Epileptic activity outlasts disinhibition after intrahippocampal tetanus toxin in the rat

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1. A single dose of tetanus toxin, injected under anaesthesia into one dorsal hippocampus of the rat, produces chronic epileptic foci involving both hippocampi. Generalized seizures occurred 1–6 weeks after injection and epileptic discharges were found in hippocampal slices *in vitro*. Here we measured the time course of decay of epileptic activity and the level of $GABA_A$ receptor-mediated inhibition in hippocampal slices 1–16 weeks after toxin injection *in vivo*.

2. Epileptic activity peaked in the dentate granule cell and CA3 pyramidal cell layers 2 weeks after toxin injection and at 4 weeks in CA1. Thresholds for evoking epileptic activity were lowest in the suprapyramidal blade of the dentate gyrus and area CA3c. Recovery from epileptic activity occurred more rapidly in the contralateral hippocampus. Polyspike activity ceased by 8 weeks and interictal activity by 16 weeks. Epileptic discharges could still be evoked from CA1 16 weeks after toxin injection.

3. The maximal monosynaptic fast inhibitory postsynaptic current (IPSC) conductance changes (g_{IPSC}) decreased to <10% of control values at the time of peak epileptic activity and remained lower than controls for 4 weeks ipsilaterally. In the contralateral hippocampus, g_{IPSC} fell to *ca* 50% of control values for the first 2 weeks. Responses to exogenous GABA remained unchanged.

4. After 8 weeks dentate granule cells had g_{IPSC} significantly larger than controls. No increase in g_{IPSC} occurred in CA3. Epileptic activity persisted 8–10 weeks after recovery from disinhibition ipsilaterally and 4 weeks contralaterally.

5. Epileptic activity was seen when monosynaptic $GABA_A$ receptor-mediated IPSCs were normal or supranormal. At these times polysynaptic inhibition was still profoundly reduced. These observations provide strong evidence for long-term changes in the pattern of synaptic excitation contributing to a chronic epileptic syndrome following disinhibitory insult, and are consistent with weakened excitation of inhibitory neurones.

A minute dose of tetanus toxin injected into the brain of laboratory animals causes profound and long-lived changes in brain function. Intracerebral tetanus toxin has been known to cause epilepsy for almost a century (Roux & Borrel, 1898). Focal application of toxin to the hippocampus leads to seizures originating in one hemisphere, associated with loss of consciousness and full motor fits on secondary generalization (Mellanby, George, Robinson & Thompson, 1977). The use of tetanus toxin provides a model of epilepsy which shares many features with the most common of clinical epilepsies, those of focal origin.

The mechanism of action of the toxin involves the blockade of exocytosis (Bevan & Wendon, 1984; Penner, Neher & Dreyer, 1986; Bittner & Holz, 1988). The toxin is synthesized by the *Clostridium tetani* bacillus and is internalized by neurones in a ganglioside-facilitated manner (Montecucco, 1989). Once internalized, the toxin acts to prevent vesicle docking, apparently by enzymatic cleavage of proteins in the synaptobrevin family (Schiavo *et al.* 1992). The toxin demonstrates some selectivity for release from inhibitory neurones (Collingridge, Thompson, Davies & Mellanby, 1981; Empson & Jefferys, 1993) while leaving postsynaptic receptors intact (Bergey, MacDonald, Habig, Hardegree & Nelson, 1983).

The principal action of tetanus toxin in generating epilepsy following intrahippocampal injection has been shown to involve functional $GABA_A$ergic disinhibition (Jordan & Jefferys, 1992). This disinhibition peaked 2 weeks after the injection of toxin, when decreases in $GABA_B$ receptor-mediated inhibition also occurred (Empson & Jefferys, 1993). However, the seizure syndrome persists for 6–8 weeks (Mellanby *et al.* 1977; Brace, Jefferys & Mellanby, 1985; Jefferys & Empson, 1990), whereas toxin-induced attenuation of GABA release had returned to normal

(Jefferys *et al.* 1991). This ability of seizures to persist after recovery from the disinhibitory effects of toxin has also been reported in the neocortex (Empson, Amitai, Jefferys & Gutnick, 1993). The seizure syndrome considerably outlasts the apparent presence of toxin. Measurement of radioactivity after injection of ^{125}I-labelled tetanus toxin into the ventral hippocampus showed that only 1·5% of the toxin remained in the brain 9 days after injection (Mellanby, 1989). In the cat, if the toxin-induced, prolonged seizure syndrome was prevented initially by anticonvulsant therapy there was complete remission (Darcey & Williamson, 1992). Failure to prevent seizures led to prolonged epilepsy lasting over 1 year. Injection into rat neocortex also leads to epileptic activity persisting >9 months (Brener, Amitai, Jefferys & Gutnick, 1990). The above observations demand a detailed study of the relationship between toxin-induced disinhibition and the development and maintenance of the seizure syndrome in order to understand the contribution made by the initial disinhibition and resulting plastic changes.

In the present study we clarified the nature of the long-term changes in neuronal excitability caused by a single tetanus toxin injection. We also concurrently recorded monosynaptic $GABA_A$ receptor-mediated IPSCs in the dentate and CA3 principal cells to provide direct evidence of an uncoupling of excitatory and inhibitory drive during the later stages of the tetanus toxin model of chronic epilepsy. The implications of these data for the understanding of processes underlying chronic epilepsy following acute lesion are discussed. A preliminary account of this work has been presented to the Physiological Society (Whittington & Jefferys, 1993).

METHODS

In vivo procedures

Sixty-two male Sprague–Dawley rats, 250–350 g (Harlan OLAC Ltd, UK) were divided into three groups; forty-two rats were anaesthetized with Hypnorm–Hypnovel mixture (8·2 mg kg^{-1} fluanisone and 0·26 mg kg^{-1} fentanyl citrate (Hypnorm); 4·1 mg kg^{-1} midazolam hydrochloride (Hypnovel)) and received a single intrahippocampal injection of a small dose (< 50 ng) of tetanus toxin (Wellcome Biotech, Beckenham, UK), or buffer under stereotaxic control. Tetanus toxin was dissolved in 0·1 M sodium phosphate buffer containing 0·2% bovine serum albumin. Twenty-two rats were given injections of 1 μl toxin solution; twenty rats were given the same volume of toxin-free buffer. Co-ordinates for injection were 2·8 mm caudal to bregma, 3·5 mm lateral to the mid-line and 3·5 mm below the cortical surface. This corresponded to a site in the dorsal hippocampal fissure adjacent to the stratum radiatum of CA3. Twenty rats received no injection.

Preparation of slices

Four rats from each treatment group (toxin injected, buffer injected and control) were stunned and killed by cervical dislocation followed by decapitation 1, 2, 4, 8 or 16 weeks after surgery. Control rats were age-matched and kept in identical conditions. Transverse dorsal hippocampal slices, 400 μm thick, were cut on a Vibroslice (Campden Instruments, Loughborough, UK), immediately transferred to a recording chamber at the interface between warm moist 95% O_2–5% CO_2 gas mixture and artificial cerebrospinal fluid (ACSF) flowing at 0·5 ml min^{-1}. The ACSF contained (mM): NaCl, 135; $NaHCO_3$, 16; KCl, 3; $CaCl_2$, 2; NaH_2PO_4, 1·25; $MgCl_2$, 1; D-glucose, 10; and was equilibrated with 95% O_2–5% CO_2, pH 7·4 at 34 °C.

Field potential recordings

Responses were recorded extracellularly using glass micropipettes filled with 2 M NaCl, resistances 5–15 MΩ, from the buried blade of the dentate granule cell layer, the pyramidal cell layer of areas CA3c and CA3a, and the subicular end of CA1 in hippocampi both ipsilateral and contralateral to the injection site. Orthodromic stimulation of the perforant path input to the dentate granule cells, the perforant path/mossy fibre input to the CA3 pyramidal cells or the Schaffer collateral/commissural input to CA1 was delivered using silver bipolar electrodes (Fig. 1). Stimuli were square waves, duration 50 μs, delivered every 10 s. Responses from each area were recorded from one slice from each hemisphere of each rat ($n = 4$ for each region at each time from each treatment group). Stimulus–response relationships were derived from orthodromic stimuli, 10–80 V (approximately 18–160 μA). The magnitude of the evoked population spike was measured at each stimulus intensity. Thresholds for evoked epileptiform activity were defined as the minimum stimulus intensity required to elicit more than one orthodromic population spike. In non-epileptic areas the threshold for epileptiform activity was defined as >100 V (the maximum stimulus used in this study). Spontaneous activity was also monitored.

Intracellular recording

Recordings were taken from one CA3c pyramidal cell and one dentate granule cell in each of 120 slices taken ipsi- and contralaterally from the injection site in each of the treatment groups. Glass microelectrodes were filled with 4 M potassium acetate, resistances 35–65 MΩ. After impalement, cells were allowed to settle for 5 min and then assessed by examining both the current–voltage relationships and the evoked compound synaptic potential. Cells with poor impalements (cell resistance < 30 MΩ) and cells not resembling the principal neurones in the two regions were rejected.

Monosynaptic $GABA_A$ receptor-mediated IPSCs

Monosynaptic $GABA_A$ receptor-mediated potentials were evoked by stimulating electrodes placed < 50 μm from the recording site in ACSF containing 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX, 20 μM), DL-2-amino-5-phosphonovaleric acid (APV, 50 μM) and 3-amino-2-(4-chlorophenyl)-2-hydroxy-propylsulphonic acid (2-OH-saclofen, 100–200 μM) (Davies & Collingridge, 1989; Davies, Davies & Collingridge, 1990). The resulting fast IPSP was clamped using discontinuous single electrode voltage clamp (Axoclamp 2A) with a gain of three for each cell recorded (no phase shift was used). Clamp efficiency, estimated by comparison of clamped with unclamped IPSP amplitude was 67–75% with no difference between treatment groups. Stimulus–response relationships for the isolated fast IPSC were measured and the potential dependence determined. From these data the maximum $GABA_A$ receptor-mediated conductance change (g_{GABA}), reversal potential, and the decay constant of the responses were calculated at each time for each treatment group ($n = 4$).

Response to exogenous GABA

To assess possible changes in the postsynaptic $GABA_A$ receptor-mediated component of the maximal IPSC, GABA was applied to the slice as a microdrop. Glass micropipettes had their tips broken back, and were filled with ACSF containing 1 mM GABA. The pipettes were lowered to within 50 μm of the slice surface at the level of the principal cell body region as close as possible to the recording site. Gentle positive pressure applied to the pipette produced a drop of the GABA solution at its tip, which increased in size until the surface of the drop touched the surface of the slice. At this point the

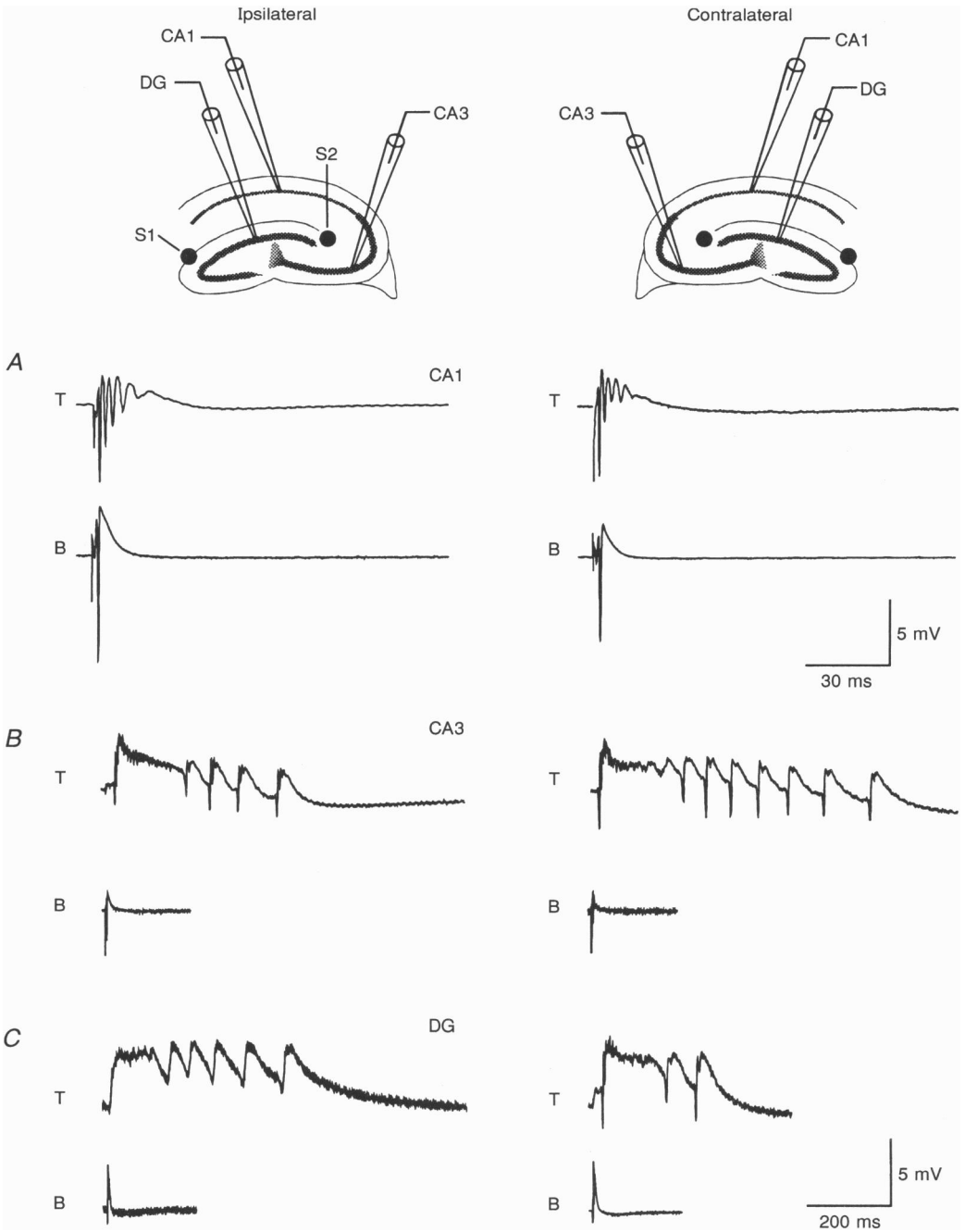

Figure 1. Pattern of epileptic activity 2 weeks after tetanus toxin injection

A, CA1 population response to Schaffer collateral/commissural stimulation. Stimulus intensity 50 V at site S2. Responses from slices from toxin-treated rats are indicated by T, and time-matched responses from slices from buffer-injected rats are indicated by B. *B*, spontaneous polyspike-like activity in CA3 seen in slices from rats injected with tetanus toxin. Time-matched control responses from buffer-injected rats were evoked by stimulation at 50 V, site S2. *C*, spontaneous polyspike-like activity occurring in the dentate granule cell layer (DG) after toxin injection. Control responses were evoked using 50 V stimulation, site S1, of the perforant path in slices from buffer-injected rats.

positive pressure was removed. With this method consistent amounts of GABA could be applied to the slice (drop diameter 50–70 μm).

Data analysis

All responses were digitized (1401, Cambridge Electronic Design (CED), Cambridge, UK) and stored on computer for off-line analysis (Sigavg, CED). All data are expressed as means ± S.E.M., or median ± interquartile range, with $n = 4$ for each group at each of the times. Statistical analysis of temporal changes in measurements over the 16 week period were performed using a two-way non-parametric analysis of variance with Dunnett's correction for multiple comparisons to identify individual differences. All statistical analyses of data from toxin-injected animals refer to comparison with buffer-injected controls. No effects of buffer injection were seen in the present observations.

RESULTS

Extent of epileptic activity induced by tetanus toxin

Epileptic field potentials could be evoked or occurred spontaneously in area CA3 and the suprapyramidal blade of the dentate gyrus of hippocampi both ipsi- and contralateral to the toxin injection site (Fig. 1). Epileptic population responses started with a small field EPSP and progressed to a large, polyspike-like, all-or-none discharge consisting of an initial positive-going wave followed by one to twelve secondary bursts. Spontaneous burst discharges occasionally occurred in area CA1 and were always preceded by bursting in area CA3. Recording in CA1 after stimulation of the Schaffer collateral/commissural pathway usually evoked a long, 40–80 ms, field EPSP of variable amplitude with two or more population spikes superimposed upon it. No epileptic activity was seen in buffer-injected or unoperated control animals.

Time course of changes in epileptic activity

Epileptic activity peaked at about 2 weeks after toxin injection and decreased in incidence and magnitude during the remainder of the 16 week experimental period. The threshold stimulus required to generate bursting (CA3 and the dentate gyrus) or multiple orthodromic population spikes (CA1) provided a measure used to quantify epileptic activity. Multiple spikes were evoked in area CA1 in slices from both injected and uninjected hippocampi throughout the 16 week period (Fig. 2*A*). The activity was most pronounced in the hippocampus ipsilateral to the injection and peaked after 4 weeks (multiple spike threshold, 36 ± 18 V, $n = 4$ ipsilateral; 56 ± 20 V, $n = 4$ contralateral). No multiple spikes were evoked in control slices or slices from buffer-injected rats (multiple spike threshold > 100 V).

In area CA3 of the injected hippocampus, threshold for burst firing was lowest at 1 week and the area remained profoundly epileptic for 4 weeks. In contrast, little spontaneous epileptic activity was seen in contralateral CA3 at 1 week and evoked activity had a significantly higher threshold than in the ipsilateral CA3 ($P < 0.05$;

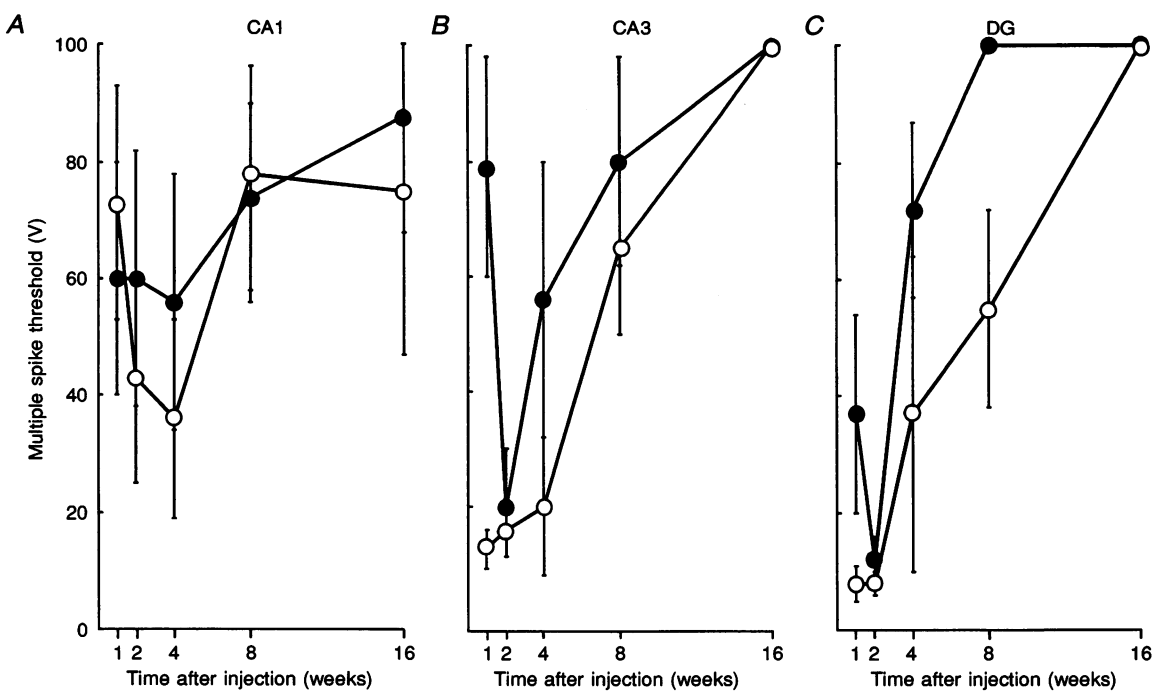

Figure 2. Threshold for evoked epileptic activity following tetanus toxin injection
A, threshold for epileptic activity in CA1 evoked by Schaffer collateral/commissural stimulation in the ipsi- (○) and the contralateral hippocampus (●). Data expressed as means ± S.E.M., $n = 4$ for each region throughout this figure. *B*, epileptic activity in CA3 evoked by mossy fibre stimulation. *C*, epileptic activity in the dentate granule cell layer (DG) evoked by perforant path stimulation.

Fig. 2*B*). However, epileptic activity then developed rapidly in contralateral CA3, with the threshold for evoked bursts being lowest at 2 weeks. Recovery from epileptic activity also appeared marginally faster in contralateral CA3, occurring by 8 weeks compared with 16 weeks ipsilaterally.

A similar time course of toxin-induced epileptic activity occurred in the dentate gyrus. The contralateral dentate gyrus was less excitable than the injected dentate gyrus 1 week after toxin injection and recovered from epileptic activity significantly faster. No burst firing was seen in the uninjected dentate gyrus after 4 weeks ($P < 0{\cdot}05$; multiple spike thresholds in ipsi- and contralateral dentate gyrus; Fig. 2*C*). Full recovery from epileptic activity was seen on both sides 16 weeks after injection.

Persistent epileptic activity recorded in area CA1 was not associated with a change in maximum population spike amplitude ($P > 0{\cdot}1$, ANOVA, 1–16 weeks; Fig. 3*A*). The two regions demonstrating the greatest degree of epileptic activity, CA3 and the dentate gyrus, were not epileptic by 16 weeks, but the maximum amplitude of orthodromically evoked population spikes remained abnormal. A dramatic, 300–500% increase in maximum population spike amplitude was seen in the ipsilateral dentate gyrus throughout the experiment ($P < 0{\cdot}001$, ANOVA, 1–16 weeks; Fig. 3*C*). These data revealed a profound, permanent change in the excitability of dentate granule cells. In contrast, maximum population spike amplitude in area CA3 was reduced by toxin injection. In the CA3 contralateral to the injection site, population spikes were smaller 1 week following toxin injection, recovered during the time of peak epileptic activity (2–4 weeks), and then dropped to 30% of buffer-injected control values by 16 weeks ($P < 0{\cdot}05$; Fig. 3*B*). A different time course was seen in the ipsilateral CA3. Population spike amplitude was significantly reduced 4–8 weeks after injection ($P < 0{\cdot}05$), but recovered towards control values after 16 weeks.

Time course of disinhibition

Analysis of the maximum amplitude of monosynaptically evoked, pharmacologically isolated $GABA_A$ receptor-mediated IPSCs revealed a profound decrease in this

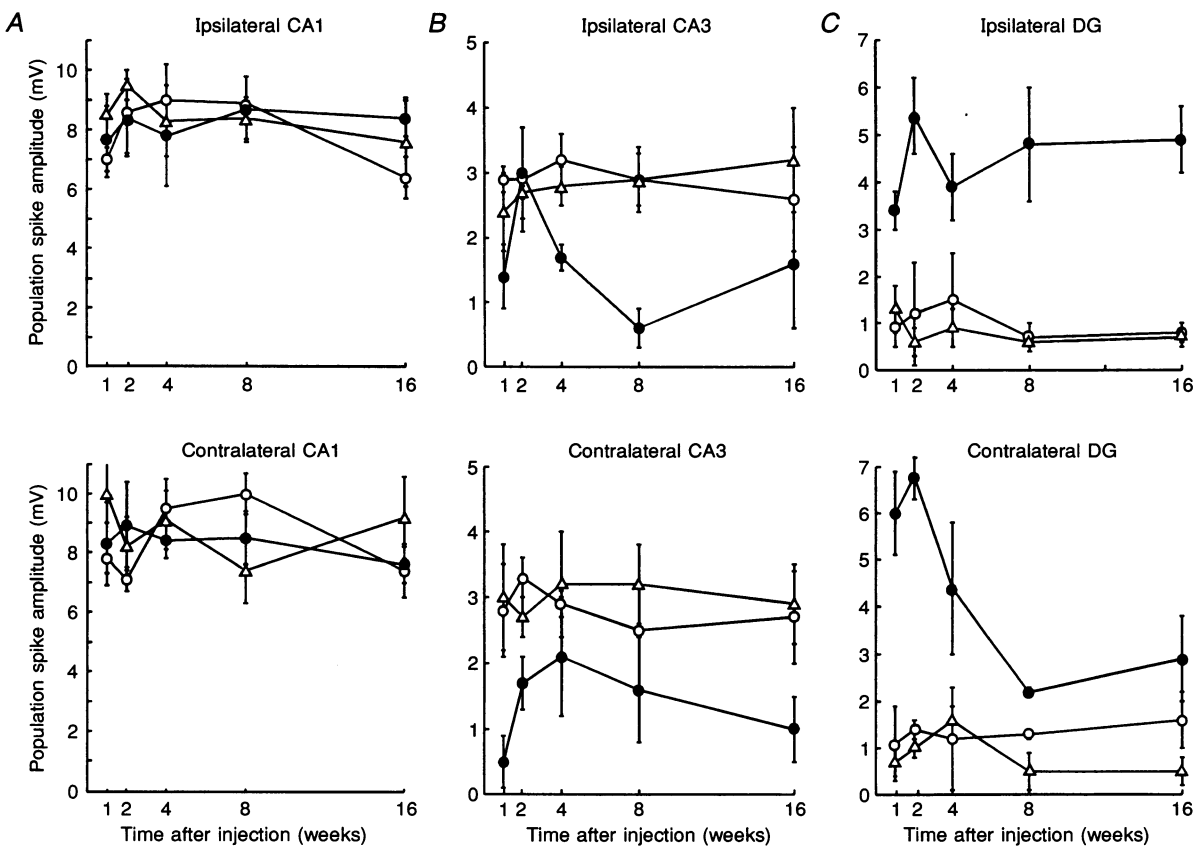

Figure 3. Maximum orthodromic population spike amplitude 1–16 weeks after tetanus toxin injection

Upper graphs show data (means ± S.E.M., $n = 4$) from slices from the ipsilateral hippocampus (left-hand hippocampus in uninjected controls). Lower graphs show data from slices from the contralateral hippocampus (right-hand hippocampus in uninjected controls). Data shown from uninjected control (△), buffer-injected (○) or toxin-injected rats (●). *A*, population spike amplitude in CA1 stratum pyrimidale following Schaffer collateral/commissural fibre stimulation. *B*, population spike amplitude in CA3 stratum pyramidale following mossy fibre stimulation. *C*, population spike amplitude in the dentate granule cell layer following perforant path stimulation.

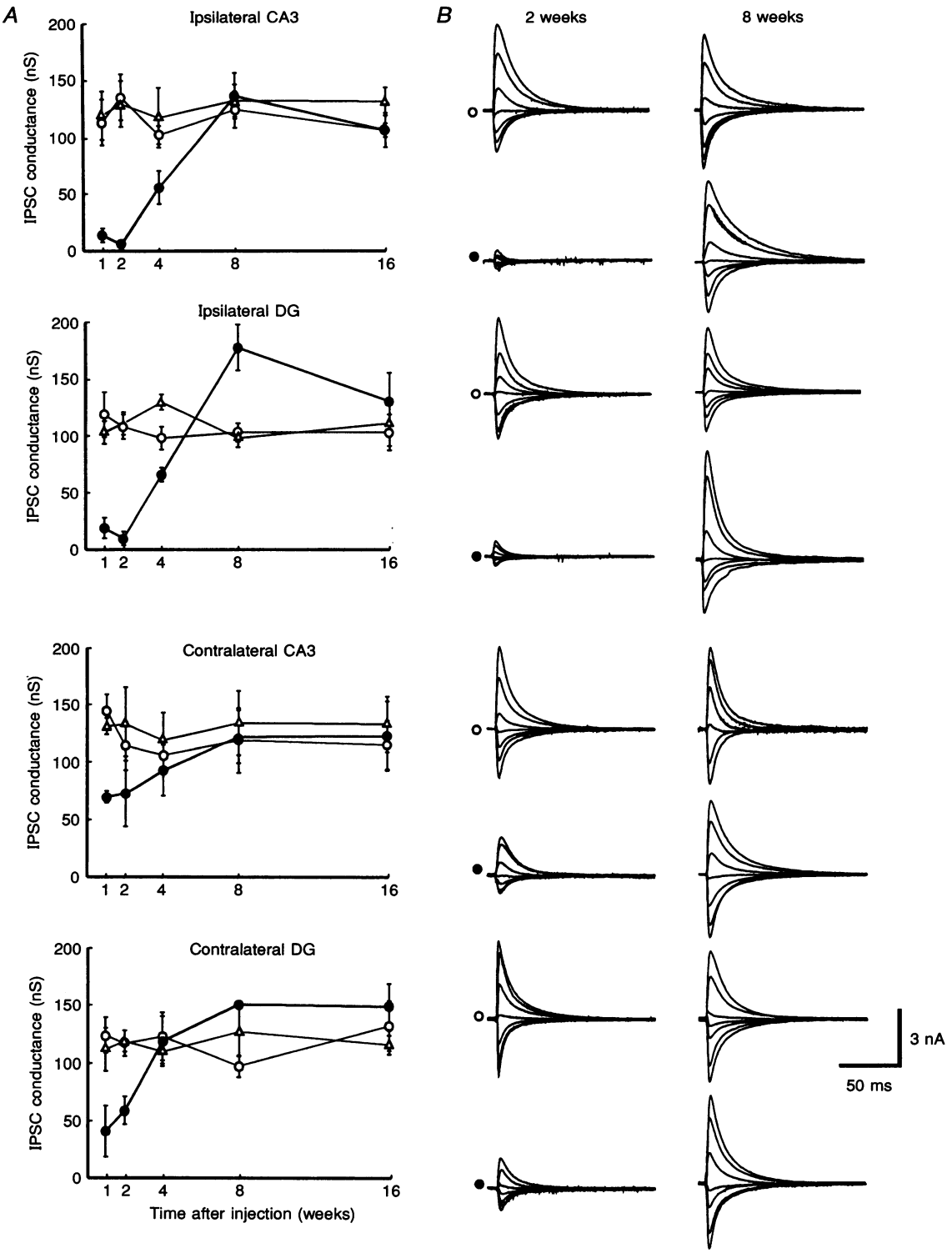

Figure 4. Time course of toxin-induced changes in IPSC conductance
Conductance was estimated from the slope of the I–V plots, in hippocampal slices from control (△), buffer-injected (○) and toxin-injected (●) rats. Data are expressed as means ± S.E.M., $n = 4$. *A*, maximal g_{IPSC} in CA3 pyramidal cells or dentate granule cells from the ipsilateral hippocampus (upper graphs) and contralateral hippocampus (lower graphs). *B*, example of maximal IPSCs from the two areas taken 2 and 8 weeks after buffer (○) or toxin (●) injection. Each family consists of 7 traces evoked from a holding potential of −40 to −100 mV in 10 mV steps.

measure of inhibition for 2 weeks following toxin injection in area CA3 and the dentate gyrus ipsilateral to the injection site (Fig. 4). Evoked IPSCs were considerably reduced at each membrane potential tested (−100 to −40 mV, $P < 0{\cdot}05$, Dunnett's multiple comparison) with no significant change in reversal potential in CA3 pyramidal cells at any time during the experiment (e.g. toxin, 66 ± 3 mV; buffer, 70 ± 2 mV at 2 weeks) or dentate granule cells (e.g. toxin, 71 ± 3; buffer, 71 ± 2 mV at 2 weeks). Partial recovery from disinhibition was seen 4 weeks after toxin injection in both regions. This recovery was complete at 8 and 16 weeks after injection in area CA3. IPSCs evoked in the ipsilateral dentate gyrus were significantly greater than corresponding currents evoked after 8 weeks in control or buffer-injected hippocampi ($P < 0{\cdot}05$). This rebound increase in monosynaptic inhibition had faded 16 weeks after injection.

The conductance change associated with the monosynaptic IPSC (g_{IPSC}) was estimated from the I–V curves and varied significantly during the course of the syndrome ($P < 0{\cdot}001$, ANOVA, 1–16 weeks). The dramatic decrease in IPSC amplitude seen in the ipsilateral CA3 region was associated with a profound decrease in g_{IPSC} 1–4 weeks after injection (Fig. 4*A*). g_{IPSC} was most affected 1–2 weeks after tetanus toxin (toxin, 6 ± 3 nS; buffer, 136 ± 20 nS). Recovery was complete from 8 weeks after injection (toxin, 137 ± 20 nS; buffer, 125 ± 16 nS). In the dentate gyrus injected with toxin, g_{IPSC} was also considerably smaller than in controls (Fig. 4*B*). The maximum difference was again seen 1–2 weeks following injection (toxin, 10 ± 6 nS; buffer, 108 ± 11 nS). By 8 weeks after injection, monosynaptic inhibition recovered to a greater than normal conductance, reaching over 160% of the control values (toxin g_{IPSC}, 178 ± 20 nS; buffer g_{IPSC}, 103 ± 8 nS).

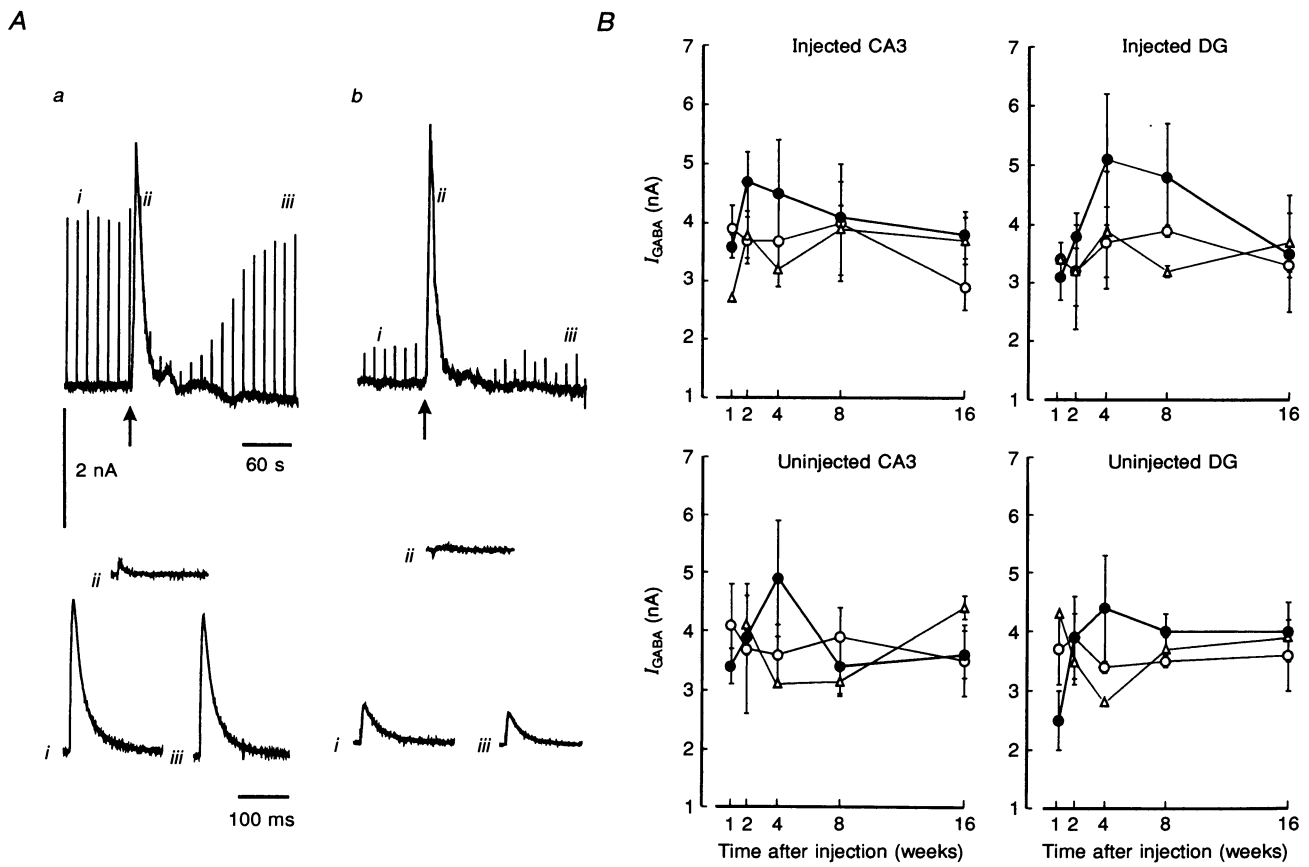

Figure 5. Response to exogenous GABA application
GABA, 1 mM, was applied to the cell body layer as a microdrop, 50–70 μm in diameter. Cells were held at −50 mV and stimulated monosynaptically every 10 s. *A a*, example of response to exogenous GABA applied to area CA3 in control, untreated slices. The time of GABA application is marked by the arrow. Monosynaptically evoked IPSCs are shown below before (*i*), during (*ii*) and after (*iii*) the response. *A b*, Example of response to exogenous GABA applied to area CA3 of the injected hippocampus 2 weeks after tetanus toxin injection. The time of GABA application is marked by the arrow. Monosynaptically evoked IPSCs are shown below before (*i*), during (*ii*) and after (*iii*) the response. *B*, graphs illustrating the lack of change in response to exogenous GABA during the 16 weeks after buffer (○) or toxin injection (●) compared with controls (△). Data are shown as means ± S.E.M., $n = 4$.

Monosynaptic IPSCs also decreased in the contralateral dentate and CA3 regions 1–2 weeks after toxin injection, but to a lesser extent than in the ipsilateral hippocampus (Fig. 4*A* and *B*). Maximum IPSC amplitude was reduced to 50% of control measurements at each of the membrane potentials studied. Again, no significant change in reversal potential was associated with this change, either in CA3 or in dentate granule cells ($P > 0{\cdot}05$). The decrease in IPSC size was entirely attributable to a drop in conductance that was maximal 1–2 weeks after injection (toxin, 71 ± 5 nS; buffer, 146 ± 14 nS; Fig. 4*A*). No significant difference was seen in g_{IPSC} 4–16 weeks after toxin injection ($P > 0{\cdot}1$), indicating a more rapid recovery from disinhibition than seen ipsilaterally. A similar time course of changes in g_{IPSC} was seen in the contralateral dentate gyrus. The initial disinhibition was less marked than that seen ipsilaterally, with recovery complete after only 4 weeks (Fig. 4*B*). The rebound increase in g_{IPSC} was again seen 8 weeks following injection (toxin, 153 ± 3 nS; buffer, 101 ± 9 nS).

Presynaptic origin of changes in GABA receptor-mediated inhibition

Application of GABA to the cell body region of both area CA3 and the dentate gyrus produced a long, 30 s, inward current with the cell clamped at −50 mV. The current was larger that the associated synaptically elicited IPSCs, probably because of a small 'depolarizing' current, which reversed at −54 mV in both regions (data not shown). Similar sized responses to GABA application were seen in CA3 2 weeks after toxin or buffer injection (Fig. 5*A*) despite marked differences in synaptically evoked responses. No significant differences in response to exogenous GABA were seen in either region of hippocampi ipsi- or contralateral to the injection site, during either the disinhibitory phase or the rebound phase of toxin effects on g_{IPSC} ($P > 0{\cdot}05$; Fig. 5*B*).

Comparison of recovery from disinhibition and epileptic activity

In the toxin-injected hippocampus, inhibition recovered more rapidly than the remission from epileptic activity. In the ipsilateral CA3, g_{IPSC} had returned to control values 8 weeks after toxin injection, whereas epileptic activity was still present (Fig. 6*A*). In the contralateral CA3, inhibition was not significantly different from control levels after 4 weeks, whereas burst discharges could be evoked up to 8 weeks after injection (Fig. 6*B*).

This phenomenon was more marked in the dentate gyrus ipsilateral to the injection site; g_{IPSC} recovered to

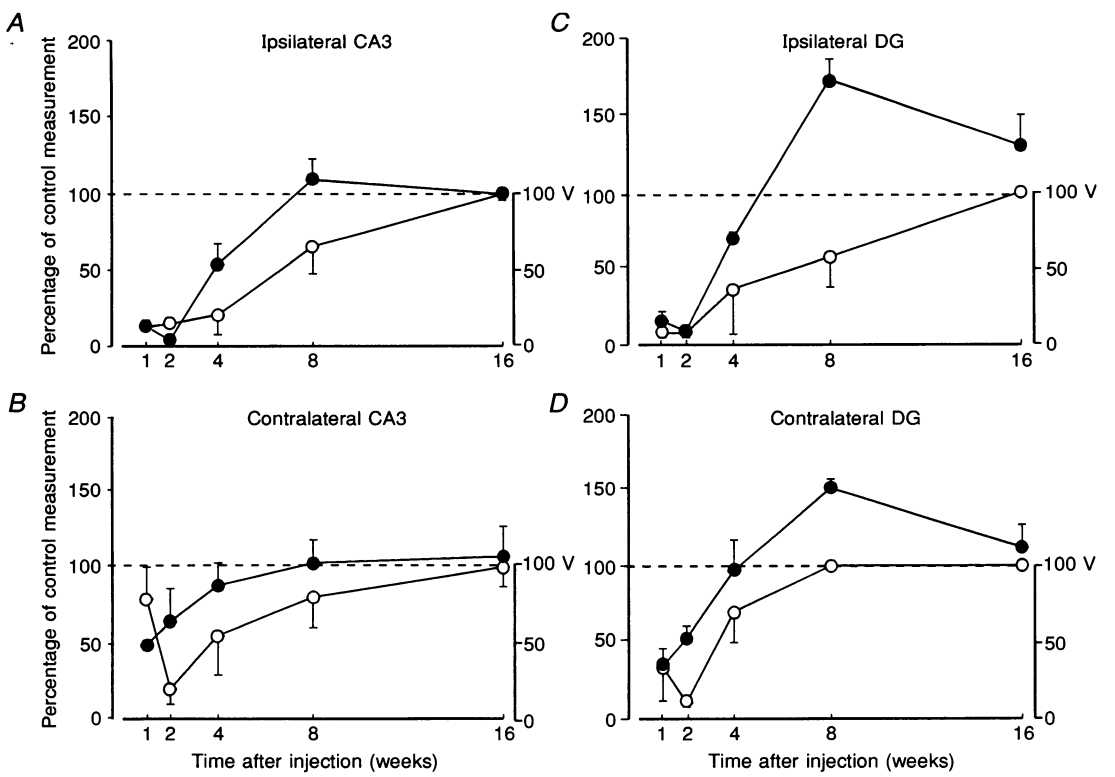

Figure 6. Comparison of the time course of changes in $GABA_A$ergic inhibition and epileptic activity following tetanus toxin

Graphs show maximal g_{IPSC} in slices from toxin-treated rats, expressed as a percentage of time- and region-matched control values from buffer-injected rats (●, left-hand axes). The corresponding measurements of evoked epileptic activity threshold are superimposed (○, right-hand axes; see Fig. 2). Comparisons of inhibition and epileptic activity are made in area CA3 both ipsilateral (*A*) and contralateral (*B*) to the toxin injection site, and in the dentate granule cell layer ipsilateral (*C*) and contralateral (*D*) to the injection site.

control levels after approximately 6 weeks and became larger thereafter. At 8 weeks, g_{IPSC} was markedly greater than in controls and yet epileptic activity was still very much apparent (Fig. 6*C*). Furthermore, population spike amplitudes in the injected dentate gyrus were also markedly larger than in controls (Fig. 3*C*). A lesser divergence of disinhibition and epileptic activity was seen in the contralateral dentate gyrus. g_{IPSC} had returned to control levels 4 weeks after injection, whereas evoked responses were still epileptic. In contrast to the ipsilateral dentate gyrus, the rebound increase in g_{IPSC} was not associated with epileptic activity.

Comparison of mono- and polysynaptic inhibitory drive

The persistence of epileptic activity after monosynaptic $GABA_A$ receptor-mediated inhibition had recovered, or reached values above control, suggested other epileptogenic mechanisms. Afferent stimuli at the threshold for a single action potential generated no observable fast or slow IPSPs in the ipsilateral CA3 up to 8 weeks after injection. In comparison, stimuli sufficient to generate a single action potential in CA3 ipsilateral to buffer injection generated a large biphasic inhibitory wave (Fig. 7*A*). Despite the 160% greater monosynaptic g_{IPSC} in ipsilateral dentate granule cells, polysynaptic potentials contained a smaller positive-going initial wave (corresponding to the depolarizing fast IPSP in the relatively hyperpolarized granule cell) 8 weeks after toxin injection. The hyperpolarizing late component of inhibition was also smaller when compared with buffer-injected controls (Fig. 7*B*).

In the contralateral hippocampus, the initial inhibitory component of the compound synaptic potential was not different from buffer-injected controls in CA3 and the dentate gyrus at 8 weeks (Fig. 7*C* and *D*). The later inhibitory component was still considerably reduced in CA3 (Fig. 7*C*).

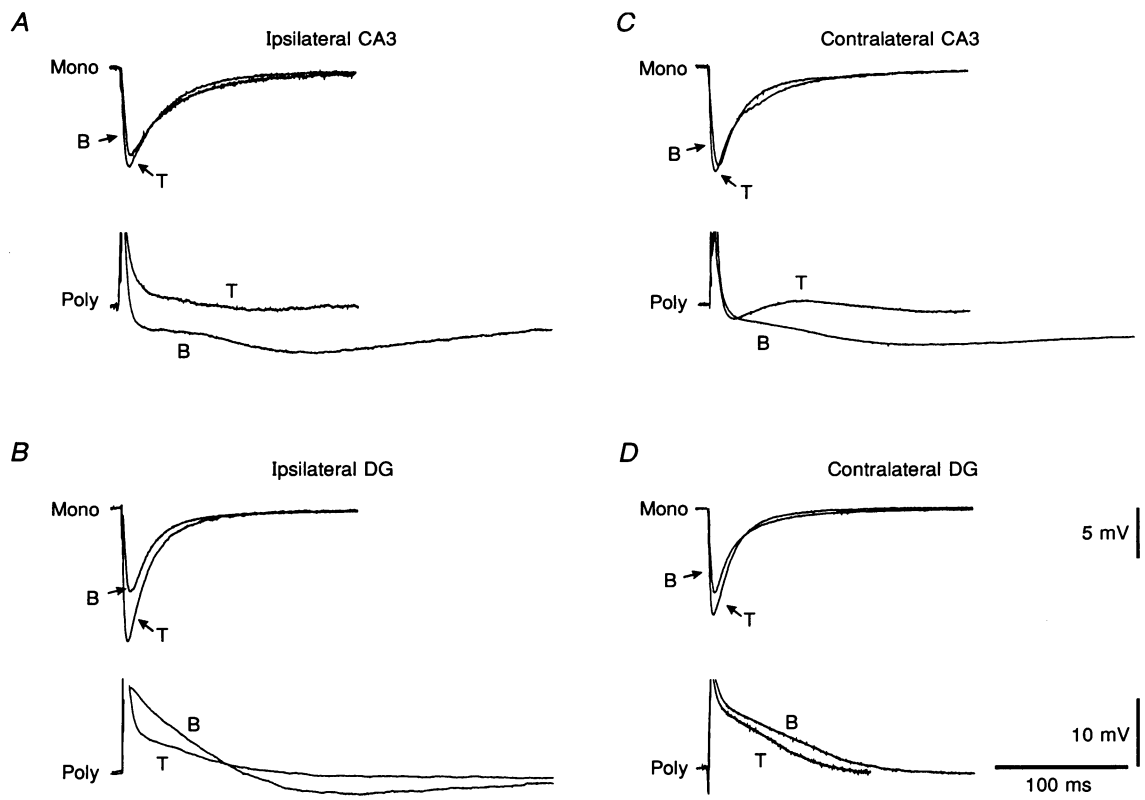

Figure 7. Comparison of monosynaptic $GABA_A$ergic inhibition and polysynaptic compound synaptic potentials following tetanus toxin injection

A, pharmacologically isolated fast inhibitory postsynaptic potentials (Mono) were evoked from a membrane potential of −50 mV in CA3 pyramidal cells ipsilateral to the injection site 8 weeks after buffer (B) or toxin (T) injection. Stimulus strength was sufficient to generate maximal IPSPs. Lower two traces are corresponding examples of compound synaptic potentials (Poly) from CA3 pyramidal cells, evoked from resting membrane potential, 8 weeks after buffer or 6 weeks after toxin injection. Stimulus strengths were adjusted to threshold for single action potential generation. Note the absence of biphasic inhibitory wave in T. *B*, isolated IPSPs evoked from −50 mV in dentate granule cells ipsilateral to buffer (B) or toxin (T) injection. Examples taken 8 weeks after injection. Lower traces are corresponding compound synaptic potentials taken at the 8 week time point. *C*, monosynaptic IPSPs and compound synaptic potentials evoked from the contralateral CA3 8 weeks after buffer or toxin injection. *D*, monosynaptic IPSPs and compound synaptic potentials evoked from the contralateral dentate gyrus 8 weeks after injection.

These polysynaptic potentials revealed an imbalance in excitatory drive, with more drive to excitatory rather than inhibitory neurones.

DISCUSSION

A single injection of tetanus toxin into one hippocampus led to almost complete loss of $GABA_A$ receptor-mediated inhibition in the injected hippocampus and an over 50% decrease in inhibition on the contralateral side. Epileptic activity occurred bilaterally and was most severe 2 weeks after injection, as reported previously (Empson & Jefferys, 1993). The key observation of the present study was the discrepancy between levels of disinhibition and severity of epileptic activity at a number of stages of the model. Epileptic activity was marginal 1 week after toxin injection in the contralateral CA3, a time when inhibition was at its lowest level. By 2 weeks epileptic activity was profound, whereas inhibition was returning to normal values. Substantial epileptic activity was seen bilaterally in all regions after recovery from disinhibition. This observation was most apparent ipsilaterally, in particular in the dentate gyrus.

Long-term changes were also seen, since CA3 responses to mossy fibre input remained decreased and granule cell responses to perforant path stimulation remained increased at 16 weeks. The depression in amplitude of the CA3 response has been reported to be associated with the long-lasting behavioural deficits associated with the tetanus toxin model (George & Mellanby, 1982; Mellanby, Renshaw, Cracknell, Rands & Thompson, 1982; Brace *et al.* 1985; Jefferys & Williams, 1987). The long-term increase in response size seen in the dentate gyrus in the present study contrasts with the decrease shown previously *in vivo* after toxin injection into the ventral hippocampus (Mellanby & Sundstrom, 1986), suggesting either differences between *in vivo* and *in vitro* measurements or different adaptive mechanisms to hyperexcitability in the dorsal and ventral hippocampi.

The primary effect of tetanus toxin is to prevent exocytosis, blocking transmitter release from, in particular, inhibitory synapses (Mellanby & Green, 1981). The presence of massive disinhibition at the early stages, 1–2 weeks, in the injected hippocampus is consistent with this mechanism. The presynaptic action of the toxin is confirmed in the present study by the lack of change in postsynaptic response to GABA (Fig. 7). The pattern of changes in inhibition in the contralateral hippocampus followed those seen ipsilaterally, suggesting the passage of toxin from the injected to the uninjected hemisphere. Although $<1\%$ of injected toxin leaves the site of injection used here (Mellanby, 1989), this very small amount of toxin could have functional effects if located at the appropriate synapses. The toxin is retrogradely and anterogradely transported along axons in smooth vesicles and may undergo trans-synaptic transfer (Schwab, Suda & Thoenen, 1979; Manning, Erichsen & Evinger, 1990). Both CA3 pyramidal cells and hilar projection neurones provide a route for the toxin to reach the contralateral hippocampus, where trans-synaptic transfer and the partial specificity for blockade of inhibitory synapses would result in the disinhibition seen. However, this interpretation does not satisfactorily explain why the peak epileptic activity occurred 1 week later than peak disinhibition seen in contralateral CA3. The difference in the primary route of administration (extracellularly on the injected side, intracellularly on the contralateral side) may expose relatively more excitatory synapses on the contralateral side to the toxin, thus amplifying the partial blockade of excitation.

Disinhibition lasted for weeks after toxin injection (*ca* 6 weeks ipsilaterally, 4 weeks contralaterally) but only very small amounts of toxin are detectable 2 weeks after injection (Habig, Bigalke, Bergey, Neale, Hardegree & Nelson, 1986; Mellanby, 1989). This observation can only be explained if the toxin causes permanent dysfunction of inhibitory synapses. Studies on the effects of tetanus toxin on the mouse neuromuscular junction (Duchen & Tonge, 1973) have shown that endplate potentials reappeared only with the creation of new endplates. The slow recovery from disinhibition seen in the present study is most likely to represent formation of new GABAergic inhibitory synapses (Najlerahim, Williams, Pearson & Jefferys, 1992). The recovery from disinhibition involved no change in postsynaptic response to GABA, indicating no net change in postsynaptic GABA receptor number. However, recovery in the dentate granule cell layer was followed by a marked rebound increase in synaptic inhibition 8 weeks after toxin injection. IPSCs were similar in size to the currents generated by exogenous GABA whereas, in controls, IPSCs were usually 60–75% of the GABA response. This suggests an increase in the utilization of $GABA_A$ receptors at active synapses with fewer extrasynaptic GABA receptors elsewhere on the target membrane. This situation differs from the long-term decrease in sensitivity of principal cells in the hippocampus to GABA seen in the kindling model of chronic epilepsy (Kamphuis, Gorter & Lopes da Silva, 1991).

Factors other than a direct impairment of GABA release must contribute to long-term epileptic activity following tetanus toxin. Prevention of seizures with anti-epileptic agents for the initial period following toxin injection can prevent the development of long-term epilepsy in the cat (Darcey & Williamson, 1992). Furthermore, GABA release recovered to normal levels by 4 weeks in rats following neocortical injection of tetanus toxin (Empson *et al.* 1993). Repeated seizures are well known to cause permanent reductions in the threshold for epileptic seizures, known as kindling (Goddard, McIntyre & Leech, 1969). It is likely that similar processes are involved in the evolution of tetanus toxin foci; indeed, the diversity of cellular and molecular mechanisms implicated in kindling are so great that this is almost inevitable (reviewed by Mody, 1993). The exposure of CNS structures to repeated epileptic events appears to act as a stimulus for reorganization of neuronal

connectivity (Cornish & Wheal, 1989; Cavazos, Golarai & Sutula, 1991; Hoffman, Salin & Prince, 1992; Bekenstein & Lothman, 1993). Neuronal death plays an intrinsic part in a number of the models used by these authors; in particular, the kainate and cortical lesion models. The model used here does not cause detectable neuronal death in the majority (90%) of rats (Jefferys, Evans, Hughes & Williams, 1992; Najlerahim *et al.* 1992). However, glial fibrillary acidic protein immunoreactivity increased substantially in CA3 stratum lucidum and the hilar region immediately below the suprapyramidal blade of the dentate granule cell layer (M. A. Whittington, T. Price & J. G. R. Jefferys, unpublished observations). These observations suggest synaptic reorganization induced by epileptic activity following tetanus toxin.

While epileptic activity persisted after the recovery of monosynaptic IPSCs, polysynaptic activation of inhibition (i.e. with intact EPSPs) onto CA3 pyramidal cells and dentate granule cells was considerably reduced. This suggested a failure of excitatory connections onto interneurones or a decrease in intrinsic interneuronal excitability. This phenomenon has been reported for models of epilepsy in area CA1, the dentate gyrus and the neocortex (Sloviter, 1987, 1991; Cornish & Wheal, 1989; Ribak, Joubran, Kesslak & Bakay, 1989; Beckenstein & Lothman, 1993) and has been termed the dormant inhibitory interneurone hypothesis. Of particular relevance from work on the dentate gyrus is the suggestion that it is particular somatostatin-containing neurones in the hilus which fail to excite inhibitory cells in the dentate area. Hilar interneurones also inhibit CA3 neurones (Hoffman *et al.* 1992), providing a link between this hypothesis and the similar pattern of changes in monosynaptic and polysynaptic inhibition following tetanus toxin in the present study. All of these studies measured inhibition of the principal cell and therefore we cannot yet exclude the possibility of selective hypertrophy of excitatory connections onto principal cells swamping available inhibition.

This study shows that tetanus toxin causes epileptic activity by different mechanisms during the initial and later stages. We propose that the epileptic activity caused by the loss of GABA release causes pathological changes in hippocampal architecture, possibly the disconnection or hypoexcitability of inhibitory interneurones, leading to further lowering of seizure threshold. This phenomenon enables the period of susceptibility to epilepsy to long outlive the presence of the initial disinhibitory epileptogenic stimulus.

REFERENCES

Beckenstein, J. W. & Lothman, E. W. (1993). Dormancy of inhibitory interneurons in a model of temporal lobe epilepsy. *Science* **259**, 97–100.

Bergey, G. K., MacDonald, R. L., Habig, W. H., Hardegree, M. C. & Nelson, P. G. (1983). Tetanus toxin: convulsant action on spinal cord neurons in culture. *Journal of Neuroscience* **3**, 2310–2323.

Bevan, S. & Wendon, L. M. B. (1984). A study of the action of tetanus toxin at rat soleus neuromuscular junctions. *Journal of Physiology* **348**, 1–17.

Bittner, M. A. & Holz, R. W. (1988). Effects of tetanus toxin on catecholamine release from intact and digitonin-permeabilized chromaffin cells. *Journal of Neurochemistry* **51**, 451–456.

Brace, H. M., Jefferys, J. G. R. & Mellanby, J. (1985). Long-term changes in hippocampal physiology and in learning ability of rats after intrahippocampal tetanus toxin. *Journal of Physiology* **368**, 343–357.

Brener, K., Amitai, Y., Jefferys, J. G. R. & Gutnick, M. J. (1990). Chronic epileptic foci in neocortex: in vivo and in vitro effects of tetanus toxin. *European Journal of Neuroscience* **3**, 47–54.

Cavazos, J. E., Golarai, G. & Sutula, T. P. (1991). Mossy fibre synaptic reorganisation induced by kindling: timecourse of development, progression and permanence. *Journal of Neuroscience* **11**, 2795–2803.

Collingridge, G. L., Thompson, P. A., Davies, J. & Mellanby, J. (1981). In vitro effect of tetanus toxin on GABA release from rat hippocampal slices. *Journal of Neurochemistry* **37**, 1039–1041.

Cornish, S. M. & Wheal, H. V. (1989). Long-term loss of paired pulse inhibition in the kainic acid-lesioned hippocampus of the rat. *Neuroscience* **28**, 563–571.

Darcey, T. M. & Williamson, P. D. (1992). Chronic/semichronic limbic epilepsy produced by microinjection of tetanus toxin in cat hippocampus. *Epilepsia* **33**, 402–419.

Davies, C. H., Davies, S. N. & Collingridge, G. L. (1990). Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *Journal of Physiology* **424**, 513–531.

Davies, S. N. & Collingridge, G. L. (1989). Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. *Proceedings of the Royal Society* B **236**, 373–384.

Duchen, L. W. & Tonge, D. A. (1973). The effects of tetanus toxin on neuromuscular transmission and on the morphology of motor end-plates in slow and fast skeletal muscle of the mouse. *Journal of Physiology* **228**, 157–172.

Empson, R. M., Amitai, Y., Jefferys, J. G. R. & Gutnick, M. J. (1993). Injection of tetanus toxin into the neocortex elicits persistent epileptiform activity but only transient impairment of GABA release. *Neuroscience* **57**, 235–239.

Empson, R. M. & Jefferys, J. G. R. (1993). Synaptic inhibition in primary and secondary chronic epileptic foci induced by intrahippocampal tetanus toxin in the rat. *Journal of Physiology* **465**, 595–614.

George, G. & Mellanby, J. (1982). Memory deficits in an experimental epileptiform syndrome in rats. *Experimental Neurology* **75**, 678–689.

Goddard, G. V., McIntyre, D. C. & Leech, C. K. (1969). A permanent change in brain function resulting from daily electrical stimulation. *Experimental Neurology* **25**, 295–330.

Habig, W. H., Bigalke, H., Bergey, G. K., Neale, E. A., Hardegree, M. C. & Nelson, P. G. (1986). Tetanus toxin in dissociated spinal cord cultures: long-term characterization of form and action. *Journal of Neurochemistry* **47**, 930–937.

Hoffman, S. N., Salin, P. A. & Prince, D. A. (1992). Chronic cortical injury and epileptogenesis in vitro. *Epilepsia* **33**, 31.

Jefferys, J. G. & Empson, R. M. (1990). Development of chronic secondary epileptic foci following intrahippocampal injection of tetanus toxin in the rat. *Experimental Physiology* **75**, 733–736.

Jefferys, J. G. R., Evans, B. J., Hughes, S. A. & Williams, S. F. (1992). Neuropathology of the chronic epileptic syndrome induced by intrahippocampal tetanus toxin in rat: preservation of pyramidal cells and incidence of dark cells. *Neuropathology and Applied Neurobiology* **18**, 53–70.

JEFFERYS, J. G. R., MITCHELL, P., O'HARA, L., TILEY, C., HARDY, J., JORDAN, S. J., LYNCH, M. & WADSWORTH, J. (1991). *Ex vivo* release of GABA from tetanus toxin-induced chronic epileptic foci decreased during the active seizure phase. *Neurochemistry International* **18**, 373–379.

JEFFERYS, J. G. R. & WILLIAMS, S. F. (1987). Physiological and behavioural consequences of seizures induced in the rat by intrahippocampal tetanus toxin. *Brain* **110**, 517–532.

JORDAN, S. J. & JEFFERYS, J. G. R. (1992). Sustained and selective block of IPSPs in brain slices from rats made epileptic by intrahippocampal tetanus toxin. *Epilepsy Research* **11**, 119–129.

KAMPHUIS, E., GORTER, J. A. & LOPES DA SILVA, F. (1991). Long term decrease in sensitivity of hippocampal pyramidal cells to GABA induced by kindling epileptogenesis. *Neuroscience* **41**, 425–431.

MANNING, K. A., ERICHSEN, J. T. & EVINGER, C. (1990). Retrograde transneuronal transport properties of fragment C of tetanus toxin. *Neuroscience* **34**, 251–263.

MELLANBY, J. (1989). I-125 labelled tetanus toxin recovery in ventral hippocampus. *Neuroscience Letters Supplement* **36**, S55.

MELLANBY, J., GEORGE, G., ROBINSON, A. & THOMPSON, P. (1977). Epileptiform syndrome in rats produced by injecting tetanus toxin into hippocampus. *Journal of Neurology, Neurosurgery and Psychiatry* **40**, 404–414.

MELLANBY, J. & GREEN, J. (1981). How does tetanus toxin act? *Neuroscience* **6**, 281–300.

MELLANBY, J., RENSHAW, M., CRACKNELL, H., RANDS, G. & THOMPSON, P. (1982). Long term impairment of learning ability after an experimental hippocampal epileptiform syndrome. *Experimental Neurology* **75**, 690–699.

MELLANBY, J. & SUNDSTROM, L. (1986). Long-term reduction in excitability of rat dentate granule cells after tetanus toxin. *Journal of Physiology* **371**, 49*P*.

MODY, I. (1993). The molecular basis of kindling. *Brain Pathology* **3**, 395–403.

MONTECUCCO, C. (1989). Thoretical considerations on the cellular mechanism of action of clostridial neurotoxins. In *Eighth International Conference on Tetanus*, ed. NISTICO, G., BIZZINI, B., BYTCHENKO, B. & TRIAU, R., pp. 71–91. Pythagora Press, Rome.

NAJLERAHIM, A., WILLIAMS, S. F., PEARSON, R. C. A. & JEFFERYS, J. G. R. (1992). Increased expression of GAD mRNA during the chronic epileptic syndrome due to intrahippocampal tetanus toxin. *Experimental Brain Research* **90**, 332–342.

PENNER, R., NEHER, E. & DREYER, F. (1986). Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature* **324**, 76–78.

RIBAK, C. E., JOUBRAN, C., KESSLAK, J. P. & BAKAY, R. A. E. (1989). A selective decrease in the number of GABAergic somata occurs in pre-seizing monkeys with alumina gel granuloma. *Epilepsy Research* **4**, 126–138.

ROUX, E. & BORREL, A. (1898). Tétanos cérébral et immunité contre le tétanos. *Annal de l'Institut Pasteur* **4**, 126–138.

SCHIAVO, G., BENFANATI, F., POULAIN, B., ROSSETTO, O., POLVERINO DE LAURETO, P., DASGUPTA, B. & MONTECUCCO, C. (1992). Tetanus and botulinum-B neurotoxins block transmitter release by proteolytic cleavage of synaptobrevin. *Nature* **359**, 832–835.

SCHWAB, M. E., SUDA, K. & THOENEN, H. (1979). Selective retrograde transsynaptic transfer of protein, tetanus toxin, subsequent to its retrograde axonal transport. *Journal of Cell Biology* **82**, 798–810.

SLOVITER, R. S. (1987). Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science* **235**, 73–76.

SLOVITER, R. S. (1991). Permanently altered hippocampal structure, excitability and inhibition after experimental status epilepticus in the rat: The 'dormant basket cell' hypothesis and its possible relevence to temporal lobe epilepsy. *Hippocampus* **1**, 41–66.

WHITTINGTON, M. A. & JEFFERYS, J. G. R. (1993). Epileptic activity outlasts disinhibition in area CA3c and the dentate gyrus *in vitro* during the tetanus toxin model of chronic epilepsy in the rat. *Journal of Physiology* **473**, 164*P*.

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