Effect of tetanus toxin on extracellular levels of neuroactive amino acids and monoamines in rat hippocampus

By

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Abstract

The effect of the neurotoxin tetanus toxin, unilaterally injected into the ventral hippocampal formation, on extracellular levels of neuroactive amino acids and monoamines was investigated in rats using intracerebral microdialysis. A single dose (1000 mouse minimum lethal doses) of tetanus toxin did not alter extracellular levels of aspartate, glutamate, and taurine 1, 2, 3, and 7 days after treatment. However, whilst extracellular GABA levels were unaffected by toxin injection 1, 2, and 3 days after treatment, they were reduced (45% of contralateral vehicle injected level) at day 7. Toxin treatment caused a progressive decline in extracellular 5-hydroxytryptamine levels over the first 3 days of dialysis (20% of contralateral control level at day 3), and a 65% reduction 7 days after injection. Two days after toxin treatment dialysate dopamine level was elevated approximately 2-fold. However, by day 7 dopamine level was reduced by 54%. The 5-hydroxytryptamine metabolite, 5-hydroxyindoleacetic acid declined in a manner similar to that of 5-hydroxytryptamine, but to a lesser extent, over the duration of investigation. Of the dopamine metabolites, extracellular 3,4-dihydroxyphenylacetic acid was decreased 2, 3, and 7 days after treatment, whilst homovanillic acid was not altered during the study.

Seven days after treatment, challenge with high K⁺ produced increases in extracellular levels of taurine, GABA, 5-hydroxytryptamine, and dopamine in both vehicle- and toxin-treated hippocampi, with evoked levels of GABA, 5-hydroxytryptamine, and dopamine being lower in toxin-treated sides. At this time, an increase in the binding of [³H]-GABA to GABA_A sites in the ventral CA1 pyramidal cell layer, and GABA_B sites in the ventral CA1 and CA2 pyramidal cell layers was observed in toxin treated hippocampi. In contrast, [³H]-paroxetine binding density was decreased in ventral CA1 and CA3 pyramidal cell layers, and in the occipital cerebral cortex.

It is proposed that tetanus toxin-induced behavioural and neuropathological effects may be related to reductions in extracellular levels of 5-hydroxytryptamine and GABA, respectively.
Acknowledgements

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<tr>
<td>ALA</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>β-ALA</td>
<td>β-Alanine</td>
</tr>
<tr>
<td>ARG</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>ASP</td>
<td>L-Aspartic acid</td>
</tr>
<tr>
<td>CGP 37849</td>
<td>DL(E)-2-amino-4-methyl-phosphono-3-pentenoic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
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<td>D-AP5</td>
<td>D-2-amino-5-phosphonopropionic acid</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalograph</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GLN</td>
<td>L-Glutamine</td>
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<td>L-Glutamic acid</td>
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<td>Glycine</td>
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<td>HIS</td>
<td>L-Histidine</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
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<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
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<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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<td>5-HTP</td>
<td>L-5-hydroxytryptophan</td>
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<tr>
<td>MLDs</td>
<td>Mouse minimum lethal doses</td>
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<tr>
<td>5-MeODMT</td>
<td>5-methoxy-N,N-dimethyltryptamine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>OPA</td>
<td>o-phthaldialdehyde</td>
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<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino)tetralin</td>
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<tr>
<td>PDC</td>
<td>L-trans-pyrrolidine-2,4-dicarboxylic acid</td>
</tr>
<tr>
<td>SER</td>
<td>L-serine</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAU</td>
<td>Taurine</td>
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<td>THR</td>
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<td>L-Tyrosine</td>
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Chapter 1

Introduction
1.1 History of tetanus

The earliest accurate description of the symptoms of tetanus was made by Hippocrates in the 4th century B.C., and subsequently reported in the Corpus Hippocratum. Although many outstanding personalities in the history of medicine repeated the original description of Hippocrates, there was no great advancement in the knowledge of the disease until 1884 when Carle and Rattone demonstrated the infectious nature of the illness. An acneic pustule, considered to be the root of entry of the illness, was taken from the neck of a patient that had died 2 hours previously from tetanus intoxication. By injecting a water homogenate of the pustule into animals they showed that tetanus could be transmitted from man to rabbit and from rabbit to rabbit (Carle and Rattone, 1884).

In the same year Arthur Nicolaier injected samples of soil into a number of animals and observed that many of the treated subjects developed tetanus. In these incidences Nicolaier microscopically studied material removed from the inoculation site. Although a mixed bacterial population was observed, he correctly identified those responsible for the production of the disease, describing them as elongated, thin bacilli (Nicolaier, 1884). He postulated that the aetiological agent remained in the inoculation site producing, by the process of fermentation, a strychnine-like substance which diffused in the blood and led to the development of the disease.
Tetanus toxin was first described by Faber in 1890. By filtering a culture of bacilli he obtained a germ free liquid which when injected in minimal doses into animals produced symptoms identical to those observed both in natural tetanus and following whole culture injection. Attempts to develop an antitoxin to tetanus in 1890 led Behring and Kitasato to publish work that is today regarded not only as a milestone in the history of tetanus, but also as a valuable insight into the field of immunohaematology (Behring and Kitasato, 1890). By obtaining vast amounts of immune serum from large animals in order to inoculate small animals such as mice, Behring and Kitasato demonstrated that the blood of animals immunized against tetanus had the ability to neutralize tetanus toxin. Roux and Vaillard were the first to apply serum antitetanus prophylaxis in wounded subjects (Roux and Vaillard, 1893).

In attempts to discover an anatoxic vaccine, the toxin was treated with a variety of different chemical substances, the most suitable of which appeared to be iodine trichloride. Vallee and Bazy (1917) attempted to vaccinate man this way, but the method proved too unsafe for use. Mixtures of toxin and antitoxin were also tested for potential anatoxic properties, whilst all the time the need to develop a safe vaccine to withstand tetanus was becoming more important. Gaston Ramon, a veterinary who had worked on milk production and animal care, was familiar with the use of formalin, which he had previously used to preserve milk. Due to this experience, Ramon found that a 0.5% solution of formalin plus heat was effective in transforming tetanus toxin into a safe and very efficient antigenic product. In 1925, Ramon and Descombey applied the anatoxic vaccine to horses, and the following year Ramon and Zoller were able to demonstrate the effect of
the anatoxic vaccine against tetanus in humans.

1.2 Effects and clinical aspects of tetanus intoxication

Tetanus intoxication is still a major problem of critical importance. Despite the implementation of a worldwide vaccination programme, incidences of the disease are extremely high in Third World countries and continue to occur in the West. Over one million people die from tetanus every year across the globe.

Infection with the bacillus *Clostridium tetani* leads to the development of characteristic clinical features. Muscle rigidity occurs in every case of tetanus intoxication: this may be followed by reflex spasms and accompanying severe convulsions in more extreme incidences. Generalized tetanus is the commonest form of the disease. Initially the patient complains of feeling unwell. This is followed by trismus (lock-jaw) due to masseter muscle spasm, muscle stiffness, and neck pain. These symptoms are usually manifested following an incubation period of between 4 and 14 days, during which the bacillus produces the exotoxin tetanus toxin under anaerobic conditions. Spasm of the facial muscles produces the characteristic grinning expression known as risus sardonicus. Stiffening of the neck, thoracic, abdominal, and limb muscles leads to the production of a characteristic ram-rod appearance. Excessive shortening of the long spinal muscles may cause the body to curve backwards producing opisthotonus (arching of the neck and back muscles). Reflex spasms develop usually within 24-72 hours of the initial symptoms. These result from simultaneous, excessive contraction of antagonistic muscle groups. The interval between the first symptom and the first
spasm is referred to as the "onset time". Spasms may occur spontaneously but are easily precipitated by tactile, auditory, or visual sensory stimuli, lasting for several seconds, often with one following the other in rapid succession. Oesophageal and urethral spasm lead to dysphagia and urinary retention respectively (Farthing and Rolston, 1990).

The most severe period of the disease usually arises approximately 3 days following onset of initial symptoms, with the patient remaining seriously ill over the next 4-5 days. In most cases, individuals who survive the first 8 days recover from the disease. Atypically, surviving patients may demonstrate chronic, often irreversible, EEG disorders, predominantly in the form of slow wave activity (Bagetta et al, 1991a).

Complications in respiratory function, including excessive secretions, pneumonia, laryngeal and oropharyngeal spasms, are invariably the factors responsible for mortality following tetanus intoxication (Creech et al, 1957; Garcia-Palmeri and Ramirez, 1957; Vakil et al, 1965; Patel et al, 1965). Autonomic dysfunction is evidenced by tachycardia, a labile blood pressure, sweating and cardiac arrhythmias (Hortnagl et al, 1979). Death usually results from aspiration, hypoxia, respiratory failure, cardiac arrest, or exhaustion.

Poor prognostic indicators are:

1. short incubation period
2. short onset time
3. cephalic tetanus
4. extremes of age
5. narcotic addicts who inject drugs subcutaneously

In local tetanus, pain and stiffness is confined to the site of the wound, with the tone of the surrounding muscle becoming increased. Recovery from local tetanus usually occurs (Farthing and Rolston, 1990).

Cephalic tetanus is an uncommon, but invariably fatal, form of the disease. It usually occurs when the portal of entry of the bacterium *Clostridium tetani* is the middle ear. This form of tetanus can lead to the production of cranial nerve abnormalities, particularly the seventh nerve, and also to the development of generalized tetanus (Farthing and Rolston, 1990).

Tetanus neonatorum occurs in neonates following infection of the umbilical stump. Each case of neonatal tetanus demonstrates multiple failures in the health system. Failure to protect the mother with tetanus toxoid coupled with unhygienic practices which bring tetanus spores into contact with the umbilical cord lead to the development of the illness. Initial symptoms include failure to thrive, poor suckling, grimacing and irritability. These symptoms are followed by the rapid development of intense rigidity and spasms, resulting in a mortality rate approaching 100% (Farthing and Rolston, 1990).
1.3 Prevention of tetanus

Tetanus is an eminantly preventable disease, with almost total protection provided by immunization with either plain or alum-adsorbed toxoid, the latter being superior (Farthing and Rolston, 1990).

1.4 Treatment of tetanus

A combined antibiotic and antitoxin drug regimen is undertaken in the treatment of individuals with tetanus, even in the absence of an obvious wound. Human antitetanus immunoglobulin is given to neutralize any circulating toxin, whilst having no effect on neuronally bound toxin. Neutralization of the bacterium is achieved by administration of penicillin. Improvement in nursing care techniques has contributed more than any other single measure to the decrease in mortality rate from 60% to nearly 20% (Farthing and Rolston, 1990).

Diazepam is the drug of choice for controlling spasms, with β-blocking agents potentially useful for the control of autonomic dysfunction. In extremely severe cases of tetanus when reflex spasms are so frequent and intense that respiration is virtually impossible, treatment with curare combined with artificial respiratory support is required (Farthing and Rolston, 1990).
1.5 Structural considerations

Tetanus toxin, the exotoxin derived from *Clostridium tetani*, is synthesized anaerobically inside the bacterium as a single peptide chain of 150 kDa. Following an autolytic process, the single chain is released as a protoxin and subsequently subjected to protease degradation, producing a double-chain structure comprising of a light chain (Fragment A; 50 kDa) and a heavy chain (Fragment BC; 100 kDa) associated by a disulphide bond (Fig 1.1). The proportion between acid and basic amino acid residues indicates that the light chain is more acidic, therefore acid-base interactions may also contribute to the forces linking the two chains of tetanus toxin (Habermann and Weller, 1989).

The double chain toxin structure can be experimentally digested with papain to yield two polypeptide fragments of unequal mass, namely the lighter fragment C, consisting of the 50 kDa COOH- terminal portion of the heavy chain, and the heavier fragment A-B, comprising the 50 kDa NH$_2$- terminal portion of the heavy chain linked to the light chain (A) by a disulphide bond (Fig 1.1).

Reducing agents can be employed to separate the light (A) and heavy (BC) chains comprising the extracellular toxin by breaking the disulphide bridge linking the two chains. In this situation, the isolated heavy chain (BC) and the light chain (A) are not toxic, however they can reassociate to generate biologically active toxin (Fig 1.1). The single chain form of toxin, which is the "stored" form, is found to be less toxic than the extracellular double chain toxin structure, both *in vivo* and *in vitro* (Habig et al, 1986; Bergey et al, 1987; Weller et al, 1988).
Fig. 1.1 Structural representation of tetanus toxin and its fragments.

a: Intracellular, intact tetanus toxin is synthesized as a 150 kDa polypeptide chain (ABC) that has at least one interchain disulphide bond. b: After cell lysis by proteases (e.g. trypsin) the holotoxin is cleaved to give a light chain (A) (50 kDa) and a heavy chain (BC) (100 kDa) with an interchain disulphide bond, designated as extracellular toxin (A-BC). c: Cleavage with papain gives two products: fragment C, which is the C terminus of the heavy chain; and fragment A-B, which is the N terminus of the heavy chain still linked to the light chain. d: Breaking the disulphide bonds with reducing agents separates the light (A) and the heavy (BC) chains in the extracellular toxin. The toxin can be divided into three domains on the basis of biological activity. The domain corresponding to fragment C contains the binding site for gangliosides. This domain is also involved in the retrograde axonal transport of the toxin molecule. The second domain is fragment A-B, involved in the ability of the toxin molecule to produce, under particular circumstances, flaccid paralysis. The third domain (B) is circumscribed by the area corresponding to the 50 kDa amino-terminal fragment of the heavy chain, and relates to the ability of the toxin to form channels across artificial asolectin membranes. The isolated heavy chain BC and the light chain are not toxic. They can reassociate to generate biologically active toxin.
trypsin

proteolysis in vitro or in vivo

papain

proteolysis in vitro

reduction

H_2N\text{---} \text{S---S---} \text{COOH}

A

B

C

A-BC

A-B

A

B

C

BC

27
Tetanus toxin can be completely inactivated by formaldehyde treatment to yield tetanus toxoid, which is the basis of the very successful vaccine in use today (for review of vaccine production see Bizzini, 1984). Inoculation with the vaccine induces the formation of protective antibodies which completely neutralize the effects of the toxin. However, the entire toxin is not essential for the development of protective immunity, as purified fragments A-B or fragment C have been used to successfully immunize animals (Helting and Zwister, 1977; Helting and Nau, 1984) indicating the possible existence of several neutralizing epitopes sited on different fragments of the toxin molecule. The isolation of many distinct monoclonal antibodies with the ability to neutralize tetanus toxin and bind to its different fragments support the theory of existence of several antibody producing epitopes (Kenimer et al, 1983; Sheppard et al, 1984; Ahnert-Hilger et al, 1983; Bizzini, 1989).

The primary structure of tetanus toxin at the DNA level has been elucidated after starting with both the plasmid (Eisel et al, 1986) and with total DNA (Fairweather and Lyness, 1986). Isolation and determination of the complete nucleotide sequence of the toxin gene has led to the deduction of its entire amino acid sequence. Portions of the toxin gene have subsequently been expressed in *Escherichia coli*, and the consequent proteins obtained used to successfully immunize animals against tetanus (Fairweather, 1989).
1.6 Mode of action of tetanus toxin

1.6.1 Binding process

In order to produce its pathogenic action, the toxin must be transported from its portal of entry, or from its site of formation in the organism, to the central nervous system (CNS) (Dimpfel and Habermann, 1973; Habermann and Wellhoner, 1974). The high toxicity of tetanus toxin indicates that its mode of action should be via specific high-affinity recognition sites. Indeed, such an interaction of the toxin with specific receptors on the surface of peripheral nerve endings in the region of the neuromuscular junction is considered to be the first step in the toxin’s pathogenic action, and is discussed in the following section.

Fixation to gangliosides

Gangliosides, the complex lipids widely distributed in most tissues, including nervous tissue, possess the ability to bind tetanus toxin. The predominating gangliosides in the nervous system are GM₁, GD₁₅, and GT₁₅ (Critchley et al, 1985, 1986), although polysialogangliosides of the GT₁₅ and GD₁₅ types are believed to be components of the receptor for tetanus toxin in neural tissue (Van Heyningen and Miller, 1961; Dimpfel et al, 1977). Mellanby and Van Heyningen (1967) demonstrated that removal of the N-acetylneuraminic acid residues for chemical modification of the carboxyl groups of the latter residues resulted in gangliosides loosing toxin fixing ability. It has been demonstrated that primary cell cultures of mouse embryonic CNS possessing long chain gangliosides exhibit a toxin binding capacity resembling that of adult brain homogenates. The toxin
binding capacity can be increased by elevating the concentration of gangliosides present in the culture medium (Dimpfel and Habermann, 1977; Dimpfel et al, 1977). Contrastingly, it is found that toxin binding is absent in cell lines lacking these long chain gangliosides (Dimpfel et al, 1977).

Fixation to subcellular fractions

The strong but reversible toxin binding capacity of CNS matter was demonstrated by Habermann (1976). As a consequence of this binding characteristic an affinity-chromatography system for purifying the toxin was developed. The subcellular structures endowed with the highest toxin-fixation capacity have been identified as synaptosomes (Mellanby et al, 1965; Mellanby and Whittaker, 1968; Choudhury et al, 1972; Haberman et al, 1973; Rogers and Synder, 1981).

A single population of saturable binding sites has been detected on rat brain neuronal membranes by Goldberg et al (1981). Binding was found to be optimal at a pH of 6.5, most effective under low-ionic strength conditions, both time- and temperature-dependent, and specific. Trypsin treatment of these synaptic membranes resulted in complete inhibition of toxin binding, whereas N-acetylneuraminidase treatment inhibited binding by only 50% (Lazarovici and Yavin, 1986).

Binding to rat brain membranes appears to be dependent on the pH and ion concentration of the medium: at pH 6.0 and low-ionic strength a single saturable binding site can be detected, with $K_D$ and $V_{max}$ values of 0.26 nM and 0.9 nmol/mg
membrane protein respectively (Critchley et al, 1986). However, under physiological salt conditions at a pH of 7.4 binding is not saturable and is found to consist of 2 phases. In this situation $K_D$ values of 0.42 nM and 146 nM and $V_{\text{max}}$ values of 0.9 pmol/mg and 179 pmol/mg, respectively, were calculated (Pierce et al, 1986).

**Fixation to neuronal cells in culture**

The capacity of nervous tissue, in particular grey matter, to fix tetanus toxin has been known for almost 100 years (Wasserman and Takaki, 1898; Marie, 1898). Using cell culture techniques, the selective fixation of tetanus toxin by neuronal, but not glial cells, has been reported by several groups of researchers (Dimpfel et al, 1975; Dimpfel and Habermann, 1977; Mirsky et al, 1978). In fact, cultured neurons from all parts of the CNS have been shown to possess toxin-fixing ability, indicating that this property is general to all neurons (Mirsky et al, 1978).

Binding of tetanus toxin to mouse neuroblastoma cells demonstrated differential toxin effects. An ineffective ganglioside-dependent binding that fails to produce any visible biological effect was distinguished from an effective ganglioside-independent binding that induces visible biological effects in culture and is both neuraminidase- and $\beta$-galactosidase-resistant (Zimmerman and Piffaretti, 1977).

As mentioned previously, lack of toxin fixation to cell lines is thought to be attributed to complete absence of long chain gangliosides, whereas primary cell cultures containing gangliosides possess a toxin-binding capacity which can be
increased by raising the concentration of gangliosides in the culture medium (Dimpfel and Habermann, 1977; Dimpfel et al, 1977). In support of these findings it has been shown that neurohybrid cells that lack detectable polysialogangliosides are devoid of toxin binding activity (Yavin, 1984).

### 1.6.2 Nature of the receptor for tetanus toxin

Specific polysialogangliosides, particularly GT\(_{1b}\) and GD\(_{1b}\) types, have the ability to fix tetanus toxin, are widely present in the mammalian CNS, and therefore are considered to be components of the receptor for tetanus toxin in neural tissue. Although there is much documented literature to support this hypothesis, the possibility of the presence of another toxin-binding receptor entity cannot be excluded. One such alternative implies that the ganglioside-dependent receptor may in fact be an ineffective, or silent receptor, with the biological effects of the toxin linked to a ganglioside-independent receptor (Zimmerman and Piffaretti, 1977).

The assumption that gangliosides do form part of the tetanus toxin receptor is supported by the following observations:

1. Neuronal cell lines devoid of long chain gangliosides do not bind tetanus toxin (Dimpfel et al, 1977; Yavin and Habig, 1984). The addition of exogenous gangliosides to these cultures does however result in cell lines with toxin fixing capacity (Dimpfel et al, 1977; Yavin, 1984; Pierce et al, 1986).
2. Neuraminidase treatment reduced ganglioside-toxin binding both at 0°C and 37°C (Rogers and Snyder, 1981; Yavin, 1984).

3. Tetanus toxin does not interact with neuron-free, glia-enriched cultures from newborn rat brain, or with a ganglioside deficient cell line (Yavin et al, 1982).

4. Gangliosides of the GT1b and GD1b type are readily able to release toxin associated with the cell in a highly specific manner (Rogers and Synder, 1981).

5. Protease treatment of ganglioside-reconstituted cultures prior to the addition of toxin has only a marginal effect in reducing subsequent binding (Yavin, 1984).

6. Radiolabelled tetanus toxin can be separated by ganglioside-affinity chromatography into two distinct populations of toxin molecules, one exhibiting high ganglioside-binding capacity and reacting avidly with neuronal membranes, the other with a low affinity for gangliosides and binding poorly to nerve cells (Lazarovici et al, 1984).

7. Tetanus toxin binding to rat brain membranes at pH 6.0 was found to be neuraminidase sensitive and protease resistant, whereas at pH 7.4 it was both protease and neuraminidase sensitive (Critchley et al, 1986).

8. The ability of rat brain neuronal membranes to fix tetanus toxin at pH 6.0 was inhibited in a dose-dependent manner by gangliosides (Rogers and Synder, 1981; Critchley et al, 1986)
Tetanus toxin does not bind to neutral glycolipids isolated from brain membranes (Critchley et al, 1986).

Suggestions for the existence of a ganglioside-independent receptor for tetanus toxin have been made (Zimmerman and Piffareti, 1977; Dumas et al, 1979a; Habermann et al, 1981), with the receptor possibly represented by a glycoprotein containing the same oligosaccharide determinant as the gangliosides (Holmgren et al, 1980).

The existence of a ganglioside-independent receptor is supported by the following observations:

1. Tetanus toxin has been demonstrated to undergo extra-axonal transport (Wellhoner et al, 1975; Erdmann et al, 1975).

2. Exogenous gangliosides fail to produce a blockade of transport of tetanus toxin (Dumas et al, 1979b).

3. The binding of tetanus toxin to nerve cells and neuronal membrane preparations is protease sensitive (Yavin and Nathan, 1986; Pierce et al, 1986; Critchley et al, 1986).

4. Neuraminidase treatment fails to suppress the effects of tetanus toxin on K+ evoked release of inhibitory or excitatory amino acids in brain slices (Bigalke et al, 1981).
5. There appears to be only a partial correlation between polysialoganglioside content of neuronal plasma membranes and tetanus toxin binding (Bigalke et al, 1981; Habermann and Dreyer, 1986; Yavin et al, 1983; Yavin and Habig, 1984; Critchley et al, 1986; Lazrovici and Yavin, 1986).

Therefore, the nature of the cellular receptor for tetanus toxin is still undefined. A model which takes into account most of the experimental findings suggests that tetanus toxin may bind via a double interaction with a protein receptor and with acidic lipids (Montecucco, 1986, 1989). With the highly enriched ganglioside population concentrating the toxin on the membrane of target cells, lateral diffusion of the molecule could occur on the membrane surface in order to encounter the protein receptor. In this model, binding of toxin to receptor would be greatly facilitated, particularly if the density of the receptor is low, because it would occur in a two dimensional solvent, the plasma membrane.

It could be that the receptor for tetanus toxin is actually a double receptor composed of a protein and a negatively charged lipid component, also in this case with negatively charged lipids contributing to the strength of binding (Montecucco, 1986, 1989).

1.6.3 Internalization of the toxin molecule

The cellular mechanism by which tetanus produces intoxication involves 3 steps: (a) binding of the toxin molecule to the cell surface; (b) membrane translocation into the cytoplasm; (c) modification of a specific target involved in the
neuroexocytosis mechanism. Although none of these steps, at present, are fully understood at the molecular level, the step involving translocation of the toxin molecule into the cytoplasm is least clear.

Once tetanus toxin has bound to its cellular receptor, the molecule becomes internalized and cannot be displaced from its binding site by antitoxin, unlabelled toxin, treatment with trypsin or detergents, high temperature, or by addition of gangliosides (Yavin et al, 1981). It has been suggested that the polysialoganglioside complex, once formed, may serve as a transport system to move the toxin from an extracellular to an intracellular compartment, thereby rendering the toxin molecule unsusceptible to chase treatment (Yavin et al, 1981; Roa and Boquet, 1985). The binding-sequestration process, a preliminary event in the internalization step, involves a neuraminidase-sensitive membrane component, whereas during internalization the toxin molecule is associated with a neuraminidase-insensitive and trypsin-resistant compartment, indicating that the toxin receptor might be associated with elements of the cytoskeleton (Yavin et al, 1982).

Proposed internalization mechanisms

1. Receptor mediated endocytosis

Binding of tetanus toxin to its sialosyl group-containing receptors is likely to trigger invagination of the cell membrane resulting in the formation of a vesicle encasing the toxin-receptor complex (Simpson, 1986, 1989; Schmitt et al, 1981).
This process is dependent upon the presence of fragment B. Aggregation of vesicles in the cytoplasm results in the formation of endosomes, with the low internal pH activating insertion of the toxin into the membrane of the vesicular endosome (Simpson, 1986, 1989; Schmitt et al, 1981; Poulain et al, 1990). If such a mechanism is actually operating in translocation of the toxin into the cell, fusion of endosomes with lysosomes is unlikely to occur. In fact, most of the labelled toxin found to accumulate in the spinal cord after retrograde intra-axonal transport was intact (Habermann et al, 1973, 1977).

It has been proposed that the low pH reached in the endosomal lumen causes a conformational transition of its structure resulting in exposure of hydrophobic surfaces, thus enabling the toxin to penetrate and cross the membrane (Boquet and Duflot, 1982). All findings supporting this hypothesis have been obtained on model systems: at low pH tetanus toxin induces the release of potassium from asolectin vesicles (Boquet and Duflot, 1982), forms ion channels across planar lipid bilayers (Hoch et al, 1985), and penetrates into the lipid bilayer as monitored by proteases (Roa and Boquet, 1985) and hydrophobic photolabelling with photoactive phospholipids (Montecucco et al, 1986).

Borochov-Neori et al (1984) have demonstrated that a tetanus toxin-ganglioside complex forms channels in a planar lipid bilayer. It was assumed that a portion of the toxin polypeptide chain should span the lipid bilayer in order to act as a channel. The tetanus toxin channel was found to be cation-selective and preferentially open. The channel forming property was found to reside in the whole toxin molecule and the fragment composed of the light chain covalently
bound to the amino terminus of the heavy chain, whilst taking place in acidified cell vesicles (Hoch et al, 1985). Single channel and macroscopic current recordings obtained from lipid bilayers exposed to either intact tetanus toxin or to the tetanus toxin B fragment indicate that fragment B contains the channel forming domain (Boquet, 1982; Roa and Boquet, 1985; Borochov-Neori et al, 1984; Hoch et al, 1985; Ahnert-Hilger et al, 1990; Gambale and Montal, 1988; Rauch et al, 1990).

Hoch et al (1985) have proposed that at acidic pH the heavy chain of tetanus toxin becomes hydrophobic forming a transmembrane channel across the lipid bilayer through which the light chain can pass, in an extended form, protected from contact with lipids (Fig 1.2a). However, Montecucco (1988), making use of a hydrophobic photolabelling technique, has demonstrated that at low pH both the light and heavy chain of tetanus toxin become hydrophobic and come into contact with hydrocarbon chains of phospholipids. It was proposed that both toxin chains penetrate into the lipid bilayer, with hydrophobic surfaces exposed to lipids and hydrophilic residues facing each other in order to minimize the energetic cost of insertion of hydrophilic groups into the hydrophobic membrane core. Penetration of this nature is not necessarily followed by the translocation of the toxin across the membrane as suggested by Hoch et al (1985). In fact, the possibility that the toxin exerts its effect whilst embedded in the plasma membrane, or in the endosome membrane with its active site facing the cytoplasm, cannot be excluded (Fig 1.2b).
Fig. 1.2  Possible mechanisms for the membrane translocation of tetanus toxin.

(a) This view envisages the formation, by the heavy chain (H), of a transmembrane channel large enough to allow the passage of the light chain (L) in an extended form across the membrane. (b) In this scenario both chains are proposed to insert into the lipid bilayer at low pH. This action may, or may not, be followed by translocation of the toxin into the cytoplasm.
2. Clathrin coated pits and non-coated membrane invaginations

It is possible that the toxin-receptor complex could be encased in clathrin-coated pits. Following endocytosis, spontaneous rupture of the clathrin structure would cause release of toxin into the cytoplasm. This mechanism for endocytosis of tetanus toxin would support the suggestion by Penner et al (1986) that the toxin most probably is processed in the cell cytoplasm. The involvement of non-coated invaginations encasing the toxin receptor complex cannot be excluded. Montesano et al (1982) observed the specific association of a tetanus toxin-gold complex with small non-coated microinvasions, with gold particles being internalized via non-coated vesicles at 37°C.

1.6.4 Retrograde intra-axonal transport of tetanus toxin to the CNS

Although axonal transport of tetanus toxin was first suggested by Marie and Morax (1902) and Meyer and Ranson (1903), approximately half a century elapsed before experimental support was given to the theory of neural ascent of tetanus toxin from its site of formation or injection to its target site in the CNS (Wright, 1955; Kryzhanovsky, 1965, 1966, 1967, 1973; Fedinec, 1965, 1967; Habermann, 1970; Dimpfel and Habermann, 1973; Hensel et al, 1973; Seib et al, 1973; Habermann and Wellhoner, 1974; King and Fedinec, 1974; Gardner and Fedinec, 1975).

The migration of tetanus toxin from the periphery to the CNS occurs mainly via intra-axonal transport, as demonstrated by autoradiographic analysis of the
distribution of $^{125}$I-labelled toxin in the CNS of rats previously injected peripherally with this substance (Dimpfel and Habermann, 1973). Tetanus toxin is known to be transported in all types of neurons (Stoeckel et al, 1975). The tissue compartments involved in its movement have been identified and the kinetic parameters of toxin transport determined. It is considered that tetanus toxin is transported intra-axonally in vesicles and cisternae of the smooth endoplasmic reticulum to the nerve cell body, with the rate of retrograde transport being about 5.0 - 7.5 mm/hour for motor neurons.

Following intramuscular injection of labelled toxin, accumulation of label occurs in the intra-axonal space of motoneurons. King and Fedinec (1974) found that toxin was localized in the epineurium and perineurium. This observation was subsequently confirmed (Erdmann et al, 1975; Fedinec, 1975), although Carroll et al (1978) failed to detect any labelling in the epineurium. Fedinec (1975) proposed that the perineural and epineural toxin was toxic and antigenic, whereas the endoneural and intra-axonal toxin was not toxic but antigenic. It was suggested by Wellhoner et al (1975) that the majority of intramuscularly injected toxin ascended in the epineurium of the supplying nerve, but the smaller amount of toxin migrating in the intra-axonal compartment is the one relevant for toxin accumulation in the spinal cord. Price et al (1975) demonstrated that systemic injection of toxin resulted in the preferential labelling of the perineurium.

1.6.5 **Form of toxin undergoing transport**

The nature of the label that accumulated in the spinal cord of rat and cat following
systemic injection of labelled toxin was investigated by Habermann et al (1977). It was found that 85% of radioactivity present in a Lubrol PX extract bound to insolubilized antitetanus antibodies, with the extracted toxin exhibiting toxic activity. It was considered that the label undergoing retrograde transport to the CNS should consist of either intact toxin molecules or of toxin polypeptides exhibiting the characteristics that enable their release from ganglionic neurons and their subsequent uptake by presynaptic terminals (Schwab and Theonen, 1977). Indeed, evidence was provided subsequently to show that the label transferred trans-synaptically did in fact represent the intact toxin molecule (Dumas et al, 1979b).

However, toxin fragments are able to undergo retrograde intra-axonal transport. Fragment C, which in itself is not toxic, is transported (Bizzini et al, 1977; An der Lan et al, 1980), accumulates in areas of the mammalian spinal cord and brain stem (Fishman and Carrigan, 1988), and competes with tetanus toxin for high-affinity binding sites in rat brain membranes (Goldberg et al, 1981). This finding led to speculation concerning the ability of such fragments to carry drugs (eg. chemotherapeutic agents, research drugs, antibodies to sites not normally accessible to antibody) to specific regions of the CNS (Bizzini et al, 1977; Goldberg et al, 1981).

Indeed, fragment C, when covalently complexed to the remainder of the molecule (fragment A-B) (see Fig 1.1) has been demonstrated to convey the complex retrogradely from the medial rectus muscle to the oculomotor nucleus, while fragment A-B alone failed to undergo retrograde transport (Bizzini et al, 1980a,
However, Weller et al (1986) have demonstrated that the retrograde transport of fragments is at least 50 - 100 times less effective than toxin transport.

Antimitotic agents such as colchicine and vinblastine are able to prevent the retrograde transport of fragment C, whilst having no effect on its ability to bind to rat brain membranes (Goldberg et al, 1981). The effect of antimitotic agents on axonal transport is considered to be due to their action on the microtubular system of nerve cells.

It is widely accepted that retrograde intra-axonal transport of tetanus toxin involves the heavy chain of the molecule. Toxin fragments comprising the heavy chain, or part of it, still possess the ability to undergo transport to the CNS, although as in the case of fragment C they lack central actions (Goldberg et al, 1981; Evinger and Erichsen, 1986; Bizzini et al, 1977, 1980a, 1981; Manning et al, 1986; Morris et al, 1980).

1.6.6 Effects of tetanus toxin on the central nervous system

Spinal neurons of arrival for tetanus toxin

Following retrograde intra-axonal transport from the periphery to the CNS, $^{125}$I-labelled tetanus toxin accumulates in the cytoplasm of large neurons (assumed to be $\alpha$-motoneurons) located in the ventral horn of the grey matter (Dimpfel and Habermann, 1973; Erdmann et al, 1975). The effect of tetanus toxin accumulation on various parameters of electrical excitability of $\alpha$-motoneurons has been investigated (Curtis et al, 1976; Kryzhanovsky et al, 1973a; Sverdllov, 1970,
Tetanus toxin was observed to assert no influence on the resting membrane potential, the membrane resistance, the various constituents comprising the action potential characteristics (threshold depolarization, latency, steepness of upstroke, time constant, refractory period, and after hyperpolarization), or the repeated discharge on continuous depolarization. The experiments of Brooks et al (1957), involving intraspinal injection of tetanus toxin, provided a valuable insight into the mode of action of the toxin, which they described as a weakening of transmission at inhibitory synapses.

Several groups of researchers have investigated the effects exerted by tetanus toxin on postsynaptic neuronal membranes. The toxin has been shown to have no influence on the postsynaptic response evoked by iontophoretic application of inhibitory or excitatory amino acids (Bergey et al, 1983; Curtis and De Groat, 1968; Guschin et al, 1971; Curtis et al, 1973).

Trans-synaptic transport of tetanus toxin

It became clear that tetanus toxin should exert its effect mainly on target neurons distinct from the neurons of arrival. It was postulated that the toxin acts at the presynaptic membrane of nerve terminals impinging on α-motoneurons, having first migrated across the synapse. Schwab and Theonen (1976) demonstrated the ability of tetanus toxin to be transported trans-synaptically from motoneurons to target neurons. Following injection of 125I-labelled toxin into the deltoid muscle of rats, it was found using electron microscope autoradiography that 125I was present over presynaptic terminals of spinal interneurons. There is now evidence
implicating the ability of tetanus toxin to be trans-synaptically transported through all synapses (Schwab and Theonen, 1977; Schwab et al, 1979; Dumas et al, 1979b), with particularly susceptible target neurons being interneurons, at which toxin is known to block the release of inhibitory neurotransmitters (GABA and glycine) (Curtis et al, 1976). This selective inhibition of GABA and glycine release is found to be unassociated with changes in cell discharge rate or in ion currents at the presynaptic terminal (Wellhoner, 1989; Dreyer et al, 1983).

The ability of tetanus toxin to be trans-synaptically transported is not reliant upon the structural integrity of the molecule. Toxin fragments containing the carboxy terminus of the heavy chain are able to be transported in this manner (Buttner-Ennever et al, 1981; Evinger and Erichsen, 1986; Manning et al, 1986). Dumas et al (1979a, 1979b) have demonstrated that native toxin does possess the ability to undergo further retrograde transport in the next higher order neurons directly following transport across the synapse. Trans-synaptic transport of tetanus toxin involves exocytosis of the molecule from the source neuron, diffusion across the synaptic cleft and subsequent fixation to receptors in the presynaptic terminal (Price et al, 1977; Price and Griffin, 1981), closely followed by endocytosis into the terminal. The reason why the toxin molecule leaves the source neuron is virtually unknown.

**Role of tetanus toxin in neuroexocytotic inhibition**

By directly injecting the toxin or toxin fragments into cells Penner et al (1986) demonstrated a blockade of exocytosis. The intact toxin molecule and a fragment
composed of the light chain covalently linked to the amino terminus of the heavy chain exhibited this property. This suggested that the active site for blocking the secretory response was somewhere within the light chain or the amino terminus of the heavy chain. It is now generally accepted that the light chain of tetanus toxin provides the mechanism for blockade of neurotransmitter release (Simpson, 1989; Niemann, 1991; Montecucco, 1986; Penner et al, 1986; Gansel et al, 1987; Bittner et al, 1989; Molgo et al, 1990; Poulain et al, 1990, 1991).

Schiavo et al (1992) have recently shown that tetanus toxin is a zinc endopeptidase, the activation of which requires reduction of the interchain disulphide bond separating the light and heavy chains. A zinc atom is bound to the light chain which possesses a protease activity specific for synaptobrevin-2 (a synaptobrevin isoform), an integral membrane protein of small synaptic vesicles. Blockade of neurotransmitter release from Aplysia neurons injected with tetanus toxin was found to be substantially delayed by peptides containing the synaptobrevin-2 cleavage site. Also proteolysis of synaptobrevin-2 was blocked by the presence of the divalent cation chelator Ethylenediaminetetraacetic acid (EDTA) and the zinc endopeptidase inhibitor captopril. It was concluded that tetanus toxin may block neurotransmitter release by cleaving synaptobrevin-2, a protein that may play a key part in neurotransmitter release.

Synapsin I, a prominent phosphoprotein in nerve terminals, has been linked with modulation of exocytosis by a mechanism involving interaction with the cytoplasmic surface of small synaptic vesicles and cytoskeletal elements in a phosphorylation dependent manner. Presek et al (1992) have demonstrated the
attenuation by tetanus toxin of a depolarization-stimulated increase in synapsin I phosphorylation in rat cortical particles and synaptosomes. Tetanus toxin also decreased the translocation of synapsin I from small synaptic vesicles into the cytosol following depolarization of synaptosomes. Phosphorylation of synapsin IIb, a known substrate of protein kinase C, was also inhibited by tetanus toxin.

In summary, tetanus toxin was shown to inhibit neurotransmitter release, the phosphorylation of a select group of phosphoproteins in nerve terminals, and the translocation of synapsin I.

Following intraventricular injection of tetanus toxin in rats, Aguilera et al (1990) observed a translocation of calcium/phosphatidy/serine-dependent protein kinase C from the inactive cytosolic compartment to a membrane-bound active form, followed by a time-dependent reduction in both total activity and enzyme protein. As protein kinase C-dependent protein phosphorylation is thought to be involved in vesicle release (Akers and Routtenberg, 1987) the authors postulated that protein kinase C may be a possible indirect target for tetanus toxin, suggesting that down regulation of the enzyme may provide a clue for tetanus neurotoxicity.

**Action of tetanus toxin on spinal motor functions and supraspinal functions**

The elevated tonus of the musculature that occurs during both local and general tetanus is not due to a direct action of the toxin on muscles, or to an enhanced release of acetylcholine at the neuromuscular junction. The tonus is brought about by an increase in the frequency of efferent impulses from the CNS which results from a disinhibitory action of the tetanus toxin rather than from a strengthening
of excitation.

The experiments of Brooks et al (1957), in which they investigated the action of
tetanus toxin on the spinal monosynaptic reflex and on inhibition of this reflex by
a preceding stimulation of inhibitory reflex arcs (conditioned inhibition), led them
to conclude that tetanus toxin did not act by augmenting transmission at excitatory
synapses. When investigating the effect of tetanus toxin on conditioned inhibition,
a reduction of inhibition transmitted through five different reflex arcs was found
indicating that tetanus toxin acted at inhibitory synapses. In an extension of these
results it was found that supraspinal inhibition was also reduced, with supraspinal
polysynaptic excitationary reflexes being augmented.

Further experiments involving the recording of mass reflex discharges and single
cell discharges gave full support to the initial findings of Brooks et al (1957) (Ado
et al, 1966; Curtis, 1959; Curtis and De Groat, 1968; Curtis et al, 1976;
Kozhechkin, 1969; Kryzhanovsk and D’yakonova, 1964; Kryzhanovsk and
Sheikhon, 1968, 1973; Kryzhanovsk et al, 1973b; Takano, 1976; Takano and

In support of the presynaptic site of action of tetanus toxin, and the inhibition of
release of inhibitory neurotransmitters, electrophysiological evidence has
demonstrated that tetanus toxin reduced the size and number of the inhibitory
postsynaptic potentials (Bergey et al, 1983; Guschin et al, 1971; Kanda and
Takano, 1983; Kryzhanovsk and Sheikhon, 1973; Kryzhanovsk et al, 1973a;
Sverdlov, 1970), with the decrease in activity found not to be due to a decrease
in the discharge rate of, or in ion currents at, the presynaptic neurons (Wellhoner, 1989; Dreyer et al, 1983).

Tetanus toxin has been shown to produce a decrease of the monosynaptic reflex in local tetanus following a prolonged incubation time (Sverdlov, 1960; Sverdlov and Berlakov, 1960; Takano et al, 1983). Not only longer exposure periods, but also much higher concentrations of toxin have been implicated to cause depression of both inhibitory and excitatory transmission (Bergey et al, 1983; Bigalke et al, 1983, Kanda and Takano, 1983). Bergey et al (1987), using neuronal cell pairs of mouse spinal cord cells in culture, demonstrated the differential blockade of inhibitory and excitatory transmission produced by treatment with tetanus toxin. In this system, the presynaptic blockade occurred at a time when excitation was unaffected, and was coincident with the onset of paroxysmal depolarization shifts.

Administration of tetanus toxin to an in vitro preparation of rat hippocampal slices is known to block the GABA-mediated feedforward and recurrent inhibition at concentrations that neither produce excitatory transmission, nor alter the intrinsic membrane properties of the recorded cells. In this same situation, a much larger dose of toxin was found to reduce excitatory as well as inhibitory synaptic transmission in the hippocampus (Calabresi et al, 1989). An in vivo selective disinhibitory action of tetanus toxin in the substantia nigra and striatum of the rat has also been reported (Davies and Tongroach, 1979). It was found that during both of these experimental paradigms, the sensitivity of the postsynaptic neuronal receptors to excitatory or inhibitory molecules remained unaffected by toxin treatment (Calabresi et al, 1989; Davies and Tongroach, 1979).
The literature therefore implicates heavily that tetanus toxin has a site of action which is presynaptic in origin, resulting in a reduction of GABA/glycine-mediated inhibition in the CNS.

It is known that intrahippocampal injection of tetanus toxin in rats produces long-lasting "partial complex" recurrent seizure activity, bearing a resemblance to human limbic epilepsy (Mellanby et al, 1977). A reduction in GABA-mediated inhibition would lead to a facilitation of excitatory mechanisms that might be responsible for inducing such a phenomenon. Other effects resulting from intrahippocampal injection of tetanus toxin include an irreversible impairment of learning and memorizing tasks which require the functioning integrity of the hippocampal formation (Brace et al, 1985).

By producing an elevation in extracellular GABA levels using drugs capable of enhancing GABAergic transmission (e.g. sodium valproate, ethanolamine-O-sulphate, γ-vinyl-GABA) De Sarro et al (1985) demonstrated that the onset and number of epileptic-like spikes produced by intrahippocampal microinfusion of tetanus toxin in rats could be delayed and reduced, respectively. This finding supports the hypothesis that the tetanus toxin-induced excitatory focus results from a time-dependent irreversible blockade of GABA release.

Although tetanus toxin seems to show selectivity for GABA/glycine containing nerve terminals, the effect of the toxin on various other transmitter systems has been demonstrated recently. Halpern et al (1990), using nerve endings of the neural lobe of rat pituitaries (neurosecretosomes), found that tetanus toxin inhibited
the depolarization-evoked release of oxytocin and vasopressin in a time- and dose-
dependent manner. Indeed, neurosecretosomes are the first system in which the
toxin has been shown to block release from peptidergic nerve terminals. The
effect of reduced tetanus toxin on similar neurosecretosomes also demonstrated the
ability of the toxin to inhibit the release of vasopressin following challenge with
micromolar calcium concentrations (Dayanithi et al, 1992).

Stecher et al (1992) have shown that noradrenaline release from rat brain cortical
synaptosomes permeabilized with streptolysin O was inhibited by tetanus toxin and
the light chain of the molecule. However, the transmitter selectivity of tetanus
toxin is lost when the toxin is administered intracellularly (Poulain et al, 1991) or
in the case of the above observations, where cells are permeabilized, if
preincubation allows internalization of the toxin in the absence of specific
receptors. Inhibition of catecholamine release from intact bovine adrenal
chromaffin cells following binding of tetanus toxin has been observed by Colville

Recent in vivo studies discovered an overall increase in tissue levels of the
neurotransmitter 5-hydroxytryptamine (5-HT) in the CNS of rats following
intraperitoneal injection of tetanus toxin. This increase was attributed to a
blockade of 5-HT release (Aguilera et al, 1987; Aguilera and Gonzalez-Sastre,
1988). It was further demonstrated that a single intraventricular injection of
tetanus toxin produced a time-dependent elevation of 5-HT level in brain and
spinal cord of rats. The tetanus toxin-induced increase was produced in areas of
high density serotoninergic innervation, such as hypothalamus, hippocampus, and
Fig. 1.3  Representation of the summary of the major neuronal elements and intrinsic connections of the hippocampal formation (except the entorhinal cortex) in a slice taken approximately midway along the septotemporal axis.

Abbreviations:  S, subiculum;  DG, dentate gyrus;  mf, mossy fibres;  pp, perforant path;  sc, Schaffer collaterals;  BC, GABAergic basket cell.
spinal cord. Direct stereotaxic injection of the toxin into the hypothalamus and hippocampus produced significant 5-HT increases in both areas (Aguilera et al, 1991). It was concluded that the increases in tissue content of 5-HT were possibly due to the inhibition of 5-HT release from presynaptic nerve terminals, or due to a direct effect of the toxin on tryptophan 5-hydroxylase activity, which had previously been observed (Aguilera et al, 1987).

1.7 Tetanus toxin induced neuronal degeneration in rat brain

It has recently been demonstrated that a single unilateral microinjection of tetanus toxin into rat hippocampus produces a time- and dose- dependent neuronal loss in the CA1 pyramidal cell layer (Bagetta et al, 1990a). Typical epileptic-like behavioural effects produced by intrahippocampal injection of tetanus toxin were also observed (Mellanby et al, 1977). Autoradiographic analysis revealed that the neuronal damage produced by tetanus toxin was accompanied by a decrease in \( \text{GABA}_A \) receptor binding density in the pyramidal cell layer, whilst the \( \text{GABA}_B \) receptor binding capacity remained unchanged (Bagetta et al, 1990b).

It was postulated that the behavioural and neuropathological effects of the toxin occurred due to a loss of neuronal inhibition, resulting in a net excitatory input involving overactivation of the NMDA-receptor complex at the site of injection. In rats subjected to a chronic unilateral surgical lesion of the Schaffer collateral-commissural pathway, through which CA1 pyramidal cells normally receive an excitatory input from the CA3 cell layer, subsequent bilateral microinjections of tetanus toxin were found to produce a loss of CA1 pyramidal cells only on the
unlesioned side (Bagetta et al., 1991b).

Similarly, prevention of the convulsant and neurodegenerative effects resulting from intrahippocampal injection of tetanus toxin has been observed in rats treated systemically with either competitive (CGP 37849) or non-competitive (dizocilpine) NMDA receptor antagonists. These agents also protected against toxin-induced mortality (Bagetta et al., 1990c, 1992).

Whilst investigating the action of GABAergic transmission enhancing drugs on the effects of toxin treatment it was observed that the anticonvulsant benzodiazepine diazepam failed to protect rats from the induced effects (Bagetta et al., 1990c). It was concluded that this failure in protective ability may be due to the fact that diazepam relies on the presence of endogenous GABA to produce its pharmacological actions (Haefely et al., 1979; Olsen, 1981). This finding indirectly confirms the ability of tetanus toxin to block GABA release at the site of injection, resulting in production of a disinhibitory focus.

To investigate the effect of injection into another part of the trisynaptic hippocampal network, tetanus toxin was also injected into the dentate gyrus of rats. This area represents the first relay of the trisynaptic circuit and receives a robust excitatory innervation from the entorhinal cortex through the perforant pathway (Anderson, 1975; Amaral and Witter, 1989). This region of the hippocampus has a functional organization similar to that of the CA1 area, with the perforant path axons utilizing glutamate as an excitatory neurotransmitter (White et al., 1977), whilst inhibitory inputs are mainly provided by basket cells
(Schwartzkroin and Knowles, 1983. Behavioural excitatory effects and neuronal damage were also observed in rats treated in this fashion. Here though, damage to granule cells of the dentate gyrus was more dramatic than that observed in CA1 pyramidal cells (Bagetta et al, 1991c).

It therefore appears that, irrespective of the site of injection, the differential action of tetanus toxin on inhibitory and excitatory transmission is responsible for disrupting the normal balance of inputs to neurons in the mammalian CNS, consequently resulting in epileptogenic and neurodegenerative effects.

1.8 Purpose of the present study

It was proposed by Bagetta et al (1991b,c) that the behavioural and neuropathological effects resulting from injection of tetanus toxin into various regions of the hippocampal formation were due to a loss of neuronal inhibition at the site of injection. Such a loss of inhibitory tone was postulated to result in a net excitatory input involving overactivation of the NMDA-receptor complex at the site. In order to investigate whether tetanus toxin injection does result in an imbalance between inhibitory and excitatory tone, the rat hippocampal formation was used as a model to examine the effect of tetanus toxin injection on extracellular levels of neuroactive amino acids, using the technique of intracerebral microdialysis. Also, in light of the recent observations that intrahippocampal injection of tetanus toxin produced an increase in tissue level of 5-hydroxytryptamine, possibly by inhibition of presynaptic 5-hydroxytryptamine release (Aguilera et al, 1991), attention was also focused onto extracellular levels of monoamines.
Chapter 2

Materials and Methods
Microdialysis is a relatively new bioanalytical sampling technique which has opened up the possibility of sampling substances from the extracellular space of essentially any tissue in the body, delivering a sample which is already clean and ready to be analyzed. The technique of microdialysis provides a way to remove chemical substances from the extracellular fluid of the body without removing liquid, and also enables substances to be introduced without directly injecting fluid. The basic principle of microdialysis involves the positioning of a membrane that allows free diffusion of water and solutes between the solution of interest and a solution lacking the substances concerned, the latter being constantly removed and sampled for further analysis. When perfusing the microdialysis probe with a physiological solution closely resembling the composition of the extracellular space various solutes on their way between cells and between cells and blood vessels will diffuse into the probe and be transported out by the perfusion fluid. Alternatively, when perfusing with a higher concentration in the perfusion medium substances will diffuse from the probe and enter the extracellular space, providing a mechanism of introducing drugs into the tissue.

From a technical standpoint certain features of microdialysis are particularly interesting: The perfusion system is simple due to the presence of a membrane bag providing a closed liquid system, hence abolishing the requirement for both pushing and pulling liquid through the probe. The membrane protects the surrounding tissue from excessive damage and irritation during perfusion as it separates the tissue from the perfusion fluid. There is no risk that tissue will be unintentionally sucked into the probe as may occur during push-pull perfusions. Dialysis probes can be delivered sterile whilst at the same time the membrane
protects the tissue from a non-sterile perfusion fluid. Microdialysis provides an opportunity to control the area investigated by varying the length of the probe membrane. Substances may be introduced and collected at the same time. For example, drug effects may be examined by including them in the perfusion medium and then analyzing the changes in the recovered endogenous substances. The dialysis membrane excludes large molecules, such as enzymes, which may brake down the low molecular substances recovered in the dialysate. Finally, it is possible to compare in vivo experiments with in vitro model experiments. Whilst it is obviously difficult to recreate the environment of the nervous system in an in vitro situation it is still possible to make estimations about the concentrations of substances in the extracellular space by extrapolating from in vitro experiments.

Whilst executing the microdialysis procedure it is of utmost importance to exclude air bubbles from the perfusing system, as air bubbles may stick inside the membrane and decrease the dialyzing surface, thereby limiting the recovery of substances. The few disadvantages of the technique include (a) relatively poor time resolution (sampling interval of minutes) resulting from the necessity to obtain a large enough dialysate sample for reliable analysis; (b) the continuous drainage of a substance from the extracellular fluid may limit its supply with time and, therefore, reduces mass transport; (c) decreased recovery across the dialysis membrane after 3-4 days in chronically implanted preparations.
2.1 Animals

Male Wistar rats (250 - 300g) were used for all experimental procedures. Animals were housed in a humidity (45 - 55%) and temperature (22 ± 1°C) controlled environment with lighting from 7.00 - 19.00 h. Access to food and water was *ad libitum*.

2.2 Microdialysis

2.2.1 Dialysis probe construction

All microdialysis probes were of a concentric design and constructed in the laboratory as represented in Figure 2.1 (a - f).

*Fig. 2.1 Construction steps involved in concentric dialysis probe manufacture.*

(a) Two lengths of vitreous silica tubing (Scientific Glass Engineering) were inserted into a 2 cm length of 24 gauge steel tubing (0.52 mm external diameter). One length of silica protruded approximately 10 mm out of the steel cannula, whilst the other length was fed approximately 3/4 of the way along the shaft of the steel tubing. The silica tubes were secured to the appropriate end of the steel cannula using a small drop of araldite adhesive which was left to set for at least 30 minutes.
b. A 1 cm length of 27 gauge steel tubing (0.38 mm external diameter) was slipped over each of the protruding lengths of silica, and the junction made secure with an appropriate amount of araldite.

c. The junction between the three steel tubes was then reinforced by the addition of quick setting acrylic dental cement.

d. The silica tubing protruding from the 24 gauge steel cannula was trimmed to a length of 4 mm, and a length of dialysis membrane (0.2 mm external diameter, 10 Kdaltons molecular cut off; ENKA AG, Germany) was carefully fitted over the silica so that it extended several mm's into the steel tubing. The dialysis membrane was trimmed so that it extended approximately 1.0 mm beyond the end of the silica tube and the end of the dialysis bag sealed with a small drop of araldite. The opposing end of the membrane was sealed to the extremity of the 24 gauge steel tubing in a similar fashion. This stage of the probe assembly required the use of a binocular microscope. The dialysis membrane used in the present study was Cuprophan capillary membrane, type F1 8 200 UFR 5.5 ± 0.5.
e. To allow the probe to be perfused with artificial cerebrospinal fluid (CSF) a 10 cm length of pp10 polyethylene tubing (O.D 0.61 mm; Portex Ltd, UK) was fixed tightly over each of the 27 gauge steel tubes.

f. The connections were secured by application of hot melt adhesive to the main body of the probe.

All probes were examined using a binocular microscope to verify that the active membrane length was 4mm. A diagrammatic representation of the concentric microdialysis probe is shown in Figure 2.2.

Prior to use, all probes were slowly perfused with an artificial cerebrospinal fluid (CSF) (125 mM NaCl, 2.5 mM KCl, 1.18 mM MgCl₂, 1.26 mM CaCl₂; pH 7.4), to test for functional integrity. If leaks were evident, or an unusually high resistance to flow was displayed, probes were discarded. The process was completed by flushing with deionised water to prevent salt precipitation.
Fig. 2.2 Diagrammatic representation of the concentric microdialysis probe used in the present study.
2.2.2 *In vitro* recoveries of amino acids and monoamines

As the technique of microdialysis does not directly obtain samples of the interstitial space, the concentrations of substances in the outflow solution are only reflections of the true brain extracellular concentrations. Therefore, dialysis probes had to be calibrated before use *in vivo*.

The *in vitro* relative recoveries of amino acids and monoamines, from standard solutions by the dialysis probes were measured as follows:

Dialysis probes were suspended in a bathing medium composed of 1 μM 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) for monoamine and metabolite recoveries or 1 μM L-aspartic acid (ASP), L-glutamic acid (GLU), L-serine (SER), L-histidine (HIS), L-glutamine (GLN), glycine (GLY), L-threonine (THR), L-arginine (ARG), taurine (TAU), β-alanine (β-ALA), L-tyrosine (TYR), L-alanine (ALA), and γ-aminobutyric acid (GABA) for amino acid recoveries (all compounds obtained from Sigma, UK). All substances were dissolved in an artificial CSF. The vessel containing the bathing medium was in turn suspended in a water bath maintained at 37 °C, in an attempt to mimic *in vivo* temperature.

Dialysis probes were connected to a microinfusion pump (Harvard Apparatus, syringe infusion pump 22; USA) and perfused with an artificial CSF at a flow rate of 0.5 μl/min. Following a 90 minute stabilisation period, four 30 minute samples were collected. These samples, together with an equal volume of
standard bathing medium, were analysed by high performance liquid chromatography (HPLC) with electrochemical or fluorometric detection for monoamines or amino acids, respectively. The relative recovery was calculated as follows:

\[
\text{Recovery}_{\text{in vitro}} = \frac{C_{\text{out}}}{C_i}
\]

where \( C_{\text{out}} \) is the substance concentration in the outflow and \( C_i \) is the substance concentration in the medium.

This relationship was used to estimate the brain extracellular concentration of substances under investigation:

\[
C_i = \frac{C_{\text{out}}}{\text{recovery in vitro}}
\]

where \( C_i \) is the extracellular concentration and \( C_{\text{out}} \) is the substance concentration in the \textit{in vivo} outflow solution.

The \textit{in vitro} relative recoveries of amino acids and monoamines from standard solutions by the dialysis probes, whilst being perfused at a flow rate of 0.5 \( \mu l/min \), are shown in Table 2.1.
Table 2.1

*In vitro* relative recoveries of monoamines and amino acids from standard solutions at a flow rate of 0.5 µl/min and a temperature of 37°C. Values are expressed as mean ± s.e.m, n=4.

<table>
<thead>
<tr>
<th>Monoamine</th>
<th>% Recovery</th>
<th>Amino acid</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>25.7 ± 7.1</td>
<td>Aspartate</td>
<td>39.9 ± 2</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>19.2 ± 1.9</td>
<td>Glutamate</td>
<td>37.2 ± 2</td>
</tr>
<tr>
<td>DA</td>
<td>40.8 ± 4</td>
<td>Taurine</td>
<td>50.7 ± 3.5</td>
</tr>
<tr>
<td>DOPAC</td>
<td>26.7 ± 6.3</td>
<td>GABA</td>
<td>47.9 ± 4.2</td>
</tr>
<tr>
<td>HVA</td>
<td>18.6 ± 3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.3 Stereotaxic injection of tetanus toxin and microdialysis probe implantation

**Central injection of tetanus toxin**

Rats were anaesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg; dissolved in 0.9% saline) and positioned into a Kopf stereotaxic frame. The scalp was incised and the skull exposed. A burr hole was drilled in the skull with use of a microdrill. Tetanus toxin, with a specific toxicity of $2.5 \times 10^7$ mouse minimum lethal doses (MLDs) per milligram of protein, was dissolved in
phosphate buffer (pH 7.4). A 1000 MLDs dose of tetanus toxin was microinjected unilaterally into the dentate gyrus region of the ventral hippocampal formation, hereinafter referred to as the ventral hippocampus, using the following coordinates:

\[
\begin{align*}
\text{Anterior from bregma} & = -5.2 \text{ mm} \\
\text{Lateral from midline} & = 4.8 \text{ mm} \\
\text{Below the dura mater} & = 6.5 \text{ mm}
\end{align*}
\]

Coordinates were according to the rat brain atlas of Paxinos and Watson (1982). The volume of injection was 1 µl for all animals, at a rate of 1 µl/min by means of a Hamilton microsyringe (5 µl) mounted on a stereotaxic micromanipulator. After injection the syringe was left in place for 5 minutes and then withdrawn slowly to reduce the possibility of tetanus toxin reflux along the injection track. Using the same procedure, phosphate buffer (vehicle) was injected into the contralateral region of the ventral hippocampus.

**Implantation of microdialysis probes**

When dialysis measurements were made up to 3 days after initial microinjections of tetanus toxin and vehicle, the following procedure was executed:

Approximately 15 minutes after intrahippocampal injection of toxin or vehicle, bilateral implantation of concentric dialysis probes was commenced. The microdialysis probe was held in place by a specialized probe holder, which in turn was mounted on a stereotaxic micromanipulator. The probe was positioned over the original injection site and then gently lowered until in contact with the dura
mater. During the implantation procedure the dialysis bag at the tip of the probe was maintained rigid by continuously perfusing the probe with an artificial CSF. This procedure helped to prevent damage of the probe during implantation. The tip of the dialysis probe was placed 8.0 mm below the dura (c.f. injection site at 6.5 mm below dura). Stainless steel screws were positioned in the skull and dialysis probes secured to the skull surface by the addition of acrylic dental cement, which in turn completely sealed the scalp incision. The sites of toxin and vehicle injections and dialysis probe placements in the ventral hippocampus can be observed in Figure 2.3.

When dialysis measurements were made 7 days after the initial microinjections of toxin and vehicle, the following procedure was undertaken:

It had previously been observed that when a microdialysis probe remained implanted in the ventral hippocampus for periods exceeding 4-5 days, perfusion of artificial CSF through the probe was often severely hindered or totally abolished. To combat this difficulty it was decided that dialysis probes would be implanted 6 days after initial microinjections, in order to enable measurements to be made at day 7. Therefore, following injection of toxin and vehicle, the scalp wound was carefully cleaned and the incision sutured. The animal was removed from the stereotaxic frame and allowed to recover from anaesthesia. After a period of 5 days, now 6 days after initial toxin and vehicle injections, rats were reanaesthetized and placed into a stereotaxic frame, ensuring that all frame settings were in accord with previous settings. Dialysis probes were implanted bilaterally and secured to the skull surface as described previously.
Fig. 2.3 Coronal section of rat brain indicating sites of microinjections and dialysis probe implantation in ventral hippocampus.

CA1-CA3 = Fields CA1-CA3 of Ammon's horn

DG = Dentate gyrus
Switching of the perfusion medium was carried out manually. The perfusion filled pp10 polythene tubing was quickly disconnected from the inflow tube of the probe and rapidly replaced with an identical length of polythene tubing filled with high K⁺ containing medium, which in turn was connected to a microsyringe mounted on the same infusion pump.
2.2.4 *In vivo* microdialysis

Throughout the duration of dialysis experiments, rats were housed singly in a purpose built perspex box (30 x 30 x 30cm), with a 2cm diameter hole in the lid which enabled passage of the perfusion and dialysate collection lines.

Twenty four hours after implantation, the inflow tube of the dialysis probe was connected to an appropriate length of pp10 polyethylene tubing (O.D 0.61mm; Portex Ltd, UK) which in turn was connected to a gas-tight 500ul Hamilton microsyringe filled with artificial CSF and mounted on a microinfusion pump (Harvard Apparatus, syringe infusion pump 22). The dialysis probe was perfused manually with artificial CSF for a short period of time in order to expel trapped air bubbles. The outlet tube of the probe was connected to a known length of low dead volume tubing (0.1 μl/cm, FEP tubing, Carnegie Medicin, Sweden) which fed into a collecting vial. Lines were weighted with Blu-tack to counterbalance the effects due to animal movement.

On commencement of dialysis, probes were perfused at a rate of 0.5 μl/min. Following a 90 minute stabilization period, hourly dialysis samples were collected into plastic vials and either analysed immediately by HPLC or frozen at -80°C and analysed at the earliest convenient time. Whilst investigating the effect of K⁺-stimulation on extracellular levels of amino acids and monoamines in rat brain, the perfusing artificial CSF was switched to an isotonic medium containing 100 mM K⁺ for a 30 minute period. This time period of perfusion with such a concentration of K⁺ had previously been shown to evoke the release of
neurotransmitters in rat brain (Kalen et al, 1988; Dietze and Kuschinsky (1992). At the end of this period the original medium was returned to the dialysis system. In this experimental paradigm, four 30 minute samples were collected before the perfusion medium was switched. Upon return of the original medium, three more 30 minute dialysis samples were collected. Dialysates were collected and analysed as described previously. All values representing hippocampal extracellular levels of amino acids and monoamines were calculated for in vitro dialysis probe recoveries (see Table 2.1).

2.3 High Performance Liquid Chromatography

2.3.1 Quantification of amino acids

The technique of reverse phase HPLC with fluorometric detection and gradient elution was used to analyse the amino acid content of dialysis samples. Dialysates were precolumn derivatized with o-phthaldialdehyde (OPA, Fluka; 27 mg OPA/0.5 ml absolute ethanol + 5 ml 0.1 M sodium teraborate dehydrate pH 9.5 + 50 μl beta-mercaptoethanol) (Lindroth and Mopper, 1979). Derivatization was carried out in a refrigerated (4°C) autosampler unit by adding 25 μl OPA to 15 μl of dialysis sample in sealed amber glass vials. Following a 2 minute reaction time, 35 μl of the mixture was injected, via a Rheodyne syringe loading sample injector, onto a 20 μl loop.

Two Gilson pumps, model 302 and model 301, fed solvent A (40 nM NaH₂PO₄.2H₂O, pH 5.5, 20% methanol) and solvent B (100% methanol), respectively into a Gilson dynamic mixer unit (model 811). All solvents were filtered through 0.2 μm nitrocellulose filters prior to use and continuously degassed with helium throughout the duration of the run. The mobile phase
gradient was determined by Gilson gradient management software, with flow rate maintained at 1.0 ml/min (Figure 2.4).

Separation of the highly fluorescent indole derivatives formed from the reaction of primary amines with OPA was produced using a microsorb column (4.6 mm I.D x 150 mm), prepacked with C_{18} 5 \mu M particles, protected by a microsorb guard column (4.6 mm I.D x 15 mm; C_{18} 5 \mu M) (both Dynamax, Rainin Instrument Co. Inc, USA). Both columns were enclosed in a column heater unit and maintained at a constant temperature of 37°C. Separated fluorescent indole derivatives were detected by a Gilson fluorescence detector at wavelengths in the range 340-455 nm. Output from the detector was channelled via a Drew data collection unit interface to a Dell System 310 personal computer where signals were subsequently converted to peaks and integrated.

**System calibration using amino acid standards.**

Standard solutions containing 2.5 \mu M L-aspartic acid, L-glutamic acid, L-serine, L-histidine, L-glutamine, glycine, L-threonine, L-arginine, taurine, \beta-alanine, L-tyrosine, L-alanine, and GABA were prepared with double distilled, deionised HPLC grade water and stored at -20°C in 1ml aliquots for periods of up to 2 months. Prior to analysis of dialysates by HPLC, varying concentrations (2-10 pmoles on the column) of amino acid standards were run in order to calibrate the system. Figure 2.5 depicts the representative chromatogram obtained from a 10 pmoles standard amino acid solution. Amino acids present in dialysis samples were identified by retention time on the column and concentrations calculated by
Fig. 2.4 Profile of HPLC gradient used to produce separation of amino acids.

Fig. 2.5 Chromatogram obtained from a 10 pmole amino acid standard solution.

1 = ASP; 2 = GLU; 3 = SER; 4 = HIS; 5 = GLN; 6 = GLY; 7 = THR; 8 = ARG; 9 = TAU; 10 = β-ALA; 11 = ALA; 12 = TYR; 13 = GABA.
comparing peak areas to calibration plots (Figure 2.6).

2.3.2 Quantification of GABA

Attempts to measure levels of GABA from rat hippocampal dialysis samples using HPLC with fluorometric detection proved highly unsuccessful due to the inability of the HPLC system to both accurately and consistently measure the extremely low GABA levels present in hippocampal dialysates. In order to circumvent this major problem a novel HPLC method, with a far greater sensitivity than the fluorometric detection system, was established in the laboratory.

A reverse phase isocratic HPLC system coupled with an ESA Coulachem II electrochemical detector (Severn Analytical, Bedfordshire) was employed. Dialysates were precolumn derivitized with OPA, as described in section 3.1. Derivatization took place manually by adding 1.5 μl of OPA to 15 μl of dialysis sample in plastic vials. Following a reaction time of 2 minutes, 15 μl of the mixture was injected manually, via a Rheodyne syringe loading sample injector, onto a 10 μl loop. A mobile phase comprised of 0.1 M NaH₂PO₄·2H₂O, pH 6.1, 27% methanol, was continuously pumped through the HPLC system by a Severn Analytical SA 6410B isocratic solvent delivery system at a constant flow rate of 1.0 ml/min. Prior to use mobile phase was filtered through 0.2 μm nitrocellulose filters. Mobile phase was recycled and continuously degassed with helium.

A microsorb column (4.6 mm I.D x 50 mm), prepacked with C₁₈ 3 μm particles (Dynamax, Rainin Instrument Co. Inc, USA.) produced separation of the
Fig. 2.6  Representative calibration plots obtained for amino acids. Peak area represents arbitrary units.
electrochemically active indole derivatives formed from the amino acid/OPA reaction. These species were detected by an ESA model 5011 analytical cell, with detector 1 set at -400 mV and detector 2 set at +600 mV. The analytical cell and column were protected by an ESA model 5020 guard cell, set at +650 mV. Output from the detector was channelled into a Hewlett Packard HP 3394 integrator, where signals were subsequently converted into peaks and integrated, and also into an on-line BBC Goerz Metrawatt SE 120 chart recorder, which allowed a further 3 fold amplification of the detector output.

**System calibration using GABA standards**

Standard solutions containing 0.5 μM GABA were prepared with double distilled, deionised HPLC grade water and stored in 1 ml aliquots for periods of up to 2 months. Prior to analysis of dialysis samples, varying concentrations (140 fmole - 4.5 pmoles on the column) of GABA standards were run in order to calibrate the system. Examples of integrator- and chart recorder-derived chromatograms obtained from a 0.57 pmole standard GABA solution are shown in Figure 2.7. Calibration plots were produced from integrated areas derived from the Hewlett Packard integrator, and also from chart recorder derived peak heights (Figure 2.8). GABA present in dialysis samples was identified by column retention time. Low level GABA concentrations were calculated from chart recorder produced calibration plots.
Fig. 2.7
Integrator (a) and chart recorder (b) derived chromatograms obtained from a 0.57 pmole GABA standard solution.
Fig. 2.8 Integrator (a) and chart recorder (b) derived GABA calibration plots.
2.3.3 Quantification of monoamines

The technique of reverse phase HPLC coupled with electrochemical detection was used to analyse the monoamine and metabolite content of microdialysis samples. A pre-filtered mobile phase composed of 90 mM sodium acetate, 35 mM citric acid, 0.34 mM EDTA, and 0.06 mM sodium octane sulphonate acid, pH 4.2, with 13% methanol, was continuously delivered through the HPLC system by a Gilson pump, model 303, at a constant flow rate of 1.0 ml/min. Throughout HPLC activity mobile phase was continuously degassed with helium and recycled. Precolumn derivatization was not required for monoamine analysis, therefore 15 μl of dialysis sample was injected, via a Rheodyne syringe loading sample injector, onto a 10 μl loop.

Separation of monoamines and metabolites was produced using a microsorb column (4.6 mm I.D x 100 mm), prepacked with C\textsubscript{18} 3 μm particles, protected by a microsorb guard column (4.6 mm I.D x 15 mm; C\textsubscript{18} 5 μm)(both Dynamax, Rainin Instrument Co. Inc, USA.). Species were detected by a ESA model 5011 analytical cell, with detector 1 set at +10 mV and detector 2 set at +460 mV, coupled to an ESA Coulachem model 5100A electrochemical detector (Severn Analytical, Bedfordshire, UK). Both column and analytical cell were protected by an ESA model 5020 guard cell, preset at +500 mV. Output from the Coulachem electrochemical detector was channelled via a Drew data collection unit interface to a Dell System 310 personal computer, where signals were converted to peaks and integrated. Detector output was also channelled directly to an on-line BBC Goertz Metrawatt SE 120 chart recorder, allowing a further 10 fold amplification.
of the signal.

**System calibration using monoamine standards**

Standard solutions containing 1 μM DA, DOPAC, 5-HT, 5-HIAA, and HVA were prepared with double distilled, deionized HPLC grade water. At the beginning of each HPLC run, varying concentrations of the standard solution were injected onto the HPLC system for calibration purposes. Figure 2.9 shows the representative computer- and chart recorder-derived chromatograms obtained from a 4 pmole and 0.125 pmole standard monoamine solution respectively. Calibration plots were obtained from computer-derived (2-10 pmoles on the column) (Fig. 2.10), and chart recorder-derived (15 fmoles - 0.25 pmoles on the column) (Fig.2.11) data. Monoamines and metabolites present in dialysis samples were identified by column retention time. Dialysate levels of 5-HT and DA were calculated from chart recorder-derived calibration plots, whereas 5-HIAA levels were obtained from computer-derived calibration plots. DOPAC and HVA levels were obtained from both sets of these plots.

### 2.4 Histology

On termination of experiments, dialysis probe placement was verified for each animal. Rats were sacrificed by overdose of pentobarbitone (Expirai 150 mg/kg i.p.). Pontamine sky blue dye was infused into the dialysis probe until it exited via the outflow tubing. After a 5 minute period, brains were removed and frozen in isopentane at -40°C. Coronal brain sections (10 μm) were cut on the cryostat and mounted on glass slides. Verification of probe placement was made by
Computer (a) and chart recorder (b) derived chromatograms obtained from a 4 pmole and 0.125 pmole monoamine standard solution, respectively.

(a) 1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = 5-HIAA; 5 = HVA.

(b) 1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = HVA.
Fig. 2.10

Computer derived monoamine calibration plots. Peak area represents arbitrary units.
Fig. 2.11 Chart recorder derived monoamine calibration plots.
visualization of the stained probe track. If the track was not easily visible, sections were stained with toluidine blue dye to readily expose the probe position. Only animals with correctly located dialysis probe tracks were incorporated into the results data. A typical photomicrograph depicting the correct positioning of a dialysis probe in the ventral hippocampal formation is shown in Figure 2.12.

Fig. 2.12  Photomicrograph illustrating the correct placement of a dialysis probe in rat ventral hippocampal formation.
2.5 mM CaCl$_2$ was included in the Tris-HCl buffer for GABA$_B$ receptor binding as the presence of divalent cations (Ca$^{2+}$ or Mg$^{2+}$) is required for the binding of $[^3]$H-GABA to GABA$_B$ receptor sites (Bowery et al., 1987).
2.5 Radioligand binding and autoradiography

2.5.1 Preparation of brain sections for autoradiographic studies

Male Wistar rats (250 - 300g) were killed by cervical dislocation. Brains were rapidly removed and frozen by immersion in isopentane at -40°C. Coronal brain sections (10 μm) were cut on a 2800 Frigocut cryostat (Reichert-Jung) at -20°C and mounted on gelatine coated glass microscope slides. After air-drying for a minimum period of 2 hours, the sections were stored at -20°C until used, or for a maximum period of up to 1 month.

2.5.2 Quantitative autoradiography of GABA<sub>A</sub> and GABA<sub>B</sub> receptor binding sites using [³H]-GABA

Upon commencement of binding studies, brain sections were thawed for 40-50 minutes at room temperature and then rinsed for 40-60 minutes, at 21-23°C, in 250 ml Tris-HCl buffer (50 mM, pH 7.4) with or without CaCl<sub>2</sub> (2.5 mM) for GABA<sub>B</sub> and GABA<sub>A</sub> receptor binding, respectively. Sections were then air-dried before carefully placing 100 μl of incubation medium containing 50 nM [³H]-GABA (91.6 Ci/mmol) (Amersham International Plc, UK.) over each section. The medium was left in contact for 20 minutes at room temperature.

Isoguvacine (40 μM) or (-)-baclofen (100 μM), for GABA<sub>A</sub> and GABA<sub>B</sub> binding, respectively were also present in the incubation medium. In the presence of (-)-baclofen and the absence of CaCl<sub>2</sub> only GABA<sub>A</sub> sites were labelled whereas in the presence of isoguvacine and CaCl<sub>2</sub> only GABA<sub>B</sub> sites were labelled. In order to
determine non-specific binding, isoguvacine (100 μM) or (-)-baclofen (100 μM) were also added to the incubation fluid for GABA\textsubscript{A} and GABA\textsubscript{B} sites, respectively.

Following incubation the radiolabelled solution was rapidly aspirated off and the section rinsed in fresh Tris-HCl buffer (2 x 4 seconds) at 21-23 °C. Sections were then dried at room temperature and placed in contact with Hyperfilm\textsuperscript{3}H (Amersham International Plc, UK) for 3-4 weeks at 21-23°C. At the end of the exposure period the film was developed in Kodak D-19 solution and allowed to air dry.

2.5.3 Quantitative autoradiography of 5-HT re-uptake sites using [\(^3\)H]-paroxetine

On initiation of binding studies, sections were thawed at room temperature for approximately 45 minutes and then pre-incubated for 15 minutes, at 21-23°C, in Tris-HCl buffer (pH 7.7, 50 mM containing 120 mM NaCl and 5 mM KCl). Brain sections were then submerged in incubation medium containing 0.25 nM [\(^3\)H]-paroxetine (21 Ci/mmol, New England Nuclear, Boston, USA.) for 120 minutes at room temperature. To determine background binding, citalopram (4 μM) was added to the incubation medium.

On termination of the incubation period the section was washed twice for 2 x 60 minutes duration in fresh Tris-HCl buffer solution at 21-23°C, dipped in chilled distilled water, and then dried under a stream of cold air. The dry sections were placed in contact with Hyperfilm\textsuperscript{3}H (Amersham International Plc, UK.) for 8 weeks at room temperature. At the end of the exposure period the film was developed in Kodak D-19 solution.
2.5.4 Densitometric analysis of autoradiograms

The autoradiographic images obtained on Hyperfilm-$^3$H were analysed using a Quantimet 970 Image analyser (Cambridge Instruments, Plc). Regional optical density measurements were made by examining 3 areas within the same brain structure on sections from at least 3 rats, and converted to the corresponding ligand concentration by reference to commercially available tritium micro-scale standards (Amersham International Plc, UK). Specific binding was determined by subtraction of background values for each area and the values expressed as fmoles [${}^3$H]-ligand/mg wet tissue. The various brain regions under investigation were identified using the rat brain atlas of Paxinos and Watson (1982).

2.6 Materials

Unless otherwise stated all reagents were obtained from Sigma or BDH and were of at least Anal AR grade. Tetanus toxin ($2.5 \times 10^7$ mouse minimum lethal doses (MLDs) mg$^{-1}$ protein) was a kind gift of Prof. B. Bizzini (Pasteur Institute, Paris.). (-)-baclofen was kindly donated by Ciba-Geigy, Switzerland. Isoguvacine and citalopram were commercially obtained from Cambridge Research Biochemicals Ltd, UK, and H. Lundbeck and Co., Denmark, respectively.
Chapter 3

Effect of tetanus toxin on extracellular amino acid levels in rat hippocampus determined by the technique of \textit{in vivo} microdialysis.
It has recently been demonstrated that a single unilateral microinjection of tetanus toxin into rat hippocampus produces a dose- and time-dependent behavioural excitation and neuropathology, with a toxin dose equivalent to 1000 MLDs producing neuronal damage 7-10 days after intrahippocampal microinjection (Bagetta et al, 1990a).

It is well documented that tetanus toxin produces an impairment of inhibitory neurotransmission within the mammalian CNS, both in vivo (see Mellanby and Green, 1981), and in vitro (Bergey et al, 1987; Jeffreys, 1989). The disinhibitory effect produced by tetanus toxin is widely considered to be due to its interaction with GABA- and glycine-mediated inhibitory synapses, resulting in an apparent reduction in inhibitory neurotransmitter release (Curtis et al, 1973; Mellanby et al, 1977; Collingridge et al, 1980, 1981; Collingridge and Davies, 1982; Calabresi et al, 1989). Therefore, it was proposed that the neuropathological effect produced by tetanus toxin may be related to a loss of neuronal inhibition, resulting in a net increase of excitatory input involving overactivation of the NMDA-receptor complex at the site of injection. In support of this hypothesis the behavioural and neuropathological effects resulting from treatment with tetanus toxin could be prevented by the NMDA-receptor antagonists dizocilpine (Bagetta et al, 1990c) and CGP 37849 (Bagetta et al, 1992).

In an attempt to determine whether a reduction in hippocampal extracellular GABA occurs following treatment with tetanus toxin, the relatively new bioanalytical sampling technique of intracerebral microdialysis (for review see Benveniste, 1989) was employed to indirectly obtain samples of the interstitial
space from the site at various times following intrahippocampal injection. This technique was combined with high performance liquid chromatography to measure levels of neuroactive amino acids in dialysis samples.

3.1 Determination of basal and K+-evoked extracellular levels of neuroactive amino acids in rat hippocampus.

Basal extracellular levels of amino acids were determined in control rats unilaterally implanted in the ventral hippocampus. Approximately 24 hours after surgery rats were perfused with an artificial CSF at a flow rate of 0.5 μl/minute (see section 2.2.4) and hourly dialysate samples were collected. This collection time resulted in a dialysate volume large enough to enable repeated HPLC determinations of extracellular amino acid concentrations to be made if necessary. Hippocampal extracellular amino acid levels derived from control unilaterally implanted rats 24 hours post-operatively are shown in Table 3.1.
Table 3.1 Basal levels of extracellular neuroactive amino acids in control rat hippocampi.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>pmoles/15 μl sample</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>7.86 ± 1.0</td>
<td>0.52</td>
</tr>
<tr>
<td>Glutamate</td>
<td>92.4 ± 24</td>
<td>6.16</td>
</tr>
<tr>
<td>Taurine</td>
<td>228.6 ± 34</td>
<td>15.24</td>
</tr>
<tr>
<td>GABA</td>
<td>1.31 ± 0.08</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Four 1 hour dialysis samples were used to evaluate a mean basal level of amino acid. Data are expressed as mean ± s.e.m of 9-11 rats.

A fluorometrically detected HPLC chromatogram depicting basal levels of extracellular amino acids in rat hippocampus is represented in Figure 3.1. Due to the amino acid peak sizes obtained in this instance, determination of the extracellular levels of aspartate, glutamate, and taurine was uncomplicated. However, as previously mentioned in section 2.3.2, attempts to measure levels of GABA from hippocampal dialysates using HPLC with fluorometric detection proved a more difficult task due to the extremely small GABA peak sizes obtained. With this method the limit of detection of GABA was approximately 250 fmoles. Therefore, basal extracellular GABA levels were measured using a more sensitive HPLC method which had been established in the laboratory and
involved electrochemical detection (see section 2.3.2). In this system, the limit of detection of GABA was approximately 50-60 fmoles. A chart recorder-derived chromatogram, obtained from an electrochemically detected HPLC sample, depicting basal GABA levels in rat hippocampus is shown in Figure 3.2.

**Fig. 3.1 Fluorometrically detected HPLC chromatogram depicting basal levels of amino acids in rat hippocampus.**

1 = aspartate; 2 = glutamate; 3 = taurine; 4 = GABA.
The effect of a 30 minute pulse with artificial CSF containing 100 mM K⁺ on hippocampal extracellular levels of amino acids in control rats is illustrated in Figure 3.3. Experiments were commenced 24 hours after completion of surgical procedures. In these rats, challenge with high K⁺ evoked the release of hippocampal aspartate, taurine, and GABA. The corresponding percentage increases from resting basal levels were 160% (p < 0.05), 493% (p < 0.001) and
4305% (p < 0.001), respectively (using Student's paired t-test). However, K+ stimulation had no effect on extracellular levels of glutamate. HPLC chromatograms illustrating K+-evoked levels of amino acids in control rat hippocampi are represented in Figure 3.4.

3.2 Dose level of tetanus toxin used in the present study

Bagetta et al (1990a) have recently demonstrated that microinjection of tetanus toxin into the CA1 pyramidal cell layer of rat hippocampus produces a dose- and time-dependent behavioural excitation and neuropathology. A toxin dose equivalent to 1000 MLDs resulted in both postural and locomotor behavioural changes within 48 hours of treatment, culminating in generalized convulsions approximately 5 - 7 days after injection. This dose of toxin produced neuronal damage 7 - 10 days after treatment and resulted in time-dependent lethal effects, with only 3 out of 10 rats surviving 10 days after injection. Treatment with a larger dose of toxin (2000 MLDs) also produced behavioural stimulation and convulsions in rats. However, the mortality rate with this dose of toxin was higher, with only 30% of injected rats surviving 4 days after treatment. A lower dose of toxin (500 MLDs) produced mild behavioural effects and in no instance were lethal effects observed. Tetanus toxin induced behavioural and neuropathological effects have also been observed following injection of toxin (1000 MLDs) into rat dentate gyrus (Bagetta et al, 1991c). Postural and locomotor behavioural changes were manifested within 3 - 4 days following treatment and resulted in generalized convulsions 5 - 7 days after injection. In comparison to CA1 pyramidal cells, dentate gyrus granular cells exhibited a higher vulnerability
Fig. 3.3  Effect of K⁺ stimulation on hippocampal basal levels of extracellular amino acids in unilaterally implanted control rats.

Rats were implanted with a single dialysis probe (see section 2.2.3 for details) unilaterally into either the left or right ventral hippocampus. At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. The first 4 dialysis samples were used to evaluate a mean basal level, which was in turn compared to the K⁺-evoked value. Data are expressed as mean ± s.e.m. of 9-11 rats. * p < 0.05, *** p < 0.001 (using paired Student’s t-test).
Fig. 3.4 HPLC chromatograms illustrating basal (a) and K⁺-evoked (b) levels of amino acids in control rat hippocampi.

1 = aspartate; 2 = glutamate; 3 = taurine; 4 = GABA.
to the neuropathological effects induced by intrahippocampl injection of tetanus toxin. However, a similar mortality rate was observed in this situation with approximately 40% of rats surviving 10 days after treatment.

Taking into account the lethality of tetanus toxin treatment it was decided that a toxin dose equivalent to 1000 MLDs would be used in the present study. This dose would be expected to produce a rapid and consistent loss of neurons in the hippocampus whilst allowing microdialysis measurements to be made 7 days after treatment, at which time the survival rate of treated rats is approximately 60% (Bagetta et al, 1990a; 1991c).

3.3 Behavioural effects in animals treated with tetanus toxin

Unilateral injection of tetanus toxin (1000 MLDs) into hippocampus of the rat produced both postural and locomotor changes characterized by piloerection, tail rigidity, hunched back, turning, and touch- and sound-evoked circling ipsilateral to the site of injection, within 48 - 72 hours of the treatment. Rats were observed to display periodic seizure activity characterized by a "freeze- like" status, chewing, vibrissal twitching, facial myoclonus, and "wet dog" shakes. Animals showed a hypersensitivity to auditory and tactile stimulation. These effects were observed in all toxin treated rats, culminating in generalized convulsions approximately 5-7 days after initial treatment.
3.4 Effect of tetanus toxin on extracellular levels of neuroactive amino acids in rat hippocampus.

The effect of tetanus toxin on extracellular concentrations of the excitatory amino acids aspartate and glutamate at days 1, 2, 3, and 7 following intrahippocampal injection is illustrated in Figures 3.5 and 3.6, respectively. During the 4 days of dialysis no significant changes in extracellular levels of aspartate and glutamate were detected in toxin-treated hippocampi when compared with the contralateral vehicle-injected sides. Throughout the duration of the experiment the extracellular levels of taurine in toxin-treated hippocampi remained unaltered when compared with the vehicle-injected contralateral sides (Figure 3.7). When comparing extracellular levels of GABA from toxin-treated hippocampi to levels from vehicle-treated sides, no significant difference was detected over the first 3 days of dialysis. On day 7 however, GABA levels in toxin-treated hippocampi were found to be significantly reduced (Figure 3.8). This reduction corresponded to 45.5% of extracellular GABA levels in the contralateral vehicle-injected sides.

Fluorometrically detected HPLC chromatograms depicting levels of amino acids in both vehicle- and toxin-treated hippocampi 7 days after initial microinjections are represented in Figure 3.9. A reduction in GABA peak size is visible in the chromatogram obtained from a tetanus toxin-treated hippocampus, when compared to the corresponding chromatogram obtained from a vehicle-treated hippocampus. This effect is more easily observed when comparing chart recorder derived chromatograms obtained from electrochemically detected HPLC samples, due to the increased sensitivity of this method of detection (see section 3.1) (Figure 3.10).
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 11-14 rats. Statistical comparison was made using Student's t-test for unpaired data.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 11-14 rats. Statistical comparison was made using Student’s t-test for unpaired data.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 11-14 rats. Statistical comparison was made using Student’s t-test for unpaired data.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 8 rats. Statistical comparison was made using Student’s t-test for unpaired data. *** p < 0.001 vs contralateral side.
Fig. 3.9 HPLC chromatograms depicting levels of amino acids in vehicle-treated (a) and toxin-treated (b) hippocampi 7 days after injection.

1 = asparate; 2 = glutamate; 3 = taurine; 4 = GABA.
Fig. 3.10  HPLC chromatograms illustrating levels of GABA in vehicle-treated (a) and toxin-treated (b) hippocampi 7 days after injection.
The extracellular concentrations of amino acids measured in vehicle-injected hippocampi over the 4 days of dialysis are in accord with the corresponding levels detected in control unilaterally implanted hippocampi (c.f. Table 3.1 in section 3.1).

3.5 Effect of K⁺ stimulation on extracellular amino acid levels in vehicle- and toxin-treated hippocampi.

Seven days after initial intrahippocampal injections, perfusion with artificial CSF containing 100 mM K⁺ failed to produce a significant evoked release of aspartate in both vehicle- and toxin-treated sides (Figure 3.11). However, it is clear from Figure 3.11 that a trend for K⁺-evoked release of hippocampal aspartate is displayed in both vehicle- and toxin-treated hippocampi. Similarly, high K⁺ failed to evoke release of glutamate in either hippocampi (Figure 3.12). However, K⁺ stimulation did produce significant increases in the extracellular concentration of taurine in the vehicle- and toxin-treated hippocampi (Figure 3.13). These increased levels corresponded to 977% (p < 0.001) and 968% (p < 0.001) of their basal levels, respectively (using Student’s paired t-test). In a similar fashion, extracellular hippocampal GABA levels were also significantly increased following challenge with high K⁺ (Figure 3.14). The vehicle-injected GABA level rose to 4653% (p < 0.001) of the basal concentration, whilst the toxin-treated GABA level was increased to 4500% (p < 0.001) of the original basal value (using paired Student’s t-test). When comparing K⁺-evoked GABA levels to basal GABA levels, the K⁺-stimulated percentage increase was similar in both sides. However, the K⁺-evoked GABA concentration in toxin-treated hippocampi was significantly
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 11 rats. Statistical comparison was made using Student's t-test for unpaired data.
Effect of K⁺ stimulation on basal levels of extracellular glutamate in both vehicle- and toxin-injected hippocampi 7 days after treatment.

Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 11 rats. Statistical comparison was made using Student's t-test for unpaired data.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 11 rats. Statistical comparison was made using Student’s t-test for unpaired data.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 7 rats. Statistical comparison was made using Student's t-test for unpaired data. * p < 0.05, ** p < 0.01 vs contralateral side. An enlarged representation of the first 2 hours of dialysis is superimposed onto this figure to emphasize the reduced extracellular GABA levels detected in toxin-treated hippocampi.
reduced when compared with corresponding levels in the contralateral vehicle-injected sides. This reduction corresponded to 39% of the evoked GABA level in vehicle-treated hippocampi.

HPLC chromatograms illustrating K⁺-evoked levels of amino acids in vehicle- and tetanus-toxin treated hippocampi 7 days following intrahippocampal injections are represented in Figure 3.15. Chromatograms derived from electrochemically detected HPLC samples specifically show the effect of high K⁺ on basal GABA levels in both vehicle- and toxin-treated sides (Figure 3.16).

Basal levels of extracellular amino acids measured in vehicle-injected hippocampi are in agreement with corresponding extracellular levels detected in control rats (c.f. Figure 3.3). Challenge with high K⁺ produced similar alterations in extracellular amino acid levels in control hippocampi and in vehicle- and toxin-treated hippocampi 7 days after treatment. K⁺ stimulation failed to produce a visible evoked release of glutamate in control hippocampi and also in vehicle- and tetanus toxin-injected hippocampi 7 days following treatment. Since glutamate is known to be efficiently removed from the synaptic cleft by cellular uptake (Garthwaite, 1985; Nicholls & Attwell, 1990), and 100 mM K⁺ did evoke the release of other amino acids in these systems, thereby suggesting that this was not due to lack of K⁺ penetration across the dialysis membrane, it was proposed that failure to observe any K⁺-evoked release of glutamate may be due to rapid removal of the amino acid from the extracellular space by high affinity transport (Fonnum, 1984). To test this hypothesis a potent and selective competitive inhibitor of L-glutamate transport, L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC)
Fig. 3.15  HPLC chromatograms illustrating K⁺-evoked levels of amino acids in vehicle-injected (a) and toxin-injected (b) hippocampi 7 days after treatment.

1 = aspartate; 2 = glutamate; 3 = taurine; 4 = GABA.
Fig. 3.16 Electrochemically detected HPLC chromatograms showing K⁺-evoked levels of GABA in vehicle-treated (a) and toxin-treated (b) hippocampi 7 days after injection.
(Bridges et al, 1991) was applied intrahippocampally via the dialysis probe to investigate its effect on basal and K⁺-evoked levels of extracellular glutamate and aspartate in rat hippocampus.

3.6 Effect of PDC on basal and K⁺-evoked extracellular levels of aspartate and glutamate in control unilaterally implanted hippocampi.

The methodology employed in this series of experiments is described in Figure 3.17. Approximately 24 hours after unilateral implantation of dialysis probes into the ventral hippocampus, rats were dialysed with an artificial CSF as previously described in section 2.2.4. Following a 90 minute stabilization period four 30 minute dialysis samples were collected. Animals were then challenged with high K⁺ for 30 minutes, after which 30 minute dialysis samples were continuously collected. A second 30 minute high K⁺ pulse was then administered, after which 3 more 30 minute dialysis samples were collected. The same process was repeated in a second set of animals. In this paradigm however, midway through the experiment the perfusing artificial CSF was switched to one containing 1 mM PDC for the duration of the experimental procedure.

Basal samples 1-4 were used to evaluate a mean basal concentration of amino acid, which was termed Sp₁. The concentration resulting from the first challenge with high K⁺ was called S₁. As with Sp₁, basal samples 9-12 were used to calculate a mean resting level of amino acid, which was named Sp₂. S₂ was defined as the concentration of amino acid resulting from the second pulse with high K⁺.
Methodology used in experiments where unilaterally implanted rats received 2 pulses with 100 mM K⁺.

At the times indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. In experiments investigating the effect of PDC on basal and K⁺-evoked levels of aspartate and glutamate, PDC (1 mM) was introduced into the perfusing CSF at the time indicated in the Figure.
The effect of duplicate pulsing with high K\(^+\) on basal levels of hippocampal extracellular aspartate, both in the absence and presence of PDC, is illustrated in Figure 3.18. Section (a) depicts the dialysate concentrations throughout the experiment. When investigating the effect of duplicate K\(^+\) pulsing in untreated control animals it was observed that the first challenge with high K\(^+\) produced a significant increase in the levels of aspartate. This increase corresponded to 198\% of basal levels (p < 0.05). The second high K\(^+\) pulse also produced a significant increase in aspartate level corresponding to 184\% of the preceding mean basal value (p < 0.05) (comparisons made using paired t-test).

In the second group of animals receiving PDC midway through the experiment, the initial challenge with high K\(^+\) produced a significant increase in extracellular aspartate level which corresponded to 206\% of the mean basal concentration (p < 0.05). With PDC present in the perfusing medium, the second pulse with high K\(^+\) produced a significant evoked release of aspartate corresponding to 330\% of the preceding mean basal value (p < 0.05) (comparisons made using paired t-test).

The ratio values obtained when multiple comparisons were made between basal and K\(^+\)-stimulated extracellular aspartate levels in both untreated and PDC treated groups are illustrated in the bar graph comprising Figure 3.18b. The ratio \(S_{p2}/S_{p1}\) represents the ratio of the respective mean basal values prior to the second and first K\(^+\)-stimulations, respectively. When comparing \(S_{p2}/S_{p1}\) ratios obtained from untreated and PDC treated groups it is evident that inclusion of PDC in the perfusion medium produced a significant increase in the \(S_{p2}/S_{p1}\) ratio value, hence indicating that the presence of PDC produced a significant increase in basal levels.
Fig. 3.18  Effect of PDC on basal and K⁺-evoked extracellular hippocampal aspartate levels in unilaterally implanted rats.

Section (a) illustrates the effect of duplicate pulsing with high K⁺ on extracellular hippocampal basal levels of aspartate in unilaterally implanted rats, both in the absence and presence of PDC. At the times indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. In experiments investigating the effects of PDC on basal and K⁺-evoked levels of aspartate, PDC was introduced into the perfusing medium at the time indicated in the Figure for the duration of the experiment. Figure 3.17, depicting the methodology and terminology used in this series of experiments, is superimposed onto this Figure.

Section (b) represents the ratio values obtained when multiple comparisons are made between basal and K⁺-evoked transmitter levels, both in the absence and presence of PDC. Data are expressed as mean ± s.e.m. of 4 rats. Statistical comparison was made using the Mann-Whitney U-test, * p < 0.05.
of extracellular aspartate.

The ratios $S_j/S_p$ and $S_2/S_p$ represent the ratio of the concentration of aspartate following $K^+$-stimulation over the mean basal level preceding stimulation, with 1 and 2 denoting the first and second $K^+$-pulses and mean basal levels, respectively. When comparing the $S_j/S_p$ and $S_2/S_p$ ratios obtained from untreated animals it is clear that there is no difference between the ratio values, indicating that the second $K^+$-evoked level of extracellular aspartate was not significantly different from the first $K^+$-evoked level (Figure 3.18b). However, when comparing the $S_j/S_p$ and $S_2/S_p$ ratios obtained from PDC treated animals it is evident that the $S_2/S_p$ ratio value is significantly larger than the $S_j/S_p$ value, indicating that in the presence of PDC challenge with high $K^+$ produced a larger stimulated release of aspartate than in its absence.

This is also demonstrated when comparing the $S_j/S_1$ (concentration following the second $K^+$-stimulation over the concentration following the first $K^+$-stimulation) ratio values. The $S_j/S_1$ ratio value obtained from PDC treated animals is significantly greater than the corresponding value obtained in untreated animals, indicating that presence of PDC in the perfusion medium results in a larger $K^+$-evoked extracellular level of aspartate.

The effect of duplicate pulsing with high $K^+$ on basal levels of hippocampal extracellular glutamate, both in the absence and presence of PDC, is shown in Figure 3.19. Section (a) illustrates the glutamate dialysate concentration throughout the experiment. As previously found with control unilaterally
Fig. 3.19  Effect of PDC on basal and K*-evoked extracellular hippocampal glutamate levels in unilaterally implanted rats.

Section (a) illustrates the effect of duplicate pulsing with high K* on extracellular hippocampal basal levels of glutamate in unilaterally implanted rats, both in the absence and presence of PDC. At the times indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K* for a 30 minute period, after which the original medium was returned. In experiments investigating the effects of PDC on basal and K*-evoked levels of aspartate, PDC was introduced into the perfusing medium at the time indicated in the Figure for the duration of the experiment. Figure 3.17, depicting the methodology and terminology used in this series of experiments, is superimposed onto this Figure.

Section (b) represents the ratio values obtained when multiple comparisons are made between basal and K*-evoked transmitter levels, both in the absence and presence of PDC. Data are expressed as mean ± s.e.m. of 4 rats. Statistical comparison was made using the Mann-Whitney U-test, * p < 0.05.
implanted animals, challenge with high $K^+$ failed to evoke the release of glutamate in untreated animals. The second $K^+$-stimulation also failed to evoke release of glutamate in this group.

In the second group of animals receiving PDC midway through the experiment, both challenges with high $K^+$ failed to produce significant increases in extracellular glutamate levels.

The ratio values obtained when multiple comparisons were made between basal and $K^+$-stimulated extracellular glutamate levels, in both untreated and PDC treated groups, are illustrated in the bar graph comprising Figure 3.19b. When comparing $S_p/S_1$ ratios obtained from untreated and PDC treated groups it can be seen that inclusion of PDC in the perfusion medium produced a significant increase in the $S_p/S_1$ ratio value, demonstrating that the presence of PDC produced a significant increase in basal levels of extracellular glutamate. When comparing the $S_1/S_p$ and $S_2/S_p$ ratios obtained from untreated animals, no difference was observed between the two ratio values, indicating a lack of difference between the first and second $K^+$-stimulated levels of glutamate. This was also found to be the case when comparing the $S_2/S_1$ and $S_2/S_p$ ratios obtained in PDC treated animals.

This finding is also apparent when comparing the $S_2/S_1$ ratio values obtained from both untreated and treated groups. It is clear from Figure 3.19b that there is no significant difference between the two ratio values, hence indicating that presence of PDC in the perfusion medium had no effect on $K^+$-stimulated extracellular levels of glutamate.
The present study is the first to monitor neurochemical changes induced by tetanus toxin treatment in freely moving animals at times ranging between 1 and 7 days after injection, using the technique of intracerebral microdialysis. It was proposed that the neuropathological effect produced by intrahippocampal injection of tetanus toxin in rats may be related to a loss of neuronal inhibition, resulting in a net increase of excitatory input at the site of injection (Bagetta et al, 1990c). In support of this proposal, 7 days after injection of tetanus toxin (1000 MLDs) when neurodegeneration is evident (Bagetta et al, 1990a), extracellular GABA levels in toxin-treated hippocampi were found to be significantly reduced (approximately 45% of contralateral vehicle injected side) whilst levels of the excitatory amino acids aspartate and glutamate remained unaltered. The possibility that an unopposed action of endogenous excitatory amino acids may lead to the production of neurodegeneration is further supported by the observations made 1, 2, and 3 days after initial injection of 1000 MLDs tetanus toxin, times at which neuronal degeneration is not evident following treatment with this dose of toxin (Bagetta et al, 1990a). Throughout this period extracellular GABA levels in toxin-treated hippocampi remained unchanged. This was also the case for the excitatory amino acids aspartate and glutamate.

It is widely accepted that tetanus toxin acts at the presynaptic level producing a reduction of Ca²⁺-dependent, K⁺ stimulated transmitter release (Osborne and Bradford, 1973a,b; Davies and Tongroach, 1979; Mellanby, 1984). In support of this, K⁺ stimulation evoked the release of GABA in both vehicle-and toxin-
treated hippocampi, with evoked levels of GABA in toxin-treated sides being significantly reduced when compared to levels in vehicle-injected hippocampi.

GABA levels in vehicle- and toxin-treated hippocampi were elevated to 4653% and 4500% of their resting basal levels, respectively. K⁺ induced outflow of GABA comparable to that observed in the present study has been reported by others (Tossman et al, 1986; Paulsen and Fonnum, 1989).

The concentration of K⁺ used in this study (100 mM) would be expected to provide a strong depolarizing stimulus. The *in vitro* relative recoveries of amino acids from standard solutions by dialysis probes employed in this study are in the range of 40-50% (see section 2.2.2). Given the small size of K⁺ ions in comparison to the size of amino acids diffusing across the dialysis membrane, it would not be unreasonable to assume that an estimated extracellular concentration of 40-50 mM K⁺ would be present in the hippocampal extracellular space within the immediate vicinity of the probe. Such a stimulus is likely to induce both Ca²⁺-dependent and Ca²⁺-independent release from amino acid containing neurons, with the possibility of glial cells contributing to K⁺-stimulated amino acid release in a Ca²⁺-independent manner (Minchin and Iversen, 1974; Bernath, 1992).

By investigating the effect of K⁺-stimulation and tetrodotoxin treatment on extracellular neurotransmitter levels in rat brain Westerink et al (1987) concluded that overflow of amino acid neurotransmitters is related to neurogenic as well as metabolic events. In synaptosomal studies of K⁺-stimulated GABA release mechanisms, Ca²⁺-dependent release appears to be exhausted within a few minutes.
of starting a constant depolarizing stimulus, and subsequent release is considered to result from the release of cytoplasmic GABA by reversal of the electrogenic GABA/sodium carrier mechanism (Sihra and Nicholls, 1987).

Using the technique of in vivo microdialysis Herbison et al (1990) have reported a reduction in basal GABA outflow from the medial preoptic area in rat following verapamil treatment. They concluded that a small but significant proportion of basal GABA outflow as measured by microdialysis results from Ca\(^{2+}\)-dependent release mechanisms, and at least 50% of the basal outflow was of neuronal origin. Similarly the Ca\(^{2+}\)-dependent GABA overflow evoked by K\(^+\) depolarization reflected, at least in part, neuronal release. In support of this, Bourdelais and Kalivas (1992) have demonstrated that at least 50% of basal extracellular GABA in the ventral pallidum is derived from Ca\(^{2+}\)-dependent mechanisms, with the release of GABA stimulated by high K\(^+\) being predominantly Ca\(^{2+}\)-dependent.

Therefore, with regard to these observations, it would not be unreasonable to assume that a significant proportion of extracellular GABA outflow measured in vehicle- and toxin-treated hippocampi in this study originates from transmitter pools.

Challenge with high K\(^+\) also produced significant increases in extracellular levels of taurine in both vehicle- and toxin-treated sides. These increases corresponded to 977% and 968% of their basal levels, respectively. It has been reported that the high levels of extracellular taurine reached during K\(^+\) stimulation may be explained by the barrier for taurine (eg. uptake) becoming virtually absent (Oja and Kontron,
1983). K⁺-stimulation failed to produce significant increases in levels of the excitatory amino acid transmitters aspartate and glutamate in both vehicle- and toxin-treated hippocampi. However, a trend for K⁺-evoked release of hippocampal aspartate was demonstrated in both sides, suggesting that an increase in experimental numbers might have made the effect significant. In support of this, the ability of high K⁺ to evoke the release of hippocampal extracellular aspartate was shown in control unilaterally implanted rats. This increase corresponded to 160% of basal levels.

The lack of effect of high K⁺ on extracellular levels of glutamate remains unclear. It seems unlikely that K⁺ ions failed to penetrate the dialysis membrane and pass into the extracellular space, as large evoked levels of taurine and GABA were detected. It was considered that high K⁺ may be evoking the release of glutamate but, due to its rapid removal from the extracellular space by glutamate transporters located both presynaptically and on glial cells, no change in extracellular level could be detected. Increased glial cell numbers around the dialysis probe would be expected as part of the pathological response to tissue damage resulting from implantation (Lehmann et al, 1983). Also, glial cells are known to play an important role in the inactivation of amino acid transmitters via their highly active uptake processes (Fonnum, 1984). In support of this, Paulsen and Fonnum (1989) demonstrated that inactivation of glial cells with the gliotoxin fluorocitrate temporarily increased the Ca²⁺-dependent, K⁺-stimulated release of glutamate in striatal dialysates.

It was demonstrated during the present study that intrahippocampal administration
of the glutamate uptake inhibitor PDC (1 mM) via the dialysis probe produced significant increases in basal levels of both aspartate and glutamate. A 1 mM concentration of PDC was used in this study because the same dose had previously been shown to elevate levels of aspartate and glutamate in rat hippocampus when administered via the dialysis probe (Millan et al, 1993). In the present study, the extracellular aspartate level resulting from challenge with high $K^+$ was larger in the presence of PDC. However, even in the presence of PDC, $K^+$-stimulation did not produce a significant increase in levels of glutamate.

It is known that at least some of the sodium-dependent high-affinity glutamate transport systems can function as exchangers across the plasma membrane (for review see Erecinska, 1987). Indeed in synaptosomes, PDC (10-100 μM) caused a transient increase of spontaneous glutamate release which was diminished in the absence of Na+, indicating that it is transported into the cytoplasm in exchange for cytosolic glutamate (Waldmeier et al, 1993). In contrast to this, the work of Robinson et al (1993) demonstrated that PDC (100 μM) potentiated glutamate toxicity in primary hippocampal cultures by slowing the clearance of extracellular glutamate rather than by participating in exchange with intracellular glutamate. It may therefore be possible that the observed increases in extracellular levels of aspartate and glutamate following PDC treatment are partly due to displacement of cytosolic transmitters by the drug.

It is not clear why the extracellular glutamate level resulting from challenge with high $K^+$ was not increased in the presence of 1 mM PDC. Interestingly, PDC has been shown to cause a significant reduction of $K^+$-stimulated glutamate release
from synaptosomes at 10-300 μM in the range of 25-50% (Waldmeier et al, 1993). The reason for this effect is not known at the present time but may have some bearing on the findings of this present study.

The responses of extracellular aspartate and glutamate to K⁺-stimulation may indicate the origin of the respective extracellular levels. As challenge with high K⁺ can evoke the release of aspartate, dialysate levels measured here may be essentially derived from the transmitter pool. The lack of effect of K⁺ on extracellular glutamate levels may indicate that dialysate glutamate levels essentially reflect release from the metabolic pool. However, the lack of effect of K⁺ stimulation may well be due to the highly active glutamate uptake carriers located on presynaptic terminals and on glial cells. Rapid removal of glutamate from the synaptic cleft may well, even in the presence of PDC, mask any subtle increase in extracellular glutamate level induced by challenge with high K⁺.

Although previous reports have shown that high concentrations of tetanus toxin can cause depression of both inhibitory and excitatory transmission (Kanda and Takano, 1983; Calabresi et al, 1989), the findings of this study do not support these observations, as extracellular levels of aspartate and glutamate remained unaltered in toxin-treated hippocampi throughout the duration of the study.

It is accepted that the dose of toxin used in this present study is high. However, it is no higher than doses previously used in other laboratories. Low doses of toxin (typically 6-20 MLDs) have been used by groups working on the medium-to long-term effects of intracranial tetanus toxin treatment (Mellanby et al, 1977;
Brace et al, 1985; Jeffreys, 1989). Such low doses have been shown to selectively disrupt neuronal inhibition in treated animals, possibly explaining why they become chronically epileptic, whilst allowing neurons to survive. However, the dose of toxin used in the present study is in accord with the dose employed by Bagetta et al (1990a) to obtain a rapid and consistent loss of neurons in the hippocampus and therefore does not necessarily invalidate the original hypothesis that a loss of GABA is primarily responsible for the neurodegeneration. The lack of effect of toxin treatment on hippocampal extracellular levels of aspartate and glutamate over the duration of the study also supports this hypothesis.

The mechanism of tetanus toxin induced neuropathology may well involve a series of alternative steps, but until contrary evidence is obtained the loss of inhibitory tone mediated via decreased GABA levels remains a reasonable hypothesis.
Chapter 4

It has been demonstrated that intraperitoneal injection of tetanus toxin in rats produces an overall increase in CNS tissue levels of the neurotransmitter 5-HT (Aguilera et al, 1987; Aguilera and Gonzalez-Sastre, 1988). This increase has been attributed to tetanus toxin-induced blockade of presynaptic 5-HT release. Recent work by Aguilera et al (1991) further demonstrated that a single intraventricular injection of tetanus toxin (150 MLDs) into rats resulted in a time-dependent elevation of 5-HT and dopamine in whole brain, and an increase in 5-HT levels in spinal cord, 24 hours following treatment. The tetanus toxin-induced increase in 5-HT level was produced in areas of dense serotoninergic innervation, such as hypothalamus, hippocampus, and spinal cord. The hippocampus was the most affected area following intraventricular injection, with 5-HT levels being increased by 75%.

Direct stereotaxic injection of tetanus toxin (50 MLDs) into the hypothalamus and hippocampus produced a significant increase in 5-HT levels in both areas, and an apparent decrease in levels of dopamine, 12 hours following treatment, whilst injection of the same dose of toxin into the dorsal or magnus raphe nuclei did not have any effect on biogenic amine levels in the brain and spinal cord, respectively. These findings indicated that tetanus toxin was required to be present at nerve terminals in order to produce 5-HT accumulation. It was concluded that the observed increases in tissue content of 5-HT were possibly due to tetanus toxin-induced inhibition of 5-HT release from presynaptic nerve terminals, or due to a direct effect of the toxin on tryptophan 5-hydroxylase activity which had previously been observed.
In order to investigate the effect of intrahippocampal injection of tetanus toxin on extracellular levels of monoamines and their metabolites at various times following treatment, the technique of *in vivo* microdialysis was used to measure extracellular concentrations of the above species at the injection site. This technique was combined with high performance liquid chromatography to estimate extracellular levels of these species.

### 4.1 Determination of basal and K⁺-evoked extracellular levels of biogenic amines and their metabolites in rat hippocampus.

Basal extracellular levels of 5-HT, dopamine, and their metabolites were determined in control rats unilaterally implanted in the ventral hippocampus. Approximately 24 hours after surgery rats were perfused with an artificial CSF at a flow rate of 0.5 µl/minute (see section 2.2.4) and hourly dialysate samples were collected following a 90 minute stabilization period. In this present study all perfusion mediums contained the 5-HT reuptake inhibitor citalopram (1 µM) in order to elevate basal extracellular 5-HT to more readily detectable levels (Sharp et al, 1989; Whitton and Fowler, 1991; Biggs et al, 1992). The collection time of 1 hour produced a dialysate volume large enough to enable repeated HPLC determinations of the extracellular concentrations of the above species to be made if necessary. Hippocampal extracellular levels of these species derived from control unilaterally implanted rats 24 hours post-operatively are shown in Table 4.1.
Table 4.1  Basal levels of biogenic amines and their metabolites in control rat hippocampi.

<table>
<thead>
<tr>
<th>Species</th>
<th>fmols/10 μl sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>96.5 ± 11.4</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>9720 ± 950</td>
</tr>
<tr>
<td>Dopamine</td>
<td>47.5 ± 8.2</td>
</tr>
<tr>
<td>DOPAC</td>
<td>768 ± 125</td>
</tr>
<tr>
<td>HVA</td>
<td>1750 ± 352</td>
</tr>
</tbody>
</table>

Four 1 hour dialysis samples were used to evaluate a mean basal level of each of the extracellular species. Data are expressed as mean ± s.e.m of 7 rats.

Electrochemically detected HPLC chromatograms depicting extracellular basal levels of 5-HT, dopamine, and their metabolites in rat hippocampus are represented in Figure 4.1. Section (a) represents a computer-derived chromatogram. With the exception of the 5-HIAA peak, it can be seen that other peak sizes in this chromatogram are very small, making determination of extracellular concentrations of monoamines and the remaining metabolites difficult. Therefore, detector output was also channelled to an on-line chart recorder which allowed a further 10 fold amplification of the signal (see section 2.3.3). The corresponding chart recorder-derived chromatogram depicting
Fig. 4.1  Computer (a) and chart recorder (b) derived HPLC chromatograms depicting extracellular basal levels of 5-HT, dopamine, and their metabolites in control rat hippocampus.

1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = 5-HIAA; 5 = HVA.
extracellular levels of monoamines and their metabolites in rat hippocampus is shown in section (b) of Figure 4.1. In this system the limit of detection of 5-HT and dopamine was approximately 1 fmole/μl of sample.

The effect of a 30 minute pulse with artificial CSF containing 100 mM K⁺ on hippocampal extracellular levels of monoamines and their metabolites in control rats is illustrated in Figure 4.2. Experiments were commenced 24 hours after completion of surgical procedures. In these rats, challenge with high K⁺ evoked the release of hippocampal 5-HT and dopamine. The corresponding increases from resting basal levels were 400% (p < 0.05), and 232% (p < 0.05), respectively (using Student’s paired t-test). In contrast to these evoked levels, K⁺-stimulation produced a reduction in extracellular levels of the 5-HT metabolite 5-HIAA. This reduction corresponded to 43% (p < 0.05) of basal levels (using Student’s paired t-test). Challenge with high K⁺ had no effect on extracellular levels of the dopamine metabolites DOPAC and HVA. Computer- and chart recorder-derived HPLC chromatograms illustrating K⁺-evoked levels of 5-HT and dopamine in control rat hippocampi are represented in Figure 4.3.

4.2 Dose level of tetanus toxin used in the present study.

A tetanus toxin dose equivalent to 1000 MLDs was used in the present study. The reasoning for employment of this dose of toxin has been previously discussed in section 3.2.
Fig. 4.2 Effect of $K^+$ stimulation on basal extracellular hippocampal levels of monoamines and their metabolites in control rats.

At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM $K^+$ for a 30 minute period, after which the original medium was returned. The first 4 dialysis samples were used to evaluate a mean basal level, which was in turn compared to the $K^+$-evoked level. Data are expressed as mean ± s.e.m. of 6-7 rats. * $p < 0.05$ (paired Student's t-test).
Fig. 4.3  Computer- and chart recorder - derived chromatograms illustrating basal (a) and K+-evoked (b) levels of monoamines and their metabolites in control rat hippocampi.

1 = DA;  2 = DOPAC;  3 = 5-HT;  4 = 5-HIAA;  5 = HVA.
4.3 Behavioural effects in animals treated with tetanus toxin.

The behavioural effects resulting from unilateral intrahippocampal injection of tetanus toxin (1000 MLDs) have been previously described in section 3.3.

4.4 Effect of tetanus toxin on extracellular levels of monoamines and their metabolites in rat hippocampus.

The effect of tetanus toxin on extracellular concentrations of 5-HT at days 1, 2, 3, and 7 following intrahippocampal injection is illustrated in Figure 4.4. Over the first three days of dialysis the extracellular levels of 5-HT in toxin-treated hippocampi progressively declined when compared to the corresponding levels in vehicle-treated sides. These reductions corresponded to 59%, 38%, and 17% of extracellular 5-HT levels in the contralateral vehicle-injected hippocampi, respectively. On day 7 however, whilst the level of 5-HT was still significantly reduced, the extent of this reduction was similar to that observed on day 2 (35% of 5-HT level in vehicle-treated sides). Extracellular concentrations of 5-HIAA were also decreased on the toxin treated side over the first 3 days of dialysis (Figure 4.5). However, after an initial rapid decline, corresponding to 25% of 5-HIAA levels in the contralateral vehicle-injected hippocampi, the level of this metabolite remained at a constant decreased level. On day 7 however, 5-HIAA levels in toxin-treated hippocampi were not significantly reduced, although a trend towards decreased extracellular levels was displayed.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data. ** p < 0.01, *** p < 0.001 vs contralateral side.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data. * p< 0.05,  ** p < 0.01 vs contralateral side.
When comparing extracellular levels of dopamine from toxin-treated hippocampi to levels from vehicle-treated sides, no significant difference was detected over the first day of dialysis (Figure 4.6). On day 2 however, dopamine levels in the toxin treated hippocampi were found to be significantly increased. This increase corresponded to 202% of extracellular dopamine levels in the contralateral vehicle-injected sides. However, this significant alteration was not maintained through day 3 of dialysis and on day 7 extracellular levels of dopamine were observed to be significantly reduced when compared to vehicle-treated sides. Despite the lack of a consistent change in the level of dopamine, tetanus toxin treatment did alter presynaptic dopamine metabolite levels since extracellular hippocampal concentrations of its metabolite DOPAC were found to be significantly reduced 2, 3, and 7 days following intrahippocampal injection. These reductions corresponded to 62%, 50%, and 45% of DOPAC levels in vehicle injected sides, respectively (Figure 4.7). Throughout the duration of the experiment the extracellular levels of the dopamine metabolite HVA in toxin-treated hippocampi remained unaltered when compared with the vehicle injected contralateral sides (Figure 4.8).

HPLC chromatograms depicting levels of 5-HT, dopamine, and their metabolites in both vehicle- and toxin-treated hippocampi 3 days, and 7 days after initial microinjections are represented in Figures 4.9 and 4.10, respectively. The extracellular concentrations of the above mentioned species measured in vehicle-injected hippocampi over the 4 days of dialysis are in accord with the corresponding levels detected in control unilaterally implanted hippocampi (c.f. Table 4.1).
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student's t-test for unpaired data.

* p < 0.05 vs contralateral side.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data.

* $p < 0.05$ vs contralateral side.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data.
Computer- and chart recorder- derived HPLC chromatograms showing extracellular levels of 5-HT, dopamine, and their metabolites in vehicle-treated (a) and toxin-treated (b) hippocampi 3 days after initial injections.

1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = 5-HIAA; 5 = HVA.
Fig 4.10 Chart recorder derived HPLC chromatograms depicting extracellular levels of monoamines and their metabolites in vehicle-injected (a) and toxin-injected (b) hippocampi 7 days after treatment.

1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = 5-HIAA; 5 = HVA.
4.5 Effect of high K\(^+\) challenge on extracellular biogenic amine and metabolite levels in vehicle- and tetanus toxin-treated hippocampi.

Seven days following initial intrahippocampal injections, perfusion with artificial CSF containing 100 mM K\(^+\) produced a significant evoked release of 5-HT in both vehicle- and toxin-treated sides (Figure 4.11). These increased levels corresponded to 374\% (p < 0.05) and 418\% (p < 0.05) of their basal levels, respectively (using Student’s paired t-test). When comparing K\(^+\)-evoked 5-HT levels to basal 5-HT levels, the K\(^+\)-stimulated percentage increase in extracellular level was similar in both sides. However, the K\(^+\)-evoked 5-HT concentration in toxin-treated hippocampi was significantly reduced when compared with corresponding levels in the contralateral vehicle injected sides. This reduction corresponded to 40\% of the evoked 5-HT level in vehicle-treated hippocampi. In contrast to this observation, K\(^+\)-stimulation produced significant decreases in the extracellular concentration of 5-HIAA in both vehicle- and toxin-treated hippocampi (Figure 4.12). These reduced levels corresponded to 46\% (p < 0.05) and 55\% (p < 0.05) of their basal levels, respectively (Student’s paired t-test).

Extracellular hippocampal dopamine levels were also significantly increased following challenge with high K\(^+\) (Figure 4.13). The vehicle-injected dopamine level rose to 184\% (p < 0.05) of the basal concentration, whilst the toxin-treated dopamine level was increased to 213\% (p < 0.05) of the original basal value (using paired Student’s t-test). When comparing K\(^+\)-stimulated dopamine levels to basal dopamine levels, the K\(^+\)-induced percentage increase in extracellular concentration was similar in both sides. However, the K\(^+\)-evoked dopamine
Fig. 4.11 Effect of K+ stimulation on basal levels of extracellular 5-HT in both vehicle- and toxin-injected hippocampi 7 days after treatment.

Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K+ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data. * p < 0.05, ** p < 0.01 vs contralateral side.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student’s t-test for unpaired data.
Fig. 4.13 Effect of K⁺ stimulation on basal levels of extracellular dopamine in both vehicle- and toxin-injected hippocampi 7 days after treatment.

Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student's t-test for unpaired data. * p < 0.05 vs contralateral side.
concentration in toxin-treated hippocampi was significantly reduced when compared with corresponding levels in the contralateral vehicle-injected sides. This reduction corresponded to 54% of the evoked dopamine level in vehicle-treated hippocampi. Perfusion with artificial CSF containing 100 mM K⁺ did not produce any significant alterations in the hippocampal extracellular levels of the dopamine metabolites DOPAC (Figure 4.14) and HVA (Figure 4.15).

Computer- and chart recorder-derived chromatograms illustrating K⁺-evoked levels of monoamines and their metabolites in vehicle- and tetanus toxin-treated hippocampi 7 days following intrahippocampal injections are represented in Figure 4.16.

Basal levels of the above mentioned species measured in vehicle-injected hippocampi are in agreement with corresponding extracellular levels detected in control rats (c.f Figure 4.2). Alterations in extracellular levels of monoamines and their metabolites resulting from challenge with high K⁺ were similar in control hippocampi and in vehicle- and toxin-treated sides 7 days following treatment.
Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K\(^+\) for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student's t-test for unpaired data. * \(p < 0.05\) vs contralateral side.
Fig. 4.15  Effect of K⁺ stimulation on basal levels of extracellular HVA in both vehicle- and toxin-injected hippocampi 7 days after treatment.

Tetanus toxin was injected unilaterally into the ventral hippocampus, whilst vehicle was injected into the contralateral side. Rats were subsequently implanted with dialysis probes (see section 2.2.3 for details). At the time indicated in the Figure the perfusing artificial CSF was switched to one containing 100 mM K⁺ for a 30 minute period, after which the original medium was returned. Data are expressed as mean ± s.e.m. of 6 rats. Statistical comparison was made using Student's t-test for unpaired data.
Fig. 4.16 Chart recorder derived HPLC chromatograms illustrating K+-evoked levels of monoamines and their metabolites in vehicle treated (a) and tetanus toxin (b) treated rat hippocampi 7 days after injection.

1 = DA; 2 = DOPAC; 3 = 5-HT; 4 = 5-HIAA; 5 = HVA.
4.6 Discussion.

The technique of \textit{in vivo} microdialysis has been used in this study to monitor changes in extracellular levels of monoamines and their metabolites following intrahippocampal injection of tetanus toxin. Using this methodology, the present investigation is the first to measure such changes in freely moving animals at times ranging between 1 and 7 days after treatment. It has been postulated that the observed increase in whole brain and spinal cord tissue content of 5-HT following intraventricular injection of tetanus toxin (150 MLDs) may be due to inhibition of 5-HT release from presynaptic nerve terminals (Aguilera et al, 1991). In support of this proposal, extracellular 5-HT levels in toxin-treated hippocampi were found to be significantly reduced 1, 2, 3, and 7 days after treatment.

The ability of tetanus toxin to elevate 5-HT tissue content has been demonstrated in adult rat brain, as mentioned previously, and also in perinatal rat brain using a toxin dose of 7.5 MLDs (Aguilera et al, 1991, 1990). However, in both these studies no measurement of levels of the 5-HT metabolite was made. It was found during the present study that extracellular levels of 5-HIAA in toxin-treated hippocampi were significantly decreased over the initial 3 days of dialysis, with a trend for decreased 5-HIAA levels demonstrated on day 7 suggesting that an increase in experimental numbers might have made the effect significant. The observed extracellular 5-HIAA decreases would suggest an overall reduction in presynaptic 5-HT metabolism. This will be discussed in more detail later.

It is possible that the decline in extracellular levels of 5-HT measured in toxin-
treated hippocampi over the duration of the study may result from toxin-induced destruction of 5-HT neurons. However, this seems unlikely since the extent of reduction of extracellular 5-HIAA levels did not parallel the corresponding 5-HT decreases on the respective days of dialysis. Also it has been demonstrated that up to three days after treatment with this dose of toxin (1000 MLDs) neurodegeneration is not evident (Bagetta et al, 1990a).

In contrast to 5-HT, there was a lack of consistent change in extracellular levels of dopamine in toxin-injected hippocampi when compared to corresponding levels in vehicle-injected sides. Whilst the dopamine concentration was unchanged on day 1, a significant increase in this level was observed on the second day of dialysis. However, this significant increase was not maintained through day 3 of the study. Seven days following injection of tetanus toxin, extracellular levels of dopamine were found to be significantly reduced. The reason for the observed alterations in extracellular dopamine concentration 1, 2, 3, and 7 days after treatment is unclear. Aguilera et al (1991) observed an apparent decrease in tissue levels of dopamine 12 hours after toxin injection (50 MLDs) into adult rat hippocampus, whilst intraventricular injection of toxin (150 MLDs) produced an elevation of dopamine in whole brain 24 hours following treatment. These findings may be related to the observations made in the present study.

The increase in extracellular levels of dopamine seen in toxin-treated hippocampi on days 2 and 3 is presumably associated with the accompanying decrease in concentration of its metabolite DOPAC. However, the underlying mechanism for this remains to be elucidated. The decreased extracellular dopamine and 5-HT
levels observed in toxin-injected sides 7 days after treatment would suggest a
toxin-induced inhibition of neurotransmitter release from presynaptic nerve
terminals. However, the possibility that the observed neurochemical changes are
a result of tetanus toxin-induced destruction of respective 5-HT and dopamine
containing neurons cannot be excluded, as this dose of toxin (1000 MLDs) is
known to produce neurodegeneration 7 days after intrahippocampal injection
Bagetta et al, 1990a).

As mentioned previously in section 3.7, it is generally accepted that tetanus toxin
acts at the presynaptic level producing a reduction of Ca\(^{2+}\)-dependent, K\(^{+}\)-
stimulated neurotransmitter release (Osborne and Bradford, 1973a, b; Davies and
Tongroach, 1979, Mellanby, 1984). In support of this, it has been demonstrated
in the current investigation that challenge with high K\(^{+}\) evoked the release of 5-HT
and dopamine in both vehicle- and toxin-treated hippocampi 7 days after initial
microinjections, with evoked levels of 5-HT and dopamine in toxin-injected sides
being significantly reduced when compared to corresponding levels in vehicle-
injected hippocampi.

It has previously been demonstrated using the technique of \textit{in vivo} microdialysis
that the extracellular dopamine level measured in rat brain is sensitive to
tetrodotoxin treatment, and dopamine release can be evoked by challenge with
high K\(^{+}\) (Westerink et al, 1987). As a consequence of these observations it was
concluded that overflow of dopamine into the extracellular space is directly related
to neurogenic events. Intracerebral microdialysis studies in rat brain have also
demonstrated that changes in extracellular levels of 5-HT are closely related to
changes in serotoninergic synaptic activity (Kalen et al, 1988). In this situation it was shown that omission of Ca\(^{2+}\) from the perfusion medium produced a significant decrease in basal extracellular 5-HT level. Treatment with 1 \(\mu\)M tetrodotoxin also resulted in decreased basal levels of 5-HT. Extracellular levels of 5-HIAA were found to be unaltered in both these situations. Challenge with high K\(^+\) evoked the release of 5-HT, but had little effect in 5,7-dihydroxytryptamine denervated striata, indicating that very little, if any, of the 5-HT present in extracellular space during steady state conditions is derived from extraneuronal sources. Therefore, with regard to these observations, it would not be unreasonable to assume that extracellular levels of dopamine and 5-HT measured in vehicle- and toxin-treated hippocampi in this study are directly derived from neurotransmission.

In contrast to the effect observed with dopamine and 5-HT, challenge with high K\(^+\) produced significant decreases in extracellular levels of the 5-HT metabolite 5-HIAA in both vehicle- and toxin-treated sides. These decreases corresponded to 46% and 55% of their basal levels, respectively. A similar K\(^+\)-induced reduction of extracellular 5-HIAA level has been previously reported by Kalen et al (1988). It is generally considered that extracellular 5-HIAA levels primarily reflect intraneuronal metabolism, and only to a minor degree the synaptic metabolism of neuronally released 5-HT (Grahame-Smith, 1971; Wolf et al, 1985; Commissiong, 1985; Kuhn et al, 1986). However, the reason why extracellular levels of 5-HIAA are reduced following challenge with high K\(^+\) is unclear. It is possible that K\(^+\)-evoked release of 5-HT from nerve terminals may lead to a reduction in the amount of 5-HT available to cytoplasmic monoamine oxidase,
which in turn might be due alteration of the equilibrium between vesicular bound and cytoplasmic 5-HT pools.

To what extent the present observations may contribute neurochemically to the behavioural and neuropathological effects resulting from intrahippocampal injection of tetanus toxin must be speculative, and weighed against the contribution of other neurotransmitters, particularly GABA and glycine. It may be that the neurochemical changes observed over the duration of the study are the result of epileptiform activity following loss of inhibitory transmission (Mellanby and Green, 1981), or to a loss of both inhibitory and excitatory neurotransmission as previously seen with relatively high doses of tetanus toxin (Bergey et al, 1983; Bigalke et al, 1983; Kanda and Takano, 1983), as used in the present study. However, although microdialysis measurements made during this time period revealed that tetanus toxin treatment produced a reduction in extracellular levels of GABA 7 days after injection, no effect on extracellular levels of the amino acids aspartate, glutamate, and GABA was detected over the first 3 days of dialysis (see section 3.4) when extracellular levels of 5-HT were found to be reduced by up to 80% and animals displayed seizure activity.

Interestingly, 5-HT deficiencies have been implicated in the development of myoclonic epilepsy (Chadwick et al, 1975; Fahn, 1978), whilst a number of studies have provided evidence that brain 5-HT plays an inhibitory role in a variety of epilepsy models (De La Torre et al, 1970; Killan and Frey, 1973; Schlesinger et al, 1969; Wada et al, 1991). It has recently been demonstrated by Wada et al (1991) that L-5-hydroxytryptophan (5-HTP), an immediate precursor
of 5-HT, has a potent anticonvulsant action in a feline model of photosensitive epilepsy at doses smaller than those shown to modify other neurotransmitter systems such as dopamine and noradrenaline (Butcher et al, 1972; Sloviter et al, 1978). This model has also been used to demonstrate that the 5-HT receptor agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) significantly suppressed photically induced myoclonus (Wada et al 1992a). Due to the differential behavioural effects observed following 5-MeODMT administration, Wada et al concluded that the anticonvulsant effect of 5-MeODMT on photosensitivity results from its action at 5-HT receptors.

It has recently been shown that the 5-HT\textsubscript{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetratin (8-OH-DPAT) (1.0 mg/kg) produced a marked suppression in hippocampal kindled seizures in cat (Wada et al, 1992b). This suppressive effect occurred in association with the appearance of behavioural signs (i.e., head weaving and flat body posture) resembling the 5-HT syndrome in rats, which is considered to reflect activation of 5-HT\textsubscript{1A} receptors (Lucki et al, 1984; Tricklebank, 1985). These findings suggest that 5-HT\textsubscript{1A} receptors play an inhibitory role in the generation of hippocampal seizures. It is known that the hippocampus has a high 5-HT\textsubscript{1A} receptor density (Kohler, 1984; Marcinkeiwicz et al, 1984; Peroutka, 1988). However, the midbrain raphe nuclei are also known to contain dense concentrations of 5-HT\textsubscript{1A} binding sites (Marcinkeiwicz et al, 1984; Peroutka, 1988) which are thought to serve as inhibitory autoreceptors (Verge et al, 1985).

With regard to this, it had previously been demonstrated that 5-HT\textsubscript{1A} agonists,
including 8-OH-DPAT, could inhibit 5-HT release and synthesis in rat hippocampus, probably by activation of somatodendritic 5-HT$_{1A}$ autoreceptors resulting in a reduction of 5-HTergic impulse flow in neurons arising from the raphe nuclei (Hutson et al, 1989; Sharp et al, 1989). However, the concentration of 8-OH-DPAT used in these studies was considerably lower than that used by Wada et al (1992b). As the participation of presynaptic 5-HT$_{1A}$ autoreceptors in the effects of systemically injected 8-OH-DPAT could not be excluded, Wada et al (1992b) suggested that 8-OH-DPAT at 1.0 mg/kg may stimulate postsynaptic 5-HT$_{1A}$ receptors more potently than presynaptic autoreceptors. Bearing in mind these recent observations, it may be possible that the decreased extracellular 5-HT level observed in toxin-treated hippocampi in the present study has some involvement in the induction of tetanus toxin seizures.

It has previously been demonstrated that the behavioural and neuropathological effects resulting from intrahippocampal injection of tetanus toxin (1000 MLDs) could be prevented by the NMDA receptor antagonists dizocilpine (Bagetta et al, 1990c) and CGP 37849 (Bagetta et al, 1992). In relation to this observation Whitton et al (1992) have shown, using the technique of intracerebral microdialysis, that dizocilpine (MK-801) increases extracellular 5-HT levels in rat hippocampus. Recent findings have also demonstrated that N-methyl-D-aspartate (NMDA) produced a concentration dependent decrease in extracellular hippocampal 5-HT levels in rat (Whitton et al, 1994). This effect was blocked when NMDA was co-infused with the specific NMDA receptor antagonist D-2-amino-5-phosphonopropionic acid (D-AP5), whilst administration of D-AP5 alone caused a significant increase in dialysate 5-HT level. These results suggest that
release of hippocampal 5-HT may be under tonic control via glutamatergic NMDA receptors.

Therefore, as the behavioural effects resulting from treatment with tetanus toxin occur before any loss of neuronal inhibition can be detected in the present study, it might be possible that the ability of dizocilpine and CGP 37849 (DL(E)-2-amino-4-methyl-phosphono-3-pentenoic acid) to prevent such behaviour may be partly related to their effect on extracellular levels of 5-HT in the hippocampus. Of course, the possibility that the observed changes may be produced in response to a primary alteration in another neurotransmitter(s) cannot be excluded.
Chapter 5

Intrahippocampal injection of tetanus toxin produces alterations in density of $\text{GABA}_A$ and $\text{GABA}_B$ receptor binding sites and 5-HT re-uptake sites in rat.
The experimental results data described so far in the present study have demonstrated that injection of tetanus toxin (1000 MLDs) into the dentate gyrus region of the ventral hippocampal formation of rat produced significant reductions in extracellular levels of the neurotransmitters GABA, 5-HT, and dopamine 7 days after treatment. It has previously been shown that injection of the same dose of tetanus toxin into the dorsal CA1 hippocampal area of rat produced a dose- and time-dependent neuronal loss in this area, accompanied by a reduction in binding of $[^3\text{H}]$-GABA to GABA$_A$ but not GABA$_B$ sites in the CA1 pyramidal cell layer (Bagetta et al, 1990b). It was proposed that the observed decrease in binding of $[^3\text{H}]$-GABA to GABA$_A$ receptor sites was a consequence of the neuronal loss occurring in the CA1 pyramidal cell layer, as it is known that GABA$_A$ receptor sites are present on these neuronal cell bodies.

The reason for the lack of effect of toxin treatment on GABA$_B$ binding site density in this region was not entirely clear. It was proposed that this effect might be explained by a different rate of disappearance for GABA$_B$ sites, or possibly that GABA$_B$ sites detected in the CA1 pyramidal cell layer are present on nerve terminals within this area rather than on pyramidal cell bodies, as it has been shown that these receptors appear to be absent from the cell soma (Newberry & Nicoll, 1984).

As a consequence of this previous study it was decided to investigate the effect of tetanus toxin injection into the ventral dentate gyrus on the binding of $[^3\text{H}]$-GABA to GABA$_A$ and GABA$_B$ receptor sites within various regions of the ventral hippocampal formation of rat brain, 7 days after injection.
As mentioned earlier, the present study has demonstrated that tetanus toxin treatment produced a significant decrease in extracellular hippocampal 5-HT levels 7 days after treatment (Chapter 4). As the dose of toxin (1000 MLDs) used in this study has previously been shown to produce neurodegeneration 7 days after intrahippocampal injection (Bagetta et al, 1990a), it is possible that the observed 5-HT decrease results from toxin-induced destruction of 5-HT containing neurons. However, this seems unlikely since the extent of reduction of extracellular 5-HIAA levels did not parallel the corresponding 5-HT decrease at this time. Therefore, in an attempt to investigate the integrity of serotoninergic nerve terminals within the hippocampus of toxin-treated rats 7 days after injection, $[^3\text{H}]$-paroxetine was employed to provide autoradiographic localization of 5-HT re-uptake sites in various regions of rat brain. $[^3\text{H}]$-paroxetine has been shown to selectively label only sites associated with the serotonin transporter complex in brain (Habert et al, 1985; Mellerup and Plenge, 1986) and provide an autoradiographic pattern of binding which is virtually identical to the reported distribution of serotonin containing nerve terminals and cell bodies (De Souza and Kuyatt, 1987).

5.1 Dose of tetanus toxin used in the present study and behavioural effects observed in treated animals.

As in Chapters 3 and 4, a tetanus toxin dose equivalent to 1000 MLDs was used in this study. The reasoning behind employment of this dose level of toxin and the behavioural effects resulting from its unilateral injection into rat hippocampus have been previously described in sections 3.2 and 3.3, respectively.
5.2 Preparation of coronal brain sections for autoradiographic investigations.

Seven days after intrahippocampal injection of phosphate buffer (toxin vehicle) or tetanus toxin, animals were sacrificed and brains removed and prepared for sectioning as described in section 2.5.1. Pilot experiments conducted by Bagetta et al (1990c) demonstrated that the neuronal damage produced by intrahippocampal injection of tetanus toxin extended at least 400 μm either side of the injection site. Therefore, brain coronal sections (10 μm) 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 taken, resulting in 6 coronal sections from each brain being used for analysis.

5.3 Effect of tetanus toxin injection on binding of $[^3]$H-GABA to GABA$_A$ and GABA$_B$ receptor sites in various regions of rat ventral hippocampal formation 7 days after treatment.

Prior to investigating the effect of tetanus toxin injection on binding of $[^3]$H-GABA to GABA$_A$ and GABA$_B$ sites in rat hippocampus it was first necessary to evaluate the effect of intrahippocampal injection of the vehicle used to dissolve tetanus toxin, namely phosphate buffer, on these respective sites. Autoradiograms of $[^3]$H-GABA binding sites in sections from rat brains unilaterally injected into the hippocampus with phosphate buffer or tetanus toxin were generated as described in section 2.5.2. Optical density measurements (see section 2.5.4) were made in areas of the autoradiogram images corresponding to the pyramidal layers of the CA1, CA2, CA3 areas and the dentate gyrus granular and molecular layers
Commercially available \[^{3}\text{H}]-\text{microscales} were used as calibration markers. These consisted of 8 tritium labelled polymer layers calibrated for the auto-absorptive features of intact brain matter, with each having varying values of tissue equivalent tritium concentration.
of the hippocampal formation, and also the occipital cerebral cortex. Measurements were made in both untreated (control) and treated sides. Brain regions were identified using the atlas of Paxinos and Watson (1982). Following densitometric analysis of autoradiogram images, [3H]-GABA binding site densities were converted into fmol/mg tissue. The density of GABA\textsubscript{A} and GABA\textsubscript{B} binding sites in these respective areas were expressed as a percentage of the binding density observed in the untreated dorsal dentate gyrus granular layer.

Seven days after unilateral injection of phosphate buffer no significant alterations in GABA\textsubscript{A} and GABA\textsubscript{B} binding site densities were observed in any of the investigated brain regions on the treated side when compared to the corresponding regions on the untreated side (Figures 5.1 and 5.2, respectively). In contrast to this, injection of tetanus toxin produced a significant increase in GABA\textsubscript{A} receptor site binding density in the ventral CA1 pyramidal cell layer 7 days after treatment (Figure 5.3). A trend towards increased GABA\textsubscript{A} binding density was also displayed in the ventral CA2 and dorsal CA1 pyramidal cell layers. The effect of tetanus toxin treatment on binding of [3H]-GABA to GABA\textsubscript{B} sites in the various brain regions at this time is illustrated in Figure 5.4. It can be seen that GABA\textsubscript{B} receptor site binding density was significantly increased in the ventral CA1 and CA2 pyramidal cell layers when compared to the binding densities observed in corresponding regions on the untreated contralateral side. A trend for increased GABA\textsubscript{B} binding density was also observed in the ventral CA3 pyramidal cell region. Mean [3H]-GABA binding densities in untreated dorsal dentate gyrus granular layers were 15.73 ± 3.5 and 8.6 ± 1.62 fmol/mg and 14.16 ± 3.0 and 7.73 ± 1.12 fmol/mg for GABA\textsubscript{A} and GABA\textsubscript{B} receptor sites in vehicle-injected and
Illustration indicating areas where optical density measurements were made in autoradiogram images.
Fig. 5.1  Effect of unilateral intrahippocampal microinjection of phosphate buffer on GABA<sub>A</sub> receptor binding density 7 days after treatment.

[<sup>3</sup>H]-GABA bound [% of binding in untreated DDGG]

Data represent mean (± s.e.m.) percentages of [<sup>3</sup>H]-GABA binding density in the untreated dorsal dentate gyrus granular layer (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using Student’s t-test for unpaired data. VCA1, VCA2, VCA3 = ventral CA1, CA2, CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers, respectively; DDGM = dorsal dentate gyrus molecular layer; DCA1 = dorsal CA1 pyramidal cell layer; OCC = occipital cerebral cortex.
Fig. 5.2  Effect of unilateral intrahippocampal microinjection of phosphate buffer on GABA<sub>\alpha</sub> receptor binding density 7 days after treatment.

[\(^3\)H]-GABA bound [% of binding in untreated DDGG]

Data represent mean (± s.e.m.) percentages of [\(^3\)H]-GABA binding density in the untreated dorsal dentate gyrus granular layer (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using Student's t-test for unpaired data. VCA1, VCA2, VCA3 = ventral CA1, CA2, CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers, respectively; DDGM = dorsal dentate gyrus molecular layer; DCA1 = dorsal CA1 pyramidal cell layer; OCC = occipital cerebral cortex.
Fig. 5.3 Effect of unilateral intrahippocampal microinjection of tetanus toxin on GABA<sub>a</sub> receptor binding density 7 days after treatment.

![Bar Chart]

[<sup>3</sup>H]-GABA bound [% of binding in untreated DDGG]

Data represent mean (± s.e.m.) percentages of [<sup>3</sup>H]-GABA binding density in the untreated dorsal dentate gyrus granular layer (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using Student's t-test for unpaired data. * p < 0.05 vs contralateral region. VCA1, VCA2, VCA3 = ventral CA1, CA2, CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers, respectively; DDGM = dorsal dentate gyrus molecular layer; DCA1 = dorsal CA1 pyramidal cell layer; OCC = occipital cerebral cortex.
Fig. 5.4  Effect of unilateral intrahippocampal microinjection of tetanus toxin on GABA<sub>a</sub> receptor binding density 7 days after treatment.

[<sup>3</sup>H]-GABA bound [% of binding in untreated DDGG]

Data represent mean (± s.e.m.) percentages of [<sup>3</sup>H]-GABA binding density in the untreated dorsal dentate gyrus granular layer (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using Student's t-test for unpaired data. * p < 0.05 vs contralateral region. VCA1, VCA2, VCA3 = ventral CA1, CA2, CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers, respectively; DDGM = dorsal dentate gyrus molecular layer; DCA1 = dorsal CA1 pyramidal cell layer; OCC = occipital cerebral cortex.
Commercially available [\(^3\text{H}\)]-microscales were used as calibration markers. These consisted of 8 tritium labelled polymer layers calibrated for the auto-absorptive features of intact brain matter, with each having varying values of tissue equivalent tritium concentration.
toxin-injected brains, respectively. These densities were in accord with previously reported values (Bowery et al., 1986). Mean GABA_A and GABA_B receptor site densities measured in regions of brains obtained from both experimental groups of animals are summarized in Tables 5.1 and 5.2, respectively. Autoradiographs illustrating [^3H]-GABA binding to GABA_A and GABA_B receptor sites in rat brain sections are represented in Figures 5.5 and 5.6, respectively.

5.4 **Effect of tetanus toxin injection on binding of [^3H]-paroxetine to 5-HT re-uptake sites in various regions of rat brain 7 days after treatment.**

As mentioned previously in section 5.3, it was first necessary to evaluate the effect of intrahippocampal injection of vehicle (phosphate buffer) on binding of [^3H]-paroxetine to 5-HT re-uptake sites. Autoradiograms illustrating [^3H]-paroxetine binding sites in sections from rat brains unilaterally injected into the hippocampus with vehicle or tetanus toxin were generated as described in section 2.5.3. Optical density measurements were made in areas of the autoradiogram images corresponding to the pyramidal cell layers of the CA1, CA3 areas and the dentate gyrus granular layer of the hippocampal formation, the occipital cerebral cortex, the central grey, the ventral tegmental area, and the substantia nigra pars reticulata. Densitometric measurements were made in both untreated (control) and treated sides and [^3H]-paroxetine binding site densities converted into fmol/mg tissue. The density of [^3H]-paroxetine binding sites in these respective areas were finally expressed as a percentage of the binding density in the untreated ventral tegmental area.
Table 5.1  GABA<sub>A</sub> receptor site binding densities in brain regions of vehicle- and toxin-treated rats.

<table>
<thead>
<tr>
<th>AREA</th>
<th>Density of GABA&lt;sub&gt;A&lt;/sub&gt; binding sites (fmol/mg tissue)</th>
<th>Vehicle injected</th>
<th>Toxin injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>VCA1</td>
<td>11.9 ± 2.3</td>
<td>11.8 ± 3.3</td>
<td>11.8 ± 1.3</td>
</tr>
<tr>
<td>VCA2</td>
<td>11.5 ± 2.4</td>
<td>12.6 ± 2.8</td>
<td>11.8 ± 2.9</td>
</tr>
<tr>
<td>VCA3</td>
<td>10.2 ± 2.4</td>
<td>10.0 ± 2.3</td>
<td>10.8 ± 1.7</td>
</tr>
<tr>
<td>VDGG</td>
<td>12.4 ± 1.7</td>
<td>13.3 ± 3.9</td>
<td>16.7 ± 3.4</td>
</tr>
<tr>
<td>DDGG</td>
<td>15.7 ± 3.5</td>
<td>18.8 ± 2.9</td>
<td>14.2 ± 1.6</td>
</tr>
<tr>
<td>DDGM</td>
<td>29.5 ± 2.2</td>
<td>34.2 ± 3.1</td>
<td>44.3 ± 2.8</td>
</tr>
<tr>
<td>DCA1</td>
<td>14.9 ± 2.2</td>
<td>17.6 ± 1.8</td>
<td>17.3 ± 1.0</td>
</tr>
<tr>
<td>OCC</td>
<td>26.9 ± 3.1</td>
<td>27.3 ± 2.5</td>
<td>31.9 ± 0.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. (n = 6 sections per rat;  n = 3 rats per treatment).
Table 5.2  GABA<sub>B</sub> receptor site binding densities in brain regions of vehicle- and toxin-treated rats.

<table>
<thead>
<tr>
<th>AREA</th>
<th>Density of GABA&lt;sub&gt;B&lt;/sub&gt; binding sites (fmol/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle injected</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>VCA1</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>VCA2</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>VCA3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>VDGG</td>
<td>10.3 ± 1.9</td>
</tr>
<tr>
<td>DDGG</td>
<td>8.6 ± 1.6</td>
</tr>
<tr>
<td>DDGM</td>
<td>15.9 ± 2.3</td>
</tr>
<tr>
<td>DCA1</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td>OCC</td>
<td>21.9 ± 0.8</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. (n = 6 sections per rat; n = 3 rats per treatment).
Fig. 5.5 Receptor autoradiographs illustrating GABA$_A$ binding in rat brain sections. Section (a) shows the effect of vehicle treatment 7 days after injection, with the corresponding background binding shown in section (b). The effect of tetanus toxin treatment 7 days after injection is represented in section (c), with non-specific binding illustrated in section (d). (T) = treated side; (C) = control untreated side.
Fig. 5.6 Receptor autoradiographs illustrating GABA$_A$ binding in rat brain sections. Section (a) shows the effect of vehicle treatment 7 days after injection, with the corresponding background binding shown in section (b). The effect of tetanus toxin treatment 7 days after injection is represented in section (c), with non-specific binding illustrated in section (d). (T) = treated side; (C) = control untreated side.
Seven days after unilateral injection of phosphate buffer no significant alterations in \[^3H\]-paroxetine binding density was observed in any of the brain regions on the treated side when compared to the corresponding densities in regions on the contralateral untreated side (Figure 5.7). However, at the same time after treatment, unilateral intrahippocampal injection of tetanus toxin resulted in a significant reduction in \[^3H\]-paroxetine labelled 5-HT re-uptake site density in ventral CA1 and CA3 pyramidal cell layers and also in cortex (Figure 5.8). A trend for reduced binding density was also produced in the granular layer of the ventral dentate gyrus. Mean \[^3H\]-paroxetine binding densities observed in the untreated ventral tegmental area were 20.32 ± 1.2 and 26.84 ± 3.3 fmol/mg in brains injected with vehicle and tetanus toxin, respectively. A summary of \[^3H\]-paroxetine binding densities measured in regions of brains from vehicle- and toxin-treated rats is shown in Table 5.3. Autoradiographs depicting \[^3H\]-paroxetine binding to 5-HT re-uptake sites in rat brain sections are shown in Figure 5.9.

The neuropathological effect resulting from injection of tetanus toxin into the ventral dentate gyrus of rat brain not used for autoradiographic analysis in this study is illustrated in Figure 5.10. An extensive loss of granule cells can be observed in the toxin treated dentate gyrus when compared with the contralateral phosphate buffer injected region.
Fig. 5.7  Effect of unilateral intrahippocampal microinjection of phosphate buffer on [3H]-paroxetine binding density 7 days after treatment.

[3H]-Paroxetine bound [% of binding in untreated VTA]

Data represent mean (± s.e.m.) percentages of [3H]-paroxetine binding density in the untreated ventral tegmental area (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using Student’s t-test for unpaired data. VCA1, VCA3 = ventral CA1, CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers, respectively; OCC = occipital cerebral cortex; CG = Central grey; VTA = Ventral tegmental area; SNR = Substantia nigra pars reticulata.
Fig. 5.8  Effect of unilateral intrahippocampal microinjection of tetanus

toxin on $[^3$H]-paroxetine binding density 7 days after treatment.

$[^3$H]-Paroxetine bound (% of binding in untreated VTA)

Data represent mean (± s.e.m.) percentages of $[^3$H]-paroxetine binding density in the untreated ventral
tegmental area (n = 6 sections per rat; n = 3 rats per treatment). Statistical comparison was made using
Student’s t-test for unpaired data. * p < 0.05 vs contralateral region. VCA1, VCA3 = ventral CA1,
CA3 pyramidal cell layers; VDGG and DDGG = ventral and dorsal dentate gyrus granular layers,
respectively; OCC = occipital cerebral cortex; CG = Central grey; VTA = Ventral tegmental area;
SNR = Substantia nigra pars reticulata.
Table 5.3  \[^3^H\]-paroxetine binding site densities in brain regions of vehicle- and toxin-treated rats.

<table>
<thead>
<tr>
<th>AREA</th>
<th>Density of [^3^H]-paroxetine binding sites (fmol/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle injected</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>VCA1</td>
<td>14.2 ± 4.3</td>
</tr>
<tr>
<td>VCA3</td>
<td>14.4 ± 5</td>
</tr>
<tr>
<td>VDGG</td>
<td>11.2 ± 2.5</td>
</tr>
<tr>
<td>DDGG</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>OCC</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>CG</td>
<td>19.5 ± 2.4</td>
</tr>
<tr>
<td>VTA</td>
<td>20.3 ± 1.2</td>
</tr>
<tr>
<td>SNR</td>
<td>20.6 ± 6.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. (n = 6 sections per rat;  n = 3 rats per treatment).
Fig. 5.9 Receptor autoradiographs illustrating \(^{3}H\)-paroxetine binding in rat brain sections. Section (a) shows the effect of vehicle treatment 7 days after injection, with the corresponding background binding shown in section (b). The effect of tetanus toxin treatment 7 days after injection is represented in section (c), with non-specific binding illustrated in section (d). (T) = treated side; (C) = control untreated side.
Fig. 5.10 Photomicrographs of a toluidine blue stained coronal brain section (10 μm) illustrating the effects of injection of tetanus toxin (1000 MLDs) into the left ventral dentate gyrus (TT) and phosphate buffer into the right dentate gyrus (PB), 7 days after treatment. (a) The tetanus toxin treated side (TT) shows an extensive loss of granule cells when compared to the contralateral phosphate buffer injected region (PB). (b) This effect is more evident at a higher magnification.

DGG = dentate gyrus granular layer; CA3 = CA3 pyramidal cell layer.
Autoradiographic techniques employed in the present study have demonstrated that injection of tetanus toxin into the dentate gyrus area of the rat ventral hippocampal formation produces alterations in density of GABA_A and GABA_B receptor binding sites and 5-HT re-uptake sites, 7 days after treatment. It has previously been shown that injection of the same dose of toxin into the dorsal CA1 hippocampal area of rat produced a dose- and time-dependent neuronal loss in this area, with a concomitant reduction in GABA_A, but not GABA_B, binding sites in the pyramidal cell layer (Bagetta et al, 1990b).

In contrast to these findings, the present study has demonstrated that injection of tetanus toxin into the ventral dentate gyrus of the hippocampal formation, a region shown to be highly vulnerable to the neuropathological effects induced by tetanus toxin (Bagetta et al, 1991c), failed to produce alterations in the binding of [3H]-GABA to GABA_A and GABA_B receptors at the site of injection. The observed lack of toxin-induced effect on GABA_A receptor density at the site of injection was unexpected. Bagetta et al (1990b) proposed that the decrease in density of GABA_A sites observed in the CA1 pyramidal cell layer following toxin injection was a direct consequence of toxin-induced neuronal loss occurring in this area, as GABA_A receptor sites are known to be present on CA1 pyramidal cell bodies.

Unfortunately, quantitative histological examinations were not performed in brain sections taken from tetanus toxin-treated rats in the present study. Therefore it is not known whether injection of tetanus toxin into the ventral dentate gyrus resulted
in the degeneration of granular cell bodies. However, injection of the same dose of toxin into rat dentate gyrus has previously been observed to produce an approximate 70% reduction in granule cell number (Bagetta et al., 1991c). In support of this, the neuropathological effect resulting from injection of tetanus toxin (1000 MLDs) into this area has been demonstrated in a separate experimental group of animals whose brains were not used for autoradiographic analysis. If tetanus toxin treatment did induce neuropathology in the dentate gyrus region in the present study, a resultant reduction in $\text{GABA}_A$ receptor site density might have been expected as $\text{GABA}_A$ receptor sites are thought to be present on the granular layer cell bodies.

The effect of toxin treatment on $\text{GABA}_B$ binding density in dentate gyrus granule cells in the present study parallels the toxin-induced effect observed by Bagetta et al. (1990b) in the CA1 pyramidal cell layer. In both these cases no significant changes were observed in $\text{GABA}_B$ binding density 7 days after treatment. Bagetta et al. (1990b) proposed that a longer period of time may be necessary for the number of $\text{GABA}_B$ sites present on pyramidal cell bodies to diminish. Alternatively it was suggested that $\text{GABA}_B$ receptor sites may be present on nerve terminals, in particular GABAergic basket cell terminals, within the pyramidal cell layer rather than on the pyramidal cells. In support of this it appears that $\text{GABA}_B$ receptors are absent from the cell soma but present on pyramidal cell dendrites (Newberry and Nicoll, 1984). Therefore, even if CA1 pyramidal cells were disappearing the $\text{GABA}_B$ receptor sites would still remain. With respect to this hypothesis, the lack of effect of toxin treatment on $\text{GABA}_B$ site density in the dentate gyrus granular layer may be explained by these cells exhibiting a similar
GABA\textsubscript{B} receptor site distribution to CA1 pyramidal cells, with GABA\textsubscript{B} sites mainly located on cell dendrites. However, it is possible that a very small number of GABA\textsubscript{B} sites are located on the dendrites of cell bodies within the granular cell layer itself. Any tetanus toxin induced reduction in binding density may therefore have been masked by sprouting of terminals where the largest number of GABA\textsubscript{B} sites is present. Of course, without appropriate histological evidence it is possible that the lack of toxin induced effect on GABA\textsubscript{A} and GABA\textsubscript{B} receptor density at the site of injection may be due to the absence of degeneration of dentate gyrus granular cells.

The present study has demonstrated that injection of tetanus toxin into the ventral hippocampal formation produces significant increases in the binding of [\textsuperscript{3}H]-GABA to GABA\textsubscript{A} sites in the ventral CA1 pyramidal cell layer and to GABA\textsubscript{B} sites in both ventral CA1 and CA2 pyramidal cell layers. The reasons for these toxin induced observations remain unclear. Whilst it has been demonstrated that tetanus toxin induced neurodegeneration extends approximately 400 \textmu m either side of the injection site (Bagetta et al, 1990c) the extent to which the toxin can diffuse within the hippocampal formation is uncertain. Following injection of \textsuperscript{125}I-labelled tetanus toxin into the ventral hippocampus of rats Mellanby et al (1977) demonstrated that 48 hours after treatment 80-90\% of the radioactivity in the brain was located in the ventral hippocampus, with the rest recovered along the course of the needle track through the cerebral cortex. Whilst this finding indicates that tetanus toxin does not diffuse to other brain structures it provides no indication of the extent to which the toxin can spread from the site of injection within the ventral hippocampus.
The approximate distances of the ventral CA1, CA2, and CA3 pyramidal cell layers from the dentate gyrus granular layer, into which the toxin was injected in the present study, are 1200 µm, 2500 µm, and 800 µm, respectively. It is accepted that neurodegeneration is unlikely to occur in cell bodies at such distances from the site of injection. However, this does not necessarily invalidate the possibility that a toxin-induced disinhibition may occur in these regions of the ventral hippocampus. Whilst such a disinhibition may not be sufficient to evoke excitotoxic neurodegeneration, it is possible that decreased levels of neuronally derived GABA may contribute to the alterations observed in GABA receptor binding site densities found in the ventral CA1 and CA2 pyramidal cell layers 7 days after tetanus toxin injection. In particular, the increased GABAA receptor binding density observed in the ventral CA1 pyramidal cell layer at this time may result from up-regulation of GABAA receptor sites located on pyramidal cell bodies as a consequence of reduced inhibitory tone in this area. This hypothesis might also explain the increase in GABAB receptor site density observed in the ventral CA1 and CA2 pyramidal cell layers, if GABAB sites are indeed present on these cell bodies (Newberry and Nicoll, 1984). If these receptor sites are absent from the cell soma but present on cell dendrites it is possible that the increased GABAB receptor density measured in toxin treated brains is a consequence of an up-regulation of GABAB sites located on the dendrites of pyramidal neurons within the CA1 and CA2 pyramidal cell layers.

Binding of [3H]-paroxetine to 5-HT re-uptake sites in the ventral CA1 and CA3 pyramidal cell layers, and in the occipital cerebral cortex was found to be significantly reduced in tetanus toxin treated rats 7 days after injection. The
underlying mechanism responsible for this observed effect remains to be elucidated. It is possible that the reduction in 5-HT re-uptake site density is a direct result of toxin-induced degeneration of serotonergic nerve terminals in these regions. However, as mentioned previously, the ability of the injected toxin to diffuse from the dentate gyrus region to the CA1 and CA3 pyramidal cell regions and induce a disinhibitory effect sufficient to produce an excitotoxic phenomenon is questionable. It has been demonstrated that most of the [3H]-paroxetine binding in rat brain is abolished after destruction of 5-HT terminals (Habert et al, 1985; Marcusson et al, 1988). Also, an autoradiographic study on rat brain with [3H]-paroxetine demonstrated that production of lesions with the potent serotonergic neurotoxin 3,4-methylenedioxyamphetamine resulted in a dramatic decrease (> 70%) in the density of the binding sites (De Souza and Kuyatt, 1987). An approximate 50% reduction in [3H]-paroxetine binding density was observed in the affected areas in the present study. Whilst this might indicate that tetanus toxin-induced degeneration of 5-HT containing nerve terminals was responsible for the reduction in [3H]-paroxetine binding density, microdialysis measurements made in toxin treated animals at this time demonstrated that the extent of reduction (approximately 22%) of extracellular 5-HIAA level did not parallel the corresponding extracellular 5-HT decrease (approximately 65%). It therefore seems unlikely that destruction of serotonergic nerve terminals was responsible for the reduction in 5-HT re-uptake site density.

A more attractive, although purely speculative, hypothesis is that the decrease in [3H]-paroxetine binding density observed in the affected areas results from a down-regulation of 5-HT re-uptake sites, occurring as a consequence of tetanus toxin-
induced reduction of neuronal 5-HT release. As mentioned previously, tetanus
toxin may have the ability to diffuse within the ventral hippocampal formation
from its site of injection to the ventral CA1 and CA3 pyramidal cell layers
and produce an inhibition of release of 5-HT from nerve terminals. Whatever the
underlying mechanism responsible for these observed effects may be, it is probable
that the decrease in [³H]-paroxetine detected in the occipital cerebral cortex of
toxin treated rats results from toxin contamination along the course of the needle
track through this structure rather than from its transport from the site of injection.
The reason for a lack of toxin-induced effect on binding density at the site of
injection remains unclear.
Chapter 6

Concluding remarks and future directions
It is well documented that tetanus toxin produces disinhibition within the mammalian central nervous system, both in vivo (see Mellanby and Green, 1981), and in vitro (Bergey et al, 1987; Jeffreys et al, 1989). The focus of action of tetanus toxin appears to be GABA- and glycine-mediated inhibitory synapses, resulting in an apparent reduction in inhibitory neurotransmitter release (Curtis et al, 1973; Mellanby et al, 1977; Collingridge et al, 1980, 1981; Collingridge and Davies, 1982; Calabresi et al, 1989). It has been considered that loss of GABAergic mechanisms within higher centres might facilitate excitatory mechanisms to produce the seizure activity associated with tetanus toxin administration (Mellanby et al, 1977).

The tetanus toxin (1000 MLDs) induced behavioural excitation and neuropathology observed by Bagetta et al (1990a) 7 days after intrahippocampal injection in rat, was proposed to be derived from a loss of neuronal inhibition, resulting in a net increase of excitatory input involving overactivation of the NMDA-receptor complex at the site of injection. Therefore, the aim of the present study was to utilize the technique of in vivo microdialysis to investigate possible changes in extracellular levels of neuroactive substances, in particular GABA and the excitatory amino acids aspartate and glutamate. As a result of these investigations the present study has demonstrated that 7 days after injection of tetanus toxin, when neurodegeneration is evident (Bagetta et al, 1990a), extracellular levels of GABA in toxin-treated hippocampi were significantly reduced (approximately 45% of contralateral vehicle-injected side concentration), whilst levels of the excitatory amino acids aspartate and glutamate remained unaltered. Microdialysis measurements made at other times (1, 2, and 3 days after initial microinjection of
1000 MLDs tetanus toxin) in the present study revealed that extracellular levels of GABA, aspartate, and glutamate in toxin-treated hippocampi remained unchanged. As neuronal degeneration has not been observed at these times after tetanus toxin treatment (Bagetta et al, 1990a), these findings provide further support to the theory that toxin-induced neuropathology may result from a disinhibition-derived net increase of excitatory input at the site of injection.

It is widely accepted that tetanus toxin acts at the presynaptic level producing a reduction of Ca²⁺-dependent, K⁺ stimulated transmitter release (Osborne and Bradford, 1973a, b; Davies and Tongroach, 1979; Mellanby, 1984). In support of this view, GABA release was evoked by challenge with high K⁺ (100 mM) in both vehicle- and toxin-treated hippocampi, with evoked levels in toxin-treated sides being significantly reduced when compared to levels in the contralateral vehicle-injected hippocampi. The ability of high K⁺ to evoke the release of aspartate and taurine in rat hippocampi was also demonstrated in the present study. However, no such effect was observed for glutamate in either control, vehicle-treated, or toxin-treated hippocampi. As discussed in section 4.6, by investigating the effect of K⁺-stimulation and tetrodotoxin treatment on extracellular neurotransmitter levels in rat brain Westerink et al (1987) concluded that overflow of amino acid transmitters is related to metabolic as well as neurogenic events. With respect to the findings of the present study, and previous studies mentioned in section 4.6, it would not be unreasonable to assume that a significant proportion of extracellular GABA and aspartate measured in rat hippocampi in this study originates from transmitter pools. However, the lack of effect of K⁺-stimulation, both alone and combined with PDC treatment, on extracellular levels of glutamate
in rat hippocampi might indicate that these levels essentially reflect release from the metabolic pool. Of course, it is possible that the lack of effect of K\(^+\) stimulation may be due to the rapid uptake of glutamate into presynaptic terminals and glial cells.

The concentration of K\(^+\) used in this study (100 mM) would be expected to provide a sufficient depolarizing stimulus likely to induce both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent release from amino acid containing neurons, with the possibility of glial cells contributing to evoked release in a Ca\(^{2+}\)-independent manner (Minchin and Iverson, 1974; Bernath, 1992). In order to investigate further the extent to which basal and K\(^+\)-evoked extracellular amino acid levels reflect overflow from the neurotransmitter pool, it would be of interest to repeat the experimental dialysis procedures using a Ca\(^{2+}\)-free perfusion medium.

Although it is indicated that high concentrations of tetanus toxin can cause depression of both inhibitory and excitatory transmission (Kanda and Takano, 1983; Calabresi et al, 1989), no evidence of this action was observed in the present study, as extracellular levels of aspartate and glutamate remained unaltered throughout the duration of the study. However, extracellular 5-HT levels in toxin-treated hippocampi were found to be significantly reduced 1, 2, 3, and 7 days after injection in the present study. This observation supports the findings of Aguilera et al (1991) who demonstrated an increase in whole brain and spinal cord tissue content of 5-HT following intraventricular injection of tetanus toxin (150 MLDs) and proposed that this effect may be due to an inhibition of 5-HT release from presynaptic nerve terminals.
Decreases in extracellular 5-HIAA levels in toxin-treated hippocampi were also observed on the various days of dialysis in this study. It is possible that the decline in extracellular levels of 5-HT measured in toxin-treated hippocampi over the duration of the study may result from toxin-induced destruction of 5-HT containing neurons. This seems unlikely however, since the extent of reduction of extracellular 5-HIAA levels did not parallel the corresponding 5-HT decreases on the respective days of dialysis. Also it has been demonstrated by Bagetta et al (1990a) that treatment with this dose of toxin does not induce a significant neuropathology until 7-10 days after treatment. In support of this, the proposed reduction in extracellular level of GABA thought to be responsible for the induction of excitotoxic events is not manifested 1, 2, or 3 days following toxin injection.

A lack of consistent change in extracellular levels of dopamine in toxin-treated hippocampi was observed throughout the present study. The reason for the observed alterations in these levels is unclear. The decreased extracellular dopamine concentration observed in toxin-injected sides 7 days after treatment would suggest a toxin-induced inhibition of neurotransmitter release. However, the possibility that the observed changes are a result of toxin-induced destruction of dopamine containing neurones cannot be excluded. In support of this, extracellular levels of the dopamine metabolite DOPAC were found to be reduced by a similar amount in toxin treated hippocampi.

The current investigation has demonstrated that challenge with high K+ evoked the release of 5-HT and dopamine in both vehicle- and toxin-treated hippocampi.
days after injection, with evoked levels of 5-HT and dopamine in toxin-injected sides being significantly reduced when compared to corresponding levels in vehicle-injected hippocampi. As discussed in section 4.6, the extracellular levels of 5-HT and dopamine detected in rat brain by intracerebral microdialysis are thought to be closely related to neurogenic events. Therefore, it was assumed that extracellular levels of these neurotransmitters detected in vehicle- and toxin-treated hippocampi in this study were derived from neurotransmission.

It is interesting that the observed decreases in extracellular levels of 5-HT in toxin-treated hippocampi were evident from as early as 1 day after treatment. All animals injected with tetanus toxin (1000 MLDs) in the present study exhibited behavioural stereotypies and seizure activity within 48 - 72 hours of the treatment, culminating in generalized convulsions 5-7 days after injection. To what extent the observed alterations in extracellular levels of 5-HT and dopamine may contribute to the behavioural and neuropathological effects of tetanus toxin treatment must be speculative, and weighed against the contribution of other neurotransmitters, particularly GABA and glycine. It is possible that these observed neurochemical changes are the result of epileptiform activity following loss of inhibitory transmission (Mellanby and Green, 1981), or to a loss of both inhibitory and excitatory neurotransmission as previously seen with relatively high doses of tetanus toxin (Bergey et al, 1983; Bigalke et al, 1983; Kanda and Takano, 1983). However, whilst it has been demonstrated that tetanus toxin treatment produced a reduction in extracellular levels of GABA 7 days after injection, no effect on extracellular levels of the amino acids aspartate, glutamate, and GABA was detected over the first 3 days of dialysis when extracellular levels
of 5-HT were found to be reduced by up to 80% and animals displayed seizure activity. As discussed previously (section 4.6), 5-HT deficiencies have been implicated in the development of myoclonic epilepsy (Chadwick et al, 1975; Fahn, 1978). Evidence that brain 5-HT plays an inhibitory role in a variety of epilepsy models has also been provided (De La Torre et al, 1970; Killan and Frey, 1973; Schlesinger et al, 1969; Wada et al, 1991), with the ability of serotonergic enhancing drugs to provide anticonvulsant action being demonstrated by various studies (Wada et al, 1991; Wada et al, 1992a, 1992b). The suppression of hippocampal kindled seizures in cat by the 5-HT<sub>1A</sub> agonist 8-OH-DPAT demonstrated by Wada et al (1992b) was suggested to be due mainly to the drugs action on postsynaptic 5-HT<sub>1A</sub> receptors in the hippocampus. As rat hippocampus is known to have a high density of 5-HT<sub>1A</sub> receptors, it is possible that the decreased extracellular 5-HT level observed in toxin treated hippocampi in this study has some involvement in the behavioural effects resulting from intrahippocampal injection of tetanus toxin.

The behavioural and neuropathological effects resulting from intrahippocampal injection of tetanus toxin (1000 MLDs) can be prevented by NMDA receptor antagonists dizocilpine (Bagetta et al, 1990c) and CGP 37849 (Bagetta et al, 1992). The studies of Whitton et al (1992, 1994) demonstrated the ability of dizocilpine and NMDA to increase and decrease extracellular 5-HT levels in rat hippocampus, respectively, suggesting that the release of hippocampal 5-HT may be under tonic control via glutamatergic NMDA receptors. As the behavioural effects resulting from treatment with tetanus toxin occur before any loss of neuronal inhibition can be detected in the present study, it is possible that the
ability of NMDA antagonists to prevent such behaviour may be partly related to their effect on extracellular levels of hippocampal 5-HT. It would be of interest therefore to conduct further dialysis experiments in toxin treated animals to investigate what effect treatment with NMDA antagonists had on extracellular levels of amino acids and monoamines, over the same time period used in the present study. Whilst it is possible that the toxin-induced behavioural effects may result from reduced levels of extracellular 5-HT, it seems likely that the neurodegeneration occurs as a result of inhibition of GABA release leading to a disinhibition induced excitotoxic phenomenon. It would be of interest to evaluate the effect of 5-HT agonists, in particular, 5-HT\textsubscript{1A} agonists, on toxin-induced behavioural effects in rat. Their ability to prevent these effects may give further insight into the extent of involvement of 5-HT in tetanus toxin seizures.

In conclusion, the findings of the present study indicate that intrahippocampal injection of tetanus toxin in rats leads to an inhibition of neuronal GABA release, whilst leaving excitatory (asparate and glutamate) neurotransmission unaffected. Tetanus toxin may therefore be useful for characterizing both the mechanisms underlying subtypes of epilepsy and the efficacy of newly developed antiepileptic drugs. The neuropathological effect of tetanus toxin previously demonstrated is considered to involve a disinhibition-derived net increase in excitatory receptor activation. Therefore, the tetanus toxin model could represent a new experimental paradigm to assess the activity of drugs with potential for use in chronic epilepsy, cerebral ischemia, and trauma, conditions in which an abnormal stimulation of NMDA receptors are thought to occur. Further use of this experimental model may reveal observations bearing therapeutic significance to the worldwide problem
of tetanus intoxication in humans. However, this approach may be delayed by the lack of clinically effective excitatory amino acid antagonists.


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