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Interplay between glutamate and γ-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons

(gene regulation/neuronal plasticity)

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ABSTRACT **In the central nervous system brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are predominantly located in neurons. Here we demonstrate that the balance between the activity of the glutamatergic and γ-aminobutyric acid (GABA)ergic systems controls the physiological levels of BDNF and NGF mRNAs in hippocampal neurons *in vitro* and *in vivo*. The blockade of the glutamate receptors and/or stimulation of the GABAergic system reduces BDNF and NGF mRNAs in hippocampus and NGF protein in hippocampus and septum. The reduction of NGF in the septum reflects the diminished availability of NGF in the projection field of NGF-dependent septal cholinergic neurons. These neurons do not synthesize NGF themselves but accumulate it by retrograde axonal transport. The refined and rapid regulation of BDNF and NGF synthesis by the glutamate and GABA transmitter systems suggests that BDNF and NGF might be involved in activity-dependent synaptic plasticity.**

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are two structurally related neurotrophic molecules (1), which are expressed by specific populations of central neurons including pyramidal neurons in the hippocampus proper and granular neurons of the dentate gyrus (2–8). NGF is produced in the hippocampus and is specifically taken up by nerve terminals of cholinergic neurons. The cell bodies of these neurons are localized in the septum, and they accumulate NGF by retrograde axonal transport (9). NGF (10–12) and probably also BDNF (13, 14) promote the survival and maintenance of specialized functions of these septal cholinergic neurons.

Glutamate is the major excitatory neurotransmitter in the mammalian brain, whereas γ-aminobutyric acid (GABA) mediates the main inhibitory input on neurons. The appropriate balance between the effects of these neurotransmitter systems appears essential for normal neuronal function, and deviations from this balance may lead to seizures and neuronal cell death (15, 16).

Ionotropic glutamate receptors can, according to pharmacological criteria, be divided into *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors, which include kainate and α-amino-3-hydroxymethylisoxazole-4-propionate (AMPA) receptors (17). The normal and pathophysiological functions of these receptors are particularly well studied in the hippocampus. The activation of NMDA receptors, for example, seems to play a critical role in long-term potentiation (18, 19), whereas uncontrolled hippocampal glutamate-receptor activation leads to seizures and irreversible neuronal damage (15, 16).

We have previously shown that depolarization of cultured hippocampal neurons (50 mM KCl) increases both BDNF and NGF mRNA levels in a calcium-dependent manner (20). Of a large number of transmitter substances tested, kainic acid proved the most potent molecule in elevating BDNF and NGF mRNAs *in vitro* and *in vivo* (20). However, the use of pharmacological doses of kainic acid (20, 21) or the induction of limbic seizures (22) represents extreme experimental conditions and does not reflect the regulation of the genes of these neurotrophic factors under physiological conditions. We have, therefore, investigated the contribution of the excitatory glutamatergic and the inhibitory GABAergic systems to the physiological regulation of BDNF and NGF synthesis. The results indicate that subtle changes in the balance between the glutamatergic and the GABAergic systems significantly alter expression of BDNF and NGF in the hippocampus and influence the amount of NGF protein available for the septal cholinergic neurons.

MATERIALS AND METHODS

Compounds. MK-801 was obtained from Research Biochemicals (Natick, MA). 2,3-Dihydroxy-6-nitrosulfanoylbenzo(*f*)quinoxaline (NBQX) was from T. Honoré from Novo Nordisk (Bagsveard, Denmark). *Taq* polymerase was obtained from Perkin–Elmer/Cetus, avian myeloblastosis virus reverse transcriptase was from Life Sciences (St. Petersburg, FL), and NuSieve/agarose was from FMC. Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GIBCO. All other reagents were obtained from Sigma.

Cell Culture. Neurons were prepared from hippocampi of 17-day-old rat embryos and cultured as described (20). Briefly, isolated hippocampi were incubated for 20 min at 37°C in calcium-free and magnesium-free phosphate-buffered saline containing 10 mM glucose, albumin at 1 mg/ml, DNase at 6 μg/ml, and papain at 12 units per ml. Hippocampal cells were carefully dissociated with a plastic pipette, collected by centrifugation (900 × *g*, 5 min), resuspended in Dulbecco's modified Eagle's medium/10% fetal calf serum, and plated onto plastic culture dishes (0.5×10^6 cells per 35 mm), which were precoated with poly(DL-ornithine) (0.5 mg/ml). Three hours after plating the medium was changed to a serum-free one containing the supplements (except glutamate) as described by Brewer and Cotman (23).

RNA Analysis. RNA was extracted by using the guanidinium thiocyanate method (24), glyoxylated, and fractionated on a 1.5% agarose gel (25). A shortened cRNA standard was

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Abbreviations: BDNF, brain-derived neurotrophic factor; GABA, γ-aminobutyric acid; NBQX, 2,3-dihydroxy-6-nitrosulfanoylbenzo(*f*)quinoxaline; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate.

added to the samples before extraction to assess RNA recovery. RNA was transferred to Hybond-N filters, which were hybridized with a ^{32}P-labeled mouse BDNF and NGF cRNA probe produced by run-off transcription (20). The filters were later hybridized with a ^{32}P-labeled β-actin probe, and the values were normalized to the β-actin signal.

Quantitative PCR. Neuronal RNA together with a recovery standard were transcribed into cDNA with avian myeloblastosis virus reverse transcriptase followed by 18 amplification cycles by using specific 5′ and 3′ oligonucleotide primers for NGF. We used 18 cycles because it has been shown (20) that in this system PCR is linear up to 20 cycles. The DNA products were separated on a 3% NuSieve/agarose 3:1 gel and then transferred to filters that were hybridized with a specific mouse NGF cRNA probe (25).

Determination of NGF Protein. NGF in hippocampus and septum was measured by a sensitive two-site ELISA (26) with described modifications (27). The mouse monoclonal antibody 27/21 (26) was used for all determinations. All NGF values obtained were corrected for the recovery of added mouse 2.5S NGF.

Treatment of Animals. Adult Wistar rats of both sexes weighing 180–200 g were injected i.p. with MK-801, NBQX, or diazepam, or i.v. with muscimol. Control animals received equivalent volumes of physiological saline. Animals treated with bicuculline (i.v.) received one injection of diazepam (10 mg/kg i.p.) after 30 min to suppress seizure activity. At the indicated times rats were decapitated, and brains were quickly removed. Hippocampus and septum were dissected, frozen on dry ice, and stored at −70°C for RNA or protein determination.

RESULTS

In a first set of experiments we investigated the time course of the levels of BDNF and NGF mRNAs in cultured hippocampal neurons. Fig. 1*A* shows that the BDNF mRNA steadily increased up to day 7 and then declined to intermediate levels. Kynurenic acid, a broad-spectrum glutamate-receptor antagonist (17), completely prevented this increase *in vitro* (Fig. 1*A*). MK-801, a noncompetitive NMDA glutamate-receptor antagonist (17), abolished the increase in BDNF mRNA similarly. Comparable results were obtained by a competitive NMDA antagonist, 2-amino-5-phosphonopentanoic acid (17) (data not shown). NBQX, a non-NMDA-receptor antagonist (28), only slightly reduced BDNF mRNA in this culture system (Fig. 1*A*). Both magnesium (10 mM), which blocks the NMDA-activated calcium channel and 7-chlorokynurenic acid, an NMDA antagonist acting on the glycine site of the NMDA receptor (17), markedly reduced the BDNF mRNA levels (data not shown). The treatments described above had no apparent effect on the morphology of the neurons or their number, and the expression of β-actin mRNA was not influenced. Thus, during the first 7 days in culture, spontaneous depolarizations of hippocampal neurons, most probably resulting from the release of endogenous glutamate, produce a steady increase in BDNF mRNA predominantly by the activation of NMDA receptors. The addition of NMDA to these cultures did not further increase BDNF mRNA but resulted in extensive neuronal cell death (refs. 15, 16, and unpublished observations). However, the decrease in BDNF mRNA after 7 days does not appear to reflect cell death because β-actin mRNA levels remained constant up to 2 weeks. The decrease could be caused by the desensitization of the glutamate receptors or the maturation of the GABAergic system. The latter interpretation is supported by the observation that the addition of bicuculline increased the levels of BDNF mRNA to a relatively higher extent in 12-day-old cultures than in 7-day-old ones (data not shown).

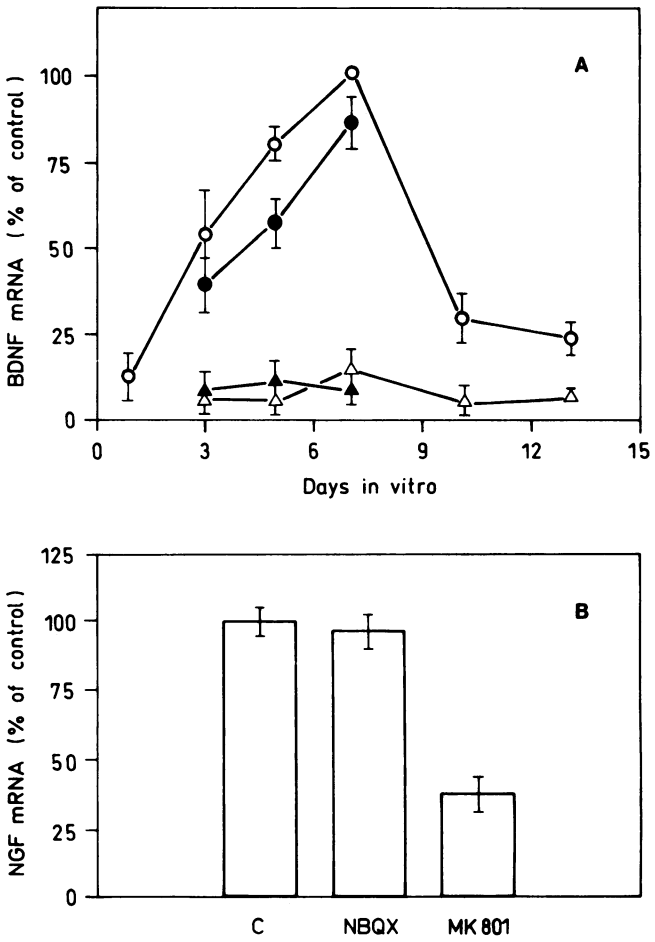

FIG. 1. (*A*) Time course of changes in BDNF mRNA in hippocampal neurons cultured in the presence of different glutamate-receptor antagonists. Cells were kept for various time periods in standard medium (○) or in the presence of 10 μM MK-801 (▲); 10 μM NBQX (●); or 1 mM kynurenic acid (△). Values represent the mean of three determinations and are expressed as percentage of the maximal level. All values for MK-801 and kynurenic acid are significantly smaller than control values ($P < 0.001$); for NBQX only the 5-hr value is smaller than the control value ($P < 0.05$). (*B*) NGF mRNA levels in hippocampal neurons cultured for 5 days in the absence or presence of NBQX or MK-801. NGF mRNA was determined by a quantitive PCR method (20). Values are means ± SEMs of three independent experiments. MK-801 differs significantly from control ($P < 0.001$).

NGF mRNA had to be determined by a quantitative PCR method (20) because its levels in hippocampus are lower by a factor of 50 than those of BDNF mRNA (6). MK-801, but not NBQX, blocked the increase of NGF mRNA (Fig. 1*B*), indicating that, at least in cultured hippocampal neurons, the synthesis of these two neurotrophins is similarly regulated by the glutamate system.

In contrast to the inhibition seen with the NMDA-receptor blockers under basal conditions, the GABA-receptor antagonist bicuculline increased the levels of BDNF mRNA (Fig. 2). Both kynurenic acid and MK-801, but not NBQX, blocked the bicuculline-mediated increase in BDNF mRNA, indicating that the bicuculline effect is predominantly mediated by an enhanced release of glutamate that then acts on NMDA receptors. This interpretation is further supported by the observation that tetrodotoxin, which blocks transmitter release, not only blocked the effect of bicuculline but also significantly ($P < 0.02$) decreased the basal levels of neuronal BDNF mRNA in 7-day-old cultures (Fig. 2).

To evaluate whether the regulatory mechanisms characterized in tissue culture are also operating *in vivo*, we

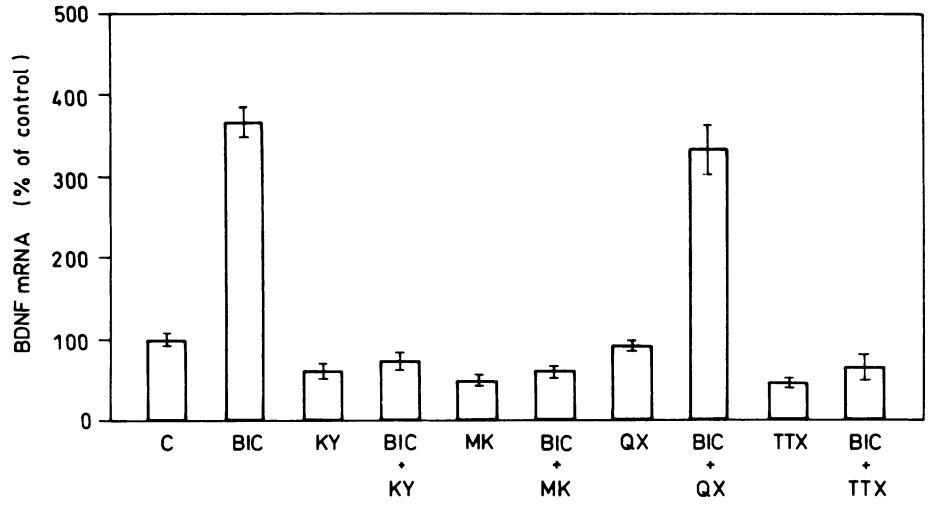

FIG. 2. Effect of bicuculline on BDNF mRNA in hippocampal neurons and its modifications by various compounds. Neurons cultured for 7 days were treated with bicuculline in the absence or presence of indicated drugs. After a 4-hr incubation, BDNF mRNA was analyzed as described. The compounds were added to the cultures 15 min before bicuculline. Values are means ± SEMs of three independent experiments. C, controls; BIC, bicuculline (50 μM); KY, kynurenic acid (1 mM); MK, MK-801 (10 μM); QX, NBQX (10 μM); TTX, tetrodotoxin (1 μM). Values for BIC, KY, MK, BIC + QX, and TTX differ significantly from the control value ($P < 0.05$).

activated the GABAergic system directly by muscimol or increased its efficiency by diazepam. The administration of these compounds *in vivo* decreased BDNF and NGF mRNA levels in hippocampus (Fig. 3). These effects were reversible, and repetitive drug administration was necessary to maintain the reduced mRNA levels. Blockade of NMDA receptors by MK-801 similarly decreased BDNF and NGF mRNA levels as from activation of the GABAergic system. NBQX, which only had a slight effect on basal BDNF mRNA levels in hippocampal neuronal cultures (Fig. 1*A*), substantially decreased BDNF and NGF mRNA in the rat hippocampus *in vivo* (Fig. 3). The effect of NBQX could either result from blockade of postsynaptic non-NMDA receptors or could be due to presynaptic inhibition of glutamate release (29).

Conversely, administration of the GABA type A-receptor blocker bicuculline, which evokes seizures, markedly increased BDNF mRNA levels in the hippocampus (Fig. 4). Pretreatment of rats with MK-801, which had no effect on the kainic acid-induced increase in BDNF mRNA (Fig. 4*A* and ref. 20) completely blocked the effect of bicuculline (Fig. 4*B*). None of these treatments influenced the levels of β-actin mRNA, indicating that the observed effects on BDNF and NGF mRNAs in the hippocampus are specific.

To evaluate whether the observed changes in NGF mRNA are also reflected in the amount of NGF protein produced in the hippocampus, the NGF protein levels were determined by a sensitive two-site immunoassay (26). NGF protein was also determined in the septum because NGF produced in the hippocampus is specifically taken up by the septal cholinergic neurons projecting to this brain area and transported retrogradely to their cell bodies (9). As shown in Table 1, the decreases in the levels of NGF mRNA from the different

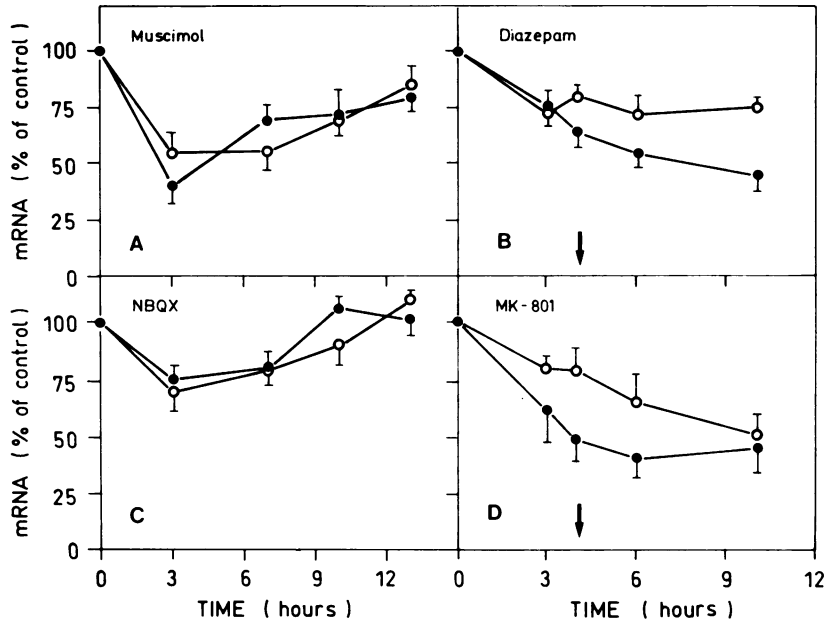

FIG. 3. Time course of changes in BDNF mRNA (●) and NGF mRNA (○) in hippocampus after administration of MK-801 (2 mg/kg), NBQX (100 mg/kg), muscimol (2 mg/kg), or benzodiazepine (10 mg/kg). Administrations of MK-801 and diazepam were repeated after the first 3.5 hr (arrows). Values are means of at least three independent experiments. In *B* and *D* all values and in *A* and *C* all values at time points 3 and 6 hr are significantly smaller than control values ($P < 0.05$).

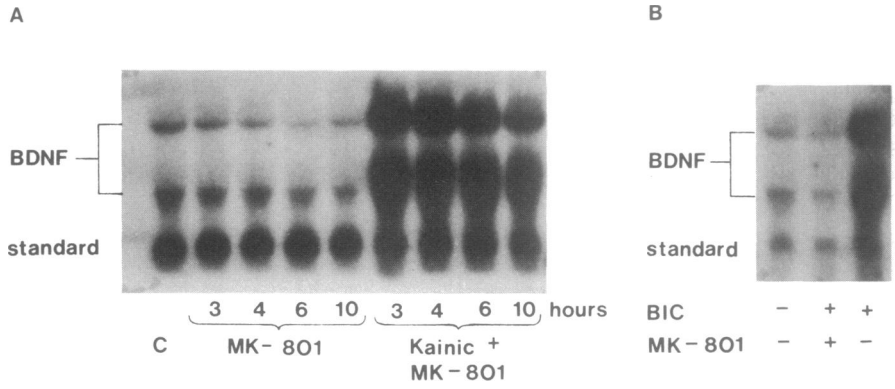

FIG. 4. Effect of MK-801 on BDNF mRNA in hippocampus. (*A*) Rats were treated with MK-801 (2 mg/kg, i.p.) 15 min before i.p. injection of saline or kainic acid (12 mg/kg). The MK-801 treatment was repeated after 4 hr. Rats were killed at indicated time points after injection of saline or kainic acid. (*B*) Bicuculline (BIC) (1.2 mg/kg) was injected i.v. 15 min after MK-801 or saline, and the rats were killed after 3 hr.

compounds were accompanied by similar reductions of NGF protein in both hippocampus and septum. The magnitude of the decrease in NGF protein in hippocampus was about the same with muscimol and with the glutamate-receptor blocker MK-801. The effect of muscimol and MK-801 together did not reduce the levels of NGF protein more than either compound alone (data not shown). In two experiments, NBQX decreased NGF in a similar manner to MK-801, but the limited quantity of the drug available precluded an analysis comparable to that done with MK-801 and muscimol (Table 1).

DISCUSSION

In the present study we have shown that the activation of glutamate receptors enhances the synthesis of BDNF and NGF in hippocampal neurons both *in vitro* and *in vivo*, whereas stimulation of the GABAergic system decreases their mRNA levels. Thus, the functional state of the hippocampal neuron seems to determine the levels of BDNF and NGF mRNA. Moreover, depending on the conditions, activation of both major types of glutamate receptors, NMDA and non-NMDA receptors, is able to up-regulate neuronal BDNF and NGF mRNA levels.

It has previously been shown that prolonged activation of neurons brought about by systemic injections of kainic acid or by electrolytic lesions that produce limbic seizures increases BDNF (20) and NGF (20–22) mRNA levels in the hippocampus. However, these extreme experimental conditions do not represent the finely tuned physiological mechanisms by which these factors are regulated *in vivo*. As an extension of our previous studies on kainic acid injections (20), we show here that the blockade of NMDA receptors by MK-801 decreases BDNF and NGF mRNA levels both *in vivo* and *in vitro*. These findings indicate that glutamate, acting via either the kainic acid (non-NMDA) or the NMDA receptor, increases the expression of these two molecules.

Table 1. Effect of various treatments on NGF protein levels

	NGF protein, pg/g of wet weight			
	Hippocampus		Septum	
Treatment	4 hr	10 hr	4 hr	10 hr
Control	1430 ± 20	1430 ± 20	1050 ± 50	1050 ± 50
MK-801	1107 ± 45*	995 ± 40**	615 ± 25**	435 ± 80**
Muscimol	875 ± 40**	ND	720 ± 25**	ND

Rats were treated for 4 hr and 10 hr with muscimol or MK-801 as described for Fig. 3. Subsequently the hippocampus and septum were isolated, and NGF protein levels were determined by ELISA. Values represent the means ± SEMs of four or more individual determinations. ND, not determined. *, $P < 0.01$ compared with control values; **, $P < 0.001$ compared with control values.

It is conceivable that the levels of BDNF and NGF mRNAs are additionally modified by the different types of glutamate and GABA receptors, which show marked differences in their regional distribution and with development (30, 31). Thus, the physiological regulation of the synthesis of BDNF and NGF may involve additional mechanisms of refinement and variability. In this context it should be remembered that in cultures of hippocampal neurons both histamine and the cholinergic agonist carbachol produce a small, though distinct, increase in BDNF mRNA. The relative importance of these neurotransmitters in the regulation of BDNF and NGF remains to be established and may be subject to regional differences.

The decreases in the levels of NGF mRNA in the hippocampus by muscimol, MK-801, and NBQX were accompanied by a corresponding change in NGF protein in the hippocampus and, most interestingly, also in the septum. The septal cholinergic neurons are responsive to NGF (10, 11) and can be rescued by NGF after fimbria fornix lesion (12). However, these neurons do not synthesize NGF themselves, but the relatively high NGF levels in septum reflect the accumulation of NGF by retrograde axonal transport from the projection fields (9). It was recently demonstrated that the cholinergic neurons are also responsive to BDNF, at least *in vitro* (13, 14). Because BDNF antibodies of sufficient specificity and affinity are not yet available, BDNF protein levels could not be determined. However, the parallel regulation of BDNF and NGF mRNAs suggests that BDNF production and release may be subject to a similar rapid regulation as those of NGF.

The surprisingly rapid decrease in septal NGF protein levels raises the question whether muscimol and MK-801, in addition to decreasing NGF synthesis, also inhibit the neuronal release of NGF. In the periphery, where NGF is synthesized exclusively by nonneuronal cells, it is released through a calcium-independent, constitutive pathway (32). For NGF produced in neurons, whether it is released through the constitutive and/or the regulated pathway is not yet established.

Recent findings indicate that the protooncogene *trk* is a component of the NGF high-affinity receptor (33–35) and that a homologous protein, trkB, binds BDNF and neurotrophin 3 (36, 37). It is still, however, unclear, whether these receptor tyrosine kinases mediate all or only a part of the effects of these neurotrophins. It is of interest to note that trkB is expressed in the hippocampus (38), which might implicate an autocrine or paracrine function in the hippocampus.

The regulation of the synthesis of neurotrophic molecules by neuronal activity and the rapidity of these changes mediated by specific transmitter systems seem to indicate that neurotrophic molecules may not only be involved in the prolonged maintenance of neuron-specific functions, as for instance the regulation of the synthesis of choline acetyltransferase (10, 11), but that they may also be involved in the regulation of activity-dependent neuronal plasticity. These findings, together with the observation that the glutamate system (in particular the NMDA receptors) are involved in long-term potentiation (18, 19), open up additional perspectives for the functions of neurotrophic molecules in the brain.

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