Published in final edited form as:

Epilepsia. 2011 September ; 52(9): 1656–1665. doi:10.1111/j.1528-1167.2011.03113.x.

Homeostatic increase in excitability in area CA1 after Schaffer collateral transection *in vivo*

Céline Dinocourt^{1,3}, Stephanie Aungst^{1,2}, Kun Yang¹, and Scott M. Thompson^{1,2}

¹Department of Physiology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD, 21201

²Program in Neuroscience, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD, 21201

SUMMARY

Purpose—Epilepsy is a significant long-term consequence of traumatic brain injury (TBI) and is likely to result from multiple mechanisms. One feature that is common to many forms of TBI is denervation. We asked whether chronic partial denervation *in vivo* would lead to a homeostatic increase in the excitability of a denervated cell population.

Methods—To answer this question, we took advantage of the unique anatomy of the hippocampus where the input to the CA1 neurons, the Schaffer collaterals, could be transected *in vivo* with preservation of their outputs and only minor cell death.

Key findings—We observed a delayed increase in neuronal excitability, as apparent in extracellular recordings from hippocampal brain slices prepared 14 days (but not 3 days) postlesion. Although population spikes in slices from control and lesioned animals were comparable under resting conditions, application of solutions that were mildly proconvulsive (high K⁺, low Mg²⁺, low concentrations of bicuculline) produced increases in the number of population spikes in slices from lesioned rats, but not in slices from unlesioned sham controls. Denervation did not produce changes in several markers of GABAergic synaptic inhibition, including the number of GABAergic neurons, $\alpha 1$ GABA_A receptor subunits, the vesicular GABA transporter, or miniature inhibitory postsynaptic currents.

Significance—We conclude that chronic partial denervation does lead to a delayed homeostatic increase in neuronal excitability, and may therefore contribute to the long-term neurological consequences of traumatic brain injury.

Keywords

traumatic brain injury; axonal injury; hippocampus; lesion; plasticity

Roughly 1.5 million people suffer traumatic brain injury (TBI) annually in the US. TBI and its sequelae are an ‘invisible epidemic’ in which patients suffer life-long cognitive, sensory, emotional, and motor deficits. Acute consequences of TBI include blood loss, hemorrhaging, thrombosis, infection, and edema. These acute complications can be

Correspondence should be addressed to: Scott M. Thompson, Department of Physiology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD, 21201, sthom003@umaryland.edu.

³Current address: IRSN, Laboratoire de RadioToxicologie expérimentale, Service de RadioBiologie et Epidémiologie Direction de la RadioProtection de l’Homme, 92262 Fontenay aux Roses cedex, France

Disclosure: None of the authors has any conflict of interest to disclose. The authors confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

managed with timely clinical care, but the long-term consequences leave TBI victims disabled.

Posttraumatic epilepsy (PTE) is one common long-term consequence of TBI, particularly after injuries involving dural penetration (Majokowski, 1990). PTE is characterized by a latent period lasting from months to years between the injury and the first seizure (Annegers *et al.*, 1998; Agrawal *et al.*, 2006). Following TBI, some slow processes are initiated that lead to a persistent state in which the threshold for seizures is lowered. A better understanding of the elementary steps in this reactive plasticity will provide insights into how to minimize the long-term adverse consequences of TBI.

Hyperexcitability occurs in several animal models of TBI (D'Ambrosio *et al.*, 2004; Nilsson *et al.*, 1994; Kharatishvili *et al.*, 2006; Lowenstein *et al.*, 1992; Coulter *et al.*, 1996; Reeves *et al.*, 1997; Toth *et al.*, 1997; Santhakumar *et al.*, 2000; 2001). These models reproduce the correlation between brain injury and hyperexcitability, but are often accompanied by considerable neuronal injury. The complexity of these models, however, makes it difficult to identify causal mechanisms.

Here we focus on one consequence of lesions that should be common to many forms of TBI: *chronic neuronal denervation*. In many head injuries axons are irreparably damaged, either through the direct physical trauma of a penetrating head wound or indirectly because of axonal shearing and stretching due to rapid acceleration and deceleration (Povlishock & Katz, 2005). Head injuries can also result in a complete or partial loss of the normal inputs to large populations of neurons due to degeneration of severed and injured axons, combined with direct and secondarily triggered (excitotoxicity, apoptosis) death of populations of cells that are presynaptic to a given region.

Neurons in many brain regions display “homeostatic” plasticity, in which cells respond to changes in the level of their activation so as to stabilize their function (Turrigiano, 1999; 2007; 2008; Turrigiano and Nelson, 2004). Most studies of homeostatic plasticity *in vivo* involve adaptive changes that compensate for developmental alterations in synapse number or strength. Maladaptive homeostatic plasticity may make an important contribution to the genesis of neurological disease, however. In the current study, we asked whether chronic denervation would result in a homeostatic increase in the activity of affected cell populations. We had addressed this issue previously using cultured hippocampal slices and observed that transection of Schaffer collateral pathway results in hyperexcitability in area CA1 (Cai *et al.*, 2007). TBI *in vivo* is more complex than *in vitro*, however, making it important to determine whether Schaffer collateral transection *in vivo* in the adult would also result in delayed increases in excitability.

MATERIALS AND METHODS

Schaffer collateral transection in vivo

All procedures involving animals were approved by the University of Maryland School of Medicine IACUC. Adult (300 – 400g) Sprague-Dawley rats were anesthetized with ketamine (40–80mg/kg)/xylazine (5–10mg/kg) mixture. The animal's were immobilized in a stereotaxic frame, a scalp incision was made, and the skin and fascia were retracted. Two holes (ca. 3 × 6mm) in the skull were made over the dorsal surface of the brain. A Ziegler ophthalmic scalpel (3mm in width), mounted on a stereotaxic carrier, was inserted into the brain to a depth of 2.5mm from the cortical surface, at a starting position 4.5mm caudal from bregma and 4mm lateral from the midline. The scalpel was left in place for 5 min before transecting the Schaffer collateral pathway along ca. one third of the rostro-caudal extent of the hippocampus (Fig. 1A) by moving the scalpel simultaneously 2.5mm rostrally, 2mm

medially. The scalpel was then left in place for 5 min before being withdrawn slowly. The procedure was then repeated in the other hemisphere. Aged matched, sham-operated control rats were treated in the same way, including the opening of the skull, but the dura was left intact and no lesion was made.

Hippocampal slice preparation

Sham-operated age matched and lesioned rats were anesthetized with Nembutal (50mg/kg, i.p.), decapitated, and the brain was placed in cold oxygenated artificial cerebrospinal fluid (ACSF), composed of (in mM): 125 NaCl, 2 KCl, 26 NaCO₃, 2 CaCl₂, and 2 MgCl₂, titrated to pH 7.4 by bubbling with 95% O₂ - 5% CO₂. 400µm-thick hippocampal sections were cut using a vibratome, incubated for at least 1 hour at room temperature (RT) before being transferred to the recording chamber.

Electrophysiological recording

Slice were continuously submerged in room temperature ACSF composed of (in mM): 125 NaCl, 5 KCl, 26 NaHCO₃, 3 CaCl₂, and 1 MgCl₂ and saturated with 95% O₂-5% CO₂. Extracellular recordings of population spikes were obtained using ACSF-filled micropipettes placed in *stratum (str.) pyramidale* of area CA1 at a distance of ca. 400–800 µm from the lesion. Responses were evoked via a concentric electrode placed in *str. radiatum* at a distance of ca. 50–100 µm from the lesion. A constant voltage stimulus (amplitude 1 – 20 V, duration 100 µs) was applied at 0.1 Hz. Voltage signals were amplified 100-fold and low pass filtered at 1 kHz (npi Electronics, Tamm, Germany) and digitized with Clampex and analyzed using Clampfit (pClamp 9.0, Molecular Devices).

To compare the population burst intensity in different conditions, we quantified the waveform by calculating a modified coastline bursting index (CBI) (Korn *et al.*, 1987). A 100 ms long epoch of the evoked response in control saline was subtracted digitally from the response of the same slice in the presence of test solution (8 mM K⁺-containing saline, Mg²⁺-free saline, or saline containing 0.1 µM bicuculline). The CBI of the resulting difference trace was then calculated as the sum of the point-to-point voltage differences during the length of the epoch. Paired-pulse ratios were determined with the stimulation intensity set to produce population spikes of half the maximum amplitude. Ratios were computed as the amplitude of the response to the second stimulus divided by the amplitude of the response to the first stimulus.

Whole-cell voltage-clamp recordings of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were made in ACSF, as above, to which was added 0.5 µM tetrodotoxin (TTX). Recording pipettes (3–6 mΩ) contained (in mM): CsCl 110, HEPES 10, MgCl₂ 1, BAPTA 10, Mg²⁺-ATP 3.6, and phosphocreatine 10, and was adjusted to pH 7.2 with CsOH. For each cell, mean amplitude and frequency were determined from continuous segments (150 – 700 mIPSCs per cell). Recordings and mIPSC analyses were performed without knowledge of the condition of the animal.

Tissue preparation for light microscopy

Anesthetized rats were perfused transcardially with fixative containing 4% paraformaldehyde in 0.12 M sodium phosphate buffer, pH 7.4 (PB). After perfusion, brains were postfixed in the same fixative for one hour at RT and rinsed, then cryoprotected in a solution of 20% sucrose in PB overnight at 4°C, quickly frozen on dry ice and sectioned coronally at 40 µm on a cryostat. Sections were rinsed in 0.1 M phosphate-buffered saline (PBS), collected sequentially in tubes containing an ethylene glycol-based cryoprotective solution (Watson *et al.*, 1986; Lu & Haber, 1992), and stored at –20°C until histological processing.

Neuronal degeneration

The Fluoro-jade B method was performed as described by Schmued and Hopkins (2000). Briefly, sections were rinsed in 0.12 M PB, mounted on silanated-coated slides and air-dried on a slide warmer at 50°C overnight. Sections were then immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 minutes, re-hydrated quickly, incubated in 0.06% potassium permanganate for 10 minutes, rinsed in distilled water and incubated in a solution of 0.0004% Fluoro-jade B (Histo-Chem Inc., Jefferson, AR, USA). Sections were then rinsed in distilled water, dried at 50°C for 5–10 minutes, immersed in xylene and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry

All free-floating sections to be processed for NeuN and GAD67 immunohistochemistry were first rinsed in 0.02 M potassium PBS (KPBS; 16 mM K₂HPO₄, 3.5 mM KH₂PO₄, 150 mM NaCl, pH 7.4), incubated in 1% hydrogen peroxide for 30 minutes to block endogenous peroxidases and rinsed. Labeling for NeuN and GAD67 was performed with unlabeled primary antibodies and standard avidin—biotin—peroxidase methods (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight at RT in NeuN or GAD67 monoclonal antibody (1:50,000 and 1:4,000, MAB377 and MAB5406, Chemicon, Millipore, Billerica, MA). Sections were rinsed and incubated for 1 hour at RT in biotinylated horse anti-mouse immunoglobulin G (1:200, Vector Laboratories). After several rinses, sections were then incubated for 1 hour at RT with an avidin—biotin—peroxidase solution prepared according to manufacturer's recommendations. Free-floating sections for vGAT immunohistochemistry were rinsed then incubated for 1 hour at RT in KPBS containing 0.3% Triton X-100 and 1% normal donkey serum. Labeling for vGAT was performed with unlabeled primary polyclonal antibody (1:5000, V5764, Sigma, St. Louis, MO) overnight at RT in KPBS containing 1% NDS. Sections were then rinsed several times and incubated with a CY3 fluorescently tagged donkey anti-rabbit secondary for 1 hour at RT. Sections were then rinsed, mounted on slides, dried, dehydrated, and coverslipped.

Quantitative analysis was performed using Neurolucida software (MicroBrightField, Williston, VT) by an investigator who was blind to the experimental group treatment. The number of NeuN positive neurons in *str. oriens* and *str. radiatum/lacunosum moleculare* of area CA1 was counted using a microscope equipped with a 3D motorized stage and a 20× objective. The total number of NeuN-positive neurons and the area were estimated separately for each region (7 sections per animal). The average total number of neurons and the mean area ± SEM were calculated for each group of sham (n = 3) and lesioned (n = 3) rats. The ratios (number of NeuN- or GAD67-positive neurons/mm²) were analyzed statistically with a mixed model analysis of variance (ANOVA) and Student's test to determine significant differences in the number of neurons between sham-operated control and lesioned rats.

Western blot

Area CA1 was dissected out of control and lesioned hippocampal slices (3–4), pooled, homogenized in cold lysis buffer (Laemmli, Sigma), boiled, and loaded into 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Multiple control lanes were included in most experiments. After running in 1X NuPage MOPS SDS running buffer, gels were transferred onto polyvinylidene difluoride membranes in 1X Nupage transfer buffer (in 10% methanol). The membrane was then probed with antibodies against the $\alpha 1$ subunit of the GABA_A receptor (06-868, Upstate Millipore, 1:3000) and the vesicular GABA transporter, vGAT (V5764, Sigma, 1:1000) at 4°C overnight. After rinses in TBS-Tween, the membranes were incubated for 1 hr at RT in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Sigma). The immunoblot was developed with enhanced chemiluminescence (Amersham,

Piscataway, NJ). Membranes were then stripped, blocked, and re-probed with antibodies against GAPDH (G8795, Sigma, 1:2000). Protein levels were determined from densitometry of exposed films and used for statistical analysis.

RESULTS

Selective neuronal damage and reactive gliosis after the Schaffer collateral transection

Many forms of TBI are characterized by an initial wave of cell death (Mattson & Scheff 1994; D'Ambrosio *et al.*, 2004; Williams *et al.*, 2006). In order to determine the extent of cell death produced by the transection, we examined Nissl stained sections. We observed that the lesions extended from the pial surface to the hippocampal fissure (Fig. 1C,D) along the border between areas CA3 and CA1 over a significant length of the rostro-caudal extent of the lesion (~2 mm, Fig. 1A). We initially controlled for the effectiveness of the lesion using electrophysiological techniques (Fig. 1B). In hippocampal slices prepared 24 hrs after the lesion, local stimulation within area CA1 elicited a field excitatory postsynaptic potential (fEPSP), as recorded with an extracellular electrode placed in *str. radiatum* of area CA1. When the stimulating electrode was repositioned on the distal side of the lesion, within area CA3, in contrast, no fEPSP was elicited. These results demonstrate that the lesion effectively transected the Schaffer collateral inputs to area CA1.

Evidence of modest local hemorrhage along the scalpel track was apparent. We observed distinct changes to neurons and glial cells that were confined to the lesion site at 24 and 48 hours after the lesion. Amidst morphologically normal cells, some shrunken cells undergoing chromatolytic changes and some small mononuclear glial-like cells with scanty cytoplasm were observed surrounding the lesion (Fig. 1C,D). Adjacent to the lesion was a zone of glial-like cells forming a thin band. Outside of this band, neurons appeared well preserved in areas CA1 and CA3, the dentate gyrus, and the hilus.

We further examined the anatomical changes in the hippocampus by using Fluoro-jade B staining of neuronal degeneration (Schmued & Hopkins, 2000). Fluorescently labeled pyramidal shaped cells were apparent at both edges of the lesion for a distance of ca. 100–200 μ m in the CA1 *str. pyramidale* when tissue was taken 24 hours after the lesion (Fig. 1E,F). In addition, many fluorescent processes, presumably degenerating dendrites, were evident in the *str. radiatum* and *str. lacunosum moleculare*, as well as fluorescent puncta, presumably degenerating axon terminals and fibers, in *str. oriens*. No damaged neurons were apparent in more distant areas of *str. pyramidale*, the dentate granule cell layer, or the hilus in the dentate gyrus. No Fluoro-jade B labeling was observed at times >7 days after the lesion.

We conclude that Schaffer collateral transection *in vivo* induced limited neuronal death and gliosis that was confined to the vicinity of the lesion.

Effects of Schaffer collateral transection on excitability in *ex vivo* hippocampal slices

Schaffer collateral transection in hippocampal slice cultures results in a delayed increase in excitability (Cai *et al.*, 2007). Does Schaffer collateral transection *in vivo* also lead to chronic hyperexcitability of the partially denervated cell population? In order to test this hypothesis, we prepared *ex vivo* hippocampal slices from animals lesioned 1–21 days previously and from sham controls. Population spikes in *str. pyramidale* of area CA1 were recorded in response to local stimulation within *str. radiatum*. We observed graded field excitatory postsynaptic potentials (fEPSPs) and, with sufficiently strong stimulation, a single population spike in all slices from sham animals (Fig. 2A–C). At 2 weeks after the lesion, local stimulation in *str. radiatum* in the vicinity of the lesion produced oscillatory field potentials comprised of 1 or 2 population spikes (mean: sham = 1.0 ± 0 , lesion = $1.25 \pm$

0.16; n.s.; n = 10 slices from 5 animals, 8 slices from 4 animals, respectively). These results indicate that the lesioned tissue was not frankly epileptic but perhaps increased in excitability.

In lesioned hippocampal slice cultures, increased excitability is revealed by manipulations that weakly perturb the balance between excitation and inhibition (McKinney *et al.*, 1997). We therefore recorded extracellular field potentials in the *str. pyramidale* of slices from lesioned and sham-operated control rats in response to challenges with three different conditions at 2 weeks after the Schaffer collateral transection: an increase in the K⁺ concentration in the extracellular saline from 4 to 8 mM (Rutecki *et al.*, 1985; Traynelis & Dingledine, 1988; McBain, 1994), removal of Mg²⁺ from the extracellular saline (Thomson and West, 1986), or application of a very low concentration of the GABA_A receptor antagonist bicuculline methochloride (0.1 μM). In order to assay the effects of the various conditions, we quantified the number of population spikes elicited with local stimulation in *str. radiatum* and the change in the coastline bursting index (CBI) (Korn *et al.*, 1987). The CBI is sensitive to changes in the number or amplitude of population spikes, and increases when the number of neurons firing action potentials increases or the number of action potentials elicited increases. All three manipulations resulted in a significant increase in the number of population spikes and a significantly greater change in CBI in slices obtained from lesioned rats compared to the slices obtained from sham-operated control animals (Fig. 2A–D).

A hallmark of human posttraumatic epilepsy is the delay between the injury and the occurrence of the first seizures. Consistent with this clinical feature, hyperexcitability after Schaffer collateral transection *in vitro* is observed after a delay of 7 days (McKinney *et al.*, 1997; Cai *et al.*, 2007). We therefore examined the excitability of *ex vivo* hippocampal slices taken from animals in which the Schaffer collateral pathway had been lesioned 1–3 days earlier. No significant difference in the number of population spikes in response to single stimuli or in the change in the CBI between lesioned (n = 5) and sham-operated control animals (n = 5) in control solution or in proconvulsive conditions (Fig. 2E,F) was observed at this shorter time point.

We conclude that the excitability of area CA1 is increased subtly with a delay of several days after chronic partial deafferentation produced by Schaffer collateral transection *in vivo*.

Effects of Schaffer collateral transection on GABA-mediated inhibition

Selective death of GABAergic interneurons has been implicated in the genesis of several forms of chronic experimental epilepsy and might therefore account for the hyperexcitability we observed (Lowenstein *et al.*, 1992; Toth *et al.*, 1997; Santhakumar *et al.*, 2000; Houser and Esclapez, 1996; Morin *et al.*, 1998; Andre *et al.*, 2001; Dinocourt *et al.*, 2003; Best *et al.*, 1993, 1994; Bouilleret *et al.*, 2000), perhaps because they are especially vulnerable to the effects of injury (Lowenstein *et al.*, 1992; Toth *et al.*, 1997; Best *et al.*, 1993; Bouilleret *et al.*, 2000; Dinocourt *et al.*, 2003). We therefore asked whether chronic partial deafferentation by Schaffer collateral transection affected interneurons in area CA1 using immunohistochemical staining with an antibody against the synthetic enzyme GAD67. We counted the number of immunoreactive neurons in *str. radiatum* and *oriens* of area CA1 in multiple tissue sections from sham controls and animals lesioned two weeks earlier (Fig. 3A,B). No significant differences were detected (Fig. 3E).

Analysis of GABAergic neuron number with the GAD67 antibody could be affected by changes in the expression of the antigen, rather than a change in interneuron number. As an independent control, we used immunohistochemical staining for the neuronal antigen NeuN and compared the number of neurons in the dendritic layers in sham-operated control (n=3)

and lesioned rats (n=3) at 2 weeks after the Schaffer collateral transection (Fig. 3C,D). No significant difference in the number of NeuN-positive neurons/mm² was observed in either *str. oriens* or *str. radiatum* (Fig. 3F). These data indicate that Schaffer collateral transection did not result in a large loss of GABAergic inhibitory interneurons.

We next asked whether more subtle changes in inhibition might be revealed by immunoblot analyses of pre- and postsynaptic markers of GABAergic synapses. We collected hippocampi from rats subjected to Schaffer collateral transection 14 days earlier and sham controls, sectioned them on a vibratome, isolated area CA1, and divided the sections into three groups: rostral to the lesion, containing the lesion, and caudal to the lesion. We found no difference in the expression of the $\alpha 1$ subunit of postsynaptic GABA_A receptor, a principal α subunit in hippocampal pyramidal cells (Prenosil *et al.*, 2006), in control and lesioned tissue (Fig. 4A). We observed no significant change in the expression of the presynaptic vesicular GABA transporting enzyme vGAT in chronically denervated tissue, compared to controls, in regions containing or lacking the lesion (Fig. 4B). We also performed immunohistochemical staining for vGAT in tissue from control and lesioned animals, but observed no striking differences (Fig. 4C). We conclude lesions do not result in large changes in postsynaptic GABA_ARs, the numbers of GABAergic terminals, or the number of synaptic vesicles at individual GABAergic synapses.

Next, we recorded an indirect measure of synaptic inhibition, paired-pulse inhibition of population spikes (Waldbaum & Dudek, 2009), over a range of inter-stimulus intervals (50 – 1000 ms) with the stimulation intensity set to produce a half maximal population spike. No detectable difference in paired-pulse ratios between slices from lesioned and control rats was observed at any interval (Fig. 4D).

Finally, we recorded spontaneous miniature inhibitory postsynaptic currents (mIPSCs), the response of the postsynaptic cell to a single quantum of GABA, from CA1 pyramidal cells in age-matched sham and 14 day post-lesion animals in the presence of TTX to block action potential-dependent release. No significant changes in either amplitude or frequency were observed (Fig. 4E). These data thus provide functional evidence that inhibition is not substantially altered after denervation.

Taken together, these results suggest that chronic denervation of area CA1 did not produce large effects on GABAergic inhibition.

DISCUSSION

Posttraumatic epilepsy (PTE) is a serious consequence of TBI, but its causes are unknown. In order to devise effective therapeutic strategies to reduce the risk of acquiring PTE, a better understanding of its biological basis is needed. TBI has many consequences, and we have focused here on one consequence that should be common to many forms of TBI: chronic neuronal denervation. In many head injuries, axons are irreparably damaged (Povlishock & Katz, 2005). Head injuries can also result in a complete or partial loss of the normal inputs to large populations of neurons due to degeneration of severed and injured axons, combined with direct and secondarily triggered (excitotoxicity, apoptosis) death of populations of cells that are presynaptic to a given region. We postulated that chronic partial denervation after TBI would alter the activity of cell populations and thereby alter function in the affected brain regions. Taking advantage of the unique anatomical arrangement of the hippocampus, we asked whether chronic denervation *in vivo* would, by itself, lead to homeostatic increases in the excitability of the denervated cell population.

We had studied the effects of Schaffer collateral transection previously using hippocampal slice cultures. We observed a delayed increase in excitability in area CA1 that was due, in

part, to a decrease in the function of SK-type Ca^{2+} -activated K^{+} channels in the dendrites of the denervated CA1 cells (Cai *et al.*, 2004; 2007). We have also shown that Schaffer collateral transection in slice cultures leads to delayed formation of new axonal collaterals by CA3 cells (McKinney *et al.*, 1997), perhaps triggered by BDNF-trkB signaling (Dinocourt *et al.*, 2006). But TBI *in vivo* is certainly more complex than *in vitro*.

One complication of TBI is cell death, characterized in other models by an initial wave of cell death due directly to physical trauma (Mattson & Scheff 1994; D'Ambrosio *et al.*, 2004; Williams *et al.*, 2006). We observed that SC transection produced only a circumscribed region of neuronal damage confined to cells within a $\sim 100\ \mu\text{m}$ of the lesion site, apparently those whose primary dendrites have been severed, with a corresponding area of gliosis in the vicinity of the lesion. Within the hippocampus, this procedure thus produces a relatively clean lesion characterized primarily by axonal injury. Small amounts of hemorrhaging were observed, however, but this might be expected to decrease excitability (Mun-Bryce *et al.*, 2004).

Altered excitability after Schaffer collateral transection

Many animal models of TBI, including closed cortical impact and fluid percussion injury, result in increased excitability, as well as behavioral and electrographic seizures (D'Ambrosio *et al.*, 2004; Nilsson *et al.*, 1994; Kharatishvili *et al.*, 2006; Jacobs *et al.*, 1996; Luhmann *et al.*, 1996; Hagemann *et al.*, 2000). Most closely related to our observations is the neocortical undercut, involving transection of both afferents and efferents from the neocortex, which also results in delayed and persistent hyperexcitability *in vivo* (Grafstein & Sastry, 1957) and in brain slices (Prince & Tseng, 1993; Hoffman *et al.*, 1994).

It is interesting to compare the altered excitability produced by Schaffer collateral transection with the PTE induced after the more widespread and diffuse fluid percussion injury (FPI) (D'Ambrosio *et al.*, 2004). Like PTE after FPI (Santhakumar *et al.*, 2000) or cortical undercutting (Prince & Tseng, 1993), the hyperexcitability of the lesioned tissue was preserved in brain slices cut from the vicinity of the lesion. We did not observe hyperexcitability in response to single stimuli, but abnormal discharge could be revealed when the tissue was challenged with proconvulsive conditions, including saline lacking Mg^{2+} , saline with a mildly elevated concentration of K^{+} , and saline containing a low concentration of bicuculline. We conclude that chronic denervation of CA1 cells does lead to an increase in their excitability and we suggest that this might be expected to lower the seizure threshold, although we have not test this directly.

Mechanisms of altered excitability after denervation

Spread of excitation in neuronal networks is enhanced when the strength of GABAergic inhibition is reduced, even by a small amount (Chagnac-Amitai & Connors, 1989). We detected no evidence of impaired GABAergic inhibition after SC transection through 1) counts of GAD67 immunoreactive neurons, as well as non-pyramidal neurons in *str. radiatum* and *str. oriens*; 2) Western blotting for $\alpha 1$ subunits of the GABA_A receptor; 3) Western blotting and immunocytochemical analyses of the vesicular GABA transporter, vGAT; 4) measurements of paired-pulse inhibition of population spikes, or 5) measurements of miniature IPSCs. We suggest that the number of inhibitory interneurons, the number of postsynaptic GABA_A receptors in pyramidal cells, the number of presynaptic GABAergic nerve terminals, and functional synaptic inhibition are not altered significantly in response to chronic denervation. In models of PTE after cortical injury, as well, only subtle defects in GABAergic inhibition (e.g. ca 25% decreases in spontaneous IPSC frequency, no change in amplitude) have been observed (Hagemann *et al.*, 2000; Li & Prince, 2002).

On the contrary, we observed that the application of a very low concentration of bicuculline led to the occurrence of epileptiform population spikes in area CA1 of lesioned slices, but not in control slices. This result suggests that GABAergic inhibition is sufficiently intact to maintain a relatively normal level of excitability. Whatever process accounts for the lesion-induced increase in excitability after Schaffer collateral transection, it remains occult until inhibition is weakened.

Further investigation will be required to determine what mechanisms underlie the increased excitability we have observed following chronic partial deafferentation. Turrigiano and colleagues have established that the expression of synaptic glutamate receptors in cortical cells is up-regulated in response to lowered activity levels (Turrigiano, 1999), not unlike the denervation supersensitivity displayed by denervated muscle (Cannon & Rosenblueth, 1949). The expression of intrinsic voltage-gated ion channels can also be affected by altered activity levels (Desai *et al.*, 1999). For example, we have reported previously that the ability of glutamatergic input to distal dendrites to trigger fast action potential discharge is significantly facilitated after chronic denervation in hippocampal slice cultures due to a down-regulation of the function of SK-type Ca^{2+} -activated K^{+} channels (Cai *et al.*, 2004; 2007).

Implications for PTE and other neurological disorders

In many head injuries there is irreparable damage to axons, either through the direct physical trauma of a penetrating head wound or indirectly because of the shearing and stretching of axons that occurs when the brain experiences rapid acceleration and deceleration (Povlishock & Katz, 2005). These injuries may not be fatal to the cell whose axon is injured (Singleton *et al.*, 2002), but the axon itself may be irreversibly damaged and ultimately degenerate. In addition, axonal injury can initiate a variety of changes in axotomized cells, including changes in the expression of ligand-gated or intrinsic voltage-gated ion channels (e.g. Abdulla & Smith, 2001). Some cell types are able to regenerate local axon collaterals after injury. Sprouting of new axon collaterals can alter the excitability of cell populations by facilitating the spread of excitation between cells (McKinney *et al.*, 1997; Nadler, 2003).

There are many neurological conditions in which deafferentation is likely to play a major role, including central pain syndrome, phantom limb pain, pain sensitization, spasticity, and tinnitus. One characteristic clinical sign in all of these conditions is a delay or latent period of months or years after the injury before symptoms appear. Another common feature is that all arise from a change in the electrical discharge of large populations of cells affected by the injury. We speculate that homeostatic upregulation of intrinsic excitability is one of the slow processes triggered by traumatic brain injury that ultimately leaves the brain in a state of permanent hyperexcitability- a form of maladaptive plasticity. It will be of clinical significance to determine what changes occur in denervated cells because the proper prophylactic intervention during the latent period might prevent these processes from occurring and thus prevent some of the neurological complications of TBI.

Acknowledgments

Supported with a postdoctoral fellowship to CD from the Epilepsy Foundation of America, and funds from the CURE Foundation (SMT), and the National Institutes of Health (SMT, NS40388). We thank Sandra Naumann for technical assistance.

References

- Abdulla FA, Smith PA. Axotomy- and autotomy-induced changes in the excitability of rat dorsal root ganglion neurons. *J Neurophysiol.* 2001; 85:630–643. [PubMed: 11160499]

- Agrawal A, Timothy J, Pandit L, Manju M. Post-traumatic epilepsy: an overview. *Clin Neurol Neurosurg.* 2006; 108:433–439. [PubMed: 16225987]
- André V, Marescaux C, Nehlig A, Fritschy JM. Alterations of hippocampal GABAergic system contribute to development of spontaneous recurrent seizures in the rat lithium-pilocarpine model of temporal lobe epilepsy. *Hippocampus.* 2001; 11:452–468. [PubMed: 11530850]
- Annegers JF, Hauser WA, Coan SP, Rocca WA. A population-based study of seizures after traumatic brain injuries. *N Engl J Med.* 1998; 338:20–24. [PubMed: 9414327]
- Best N, Mitchell J, Baimbridge KG, Wheal HV. Changes in parvalbumin-immunoreactive neurons in the rat hippocampus following a kainic acid lesion. *Neurosci Lett.* 1993; 155:1–6. [PubMed: 8361655]
- Best N, Mitchell J, Wheal HV. Ultrastructure of parvalbumin-immunoreactive neurons in the CA1 area of the rat hippocampus following a kainic acid injection. *Acta Neuropathol.* 1994; 87:187–195. [PubMed: 8171969]
- Bouillieret V, Loup F, Kiener T, Marescaux C, Fritschy JM. Early loss of interneurons and delayed subunit-specific changes in GABA(A)-receptor expression in a mouse model of mesial temporal lobe epilepsy. *Hippocampus.* 2000; 10:305–324. [PubMed: 10902900]
- Cai X, Liang CW, Muralidharan S, Kao JPY, Tang CM, Thompson SM. Unique roles of SK and Kv4.2 potassium channels in dendritic integration. *Neuron.* 2004; 44:351–364. [PubMed: 15473972]
- Cai X, Wei DS, Gallagher SE, Bagal A, Mei YA, Kao JP, Thompson SM, Tang CM. Hyperexcitability of distal dendrites in hippocampal pyramidal cells after chronic partial deafferentation. *J Neurosci.* 2007; 27:59–68. [PubMed: 17202472]
- Cannon, WB.; Rosenblueth, A. The supersensitivity of denervated structures. New York, NY: Macmillan Press; 1949.
- Chagnac-Amitai Y, Connors BW. Horizontal spread of synchronized activity in neocortex and its control by GABA-mediated inhibition. *J Neurophysiol.* 1989; 61:747–758. [PubMed: 2542471]
- Coulter DA, Rafiq A, Shumate M, Gong QZ, DeLorenzo RJ, Lyeth BG. Brain injury-induced enhanced limbic epileptogenesis: anatomical and physiological parallels to an animal model of temporal lobe epilepsy. *Epilepsy Res.* 1996; 26:81–91. [PubMed: 8985690]
- D'Ambrosio R, Fairbanks JP, Fender JS, Born DE, Doyle DL, Miller JW. Post-traumatic epilepsy following fluid percussion injury in the rat. *Brain.* 2004; 127:304–314. [PubMed: 14607786]
- Desai NS, Rutherford LC, Turrigiano GG. Plasticity in the intrinsic excitability of cortical pyramidal neurons. *Nat Neurosci.* 1999; 2:515–520. [PubMed: 10448215]
- Dinocourt C, Gallagher SE, Thompson SM. Injury-induced axonal sprouting in the hippocampus is initiated by activation of trkB receptors. *Eur J Neurosci.* 2006; 24:1857–1866. [PubMed: 17040478]
- Dinocourt C, Petanjek Z, Freund TF, Ben-Ari Y, Esclapez M. Loss of interneurons innervating pyramidal cell dendrites and axon initial segments in the CA1 region of the hippocampus following pilocarpine-induced seizures. *J Comp Neurol.* 2003; 459:407–425. [PubMed: 12687707]
- Grafstein B, Sastry PB. Some preliminary electrophysiological studies on chronic neuronally isolated cerebral cortex. *Electroencephalogr Clin Neurophysiol.* 1957; 9:723–725. [PubMed: 13480247]
- Hagemann G, Redecker C, Witte OW. Intact functional inhibition in the surround of experimentally induced focal cortical dysplasias in rats. *J Neurophysiol.* 2000; 84:600–603. [PubMed: 10899234]
- Hoffman SN, Salin PA, Prince DA. Chronic neocortical epileptogenesis in vitro. *J Neurophysiol.* 1994; 71:1762–1773. [PubMed: 8064347]
- Houser CR, Esclapez M. Localization of mRNAs encoding two forms of glutamic acid decarboxylase in the rat hippocampal formation. *Hippocampus.* 1994; 4:530–545. [PubMed: 7889124]
- Jacobs KM, Gutnick MJ, Prince DA. Hyperexcitability in a model of cortical maldevelopment. *Cereb Cortex.* 1996; 6:514–523. [PubMed: 8670677]
- Kharatishvili I, Nissinen JP, McIntosh TK, Pitkänen A. A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. *Neuroscience.* 2006; 140:685–697. [PubMed: 16650603]

- Korn SJ, Giacchino JL, Chamberlin NL, Dingledine R. Epileptiform burst activity induced by potassium in the hippocampus and its regulation by GABA-mediated inhibition. *J Neurophysiol.* 1987; 57:325–341. [PubMed: 3559679]
- Li H, Prince DA. Synaptic activity in chronically injured, epileptogenic sensory-motor neocortex. *J Neurophysiol.* 2002; 88:2–12. [PubMed: 12091528]
- Lowenstein DH, Thomas MJ, Smith DH, McIntosh TK. Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. *J Neurosci.* 1992; 12:4846–4853. [PubMed: 1464770]
- Lu W, Haber SN. In situ hybridization histochemistry: a new method for processing material stored for several years. *Brain Res.* 1992; 578:155–160. [PubMed: 1354996]
- Luhmann HJ, Mittmann T, Schmidt-Kastner R, Eysel UT, Mudrick-Donnon LA, Heinemann U. Hyperexcitability after focal lesions and transient ischemia in rat neocortex. *Epilepsy Res Suppl.* 1996; 12:119–128. [PubMed: 9302510]
- Majkowski, J. Posttraumatic epilepsy. In: Mogens, D.; Lennart, G., editors. *Comprehensive Epileptology*. New York, NY: Raven Press; 1990. p. 281–288.
- Mattson MP, Scheff SW. Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. *J Neurotrauma.* 1994; 11:3–33. [PubMed: 8201625]
- McBain CJ. Hippocampal inhibitory neuron activity in the elevated potassium model of epilepsy. *J Neurophysiol.* 1994; 72:2853–2863. [PubMed: 7897494]
- McKinney RA, Debanne D, Gähwiler BH, Thompson SM. Lesion-induced axonal sprouting and hyperexcitability in the hippocampus *in vitro*: Implications for the genesis of posttraumatic epilepsy. *Nature Med.* 1997; 3:990–996. [PubMed: 9288725]
- Morin F, Beaulieu C, Lacaille JC. Selective loss of GABA neurons in area CA1 of the rat hippocampus after intraventricular kainate. *Epilepsy Res.* 1998; 32:363–369. [PubMed: 9839776]
- Mun-Bryce S, Roberts LJ, Hunt WC, Bartolo A, Okada Y. Acute changes in cortical excitability in the cortex contralateral to focal intracerebral hemorrhage in the swine. *Brain Res.* 2004; 1026:218–226. [PubMed: 15488483]
- Nadler JV. The recurrent mossy fiber pathway of the epileptic brain. *Neurochem Res.* 2003; 28:1649–1658. [PubMed: 14584819]
- Nilsson P, Ronne-Engström E, Flink R, Ungerstedt U, Carlson H, Hillered L. Epileptic seizure activity in the acute phase following cortical impact trauma in rat. *Brain Res.* 1994; 637:227–232. [PubMed: 8180800]
- Povlishock JT, Katz DI. Update of neuropathology and neurological recovery after traumatic brain injury. *J Head Trauma Rehabil.* 2005; 20:76–94. [PubMed: 15668572]
- Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM, Vogt KE. Specific subtypes of GABA_A receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J Neurophysiol.* 2006; 96:846–857. [PubMed: 16835366]
- Prince DA, Tseng GF. Epileptogenesis in chronically injured cortex: in vitro studies. *J Neurophysiol.* 1993; 69:1276–1291. [PubMed: 8492163]
- Reeves TM, Lyeth BG, Phillips LL, Hamm RJ, Povlishock JT. The effects of traumatic brain injury on inhibition in the hippocampus and dentate gyrus. *Brain Res.* 1997; 757:119–132. [PubMed: 9200506]
- Rutecki PA, Lebeda FJ, Johnston D. Epileptiform activity induced by changes in extracellular potassium in hippocampus. *J Neurophysiol.* 1985; 54:1363–1374. [PubMed: 2416891]
- Santhakumar V, Bender R, Frotscher M, Ross ST, Hollrigel GS, Toth Z, Soltesz I. Granule cell hyperexcitability in the early post-traumatic rat dentate gyrus: the 'irritable mossy cell' hypothesis. *J Physiol.* 2000; 524:117–134. [PubMed: 10747187]
- Santhakumar V, Ratzliff AD, Jeng J, Toth Z, Soltesz I. Long-term hyperexcitability in the hippocampus after experimental head trauma. *Ann Neurol.* 2001; 50:708–717. [PubMed: 11761468]
- Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.* 2000; 874:123–130. [PubMed: 10960596]
- Singleton RH, Zhu J, Stone JR, Povlishock JT. Traumatically induced axotomy adjacent to the soma does not result in acute neuronal death. *J Neurosci.* 2002; 22:791–802. [PubMed: 11826109]

- Thomson AM, West DC. N-methylaspartate receptors mediate epileptiform activity evoked in some, but not all, conditions in rat neocortical slices. *Neuroscience*. 1986; 19:1161–1177. [PubMed: 3029626]
- Toth Z, Hollrigel GS, Gorcs T, Soltesz I. Instantaneous perturbation of dentate interneuronal networks by a pressure wave-transient delivered to the neocortex. *J Neurosci*. 1997; 17:8106–8117. [PubMed: 9334386]
- Traynelis SF, Dingledine R. Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *J Neurophysiol*. 1988; 59:259–276. [PubMed: 3343603]
- Turrigiano GG. Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. *Trends Neurosci*. 1999; 22:221–227. [PubMed: 10322495]
- Turrigiano G. Homeostatic signaling: the positive side of negative feedback. *Curr. Opin. Neurobiol*. 2007; 17:318–324. [PubMed: 17451937]
- Turrigiano G. The self-tuning neuron: homeostatic synaptic scaling of excitatory synapses. *Cell*. 2008; 135:422–435. [PubMed: 18984155]
- Turrigiano GG, Nelson SB. Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci*. 2004; 5:97–107. [PubMed: 14735113]
- Waldbaum S, Dudek FE. Single and repetitive paired-pulse suppression: a parametric analysis and assessment of usefulness in epilepsy research. *Epilepsia*. 2009; 50:904–916. [PubMed: 19170733]
- Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides*. 1986; 7:155–159. [PubMed: 3520509]
- Williams AJ, Hartings JA, Lu XC, Rolli ML, Tortella FC. Penetrating ballistic-like brain injury in the rat: differential time courses of hemorrhage, cell death, inflammation, and remote degeneration. *J Neurotrauma*. 2006; 23 1828-1246.

Figure 1.

Morphological consequences of Schaffer collateral transection. (A) Fixed brain showing the location of the lesion in the hippocampus (dashed line) after dissecting away the overlying neocortex. The lesion pathway extends ca. one third of the rostro-caudal extent of the hippocampus. Scale bar = 1 mm. (B) Extracellular recording from *str. radiatum* (SR) of area CA1 24 hrs after transecting the Schaffer collateral pathway. In the top trace, a field excitatory postsynaptic potential (fEPSP, arrow head) is elicited by stimulation in SR on the CA1 side of the lesion. When the stimulating electrode was repositioned in SR on the CA3 side of the lesion, however, no fEPSP could be evoked (bottom trace), demonstrating the effectiveness of the lesion. (C, D) Cresyl violet-stained section of the lesioned hippocampus. In the low power image (C), the lesion (black line) can be seen to extend from the pial surface to the hippocampal fissure along the border between areas CA1 and CA3. In the higher power image (D), some clotted blood and numerous cells with small compact nuclei, presumably glial cells, were observed surrounding the lesion. (E, F) Neuronal degeneration in the lesioned hippocampus visualized with Fluoro-Jade B labeling. Cells and their processes were observed at the both side of the lesion (white line) for a distance of 100 to 200 μm . Scale bar, 200 μm (C and E), 50 μm (D) and 20 μm (E). SO: *str. oriens*; SP: *str. pyramidale*; SR: *str. radiatum*; SLM: *str. lacunosum-moleculare*; DG: dentate gyrus.

Figure 2.

Increase of excitability in area CA1 at 2 weeks after the lesion. **(A, B)** Extracellular recordings of fEPSPs in SR of area CA1 in response to stimulation in SR on the CA1 side of the lesion in hippocampal slices from sham operated control rats (top) and rats in which the Schaffer collateral transection had been lesioned 14 days earlier (bottom). Recordings were made in one slice **(A)** in control saline and then after increasing the extracellular K^+ concentration from 4 to 8 mM (high K^+) or removing Mg^{2+} (low Mg^{2+}). In another slice **(B)**, recordings were made before and after application of 0.1 μ M bicuculline. In all cases in the slices from lesioned animals, there was a robust increase in the number of population spikes compared to the control saline, but not in the slices from the sham rats. Extra population spikes are indicated with small arrows. **(C, D)** Comparisons of population spikes in slices from sham control rats (white bars) and rats in which the Schaffer collateral pathway had been transected 14 days earlier (black bars). There was a statistically significant difference in the number of population spikes **(C)** and in the coastline bursting index (CBI, mV*ms) **(D)** in slices from lesioned rats ($n = 10$ slices) compared to the slices from sham rats ($n = 8$ slices) in all three conditions (**, $p < 0.01$; *, $p < 0.05$; paired t-tests). **(E, F)** Comparisons of population spikes in slices from sham control rats (white bars) and rats in which the Schaffer collateral pathway had been transected three days earlier (gray bars). No was no statistically significant difference in the number of population spikes **(E)** or CBI **(F)** in slices from lesioned rats compared to the slices from sham rats in any of the three conditions ($n = 4$ sham and 5 lesioned slices). Error bars indicate SEM.

Figure 3.

GAD67- and NeuN- containing neurons in area CA1 of the hippocampus in sham-operated (A, C) and lesioned (B, D) rats at 2 weeks postlesion. (A) In a sham operated control rat, GAD67-immunoreactive (IR) cell bodies were observed in all layers in area CA1. A dense plexus of GAD67-IR terminals was apparent in SP and also present in SO and SR. (B) In a lesioned rat, both the distribution of GAD67-IR cells bodies and the density of GAD67-IR terminals were similar to those observed in sham operated controls. (C, D) No significant difference in the distribution of NeuN-IR neurons is observed in hippocampal sections from lesioned (D) and sham control (C) rats. In B and D, the lesion site is indicated with a black line. Scale bar, 50 μm (A, B, C and D). SO: *str. oriens*; SP: *str. pyramidale*; SR: *str. radiatum*. (E, F) Bar graphs comparing the mean number of GAD67 (E) and NeuN (F) IR neurons in SO and SR of area CA1 of sham control (white bars) and lesioned (black bars) rats. No statistically significant difference in the mean number of IR neurons/mm² was observed, (unpaired t-test, $n = 3$ animals each). Error bars indicate SEM. Areas analyzed (mm²): SO sham = 2.55 ± 0.18 ; SO lesion = 2.35 ± 0.17 ; SR sham = 5.41 ± 0.55 ; SR lesion = 4.65 ± 0.40 .

Figure 4.

Analysis of GABAergic synaptic transmission by immunoblotting. Expression of neither the $\alpha 1$ subunit of the GABA_A receptor (**A**) nor the vesicular GABA transporter vGAT (**B**) were significantly different in hippocampal tissue taken from rats in which the Schaffer collateral pathway had been transected 14 days earlier (black bars) than in tissue from sham operated control rats (white bars). Tissue was harvested separately from the dorsal and medial levels of the hippocampus, containing the lesion, and the ventral hippocampus that does not include the lesion (n=3 animals each). Representative gels are shown at right, along with loading controls labeled with an antibody against the housekeeping gene GAPDH. S, sham; L, lesion; D, dorsal; M, medial; V, ventral. (**C**) Immunohistochemical staining with an antibody directed against vGAT in area CA1 from a sham rat (left) and a rat 14 days after SC transection. No striking differences were detected. (**D**) Paired-pulse ratios of population spikes in area CA1 were not different in slices from rats lesioned 14 days earlier (black bars) compared to sham operated controls (open bars), suggesting that functional inhibition is not substantially altered after denervation. (**E**) Mean mIPSC amplitude and frequency were not significantly different (unpaired t-test) in CA1 cells recorded from sham operated control

slices (n= 11 cells, 4 animals) and slices taken from animals that had received a Schaffer collateral transection 14 days earlier (n= 17 cells, 3 animals).