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Seizure-Suppressant and Neuroprotective Effects of Encapsulated BDNF-Producing Cells in a Rat Model of Temporal Lobe Epilepsy

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Brain-derived neurotrophic factor (BDNF) may represent a therapeutic for chronic epilepsy, but evaluating its potential is complicated by difficulties in its delivery to the brain. Here, we describe the effects on epileptic seizures of encapsulated cell biodelivery (ECB) devices filled with genetically modified human cells engineered to release BDNF. These devices, implanted into the hippocampus of pilocarpine-treated rats, highly decreased the frequency of spontaneous seizures by more than 80%. These benefits were associated with improved cognitive performance, as epileptic rats treated with BDNF performed significantly better on a novel object recognition test. Importantly, long-term BDNF delivery did not alter normal behaviors such as general activity or sleep/wake patterns. Detailed immunohistochemical analyses revealed that the neurological benefits of BDNF were associated with several anatomical changes, including reduction in degenerating cells and normalization of hippocampal volume, neuronal counts (including parvalbumin-positive interneurons), and neurogenesis. In conclusion, the present data suggest that BDNF, when continuously released in the epileptic hippocampus, reduces the frequency of generalized seizures, improves cognitive performance, and reverts many histological alterations associated with chronic epilepsy. Thus, ECB device-mediated long-term supplementation of BDNF in the epileptic tissue may represent a valid therapeutic strategy against epilepsy and some of its co-morbidities.

INTRODUCTION

Because one-third of the epilepsies are refractory to medical treatment, it is highly important that new therapies with novel mechanisms of action are developed.[1] Neurotrophic factors like brain-derived neurotrophic factor (BDNF) represent interesting therapeutic candidates, because an extensive literature demonstrates their involvement in the cellular alterations observed in the epileptic tissue. In fact, the trophic effects of BDNF suggest an involvement in cell death, neurogenesis, and axonal sprouting; in addition, BDNF exerts effects at the synaptic level, with distinct modulatory actions at excitatory and inhibitory synapses.[2] Moreover, an important function of BDNF includes the control of short- and long-lasting synaptic interactions that influence memory and cognition.[3] With specific reference to chronic epilepsy, electrophysiological experiments in a model of neocortical epileptogenesis support the notion that reduction in trophic support by BDNF may contribute to regressive changes in axons and dendrites of fast-spiking interneurons and decreased GABAergic inhibition, suggesting that supplying BDNF to the injured brain may reverse structural and functional abnormalities in parvalbumin interneurons and provide an antiepileptic therapy.[4]

The development of BDNF-based therapeutic approaches for epilepsy, however, is complicated, because it exerts both beneficial and deleterious effects in models of epilepsy.[2] These variable results may depend on multiple factors, including the period of BDNF therapy in the natural history of the disease; specific alterations in some of its biological properties including biosynthesis, processing, and subcellular localization, and the method of delivery.[5–7] In particular, the method of delivering BDNF to the brain is a critical issue, given that its optimal effectiveness likely requires a specific targeting of the temporal lobe in a robust and prolonged manner. No traditional small-molecule drug with suitable pharmacokinetics and capability to act as either a selective agonist or antagonist to high-affinity BDNF receptors (the tropomyosin receptor kinase B [TrkB] receptors) has been developed and, in any event, such drugs would not act only in the epileptogenic region but throughout the brain, with risk of unwanted side effects. Other delivery strategies, based on cell grafts or viral vectors, may only provide a relatively short-term treatment, whereas, by their very nature, chronic diseases like epilepsy require long-term treatments. In addition, cell or gene therapy approaches do not generally offer a reversible strategy after inoculation in case of undesired effects.

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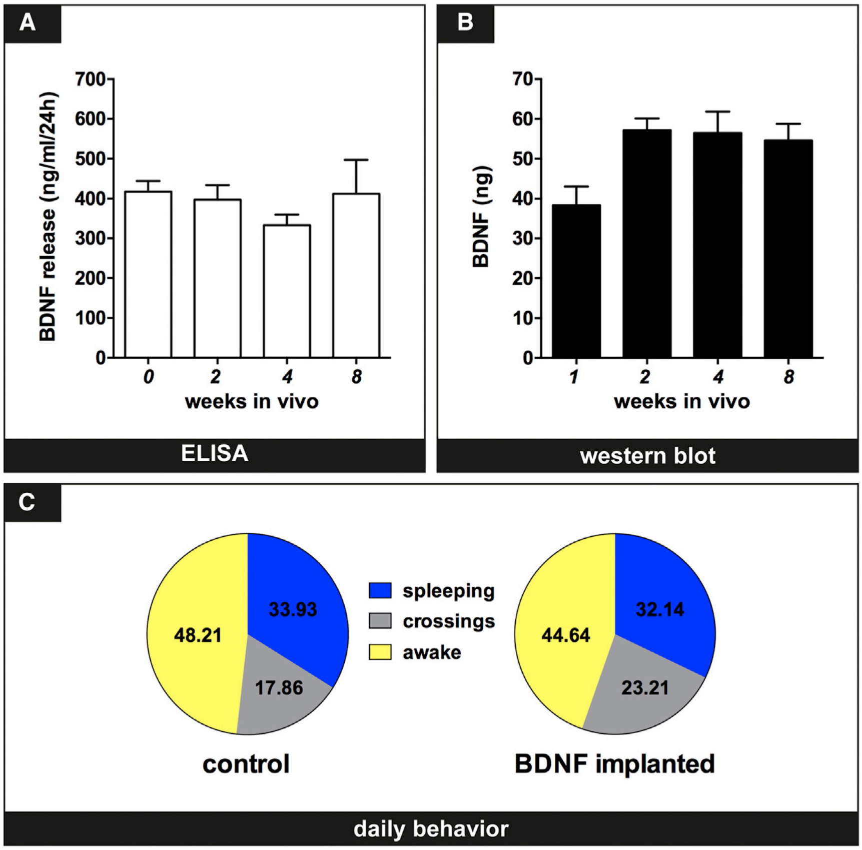

Figure 1. Long-Term BDNF Delivery Does Not Impact General Activity of Naive Rats

(A) BDNF release from devices (as measured using ELISA) prior to implantation and after 2, 4, and 8 weeks *in vivo*. See Materials and Methods for details on experimental design. (B) Levels of BDNF protein in the implanted hippocampus (as measured using ELISA) after 2, 4, and 8 weeks *in vivo*. (C) Daily behavioral activity of naive rats (left) and BDNF-treated rats (right), measured as percentage of the time spent awake, sleeping, or moving (crossings) for 20 min a day over a 24-week period. Data in (A) and (B) are expressed as mean ± SEM of eight devices and hippocampi per group. Data in (C) were obtained from 24 rats per group.

Here, we describe the beneficial effects of encapsulated cell biodelivery (ECB) devices loaded with BDNF-secreting cells and implanted into the hippocampus of pilocarpine-treated rats. In this approach, a human cell line is engineered to secrete BDNF, encapsulated in a biocompatible matrix and kept separated from the adjacent host brain tissue by a thin polymer membrane. The membrane possesses pores that allow BDNF to diffuse into the surrounding tissue and also allow oxygen and nutrients to enter from the surrounding brain to nourish the encapsulated cells. Immunological reactions are obviated because the semipermeable membrane prevents the host immune system from gaining access to cells, thereby preventing their rejection. Not only do ECB devices offer the advantage of long-term, local delivery of BDNF, but they also offer the possibility of easy removal if necessary or desired. We report here that these features were associated with a dramatic reduction of seizures and associated cognitive impairment, as well as normalization of many histological alterations associated with chronic epilepsy. These data provide support for continuing the development of this approach as a potential treatment for drug-resistant patients affected by focal epilepsy.

RESULTS

Long-Term BDNF Secretion and Tissue Levels of BDNF

We first evaluated the potential for long-term delivery of BDNF from encapsulated cells after implantation of ECB devices into the hippocampus of naive rats. Devices were assessed for BDNF output both before implantation and following retrieval after 2, 4, or 8 weeks *in vivo*. The implanted devices were easily retrieved from the brain with no host tissue adhering to the capsule wall. All capsules remained intact, with no evidence that any capsule broke either during implantation, while *in situ* or during the retrieval procedure. As described in the Materials and Methods section, devices were then transferred to culture medium for quantitation of BDNF secretion. As shown in Figure 1A, BDNF levels in the medium (that is, BDNF release capacity) were very stable, ranging from approximately 350–400 ng/device/24 hr at all time points.

This continuous delivery of BDNF to the hippocampus significantly elevated tissue concentrations of BDNF, as determined by ELISA (Figure 1B). Tissue levels of BDNF appeared to increase in the first 2 weeks following implantation and thereafter to remain relatively constant. Parallel studies (data not shown) confirmed that the secretion of BDNF from the devices and the elevated tissue levels of BDNF remained relatively stable for at least 6 months (the longest time period evaluated). Within this time frame, no differences in activity or sleep/wake patterns (Figure 1C) or body weight were found between implanted and unimplanted control animals.

Effect on Spontaneous Seizures

A schematic representation of the *in vivo* experiments is shown in Figure 2. All animals were continuously video monitored between day 10 and day 20 after status epilepticus (SE) (early chronic period) to verify occurrence of spontaneous generalized seizures.[8] Twenty days after SE, at the end of the first monitoring epoch, all animals were randomly assigned to one of four experimental groups: one group was not treated at all (no device), the second group was bilaterally implanted with empty ECB devices, the third group with two devices filled with parental ARPE-19 cells, the last group with ECB

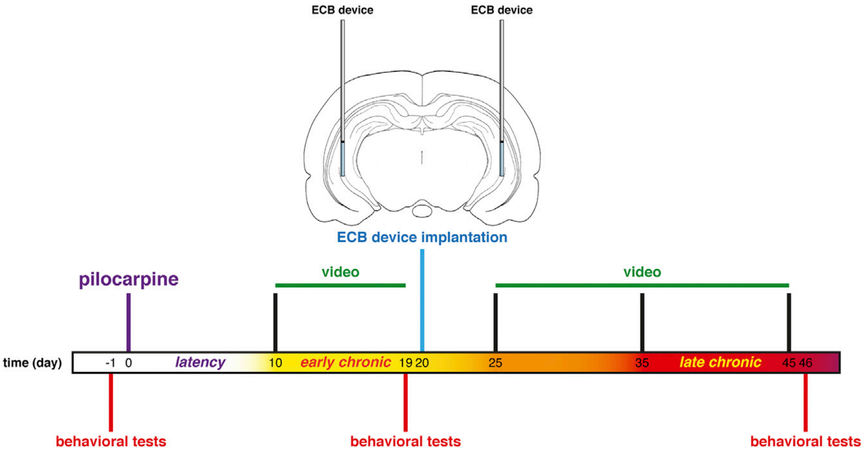

Figure 2. Timeline and Schematic Representation of the *In Vivo* Experiments

The top coronal brain slice illustrates the ECB device implant location. The bottom timeline depicts the sequence of behavioral testing (OF, NOR), video monitoring, and device implantation. In all cases, timing (days) is relative to pilocarpine treatment.

devices filled with ARPE-19-BDNF cells. Randomization was based on seizure frequency.

Surgical implantation did not impact seizure frequency. Between day 25 and 35 after SE, control animals (no device, empty device, or device with parental cells) displayed about three generalized seizures per day (Figure 3A). No difference in any of the parameters analyzed in this study were observed between the no-device and control implant groups, and therefore they were pooled together for statistical analysis and collectively termed the "control" group. In contrast, animals treated with BDNF devices exhibited a marked and significant reduction in seizures, displaying on average less than one seizure per day (Figure 3A). This benefit became even more apparent between days 35 and 45 after SE (late chronic period) as control rats exhibited a progression of the disease with an increased seizure frequency that was not observed in treated animals. In this time frame, treated animals exhibited a 90% reduction in seizure frequency. In contrast, the forelimb clonus duration was only moderately, but not significantly, reduced (Figure 3B).

At the conclusion of video monitoring, devices were removed and BDNF secretion was confirmed. Pilocarpine treatment did not impact device secretion. Before implantation, the average BDNF concentration in the medium was 206 ± 11 ng/24 hr, while after 2 weeks *in vivo* it was increased to 463 ± 43 ng/24 hr incubation (Figure 4A). Moreover, hippocampal levels of human mBDNF were investigated by western blot and expressed as BDNF protein levels relative to recombinant BDNF. Tissue levels of human mBDNF were elevated within hippocampi implanted with the ARPE-19 BDNF cell-loaded device (37.56 ± 4.59 relative BDNF protein level) whereas, as expected, they were negligible in all controls (Figure 4B).

Behavioral Effects

The effects of ECB-released BDNF were further evaluated on behavioral tests. In the open-field test, the control group spent increasing amounts of time in the center of the arena with the progression of the disease. In contrast, the BDNF-implanted animals remained in the center region for significantly shorter times even 46 days after SE, i.e., 4 weeks after the bilateral implant of the ARPE-19 BDNF-filled devices (Figure 5A). Moreover, the control rats displayed a progressively increased number of entries into the central area that was not evident in BDNF-treated animals. This difference became statistically significant in the late chronic period (32 ± 7 versus 18 ± 3 entries to center; Figure 5B). No difference was observed between groups in the distance covered during the test period. In sum, epileptic BDNF-treated animals appeared more "normal" than epileptic controls, because they displayed a behavior indistinguishable from that of naive rats.

Recognition memory was evaluated using the novel object recognition (NOR) test (Figure 6A). As expected, all animals spent more time exploring the novel object during the baseline phase, before the epileptogenic insult (SE). Establishment of an epileptic condition (early chronic phase) was associated with impairment of memory in this test (Figure 6B, yellow bars). However, increased exploration of the novel object (that is, improved memory) was observed in epileptic BDNF-treated rats but not in controls in the late chronic time point ($p < 0.01$; Figure 6B, purple bars). In addition, whereas a decrease in the total interaction time with the objects was observed in control rats from baseline to the early chronic to the late chronic time point, no such progression was observed in BDNF-treated animals (Figure 6C). Together, these findings suggest that the treatment with BDNF significantly improved memory function.

Histology

Neuron Survival and Hippocampal Volume

Neuronal survival was estimated by counting NeuroTrace-positive cells. Quantification of NeuroTrace-positive cells in the hippocampus revealed that pilocarpine-induced epilepsy led to significantly reduced neuronal numbers in the control group (55.2% ± 8.6% compared to naive animals). In contrast, BDNF-treated rats displayed only a modest, non-significant decrease (Figures 7A and 7B). No statistical differences were found between the hippocampi, nor in hippocampal subareas (Table S1), even when ECB devices were implanted unilaterally. In fact, we implanted a subset of epileptic animals in a single hippocampus with the intent of using the contralateral one as an internal control but, unexpectedly, all histological examinations described in this section (not only NeuroTrace) underwent identical changes in both the implanted and the non-implanted hippocampus.

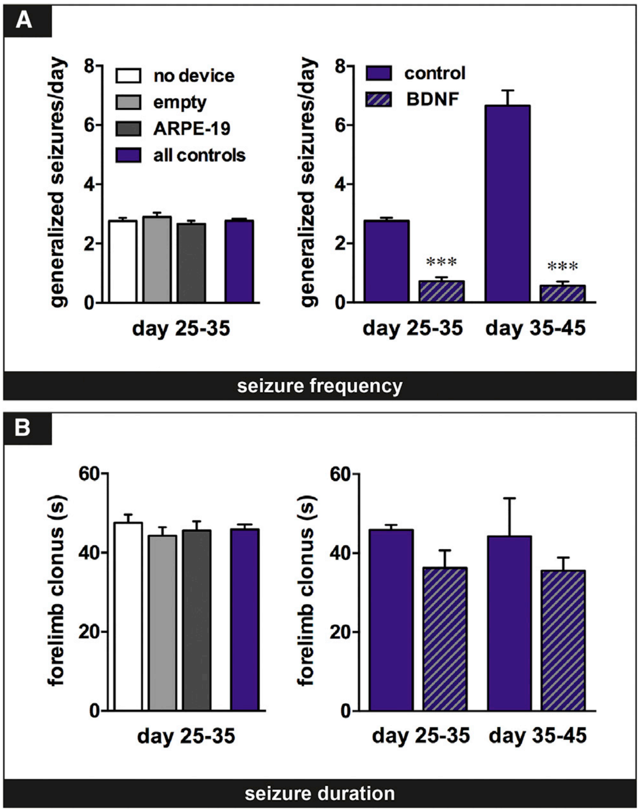

Figure 3. BDNF-Secreting Devices Reduce Frequency of Spontaneous Seizures

(A) Average daily frequency of spontaneous generalized seizures (class 4 or 5) in the chronic period (25–35 days after pilocarpine/SE and 5 days after device implantation) and in the late chronic period (35–45 days after SE). Controls received either no device, empty devices, or devices loaded with non-modified parental cells. To facilitate graphical and statistical representation, the controls were combined into a single control group. Interaction F(1,18) = 47.74; treatment F(1,18) = 223.90; time F(1,18) = 41.08. ***$p < 0.001$ versus control; two-way ANOVA and Sidak post-hoc test. (B) Average daily forelimb clonus duration of spontaneous generalized seizures in the chronic and late chronic period expressed in seconds. Data are expressed as mean ± SEM of 10 animals per group.

A pronounced hippocampal atrophy was observed in control epileptic animals, with a volume reduction of about 30% compared with naive rats. In contrast, no significant change in this parameter was observed after BDNF treatment.

Astrocytosis

Epilepsy-associated astrocytosis was evaluated using GFAP immunofluorescence. The density of GFAP-positive cells in the hippocampus was not altered in chronically epileptic animals, not even after implantation of BDNF devices (Figure 8A). However, whereas many of the GFAP-positive cells in epileptic controls displayed short, thick processes, an indication of active astrocytosis (Figure 8C, inset), GFAP-positive cells of BDNF-treated rats were similar to those of naive animals, with a small cell body and thin processes (compare Figures 8B and 8D insets).

Inhibitory Interneurons

Consistent with previous reports, pilocarpine-induced epilepsy was associated with a significantly reduced number of parvalbumin-positive interneurons in the hippocampus (Figure 9A; 49.8% ± 1.5% as compared with naive). BDNF treatment partially reverted this loss, parvalbumin-positive cells being 77.9% ± 4.6% of those found in naive hippocampi. This effect was especially prominent in the dentate gyrus (Table S2).

Neurodegeneration and Neurogenesis

BDNF-induced reversal of neuronal death may depend on reduction of continued, ongoing neurodegeneration in the chronically epileptic brain and/or on induction of neurogenesis. To explore the first possibility, we used Fluoro-Jade C (FJC) staining. FJC identified numerous degenerating cells in CA1, CA3, and in the hilus of the dentate gyrus in control epileptic rats (Figures 10A and 10C), while a very limited number of FJC-positive cells were observed after BDNF treatment (Figures 10A and 10D). Similar results were obtained analyzing the entire hippocampus or the single subareas (Table S3).

To evaluate neurogenesis, we counted doublecortin (DCX)-positive cells. The effect of the BDNF devices on neurogenesis was remarkable, as shown in Figure 11A. While pilocarpine alone significantly decreased the numbers of DCX-positive cells, this effect was largely reverted by BDNF. An increased number of DCX-positive cells was observed with BDNF especially in the dentate gyrus, where they were almost double the number found in control epileptic animals, which in turn were about 40% of those in naive rats (Figure 11C). Moreover, DCX-positive cells in control epileptic animals did not have elaborate elongations and tended to aggregate into clusters (morphological aspects of aberrant neurogenesis; Figure 11Ab). In contrast, DCX-positive cells in BDNF-treated epileptic hippocampi had more elongations projecting across the granular layer and did not aggregate in clusters (Figure 11Ac).

Target Engagement

Finally, we verified that the BDNF treatment with ECB devices could indeed lead to activation of TrkB, the high-affinity BDNF receptors. To do so, we compared the expression of TrkB with that of its phosphorylated (activated) form (p-TrkB^{Y515}) and calculated the ratio between p-TrkB^{Y515} and TrkB in the different conditions (naive, control epileptic, and BDNF-treated epileptic). BDNF-treated epileptic rats exhibited a highly significant increased TrkB^{Y515}/TrkB ratio compared to naive and control epileptic rats (Figure 12C). Similar results were obtained in each hippocampal subarea (Table S4), arguing that the effects observed in this study are likely dependent on TrkB engagement.

DISCUSSION

We show here that intrahippocampal implants of ECB devices secreting BDNF highly significantly decrease the frequency of spontaneous seizures in chronically epileptic rats. Considering that only a subset of spontaneous seizures originate from the hippocampus in the pilocarpine model,[9] this effect may be even greater than reported

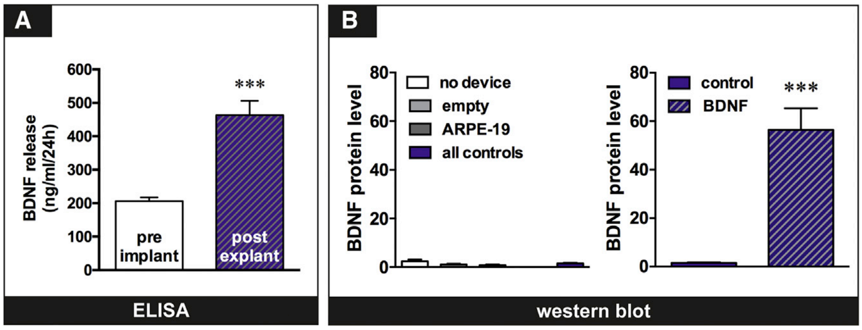

Figure 4. BDNF Release from Devices Explanted from Pilocarpine-Treated Animals

(A) BDNF release (ELISA) from devices explanted after 2 weeks *in vivo*. (B) Concentrations of BDNF protein (western blot) in the hippocampus. The mature BDNF (mBDNF) signal was normalized to α-actin for quantification. As in Figure 3, controls received either no device, empty devices, or devices loaded with non-modified parental cells devices. Data are expressed as mean ± SEM of 10 animals per group. ***$p < 0.001$ versus control; Mann-Whitney U test.

here. An accurate EEG mapping of electrical seizure activity in multiple brain areas will be needed to clarify this point. In this study, however, we decided to avoid inserting electrodes, because inserting two ECB devices is an invasive procedure for the rat brain, and we did not want to take the risk of altering behavior because of an overload of materials in the brain. In fact, the decrease in seizure frequency associated with dramatic improvements in recognition memory and with normalization of the hippocampal architecture.

BDNF-mediated TrkB activation has been found to exert contrasting effects on seizures, depending on the epilepsy model, the time of administration in the natural history of the experimental disease, and the TrkB activation or inhibition strategy. For example, many studies support a pro-epileptogenic effect of TrkB activation,[10,11] but others suggest an anti-epileptogenic effect of BDNF supplementation.[12,13] More relevant to the present study, i.e., in the chronic epileptic period, BDNF was found to reduce GABA_A receptor desensitization in the human and in the murine epileptic hippocampus[14,15] but, in contrast with these and with the present findings, a herpes simplex vector-mediated supplementation of BDNF in the hippocampus of epileptic rats did not alter spontaneous seizure frequency or severity.[12] However, some major differences exist between this previous report and the present study. First, the BDNF delivery method differed (endogenous cells infected by the vector producing and secreting BDNF versus ECB). Second, in the study by Paradiso et al.,[12] BDNF was expressed together with fibroblast growth factor 2 (FGF-2) by the viral vector. Third, the viral vector induced expression of the transgenes (BDNF and FGF-2) only in the dorsal hippocampi, whereas ECB devices were implanted bilaterally in the ventral hippocampus and likely released BDNF in a wider area. Therefore, an insufficiently broad supplementation of BDNF with the viral vector may have led to an apparent lack of effect.

In the present experimental settings, we also found that BDNF delivery improved the performance in behavioral tests of memory and spontaneous activity. This is an important finding, because cognitive and behavioral abnormalities are the most common and severe co-morbidities of epilepsy,[16] and can greatly reduce the quality of life of patients.[17]

Several studies have shown that spontaneous recurrent seizures seriously affect cognitive ability[17] in animal models of epilepsy. On the other hand, it is also known that hippocampal BDNF is implicated in spatial and recognition memory,[18,19] and that BDNF delivery to the entorhinal cortex prevents learning and memory impairment in rodent and primate models of Alzheimer disease.[20] Our data confirm the involvement of BDNF in memory functions, showing that ECB devices secreting BDNF, when implanted in the hippocampus of chronically epileptic rats, significantly improve recognition memory, reverting the learning and memory deficits of epileptic rats.

Exploratory behavior was tested using the open-field test. Rodents are spontaneously thigmotaxic and prefer the safer and darker periphery of the arena over the central and bright part of the apparatus. In accordance with previous studies,[21,22] we found that epileptic animals spent a progressively increasing amount of time in the aversive central part of the field, indicating a hyperactive and disinhibited state. In contrast, rats implanted with BDNF devices had a behavior indistinguishable from that of naive rats.

All together, these data support a strongly positive effect of ECB-mediated supplementation of BDNF in the epileptic hippocampus. However, a difficult issue to clarify is the genuine BDNF-dependence of the observed effects. Unfortunately, no small molecule TrkB antagonist with suitable pharmacokinetics for peripheral administration is currently available, and the ECB device does not allow practical space for intra-hippocampal injection in the area where it releases BDNF. However, the mature BDNF isoform, binding to the high-affinity TrkB receptor, initiates its dimerization and auto-phosphorylation of intracellular tyrosine residues, which results in formation of phosphorylated-TrkB receptors that activate intracellular signaling cascades.[23] Therefore, to begin to explore the mechanistic basis of our data, we analyzed the activation of TrkB by measuring the ratio of phosphorylated to non-phosphorylated TrkB. We found that TrkB activation was increased in all subareas of the hippocampi implanted with BDNF releasing devices. These data do not directly demonstrate that the observed effects are TrkB dependent, but they do clearly show that the employed procedure leads to target engagement.

What then are the consequences of TrkB receptor activation in the epileptic hippocampus that lead to reduced frequency of seizures and amelioration of co-morbidities? The present data support the idea that neurotrophic effects play a key role. A prevention of further

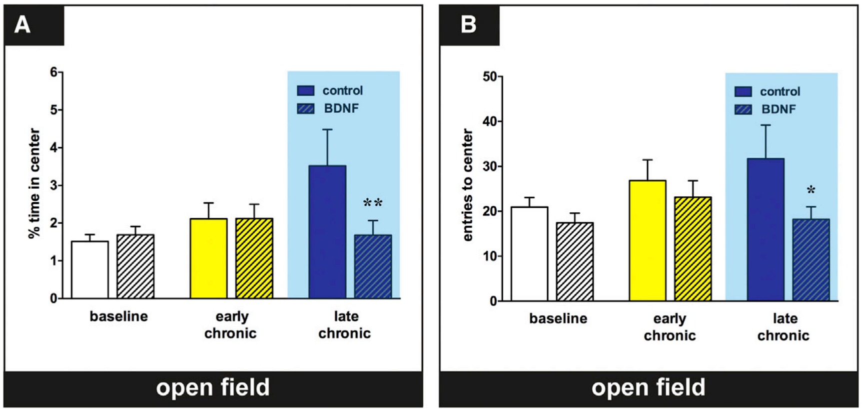

Figure 5. BDNF Normalizes Exploratory Behavior in an Open-Field Test

Shown here are the times spent in the center of an open-field arena (A) and the number of entries into the center of the arena (B) for epileptic control (empty bars) and BDNF-treated rats (hashed bars). Note that animals were not yet holding devices (either control or BDNF) at baseline and in the early chronic period. Because randomization was based on seizure frequency, the two bar types (empty and hashed) are shown also at these time points to show data on animals that will be subsequently allocated in the control or BDNF group. Over time, pilocarpine controls displayed a tendency toward increased time spent in the center and entries in the center (compare empty bars), which was not evident in BDNF-treated animals. Data are expressed as mean ± SEM of 14 or 15 animals/group. Data in (A), but not in (B), were found to be normally distributed based on the D'Agostino-Pearson and the Shapiro-Wilk tests. $^{**}p < 0.01$ versus control; Student's t test for unpaired data. $^{*}p < 0.05$ versus control; Mann-Whitney U test.

damage that is ongoing in this epileptic model, together with an increased neurogenesis (and, in particular, with increased production of GABA cells) may favor a normalization of hippocampal cytoarchitecture and circuitries, including regression of astrocytosis. Although there may be a key basic mechanism that leads to all these positive outcomes, it would be quite difficult to track it down with the currently available tools. An alternative interpretation may be that all of the identified effects are important and combine in a complex and non-linear way to impact the behavior of the neural network. This alternative view is in line with the emerging concept of network neuroscience.[24,25]

The SE-damaged hippocampus exhibits a loss of neuronal cells.[26] We found that, while pilocarpine-induced epilepsy was associated with a dramatic reduction of hippocampal volume, reduction in the total number of neurons (as estimated using NeuroTrace) and continuing neuronal degeneration (as estimated using FJC), all these parameters were attenuated or normalized after ECB-mediated BDNF supplementation. In addition, chronic epilepsy is known to lead to reduced neurogenesis,[27] and in fact we observed that pilocarpine-induced epilepsy resulted in a dramatic decrease in DCX-positive cells. Again, this was reversed by BDNF treatment.

BDNF has been reported to exert a specific role in the development and maturation of GABAergic cells, in particular of parvalbumin-positive interneurons.[28] Indeed, we found that ECB device-mediated BDNF supplementation can lead to a robust increase in the number of parvalbumin-positive cells in the hippocampus, returning them to nearly control levels. Parvalbumin interneurons are known to degenerate in both patients[29] and animal models of temporal lobe epilepsy (TLE)[30–33] recently provided experimental evidence that selective, permanent inhibition of parvalbumin interneurons reduce perisomatic feed-forward inhibition *in vivo*, resulting in a decrease in seizure threshold and the development of spontaneous seizures (i.e., epilepsy). Based on our data, it is intriguing to hypothesize that ECB device-mediated BDNF supplementation can activate neurogenesis to produce (among others) new parvalbumin interneurons that contribute to decreased seizure frequency.

Implantation of BDNF devices did not alter the density of GFAP-positive cells in chronically epileptic rats but normalized their morphology, indicating an attenuation of reactive astrogliosis. Whether this effect is an indirect consequence of the other effects discussed above (i.e., of a general amelioration/normalization of hippocampal cytoarchitecture) or, vice versa, those effects are a consequence of a primary action on astrocytes, remains uncertain. Future studies will be needed to define the precise mechanism of the therapeutic effects of direct BDNF delivery to the hippocampus.

Another interesting observation that deserves further investigation is that all histological benefits were observed in both hemispheres even when the implants were performed unilaterally, suggesting a complex interplay between hemispheres during epilepsy. While the spread of epileptiform activity to the contralateral side is well known in human and experimental epilepsy, the symmetrical amelioration of histological impairments after treatment of a single site is more difficult to explain. Even if it is known that BDNF and other neurotrophic factors can undergo anterograde transport in neurons, thereby potentially reaching distant areas, the existence and precise nature of such mechanisms in the present experimental settings remain a mere hypothesis.

In spite of uncertainties regarding a mechanistic interpretation of the effects downstream target activation, a strength of the present findings is their clinical translatability. These findings demonstrate that ECB devices represent an effective means of exogenous long-term delivery of BDNF to the hippocampus,and that this strategy can reduce the frequency of seizures and the epilepsy comorbidities. The ECB device approach has been developed into a practical, clinically validated means of overcoming the obstacles associated with delivering to the brain molecules that cannot cross the blood-brain barrier.[34] This system has

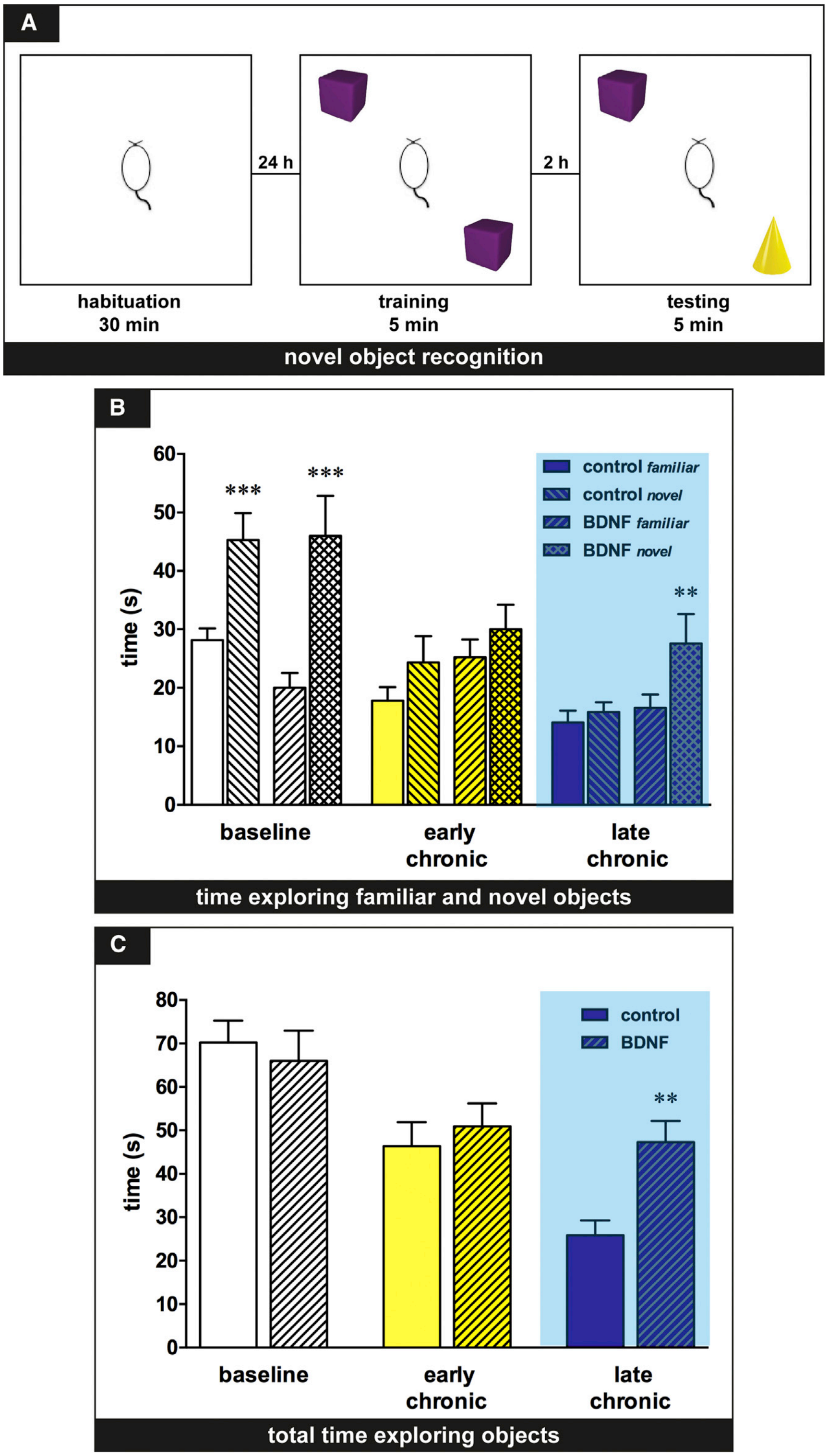

Figure 6. BDNF Improves Recognition Memory

(A) A schematic representation of the Novel Object Recognition Test (see Materials and Methods for additional details). Twenty-four hours after the habituation phase, animals were allowed to explore two identical objects for 5 min. After a subsequent interval of 2 hr, animals were exposed to two different objects: one familiar from the training phase and one novel object (testing phase). As shown in (B) and (C), a progressive impairment in recognition memory occurred in controls (empty bars), as evidenced by a reduced amount of time spent exploring the novel object (B) as well as the overall time exploring both objects (C). In contrast, relative to controls, the BDNF-treated animals (hashed bars) exhibited an increased exploration of the novel object. Note that animals were not yet holding devices (either control or BDNF) at baseline and in the early chronic period. Because randomization was based on seizure frequency, the two bar types (empty and hashed) are shown also at these time points to show data on animals that will be subsequently allocated in the control or BDNF group. Data are expressed as mean ± SEM of 14 or 15 animals per group. Data were found to be normally distributed based on the D'Agostino-Pearson and the Shapiro-Wilk tests. ***$p < 0.001$, **$p < 0.01$ versus control; Student's t test for unpaired data.

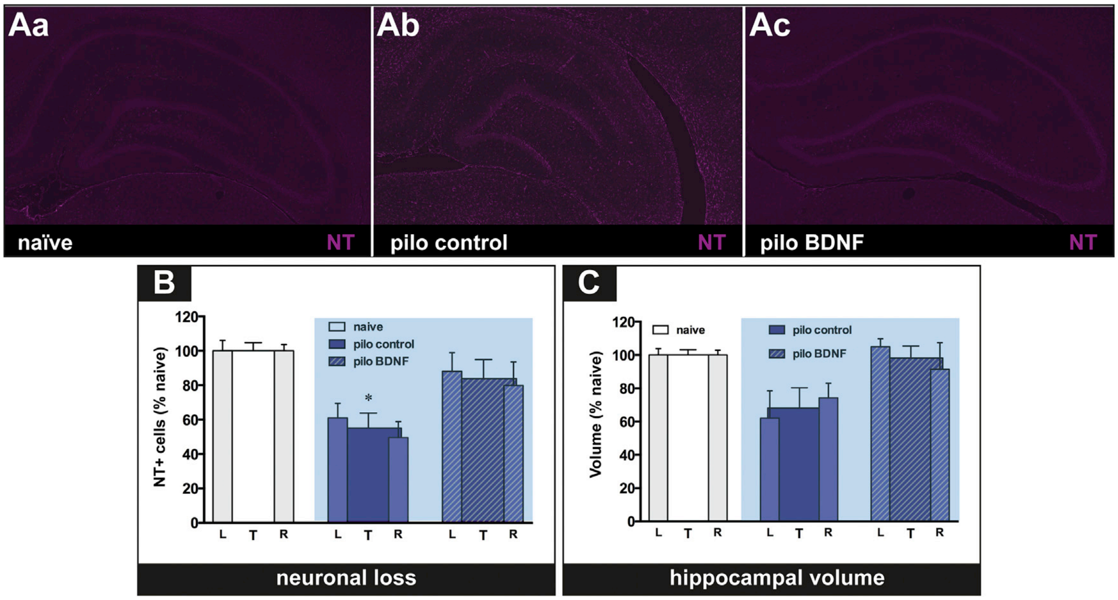

Figure 7. BDNF Treatment Reduces Hippocampal Atrophy and Neuronal Cell Loss

(A) Representative hippocampal sections stained for NeuroTrace from naive rats (a), control epileptic rats (b), and BDNF-treated epileptic rats (c) illustrates pronounced neuronal cell loss and hippocampal atrophy in control epileptic, but not in BDNF-treated epileptic rats. (B) Quantification of the NeuroTrace-positive cells confirmed this observation in both hemispheres. While the control group exhibited a 44.8% loss of cells, the BDNF-treated animals showed only a 16.1% loss. (C) The normalization of neuron numbers was associated with a normalization of the overall hippocampal volume, as control epileptic rats exhibited a 28.6% volume reduction, whereas the BDNF-treated animals did not show any change in hippocampal volume. Data are expressed as mean ± SEM percentage of naive animals obtained from a total of five animals/group. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined). **p < 0.01 versus naive; ANOVA and post-hoc Dunnett test.

already been tested for safety in large animal models and, most importantly, has been tested clinically in Alzheimer patients using NGF-secreting cells. In this clinical study, up to four devices were well tolerated when implanted bilaterally into the cholinergic basal forebrain and then easily and safely retrieved intact 12 months later.[35,36]

Several other aspects of the present findings are worthy of note with regard to human translation. First, the implantation of ECB devices was performed under conditions that reproduce the clinical situation: chronic patients with surgically treatable TLE. Patients who planned to undergo a two-step surgery may be an ideal population to clinically test this approach because the ECB device could be implanted together with recording electrodes and, should it prove ineffective, it could be removed and the patient would undergo surgery as originally planned. The use of conventional stereotactic procedures for ECB implantation inherently provides a means of selectively targeting those areas of the brain where BDNF will be therapeutic, while reducing exposure of other anatomical regions of the brain where it could produce side effects. Because multiple implants can be used within the same target region, it is possible to achieve far greater spread of protein throughout the targeted region than can be achieved with crude infusion of protein.

In conclusion, the present data suggest that BDNF, continuously released in the epileptic hippocampus, reduces the frequency of generalized seizures and improves co-morbidities while producing a robust neuroprotective effect. This approach may be directly applicable to patients that are selected for surgical resection of the epileptic hippocampus and are undergoing implantation of depth electrodes to define the epileptogenic area before respective surgery. ECB device(s) may be implanted together with these electrodes: if ineffective, they would be removed and the patient would undergo surgery as originally planned; if effective, the patient would have the option of avoiding surgery.

MATERIALS AND METHODS

Cells and Devices

Cell Culture

ARPE-19 cells, a spontaneously immortalized human retinal pigment epithelial cell line, were cultured using standard plating and passaging procedures in T-175 flasks with growth medium; DMEM + glutamax (1×) supplemented with 10% fetal bovine serum (Gibco). Routine culture consisted of feeding the cells every 2–3 days and passaging them at 70%–75% confluence. Cells were incubated at 37°C, 90% humidity, and 5% CO_2.

Human BDNF-Secreting Cell Line Establishment

We generated clonal BDNF-secreting ARPE-19 cell lines using the sleeping beauty (SB) transposon expression system, as described elsewhere.[37] In brief, ARPE-19 cells were co-transfected with the plasmid pT2.CAn.hopp.BDNF, containing the entire pre-pro BDNF sequence, and the SB vector pCMV-SB-100x. Clones were selected using G418 (Sigma-Aldrich, Germany), and cells were isolated and

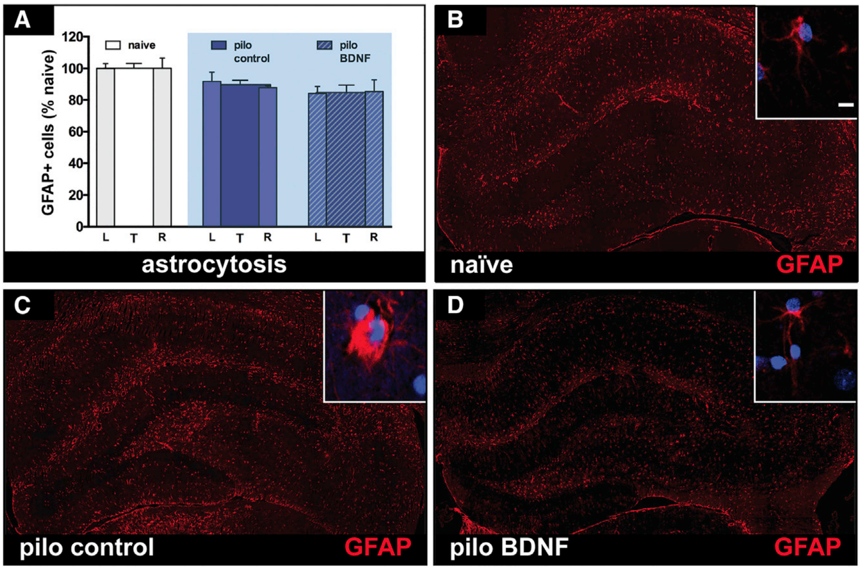

Figure 8. Effects on Astrocytosis

(A) GFAP-positive cells in the hippocampus of naive, epileptic control, and BDNF-treated epileptic rats. While no significant changes in the total number of GFAP-positive cells were found following either pilocarpine alone or pilocarpine + BDNF, clear qualitative differences were apparent (higher magnification insets). Numerous GFAP-positive cells in control epileptic animals (C) displayed short, thick processes, while the cells of BDNF-treated rats (D) were notably similar to those of the naive group (B) with a small cell body and long, thin processes. Nuclei were counterstained using DAPI (blue). Horizontal bar: 10 μm. Data are expressed as mean ± SEM of five animals per group. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined).

expanded based on their BDNF release levels. Clonal cell lines producing high and stable levels of BDNF were further characterized *in vitro* and *in vivo* in ECB devices and the BDNF clone used in the experiments was selected based on high BDNF secretion and long-term function in ECB devices *in vivo*.

Encapsulation of Cells in the ECB Device

Devices for cell culture experiments were built as follows: 7-mm-long semipermeable polysulfone hollow fibers (NsGene, USA), with an inner diameter of approximately 500 μm, were internally fitted with filaments of polyethylene terephthalate yarn scaffolding for cell adhesion. Prior to filling, ARPE-19-BDNF cells were cultured in growth medium. Prior to encapsulation, cells were dissociated and suspended in human endothelial serum-free medium (HE-SFM; Invitrogen) at a density of 100,000 cells/μL. Five microliters of cell solution (5×10^4 cells in total) were injected into each device using a custom manufactured automated cell-loading system. Devices were kept in HE-SFM at 37°C and 5% CO_2 for 2–3 weeks prior to surgical implantation. Devices loaded with non-modified ARPE-19 cells or without cells were treated in the same manner and included as negative controls.

These cells replicate until contact inhibited and remain stable thereafter. They do not have tumorigenic potential *in vivo* when injected naked into the brain. Membrane and cell scaffolds were prepared under rigorous, well-controlled manufacturing processes, and all the devices were removed at the end of the experiments and proved to be intact.

BDNF Release In Vitro

The amount of BDNF released by each capsule over a 24-hr period in HE-SFM was measured using the Human BDNF Quantikine ELISA Kit (R&D systems, Minneapolis, USA). Standards and samples were assayed in duplicate according to the manufacturer instructions, and results were expressed in ng/24 hr.

BDNF Release In Vivo

Following device removal, the left and right hippocampi were dissected and processed to extract total RNA, genomic DNA, and proteins using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Germany). RNA extraction was performed following the manufacturer instructions. Proteins and genomic DNA were isolated after RNA extraction using the phenol phase. In brief, genomic DNA was precipitated from the phenol phase with ethanol, and pellets were washed with sodium citrate ethanol solution and stored in 75% ethanol at −80°C. After DNA precipitation, proteins were isolated from the supernatant ethanol-phenol by isopropanol precipitation. Proteins were then washed several times with 0.3 M guanidine HCl-95% ethanol solution before being air-dried and resuspended in a rehydration buffer (62 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 12.5 mM EDTA, 50 mM DTT, β-mercaptoethanol, protease inhibitor cocktail) by a 20 min incubation at 95°C and three rounds of 30 s sonication.

Proteins were quantified using the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA) and a bio-spectrometer (Eppendorf, Germany) and then analyzed by western blotting. Protein samples (2 μg) were diluted in SDS-gel loading buffer, boiled for 10 min, and centrifuged before loading. Samples were then electrophoretically separated onto a 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocking in a buffer (PBS-Tween 20) containing 5% dried milk, membranes were incubated with the primary antibody in another buffer containing 2.5% dried milk overnight at 4°C. After three washings, incubations were performed with the secondary antibody in buffer/dried milk at room temperature for 1 hr. The mature BDNF protein was revealed using a polyclonal chicken anti-hBDNF antibody (Promega, WI, USA; dilution 1:500) and actin using a rabbit anti-actin monoclonal antibody (Sigma, MO, USA; 1:1,000). The chicken polyclonal antibody was revealed using a rabbit anti-chicken horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Denmark; dilution 1:1,250) and the rabbit monoclonal antibody by a swine anti-rabbit HRP-conjugated secondary antibody (Dako; dilution 1:3,000). The

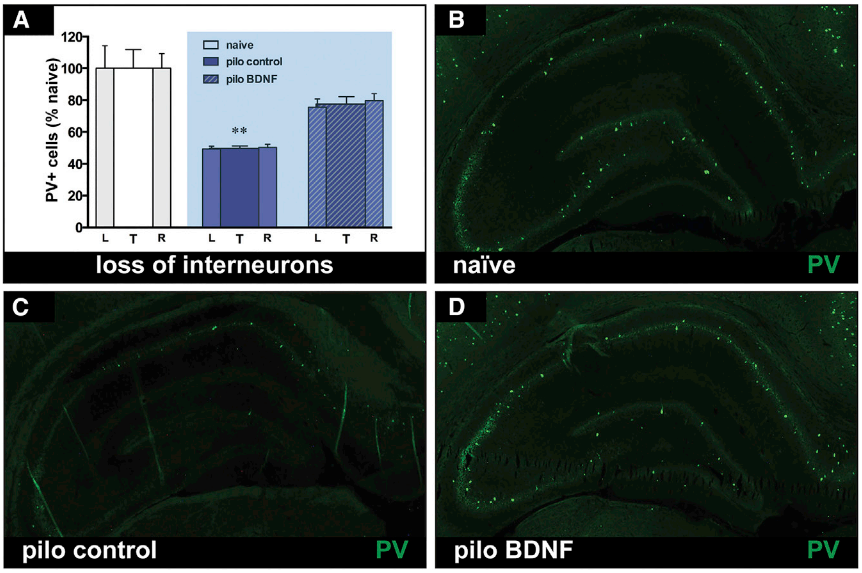

Figure 9. BDNF Preserves Hippocampal Parvalbumin Interneurons

(A) Quantification of parvalbumin-positive cells in the hippocampus of naive, control epileptic, and BDNF-treated epileptic rats. While pilocarpine significantly reduced the numbers of parvalbumin cells throughput the hippocampus (A and C) relative to naive controls (B), BDNF treatment substantially reversed this pathological effect (A and D). While the control epileptic group exhibited a 50.3% loss of cells, BDNF-treated animals displayed only a 22.5% loss. Data are expressed as mean ± SEM percentage of naive animals and were obtained from five animals/group. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined). ***p < 0.001 versus naive; ANOVA and post-hoc Dunnett test.

immunocomplexes were detected using the ECL western blot detection kit (GE Healthcare, NJ, USA) and ChemiDoc XRS (Bio-Rad) for electronic blot pictures. Quantification was performed using the Image Lab software (Bio-Rad).

Animals

A total of 78 animals were employed in this study. Male Sprague-Dawley rats (250–350 g; Harlan, USA) were used for all experiments. The experiments involving animals were conducted in accordance with European Community (EU Directive 2010/63/EU) and national and local laws and policies. The IACUC of the University of Ferrara approved this research that was authorized by the Italian Ministry for Heath (D.M. 246/2012-B). The ARRIVE (Animal Research: Reporting *In Vivo* Experiments), the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animal Research), and the National Institutes of Health guidelines were followed.[38,39] Animals were housed under standard conditions: constant temperature (22°C–24°C) and humidity (55%–65%), 12 hr light/dark cycle, free access to food and water.

Long-Term BDNF Secretion

Separate sets of naive animals (n = 16) were used to verify the long-term, continued secretion of BDNF. Devices were bilaterally implanted into the hippocampus, as described below, removed 1, 2, 4, and 8 weeks post-implant, and immediately incubated at 37°C in HE-SFM prior to measuring BDNF levels using ELISA.

Immediately after device removal, the previously implanted hippocampi were dissected, placed in 1 mL of Tissue Protein Extraction Reagent (T-PER; Thermo Scientific, Rockford, IL, USA), and flash frozen in liquid nitrogen. For ELISA analysis, samples were thawed and placed in 1.5 mL tissue protein extraction reagent (T-PER) plus 0.5 mL of a modified buffer[40] containing 100 mM Tris-HCL (pH 7.2), 400 mM NaCl, 4 mM EDTA, 0.05% sodium azide, 0.5% gelatin, 0.2% Triton X-100, 2% BSA, and complete protease inhibitor cocktail (Sigma, P8340). Tissues were homogenized with a polytron for 10 s and supernatants from pulverized and centrifuged tissue samples were assessed for BDNF levels using ELISA.

The same naive animals implanted with BDNF-secreting devices were monitored over the implant period for alterations in body weights and general activity, as rated by a blind observer using the Ellinwood and Balster[41] behavioral rating scale.

Pilocarpine Treatment

Pilocarpine was administered intraperitoneally (i.p.) (340 mg/kg), 30 min after a single subcutaneous injection of methyl-scopolamine (1 mg/kg, to prevent peripheral effects of pilocarpine), and the rats' behavior was monitored for several hours thereafter, using the scale of Racine:[8] (1), chewing or mouth and facial movements; (2), head nodding; (3), forelimb clonus; (4), generalized seizures with rearing; (5), generalized seizures with rearing and falling. Within the first hour after injection, all animals developed seizures evolving into recurrent generalized (stage 4 and higher) convulsions (SE). SE was interrupted 2 hr after onset by administration of diazepam (10 mg/kg, i.p.). All animals began experiencing spontaneous behavioral seizures 10 ± 1 days after SE.

Surgery

In all efficacy studies, surgery for ECB device implantation was performed 20 days after SE (Figure 2), between two video monitoring sessions (as described below). Rats were anaesthetized using isoflurane (3%–4%) and positioned in a stereotaxic frame (Stoelting, Dublin, Ireland). A midline incision was made in the scalp, and two bilateral holes were drilled through the skull. Devices filled with ARPE-19 BDNF cell (n = 20), filled with non-modified ARPE-19 cells (n = 20) and empty devices (n = 20) were bilaterally implanted in hippocampus in a vertical position using a cannula mounted to the stereotaxic frame. The implantation coordinates, with respect

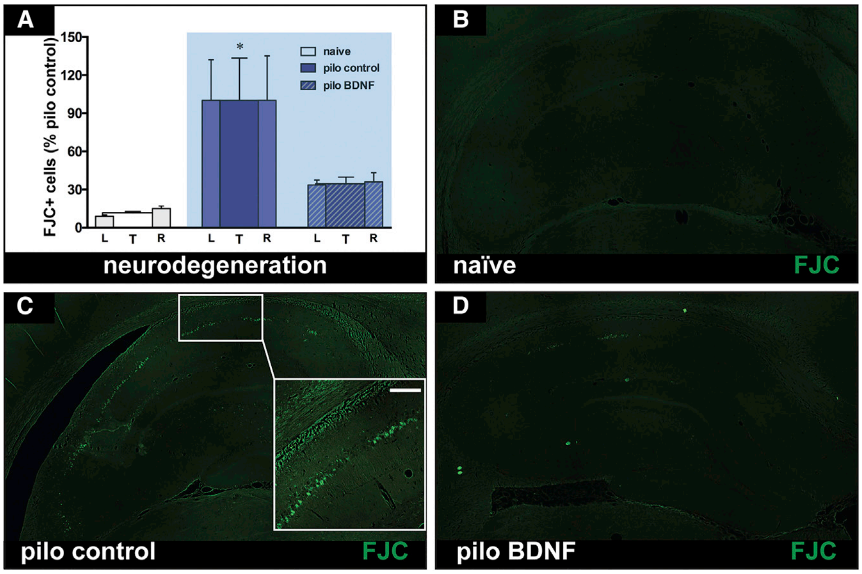

Figure 10. BDNF Reduces Ongoing Neuronal Degeneration

Quantitative analysis of Fluoro-Jade C (FJC)-positive cells (A) revealed that the numbers of degenerating cells were highly increased in epileptic controls (C) but not in the BDNF-treated animals (D) and naive animals (B). Data are expressed as mean ± SEM percentage of naive animals and were obtained from five animals/group. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined). $^*p < 0.05$ versus naive; ANOVA and post-hoc Dunnett test. Scale bar in (C) inset, 150 μm.

to bregma, were as follows: AP, −4.8; ML, ±4.6; and DV, −7.0. A subset of epileptic rats was unilaterally implanted with devices filled with ARPE-19 BDNF cells (n = 5) for histological analyses. After implantation, the skin was sutured closed. Continued secretion of BDNF was verified at the end of all experiments. Devices were removed by placing the anesthetized animal into the stereotactic frame, visualizing the proximal tip of the implant and gently removing it using microforceps. Devices were immediately incubated at 37°C in HE-SFM prior to processing for BDNF levels using ELISA.

Video Monitoring

Video monitoring was performed using a Swann 4 channel system (Swann, Santa Fe Springs, California USA). The first video-monitoring session was between day 10 and day 20 after SE (Figure 2), when animals began experiencing spontaneous seizures.[12] The second video-monitoring session was after implantation of the ECB devices, between days 25 and 45 after SE. Seizure severity was scored using the scale of Racine[8] by investigators that were blind of the treatment administered to the different rats.

Open-Field Test

Rats were placed for 30 min in an open-field arena measuring 75 cm in length, 75 cm in width, and 45 cm in height. The whole area was divided into 36 squares of 12.5 × 12.5 cm by black lines and the four central squares (25 cm from the walls) were defined as the central area. Each rat was placed at the center of the apparatus and, using the ANY Maze video software, we counted the total number of crossings in the central area and measured the time (in seconds) spent in the center of the arena by each rat over 30 min. The test was repeated at three different time points: baseline (1 day before pilocapine-induced SE), at the end of early chronic period (i.e., before surgery, at day 19 after SE), and at the end of late chronic period; 46 days after SE (Figure 2). The apparatus was cleaned with 5% ethanol between each animal testing.

Novel Object Recognition Test

The novel object recognition test (NOR) was used to assess recognition memory at three different time points, as above (Figure 2). The open-field squared box was used. NOR testing consisted of three parts: habituation, training/object familiarization, and novel object recognition testing, which were recorded using a video camera placed above the box. All objects and the open-field box were cleaned with 5% ethanol between each rat testing. During the habituation session, rats were placed in the empty arena, in the absence of objects, and allowed to move freely and explore the environment. On the next day, rats were put in the arena containing two identical objects and the total time spent exploring each object was recorded for 5 min. Then, after a 2-hr interval, animals were returned to the apparatus with a familiar and a novel object. Object recognition was assessed as more time spent interacting with the novel rather than the familiar object.

All behavioral testing was performed only if no spontaneous seizures were observed for at least 2 hr before the test; if seizures occurred during this pre-test period, the rat was placed back into its home cage and the trial was repeated after 2 hr.

Histology

Immunohistochemistry

Brains were rapidly removed, immersed in 10% formalin, and paraffin embedded after 48 hr. Coronal sections (8 μm thick) were cut with a Microtome (Leica RM2125RT, Germany) across the entire hippocampus, and mounted onto polarized slides (Superfrost slides, Diapath). One section every 500 μm was used for each stain. These sections were dewaxed (two washes in xylol for 10 min, 5 min in ethanol 100%, 5 min in ethanol 95%, 5 min in ethanol 80%) and re-hydrated in distilled water for 5 min. All antigens were unmasked using a commercially available kit (Unmasker, Dia-path), according to the manufacturer's instructions. After washing in PBS, sections were incubated with Triton x-100 (Sigma; 0.3% in 1× PBS, room temperature, 10 min), washed twice in 1× PBS, and incubated

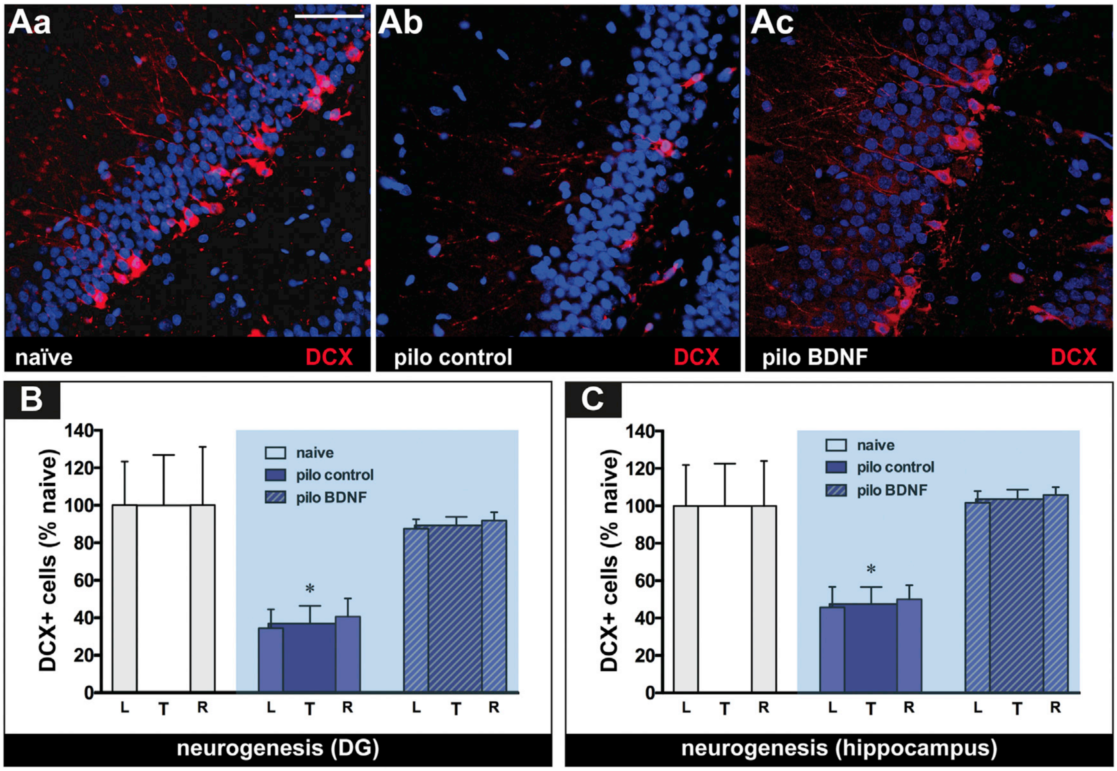

Figure 11. BDNF Normalizes Hippocampal Neurogenesis

Pilocarpine significantly reduced the numbers of doublecortin-positive (DCX) cells in the dentate gyrus and hippocampus (Ab, B, and C) relative to naive animals. When quantified, this effect manifested as a loss of, respectively, 63.0% and 52.4% in the dentate gyrus and in the whole hippocampus of epileptic controls versus naive animals. In contrast, the loss was only 10.6% in the dentate gyrus, and no loss in the whole hippocampus of BDNF-treated rats. The benefits of BDNF were also apparent on qualitative morphological observation, with the DCX-positive cells of BDNF-treated animals having a normal morphology (Ac), in contrast with those of epileptic controls (Ab). Data are expressed as mean ± SEM percentage of naive animals and were obtained from five animals/group. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined). *$p < 0.05$ versus naive; ANOVA and post-hoc Dunnett test. Immunofluorescence (dentate gyrus) for DCX is shown in red with nuclei labeled in blue with DAPI. Scale bar in (Aa), 75 μm.

with 5% BSA and 5% serum of the species in which the secondary antibody was produced, for 30 min. Sections were incubated overnight at 4°C in a humid atmosphere with a primary antibody specific for different cellular markers: glial fibrillaryacid protein (GFAP; mouse polyclonal, Sigma) 1:100; DCX (rabbit polyclonal, Cell Signaling, MA, USA) 1:400; parvalbumin (mouse monoclonal, Swant, Switzerland) 1:100; TrkB (rabbit polyclonal, Santa Cruz) 1:50; phosphor Y515-TrkB (rabbit polyclonal, AbCam) 1:100. After 5-min rinses in PBS, sections were incubated with Triton (as above, 30 min), washed in PBS, and incubated with a goat anti-mouse Alexa 594 or Alexa 488 secondary antibody (1:250, Invitrogen) for mouse primary antibodies, or with a goat anti-rabbit, Alexa 488 or Alexa 594 secondary antibody (1:250; Invitrogen) for rabbit primary antibodies, at room temperature for 3 hr. NeuroTrace (1:250; Invitrogen) was included in the secondary antibody incubation. After staining, sections were washed in PBS, counterstained with 0.0001% DAPI (Santa Cruz, Texas, USA) for 15 min, and washed again. Coverslips were mounted using anti-fading, water-based Gel/Mount (Sigma).

FJC Staining

For FJC (Millipore, Massachusetts, USA) staining, slides were first dewaxed as described above and then immersed for 5 min in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol. They were then rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were then transferred for 20 min to a 0.001% solution of FJC followed by three washes in distilled water for 1 min each. Finally, the slides were air dried on a slide warmer at 50°C for 5 min and cleared in xylene for 1 min. Coverslips were mounted using DPX (Sigma) mounting media.

Quantitative Analysis of Histological Staining

All quantifications were performed by two investigators that were blinded to the experimental condition. The analysis included 10 coronal sections cut at 500-μm intervals across the entire hippocampus, between −1.8 mm and −6.3 mm from bregma (Figure S1A; Paxinos[42]). Quantifications of cell numbers were performed in the whole hippocampus and in regions of interest (ROI) corresponding to the

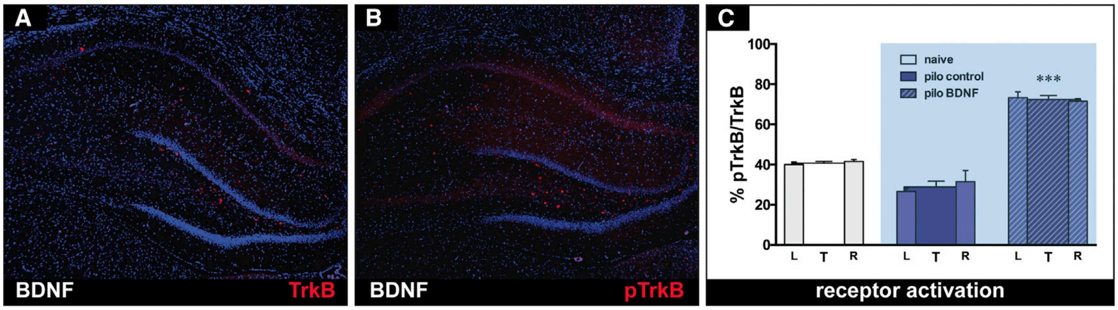

Figure 12. Hippocampal TrkB Is Activated following ECB Device-Mediated BDNF Treatment

Representative sections illustrating immunofluorescence (in red) of TrkB (A) and pTrkB^{Y515} (B). DAPI was used to counterstain nuclei in blue. (C) Percent ratio between phosphorylated and non-phosphorylated TrkB receptors. L, left hippocampus; R, right hippocampus; and T, total (both hippocampi combined). Data are expressed as mean ± SEM of five animals per group. ***$p < 0.001$ versus naive; ANOVA and post-hoc Dunnett test.

various hippocampal subareas (dentate gyrus; CA3; CA1–2), as shown in Figures S1B and S1C. All images were captured using a Leica microscope (DM RA2, Leica), and analyses were performed using the ImageJ software (NIH) and the MetaMorph Image Analysis software, respectively, for cell quantification and for hippocampal volume estimation.

To quantify the cells positive for the different markers (NeuroTrace, Parvalbumin, FJC, DCX, TrkB, and p-TrkB), we employed a method based on the thresholding of each digital image. The 10 full-color images of both hippocampi of each rat were captured using a Leica microscope, and transformed into grayscale. A fixed threshold for each staining was set to allow the software to recognize positive pixels and calculate the number of positive cells based on pre-defined recognition parameters. As stated above, one section every 500 μm across the entire hippocampus was quantified; thus, ten coronal sections regularly spanning 5-mm were examined for each rat. The number of positive cells obtained from the 10 coronal sections was summed to obtain a single estimate for each animal. All analyses were independently performed by two investigators, and the two estimates were averaged to obtain a single number for each animal. Finally, data have been reported as percentage of average cell number in naive rats.

The volume of the hippocampus was calculated using the 10 coronal sections from each rat (from −1.8 mm to −6.3 mm from bregma), stained with DAPI. The hippocampal area in each section was drawn and calculated using the MetaMorph Image Analysis software. The volume of the cone between two progressive sections was then calculated using the Cavalieri's principle. The sum of these values provided an estimation of the hippocampal volume for each rat. Finally, data were expressed as percentage of the average volume in naive rats.

Statistical Analysis

Results were expressed as the mean ± SEM. *In vitro* data (ELISA; western blot) were statistically examined using the non-parametric Mann-Whitney U test. Statistical evaluation for *in vivo* data was performed using two-way ANOVA and post-hoc the Sidak test, the Student's t test for unpaired data, or the Mann-Whitney U test, as appropriate and as indicated in the figure legends. Statistical analysis for histological quantification was conducted using one-way ANOVA and post-hoc the Dunnett test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and four tables and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.03.001.

AUTHOR CONTRIBUTIONS

C.F., D.F.E., L.U.W., and M.S. conceived and designed the experiments. C.F., F.L., and G.P. performed the experiments. C.F. and M.S. analyzed the data. T.F and W.J.B. contributed reagents/materials/analysis tools. C.F. and M.S. wrote the paper.

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