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Prevention by the NMDA receptor antagonist, MK801 of neuronal loss produced by tetanus toxin in the rat hippocampus

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1 The behavioural and neuropathological effects of tetanus toxin, microinjected directly into the hippocampus, were studied in rats.

2 A single dose (1000 minimum lethal doses, MLDs) of tetanus toxin, injected unilaterally into the hippocampus produced a time-dependent neuronal loss in the CA1 pyramidal cell layer. In comparison with the contralateral, untreated side these effects became statistically significant ($P < 0.05$) 7 days (22.0 ± 1.1% reduction) and 10 days (29.2 ± 1.7% reduction) after the injection. No significant changes were observed 7 days after treatment with 500 MLDs whereas a reduction of 37.5 ± 3.1% in the CA1 area cell number was produced 4 days after the injection of 2000 MLDs.

3 Behavioural stimulatory effects were also induced by tetanus toxin (1000 MLDs) within 48 h of the injection and these culminated in generalized convulsions 5–7 days later. Convulsions were observed after a shorter period of latency in rats receiving 2000 MLDs tetanus toxin whereas 500 MLDs were ineffective.

4 No behavioural and neuropathological effects were observed in rats treated with neutralized tetanus toxin (1000 MLDs), bovine serum albumin or phosphate buffer.

5 Pretreatment with MK801 (0.3 mg kg^{-1}, i.p., given 1 h before and after the injection with tetanus toxin and then once daily for 4 or 7 days) prevented the behavioural and neuropathological effects induced by tetanus toxin (1000–2000 MLDs). In addition, such treatment fully protected the animals from the lethal effects induced by 1000 MLDs tetanus toxin. By contrast, pretreatment with diazepam (3.0 mg kg^{-1}, i.p.) using the same schedule as for MK801 did not antagonize the effects of tetanus toxin (1000–2000 MLDs).

6 In conclusion, the present experiments have demonstrated that the intrahippocampal injection of tetanus toxin produces in rats a dose- and time-dependent behavioural stimulation and neuronal loss in the CA1 pyramidal cell layer which can be prevented by the non-competitive NMDA antagonist, MK801.

Introduction

Excitatory amino acid receptors have been implicated in the mediation of neuronal degeneration produced by transient ischaemia (Simon *et al.*, 1984; Rothman & Olney, 1986). In particular, activation of the N-methyl-D-aspartate (NMDA) receptor subtype appears to play a major role since antagonists of this receptor complex, e.g. 2-aminophosphonovaleric acid and MK801, prevent ischaemic damage as well as the degeneration produced by administration of NMDA receptor agonists (Simon *et al.*, 1984; Wong *et al.*, 1986; Foster *et al.*, 1987; Gill *et al.*, 1987). In normal brain, neuronal cell activity is maintained by the integrated actions of excitatory and inhibitory afferents. In many brain regions, such as the hippocampus, glutamic acid and γ-aminobutyric acid (GABA) are the neurotransmitters which probably subserve these functions (Fagg & Foster, 1983; Taylor, 1988). Under abnormal conditions when neurodegeneration occurs the synaptic extracellular concentration of glutamate increases (Beneviste *et al.*, 1984) producing neuronal excitation (Suzuki *et al.*, 1983). However, excitation and perhaps degeneration could also arise without any net change in glutamate levels if the GABA-mediated inhibitory input is reduced. We have tested this hypothesis using tetanus toxin which selectively blocks the GABAergic inhibitory tone in the CNS (Davies & Tongroach, 1979) and the release of GABA in rat hippocampal slices (Collingridge *et al.*, 1981).

Methods

Injections and histology

Adult male Wistar rats (280–300 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and tetanus toxin microinjected unilaterally into the CA1 hippocampal area (coordinates A = −4 mm from the bregma, L = 2 mm from the midline, V = 2.4 mm below the dura mater according to the rat brain atlas of Paxinos & Watson, 1982; the volume of injection was 1 μl min^{-1}) by means of a Hamilton microsyringe (5 μl) mounted on a stereotaxic frame. Animals injected with bovine serum albumin (BSA), phosphate buffer (pH = 7.0; used to dissolve tetanus toxin) and neutralized tetanus toxin (the neutralization was carried out with a F(ab)' fragment of the native IgG antitetanus toxin as previously described by Gawade *et al.*, 1985) were used as controls. After 1, 4, 7, 10 days the animals were anaesthetized and perfused – fixed by intracardiac administration of 100 ml 0.1% paraformaldehyde in phosphate buffered (pH = 7.0) saline. Brain coronal sections were cut from a 1 mm brain block which included the needle track and every tenth slice for 300 μm either side of the track was stained with cresyl fast violet, toluidine blue or a silver stain procedure described by Gallyas *et al.* (1980). In pilot experiments we have determined that the damage produced by tetanus toxin extended at least 400 μm either side of the injection site. The number of cells was counted in areas of 3700 μm^2 in each of the pyramidal cell layers of CA1, CA2 and CA3 hippocampal regions and granule cell layer of the dentate gyrus, stained with cresyl fast violet. Cell counting was always performed 'blind' at the same location for all slices ($n = 6$ sections per brain; the site of injection was the same for both control and tetanus toxin-treated animals). Only cells showing normal morphological characteristics were included. Pyknotic cells or cells with cytoplasmic vacuolization and swollen membranes were excluded from the count.

The mean number of neurones from each area was pooled and expressed as mean (±s.e.mean) neurones per brain area per treatment group ($n = 3$–6 brains per treatment group; 6 sections per brain). The pooled mean cell number counted in the treated side was compared with the corresponding contralateral area (control) and the statistical difference within each group was evaluated by Student's *t* test.

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Behavioural and survival study

For the behavioural and survival study, 10 rats from each group of treatment were used. Postural, locomotor and lethal effects induced by the treatments were studied twice daily in blind conditions. The mortality rate was stored and is reported as a percentage of survivors.

Drugs

Tetanus toxin (2.5×10^7 mouse minimum lethal doses, MLDs mg^{-1} of protein) and the F(ab)' fragment were kind gifts of Prof. B. Bizzini (Pasteur Institute, Paris) and were dissolved in phosphate buffer (pH = 7.0). Bovine serum albumin (Sigma, U.S.A.) and MK801 (Dizocilpine, ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, SEMAT, U.K.) were dissolved in twice distilled, pyrogen-free H_2O. The commercially available vials of diazepam (Valium, Roche, Switzerland) were used.

Results

Neuropathological effects

A single dose of tetanus toxin (1000 MLDs; $n = 6$ rats) injected into the right hippocampus failed to produce any neuropathological effects 24 h after the treatment. By contrast, a statistically significant ($P < 0.05$) reduction in the number of cells in the CA1 region was observed after 7 and 10 days ($n = 4$–6 rats respectively). The number of cells was reduced by 22.0 ± 1.1% and 29.2 ± 1.7% at 7 and 10 days, respectively (Table 1). A lower dose of toxin (500 MLDs; $n = 6$ rats) did not produce any apparent reduction in cell number 7 days after treatment whereas a higher dose, 2000 MLDs ($n = 3$ rats) produced a significant ($P < 0.05$) reduction (37.5 ± 2.3%) in the CA1 area cell number. A summary of the quantitative changes produced by these three doses of tetanus toxin at various times is shown in Table 1. The corresponding morphological changes are illustrated in Figure 1 and Figure 2. In rats treated with bovine serum albumin (BSA; 300 ng), phosphate buffer (vehicle for tetanus toxin) or neutralized tetanus toxin (1000 MLDs; by using a 3 fold excess of a non precipitable F(ab)' fragment of the native specific IgG, see Gawade *et al.*, 1985) no morphological or quantitative changes in neuronal cell numbers were observed ($n = 6$ rats per each treatment); (Table 1).

Behavioural effects

Tetanus toxin (1000 MLDs; $n = 10$ rats) produced both postural and locomotor changes characterized by tail rigidity, hunched back, turning and touch and sound-evoked circling, ipsilateral to the site of injection, within 48 h of the treatment. These effects were observed in all of the toxin-treated rats and culminated 5–7 days later in generalized convulsions. Similar effects were evoked by 2000 MLDs tetanus toxin ($n = 10$ rats) although the animals were only observed for a shorter period of time (4 days) due to the high mortality rate (see Figure 3). Minor behavioural changes (i.e. piloerection, tail rigidity and occasionally touch-evoked ipsilateral circling) were produced by 500 MLDs ($n = 10$ rats).

Table 1 Neuronal loss induced by tetanus toxin, directly microinjected into the rat CA1 hippocampal area and its antagonism by MK801

Treatment group	*Number of animals*	CA1		CA2		CA3		DG	
		L	R	L	R	L	R	L	R
Tetanus toxin (1000 MLDs) 1 day after	6	36.5 ± 0.7	32.6 ± 0.6	31.7 ± 0.7	31.0 ± 0.7	16.7 ± 0.5	15.6 ± 0.3	79.2 ± 0.7	78.1 ± 1.1
Tetanus toxin (1000 MLDs) 7 days after	4	44.5 ± 1.7	34.7 ± 1.7*	38.4 ± 1.7	35.9 ± 2.1	20.3 ± 0.5	18.8 ± 0.5	92.0 ± 5.2	95.5 ± 5.7
Tetanus toxin (1000 MLDs) 10 days after	6	37.2 ± 0.9	26.3 ± 0.9*	34.4 ± 0.5	31.5 ± 0.5	19.7 ± 0.5	18.0 ± 0.4	76.1 ± 0.9	74.2 ± 0.7
Neutralized toxin (1000 MLDs) 7 days after	6	44.9 ± 1.8	44.7 ± 1.6	40.3 ± 0.6	39.4 ± 0.5	25.1 ± 0.6	24.6 ± 0.7	82.9 ± 1.8	82.6 ± 1.6
Tetanus toxin (500 MLDs) 7 days after	6	37.7 ± 0.5	35.5 ± 0.4	28.1 ± 0.6	26.1 ± 0.5	22.0 ± 0.4	20.4 ± 0.7	71.7 ± 0.9	68.5 ± 0.6
Tetanus toxin (2000 MLDs) 4 days after	3	39.7 ± 0.7	24.8 ± 1.0*	29.7 ± 0.7	27.4 ± 0.4	24.6 ± 0.5	22.3 ± 0.7	68.9 ± 0.9	65.4 ± 0.9
Tetanus toxin (1000 MLDs) + MK801 (7 days after)	6	39.5 ± 1.0	37.9 ± 0.9	31.9 ± 0.3	30.5 ± 0.4	22.7 ± 0.3	21.7 ± 0.3	76.6 ± 0.3	74.0 ± 0.3
Tetanus toxin (2000 MLDs) + MK801 (7 days after)	3	39.9 ± 1.2	35.8 ± 1.2	35.2 ± 1.2	32.2 ± 1.3	21.2 ± 0.9	19.3 ± 0.6	73.4 ± 2.0	71.6 ± 2.3
Tetanus toxin (2000 MLDs) + Diazepam (4 days after)	3	40.5 ± 0.3	27.9 ± 0.6*	36.7 ± 0.5	34.3 ± 0.6	24.2 ± 0.7	21.3 ± 0.6	78.6 ± 0.8	75.8 ± 0.9

Brain sections (10 μm) of perfused-fixed rats ($n = 3$–6 per treatment group) were analysed under light microscopy (Leitz 40×). Cell counting was performed blind and the analyses concerned the pyramidal layers of CA1, CA2 and CA3 hippocampal areas and the dentate gyrus (DG). Quantitation of the cell numbers (dark degenerating neurones were excluded from the counting) was performed with a 3700 μm^2 box positioned in corresponding areas of control and treated hippocampus within each section ($n = 6$ sections per brain). Data are represented as means ± s.e.mean of cells. Statistically significant changes within the means of treated (R) vs control (L; untreated) side were evaluated by using Student's t test (unpaired data). Due to the interanimal variations in the cell numbers within the same areas, comparison between groups of treatment was not allowed. * denotes $P < 0.05$.

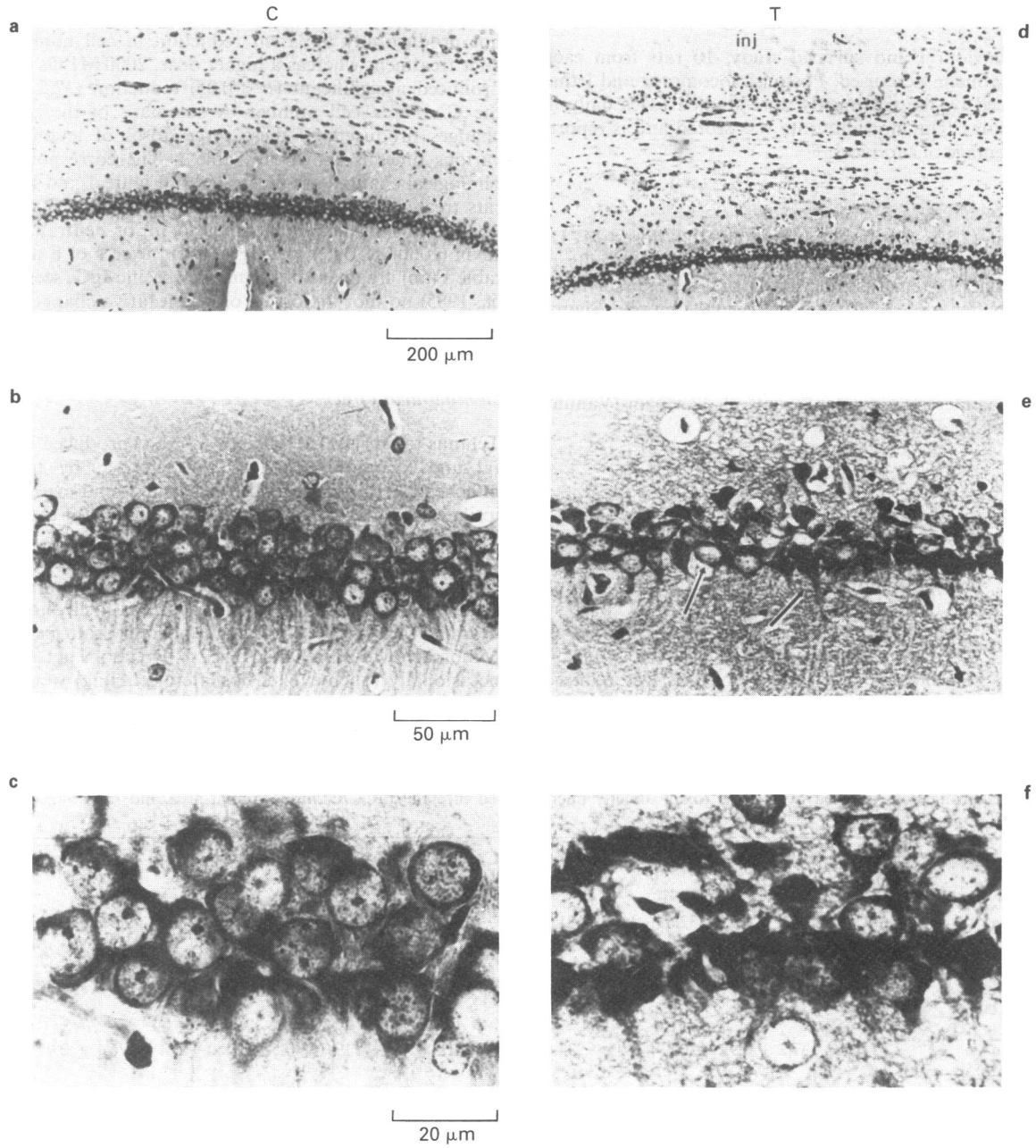

Figure 1 Light photomicrographs of a rat brain coronal section (10 μm; toluidine blue stain) depicting a typical pattern of neuronal damage in the CA1 pyramidal cell layer seven days after a single injection of tetanus toxin (1000 MLDs; d, e and f) compared with the contralateral control (uninjected) side (a, b and c). The same area of damage in (d) (10 × magnification) is also shown at higher magnification in (e) (40 ×) and (f) (100 ×). Note the dark somatodendritic staining (right arrow) and the chromatolytic phase in some neurones (left arrow). T = tetanus toxin-treated side; C = control (untreated) side; Inj = injection tract.

Antagonism study

In an attempt to test our hypothesis that excitatory amino acid receptor activation was responsible for the neuropathological and behavioural response to tetanus toxin, rats were treated with either diazepam at a concentration known to be active in blocking generalized seizures (Goodman & Gillman, 1985) or with MK801, a selective NMDA receptor antagonist (Wong *et al.*, 1986; Kemp *et al.*, 1987) which is able to cross the blood brain barrier after systemic administration (Clineschmidt *et al.*, 1982). Diazepam (3.0 mg kg^{-1}, i.p., given 1 h before and after the toxin injection and once a day for 4 or 7 days after) did not show any protective effect against the neuropathological (Table 1) or behavioural effects induced by 1000 or 2000 MLDs tetanus toxin (n = 10 rats per each dose), respectively. By contrast, MK801 (0.3 mg kg^{-1} i.p., given 1 h before and after the toxin injection and once a day for 4 or 7 days, n = 10 rats per each group of treatment) completely abolished the neuropathological effects of both 1000 and 2000 MLDs tetanus toxin (Table 1 and Figure 4). In neither group was any convulsive behaviour observed. At the end of the study period (day 10) there was a significantly greater survival of animals treated with tetanus toxin and MK801 compared to those treated with tetanus toxin alone ($P < 0.001$ by G-test of independence with Williams correction) (Figure 3).

Discussion

The present results have shown that the injection of tetanus toxin into the rat hippocampus produces potent behavioural stimulation accompanied by neuronal loss in the CA1 pyramidal cell layer. Control experiments carried out with neutralized tetanus toxin indicate that the toxin itself and not contaminants was responsible for the behavioural and neuropathological effects. The behavioural effects of tetanus toxin

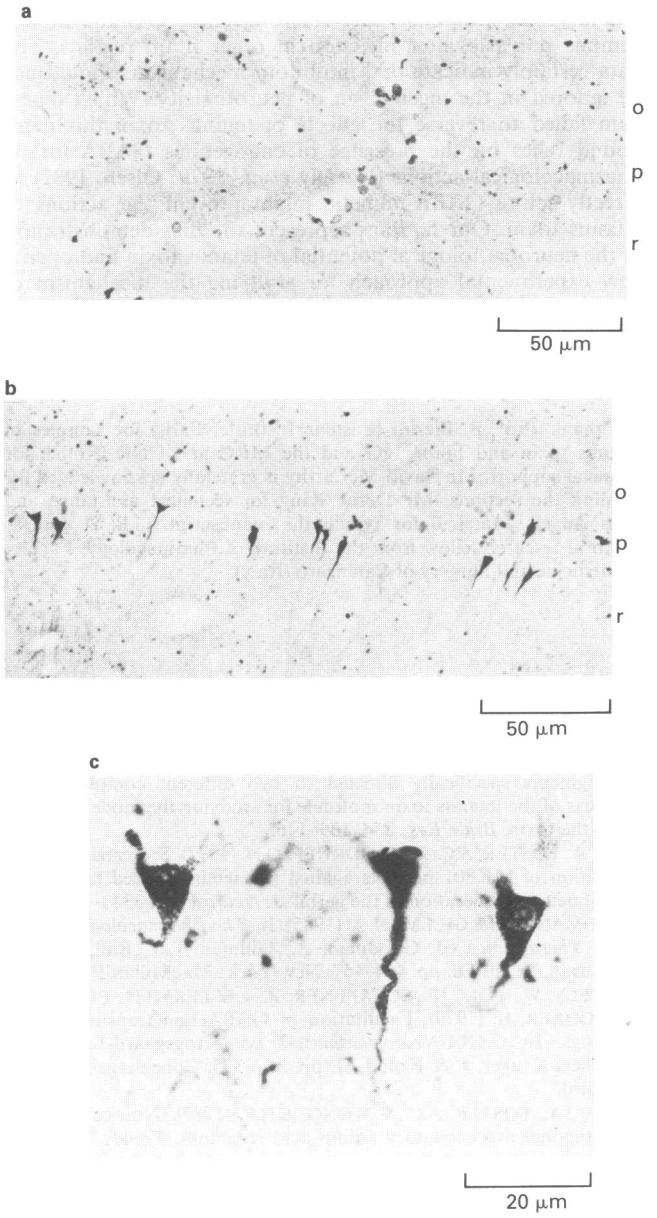

Figure 2 Rat brain section (14 μm) showing the CA1 hippocampal areas, stained by the procedure of Gallyas *et al.* (1980) to illustrate the effect of tetanus toxin (1000 MLDs) injected ten days earlier. In (b) and (c) (40 × and 100 × magnification respectively), tetanus toxin was injected and in this hippocampus argyrophilic neurones were observed in the pyramidal cell layer. In the contralateral side (a) no silver impregnation was observed. ○ = stratum oriens; p = stratum pyramidale; r = stratum radiatum.

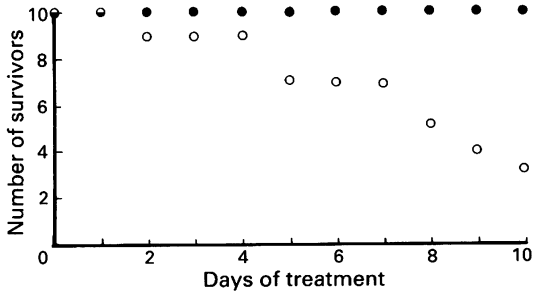

Figure 3 Time course of the lethality induced by tetanus toxin (1000 MLDs; ○) and its antagonism by systemic treatment with MK801 (0.3 mg kg^{-1}, i.p.; ●). Note that MK801 abolished the lethal effects even ten days after tetanus toxin injection. Treatment with diazepam (3.0 mg kg^{-1}), with the same schedule as for MK801, failed to show any protective effects and the lethality was not significantly different from tetanus toxin alone (data not shown).

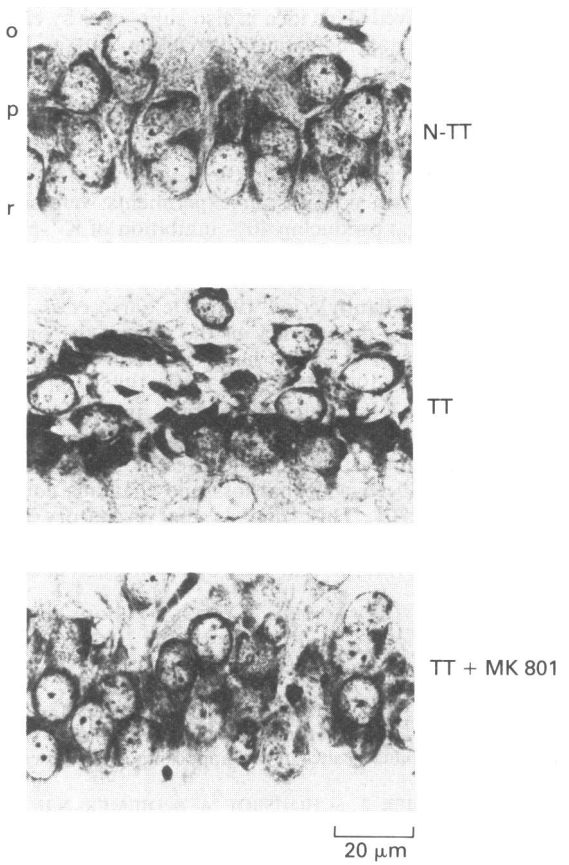

Figure 4 Light micrographs of brain coronal sections from 3 rats showing the protective effect of MK801 (TT + MK801, see text for dosage) against the neuronal damage induced by tetanus toxin (TT; 1000 MLDs). Tetanus toxin was injected seven days earlier and only the regions where the injections were made are shown. In the top panel, neutralized tetanus toxin (N-TT 1000 MLDs equivalent) was injected and no degeneration is evident. ○ = stratum oriens; p = stratum pyramidale; r = stratum radiatum. Magnification 100 ×. Toluidine blue stain.

observed in our study have been described previously by other groups (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985). However, prevention of these effects by an NMDA antagonist has never been demonstrated before. Interestingly the inability of an enhancer of GABAergic function to suppress the behavioural effects has already been described. De Sarro *et al.* (1985) failed to obtain any reversal in behaviour with sodium valproate. The neuropathological effects of tetanus toxin under our experimental conditions were limited to the area close to the injection site which confirms the poor penetration of tetanus toxin within brain tissue after its focal injection, as described by Mellanby & Thompson (1977) using ^{125}Iodine-labelled toxin. This has also been confirmed by an autoradiographical study in which a significant reduction in $GABA_A$ receptor binding sites has been observed in the CA1 pyramidal cell layer but not in other hippocampal regions, 7 and 10 days after the focal application of tetanus toxin (Bagetta *et al.*, 1990). The neuronal population most vulnerable to the effects of tetanus toxin appeared to be the pyramidal cells but other experimental studies, such as detection of GAD-immunoreactive neurones, are required. The effects of the toxin were time- and dose-dependent although the dose-response curve appeared to be very steep. The minor behavioural effects observed with 500 MLDs indicated that the toxin had exerted some effect but this was insufficient to produce neurotoxicity. If, as we suspect, the neurotoxicity results from an unopposed action of excitatory transmitter, a major reduction in GABA release would be required. Thus partial inhibition might be expected to produce some behavioural changes without allowing the threshold for glutamate

toxicity to be achieved. This idea is also supported by electrophysiological evidence indicating that tetanus toxin blocks inhibitory transmission (mainly GABAergic) at the presynaptic level, leaving unaffected the excitatory input within the CNS (Curtis & De Groat, 1968; Curtis *et al.*, 1973; Davies & Tongroach, 1977; Calabresi *et al.*, 1989) and in foetal mice dissociated spinal cord neurones (Bergey *et al.*, 1987). The doses of tetanus toxin used in our experiments were smaller than that (10^4 LD_{50}) producing 40% inhibition of K^+-evoked GABA release in hippocampal slices (Collingridge *et al.*, 1981) but similar to those used for producing an excitatory focus in the same region (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985). In addition, the dose-dependency of the rate of the onset of the tetanus toxin-evoked effects has been confirmed both *in vivo* (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985) and *in vitro* (Bergey *et al.*, 1987) although differences in the purity of separate batches of toxin may invalidate such comparisons. The most important aspects of the present study are that tetanus toxin produced neuronal loss and that this could be prevented by MK801. This suggests that excitatory amino acid transmission might play an important role in the mediation of the behavioural and neuropathological effects of tetanus toxin not only after microinjection into the hippocampus but also after injection in other brain regions. The data certainly indicate that inhibitory mechanisms should not be ignored in the mechanism of neurotoxicity. Whilst diazepam failed to reverse the effects of tetanus toxin this compound relies on the presence of endogenous GABA for its pharmacological actions (Haefely *et al.*, 1979; Olsen, 1981). A directly-acting GABA-mimetic might prevent the actions of tetanus toxin. Our findings represent the first demonstration of the neuropathological potential of tetanus toxin and open a new experimental approach for studying the mechanism of action of the toxin as well as of neurotoxicity. In addition, the excitatory amino acid receptor antagonism may provide a therapeutic strategy for the treatment of human tetanus.

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