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# CHEMICALLY INDUCED DAMAGE TO THE HIPPOCAMPAL FORMATION

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Liv Unni Naalsund

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8) ABSTRACT (continue on reverse side if necessary) <p style="text-align: center;"><i>Hippocampus</i></p> <p>In the present work it was shown that degeneration of the hippocampal formation after trimethyltin (TMT) administration can be detected at an early phase by electric recordings from intrahippocampal electrodes. The anatomical distribution of the damage can be studied by histochemistry, and was shown to include the CA3 and CA4 subfields in the initial period and spreading to the CA1 after a complete destruction of the pyramidal cells in CA3 and CA4. The degeneration was shown to be specific for glutamergic neurons in the hippocampus, high affinity uptake of [<sup>3</sup>H] glutamate being the only biochemical parameter which was changed. <u>In vitro</u> TMT increased the efflux of [<sup>3</sup>H] glutamate from synaptosomes in the resting state, but had no effect on the efflux of [<sup>3</sup>H] GABA. The synaptosomal reuptake of transmitter and receptor binding were both inhibited in the presence of TMT. In relation to reports on TMT as an</p>				
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## ABSTRACT (continued)

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The thesis is based on the following publications, which will be referred to in the text by Roman numerals:

PAPER I: Naalsund, L U, Allen, C N and Fonnum, F, 1985, Changes in neurobiological parameters in the hippocampus after exposure to trimethyltin, *Neuro Toxicology* 6(3), 145-58.

PAPER II: (manuscript)  
Naalsund, L U and Fonnum, F, 1986, The effect of trimethyltin on three glutamergic and GABAergic transmitter parameters in vitro: high affinity uptake, release and receptor binding, *Neuro Toxicology* (in press).

PAPER III: (manuscript)  
Naalsund, L U and Fonnum, F, 1986, Differences in anionic dependence of the synaptic efflux of D-aspartic acid and  $\gamma$ -amino butyric acid, *J Neurochem* (in press).

PAPER IV: (manuscript)  
Naalsund, L U, 1986, Hippocampal EEG in rats after chronic toluene inhalation, *Acta Pharmacol et Toxicol* (submitted).

## CHEMICALLY INDUCED DAMAGE TO THE HIPPOCAMPAL FORMATION

SUMMARY

In the present work it was shown that degeneration of the hippocampal formation after trimethyltin (TMT) administration can be detected at an early phase by recordings from electrodes implanted in the hippocampal formation. The distribution of the damage can be studied by histology, and was shown to include the pyramidal cells in the CA3 and CA4 subfields in the initial period and spreading to the CA1 after a complete destruction of the pyramidal cells in CA3 and CA4. Neurochemical methods showed that the degeneration was specific for glutamergic neurons in the hippocampus, and not affecting gabaergic or cholinergic neurons. High affinity uptake of [ $^3\text{H}$ ] glutamate was the only biochemical parameter investigated which was changed. In vitro low concentrations of TMT increased the efflux of [ $^3\text{H}$ ] glutamate from synaptosomes in the resting state, but had no effect on the efflux of [ $^3\text{H}$ ] GABA. The synaptosomal reuptake of transmitter and receptor binding were both inhibited in the presence of TMT. In relation to reports on TMT as an anion conductor, the effect of changes in extracellular  $\text{Cl}^-$  concentration on transmitter release was investigated. A reduction in the external  $\text{Cl}^-$  concentration was shown to have a much larger effect on the efflux of transmitter from glutamergic than from GABAergic synaptosomes. The sensitivity of the hippocampal electric activity to changes after exposure to toxic compounds was applied for the investigation of a putative neurotoxic effect of long term exposure to low levels of toluene. An increased synchronization together with an increased frequency in the theta waves was observed during the first period of toluene exposure. The later phase was characterized by a gradually increasing disruption in regular waves and a reduction in the theta frequency. Small improvements regarding regularity, but no increase in frequency was seen during one month after termination of exposure, indicating that irreversible changes in neuronal functions have occurred.

1 INTRODUCTION1.1 Hippocampal function

The hippocampal formation is one of the most extensively studied areas of the brain. The first theory of the hippocampus as an important olfactory centre, based on its anatomical location in the rhinencephalon or "smell brain" (Ramon y Cajal, 1903; Brodal, 1947), was

displaced by a hypothesis suggesting that the hippocampus is a part of a circuit forming the neural substrate of emotional behavior proposed by Papez in 1937. This hypothesis was extended by MacLean (1952) who introduced the term limbic system, which included the cerebral cortex surrounding the medial part of the cerebral hemisphere and the subcortical nuclei to which it is connected, and suggested that these areas are related to non-visceral functions. Since the introduction of the term limbic system, many brain regions have been shown to have connections with the limbic cortex, and it is now clear that essentially all of the major functional systems in the brain share direct connections with the limbic lobe (Brodal, 1981). This has made it particularly difficult to define the limits of the limbic system, and the old assumption upon which the limbic system concept was based, namely that visceral and non-visceral systems in the brain can be clearly distinguished, seems to be disproved.

Present knowledge suggests that the hippocampal formation may be responsible for intergration of information from the sensory modalities, and transformation of these informations to complex association areas and visceral and motoric control systems. In the broadest functional terms it seems to be in the unique position of influencing somatomotor, visceral, motivational and affective and cognitive mechanisms (Swanson, 1983).

## 1.2 Hippocampal anatomy

The hippocampal formation has a unique structural organization. The major fiber connections are located in sharply demarcated regions, and cells and fibers form distinct laminae (Andersen et al, 1971). The relative simplicity of its intrinsic neuronal architecture renders the hippocampus a popular model system for the study of basic cell biological functions.

The hippocampal region consists of the area dentata, the hippocampus and the subiculum, see Figure 1.1, (Blackstad, 1956). The principal cells of the area dentata are the granule cells with its excitatory

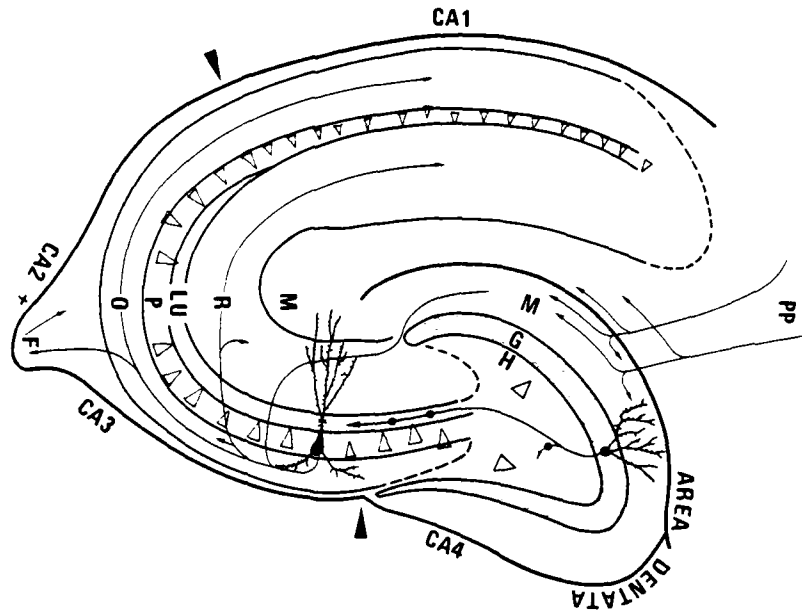


Figure 1.1 Schematic drawing showing the main layers and their connections in the hippocampal formation

Labelling: Hippocampus: F=fimbria, O=stratum oriens, P=stratum pyramidale (with the indicated subfields CA1-4), R=stratum radiatum, M=stratum lacunosum moleculare, LU=stratum lucidum (mossy fibre layer). Area dentata: H=hilus fascia dentata, G=stratum granulosum, M=stratum moleculare. PP=perforant path axons from the entorhinal cortex distributing to the molecular layer of area dentata. Granular cells give rise to the mossy fibres, which terminates in the hilus of fascia dentata and in stratum lucidum of CA3 and CA4

axons, the mossy fibers, (Blackstad et al, 1970). The pyramidal cells are the major neurons of the hippocampus. They are arranged in a dense, continuous layer, the stratum pyramidale, and their dendrites are located in the stratum oriens, stratum radiatum and the stratum lacunosum moleculare (Andersen et al, 1971). The hippocampus is

divided into the subfields CA1-4 (Lorente de No, 1934), in the rat the CA2 is usually included in the CA3 (Blackstad, 1956).

The main inputs to the hippocampus are the perforant path (Lorente de No, 1934; Hjort-Simonsen, 1972), which originates in the entorhinal cortex and terminates in the area dentata, and the septo-hippocampal projection, which originates in the medial septal nucleus and distributes to the whole hippocampal formation and the adjacent cortical areas (Swanson and Cowan, 1979). Impulses entering the granule cells through the perforant path are further processed through the hippocampal formation by a three-membered neuronal chain, i.e. the mossy fibers, the CA3 axons and the CA1 axons. The CA3 neurons receive impulses from the mossy fibers (Blackstad et al, 1970) and propagate these impulses through the Schaffer collaterals to the ipsilateral CA1 region (Schaffer, 1892) and through the commissural fibers to the contralateral CA1 and CA3 region (Blackstad, 1956; Laurberg, 1979). The excitatory CA1 pyramidal cells send most of their axons caudally into the subicular complex (Andersen et al, 1973). All these excitatory pathways appear to be monosynaptically connected (Andersen, 1975), but they are also influenced by recurrent inhibition at each step (Andersen et al, 1964). Local interneurons are present both in the area dentata and in the stratum pyramidale of the hippocampus (Storm-Mathisen, 1972; Fonnum and Walaas, 1978).

The septo-hippocampal connections are mediated through the fimbria and fornix. Both CA1 and CA3 pyramidal cells project to the lateral septum via fimbria (Swanson and Cowan, 1977). The lateral septum projects to the medial septum, which in turn projects back to the hippocampal subfields as well as to the dentate granular cells and the subicular complex (Swanson, 1978; Swanson and Cowan, 1979).

The summarized connections represent the quantitatively important part of the hippocampal circuitry. More detailed descriptions are given in reviews by Andersen, 1975, and Walaas, 1983.

### 1.3 Neurotransmitters in the hippocampus

The unique anatomy of the hippocampal formation, described above has made it an attractive brain area for neurotransmitter studies. The acidic amino acids, glutamate and aspartate are probably the most important neurotransmitters in the hippocampus. The entorhinal perforant path is a well documented glutamergic or aspartergic pathway (Nadler et al, 1976; 1978; Cotman and Nadler, 1981; Storm-Mathisen, 1977; 1981), while the role of acidic amino acids in the hippocampal mossy fibers is yet uncertain. Some evidence do however exist for this fibers utilizing glutamate or aspartate as well (Storm-Mathisen and Iversen, 1979; Aamodt et al, 1984). Both the CA3 and CA1 excitatory fibers may also utilize glutamate or aspartate as transmitter (Nadler et al, 1976; Storm-Mathisen, 1977; 1978; Fonnum and Walaas, 1978; Fonnum et al, 1979).

Acetylcholine is the excitatory transmitter of about 50% of the afferents from medial septum entering the hippocampus through fimbria (Lewis and Shute, 1967; Lewis et al, 1967; Storm-Mathisen, 1972; Wainer et al, 1985). The transmitter of the non-cholinergic fimbrial fibers are at present not identified. GABA neurons in the hippocampal formation are mainly local neurons (Fonnum and Storm-Mathisen, 1969; Storm-Mathisen and Fonnum, 1971) probably involved in both recurrent inhibition of the principal projection neurons in the region and regulation of local excitability in the dendritic tree that receives the major excitatory inputs (Storm-Mathisen and Fonnum, 1971). A serotonergic input, mainly derived from median raphe nucleus (Lorenz and Guldberg, 1974) exerts an inhibitory action on the hippocampus (Stefanis, 1964; 1968). The catecholamine level in the hippocampus is low and is mainly located in noradrenergic fibers, which originate in the brainstem, and releases noradrenaline exerting a depressant action on the pyramidal cells (Segal and Bloom, 1974; 1976). The dopamine content of the hippocampus is less than 5% of the noradrenaline level (Bischoff et al, 1979). A restricted dopamine input arising in the substantia nigra (Swanson, 1982) to the hippocampus and area dentata is however believed to exist (Scatton, 1980).

#### 1.4 Toxic damage to the hippocampal formation

Hippocampus appears to be extremely sensitive to the action of several neurotoxic compounds such as kainic acid (Olney et al, 1974), 3-acetylpyridin (Cogeshall and Mac Lean, 1958), glucocorticoids (Sapolsky, 1985), trimethyltin (Brown et al, 1979) as well as to cell loss induced by ischemia (Kirino, 1984), hypoglycemia (Auer et al, 1984), seizures (Folbergrova et al, 1981) and Alzheimers disease (Henke and Lang, 1983).

The selective vulnerability of the hippocampus was recognized early in the history of neuroscience, and the differences in vulnerability of the hippocampal subfields has been well described (Friede, 1966). However, so far it has not been possible to draw any conclusions about the mechanisms behind selective vulnerability of neuronal tissue. As no correlation has been demonstrated between regional cerebral blood flows and regional neuronal necrosis (Wieloch, 1985) the vulnerable neurons are thought to be subject to local stress, or to possess particular properties making them prone to insults. The pyramidal cells in the hippocampus and cortical regions as well as the cerebellar Purkinje cells are thought to possess a high vulnerability to anoxic damage due to their large soma and high metabolic activity (Norton, 1980). A striking common property of the brain areas selectively vulnerable to an ischemic insult, CA1 and subicular region of the hippocampus, hilus of area dentata, lateral septum, the amygdaloid nuclei, entorhinal cortex, the olfactory tubercle and the reticular nucleus of the thalamus (Wieloch, 1985), is that they all receive a dense excitatory aminoacidergic innervation (Foster and Fagg, 1984, Wieloch, 1985). The density and subtype distribution of excitatory amino acid receptors on postsynaptic membranes have been shown to possess patterns related to selective vulnerability (Monaghan et al, 1983). The intracellular second messenger used by the excitatory amino acids is not yet identified (Wieloch, 1985), however ionic movements over the plasma membrane have been suggested to be possible mediators of the neurotoxic actions of these substances. Hypotheses regarding such phenomena include either an excessive influx of calcium (Siesj , 1981) or an enhanced chloride influx into cells (Wieloch, 1985).

### 1.5 The aim of the study

The aim of the present study was to characterize the development of the toxic damage in hippocampus induced by trimethyltin. Previous studies have shown that trimethyltin may produce selective damage to the hippocampal pyramidal cells (Brown et al, 1979). It was therefore interesting to study this damage as a model for neurotoxic degeneration in the hippocampus. By using neurochemical, histochemical and neurophysiological techniques we wanted to compare the time course of changes in the different parameters and then study in detail the effect of this compound on the biochemical events of synaptic transmission. Based on the results from these experiments we chose to study neurophysiological changes in the hippocampus in an investigation of a possible neurotoxic action of the organic solvent toluene.

## 2 RESULTS AND DISCUSSION

### 2.1 Trimethyltin

Trimethyltin induced hippocampal damage was detected by neurochemical, histochemical and neurophysiological methods at different developmental stages of the degenerative process (paper I). In vitro studies showed that the neurotransmitter release, high affinity uptake and receptorbinding are interrupted at low concentrations of trimethyltin (paper II). The effect on release was quantitatively different for GABA and L-glutamate (paper II). A similar quantitative difference between the release of GABA and D-aspartate was observed when varying concentrations of  $\text{Cl}^-$  was included in the perfusion buffer (paper III).

### 2.1.1 Neurochemistry

Neurochemical markers specific for a certain cell population is frequently chosen as a measure for the number of actively functioning cells of the population under study in a brain region. Glutamate decarboxylase (GAD) is located in the nerve endings of GABAergic neurons while choline acetyltransferase is located in the cholinergic nerve endings. As there is no enzyme specific for glutamergic terminals, the high affinity uptake of glutamate, specifically present in glutamergic synaptic terminals, is used as marker for glutamergic neurons. A reduction in the activity of the markers is seen when neurons are completely degenerated (Fonnum, 1977).

The injection of TMT in rats produced a gradually increasing loss of activity of the high affinity uptake of glutamate in the hippocampal CA1 region (paper I). Five weeks after the first of three weekly injections of 3 mg/kg TMT, a 68% reduction in high affinity uptake of glutamate was found. The first significant change was detected four weeks after the first injection (paper I). These results indicate that a complete degeneration of a fraction of the glutamergic nerve terminals was obtained after four weeks. A single large dose of TMT (8 mg/kg) produced a significant loss of glutamergic neurons (33%) seven days after the injection and increasing to a 42% reduction after 3 weeks (paper I), suggesting a faster destruction process after a single, large dose of TMT than after several smaller doses.

The GABAergic and cholinergic markers were not reduced after the TMT treatment of the rats (paper I). A selective destruction of glutamergic neurons seems to take place. Previous studies have reported changes also in the GABAergic markers, a reduced concentration of GABA within the hippocampus (Mailman et al, 1983) and a reduced high affinity uptake of [ $^3\text{H}$ ] GABA has been reported (Doctor et al, 1982). The reduced uptake of GABA was detected 2 hours following administration of 4 mg/kg TMT i p (Doctor et al, 1982) suggesting a direct effect on the active transport rather than a cellular degeneration of the basket cells utilizing GABA as their transmitter. The cholinergic activity was increased five weeks after the multiple injection schedule

(paper I). This may represent sprouting of surviving axons, previously described after lesions in the area dentata (Matthews et al, 1976) and in the hippocampus (Gage et al, 1983; Dravid and Van Deusen, 1984). The phenomenon termed reactive synaptogenesis in many cases represents heterotypic reconstruction not associated with functional recovery (Mc Couch et al, 1958).

### 2.1.2 Histology

Histological examinations of the brains of TMT intoxicated rats show that the CA4 and ventral CA3 pyramidal cells are the most vulnerable to toxic damage produced by TMT (paper I; Brown et al, 1979; Chang and Dyer, 1983; Valdes et al, 1983). The CA1 pyramidal cells are less sensitive to TMT induced degeneration, they do not disappear completely during the time course of our study as do the CA3 and CA4 pyramidal cells. Differences in vulnerability of the hippocampal subfields has been described in several instances (Friede, 1966) and has been proposed to be due to differences in their chemical composition (Friede, 1966). A sharp chemical border exists between CA1 and CA2-3 (Friede, 1966), corresponding well with their different reaction to toxic damage.

Degenerating neurons are found in the CA3 and CA4 subfields 21 days after the first of three weekly injections of TMT (3 mg/kg) (paper I). At this time no change is found in the biochemical parameters. Similarly after a single injection of TMT (8 mg/kg), degenerating neurons are found in CA1, CA3 and CA4 hippocampal subfields three days post injection, that is four days prior to any reduction in high affinity uptake of glutamate (paper I).

### 2.1.3 Neurophysiology

The electrical activity in the hippocampus is a well described characteristic of this region, consisting of two distinguishable wave patterns closely related to the behavior of the rat. The electrographic record of a motionless rat consists of low frequency asynchronous

waves and includes variably occurring large amplitude waves (Vanderwolf, 1969). This EEG pattern is referred to as irregular activity. Rhythmic slow activity (RSA) accompanies voluntary movements such as walking, rearing and moving the body. RSA is typically regular synchronous electrical waves with a frequency of 7-8 Hz (theta-activity) in the walking rat (Vanderwolf, 1969). A reduction in the frequency of the theta activity occurs two weeks after the first of three weekly injections of TMT (3 mg/kg), and further frequency reduction occurs between day 14 and day 21 together with a total loss of regular waves (paper I). A single dose of TMT (8 mg/kg) produces a reduction in the theta frequency three days after administration of the TMT (paper I; Ray, 1981). Both amplitude, frequency and incidence of theta are reduced. A total loss of regular waves occurs during the third week after a single TMT injection (paper I). Ray (1981) observed that the decreased theta was preceded by an initial period of increased incidence and increased frequency of the theta activity and a presence of theta waves in the immobile rats. Human intoxications with organic tin compounds also produces general disturbance and dysrhythmia of the EEG-recording (Prüll and Rompel, 1970).

The hippocampal response to evoked potentials increases during the first 2-4 days after TMT administration and falls gradually from day 4-6 onward (Ray, 1981; Hasan et al, 1982). In the initial period of TMT intoxication an increased vulnerability to production of seizures is also seen (Dyer et al, 1982b). A phase of increased neuronal excitability is suggested to precede neuronal damage (Ray, 1981). The increased activity of the pyramidal cells may also stem from an increased activity of the mossy fibers due to a reduction in the inhibitory activity of the basket cells on the granule cells, showed by Dyer and Boyes (1984). The possibility of increased occurrence of seizures as the cause of neuronal damage in the hippocampal formation was disproved by Zimmer et al (1985), showing that continuously treatment with an anticonvulsant agent did not affect the pathological changes after TMT treatment. Doctor and Fox (1982) even found a reduced responsiveness to electroshock seizures following i p injection of 0.7 and 3.5 mg/kg TMT to mice. These results indicate that the neuronal mechanisms of action of TMT differs from the action

of kainic acid, a neurotoxic compound with a similar selective neuronal damage as TMT. Kainic acid is dependent upon seizure activity for production of pathological changes in the hippocampus (Ben-Ari et al, 1980). In contrast to the observations on increased excitability of the hippocampus, TMT has a depressive effect on hippocampal pyramidal cells in vitro, suggesting a decreased excitability of the postsynaptic neuron, or a decreased release of transmitter during stimulation of the pyramidal neuron (Allen and Fonnum, 1984).

#### 2.1.4 In vitro measurements of chemical transmission

Several neurochemical mechanisms may be involved in the observed neuronal changes during TMT intoxication. In vitro alkyltin compounds causes a disruption of mitochondrial function (Aldridge, 1958; Selwyn, 1976) including an inhibition of oxidative phosphorylation (Selwyn, 1976). The ability of neuronal cells to perform chemical transmission makes them vulnerable to any change in biochemical activity. Both the release, high affinity uptake and specific receptor binding of the neurotransmitter is affected by TMT at different concentration levels. The release of L-[<sup>3</sup>H] glutamic acid and D-[<sup>3</sup>H] aspartic acid from synaptosomes in vitro is enhanced by 20% at TMT concentrations from 50  $\mu$ M to 1 mM (paper II). At the 50  $\mu$ M concentration level a 25 - 30% reduction in the high affinity uptake (paper II) and in specific receptorbinding of glutamate (paper II) is observed. The release of [<sup>3</sup>H] GABA is unaffected by the presence of TMT at concentrations up to 1 mM (paper II), while the high affinity uptake of [<sup>3</sup>H] GABA is reduced at the highest TMT concentrations (0.5 - 1 mM) and receptor binding at 50 nM ligand concentration is inhibited with 100  $\mu$ M or more TMT in the medium. The lowest concentration of TMT which interrupts the neurotransmission, 50  $\mu$ M (= 50 nmol/ml), is about five times the in vivo concentration after injection of a neurotoxic dose of TMT. Brown et al (1979) reports that the minimum amount of TMT in brain tissue to cause neuronal necrosis is 8.6 nmol/g wet wt.

The TMT stimulated release of glutamate is much smaller but has the same characteristics of the K<sup>+</sup> stimulated release in that it is Ca<sup>++</sup> dependent and TTX independent (paper II). The effect of TMT on gluta-

mate release is quite different from the effect on ACh release, which is inhibited during depolarization but unaffected at low  $K^+$  concentrations (5 mM) (Morot-Gaudry, 1984) in the presence of high concentrations of TMT (0.5 - 3.5 mM). Morot-Gaudry showed that the alkyltin was unable to inhibit transmitter release induced by a calcium ionophore, and suggested that the action of TMT is an interference with calcium conductance. The effect on ACh transmission could also be secondary to an inhibition of mitochondrial function (Aldridge, 1958; Selwyn, 1976), since the synthesis of ACh in the cytosol is dependent upon acetyl-Co A formed in the mitochondria (Quastel, 1977).

Organotin compounds have been shown to induce several effects on membrane systems. They are associated with  $Cl^-/OH^-$  exchanges in mitochondria (Selwyn et al, 1970), erythrocytes and liposomes (Selwyn et al, 1970; Motais et al, 1977; Wieth and Tosteson, 1979) and in pancreatic  $\beta$ -cells (Pace and Tarvin, 1983). This exchange could possibly explain the effect of TMT on glutamate release through a change in the relation between internal and external  $Cl^-$  concentration. A reduction in the extracellular  $Cl^-$  concentration induces a glutamate release similar to that induced by high potassium (paper III; Hardy et al, 1984), but do not have the same stimulatory effect on the release of the inhibitory transmitter GABA (paper III). A reduction in the extracellular  $Cl^-$  concentration from 145 mM to 125 mM is sufficient to produce a significant release of transmitter from glutamergic and aspartergic neurons (paper III), while the release of GABA is unaffected by the same change in  $Cl^-$ -concentration.

The putative neurotransmitter amino acids glutamate and aspartate can cause neurotoxic effects when present in excess in nervous tissue (Olney et al, 1971; Olney, 1976). A delicate balance may exist between the function of these substances as excitatory transmitters and their capacity to cause nerve cell death. The increased leakage of tritiated transmitter together with a reduced uptake (paper II) should give an increase in the extracellular level of the neurotransmitter in the synaptic cleft. This effect seems to be specific for glutamergic and aspartergic neurons at low doses (paper II and III). This is in agreement with Dyer et al (1982a) who suggested that TMT may induce an

excitotoxic action. The reduced post-synaptic activity in the presence of TMT (paper II; Allen and Fonnum, 1984), should however, reduce the effect of an excess glutamate efflux into the synaptic cleft. Another possibility of the site of primary toxic damage is that it occurs pre-synaptically in the terminal region as a result of ionic disturbances and transmitter depletion (paper II; Selwyn, 1970). The resulting degeneration process could be a retrograde degeneration of the axon. Electronmicroscopic examinations of early damage shows degenerating axons in chronically intoxicated rats (Bouldin et al, 1981), but no descriptions on terminal structures have been given in the literature.

## 2.2 Toluene

On the background of the results from TMT studies we chose the hippocampal electric activity as the parameter in an investigation of possible neurotoxic effects of the organic solvent toluene. Toluene inhalation produces disruptions and frequency changes in the hippocampal theta activity (paper IV; Takeuchi and Hisanaga, 1977). The time course of changes in the hippocampal electric activity follows the same phases as described for TMT (Ray, 1981; Hasan et al, 1982; paper I). After an initial increase in the frequency and an increase in the occurrence of theta waves, there is a reduction in the frequency and a gradually increased disruption of the theta waves (paper IV). High toluene concentrations (4000 ppm) will produce acute frequency reduction in the theta waves, when recorded during and within two hours after exposure (Takeuchi and Hisanaga, 1977).

One month of recovery after the termination of toluene exposure do not allow any improvements to occur in the theta-activity, suggesting that an irreversible brain damage is developed (paper IV). A neuronal damage causing disruption in theta activity could be located in any of the brain nuclei involved in generating or regulating the theta rhythm. The main source for hippocampal desynchronization is thought to be the median raphe nucleus (Vertes, 1982), while the medial pontine region of the reticular formation seems to be the source of hippocampal syn-

chronization (Vertes, 1982). Brain stem generation of hippocampal activity is mediated by the medial septum (Vertes, 1982). By use of surgical lesions it has been shown that lesions in the medial septum can give a complete loss of hippocampal rhythmic slow activity (theta) (Andersen et al, 1979), while lesions in the brain stem and pontine area give a reduction in frequency without any qualitative disturbances (Vertes, 1982). The reason for this lack of effect of brain stem lesions in abolishing theta activity is thought to be the diffuse organization of the ascending brain stem systems and the fact that several pathways maintain the theta activity (Vertes, 1982). Studies on pharmacologic manipulations of the theta-rhythm describes acute effects, and our experiments can therefore not be readily compared to any of these studies (Stumpf, 1965). The toluene level in the brain tissue declines with the same rate as the bloodlevel, and 24 hours after an inhalation exposure the brain level is 0.1% of the concentration at the termination of exposure (Pyykkö et al, 1977). We have measured the hippocampal EEG 48 hours after exposure each week and hence no acute effects have been detected.

Toluene produces alterations in CNS excitability (Benignus, 1981), sensory function (Pryor et al, 1983a; 1983b; Dyer et al, 1984) and abnormalities in EEG recordings (Takeuchi and Hisanaga, 1977), all of which might be previous effects to the observed total loss of hippocampal rhythmic activity (paper IV). Several cases of abnormal EEG in humans, either occupationally or voluntarily exposed to toluene is reported (Andersen and Kaada, 1953; Knox and Nelson, 1966; Seppäläinen et al, 1980; Grasso et al, 1984).

Changes in biochemical parameters related to aminergic and cholinergic neurons occurs in hippocampus (Yamawaki et al, 1982; Honma, 1983), brain stem (Yamawaki et al, 1982; Rea et al, 1984) and hypothalamic nuclei (Andersson et al, 1980) after short term exposure of rats to toluene levels from 500 to 8000 ppm, supporting the suggestion of a neuronal damage in the theta-generating neural axis from the brain stem through midbrain and septal nuclei to the hippocampus.

### 3 CONCLUSIONS

- 1) Trimethyltin (TMT) caused a selective neuronal damage to the hippocampal subfields CA3, CA4 and partly to CA1. The cell loss in these areas increased gradually during one month after the first administration of TMT.
- 2) The hippocampal damage produced by TMT could be detected by either neurophysiological, histological or biochemical methods, the neurophysiological being the most sensitive.
- 3) Degenerating cells in the hippocampus after TMT-intoxication are glutamergic.
- 4) TMT induced release of glutamate from resting cells, and inhibited reuptake and receptor binding of this neurotransmitter. Release of GABA was not induced by TMT, however reuptake and receptor binding of GABA were inhibited.
- 5) The synaptosomal efflux of D-aspartate is much more sensitive than the efflux of GABA to changes in extracellular  $Cl^-$  concentrations.
- 6) Toluene produced irreversible loss of hippocampal theta activity after exposure to 500 ppm of the solvent for 8 hours/day, 5 days/week in 12 weeks.
- 7) Following toluene exposure an initial period of increased synchronous electric activity in the hippocampus was followed by a gradually increased disruption and a reduction in frequency of the theta waves.

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PAPER I - IV







## Changes in Neurobiological Parameters in the Hippocampus After Exposure to Trimethyltin

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**ABSTRACT:** *The effects of trimethyltin (TMT) on neurotransmitters, morphological changes and physiological activity of the hippocampus were studied. A single injection of TMT (8 mg/kg) decreased the high affinity uptake of glutamate (HA-Glu), which is a marker for glutamergic nerve terminals, after 7 days. The maximal reduction of HA-Glu was 42% and was obtained on postinjection day 21. Glutamate decarboxylase (GAD) and choline acetyltransferase (ChAT), markers for GABAergic and cholinergic structures, were not affected. The electrical activity of the hippocampus recorded through chronically implanted electrodes was altered by day three postinjection. The amplitude of the hippocampal electrographic record gradually decreased and the EEG ceased to be correlated with the rats' behavioral state. Fink-Heimer staining showed degenerating neurons within the subiculum, CA1, ventral CA3 and CA4 hippocampal subfields.*

*TMT (3 mg/kg) injected once a week for three weeks decreased the HA-Glu significantly 21 days after the first injection. The HA-Glu was reduced by a maximum of 68%. The activity of ChAT was slightly increased only at day 35 postinjection while the GAD activity was not significantly reduced over a 21 day period beginning on day 14. Fink-Heimer staining showed degeneration of nerve cells within the CA1, ventral CA3 and CA4 hippocampal subfields.*

*Both injection schedules produced degenerating neurons in the entorhinal cortex. The neurons of the dorsal CA3 region and the granule cells of the dentate gyrus were not lesioned by either TMT injection. The relationship between the behavioral, physiological and neurochemical changes induced by TMT will be discussed.*

**Key words:** Trimethyltin, Hippocampal EEG, High Affinity Glutamate Uptake, Neurotransmitters

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## INTRODUCTION

Trimethyltin (TMT), an organotin compound, is a byproduct in the manufacture of dimethyltin chloride, a stabilizing agent for certain plastics. Exposure to TMT causes neuropathological changes in the limbic system of the brain (Bouldin *et al.*, 1981; Brown *et al.*, 1979). Several studies have demonstrated that the hippocampal pyramidal cells which are limbic system neurons, degenerate after exposure to TMT (Bouldin *et al.*, 1981; Brown *et al.*, 1979; Dyer *et al.*, 1982a; Mushak *et al.*, 1982). Degeneration of these neurons would be expected to produce changes in the neurochemical markers utilized by the affected pathways. For example, the pyramidal cells of the ventral CA3 and CA4 hippocampal subfields appear to be particularly sensitive to TMT's cytotoxic effects (Bouldin *et al.*, 1981; Brown *et al.*, 1979). These neurons project axons which synapse within the stratum radiatum of the hippocampal CA1 subfield (Hjort-Simonsen, 1973). This projection (the Schaffer collaterals) is believed to release glutamate as a neurotransmitter (Nadler *et al.*, 1976; Malthé-Sorensen *et al.*, 1979). The high affinity uptake of glutamate (Fonnum and Walaas, 1979), which is a marker for glutamergic neurons (Fonnum *et al.*, 1981), was examined in the TMT lesioned animals. The hippocampus also contains inhibitory interneurons, the basket cells, which are believed to release gamma-aminobutyric acid (GABA) as a neurotransmitter (Storm-Mathisen, 1977). In addition the hippocampus contains cholinergic terminals derived from the medial septum and the nucleus of the diagonal bank (Fonnum *et al.*, 1970; Storm-Mathisen, 1977). Electrophysiological evidence suggests that TMT will decrease inhibition within the hippocampus (Dyer *et al.*, 1982b). It is not clear whether this reduction is due to a loss of the basket cells or their functional inhibition. Such

an effect could also be mediated via the cholinergic input on the basket cells. We, therefore, utilized neurochemical assays to examine the effects of TMT on the CA3-CA4 Schaffer collateral pathway and the hippocampal inhibitory interneurons.

The hippocampal electrical activity is a well described characteristic of the hippocampus (Vanderwolf, 1969; Winsor, 1974). The destruction of hippocampal pyramidal cells should alter the physiological activity of the hippocampus. In fact TMT first increases, then decrease the amplitude of hippocampal evoked potentials (Zimmer *et al.*, 1982). Hence, we recorded hippocampal electrographic activity to establish the correlation between degeneration of the pyramidal cells and physiological alterations of the hippocampus.

## MATERIALS AND METHODS

### Experimental Animals

Male Wistar rats were used in all experiments. The animals were housed in group cages containing 4-7 rats. Rats implanted with electrodes were placed in individual cages. The animal room was maintained on a 12 hour light-dark cycle. The rats were given food and water *ad libitum*.

### Drug Injection

Trimethyltin chloride was injected on two dosing schedules. The "single" injection schedule consisted of a solitary injection of TMT chloride (8 mg/kg, ip) in corn oil on experimental day 0. The "multiple" injection schedule involved three injections of TMT chloride (3 mg/kg, ip) in corn oil at one week intervals. The injections were made on days 0, 7, 14. The injection volume in all cases, was 1 ml/kg. Trimethyltin chloride was purchased from Aldrich-Europe (Belgium).

### Neurochemical Assays

For uptake and enzyme activity studies the rats were decapitated, the brains removed and placed on ice. The hippocampi were dissected out and rinsed in cold physiological saline. Slices (800  $\mu\text{m}$ ) were cut transverse to the long axis of the hippocampus with a Sorvall tissue chopper (Model TC-2). The CA1 subfield and subiculum including stratum oriens, pyramidale, radiatum and moleculare (Fig. 1) were dissected free and homogenized in cold 0.32 M sucrose (0.5 ml) with a glass-Teflon homogenizer. Samples for the HA glutamate uptake were analyzed immediately. The homogenates were kept frozen ( $-20^{\circ}\text{C}$ ) and enzyme analysis performed within two days after activation of the enzyme.

The HA uptake of glutamate was chosen as a marker for glutamergic nerve terminals (Fonnum *et al.*, 1981). The determination of the HA uptake of glutamate has been described in detail elsewhere (Fonnum *et al.*, 1977). Briefly 2  $\mu\text{l}$  of

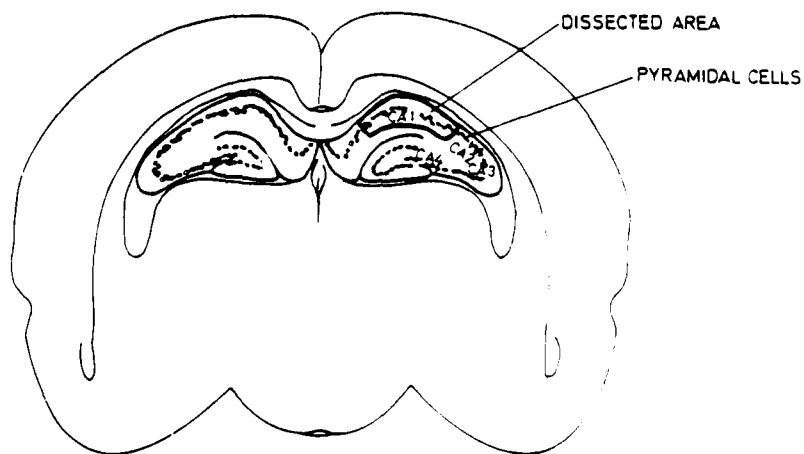
homogenate was added to 0.5  $\mu\text{l}$  Tris Krebs medium, incubated with L-3H-glutamate for 3 min at  $25^{\circ}\text{C}$  and the uptake terminated by Millipore filtration. The amount of labelled glutamate accumulated in the tissue was measured by liquid scintillation counting.

Choline acetyltransferase (ChAT; EC 2.3.1.6) and glutamate decarboxylase (GAD; EC 4.1.1.15) were chosen as neurochemical markers for cholinergic and GABAergic nerve terminals, respectively. Previously described methods were used for determination of GAD (Fonnum *et al.*, 1970) and ChAT (Fonnum, 1975). Protein content was measured by the method of Lowry (Lowry *et al.*, 1951).

### Neurophysiology

Surgical preparation of the rats (275-325 g) was conducted under diazepam (Valium, Roche) and fentanyl, fluanisone (Hypnorm, Mekos) anesthesia. The rat was placed in a stereotaxic frame, an incision made in the skin overlying the skull

### DISSECTION



**FIG. 1.** The dissection for uptake and enzyme activity studies was performed on a 800  $\mu\text{m}$  slice cut out at the level A 3400  $\mu\text{m}$  (Konig and Klippel, 1963). The figure shows the hippocampus omitting other structures in the plane. The dissected area, which is marked in the figure consist of the CA1 subfield, including stratum oriens, pyramidale, radiatum and moleculare, and the subiculum.

and the connective tissue scraped away. Stainless steel screws were set in the skull to serve as anchors. A bipolar electrode of twisted stainless steel wire (200  $\mu\text{m}$ ) insulated except for the tip was implanted in each hippocampus (coordinates AP + 3.2; L + -2.5; P -2.1 bregma dura, Konig and Klippel, 1963). The electrodes were covered with dental acrylic which secured the electrodes to the stainless steel screws. The rats were allowed 10 days to recover before experimentation.

The hippocampal electrical activity from awake freely moving rats was recorded through an EEG preamplifier (Grass model 7PIA) and recorded on a Grass model 7 polygraph. The signal was amplified and filtered (highpass 1 Hz, lowpass 35 Hz). Forty seconds of electrical activity were recorded every ten minutes during each hour long recording session. The single injection schedule rats were recorded on days 0, 3, 7, 14 and 21. The multiple injection rats were injected on days 0, 7, 14 and recorded prior to the injection of TMT and they were recorded on days 21 and 28. The rat's behavior was simultaneously noted on the paper record.

### Histology

The rats were killed by decapitation and the brain removed. The brain was frozen using liquid carbon dioxide and sectioned (20  $\mu\text{m}$ ) using a refrigerated cryostat. The slices were allowed to air dry for two hours then fixed overnight using a  $\text{NaPO}_4$  buffered 10% formalin solution. The slices were then stained by a modification of the Fink-Heimer method (Hjort-Simonsen, 1976). Adjacent sections were stained with Cresyl-Violet.

### Statistics

The neurochemical data were initially analyzed for differences of means by an analysis of variance (ANOVA). Groups of data which contained unequal means were further analyzed by the Neuman-

Keuls multiple range test. A probability of  $p < 0.05$  was accepted as significant in all cases (Zar, 1974).

## RESULTS

### Neurochemistry

**Single injection schedule.** The single injection of TMT significantly altered the HA uptake of glutamate within the hippocampal CA1 subfield (Table 1). The HA-uptake was significantly reduced on day 7 and continued to decrease for the next 14 days. The choline acetyltransferase and glutamic acid decarboxylase activities were not affected during this period.

**Multiple injection schedule.** The "multiple" injection schedule of TMT produced a progressive reduction in the high affinity uptake of glutamate (Table 2). However, this decrease did not appear until day 28. The GAD activity was not affected during the experimental period. ChAT activity was significantly increased only on day 35.

**TABLE 1.** The Effects of a Single Trimethyltin Chloride (8 mg/kg) Injection on Hippocampal Neurotransmitter Markers

Days After Injection	HA-Glu	GAD	ChAT
3	107 $\pm$ 4(12)	114 $\pm$ 5(7)	95 $\pm$ 8(7)
7	77 $\pm$ 5(13)*	94 $\pm$ 6(13)	110 $\pm$ 4(11)
14	68 $\pm$ 5(13)*	91 $\pm$ 5(13)	108 $\pm$ 4(13)
21	58 $\pm$ 5(13)**	115 $\pm$ 6(13)	105 $\pm$ 5(13)

All values are presented as percent of non-injected controls  $\pm$  S.E.M.

\*Denotes values which are significantly different from 3 day values.

\*\*Indicates values significantly different from 3 and 7 day values (Neuman-Keuls multiple range test  $p < 0.05$ ).

The parenthesis ( ) indicate the number of animals tested. Abbreviations: HA-Glu—high affinity glutamate uptake; GAD—glutamate decarboxylase; ChAT—choline acetyltransferase.

**TABLE 2.** Hippocampal Neurochemical Markers Following 3 weekly Injections (3 mg/kg) of Trimethyltin Chloride

Days After First Injection	HA-Glu	GAD	ChAT
22	96±6(9)	102±1(8)	86±10(9)
28	54±9(8)*	94±4(8)	99±7(8)
35	32±3(8)**	104±8(8)	131±15(8)**

The results are expressed as percent of non-injected controls ±S.E.M.

\*Denotes values which are significantly different from 1 week values.

\*\*Denotes values significantly different from 1 and 2 week values (Neuman-Keuls multiple range test,  $p < 0.05$ ).

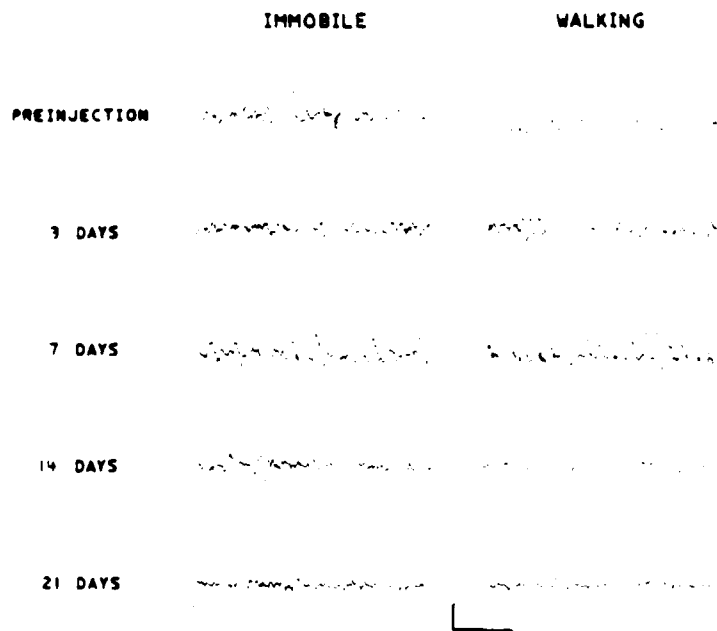
The parenthesis ( ) indicate the number of animals in each sample.

Abbreviations: HA-Glu—high affinity glutamate uptake, GAD—glutamate decarboxylase, ChAT—choline acetyltransferase.

## Neurophysiology

**Preinjection.** The hippocampal electrical activity of awake rats consists of two distinguishable wave patterns (Fig. 2). The electrographic record of a motionless rat consists of low frequency, asynchronous waves and includes variably occurring large amplitude waves (Vanderwolf, 1969). This EEG pattern is referred to as irregular activity (IA). Rhythmic slow activity (RSA) accompanies voluntary movement and is comprised of regular synchronous electrical waves (Vanderwolf, 1969). The RSA of a walking rat has a frequency of 7-8 Hz.

**Single injection schedule.** The "single" injection of TMT produced progressive changes in the hippocampal electrical

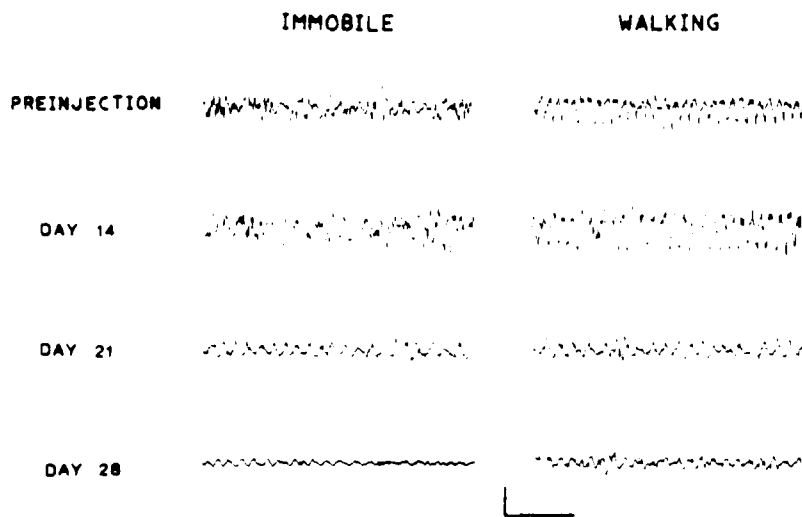


**FIG. 2.** The hippocampal electrical activity recorded before and after a single injection of TMT. The records are from a single rat recorded during two different behavioral states. Immobile describes a motionless but awake, alert rat. Walking refers to active movement within the recording chamber. The number of days indicate the survival time following a single injection of TMT (8 mg/kg). Note the gradual decrease in EEG amplitude with increasing survival time and also the small spikes present during immobility on day 21. The horizontal bar and vertical bar represent 1 sec and 500  $\mu$ V, respectively.

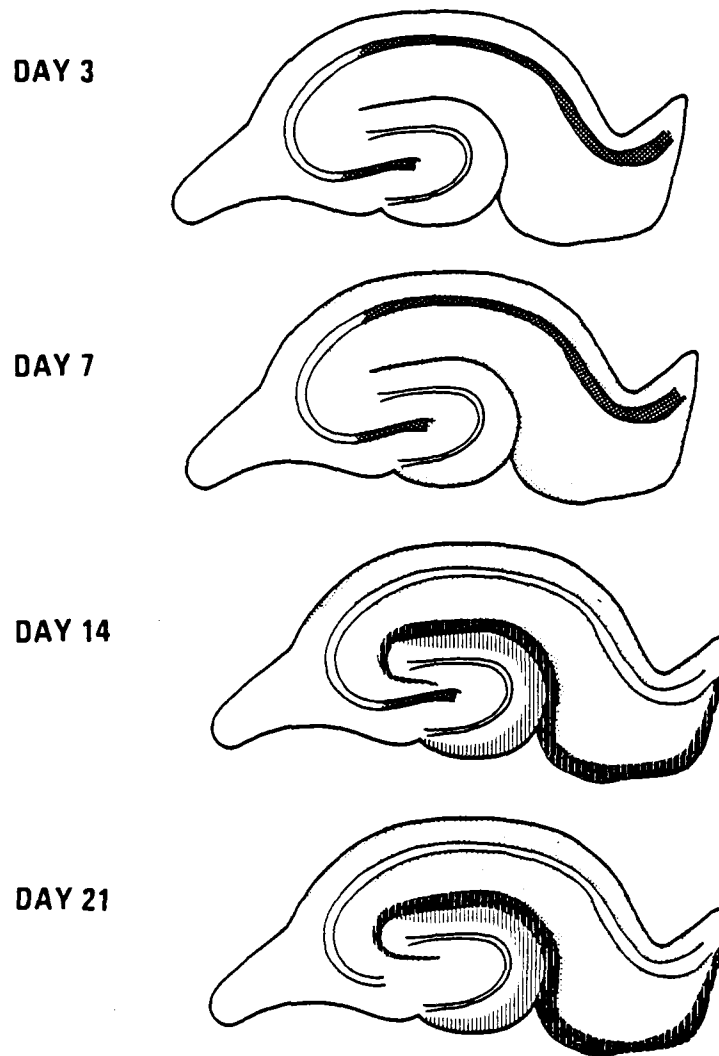
activity of the treated rats (Fig. 2). Three days after the TMT injection both the IA and the RSA amplitude were decreased and the frequency reduced. By day 7 the RSA frequency during walking had decreased from 7-8 Hz to 5-6 Hz. At the same time the electrographic record during alert motionless behavior showed an increased number of slow waves. The RSA amplitude on day 14 was decreased 50% to 60% compared to preinjection controls although regular waves were present. The large slow waves characteristic of IA were almost completely absent having been replaced by low amplitude fast waves. On day 21, the electrical activity recorded during voluntary movement consisted of very low amplitude waves which did not change when the rat became motionless. However, the EEG of a

still rat contained spikes which occurred every 5 to 20 seconds.

**Multiple injection schedule.** The multiple injection schedule of TMT produced gradual changes in the electrical activity of the treated rats (Fig. 3). The electrographic records on day 7 contained RSA and IA which appeared normal. On day 14 the RSA amplitude appeared to be slightly increased and the frequency during walking decreased to 5-6 Hz. The irregular activity during motionless but alert behavior was also increased in amplitude and contained more large slow waves. The EEG characteristics changed dramatically between day 14 and day 21. The hippocampal electrical activity consisted of low amplitude slow waves which did not change as the rats behavior



**FIG. 3.** The hippocampal electrical activity before and after a multiple TMT injection schedule. The records are from a single rat recorded during two different behavior states. The animals received 3 TMT injections (3 mg/kg) spaced one week apart. On days of injections the recordings were done prior to injection. Immobile describes a motionless but awake and alert rat. Walking refers to active movement within the recording chamber. The times indicate the duration since the first TMT injection. Beginning with day 21 the EEG does not change as the rat's behavior changes. The horizontal bar and vertical bar represent 1 sec and 500  $\mu$ V.



**FIG. 4.** Patterns of staining for degenerating neurons following a single TMT injection. Cross-hatching refers to degenerating pyramidal cells within the CA1, subiculum, ventral CA3, and CA4. The fine stippling indicates those dendritic areas showing evidence of heavy degeneration. The white vertical lines with a black background are degenerating axons within the perforant pathway. The staining in this area is lighter than that in the stippled area. The dark vertical lines with a white background is a lighter staining region in the outer 2/3 of the molecular layer of the dentate gyrus. These are probably degenerating terminals of the perforant pathway.

changed. The waves had a frequency of 4-6 Hz and an amplitude approximately half of the control RSA amplitude. The EEG of walking and immobile rats on day 28 was comprised of only low ampli-

tude (<150  $\mu$ V) fast waves which showed no correlation to the animals behavior.

#### Histology

**Single injection schedule.** Three days

after the injection of a single large dose of TMT Fink-Heimer staining provided evidence of degenerating axons within CA1, ventral CA3 and CA4 hippocampal subfields. The subiculum showed only occasionally damaged structures. The resistance of the dorsal CA3 pyramidal cells to TMT's neurotoxic effects is similar to that reported previously (Bouldin *et al.*, 1981; Brown *et al.*, 1979; Valdes *et al.*, 1983). A slight degeneration was present in the stratum radiatum and the stratum oriens of CA1. The entorhinal cortex contained neuronal cell bodies showing signs of degeneration.

Seven days postinjection the ventral CA3 and the CA4 subfields still contained the largest amount of degeneration (Fig. 4). There were signs of increased degeneration within the CA1 subfields. The stratum oriens, stratum radiatum and the stratum lacunosum-moleculare showed signs of degeneration. Additionally there was evidence of damaged nerve tissue in the outer portion of the molecular layer of the dentate gyrus. This may represent degenerating perforant pathway terminals originating from entorhinal cortex neurons (Hjort-Simonsen and Jeune, 1972). The cresyl-violet stained sections of day 21 showed the absence of neurons in the ventral CA3 and CA4 subfields.

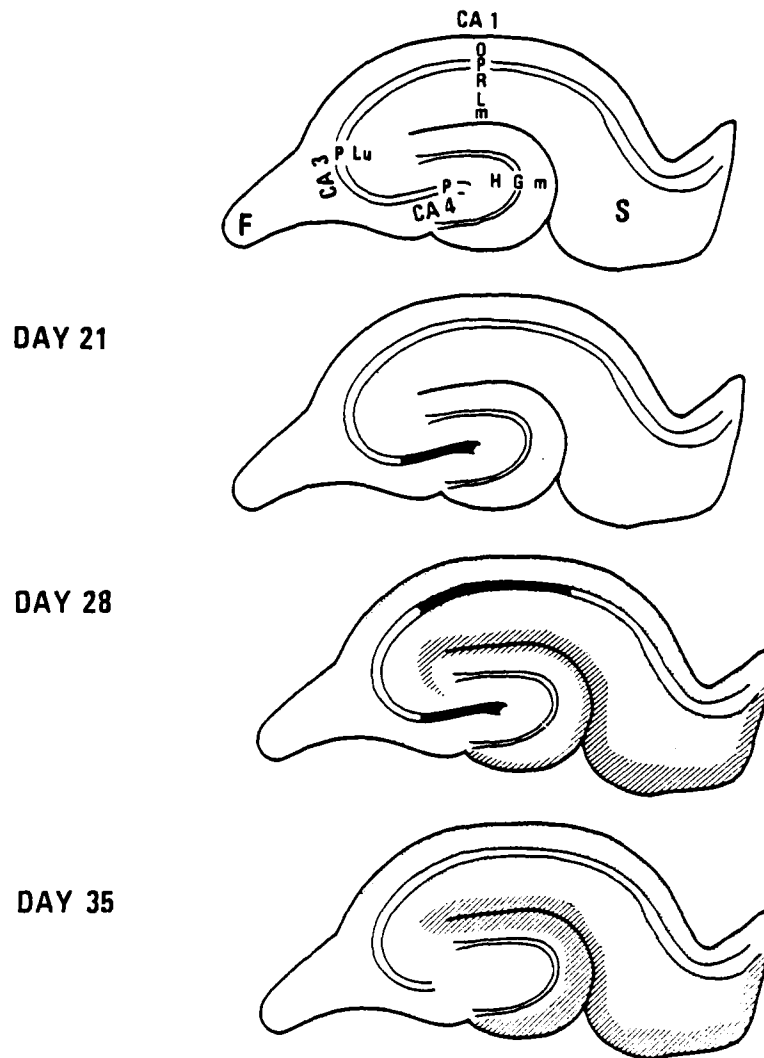
**Multiple injection schedule.** The multiple TMT injection schedule produced a pattern of degenerating neurons different from the single injection schedule (Fig. 6, 7). On day 21, the ventral CA3 and the CA4 subfield contained evidence of degenerating neurons (Fig. 5). A week later, evidence of damaged neurons was found in the CA1 in addition to the ventral CA3 and CA4 subfields. The neurons within the subiculum and the dorsal CA3 appeared to be insensitive to TMT's neurotoxic effects. The perforant pathway and its terminal field, the outer 2/3 of the molecular layer of the dentate gyrus and molecular layer of hippocampus

showed signs of damaged nervous tissue. On day 35 only the dendritic regions showed darkly staining areas. The cresyl violet stained sections showed an absence of nerve cell bodies in the ventral CA3 or the CA4 pyramidal cell areas and also a loss of CA1 neurons (Fig. 7).

## DISCUSSION

Trimethyltin produces physiological, morphological and biochemical changes within the hippocampus. Following a single TMT injection the histological and physiological changes occurred within 3 days while the biochemical alterations occurred after 7 days. With the multiple injection schedule the EEG was changed after 14 days while the histological and biochemical parameters were changed after 28 days.

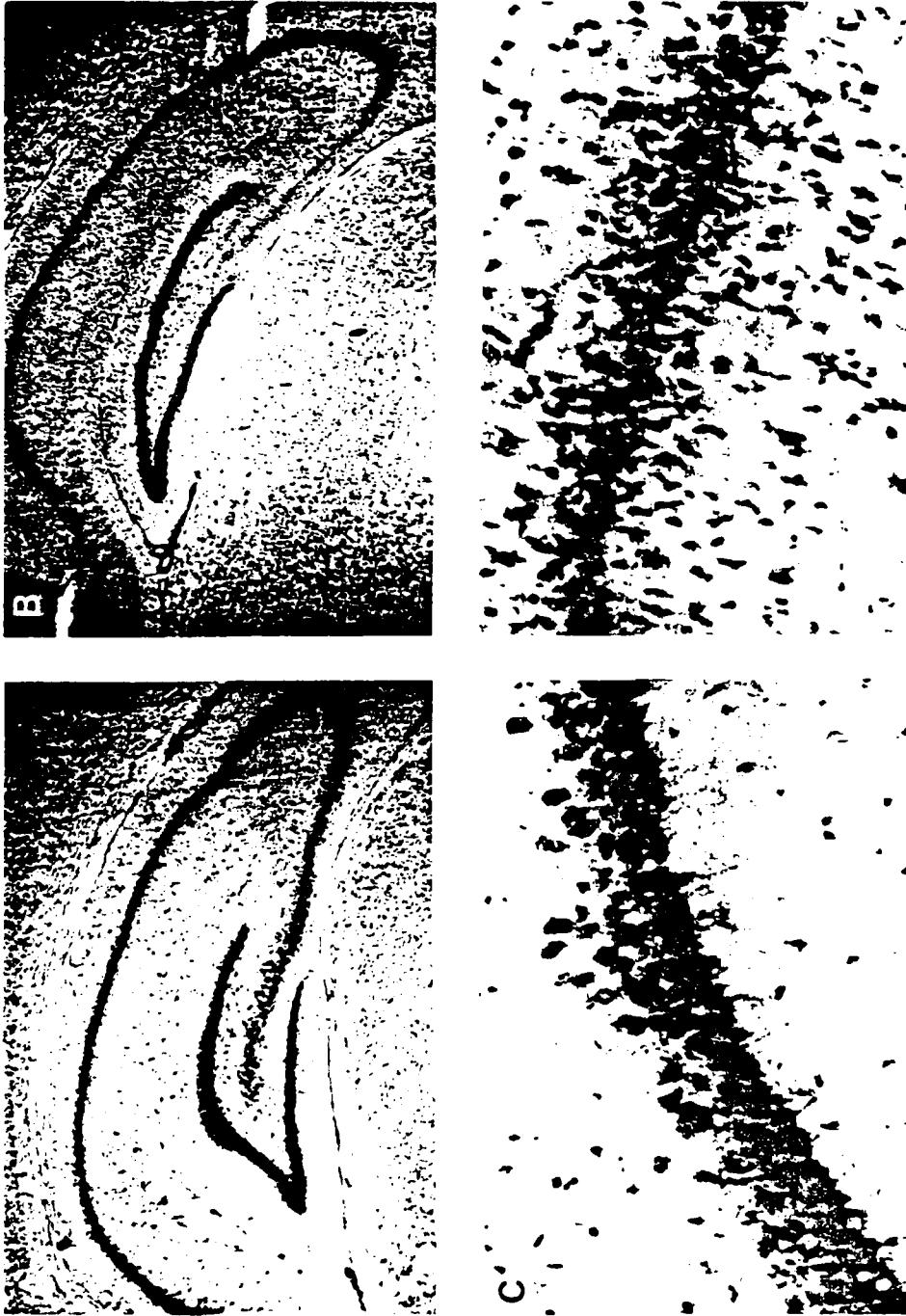
The CA4 pyramidal cells and the ventral CA3 pyramidal cells are the most sensitive to the toxicant actions of TMT. These pyramidal cells have almost completely disappeared by 21 days after a single TMT injection or by 35 days after beginning the multiple injection schedule. The biochemical or morphological characteristics which make this subfield so sensitive to TMT exposure are unknown. The CA1 pyramidal cells are less sensitive to TMT induced degeneration. Although neurons within this subfield degenerate they do not completely disappear (Fig. 6; Dyer *et al.*, 1982a). The pattern of TMT induced degeneration of the CA1 and CA4 pyramidal cells is similar to that of previous investigations (Brown *et al.*, 1979, Bouldin *et al.*, 1981, Mushak *et al.*, 1982, Valdes *et al.*, 1983). The sparing of the dorsal CA3 neurons observed in the present study has been observed by these research groups although a progressive degeneration of the pyramidal cells beginning in the CA4 region and extending as far as the CA2 (Dyer *et al.*, 1982) has also been reported. A combination of differences between dosing regimens, injec-



**FIG. 5.** Schematic drawing of the hippocampus showing the degenerating pattern in major subdivisions after multiple TMT injections. The black regions indicate areas containing degenerating pyramidal cells. The stippling refers to areas heavily stained for the presence of degenerating fibers. The dark slanted lines indicate lighter staining degenerating areas. Abbreviations: S—subiculum, F—fimbria, P—pyramidal cell body layer, O—stratum oriens, R—stratum radiatum, L,m—stratum lacunosum-moleculare, Lu—stratum lucidum, H—hilus of fasciae dentatae, G—dentate granule cells, m—molecular layer of the dentate gyrus, CA1, CA3 and CA4—subfields of the hippocampal pyramidal cells

tion vehicle and time of sacrifice probably produce different absorption rates and a final brain TMT concentration differentially affecting the dorsal CA3 region.

The degeneration of pyramidal cells will reduce the markers for the neurotransmitters utilized by these neurons. The CA3 and CA4 pyramidal cells pro-



**FIG. 6.** Cresyl-violet stained sections before (A and C) and 35 days after (B and D) the multiple TMT injections schedule (3 x 3 mg/kg). Note the decreased width in the CA1 pyramidal cell subfield and the absence of pyramidal cells in the ventral CA3 and CA4 regions. C and D shows the CA1 area. D shows a reduction in number of pyramidal cells and an increase in glial cells in the dendritic region.

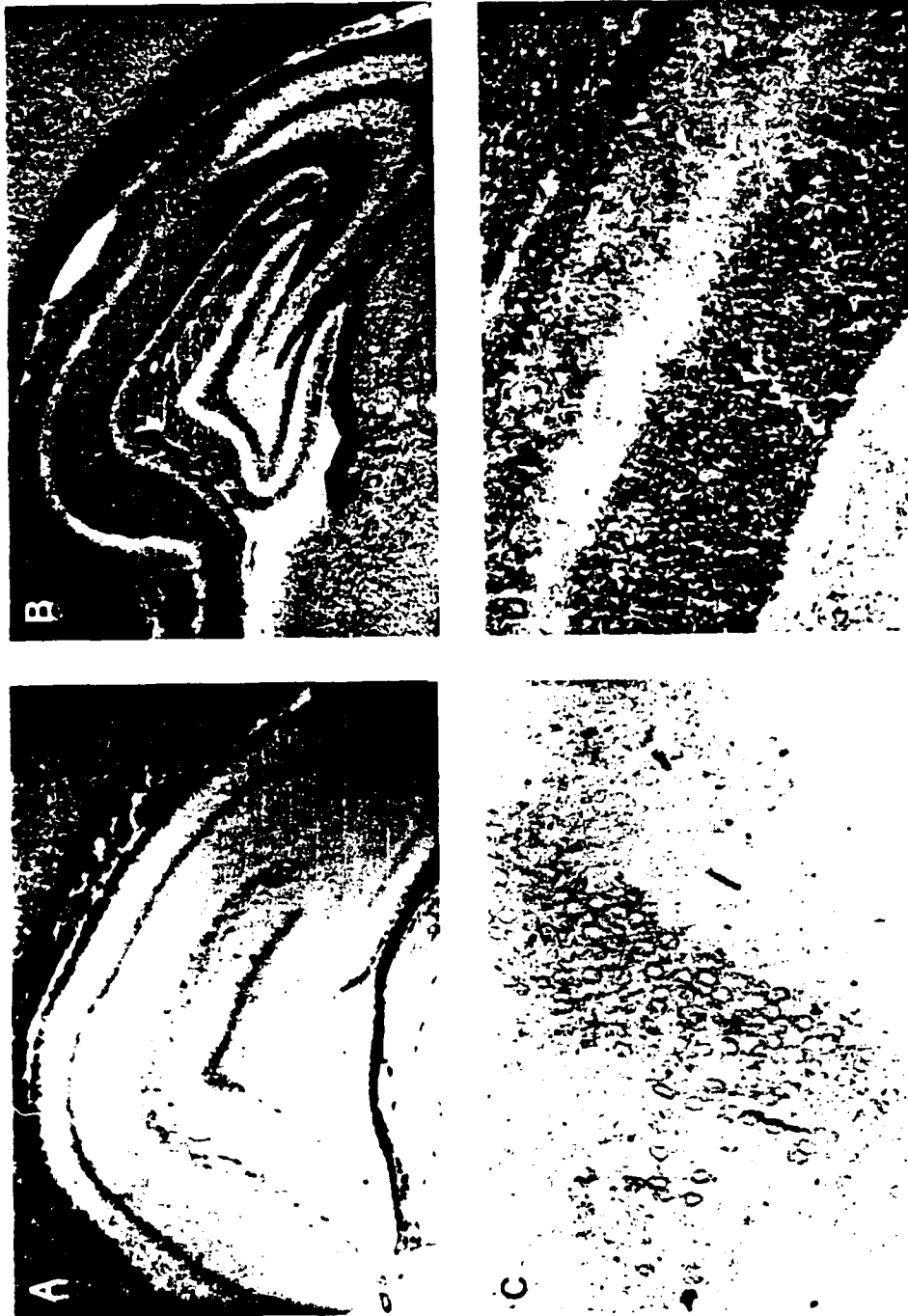


FIG. 7. Fink-Heimer stained sections before (A and C) and 35 days after (B and D) a multiple TMT injection schedule (3 x 3 mg/kg). The dark staining in the dendritic sections indicate massive degeneration. The cell body layer of CA1, CA3 and CA4 subfields seems completely absent. C and D shows the CA1 area

ject axons, the Schaffer collaterals, which synapse on dendrites of the CA1 neurons (Hjort-Simonsen, 1973). The terminals of the CA3 and CA4 neurons are believed to release glutamate as a neurotransmitter (Nadler *et al.*, 1976; Malthé-Sorensen *et al.*, 1979). The first signs of degenerating cell bodies appear in the CA3 and CA4 region 3 days after a single dose of TMT (8 mg, kg, ip). At this time the high affinity glutamate uptake, a marker for glutamergic terminals, remains at control levels. The HA Glu is reduced 25% seven days postinjection (Table 1). Simultaneously there is evidence of degeneration within the stratum radiatum, the terminal zone for the Schaffer collaterals. Although it would be nice to say we can identify degenerating terminals the staining within the stratum radiatum probably includes degenerating dendrites of the CA1 neurons. However, the data clearly demonstrates that the CA3 and CA4 cell bodies show the first degeneration after TMT exposure followed by degeneration in the terminal fields. The data reported here supports the conclusion of Valdes *et al.*, (1983) that the decreased high affinity uptake of glutamate is secondary to the loss of pyramidal cell neurons.

The activity of glutamic acid decarboxylase, a specific marker for GABAergic terminals is not altered by TMT exposure. These observations suggest that the basket cells, inhibitory interneurons within the CA1 subfield are not lesioned by TMT. Several authors have suggested based on physiological and biochemical data that TMT alters the activity of the inhibitory interneurons. For example, TMT exposure reduces the concentration of GABA within the hippocampus (Mailman *et al.*, 1983) suggesting that TMT will affect the GABAergic systems. The decreased GABA concentration may be secondary to a reduction in the high affinity uptake of 3H-GABA which appears to occur in a nonspecific manner (Doctor *et al.*, 1982). The decreased GABA levels

may be the mechanism which reduces the recurrent inhibition of the CA3 region following exposure to TMT (Dyer *et al.*, 1982b).

Trimethyltin in addition to killing pyramidal cells will alter the physiological activity of the hippocampus (Allen and Fonnum, 1984). TMT reduces the amplitude of dentate granule cell evoked potentials generated by stimulation of the pyriform cortex (Zimmer *et al.*, 1982). The activation of the granule cells by pyriform cortex stimulation is via a pathway which projects from the pyriform cortex and synapses upon neurons of the entorhinal cortex (Hjort-Simonsen, 1973). The entorhinal neurons project axons via the perforant pathway synapsing on the granule cells of the dentate gyrus. Zimmer and co-workers (Zimmer *et al.*, 1982) postulated that the transmission failure occurred within the entorhinal cortex. The histological data presented here supports the idea that the reduction in granule cell evoked potentials is due to a degeneration of entorhinal cortex neurons and axons of the perforant pathway. The degeneration within the outer 2/3 of the molecular layer represents terminal degeneration of the perforant pathways since this is the area where they are known to synapse (Hjort-Simonsen, 1973). The evoked potentials reach a very low amplitude between 8 to 20 days post injection. Using a similar dose (8.0 mg/kg vs. 7.5 mg/kg by Zimmer *et al.*, 1982) we observed degenerating axons beginning on day 14. However, neurons of the fascia dentata are not immune to TMTs effects. Bouldin *et al.*, (1981) reported that short term high doses (5 mg/kg day, 3 days) produced damage which is limited to granule cell neurons in the fascia dentata while in low dose long term (1 mg/kg on alternate days, 14 injections) animals the neuronal loss was much more marked in the pyramidal neurons of the hippocampus. Brown *et al.* (1979) have also reported

damaged cells of the fascia dentata after a single high dose injection. Damage to the granule cells contributing to the degeneration seen in the molecular layer could not be ruled out.

The hippocampal electrical activity recorded in these experiments is an extracellular correlate of the activity of intracellular potentials. For example, the rhythmic slow activity generator appears to be within the cell body layer (Bland *et al.*, 1976; Green *et al.*, 1979). Fujita and Sato (1964) demonstrated the presence of an intracellular RSA which was composed of excitatory postsynaptic potentials. The generation of the electrical waves is thought to be a combination of excitation of the pyramidal cells and an inhibition by the basket cells (Kandel *et al.*, 1961; Anderson and Eccles, 1962). These observations show that an intact hippocampal neuronal circuitry is necessary for the generation of normal hippocampal electrical activity. The loss of pyramidal cells after TMT exposure would be expected to alter the physiological activity of the hippocampus. TMT exposure alters the electrical activity of the hippocampus within 3 days (Fig. 2; Ray, 1981). These alterations occur at a time when the first signs of degeneration appear in the hippocampus (Fig. 4). Ray (1981) recorded an increased RSA amplitude from the granule cells on days 2-4 following a single 10 mg/kg oral dose of TMT. These observations contrast with the decreased amplitude of the present studies (Fig. 2). These opposite findings suggest differential effects of TMT on the CA1 pyramidal cells and the granule cell neurons. The dysfunction of the hippocampus electrical activity may provide a clue to the deficits in behavioral task performance following TMT treatment (Miller *et al.*, 1982; Swartzwelder *et al.*, 1982; Walsh *et al.*, 1982).

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THE EFFECT OF TRIMETHYLTIN ON THREE GLUTAMERGIC AND GABAERGIC  
TRANSMITTER PARAMETERS IN VITRO; HIGH AFFINITY UPTAKE, RELEASE  
AND RECEPTOR BINDING.

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## ABSTRACT

The effects of trimethyltin (TMT) on high-affinity uptake, release and sodium-independent binding of glutamic acid and  $\gamma$ -aminobutyric acid (GABA) were studied in vitro in homogenates of hippocampal tissue. TMT (10  $\mu$ M) increased the release of glutamic acid from synaptosomes in the resting state (5 mM  $K^+$ ), whereas the release of GABA was only slightly affected. High affinity uptake of glutamate was inhibited by TMT in the same concentration range as release. The uptake of GABA was only affected by TMT-concentrations from 500  $\mu$ M to 5 mM. The sodium independent binding of both glutamate and GABA, usually assumed to be binding to receptor sites, were inhibited with 50  $\mu$ M or more TMT in the incubation medium. The results indicate that TMT can interfere with several different events of the neurotransmission process in the central nervous system at concentrations which can be obtained in the brain of rats after a sublethal dose of the compound.

Running title: In Vitro effects of trimethyltin

Key words: Trimethyltin, Hippocampus, In Vitro-study, Glutamate Release.

## INTRODUCTION

Many organic tin compounds are now in widespread use. In industry organotins are used as stabilizers in certain plastic polymers, as catalysts for polyurethane formation, silicone curing, esterification, epoxy curing, olefine polymerization, etc. Some organotin compounds are known to have biocidal effects and are used as fungicides, bactericides, algicides, helminthics, insecticides and chemosterilants, (Barnes and Stoner, 1959; Environmental Protection Agency, 1976).

The most toxic organotin analoges are triethyltin (TET) and trimethyltin (TMT), which are absorbed via the skin and gastrointestinal tract. The other alkyl and aryl tin compounds are poorly absorbed. TET-acetate has a LD50 value of 4 mg/kg when given orally to rats, while for TMT-acetate the value is 9.1 mg/kg. The corresponding values for higher tin analoges are for propyl, 118.3; butyl, 380.2; hexyl, 1000; octyl > 1000 (Watanabe, 1980).

Exposure to triethyltin (TET) is known to produce cerebral edema and demyelination in the central nervous system (Barnes and Stoner, 1959; Aleu et al., 1963). Unlike the other alkyltins TMT produces neuronal necrosis rather than intramyelinic edema (Brown et al., 1979). At low doses the hippocampal formation and pyriform cortex seem to be the most vulnerable regions (Brown et al., 1979; Bouldin et al., 1981). The hippocampal pyramidal cells have been demonstrated to degenerate selectively after TMT-exposure (Brown et al., 1979; Bouldin et al., 1981; Naalsund et al., 1985; Dyer et al., 1982). We have previously measured different neurobiological parameters in the hippocampus of TMT-exposed rats and found that the glutamergic pathways are degenerating while the cholinergic and gabaergic pathways are not affected (Naalsund et al., 1985). Histological changes were detected three days after injection of a single dose (8 mg/kg i.p.) and the hippocampal EFC changed gradually from day three after the TMT-injection (Naalsund et al., 1985). A reduction in a glutamate transmitter parameter (high affinity uptake) as an indicator of degeneration of glutamergic terminals, was observed 8 days after injection.

In an in vitro recording of the electrically evoked potential of the pyramidal cells in the hippocampus TMT was shown to depress the activity of the postsynaptic CA1 neurons in a dose dependent manner at concentrations from 3.6  $\mu\text{g}$  to 10  $\mu\text{g}/\text{ml}$  (Allen and Fonnum, 1984). In the present study the aim was to investigate the in vitro effects of TMT on the biochemical processes of transmission in neuronal tissue. The neuronal degeneration seen in the hippocampus after trimethyltin exposure could be a result of the disruption of neurotransmitter functions through an effect on some basic cellular functions. We present here the results of the effect of trimethyltin in vitro on the high affinity uptake, spontaneous release from synaptosomes and on receptor binding of tritiated L-glutamic acid and  $\gamma$ -aminobutyric acid.

## MATERIALS AND METHODS

### CHEMICALS

Trimethyltin-chloride, 9%, (Aldrich-Europe) was dissolved to 50 mM concentration in distilled water and added in suitable volumes to the test media to give the desired concentrations. L-[2,3- $^3\text{H}$ ] glutamic-acid, 20 Ci/mmol, D-[2,3- $^3\text{H}$ ] aspartic acid, 14 Ci/mmol,  $\gamma$ -[2,3- $^3\text{H}(\text{N})$ ] aminobutyric acid (GABA), 30 Ci/mmol, were all obtained from New England Nuclear.

### TISSUE PREPARATION

Tissue homogenates were prepared from the hippocampus dissected from the brain of young adult male Wistar rats (150-250 g). The rats were killed by decapitation, the brains were removed and placed on ice. The hippocampi were dissected and homogenized in cold sucrose (0.32 M) at 800 rev/min with a glass-teflon homogenizer. These homogenates were used for uptake and release experiments.

For the experiments on synaptosomal and mitochondrial fractions, a modification of the procedure described by Gray and Whittaker (1962)

was used. Homogenates in 0.32 M sucrose were centrifugated at 1000 xg for 10 min and the pellets washed once. The combined supernatants were then centrifugated at 20 000 xg for 20 min and the P2-pellets were resuspended in 0.32 M sucrose and placed on a discontinuous density gradient consisting of 0.8, 1.2 and 1.6 M sucrose. Separation was achieved by centrifugation on a swing-out head in an ultracentrifuge at 150 000 xg for 30 min. The gradients were carefully collected through a hole at the bottom of the tube. The layers between 1.6 and 1.2 M were collected as the mitochondrial fraction and the layers between 0.8 and 1.2 M as the synaptosomal fraction.

#### NEUROTRANSMITTER RELEASE

Release of [ $^3\text{H}$ ] glutamic acid and [ $^3\text{H}$ ] GABA was measured by a modification of the method described by Hammerstad et al. (1979). Briefly 2  $\mu\text{l}$  homogenate containing about 2  $\mu\text{g}$  protein were absorbed to a small piece of millipore filter. The filters were placed in a small tissue culture dish, four filters in each dish, and 4 ml Krebs-Hepes buffer containing 27,5 mM Hepes, 138 mM NaCl, 2,4 mM KCl, 1 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 5 mM  $\text{KH}_2\text{PO}_4$ , 1,6 mM glucose were added. The synaptosome pools were labelled with [ $^3\text{H}$ ] glutamic acid, [ $^3\text{H}$ ] aspartic acid or [ $^3\text{H}$ ] GABA by incubation in the Krebs-Hepes buffer containing 5  $\mu\text{M}$  labeled transmitter (20-30 Ci/mmol) for 20 min at 25°C. The filters were washed four times in Krebs-Hepes buffer and then each one placed in a small perfusion chamber made of two disposable plastic tips. The chambers were continuously perfused with Krebs-Hepes buffer. The liquid flow was held constant at 0.4 ml/min and perfusate was collected in fractions of 0.8 ml. The chambers were washed for 20 min with Krebs-Hepes buffer and the release studied with Krebs-Hepes buffer containing 50 mM  $\text{K}^+$  in stead of an equivalent amount of NaCl or Krebs-Hepes buffer containing different concentrations of trimethyltin ( $10^{-5}$  -  $10^{-3}$  M) for 4 min periods. The fractions (0.8 ml) of the perfusate were added 10 ml Insta-Gel (Packard) and the radioactivity were counted. The filters were dissolved in Filter Count (Packard) and the rest activity measured. The results were calculated as percentage fractional release. The fractional release during perfusion with wash

buffer (5 mM  $K^+$ ) immediately before TMT perfusion were set to 100% and changes in fractional release were expressed in per cent of this normal spontaneous release. Eight superfusion assays were run parallel by the use of an eight channel peristaltic pump. Two assays with potassium stimulation (50 mM) were run each time as positive controls of the method. The other six were subject to TMT-perfusion for four minutes pulses.

#### HIGH AFFINITY UPTAKE OF NEUROTRANSMITTER

High affinity uptake of [ $^3H$ ] glutamic acid and [ $^3H$ ] GABA was measured by the method described by Fonnum et al., 1980. 2  $\mu$ l homogenate (containing ca 6  $\mu$ g protein) were added to 0.5 ml Tris-Krebs medium containing 15 mM Tris, 140 mM NaCl, 5 mM KCl, 1.2 mM  $CaCl_2$ , 1.2 mM  $MgCl_2$ , 1.2 mM  $Na_2HPO_4$ , 10 mM glucose and pH adjusted to 7.4. The TMT was added to the Tris-Krebs buffer before addition of the tissue. The mixtures were preincubated for 15 min before incubation with 70 nM tritiated transmitter (20-30 Ci/mmol) for 3 min at 25°C. The uptake was terminated by filtration in a Titertek cell harvester with a Titertek filtermat. The filters were dissolved in 5 ml Filter Count (Packard) and the radioactivities were counted. For each animal triplicates of the high affinity uptake were performed at each TMT concentration and of TMT-free controls.

#### RECEPTORBINDING

We have measured the sodium-independent binding of transmitter to synaptosomal membranes. This binding is commonly referred to as the receptor binding (Foster and Fagg, 1984). Binding of [ $^3H$ ] glutamic acid and [ $^3H$ ] GABA was measured by a modification of the method described by Hill et al. (1984). The hippocampi were homogenized in 20 volumes 0.32 M sucrose, centrifugated at 1000 xg for 10 min and the pellets were washed once. The combined supernatants were then centrifugated at 20 000 xg for 20 min and the pellets were resuspended to the same volume with cold, distilled water and kept on ice for one hour. After centrifugation at 8000 xg for 20 min the supernatants were gently decanted and the upper layer of the pellets were rinsed off and added

to the supernatant. These combined supernatants were recentrifuged at 48 000 xg for 20 min, and the pellets were stored frozen at -20°C prior to use.

For the binding assay, membranes were thawed and resuspended in Tris-HCl buffer (50 mM, pH 7.4) and incubated for 45 min at room temperature before centrifugation (8500x g for 10 min). The membrane suspensions were further washed three times and incubated with the Tris-buffer for 15 min at room temperature between each centrifugation. The final pellets were resuspended to give a protein concentration of about 20-80 µg per assay. Binding of [<sup>3</sup>H] glutamic acid or [<sup>3</sup>H] GABA (final concentrations 100 and 50 nM with a specific activity of 20 and 30 Ci/mmol respectively, or varying ligand concentrations in saturation studies) were assayed in absence or presence of 1 mM unlabeled transmitter, to determine the specific transmitter binding. Varying concentrations of trimethyltin were added to the mixture before a preincubation period of 15 min, and then after addition of the ligand the mixtures were incubated for 15 min at 30°C for glutamic acid (Raudry and Lynch, 1980) and for 10 min at 20°C for GABA (Hill et al., 1984). Binding was terminated by Titertek filtration. The filters were dissolved in Filter Count (Packard) and the radioactivities were counted. For each animal were run four assays with total binding and two assays with unspecific binding at each TMT concentration as well as of TMT-free controls.

#### PROTEIN MEASUREMENTS

Protein content in the homogenates were measured by the method of Lowry (Lowry et al., 1951).

#### SCINTILLATION COUNTING

Counting efficiency was 40% for the perfusates and 50% for filters dissolved in Filter Count.

## STATISTICS

In release experiments each assay was used as its own control. For high affinity uptake and binding studies the results were compared in groups and each group compared with one common control group. The results were analyzed for differences of means by an analysis of variance (ANOVA). Groups of data which contained unequal means were further analyzed by Dunnet's test. A probability of  $p < 0.05$  was accepted as significant in all cases.

## RESULTS

### TRANSMITTER RELEASE

[<sup>3</sup>H] glutamate was released by perfusion with TMT at concentrations from 0.05 mM to 1 mM (table 1). The release was measured by exposure of homogenates in the resting state to TMT, i.e. at a potassium concentration of 5 mM. D-[<sup>3</sup>H] aspartate, commonly used as a false transmitter for glutamate (Fonnum, 1984) behaved similar. This compound which is not metabolised, gave as expected higher releases. The release of [<sup>3</sup>H] GABA was studied in parallel and was not significantly higher than normal spontaneous release in the presence of TMT in the perfusionbuffer at concentrations from 10 mM to 1 mM. The addition of different concentrations of TMT in the potassium depolarization buffer (50 mM K<sup>+</sup>) did not give significant changes in the release of transmitters investigated (data not shown).

### REPETITIVE STIMULATION

Several stimulations with high potassium concentrations (50 mM) reduced the fractional release of glutamate (figure 1). The effect of TMT induced release was similar. A previous potassium stimulated release did not affect the amount of glutamate released by a TMT stimulation (0.1 mM), and the potassium stimulated release was not reduced by a previous TMT stimulation (figure 1).

#### Ca-DEPENDENCE

The release was normally performed in an assay medium containing 2 mM calcium. A replacement of the calcium chloride with magnesium sulfate (10 mM) resulted in a  $45\% \pm 7\%$  (N=10) decrease in release after depolarization with potassium. The effect of the calcium concentration in the perfusion buffer on the release induced by TMT was a  $24\% \pm 12\%$  (N=9) decrease in release.

#### EFFECT ON SUBCELLULAR FRACTIONS

The mitochondria and synaptosomes were isolated in two fractions to see whether the transmitter released by TMT exposure might be derived from the mitochondria rather than from the synaptosomes. The amount of uptake and release of tritiated transmitter in the mitochondrial fraction was 12% of the amount in the synaptosomal fraction. Fractional release from the two fractions were similar both after exposure to 0.5 mM TMT and potassium stimulation. The small release from mitochondrial fraction may well be due to the presence of contaminating synaptosomes.

#### Na-CHANNELS

Tetrodotoxin (TTX) was used to study the importance of Na-channels (Narahashi, 1984; Catterall, 1984) in the TMT induced transmitter release. The results showed that the release by veratridine was reduced to  $9\% \pm 2\%$  (N=4) by TTX as expected. In contrast potassium (50 mM) stimulated and TMT (0.5 mM) stimulated release were not affected by TTX. These results suggest that the Na-channel is not involved in the release of transmitter mediated by TMT.

#### TISSUE CONCENTRATION

The effect of tissue concentration on the amount of [ $^3\text{H}$ ] glutamate released during perfusion of the tissue homogenate with 0.5 mM TMT was measured. At tissue concentrations from 2 - 6% the fractional release of transmitter was in the same range.

#### BRAIN REGION

The effect of TMT on transmitter release is here demonstrated on hippocampal homogenate. Similar results were obtained by using homogenate from neostriatum.

#### HIGH AFFINITY UPTAKE

The high affinity uptake of [ $^3\text{H}$ ] glutamate into synaptosomes was significantly reduced (20 - 30%) with TMT concentrations from 50  $\mu\text{M}$  to 1 mM in the incubation mixture (table 1). At 5 mM TMT the reduction was enhanced to 60%. The high affinity uptake of [ $^3\text{H}$ ] GABA was reduced by 50 - 85% at TMT-concentrations from 0.5 mM to 5 mM.

#### RECEPTOR BINDING

The receptor binding of [ $^3\text{H}$ ] glutamate and [ $^3\text{H}$ ] GABA was inhibited by TMT (Table 1). At a ligand concentration of 100 nM the binding of [ $^3\text{H}$ ] glutamate was reduced by 25% in a medium with 50  $\mu\text{M}$  TMT compared to TMT-free controls. The binding of [ $^3\text{H}$ ] GABA was not significantly altered at this TMT concentration and a 50 nM ligand concentration. 100 mM and higher concentrations of TMT produced a large inhibition (50 - 90%) of binding of both ligands. Figure 2 shows the saturation of the glutamate and GABA receptors at different TMT-concentrations. The effect of the tin-compound seemed to be a reduction in the number of sites ( $B_{\text{max}}$ ) for glutamate binding (Table 6) while  $B_{\text{max}}$  for GABA binding and affinity constants ( $K_d$ ) for both ligands were not significantly altered.

#### DISCUSSION

The release of L-[ $^3\text{H}$ ] glutamic acid and D-[ $^3\text{H}$ ] aspartic acid from synaptosomes have been shown to be enhanced by TMT at concentrations from 50  $\mu\text{M}$  to 1 mM, while the release of [ $^3\text{H}$ ] GABA was not significantly changed. The enhanced release of glutamate produced by

TMT has been shown to be calcium dependent, suggesting a synaptic release. High affinity uptake of transmitter is inhibited at concentrations from 50 mM to 5 mM for [ $^3\text{H}$ ] glutamate and from 500 mM to 5 mM for [ $^3\text{H}$ ] GABA. Binding of glutamate and GABA to synaptic membranes was reduced by concentrations of TMT from 50 mM to 1 mM (Table 1, Figure 2).

TMT has been reported to produce neuronal necrosis in the hippocampal formation after intraperitoneal administration of two doses of 4 mg/kg body weight to rats (Brown et al, 1979). The amount of TMT in the hippocampus after injection of the neurotoxic dose has been measured to 9.1 nmol/g wet weight (Aldrich, personal communication). The lowest concentration of TMT reported here to produce an interruption of neurotransmission (50 mM = 50 nmol/ml) in vitro is about five times higher than in the in vivo situation. This concentration is also in the same range as the concentration (3.5 mg/ml = 17.5 nmol/ml) required to depress the electric activity of the hippocampal neurons in vitro (Allen and Fonnum, 1984).<sup>1</sup>

The TMT stimulated release of glutamate was much smaller but had the same characteristics of the  $\text{K}^+$  stimulated release in that it was  $\text{Ca}^{++}$ -dependent and TTX independent. The induction of release by TMT seems to be specific for glutamate, since neither GABA (Table 1) nor acetylcholine release (Morot-Gaudry, 1984) is induced by TMT. The release of acetylcholine was inhibited by TMT during depolarization (Morot-Gaudry, 1984) when tested at a much higher TMT concentration, namely from 0.5 to 3.5 mM. The effect of TMT on acetylcholine and glutamate is therefore probably quite different.

Organotincompounds have been shown to induce several effects on membrane systems. They are associated with  $\text{Cl}^-/\text{OH}^-$  exchanges in mitochondria (Selwyn et al., 1970), erythrocytes and liposomes (Selwyn et al., 1970; Motais et al., 1977; Wieth and Tosteson, 1979) and in pancreatic  $\beta$ -cells (Pace and Tarvin, 1983). This exchange could produce a change in the internal and external  $\text{Cl}^-$ -concentration. A reduction in the extracellular  $\text{Cl}^-$ -concentration has been shown to

induce a glutamate release in vitro similiar to that induced by high potassium concentration (Hardy et al., 1984; Naalsund and Fonnum, 1986). This release takes place even at low changes in  $\text{Cl}^-$  concentration (Naalsund and Fonnum, 1986). A TMT induced chronic increased efflux of glutamate might be due to an alteration in external  $\text{Cl}^-$  concentration. Reduction in chloride concentration do not have the same stimulatory effect on the release of the inhibitory

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<sup>1</sup>According to the authors the actual concentrations used in these experiments are given in the discussion section of the paper. An error in the calculations has produced disagreement between molar and  $\mu\text{g}$ -concentrations.

transmitter GABA. (Naalsund and Fonnum, 1986). The lack of effect of TMT on GABA release supports a theory on  $\text{Cl}^-$  ions as mediators of TMT induced release. Measurements on anion exchanges in synaptosomes in the presence of TMT are at present lacking.

Our results on inhibition of uptake of GABA are in agreement with Doctor et al. (1982), who reported a 50% inhibition of GABA uptake in mouse forebrain synaptosomes at a TMT concentration of 75  $\mu\text{M}$ . We found an inhibition of uptake of both GABA and glutamate with a slightly lower sensitivity of the GABA uptake to the TMT exposure than reported by Doctor et al.

The binding of glutamate and GABA to CNS membranes has been described by a number of different methods (for review see Foster and Fagg, 1984). The values for the equilibrium dissociation constant ( $K_d$ ) and binding capacity ( $B_{\text{max}}$ ) in the literature varies widely. We have measured the binding to a specific population (saturable) of high to medium affinity binding sites ( $K_d < 500 \text{ nM}$ ) in a preparation of crude synaptic membranes.  $\text{Na}^+$ -ions were not present in the incubation mixture to exclude binding to high affinity uptake sites. The ability of TMT to inhibit the binding of transmitter and reduce the  $B_{\text{max}}$  should reflect an interruption with the structural or chemical composition of the binding sites. The postsynaptic effect of TMT is in general agreement with the blocking of neurotransmission found by Allen and Fonnum (1984). According to Allen and Fonnum (1984) TMT exposure leads to a reduction in the number of action potentials produced by the CA1 pyramidal cells in response to stimulation of the Schaffer collaterals.

In conclusion our in vitro studies show a specific effect of TMT on the release of glutamate possibly due to anion exchange, and an effect of TMT on both glutamate and GABA receptor sites. It is not possible at the present stage to say which effect is linked to the neuronal damage in the hippocampus. It is well established that glutamate is an excitotoxic amino acid (Olney et al., 1971), however the effect on the postsynaptic receptor might decrease such an excitotoxic effect.

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TABLE 2

THE EFFECT OF TRIMETHYLTIN ON THE BINDING CHARACTERISTICS OF GLUTAMATE AND GABA

LIGAND	TRIMETHYLTIN CONC	Bmax (pmol)	Kd (nmol)
[ <sup>3</sup> H] glutamate	0	136 ± 9	503 ± 46
	10 μM	99 ± 5*	415 ± 29
	100 μM	22 ± 1*	178 ± 40
	1 mM	2 ± .5*	279 ± 103

LIGAND	TRIMETHYLTIN CONC	Bmax (fmol)	Kd (nmol)
[ <sup>3</sup> H] GABA	0	335 ± 28	83 ± 17
	10 μM	205 ± 18	44 ± 6
	100 μM	240 ± 41	67 ± 16
	1 mM	179 ± 22	83 ± 17

Dissociation constants (Kd) and maximum number of binding sites (Bmax) for the binding of glutamate and GABA in the presence of different concentrations of trimethyltin. The values were determined by linear regression analysis on a computer of the Scatchard plots derived from mean values of four assays in each of three saturation experiments. Ligand concentrations in saturation studies were between 10 nM and 500 nM. Calculations are made according to the assumption that one type of binding sites is dominating in this concentration range. \*Significantly different from TMT-free controls,  $p < 0.05$  (Dunnett's test).

FIGURE 1 TIME COURSE AND INDEPENDENCE OF TRANSMITTER RELEASE AFTER STIMULATION WITH  $K^+$  AND TMT.

Time course of release of L-[ $^3H$ ] glutamate from hippocampal tissue in homogenate, induced by sequential stimulation with: A. 50 mM potassium ( $K^+$ ), B. 0.1 mM trimethyltin (TMT). C and D shows that a previous stimulation with potassium does not influence the TMT-induced release and vice versa. The results are expressed as fractional release of tritiated transmitter in ten minutes during and after a four minutes perfusion with the indicated stimuli. Results are mean of three to six experiments. Error bars represent SEM.

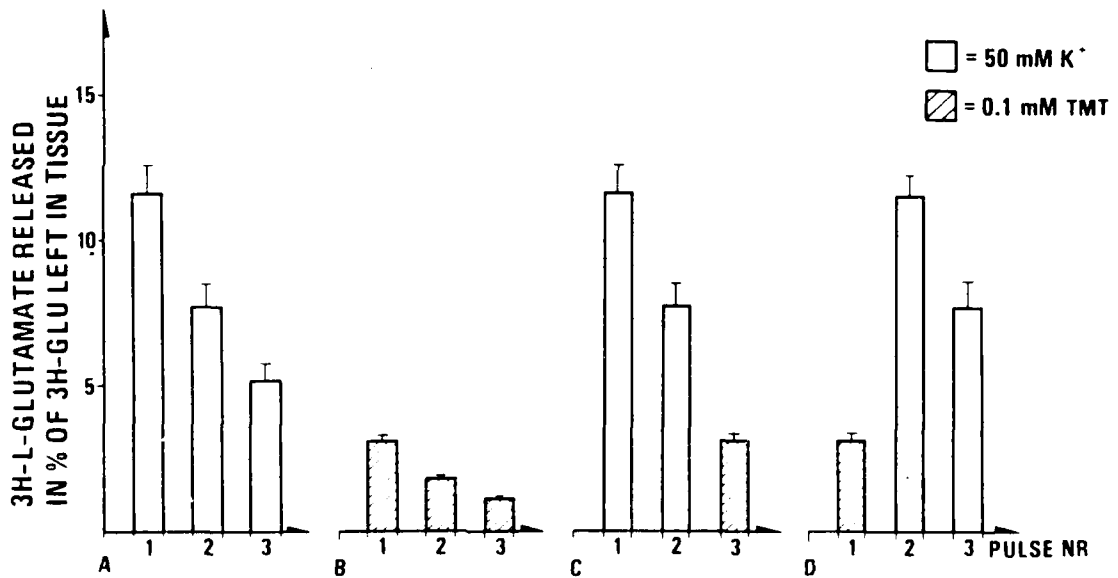
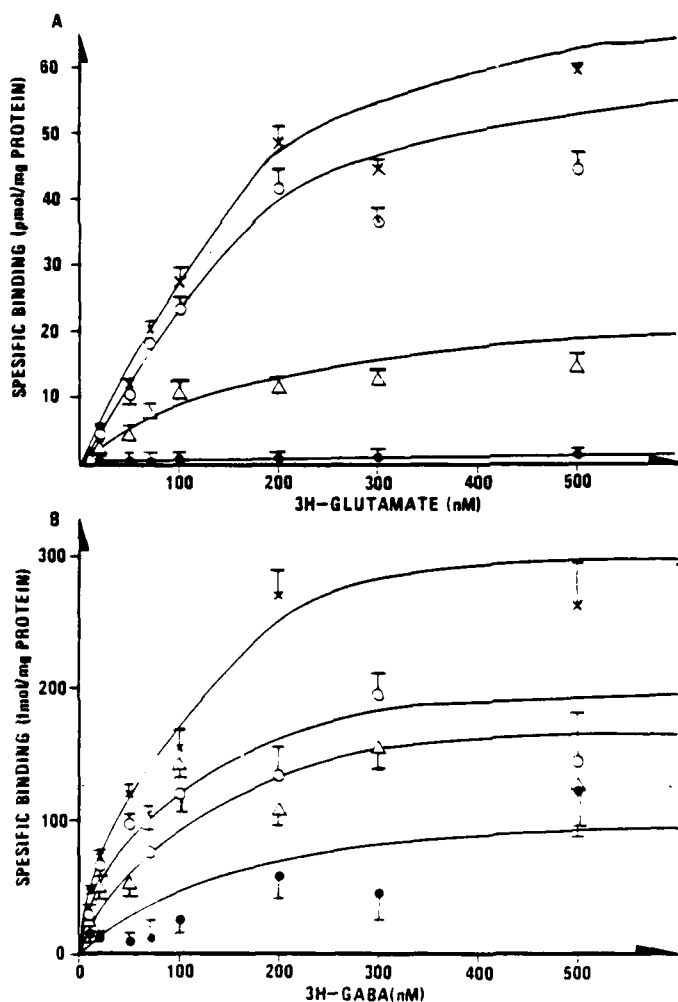


FIGURE 2. THE EFFECT OF TRIMETHYLTIN ON THE SATURATION OF GLUTAMATE AND GABA RECEPTORS.

Saturation plots of A. L-[ $^3\text{H}$ ] glutamate and B. [ $^3\text{H}$ ] GABA binding. Ligand concentrations between 10 nM and 500 nM were used in saturation studies. Each point represent spesific binding (the average of four assays in each of three experiments minus the measured binding in the presence of 1 mM unlabelled ligand), bars indicating SEM. x-x = TMT-free control; o-o = 10  $\mu\text{M}$  TMT,  $\Delta$ - $\Delta$  = 100  $\mu\text{M}$  TMT,  $\bullet$ - $\bullet$  = 1 mM TMT.







DIFFERENCES IN ANIONIC DEPENDENCE OF THE SYNAPTIC EFFLUX OF  
D-ASPARTIC ACID AND  $\gamma$ -AMINO BUTYRIC ACID.

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## ABSTRACT

The synaptosomal efflux of D-aspartate and GABA induced by a substitution of the  $\text{Cl}^-$ -ions with propionate in the incubation medium were measured in preparations of hippocampal tissue homogenates. The efflux of aspartate was significantly higher than spontaneous efflux at 125 mM  $\text{Cl}^-$  (normal = 144 mM) and was increased with decreasing  $\text{Cl}^-$ -concentration. GABA efflux was much less sensitive to a reduction in  $\text{Cl}^-$ -concentration than D-aspartate. The efflux was  $\text{Ca}^{2+}$ -dependent in both cases.

Key words: GABA, D-aspartate, transmitters, chloride, efflux.

Running title: Anion changes induce transmitter efflux

## INTRODUCTION

The synaptic efflux of endogenous transmitters or exogenously labelled transmitters and analogues have been studied by several different depolarization methods (reviews: Szerb, 1983, Fonnum, 1984). The transmitter amino acids are also secreted by depolarization of glial cell preparations, but this efflux can be separated from the synaptic efflux by its lack of  $\text{Ca}^{2+}$ -dependence (Sellström and Hamberger, 1977).

It is well established that a sudden change in the external  $[\text{K}^+]$  or  $[\text{Cl}^-]$  will depolarize the membrane of a muscle fibre (Hodgkin and Horowitz, 1960). Stimulation of synaptosomal release by high  $\text{K}^+$ -concentration is a frequently used tool in neurochemical research. Recently it has been shown that a severe reduction in the  $\text{Cl}^-$ -concentration in the perfusion medium was accompanied by an efflux of the transmitter amino acids glutamate and aspartate (Hardy et al., 1984). We are interested in studying the differences in efflux mechanisms for different transmitters.

The neurotoxic agent trimethyltin has been shown to induce the efflux of glutamate, but has no effect on the efflux of GABA (Naalsund and Fonnum, submitted). Trimethyltin is able to induce  $\text{Cl}^-/\text{OH}^-$  exchanges across cellular membranes (Métais et al., 1977). It has therefore been suggested that changes in  $\text{Cl}^-$  equilibrium could explain the observed effects on efflux. If trimethyltin is able to produce a change in the  $\text{Cl}^-$  equilibrium in the nerve terminal this could probably result in transmitter efflux.

GABA is known to act as a  $\text{Cl}^-$ -ionophore (McBurney and Barker, 1978). We have therefore suggested that the reaction of the GABAergic nerveterminal to changes in extracellular  $\text{Cl}^-$ -concentration might be different from that of the glutamergic terminals. If this is the case, it would give an explanation for the selective effect of trimethyltin on glutamergic neurons. This could also represent a basic difference

between the excitatory glutamergic and inhibitory GABAergic neurons which could be helpful during discussions on different vulnerability to neurobiological tools. In this report we present the results of a comparative study of the efflux of D-aspartate and GABA during pulses of low  $\text{Cl}^-$ -concentration.

#### METHODS

Tissue homogenates were prepared from the hippocampus dissected from the brain of young adult male Wistar rats (150-200 g). The hippocampi were homogenized in cold sucrose, 0.32 M, to give a protein concentration of 6 mg/ml. Efflux of D-[2,3- $^3\text{H}$ ] aspartic acid and [2,3- $^3\text{H}(\text{N})$ ] GABA was measured by a modification of the method described by Levi et al., 1982. Briefly 2  $\mu\text{l}$  homogenate containing about 12  $\mu\text{g}$  protein was absorbed to a small piece of millipore filter. The filter was placed in a small tissue culture dish and 2 ml normal wash buffer (1st row table 1) was added. After preincubation at 25°C for 15 min the synaptosome pools were labelled with D-[ $^3\text{H}$ ] aspartic acid or [ $^3\text{H}$ ] GABA by incubation in the wash buffer containing 5  $\mu\text{M}$  labelled transmitter (10-30 Ci/mmol) for 20 minutes at 25°C. The filters were washed four times in Krebs buffer and then placed in a small perfusion chamber made of two disposable plastic tips. The chambers were continuously perfused with buffer at a constant flow of 0.4 ml/min. The perfusate was collected in fractions of 0.8 ml in counting vials. After a washperiod of 30 minutes the collection of fractions was initiated. An additional wash period of 20 minutes was followed by a 4 minutes perfusion period with one of the buffers numbered 2-7. The chambers were then washed with buffer 1 for 16 minutes before the experiment was terminated. To each fraction of the perfusate were added 10 ml Opti-fluor (Packard) and the radioactivities were counted. The filters were dissolved in Filtercount (Packard) and the rest activity measured.

For measurements of the  $\text{Ca}^{2+}$ -dependence the buffers were substituted with "low- $\text{Ca}^{2+}$ "-buffers containing 0.1 mM  $\text{Ca}^{2+}$ , (nr. 7-9) before the

collection of the fractions started. All the GABA-efflux experiments were performed in the presence of 0.1 mM of the GABA-transaminase inhibitor, amino oxyacetic acid.

High affinity uptake of transmitter was measured in the presence of either 70 nM D-[<sup>3</sup>H] aspartic acid or 70 nM [<sup>3</sup>H] GABA. Crude homogenates were diluted with Krebs buffer containing either 150 mM Cl<sup>-</sup> or 50 mM Cl<sup>-</sup> and 100 mM propionate. (For details on the method see Fonnum et al, 1980).

#### RESULTS AND DISCUSSION

The present study shows that the efflux of the glutamate analogue D-aspartic acid is much more sensitive than the efflux of the inhibitory transmitter GABA to reduction in the Cl<sup>-</sup>-concentration. The efflux of D-aspartic acid was inversely dependent on the concentration of Cl<sup>-</sup> and significantly higher than normal spontaneous efflux at all the Cl<sup>-</sup>-concentrations tested (Table 2). A reduction in the [Cl<sup>-</sup>] to 6 mM gave an efflux which was four times the efflux stimulated by 50 mM K<sup>+</sup>. The GABA efflux during exposure to low Cl<sup>-</sup> was approximately 50% lower than the D-aspartate efflux at Cl<sup>-</sup>-concentrations from 6 mM to 50 mM and not significantly different from the normal spontaneous efflux for chloride concentrations above 100 mM.

The Cl<sup>-</sup>-ions were substituted with propionate, which does not pass through the Cl<sup>-</sup>-channel according to Eccles (1964). This substitution should therefore produce a change in the relation between intracellular and extracellular Cl<sup>-</sup>-concentration. According to the Goldman constant-field equation, which states that the membrane potential is dependent upon the distribution of K<sup>+</sup>, Na<sup>+</sup> and Cl<sup>-</sup>, this will create a shift in the membrane potential towards depolarization, and thereby result in an efflux of transmitter. The duration of the depolarization and hence the amount of transmitter released will depend on diffusion and the cell's ability to transport Cl<sup>-</sup> out of the cell to restore an equilibrium. This hypothesis was supported by our results which are an extension of the previous findings by Hardy et al. (1984).

It is well known that the synaptosomal high affinity uptake of GABA is dependent upon a high concentration of extracellular  $\text{Cl}^-$  (Kanner, 1978), while the uptake of glutamate is less dependent on high  $[\text{Cl}^-]$  (Kanner and Sharon, 1978). The effect on reuptake of transmitter produced by changes in the anion composition was measured for both D- $[\text{}^3\text{H}]$  aspartic acid and  $[\text{}^3\text{H}]$  GABA. Uptake of D- $[\text{}^3\text{H}]$  aspartic acid was reduced by  $20\% \pm 2\%$  (mean  $\pm$  SEM,  $n=4$ ) after replacing  $100 \text{ mM Cl}^-$  with propionate, while  $[\text{}^3\text{H}]$  GABA uptake showed  $24\% \pm 4\%$  (mean  $\pm$  SEM,  $n=6$ ) reduction. With the same ionic composition the observed increase in efflux was 300% for D-aspartic acid and 200% for GABA. These results suggests that the measured efflux was not merely due to a reversal of the uptake mechanism that could possibly have been caused by the changes in ionic gradients. It also shows that the observed increase in tritiated transmitter in the perfusate was mainly due to an increased efflux rather than to a reduced reuptake. Additional evidence for this is provided by earlier reports stating that continuous perfusion at the rate used here ( $0.4 \text{ ml/min}$ ) minimizes reuptake of the excreted transmitter (Raiteri et al, 1975).

The measured efflux was considered to be of synaptosomal origin. Glial cell uptake and release of transmitter are very labile to mechanical disruption such as homogenization (Fonnum, 1984). Glial release is therefor normally not a major part of the efflux measured by the method used here. This was verified by a demonstration of the  $\text{Ca}^{2+}$ -dependence of the efflux. D-Aspartic-acid efflux induced by  $50 \text{ mM K}^+$  was reduced by  $40\% \pm 2\%$  (mean  $\pm$  SEM,  $n=4$ ) by a reduction of calcium in the medium from  $2 \text{ mM}$  to  $0.1 \text{ mM}$ . The efflux induced by  $50 \text{ mM Cl}^-$  was reduced by  $46\% \pm 2\%$  (mean  $\pm$  SEM,  $n=4$ ) in the low-calcium medium. GABA-efflux was reduced by  $67\% \pm 5\%$  (mean  $\pm$  SEM,  $n=4$ ) and  $71\% \pm 4\%$  (mean  $\pm$  SEM,  $n=4$ ) for high potassium- and low chloride-induced efflux respectively. The high  $\text{Ca}^{2+}$ -dependence of the chloride stimulated efflux and the potassium stimulated efflux suggests that large parts of the measured effluxes were associated with a depolarization of the synaptosomal membrane.

$\text{Cl}^-$ -ions are essential for ATP-dependent proton transport across the membranes of catecholamine storage vesicles (Pollard et al, 1979) and

the microsomal vesicles in plant cells (Bennet and Spanswick, 1983). Reduction in  $[Cl^-]$  will reduce proton transport and thereby collapse the pH-gradient across these vesicle membranes, resulting in leakage of the stored molecules. Some evidence exists for the storage of glutamate in synaptosomal vesicles (Naito and Ueda, 1985), while the existence of a vesicular storage of GABA remains unclear. The possibility of a vesicular storage of the transmitter amino acids raises the question of whether the observed efflux reported in the present paper is the result of a change in the pH-gradient across vesicle membranes. Changes in intracellular  $[Cl^-]$  is very slow following an extracellular reduction in  $[Cl^-]$  (Kelly et al, 1969), and could not produce a pH-change resulting in an immediate efflux of transmitter after a change in the anion composition of the perfusion buffer. Diffusion of propionate in the free acid form across the cell membrane is known to take place at a slightly faster rate than the  $Cl^-$  diffusion (Carvalho and Carvalho, 1979). This may represent a cause of a reduction in the intracellular pH resulting in vesicular leakage of transmitter, although this diffusion also seems to need about 1 - 2 minutes before equilibrium is achieved (Carvalho and Carvalho, 1979) and hence should give a lag time before any transmitter efflux.

GABA is known to increase the membrane permeability to  $Cl^-$ -ions (McBurney and Barker, 1978). A possible explanation for the lack of effect of a 45 mM reduction in the  $Cl^-$ -concentration on the measured GABA efflux could possibly be due to the GABA-ergic neuron having a greater ability to restore a transmembrane equilibrium of  $Cl^-$ . The presence of presynaptic GABA receptors may increase the synaptosome's ability to regulate GABA efflux (Mitchell and Martin, 1978).

Our results showing that a 20-40 mM reduction in  $Cl^-$ -concentration produced an efflux of D-aspartic acid which was twice the amount of the spontaneous efflux, while the same  $Cl^-$ -change had no effect on the GABA efflux, is an interesting observation, which should be taken into account in studies of drugs that may act on  $Cl^-$ -channels.

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TABLE 1. COMPOSITION OF MEDIA (mM)

Number	1	2	3	4	5	6	7	8	9	10
	normal									
Name:	wash	125 Cl <sup>-</sup>	100 Cl <sup>-</sup>	50 Cl <sup>-</sup>	25 Cl <sup>-</sup>	6 Cl <sup>-</sup>	50 K <sup>+</sup>	wash	50 Cl <sup>-</sup>	50 K <sup>+</sup>
								low Ca <sup>2+</sup>	low Ca <sup>2+</sup>	low Ca <sup>2+</sup>
Na <sup>+</sup>	137.6	137.6	137.6	137.6	137.6	137.6	85.6	137.6	137.6	85.6
K <sup>+</sup>	7.4	7.4	7.4	7.4	7.4	7.4	51.8	7.4	7.4	51.8
Mg <sup>2+</sup>	1	1	1	1	1	1	1	10	10	10
Ca <sup>2+</sup>	2	2	2	2	2	2	2	0.1	0.1	0.1
SO <sub>4</sub> <sup>2-</sup>	1	1	1	1	1	1	1	10	10	10
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	5	5	5	5	5	5	5	5	5	5
Cl <sup>-</sup>	144	125	100	50	25	6.4	146.4	144	50	146.4
Propionate	-	18	44	94	119	137.6	-	-	94	-
Glucose	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Hepes	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5

TABLE 2. RELEASE OF TRANSMITTER AMINO ACIDS

Ruffer used to normal induce release wash	50 K <sup>+</sup>	125 Cl <sup>-</sup>	100 Cl <sup>-</sup>	50 Cl <sup>-</sup>	25 Cl <sup>-</sup>	6 Cl <sup>-</sup>
Preloaded amino acid:						
D-[ <sup>3</sup> H]aspartic acid	4 ± 0.7	10 ± 1*	3 ± 0.4*	5 ± 1*	13 ± 1*	15 ± 1*
[ <sup>3</sup> H] GABA	3 ± 0.4	9 ± 1*	0 ± 0	1 ± 0	7 ± 1*	6 ± 9*
						18 ± 1*

Increase in amino acid release from rat hippocampal synaptosomes into the perfusion medium in per cent of total content of tritiated amino acid in tissue after replacement of the normal wash buffer (1st row) with a buffer with the indicated composition. Results are means ± SEM of eight experiments. \*Increase is significant,  $p < 0.01$  (Wilcoxon's two sample test). Potassium induced release is not significantly different for D-[<sup>3</sup>H] aspartic acid and [<sup>3</sup>H] GABA. All Cl<sup>-</sup>-concentrations tested induce a significantly lower release of [<sup>3</sup>H] GABA than of D-[<sup>3</sup>H] aspartic acid,  $p < 0.01$  (Student's t-test).





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HIPPOCAMPAL EEG IN RATS AFTER CHRONIC TOLUENE INHALATION

by

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Running title: Toluene-induced changes in hippocampal EEG.

ABSTRACT

Rats were chronically implanted with bipolar electrodes in the hippocampal regions containing cells generating electric theta-activity. The animals were exposed to 500 ppm of toluene in inhalation chambers, for 8 or 16 hours per day for 5 days per week in 12 weeks. The hippocampal electric activity was recorded 48 hours after each weekly exposure, ensuring a minimal amount of toluene in the tissue during the recordings. The eight hours daily exposed group showed an initial period of increased frequency of the regular theta waves together with an increased incidence of theta activity after 1 - 2 weeks of exposure. In the sixteen hours daily exposed rats two weeks of toluene inhalation produced a significant reduction in the theta wave frequency. This change was also reached after eight weeks of exposure in the eight hours daily exposed group. At this moment the theta activity was frequently disrupted by short amplitude irregular waves, a phenomenon which increased gradually throughout the rest of the exposure period. The average blood concentration of toluene was 16.7 mg/ml and 17.7 mg/ml and not significantly different for the eight and sixteen hours exposed groups respectively.

Key-words: Hippocampal EEG, Inhalation, Toluene.

## INTRODUCTION

Neurophysiological tests allow relatively direct assessment of the functional integrity of specific neuronal systems. These tests also have the advantage that they can be carried out several times in the same animal, and give valuable information concerning the development of a brain dysfunction.

EEG as a screening test for solvent induced brain damage in humans has not been recommended because of a large variation in the normal EEG's. In a normal population 10 - 15% may have EEG abnormalities (Seppäläinen 1985). In animals however, the use of intracerebral implantation of electrodes in defined brain areas enables recordings with a high specificity and with a minimal interindividual variation.

Hippocampal EEG is characterized by a very prominent rhythm designated the theta rhythm, with an approximately sinusoidal pattern of electrical activity of different frequency ranges in various species (6-12 Hz in the rat). Theta rhythm can be recorded from the hippocampus of several mammalian species, it possess a strong biological potential (1-3 mV in amplitude), and can readily be recorded by electrodes properly implanted within the hippocampus. The theta rhythm is selectively present during specific behaviors (Vertes 1982).

We have previously used hippocampal EEG to study the development of a degeneration process after injection of the well known neurotoxic substance, trimethyltin (Naalsund et al. 1985). This study showed that the hippocampal electric activity was a more sensitive parameter than histological and neurochemical parameters for early detection of damage.

Toluene has been reported to produce alterations in CNS excitability (Benignus 1981) and sensory function (Pryor et al. 1983a; 1983b; Dyer et al. 1984) in animal studies and in humans (Metric and Brenner 1982; Cooper et al. 1985). Abnormal electroencephalographic (EEG) activity

after toluene inhalation has been found in several cases after both abuse and occupational exposure (Andersen and Kaada 1953; Knox and Nelson 1966; Seppäläinen et al. 1980; Grasso et al. 1984), as well as in animal experiments (Takeuchi and Hisanaga 1977).

The present paper describes neurophysiologic studies designed to detect alterations in the hippocampal theta cells or their activating or inhibitory connections following exposure to 500 ppm toluene at various time intervals. This level is above the recommended TLV (threshold limit value) of 75 ppm for an 8 hr exposure (Norwegian Labour Inspection 1984), but is within the range of some short-term exposure situations (Benignus 1981) and far below the exposure level for "sniffers" (Benignus 1981).

#### MATERIALS AND METHODS

Surgery. The hippocampal EEG was recorded in male Wistar rats with chronically implanted electrodes. Initial body weights were between 300 and 400 g. Surgical preparation of the rats were performed under diazepam (5 mg/kg i.p.) and fluanizone (7.5 mg/kg s.c.) anesthesia. The rats were placed in a stereotaxic frame, and the skin and connective tissue on the skull were removed. Stainless steel screws were set in the skull to serve as anchors for the electrodes. The electrodes were made of a silverplated copperalloy conductor (127  $\mu$ m diam.) insulated with teflon and each soldered to a male golden pin component (220-P02100 Bunker Ramo, Amphenol North America). The electrodes were twisted and cut so that one tip was 0.7 mm shorter than the other. Only the tips were bared of insulation and they were spaced 1.5 mm from one another. The electrodes were fixed to the anchors with dental cement.

Placement of such electrodes in the dorsal hippocampus with one in the subfield CA1 of the pyramidal cell layer and the other in the granular layer of fascia dentata (FD), permits the recording of clear and large amplitude theta activity either bipolarly or unipolarly (Sainsbury and

Bland 1981). One of the screws served as a zero level during unipolar recordings. The implantation coordinates were the following: 3.2 mm behind bregma, 2.0 mm lateral to the midline and 3.0 mm depth from the dura (fig. 1). The electrode pair was longitudinally oriented with the deepest electrode in posterior position.

Recording. After the surgery the rats were allowed ten days to recover before any recordings were taken. At least two recordings were carried out before the exposure period, and one recording per week during exposure time. The rats were allowed two days of recovery from exposure before recording at any time, ensuring no solvent to be present in the tissue (Pyykkö et al. 1977). During recording the animals were situated in cages (50 x 30 x 15 cm) without cover and connected to the graph (Grass Electroencephalograph model IIIc) through a female plug. The behavior of the rats were coded on the records throughout the test period. Each rat was tested for five to ten minutes, assuring that different behavior types were seen.

Exposure. Exposure to toluene was performed in an inhalation chamber, volume 300 litre, with constantly supply of vaporized toluene diluted to 500 ppm in the inlet air, and a ventilation of 3000 l per hour. The toluene concentration was measured continuously with a Miran IR spectrophotometer connected to a recorder. A second chamber was ventilated with fresh air for control experiments. Temperature and humidity in the animal room were controlled according to OECD guidelines ( $22 \pm 3^{\circ}\text{C}$ , 30-70% relative humidity).

The animals were divided into four groups: two groups spent eight hours per day in the toluene and control chamber, respectively, while the other two groups were kept in the chambers for 16 hours per day. The exposures were carried out for five days per week. The eight hours exposure was carried out during daylight hours and in an illuminated room, while the sixteen hours was performed during the dark hours and without illumination. This was done for practical reasons. The effect of the activity level of the animals on the uptake of toluene was

tested by blood-concentration measurements (gass-chromatography, headspace technique).

## RESULTS

The daylight exposed group had an average blood-concentration of toluene of  $16.7 \pm 2.7$   $\mu\text{g/ml}$  (N=3), while for the dark-exposed group the concentration was  $17.7 \pm 1.8$   $\mu\text{g/ml}$  (N=3), measured at the end of the fifth exposure day. There was no statistical difference between the two groups.

Clear theta waves were recorded during type 1 behaviors (walking, rearing, changes in posture, head movements). Frequencies of the theta waves were estimated from at least five 1 sec segments of graph records from each brain half during walking or rearing. Average values of these estimates are presented as a function of exposure time in fig. 2. The animals exposed for eight hours per day show an initial increase in the frequency followed by a decline. The animals exposed for sixteen hours showed a faster and more pronounced decline in frequency. Ten days of toluene inhalation produced a significant reduction in the theta-wave frequency in the rats with longest exposure intervals, whereas forty days of exposure produced the same change in the eight hours exposed group.

Toluene exposure was terminated after 60 days of exposure. Two animals from each group were allowed a recovery period of one month before a last recording was performed. No increase in the theta wave frequency had occurred during this recovery period, which indicate that the observed changes was irreversible. Neither of the control groups showed any change in frequency or quality of the theta activity during and after the treatment period.

Parallell to the changes in frequency of the theta waves, qualitative changes also occurred. The first period of increased frequency was not

accompanied by any disruptions in the regular waves during type 1 behavior. During immobility, which is normally associated with irregular waves, however theta waves were in some instances seen in this period (fig. 3). The reduction in theta frequency was followed by a disruption of the regular waves. After 60 days of exposure regular theta activity were absent in the sixteen hours daily exposed group (fig. 4) while the eight hours daily exposed group had frequent disruptions with short amplitude waves during the theta-activity (fig. 3). Only slight improvement in the quality of the theta waves are seen after the one month recovery period.

#### DISCUSSION

Toluene inhalation produced disruptions and frequency changes in the hippocampal theta activity. After an initial increase in the frequency and an increase in the occurrence of theta waves, there was a reduction in the frequency and a gradually increased disruption of the theta waves. The degree of changes were related to the length of the exposure period and to the daily exposure intervals. Sixteen hours per day with 500 ppm toluene produced frequency reduction after ten days, while eight hours daily exposure to the same concentration gave frequency reduction after forty days. Recovery for one month gave small qualitative improvements but no increase in frequency towards normal theta waves. The initial increase in frequency was only seen in the eight hours exposed group. It is likely that a frequency increase in the sixteen hours exposed group could have occurred during the first five days of exposure and therefore have not been detected.

Previously it has been shown that very high doses of toluene (4000 ppm) in short exposure intervals (four hours) will reduce hippocampal theta wave frequency and simultaneously give an increase in the theta component of the hippocampal EEG (Takeuchi and Hisanaga 1977). The present report confirms and extends the nature of these observations and suggest that also low concentrations (500 ppm) of toluene can induce such changes during chronic exposure.

In the rat there are only two rather well-defined behaviors during which theta activity is present, i.e. voluntary motion and paradoxical sleep (Winson 1974). This is not true for other species, eg. cats and rabbits, in which extensive behavioral observations have been made (Winson 1972). During ether or urethane anesthesia spontaneous theta waves have been observed in the rat (Bland and Whishaw 1976). A similar anesthetic effect could possibly explain the observed theta activity during immobility in the toluene exposed rat.

Several nuclei at different levels of the neural axis from the brain stem to septum appear to be involved in hippocampal theta generation. With few exceptions, studies indicate that the medial pontine region of the reticular formation is the main source for hippocampal synchronization and that the median raphe nucleus is the brain stem source for hippocampal desynchronization (Vertes 1982). The communication between the brain stem and the hippocampus are mediated via the medial septum/diagonal band (Vertes 1982). Septal lesions can give complete loss of hippocampal theta-activity (Andersen et al. 1979). The effects of brain stem lesions on the hippocampal EEG are less straightforward, possibly because ascending brain stem systems are more diffusely organized than the septal- hippocampal system. Increased occurrence of hippocampal theta, as were seen in the rats in a period during toluene exposure, may reflect damage to desynchronizing systems. Lesions in the median raphe nucleus and the lateral hypothalamus (Vertes 1982) shows the characteristics of damage to such pathways: the occurrence of a lowfrequency theta (5.8 Hz) during immobility. Several synchronizing pathways maintain the theta activity (Vertes 1982), an interruption of one synchronizing system would therefore have the effect of slightly reducing the theta frequency but not abolishing theta (Vertes 1982). From these data it follows that our observations could be associated with both a damage to the desynchronizing and to the synchronizing pathways.

Increased frequency and improved quality of hippocampal theta waves during type 1 behavior is also reported in rats with severe motor deficits after surgically prepared cerebellar lesions (Myhrer and

Allen 1985). It is suggested that activation of compensatory mechanisms due to adoption of new behavioral strategies could be reflected in this way.

Hippocampal theta activity may be manipulated by systemic or iontophoretic application of pharmacologically active substances. A large number of drugs depress the theta rhythm of the hippocampus, among these are anesthetics, hypnotics, tranquilizers and related drugs (Stumpf 1965). In situ injection in the medial septal nucleus, of atropine or GABA, inhibits theta activity (Kolb and Whishaw 1977; Allen and Crawford 1984) and the same effect can also be achieved by blocking the excitatory action of glutamate in the hippocampus (Fontain et al. 1984). Cholinergic agents are known to produce theta waves, provided the septal area is intact (Stumpf 1965). The mechanism by which toluene can produce an irreversible depression of the hippocampal theta activity should be quite different from the acute membrane fluidizing action of organic solvents (Franks and Lieb 1978) for which the membranes seem to develop a tolerance (Chin and Goldstein 1977). According to Pyykkö et al. (1977) the toluene concentration in brain tissue is reduced to 0.1% of its level during exposure, 24 hours after termination of the exposure. This indicates that acute effects have not been measured in our experiments. In view of the results from surgical and pharmacological manipulations of the theta rhythm it is clear that the observed changes in hippocampal EEG after toluene inhalation could be the result of a toxic action on any part of the neural system generating or regulating the theta activity.

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CHEMICALLY INDUCED DAMAGE TO THE HIPPOCAMPAL FORMATION  
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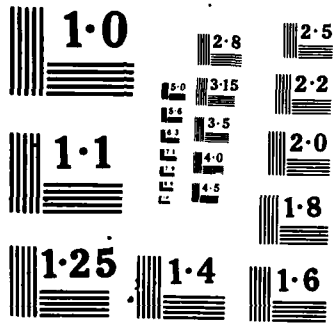
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MICROCOPY RESOLUTION TEST

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FIGURE 1.

Representative example of location of electrodes. The implantation coordinates were for the dentate electrode (FD): 4.7 mm behind bregma, 2.0 mm lateral to the midline and 3.7 mm below the dura, and for the pyramidal electrode (CA1): 3.2 mm, 2.0 mm and 3.0 mm given in the same order. The section shown is a frontal view 3.3 mm behind bregma (Paxinos and Watson, 1982).

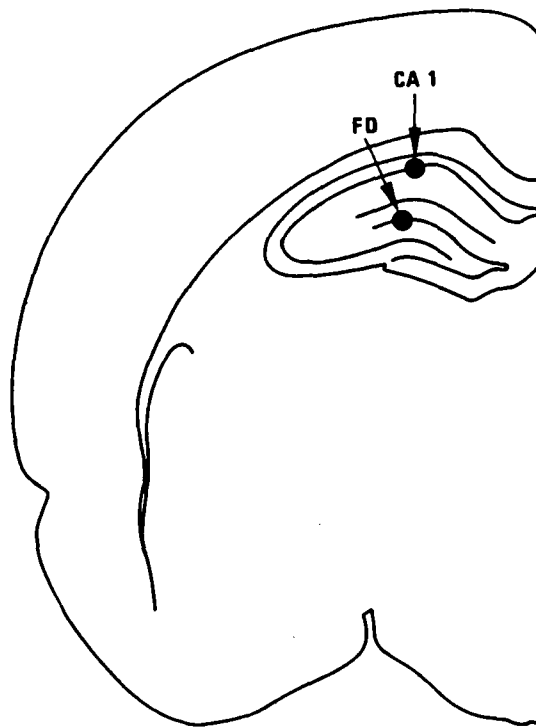


FIGURE 2.

Frequency of theta waves during walking or rearing after exposure to 500 ppm toluene for eight (x) or sixteen (o) hours per day, five days per week. Toluene inhalation was started at point zero on the horizontal axis, days without exposure are not counted in the exposure period (5 days = 1 week). Each point represent an average of three to eight animal records (one brain half give one record), and the bars indicate the standard error of mean. \* Significant different from controls ( $p < 0.05$ , Student's T-Test).

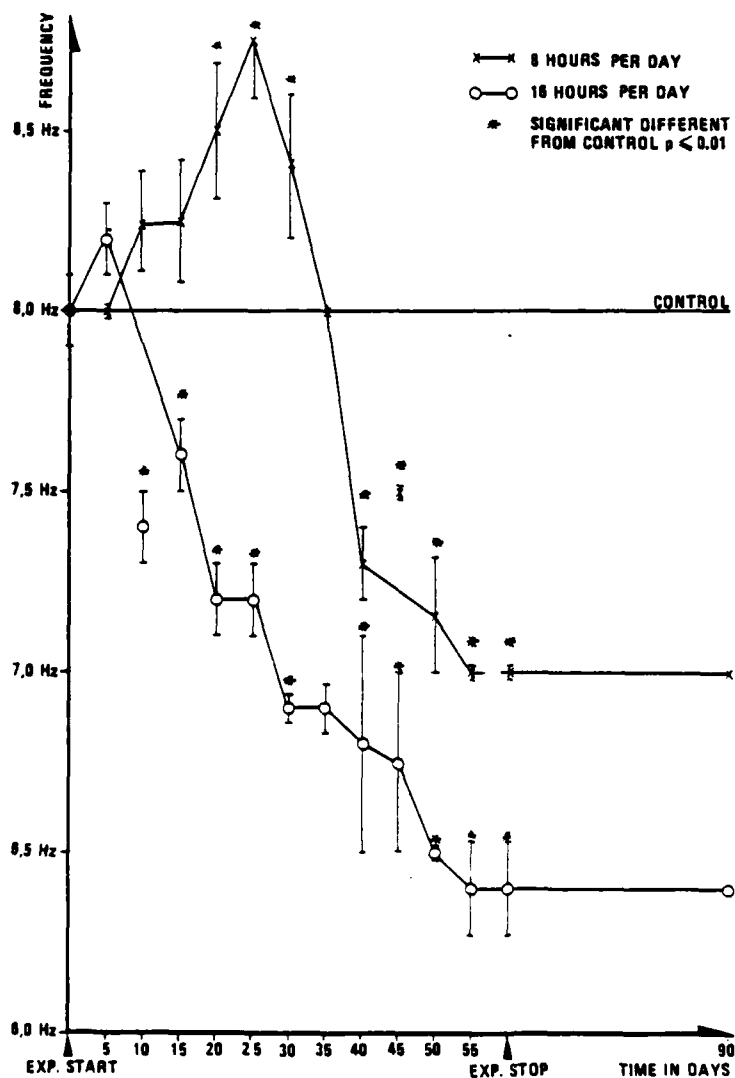


FIGURE 3.

Electric activity in the dorsal hippocampal formation before, during and after a 60 day period with toluene inhalation, 500 ppm for eight hours per day, five days per week. Days without exposure are not counted in the exposure period. The records are bipolar registrations from a single rat.

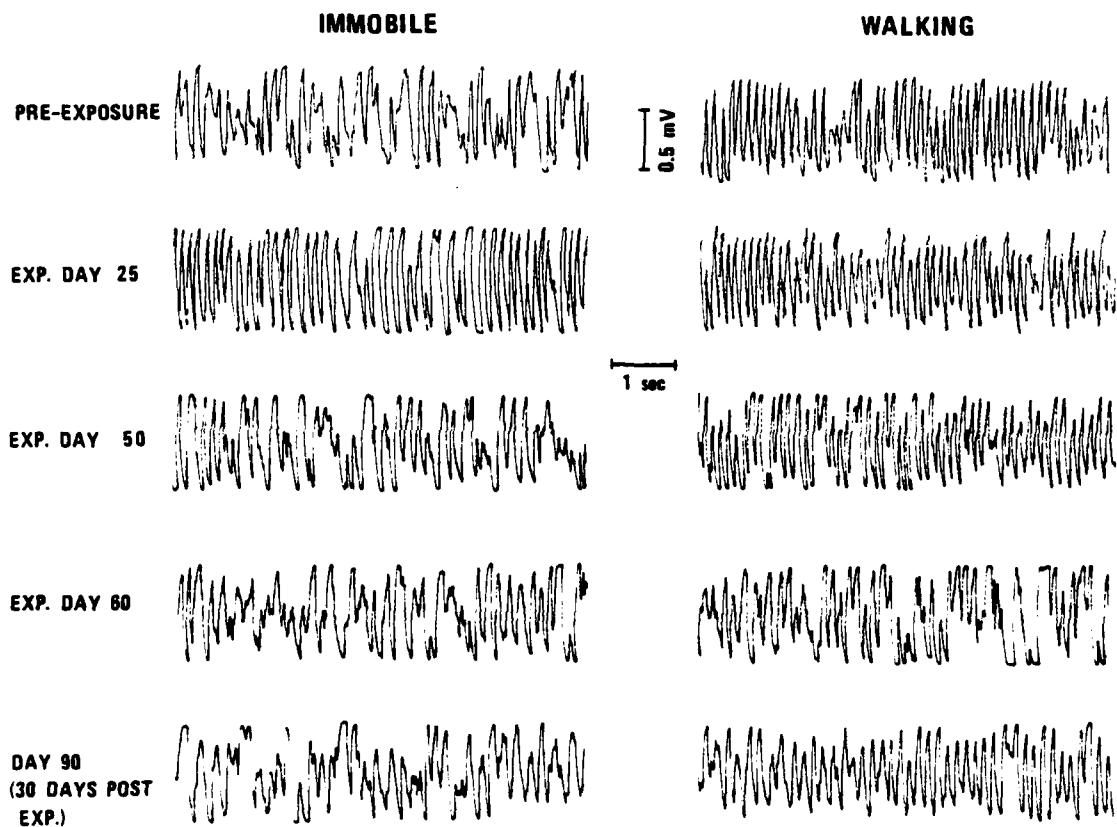
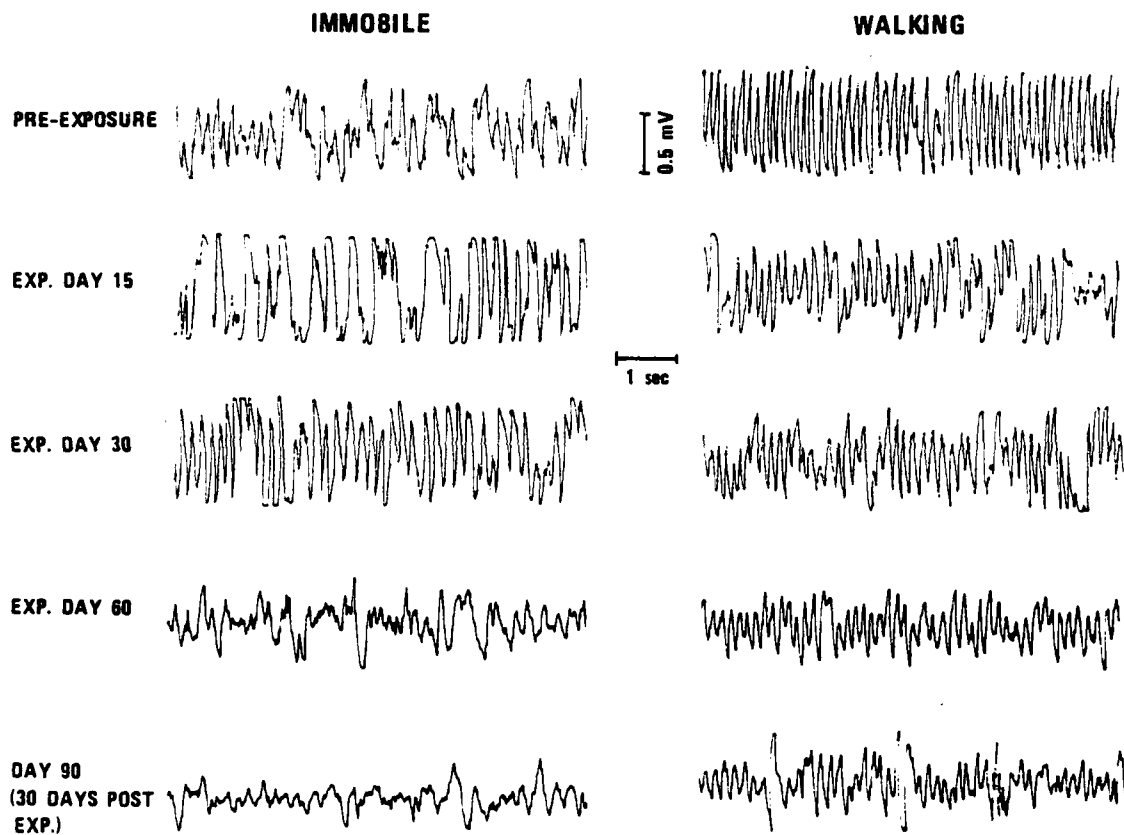


FIGURE 4.

Electric activity in the dorsal hippocampal formation before, during and after a 60 day period of toluene inhalation, 500 ppm for sixteen hours per day, five days per week. Days without exposure are not counted in the exposure period. The records are from bipolar registrations from a single rat.



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