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Depolarizing stimuli regulate nerve growth factor gene expression in cultured hippocampal neurons

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ABSTRACT **Although trophic factors and neuronal activity have been implicated in regulating functional synaptic circuits, the relationship of trophic interaction to impulse activity in synaptogenesis remains unclear. Using cultured hippocampus as a model system, we provide direct evidence that depolarization and impulse activity specifically increase nerve growth factor gene expression in neurons. Depolarizing stimuli, such as a high K^+ concentration or the Na^+ channel agonist veratridine, elicited a 3-fold increase of nerve growth factor mRNA levels in both explant and dissociated cultures. Blockade of depolarization by tetrodotoxin prevented the increase of neuronal nerve growth factor mRNA. Further, nerve growth factor gene expression was stimulated by picrotoxin, a γ-aminobutyric acid antagonist frequently used to enhance hippocampal neuronal activity. Impulse regulation of trophic gene function may be relevant to developmental synaptogenesis and synaptic strengthening in learning and memory.**

A central question in neuroscience concerns mechanisms through which experience and consequent impulse activity regulate the functional architecture of synaptic circuits. Impulse activity modulates neuronal connectivity during development (1–3) and enhances synaptic efficacy in learning and memory during maturity (4–6). However, underlying molecular mechanisms remain obscure. It has been suggested that target cells may convey signals to presynaptic terminals in response to frequent synaptic use, thereby stabilizing active synaptic connections (6, 7). What molecular signals might be involved?

Trophic factors that govern neuronal survival during development are elaborated by targets and play a role in connectivity as well (8, 9). Nerve growth factor (NGF), the most fully-characterized agent, regulates the formation and maintenance of synaptic connections between peripheral neurons and their targets (10, 11). Moreover, innervating sympathetic and sensory neurons compete for limiting quantities of NGF synthesized by targets; the outcome of competition apparently determines patterns of synaptic connectivity (12, 13). Nevertheless, the relationship of trophic interactions to impulse activity in the periphery is undefined. Clues to the relationship have been derived from study of NGF in the central nervous system. For example, fimbrial transection increased NGF expression in neonatal hippocampus, suggesting that afferent innervation regulates target elaboration of the trophic factor (14). Limbic seizures, leading to a generalized increase in impulse activity, dramatically elevate NGF mRNA in hippocampal neurons (15). These observations are of particular interest, since hippocampal neurons normally elaborate the factor that interacts with receptors on innervating basal forebrain terminals (16–18) and since the basal forebrain–hippocampal system has been the primary brain model used to study central actions of NGF (19, 20). Using cultured hippocampus as a model system, we provide direct evidence that depolarization and impulse activity specifically increase NGF gene expression in neurons. Moreover, neuronal activity appears to be responsible for the developmental increase of NGF mRNA in the hippocampus.

MATERIALS AND METHODS

Tissue Dissection. Time-mated pregnant Sprague–Dawley rats were sacrificed by CO_2 asphyxiation. The day of discovery of vaginal plug was considered as embryonic day (E) 1, and the day of birth as postnatal day (P) 0. The brains of fetuses or pups were dissected in ice-cold phosphate-buffered saline under sterile conditions. Particular care was taken to avoid contamination from neighboring brain areas, blood vessels, and meninges during dissection.

Explant Cultures. The explant culture method used in this study is a modification of Maximov lying-drop explant system as described (21). Briefly, a layer of 0.2- to 0.5-mm-thick depolymerized collagen was spread onto round coverslips and photoreconstituted. The collagen-coated coverslips were placed in each well of a 6-well plastic dish. Several postnatal hippocampal slices (P2–4, ≈1 mm^3 in size) were transferred onto the coverslips. Each coverslip received two or three drops of nutrient medium [33% (vol/vol) house serum/10% (vol/vol) chicken embryo extract/10 mM Hepes/glucose (6 mg/ml)/achromycin (1.2 μg/ml) with ascorbic acid]. The cultures were grown in a CO_2 incubator at 37°C and fed every 3 to 5 days. This procedure allowed us to maintain healthy explants in large quantity for at least 2 weeks, facilitating our study of NGF gene expression *in vitro*.

Dissociated Culture of Hippocampal Neurons. The dissected E18 hippocampi were minced into small pieces in a serum-free medium and dissociated by mechanical trituration. Serum-free medium consisted of a 1:1 (vol/vol) mixture of Ham's F12 and Eagle's minimum essential medium and supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), selenium (30 nM), glucose (6 mg/ml), penicillin (0.5 unit/ml), and streptomycin (0.5 μg/ml). Cells were counted and plated in serum-free medium at 1×10^7 cells per dish on 100-mm Petri dishes coated with poly(D-lysine) (0.1 mg/ml). The cultured cells were maintained in a 37°C incubator with 100% humidity and an atmosphere of 5% CO_2/95% air. These cultures contain virtually pure neurons as demonstrated by neuron-specific enolase immunocytochemistry (22).

RNA Extraction. Total RNA was extracted by the method of Chirgwin *et al.* (23) and purified through CsCl gradient. For brain tissues or explant cultures of appropriate ages, the tissues were homogenized in a glass Dounce homogenizer

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Abbreviations: NGF, nerve growth factor; E*n*, embryonic day *n*; P, postnatal day; TTX, tetrodotoxin.

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with lysis buffer (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl/0.1 M 2-mercaptoethanol). For dissociated cultures, the lysis buffer was added to the culture dishes after removing the medium and scraping with an RNase-free rubber policeman. The lysates were immediately loaded onto the CsCl gradient and centrifuged overnight at 35,000 rpm in a SW 50.1 rotor. The pellets were ethanol-precipitated twice. The quality of the RNA was monitored by the ratio of UV absorbance at 260 and 280 nm and by an RNA denaturing minigel.

RNase Protection Assay. RNase protection experiments were performed as described (24). Briefly, a 455-base ^{32}P-labeled antisense NGF RNA probe was generated by *in vitro* transcription from a rat cDNA construct, pBSrNGF. Equal amounts of total RNA samples from control and drug-treated cultures were hybridized with the probe, and single-stranded sequences were digested by RNase T2. A 411-base-pair NGF-specific protected fragment was resolved on a polyacrylamide gel, which was then exposed to x-ray film. This assay is sensitive, quantitative, and specific for NGF mRNA (24).

Hybridization signals were assigned arbitrary numerical values based on densitometric scanning of autoradiograms. The results represent comparisons of relative NGF mRNA levels, among various conditions. Each experiment was repeated at least three times with independent RNA preparations, and the relative levels of NGF mRNA observed in these experiments were entirely consistent among repetitions. To ensure that densitometric values faithfully reflected relative levels of mRNA expression, we (22) reduced the potential error within each repetition of a particular experiment by combining three to seven dishes. Results are expressed either as percent control, as in Fig. 1, or in densitometric units, as in Figs. 2 and 3, to most accurately reflect the raw data.

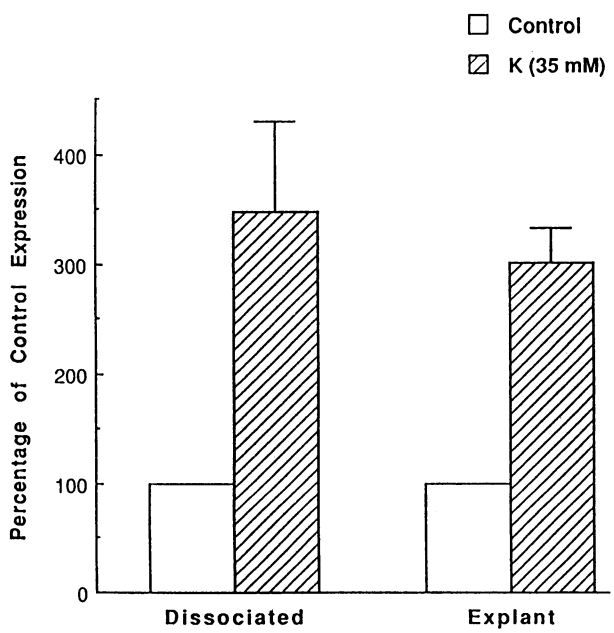

FIG. 1. Effect of a high K^+ concentration on NGF mRNA expression in hippocampal cultures. For explant cultures, P2 hippocampal slices were grown in nutrient medium for 4 days. For dissociated neuronal cultures, dissociated E18 hippocampal neurons were cultured for 7 days in serum-free medium at 1×10^7 cells per 100-mm dish. These cultures were then treated with or without 35 mM K^+ for 2 days and harvested for NGF mRNA measurement. Autoradiograms of NGF RNase protection were analyzed densitometrically and data are expressed as percentage of control expression. Explant, $n = 3$; dissociated cells, $n = 4$. Since various amounts of RNA were employed in each repetition of an individual experiment and since exposure times for the autoradiograms differed, drug-treated groups were compared to a normalized control; thus standard deviation was only presented for those drug-treated groups. The same is true for Fig. 4.

RESULTS

Depolarizing Influences and NGF Gene Expression. To begin studying the regulation of NGF gene expression in brain target cells, we used virtually pure neuronal dissociates derived from rat hippocampus (21, 22). A sensitive RNase protection assay was employed to measure NGF mRNA in culture (24). The potential role of membrane depolarization was assessed by exposing neurons to high concentrations of potassium (35 mM), a commonly employed depolarizing stimulus (25). Exposure to elevated concentrations of potassium elicited a 3-fold increase in NGF mRNA expression in the cultured hippocampal neurons (Fig. 1), suggesting that depolarization induces NGF gene expression.

To approximate *in vivo* function more closely, we also used explant cultures of hippocampal slices, which retain physiologic neuronal circuitry and cytoarchitectonics (26). Elevation of extracellular potassium, which is known to evoke synchronous neuronal discharge in this preparation (27), also dramatically increased NGF mRNA (Fig. 1).

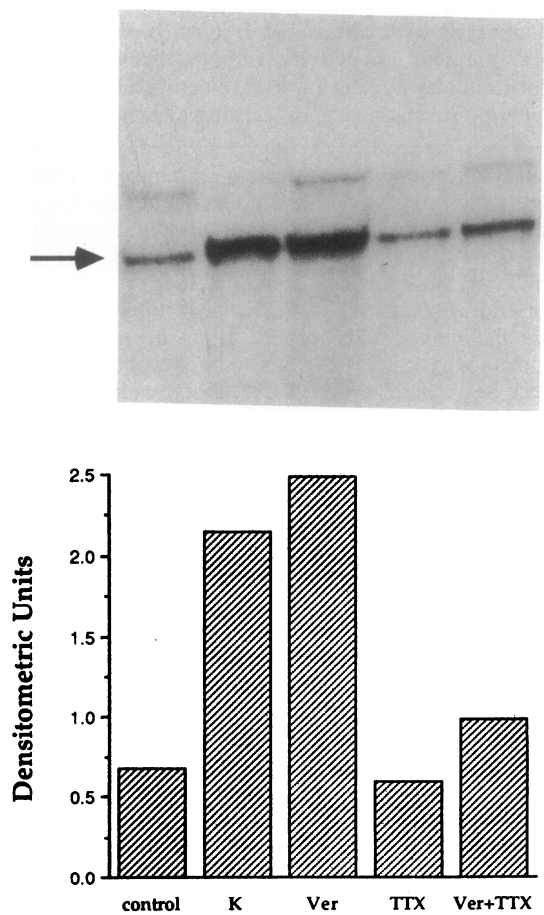

FIG. 2. Regulation of NGF mRNA expression in hippocampal neurons by depolarization. E18 hippocampal neurons were cultured for 6 days in serum-free medium. These cultures were then treated with the following agents for an additional 2 days and harvested for NGF mRNA measurement: K^+, 35 mM; veratridine (Ver), 1 μM; TTX, 1 μM. (*Upper*) Autoradiogram with 40 μg of total RNA per lane. Arrow indicates the 411-base-pair NGF mRNA fragment protected by RNase T2. (*Lower*) Densitometric plot of the autoradiogram. The result represents three experiments with independent RNA preparations.

High potassium concentration is known to enhance survival of some neuronal populations cultured in serum (28, 29). Although our dissociates were grown in serum-free medium, we examined neuronal number in our experiments to determine whether increased mRNA might have been attributable to increased survival. The number of neurons per dish, identified with neuron-specific enolase, was unchanged in dissociates after exposure to 35 mM potassium for 2 days (159,950 ± 9009 vs. 152,259 ± 17,720 cells per dish). Moreover, total protein per culture was also unaffected by increased potassium (195 ± 61 vs. 189 ± 57 μg per dish).

To determine whether depolarization *per se* regulated NGF gene expression, the specific depolarizing agent veratridine was employed. The alkaloid binds to specific ion channels, allowing sodium influx and eliciting membrane depolarization (30). Veratridine evoked a 3-fold increase in NGF mRNA in dissociated hippocampal neurons, reproducing the effect of elevated levels of potassium (Fig. 2). Moreover, the increase in mRNA was blocked by the specific sodium channel antagonist tetrodotoxin (TTX; Fig. 2, lane 5), indicating that the veratridine effect was, indeed, mediated by voltage-dependent sodium channels. Interestingly, exposure to TTX alone for 2 days slightly reduced the level of mRNA, suggesting that spontaneous neuronal discharge may also regulate NGF gene expression (Fig. 2, lane 4).

Impulse Activity and Developmental Expression of the NGF Gene. The frequency of spontaneous discharge of hippocampal neurons increases with age; additionally, responses progress from single spike to complex slow wave forms with time in culture (26). In our experiments, the developmental increase of hippocampal NGF mRNA paralleled this rise, attaining adult plateau levels by postnatal day 20 *in vivo* (Fig. 3). Further, both explant and neuronal dissociates exhibited a similar progressive increase in mRNA correlating with the ontogenetic increase in impulse activity (Fig. 3). To begin ascertaining whether impulse activity normally regulates NGF gene expression during development, several approaches were employed.

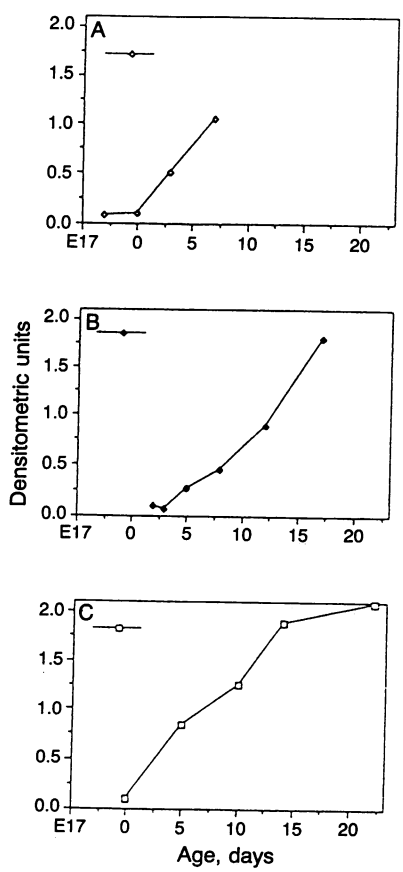

FIG. 3. Ontogeny of NGF mRNA expression in hippocampus *in vivo* and *in vitro*. For each developmental time course, equal amounts of total RNA from different ontogenetic stages of the hippocampus were assayed by the NGF RNase protection (15). Each curve was repeated two to four times and representative densitometric analyses of the autoradiograms were plotted together for relative comparison. (*A*) Neuron culture. (*B*) Explant culture. (*C*) *In vivo*.

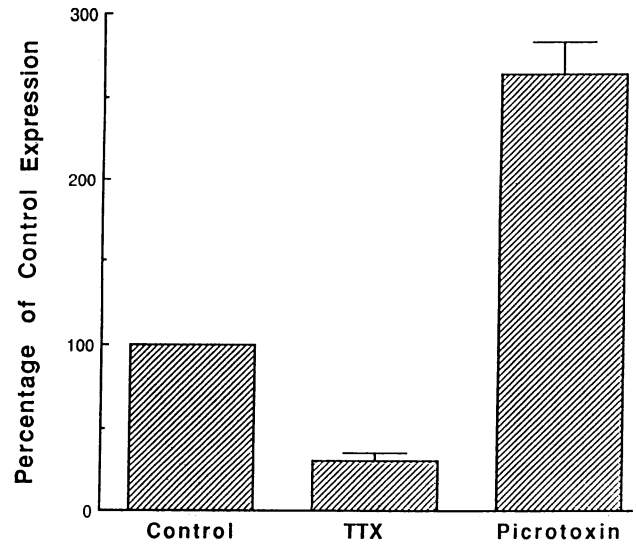

FIG. 4. Regulation of NGF gene expression by spontaneous neuronal activity. E18 hippocampal neurons were cultured in serum-free medium. The control group was cultured for 8 days. TTX group was cultured for 2 days and treated with 1 μM TTX for 6 days. Picrotoxin group was cultured for 8 days and treated with the γ-aminobutyric acid antagonist picrotoxin (1 μM) for 6 hr on the last day. All groups were cultured for the same amount of time before harvest for NGF RNase protection. Autoradiograms were analyzed densitometrically and numerical values were normalized to percentage of control, as in Fig. 1. (n = 3.)

We exposed neuronal dissociates to TTX for an extended period to inhibit spontaneous discharge during the developmental time frame. Exposure for 6 days prevented the ontogenetic rise of NGF mRNA (Fig. 4), suggesting that activity regulates NGF gene expression during development.

We employed an entirely different approach to increase spontaneous activity in the dissociates: inhibitory hippocampal γ-aminobutyric acid interneurons were blocked with picrotoxin (27). Exposure to picrotoxin, the γ-aminobutyric acid antagonist, for 6 hr markedly increased NGF mRNA (Fig. 4), suggesting that enhancement of spontaneous discharge facilitates developmental NGF gene expression.

DISCUSSION

Our results indicate that membrane depolarization, elicited by elevated extracellular K^+ concentration or by the Na^+ channel agonist veratridine, increased the levels of NGF mRNA. Consequently, afferent impulse activity, driven by environmental stimuli, may stimulate the elaboration of the target trophic factor that enhances growth of the very same afferent fibers. These observations may be relevant to synaptic regulation occurring in a number of contexts.

During development, impulse activity is known to increase, and the complexity of electrophysiologic responses increases markedly in the hippocampus and other central nervous system areas (26, 31), paralleling the increase in NGF mRNA (ref. 17 and Fig. 3). Moreover, recent work indicates that elaboration of NGF protein is faithfully re-

flected by mRNA levels (32). Since both impulse activity (1–3) and NGF (10–12) play important roles in synapse formation, it is possible that activity regulation of neuronal connectivity is mediated by the trophic agent. It has been hypothesized that afferent activity may regulate target production of trophic factors during development, which in turn feeds back on presynaptic terminals to selectively stabilize the connections (7). The results described here provide evidence for a causal link between impulse activity and production of the trophic factor, thereby supporting the hypothesis. Thus, the activity-dependent enhancement of NGF gene expression has potential significance for synaptogenesis during neuronal ontogeny.

Regulation of NGF gene expression by neuronal activity may also have implications for learning and memory during maturity. Repetitive afferent activity may enhance trophic factor production by targets (7, 33), thereby strengthening synaptic connections (4). Indeed, impulse regulation of trophic function may constitute one mechanism through which transient environmental events are translated into long-lasting genomic, synaptic, and circuit changes. Finally, impulse regulation of NGF may be relevant to the pathogenesis of various disease processes. For example, limbic seizures, which lead to a generalized increase in impulse activity and elevated NGF mRNA in hippocampal neurons (15), may perpetuate epileptic activity through synaptic strengthening.

Note. During the preparation of this manuscript, a paper describing the activity-dependent regulation of mRNAs for two neurotrophic factors was published (34).

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