Epileptogenesis in vitro: Sprouting and Neurotrophins

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Summary

Epilepsy is one of the most common neurological conditions with an estimated one in 20 people experiencing epileptic seizures within their lifetime. Normally in the brain there is a delicate balance between excitation and inhibition among the billions of neurons in the nervous system and action potential discharge of individual cells is usually not synchronized. A disturbance of this equilibrium is thought to be critical in the development of epileptic seizures and is common for all epilepsies. Since only some of the patients suffering from epileptic seizures can be effectively helped by anticonvulsants treatment, it is of considerable interest to better understand the complex sequence of events leading to epileptic discharge in order to find new and better therapeutic approaches. I am particularly interested in the recurring seizures that are a common consequence of traumatic brain injury. In order to investigate this phenomenon we have developed an *in vitro* model for posttraumatic epilepsy. To simulate brain injury we transected morphologically stable CA3 pyramidal cell axons in hippocampal slice cultures. The lesioned cultures exhibited an increase in excitability. We demonstrated that axon collaterals are newly sprouted by CA3 pyramidal cells as a response to axonal injury and suggest this underlies the development of posttraumatic epilepsy. We are currently trying to identify the factor(s) that are activated by injury and that induce axonal sprouting in the hope that interrupting this process will prevent the development of hyperexcitability in lesioned hippocampal slice cultures and, ultimately, the occurrence of human posttraumatic epilepsy.

Epileptogenese *in vitro*: Regeneration von Nervenfasern und Neurotrophine

Epilepsie ist eine der häufigsten neurologischen Erkrankungen, wobei schätzungsweise 5% der Bevölkerung im Verlauf ihres Lebens epileptische Anfälle bekommen können. Zwischen den Milliarden von Neuronen im Gehirn besteht normalerweise ein komplexes Gleichgewicht zwischen Erregung und Hemmung, und die elektrische Entladung einzelner Zellen ist normalerweise nicht synchronisiert. Es wird angenommen, dass eine Störung dieses Gleichgewichts eine kritische Komponente in der Entstehung von epileptischen Anfällen und allen Epilepsien gemein ist. Da einem grossen Anteil der Epilepsie-Patienten mit den gegenwärtig verfügbaren antiepileptischen Medikamenten nicht effektiv geholfen werden kann, ist es von besonderem Interesse, die komplexe Folge von Ereignissen, die zu einer epileptischen Entladung führen, besser zu verstehen. Dies ist eine Voraussetzung, um neue und bessere therapeutische Ansätze zu finden. Mich interessieren im Besonderen die wiederkehrenden Anfälle, die als eine häufige Folge von traumatischen Hirnverletzungen auftreten können. Zur Untersuchung dieses Phänomens haben wir ein *in vitro*-Modell für posttraumatische Epilepsie entwickelt. Zur Simulation einer Hirnverletzung wurden in Kulturen von Hippokampus-Schnitten die morphologisch erkennbaren stabilen CA3-Pyramidenzell-Axone durchtrennt. Dieser Eingriff führte zur Ausbildung neuer CA3-Nervenfasern und einer Zunahme der Erregbarkeit. Wir konnten zeigen, dass diese Axone von Pyramidenzellen neu gebildet wurden und zwar als Antwort auf die axonale Läsion, und vermuten, dass dies ein Grund für die Entstehung der posttraumatischen Epilepsie ist. Zur Zeit versuchen wir, die Faktoren zu identifizieren, die durch die Verletzung aktiviert werden und das axonale Wachstum auslösen, in der Hoffnung, dass eine Unterbrechung dieses Prozesses die Entwicklung der Übererregbarkeit in läsionierte Schnittkulturen des Hippokampus und letztendlich die Entstehung der humanen posttraumatischen Epilepsie verhindert.

Epileptogenèse *in vitro*: pousse neuritique et neurotrophines

L'épilepsie est le trouble neurologique le plus répandu, touchant environ une personne sur 20 au cours de sa vie. Dans des conditions normales, il existe un équilibre entre l'excitation et l'inhibition des milliards de neurones du système nerveux central, et les décharges de potentiels d'actions ne sont habituellement pas synchronisées. Une perturbation de cet équilibre est susceptible d'induire des crises d'épilepsie. Seule une fraction des patients développant des crises d'épilepsie peuvent être efficacement traités au moyen d'anticonvulsifs, il est donc essentiel d'améliorer notre compréhension des événements complexes dont l'épilepsie résulte, afin de développer de nouvelles stratégies thérapeutiques. Je m'intéresse en particulier aux crises récurrentes, conséquence fréquente de lésions cérébrales d'origine traumatique.

Pour étudier ce phénomène, nous avons développé un modèle *in vitro* d'épilepsie post traumatique en simulant la lésion cérébrale par transection des axones de neurones pyramidaux de la région CA3 dans des cultures organotypiques d'hippocampe. Cette lésion induit la formation de nouvelles collatérales des axones

des neurones pyramidaux de CA3, et augmente l'excitabilité des neurones de la culture.

Nous avons démontré que ces nouvelles collatérales bourgeonnent des cellules pyramidales en réponse à la lésion des axones, suggérant que ce mécanisme soit à l'origine du développement de l'épilepsie posttraumatique.

Nous essayons actuellement d'identifier les facteurs activés lors de lésions et pouvant induire le bourgeonnement axonal. Interférer avec ce mécanisme pourrait prévenir le développement de l'hyperexcitabilité dans les cultures organotypiques d'hippocampe, et à terme, prévenir l'apparition d'épilepsie posttraumatique chez le patient humain.

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Introduction

A relatively common type of epilepsy occurring as a consequence of acute damage to the brain is posttraumatic epilepsy. The severity of the head injury, particularly the occurrence of dural penetration, is correlated with the likelihood of developing posttraumatic epilepsy. Seizures can occur with various delays after the head trauma and different mechanisms may underlie the genesis of these different phases. Although a seizure may occur within the first hours or weeks after injury, epilepsy typically begins from 1 week to 3 years after the trauma. Unfortunately at present, little is known about the mechanisms underlying the generation of posttraumatic epilepsy.

The most probable injuries leading to epileptic seizures are hemiplegia, dural penetration, missile wounds or intracerebral hematomas. These injuries are more frequent in young adults and military personnel. Brain injury not only consists of mechanical damage but is also associated with neurochemical changes which facilitate the injury process and may contribute to generation of seizures. Liberated ions from hemoglobin of the blood can lead to the formation of free radicals disrupting membranes, subcellular organelles and normal flux of calcium, sodium and potassium. This leads to a progressive depolarization of the membrane, excessive glutamate release and subsequent opening of voltage-dependent calcium channels. The elevated intracellular calcium concentration then causes the release of even more glutamate thereby sustaining the increase in neuronal excitation. In addition, a reduction of synaptic inhibition by γ-aminobutyric acid (GABA) in cortical structures leads to the release of powerful recurrent excitation and the generation of synchronous epileptiform discharge in numerous experimental models of epilepsy [1,2]. Decreases in GABA immunoreactivity have also been reported following cortical injury [3].

Alternatively, Prince and colleagues [4,5] have demonstrated the delayed development of epileptiform discharge after a form of neuronal injury, produced by undercutting a portion of neocortex, which is maintained in acutely prepared brain slices containing the lesion. It was suspected that a reorganization in excitatory and/or inhibitory synaptic circuits occur in such injured tissue, leading to the generation of posttraumatic epilepsy. Nonetheless it remained to be shown whether new axon collaterals are generated *de novo* or the existing axons become modified after injury. It was also not known whether the new axons are generated by cells whose axons are injured or by uninjured cells in response to the availability of vacant synaptic territory.

We have created an *in vitro* model to investigate what changes can take place slowly in the brain as a result of the insult, yet remain for the life of the patient, in order to give rise to epilepsy. As the situation is too complicated to analyze in a patient or in animal experiments, we were successful in developing a system in which we can simulate an injured brain developing post-traumatic epilepsy, using brain tissue in a test-tube. In order to answer these problems we produced axonal injury in an *in vitro* system of slices of rat hippocampus cultures and investigated possible mechanisms involved in axonal reorganization. From this model we have been able to identify the causes of posttraumatic epilepsy, a question, which has awaited an answer for many years. Currently we are investigating the role of neurotrophins in axonal sprouting after brain injury. Neurotrophins are a subfamily of neurotrophic factors, which are small soluble peptides that play crucial roles in inducing and supporting normal development and maintenance of neurons. The best-characterized neurotrophin is nerve growth factor. Other members of this family are called "brain-derived neurotrophic factor" (BDNF), NT-3, and NT-4/5. They all act by binding receptors on the surface of cells that act by phosphorylating target proteins. These receptors are called Trk (the abbreviation of "Tyrosine kinase receptor"). TrkA is the receptor for nerve growth factor, TrkB the receptor for BDNF and NT-4/5. Several neurotrophins, in particular BDNF, have been shown to be expressed after experimentally induced epileptic seizures [6,7]. It may therefore be that an increase in neurotrophin secretion in response to injury might trigger axonal sprouting.

Methods

Posttraumatic epilepsy model

Organotypic hippocampal slice cultures were prepared from six-day-old rat pups and maintained in culture for 14 days before starting the experiments. During this time cells will differentiate and form an extensive network of synaptic connections [8,9]. Lesions were made of the so-called Schaffer collateral axons between

area CA3 and CA1 and changes in both axonal sprouting and excitability were assessed at different time points post-lesion.

During axonal sprouting and regeneration, a number of genes typically expressed during development become reactivated, such as growth-associated protein GAP-43 [10]. GAP-43 is a nervous system specific protein that is highly expressed in axons during development and regeneration [11,12, 13]. During the first 2 weeks of development high levels of the protein are expressed in the cell body and immediately transported into the axon and growth cones. As soon as the neurons mature, GAP-43 expression is reduced [14,15]. At later stages, GAP-43 reappears in axons that regenerate after a lesion. GAP-43 thus serves as an excellent marker with which to monitor axotomy-induced neuronal regeneration and rearrangement of the mature CNS. In the present study, GAP-43 immunohistochemistry was employed to visualize regenerating axons in our slice cultures [16] after lesioning the Schaffer collaterals. In addition, the morphological analysis was correlated with a functional alteration induced by the lesion: cells were recorded electrophysiologically, using a protocol facilitating the distinction of excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). Pairs of monosynaptically connected CA3 pyramidal neurons were recorded and identified as previously described [17].

Neurotrophin involvement

After 14 days in vitro the cultures were lesioned and then chronically treated with antibodies (200 ng/ml) against TrkB, the receptor for BDNF and NT-4/5, to block their function for 7 or 14 days in order to test whether reactive axonal sprouting can be prevented. Unlesioned sister cultures received the TrkB blocking antibody to control for the effects of the antibody. The cultures were then analyzed for axonal sprouting by GAP-43 immunohistochemistry. In the future electrophysiological analysis will also be performed to see if there is an increase of electrical activity.

Results

Three to 7 days after the Schaffer collateral lesions, numerous short immunopositive axons and growth cones were evident within area CA3. However by 14 days post-lesion there was a dramatic increase in both the number and length of immunopositive GAP-43 axons within area CA3 but no GAP-43 upregulation was detected in the CA1 region. Some immunoreactive axons were visible crossing the lesion into area CA1. In contrast, by 21 days after the lesion, GAP-43 immunopositive axons were no longer visible. We can conclude from this finding that new axon branches were created by the injured CA3 pyramidal cells within 3 days of the lesion. These axons then ramified within area CA3 and became mature by 21 days after the lesion, as determined by the lack of staining for GAP-43 at this stage. The functional consequences of these morphological changes were examined with electrophysiological recordings from CA3 pyramidal cells. The frequency of spontaneous synaptic potentials in the control cultures was low (<10Hz) compared to 15Hz in 2-week post-lesion cultures. No decrease in the strength of synaptic inhibition was apparent in lesioned cultures. As there was an increase in the amount of axonal sprouting in area CA3, we could expect an increase in the probability that one CA3 cell will excite neighboring cells. Simultaneous recordings from pairs of CA3 cells were used to test this hypothesis. Normally, as tested in control cultures, the probability that any CA3 cell excites another CA3 cell is about 56% but this was significantly increased to 83% of cell pairs in cultures in which the Schaffer collateral pathway had been lesioned for 14 days. Epileptiform burst discharges could be evoked in several cultures examined 14 days post-lesion but in none of the sister control cultures. Although many of the cultures were not spontaneously epileptic, we investigated if they were more epilepsy-prone by applying a low concentration (0.1 μM) of bicuculline methochloride, a $GABA_A$ receptor antagonist. This treatment led to the generation of spontaneous and evoked epileptiform burst discharges in lesioned cultures. Application of the same concentration of bicuculline to control cultures did not induce epileptiform discharge.

Neurotrophin involvement

Addition of specific receptor binding antibodies to the receptor of the neurotrophins BDNF and NT-4/5, i.e., TrkB antibodies for 14 days to unlesioned cultures did not lead to the formation of GAP-43 immunopositive fibers. However GAP-43 fibers were evident in lesioned cultures that were not treated with TrkB antibodies, showing that the GAP-43 immunohistochemistry was working. Interestingly, addition of the TrkB antibodies to the lesioned cultures for 14 days prevented the upregulation of GAP-43 immunopositive fibers which could be seen in sister cultures which were lesioned but untreated during the 2 week period. This suggests that activation of the TrkB receptor is necessary for the upregulation of GAP-43 and axonal reorganization after brain injury.

Discussion

Lesion-induced axonal sprouting

We have successfully produced an *in vitro* model for posttraumatic epilepsy. We have demonstrated that

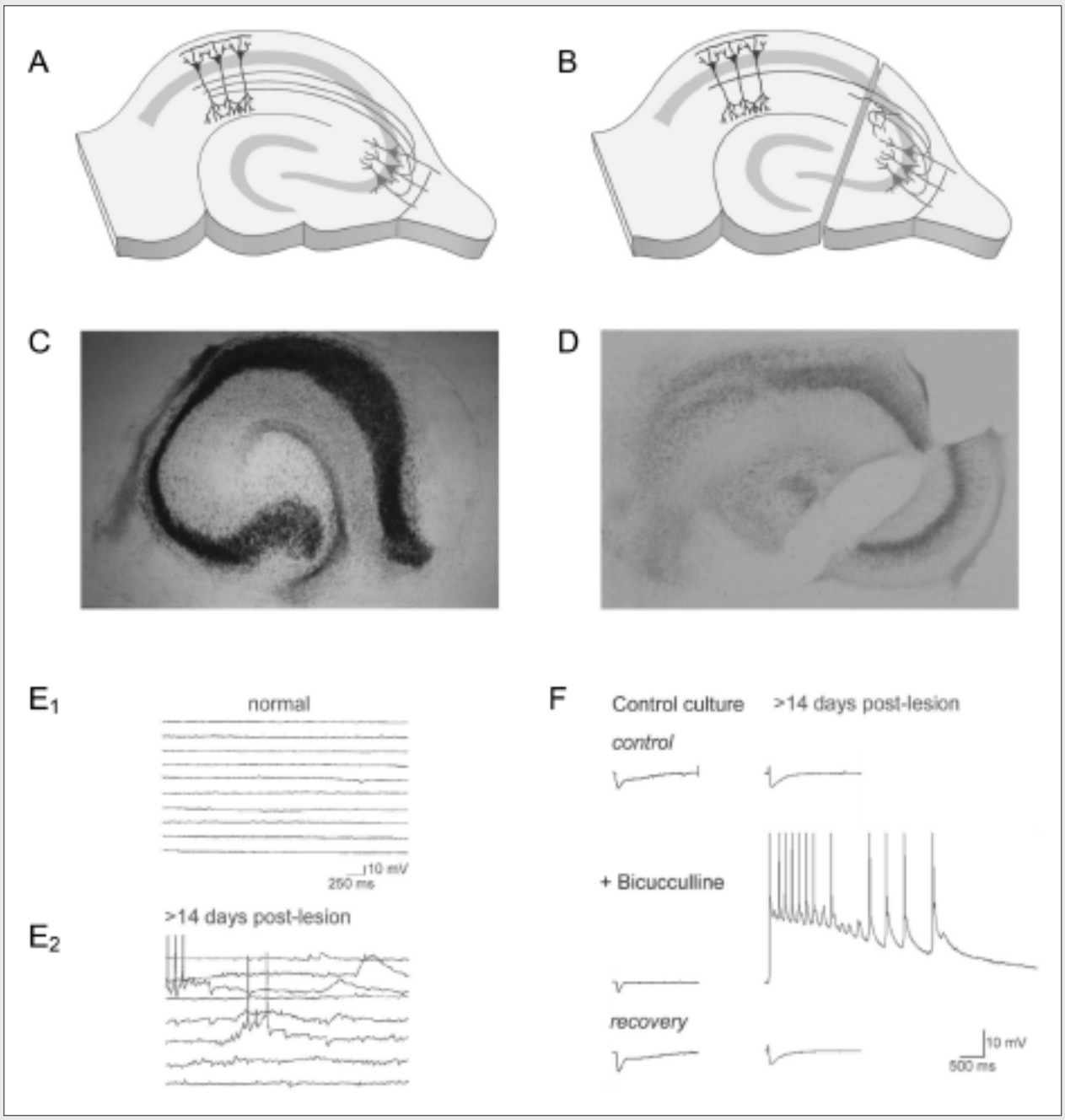

Figure 1: Slices of hippocampus (A, C) were used, as this is the area of the brain in which the synchronized electrical activity in epilepsy often originates. To simulate a brain injury we cut the nerve fibers with a scalpel (B, D). C and D show Nissl stained (revealing RNA within the cell bodies) hippocampal cultures *in vitro* for 14 days. CA3 pyramidal cells are shown in green, with their axons, the Schaffer collaterals innervating CA1 pyramidal cells (blue). The lesion of the Schaffer collaterals after the cut led into axonal sprouting in CA3 (red), but not to the replacement of the Schaffer collaterals. To determine whether this axonal sprouting was resulting in increased excitation and epileptic discharges in CA3, nerve cells in the cultures were tested after varying periods of time using electrophysiology (E). Cultures exhibited an increase in excitation two weeks after the injury (E_2). When inhibition was slightly reduced by chemical means (bicuculline), which had no effect on unlesioned cultures, all cultures with the injury had epileptic discharge (F).

new axon collaterals are generated by CA3 pyramidal cells at 7 and 14 days after the lesion of the Schaffer collateral pathway. These axons were GAP-43 immunopositive permitting us to state that they are indeed newly regenerated axons. As axons stained for GAP-43 were only visible in area CA3 and not in area CA1, this observation strongly suggests that the cells do not sprout new axonal branches to occupy postsynaptic sites that have become vacant in CA1 following the degeneration of the lesioned Schaffer collateral pathway. This is quite different to what has been previously reported for the axons of the dentate granule cells and the septohippocampal cholinergic cells, which sprout after lesions that remove input to the dentate gyrus.

We also observed that the newly formed axons increase the connectivity of cells within area CA3. There was a high level of spontaneous synaptic activity and polysynaptic EPSPs in lesioned cultures, a phenomena never seen in sister control cultures. This increase in synaptic activity rendered the CA3 region more susceptible to generate epileptiform discharges. The hippocampal neuronal network generates epileptiform discharge when the balance between synaptic excitation and inhibition is disrupted in favor of excitation. We observed no functional or morphological change in GABAergic activity in the lesioned cultures to account for the observed increase in excitability in area CA3. Therefore, the increase in connectivity of the CA3 pyramidal cells, as seen in the paired recordings, could account for the observed increase in excitability. The spread of excitation between cells will be facilitated, as there is an increased chance that an action potential in another cell will successfully lead to the generation of an action potential in another cell. Furthermore, the facilitated spread of excitation via the newly sprouted axons will be even more prominent when the strength of GABAergic inhibition is reduced, thereby accounting for the observed occurrence of bursting in low concentration of the $GABA_A$ antagonist.

Axonal sprouting and posttraumatic epilepsy

Anatomical studies of tissue from patients with intractable temporal lobe epilepsy have shown with Timm´s stain, that granule cell axons (which are called mossy fibers) sprout collaterals into the inner molecular layer of the dentate gyrus in animal models and surgically resected human tissue [18,19]. These anatomical findings lead to the hypothesis that synaptic reorganization of the dentate gyrus resulting from mossy fiber sprouting leads to the formation of new excitatory synapses among granule cells. Hence, mossy fiber sprouting and the hypothetical formation of new recurrent excitatory circuits have been proposed as mechanisms for increased seizure susceptibility of the dentate gyrus [20,21]. Our data indicate that axonal sprouting is not limited to the granule cells. Previously, direct evidence that axons of hippocampal or cortical pyramidal cells can sprout in response to injury was impossible to establish due to lack of a specific marker.

These observations permit the formulation of a hypothesis for the development of epilepsy after traumatic brain injury in humans. We suggest that axonal sprouting from neocortical or hippocampal pyramidal cells are initiated by axonal injury. The time required for the elaboration of these new axonal collaterals and the formation of the new synapses may underlie the delay between the initial injury and the development of posttraumatic injury.

Neurotrophin involvement in axonal sprouting

We have begun to identify the factor(s) that are activated by injury to induce axonal sprouting in the hope that interrupting this process will prevent the development of hyperexcitability in lesioned hippocampal slice cultures. We hypothesized that an increase in neurotrophin secretion in response to the injury might trigger the axonal sprouting. We could successfully prevent the upregulation of GAP-43 immunoreactivity in lesioned cultures by adding the receptor blocker for TrkB. Although this is a very promising result, we still must determine whether blockade of the action of neurotrophins prevents the increase in excitability observed in lesioned cultures.

It has been previously reported that the expression of several neurotrophic factors is increased after experimentally induced epileptic seizures [22]. Chronic temporal lobe epilepsy is associated with neuronal cell loss, neurogenesis, mossy fiber sprouting and reactive gliosis. Clinical studies on brain tissue removed during surgical intervention as therapy for intractable temporal lobe epilepsy revealed enhanced mRNA neurotrophin levels in the dentate granule cells, suggesting a correlation between these levels and the extent of mossy fiber sprouting [23].

Seizure activity leads to an increase of BDNF mRNA and protein level and activation of TrkB receptors, mainly in the granule cells of the dentate gyrus as well as in pyramidal cells of area CA1 and CA3 in the hippocampus. Additionally BDNF has been reported to potentiate neuronal excitability at the mossy fiber pathway, thereby leading to an imbalance between excitatory and inhibitory synaptic transmission [24,25]. The hyperexcitability induced by BDNF could be blocked by the administration of the general tyrosine kinase receptor antagonist K252a [26] thereby showing the feasibility of interfering with the action of neurotrophins to prevent epileptiform activity.

Conclusions

The observations permitted the formation of a hypothesis for the development of epilepsy after traumatic brain injury in humans. Axonal sprouting from neocortical or hippocampal pyramidal cells is initiated by axonal injury indirectly in the absence of dural penetration or directly in the case of penetrating wounds. The time required for the elaboration of these new axons and the formation of new synapses might underlie the delay between the initial injury and the development of posttraumatic epilepsy. This hypothesis suggests novel therapeutic possibilities. In particular, we are continuing to investigate the role of neurotrophins as the factors involved in axonal sprouting. If the release of growth factors promotes axonal sprouting in the vicinity of an injury, then interfering with their

action immediately after cortical injury in patients at risk of epilepsy may reduce posttraumatic axonal sprouting and thus reduce the risk of developing epilepsy.

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