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Chapter VI

Immunohistochemical Analysis of Trk Receptor Activation in Epilepsy

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Introduction

BDNF and Epilepsy

The discovery that limbic seizures increase nerve growth factor (NGF) mRNA levels [34] led to the idea that seizure-induced expression of neurotrophic factors may contribute to the lasting structural and functional changes underlying epileptogenesis [33, 36, 47]. Multiple findings have since implicated the neurotrophin brain-derived neurotrophic factor (BDNF), more than NGF, in epileptogenesis [8]. BDNF mRNA and protein are markedly upregulated in the hippocampus by seizure activity in animal models [28, 46, 56, 69]. The development of kindling is partially inhibited in heterozygote knockout BDNF mice [53] or mice with conditional BDNF gene deletion [42]. Interestingly, conditional homozygous deletion of trkB appears to prevent kindling [42]. Intraventricular infusion of trkB-Fc, which would sequester and limit the activity of endogenous BDNF, inhibits kindling development [11]. Mice overexpressing a truncated trkB display reduced seizure susceptibility [55]. Conversely, direct application of BDNF induces hyperexcitability *in vitro* [79, 80]; overexpression of BDNF in transgenic mice leads to spontaneous seizures [21]; and intrahippocampal infusion of BDNF is sufficient to induce seizure activity *in vivo* [81] (but see [74]). Furthermore, increased BDNF expression in the hippocampus is found in specimens from patients with temporal lobe epilepsy [62, 93].

The above results implicated BDNF and trkB receptor signaling in kindling development and hyperexcitability but did not directly address where and when trk receptors are activated during limbic epileptogenesis. Therefore, we aimed to create an assay to directly analyze trk receptor activation *in vivo* [10]. Defining the anatomy, time course, and threshold of trk

receptor activation *in vivo* would provide a more mechanistic understanding of the involvement of neurotrophins in kindling, and combined with the functional data pinpoint important sites of epileptogenesis in the brain.

Trk Receptor Signaling

How might one measure trk receptor activation? Trk proteins are transmembrane receptor tyrosine kinases (RTKs) homologous to other RTKs such as the EGF receptor and insulin receptor family [4, 94]. Signaling by receptor tyrosine kinases is known to involve ligand-induced receptor dimerization and dimerization-induced trans-autophosphorylation [39, 82]. Receptor autophosphorylation on multiple tyrosine residues creates specific binding sites for intracellular target proteins, which bind to the activated receptor via SH2 domains [82] (Figure 1). For the neurotrophin family, these target proteins have been shown to include PLCγ1 (phospholipase C gamma 1), p85 (the noncatalytic subunit of phosphatidyl inositol (PI)-3 kinase), and Shc (SH2-containing sequence); activation of these target proteins can then lead to a variety of intracellular signalling cascades such as the Ras-MAP kinase cascade and phosphorylation of CREB [44, 49, 64, 85, 87] (Figure 1). Binding specificity to the various ligands of the neurotrophin family is conferred via the juxtamembrane Ig-like domain of the extracellular portion of the receptor (98) in the following pattern: trkA is the receptor for NGF (with lower-affinity binding by NT-3 in some systems), trkB is the receptor for BDNF and NT-4/5 with lower-affinity binding by NT-3, and trkC is the receptor for NT-3 [4]. In addition, all of the neurotrophins bind to the p75 receptor [19, 23].

Ligand-induced receptor tyrosine phosphorylation is necessary for cellular responses to trk activation [4] (but see [72]). For example, cooperative interaction between tyrosines in trkA mediates the neurite outgrowth effect of NGF [45]. Thus, receptor tyrosine phosphorylation seems a logical measure of the biologic level of neurotrophin activity. Indeed, receptor tyrosine phosphorylation has been used as an index of neurotrophin receptor activation by several investigators [48, 50-52, 78]. For example, dexamethasone or NGF given ICV to rats led to a marked increase in trk phosphorylation in septum but not hippocampus (not surprising given lack of trkA in hippocampus) maximum at 30 minutes after ICV NGF [78].

Trk receptors exist in both a full-length (trkB.FL) form as well as truncated (trkB.T1, trkB.T2) forms lacking the kinase domain [25, 32]. Although most functions attributed to BDNF are associated with full-length trkB, several roles have been suggested for truncated receptors, including growth and development [32, 60, 104] and negative modulation of trkB receptor expression and function [25, 40, 41].

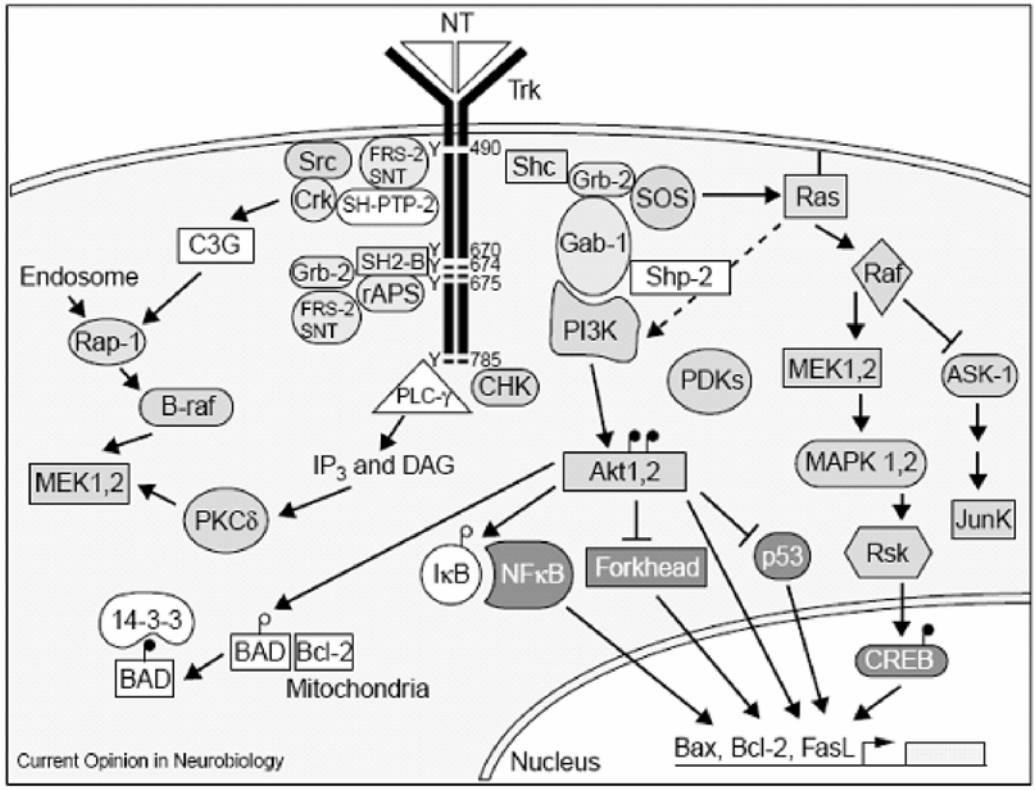

Figure 1. Schematic of trk signal transduction pathways. Reproduced with permission from Patapoutian and Reichardt, *Curr. Op. Neurobiol.*11:272-80 (2001).

Phospho-Specific Trk Antibodies

Using trk receptor phosphorylation as a surrogate measure of (full-length) trk receptor activation, the development of phospho-specific trk antibodies that selectively detect phosphorylated trks provides the opportunity to directly assess trk receptor activation. Rosalind Segal and colleagues developed an antibody directed against a tyrosine-phosphorylated peptide (VIENPQY*FGITNS, from mouse trkB sequence data) surrounding the tyrosine 490 residue in the catalytic domain of the trk receptors [85, 86]. A distinct pY490 antibody based on human trkA sequence data was developed at New England Biolabs (now Cell Signaling Technology, CST) (Table 1). Tyrosine-490 is phosphorylated following neurotrophin application and is known to couple trk receptors to Shc binding and activation of the ras-MAP kinase cascade [70, 87] (Figure 1). The pY490 antibody detects phosphorylated trks on Western blots from cell lysates [86] and has been used in immunohistochemical assays to detect phosphorylated trks [7, 84] (Table 1). While the original peptide sequence for the Segal pY490 antibody was derived from mouse trkB sequence data, this and the CST pY490 antibody do not distinguish between phosphorylated trkA, trkB, and trkC [86]. This is not surprising given the extensive sequence identity in the intracellular tyrosine kinase domain between trkA, trkB, and trkC [89]. Furthermore, human, rat, and mouse trkA, trkB, and trkC share nearly identical sequences within the tyrosine

kinase domain, and therefore phosphotrk antibodies would be expected to detect trks from all species [89]. It is important to emphasize that these pY490 antibodies only detect trk phosphorylation at the Shc site (Y490) [3] and not at other trk autophosphorylation sites (*e.g.* Y674/675, Y785 [PLC site]). Distinct phosphospecific antibodies will be needed to specifically address activation of these sites in the future.

Table 1. Comparison of Segal *vs.* CST pY490 antibodies

	Segal pY490	**Cell Signaling Technology pY490**
Peptide immunogen	VIENPQY*FGITNS (coupled to KLH)	IENPQY*FSD (coupled to KLH)
Sequence data	Mouse trkB residues 509-521[1] Cognate trkA sequence: IIENPQYFSDACV Cognate trkC sequence: VIENPQYFRQGHN	Human trkA residues 491-499[2] Cognate trkB sequence: IENPQYFGI Cognate trkC sequence: IENPQYFRQ
Method of purification	Protein A chromatography[1] Unphosphopeptide column[1] KLH column[1] Phosphopeptide column[1]	Protein A chromatography[3] Unphosphopeptide column (twice)[3] Phosphopeptide column[3]
Western blot results	appropriate band from NGF-stim. PC12 cells[1] BDNF-stim. 3T3 cells expressing trkB[1] NT-3-stim. 3T3 cells expressing trkC[1] NGF and BDNF-stim. DRG cells[4]	NGF-stim. PC12 cells[3,5] BDNF and NT-3- stim. cells transfected with trkB and trkC respectively[3] BDNF and NT-3-treated primary cortical cells[6]
Western blot specificity controls	blocked by phosphopeptide immunogen[1] not blocked by unphosphopeptide, phosphopeptide pY674/5, erbB2 phosphopeptide[1]	
Immunocytochemistry (on cells)	neurotrophin-stimulated 3T3 cells expressing trkA, trkB, or trkC[7] neurotrophin-stimulated DRG cells[4]	recognizes NGF-stim. PC12 cells[3]
Immunocytochemistry specificity controls	blocked by phosphopeptide immunogen[7] not blocked by unphosphopeptide, phosphopeptide pY674/5, erbB2 phosphopeptide[7]	
Immunohistochemistry (tissues)	sciatic nerve[7] developing cerebellum[8] ferret visual cortex[9] adult brain (before and after seizure)[10]	developing cerebellum[11] adult brain (before and after seizure)[12]
Immunohistochemistry specificity controls	does not stain BDNF -/- cerebellum[8] sciatic nerve staining blocked by phosphopeptide immunogen but not unphosphopeptide or erbB2 phosphopeptide[7] sciatic nerve extracts recognized on Western blot[7] cerebellum staining blocked by phosphopeptide but not unphosphopeptide[8] ferret visual cortex staining blocked by infusion of trkB-Fc[9]	basal staining in developing cerebellum[11] and adult brain (this chapter) as well as seizure-induced mossy fiber staining[12] (this chapter) blocked by phosphopeptide immunogen but not by unphosphopeptide or pY674/5 phosphopeptide

References:

1. (86).
2. (89).
3. New England Biolabs (Cell Signaling Technology) company data, Figure 2A.
4. (102).
5. (18).
6. Figure 2B (this chapter).
7. (7).
8. (84).
9. (15).
10. R. Segal, unpublished results.
11. D. Binder, data not shown.
12. (10).

Validation of CST pY490 Antibody

For the immunohistochemical analysis presented below, a pY490 antibody (CST) raised to a tyrosine-phosphorylated peptide (IENPQY*FSD, from trkA sequence data) with an overlapping but distinct sequence from that of the Segal pY490 antibody was employed (Table 1). The summary of methods of validation used and results obtained to date for both Segal and CST pY490 antibodies are shown (Table 1) and are a compilation of results from Segal and colleagues' published work [7, 15, 84, 86], CST company data (H. Ruan, personal communication), and our published work [10] (Western blots courtesy of M. Routbort).

The CST pY490 detects phosphorylated trks on Western blots, and like Segal pY490 does not distinguish between trkA, trkB, and trkC. First, treatment of PC12 cells with NGF leads to the induction of a pY490-immunoreactive band of the appropriate molecular size (approximately 140 kD), whereas no band is detected from untreated lysates (Figure 2A).

Second, the CST pY490 antibody detects activated trk from BDNF and NT-3-stimulated cells transfected with trkB and trkC, respectively (H. Ruan, personal communication). Third, treatment of embryonic cortical cells with 100 ng/ml BDNF or NT-3 induces a strong pY490-immunoreactive band at an appropriate molecular size (approximately 145 kD) (Figure 2B top) that co-migrates with a pan-trk immunoreactive band (Figure 2B bottom). Furthermore, pre-incubation of the neurotrophin with 20 μg/ml trkA-Fc, trkB-Fc, or trkC-Fc blocks pY490 immunoreactivity with expected neurotrophin-receptor body specificity (Figure 2B top). Receptor bodies (trk-Fcs) are soluble fusion proteins of trk extracellular domains and human IgG Fc, and would be expected to sequester cognate neurotrophins (as they have been used *in vivo*) [11]. That is, trkB-Fc but not trkA-Fc blocks BDNF-induced pY490 immunoreactivity, and trkC-Fc but not trkA-Fc blocks NT-3-induced pY490 immunoreactivity (Figure 2B top). Untreated cortical cell lysates are not immunoreactive with pY490 (data not shown).

Selection of Seizure Paradigm

Using these pY490 antibodies, an *ex vivo* method was developed to assess phosphorylation of trk receptors following seizures (Figure 3). One important issue remained the selection of seizure paradigm to use in this study. A potential advantage of chemoconvulsant (*e.g.* kainate)-induced status epilepticus (SE) is that it induces intense limbic motor seizures lasting hours, potentially giving a large trk activation signal. By contrast, a single kindling (electrically-induced seizure) stimulation in a naïve animal induces a much more mild seizure (a comparatively short electrographic seizure (ES) with little or no behavioral change) [9]. If there is a seizure duration threshold for trk receptor activation, it is possible that the magnitude of trk phosphorylation would be more robust following SE (which involves hours of seizure activity) than a single ES (which lasts only seconds and may involve just a focal brain area). Both kainate-induced SE and electrical stimulation-induced ESs were employed so that these quite different paradigms could be compared. Furthermore, if a single ES can increase trk receptor activation, it suggests that seizure-induced trk receptor activation occurs during kindling development, tying trk receptor activation to the previous functional studies of effects of trk-Fcs on kindling development [11].

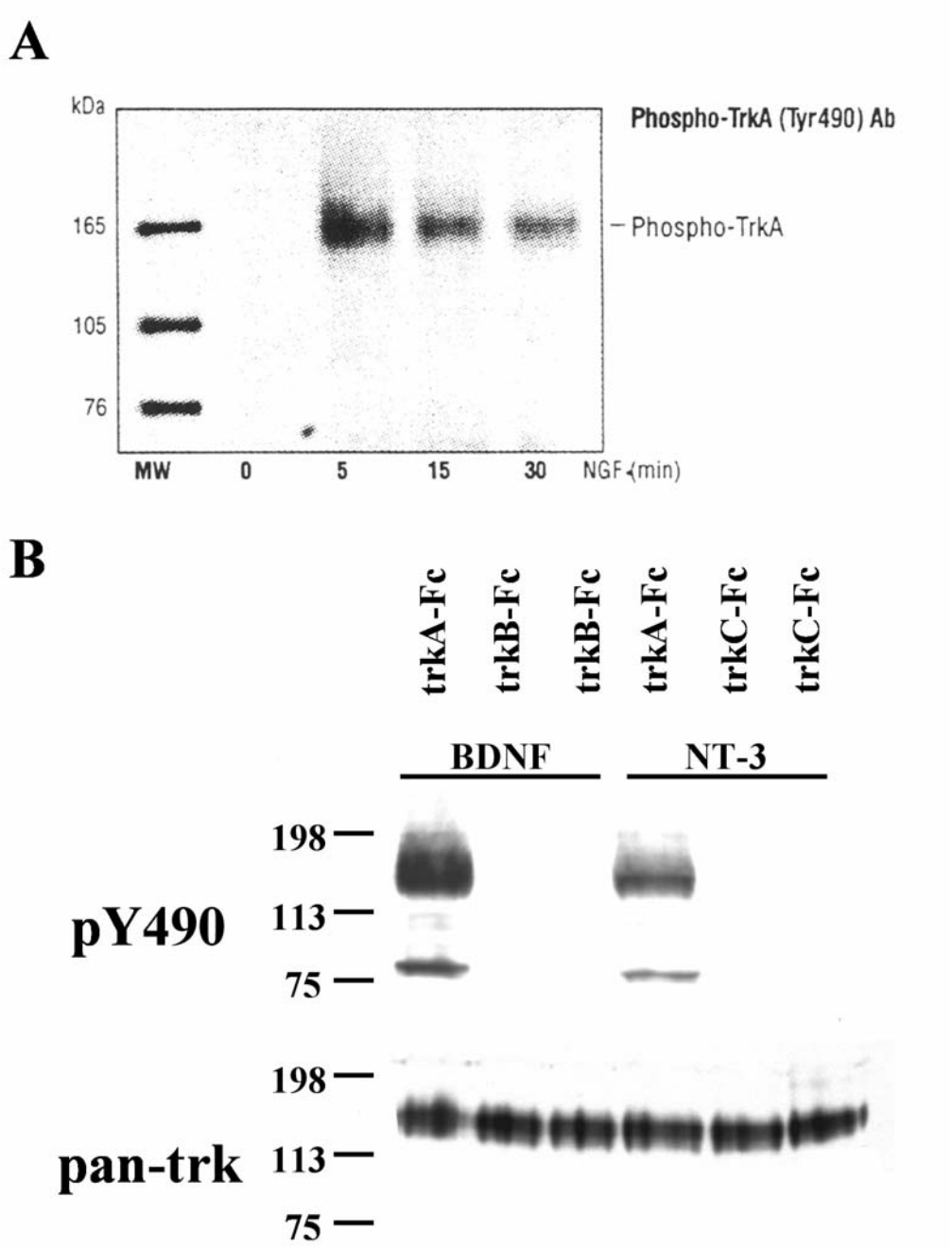

Figure 2. Specificity of CST pY490 antibody on Western blots. A. CST pY490 detects phosphorylated trk from PC12 cell lysates. PC12 cell lysates from untreated cultures or cultures treated with 100 ng/ml NGF. Figure courtesy of CST. B. CST pY490 detects phosphorylated trk from cortical cell lysates. 100 ng/ml neurotrophin was preincubated for 1 hr. with/without 20 μg/ml receptor body (trkA-Fc, trkB-Fc, trkC-Fc). This mixture was incubated with E18 cortical cells (6 DIV) for 5 minutes, and a Western blot using CST pY490 was performed on cell lysates (top), stripped and re-probed with anti-pan-trk antibody (bottom). TrkB-Fc (lanes 2 and 3) but not trkA-Fc (lane 1) blocked BDNF-induced phosphotrk immunoreactivity; and trkC-Fc (lanes 5 and 6) but not trkA-Fc (lane 4) blocked NT-3-induced phosphotrk immunoreactivity. Data courtesy of M. Routbort.

The particular version of stimulation-induced ESs employed was the rapid hippocampal stimulation model developed by Lothman, Lindvall and colleagues in which up to 40 consecutive ventral hippocampal stimulations are given at 5-minute intervals [26, 27, 57, 58, 63]. In contrast to the maximum of 40 stimulations used by Lindvall and colleagues, the present experiments utilized many fewer stimulations, inducing either a single ES or 7 ESs. The fact that the anatomy and time course of BDNF protein upregulation following one or seven ESs in an identical hippocampal stimulation paradigm had already been reported by Elmer *et al.* [27] provided the opportunity for comparison of the anatomy and time course of increases in BDNF protein and phosphotrk immunoreactivity.

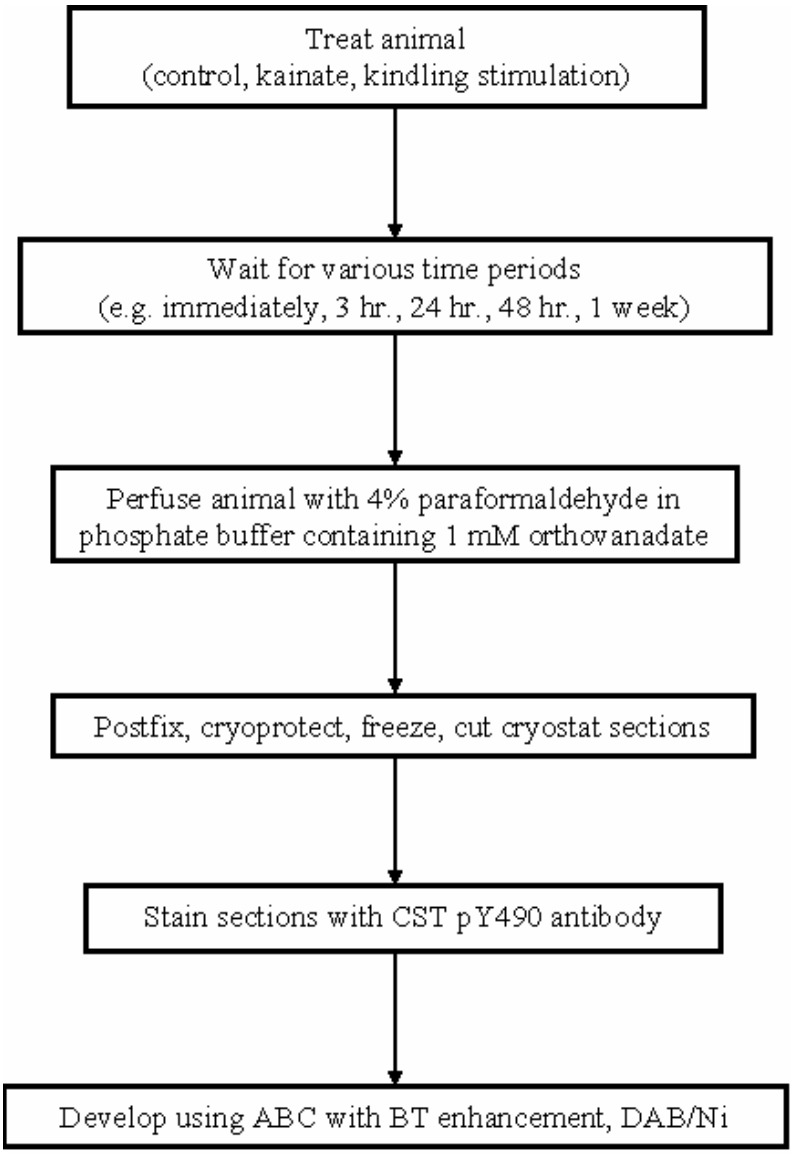

Figure 3. Schematic of phosphotrk immunohistochemical assay. ABC, avidin-biotin conjugate; BT, biotinyl tyramide; CST, Cell Signaling Technology; DAB, diaminobenzidine; Ni, nickel.

Time Points after Seizure

What time points after seizure would be expected to show maximal trk receptor activation? Importantly, the time course of increases in neurotrophin mRNA and protein content appears to be similar whether the stimulus is kainate-induced SE or electrically-induced afterdischarge [27, 76]. Increase in BDNF protein in the rapid hippocampal stimulation model is maximal in dentate gyrus at 12 hours and CA3 at 24 hours, and is back to control levels 1 week after stimulation [27]. This time course is consistent whether 1 stimulation, 7 consecutive stimulations, or 40 stimulations are given [27]. Similarly, other investigators have found maximal BDNF content at about 24 hours after hilus lesion-induced [68] or kainate-induced [76] seizures. Depending on the kinetics of BDNF transport and release, one might expect the maximum time course of phosphotrk immunoreactivity to parallel this BDNF protein time course. In addition, we aimed to determine whether trk receptor activation occurs during or immediately following seizure activity, perhaps reflecting release of preformed neurotrophin [13]. Thus, the following time points were chosen: immediately following seizure activity, 3 hours, 12 hours, 24 hours, 48 hours, and 1 week after kainate or kindling stimulation.

Methods

Phospho-specific trk antibodies (pY490) were obtained by immunizing rabbits with a synthetic phospho-tyr490 peptide (coupled to KLH, keyhole limpet hemocyanin) corresponding to residues 485 to 493 (IENPQY*FSD) of human trkA (**TABLE 1**). Polyclonal antibodies were purified sequentially by protein A chromatography, two rounds of nonphosphopeptide affinity chromatography, followed by elution from a phosphopeptide affinity column. A pY674/5 antibody raised to a dually-phosphorylated peptide (STDY*Y*RVGG, residues 671-679 of human trkA) [89] and purified as above was also employed (gift of CST). The phosphopeptide immunogens and cognate unphosphopeptides were used in peptide competition experiments as described below.

Kainic Acid-Induced Status Epilepticus

250-300 g adult male Sprague-Dawley rats were injected with 15 mg/kg kainic acid i.p. or served as uninjected controls. During the injection period, the animals were observed continuously for tonic-clonic seizure activity. Animals were injected with 5 mg/kg kainic acid each half hour starting one hour after the original 15 mg/kg injection until they exhibited continuous tonic-clonic seizure activity (status epilepticus). Following at least four hours of continuous seizure activity, status epilepticus was terminated with 50 mg/kg i.p. pentobarbital. Animals were sacrificed immediately or at varying intervals (3 hours, 12 hours, 24 hours, 48 hours, 1 week) after pentobarbital treatment.

Hippocampal Kindling Protocol

250-300 g adult male Sprague-Dawley rats were anesthetized with sodium pentobarbital (60 mg/kg) and placed in a stereotaxic frame. Bipolar electrodes made from teflon-coated stainless steel wire were implanted into the right ventral hippocampus (bregma as reference: coordinates –4.8 mm AP, +5.2 mm lateral, 6.5 mm ventral to dura) [71]. Electrodes were firmly secured to the skull with dental cement and anchor screws, and a ground wire was attached to one anchor screw. Animals were allowed to recover for 4 days following surgery before initiation of kindling stimulations.

Each stimulation consisted of a 400 μA 10-Hz 10-second train of 1 msec biphasic rectangular pulses with an interstimulus interval of 5 min. Behavioral (seizure class) and electrophysiologic (electrographic seizure duration, ESD) parameters were recorded for each stimulation. EEG was recorded before, during and for several minutes after each stimulation-induced afterdischarge. Behavioral seizure class was scored according to Racine's classification [73]: Class 0–no behavioral change; Class 1–facial clonus; Class 2–head nodding; Class 3–unilateral forelimb clonus; Class 4–rearing with bilateral forelimb clonus; Class 5–rearing and falling (loss of postural control). Animals were stimulated until either 1 or 7 hippocampal electrographic seizures (ESs) were elicited and then were sacrificed at varying intervals (10 minutes, 3 hours, 12 hours, 24 hours, 1 week). Sham-stimulated animals were treated identically but no stimulation was given.

Perfusion and Histology

At various times after kainate status epilepticus or hippocampal stimulation, animals were perfused intracardially with ice-cold 4% paraformaldehyde in 1X phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate (PBSV) for 5 min. at 50 ml/min. Brains were dissected, postfixed overnight at 4^{o}C in fixative, cryoprotected in 20% sucrose/1X PBV until they sunk, and then frozen in isopentane in a dry ice/methanol bath. 40 μm coronal frozen sections were cut and 2 sections/slide were wet-mounted in PBSV onto Superfrost (Corning) slides, air dried and stored frozen at –70^{o}C.

Phosphotrk Immunohistochemistry

Slides (2 sections/slide) were thawed in room-temperature PBSV (10 min.), endogenous peroxidase activity was quenched with 0.3% H_2O_2/MeOH (30 min.), then slides were washed in PBSV (10 min.), blocked/permeabilized in PBSV/5% normal goat serum/0.5% NP-40 (1 hr.), then washed in PBSV (10 min.). 20 μl 1^{o} antibody (1:10 CST anti-pY490 diluted in PBSV/5%NGS) was applied to each slide, and slides were coverslipped and stored in a humidified chamber at 4^{o}C overnight. For peptide competitions, phosphopeptide immunogen, cognate unphosphopeptide, and unrelated phosphopeptide were incubated at RT with the 1^{o} antibody solution at indicated concentrations for at least 30 min. before application to slides. The following day, coverslips were removed and slides were washed in PBSV/5% NGS (2 x 10 min.), exposed to 2^{o} Ab (1:200 biotinylated anti-rabbit IgG (Jackson ImmunoResearch)

diluted in PBSV/5% NGS) (1 hr.), washed in PBSV/5% NGS (2 x 10 min.), exposed to ABC reagent (Vectastain Elite, Vector) (30 min.), washed in PBSV/5% NGS (2 x 10 min.), exposed to biotinyl tyramide solution (1:100 BT stock solution, Bio-Rad) (30 min.), washed in PBSV/5% NGS (2 x 10 min.), exposed again to ABC reagent (30 min.), washed in PBSV/5% NGS (2 x 10 min.), and developed 10-30 min. in DAB solution containing 0.03% H_2O_2 and 0.04% nickel ammonium sulfate. Slides were then rinsed in PBS, dehydrated in EtOHs, cleared in xylene, and coverslipped with Permount.

Densitometric Quantification

Sections at equivalent coronal levels (-3.60 mm from bregma) [71] from control, kainate-treated, and kindling stimulation-treated animals were analyzed. Nissl-stained alternate sections were used to verify identity of structures. For quantitative analysis of staining intensity, sections from each animal from the hippocampal kindling protocol were analyzed by densitometry. Four hippocampi per animal (one slide per animal containing two adjacent sections each with two hippocampi) were analyzed blinded to treatment. CA3 stratum oriens, pyramidale, lucidum, and radiatum were viewed under a 10× objective using Image-1 (Figure 4B). White and black reference images were obtained, and an identical box was placed at the tip of CA3 to measure the average gray value for each stratum in individual hippocampi. Because the stratum pyramidale had the highest gray value (least immunoreactive), results are presented as % reduction in gray value compared to stratum pyramidale for stratum oriens, lucidum, and radiatum.

Results

Seizure Characteristics

In the ventral hippocampal kindling experiments Seizure behavior during all ESs consisted of wet dog shakes with no to mild (Class 0-2) behavioral change, and no significant behavioral seizure development occurred during the 7 ESs. In addition, electrographic seizure amplitudes were similar. Representative electrographic seizure durations for the animals with 1 vs. 7 ventral hippocampal ESs are shown in Table 2. Electrode placement was verified to be in the ventral hippocampus in all cases. In the kainic acid status epilepticus experiments, all animals experienced at least 4 hours of status epilepticus (continuous class 4 or 5 seizure).

Seizures Increase Phosphotrk Immunoreactivity in Hippocampus

Stimulation-induced seizures increased phosphotrk immunoreactivity in hippocampus. Basal hippocampal phosphotrk immunoreactivity in untreated or sham-stimulated controls was confined to the neuropil whereas the cell body layers (dentate granule cells and CA1-CA3 pyramidal cells) were not immunoreactive (Figure 4D). Twenty-four hours after kainate-

induced status epilepticus (SE) or 7 hippocampal ESs, phosphotrk immunoreactivity was increased in hippocampus (Figure 4F). Furthermore, the increase in hippocampal immunoreactivity was largely confined to the dentate hilus and stratum lucidum of CA3 (Figure 4F) and was quite similar between kainate SE and 7 hippocampal ES groups (Figure 6). The remaining neuropil in hippocampus (e.g. CA3 stratum oriens and stratum radiatum) also appeared slightly more immunoreactive than the control sections (Figure 4F *vs.* 4D). In neither control nor stimulated conditions was immunoreactivity observed on cell bodies of dentate granule cells or CA1-3 pyramidal cells. While this increase in the hippocampus was dramatic and consistent, phosphotrk immunoreactivity did not appear to be altered in other areas of the brain (data not shown). Of course, it is possible that other areas of the brain exhibit increased trk receptor activation following seizures at a lower level or at different time points not detected by the current assay.

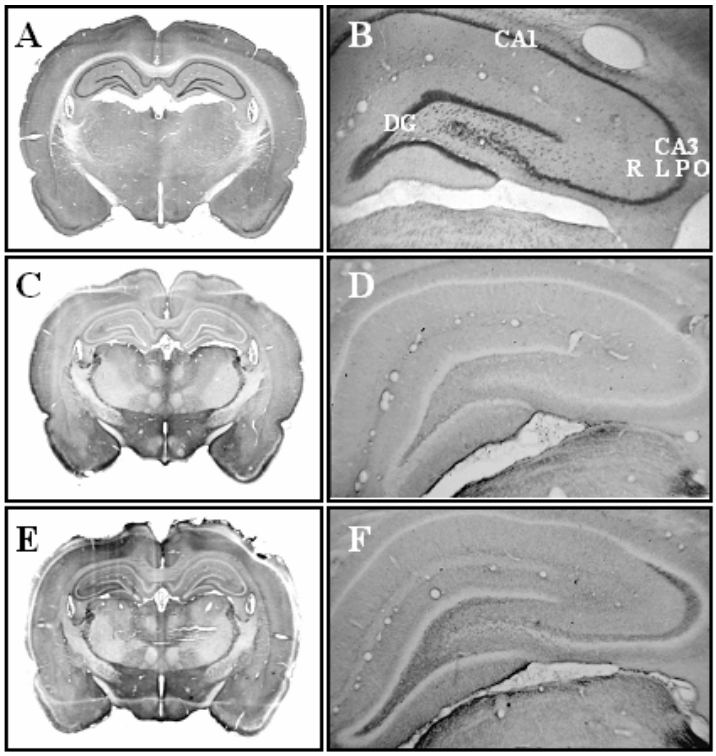

Figure 4. Seizures increase phosphotrk immunoreactivity in hilus and CA3 stratum lucidum. A-B. Nissl-stained coronal section of whole brain (A) and hippocampus (B) showing cell body layers (DG, CA1-CA3). In B, DG=dentate gyrus, CA1=cornu Ammonis 1, CA3=cornu Ammonis 3. Within CA3, R denotes stratum radiatum, L stratum lucidum, P stratum pyramidale, and O stratum oriens (area used for densitometric analysis by stratum). C-D. Phosphotrk immunoreactivity in sham-stimulated animal. Note presence of light immunoreactivity in neuropil of hippocampus (D) but absence in cell body layers. Specific immunoreactivity is also seen at baseline throughout the coronal section (C). E-F. Phosphotrk immunoreactivity in animal 24 hours after 7 ventral hippocampal ESs. Note marked increase in immunoreactivity in dentate hilus and stratum lucidum of CA3 (arrows); remainder of hippocampal neuropil also appears slightly more immunoreactive whereas cell body layers still display absence of immunoreactivity. Panels B, D, F reproduced with permission from Binder *et al.*, *J. Neurosci.* 19:4616-4626, copyright 1999 by the Society for Neuroscience.

Table 2. Relationship between electrographic seizure duration and increases in phosphotrk immunoreactivity in hilus and CA3 stratum lucidum.

Stimulation site	# of seizures	ESD (seconds)	Induction of phosphotrk
Ventral hippocampus	7	338	+
		275	+
		273	+
		272	+
		244	+
	1	71	+
		39	-
		30	-

Individual animals sacrificed 24 hours after 1 or 7 electrographic seizures are shown. ESD=electrographic seizure duration.

Specificity of Phosphotrk Immunoreactivity

Both the basal phosphotrk immunoreactivity in control sections as well as the seizure-induced increase in hippocampal phosphotrk immunoreactivity could be selectively eliminated by preincubation of the pY490 antibody with the phosphopeptide immunogen. Figure 5 shows a series of adjacent sections from the same animal 24 hours after 7 ventral hippocampal ESs processed together. Incubation of the pY490 antibody without any peptide demonstrates the typical pattern of seizure-induced phosphotrk immunoreactivity in hilus and CA3 stratum lucidum (Figure 5A). Pre-incubation with 300 nM of the phosphopeptide 490 immunogen abrogated both basal (not shown) and seizure-induced (Figure 5B) phosphotrk immunoreactivity. In contrast, neither pre-incubation with 300 nM of the corresponding unphosphorylated peptide (Figure 5C) nor pre-incubation with 30 μM of the unrelated tyrosine phosphopeptide 674/5 (see Methods) diminished basal (not shown) or seizure-induced (Figure 5D) phosphotrk immunoreactivity. In addition, omission of the primary antibody was carried out on sections from each animal to verify lack of nonspecific immunoreactivity (not shown).

In addition to the fact that the CST pY490 antibody was validated on Western blots (Figure 2) and with peptide competitions, a distinct phosphotrk antibody was employed in this immunohistochemical assay. The Segal pY490 antibody (Table 1) showed patterns of basal immunoreactivity and seizure-induced increases in stratum lucidum of CA3 similar to the CST pY490 antibody, albeit with much lower signal/noise ratio (data not shown). In addition to the peptide competition experiments described above, the observation that two affinity-purified polyclonal antibodies raised against distinct phosphotrk peptide immunogens seem to display a similar pattern of immunoreactivity supports the hypothesis that the epitope recognized by these antibodies is indeed phosphorylated trk protein.

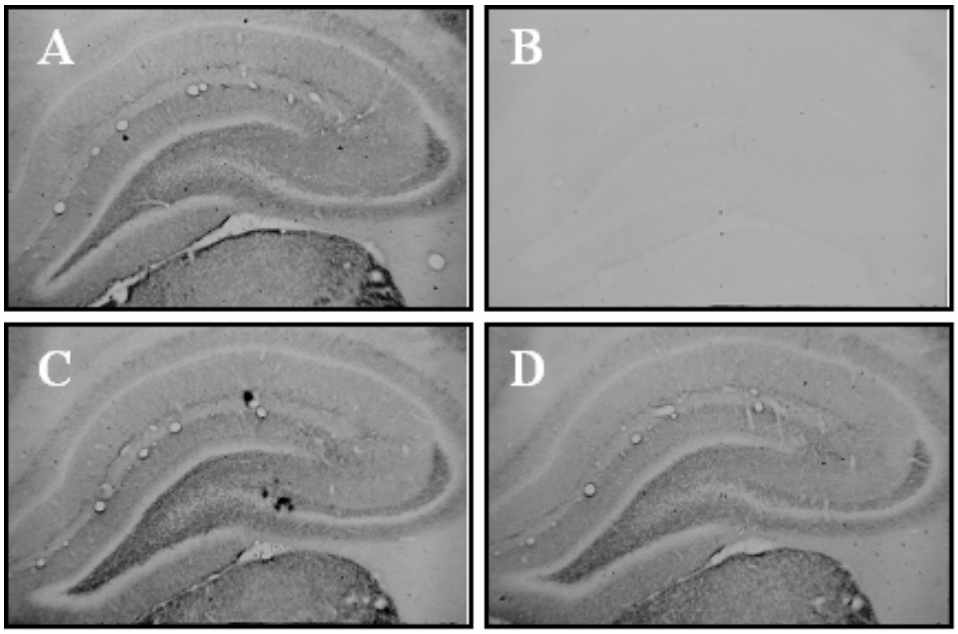

Figure 5. Peptide competition of phosphotrk immunoreactivity. Phosphotrk immunoreactivity in coronal sections of hippocampus from a single animal sacrificed 24 hours after 7 ventral hippocampal ESs. A. No peptide. B. Pre-incubation of pY490 antibody with 300 nM phosphopeptide 490 immunogen. C. Pre-incubation with 300 nM unphosphopeptide 490. D. Pre-incubation with 30 μM phosphopeptide 674/5. Reproduced with permission from Binder *et al.*, *J. Neurosci.* 19:4616-4626, copyright 1999 by the Society for Neuroscience.

Anatomy and Time Course of Phosphotrk Immunoreactivity Following Kainate Status Epilepticus

Sections from animals 3 hours (n=4 rats), 24 hours (n=5), 48 hours (n=4), or 1 week (n=3) after kainate-induced status epilepticus or control animals (n=6) were analyzed to determine the time course of kainate-induced increases in phosphotrk immunoreactivity. While untreated animals showed the typical pattern of phosphotrk immunoreactivity in hippocampal neuropil, sections from animals 24 or 48 hours after kainate uniformly showed dramatic increases in immunoreactivity in hilus and stratum lucidum of CA3 bilaterally (Figure 6). This increase was most obvious in the hippocampus perhaps because of its organized laminar structure. In contrast, sections from animals 3 hours or 1 week after kainate did not appear different from control sections (Figure 6), with the exception that 1/3 of the animals 1 week after kainate demonstrated increases in phosphotrk immunoreactivity in hilus and CA3 stratum lucidum. Thus, kainate-induced status epilepticus leads to a dramatic but transient increase in phosphotrk immunoreactivity in the hippocampus and in particular to the hilus and stratum lucidum of CA3.

Anatomy and Time Course of Phosphotrk Immunoreactivity Following 7 Ventral Hippocampal Electrographic Seizures

Sections from animals 3 hours (n=5 rats), 12 hours (n=5), 24 hours (n=5), or 1 week (n=5) after 7 ventral hippocampal electrographic seizures or control sham-stimulated animals (n=5) were analyzed to determine the time course of rapid hippocampal kindling-induced phosphotrk immunoreactivity. While sham-stimulated animals showed the typical pattern of phosphotrk immunoreactivity in hippocampal neuropil, sections from all 8 animals sacrificed 24 hours after 7 ventral hippocampal ESs uniformly showed dramatic increases in immunoreactivity in hilus and stratum lucidum of CA3 (Figure 7). As with kainate, this increase in immunoreactivity was bilateral and was most obvious in the hippocampus. In contrast, sections from animals at 3 hours, 12 hours, or 1 week time points did not appear different from control sections (Figure 7). Thus, 7 ventral hippocampal ESs consistently led to a dramatic but transient increase in phosphotrk immunoreactivity confined to the hippocampus and in particular to the hilus and stratum lucidum of CA3.

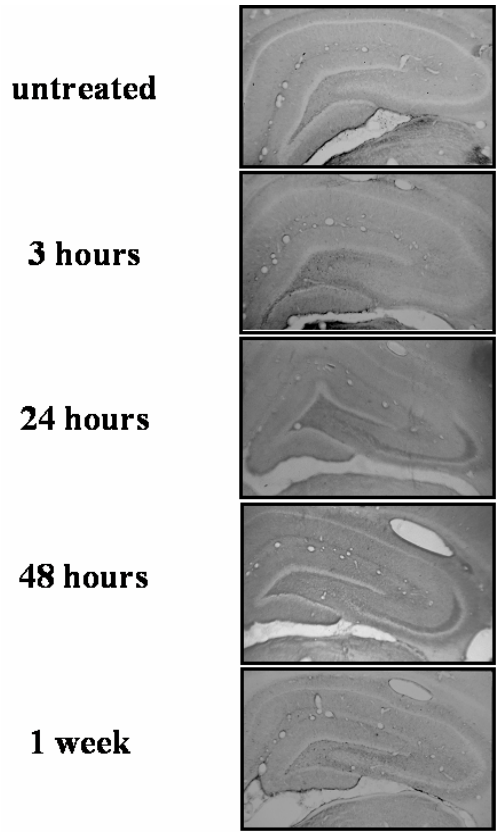

Figure 6. Time course of phosphotrk immunoreactivity in hippocampus following kainate-induced status epilepticus**.** Phosphotrk immunoreactivity in representative coronal sections of hippocampus from uninjected animal and animals sacrificed 3 hours, 24 hours, 48 hours and 1 week following kainate-induced status epilepticus. Note increased phosphotrk immunoreactivity in hilus and stratum lucidum of CA3 at 24 and 48 hours.

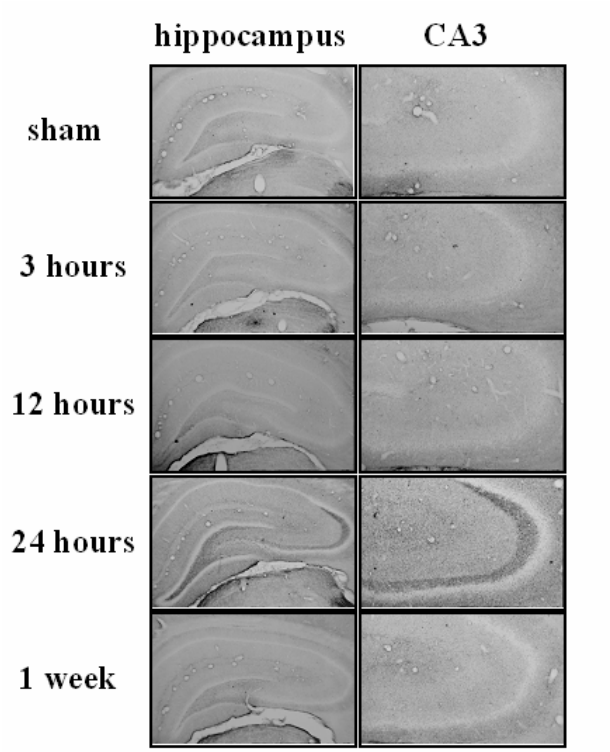

Figure 7. Time course of phosphotrk immunoreactivity in hippocampus and CA3 following 7 ventral hippocampal electrographic seizures. Phosphotrk immunoreactivity in representative coronal sections of hippocampus from sham-stimulated animals and animals sacrificed 3 hours, 12 hours, 24 hours, and 1 week following 7 ventral hippocampal electrographic seizures. Whole hippocampus is shown on left and CA3 region on right. Note temporal (24 hours only) and spatial (hilus and stratum lucidum of CA3) pattern of increase in phosphotrk immunoreactivity. Reproduced with permission from Binder *et al.*, *J. Neurosci.* 19:4616-4626, copyright 1999 by the Society for Neuroscience.

Densitometric Analysis of Phosphotrk Immunoreactivity in CA3 Following 7 Ventral Hippocampal Electrographic Seizures

In order to more quantitatively assess the anatomy and time course of electrographic seizure-induced changes in hippocampal phosphotrk immunoreactivity, densitometric analysis of CA3 in sections from each animal included in the kindling data was performed. Stratum pyramidale, oriens, lucidum, and radiatum were analyzed as described in Methods (above) (Figure 8). Data are expressed as % reduction in gray value compared to stratum pyramidale (see Methods), although absolute gray values showed a similar pattern (data not shown).

Using this measure, the increase in hippocampal phosphotrk immunoreactivity following 7 hippocampal ESs was found to be anatomically and temporally specific. An increase in mean immunoreactivity compared to sham controls was detected only in stratum lucidum and only at the 24 hour time point (Figure 8 top; one-way ANOVA, $p<0.01$). By contrast, mean immunoreactivity at no other time point in any stratum was different from sham controls.

Thus, there is an anatomically and temporally specific increase in phosphotrk immunoreactivity in CA3 stratum lucidum at 24 hours following 7 hippocampal ESs. In addition, there was no difference in the magnitude of seizure-induced phosphotrk immunoreactivity between hippocampi ipsilateral vs. contralateral to the stimulating electrode (data not shown), confirming that the effect is bilaterally symmetric.

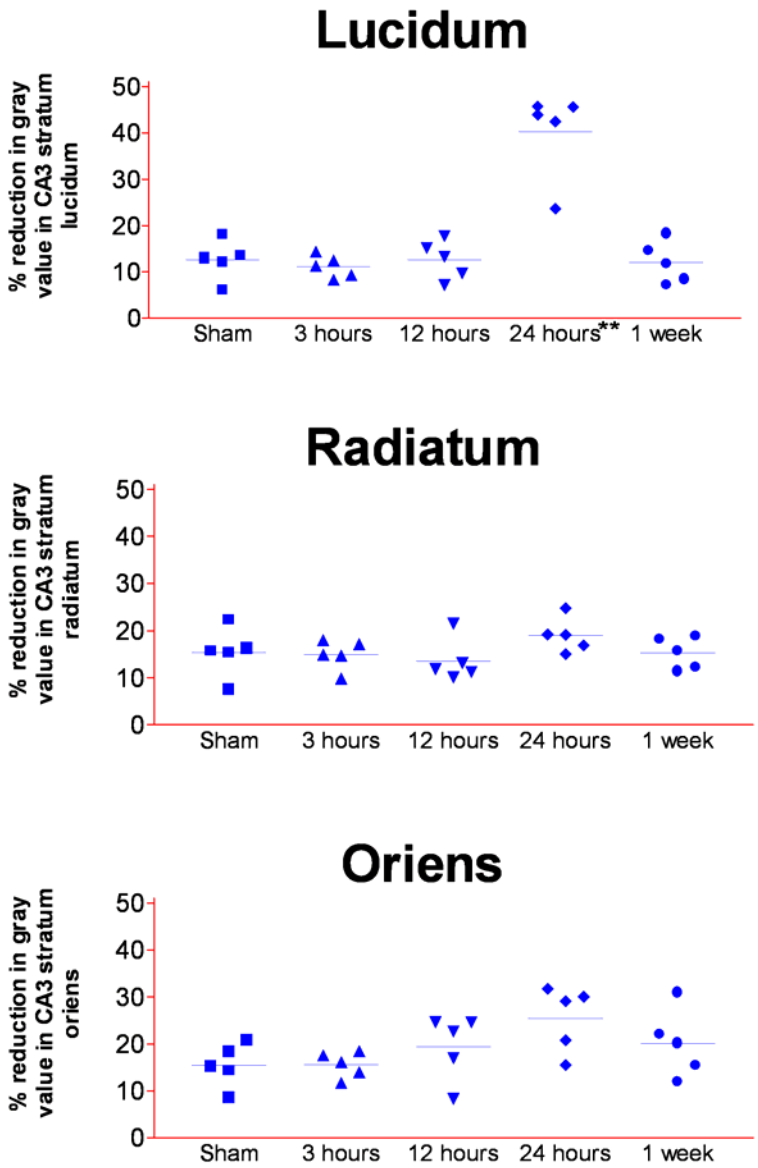

Figure 8. Densitometric analysis of phosphotrk immunoreactivity in CA3 stratum lucidum, radiatum, and oriens following 7 ventral hippocampal kindling stimulations. Data are expressed as % reduction in gray value in given stratum compared to stratum pyramidale (see Methods); thus, higher values reflect more intense immunoreactivity. Each symbol corresponds to one animal. Horizontal lines denote mean values. ** denotes $p<.01$ compared to all other time points by ANOVA with post-hoc Bonferroni's test. Modified with permission from Binder *et al.*, *J. Neurosci.* 19:4616-4626, copyright 1999 by the Society for Neuroscience.

Seizure Duration Threshold of Increase in Phosphotrk Immunoreactivity

The next aim was to determine whether there was an identifiable seizure duration threshold for increases in phosphotrk immunoreactivity using different stimulation parameters. The total electrographic seizure duration of all animals (n=20) exhibiting 7 ventral hippocampal ESs was at least 163 seconds (range 163-368) and the subset of this group sacrificed at 24 hours (n=5) which all displayed increases in phosphotrk immunoreactivity fell within this range (244-338 seconds) (Table 2) (Figure 9). Would fewer or shorter seizures lead to similar increases in phosphotrk immunoreactivity at 24 hours? A single ventral hippocampal ES was evoked in three animals which were sacrificed 24 hours later. Only the animal with the longest seizure (71 seconds) displayed an increase in phosphotrk immunoreactivity in hilus and CA3 stratum lucidum; the other two had seizure durations of 39 and 30 seconds and were phosphotrk-negative (Table 2) (Figure 9).

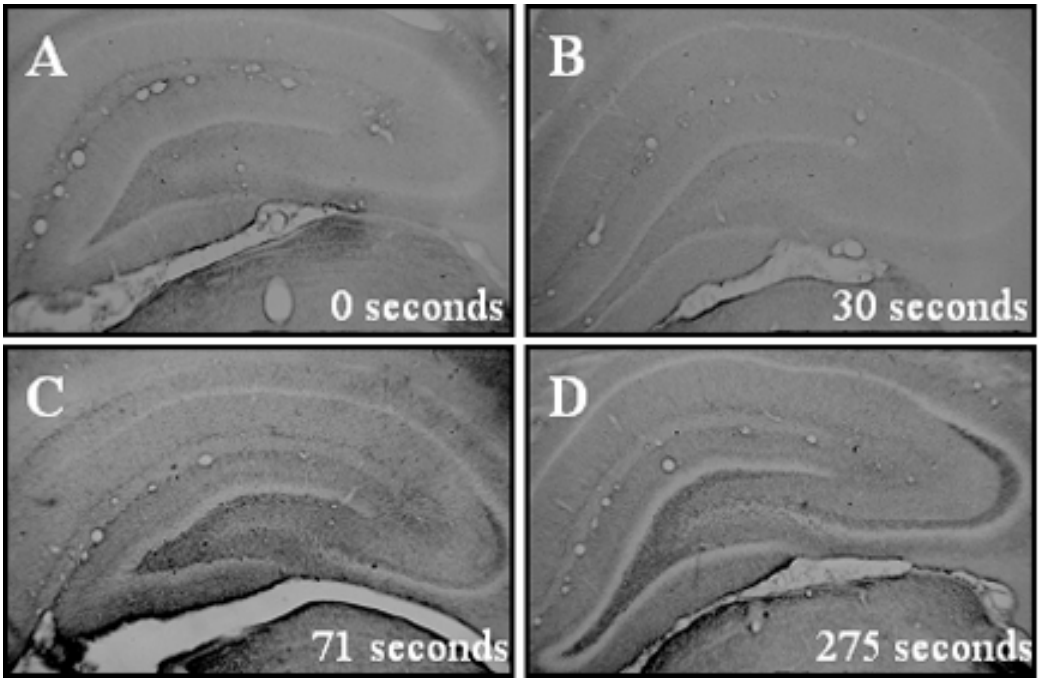

Figure 9. Threshold for increases in phosphotrk immunoreactivity. A. Sham-stimulated animal. B. 24 hours after 1 ventral hippocampal stimulation (ESD: 30 seconds). C. 24 hours after 1 ventral hippocampal stimulation (ESD: 71 seconds). D. 24 hours after 7 ventral hippocampal stimulations (total ESD: 275 seconds).

Phosphotrk Immunoreactivity is not Increased Immediately after Seizure Activity

Recent work demonstrating neurotrophin release following depolarization *in vitro* [12, 13, 37] suggests that release of preformed neurotrophins may occur during seizure activity. If such neurotrophin release is quantitatively important *in vivo*, one might expect to detect

seizure-induced changes in phosphotrk immunoreactivity during or immediately following a seizure. Nevertheless, no changes in phosphotrk immunoreactivity were observed in a single animal sacrificed during kainate status epilepticus, nor in any animals sacrificed 10 minutes after a single dorsal hippocampal kindling stimulation (n=3) (Figure 10). Furthermore, no changes in immunoreactivity were observed 3 hours after 7 hippocampal ESs (n=5; Figure 7). These data do not support the idea of acute neurotrophin release by seizure activity, at least as detected in the current assay.

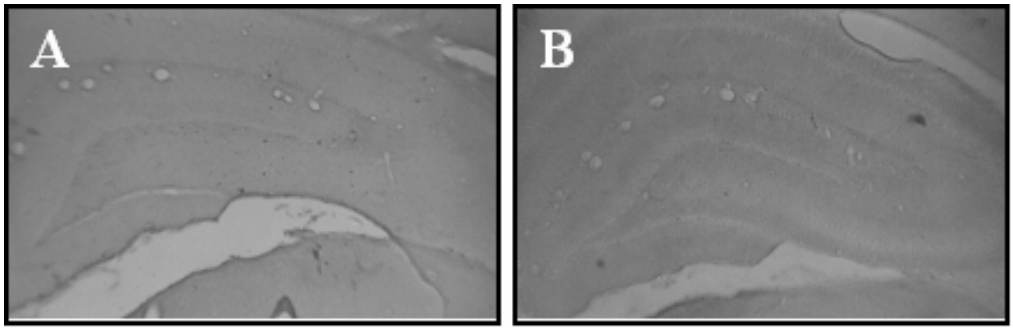

Figure 10. Phosphotrk immunoreactivity is not increased immediately after seizure activity. Coronal sections of hippocampus showing phosphotrk immunoreactivity in animals sacrificed 10 minutes after a single ventral hippocampal electrographic seizure (A) or during kainate status epilepticus (B). In neither condition is there a detectable increase in phosphotrk immunoreactivity compared to controls.

Pattern of Basal Phosphotrk Immunoreactivity

In addition to the pattern in hippocampus previously described, there was a consistent pattern of phosphotrk immunoreactivity in other brain structures (Figure 11). Diffuse immunoreactivity was detected throughout the neocortex with no strong laminar distribution (Figure 11A). Diffusely higher basal immunoreactivity was detected in the amygdaloid complex and piriform cortex, especially in the medial amygdaloid nucleus (asterisk, Figure 11A). Discrete patterns of immunoreactivity were consistently observed in distinct thalamic nuclei, with strong immunoreactivity apparent in lateral geniculate nucleus, reticular thalamic nucleus, and medial habenula (Figure 11B). Diffuse immunoreactivity was also detected in hypothalamus in a uniform pattern sparing white matter tracts (Figure 11C). Intense immunoreactivity in a fiber-like distribution was also observed in portions of the internal capsule, presumably corresponding to axons (Figure 11D). Similar to seizure-induced increases in phosphotrk immunoreactivity, all of the phosphotrk immunoreactivity described here was eliminated by preincubation of the pY490 antibody with the phosphopeptide immunogen but not by the cognate unphosphopeptide or an unrelated phosphopeptide (data not shown).

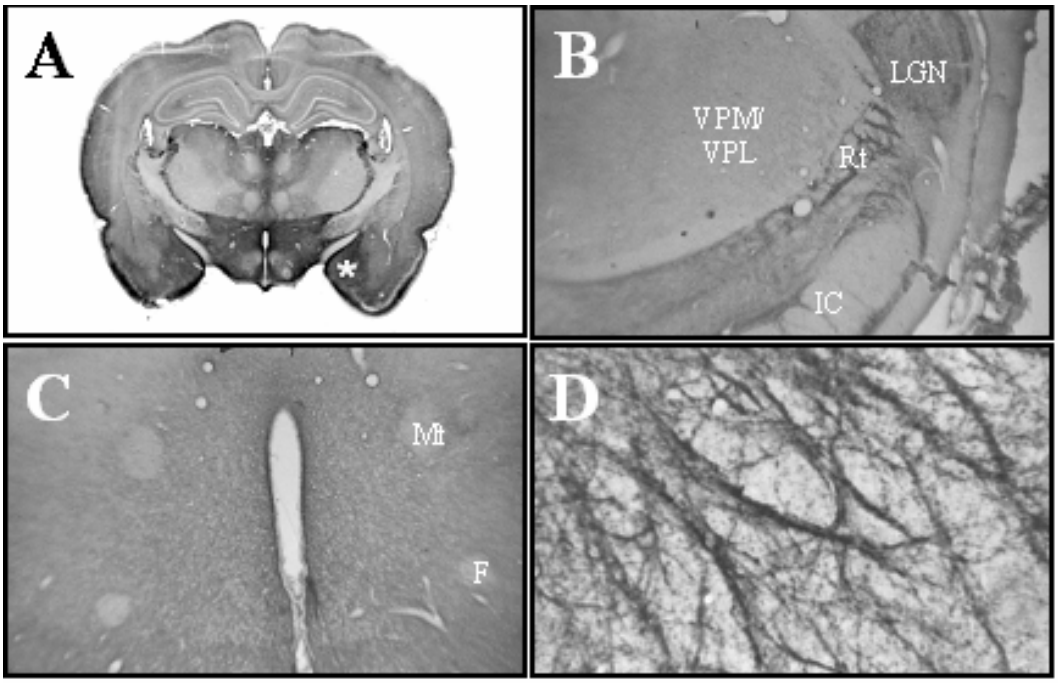

Figure 11. Discrete localization of phosphotrk immunoreactivity in subcortical structures. A. Representative coronal section through forebrain from sham-stimulated animal developed for phosphotrk immunoreactivity. Asterisk denotes medial amygdaloid nucleus. B. Lateral thalamus (10×). Note relatively intense immunoreactivity in lateral geniculate nucleus (LGN) and reticular thalamic nucleus (Rt) compared to ventral posterior medial and lateral thalamic nuclei (VPM/VPL). IC=internal capsule. C. Hypothalamus (10×). Note uniform intense immunoreactivity sparing white matter tracts. F=fornix. Mt=mammillothalamic tract. D. Internal capsule (40×). Note immunoreactivity pattern in fiber-like distribution.

Discussion

Four principal findings emerge from this work. First, kainate-induced status epilepticus or hippocampal electrographic seizures increase phosphotrk immunoreactivity selectively in the hippocampus, primarily confined to the dentate hilus and CA3 stratum lucidum. Second, this seizure-induced phosphotrk immunoreactivity is marked but transient, maximal at 24-48 hours but back to baseline by 1 week. Third, the seizure duration threshold for increase in phosphotrk immunoreactivity appears to correspond to the previously reported threshold for increase in BDNF gene expression. Fourth, phosphotrk immunoreactivity does not appear to be increased immediately following seizure activity. A portion of this work has already been published [10].

Anatomy of Seizure-Induced Phosphotrk Immunoreactivity

Following seizure activity, phosphotrk immunoreactivity was selectively increased in dentate hilus and CA3 stratum lucidum of hippocampus. This distribution precisely coincides with the 'mossy fiber' pathway of dentate granule cell axon terminals. In addition, this anatomic pattern coincides with the distribution of both basal and seizure-induced BDNF protein. Basal BDNF protein is also localized in hilus and CA3 stratum lucidum [20], and seizures increase levels of BDNF protein in dentate gyrus and CA3 [27] and BDNF immunoreactivity in hilus and CA3 stratum lucidum [76, 91, 99, 106]. This precise anatomic colocalization of increased phosphotrk immunoreactivity and increases in BDNF protein suggests that the phosphotrk immunoreactivity is caused by seizure-induced increases in BDNF. BDNF but not NGF is known to increase levels of neuropeptide Y [22], and kindling and kainate-induced seizures increase neuropeptide Y immunoreactivity in hilus and CA3 stratum lucidum [61, 96], further implicating seizure-induced BDNF acting in the mossy fiber pathway. While NGF mRNA content is upregulated by seizures, the anatomic distribution of increased NGF protein is not known. Thus, these anatomic considerations are most consistent with a role for BDNF.

Time Course of Seizure-Induced Phosphotrk Immunoreactivity

The time course of known BDNF upregulation following seizures coincides temporally with increased phosphotrk immunoreactivity. Using hippocampal microdissection and a two-site ELISA for BDNF, Elmer *et al.* showed that after 7 ventral hippocampal electrographic seizures (identical to our protocol), the maximum increase in BDNF protein occurs at 12 hours in dentate gyrus and 24 hours in CA3 [27]. Similarly, maximum increases in BDNF protein following hilus lesion-induced (68) or kainate-induced [76] seizures occur at about 24 hours in hippocampus. Importantly, BDNF protein levels in both of these studies returned to baseline after 1 week, similar to phosphotrk immunoreactivity. In contrast, Bengzon *et al.* found maximal NGF protein content (measured by two-site immunoassay) 7 days after a similar rapid kindling protocol [6] and did *not* see NGF protein increases at earlier time points. Similarly, Lowenstein *et al.* found maximal NGF-like neurotrophic activity of hippocampal extracts from animals one week after KA treatment [59]. Thus, the time course data favor a role for BDNF rather than NGF in seizure-induced phosphotrk immunoreactivity.

Seizure Duration Threshold for Increased Phosphotrk Immunoreactivity

The seizure duration threshold for increase in phosphotrk immunoreactivity further supports a role for BDNF. We observed consistently increased phosphotrk immunoreactivity only in animals with ESD>~70 sec (Figure 9, Table 2). In a similar ventral hippocampal stimulation protocol, Bengzon et al. observed increases in BDNF mRNA content in dentate granule cells in an all-or-none manner above an electrographic seizure duration of about 70

seconds [5]. Like the increases in mRNA content, increases in phosphotrk immunoreactivity appeared to be 'all-or-none' as no differences were noted in intensity of immunoreactivity between kainate-treated and 7 hippocampal ES-treated animals in the present study despite marked differences in seizure duration (hours for kainate vs. seconds for 7 hippocampal ESs). This strong similarity between thresholds as well as all-or-none characteristics suggests that such prior increases in BDNF mRNA content may not only be necessary for any increase in phosphotrk immunoreactivity but also sufficient for maximal increase in phosphotrk immunoreactivity following seizures.

Evidence that the trk Receptor Activated By Seizures is trkB

Indirect evidence suggests that BDNF-induced trkB activation is responsible for the increased phosphotrk immunoreactivity following seizures. First, the mRNA content of NGF and BDNF is increased following seizures [28, 46, 56, 69] whereas dentate granule NT-3 mRNA content is decreased [5, 33, 35, 67, 83]. Second, protein levels of NGF and BDNF increase after seizure activity [6, 27]. Third, the time course data described above implicate BDNF rather than NGF. Fourth, mRNA levels of the other neurotrophin known to activate trkB, NT-4, are very low in adult brain [95] and do not increase after seizures [67]. Fifth, unlike trkB and trkC, levels of expression of trkA in hippocampus are barely detectable [4, 17], suggesting that trkA is unlikely to mediate seizure-induced increases in phosphotrk immunoreactivity.

In order to more directly analyze the role of the trkB receptor in seizure-induced trk receptor activation, He *et al.* studied trk receptor phosphorylation in a mouse mutant with a single point mutation at the shc site (Y490 in humans, Y515 in mice) of the trkB receptor [43]. Homozygous $trkB^{shc/shc}$ (Y515F) mice were generated by Minichiello *et al.* and interestingly display loss of NT-4-dependent neurons but no major effects on BDNF responses [66]. He *et al.* found that following amygdala kindling stimulation, phosphotrk immunoreactivity is increased in wild-type mice in a similar pattern (hilus and CA3 stratum lucidum) to that seen in the rat experiments (above). The $trkB^{shc/shc}$ homozygous mice displayed absence of seizure-induced phosphotrk immunoreactivity, and the heterozygotes displayed intermediate immunoreactivity [43]. These experiments suggest that the trk receptor activated during kindling stimulation is indeed trkB.

Interestingly, the Y515F point mutation had no effect on kindling development in the same study [43]. This is remarkably consistent with the lack of effect of this mutation on synaptic long-term potentiation (LTP) (54). More recently, this group has generated a distinct mouse with point mutation at the PLC site (see Figure 1). Unlike $trkB^{shc/shc}$ mice, $trkB^{PLC/PLC}$ mice exhibit impaired LTP (65). This direct comparison of distinct trkB tyrosine mutants implicates the PLC signaling pathway as opposed to the MAPK pathway in trkB activation-induced synaptic plasticity.

Similarly, other studies have shown that specific stimuli may cause tyrosine-specific phosphorylation of the trkB receptor (*i.e.* at other tyrosines but not at the shc site). For example, Saarelainen *et al.*, in studying the role of endogenous BDNF and trkB signaling in the mechanism of action of antidepressant drugs, found that acute and chronic antidepressant

treatment caused trkB receptor phosphorylation and activation, but the pY674/5 site was selectively phosphorylated compared to the pY490 (shc) site [77]. The further development of phosphorylation state-specific antibodies to distinct tyrosines (pY674/5, pY785) may prove to be of use in dissecting tyrosine site-specific trkB signal transduction *in vivo* in a variety of paradigms. Furthermore, these results can be compared with antibodies that recognize activated intracellular signaling pathways (*e.g.* phosphoCREB) [30].

Cellular Site of Seizure-Induced Phosphotrk Immunoreactivity

What is the likely cellular site of seizure-induced phosphotrk immunoreactivity? The light microscopic distribution of phosphotrk immunoreactivity after seizure (dentate hilus and CA3 stratum lucidum of hippocampus) corresponds to the mossy fiber pathway of dentate granule cell axon terminals. This suggests that the cellular site of phosphotrk immunoreactivity is either on mossy fiber axons and/or targets. Localization on mossy fiber axons represents a parsimonious explanation for both hilar and CA3 stratum lucidum immunoreactivity. In contrast, localization on targets requires immunoreactivity on both targets in hilus (hilar interneurons) and in CA3 stratum lucidum (pyramidal cell dendrites and/or stratum lucidum interneurons).

Recent anatomic studies of trkB-like immunoreactivity may lend insight into the likely cellular site of phosphotrk immunoreactivity. In these experiments, an affinity-purified antibody directed against an extracellular trkB peptide sequence was used, which does not distinguish between full-length and truncated [4] trkB receptors. The earlier studies (using light microscopy) demonstrated that trkB-like immunoreactivity is preferentially distributed on cell bodies and dendrites of both cortical and hippocampal neurons [31, 105]. Pyramidal neurons in hippocampus in particular demonstrate marked trkB immunoreactivity on cell bodies and dendrites in comparison with axons [31, 105]. These studies utilized an antibody raised against the extracellular portion of trkB ($trkB_{23-36}$) common to both full-length and truncated forms. A more recent and comprehensive study of cellular and subcellular localization of trkB immunoreactivity was carried out by Drake *et al*. [24]. These investigators used a cytoplasmic-domain antibody (trkB-in) to selectively label the full-length form of trkB and carried out both light and electron microscopic analysis. Their conclusion was that full-length trkB immunoreactivity exists in glutamatergic granule and pyramidal cells and was most intense in axons, axon terminals, and dendritic spines and to a lesser extent in somata and dendritic shafts. Occasionally, interneurons were also labeled. Thus, phospho-trkB immunoreactivity could represent pre- and/or postsynaptic activation of trkB receptors in the mossy fiber pathway.

Potential Models for Induction of Phosphotrk Immunoreactivity by Seizure Activity

Throughout the brain, BDNF immunoreactivity appears to be preferentially localized in cell bodies and axons compared to dendrites [20]. In addition, unlike the classical target-

derived trophic factor model in which neurotrophins are retrogradely transported, abundant recent evidence suggests that CNS BDNF appears to be anterogradely transported [2, 20, 29, 97, 100, 108]. This evidence, together with the anatomic distribution of BDNF immunoreactivity in hippocampus in a mossy fiber-like pattern, suggests that BDNF protein in hilus and CA3 stratum lucidum was synthesized in granule cell bodies and anterogradely transported to mossy fiber terminals.

Furthermore, following seizures there may be increased anterograde transport of BDNF. First, using hippocampal microdissections of dentate gyrus (which contained hilus) and CA3 (which contained stratum lucidum), Elmer *et al.* showed that maximal BDNF protein levels after seizures were at 12 hours in dentate gyrus but 24 hours in CA3 [27]. This suggests anterograde transport of seizure-induced BDNF protein. More recent evidence regarding the time course of BDNF immunoreactivity following seizures demonstrates that there is increased BDNF immunoreactivity in dentate granule cells at 4 hours followed by subsequent increases in hilus and finally increases in CA3 stratum lucidum at about 24 hours [99] (C. Gall, personal communication). Furthermore, this anterograde 'movement' of BDNF immunoreactivity was abrogated by the axonal transport inhibitor colchicine (C. Gall, personal communication).

These considerations lead to a model in which CA3 stratum lucidum phosphotrk immunoreactivity is a consequence of seizure-induced BDNF release from mossy fiber axons activating trkB receptors on dendrites of CA3 pyramidal cells and hilar interneurons. Supporting a postsynaptic site for trk receptor activation is the evidence that full-length trkB receptors are localized to the postsynaptic density [103]. A somewhat surprising finding given this model, however, is the lack of phosphotrk immunoreactivity during or immediately after seizure activity (Figure 10). If resting BDNF in the mossy fibers is in a releasable pool, one might have expected that seizure activity would lead to immediate release of mossy fiber BDNF onto postsynaptic targets and induction of phosphotrk immunoreactivity. The possibility of a fleeting increase in phosphotrk immunoreactivity in a mossy fiber-like distribution following seizures cannot be excluded. However, these findings raise the possibility that the seizure itself may not trigger BDNF release from mossy fiber axons but rather synthesis, transport, and release of new BDNF in sufficient quantities to lead to detectable trk receptor activation with a latency of approximately 24 hours.

Other models include autocrine release of BDNF from mossy fibers and activation of mossy fiber terminal trkB receptors, the analogous possibility for CA3 pyramidal cell dendrites, release of BDNF from CA3 dendrites onto mossy fiber terminal trkB receptors, or effects on other cell types. Indeed, dendritic BDNF mRNA targeting may underlie another potential cellular mechanism for BDNF translation, release, and trk receptor activation [90]. Determining the ultrastructural distribution of phosphotrk immunoreactivity will be necessary to distinguish these possibilities.

Since the other primary target of mossy fiber axons in CA3 is dendrites of stratum lucidum interneurons [92], it is possible that phosphotrk immunoreactivity in stratum lucidum could reflect activation of trk receptors on interneurons as well as CA3 pyramidal cell dendrites. Indeed, quantitative analysis of mossy fiber targets in CA3 suggests that the number of synaptic contacts onto GABAergic interneurons vastly outnumbers those onto CA3 dendrites [1]. Indeed, any interneuron with dendrites traversing stratum lucidum could

be a target of mossy fiber axons. However, it is unclear whether functional trkB receptors exist on stratum lucidum interneurons, as *in situ* hybridization studies show trkB mRNA localization predominantly in granule and pyramidal cells of hippocampus [5] and only occasional interneurons were found to be trkB-immunoreactive in the EM study (24).

Furthermore, recent evidence indicates that activated trk receptors may be endocytosed and retrogradely transported while still tyrosine phosphorylated [7, 38, 75, 88, 101]. Therefore, mossy fiber-like phosphotrk immunoreactivity could in part reflect not only distal synaptic sites of trk activation but also in-progress retrograde transport of activated trk from CA3 within the mossy fibers. Thus, the increase in phosphotrk immunoreactivity observed in the dentate hilus may represent activated trk from mossy fiber terminals in hilus or CA3. However, the hypothesis of retrograde transport of tyrosine-phosphorylated trk within the mossy fibers would predict that phosphotrk immunoreactivity might increase first in CA3 stratum lucidum and later in hilus and perhaps reach the dentate granule cell bodies, which was not observed given the time points examined.

Comparison of Basal Phosphotrk Immunoreactivity to trkB-like Immunoreactivity

The basal distribution of activated trk receptors in the adult brain is of interest based on recent detailed descriptions of BDNF and trkB protein distributions [20, 105-107]. Precise immunohistochemical distributions of NGF, NT-3, NT-4, trkA, and trkC are not known. Basal phosphotrk immunoreactivity appears to correspond to a subset of the distribution of trkB-like immunoreactivity [105, 107]. This may be due to the fact that the trkB antisera used in these studies recognized both the full-length and truncated forms of the trkB receptor, to recognition of trkA and trkC by pY490, and/or to relatively low levels of basal trk receptor activation in certain structures. Phosphotrk immunoreactivity in neocortex is widespread and diffuse, generally similar to the distribution of trkB protein [105]. Phosphotrk immunoreactivity in amygdala and piriform cortex (Figure 11) was more intense than neocortex. In thalamus, the most strongly phosphotrk-immunoreactive nuclei are the lateral geniculate nucleus (LGN), reticular thalamic and medial habenula (Figure 11); interestingly, while both of these nuclei are strongly immunoreactive for trkB protein [14, 105, 107], other thalamic nuclei are also similarly trkB-immunoreactive but relatively less phosphotrk-immunoreactive. It is conceivable that ongoing physiologic activity in the visual system that is known to increase BDNF mRNA content [16] may maintain LGN BDNF activity [20] and thus a certain basal level of trk receptor activation (Figure 11), an idea which could be tested by examining the effect of dark-rearing on phosphotrk immunoreactivity in LGN and visual cortex.

Summary

In summary, these data demonstrate that trk receptors undergo activation in dentate hilus and CA3 stratum lucidum in adult brain (the ‘mossy fiber pathway’) following seizure

activity. Trk receptors are activated in an anatomic distribution corresponding to BDNF protein; with a time course consistent with the time course of maximum BDNF content; and with an ESD threshold consistent with the requirement for BDNF gene regulation [10]. Furthermore, a Shc site mutation in mouse trkB abolishes seizure-induced phosphotrk immunoreactivity [43]. These findings imply that the phosphotrk immunoreactivity reflects activation of trkB receptors by seizure-induced BDNF. Since BDNF has been functionally implicated in kindling epileptogenesis, trk receptor activation in hilus and CA3 stratum lucidum may be a critical molecular step in kindling development. It is hoped that the phosphotrk immunohistochemical assay will be of use in defining the anatomy and time course of trk receptor activation in other seizure paradigms and in response to diverse physiological stimuli.

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