Experimental Neurology **174,** 201–214 (2002)
doi:10.1006/exnr.2002.7869, available online at http://www.idealibrary.com on IDEAL®

Spontaneous Limbic Seizures after Intrahippocampal Infusion of Brain-Derived Neurotrophic Factor

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Received August 31, 2001; accepted December 14, 2001

The results of several studies have contributed to the hypothesis that BDNF promotes seizure activity, particularly in adult hippocampus. To test this hypothesis, BDNF, vehicle (phosphate-buffered saline, PBS), or albumin was infused directly into the hippocampus for 2 weeks using osmotic minipumps. Rats were examined behaviorally, electrophysiologically, and anatomically. An additional group was tested for sensitivity to the convulsant pilocarpine. Spontaneous behavioral seizures were observed in BDNF-infused rats (8/32; 25%) but not in controls (0/20; 0%). In a subset of six animals (three BDNF, three albumin), blind electrophysiological analysis of scalp recordings contralateral to the infused hippocampus demonstrated abnormalities in all BDNF rats; but not controls. Neuronal loss in BDNF-treated rats was not detected relative to PBS- or albumin-treated animals, but immunocytochemical markers showed a pattern of expression in BDNF-treated rats that was similar to rats with experimentally induced seizures. Thus, BDNF-infused rats had increased expression of NPY in hilar neurons of the dentate gyrus relative to control rats. NPY and BDNF expression was increased in the mossy fiber axons of dentate gyrus granule cells relative to controls. The increase in NPY and BDNF expression in BDNF-treated rats was bilateral and occurred throughout the septotemporal axis of the hippocampus. Mossy fiber sprouting occurred in five BDNF-treated rats but no controls. In another group of infused rats that was tested for seizure sensitivity to the convulsant pilocarpine, BDNF-infused rats had a shorter latency to status epilepticus than PBS-infused rats. In addition, the progression from normal behavior to severe seizures was faster in BDNF-treated rats. These data support the hypothesis that intrahippocampal BDNF infusion can facilitate, and potentially initiate, seizure activity in adult hippocampus.

Key Words: **epilepsy; dentate gyrus; neurotrophin; growth factor; osmotic pump; pilocarpine; mossy fibers; neuropeptide Y; hippocampus; sprouting.**

INTRODUCTION

BDNF is a member of the neurotrophin family, which also includes nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). BDNF binds with high affinity to the tyrosine kinase receptor trkB and with low affinity to the p75 receptor. BDNF has been shown to have important effects on the growth and development of the nervous system; in addition, it is neuroprotective under various conditions (5, 15, 16, 19, 57, 83, 92). In addition to the trophic and protective effects, BDNF has also been shown to have other actions in the adult brain. BDNF exposure in adult hippocampal slices leads to increased excitatory transmission (1, 7, 8, 13, 29, 41, 42, 57, 61, 70, 71, 79). There appear to be several potential mechanisms, including increased glutamate release (17, 33, 50, 53, 54, 58, 59), protein synthesis (42), changes in inhibition (9, 11, 30, 60, 67, 90), and modulation of NMDA receptors (38, 51, 52, 55, 87) and/or sodium channels (40).

Studies of area CA3 and the entorhinal cortex in hippocampal slices demonstrated that BDNF exposure can lead to epileptiform activity (79). *In vivo* studies using the kindling model of epilepsy are consistent with an epileptogenic effect of BDNF because transgenic mice with less BDNF (heterozygote knockouts, i.e., +/−) kindled more slowly than wild type mice (47), and infusion of an inhibitor of BDNF's actions inhibited kindling (6). However, other studies have found that infusion of BDNF does not facilitate kindling; i.e., when BDNF was infused into hippocampus, kindling was delayed (49, 68, 76). *In vitro* studies are consistent with a proconvulsant effect of BDNF, because transgenic mice which overexpress BDNF had increased seizure sensitivity (20) and epileptiform evoked responses in hippocampus (20). It has also been shown that exogenous BDNF exacerbated damage produced by the convulsant kainic acid (78).

These studies suggest that, in the adult hippocampus, BDNF may increase excitability. If excitability increased enough, one would predict that seizures

0014-4886/02 $35.00

might occur. To test this hypothesis, BDNF was infused directly into the hippocampus *in vivo* using osmotic minipumps and compared to animals infused with control solutions.

All rats were examined immunocytochemically to detect changes in hippocampus that might have been caused by BDNF infusion. Markers were chosen that could detect changes that are commonly associated with seizures. For example, an antibody to a neuronal nuclear protein, NeuN, was used to determine whether there was neuronal loss in the hippocampus, since this occurs in several animal models of epilepsy (84). Neuropeptide Y (NPY)-immunoreactivity was tested because it is increased in dentate hilar neurons and dentate granule cell axons, the mossy fibers, after seizures. Thus, comparing NPY staining in rats with and without seizures can be used to evaluate whether seizures were likely to have occurred. However, increased NPY staining after seizures is not always long lasting, and increased NPY staining may occur after activity that is not as extensive as seizures. In addition, NPY was of interest because BDNF induces NPY expression (21, 60, 75, 77, 95).

Another well-accepted marker of seizures is "mossy fiber sprouting," which occurs in several animal models of epilepsy (91, 93) and human epilepsy (88). Mossy fiber sprouting refers to the development of new axon collaterals of granule cell axons, the mossy fibers, that project to an abnormal location, the inner molecular layer (88, 91). Sprouting often occurs after seizures, and it was also of interest because sprouting may be influenced by BDNF (3, 56, 69, 73).

Another marker of increased neuronal activity is BDNF itself, which is ordinarily expressed in mossy fibers (18, 99), and increases its expression after various types of seizures (2, 4, 26–28, 34–37, 44, 48, 63–65, 82, 94). However, BDNF expression in granule cells also increases under conditions that do not necessarily involve seizures, such as long-term potentiation (LTP; 10, 14, 23, 32, 71, 72), learning (45), stress (65), exercise (66), and dietary restriction (25). Thus, given that each marker has its limitations, several were used to assess anatomical changes that would be consistent with a history of abnormal neuronal activity.

MATERIALS AND METHODS

All experiments were conducted in compliance with guidelines established by the National Institutes of Health and the New York State Department of Health. Procedures conducted at Regeneron Pharmaceuticals or Helen Hayes Hospital were approved by each institution's Animal Care and Use Committee.

The animals used were adult male Sprague–Dawley rats (300–350 g). Animals were housed in pairs at standard temperature and humidity-controlled colony conditions at Regeneron Pharmaceuticals and then were transferred to Helen Hayes Hospital, where they were housed individually. Food and water were available *ad libitum*. Animals were maintained on a 12:12 light:dark cycle (lights on 06:00h). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted.

Implantation and Use of Osmotic Minipumps Containing BDNF, Phosphate-Buffered Saline (PBS), or Albumin

A cannula was implanted in the dorsal hippocampus, with the cannula tip near the hilus (3.8 mm posterior and 2.7 mm lateral measured from bregma). Surgery was performed under aseptic conditions using chloral hydrate (170 mg/kg) and pentobarbital (35.2 mg/kg) anesthesia. All cannulae (30 gauge, 4 mm length; Plastics One, Roanoke, VA) were cemented (Cranioplastic cement; Plastics One) to the skull, anchored with machine screws (Plastics One), and connected to an Alzet osmotic minipump (model 2002; Alza Corp., Palo Alto, CA) by medical grade vinyl tubing (Biolab Inc., Decatur, GA). Pumps were filled with BDNF or control solutions. One control solution was PBS, the vehicle used to dissolve BDNF. The other solution was albumin, a large protein (like BDNF) that could be infused at a similar dose to BDNF (i.e., 3 μg/μl).

Pumps were filled with BDNF, sterile PBS (Gibco; Invitrogen Co., Carlsbad, CA), or bovine serum albumin (BSA) the day before surgeries were performed and placed at 4°C in sterile PBS overnight. All pumps were implanted subcutaneously in the nape of the neck, and cannula were lowered after the pumps began pumping. The scalp incision was closed with surgical staples and treated with povidone iodine. Rats were infused continuously at a rate of 0.5 μl/h with either PBS, recombinant human BDNF (1 or 3 μg/μl; Amgen-Regeneron Partners), or BSA (1 or 3 μg/μl) for 14 days. Animals were transferred 3–4 days after surgery to Helen Hayes Hospital.

Seven animals were reanesthetized at 14 days after pump implantation, and the wound clips were removed. The old pump was removed and a new pump was placed into the area between the shoulders where the old pump was located. The tubing from the new pump was sealed onto the end of the old tubing. The wound was closed and treated with betadine solution. Upon sacrifice, it was observed that the tubing had broken off from the second pump in all animals except two. Therefore, in the results, only two animals are listed as having had infusions for 4 weeks. The other animals were pooled with the animals which had pumps for only 2 weeks because the data from these animals could not be distinguished.

Scalp recordings. For scalp recordings, animals were first anesthetized with a half-dose of chloral hydrate (175 mg/kg) i.p. Animals were awake but not

moving during scalp recordings. Recordings were made using a Grass AC Preamplifier (Model P55, Astro-Med Inc., West Warwick, RI) with amplification set on 10,000 and filters at 1 Hz and 0.1 kHz. The active lead was clipped to the exposed end of a 26-gauge copper wire that was wrapped around a machine screw in the skull overlying the hippocampus contralateral to the infusion site. The wire and screw were placed at the time of pump implantation, at which time a small extension of the wire was allowed to emerge from the scalp incision and then secured with standard wound clips. The reference lead was clipped to a different wound clip over the scalp incision that was not touching the other wound clips. Recordings were displayed on a chart recorder (Model 42-8240-10, Gould Inc., Valley View, OH), digitized (Neurocorder Model DR-484, Cygnus Technology, Delaware Water Gap, PA), and stored on videotape.

Anatomical analysis. Animals were perfused with 4% paraformaldehyde except for those that died prematurely, in which case the brain was removed shortly after death and immersion-fixed in 4% paraformaldehyde. The animals that died prematurely were BDNF-infused ($n = 4$) and controls (PBS, $n = 1$; albumin, $n = 1$). The data from perfusion and immersion fixation are pooled in the results because they could not be distinguished.

Using a vibratome (Ted Pella, Redding, CA), 50-μm sections were cut in the coronal plane. Sections were stained for Nissl substance using cresyl violet. Animals which had damage near the cannula track, i.e., a track exceeding 750-μm width (far greater than the width of the cannula, which was 30-gauge; Fig. 2), are excluded from the results. Tissue sections from BDNF-infused rats and PBS- or albumin-infused rats were processed together.

Immunocytochemistry followed a procedure that has been reported (85). Briefly, free-floating sections were washed in 0.1 M trishydroxymethyl-aminomethane (TRIS) buffer (pH 7.6) followed by incubation in 1% H_2O_2 (dissolved in TRIS) for 30 min. After a 5-min wash in Tris, sections were washed in TRIS containing 0.1% Triton X (Tris A) for 10 min, followed by a 10-min wash in Tris A containing 0.005% bovine serum albumin (Tris B). Sections that were processed with polyclonal antibodies were preincubated in 10% normal goat serum and sections that were processed with monoclonal antibodies were preincubated in 10% normal horse serum. The sections were incubated for 48 h at 4°C in antisera to a neuronal nuclear marker (NeuN, monoclonal, 1:5,000, Chemicon) or neuropeptide Y (NPY; polyclonal; 1:30,000; Peninsula), diluted in TRIS B. On the second day, sections were washed for 10 min in Tris A followed by 10 min in Tris B. The sections were then incubated for 45 min in a biotinylated secondary antibody against rabbit IgG made in goat (1:1000; Vector) diluted in TRIS B for polyclonal antibodies' and a biotinylated secondary antibody against mouse IgG made in horse (1:400, Vector) for monoclonal antibodies. Following a 10-min wash in Tris A and a 10-min wash in Tris D (0.5 M TRIS containing 0.1% Triton and 0.005% bovine serum albumin), sections were incubated for 1 h in avidin–biotin–horseradish peroxidase complex (ABC, 1:1000 dilution, Elite kit, Vector) diluted in Tris D. Sections were washed in Tris (3×5 min) and developed in diaminobenzidine tetrahydrochloride (DAB; 50 mg/100 ml TRIS, Polysciences) plus 200 mg β-D-glucose, 0.3 mg glucose oxidase, and 40 mg ammonium chloride. Sections were mounted on slides, dehydrated, and coverslipped.

Sections for BDNF immunocytochemistry were processed as described previously (78, 80). Sections were washed in 0.05 M potassium phosphate buffered saline (KPBS) and then treated with 0.5% H_2O_2 in KPBS for 30 min. After a 5-min wash in KPBS, sections were incubated in 10% normal goat serum, 1% bovine serum albumin (BSA), and 0.4% Triton-X in KPBS for 20 min. Sections were incubated in an antibody made in rabbit that recognizes rat BDNF (1:30,000, polyclonal, Amgen-Regeneron Partners) in 1% BSA and 0.4% Triton-X in KPBS overnight at 4°C. Sections were then rinsed 10 times for 10 min in 0.25% BSA and 0.02% Triton X in 0.01 M KPBS. Then sections were incubated in a biotinylated secondary antibody made in goat (1:1000; Vector) in 1% BSA and 0.02% Triton-X in KPBS for 1 h, followed by ABC (1:1000, Elite kit, Vector) in 1% BSA in KPBS for 1 h. Sections were rinsed in KPBS and then Tris buffer and reacted with DAB (50 mg/100 ml TRIS; Polysciences) in 50 mM $NiCl_2$.

Evaluation of spontaneous seizures. Seizures were defined using the rating scale of Racine (74). Observations of spontaneous seizures were made by investigators who were blind to the treatment of the animal (i.e., BDNF, PBS, or albumin). Observations began on the 4th day of infusion and continued on an intermittent basis thereafter (3–4 times per day for 5–30 min, between 08:00 and 20:00). Thus, the extent of seizure behavior that is reported in the Results is likely to underestimate the number of seizures that occurred.

Pilocarpine administration. Pilocarpine administration was conducted by an investigator who was blind to the treatment of the animal (i.e., BDNF or PBS). Rats were first injected subcutaneously with atropine methylbromide (1 mg/kg). After 30 min they were injected i.p. with pilocarpine hydrochloride (380 mg/kg). The onset of status epilepticus was defined as the time that stage 5 seizures became continuous. After 1 h of status epilepticus, rats were treated with diazepam i.p. (5 mg/kg; Wyeth-Arced). There were only two animals that failed to exhibit status (both were PBS-infused rats); one never demonstrated seizure-

associated behavior and the other exhibited only facial automatisms.

Data analysis. Significant differences were determined using either a Student's *t* test (PSI plot software, Polysoft International) or $\chi 2$ analysis. Significance was set at $P < 0.05$ prior to experiments.

RESULTS

A total of 52 rats were used. Thirty-five (BDNF, $n = 22$; PBS, $n = 6$; albumin, $n = 7$) were examined behaviorally for evidence of spontaneous seizures between days 4 and 14 after pumps were implanted. The other 17 (BDNF, $n = 10$; PBS, $n = 7$) were also examined behaviorally, but this observation period was truncated in order to test for sensitivity to pilocarpine during the period of time when BDNF was being infused. Thus, spontaneous seizures in the pilocarpine-treated group were more likely to have been underestimated because the observation period was brief relative to the group that did not receive pilocarpine.

Within the group of animals that were not tested for pilocarpine sensitivity, two doses of BDNF were used: 1 or 3 μg/μl (12 or 36 μg/day). Thus, of the 22 BDNF-treated animals, 8 received 1 μg/μl and the other 14 received 3 μg/μl. These rats were compared to albumin-infused rats that received 1 ($n = 3$) or 3 ($n = 4$) μg albumin/μl. In the results presented below, 1 μg/μl is referred to as the "low" dose and 3 μg/μl is referred to the "high dose."

Spontaneous Behavioral Seizures in BDNF-Infused Rats

In 8 of 32 rats (25%) that were infused with BDNF, spontaneous behavioral seizures occurred. All of these rats were infused with the higher dose of BDNF. No behavioral seizures were observed in any of the PBS (0/13)- or albumin (0/7)-infused rats (total, 0/20, 0%), which were interspersed among the cages with BDNF-infused rats, so they would be noticed equally well. Investigators observing the animals were blind to the type of treatment (i.e., BDNF, PBS, or albumin). The difference (25% vs 0) was statistically significant ($\chi^2 = 5.66$; $P < 0.05$).

The seizures observed in BDNF-treated rats were classic limbic seizures that reached stage 5 ($n = 6$) or stages 3–4 ($n = 2$). In two of the animals that exhibited stage 5 seizures, more than one stage 5 seizure was observed. In one of these animals two seizures were observed on the same day, separated by several hours. In the other animal, 3 seizures were observed, 2 on the same day (again, separated by several hours) and 1 on a different day. All seizures occurred between the 6th and 14th day of infusion, and were not detected thereafter.

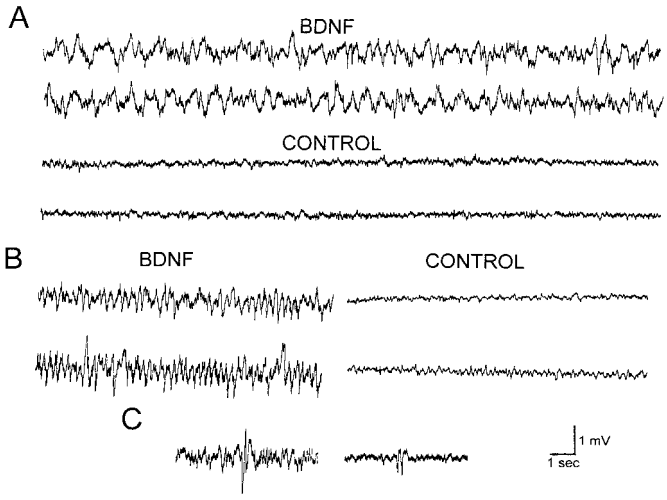

FIG. 1. Scalp recordings from BDNF-infused rats vs. PBS-infused rats. (A) Recordings from the contralateral hippocampus of a BDNF-treated ("BDNF") and PBS-treated ("Control") rat. Recordings were made using a wire attached to a skull screw and were made with reference to a screw over the frontal cortex. Calibration is in part C. (B) Recordings from other BDNF-treated and PBS-treated rats. Traces from two different BDNF-treated rats are shown at left and two different PBS-infused rats are shown at right. Calibration is in part C. (C) Recordings of isolated sharp wave-like activity (12) in two different BDNF-treated rats. Calibration is on the right.

Scalp Recordings

Six animals had scalp recordings made from the scalp overlying the hippocampus contralateral to the infusion site. Three rats were infused with BDNF (high dose) and the other three were infused with albumin (high dose). The recordings were made by an investigator who was blind to the treatment of the animals. All recordings were made during the week after pump infusion ceased, and at a similar time of day (between 12:00 and 16:00 h). The length of the recording period was approximately 45 min. Within that time, the frequency and amplitude of activity did not appear to change.

All animals that were identified as having large amplitude, irregular activity were animals that had received BDNF. Figure 1 shows examples of records from these animals. In addition to large amplitude potentials in BDNF treated rats (Figs. 1A and 1B), some fast spikes were also observed, similar to "sharp waves" (Fig. 1C; 12). No electrographic seizures were observed.

Histology and Immunocytochemistry

Confirmation of cannula sites. All animals were evaluated with Nissl staining to determine the specific site of the cannula. All cannulas descended into CA3b or CA3c. The tip of the cannula reached the cell layer of CA3b, CA3c or the lower blade of the dentate gyrus (Fig. 2). In 16 cases (BDNF, $n = 12$; PBS, $n = 4$) there was evidence that the tip of the cannula appeared to

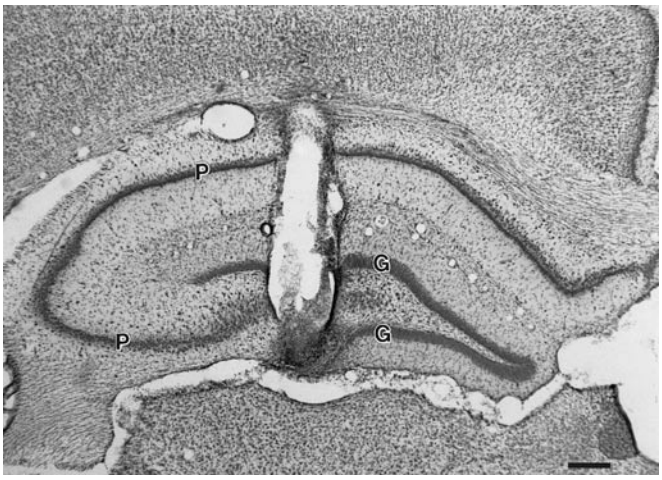

FIG. 2. Nissl staining in a tissue section from the center of the cannula track in the hippocampus of a BDNF-infused rat. A Nissl-stained section of the dorsal hippocampus is shown, illustrating the cannula track of a rat that was infused with BDNF. The cannula passed through area CA1 and the dentate gyrus. Its tip reached the lateral edge of the lower blade of the dentate gyrus. P, pyramidal cell layer. G, granule cell layer. Calibration, 300 μm.

touch the dorsal border of the thalamus, just ventral to the dentate gyrus.

Exogenous BDNF. BDNF immunocytochemistry was conducted to (1) confirm BDNF-infusion from BDNF-containing pumps, and (2) determine how far BDNF spread from the cannula tip.

Thirteen animals were examined. Nine received BDNF (high dose) and the others were infused with PBS. In BDNF-infused animals that died while the pump was still infusing BDNF, exogenous BDNF staining was intense, and distributed throughout the dorsal hippocampus (Fig. 3). In two animals that were examined throughout the hippocampus, BDNF did not stain ventral hippocampus. Therefore, BDNF distribution was likely to have only spread throughout the dorsal ipsilateral hippocampus at high concentration. However, there was some BDNF staining in dorsal thalamus, and hence BDNF could have entered the ventricles and spread to other areas at a diluted concentration. In animals that were perfused after the pump stopped, BDNF staining was not apparent anywhere in the brain except for the tip of the cannula track (Fig. 3). In PBS-treated animals, there was no evidence of BDNF near the cannula except for weak staining in two cases on the edge of the track made by the cannula, which could represent nonspecific staining, endogenous peroxidase, or local upregulation of BDNF due to tissue injury (Fig. 3).

Comparison of BDNF-infused rats with epileptic rats. In 35 animals that received BDNF (n = 22), PBS (n = 6), or albumin (n = 7) infusion, and were not treated with any other drug (i.e., pilocarpine), NeuN staining revealed similar hippocampal neuronal distribution in all rats (data not shown, but see Fig. 2 for a Nissl-stained section of a BDNF-infused rat showing normal hippocampal structure). However, 17 of 22 (77%) BDNF-treated rats had anatomical abnormali-

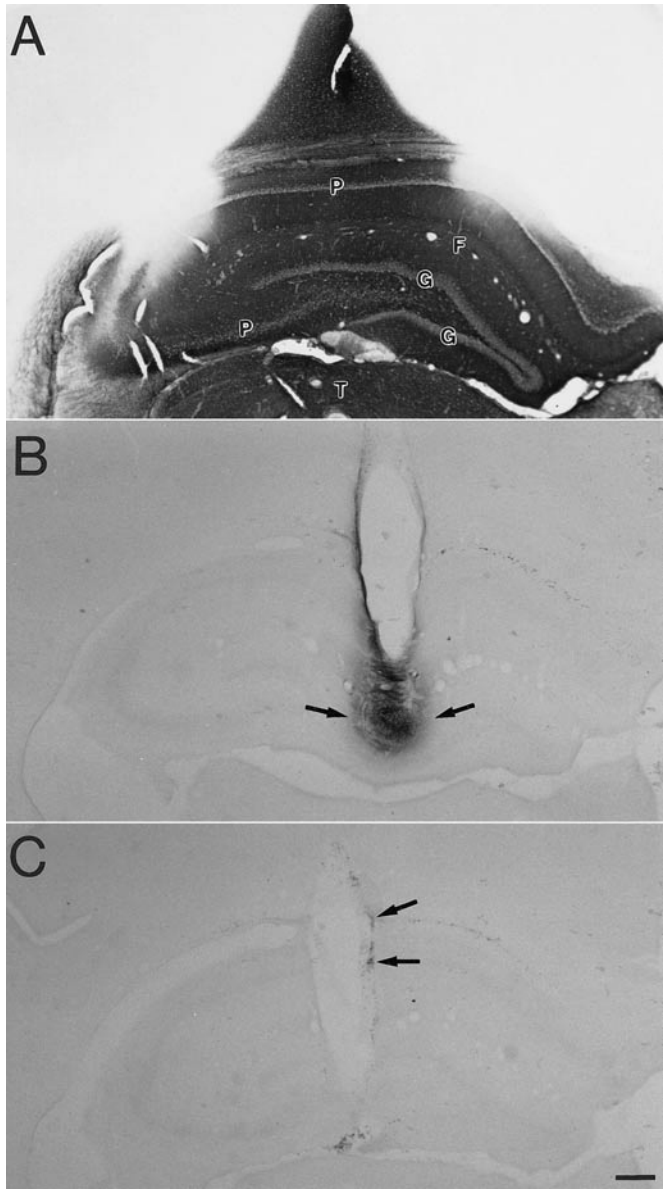

FIG. 3. The distribution of exogenous BDNF after infusion into the hippocampus. (A) In a BDNF-infused animal that died during pump infusion, immunocytochemistry using an antibody to BDNF illustrates the extent that exogenous BDNF spread from the cannula into the hippocampus. In addition to the hippocampus, BDNF also appeared to reach the thalamus. P, pyramidal cell layer; G, granule cell layer; F, fissure; T, thalamus. Calibration (300 μm, in C) applies to A–C. (B) In an animal that was sacrificed several weeks after the infusion of BDNF ended, staining is only apparent at the end of the cannula track (arrows). (C) In a PBS-infused rat, little staining is evident. Staining at the edge of the cannula track (arrows) could reflect BDNF that leaked into the track from the serum, BDNF upregulation in injured tissue, or nonspecific staining of damaged tissue.

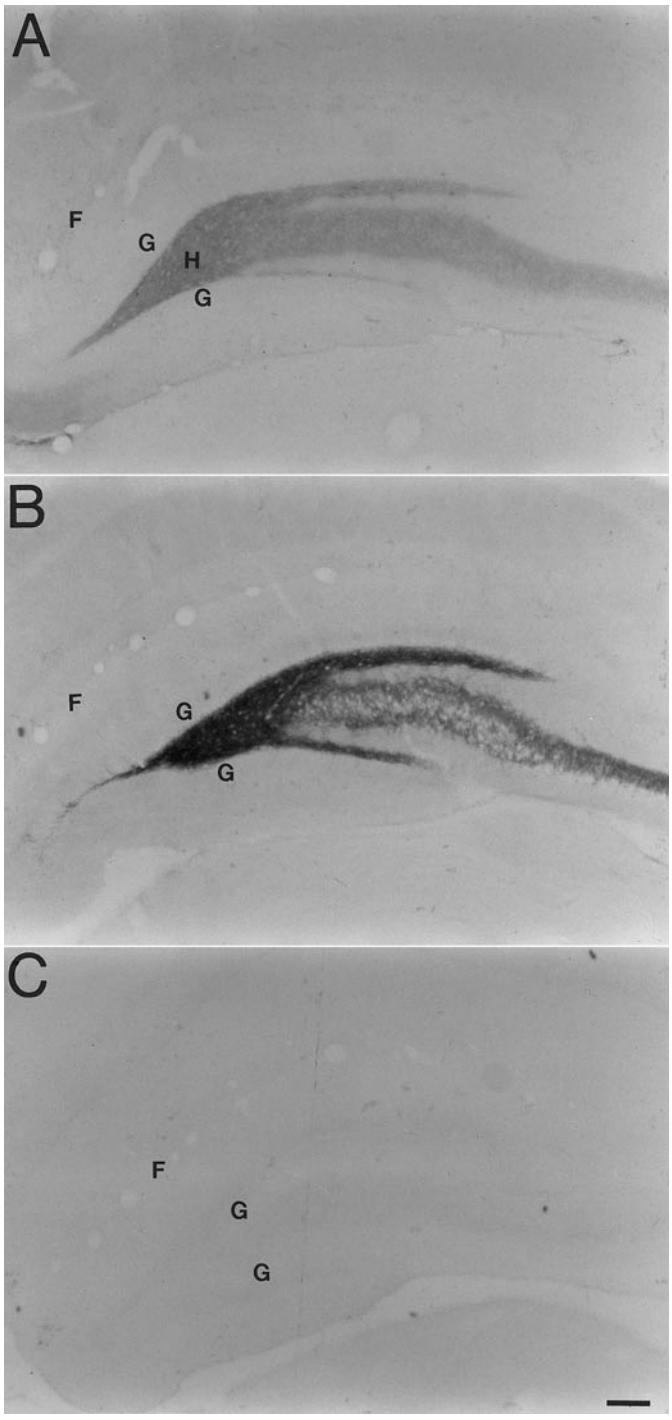

FIG. 4. Increased endogenous BDNF in the hippocampus of BDNF-infused rats with seizures. (A) In a BDNF-infused rat that had an observed spontaneous seizure, BDNF immunoreactivity was increased in the mossy fibers. The hippocampus contralateral to the infusion site is shown. This animal was examined 2 weeks after the start of pump infusion. F, fissure; G, granule cell layer; H, hilus. Calibration (300 μm, in C) applies to A–C. (B) In a PBS-treated rat that was perfused 1 day after pilocarpine-induced status epilepticus, BDNF immunoreactivity was increased in the mossy fibers contralateral to the cannula. Note the similarity in the distribution of the staining to the section shown part A. The staining is likely to be much darker in B because the animal had much more severe seizures (status epilepticus). Note that the sections shown in parts A–C were processed together. (C) Staining for BDNF in the contralateral hippocampus in a PBS-treated rat. This rat did not receive a pilocarpine injection. It was examined 2 weeks after the start of pump infusion. Little endogenous staining is apparent for BDNF compared to the tissue from animals which had seizures (parts A and B).

ties that resembled the hippocampus in other studies of epileptic rats. Six of 8 animals that had the lower dose of BDNF demonstrated these abnormalities, as well as 11 of 14 rats that received the higher dose. Seizures were never observed in 14 of the 17 BDNF-treated rats, which demonstrated anatomical abnormalities.

Endogenous BDNF staining. Figure 4 provides an example of a change in the BDNF-infused rats that resembled rats after seizures. This change was an increase in BDNF expression in mossy fibers. Mossy fiber staining was increased both ipsilateral and contralateral to the infusion site. It was increased in the 2 BDNF-treated animals that had spontaneous seizures, but not in the 3 BDNF-treated rats without seizures, nor 3 PBS-treated rats (Fig. 4). The upregulation was similar qualitatively to PBS-infused rats that had status-epilepticus after pilocarpine (Fig. 4), and is consistent with previous reports of increased mossy fiber expression of BDNF after pilocarpine-induced seizures (59, 64, 78, 80, 82).

Surprisingly, we observed a lack of staining for endogenous BDNF in PBS-infused rats, because BDNF is normally expressed in mossy fibers of the adult rat and can be revealed with the antibody that was used (18, 78, 80, 99). It is possible that endogenous BDNF was not well stained because processing was conducted to optimize staining for exogenously administered BDNF; endogenous staining requires different conditions (78, 80). Thus, the results are likely to have underestimated the extent of mossy fiber BDNF expression, both in the control and BDNF-treated tissue.

NPY. NPY expression was examined in 13 control and 22 BDNF-treated rats that were not injected with pilocarpine. Figure 5 illustrates NPY immunoreactivity in the dorsal dentate gyrus after BDNF- vs PBS-infusion. The sections chosen are from the hippocampus contralateral to the cannula, but similar results were obtained using ipsilateral tissue (data not shown). In the PBS-treated rat (Fig. 5A), normal staining is apparent: a variety of interneurons in the hilar region are immunoreactive, as well as fibers in several different laminae. Fiber staining is apparent in the outer molecular layer, where many of the NPY-immunoreactive hilar neurons project (22, 46, 62). NPY immunoreactivity of all 13 control rats (6 PBS, 7 albumin) were similar. In contrast, 11 of 22 (50%) BDNF-treated rats demonstrated increased immunoreactive hilar cells (Fig. 5B) relative to control rats (Fig. 5A). Increased staining for NPY was primarily observed when

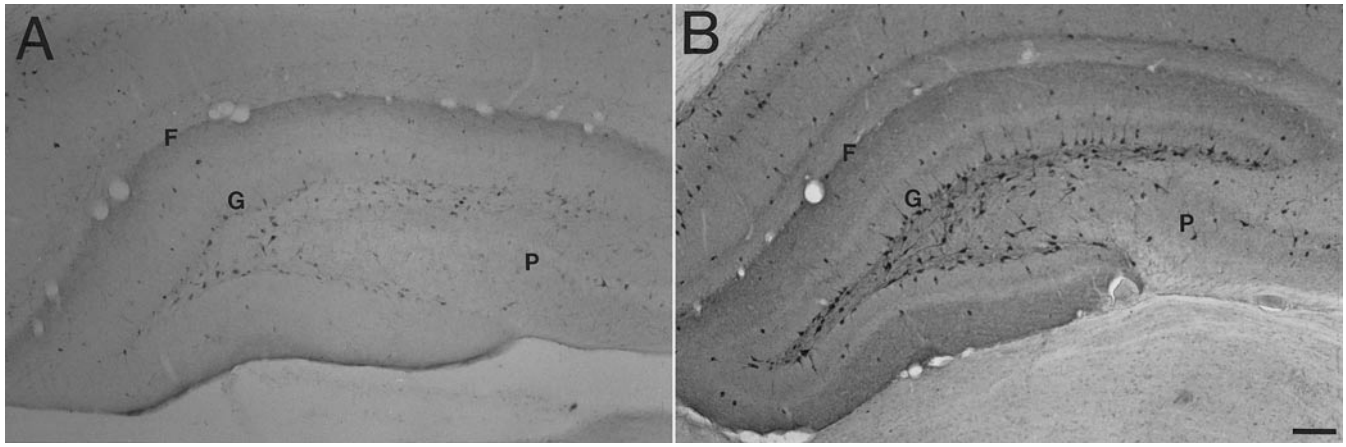

FIG. 5. Neuropeptide Y immunoreactivity in BDNF- and PBS-treated rats. (A) In a PBS-treated rat, neuropeptide Y (NPY) immunoreactivity is evident in hilar neurons. Fibers are stained also, predominantly in the outer molecular layer, where the axons of the stained hilar neurons terminate. Such staining is similar to what has been previously reported in normal, untreated rats (22, 46, 62). This animal was examined 14 days after the pump was implanted. P, pyramidal cell layer; G, granule cell layer. F, fissure. Calibration (300 μm, in B) applies to A and B. (B) In a BDNF-infused rat, NPY staining was increased relative to PBS-treated rats. This animal was examined 12 days after the pump was implanted. Staining is stronger in hilar neurons and fiber staining is also increased relative to the tissue section from the PBS-infused rat shown in A. To allow comparison, the sections in part A and B were processed at the same time.

rats were sacrificed within 3 weeks of pump implantation, and is consistent with studies showing that NPY expression increases in dentate gyrus interneurons after acute, rather than chronic seizures (95, 96).

In 10/22 (45%) BDNF-treated rats that were examined weeks or months after pump infusion (7/14 high dose BDNF, 3/8 low dose BDNF), NPY staining of mossy fibers was present throughout the septotemporal axis of both hemispheres (Figs. 6A–6C). In control rats, NPY staining of interneurons predominates and mossy fiber staining is minimal (22, 46, 62, 81). The increased mossy fiber staining of BDNF-treated rats (Figs. 6A–6C) was similar to PBS-infused rats that had status epilepticus and chronic seizures following pilocarpine injection (Fig. 6D). It was also similar to previous reports of increased mossy fiber NPY in other experimental models of chronic epilepsy (96, 97). PBS- or albumin-treated rats (3 low dose, 4 high dose) demonstrated relatively weak mossy fiber NPY staining at all time points examined. Interestingly, only 3 of the 10 BDNF-treated rats with increased mossy fiber NPY staining had an observed seizure. These data suggest that all animals might have had increased activity in hippocampus, and perhaps seizures, even though overt seizures were only observed in a few.

Figure 6A also shows that there was strong NPY staining in the inner molecular layer of some BDNF-treated rats. We interpret this staining as mossy fiber sprouting because the NPY antibody that was used stains the sprouted mossy fibers in pilocarpine-treated rats in a similar way, although the degree of sprouting after pilocarpine appears greater (81). Furthermore, granule cells are the only NPY-immunoreactive neurons known to innervate the inner molecular layer robustly after seizures. Other NPY-immunoreactive neurons preferentially target the outer molecular layer (22, 46, 62).

NPY-immunoreactivity in the inner molecular layer occurred in 5 animals (16%; all received the higher dose of BDNF), but among these 5 animals the staining pattern differed along the hippocampal axis. In 3 animals it was only detected in ventral hipocampus. In the other two rats sprouting was present bilaterally in the dorsal hippocampus, but not in ventral hippocampus.

Effects of Pilocarpine

A subset of 17 animals (10 high dose BDNF, 7 PBS) were injected with pilocarpine using a dose that usually produces status epilepticus in normal adult Sprague–Dawley rats (86). Investigators administering pilocarpine and evaluating status epilepticus were blind to the treatment of each animal. Pilocarpine injection occurred either on day 4, 5, 13, or 14 after pump implantation; data are pooled below because data for each group were similar. In addition to the animals injected with pilocarpine, 2 BDNF- and 1 PBS-treated animal were injected with atropine followed by saline (instead of atropine followed by pilocarpine). No animal had behavioral signs of seizures following saline injection.

Several measurements were made: (1) the time from pilocarpine injection to the first stage 5 seizure, (2) the number of stage 5 seizures that occurred before status epilepticus began, and (3) the latency to the onset of status epilepticus. In addition, it was noted whether the first seizure behavior that occurred was severe (stage 5) or not (stages 1–2).

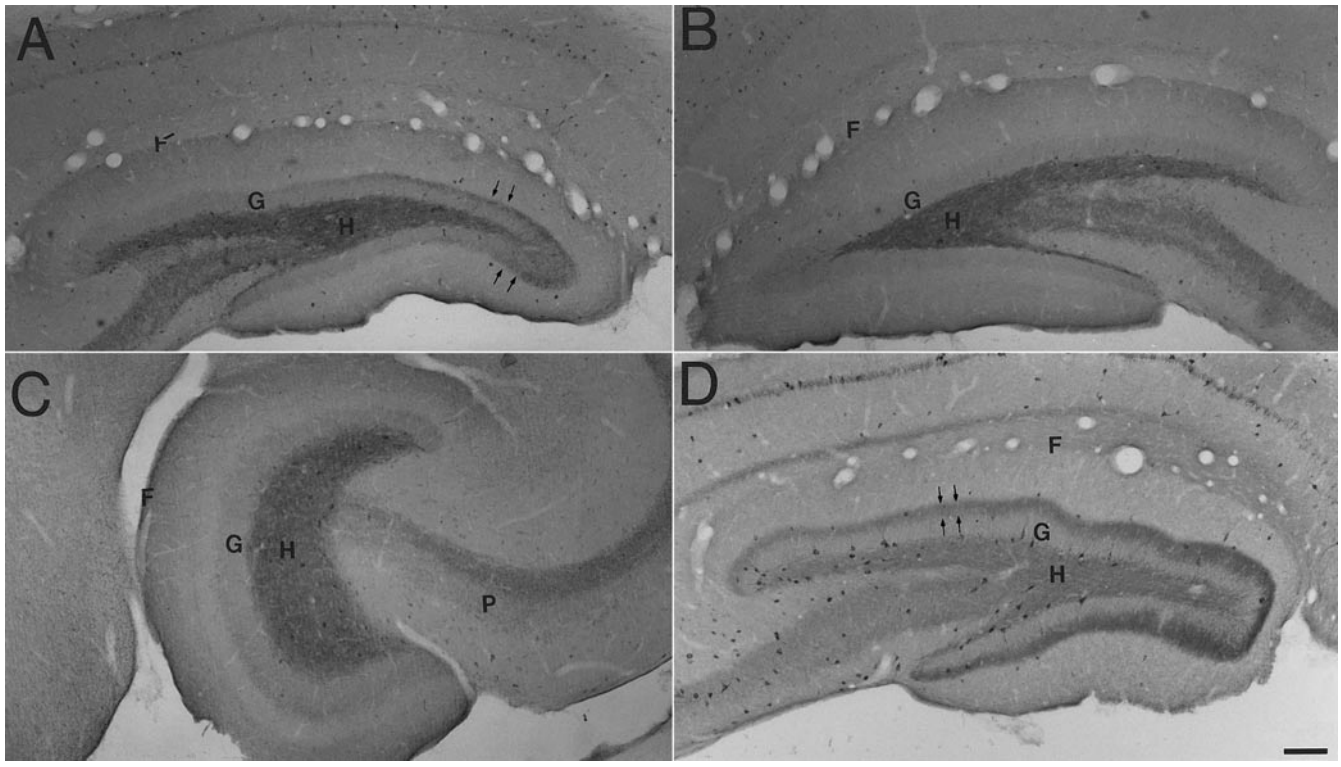

FIG. 6. NPY expression in the mossy fibers of BDNF-treated rats. (A–C) In a BDNF-treated rat that was killed 3 months after pump infusion, NPY immunoreactivity was robust in the mossy fibers. A illustrates staining ipsilateral to the cannula; B is the contralateral side of the section shown in A, and C illustrates the ventral hippocampus, ipsilateral to the cannula. Mossy fiber expression of NPY was strong throughout the hippocampus of both hemispheres. In control rats, mossy fiber expression is minimal (see Fig. 5A). Note that mossy fiber sprouting was evident ipsilateral to the cannula, in the dorsal hippocampus (part A, arrows). G, granule cell layer; P, pyramidal cell layer; F, fissure. Calibration (500 μm, in D) applies to A–D. (D) NPY staining in a PBS-treated rat that was injected with pilocarpine and perfused 2 months after the pump was implanted. This animal had status epilepticus and chronic spontaneous seizures. NPY expression was evident in mossy fibers and there was sprouting (arrows). Note that this section was not processed at the same time as the sections in A–C so the differences in background staining should be interpreted with caution.

Every rat entered status epilepticus within 60 min of pilocarpine injection except 2 PBS-treated rats. Of the animals that had status epilepticus ($n = 10$ BDNF, $n = 5$ PBS), BDNF-infused animals had a significantly shorter latency to the onset to status epilepticus than PBS-infused animals (Student's t test, $P < 0.05$; Fig. 7, Table 1). Although the means for latency to the first stage 5 seizure and the number of stage 5 seizures prior to status were shorter for BDNF-infused rats relative to PBS-treated rats, these differences were not statistically different (Student's t tests, $P > 0.05$; Fig. 7; Table 1).

BDNF-treated rats that had status epilepticus had a different progression of seizure behavior than PBS treated rats. In PBS-infused animals, similar to normal rats (80), the first seizure activity after pilocarpine injection was usually mild, i.e., stages 1–2. Only after the first mild seizure did stage 5 seizures occur. In contrast, the first seizure of BDNF-treated rats was often stage 5, i.e., quite severe. Thus, the first seizure was a stage 5 seizure in 8 of 10 rats (80%) treated with BDNF. In contrast, only 1 of 5 animals (20%) treated with PBS had their first seizure behavior reach stage 5. This difference (BDNF, 80%; PBS, 20%) was statistically significant ($\chi^2 = 4.97$, $P < 0.05$).

DISCUSSION

Summary

The results suggest that infusion of the hippocampus with BDNF can lead to seizures in adult rats. It can also lead to some of the anatomical changes that are associated with seizures. In addition, animals with intrahippocampal infusion of BDNF have increased sensitivity to the convulsant pilocarpine. The results support the hypothesis that hippocampal BDNF can be proconvulsant in adult rats.

Spontaneous Seizures after Intrahippocampal BDNF

There have been several studies of rats after BDNF infusion into the brain, yet, to our knowledge, there has been no previous report of spontaneous seizures.

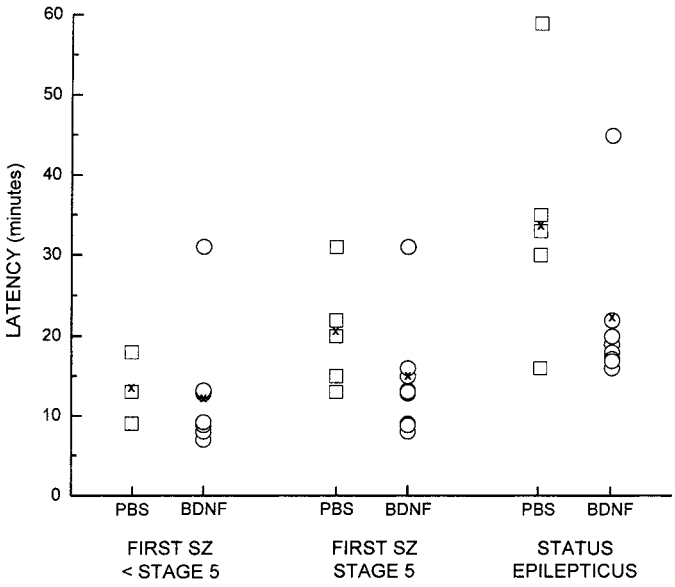

FIG. 7. Seizure sensitivity of BDNF- and PBS-infused rats to the convulsant pilocarpine. A scatter plot illustrates the responses of BDNF- and PBS-treated rats to intraperitoneal administration of pilocarpine. Each symbol represents data from a single animal. Means are designated by X. Measurements were made of the latency from pilocarpine injection to the first seizure that was less than stage 5 severity (first sz <stage 5; left), the latency to the first stage 5 seizure (first sz stage 5; center), and the latency to status epilepticus (status epilepticus; right). BDNF-treated rats (circles) had a significantly shorter latency to status epilepticus than PBS-infused rats (squares; Student's *t* test, $P > 0.05$). Other differences between the means were not statistically significant.

This could be due to differences in dose or site from the present study (21, 49, 68). We observed seizures only in those animals that received the high dose of BDNF. However, it is difficult to be certain of the number of BDNF-infused animals that had seizures. Seizures may have occurred more frequently, but were not detected because animals weren't continuously monitored, or because some seizures did not have behavioral signs (i.e., clonus, rearing, etc.). One reason we think that more seizures were likely to have occurred was that animals without observed seizures demonstrated altered expression of NPY and BDNF in a pattern similar to what was found in control animals that had pilocarpine-induced seizures. In addition, other studies have shown that after pilocarpine-induced seizures (59, 64, 80, 82), kainic acid-induced seizures (2, 26, 34, 37, 44, 78, 94), kindled seizures (4, 27, 28, 35, 48, 63), or electroconvulsive shock (65, 93), BDNF expression increases in mossy fibers, as does NPY (81, 95, 96). However, lesser forms of increased activity can also lead to increased NPY and BDNF expression. For example, tetanic stimulation can increase expression of BDNF in mossy fibers (10, 14, 23, 32, 63, 71, 86). Perhaps the best argument that seizures were underestimated lies in the demonstration of mossy fiber sprouting in animals that did not have observed seizures, because mossy fiber sprouting has rarely been reported in normal rats.

Anatomical Changes after BDNF Infusion

Given that anatomical changes were only present in BDNF-infused rats, it is likely that they were mediated by BDNF, rather than trauma associated with cannula implantation or infusion per se. However, it is not clear whether BDNF directly led to anatomical changes or whether the changes were indirect, for example by increased neuronal activity produced by BDNF. Regarding NPY, it has been suggested that BDNF can directly influence NPY expression (21, 75, 77). In support of this hypothesis, BDNF heterozygote knockouts with diminished BDNF have decreased levels of corti-

TABLE 1

Effects of Pilocarpine Injection in BDNF-Infused and PBS-Infused Rats

	Latency to first seizure stage 5 activity	Latency to first status seizure	Latency to epilepticus	No. of stage 5 seizures to status epilepticus	The first seizure was a stage 5 seizure
BDNF-infused					
Mean	12.5	14.0	20.8*	3.5	8/10**
SD	6.6	6.5	8.7	1.0	
SEM	1.9	2.1	2.8	0.3	
N	10	10	10	10	
PBS-infused					
Mean	13.3	20.2	34.6	4.0	1/5
SD	4.5	7.0	15.5	1.0	
SEM	2.6	3.2	7.0	0.4	
N	3	5	5	5	

Note. Measurements were made relative to the time of an i.p. injection of pilocarpine (see Materials and Methods). For definition of seizure behavior and status epilepticus, see Materials and Methods. Abbreviations: SD, standard deviation; SEM, standard error of the mean; *statistically significant from PBS-infused group (Student's *t* test, $P < 0.05$); **statistically significant from PBS infused group ($\chi^2 = 4.97$, $P < 0.05$).

cal NPY (39). Our studies of transgenics overexpressing BDNF demonstrated increased NPY relative to wild-type controls also (Scharfman and Croll, unpublished data).

In the present experiments, one could argue that BDNF had indirect effects because some changes in anatomy were distributed far outside the area where BDNF was infused. Thus, changes were observed in the contralateral and ventral hippocampus, target zones of neurons located at the infusion site. These distant changes could be explained by an effect of infused BDNF to increase activity in neurons near the infusion site which project to neurons outside the infusion site. There was little anatomical evidence of a direct effect of BDNF because the ipsilateral and contralateral hippocampi showed very similar anatomical changes. However, a direct effect can not be excluded because contralateral changes could have been a direct result of BDNF uptake by terminals at the infusion site and retrograde transport to contralateral hippocampus. However, this would not explain all of the contralateral anatomical changes, such as the increase in BDNF in mossy fibers contralateral to the infusion site, because mossy fibers do not have commissural projections. Thus, it appears likely that at least some effects were indirect.

Relevance to Seizure Generation in the Normal Rat

If BDNF infusion caused chronic seizures, as the results suggest, it is of interest to clarify the mechanisms underlying generation of seizures in this experimental paradigm. One possibility is that BDNF potentiated glutamatergic synapses in hippocampus, specifically those of the trisynaptic pathway. Kang and Schuman (41–43), and subsequently others (1, 7, 8, 13, 29, 50, 57, 58, 61, 70, 71, 79) have shown that BDNF exposure leads to a long lasting increase in transmission from CA3 to CA1 pyramidal cells. Messauodi *et al.* (61) showed *in vivo* that BDNF application potentiated perforant path transmission to granule cells (see also (1)). Scharfman and colleagues showed that BDNF potentiates mossy fiber transmission of the granule cell input to CA3 neurons, and BDNF can lead to seizure-like activity in convulsant-treated rats (79, 80). Others have shown that BDNF exposure alters inhibitory transmission (9, 11, 30, 67, 90) and increases NMDA receptor function (51, 52, 55, 87). Thus, BDNF infusion could have led to initial increases in excitability due to several mechanisms. If the increase in excitability were sufficient, perhaps brief periods of high frequency neural activity would follow. Because high frequency activity leads to increased BDNF expression (10, 32, 63, 86), a positive feedback loop could begin, culminating in repetitive seizures. If true, this would support the arguments of some clinical epileptologists that "seizures beget seizures," a controversial topic that has received much attention because of its clinical relevance.

This hypothesis is supported by EEG data from BDNF-infused rats, which showed higher amplitude activity relative to controls. This difference is likely to signify increased neuronal activity (although increased inhibition can not be ruled out). However, no classic electrographic seizures were recorded from BDNF rats. This could have been due to the fact that seizures were missed because they didn't occur during the recording sessions. This is highly likely given the fact that most hippocampal seizures are intermittent and recordings were extremely brief. Furthermore, recordings were made under anesthesia, which would potentially inhibit seizures.

Understanding the Role of Endogenous BDNF

Although the results indicate that unilateral exposure of the hippocampus to exogenous BDNF facilitates seizures, whether endogenous BDNF might have this role is not clear from the present study. The release characteristics of BDNF from endogenous stores and the endogenous concentration are not well understood, and could be very different from the infusion paradigm. However, the site of the cannula was in the mossy fibers, the area of the brain with perhaps the greatest concentration of endogenous BDNF (18, 99). Furthermore, trkB receptors are prevalent in this part of the brain (24, 31, 98). Therefore, if there was a site where infusion of BDNF would best mimic the normal condition, it would be the site that was chosen, the area around the mossy fibers.

Several studies support a potential proconvulsant influence of endogenous BDNF. For example, transgenic mice with increased expression of endogenous BDNF had increased seizure sensitivity and spontaneous epileptiform activity (20). In some of these transgenics, spontaneous limbic seizures were observed (Croll and Scharfman, unpublished results). However, these mice had increased expression of BDNF throughout development, and increased expression was triggered by the β-actin promoter, so how much this applies to normal endogenous BDNF function is unclear. However, other studies are also consistent with a proconvulsant role of endogenous BDNF. For example, inhibiting the actions of endogenous BDNF by infusing a scavenger, trkBIgG (6), or by decreasing endogenous BDNF (47), can inhibit kindling. While the results show that exogenous BDNF administration into the adult hippocampus has dramatic effects on excitability, making it tempting to speculate that endogenous BDNF plays a role in seizure initiation or seizure susceptibility, further studies will be necessary to address whether endogenous BDNF has these effects.

ACKNOWLEDGMENTS

We thank Ning Cai, Annmarie Curcio, and Qing Zhang for technical assistance and Ruth Marshall for secretarial assistance. We appreciate the comments of Dr. Solomon Moshe and Dr. Gyuri Buzsaki about scalp recording data. This study was supported by NIH Grant NS 39562 to H.E.S.

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