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Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation

(kainic acid/transcriptional regulation/*in situ* hybridization/glutamate receptors)

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ABSTRACT **The rat brain-derived neurotropic factor (BDNF) gene consists of four 5′ exons linked to separate promoters and one 3′ exon encoding the prepro-BDNF protein. To gain insights into the regulation of BDNF mRNA expression, probes specific for the different 5′ exons were used to study the expression of BDNF mRNA in the brain. Following a systemic injection of the glutamate analog kainic acid, exon I, II, and III mRNAs increased transiently in hippocampus and cerebral cortex. A modest increase was seen for exon IV, where a new transcription initiation site was induced by this treatment. Pretreatments with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK801 or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 2,3-dihydroxy-6-nitrosulfanoylbenzo(*f*)quinoxaline revealed two region-specific patterns of glutamate receptor-mediated regulation. The first pattern found in neocortex, piriform cortex, and amygdala involves regulation of BDNF exon I, II, and III mRNAs through NMDA and AMPA/kainate receptors. The second pattern found in the hippocampus involves regulation of BDNF exon I, II, and III mRNAs by high-affinity kainate or metabotropic receptors. Treatment with the γ-aminobutyric acid subtype A ($GABA_A$) receptor antagonist bicuculline increased exon I and III mRNAs in the dentate gyrus, and the muscarinic receptor agonist pilocarpine increased exon I mRNA mainly in the neocortex. These data show that the four BDNF promoters allow multiple points of BDNF mRNA regulation and suggest that the activation of different subtypes of glutamate receptors differentially regulates the expression of BDNF exon-specific mRNAs in the brain.**

Brain-derived neurotrophic factor (BDNF; refs. 1 and 2) is a member of the neurotrophin family, which also includes nerve growth factor (NGF) (3), neurotrophin-3 (NT-3) (4–9), and neurotrophin 4/5 (NT-4) (10–12). BDNF promotes the survival of retinal ganglion cells (13), basal forebrain cholinergic neurons (14), and embryonic mesencephalic dopaminergic neurons (15, 16) in cell culture. *In vivo* BDNF has been shown to increase the survival of embryonic sensory neurons (17) and developing as well as injured motoneurons (18–20).

BDNF mRNA is expressed in neurons throughout the brain with the highest level in the hippocampus (8, 21–24). The findings that the glutamate analog kainic acid (KA) and the γ-aminobutyric acid subtype A ($GABA_A$) receptor antagonist bicuculline increase BDNF mRNA levels in hippocampal neurons have led to the hypothesis that the balance between the activity of the GABAergic and glutamatergic systems controls the level of BDNF mRNA in the hippocampus (25–28). Moreover, stimulation of the septo-hippocampal pathway transiently increases BDNF mRNA in the hippocampus (29). Similarly, seizure activity induced by focal electrolytic lesions (30) or repeated subconvulsive electrical stimulations (31) in the hippocampus lead to marked and transient increases of BDNF mRNA in the dentate gyrus, neocortex, and piriform cortex.

The increase of BDNF mRNA following KA treatment has been suggested to be due to a glutamate receptor activation. The glutamate receptor family has been classified into two main groups: the metabotropic receptors, which exert long-lasting actions through the modulation of intracellular signals, and the ionotropic receptors, which contain glutamate-gated cation channels providing fast synaptic responses (32). Ionotropic receptors, further subdivided according to pharmacological criteria into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) low-affinity kainate receptor, and high-affinity kainate receptors, have been implicated in the increase of BDNF mRNA after treatment with KA (25, 26) and after electrical stimulations (31).

The recent molecular cloning of the rat BDNF gene has revealed a complex gene structure with four short 5′ exons and one 3′ exon encoding the mature BDNF protein. A separate promoter is present upstream of each 5′ exon (33). Alternative usage of these promoters and differential splicing result in four BDNF mRNAs with different 5′ untranslated exons. The regulation of BDNF exon-specific mRNAs by neuronal activation was unknown until now since previous studies have used probes from exon V that detect a pool of all BDNF mRNAs.

In this report we have used exon specific probes from the rat BDNF gene to demonstrate a differential activation of the four BDNF promoters in specific brain regions following changes in neuronal activation. NMDA and AMPA/kainate receptors are involved in mediating the promoter activation with different patterns of regulation in different brain regions.

MATERIALS AND METHODS

Animals and Pharmacological Treatments. Adult male Sprague–Dawley rats (body weight, 200–230 g; Alab, Stockholm) were used in all experiments. KA (12 mg/kg of body weight), bicuculline (2 mg/kg), and pilocarpine (400 mg/kg) were injected i.p. and the animals were sacrificed at the indicated times after the injections. For pretreatment with glutamate receptor antagonists, rats were injected i.p. with MK801 (2 mg/kg) 10 min before KA injection and with 2,3-dihydroxy-6-nitrosulfanoylbenzo(*f*)quinoxaline (NBQX; 30 mg/kg) 30 and 10 min before KA injection and 10 min after KA injection.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin 3; NT-4, neurotrophin 4; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid; KA, kainic acid; NBQX, 2,3-dihydroxy-6-nitrosulfanoylbenzo(*f*)quinoxaline; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

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RNA Preparation and Northern Blot Analysis. Total and poly(A) RNA from the indicated tissues were purified and analyzed by Northern blot as described (33). The filters were hybridized to the following BDNF exon-specific probes: for exon I, a 0.35-kb *Sac* I–*Hin*dIII genomic fragment; for exon II, a cDNA fragment covering the sequence 2067–2229 in the rat BDNF gene; for exon III, a cDNA fragment covering sequence 782–975 in the rat BDNF gene; and for exon IV, a cDNA fragment covering sequence 1790–2056 in the rat BDNF gene (33). The same filters were also rehybridized with a mouse β-actin cDNA probe to standardize for the amount of mRNA in each lane. Appropriate exposures of all autoradiograms were quantified using a Shimadzu CS-9000 densitometer. The levels of the different BDNF mRNAs were then normalized relative to the levels of actin mRNA.

***In Situ* Hybridization and Image Analysis.** The same DNA fragments as used for the Northern blots were used to synthesize UTP[α-^{35}S]-labeled complementary RNA probes. Coronal sections (14 μm) from fresh frozen adult Spraque–Dawley rat brain were analyzed by *in situ* hybridization as described (33). To quantify the relative levels of the different BDNF transcripts in control and treated brains, the optical density values of the autoradiograms were measured using a Quantimet 570 image processing and analysis system (Cambridge Instruments, Cambridge, U.K.). For calibration, an autoradiographic microscale (Amersham) was coexposed on all x-ray films. The number of neurons per unit area in the different brain regions was determined by counting on cresyl violet-stained sections. The optical density values were divided by the neuron density, thus providing an estimate of the labeling intensity over individual neurons in the areas measured. All measurements were performed on at least six different tissue specimens. An overall statistical test using ANOVA was used to determine the significance of differences among the various groups of animals.

RESULTS

Levels of Exon-Specific BDNF mRNAs After Treatments with KA. BDNF exon I mRNA increased 50-fold in the hippocampus 3 hr after a systemic injection of KA (Fig. 1). The level was the same at 4.5 hr but tapered off to 10-fold higher than control 6 hr after the injection. A 10-fold increase was seen in the cerebral cortex at 3 hr, which remained at the same level at the later time points. Exon II mRNA increased 5-fold in hippocampus at 3 hr, declined at 4.5 hr and reached 3-fold higher than control at 6 hr (Fig. 1). Only low levels of exon II mRNA were detected in the cerebral cortex with 3-fold higher levels than control at all time points after the KA injection. Exon III mRNA showed similar increases in hippocampus and cerebral cortex, with ≈20-fold higher levels at 3 hr declining to 10- and 3-fold more than control at 4.5 and 6 hr, respectively. In hippocampus and cerebral cortex, exon IV mRNA increased 2-fold 3 hr after the KA injection and returned to control levels at 6 hr. RNase protection assays showed that in hippocampus and cerebral cortex the same major initiation sites (cap sites) were used for exon I, II, and III mRNAs in control animals and in animals analyzed 3 hr after KA treatment, whereas for exon IV mRNA the KA treatment induced a cluster of cap sites not seen in the control located 218–222 bp upstream of the 3′ end of exon IV (data not shown).

Exon-Specific BDNF mRNA Analyzed by *In Situ* Hybridization. *In situ* hybridization revealed a more pronounced increase of exon I mRNA than exon II mRNA after the treatment with KA, although the regional distribution of the increases was similar for the two exons (Fig. 2 *B* and *G*). The highest increases were seen in the granular cell layer of the dentate gyrus, pyramidal cell layers of the neocortex and piriform cortex, dorsal endopiriform nuclei, and lateral and dorsolateral amygdaloid nuclei. Less pronounced increases

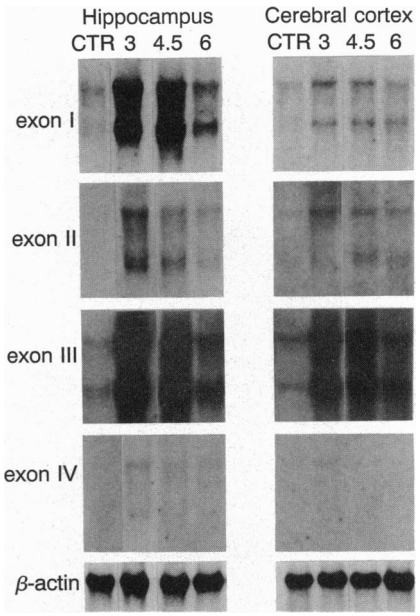

FIG. 1. Northern blots of BDNF exon-specific mRNAs after treatment with KA. Poly(A) RNA was isolated from adult rat hippocampus (*Left*) and cerebral cortex (*Right*) of control animals (CTR) or animals sacrificed at the indicated time (in hours) after a systemic injection of KA. The RNA (20 μg per slot) was electrophoresed in an agarose gel, transferred to a nitrocellulose filter, and hybridized to the indicated BDNF exon-specific probes. The same filter was used for all probes. The filter was also hybridized with a probe for β-actin to standardize for the amount of mRNA in each lane. The filter was washed at high stringency followed by exposure to x-ray film.

were seen in the pyramidal layers of the hippocampus, posterior paraventricular thalamic nuclei, and ventromedial hypothalamic nuclei. The increase of exon I mRNA in neocortex was completely blocked by MK801 and reduced by 70% by NBQX (Figs. 2 *C* and *D* and 3). No statistically significant effect of MK801 was seen in piriform cortex, whereas NBQX attenuated the increase by 70%. No blocking effect was seen in the hippocampus after pretreatment with MK801. Instead, MK801 potentiated the KA-mediated increase of exon I mRNA in the CA1 and CA3 regions 3- and 2-fold, respectively. No effect of NBQX was seen in the pyramidal layer of hippocampus, whereas the increase in dentate gyrus was reduced by 50%.

MK801 and NBQX reduced the increase of exon II mRNA in the neocortex and piriform cortex by 50% (Figs. 2 *H* and *I* and 3). NBQX had no significant effect in the hippocampus, whereas MK801 potentiated the increase of exon II mRNA 2- and 3-fold in the CA3 and CA1 regions, respectively, and 1.5-fold in the dentate gyrus (Figs. 2*H* and 4).

Following KA treatment, exon III mRNA increased markedly in the granular layer of the dentate gyrus, the CA1 and CA2 regions of the hippocampus, neocortex, and piriform cortex and the lateral amygdaloid complex (Fig. 2*L*). A moderate increase was seen in the CA3 region of the hippocampus, the internal pyramidal layer of neocortex, medial amygdaloid nuclei, ventral paraventricular thalamic nuclei, and ventromedial hypothalamic nuclei. MK801 reduced the increase of exon III mRNA in neocortex and piriform cortex by 80 and 50%, respectively (Figs. 2*M* and 4). Similarly, NBQX reduced the increase in neocortex and piriform cortex by 70% and 50%, respectively (Figs. 2*N* and 3). The increase in the CA3 region and dentate gyrus was not blocked by MK801 or NBQX, whereas both antagonists reduced the increase in CA1 region by ≈50% (Fig. 3). The KA treatment induced a modest increase in exon IV mRNA, predominantly

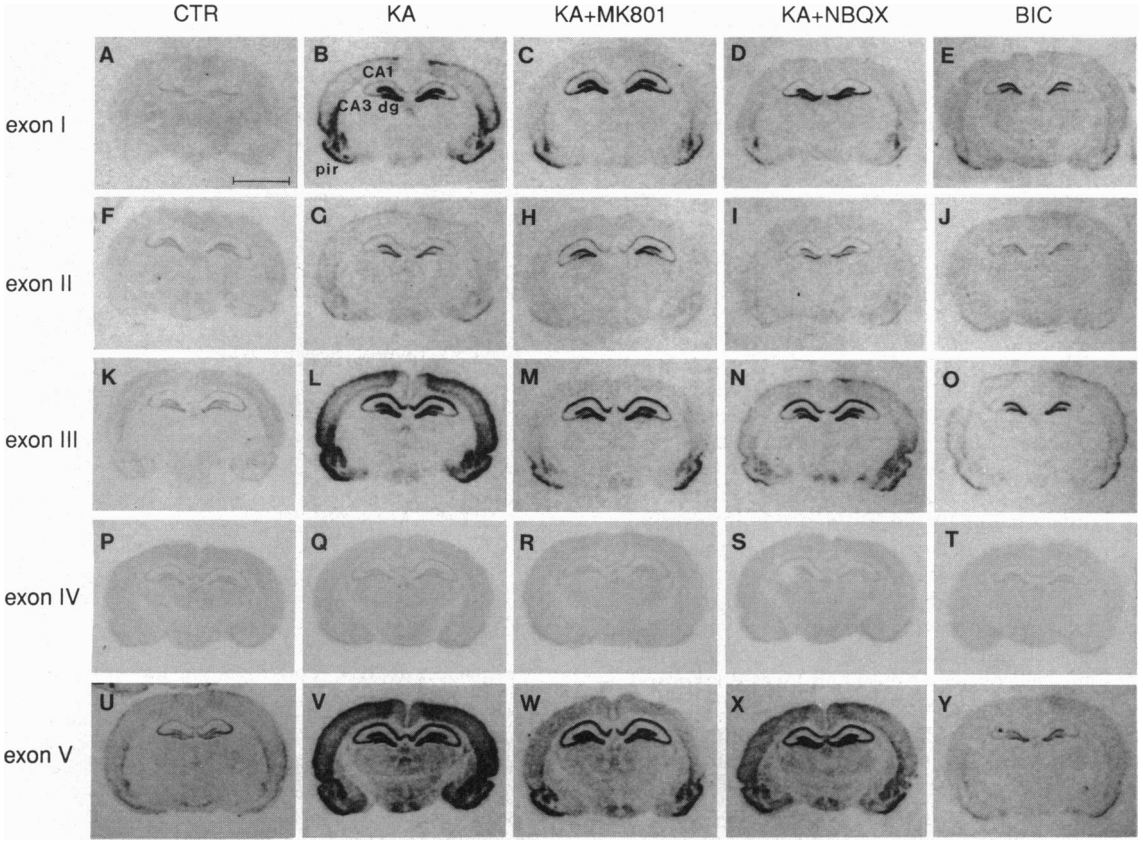

FIG. 2. *In situ* hybridization with exon-specific anti-sense complementary RNA probes after pharmacological treatments. Coronal sections at the level of the dorsal hippocampus were prepared from adult rat control brain (CTR) or from animals 3 hr after systemic injection of KA or bicuculline (BIC). KA-injected animals pretreated with MK801 (KA+MK801) or NBQX (KA+NBQX) were also included in the analysis. The sections were hybridized to the indicated BDNF exon-specific anti-sense complementary RNA probes. Shown are images of autoradiograms obtained after hybridization and exposure to x-ray films for 2 weeks (all treated animals) or 4 weeks (control animals). (Bar in upper left = 5 mm and is the same for all panels.) dg, Dentate gyrus; CA1 and CA3, pyramidal layers CA1 and CA3 of the hippocampus; pir, piriform cortex.

in the hippocampus and neocortex. This increase was not blocked by MK801 or NBQX (Fig. 2 *R* and *S*).

Three hours after a systemic injection of bicuculline, exon I and III mRNAs increased 15- and 5-fold, respectively in the granule cell layer of the dentate gyrus (Fig. 2 *E* and *O*). A modest increase was also seen for exon I and III mRNAs in the neocortex and piriform cortex. In contrast, no change or only small changes were seen for exon II and IV mRNAs in these brain regions (Fig. 2 *J* and *T*).

Three hours after a systemic injection of the muscarinic receptor agonist pilocarpine, exon I mRNA increased markedly in neurons of layer VI of the parietal cortex (compare Fig. 4 *A* and *C*). A modest increase was also seen in neurons of layer V, whereas no change was seen in the other brain regions examined. The pilocarpine treatment had no significant effect on exon II, III, and IV mRNAs.

DISCUSSION

In this study we have used BDNF exon-specific probes to demonstrate that BDNF mRNAs containing different 5′ exons are differentially expressed in the adult rat brain. A separate promoter is present upstream of each of the four 5′ exons as revealed by transfection of promoter–reporter gene constructs in cell culture (33) and by the expression of these constructs in transgenic mice (unpublished data). It is therefore likely that the changes in exon-specific mRNAs reported here are due to a differential activation of the four different BDNF promoters. All exon-specific BDNF transcripts contain the coding sequence for the prepro-BDNF protein, although sequences within the untranslated 5′ exons could form secondary structures that may affect their translatability (33). Analysis of BDNF exon-specific sequences in polysomal RNA from hippocampus and cerebral cortex of normal and KA-treated rats showed that all BDNF exon-specific mRNAs are associated with polysomes (data not shown). This implies that all BDNF exon-specific mRNAs are used for translation of the prepro-BDNF protein.

Treatment with the $GABA_A$ receptor antagonist bicuculline increases BDNF mRNA in hippocampal neurons (26). Our results provide evidence that this increase is mediated by a selective activation of BDNF promoters I and III and is mostly restricted to the dentate gyrus, a structure highly enriched in GABAergic nerve terminals and GABA receptors. A marked exon-specific response was also found after treatment with the muscarinic receptor agonist pilocarpine, which caused a significant increase of exon I mRNA in the parietal cortex. The regional restriction in the response to pilocarpine indicates that only a subset of muscarinic receptors regulates the expression of BDNF exon I mRNA.

Previous studies using probes from the protein coding part of the BDNF gene (exon V), detecting a pool of all BDNF exon mRNAs, have revealed marked increases of BDNF mRNA after treatment with KA (25–28). As shown here, these increases are the sum result of a differential usage of BDNF 5′ exons. Comparison of the time course for the increases of exon-specific mRNAs revealed that exon I and II mRNAs remained elevated for longer times after the KA treatment compared to exon III and IV mRNAs. This may reflect the fact that promoters I and II and promoters III and IV, respectively, are located close to each other within the BDNF gene (33) and therefore may share regulatory sequences.

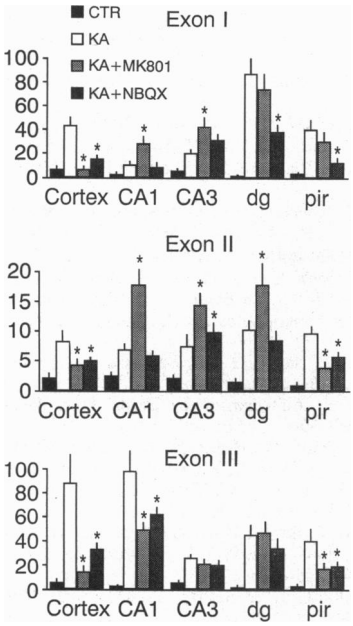

FIG. 3. Levels of exon-specific BDNF mRNAs in different brain regions after KA treatment alone or in the presence of glutamate receptor antagonists. Computerized image analysis was used to measure the optical density on autoradiograms obtained after hybridization of the sections shown in Fig. 2. The regions analyzed are indicated. The number of neurons per unit area was calculated for each region and the optical density values were divided with the neuronal density. Thus the bars represent a measure of the relative labeling intensity over individual neurons in different brain areas. The level of exon III mRNA in the CA1 region after KA treatment was arbitrarily set at 100. Six measurements were made for each probe and brain area and the results shown are the mean values ± standard deviation. Asterisks indicate significant effects of pretreating the animals with glutamate receptor antagonists compared to KA alone ($P < 0.05$ using ANOVA). Abbreviations as in Fig. 2.

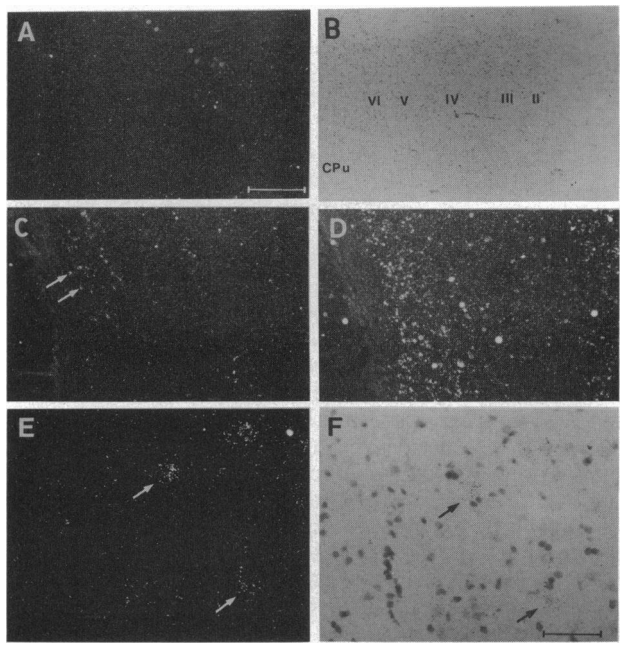

FIG. 4. Pilocarpine-mediated increase of BDNF exon I in the parietal cortex. Shown are emulsion autoradiograms obtained after hybridization of sections to a BDNF exon I mRNA-specific probe. The sections were prepared from control brain (*A*) or 3 hr after systemic injections of pilocarpine (*C*, *E*, and *F*) or KA (*D*). (*B*) Bright-field illumination included as an overview of the area analyzed. Note labeled cells (some of which are indicated by arrows) in layer IV of the parietal cortex in the pilocarpine-treated animal (*C*) but not in the control brain (*A*). (*F*) Bright-field illumination of labeled neurons in layer IV of the parietal cortex after pilocarpine treatment. (Bar in *A* = 500 μm and is the same for *B–D*; bar in *E* = 125 μm and is the same for *F*.) CPu, caudate-putamen; II, III, IV, V, and VI, layers of the parietal cortex.

The position of the cap sites for exon I, II, and III mRNAs was the same in control and KA-treated animals, suggesting that the structure and the translatability of these mRNAs were not changed after the KA treatment. However, for exon IV a new major cap site was induced by KA and this shift removes part of the 5′ end of exon IV mRNA, which may improve the translatability of this mRNA (33). Thus, for exon IV mRNA, transcriptional and translational changes may control the amount of prepro-BDNF protein synthesized after KA treatment.

In addition to the direct activation of kainate receptors, KA causes a release of glutamate in the brain and this has been suggested to mediate the increase of BDNF mRNA (25). The different regional distributions in the response comparing the four exons could be due to the fact that the BDNF promoters are differentially induced following activation of various subsets of glutamate receptors. The results obtained by the use of specific glutamate receptor antagonists revealed two main patterns of region-specific, glutamate receptor-mediated regulation of exon-specific BDNF mRNAs.

The first pattern was seen in the neocortex, piriform cortex, and amygdaloid complex, where the increases of exon I, II, and III mRNAs were partially blocked by MK801 and NBQX. This suggests that NMDA and AMPA/kainate receptors are involved in the regulation of promoters I, II, and III in these brain regions. The only exception was the increase of exon I in the piriform cortex, which was blocked only by NBQX, indicating that NMDA receptors are not involved. The finding that NMDA and AMPA/kainate receptors are involved in the KA induction of exon-specific BDNF mRNA suggests that (*i*) polysynaptic pathways using different glutamate receptors are involved or (*ii*) presynaptic kainate receptors stimulated by KA induce glutamate release and activate postsynaptic glutamate receptors in a similar fashion to that described in the hippocampus (34, 35). Correlation of the glutamate receptor subtype distribution in the piriform cortex and amygdaloid complex (36–39) and the blockade of the kainate response by NBQX suggest that the kainate effect might be triggered by either homodimeric GluR5Q (unedited) or heterodimeric KA2/GluR6 or KA2/GluR5 receptors. The increases of exons I, II, and III in neocortex may be due to an activation of heterodimeric GluR6/KA receptors that are sensitive to 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and mainly localized in cortical layers II and IV (37, 38, 40). In addition, glutamate receptor subunits 1, 2, and 3 forming AMPA low-affinity kainate receptors (41–43) and the NMDA receptors formed by subunits 1, 2A, and 2B (44), also present in these brain areas, may participate in the regulation of BDNF exon-specific mRNA expression.

The second main pattern of BDNF mRNA regulation by KA was found in the hippocampus. In this region the increases of BDNF exon I, II, and III mRNAs did not seem to be mediated through NMDA receptors since MK801 did not block the kainate induction. On the contrary, pretreatment with MK801 potentiated the kainate-induced increase of exon I mRNA in the CA1 and CA3 regions and exon II in CA1, CA3, and dentate gyrus. This suggests that activation of NMDA receptors, formed by subunits 1 and 2A or 1 and 2B in these hippocampal regions (44), could be negatively coupled to the expression of exon I and II mRNAs. In most of the hippocampal regions the induction of BDNF exon I, II, and III mRNAs was not blocked by NBQX. This finding is

intriguing since it has recently been shown (37) that the glutamate receptor subunit GluR6 is enriched in the dentate gyrus and CA3 region and forms homodimeric, CNQX low-sensitivity, high-affinity kainate receptors. Although we cannot rule out the involvement of metabotropic receptors due to the lack of selective antagonists, our data suggest that the KA-mediated increases of exon I and III mRNAs in the hippocampus may occur through the GluR6 subtype of kainate receptors.

Exceptions to the pattern of regulation in the hippocampus were seen in the dentate gyrus, where the increase of exon I mRNA was blocked by NBQX, and in the CA1 region, where the increase of exon III mRNA was blocked by MK801 and NBQX. This suggests that NMDA receptors are not regulating exon I mRNA in the dentate gyrus, whereas NMDA and AMPA/kainate receptors could be involved in the increase of exon III mRNA in the CA1 region. In this case, in addition to a postsynaptic activation of AMPA/kainate receptors, presynaptic kainate receptors (34) could induce glutamate release from hippocampal mossy fibers (35) and activate postsynaptic NMDA receptors. Given that in both cases the kainate action could be blocked by NBQX, it may be possible that either the heterodimeric AMPA high-affinity kainate receptor GluR6/KA2 (38) or AMPA low-affinity kainate receptors formed by subunit 1, 2, 3, or 4 (41, 43, 46) may be involved since they are sensitive to CNQX and expressed in the CA1 region and dentate gyrus.

Since all treatments in this study were applied systemically, we cannot rule out more complex indirect pathways leading to the differential activation of BDNF promoters. However, the significant correlation of these effects with glutamate receptor subtype distribution leads us to suggest that some particular combination of glutamate receptor subunits, if functional in that particular region, may be responsible for the specific induction of the different BDNF promoters.

The regional variations in the brain in the expression of the four BDNF 5′ exon mRNAs in response to changes in neuronal activation suggest that the four transcription units within the BDNF gene are independently regulated. The blocking effects of glutamate receptor antagonists on BDNF exon-specific mRNAs suggest that a broad spectrum of different glutamate receptors regulates, in a precise manner, the expression of BDNF mRNA in different brain areas. The functional consequence of this regulation is not known, but the fact that the treatments used in this study enhance memory, at low doses, or induce seizures, at high doses (45, 47, 48), suggests that BDNF may provide a trophic feedback to the afferent nerve terminals, which results in a reinforcement of these inputs.

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