in the rat cerebral cortex (Kokaia et al., 1993). Next, we investigated if SD increases CAT expression in the cerebral cortex of transgenic lines where transgene induction by KA was seen. In one out of two different analyzed lines of BDNF I + II CAT and BDNF IV CAT, KCl depolarization increased CAT mRNA in the cerebral cortex through preferential usage of promoters I and III, respectively (Table III and Fig. 5). Induction of CAT mRNA was also analyzed and observed in one founder line of BDNF III + IV CAT and it was predominantly mediated by promoter III (Table III and Fig. 5). The transgene mRNA levels after KCl depolarization were up to eightfold higher than in control animals, but no changes were seen in the CAT activities. These results demonstrate that CAT mRNA transcribed from BDNF promoter constructs was regulated in the transgenic mouse brain by two different types of neuronal activation in a manner similar to the regulation of rat BDNF mRNA in the same experimental models.

Transgenic Expression of BDNF I + II CAT and BDNF III + IV CAT in the Mouse Brain Is Neuron-specific and Overlaps with the Basal and Kainate-induced Expression Pattern of Endogenous Rat BDNF mRNA

The founder lines of BDNF I + II CAT and III + IV CAT that expressed the reporter gene at the highest levels (P4 and Y7, respectively) were used to analyze the transgene expression in the brain of control and and KA-treated animals by in situ hybridization using ^{35}S-UTP labeled cRNA probe of CAT coding sequence. To check if the KA treatment had in-

Figure 7. CAT mRNA expression in the hippocampus of P4 founder line of I + II CAT. Shown are emulsion autoradiograms obtained after hybridization of coronal sections from adult control transgenic brain or from transgenic animals 3 h after kainic acid treatment to a cRNA probe specific for CAT. Dark-field photomicrographs of the hippocampus are shown above. Note the sharp decrease of labeling at the end of CA3 region in the kainate-treated animal. Below are presented bright-field photomicrographs of higher magnification showing labeled neurons in CA1, CA3, and hilar region and in the granular layer of dentate gyrus, respectively. Note that some of the neurons have greater intensity of labeling than neighboring ones in the CA3 and hilar regions of both control and kainate-treated animals. Note also that in contrast to the induction of CAT mRNA by kainate in the CA3, hilus, and dentate gyrus there is no increase in the grain density in the neurons of CA1 region.


I+II control
I+II kainate
CA1
CA3
hi
dg

duced endogenous BDNF expression, sections from the same brain were also hybridized with a mouse BDNF-specific probe (coding exon). BDNF mRNA was induced in both of the analyzed animals and the distribution of BDNF in the brain of the analyzed animal of P4 founder line is shown in Fig. 6, *E* and *F*.

The transgenic line P4, expressing the BDNF I + II CAT, showed the highest levels of CAT mRNA in the pyramidal layer of CA3 and in the hilar region of the hippocampus (Fig. 6 *A*). Labeling was also seen in the granular layer of dentate gyrus, regions CA1 and CA2, external layers of the cerebral cortex, and in the piriform cortex. Lower levels of CAT mRNA were detected in other brain regions. Different neurons of the same region expressed the transgene at different levels. In the hippocampus, the granule neurons of dentate gyrus and the pyramidal neurons of CA1 and CA2 regions were labeled in a diffuse and uniform manner that contrasted with the different densities of grains found in the neurons of CA3 and especially of the hilus (Fig. 7). 3 h after systemic injection of KA, more than fivefold increases were seen in the CA3 and hilar region, and in the granular cell layer of dentate gyrus (Figs. 6 *B* and 7). Marked increases (about threefold) were also seen in the external layers of the cerebral cortex, in the piriform cortex, and in several nuclei of the amygdaloid complex, such as the medial and posteromedial amygdaloid nuclei. Examination of emulsion autoradiograms revealed that labeling was restricted to neurons in control and in kainate-treated animals. These results are in agreement with the induction of rat BDNF exon I and exon II mRNAs by KA, but the relative levels of the endogenous mRNA, particularly in the dentate gyrus, are higher (Metsis et al., 1993; Timmusk et al., 1993). On the cellular level, a differential response to KA was seen in all these regions. In addition to the uniform increase in the labeling pattern, individual cells were found to be more densely labeled than neighboring ones (Fig. 7). In the internal layers of cerebral cortex and in the CA1 region of the hippocampus, no increase of CAT mRNA levels were seen.

The transgenic line Y7 was used for a more detailed study of the expression of BDNF III + IV. In this founder line, the levels of CAT mRNA were more than threefold higher than the levels of endogenous mouse BDNF mRNA in the same animal. Several differences were seen in the BDNF III + IV CAT expression pattern as compared to BDNF I + II CAT (Fig. 6 *C*). In the hippocampus, the pyramidal neurons of CA1, CA2, and CA3, the neurons of hilar region, and granular layer of dentate gyrus expressed CAT mRNA with no significant differences in the labeling intensities. Furthermore, in contrast to differential induction pattern of transgene in P4 founder line of BDNF I + II CAT, KA induced the transgene levels about threefold in all these regions of the Y7 founder line (Figs. 6 *E* and 8). Similar levels of CAT expression were observed in the layers II-VI of cerebral cortex and also in the piriform cortex. Moderate labeling was seen in other brain regions. On the cellular level diffuse, uniform grain density predominated in hippocampal and cortical neurons of Y7 control and kainate-treated transgenic animal and labeling was not seen in glial cells (Fig. 8). The transgenic exression of BDNF III + IV CAT overlapped with the endogenous expression pattern. In the rat brain, BDNF exon III and IV mRNAs are expressed at relatively equal levels in the dentate gyrus and also in the CA1, CA2, CA3, and hilar regions of hippocampus. KA treatment does not elevate exon IV mRNA levels substantially, but it does enhance the levels of exon III mRNA predominantly in dentate gyrus and CA1 region of hippocampus and also in CA3 and hilus (Metsis et al., 1993; Timmusk et al., 1993).

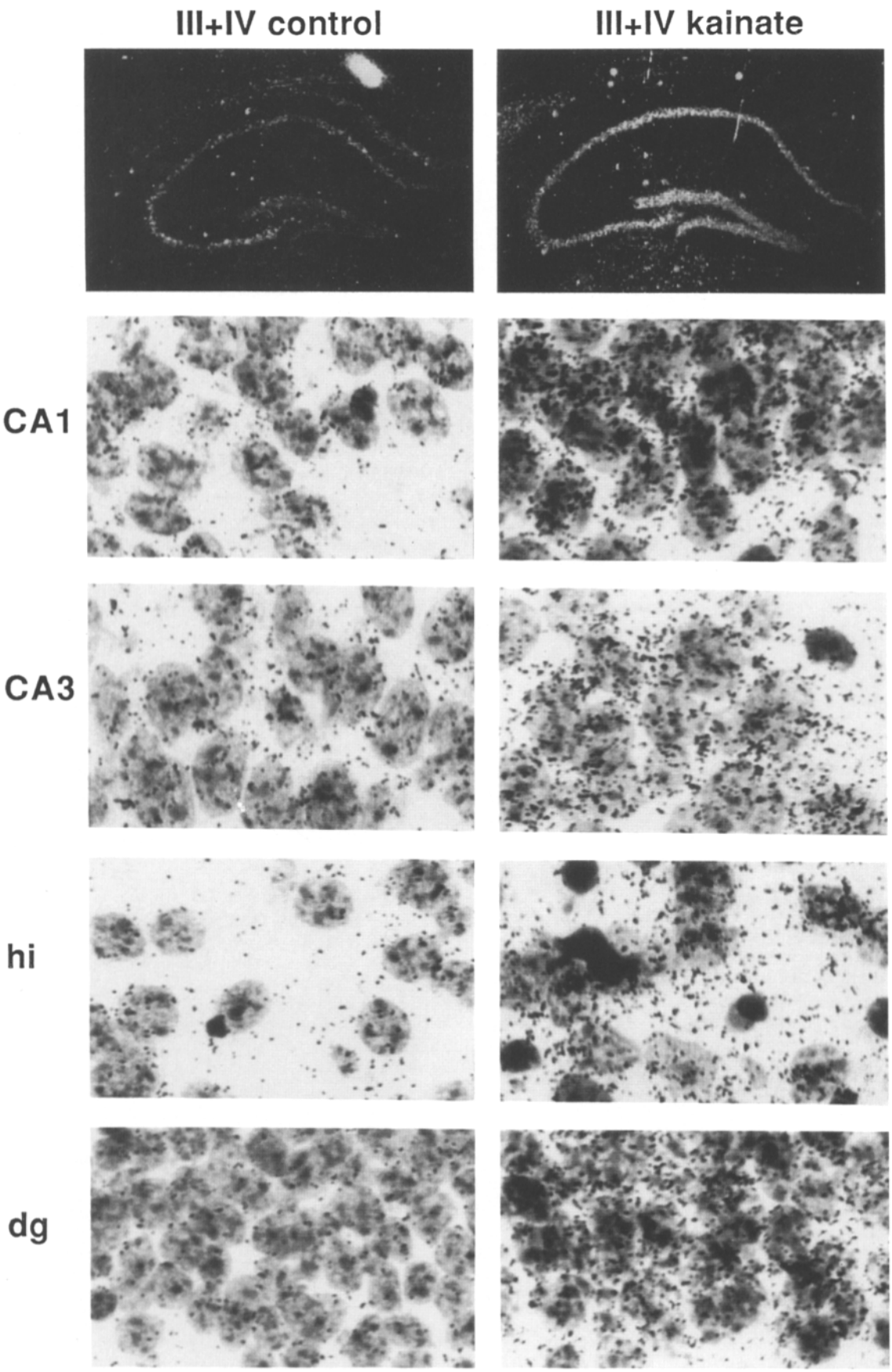
In conclusion, both BDNF I + II CAT and BDNF III + IV CAT expression was detected only in the neurons with a cellular distribution and induction pattern similar to those of endogenous rat BDNF promoter-specific mRNAs.

Discussion

In the present study, we have analyzed more than 60 different transgenic mice founder lines that express CAT under the control of seven different rat BDNF promoter constructs. We show that all four promoters of the rat BDNF gene are capable of driving the expression of reporter gene in transgenic mice, but high level expression of the reporter gene in correct neuronal populations of mouse brain was achieved only by using 9 kb of genomic sequences covering the promoter regions that lie close to each other in the genome and by including BDNF mini-introns and 3′ UTR in the constructs. We provide evidence that SD- and KA-responsive *cis*-acting elements of rat BDNF gene are separated and that the genomic region covering promoters I and II and the region covering promoters III and IV have distinct KA- and SD-responsive sequences. We also show that 5.5 kb of BDNF promoter IV region directs axotomy-induced reporter gene expression in the sciatic nerve of transgenic mice in a manner similar to the regulation of endogenous BDNF mRNA in the same paradigm.

In many transgenic mouse studies, expression from the transgene has been found to vary among lines derived from different founder animals. In addition, the transgene expression levels are not correlated with the transgene copy number. These differences have been believed to reflect a position effect resulting from random integration of the transgene into the mouse genome (Palmiter and Brinster, 1986). Locus control regions are *cis*-acting DNA elements that mediate position-independent and copy number–dependent expression of genes (reviewed in Evans et al., 1990). Several BDNF-CAT fusion genes, such as BDNF I CAT, BDNF II CAT, BDNF I + II CAT, and BDNF III + IV CAT, had very similar expression patterns in all the different founder lines of the same construct, suggesting the presence of regulatory regions that protect the reporter gene from the *cis*-acting effects of regulatory elements flanking random sites of integration. On the other hand, there was no strict correlation

Figure 8. CAT mRNA expression in the hippocampus of Y7 founder line of III + IV CAT. Shown are emulsion autoradiograms obtained after hybridization of coronal sections from adult control transgenic brain or from transgenic animals 3 h after kainic acid treatment to a cRNA probe specific for CAT. Above are shown dark-field photomicrographs of the hippocampus. Below are presented bright-field photomicrographs of higher magnification showing labeled neurons in CA1, CA3, hilus, and dentate gyrus, respectively.


III+IV control
III+IV kainate
CA1
CA3
hi
dg

between the integrated copy number of transgene and its expression levels (data not shown). It is possible that a locus control region exists also in the BDNF gene and only part of it was included in these BDNF promoter constructs, expressed in a position-independent manner, but with expression levels that were not correlated with the transgene copy number.

Tissue-specific Expression of BDNF-CAT Fusion Genes in Transgenic Mice

When fused separately to bacterial CAT gene the upstream regions of exons I, II, and III of rat BDNF gene were able to direct the transgene expression in tissues that partially overlapped with the endogenous sites of particular BDNF promoter activities. 2.6 kb of exon I 5′ flanking sequence recapitulated BDNF expression in the thymus, but not in the brain and spleen. Similarly, 4.5 kb of exon III 5′ flanking region was insufficient to restrict transgene expression to correct tissues, but predominant transgenic expression in the brain and muscle suggests that some regulatory elements are present in the analyzed promoter III sequence. And finally, 5.5 kb of promoter IV recapitulated endogenous expression in the brain, but not in the heart and lung. These results suggest that *cis*-acting elements that are regulating the expression of rat BDNF gene in different tissues (cell populations) are separated in the genome. Examples of genes for which similar regulation strategy is used are Thy-1, where elements responsible for the brain, thymus, kidney, and spleen expression are all separated (Vidal et al., 1990), and nestin, where independent regulatory elements control the expression in the neural stem cells and muscle precursors (Zimmerman et al., 1994). An exceptional case was the 3.7-kb of the exon II 5′ sequence, which, when linked to the reporter gene, was silent in the transgenic mice. This may be explained by the presence of a negative control element as a neural-restrictive silencer element (NRSE) has been described in the SCG10 gene (Mori et al., 1992) and the rat type II sodium channel gene (Kraner et al., 1992) and a highly homologous sequence is present in the 5′ region of rat BDNF exon II (Timmusk et al., 1993). Therefore, it is possible that because of the absence of positive neural-specific regulatory sequences in the BDNF promoter II construct, this NRSE is involved in the suppression of transgene expression.

Neuronal-specific and high level expression of the reporter gene was achieved by introducing two new fusion genes, BDNF I + II CAT and BDNF III + IV CAT, into transgenic mice. These constructs contain 9 kb of genomic sequences covering the BDNF promoter regions that lie close to each other in the genome, and sequences of BDNF intron-exon splice junctions and 3′ UTR. In the brain, no significant differences from the pattern of endogenous BDNF expression were seen for either of these constructs with the exception of higher levels of CAT in striatum and lower in cerebellum. Although the expression levels of transgene in the same brain region varied from one founder line to another of the same construct, the highest expression was always in the hippocampus and the absolute levels were comparable, or even higher, than the endogenous levels of mouse BDNF mRNA. The cellular expression patterns were similar to those of rat BDNF promoter specific mRNAs. In the brain, BDNF I + II CAT and BDNF III + IV CAT were expressed only in the neurons with the highest levels in the hippocampal pyramidal, granular, and hilar neurons, as well as in the cortical neurons. Transgene expression was not detected in glial cells. In nonneural tissues, BDNF I + II CAT correctly mimicked the expression pattern of BDNF exon I and exon II mRNAs, but BDNF III + IV CAT transgenic expression was comparatively low in heart and lung, where high levels of BDNF mRNA are transcribed from promoters III and IV in the rat.

We cannot precisely determine which regions of BDNF I + II CAT and BDNF III + IV CAT were responsible for the high level and accurate pattern of expression in brain neurons, because 5′ flanking regions, introns, and 3′ UTR, not present in the first four analyzed BDNF constructs, were included in these BDNF-CAT fusion genes. Regulatory elements that drive neuron-specific expression in transgenic mice have been shown to be located in the 5′ flanking regions and in the intragenic regions (reviewed in Belecky, 1993; Beaudet, 1992). Correct expression pattern in transgenic mice has often been achieved when the entire region covering the gene, as well as its 5′ and 3′ flanking sequences, has been included in the transgene (Patil et al., 1990; Kaneda et al., 1991). For several neural genes, comparatively short genomic regions of 5′ flanking sequences have been shown to drive the expression to all neurons, or neurons and some ectopic tissues, recapitulating partially the endogeneous expression pattern. Furthermore, inclusion of longer 5′ sequences (Vandaele et al., 1991) or intragenic regions (Ang et al., 1993; Belecky et al., 1993; Vanselow et al., 1994) has restricted the expression to correct neurons. Similarly, the shorter BDNF promoter–CAT fusion genes partially recapitulated the expression of endogenous BDNF gene, but higher level and more precise expression patterns were achieved with the longest constructs. Although both BDNF I + II and BDNF III + IV CAT contained ~15 kb of genomic sequences, they did not completely mimick the complex tissue-specific pattern of BDNF gene. Therefore, it is reasonable to assume that additional regulatory regions are involved in the regulation of the rat BDNF gene.

5.5 kb of BDNF Promoter IV Region Directs Axotomy-induced Expression of Reporter Gene in the Sciatic Nerve

Transection of the rat sciatic nerve leads to a marked increase of BDNF mRNA in the distal segment of nerve, probably in Schwann cells, which is thought to play a role in providing trophic support to injured peripheral nerves. The increase is seen only 1 wk after lesion, with progressively higher levels until 3 wk after lesion (Meyer et al., 1992; Funakoshi et al., 1993). Only BDNF exon IV mRNA is increased in the lesioned sciatic nerve, suggesting that the induction is caused by selective activation of BDNF promoter IV (Funakoshi et al., 1993). Here, we show that 5.5 kb of the 5′ flanking sequence of BDNF exon IV directs axotomy-induced expression of CAT mRNA and CAT activity in the sciatic nerve of two different founder lines that were analyzed. The time course and spatial pattern of transgene expression were similar to BDNF expression in rat and in mouse. Both CAT and endogenous BDNF were induced predominantly in the distal segment of sciatic nerve and the upregulation was observed first at 7 d after lesion, peaking at 2 wk, and remained elevated also 3 wk after lesion. As previously shown, there is also a marked increase of NGF mRNA

in the nonneuronal cells of lesioned sciatic nerve. In contrast to BDNF mRNA, the increase in NGF mRNA is biphasic with a rapid transient increase followed by a second increase that lasts for several weeks (Heumann et al., 1987). It has been suggested that the induction of NGF expression in the lesioned sciatic nerve is mediated by the immediate early gene c-*fos* through a functional AP-1–binding site in the NGF gene (Hengerer et al., 1990). The delayed induction of BDNF and promoter IV-CAT fusion gene suggests that c-*fos* and other transcription factors encoded by immediate early genes are not involved in the regulation of BDNF expression after sciatic nerve transection. We have shown that adrenalectomy attenuated the increase of BDNF mRNA in the lesioned nerve and that a putative binding site for the glucocorticoid receptor is present 420 bp upstream from the cap site of promoter IV, suggesting that glucocorticoids could be involved in the upregulation of BDNF mRNA (Funakoshi et al., 1993). In this study, we show that 0.9 kb of the exon IV 5′ sequence is not sufficient to mediate the induction of the reporter gene in lesioned sciatic nerve, suggesting that this putative binding site for the glucocorticoid receptor is not involved in the regulation of BDNF induction in this experimental model. Work is in progress to determine which particular sequence elements are involved in the regulation of BDNF expression in lesioned sciatic nerve.

Regulation of the Expression of BDNF-CAT Fusion Genes in the Hippocampus and Cerebral Cortex after KA Seizures and KCl-mediated Depolarization

BDNF mRNA levels in the hippocampus and cerebral cortex of adult rat brain have been shown to be markedly but transiently induced by several stimuli such as seizures induced by administration of KA (Zafra et al., 1990; Ballarin et al., 1991; Dugich-Djordejevic et al., 1992*a*, *b*), pentylentetrazol (Humpel et al., 1993), pilocarpine (Berzaghi et al., 1993; Metsis et al., 1993) focal electrolytic lesions (Isackson et al., 1991), kindling after electric stimulation (Ernfors et al., 1991), ischemic insults and insulin-induced hypoglycemic coma (Ernfors et al., 1991; Lindvall et al., 1992; Comelli et al., 1993), and cortical SD induced by application of KCl (Kokaia et al., 1993). These data have led to the hypothesis that BDNF protein could play a neuroprotective role after brain insults.

We investigated if the expression of BDNF-CAT fusion genes is induced by systemic kainic acid treatment, which is a potent pharmacological regulator of BDNF mRNA expression. CAT mRNA was under the detection limit in the founder lines possessing BDNF I CAT, BDNF II CAT, and BDNF III CAT. All the other BDNF promoter constructs analyzed, BDNF IV CAT, BDNF I + II CAT, and BDNF III + IV CAT, directed kainate-inducible expression of reporter gene mRNA in the transgenic mice by preferential usage of exons I and III in these constructs. The relative increases of transgene mRNA varied in different founder lines of the same construct but were always smaller than for particular BDNF exon mRNAs. Striking differences were seen in the regional distribution of increases of CAT mRNA after treatment with kainic acid. In the hippocampus, transgene mRNA transcribed from BDNF I + II CAT of P4 founder line was increased in the hilar region, in the granular cells of dentate gyrus and in the CA3 region. No significant induction was observed in the CA1 region. Rat BDNF exon I and exon II mRNAs are induced in similar hippocampal neuronal populations, but with a more marked relative increase in the granular cell layer of dentate gyrus. CAT mRNA transcribed from BDNF III + IV CAT was increased in CA1, CA2, and CA3 regions and in dentate gyrus with no significant differences in the amplitude of induction. Rat BDNF exon III mRNA is increased in the same hippocampal neuronal populations, but the increase is more marked in CA1 region.

SD, caused by KCl depolarization of cortical neurons, was tested in those founder lines that showed a marked induction of transgene mRNA after KA treatment. SD induced CAT mRNA expression in the cerebral cortex of one analyzed founder line of BDNF III + IV CAT and in one out of two analyzed founder lines of BDNF IV CAT. Furthermore, SD increased CAT mRNA expression in one out of two analyzed founder lines of BDNF I + II CAT, while KA induced transgene expression in both of them. The results indicate that both KA- and SD-responsive *cis*-acting elements are present in the BDNF 5.5-kb sequence of BDNF IV CAT (containing both exon III and exon IV, and their 5′ flanking sequences), and also in the BDNF genomic regions of BDNF I + II CAT. As these BDNF-CAT fusion genes do not have overlapping BDNF sequences, it is highly likely that different signaling pathways, linked to the responsive *cis*-acting elements in the cluster of promoters I and II, and to the sequences in the cluster of promoters III and IV, mediate the kainate induction of rat BDNF gene. The variation of induction of BDNF I + II CAT and BDNF IV CAT by KA and SD could be the result of a different transgene integration site where the SD- but not KA-responsive *cis*-acting elements lost the ability to mediate transgene induction in one founder line, suggesting that KA and SD elements are different and located in different sites of the sequence of these BDNF-CAT fusion genes. Taken together, our data suggest that (*a*) SD- and KA-responsive *cis*-acting elements of rat BDNF gene are separated; and (*b*) genomic region covering promoters I and II and genomic region covering promoters III and IV have distinct KA- and SD-responsive sequences.

Concerning the signaling pathways leading to the induction of BDNF mRNA, it has recently been shown that in cultured embryonic cortical neurons, KCl and glutamate induce BDNF expression by activation of distinct calcium channels, L-type voltage-sensitive calcium channels, and *N*-methyl-D-aspartate receptors respectively (Ghosh et al., 1994). Furthermore, in cultured hippocampal neurons L-type calcium channels and NMDA receptors are coupled to two different signaling pathways that activate transcription through different *cis*-acting regulatory elements in the c-*fos* promoter (Bading et al., 1993). Since KCl and KA in this study were applied systemically, the induction of CAT expression was probably caused not only by direct activation of one particular glutamate receptor, but also by indirect activation of different glutamate receptor subtypes by endogenous glutamate release that is thought to mediate the increase of BDNF expression in these experimental models (Zafra et al., 1990; Kokaia et al., 1993). It remains to be determined to what extent the increase of BDNF gene expression is causally related to the activation of immediate-early genes, such as c-*fos*, c-*jun*, zif/268, and others, in KA-mediated seizures and SD (reviewed in Morgan and Curran, 1991).

KA and SD upregulated CAT mRNA levels in transgenic mice of different BDNF-CAT fusion genes up to 10-fold, but mostly it was not paralleled by an increase of CAT protein,

as determined by CAT assay 3 or 6 h after the treatment. Recently, it was reported that the kainate-induced increase of BDNF mRNA was not paralleled by alterations of equal magnitude and distribution of BDNF immunoreactivity (Wetmore et al., 1994). The increase of BDNF immunoreactivity was observed in restricted neuronal subpopulations of hippocampus, with most significant changes in the CA3 region, and to a lesser degree in other cortical areas. Similarly, it has been reported that during the acute phases of pentylentetrazol seizures, the increase of BDNF mRNA levels in the hippocampus is not paralleled by an increase of BDNF-like immunoreactivity (Humpel et al., 1993). This discrepancy between the induction level of CAT (and also BDNF) mRNA and protein could be explained by the results obtained using hippocampal slice culture showing that high concentrations of glutamate, KA, NMDA, and quisqualate inhibit neuronal protein synthesis by ⩽90% (Vornov and Coyle, 1991). We cannot rule out that CAT protein, like BDNF protein, is induced only in some neuronal populations, and that the CAT assay is not a suitable method to detect these subtle changes. Finally, the finding that axotomy induced CAT activity in transgenic lines with BDNF IV CAT suggests that only moderate BDNF mRNA induction by focal lesions, like axotomy, or by more physiological stimuli like light (Castrén et al., 1992), long-term potentiation (Patterson et al., 1992; Castrén et al., 1993), improved spatial memory, and enriched environment (Falkenberg et al., 1992), may result in elevated BDNF protein levels.

Taken together, this study is one of the first steps towards the understanding of the regulatory mechanisms governing BDNF gene expression. Our results show that the multipromoter structure of the BDNF gene supports neuronal function through a complex regulation of gene expression in different tissues and cell populations. The transgenic mice with the various BDNF-CAT fusion genes provide the opportunity to study the relative contributions of BDNF promoters and their particular sequence elements in the regulation of BDNF gene expression during development, as well as in different lesion models or after neuronal activation. Finally, targeted expression of neurotrophins and other heterologous genes under the control of the described BDNF promoter constructs could be used to study their function and regulation in the nervous system and nonneural tissues.

Prof. Håkan Persson died during the preparation of this study and his premature death is a great loss for all of us. We thank E. Nilsson for excellent technical assistance and Dr. N. Belluardo for help and advice in animal experiments.

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