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REGULAR PAPER

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Loss of hilar somatostatin neurons following tetanus toxin-induced seizures

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Abstract A loss of inhibitory interneurons has been reported in the hippocampus following seizure activity in various animal models of epilepsy and in human epileptic tissue. The question of whether particular populations of inhibitory neurons are similarly affected by the chronic block of inhibition that results after tetanus toxin injections directly into the brain has not previously been addressed. In the present study a unilateral intrahippocampal injection of tetanus toxin into the ventral hippocampus was used to produce a chronic epileptic syndrome characterised by brief seizures that recurred intermittently for 6–8 weeks. The results reveal, for the first time, the morphological changes in somatostatin interneurons following tetanus toxin-induced seizures in the rat. A bilateral short-term increase in immunoreactivity of somatostatin neurons is present 1 week after injection. This is accompanied by an increased intensity of somatostatin-immunoreactive axon terminals in the outer molecular layer of the dentate gyrus, which is more marked on the contralateral side. A chronic and significant loss of somatostatin-immunoreactive neurons was noted in the hilus of the dentate gyrus 2 months later. The significance of the chronic loss of the hilar somatostatin neurons in the control of excitatory activity in the dentate gyrus and whether the acute morphological changes are due to a direct action of the toxin on release mechanisms or as a result of seizure activity are discussed.

Key words Epilepsy · Hippocampus · Rat · Somatostatin · Tetanus toxin

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Introduction

The likelihood that a decrease in inhibition participates in epileptogenesis has been a central theme in the investigation of mechanisms of chronic epilepsy [21, 29]. Early investigations focused mainly on models in which an acute block of -aminobutyric acid (GABA)-mediated inhibition was present; however, more recently models have become available in which a chronic block of inhibition is possible [16, 23]. Injection of minute amounts of tetanus toxin directly into the brain generates an epileptiform syndrome, which is presumed to result from a chronic block of inhibition [14, 38], and which allows the study of the consequences of a period of relatively brief but intense seizure activity on hippocampal structure and function.

So far, very few neuropathological consequences have been reported to result from the chronic block of inhibition produced by low doses of tetanus toxin. Unlike toxins acting directly on excitatory transmission, such as kainic acid, overt lesions of pyramidal cells do not appear to result with tetanus toxin [18]. In a recent study, Jefferys et al. [16] have reported that only 3 out of 36 tetanus toxin-injected rats showed a small loss of CA1 pyramidal cells contralateral to the injection site. Other studies have revealed activated microglial cells along the CA1 pyramidal cell dendrites [32], which might be indicative of previous cellular damage in CA1. However, this remains fairly limited compared to damage produced by "excitotoxins" such as agonists of glutamate receptors.

A loss of GABAergic interneurons has frequently been postulated to be at the origin of chronic hyperexcitability in epilepsy. Evidence for a significant loss of GABAergic interneurons, from immunocytochemical studies using glutamate decarboxylase (GAD) as a marker, have been somewhat controversial (for review see [13]). More recent studies have attempted a more refined analysis and have focused on identifying particular subsets of specific inhibitory neurons which are lost or malfunctioning following seizure activity [2, 3, 17, 19, 25, 34, 36, 37]. Hilar interneurons containing the neuropeptides somatostatin and

neuropeptide Y would appear to be the most vulnerable population, losses having been reported following kainic acid treatment [37], perforant path stimulation [34] and in human temporal lobe epilepsy [28]. The question of whether this particular population of inhibitory neurons is similarly affected by the seizure activity in the tetanus toxin model has not previously been addressed.

The present study describes, for the first time, bilateral morphological changes in, and the subsequent loss of, somatostatin-immunoreactive (SI) neurons following a unilateral injection of tetanus toxin into the hippocampus.

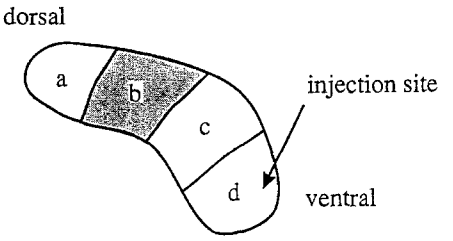

Fig. 1 Diagram shows how the hippocampus was sliced. The *shaded area* (*b*) was used for somatostatin-immunoreactive neuronal cell counts

Materials and methods

Animals

Wistar rats (n = 54) weighing between 180 and 200 g body weight were anaesthetised with an intraperitoneal injection of sodium pentobarbitone (Sagatal 60 mg/kg) and placed in a stereotaxic frame. Animals were separated into groups: group A (n = 44) received a unilateral injection of 0.5 µl (10 ng) tetanus toxin (a gift from Wellcome Biotech) into the CA3 region of the ventral hippocampus (co-ordinates: +3.2 mm from interaural line; +5.3 mm lateral; +6.0 mm ventral from dura: [26]); and group B (n = 10) received a unilateral injection of physiological saline (0.5 µl) into the ventral hippocampus. Animals in group B were killed by perfusion (see below) 2 months later. Animals in group A were killed at 24 h (n = 2); 48 h (n = 2); 96 h (n = 2); 5 days (n = 2); 6 days (n = 2); 7 days (n = 2); 9 days (n = 2); 12 days (n = 2); 14 days (n = 6); 1 month (n = 10) and 2 months (n = 12) after the injection of tetanus toxin. Each tetanus toxin-injected animal was continuously monitored throughout the first 7 days by means of a video camera to establish which animals were experiencing spontaneous seizures.

Fixation

While under deep Sagatal anaesthesia, the animals were perfused with 100 ml of physiological saline via a cannula inserted into the left ventricle followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The hippocampi were removed from each animal and postfixed in 4% paraformaldehyde for a further 4 h. Each hippocampus was sliced into four blocks (Fig. 1) and transferred into 0.1 M phosphate-buffered saline (PBS) pH 7.4 overnight at 4°C. Serial 40-µm-thick vibratome sections from each block were collected in PBS prior to further treatment.

Histology and immunocytochemistry

Serial sections of hippocampal tissue from block d (Fig. 1) were stained with 1% cresyl violet and used to confirm the position of the injection site, and to establish any gross pathology or obvious cell loss. Serial 40-µm sections of hippocampus from block b (Fig. 1) were processed for somatostatin immunoreactivity using the avidin-biotin method with Vectastain kits. To avoid any variability in immunostaining sections from all animals were incubated at the same time and with the same reagents. In brief: endogenous peroxidase activity was blocked by treating sections with a methanol/hydrogen peroxide solution (11.8:0.2, v/v) for 10 min. After rinsing in PBS, sections were treated with 1% bovine serum albumin in distilled water for 5 min, rinsed in PBS and incubated in the primary somatostatin antibody (a kind gift of G. Sperk, University of Innsbruck) at a dilution of 1:1000. This antibody, raised in rabbits against the synthetic peptide, has been extensively characterised by radioimmunoassay; it recognises on an equimolar basis somatostatin 14, 28 and prosomatostatin [35]. In control sections specific immunoreactivity was blocked by preadsorbing the antiserum with 10 nM somatostatin. Sections were then treated with biotinylated goat anti-rabbit IgG (1:200; 90 min at 20°C), avidin-biotin horseradish peroxidase complex (1:100; 120 min at 20°C). Immunoreactivity was visualised using 0.05% diaminobenzidine and 0.01% hydrogen peroxidase (2–6 min) in 0.02 M PBS pH 7.6.

Cell counts

The total number of SI neurons was counted in the dentate hilus of each animal. A minimum of 30 (average of 40) serial 40-µm sections were used from each hippocampus. To minimise the possibility of counting the same neuron in adjacent sections only neurons with an obvious nucleus were counted. Statistical analyses (unpaired Student t-test) were performed on the data from hippocampi of both sides.

Results

Animal behaviour

Each tetanus toxin-injected animal exhibited the characteristic epileptic syndrome which has been described in detail elsewhere [12, 23]. The first seizures occurred usually between 4 and 6 days after injection and then on a chronically recurrent basis over several weeks. These seizures typically begin with arrest of activity, followed by myoclonic jerks of the front limbs and, in some animals, generalised tonic clonic seizures. During the final day of continuous monitoring (day 7 after injection) all of the tetanus toxin-injected animals exhibited myoclonic jerks of the front limbs and in 15 animals this activity developed into a generalised seizure. The number of seizures ranged from 6 to 12, but in one animal 22 seizures were recorded. The duration of the seizures varied between 5 and 73 s. None of the animals in group B showed any evidence of seizure activity.

Cresyl violet staining

Although neuronal counts were not performed on the cresyl violet-stained sections, there was no obvious loss of neurons observed in the tetanus toxin- or saline-injected hippocampi when compared to the contralateral side at any of the time periods examined. In those tetanus toxin-injected animals in which the injection site was positively identified, it was found to be on target.

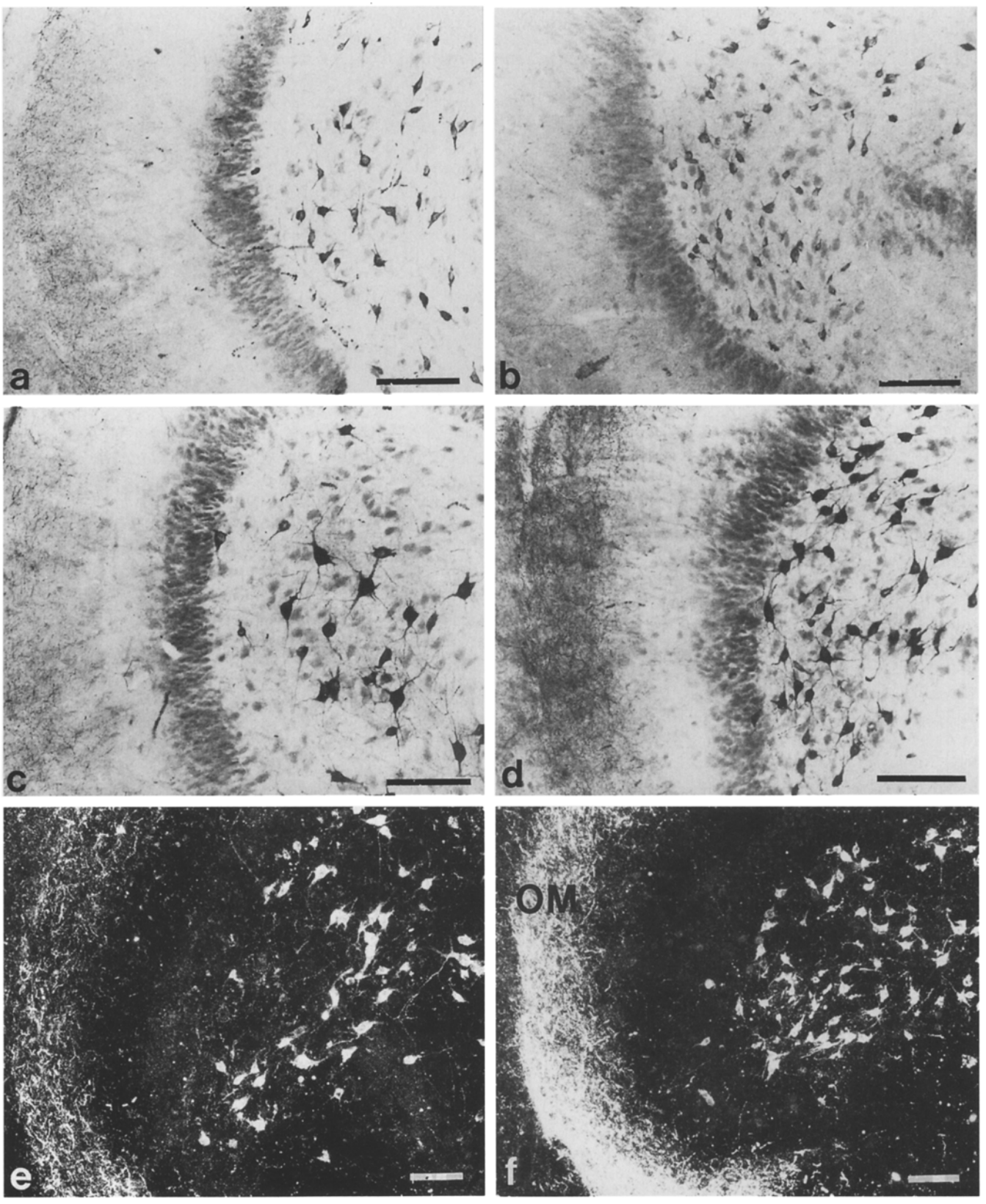

Fig. 2 SI neurons in the dentate hilus and their terminal arborisation in the outer molecular layer (*OM*) of the injected (**c, e**) and contralateral (**b, d, f**) hippocampi. **a–d** Counterstained lightly with thionin. **e, f** Dark-ground microscopy. **a** Saline-injected control; **b** 5 days; **c, d** 7 days; **e, f** 9 days after injection of tetanus toxin. *Scale bar* = 100 μm

Table 1 Mean number and standard deviation of hilar somatostatin-immunoreactive neurons per section. Significance levels from saline-injected controls

No. of animals	Group	Mean and SD Left side	*P*	Mean and SD Right side	*P*
10	Saline	35.4 + 3.7	–	35.4 + 3.7	–
22	2 weeks	38.3 + 7.0	NS	39.9 + 7.1	NS
10	4 weeks	35.4 + 6.7	NS	37.8 + 6.8	NS
12	8 weeks	25.6 + 4.5	***	24.1 + 4.8	***

NS Not significant; *** $P = < 0.001$

Somatostatin staining

At post-injection times of less than 1 week, no obvious difference was apparent in the morphology or the intensity of the hilar SI neurons in the tetanus animals when compared to those observed in sections from the saline-injected animals. However, between 7 and 14 days after injection there was an obvious bilateral increase in the intensity of staining of the SI neurons (Fig. 2a–d). This was accompanied by a marked increase in the intensity of the SI terminal plexus in the outer molecular layer (OML) of the dentate gyrus, which was more marked in the contralateral hippocampus (Fig. 2e, f). At 1 and 2 months after injection of the tetanus toxin the SI neurons appeared normal.

Number of hilar SI neurons

There was no significant difference between the number of SI neurons in the saline-injected hippocampus and that in the contralateral hippocampus of the same saline-injected animals and, therefore, the results are pooled. The number of SI neurons in the dentate hilus of animals killed at periods of less than 2 weeks after tetanus injections did not differ significantly from those killed at 2 weeks, and for convenience the results are pooled (Table 1).

As can be seen from Table 1 there was no significant difference between the number of SI neurons in the dentate hilus of the tetanus toxin- injected animals killed at 2 and 4 weeks after injection and the number counted in the hilus of the saline-injected animals. However, at 8 weeks after an injection of the tetanus toxin, there was a highly significant bilateral reduction in the number of hilar SI neurons per section when compared to the saline-injected controls.

Discussion

This study demonstrates, for the first time, changes in SI interneurons following tetanus toxin-induced seizures in the rat. An acute increase in immunoreactivity of SI neurons and their axon terminals in the OML of the dentate gyrus is followed by a chronic loss of SI neurons in the dorsal dentate hilus after a unilateral injection of tetanus toxin into the ventral hippocampus.

This discussion addresses the following points: the significance of the short-term morphological changes and in particular the more marked increased activity of SI axon terminals in the OML of the contralateral dentate gyrus; and how the chronic loss of SI hilar neurons compares with that seen in other animal models of epilepsy and with that seen in biopsy material from human epileptic patients.

Significance of the short-term morphological changes in the SI neurons

The bilateral increase in somatostatin immunoreactivity seen around 7 days could result from direct actions of the toxin on release mechanisms or indirectly from the seizure activity.

The acute mechanism of action of tetanus toxin involves a heavy chain responsible for specific binding to neuronal cells and a light chain which blocks neurotransmitter release through cleaving synaptobrevin 2, an integral membrane protein of small synaptic vesicles [30]. The mechanism through which the toxin initially generates a block of inhibition involves a depression of the evoked release of the inhibitory transmitter GABA [5, 15]. Since the majority of the hilar SI cells also contain GABA, it would seem reasonable to assume that the tetanus toxin is acting on these cells. Somatostatin, like other neuropeptides, is derived from precursor molecules which are synthesised in the perikaryon, stored in vesicles and then transported to the nerve terminals [12]. An accumulation of somatostatin immunoreactivity, as seen in the ipsilateral hilar neurons of the present study, could be explained by the toxin blocking the release of the peptide. The increased somatostatin immunoreactivity observed in the contralateral hilar neurons might be the result of the anterograde or retrograde transport of the toxin [31]. Both CA3 pyramidal cells and hilar projection neurons provide possible routes for the toxin to reach the contralateral hippocampus. However, since the tetanus toxin remains in the injected hippocampus [22], as does the block of Ca^{2+}-dependent K^+-evoked release of GABA [8, 9], this explanation seems unlikely.

An alternative explanation could be that the increased immunoreactivity of the SI neurons both ipsilaterally and contralaterally may be a reflection of increased synaptic activation due to seizure activity. This latter explanation seems more likely in view of the fact that the toxin blocks inhibition within a few hours [38] but the increased immunoreactivity appears after approximately 7 days, when overt seizures are apparent. A similar explanation has been offered to explain the increased levels of GAD mRNA present in the hilar cells in this tetanus toxin model [24].

The increased somatostatin immunoreactivity may be a reflection of increased somatostatin synthesis, in an attempt to further increase inhibitory potency as a result of seizure activity. It has been suggested that, by analogy with peripheral systems, neuropeptides, which are stored

in large dense-cored vesicles, are preferentially released during the increased activity that may occur during epileptiform bursting [12, 37]. The hippocampus is able to cope with the initial disinhibition in the injected hippocampus but, as the hilar mossy cells are uncoupled from their inhibitory control and the commissural/associational excitation increases, the epileptiform focus "matures" [24] and further morphological changes such as axonal sprouting and synaptic reorganisation might be necessary.

A considerable degree of anatomical plasticity appears to occur following seizure activity in the hippocampi in several animal models [4, 7, 27, 37, 39] and in humans [1, 40]. The increase in somatostatin immunoreactivity observed in OML of the present study could be a reflection of sprouted somatostatin terminals. The more marked increase observed in the contralateral OML may be an attempt to reduce afferent excitation of contralateral granule cells in the face of a developing mirror focus. This would also indirectly result in a reduction of reverberating commissural activity back to the ipsilateral side, which would presumably have a reduced seizure threshold at this point.

The long-term loss of SI neurons

The initial observation by Sloviter [33] of a specific reduction in SI neurons, similar to that observed in the present study, following repeated perforant path stimulation has also been reported in other animal models [6, 37] and in human temporal lobe epilepsy [19, 28]. These neurons also appear to be vulnerable to traumatic brain injury [20] and cerebral ischaemia [17]. These SI neurons seem, therefore, to be much more vulnerable to excitotoxic damage (caused by excessive release of glutamate) than, for example, the basket cell interneurons, which appear to survive perforant path stimulation [33], kainic acid seizures [2, 7] and are still present in hippocampi from human epileptic brains [35]. There is some evidence to suggest that the cells that are resistant to excitotoxic damage in the dentate gyrus may be protected by the presence of calcium binding proteins in their cytoplasm [35], although this has been questioned recently [10]. In contrast to other epilepsy models, the tetanus toxin-induced seizures are caused by a complete failure of inhibitory GABAergic mechanisms and not of direct stimulation. Nonetheless, the end-result remains a loss of these vulnerable SI neurons.

In conclusion the present study provides evidence of acute bilateral morphological changes in, and a chronic loss of, hilar SI neurons following a block of inhibition induced by a unilateral intrahippocampal injection of tetanus toxin.

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