NEUROTOXICITY OF TOLUENE AND AROMATIC WHITE SPIRIT

A study of rat brain neurochemistry

Ph.D. Thesis

Henrik Rye Lam

Institute of Toxicology
Division of Biochemical and Molecular Toxicology
Laboratory of Biochemical and Genetic Toxicology
National Food Agency of Denmark

and

Institute of Life Sciences and Chemistry
Roskilde University Center
Denmark

DATA SHEET

Title:	Neurotoxicity of toluene and aromatic white spirit.
Subtitle:	A study of rat brain neurochemistry.
Author:	Henrik Rye Lam.
Publisher:	National Food Agency of Denmark.
Affiliation:	Institute of Toxicology, Division of Biochemical and Molecular Toxicology, Laboratory of Biochemical and Genetic Toxicology.
Address:	Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
Telephone/fax:	+45 39696600/+45 39660100

Abstract:

Male Wistar rats were inhalation exposed to 0, 500 (400), or 1500 (800) ppm toluene (aromatic white spirit) 6 h/day for 3 weeks (aromatic white spirit) or 6 months (toluene, aromatic white spirit).

Regional and whole brain NA, DA, and 5-HT concentrations were statistically significantly changed by 6 months exposure to toluene. These changes were long-lasting.

A 3 weeks aromatic white spirit study suggested that the threshold capacity for the elimination of aliphatic components was exceeded. Whole brain NA, DA, and 5-HT concentrations were statistically significantly increased.

In a 6 months aromatic white spirit study, regional NA, DA, and 5-HT concentrations were statistically significantly changed. The level of 5-HT increased in 6 of 7 investigated regions. These changes were long-lasting.

Following 3 weeks or 6 months of aromatic white spirit exposure, the yield of synaptosomal protein was statistically significantly reduced, whereas synaptosomal NA, DA, and 5-HT concentrations, high-affinity 5-HT uptake rate and uptake capacity were statistically significantly increased.

Regional GFAP concentrations were not affected following 3 weeks or 6 months of aromatic white spirit exposure.

The long-lasting toluene- and aromatic white spirit-induced changes of NA, DA, and 5-HT metabolism are serious findings which should be taken into consideration by regulators.

Key words: Aromatic white spirit, brain regions, GFAP, neurochemistry, neurotoxicity, neurotransmitter metabolism, synaptosomes, toluene.

Please quote: Henrik Rye Lam (1996): Neurotoxicity of toluene and aromatic white spirit. A study of rat brain neurochemistry. Ph.D. Thesis. National Food Agency of Denmark, DK-2860, Søborg, Denmark. 138 pp.

ISBN: 87-601-5591-4

Front cover: Section in the sagittal plane through the rat brain.

PREFACE

For several years, exposure to some organic solvents has been correlated with the induction of acute and long-lasting (i.e. long-term reversible or true irreversible) neurotoxicity in man. It is important to gain insight into this relationship in order to ensure sufficient protection of the population against these unwanted effects. Investigations in exposed laboratory animals can contribute to increase this knowledge and provide valuable information about the neurochemical basis of neurotoxicity in man.

The present neurochemical studies constitute an integrated part of a collaborative multicenter neurotoxicology research project investigating the effects induced by organic solvents on the nervous system. The project was initiated in the mid-80s. Presently, it includes the following disciplines: general toxicology, behaviour, neuroelectrophysiology, neurochemistry, and neuropathology.

The studies which constitute the experimental part of this thesis (Ladefoged et al., 1991; Lam et al., 1992; Østergaard et al., 1993; Lam et al., 1995) applied general toxicological, behavioural, clinical biochemical, neurochemical, neuropathological, and neurostereological approaches to study the effects induced by inhalation exposure of rats to toluene or aromatic white spirit. The neurochemical and clinical biochemical investigations were carried out by the author.
Analysis of aromatic white spirit components in the rat brain was performed by Agneta Löf, Arbetslivsinstitutet, Solna, Sweden.
Other collaborators contributed with the additional approaches: pathological and stereological studies were carried out by Ole Ladefoged, Arne Møller, and Poul Strange, and the behavioural investigations were carried out by Grete Østergaard and Jens-Jørgen Larsen.

The neurochemical parameters include global, regional, and subcellular CNS neurotransmitter (noradrenaline, dopamine, and 5-hydroxytryptamine) metabolism and regional glial fibrillary acidic protein concentrations.

Findings are discussed in terms of relevant neurochemical investigations published in the peer reviewed literature and in terms of the neurotoxicity of the 2 solvents in man. The applicability of the rat as a neurochemical research model for studying neurotoxicity in man is also discussed.

I wish to thank professor Jørgen Clausen, Institute of Life Sciences and Chemistry, Roskilde University Center, Denmark, for accepting to be my supervisor and for his interest and fruitful comments during the writing phase. Thanks also to Jens-Jørgen Larsen, head of Department of General Toxicology at the Institute of Toxicology, for being my adviser.

I am glad to make use of this opportunity to thank all project collaborators: Peter Arlien-Søborg, Stephen C. Bondy, Shirley X. Guo, Ulla Hass, Lise Korbo, Ole Ladefoged, Søren P. Lund, Agneta Löf, Arne Møller, Kirsten Pilegaard, Leif Simonsen, Poul Strange, and Grete Østergaard for excellent collaboration within the various fields of neurotoxicology.

Also thanks to Sven Edelfors and Dorrit Glendrup at the Department of Pharmacology, University of Copenhagen, for personal advice about the rat brain dissection procedure.

I am grateful for the assistance of James P. O'Callaghan during the implementation phase of 'his' glial fibrillary acidic protein sandwich enzyme-linked immunosorbent assay technique and for the offered opportunity for inter-laboratory 'calibration' studies.

I wish to thank Lejf Burkal, Morten Falding, Joan Gluver, Bo Herbst, Vibeke Kegel, Bo L. Jensen, Karen Roswall, Lonni Sand, and Berit Tonnesen for skilful technical assistance throughout the studies.

Especially, I am in great debt to Annette Landin for more than 10 years of pleasant and productive, high-quality laboratory collaboration and for her sense of humour and patience, often needed to overcome episodes of pronounced stress.

Other colleagues at the Institute of Toxicology and the National Food Agency of Denmark are thanked for he'p throughout the work.

At last a thank to the Danish Environmental Protection Agency for support, interest, and encouragement in relation to the neurotoxicology research project.

Søborg, May 1996.

Henrik Rye Lam

CONTENTS

LIST OF PUBLICATIONS INCLUDED

This Ph.D. thesis is mainly based upon the neurochemical investigations as published in 4 research papers, which will be referred to throughout this report by their Roman bold numerals (**I**; **II**; **III**; **IV**):

I
Ladefoged, O., P. Strange, A. Møller, H.R. Lam, G. Østergaard, J.-J. Larsen & P. Arlien-Søborg: Irreversible effects in rats of toluene (inhalation) exposure for six months. *Pharmacology & Toxicology* 1991, **68,** 384-390.

II
Lam, H.R., A. Löf & O. Ladefoged: Brain concentrations of white spirit components and neurotransmitters following a three week inhalation exposure of rats. *Pharmacology & Toxicology* 1992, **70,** 394-396.

III
Østergaard, G., H.R. Lam, O. Ladefoged & P. Arlien-Søborg: Effects of six months' white spirit inhalation exposure in adult and old rats. *Pharmacology & Toxicology* 1993, **72,** 34-39.

IV
Lam, H.R., G. Østergaard & O. Ladefoged: Three weeks' and six months' exposure to aromatic white spirit affect synaptosomal neurochemistry in rats. *Toxicol. Lett.* 1995, **80,** 39-48.

The neurochemical and clinical biochemical investigations published in these research papers were performed strictly by the author of this thesis and independently of the co-authors of the publications **I**, **II**, **III**, and **IV**.
These neurochemical studies are supplemented with unpublished results of studies of the effect induced by 3 weeks' or 6 months' inhalation exposure to aromatic white spirit on regional rat brain concentrations of glial fibrillary acidic protein. These investigations were also carried out strictly by the author of this thesis.

ABBREVIATIONS

The abbreviations as defined below are used in the subsequent part of this thesis. It is emphasized that these abbreviations, apart from the summaries, will not be defined elsewhere in the text.

Acc. dif.	Diffuse type of dopamine fluorescence in anterior nucleus accumbens in the forebrain.
Acc. dot.	Dotted type of dopamine fluorescence in posterior nucleus accumbens in the forebrain.
AChE	Acetylcholinesterase.
ALAT	Alanine aminotransferase.
ANOVA/GLM	Analysis of variance by use of least squares to fit a general linear model.
ATPase	Adenosine triphosphatase.
BBB	Blood-brain barrier.
BCB	Blood-cerebrospinal fluid barrier.
b.w.	Body weight.
CA2/3	Regio inferior of the hippocampus.
CA	Catecholamine(s).
Caud. cent.	Central parts of the caudate nucleus in the forebrain.
Caud. marg.	Marginal zone of the caudate nucleus in the forebrain.
Caud. med.	Medial part of the caudate nucleus in the forebrain.
CNS	Central nervous system.
CT	Computerized tomography.
CV	100 x SD/mean.
DA	Dopamine.
DOPAC	3,4-Dihydroxyphenylacetic acid.
DPH	Diphenyl-1,3,5-hexatriene.
EEG	Electroencephalogram.
ELISA	Enzyme-linked immunosorbent assay.
FURA-2	1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid.
GFAP	Glial fibrillary acidic protein.
GSH	Glutathione (reduced state).
5-HIAA	5-Hydroxyindoleacetic acid.
HPLC-ECD	High performance liquid chromatography with electrochemical detection.
h	Hour(s).
5-HT	5-Hydroxytryptamine (serotonin).
i.p.	Intraperitoneal.
HVA	Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid).
LDH	Lactate dehydrogenase.
LPZ	Lateral palisade zone of the median eminence in hypothalamus.
Med. obl.	Medulla oblongata.
MHPG	3-Methoxy-4-hydroxyphenylglycol.
min	Minute(s).

Mol:WIST	Wistar rat strain from Møllegaard Breeding Center.
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
MPZ	Medial palisade zone of the median eminence in hypothalamus.
NA	Noradrenaline (norepinephrine).
NPA	Propylnorapomorphine.
PAPF	Parvocellular part of the paraventricular hypothalamic nucleus.
PAFM	Magnocellular part of the paraventricular hypothalamic nucleus.
PCBs	Polychlorinated biphenyls.
ppm	Parts per million.
PV I	Anterior periventricular hypothalamic region.
PV II	Posterior periventricular hypothalamic region in hypothalamus.
ROS	Reactive oxygen species.
SDH	Succinate dehydrogenase.
SEL	Subependymal layer of the median eminence in hypothalamus.
SD	Standard deviation.
SDS	Sodium dodecylsulfate.
TLV	Threshold limit value.
TMA-DPH	1-(4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene.
Tub. dif.	Diffuse type of dopamine fluorescence in lateral-posterior tuberculum olfactorium in the forebrain.
Tub. dot.	Dotted type of dopamine fluorescence in medial-posterior tuberculum olfactorium in the forebrain.
USEPA	US Environmental Protection Agency.
VMA	Vanillymandelic acid.

SUMMARY

Evidence is available for a causal relationship between occupational inhalation exposure to some organic solvents and the induction in the nervous system of effects which impair health and life quality of man. Two of these solvents are toluene and white spirit. A brief presentation of the toxicokinetics and the effects on the nervous system of these 2 solvents is given.

There is no internationally accepted definition of neurotoxicity. In the scientific community, it is generally agreed that neurotoxicity is '*any adverse effect on the chemistry, structure, and/or function of the nervous system during development or at maturity*'. It is also agreed that '*any long-lasting (long-term reversible or true irreversible) effect should always be regarded as serious*'.
The problem is how to define adversity on the basis of neuroactivity.

Neurochemical parameters measured in laboratory animals (mammalian species) are useful for studying the neurotoxicity of chemicals in man because of inter-species similarity of the underlying biology.
The following working hypothesis is put forward: *any change of any neurochemical parameter induced by a chemical demonstrates - directly or indirectly - the neuroactivity of that chemical.*

There was an obvious lack of long-term studies of toluene and white spirit in laboratory animals. Such studies are relevant for occupational exposure settings. In order to gain insight into the neurotoxicity of organic solvents a series of multidisciplinary neurotoxicity studies in laboratory animals was initiated. At present, the disciplines include: general toxicology, neuropathology, behaviour, neurochemistry, electrophysiology, and NMR spectroscopy.

The aim of the present studies is to investigate whether inhalation exposure of rats to toluene or aromatic white spirit affect important neurochemical parameters.
Focus is placed on global, regional, and subcellular rat brain concentrations of the CNS neurotransmitters noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT). The analysis is performed by HPLC with electrochemical detection.
Glial fibrillary acidic protein (GFAP) has been proposed as a sensitive and specific biomarker for neurotoxicity. To challenge this proposal, the effects on regional rat brain GFAP concentrations are investigated following exposure to aromatic white spirit for 3 weeks or 6 months. GFAP is analyzed applying an ELISA technique.

Male Wistar rats (Mol:WIST) are used in all the studies. The exposure schedule is similar in all investigations: one group is sham-exposed (control), a second group is exposed to 500 (400) ppm and a third group to 1500 (800) ppm toluene (aromatic white spirit) in the inhaled air, 6 h/day for 3 weeks (aromatic white spirit) or 6 months (toluene, aromatic white spirit). In the

6 months exposure studies, exposure is followed by an exposure-free period of 4 months' duration prior to sacrifice to assure that findings are long-lasting changes.

In the 6 months toluene study, the weight of the hippocampus is statistically significantly reduced when exposed to 1500 ppm. The NA, DA, and 5-HT concentrations in various brain regions and in the whole brain are statistically significantly changed. The NA and DA concentrations change in the same direction in 3 regions: both increase in pons and thalamus, both decrease in medulla oblongata. This indicates a common underlying mechanism. Another characteristic pattern is statistically significantly increased 5-HT concentration in 3 regions (hemisphere, hippocampus, pons) and in the whole brain, indicating a common underlying mechanism. These findings are discussed on the basis of a review of relevant data in the existing literature. This procedure gives rise to the hypothesis: *the generally toluene-increased regional 5-HT concentration is part of a compensatory mechanism acting to overcome a toluene-reduced potential for serotonergic activity.*

In a 3 weeks aromatic white spirit study, aromatic and aliphatic components of aromatic white spirit are present in the whole brain. Data indicate that a threshold capacity for the elimination of aliphatic components is exceeded. Whole brain NA, DA, and 5-HT concentrations are statistically significantly increased.
In a 6 months aromatic white spirit study, NA and DA concentrations are statistically significantly changed in various regions. The 5-HT concentration statistically significantly increases in hemisphere, hippocampus, hypothalamus, pons, thalamus, medulla oblongata, and the whole brain, suggesting a general underlying mechanism.
Both 3 weeks and 6 months of aromatic white spirit exposure statistically significantly decrease the yield of synaptosomal protein whereas synaptosomal NA, DA, and 5-HT concentrations, high-affinity 5-HT uptake rate and uptake capacity are statistically significantly increased.
It is hypothesized that *the aromatic white spirit-increased synaptosomal NA, DA, and 5-HT concentrations are indices of increased neurotransmitter concentrations in respective presynaptic nerve terminals in situ acting to overcome reduced transmission capacity caused by an aromatic white spirit-reduced number of noradrenergic, dopaminergic, and serotonergic synapses in situ* and that *aromatic white spirit-increased 5-HT uptake rate and uptake capacity in serotonergic neurons in situ cause the generally increased global and regional 5-HT concentrations.*
The use of the synaptosomal model for studying the neurotoxicity of aromatic white spirit is supported.

Regional GFAP concentrations are not affected following 3 weeks or 6 months of aromatic white spirit exposure. This does not add evidence for the proposed use of GFAP as a biomarker for neurotoxicity.

In conclusion, the demonstrated changes of NA, DA, and 5-HT metabolism induced by exposure to toluene or aromatic white spirit demonstrate that

both these solvents are neuroactive. The long-lasting changes are serious effects, which should be taken into consideration by regulators.
At the present stage of knowledge, however, it is not possible to extrapolate from organic solvent affected neurochemical parameters in laboratory animals to the integrated outcome in the human being or *vice versa*.

The neurochemical findings taken together with the epidemiological evidence for toluene and white spirit neurotoxicity support the applicability of the proposed working hypothesis and the rat as a neurochemical research model for studying the mechanisms underlying/reflecting the neurotoxicity of these 2 solvents in man.

DANSK SAMMENDRAG

Der foreligger evidens for en årsagssammenhæng mellem erhvervsmæssig eksponering gennem indåndingsluften for visse organiske opløsningsmidler og fremkomsten i nervesystemet af effekter, som er sundhedsskadelige for mennesker og reducerer disses livskvalitet. To sådanne opløsningsmidler er toluen og terpentin. Der gives en kortfattet præsentation af disse 2 opløsningsmidlers toksikokinetik og effekter på nervesystemet.

Der findes ingen internationalt accepteret definition på neurotoksicitet. I videnskabelige kredse er der almindelig enighed om, at neurotoksicitet er *"enhver skadelig effekt på nervesystemets kemi, struktur og/eller funktion under individets udvikling eller som udvokset"* og om, at *" enhver langvarig effekt, det være sig en langtids-reversibel eller en sand irreversibel effekt, altid bør betragtes som alvorlig".*
Problemet er at definere "skadelig" på basis af neuroaktivitet.

Neurokemiske parametre målt i laboratoriedyr (pattedyr) er nyttige til studiet af kemiske stoffers neurotoksicitet i mennesker pga art-til-art lighed i tilgrundliggende biologi. Den følgende arbejdshypotese fremsættes: *Enhver ændring af enhver neurokemisk parameter induceret af et kemisk stof demonstrerer - direkte eller indirekte - det pågældende stofs neuroaktivitet.*

Der var en åbenbar mangel på langtidsstudier af toluen og terpentin i laboratoriedyr. Sådanne studier er relevante i relation til erhvervsmæssig eksponering. For at øge forståelsen for organiske opløsningsmidlers neurotoksicitet iværksattes en serie af multidisciplinære neurotoksicitets studier i laboratoriedyr. For indeværende inkluderer disciplinerne: Almen toksikologi, neuropatologi, adfærd, neurokemi, elektrofysiologi og NMR spectroskopi.

Sigtet med nærværende studier er at undersøge, hvorvidt inhalations-eksponering af rotter for toluen eller aromatholdig terpentin påvirker vigtige neurokemiske parametre.
Opmærksomheden rettes mod globale, regionale og subcellulære rottehjerne koncentrationer af CNS neurotransmitterne noradrenalin (NA), dopamin (DA) og 5-hydroxytryptamin (5-HT). Analyse heraf udføres ved HPLC med elektro-kemisk detektion.
Glia fibrillært surt protein (GFAP) er blevet foreslået som en sensitiv og specifik biomarkør for neurotoksicitet. For at undersøge dette forslag nærmere undersøges effekten af 3 ugers eller 6 måneders eksponering for aromatholdig terpentin på regionale GFAP koncentrationer i rottehjernen. GFAP analyseres ved ELISA teknik.

Wistar hanrotter (Mol:WIST) anvendes i alle studierne. Eksponeringsplanen er analog i alle undersøgelser: En kontrolgruppe eksponeres for ren luft, en anden gruppe eksponeres for 500 (400) ppm og en tredje for 1500 (800) ppm toluen (aromatholdig terpentin) i indåndingsluften i 6 timer/dag i 3 uger (aromatholdig terpentin) eller 6 måneder (toluen, aromatholdig terpentin). I 6 måneders eksponeringsundersøgelserne efterfølges eksponering af en 4

måneders eksponeringsfri periode inden aflivning. Hermed tilsikres, at iagttagne effekter er langvarige effekter.

I 6 måneders toluen studiet reduceres hippocampus' vægt statistisk signifikant ved eksponering for 1500 ppm. NA, DA og 5-HT koncentrationerne ændres statistisk signifikant i forskellige hjerneregioner og i totalhjernen. NA og DA koncentrationerne ændres i samme retning i 3 regioner: Begge øges i pons og thalamus, begge reduceres i medulla oblongata. Dette indicerer en fælles tilgrundliggende mekanisme. Et andet karakteristisk mønster er statistisk signifikant forøgede 5-HT koncentrationer i tre regioner (hemisfære, hippocampus, pons) og i totalhjernen, hvilket indicerer en fælles tilgrundliggende mekanisme. Disse fund diskuteres på basis af et review af relevante data i den eksisterende litteratur. Dette giver anledning til følgende hypotese: *De generelt toluen-forøgede regionale 5-HT koncentrationer er del af en kompensatorisk mekanisme, som virker for at overvinde et toluen-reduceret potentiale for serotoninerg aktivitet.*

I et 3 ugers studium af aromatisk terpentin findes aromatiske og alifatiske terpentin komponenter tilstede i totalhjernen. Data indicerer, at en tærskel for eliminationskapaciteten for alifatiske komponenter overskrides. Totalhjernens NA, DA og 5-HT koncentrationer er statistisk signifikant forøgede.
I et 6 måneders studium af aromatisk terpentin ændres NA og DA koncentrationerne statistisk signifikant i forskellige regioner. 5-HT koncentrationen forøges statistisk signifikant i hemisfære, hippocampus, hypothalamus, pons, thalamus, medulla oblongata og i totalhjerne, hvilket antyder en generel tilgrundliggende mekanisme.

Både 3 ugers og 6 måneders eksponering for aromatholdig terpentin reducerer statistisk signifikant udbyttet af synaptosomalt protein, hvorimod synaptosomale koncentrationer af NA, DA og 5-HT, høj-affinitets 5-HT optagelses-hastighed og -kapacitet forøges statistisk signifikant.
Der fremsættes hypotetisk, at *aromatisk terpentin-forøgede synaptosomale NA, DA og 5-HT koncentrationer er indicier på øgede neurotransmitterkoncentrationer i respektive presynaptiske nerveterminaler in situ, som virker for at overvinde reduceret transmissionskapacitet forårsaget af et aromatisk terpentin-reduceret antal af noradrenerge, dopaminerge og serotonerge synapser in situ* og, at *aromatisk terpentin-forøget 5-HT optagelses-hastighed og -kapacitet i serotonerge neuroner in situ forårsager de generelt forøgede global og regionale 5-HT koncentrationer.*
Synaptosommodellens anvendelighed til studiet af aromatisk terpentins neurotoksicitet understøttes.

Regionale GFAP koncentrationer påvirkes ikke som følge af 3 ugers eller 6 måneders eksponering for aromatisk terpentin. Dette øger ikke evidensen for den foreslåede anvendelse af GFAP som biomarkør for neurotoksicitet.

Det kan konkluderes, at de påviste ændringer af NA, DA og 5-HT metabolisme induceret ved eksponering for toluen eller aromatisk terpentin demonstrerer, at begge disse opløsningsmidler er neuroaktive. De langvarige ændringer er alvorlige effekter, som bør tages i betragtning af regulatorer.

Med den nuværende viden er det imidlertid ikke muligt at ekstrapolere fra opløsningsmiddel inducerede påvirkninger af neurokemiske parametre i laboratoriedyr til den integrerede resultant i mennesker eller *vice versa.*

De neurokemiske fund sammenholdt med epidemiologisk evidens for toluens og aromatisk terpentins neurotoksicitet støtter den foreslåede arbejdshypoteses anvendelighed, og at rotten kan anvendes som neurokemisk forskningsmodel ved studiet af de mekanismer, som ligger til grund for/afspejler disse 2 opløsningsmidlers neurotoksicitet i mennesker.

I. INTRODUCTION

1. THE NERVOUS SYSTEM

A. Structure and function

The mammalian nervous system consists of the brain, spinal cord, peripheral nerves, nerve endings, and some parts of the sensory organs. The CNS includes the brain and the spinal cord.

In the adult human being, the weight of the brain corresponds to approximately 2% of the total body weight. The brain receives 15% of the cardiac output and accounts for 20% of the oxygen utilized by the body at rest (Ginsberg, 1992).

Neurons and glial cells are the fundamental cellular elements of the CNS. The principal functions of neurons are the transmission and processing of information. To perform these functions, neurons receive, integrate, and respond to stimuli. These properties have been known for many years. Therefore, focus has traditionally been placed on the neurons.

The glial cells include oligodendrocytes, microglia, and astrocytes. Previously, glial cells were looked upon as only being structural elements supporting neurons. New knowledge, however, has stressed the integrative participation of glial cells in brain function. Oligodendrocytes are responsible for elaborating short lengths of myelin around axons speeding up their electrical impulse propagation velocity. Furthermore, they are able to synthesize neurotrophic factors (Raine, 1989; Travis, 1994). Microglia cells possess phagocytic functions (Raine, 1989). Astrocytes have intimate correlation with neighbouring neurons supporting their action by providing nutrients and precursors. They also take up ions and neurotransmitters released at nerve impulse propagation and thereby maintain the signalling capacity of the neurons. Astrocytes may play part of learning and memory processes and they are able to produce neurotrophic factors, which may help the neurons to recover after insults. Growing evidence suggests that glial cells by themselves may be communicative (Travis, 1994).

The nervous system is essential for the physical, psychological and social performance of the individual.

B. Protection

The maintenance of a constant internal environment is very important for the function of the brain.

The penetration of chemical substances from the blood into the nervous tissue in CNS has been studied since late in the nineteenth century. The results led to the development of the concepts of the BBB and the BCB (Betz et al., 1989).

Endothelial cells in brain capillaries differ from endothelial cells of capillaries in other organs by the presence of continuous tight junctions. In most areas of the CNS these junctions prevent transcapillary movement of many chemical substances. Furthermore, there is an absence of transcellular channels as well as a paucity of plasmalemmal and intracellular vesicles (Betz et al., 1989). These features make up the BBB. The foot plates of astrocytes cover almost the entire outer surface of the capillaries in the brain

and contribute to the development and maintenance of the BBB, which covers approximately 99% of the brains capillary surface (Ladefoged et al., 1995).
The BBB contains different transport systems each of selective affinity to endogenous substances such as glucose, lactate, amino acids, choline, amines, purines, nucleosides, and thyroid hormone. These transport systems are not totally specific and allow entry of exogenous substances possessing some carrier affinity. Penetration mechanisms include: diffusion, pinocytosis, and carrier-mediated transport (Betz et al., 1989).
In some areas of the brain, the BBB is weak/absent (pituitary gland, median eminence, area postrema, preoptic recess, paraphysis, pineal gland, and the endothelium of the choroid plexus) (Betz et al., 1989).

The BCB is located at the epithelial cells of the capillaries in the choroid plexus, which are situated in the cerebral ventricles. The CSF is secreted by diffusion, facilitated diffusion, and active transport, so it is not a simple protein-free ultrafiltrate of the plasma (Betz et al., 1989).

The BBB and the BCB separate the CNS from the bloodstream and protect the brain from entry of a.o. exogenous substances.
Generally, these barriers inhibit influx of most hydrophillic substances along their electrochemical gradients, whereas lipophilic substances may cross. The function of these barriers is not constant throughout life and may be disturbed by different external stimuli. Individual and inter-species variations in BBB and BCB function may exist.

C. Vulnerability

The brain is very susceptible to many chemical substances. This is mainly a consequence of its structural and functional integral complexicity, its metabolic characteristics, and its small capacity for compensation and repair:

-The brain requires a continuous and rich supply of oxygen and glucose, which are both essential for its function.
-Intensive intracellular transport and intercellular communication are special characteristics of nerve tissue.
-Neuronal cells, with their long processes, provide a vast surface area for chemical attack and are, therefore, inherently susceptible to chemicals.
-Neuronal cells in the CNS are capable of some repair, but dead neurons can not be replaced.

Thus, the risk for permanent damage after toxic injury is greater in the nervous system than in other organ systems. The manifestations of toxic damage to the brain are particularly serious because of the brains role as a superior controlling unit of the individual.

2. NEUROTOXICITY OF CHEMICAL SUBSTANCES

The concern for induction of toxic damage to the nervous system has increased during the last decades because of outbreaks of neurotoxic episodes induced by accidental, intended, occupational, or environmental exposure.

Increasing evidence has become available connecting exposure to certain classes of chemicals with the appearance of various neurotoxic effects, which impair human health, life quality, and even may increase the mortality rate.

It is not known for certain, how many of the 50000 to 100000 chemicals in use, that are neurotoxic (Tilson et al., 1995). This is mainly because the neurotoxicity of most of these chemicals has never been investigated. However, it has been reported that more than 850 chemicals produce behavioural disorders (Anger & Johnson, 1985) and that more than 20000 chemicals are considered to be able to interfere with the nervous system (McMillan, 1987). Focus is placed on specific chemicals and classes of chemicals, including: inorganic heavy metals, organometals, pesticides, herbicides, carbon monoxide, MPTP, PCBs, toxins, contaminants of food and water, ethanol, pharmaceuticals, and organic solvents.

3. ORGANIC SOLVENTS

A. Organic solvents in general

a. Definition and some physical and chemical properties of organic solvents

'Organic solvents are often called industrial solvents or just simply solvents. The term is a generic name for a group of organic chemicals or mixtures thereof, which typically are liquid in the temperature range of 0-250 °C. In traditional technical applications solvents have been used for extracting, dissolving or suspending materials, which are not soluble in water, e.g. fats, lipids, resins and polymers. Thus, the organic solvents are lipophilic, and they are often volatile. Furthermore, they do not react chemically with the materials' (Cohr, 1985).

Organic solvents is a very heterogenous class of chemical substances, a common feature of which is a high degree of solubility in lipids.

b. Historic overview

Organic solvents have been used in industry since the 1840s. Along with the growing industrialization their use also increased. In the 1970s and 1980s, an increasing number of reports connected exposure to organic solvents with the induction of neurotoxicity in man, especially reports from the Nordic countries. Hexacarbons, toluene, acrylamide and various solvent mixtures have been studied intensively. From around the mid-1980s their use has been reduced (For review see Arlien-Søborg, 1992).

c. Exposure

Inhalation is the main occupational exposure route. High-concentration exposure outside the workplace occurs when chemicals are abused, e.g. by sniffing, and from the use of household products. The entire population may

be subjected to low-dose exposure from contamination of air, soil, food, and drinking water.

d. Uptake

In Denmark, there is a tradition for brain research. The diffusion of organic non-electrolytes, including some organic solvents, from blood to brain tissue was studied by Christian Crone back in 1961 (Crone, 1961). Owing to lipophilicity, an organic solvent may cross the BBB by simple diffusion i.e. movement of solvent molecules from compartments with higher concentrations of free solvent to compartments with lower. The flux is proportional to the concentration gradient of free solvent across the BBB.

Many factors are important for the uptake of organic solvents from the inhaled air into the brain: concentration in air, respiratory volume, duration of exposure, blood perfusion, partition coefficients (blood/air; brain/blood), and the rate of breakdown and excretion. Even a light work load may double or triple the pulmonary uptake when compared with the level at rest (Åstrand, 1983).

e. General toxicity

It is not the scope of this thesis to go into details with the publications concerning solvent-induced general toxicity in laboratory animals or man. General reviews have been published concerning toluene (WHO, 1985; Jelnes, 1989; Arlien-Søborg, 1992; US Department of Health and Human Services, 1992) and white spirit (Hass & Prior, 1986; Arlien-Søborg, 1992; IPCS, 1995).

B. Neurotoxicity

In the scientific community there is international agreement that long-term exposure to organic solvents can cause long-lasting (i.e. long-term reversible or true irreversible) brain damage in man. Information originates from case stories, epidemiological studies, studies with laboratory animals, *in vitro* studies, and studies in human volunteers exposed in exposure chambers under controlled exposure conditions. A number of international conferences held in Denmark during the last decade (World Health Organization/Nordic Council of Ministers in 1985; Commission of the European Communities/Danish Ministry of the Environment in 1990; International Neurotoxicology Association in 1993; and Nordic Council of Ministers in 1995) have also stressed the causative relationship between exposure to organic solvents and the induction of reversible and long-lasting neurotoxic effects in man.

In Denmark, from 1978 to 1989, 5157 persons were awarded disability pension because of brain damage after exposure to organic solvents (Rohde, 1993). In the 1990s, the number of cases have decreased, mainly because of reduction of the use and the exposure levels, and owing to increased information and protection.

C. Protection from insults

Toxicology is a scientific discipline with a special status compared with most other biomedical disciplines, because its results are mandatory for regulatory authorities. Neurotoxicology is a relatively new field within toxicology.

By regulation, labelling, and information, regulatory agencies make efforts to protect the population against a.o. neurotoxic insults. In October 1986, the Danish Environmental Protection Agency sent a list of 13 organic solvents to the EU-commission proposing labelling of these solvents. The compounds were: chloromethane, dichloromethane, n-hexane, methylethyl-ketone, methyl-n-butylketone, white spirit, styrene, toluene, tetrachloroethylene, 1,1,1-trichloroethane, trichloroethylene, 1,1,2-trichloro-1,2,2-trifluorethane, and xylene.
These proposals were not accepted because of the lack of 'proofs'. The only substances, which were R-48 labelled ('Danger of serious damage to health by prolonged exposure') because of neurotoxicity in man were n-hexane, chloromethane, and methyl-n-butylketone.

The Danish Working Environmental Service every second year publishes a list of a.o. organic solvents together with their Danish TLV. The present list was published in 1994 (Arbejdstilsynet, 1994).

In order to gain insight into the neurotoxicity of organic solvents a series of neurotoxicity studies in rats was initiated at the Institute of Toxicology in the mid 1980s. To increase the strength of evidence for neurotoxicity, this research project was set up as a multidisciplinary approach comprising general toxicity, pathology, behaviour, neurochemistry, and electrophysiology. To be able to cover all these disciplines, collaboration was established with national and international scientists from various institutions and universities. It was decided to initiate the project by studying 2,5-hexanedione (1986), which is the neurotoxic metabolite of n-hexane and methyl-n-butylketone. Later on, investigations of toluene (1986), aromatic white spirit (1989), dearomatized white spirit (1992), *p*-cymene (1993), *tert*-butyltoluene (1994), and phenylglyoxylic acid (1994), were started.

II. DEFINITION OF NEUROTOXICITY AND WORKING HYPOTHESIS

There is no internationally accepted definition of neurotoxicity. In the scientific community, it is generally agreed that neurotoxicity is '*any adverse effect on the chemistry, structure, and/or function of the nervous system during development or at maturity, induced by biological, chemical, or physical influences*' (Johnsen et al., 1992; Eisenbrandt et al., 1994; Ladefoged et al., 1995; Simonsen et al., 1995; Tilson et al., 1995). The problem is how to define and set a threshold for adverse. This problem is not unique for neurotoxicity.
Furthermore, it is agreed that '*any long-lasting (long-term reversible or true irreversible) effect should always be regarded as serious*'.
Neurotoxicity may be a direct effect on cells in the nervous system or an indirect effect induced via damage to another organ e.g. liver or the kidneys (Ladefoged et al., 1995).

The function of the nervous system is essential for the physical, psychological, and social performance of the individual.
The normal function of the nervous system is an intimate balance between numerous chemical and physical parameters. This balance is essential for uncompromised nerve signal generation, integration, propagation, and cell-to-cell transmission. Any imbalance can disrupt the normal function, and may be expressed as affected performance: as neurotoxicity. Regaining the balance, either by reversing the disturbed parameter to the normal or by functionally compensating the disturbance, may reverse the performance of the individual to an unaffected or apparently unaffected state, respectively.

Generally, chemicals that induce neurotoxicity in laboratory animals, including the rat, also induce neurotoxicity in man and *vice versa*, although a few exceptions are known, including: the lack of organophosphate-induced delayed neuropathy and MPTP-induced motor dysfunction in the rat (Tilson et al., 1995). Such differences may be caused by inter-species variations in toxicokinetics, toxicodynamics, or susceptibility.
Neurochemical parameters measured in laboratory animals (mammalian species) are useful for studying the neurotoxicity of chemicals in man because of inter-species similarity of the underlying biology.
The following working hypothesis is put forward for the neurochemical part of the neurotoxicology research project: *any change of any neurochemical parameter induced by a chemical demonstrates - directly or indirectly - the neuroactivity of that chemical.*
Any change may cause neurotoxicity, reflect a compensatory mechanism, or may be unrelated to neurotoxicity. The problem is how to define and assess adversity on the basis of neuroactivity.

III. OBJECTIVES

This thesis reviews own neurochemical studies (**I**; **II**; **III**; **IV**; unpublished GFAP studies) in the rat brain of effects caused by inhalation exposure to toluene or aromatic white spirit.

In order to understand the neurochemical mechanisms underlying or reflecting neuroactivity of organic solvents, it is important to study their effects on every single step of the transmission line. In this thesis, focus is placed on global (**II**), regional (**I**; **III**), and subcellular (**IV**) rat brain NA, DA, and 5-HT metabolism, as these substances are important CNS neurotransmitters. Determination of neurotransmitter concentrations in the whole brain (**II**) was used to screen for effects, although even large changes in one region may be concealed in the whole brain by opposite changes in other regions.
Owing to the regional heterogeneity in the brain, it was decided to dissect it into discrete regions to obtain more specific information (**I**; **III**). The brain was dissected into the following 7 regions: cerebellum, hemisphere, hippocampus, hypothalamus, pons, thalamus, and med. obl. Various aspects of brain function can be ascribed to these regions.
Effects found at the subcellular level may provide more specific information than effects shown at the regional level. Synaptosomes constitute a well-documented *ex situ* model system for the presynaptic nerve terminal complex *in situ*. The synaptosomal preparation was used (**IV**) for more detailed studies of the mechanisms underlying the effect of aromatic white spirit on global (**II**) and regional (**III**) neurotransmitter concentrations.

New knowledge has stressed the integrative role of glial cells in the function of the brain. Reactive astrogliosis may take place in response to any kind of CNS damage irrespective of its cause: biological, chemical, or physical (Duchen, 1984; Eng, 1988). Characteristic features of reactive astrogliosis are hypertrophy and hyperplasia, accompanied by the accumulation of glial filaments (Eng, 1988). GFAP is the major cytoskeletal protein of glial filaments in the differentiated astrocyte. O'Callaghan and colleagues from USEPA have proposed the use of GFAP as a sensitive and specific biomarker for neurotoxicity (O'Callaghan, 1991a). It is important to test this proposal with many non-neurotoxicants and neurotoxicants, preferably neurotoxicants with different targets and mechanisms of action. Therefore, the effects of aromatic white spirit exposure for 3 weeks and for 6 months on regional GFAP concentrations in the rat brain, were examined.

Own findings (**I**; **II**; **III**; **IV**; the GFAP studies) are discussed in the context of reviews of relevant data already published in peer reviewed literature and in terms of the neurotoxicity of toluene and white spirit in man.
The applicability of the working hypothesis and the rat as a laboratory animal for neurochemical studies of the mechanisms underlying/reflecting toluene- and aromatic white spirit-induced neurotoxicity in man, are also discussed.

IV. BASIC CHEMICAL, PHYSICAL, BIOCHEMICAL, AND NEUROTOXICOLOGICAL PROPERTIES OF TOLUENE AND WHITE SPIRIT

It is outside the scope of this thesis to make a detailed presentation of these topics. Only a brief summary of the most relevant data is given. Reference **I, II, III, IV**, and own GFAF studies are excluded from this summary. For more detailed information, reviews and original references should be consulted.

1. TOLUENE

A. Identity and some chemical and physical properties

Chemical formula:	C_7H_8 (Structure, see p. 19).
Molecular weight:	92.13 g/mol.
CAS registry number:	108-88-3.
Boiling point at 760 mm Hg:	110.6 °C.
Concentration in air at saturation at 25 °C:	37000 ppm, corresponding to 138.8 mg/l.
Solubility in water at 25 °C:	535 mg/l, corresponding to 5.81 mM.
Partition coefficients:	
Blood/air	11-16.
Fat/blood	81-83.
Octanol/water	62.
Present Danish TLV:	35 ppm (Arbejdstilsynet, 1994).

A brief presentation is given of the toxicokinetics and the effects on the nervous system. More detailed and specific data concerning toxicokinetics, general toxicity, and neurotoxicity of toluene are reviewed by WHO (1985), Jelnes (1989), Arlien-Søborg (1992), and US Department of Health and Human Services (1992).

B. Toxicokinetics

Humans are exposed to toluene through the air, in the form of liquid toluene, and as a contaminant of water and food. Exposure takes place both at work and at home. Occupational exposure is the most frequent and quantitatively most important kind of exposure.

a. Uptake

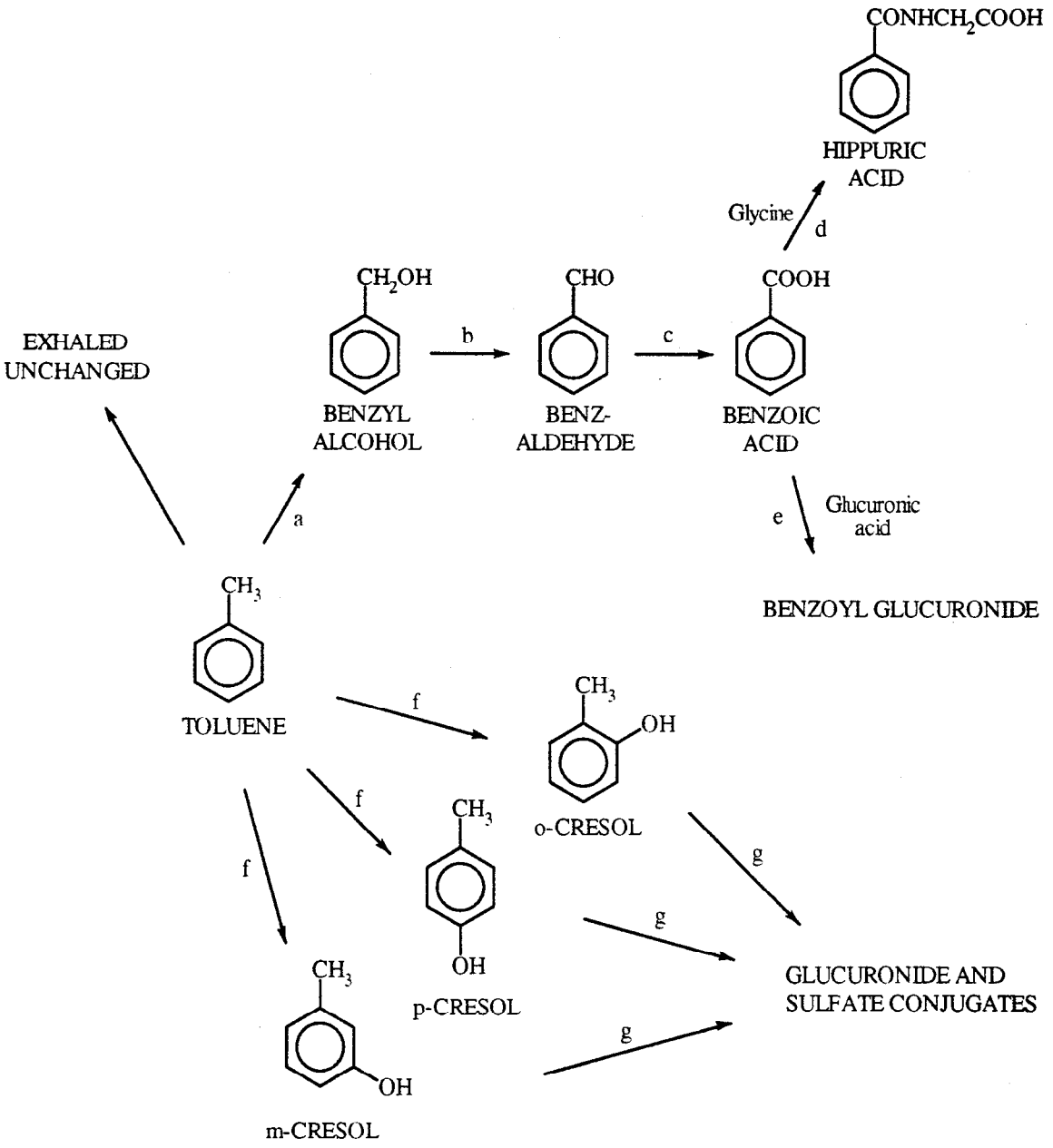
Toluene in the inhaled air is rapidly taken up via the lungs. This constitutes the principal uptake route when occupationally exposed. Even a light work load increases the pulmonary uptake.
Transdermal uptake of toluene present (as gas) in the air has been estimated to constitute approximately 1% of the concomitant pulmonary absorption. If the skin gets into direct contact with liquid toluene, especially hydrated, disrupted, or diseased skin, transdermal absorption may reach higher levels.
Accidental or intended oral intake have shown that gastrointestinal uptake is almost complete and that the plasma concentration peak value appears later than when inhalation exposed.

b. Distribution

After systemic uptake, toluene is distr'buted to the different organs, a.o. liver, adipose tissue, and the brain. Different concentrations in various brain parts arise, the highest concentration has been observed in pons, the lowest in the olfactory bulb (Kiriu et al., 1990).

c. Biotransformation

After absorption via the lungs, an unknown fraction of the absorbed toluene gets to the brain unmetabolized before passing the liver, which is the principal organ for toluene detoxification.
The liver and brain biodegradation of toluene in a.o. rat and man is:



Biodegradation and elimination of toluene.

In the human being and in laboratory animals, including the rat, toluene is mainly metabolized by side chain oxidation by the cytochrome P450 system (step **a**, where cytochrome P450 IIE1, IIC11/6, and IIB1/2 are known to contribute, Nakajima et al., 1993) to benzyl alcohol, which is further oxidized to benzaldehyde by alcohol dehydrogenase (step **b**), and to benzoic acid by aldehyde dehydrogenase (step **c**). Benzoic acid is conjugated with glycine to form hippuric acid (step **d**) (60-70% of the dose) or with glucuronic acid to form benzoyl glucuronide (step **e**) (10-20% of the dose). The degradation seems to be limited by the maximum capacity of the initial hydroxylation system.
Minor oxidative pathways are ring hydroxylation to *o*-, *m*- and *p*-cresol (steps **f**, where cytochrome P450 IIE1, IIC11/6, IIB1/2, and IA1/2, are known to contribute, Nakajima et al., 1993). These hydroxylation products can be conjugated (steps **g**).
The clearance of toluene from rats can be increased by ethanol or phenobarbital pretreatment (Pyykkö, 1984; Wang & Nakajima, 1992).
Toluene pre-exposure accelerates toluene metabolism *in vivo* by increasing P450 dependent microsomal monooxygenase activity (Pyykkö, 1984). Toluene has been shown to increase the P450 IIEI and IIB1/2 activities and to decrease P450 IIC11/6 activity in rat liver microsomes *in vitro* (Nakajima et al., 1993).

d. Excretion

The elimination of toluene from lipid tissue, a.o. the brain, is slow. This may lead to toluene accumulation in the brain as a consequence of repeated exposure.
Urinary excretion of hippuric acid and benzoyl glucuronide is the major elimination route of toluene. Minor elimination pathways are exhalation of toluene as such, urinary excretion of *o*-, *m*-, and *p*-cresol as such and as *o*-, *m*-, and *p*-cresol conjugates.
Determination of the urinary excretion of hippuric acid has been proposed as a marker for toluene exposure. However, the applicability of this parameter is hampered by serious drawbacks such as the relatively high basal hippuric acid excretion originating from endogenous sources and the variation of the excretion among individuals even after similar toluene exposure. Furthermore, hippuric acid is not specific for toluene exposure, because other organic solvents such as ethylbenzene and styrene, and the intake of benzoic acid with the food may contribute significantly to the total excretion of hippuric acid. In addition, formation of benzoic acid takes place from the activity of the bacterial flora in the gut.
The urinary excretion of *o*-cresol has also been proposed as a marker for toluene exposure. The use of this parameter, however, has drawbacks too: lifestyle, such as smoking and alcohol drinking are confounders, which should be considered because both affect *o*-cresol excretion (Døssing et al., 1983a; Bælum et al., 1987; Inoue et al., 1994).
The applicability of hippuric acid and *o*-cresol (alone or in combination) as markers for toluene exposure has not been finally confirmed (Skender et al., 1993) especially not when the exposure level is below 0.20 mg/l, corresponding to 53 ppm (Nise, 1992).

C. Effects on the nervous system

Toluene affects the CNS. The effects induced in laboratory animals and man include acute toxicity (narcosis, death) and morphological, neurochemical, and behavioural changes. In humans, neuropsychological disorders is also a frequent consequence of exposure as revealed by epidemiological studies.

a. Animal studies

Pathology

Only a limited number of neuropathological studies have been performed. In the area dentata of the hippocampus the volume of the granule cell layer was reduced at the end of 28 days of exposure from postnatal day 1 to day 28. The reduction was 6% in rats exposed to 100 ppm and 13% in rats exposed to 500 ppm (Slomianka et al., 1990). In this study, also the volume of the hilus and the commissural-associational zone of the dentate molecular layer was reduced. However, the same authors were unable to demonstrate these changes at postnatal day 120 following cessation of exposure to 500 ppm at postnatal day 1-28 (Slomianka et al., 1992). This may be an indication of reversibility. Korbo et al. (1993, 1996) found a 16% reduced number of neurons in regio inferior of hippocampus of rats exposed to 1500 ppm toluene for 6 months followed by an exposure-free period of 4 months' duration.

Biochemical changes

Reduced rat plasma L-tyrosine and L-tryptophan concentrations have been demonstrated 60 min after an i.p. injection of a single dose of 30 mmol toluene/kg b.w. and immediately after inhalation exposure to 30000 ppm toluene for 2 to 16 min (Voog & Eriksson, 1984). The decrease in the plasma concentration of L-tryptophan was more pronounced than that of L-tyrosine, irrespective of the route of administration. Reduced L-tyrosine and L-tryptophan plasma concentrations suggest reduced supply to the brain. L-tyrosine and L-tryptophan are CNS neurotransmitter precursors.

Neurochemical studies

Many investigations have shown effects induced by toluene on brain neurochemistry. Effects have been located to the whole brain, various brain regions, and to the subcellular level.

The effects include changes of transmitter concentrations and -turnovers (predominantly NA, DA, 5-HT, and amino acids) in the brain. Ca^{2+}-uptake rate and induction of oxidative stress have been studied in synaptosomes. Additionally, neurotransmitter-synthesizing and -degradating enzyme activities, neuron and glial cell markers (neuron specific enolase, S-100 protein, creatine kinase), membrane ATPase activities, and membrane fluidity as well as receptor functions, have been studied. Many of these findings will be reviewed in Chapter VIII.

Pre-exposure postnatal day 1-7 affected the effects of subacute exposure postnatal week 8 on CA levels and CA depletions in forebrain, hypothalamus, and substantia nigra (von Euler et al., 1989a).

Behaviour

Behavioural effects of toluene have been studied particularly in rats and mice following short-term exposure. As is the case for other substances with narcotic properties, a biphasic effect of toluene exposure has been found: CNS-stimulation following low-dosing, whereas higher doses have a CNS-depressant effect.

Studies in rats and mice suggest that toluene possess anxiolytic properties as expressed as reinstation of behaviour that had been suppressed by punishment. It is suggested that toluene acts alone and in a synergistic manner in combination with diazepam (Geller et al., 1983). In various respects its action resembles the action of benzodiazepines (Wood et al., 1984).

Neurophysiological changes

Neurophysiological examinations have revealed many functional changes of the nervous system including affected sleep pattern, abnormal light flash evoked potentials, permanent high frequency hearing loss, which is enhanced by concomitant noise exposure, and reduced peripheral nerve impulse conduction velocity (For review see Arlien-Søborg, 1992).

b. Studies in man

Biochemical studies

The *in vitro* rate of 5-HT uptake (V_{max}) into platelets isolated from car painters exposed to mixtures of organic solvents and organic isocyanates was elevated in 9 of 12 workers (Beving et al., 1983).

Exposure chamber studies

Studies in volunteers exposed in exposure chambers under controlled conditions demonstrate acute narcotic effects. Initially excitatory effects are observed, followed by sedation including fatigue, sleepiness, headache, nausea, decreased manual dexterity, psychiatric symptoms, and impaired performance in neuropsychological tests, affected visual vigilance, vestibular function, and tone detection (For review see Arlien-Søborg, 1992).

Abuse

Toluene is in widespread use among sniffers. Studies of abuse may provide important information of short- and long-lasting neurotoxic effects. Most often, abuse involves much higher exposure levels than those encountered in the working environment. Studies have revealed chronic toxic encephalopathy, frequently with cerebral atrophy, and affected EEG and performance.

Solvent-induced chronic toxic encephalopathy is a symptom complex characterized by deficits in psychomotor, perceptual, and memory functions with associated disturbances in the mood (WHO/Nordic Council of Ministers, 1985).

Epidemiological findings

Case stories indicate short- and long-lasting disturbances of CNS functions, including an increased frequency of psychiatric symptoms and affected psychological functions.

There is supportive evidence for a causal relationship between long-term exposure to toluene and increased frequency of the development of chronic toxic encephalopathy in man. However, no longitudinal epidemiological study of toluene exposed workers exists to give the ultimate proof (For review see Arlien-Søborg, 1992).

D. General conclusions

Studies in laboratory animals and in humans support the indication that toluene induces CNS neurotoxicity at both high and low concentrations in the inhaled air. No longitudinal study exists to provide the final evidence in man.

2. WHITE SPIRIT

White spirit is a widely used organic solvent in paints, printing inks, and varnishes. Various products are manufactured as distillation or chemically modified fractions of crude oil. All are complex mixtures defined on the basis of their content of aliphatic, aromatic, alkyl aromatic and naphthenic hydrocarbons.
For several years, aromatic white spirit has been the most frequently used type of white spirit in the Nordic countries. It is typically composed of numerous different components, as depicted in Appendices III A,B.

The following is a brief presentation of the present knowledge concerning white spirit toxicokinetics and neurotoxicity. More detailed and specific data concerning toxicokinetics, general toxicity, and neurotoxicity of white spirit are given in 3 reviews: Hass & Prior (1986), Arlien-Søborg (1992), and IPCS (1995).

A. Some chemical and physical properties

Synonyms:	Mineral spirit, mineral turpentine, stoddard solvent, varnolene, petroleum naphtha, petroleum spirit.
Average molecular weight:	135-145 g/mol.
Boiling point at 760 mm Hg:	In the interval from 140 to 215 °C.
Concentration in air at saturation:	Approximately 5000 ppm.
Present Danish TLV:	25 ppm (Arbejdstilsynet, 1994).

The solubility in water is poor and differs for the various components.

Partition coefficients between blood and air, and between fat tissue (brain) and blood also differ for the various components. Typically, aromatic compounds possess higher blood/air partition coefficients than those of the aliphatics, whereas the opposite seems to be the case for the distribution between brain and blood.

B. Toxicokinetics

a. Uptake

White spirit is taken up through the lungs as the principal route of uptake when occupationally exposed. It has been estimated that the fractional uptake from the inhaled air of the aromatic part of aromatic white spirit (58-70% of total uptake) is greater than that of the aliphatic part (46-59% of total uptake), when considering that the content of aromatic compounds is only in the range of 15-20% (w/w) in the inhaled air (Appendix III B). From accidents and intended misuse it is known that uptake also takes place from the gastrointestinal tract. Transdermal uptake may take place especially through hydrated, disrupted, or diseased skin. No controlled studies of these alternative routes of uptake have been performed.

b. Distribution

Following uptake, the components are distributed to the different organs especially fat tissue, liver, and the brain. Aliphatic and naphthenic components of aromatic white spirit are present in the brain in higher concentrations than those of the aromatic components when single-solvent exposed for the same time to the same concentrations of the pure components. Accumulation may occur following daily exposure (For review see IPCS, 1995).

c. Metabolism and elimination

After absorption via the lungs, an unknown fraction of the absorbed white spirit components reach the brain unmetabolized. No study of the metabolism of white spirit as such has been performed. Information has been gathered from studies where single components have been examined. The extent to which white spirit components are eliminated by exhalation is not known but this elimination route is presumed to contribute to the total elimination (For review see IPCS, 1995).

The liver is supposed to be the principal detoxification organ. Hydrocarbons undergo oxidative conversion to alcohols by the cytochrome P450 system. Oxidation of n-alkanes, $\leq C_7$, results in secondary mono- or dialcohols. Higher n-alkanes only undergo oxidation at the terminal carbon atoms. Branched aliphatic components are predominantly oxidized to secondary or tertiary alcohols. Monocyclic and polycyclic components are mainly oxidized by ring-hydroxylation as also known to be the case for toluene. The potential for epoxide formation exists. Subsequently, conjugation of the hydroxy groups with glucuronic acid or sulphate may occur. Some metabolites undergo further oxidation to aldehydes/ketones and carboxylic acids. The carboxylic acids generated from the n-alkanes may undergo ß-oxidation (For review see IPCS, 1995).

d. Markers for exposure

It has been reported that the urinary excretion of dimethylbenzoic acid isomers (expressed as the sum in mg of dimethylbenzoic acid isomers per g of creatinine) is linearly related to the exposure to white spirit (Pfäffli et al., 1985). These dimethylbenzoic acid isomers may be metabolites of trimethylbenzenes, which are components of aromatic white spirit, albeit in low concentrations (Appendix III B; **II**). The urinary excretion of dimethylbenzoic acid isomers may be used as marker for the exposure to aromatic white spirit. The use of this marker must be performed with reservations.

C. Animal experiments

a. Neurotoxic effects

Few laboratory animal experiments investigate the toxicity of white spirit. These studies report general neurotoxic effects such as increased mortality, dyscoordination, tremor, seizure, and decreased pupillary light reaction.

Special attention should be paid to a specific white spirit component: n-nonane, because of its potential ability to damage nerve tissue (Verkkala et al., 1984; Nilsen et al., 1988), and because n-nonane accumulates in the brain to high concentrations (Nilsen et al., 1988; Zahlsen et al., 1990, 1992).

b. Pathology

In a satellite study to study **III**, Pilegaard (1993) applied unbiased stereological methods for the estimation of the number of astrocytes in the molecular layer of the dentate gyrus of the hippocampus. There was no treatment related effect on the number of GFAP-positive astrocytes, GFAP-volume, or GFAP-volume per astrocyte in this subregion of the hippocampus.

c. Biochemical effects

One study reports reduced rat muscle membrane sialic and uronic acid contents in proportion to lipid phosphorous or total membrane protein (Savolainen & Pfäffli, 1982).

d. Neurochemical studies

A limited number of neurochemical studies exists. They report increased activity of brain acidic proteinase and cerebellar creatine kinase. The cerebellar GSH concentration increased following exposure to 500 and 1000 ppm aromatic white spirit for 4 weeks whereas it decreased following 8 and 17 weeks of exposure at 1000 ppm (Savolainen & Pfäffli, 1982).

Three studies (other than **IV**) have been performed on synaptosomes (Edelfors & Ravn-Jonsen, 1985, 1992; Bondy et al., 1995). These studies will be discussed in Chapter VIII part 4.

We have applied *in vivo* NMR spectroscopy to the hippocampus and surrounding regions of rats exposed for 3 weeks for 0, 400, or 800 ppm aromatic white spirit (Stensgaard et al., 1995, 1996). We were not able to detect any aromatic white spirit-induced effect on the content of N-acetylaspartate, total creatine containing compounds, or choline.

e. Behavioural effects

Behavioural effects are reported including dose related decreased speed of learning and visual discrimination (For review see Arlien-Søborg, 1992).

D. Human experience

a. Biochemical studies

The *in vitro* rate of 5-HT uptake (V_{max}) into platelets isolated from car painters exposed to mixtures of organic solvents and organic isocyanates was elevated in 9 of 12 workers (Beving et al., 1983).

b. Exposure chamber studies

Visual reaction time, vigilance, and attention were the first parameters to be impaired, followed by manual dexterity and affected long-term memory. In some persons, cognitive functions were impaired even when exposed for low concentrations (at 50 ppm) for 7 h (For review see Arlien-Søborg, 1992).

c. Neuroradiological findings

Painters complaining of psychiatric symptoms have been CT scanned: cerebral atrophy was demonstrated in 8 reports, whereas one study failed to find such atrophy (For review see Arlien-Søborg, 1992).

d. Epidemiological studies

A number of cross-sectional and longitudinal studies indicate a causal relationship between exposure to white spirit and the development of chronic toxic encephalopathy. Most of the cross-sectional studies revealed an excess of psychiatric, neurophysiological and neuropsychological impairments. The results of longitudinal studies support the relation between long-term exposure to white spirit (alone or in combination with other organic solvents) and an increased risk of being awarded disability pension due to psychiatric diseases, in particular presenile dementia (For review see Arlien-Søborg, 1992).

V. DRAWBACKS OF EPIDEMIOLOGICAL STUDIES. NEED AND LIMITATIONS FOR STUDIES IN LABORATORY ANIMALS

Epidemiological data are highly relevant because they deal with the species of interest and concern: the human being.
Methological problems disfavour the interpretation of epidemiological studies of the neurotoxicity of organic solvents. The drawbacks include the uncertainties of confounding factors and the nature of exposure e.g. duration, levels, co-exposure to other neurotoxicants (a.o. alcohol), and the differentiation between acute narcotic effects and long-lasting effects.

Because of these drawbacks and because ethical reasons limit investigations in man, the use of laboratory animals under controlled circumstances are important supplements to studies in man. In the design of animal studies, it is necessary to identify a suitable animal species, exposure schedules, and effect parameters.
Because of the complexity of the nervous system, different animal species and research disciplines may be necessary to ascertain neurotoxicity. Generally, documentation can not be expected to be reduced to a single parameter in a single animal species. It is necessary to optimize, standardize, validate, and integrate several research techniques and disciplines.

Obvious advantages of laboratory animal studies compared to *in vitro* investigations include intact homeostasis, barrier functions, integrated functions, potential targets, and the possibilities for compensatory mechanisms and reversibility. Furthermore, 'physiological' concentrations of test substance (parent compound, metabolites) are present at potential targets not 'unphysiological' concentrations, as often used in *in vitro* studies. Questions regarding indirect neurotoxicity are generally best investigated in *in vivo* experiments.

In the studies included in this thesis the rat was chosen as the laboratory animal. This species is easily handled in inhalation chambers and in behavioural tests as well. Furthermore, at the institute there is great experience in conducting studies with this species, in its anatomy, and in its histopathology.
However, '*man is not a big rat*' and results obtained from *in vivo* studies in the rat should not be extrapolated to effects in man without reservations because of potential inter-species differences in a.o. growth, development, physiology, biochemistry, toxicokinetics, toxicodynamics, and susceptibility. Generally, the liver is regarded to be the principal detoxification organ for xenobiotics. As an example of inter-species differences in relation to neurotoxicity, the brain/liver weight ratio can be calculated to approximately 1.1 and 0.15 for adult man and rat, respectively. Supposing the same detoxification capacity per g liver, the brain/liver weight ratio difference *per se* implies a more than 7-fold greater capacity of the rat to detoxify neurotoxicants per g brain as compared to man for chemicals passing the liver.
Animal care considerations is another important issue to consider when conducting studies in laboratory animals.

VI. STUDIES OF THE EFFECTS OF TOLUENE AND WHITE SPIRIT EXPOSURE ON BRAIN NA, DA, AND 5-HT METABOLISM

Neurotransmitter metabolism, defined in its widest sense, is considered to be an important index of CNS function and affection by neurotoxicants.

1. TOLUENE

A literature survey in CD-ROM Silverplatter[R] Medline (1/1984-12/1995) and Toxline (1/1981-9/1995) databases was performed. The string was restricted to: (toluene or methylbenzene, or 108-88-3) and (noradrenaline or norepinephrine or dopamine or 5-hydroxytryptamine or serotonin).

Twenty-six relevant references, one of which was **I**, were found dealing with toluene and NA, DA, or 5-HT. From these studies it is apparent that there is an obvious lack of consensus about which exposure conditions should be used and which brain regions that should be examined. In an attempt to clarify the discussion of the effects caused by these different toluene exposure schedules, the 26 studies were classified into 4 main categories according to the duration of exposure as defined below (A-D).
This procedure resulted in the corresponding Appendices I A-D, in which demonstrated effects are summarized. It is emphasized that all these effects are statistically significant effects induced by toluene on the metabolism of NA, DA, or 5-HT in the CNS. The general level of significance was set to $P<0.05$. For details, the Appendices (I A-D) should be consulted.

The appendices reveal that the rat was the predominant laboratory animal species (21 references) and that mainly the Sprague-Dawley strain (14 references) was used. In 17 of the 21 studies dealing with the rat, exposure was by inhalation. This reflects the easy handling of this species for inhalation exposure investigations.
In only 7 rat inhalation studies, the exposure was specified in relation to the light/dark-cyclus: in 4, the exposure was only in the light and in 2 the exposure was both in the light and the dark phase. Only one study, **I**, performed exposure exclusively in the dark-phase. This is regarded to be the most relevant exposure period of rats in relation to occupational exposure of man because this is the active period of the rat.
Mice, all receiving toluene in the drinking water, were investigated in 3 studies, and rabbits in 2 inhalation studies.
Measurement by quantitative microfluorimetry on slides for microscopy was applied in 6 investigations, HPLC techniques in 17.

Toluene inhalation-induced effects were shown in the concentration range from 80 to 40000 ppm toluene in the inhaled air. In 2 studies (Yamawaki & Sarai, 1982; Yamawaki et al., 1982), the exposure level was 0.7% but not otherwise specified. Therefore, it was not possible to convert to ppm.
The duration of the exposure ranged from 30 min as a single-exposure to long-term exposure for 6 months.

A. Single-exposure, see Appendix I A

Definition: Studies in which the animals were exposed for a single period shorter than or equal to 24 h.

Six references met these criteria, they all used the rat as the laboratory animal. The animals were exposed via inhalation in 3 studies; in 2 studies, i.p. injection was applied, in one gavage was used.

The toluene concentration in the inhaled air ranged from 100 to 10000 ppm. The lowest concentration in the inhaled air at which effects were shown was 1000 ppm (Rea et al., 1984; Stengård et al., 1994). Animals were exposed from 30 min to 8 h.

There was increased NA, DA, and 5-HT levels in various parts of the brain and in the whole brain (Arito et al., 1984; Rea et al., 1984). The study by Kiriu et al. (1990) showed decreased NA level in the dorsal part of pons when exposed to 1500 and 10000 ppm and decreased DA level in hypothalamus and ventral part of midbrain when exposed to 1500 and 10000 ppm. Kanada et al., (1994) showed decreased DA and 5-HT level in midbrain, increased DOPAC in hypothalamus, and decreased HVA in med. obl. following administration by gavage. The study by Kiriu et al. (1990) revealed increased DA level in hippocampus and cerebellum when exposed to 10000 ppm. The MPHG and 5-HIAA levels were changed in frontal cortex and pons+med. obl. (Arito et al., 1984) following i.p. injection. In the study by Stengård et al. (1994) applying microdialysis, an increased level of extracellular DA was shown in corpus striatum (part of thalamus) when exposed to 1000 and 2000 ppm. This is in agreement with the increased striatal DA level (at 1000 ppm) in this region as demonstrated by Rea et al. (1984). These findings are in accordance with increased dopaminergic activity in this part of the brain provided that the B_{max} for DA binding is not decreased and the K_d is not increased owing to single-exposure. However, interpretation of concentrations in terms of neuronal activity is mainly speculative, especially, if the effects on binding characteristics as here are unknown. Also applying microdialysis, Kondo et al. (1995) found unchanged extracellular DA, DOPAC, HVA, and 5-HIAA levels in striatum following i.p. inj. of 80, 250, or 800 mg/kg b.w. This discrepancy with the findings of Stengård et al. (1984) may be a consequence of a different route of exposure.

There seems to be a trend towards generally increased NA, DA, and 5-HT levels in the various brain parts suggest a common underlying mechanism, which will be discussed in Chapter VIII part 1.

B. Short-term exposure a, see Appendix I B

Definition: Studies in which the animals were exposed for more than 24 h but for no more than 7 days.

Nine references met these criteria. In 7 of these, the rat was used as the laboratory animal, in 2 the rabbit. All animals were exposed via inhalation.

The toluene concentration in the inhaled air ranged from 80 to 3000 ppm. The lowest concentration at which effects were shown was at 80 ppm (Fuxe et al., 1982; Andersson et al., 1983; von Euler et al., 1989a).

The CA level and depletion (at 80, 500, 1000, 1500, and 3000 ppm) (measured as unspecific CA fluorescence) were increased in various

subregions of hypothalamus (Andersson et al., 1980, 1983).
The depletion of CA was measured by determination of the CA content after *in vivo* administration of a substance (generally α-methyl-tyrosine methylester, H44/68), which terminates the CA synthesis. Neurotransmitter depletion is used as an index of the turnover and may represent an indication of the activity of corresponding neurons. Increased depletion indicates increased turnover/activity. Reduced depletion indicates decreased turnover/activity.

An increased DA level was shown in the whole brain minus cerebellum following exposure to 0.7% toluene in the air (Yamawaki & Sarai, 1982). The DA level and depletion decreased in various subregions of the forebrain at low concentrations of toluene in the inhaled air (80 and 500 ppm). At higher concentrations (1500 and 3000 ppm), the DA level increased and the depletion remained decreased in caud. cent., whereas depletion increased in acc. dot. and tub. dot. at exposure to 3000 ppm (Fuxe et al., 1982).
In the study by von Euler et al. (1989a) a decreased NA level and decreased NA and DA depletion were observed in substantia nigra at an exposure level of 80 ppm. The DA level was increased and the DA depletion decreased in a subregion (tub. dif.) of the forebrain, whereas CA (unspecific) depletion was increased in subregions (SEL, PAFM) of the hypothalamus (at 80 ppm).

One study revealed decreased B_{max} for 5-HT binding to P2-fraction membranes prepared from different brain regions when exposed to 0.7% in the air (Yamawaki et al., 1982). Another study showed increased K_d for 5-HT association to cortical membranes (Celani et al., 1983) following exposure to 3000 ppm. These effects may indicate reduced capacity for serotonergic transmission. No effects on brain 5-HT concentrations are reported in Appendix I B.
There was also an increased K_d for the association of the dopamine D_2 receptor ligand spiperone to striatal membranes (at 3000 ppm) (Celani et al., 1983). This finding suggests that the increased DA level found in this region following single-exposure (Rea et al., 1984; Stengård et al., 1994) may be linked with a decreased DA binding to the dopamine D_2 receptor in this region. This is in accordance with a compensatory mechanism.

Pre-exposure postnatal day 1-7 to 80 ppm affected the effects of exposure in week 8 to 80 ppm: effects on the CA level and depletion in forebrain, hypothalamus, and substantia nigra were changed (von Euler et al., 1989a).

Two studies (Romanelli et al., 1986; Mutti et al., 1988) will be discussed in Chapter VIII.

C. Short-term exposure b, see Appendix I C

Definition: Studies in which the animals were exposed for more than 7 days but for no more than 30 days.
Eight references met these criteria, 5 of which used the rat and 3 the mouse as the laboratory animal. The rats were exposed by inhalation in all but one, applying i.p. injections.

In the 3 studies in mice the animals received toluene through their drinking water (Hsieh et al., 1990a,b, 1991). These data are not easily interpreted in relation to inhalation exposure (see below: **E. General conclusions**). Therefore, they are excluded from the discussion in this section.
The toluene concentration in the inhaled air ranged from 80 to 40000 ppm. The lowest concentration in the inhaled air at which effects were shown was 80 ppm (von Euler et al., 1988a).

The CA depletion increased in subregions of hypothalamus at 80 ppm (von Euler et al., 1988a).

The NA level increased in ventral cortex and decreased in olfactory cortex at 400 ppm (Ikeda et al., 1986).
Different effects on the NA concentration in the hypothalamus were observed: decreased NA level was shown at 400 ppm by Honma et al. (1983) and Ikeda et al. (1986), whereas an increased level was demonstrated by Arito et al. (1985) after i.p. injections.

The DA depletion was increased in frontal cortex at 80 ppm (von Euler et al., 1988a). The DA level increased in olfactory cortex and decreased in corpus striatum at 400 ppm (Ikeda et al., 1986). This decreased striatal DA level is not in accordance with the above suggested compensatory mechanism acting in this brain region.

Decreased 5-HT level was shown in various brain regions after i.p. injections (Arito et al., 1985). The K_d for 5-HT association to crude synaptic membranes increased (Castilla et al., 1993). The effects induced on 5-HT concentrations and K_d are in accordance with a lower potential for serotonergic activity.

The levels of the neurotransmitter metabolites: 5-HIAA, MHPG, DOPAC, and HVA were changed in various brain parts (Arito et al., 1985) after i.p. injections: the 5-HIAA level decreased in midbrain and hypothalamus; the MPHG level increased in hypothalamus; the DOPAC and HVA level increased in corpus striatum.
5-HIAA is a 5-HT metabolite. DOPA is a CA precursor. MHPG, HVA, and DOPAC are CA metabolites.
The effects on 5-HIAA and MHPG in hypothalamus will be discussed in Chapter VIII part 1.

D. Long-term exposure, see Appendix I D

Definition: Studies in which the animals were exposed for more than 30 days.
Only 2 other references than **I** met this criterium. In the 2 other studies, rats were inhalation exposed to toluene concentrations at the range from 80 to 1000 ppm. The lowest concentration at which effects were shown was 80 ppm (von Euler et al., 1988b).
The results of these 3 studies will be discussed in Chapter VIII.

E. General conclusions

As can be noticed from above, it was only possible to draw some fragmentary conclusions from the available studies because of conflicting findings, different exposure schedules with respect to toluene concentration in the inhaled air, and the duration of exposure and because the various parameters and regions were studied in different manners.

It is mainly speculative to interpret solvent-affected concentrations of CNS transmitters and their metabolites following oral dosing in terms of occupational exposure. This is because of a slower rate of absorption, the possibility for microbial degradation in the gut, and extensive first-pass metabolism in the liver before the solvent gets access to the brain.

After absorption via the lungs, a fraction of the absorbed solvent may reach the brain unmetabolized before passing the liver, whereas another fraction may be first-pass metabolized in the liver before getting access to the brain. When a solvent is administered by i.p. injection, the rate of absorption is faster than by oral dosing. First-pass metabolism in the liver takes place before the solvent gets access to the brain. If the solvent enters the lymphatic system in the peritoneum it may reach the brain unmetabolized before passing the liver.
Therefore, studies applying i.p. administration of a solvent are of greater relevance in relation to occupational exposure than studies using the oral route.

Conclusions based on effects on neurotransmitter depletion and neurotransmitter metabolite concentrations induced by inhalation exposure and by i.p. injections may provide some knowledge about the rate of release, uptake, and turnover of respective neurotransmitters. Conclusions and interpretation in terms of transmitter concentrations and activity of corresponding neurons should be considered with care.

There was an obvious lack of long-term toluene exposure studies, which are considered to be highly relevant in relation to occupational exposure and the neurotoxicity in man. Originally, this lack of long-term studies was the reason for initiating the 6 months' inhalation study in rats (**I**).
The dissection procedure of the rat brain as described by Glowinski & Iversen (1966) was the most frequently applied procedure (Appendices I A-D). In order to compare with existing data, we decided also to use this dissection procedure.
The regional NA, DA, and 5-HT concentrations were selected as the parameters of interest because these concentrations are considered to be important parameters for CNS function and solvent neuroactivity.

2. WHITE SPIRIT

A literature survey in CD-ROM Silverplatter^{R} Medline (1/1984-12/1995) and Toxline (1/1981-9/1995) databases using the string: (white spirit or turpentine or stoddard solvent or varnolene) and (noradrenaline or norepinephrine or dopamine or 5-hydroxytryptamine or serotonin or GFA or GFAP or glial fibrillary protein or tyrosine or tryptophan) failed to reveal any other publications than **II**, **III**, and **IV**. Originally, this was the reason for initiating the 3 weeks and the 6 months inhalation studies.

VII. MATERIALS AND METHODS

It is considered outside the scope of this thesis to give a detailed presentation of the applied materials and methods. Therefore, the following is only a brief presentation of the general materials and methods used in the studies **I, II, III, IV**, and in the additional GFAP investigations. For further details, the references **I, II, III, IV**, and O'Callaghan (1991b) should be consulted.

1. CHEMICALS

Toluene was purchased from Merck, Germany (Cat. No. 8325). The purity was >99.5%.

Aromatic white spirit of the North European type was from Shell, Denmark [Mineralsk terpentin K-30, CAS 64742-88-7, boiling point in the range from 148 to 200 °C, 20% (w/w) aromatic components]. Toluene could not be detected in the product by the GLC-method used in study **II**. The detection limit was a concentration of toluene at approximately 50 nM in the aromatic white spirit.

5-hydroxy[side chain-2 ^{14}C]tryptamine creatinine sulfate] (^{14}C-5-HT) (Cat. no. CFA 170, specific activity: 57 mCi/mmol; 2.11 GBq/mmol) was from Amersham. All other chemicals were of analytical grade.

2. ANIMALS AND EXPOSURE

Male rats (Mol:WIST) were used in all studies. They were obtained from Møllegaard Breeding Center Ltd., DK-4623 Ll. Skensved, Denmark.

When the exposure was initiated, the rats were 3 months old in study **I, II, III**, and **IV**. It is emphasized, that the effects of 6 months inhalation exposure to aromatic white spirit on regional GFAP concentrations were measured in the very same samples of brain tissue as also used for neurotransmitter analyses in study **III**. Furthermore, GFAP was measured in the hemisphere and hippocampus of young adult and 'middle-aged' rats, 5 and 14 months old, respectively, at the beginning of the 3 weeks of exposure.

The rats were housed in stainless steelwire cages, 2 animals/cage, in animal rooms with automatic control of temperature (22±1 °C), relative humidity (55%±5%), air exchange (8 times/h), and fluorescent light (21:00 to 09:00 h) with free access to food and drinking water. The animals were exposed in the dark-phase of the light/dark cyclus. Because this is the active period of rats, exposure in the dark-phase is regarded to be the most relevant exposure period of rats in relation to occupational exposure of man. During the daily exposure, the food was removed whilst drinking water was still available.

The animals were placed in inhalation chambers (Holten Luftteknik A/S, Denmark) in a flow-through of filtered atmospheric air. The concentrations of solvent in the chambers were followed continuously and calibrated by means of an infrared spectrophotometer (Miran 402, Foxboro, England). The solvent vapour was generated by an evaporation equipment (Microlab A/S,

Denmark) (Ladefoged et al., 1990).

The exposure schedule was similar in all the studies: one group was sham-exposed and served as the control, a second group was exposed to 500 (400) ppm, and a third group to 1500 (800) ppm toluene (aromatic white spirit) in the inhaled air for 6 h/day, 5 days/week (7 days/week in the GFAP study in young adult and 'middle-aged' rats) for either 3 weeks (**II**; **IV**; the GFAP study in young adult and 'middle-aged' rats) or 6 months (**I**; **III**; **IV**; the GFAP study i 7 brain regions). In the 6 months exposure studies, exposure was followed by an exposure-free period of 4 months' duration prior to sacrifice at an age of 13 months.

3. NEUROTRANSMITTER METABOLISM

Rats were decapitated in CO_2/O_2-narcosis.

A. Dissection procedure and preparation of synaptosomes

a. Dissection of the brain

The brain was dissected into cerebellum, hemisphere, hippocampus, hypothalamus, pons, thalamus, and med. obl. according to the method described by Glowinski & Iversen (1966), Edelfors (1975), and Edelfors (personal communication).

b. Preparation of synaptosomes

Whole brain minus cerebellum was quickly transferred to ice-cold 0.32 M sucrose and weighed. A 10% (w/v) homogenate was made, all of which was processed. Synaptosomes were prepared at 0-4 °C by 'conventional' homogenization, differential and discontinuous sucrose density-gradient centrifugation techniques (Gray & Whittaker, 1962).

The synaptosomal band obtained after gradient centrifugation as the 0.8-1.2 M sucrose interphase-band was isolated, diluted by slowly addition of ice-cold incubation buffer (1+1), and centrifuged at 10000 g for 15 min at 0-4 °C. The synaptosomal sediment was washed by resuspension in 10 ml ice-cold incubation buffer and subsequent centrifugation. The final sediment was resuspended in 10 ml ice-cold incubation buffer.

Samples were taken and prepared for protein and neurotransmitter analyses. The composition of the 'extracellular' incubation buffer was: NaCl (132 mM); KCl (6 mM); $CaCl_2$ (0.75 mM); $MgSO_4$ (1.3 mM); NaH_2PO_4 (1.3 mM); and glucose (10 mM), pH 7.4.

B. Sample preparation

a. Whole brain and regional studies

The whole brain (**II**) or brain regions (**II**; **III**; the 2 GFAP studies) were transferred to ice-cold 0.32 M sucrose as fast as possible, weighed and thoroughly homogenized by an Ultra-Turrax T-25 homogenizer using an ice-cooling jacket. Immediately after homogenization an aliquot of homogenate was deproteinized by the addition of ice-cold 0.2 M perchloric acid (1+1) and subsequent centrifugation. Supernatants were stored at -20 °C until neurotransmitter analyses:

-one aliquot of supernatant was used unprocessed for the determination of 5-HT (**I**; **II**; **III**), and

-another aliquot was used for NA and DA determinations after purification on aluminum oxide (Anton & Sayre, 1962) (**I**; **II**; **III**).

N-ω-methyl-5-HT (Sigma, M 1514) was the internal standard for the 5-HT determinations whilst 3,4-dihydroxy-benzylamine (Sigma, D 7012) was the internal standard for NA and DA determinations.
The recoveries of NA, DA, and 3,4-dihydroxy-benzylamine during the aluminum oxide purification procedure were similar, 86±4%, 84±3%, and 85±3% (mean±SD, n=10), respectively.

The neurotransmitter concentrations in a whole brain (**I**; **III**) was calculated from the concentrations and weights of its corresponding regions.

In **III** and the GFAP study in young adult and 'middle-aged' rats, an aliquot of homogenate was taken for GFAP analysis and stored at -20 °C until analysis.

b. Synaptosomal neurotransmitter and protein concentrations
Immediately after the final resuspension, 4 volumes of resuspended synaptosomes were deproteinized by the addition of 1 volume ice-cold 0.5 M perchloric acid and subsequent centrifugation. Aliquots of supernatant were used as described above for the determination of 5-HT, NA and DA concentrations. When purified on aluminum oxide (Anton & Sayre, 1962), samples were concentrated 5-fold. A 75-µl sample was injected for NA and DA analysis and a 200-µl sample for 5-HT analysis.
A sample of each resuspension was taken for protein analysis. These samples were stored at -20 °C until analysis.

C. Neurotransmitter concentrations

Neurotransmitter (NA, DA, and 5-HT) analyses were performed by HPLC-ECD. The analytical system consisted of a Hewlett-Packard 1084B liquid chromatograph equipped with a 250x4 mm LiChrospher^R 100 RP-18 (5 µm) analytical column protected by a 20x4 mm (5 µm) RP-18 guard column (both from Merck, Germany). Separation was achieved at ambient temperature by use of an acetonitrile modified citrate/octenyl sulphate buffer (Lin & Blank, 1983). The composition of the buffer was: citric acid (100 mM); octyl sodium sulfate (0.225 mM); disodium-EDTA (0.05 mM); acetonitrile (6%, v/v); and diethylamine (0.06%, v/v). The pH was adjusted to 2.5-2.6 before the addition of acetonitrile. The flow rate was 1 ml/min. The detector was a Waters M 460 electrochemical detector applying an oxidation potential of +0.6 V.
There was a linear concentration-response relationship in the concentration range of the samples. When NA, DA, and 5-HT analyses were performed on a pooled brain sample, the intra-sample CV of the concentration was below 5% for each transmitter over a time period of at least 6 months. By changing

buffer composition, flow rate, or column temperature when analyzing the pooled brain sample, it was not possible to disclose contaminants of NA, DA, or 5-HT.

It was decided to express global and regional neurotransmitter concentrations on the basis of the wet weight of the tissue, not on the basis of DNA or protein. The rationale for this decision was:

-neuronal and glial cells each contain one nucleus. Organic solvents may induce neuronal death, which may be followed by proliferation of glial cells. Because one neuron may be replaced by an unknown number of glial cells, the possibility to express the neurotransmitter concentrations on the basis of DNA was excluded, and

-protein was excluded because technical problems with the protein determination resulted in larger intra-sample and inter-sample CV's of the neurotransmitter concentrations than when expressed on the basis of the wet weight,

-the use of the tissue wet weight as the basis for the calculation of neurotransmitter concentrations offers the largest and most consistent literature data base with regard to rat brain neurotransmitter concentrations and the effect of organic solvents, and

-this procedure has the advantages of being independent of the choice of method and standard and the potential interference of the solvent on the analysis.

The regional and whole brain NA, DA, and 5-HT concentrations (nmol/g wet weight) in tissue from untreated rats as presented in reference **I**, **II**, and **III** are in the range of values published in the literature.

D. High-affinity synaptosomal 5-HT uptake

a. Storage and protection

Storage was at 0 °C and the incubation was performed as soon as possible on the day of preparation.
To avoid metabolic degradation of 5-HT during storage and incubation, the resuspended synaptosomes were added the MAO-inhibitors pargyline (MAO-B inhibitor, Sigma P 8013) and clorgyline (MAO-A inhibitor, Sigma M 3778) to final concentrations of 100 µM in the incubate.

b. High-affinity 5-HT uptake rate and 5-HT uptake capacity

Incubation was performed in 10 ml polypropylene tubes in a metabolic shaker with free access of atmospheric air. Each synaptosome preparation was incubated in triplicate at both 37 and 0 °C.
The protein concentration was approximately 100µg/250µl (amount in samples) and the final incubation volume was 2000 µl.

After 10 min of preincubation at 37 °C, to time zero, ^{14}C-5-HT/5-HT (specific activity: 57 mCi/mmol; 2.11 GBq/mmol) was added to a final neurotransmitter concentration of 100 nM. Incubation was continued at 37 °C. After 1 and 15 min of incubation, samples of 250 µl were taken. Immediately after each sampling, synaptosomes were trapped by rapid vacuum filtration on 0.45 µm disposable filters (Millipore, Cat No. HAWP01300) installed in Millipore filterholders (Cat No. SX00 013 00). Filters were washed twice by the addition 1000 µl ice-cold incubation buffer and filtration. After the second wash, filters were transferred to counting vials containing 4 ml Optifluor^R (Packard Instruments). Radioactivity was determined in a Packard Tri-Carb 460 CD Liquid Scintillation System^R equipped with luminescence- and quench-correction options.

Unspecific 5-HT binding was determined analogously after 1 and 15 min incubation at 0 °C.
The high-affinity uptake at 1 and 15 min was calculated as the difference between the mean of respective determinations at 37 and 0 °C of each preparation of synaptosomes.

By this method, 2 synaptosomal 5-HT uptake parameters were measured:

-the high-affinity 5-HT uptake rate as reflected by the uptake during 1 min of incubation, and

-the maximum 5-HT uptake capacity as reflected by the steady-state 5-HT uptake attained after 15 min of incubation.

4. GFAP ANALYSIS

Samples for GFAP analysis were brought to ambient temperature and added SDS to a final SDS concentration of 1% (w/v), heated to 90-95 °C and sonicated for 3 min. One aliquot of sonicated sample was used for GFAP analysis. It was diluted properly, depending on the brain region, to a GFAP concentration in the range of the GFAP standard curve i.e. 1 to 10 µg GFAP/100 µl sample. Another aliquot was stored at -20 °C until protein analysis.
GFAP was analyzed applying the 96-well approach as described by O'Callaghan (1991b; O'Callaghan personal communication) by use of purified cow GFAP (Dako A/S, Denmark, Code no. Z-0334) as the standard. Quantification was done by means of the programme: Immunofit^R (Beckman).
The GFAP concentrations were expressed in terms of µg GFAP per mg of SDS-soluble protein.
The GFAP concentration in each whole brain was calculated from its corresponding regions.
The detection limit was below 0.5 ng GFAP per well (0.5 ng/100 µl). When the GFAP analysis was performed on pooled brain samples the intra-sample CV of the concentration was below 8% over a time period of at least 6 months.

The global and regional GFAP concentrations were in the range of those found by O'Callaghan, with whom an inter-laboratory 'calibration' study is in progress at the Institute of Toxicology.

5. PROTEIN ANALYSES

a. Synaptosomal protein

The concentration of synaptosomal protein in the resuspension was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

b. SDS-soluble protein

The concentration of SDS-soluble protein in extract used for the GFAP analysis was determined by use of a commercially available kit: Pierce, BCA Protein Assay Reagent, Cat. no. 232225. The analysis was not interfered by the SDS in the samples. When the protein analysis was performed on a pooled brain sample the intra-sample CV of the concentration was below 4% over a period of at least 6 months.

6. CLINICAL BIOCHEMISTRY

Urine and plasma analyses were carried out as described in study **III**.

7. CALCULATIONS AND STATISTICAL ANALYSES

Data were entered into Quattro Pro^{R} (Borland) spreadsheet version 2.0 for calculations and transferred to the SAS PC-version software package (SAS Institute Inc., 1988) for statistical analyses.

Data were analyzed by analysis of variance (PROC ANOVA/GLM) followed by Dunnett's two-tailed t-test when indicated. The general level of significance was set to $P<0.05$. It is emphasized throughout this thesis, that any effect on any parameter referred to from reference **I**, **II**, **III**, and **IV** is **always statistically significant**.

In the literature referred to in this thesis, the general level for a statistically significant effect was always set to $P<0.05$. It is emphasized throughout this thesis, that any effect referred to from the literature is **always statistically significant**.

8. PATHOLOGICAL AND STEREOLOGICAL METHODS

In study **I**, these methods were carried out by Ole Ladefoged, Arne Møller and Poul Strange, and in study **III** by Ole Ladefoged.

9. BEHAVIOURAL METHODS

In study **I**, these methods were carried out by Grete Østergaard and Jens-Jørgen Larsen, and in study **III** by Grete Østergaard.

10. ANALYSIS OF AROMATIC WHITE SPIRIT COMPONENTS

In study **II**, the head-space gaschromatographic analysis was carried out by Agneta Löf.

VIII. RESULTS AND DISCUSSION

In this chapter neurochemical effects found to be induced by exposure to toluene (**I**) and aromatic white spirit (**II**; **III**; **IV**; the GFAP studies) are outlined and discussed in the context of relevant data published in the peer reviewed literature. Also, potential mechanisms related to the effects found in study **I**, **II**, **III**, and **IV** are discussed in this context.
It is emphasized that any effect referred to is **always statistically significant.**

1. TOLUENE, REFERENCE I

Long-term exposure studies are considered to be relevant in relation to occupational exposure. In contrast to the 2 other published long-term exposure studies (Granholm et al., 1988; von Euler et al., 1988b), in study **I**, special attention was paid to the long-lasting effects, because such disturbances are characteristics of chronic toxic encephalopathy in man following occupational exposure. This was the reason for inserting the exposure-free period of 4 months' duration prior to analysis. Thereby, short-term reversible effects were excluded. Such effects remained undiscovered by the applied design.
No behavioural (motor activity, exploratory activity, diurnal activity, passive avoidance, Morris maze, radial maze) effects were found.
Besides a reduced weight of the hippocampus following exposure to 1500 ppm toluene, no macroscopic pathological changes, which could be attributed to the toluene exposure, were found in study **I**.

A. The brain regions

The concentrations of NA, DA, and 5-HT in various brain regions and in the whole brain (reconstructed by calculation) were changed.

In this section (1A, a-i), the observed effects induced by toluene on regional brain neurochemistry (**I**) are discussed in the context of the available relevant literature on NA, DA, and 5-HT metabolism. To ease this discussion, the previously mentioned 25 studies published by others and reference **I** were rearranged according to the 7 brain regions (cerebellum, hemisphere, hippocampus, hypothalamus, pons, thalamus, and med. obl.) and the whole brain as examined in study **I**. Neurochemical studies performed in subregions of these 7 brain regions are excluded.

Intraperitoneal injections may be of some relevance and provide results comparable with results obtained after inhalation exposure. In 3 studies, however, mice received toluene through the drinking water or by gavage (Hsieh et al., 1990a,b, 1991; Kanada et al., 1994). These data are not easily interpreted in relation to occupational exposure. Consequently, these studies are excluded from the discussion below and the Appendices II A-H. These procedures resulted in Appendices II A-H where statistical significant effects are summarized together with the respective exposure category (A-D).
Some investigators calculate a ppm-h value by multiplying the exposure

level in ppm with the duration in hours of exposure. This index of exposure is considered to be of dubious toxicological relevance as low-level exposure for a long period will induce other effects than high-level exposure for a short period, irrespective of identical ppm-h values.

Effects found in regional brain studies may give more specific information than studies in the whole brain. However, regional effects may be interpreted incorrectly because neurons may have cell body in one region and neurotransmitter containing nerve terminals in other regions. i.e. affection of one part of a neuron in one region may give rise to changed neurotransmitter concentrations in another region.

a. Cerebellum, see Appendix II A

In study **I**, both the NA and the 5-HT level were unaffected by the exposure (DA was undetectable). In a single-exposure study (10000 ppm for 30 min) an increased level of DA was observed (Kiriu et al., 1990).

An increased 5-HT concentration was shown by Rea et al. (1984) following inhalation exposure to 1000 ppm for 8 h. Long-term inhalation exposure at 1000 ppm of cerebellar transplants *in oculo* increased the NA and MHPG (a NA metabolite) levels in the transplants and the MHPG concentration in the host cerebellum, but failed to affect the NA concentration in the host cerebellum (Granholm et al., 1988). This latter observation is in accordance with the results presented in reference **I**.

Irrespective of the unchanged NA level, the increased MHPG level in the host cerebellum may indicate increased activity of noradrenergic neurons.

b. Hemisphere, see Appendix II B

Our publication (**I**) was the only published paper concerning NA, DA, or 5-HT levels in the hemisphere. Long-lasting increased DA (at 500 and 1500 ppm) and 5-HT (at 1500 ppm) concentrations were documented following the exposure.

At 500 ppm, the mean nuclear volume and mean perikaryonal volume in neocortex were increased (**I**). The CV's of these parameters increased in the 1500 ppm exposed group compared to the controls. This may be an indication of effects also at 1500 ppm.

Neither the neocortical volume nor the number of neurons in the neocortex were affected by exposure to 500 or 1500 ppm for 6 months (**I**). This is in accordance with the results of Korbo et al. (1990) who investigated animals exposed perorally with doses of 0, 200, 400, and 800 mg/kg b.w./day for 12 weeks followed by an exposure-free period of 4 weeks. This study failed to find any neuron loss in rat cerebral cortex. However, as stressed, the relevance of this route of administration in relation to occupational exposure is mainly speculative.

In conclusion, long-lasting morphological changes and increased DA and 5-HT concentrations were demonstrated in the hemisphere. Such effects have to be considered with concern. Affection of this region is in accordance with some of the symptoms recognized in persons long-term occupationally exposed to organic solvents.

c. Hippocampus, see Appendix II C

Long-term exposure decreased NA (at 500 and 1500 ppm) and increased 5-HT (at 1500 ppm) levels (**I**).

In a single-exposure study (10000 ppm for 30 min), an increased level of DA was observed (Kiriu et al., 1990).

Short-term inhalation exposure decreased the B_{max} for 5-HT binding to P2 membranes isolated from various brain regions, including the hippocampus (0.7% in the air) (Yamawaki et al., 1982). Short-term i.p. injections decreased the 5-HT level in this region (Arito et al., 1985). These changes of 5-HT parameters (B_{max}, concentration) indicate a reduced ability for serotonergic activity following short-term exposure.

The weight of the hippocampus was reduced at 1500 ppm (**I**). Furthermore, we found that this weight loss was accompanied by a 16% neuron loss in subdivision CA2/3 of hippocampus of rats exposed to 1500 ppm for 6 months (Korbo et al., 1993, 1996). These animals were a part of the animals exposed in study **I**. The cell loss in CA2/3 was accompanied by a reduced NA concentration. There may be a relation between these findings.

The long-lasting effects: decreased weight of hippocampus, loss of neurons in CA2/3, and affected NA and 5-HT levels show that the hippocampus may be an important target for toluene neuroactivity.

The affection of hippocampal parameters is in accordance with some of the symptoms that characterize chronic toxic encephalopathy e.g. decreased memory ability, concentration difficulties, and disturbance of the mood.

d. Hypothalamus, see Appendix II D

Short-term inhalation exposure (at 400 ppm) decreased the NA level in this region (Honma et al., 1983; Ikeda et al., 1986). Contrary to these findings, the NA level was increased following short-term administration by i.p. injections (Arito et al., 1985).

The decreased NA levels (Honma et al., 1983; Ikeda et al., 1986) are in accordance with the decreased NA level observed in study **I** by exposure to 500 ppm and with the increased MHPG level following i.p. injections as shown by Arito et al. (1985). This increased MHPG level and the decreased NA concentration (**I**) indicate increased degradation perhaps caused by increased activity of noradrenergic neurons.

Single inhalation exposure (at 1500 and 10000 ppm for 30 min) decreased the DA concentration in hypothalamus (Kiriu et al., 1990), whereas the long-term exposure (at 1500 ppm) increased the DA level (**I**).

Long-term exposure followed by an exposure-free period decreased the 5-HT level (at 500 and 1500 ppm) (**I**). Arito et al. (1985) showed decreased 5-HIAA level following i.p. injections.

In conclusion, long-lasting changes of the neurochemistry in hypothalamus were demonstrated. Such effects should be considered with concern. The physiological outcome of these changes, if any, is unpredictable.

e. Pons, see Appendix II E

Paper **I** was the only published paper studying this brain region. Here, increased NA (at 1500 ppm), DA (at 500 ppm), and 5-HT (at 1500 ppm) concentrations were documented following long-term exposure. These changes were long-lasting effects.

f. Thalamus, see Appendix II F

Paper **I** was the only published paper studying this brain region. Here, increased NA (at 500 ppm) and DA (at 500 ppm) levels were documented following the long-term exposure. These changes were long-lasting effects.

g. Med. obl., see Appendix II G

Single inhalation exposure increased NA and 5-HT (at 1000 ppm) levels (Rea et al., 1984). Paper **I** was the only published paper concerning long-term exposure. Here, decreased NA (at 500 ppm) and DA (at 500 ppm) concentrations were documented. These changes were long-lasting effects.

h. Whole brain, see Appendix II H

Single-exposure by inhalation (at 1000 ppm) increased the DA level (Rea et al., 1984). Short-term inhalation exposure decreased the B_{max} for 5-HT binding to P2 membranes isolated from various brain regions, including the whole brain (0.7% in the air) (Yamawaki et al., 1982).

Paper **I** was the only published paper concerning long-term exposure. Here, increased DA (at 500 and 1500 ppm) and 5-HT (at 1500 ppm) concentrations were documented. These changes were long-lasting effects.

i. Metabolites of neurotransmitters

Changes in neurotransmitter metabolite concentrations were observed in cerebellum following long-term inhalation exposure to 1000 ppm (Granholm et al., 1988) and in the hypothalamus after short-term i.p. injections (Arito et al., 1985). Such changes may be interpreted in terms of the concentrations of the parent neurotransmitters, neurotransmitter release, -uptake, and -turnover and may be correlated with the activity of corresponding neurons. Such extrapolations are mainly speculative.

B. Mechanisms potentially related to the observed general effects on neurotransmitter concentrations

In study **I**, both the NA and the DA concentrations were changed in 4 brain parts (hypothalamus, pons, thalamus, and med. obl.). Interestingly, the NA and DA concentrations changed in the same direction in 3 of these 4 regions: both neurotransmitter concentrations increased in pons and thalamus, both decreased in med. obl., suggesting a common underlying mechanism. Generally, the regional DA concentrations seem to be increased. A third general observation in study **I** was the increased 5-HT concentrations found in 3 regions (hemisphere, hippocampus, pons) and in the whole brain.

In this section (1 B, a-c), the results from investigations of mechanisms potentially related to these general effects of toluene on brain NA, DA, and 5-HT concentrations (**I**) are reviewed. The investigations include: mechanisms studied in intact laboratory animals and in man (a), mechanisms studied mainly in synaptosomes (b), and receptor functions (c).

a. Mechanisms studied in intact laboratory animals and in man

Ad. The general effects on regional NA and DA concentrations

Concentrations of NA and DA precursor and related compounds

In one study, the effects of toluene exposure on plasma L-tyrosine and L-tryptophan concentrations were studied (Voog & Eriksson, 1984). Reduced rat plasma concentrations of both these amino acids (L-tyrosine is essential if L-phenylalanine is not available for *p*-hydroxylation, L-tryptophan is essential) were observed after a single i.p. injection of 30 mmol toluene/kg b.w. and by inhalation of 30000 ppm for 2 to 16 min.

L-tyrosine is the precursor for both NA and DA. It is not possible from these results directly to extrapolate to the regional L-tyrosine concentrations in the brain following the exposure schedule as used in study **I**.

In theory, if the plasma L-tyrosine concentration was also reduced in **I**, and provided that a reduced plasma L-tyrosine concentration implies reduced regional levels, this can explain the decreased concentrations of both NA and DA as observed in med. obl. (**I**), not the increased NA and DA concentrations as noticed in pons and thalamus. Contrary, these may be caused by increased L-tyrosine transport across BBB as discussed below.

In another study, i.p. administration of mice with 0.5 ml toluene/kg b.w. increased the level in striatum (the only investigated region and a part of thalamus) of *p*-tyramine from 15 min to 2 h after the administration (Juorio & Yu, 1985). *p*-Tyramine originates from L-tyrosine by decarboxylation. An increased *p*-tyramine level indicates an increased striatal L-tyrosine concentration and/or increased L-tyrosine decarboxylation activity.

-Increased L-tyrosine transport across BBB could explain the reduced plasma L-tyrosine concentration as observed by Voog & Eriksson (1984) and could give rise to increased brain L-tyrosine and *p*-tyramine concentrations. If an increased L-tyrosine concentration was present in study **I** in pons and thalamus this may explain the increased NA and DA concentrations in these regions caused by increased synthesis.

-Increased L-tyrosine decarboxylation activity may act in the opposite direction and decrease the NA and DA concentrations by causing a decreased amount of L-tyrosine available for neurotransmitter synthesis. This phenomenon may explain the decreased concentrations in med. obl. (**I**).

-*p*-Tyramine may displace the catecholamines from their stores in the presynaptic nerve terminals via competition with catecholamines for vesicular storage sites (Weiner & Molinoff, 1989). So, an increased *p*-tyramine concentration as observed by Juorio & Yo (1985) may give rise to decreased NA and DA levels as observed in med. obl. following toluene exposure.

These explanations/extrapolations are hypothetic. The resultant of this symphony of potential, concomitantly acting mechanisms on regional NA and DA concentrations is unpredictable.

Ad. The generally increased regional 5-HT concentrations

This may be explained by increased regional L-tryptophan concentrations, increased synthesis, decreased 5-HT release, decreased breakdown, and/or increased 5-HT uptake rate and -capacity into presynaptic serotonergic nerve terminals.

Nothing is known about the effect of toluene exposure on 5-HT synthesis, release, or breakdown.

Concentration of L-tryptophan

L-tryptophan is the precursor for 5-HT. The amount of L-tryptophan available for hydroxylation seems to be the rate limiting parameter for 5-HT synthesis in the brain, as the involved enzyme (L-tryptophan-5-hydroxylase) is not saturated at the precursor concentration present in the brain (Green, 1989).

The study by Voog & Eriksson (1984) demonstrated a decreased plasma L-tryptophan concentration following both i.p. injection and inhalation exposure. It is not possible from these results directly to extrapolate to the regional L-tryptophan concentrations in the brain following the exposure schedule as used in study **I**.

In theory, if the plasma L-tryptophan concentration was also reduced in **I**, and provided that a reduced plasma L-tryptophan concentration implies reduced regional levels, this can only explain the decreased concentration of 5-HT in hypothalamus (**I**) not the generally increased 5-HT concentrations in hemisphere, hippocampus, pons, and the whole brain (**I**).

Contrary, increased L-tryptophan transport across BBB could explain the reduced plasma L-tryptophan concentration as observed by Voog & Eriksson (1984) and could give rise to increased regional brain L-tryptophan concentrations. If increased regional L-tryptophan concentrations were present in study **I** in hemisphere, hippocampus, and pons these may explain the increased 5-HT concentrations observed in these regions, and in the whole brain as well, because of increased synthesis.

5-HT uptake into platelets

An interesting observation was that the *in vitro* uptake of 5-HT into platelets isolated from car painters exposed to mixtures of organic solvents and organic isocyanates showed elevated 5-HT uptake rate (V_{max}) in 9 of 12 workers compared to controls (Beving et al., 1983). Platelets possess a 5-HT uptake system and 5-HT storage characteristics similar to those of the presynaptic serotonergic nerve terminal. Therefore, platelets are used as models for the presynaptic serotonergic CNS nerve terminal (For review see Jensen, 1995). If the 5-HT uptake rate is also increased *in vivo* in presynaptic serotonergic nerve terminals this can explain the increased 5-HT concentrations as observed in hemisphere, hippocampus, pons, and in the whole brain (**I**). This suggestion could be investigated by the determination of the *in vitro* 5-HT uptake into synaptosomes isolated from control and toluene exposed rats as performed in **IV** for aromatic white spirit. However, such a study has not been performed.

b. Mechanisms studied mainly in synaptosomes

Other targets and mechanisms for toluene affection of neurons have been investigated, mainly by use of synaptosomes. The rationale for investigations performed in synaptosomes is given in part 5 of this chapter.

The investigated targets and mechanisms are located in the plasma membrane and in intrasynaptosomal compartments as well. Only studies in which toluene is administered *in vivo* by i.p. injection or inhalation prior to synaptosome preparation are considered in the review. Studies in which toluene was exclusively added *in vitro* have been excluded.

The topics include:

-interference with membrane structure and function, and

-the generation of reactive intermediates.

Ad. Interference with membrane structure and function

Being an organic solvent, toluene is lipophilic. The apolar and planar toluene molecule may dissolve in lipophilic regions of the cell membrane and other cell constituents and cause structural and/or functional changes. These lipophilic targets include lipids and lipophilic regions of proteins.

The studies of synaptosomes reviewed below deal with toluene-induced effects on membrane lipid composition and metabolism, membrane fluidity, membrane leakage, activity of membranal enzymes, Ca^{2+}-uptake and -levels, and membrane potential.

Synaptosomal membrane lipid composition and lipid metabolism

Le Bel & Schatz (1988) injected rats i.p. with doses of 0.5, 1, or 1.5 g toluene/kg b.w. 1 h before sacrifice. Doses of 1 and 1.5 g/kg b.w. decreased the synaptosomal phospholipid and phosphatidylethanolamine levels. Phospholipid methylation, a reaction which uses phosphatidylethanolamine as its initial substrate, was decreased by the toluene treatment (1 g/kg b.w., the only investigated dose) provided that [^{3}H]-methionine was the methyl donor, but was unaffected with [^{3}H]-adenosylmethionine as the methyl donor. These observations were confirmed in another study by the same authors (LeBel & Schatz, 1989) following i.p. injection of rats with a dose of 1 g/kg b.w. 1 h before sacrifice.

There may be a relation between phospholipid methylation and membrane fluidity.

In a third study, LeBel & Schatz (1990) investigated the mechanism by which toluene decreased the synaptosomal phosphatidylethanolamine concentration by measuring the synaptosomal activity of enzymes degradating and synthesizing phospholipids. Rats were injected i.p. with a dose of 1 g/kg b.w., 15, 30, or 60 min before sacrifice. Toluene stimulated phosphatidylethanolamine-specific phospholipase C. This may explain the toluene-reduced phosphatidylethanolamine concentration. Phospholipase A, phospholipase D, and base exchange enzymes were unaffected. Toluene increased the synthesis of phosphatidylethanolamine when measured as [^{3}H]-ethanolamine incorporation into [^{3}H]-phosphatidylethanolamine. This may

represent a compensatory mechanism in response to the depletion of phosphatidylethanolamine. Toluene rapidly increased phospholipid methylation (15 min after exposure), which was followed by a decrease 1 h after exposure (LeBel & Schatz, 1990).

In conclusion, the reduced phosphatidylethanolamine and phospholipid concentrations and the reduced phospholipid methylation (1 h after administration) caused by single i.p. administration may change the structure/function of the lipids in the neuronal membrane and thereby affect its properties, including its fluidity. This may explain the acute narcotic effect of toluene as anaesthetics are known to dissolve in nerve membrane lipids and change the structure of the membrane (membrane swelling) (McMurray, 1982). Other membrane functions as discussed below may also be changed either directly or indirectly.
However, to extrapolate from these effects induced by single i.p. administration to effect induced by the exposure schedule as applied in study I, must be performed with reservations.

Membrane fluidity
In a study of rat erythrocytes, the *in vivo* exposure to 2000 ppm toluene 6 h/day for 7 or 21 days increased the resistance of erythrocytes to haemolysis when incubated in a hypotonic medium *in vitro* (Korpela et al., 1983). In this study, toluene was also added to erythrocytes *in vitro* in the concentration range of 500 to 1400 ppm. Hereby, a concentration-related antihaemolytic effect was observed up to 1000 ppm, the maximum being at 300 ppm. Above 1000 ppm an increase in the haemolysis was seen. It was suggested that the increased haemolytic resistance was due to a change in membrane fluidity, which might be associated with the narcotic property of toluene.

In a study by Edelfors & Ravn-Jonsen (1989) rats were exposed to 500 ppm toluene for 18 h, 2, 4, 8, 12, 26, 52, or 78 weeks. No treatment related effects were found on synaptosomal membrane fluidity as measured with DPH as the probe.

Le Bel & Schatz (1988) injected rats i.p. with doses of 0.5, 1, or 1.5 g toluene/kg b.w. 1 h before sacrifice. Phospholipid/cholesterol ratios (indirect index of membrane fluidity) were unchanged by all the doses. This was confirmed by fluorescence polarization studies with DPH as the probe, showing that toluene treatment (1 g/kg b.w.) did not alter synaptosomal membrane (from whole brain) fluidity.

Later, LeBel & Schatz (1989) injected rats i.p. with a dose of 1 g/kg b.w. 1 h before sacrifice. Membrane fluidity studies demonstrated that toluene increased the fluidity of the outer core of the synaptosomal membrane (from whole brain) (TMA-DPH as the probe), whereas the dynamic of the central acyl-side chain region of the membrane was unaffected when measured with DPH as the probe.

Finally, LeBel & Schatz (1990) injected rats i.p. with a dose of 1 g/kg b.w.,

15, 30, or 60 min before sacrifice. Toluene increased the fluidity of the outer core of the synaptosomal membrane (from whole brain) (15, 30, and 60 min after dosing) using TMA-DPH as the probe, whereas no effect was observed on the central core fluidity when measured with DPH as the probe.

In an investigation by von Euler et al. (1991), the effects on rats of low concentrations of toluene (80 ppm, 3 days, 6 h/day) were studied. Toluene exposure did not affect the fluidity of the membrane of synaptosomes isolated from frontoparietal cortex, neostriatum or the subcortical limbic area, neither when TMA-DPH nor when DPH was used as the probe.

Generally, all 3 groups studying membrane fluidity found no effect on the fluidity of the central region of the synaptosomal membrane using DPH as the probe.
There are contradictory toluene-induced effects on the fluidity of the outer core of the synaptosomal membrane: LeBel & Schatz (1989, 1990) found increased fluidity following a single i.p. injection of toluene, whereas von Euler et al. (1991) could not confirm this effect following inhalation exposure for 3 days. Both groups used TMA-DPH as the probe. This controversy may be explained by different routes of exposure, doses, duration of exposure, and brain regions used for synaptosome preparation.

Theoretically, increased fluidity of the outer core of the synaptosomal membrane may alter the properties of the membrane and cause changes of membrane stability and of the activities of various membranal enzymes, ion-channels, and carriers. Also binding characteristics (K_d and B_{max}) of the various neurotransmitter receptors may be altered. Such changes, which are discussed below, may compromise the function of the neuron. However, it is limited by reservations to extrapolate from the effects shown on membrane fluidity induced by single i.p. administration to effects induced by the exposure schedule as applied in study **I**.

Membrane leakage

In a study by von Euler et al. (1991), the effects in rats of low concentrations of toluene (at 80 ppm, 3 days, 6 h/day) were investigated. The rats were killed 18 h after the last exposure. Following toluene exposure, membrane FURA-2 leakage *in vitro* was increased from synaptosomes isolated from the neostriatum, whereas there was no effect on the leakage from synaptosomes isolated from frontoparietal cortex, neostriatum, or the subcortical limbic area. The increased leakage from synaptosomes isolated from neostriatum indicates that this region may be selectively vulnerable to toluene exposure.

Activity of membranal enzymes

Korpela & Tähti (1988) showed that 2 h exposure of rats to 2000 ppm toluene inhibited the activity of AChE, total-ATPase, and Mg^{2+}-ATPase when compared to membranes prepared from untreated controls.

In a study by Edelfors & Ravn-Jonsen (1989), rats were exposed to 500 ppm toluene for 18 h, 2, 4, 8, 12, 26, 52, or 78 weeks. Ca^{2+}/Mg^{2+}-ATPase

activity in isolated synaptosomal membranes decreased as compared with controls by exposure for 18 h, whereas exposure to 8, 12, 26, 52, and 78 weeks increased the activity.

In a study by LeBel & Schatz (1990), rats were injected i.p. with a single dose of 1 g/kg b.w., 15, 30, or 60 min before sacrifice. Toluene treatment stimulated synaptosomal Na^+/K^+-ATPase activity 15 and 30 min after injection, whereas Ca^{2+}- and Mg^{2+}-ATPases were unaffected by all the treatments.

Edelfors & Ravn-Jonsen (1991) studied the effect of toluene treatment on synaptosomal Ca^{2+}/Mg^{2+}-ATPase activity. Rats were exposed to 500 ppm toluene by inhalation for 12 h/day during 4 weeks. Ca^{2+}/Mg^{2+}-ATPase activity in isolated synaptosomal membranes was unchanged.

In conclusion, single inhalation exposure seems to decrease the activity of membranal enzymes [AChE, various ATPases (total-, Mg^{2+}-, Ca^{2+}/Mg^{2+}-ATPase)], whereas 4 weeks of inhalation exposure normalized the Ca^{2+}/Mg^{2+}-ATPase activity and long-term inhalation exposure (8-78 weeks) increased Ca^{2+}/Mg^{2+}-ATPase activity in isolated synaptosomal membranes. Synaptosomal membrane Ca^{2+}/Mg^{2+}-ATPase activity may be linked with active transport of Ca^{2+} out of intact synaptosomes.

Calcium-uptake and calcium-levels

Edelfors & Ravn-Jonsen (1985) investigated the ½ to 8 min Ca^{2+}-uptake into high $[K^+]$-stimulated synaptosomes. The synaptosomes were isolated from 18 h toluene exposed rats and untreated controls. Exposure to 500 ppm toluene increased the Ca^{2+}-uptake rate, whereas exposure to 1000 ppm had no effect on the Ca^{2+}-uptake rate.

In a second study the same authors (Edelfors & Ravn-Jonsen, 1987) extended the toluene exposure (500 ppm, 12 h/day for up to 80 weeks). Both 4 and 12 weeks of exposure increased 2-16 min Ca^{2+}-uptake into unstimulated synaptosomes compared with synaptosomes from controls, whereas the unstimulated uptake was normalized after 30 and 80 weeks of exposure. There was no treatment related effects on the high $[K^+]$-stimulated Ca^{2+}-uptake.

In a third study, Edelfors & Ravn-Jonsen (1991) investigated the effect of inhalation exposure of rats to 500 ppm toluene for 12 h a day during 4 weeks. The toluene exposure caused a higher unstimulated (0.5 and 2 min uptake) and high $[K^+]$-stimulated synaptosomal Ca^{2+}-uptake (0.5, 2, and 4 min uptake).

In an investigation by von Euler et al. (1991), the effects on rats of low concentrations of toluene (at 80 ppm, 3 days, 6 h/day) were studied. The rats were killed 18 h after the last exposure. Toluene exposure did not affect Ca^{2+} levels in synaptosomes isolated from frontoparietal cortex, neostriatum, or the subcortical limbic area.

In conclusion, the unstimulated Ca^{2+}-uptake increased following exposure to 500 ppm toluene for 4 and 12 weeks, whereas it was at control levels following 30 and 80 weeks of exposure.
Single and 4 weeks (one of 2 studies) inhalation exposure to 500 ppm toluene increased high $[K^+]$-stimulated Ca^{2+}-uptake, whereas a longer exposure time had no effect.
If present *in situ*, an increased stimulated Ca^{2+}-uptake would be in accordance with a potential for increased release of various neurotransmitters a.o. NA, DA, and 5-HT. No data were available of the effect of *in vivo* toluene exposure on the synaptosomal release of these neurotransmitters to confirm this indication.

Membrane potential
In a study by Bondy & McKee (1991), rats were sacrificed 1 h after i.p. injection of 1 g toluene/kg b.w. No changes were detected in synaptosomal transmembranal potential by this treatment when compared with untreated controls.

Ad. Generation of reactive intermediates
The brain is known to require a continuous and rich supply of oxygen to function. The high rate of oxidative metabolism may give rise to a high rate of ROS generation in this organ. Protective systems compete to minimize damage caused by ROS. However, because the brain has relatively low levels of activity of such systems, it may be a prime target for free-radical damage (LeBel & Bondy, 1991). It has been proposed that several diverse neurotoxic events may be related to excess formation of ROS because the brain contains large amounts of substrates, which are susceptible to ROS (LeBel & Bondy, 1991). These substrates include unsaturated lipids, NA, DA, and 5-HT.

Lipid peroxidation
Mattia et al. (1991) studied the effect of toluene exposure on lipid peroxidation in crude synaptosomal fractions from cortex. Rats were dosed i.p. with 1 g toluene/kg b.w. 1 h before sacrifice. Toluene exposure did not stimulate lipid peroxidation as evaluated by measurement of conjugated dienes. In a second study by Mattia et al. (1993), rats were injected i.p. with toluene (0.5 or 1.0 g/kg b.w.). Striatal and hippocampal lipid peroxidation were measured as the formation of thiobarbiturate reactive substances. Lipid peroxidation was increased in the crude synaptosomal fraction of hippocampus after administration of 1 g/kg b.w.

Reactive intermediates
Mattia et al. (1991) studied the effects of toluene exposure on the rate of ROS formation in crude synaptosomal fractions from cortex. Rats were dosed i.p. with 1 g toluene/kg b.w. 1 h before sacrifice. Toluene caused an elevated ROS formation within the cortical crude synaptosomal fraction when compared with controls. The ROS-inducing properties of toluene were blocked by i.p. administration of a mixed-function oxidase inhibitor,

metyrapone (200 mg/kg b.w), 1 h prior to toluene administration. Both toluene and benzaldehyde added *in vitro* to incubates increased ROS generation in a concentration related manner, by far most pronounced by benzaldehyde addition. Preincubation with metyrapone blocked the stimulation of ROS generation. Both benzyl alcohol and benzoic acid added to incubates *in vitro* were without any effect on the rate of ROS generation. These results suggest that benzaldehyde may be central for the stimulating effect of toluene on ROS generation in CNS *in situ* and may contribute to the neurotoxicity of toluene.

In a second study by Mattia et al. (1993) rats were injected i.p. with various doses of toluene (0.5, 1.0, or 1.5 g/kg b.w.). The authors found an elevated ROS generation rate in the crude synaptosomal fractions prepared from hippocampus and striatum of rats exposed to 0.5, 1.0, or 1.5 g/kg b.w., whereas the ROS generation was only elevated in cerebellum by the administration of 1 and 1.5 g/kg b.w. For all 3 regions, the increased ROS generation persisted even 24 h after the injection of 1.5 g/kg b.w., at a time where blood toluene levels were negligible.

Intraperitoneal injection of toluene, 1 ml/kg b.w./day for 21 days did not affect brain cytochrome P450 activity but increased GSH S-transferase activity in the cytosol isolated from the brain (Chand & Clausen, 1982). This may be a protective mechanism against increased ROS generation.

In conclusion, increased ROS generation seems to be a general effect following single i.p. administration of toluene. If NA, DA, and 5-HT are oxidized in defence against the increased ROS generation this may give rise to reduced neurotransmitter concentrations.

c. Receptor functions

The effects of toluene have been studied on NA, DA, and 5-HT receptors.

Treatment of rats with 80, 500, or 1500 ppm toluene for 6 h per day for 3 consecutive days increased both the K_d and the B_{max} for the β-adrenergic ligand, [^{3}H]-dihydroalprenonol, (at all exposure levels) to membranes isolated from the frontoparietal cortex, whereas the α-1- and α-2-adrenergic binding sites in this region were unaffected by the treatments (Fuxe et al., 1987). The results suggest that the cortical β-adrenergic receptors are particularly vulnerable to toluene although the outcome of increased K_d and the B_{max} is unpredictable.

There was an increased K_d for the association of the dopamine D_2 receptor ligand spiperone (Celani et al., 1983) to striatal membranes following inhalation exposure to 3000 ppm toluene 6 h/day for 3 days.

In a series of studies, von Euler et al. (1987, 1989b, 1991, 1993) investigated the effect of toluene exposure on the binding characteristics of NPA (a dopamine D_2 receptor ligand) to striatal membranes:

-Rats were exposed to 80 ppm toluene 6 h/day for 3 days. Toluene exposure increased both the K_d and the B_{max} (von Euler et al., 1987).

-Rats were exposed to 80 ppm toluene 6 h/day for 3 days. Toluene exposure increased K_d without affecting the B_{max} (von Euler et al., 1989b).

-Rats were exposed to 40 or 80 ppm toluene, 6 h/day for 3 days and killed 18 or 42 h after the last exposure. Toluene increased the K_d value of NPA association to membranes isolated 18 h after the end of exposure to 80 ppm without affecting the B_{max}. The other treatments did not affect neither K_d nor B_{max} values. (von Euler et al., 1991).

-In a fourth study (von Euler et al., 1993), the effect of exposure to 80 ppm toluene, 5 days/week, 6 h/day for 4 weeks were studied. The toluene treatment produced persistent increased B_{max} and K_d values of NPA binding to striatal membranes isolated 17 days after the last exposure.

Rats were inhalation exposed to 40, 80, 160, or 320 ppm toluene, 6 h/day, 5 days/week for 4 weeks followed by an exposure free period of 29-40 days. Toluene exposure ≥80 ppm is indicated to induce persistent increase in the affinity of dopamine D_2 ligand binding in the rat caudate-putamen area (part of thalamus), without affecting the number of D_2 receptors (Hillefors-Berglund et al., 1995).

One study revealed decreased B_{max} for 5-HT binding to P2-fraction membranes prepared from the whole brain, hippocampus, and med. obl.+pons after inhalation exposure of rats to 0.7% toluene for 15 min/day for 14 days. The study reported no effects on K_d (Yamawaki et al., 1982).
In another study, increased K_d was shown for 5-HT association to cortical membranes (Celani et al., 1983) following inhalation exposure to 3000 ppm for 3 days without effect on B_{max}.
The K_d for 5-HT association to crude synaptic membranes highly increased following 30000-40000 ppm for 30 days, without affecting B_{max} (Castilla et al., 1993).

In conclusion, short-term inhalation exposure to toluene increased the K_d for NPA and spiperone association to the striatal dopamine D_2 receptor probably without affecting the B_{max}, whereas 4 weeks of exposure to 80 ppm followed by an exposure free period of 17 days' duration induced persistent increased B_{max} and K_d values of NPA binding to striatal membranes. Toluene exposure to ≥80 ppm for 4 weeks induced persistent increases in the affinity of D_2 ligand binding in the caudate-putamen area without affecting B_{max}. These changes may give rise to affected dopaminergic activity in these subregions of the thalamus. In thalamus the concentration of DA increased following exposure to 500 ppm toluene (**I**).
Inhalation exposure to toluene for 3 or 30 days increased the K_d for 5-HT association. Fourteen days of exposure decreased the B_{max}, whereas 3 or 30 days of exposure were reported to be without any effect. If present *in situ*,

an increased K_d and a decreased B_{max} for 5-HT binding to its receptor(s) would be in accordance with a decreased potential for serotonergic activity. This may be compensated for by increased 5-HT concentration.

C. Overall conclusions on mechanisms underlying the demonstrated effects of toluene exposure on neurotransmitter concentrations

The targets and mechanisms discussed in section 1 B, a-c of this chapter are directly or indirectly related to neurotransmitter metabolism and could theoretically explain why toluene exposure induced long-lasting changes of neurotransmitter concentrations as observed in study **I**. However, the data which support each of these potential targets and mechanisms are not complete to provide a final proof.
The findings that deserve most attention are:

-the increased *in vitro* 5-HT uptake rate (V_{max}) of platelets isolated from occupationally solvent exposed car painters,

-the increased K_d for 5-HT association to its receptor(s) following inhalation exposure for 3 and 30 days, along with the decreased B_{max} for 5-HT binding following 14 days of inhalation, and

-the increased K_d-value for NPA association to the striatal membrane dopamine D_2 receptor following short-term and extended inhalation exposure.

Single toluene inhalation exposure decreased Ca^{2+}/Mg^{2+}-ATPase activity in isolated synaptosomal membranes, whereas the activity was normalized after 4 weeks of exposure and even increased following long-term exposure (8-78 weeks).
Provided that the increased synaptosomal membrane Ca^{2+}/Mg^{2+}-ATPase activity implies increased potential for active transport of Ca^{2+} out of neurons, this exposure-time related response suggests an increased ability for active transport of Ca^{2+} out of neurons following long-term exposure. This may be acting in response to an increased Ca^{2+}-influx following long-term exposure. Although not contradicted, this mechanism seems not to be supported by the reported toluene-induced effect on Ca^{2+}-uptake following long-time exposure:

-The unstimulated Ca^{2+}-uptake increased following exposure to 500 ppm for 4 and 12 weeks, whereas it was at control levels following 30 and 80 weeks of exposure.
Single and 4 weeks (one of 2 studies) inhalation exposure to 500 ppm toluene increased high $[K^+]$-stimulated Ca^{2+}-uptake, whereas a longer exposure time had no effect.

The increased *in vitro* 5-HT uptake rate (V_{max}) of platelets isolated from car

painters is of great interest as it is documented in man (Beving et al., 1983). The increased K_d for 5-HT association to its receptor(s) were induced after inhalation exposure for 3 or 30 days. Thirty days of exposure is at the threshold limit between short-term exposure B and long-term exposure, as defined previously, Appendices I C,D.

A pattern of changes seems to arise: increased $[K^+]$-stimulated Ca^{2+}-uptake, increased 5-HT uptake rate, increased K_d, and decreased B_{max} for 5-HT receptor association. If present in the brain *in situ* following the exposure schedule as applied in study **I** this consistent pattern of changes can explain the generally increased regional 5-HT concentrations demonstrated in study **I** as a part of a compensatory mechanism: *Toluene causes a reduced potential for activity of serotonergic neurons by increasing K_d and decreasing B_{max} for 5-HT association to its receptor(s). This is compensated for by increased 5-HT release caused by increased Ca^{2+}-influx and by an increased 5-HT concentration, maintained (in part) by an increased 5-HT uptake rate.* This proposal needs further evaluation.

This mechanism implies that: *the generally toluene-increased regional 5-HT concentration is part of a compensatory mechanism acting to overcome a toluene-reduced potential for serotonergic activity.*
This hypothesis remains to be tested.

Important questions to be answered are:

-whether toluene-reduced serotonergic activity is an element of toluene CNS neurotoxicity in rat and man,

-whether the compensatory mechanism is actually acting as proposed, and

-whether the proposed compensatory mechanism if acting is fully compensatory.

Obviously, a fully compensatory mechanism can not explain neurotoxicity, but it demonstrates neuroactivity of toluene.
Finally, until yet, the effect of toluene on brain neurochemistry, has been looked upon through a narrow window: unstudied parameters may be the most important.

If the increased K_d for NPA association to the striatal dopamine D_2 receptor following short-term and 4 weeks inhalation exposure to toluene implies reduced affinity for DA to its receptor this is in accordance with a reduce potential for dopaminergic activity in this brain region. This may be (partly) compensated for by the persistently increased B_{max} due to 4 weeks of exposure (defined as long-term exposure, Appendix I C).
However, if a toluene-reduced potential for dopaminergic activity is generally present in the brain in study **I**, it may be compensated for by a mechanism similar to that proposed for 5-HT: increased DA release caused

by increased Ca^{2+}-influx and increased DA concentration.
This could theoretically explain the generally increased regional DA concentrations demonstrated in study **I** as a part of the compensatory mechanism. Nothing is known about the effect of toluene on DA reuptake in dopaminergic CNS terminals.

2. AROMATIC WHITE SPIRIT, REFERENCE II

Both aromatic and aliphatic components of aromatic white spirit were present in the whole brain. The brain concentration of the aromatic constituents increased proportionally (2.1 times) in brains from rats exposed to 800 ppm compared to brains from rats exposed to 400 ppm, whereas the concentration of the aliphatic fraction more than tripled (ratio: 3.6). This indicates that a threshold capacity for the elimination of aliphatic components was exceeded.
The greater accumulation of aliphatic compounds is in accordance with findings reported in studies applying single-substance exposure at identical concentrations and time to various aliphatic, naphthenic, and aromatic compounds (Zahlsen et al., 1990, 1992). Special attention should be paid to n-nonane because of the high concentration of C_9 aliphatic components in aromatic white spirit (10%, w/w, Appendix III A), the ability of n-nonane to cause damage to nerve tissue (Verkkala et al., 1984; Nilsen et al., 1988), and because n-nonane accumulates in the brain in high concentrations (Zahlsen et al., 1990, 1992). The qualitative and quantitative composition of the aromatic white spirit used in study **II** is not known, but it was shown that nonane (unspecified) was present in the brain (**II**).

Aromatic white spirit did not affect the weight of the whole brain, whereas an increased NA concentration was demonstrated in the whole brain by exposure to 400 ppm and increased NA, DA, and 5-HT concentrations were shown in the whole brain following exposure to 800 ppm (**II**).

3. AROMATIC WHITE SPIRIT, REFERENCE III

No behavioural (functional observational battery, motor activity, diurnal activity, passive avoidance, Morris maze, radial arm maze), macroscopic, or histopathological changes were found. In contradiction to the results of Døssing et al. (1983b), who reported increased ALAT activity in serum from 23 of 156 solvent-exposed house painters, the present study (**III**) demonstrated reduced rat plasma ALAT activity. The cause and physiological consequences of this reduction are unknown. The urea and creatinine concentrations were increased (**III**), in accordance with affected liver and kidney functions.
No effect was shown on the weight of any brain region. The concentrations of NA, DA, and 5-HT in various brain regions and in the whole brain (reconstructed by calculation) were changed in study **III**.

The concentration of NA increased in the hemisphere (at 400 and 800 ppm)

and hippocampus (at 800 ppm), whereas it decreased in the cerebellum (at 400 ppm). The concentration of DA increased in hemisphere (at 400 and 800 ppm), thalamus (at 800 ppm), and whole brain (at 400 and 800 ppm), whereas it only decreased in the hippocampus (at 800 ppm). The level of 5-HT increased in hemisphere, hippocampus, hypothalamus, pons, thalamus, med. obl., and whole brain when exposed to 800 ppm. The 5-HT concentration was only reduced in the cerebellum (at 400 and 800 ppm). Thus, the increased concentrations in the whole brain of DA and 5-HT following 3 weeks of exposure (**II**) were also present following 6 months exposure succeeded by an exposure-free period of 4 months' duration (**III**), indicating that these changes were also present immediately after the end of the 6 months of exposure.
The literature survey failed to reveal any other investigations of the effect of white spirit exposure on DA, NA, or 5-HT concentrations than the references **II**, **III**, and **IV**.

4. MECHANISMS POTENTIALLY RELATED TO THE GENERALLY INCREASED 5-HT CONCENTRATIONS AS OBSERVED IN STUDY II AND III

This section reviews the potential mechanisms related to the observed effects of aromatic white spirit on rat brain neurotransmitter concentrations (**II**; **III**) with special focus placed on the 5-HT concentrations.

Only one region, the hemisphere, revealed changes of NA and DA concentrations in the same direction, as noticed in 3 regions following toluene exposure (**I**).
Toluene caused an increased 5-HT concentration in 3 regions (hemisphere, hippocampus, pons) and in the whole brain (**I**). The same but even more pronounced general phenomenon of increased regional 5-HT levels was recognized following aromatic white spirit exposure: except for the cerebellum, the 5-HT concentration increased in all 7 brain regions and in the whole brain as well when exposed to 800 ppm (**III**). This suggests a common underlying mechanism. The same mechanisms may be responsible for the increased regional 5-HT concentrations following aromatic white spirit exposure as those discussed for toluene.

As already mentioned, the L-tryptophan concentration seems to be the rate limiting parameter for 5-HT synthesis in the brain. Thus, an increased brain L-tryptophan concentration might explain an increased 5-HT concentration. No data were revealed in the literature survey on the effect of white spirit on the blood/plasma/serum or brain concentrations of L-tryptophan.

Only a few other investigations than **II** and **III** have been performed concerning the effect of white spirit on brain neurochemistry. These studies were carried out on synaptosomes (4 a,d, and study **IV**). However, some other studies may also be of interest (4 b,c,d).

a. Some studies on synaptosomes

Edelfors & Ravn-Jonsen (1985) investigated the 0.5 to 8 min Ca^{2+}-uptake

rate into high [K^+]-stimulated synaptosomes. The synaptosomes were isolated from 18 h white spirit (type not specified) exposed rats (at 500 or 1000 ppm) and untreated controls. Exposure to 500 ppm increased the Ca^{2+}-uptake rate, whereas exposure to 1000 ppm white spirit depressed the uptake rate compared with synaptosomes isolated from control animals.
It is limited by reservations to extrapolate from this single-exposure study to the situation *in situ* following the exposure schedule applied in **III**.

Another study used *in vitro* addition of non-aromatic white spirit to investigate the effects on ATPase activity and fluidity. Edelfors & Ravn-Jonsen (1992) added several organic solvents *in vitro* to isolated synaptosomal membranes. Non-aromatic white spirit decreased synaptosomal membrane Ca^{2+}/Mg^{2+}-ATPase activity in a concentration related manner and increased fluidity when measured with DPH as the probe. However, non-aromatic white spirit is another product than aromatic white spirit.

b. Studies on platelets

As mentioned previously, platelets are used as models for the presynaptic serotonergic nerve terminal. The increased 5-HT uptake rate (V_{max}) into platelets shown by Beving et al. (1983) in car painters exposed to mixtures of organic solvents may be extrapolated to the brain. If also present in the brain, *an increased 5-HT uptake rate and uptake capacity in serotonergic nerve terminals in the brain in vivo following aromatic white spirit exposure can be a mechanism underlying the generally increased global and regional 5-HT concentrations following aromatic white spirit exposure.* This proposal was challenged *in vitro* by the determination of the 5-HT uptake into synaptosomes isolated from rats exposed to aromatic white spirit for 3 weeks or 6 months (**IV**). The results obtained in **IV** are discussed in part 5 and 6 of this chapter.

c. Studies on some aromatic white spirit components and their metabolites

It has been shown that ethylbenzene, a component of aromatic white spirit, vinyltoluene, and styrene all deplete striatum and the tuberoinfundibular region for DA in rabbits after inhalation exposure to 750 ppm for 12 h/day for 7 days (Romanelli et al., 1986; Mutti et al., 1988), whereas other organic solvents: toluene, xylenes, and 7-methylstyrene did not reduce the DA level in these regions.
In the 2 studies, reduced DA levels in the 2 regions were also shown after i.p injections of 4 mmol/kg b.w./day for 3 days of mandelic acid and phenylglyoxylic acid (metabolites of ethylbenzene styrene and styrene). No effects were induced by injections of hippuric acid (toluene metabolite), methylhippuric acid, or 7-methylmandelic acid (metabolites of aromatic hydrocarbons).
No effect on the NA concentration in the 2 regions was produced by the 3 solvents or their metabolites.
It was concluded, that the DA depletion could not be ascribed to a 'solvent effect', but depended on the interference of aromatic metabolites whose side chain was biotransformed into an α-ketophenylic acid (Romanelli et al.,

1986; Mutti et al., 1988).
This effect of phenylglyoxylic acid may be a chemical effect based on a reaction between phenylglyoxylic acid and DA, because *in vitro* experiments have shown that phenylglyoxylic acid can condense non-enzymatically with DA (Mutti et al., 1988). It may also be a pharmacological action caused by phenylglyoxylic acid induced displacement of DA from stores in dopaminergic neurons.
The 'α-ketophenylic acid hypothesis' may be extended to other organic solvents, which are metabolized to phenylglyoxylic acid or another α-keto-phenylic acid derivative. Such solvents may be neuroactive by reducing the DA content in dopaminergic neurons. In Appendix III B it is shown that potential α-ketophenylic acid parent compounds, e.g. the various ethyl-benzenes, are present in aromatic white spirit. Therefore, a reduced DA level as observed in hippocampus (**III**) may be explained by this mechanism. The effect of oral administration of phenylglyoxylic acid for 90 days on regional and synaptosomal neurotransmitter metabolism is about to be investigated in our laboratory.

d. Reactive oxygen species, ROS

As already mentioned, oxidative processes are characteristic features of the catabolism/detoxification of white spirit components as also stressed by the highly reduced state, especially of the aliphatic components. As proposed for toluene neurotoxicity, damage caused by reactive intermediates may also be an underlying mechanism for white spirit neurotoxicity. However, in one study of 3 weeks exposure of rats to aromatic white spirit (at 400 or 800 ppm, 6 h/day, 7 days/week) we were not able to confirm oxidative stress to take place in the synaptosomal fraction prepared from the hemisphere or the hippocampus (Bondy et al., 1995).
The cerebellar GSH concentration increased following exposure to 500 and 1000 ppm aromatic white spirit for 4 weeks whereas it decreased following 8 and 17 weeks of exposure at 1000 ppm (Savolainen & Pfäffli, 1982), reflecting increased and decreased potential, respectively, for protection against ROS.

5. AROMATIC WHITE SPIRIT, REFERENCE IV

To further study the mechanisms underlying the aromatic white spirit-induced effects on global and regional neurotransmitter concentrations (**II**; **III**), it was decided to study whether effects were induced at the subcellular level or not. Synaptosomes were selected as a model for the situation at the presynaptic CNS nerve terminal level *in situ*. The rationale for this choice is:

-synaptosomes are resealed presynaptic nerve terminal complexes surrounded by a well-functioning membrane. They constitute a well-documented *ex situ* model system for the nerve terminal *in situ*,

-it has been shown that the homogenization, differential-, and sucrose density-gradient centrifugation procedures applied in our laboratory provide main and subfractions of brain tissue, including synaptosomes, with marker

enzyme distributions (LDH for cytosol; SDH for mitochondria) (Lam, 1980) as those reported in the literature and verified by electron microscopy,

-isolated synaptosomes when prepared and kept under ice-cold conditions from decapitation until neurochemical analyses have been proposed to represent a 'freezing' of the conditions in the presynaptic nerve terminal *in situ* at the time of sacrifice (Lam & Christensen, 1992), and

-when incubated at 37 °C in an 'extracellular' medium with glucose as substrate, our preparation procedure provides synaptosomes that have been demonstrated to respire in a linear fashion for hours (Lam, 1980) and to establish cation concentration gradients and a membrane potential comparable to those of neurons *in vivo* (Lam & Christensen, 1992).

There was no aromatic white spirit-induced effect on the weight of whole brain minus cerebellum after neither 3 weeks nor 6 months exposure to aromatic white spirit. This is in accordance with the previous findings (**II**; **III**).

Both the relative (expressed as mg synaptosomal protein obtained per g whole brain minus cerebellum) and the absolute (expressed as mg synaptosomal protein obtained per whole brain minus cerebellum) yield of synaptosomal protein were reduced in the 2 exposed groups following both 3 weeks and 6 months of exposure. Such effects induced by an organic solvent have not previously been published.
It can be hypothesized that aromatic white spirit exposure for 3 weeks and 6 months both reduced the density (expressed as the relative yield of synaptosomal protein) and the total number (expressed as the absolute yield of synaptosomal protein) of inter-neuron connections *in situ*, provided that the synapse protein content is unchanged and that the yield of synaptosomes during the homogenization and isolation procedures is uncompromised by aromatic white spirit.

The relative synaptosomal NA, DA, and 5-HT concentrations (expressed as the pmol neurotransmitter per mg synaptosomal protein) were increased in both experiments at both exposure levels.
The absolute amount (expressed as the pmol neurotransmitter in synaptosomes obtained per whole brain minus cerebellum) of synaptosomal NA was increased at both exposure levels in the 6 months study. The absolute amount of synaptosomal DA was increased in the highest exposed group in both experiments. The absolute amount of synaptosomal 5-HT was increased in the 6 months investigation at both exposure levels. The increased synaptosomal 5-HT concentration is in agreement with the increased global and regional 5-HT concentrations and supports the use of synaptosomes as model for testing the proposal as put forward p. 58.
Such effects induced by an organic solvent have not previously been reported. These findings are in agreement with increased ability for neurotransmitter release when the nerve impulse has to be transmitted over the synaptic cleft of noradrenergic, dopaminergic, and serotonergic neurons.

The relative (expressed as the pmol 5-HT/mg synaptosomal protein/min) and absolute (expressed as the pmol 5-HT/min in synaptosomes obtained per whole brain minus cerebellum) high-affinity 5-HT uptake rate and -capacity (the steady-state 5-HT uptake measured after 15 min expressed analogously) were increased following 3 weeks and 6 months of exposure to both 400 and 800 ppm aromatic white spirit.
Increased 5-HT uptake rate and -capacity may be the mechanisms underlying the increased intrasynaptosomal 5-HT concentration (**IV**) and they support the proposal put forward p. 58 regarding the mechanism for increased global and regional 5-HT concentrations as found in study **II** and **III**.

Similar effects might also be induced on high-affinity NA and DA uptake rates and -capacities. These parameters were not investigated in this study (**IV**). If present, such increases could also explain increased global, regional, and synaptosomal NA and DA concentrations when observed in **II**, **III**, and **IV**.

The increased synaptosomal 5-HT uptake rate is in agreement with results in a study by Beving et al. (1983), who demonstrated increased 5-HT uptake rate into platelets isolated from occupationally solvent exposed car painters. However, the increased synaptosomal 5-HT uptake capacity and -content (**IV**) are not in agreement with the results of a study we performed in solvent-intoxicated workers (Lam et al., 1983), demonstrating unaffected platelet 5-HT concentration. Thus, the use of the platelet as an alternative human model for the human presynaptic serotonergic CNS nerve terminal needs further evaluation.
Mood disorders with symptoms of depression may be treated with pharmaceuticals that inhibit platelet 5-HT uptake rate. So, a relation between the aromatic white spirit-increased synaptosomal 5-HT uptake rate and the development of symptoms of depression as observed in patients suffering from solvent-induced chronic toxic encephalopathy may be tempting to propose. However, such a relation is very complex and at the present stage of knowledge it is premature to make any conclusion. Anyway, it is an important issue that deserves clarification.

It is well-known that synaptosomes spontaneously release 5-HT during incubation. Liberated endogenous 5-HT is substituted by labelled 5-HT during the incubation and a steady state for labelled 5-HT uptake was attended when the incubation was extended to 15 min. Therefore, the increased uptake capacities (relative and absolute) suggest increased synaptosomal compartments of 5-HT that can be spontaneously released owing to aromatic white spirit exposure for 3 weeks and 6 months.
Theoretically, if the compartment of 5-HT that can be released by neuron stimulation is increased in serotonergic neurons *in situ*, this may increase the ability for serotonergic activity in CNS. To investigate this suggestion, the high $[K^+]$-stimulated synaptosomal 5-HT release should be determined in synaptosomes isolated from control and aromatic white spirit exposed rats. However, this was not performed in study **IV**.

6. OVERALL CONCLUSIONS ON MECHANISMS UNDERLYING THE DEMONSTRATED EFFECTS OF AROMATIC WHITE SPIRIT EXPOSURE ON RAT BRAIN NEUROTRANSMITTER METABOLISM

The changed parameters as discussed in section 4 and 5 of this chapter could be the underlying mechanisms for the aromatic white spirit-changed neurotransmitter concentrations following exposure for 3 weeks (**II**) and 6 months (**III**; **IV**).
The components of aromatic white spirit are lipophilic. Theoretically, the same targets and mechanisms as discussed for the actions of toluene could be extrapolated to aromatic white spirit.

The data obtained in reference **II**, **III**, and **IV** are in agreement with the hypotheses:

-aromatic white spirit-increased synaptosomal NA, DA, and 5-HT concentrations are indices of increased neurotransmitter concentrations in respective presynaptic nerve terminals in situ acting to overcome reduced transmission capacity caused by an aromatic white spirit-reduced number of noradrenergic, dopaminergic, and serotonergic synapses in situ, and

-aromatic white spirit-increased 5-HT uptake rate and uptake capacity in serotonergic neurons in situ cause the generally increased global and regional 5-HT concentrations.

These proposed hypotheses remain to be tested. It still has to be investigated whether aromatic white spirit exposure reduces the number of inter-neuron connections *in situ* or not. It is not known whether such a reduction is an element of white spirit CNS neurotoxicity in rat and man.
Other important questions to be answered are:

-whether aromatic white spirit-reduced noradrenergic, dopaminergic, and serotonergic activity is an element of white spirit CNS neurotoxicity in rat and man,

-whether the compensatory mechanisms are actually acting as proposed,

-whether the proposed compensatory mechanisms if acting are fully compensatory.

Obviously, a fully compensatory mechanism can not explain neurotoxicity, but it demonstrates neuroactivity of aromatic white spirit.
Finally, until yet, the effect of aromatic white spirit on brain neurochemistry, has been looked upon through a narrow window: unstudied parameters may be the most important.

Presently, it is not possible to identify one or more white spirit components as particularly active. n-Nonane is proposed to be of particular interest

(Verkkala et al., 1984; Nilsen et al., 1988; Zahlsen et al., 1990, 1992).
The identification of (a) causative component(s) is complicated by the possibility for interaction between various components of this complex mixture composed of more than 200 substances (Henriksen, 1980). Thus, effects caused by exposure to one single component may not be comparable to effects caused by that component in the mixture.

Furthermore, the use of the synaptosomal model for studying the neurotoxicity of aromatic white spirit is supported by the consistent pattern of affected neurochemistry as demonstrated in **II**, **III**, and **IV**.

7. ADDITIONAL STUDIES OF THE EFFECT OF AROMATIC WHITE SPIRIT EXPOSURE ON REGIONAL RAT BRAIN GFAP CONCENTRATIONS

As already mentioned, O'Callaghan and colleagues from USEPA have proposed the use of GFAP as a sensitive and specific biomarker for neurotoxicity (O'Callaghan, 1991a). It is important to challenge this proposal by studying the effect on the GFAP concentration of many non-neurotoxicants and neurotoxicants, preferably neurotoxicants with different targets and mechanisms of action. This was the rationale for investigating the effects of aromatic white spirit exposure for 3 weeks and for 6 months on regional GFAP concentrations in the rat brain.

A. Three weeks of exposure

There was no effect on the GFAP concentration in hippocampus or hemisphere in young adult or 'middle-aged' rats following 3 weeks of exposure to aromatic white spirit (Table I).
There was no age related effect on the GFAP concentration in hippocampus or hemisphere of control animals (Table I). This is not in accordance with the results of O'Callaghan & Miller (1991) who demonstrated increased GFAP-concentrations in all studied brain areas, including the hippocampus and the hemisphere, of 24-months old male Long-Evans rats compared with 3-month old male Long-Evans rats. The conflicting results may be caused by strain and/or age differences as our young adult and 'middle-aged' male Wistar rats were 5 and 14 months old at the beginning of the 3 weeks of exposure, respectively.

B. Six months of exposure

There was no long-lasting effects on the GFAP concentration in any brain region or in the whole brain following 6 months of exposure to aromatic white spirit (Table II). Reversible effects remain undiscovered by the applied design.
In a satellite study to the present study, Pilegaard (1993) exposed 'middle-aged' rats (13 months old at sacrifice) and 'old' rats (25 months old at sacrifice) to 0 or 800 ppm aromatic white spirit, 6 h/day, 5 days/week for 6 months followed by an exposure-free period of 4 months' duration. This satellite study applied unbiased stereological methods for the estimation of

the number of astrocytes in the molecular layer of the dentate gyrus of the hippocampus. The study failed to find any treatment related effect on the number of GFAP-positive astrocytes, GFAP-volume, or GFAP-volume per astrocyte in this subregion of the hippocampus. A possible relation between the parameters measured by Pilegaard (1993) and the GFAP concentrations as given in Table I and II is presently under investigation. The establishment of any relation is important because neurochemical analyses are much faster than the stereological methods and, therefore, can be used to guide the pathologist to which brain region or even to which subregion more efficiently to perform the stereological studies.

GFAP metabolism in the living organism is influenced by a variety of stimuli from outside the brain. These include hormones, aging, and kidney and liver function, which ideally all should be taken into consideration when evaluating GFAP results. The results of the clinical biochemical analyses indicate affected liver and kidney functions (**III**). However, no macroscopic or microscopic changes, which could be attributed to exposure were found. In other studies, however, aromatic white spirit is reported to affect rat kidney structure and function (For review see IPCS, 1995).

If the function of the liver and kidneys were affected in study **III** as indicated by the affected clinical chemical parameters (ALAT, urea, creatinine), such damages either did not influence the GFAP status or they reversed an aromatic white spirit-changed GFAP concentration to the normal. The study was not designed to control other parameters of origin outside the CNS.

The underlying mechanism for increased GFAP immunoreactivity is presently unknown. It may be caused by increased synthesis, decreased breakdown, unmasking of immunoreactive epitopes, or increased GFAP solubility. The use of GFAP as a general biomarker is problematic:

-Neuroactive/neurotoxic pharmacological agents (reserpine, phenobarbital, pentobarbital, ketamine, apomorphine, muscimol, scopolamine, atropine, and MAO-inhibitors) in small doses did not alter GFAP concentrations although these substances are neurotoxic in higher doses (O'Callaghan, 1991a). Therefore, the selected dose is important.

-The time-course of the GFAP response may differ from neurotoxicant to neurotoxicant, so the time of investigation in relation to exposure is important. This is reported to be the case for MPTP and trimethyltin:

> -A single subcutaneous injection of mice with MPTP (a substance known to be toxic to dopaminergic nigrostriatal neurons) increased the striatal GFAP concentration 24 and 48 h after administration, whereas the GFAP concentration gradually declined to control values 14 to 21 days after administration. This effect was also noticed following dosing for 5 consecutive days (O'Callaghan et al., 1990).

-A similar reversible effect is reported following a single intravenous injection of rats with trimethyltin (a substance destructing the hippocampal pyramidal cells) on the GFAP concentration in hippocampus and frontal cortex (Brock & O'Callaghan, 1987).

-Some neuroactive/neurotoxic organic solvents [1,1,1-trichloroethane, xylene (mixture of *o*-, *m*-, and *p*-xylene), dichloromethane, and styrene] are known to increase the GFAP concentration in various brain regions of mongolian gerbils and rats 4 months after the end of 3 months of exposure (Rosengren et al., 1985, 1986 a,b; Rosengren & Haglid, 1989).
Contrary, exposure to another neuroactive/neurotoxic organic solvent, perchloroethylene, has been shown to reduce frontal cerebral cortex GFAP concentration in the rat following 12 weeks of exposure to 600 ppm (Wang et al., 1993).
Therefore, the GFAP-response to organic solvents may not always be an increased concentration.

-Other potential inflicting factors may be: the duration of exposure, the animal species or even strain, sex, age, effects on the levels of regulating hormones, and affected kidney and liver function.

There was no aromatic white spirit-induced effect on regional GFAP concentrations following 3 weeks (GFAP concentrations immiatedly after end of exposure) or 6 months (GFAP concentrations 4 months after end of exposure) of exposure. From study **II**, **III**, and **IV** it is known that aromatic white spirit is neuroactive when GFAP was measured, because whole brain, regional, and synaptosomal NA, DA, and 5-HT metabolism was changed at these time points.

In conclusion, the lack of aromatic white spirit-changed GFAP concentrations in the 2 studies do not add evidence for the hypothesis posed by O'Callaghan (1991a).

IX. INTERPRETATION OF THE NEUROCHEMICAL FINDINGS IN RATS IN TERMS OF TOLUENE AND AROMATIC WHITE SPIRIT NEUROTOXICITY IN MAN

The ideal model for human neurotoxicity is of course the human being itself. Ethical reasons, however, limit the examinations that can be carried out, and epidemiological studies and *in vitro* methodology are both hampered by several drawbacks. These facts necessitate the use of laboratory animals.
Neurotoxicity testing in laboratory animals can be divided into 4 general disciplines: behaviour, neuroelectrophysiology, neurochemistry, and neuropathology. The present studies (**I**; **II**; **III**; **IV**) applied behavioural, neurochemical, neuropathological including neurostereological methods. Assessment of effects from all disciplines of neurotoxicology are preferable/necessary to establish a full neurotoxicological profile of a chemical.

At the present stage of knowledge, neurochemical findings are of value in the interpretation of behavioural and structural findings. Neurochemical changes provide supportive evidence for neurotoxicity. In its own they can not be used to detect or assess neurotoxicity. The main use of neurochemistry within neurotoxicology is for research purposes and the elucidation of underlying mechanisms, not for routine screening.

At 500 ppm toluene, the mean nuclear volume and mean perikaryonal volume in neocortex were increased (**I**). The weight of the hippocampus was reduced following exposure to 1500 ppm toluene (**I**). In the satellite study to study **I**, a 16% loss of neurons was demonstrated in a subdivision, CA2/3, of the hippocampus when exposed to 1500 ppm (Korbo et al., 1993, 1996). These finding are important observations.
No behavioural changes were found (**I**; **III**), whereas the concentration of NA, DA, and 5-HT in the whole brain (**II**) and in various brain regions (**I**; **III**) were affected following inhalation exposure to toluene (**I**) and aromatic white spirit (**II**; **III**). The yield of synaptosomal protein was reduced and synaptosomal NA, DA, and 5-HT metabolism was affected following exposure to aromatic white spirit (**IV**). It is emphasized that findings in study **I**, **III**, and **IV** are long-lasting effects.
Neurochemical parameters seem to be more sensitive to detect neuroactivity of toluene and aromatic white spirit in rats than are the parameters applied within the other disciplines. However, there is a need to establish the relations between affected rat brain neurochemistry and findings/lack of findings within the other disciplines of neurotoxicology.

It is essential to evaluate whether these neurochemical findings can be regarded as adverse. Any statistical deviation from normal is not necessarily adverse. On the other hand, any change of any neurochemical parameter may give rise to or reflects neurotoxicity. In fact, presently, in neurotoxicology there is not any agreement as to what constitutes an adverse effect.

In the regulatory setting, the lowest level of concern is when one or a few unrelated parameters are affected. Concern is increased if several related

parameters are consistently affected, as observed in **I**, **II**, **III**, and **IV**, and if the effects seem to be dose- and time dependent.

The affected rat brain neurochemistry demonstrates that toluene and aromatic white spirit are both neuroactive. It is generally accepted that changed NA, DA, and 5-HT metabolism may have effects on mood, emotional state, and to some extent on aggressive behaviour of the individual. *Although not dose-dependent and not accompanied by behavioural changes, the actual long-lasting affected NA, DA, and 5-HT metabolism are serious findings, which add to the weight of evidence for toluene and aromatic white spirit neurotoxicity in man.*

Solvent-induced chronic toxic encephalopathy is a symptom complex characterized by deficits in psychomotor, perceptual, and memory functions with associated disturbances in the mood. Theoretically, the toluene- and aromatic white spirit-affected global, regional, and synaptosomal neurotransmitter metabolism (**I**; **II**; **III**; **IV**) may explain parts of these symptoms. At the present stage of knowledge it is not possible for sure to correlate the solvent-affected neurochemical parameters found in laboratory animals to the integrated outcome in the human being or *vice versa*.

At present, the interpretation of the neurotoxicological significance of a statistically significantly changed neurochemical parameter induced by toluene or aromatic white spirit in the rat brain in terms of neurotoxicity in man will depend upon a scientific and/or political judgement. An outcome, if any, of such an interpretation can be used to protect the population against neurotoxic effects of the substance. This can be achieved by regulation, labelling, and information.

X. CONCLUSIONS

Long-term toluene and aromatic white spirit exposure studies in laboratory animals are relevant for occupational exposure settings. Long-lasting (i.e. long-term reversible or true irreversible) effects are serious findings.

No behavioural changes were found to be caused by 6 months exposure to toluene followed by a 4 months exposure-free period. At 500 ppm, the mean nuclear volume and mean perikaryonal volume in neocortex were statistically significantly increased. A statistically significantly decreased weight of hippocampus was shown after exposure to 1500 ppm (**I**). In a satellite study to study **I**, the decreased hippocampal weight was accompanied by a statistically significantly reduced number of neurons in the subdivision CA2/3 of the hippocampus when exposed to 1500 ppm (Korbo et al., 1993, 1996).
The concentrations of NA, DA, and 5-HT in various brain regions and in the whole brain were statistically significantly changed (**I**).
Generally, there were increased DA and 5-HT levels in various brain regions. Both the concentration of NA and DA changed in 4 brain regions (hypothalamus, pons, thalamus, and med. obl.). The changes were in the same direction in 3 (pons, thalamus, and med. obl.) of these 4 regions for both transmitters. These general effects were discussed on the basis of existing relevant peer reviewed literature. This gave birth to the hypothesis that *the generally toluene-increased regional 5-HT concentration is part of a compensatory mechanism acting to overcome a toluene-reduced potential for serotonergic activity*. This hypothesis needs to be tested.

After 3 weeks of exposure to aromatic white spirit, both aromatic and aliphatic components were present in the whole brain (**II**). Especially, aliphatic components accumulated indicating that a threshold capacity for the elimination of aliphatic components was exceeded. The weight of the whole brain was not affected, whereas statistically significantly increased NA, DA, and 5-HT concentrations were found in whole brain.

In the 6 months aromatic white spirit exposure study (**III**), no behavioural, macroscopic or histopathological structural changes were found.
The concentrations of NA, DA, and 5-HT in various brain regions and in whole brain were statistically significantly changed. Except for the cerebellum, increased 5-HT levels were recognized in all 7 brain regions and in the whole brain.

Synaptosomal neurochemistry was investigated as index of the *in situ* conditions in the presynaptic nerve terminal. Following 3 weeks or 6 months of exposure to aromatic white spirit, the relative and absolute yield of synaptosomal protein were statistically significantly reduced whereas the synaptosomal NA, DA, and 5-HT concentrations, high-affinity 5-HT uptake rate and -uptake capacity were statistically significantly increased (**IV**).

It is hypothesized that *aromatic white spirit-increased synaptosomal NA, DA, and 5-HT concentrations are indices of increased neurotransmitter*

concentrations in respective presynaptic nerve terminals in situ acting to overcome reduced transmission capacity caused by an aromatic white spirit-reduced number of noradrenergic, dopaminergic, and serotonergic synapses in situ, and that *aromatic white spirit-increased 5-HT uptake rate and uptake capacity in serotonergic neurons in situ cause the generally increased global and regional 5-HT concentrations.*

The use of the synaptosomal model for studying the neurotoxicity of aromatic white spirit is supported. This may be extended to other solvents as well.

There was no effect on the GFAP level in the hippocampus or hemisphere neither in young nor in 'middle-aged' animals following 3 weeks of exposure to aromatic white spirit. No long-lasting effect was found on the GFAP level in any brain region or in the whole brain following 6 months of exposure to aromatic white spirit followed by an exposure-free period of 4 months' duration. At these time points, global, regional and synaptosomal NA, DA, and 5-HT metabolism are known to be altered by aromatic white spirit (**II**; **III**; **IV**).
The GFAP results in the 3 weeks' and 6 months' exposure studies do not add evidence in favour to the proposed use of GFAP as biomarker for neurotoxicity.

The structural changes induced by toluene in neocortex and hippocampus and the changes induced by toluene and aromatic white spirit on rat brain neurochemistry demonstrate the neuroactivity of these compounds. The affected neurochemical parameters may be manifested as compromised nerve cell function and be expressed as affected performance of the individual: as neurotoxicity of the 2 organic solvents.
Although not dose-dependent and not accompanied by behavioural changes, these long-lasting effects are serious findings, which add to the weight of evidence for toluene and aromatic white spirit neurotoxicity in man. Such long-lasting effects should be taken into consideration by regulators.

Theoretically, the demonstrated affected global, regional, and synaptosomal neurotransmitter metabolism (**I**; **II**; **III**; **IV**) in the rat may explain some of the symptoms of solvent-induced chronic toxic encephalopathy induced in man. At our present stage of knowledge, however, it is not possible to extrapolate from these findings demonstrated in laboratory animals to the integrated outcome in the human being or *vice versa.*

The neurochemical findings together with the epidemiological evidence for toluene- and white spirit-induced neurotoxicity support the applicability of the working hypothesis and the rat as a laboratory animal for neurochemical studies of the mechanisms underlying/reflecting toluene- and aromatic white spirit-induced neurotoxicity in man.

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XII. APPENDICES

Appendices I (A-C) and II (A-H) summarize the statistically significant ($P<0.05$) toluene-induced effects on NA, DA, and 5-HT metabolism in the CNS. The animals were exposed *in vivo* to toluene.

The references were found by a literature survey in CD-ROM Silverplatter^{R} Medline (1/1984-12/1995) and Toxline (1/1981-9/1995) databases. The string was restricted to: (Toluene or methylbenzene, or 108-88-3) and (noradrenaline or norepinephrine or dopamine or 5-hydroxytryptamine or serotonin).

In bracket is given the age of the animals as estimated from their weight according to Harlan.

Appendix I A

Studies in which the animals were exposed for a single period shorter than or equal to 24 h.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 8 weeks old	I.p. inj. 200, 400, or 600 mg/kg b.w. Sacrificed 1 h or 17 h after inj. Inj. in the beginning of the 12 h light period	HPLC-ECD	Glowinski & Iversen, 1966	200 mg/kg b.w. a. 1 h after inj Increased 5-HT, NA, and MHPG levels in frontal cortex Increased 5-HT level in pons+med. obl. 400 mg/kg b.w.: a. 1 h after inj.: Increased 5-HT, NA and MHPG levels and decreased 5-HIAA level in frontal cortex Increased 5-HT level in pons+med. obl. b. 17 h after inj.: Increased 5-HIAA level in pons+med. obl. 600 mg/kg b.w.: b. 17 h after inj.: Increased 5-HIAA level in frontal cortex and pons+med. obl. Decreased MHPG level in frontal cortex	Arito et al., 1984
Rat Sprague-Dawley Male 220-240 g (6-7 weeks old)	Inhalation 100, 300, or 1000 ppm, 8 h Sacrificed immedia-tely after end of exposure	Spectro-photo-meter	Glowinski & Iversen, 1966	1000 ppm: Increased DA levels in whole brain and corpus striatum Increased NA levels in med. obl. and midbrain Increased 5-HT levels in cerebellum, med. obl., and corpus striatum	Rea et al., 1984

Appendix I A (Single-exposure, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat	Inhalation 1500 or 10000 ppm, 30 min		Glowinski & Iversen, 1966	1500 ppm: Decreased NA level in dorsal part of pons Decreased DA levels in hypothalamus and ventral part of midbrain 10000 ppm: Decreased NA level in dorsal part of pons Decreased DA levels in hypothalamus and ventral part of midbrain. Increased DA levels in hippocampus and cerebellum	Kiriu et al., 1990
Rat Sprague-Dawley Male 4 weeks old	Gavage 1250 mg/kg b.w.	HPLC separation, fraction collection, derivatization, and fluorimetry	Glowinski & Iversen, 1966	1250 mg/kg b.w.: Decreased DA and 5-HT in midbrain. Increased DOPAC in hypothalamus. Decreased HVA in med. obl.	Kanada et al., 1994
Rat Sprague-Dawley Male 290-310 g (9 weeks old)	Inhalation 500, 1000, or 2000 ppm, 2 h	Microdialysis, HPLC-ECD	Probe implanted into corpus striatum	1000 ppm: Increased extracellular DA level in corpus striatum within 30 min of exposure persisting 2 h after end of exposure 2000 ppm: Increased extracellular DA level in corpus striatum within 30 min of exposure persisting 2 h after end of exposure	Stengård et al., 1994
Rat Wistar Male 200-300 g (7-9 weeks old)	I.p. inj. 80, 250, or 800 mg/kg b.w.	Microdialysis, HPLC-ECD	Probe implanted into corpus striatum	No effect on the extracellular concentration of DA, DOPAC, HVA, or 5-HIAA in corpus striatum 0-240 min after inj.	Kondo et al., 1995

Appendix I B

Short-term exposure a.

Studies in which the animals were exposed for more than 24 h but for no more than 7 days.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male	Inhalation **1.** 500 ppm, 6 h/day for 3 days Sacrificed 16-18 h after end of exposure **2.** 1000 ppm, 6 h/day for 5 days Sacrificed 4 h after end of exposure	Quantit-ative microfluo-rimetry	Measured on slides for microsco-py	500 ppm: Increased CA level in LPZ in hypothalamus 1000 ppm: Increased CA level in SEL in hypothalamus Increased CA depletions in SEL, MPZ, PV I, PAFP, and PAFM in hypothalamus	Andersson et al., 1980

Appendix I B (Short-term exposure a. continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 250 g (7 weeks old)	Inhalation 80, 500, 1500, or 3000 ppm, 6 h/day for 3 days Sacrificed 16-18 h after end of exposure	Quantitative microfluorimetry	Measured on slides for microscopy	80 ppm: Decreased DA levels in caud. marg. and acc. dif. in forebrain Decreased DA depletions in caud. marg., caud. med., and caud. cent. in forebrain 500 ppm: Decreased DA level in acc. dif. in forebrain Decreased DA depletions in caud. marg., caud. med., caud. cent., and acc. dif. in forebrain 1500 ppm: Increased DA level in tub. dot. in forebrain Decreased DA depletion in caud. cent. in forebrain 3000 ppm: Increased DA level in tub. dot. in forebrain Decreased DA depletion in caud. cent. Increased DA depletions in acc. dot. and tub. dot. in forebrain	Fuxe et al., 1982

Appendix I B (Short-term exposure a. continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 200-300 g (7-<12 weeks old)	Inhalation 0.7%, 15 min/day for 14 days Sacrificed 3 h after end of exposure	5-HT binding studies	Glowinski & Iversen, 1966	0.7%: Decreased B_{max} for 5-HT binding to P2 membranes from whole brain, med. obl.+pons, and hippocampus	Yamawaki et al., 1982
Rat Wistar Male 200-250 g (7-8 weeks old)	Inhalation 0.7%, 15 min/day for 14 days Sacrificed 10 min after end of exposure	HPLC		0.7%: Increased DA level in whole brain minus cerebellum	Yamawaki & Sarai, 1982
Rat Sprague-Dawley Male 250-300 g (7-9 weeks old)	Inhalation 80, 500, 1500, or 3000 ppm, 6 h/day for 3 days Sacrificed 16-18 h after end of exposure	Quantitative microfluorimetry	Measured on slides for microscopy	80, 500, 1500, and 3000 ppm: Increased CA levels in SEL, MPZ, LPZ, PV I, PV II, PAFP, and PAFM in hypothalamus 80, 500, 1500, and 3000 ppm: Increased CA depletions in SEL, MPZ, PV I, PV II, PAFP, and PAFM in hypothalamus	Andersson et al., 1983

Appendix I B (Short-term exposure a, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 150-200 g (5-6 weeks old)	Inhalation 80, or 3000 ppm, 6 h/day for 3 days Sacrificed 16-18 h after end of exposure Exposed in the light period	Spiperone and 5-HT binding studies		3000 ppm: Increased K_d's for spiperone association to striatal membranes and for 5-HT to dorsal cortical membranes	Celani et al., 1983
Rabbit New- Zealand Male Adult	Inhalation 750 ppm, 12 h for 7 days Sacrificed 12 h after end of exposure	HPLC-ECD	Mutti et al., 1985	No effect of toluene on striatal or tuberoinfundibular NA, DA, or HVA levels	Romanelli et al., 1986
Rabbit New Zealand Male Adult	Inhalation 750 ppm, 12 h/day for 7 days Sacrificed 12 h after end of exposure	HPLC-ECD	Romaneli et al., 1986	No effects	Mutti et al., 1988

Appendix I B (Short-term exposure a. continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male Postnatal exposure Some postnatally exposed animals were also exposed at 8 weeks of age	Postnatal inhalation 80 ppm 6 h/day, day 1-7 postpartum Some rats additionally exposed for 3 days week 8 post partum Sacrificed 16-18 h after end of exposure Exposure in the light period	Forebrain and hypothalamus: Quantitative microfluorimetry Substantia nigra: HPLC-ECD	Measured on slides for microscopy Andersson & Eneroth, 1987	80 ppm: Increased DA level and decreased DA depletion in tub. diff. in forebrain Increased CA depletions in SEL and PAFM in hypothalamus Decreased NA level and decreased NA and DA depletions in substantia nigra Postnatal exposure day 1-7 affected the effects of subacute exposure in week 8 on CA levels and on CA depletions in forebrain, hypothalamus, and substantia nigra	von Euler et al., 1989a

Appendix I C

Short-term exposure b.

Studies in which the animals were exposed for more than 7 days but for no more than 30 days.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 8 weeks old	Inhalation 200, 400, or 800 ppm, 24 h/day for 30 days Exposed 12 of 24 h in the light period Sacrificed immedia-tely after end of exposure	HPLC separation, fraction collection derevatiza-tion and fluorimet-ry	Glowinski & Iversen, 1966	400 ppm: Decreased NA level in hypothalamus	Honma et al., 1983
Rat Sprague-Dawley Male 8 weeks old	I.p. inj. 100, or 200 mg/kg b.w./day for 14 days Sacrificed 6 or 18 h after last inj. Inj. in the beginning of the 12 h light period	HPLC-ECD	Glowinski & Iversen, 1966	200 mg/kg b.w.: a. 6 h after last inj.: Decreased 5-HT levels in frontal cortex, hippocampus, and midbrain Decreased 5-HIAA levels in midbrain and hypothalamus Increased MHPG level in hypothalamus b. 18 h after last inj.: Decreased 5-HT levels in frontal cortex, hippocampus, and midbrain Decreased 5-HIAA levels in midbrain and hypothalamus Increased MHPG level in hypothalamus Increased DOPAC and HVA levels in corpus striatum Increased NA level in hypothalamus	Arito et al., 1985

Appendix I C (Short-term exposure b. continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 230 g (8 weeks old)	Inhalation 200, or 400 ppm, 24 h/day for 30 days Sacrificed immedia-tely after end of exposure	HPLC-FD	Glowinski & Iversen, 1966	400 ppm: Increased NA level in ventral cortex Decreased NA level in olfactory cortex Increased DA level in olfactory cortex Decreased DA level in corpus striatum Decreased NA level in hypothalamus	Ikeda et al., 1986
Rat Sprague-Dawley Male 250 g (7 weeks old)	Inhalation 80 ppm, 6 h/day for 2 weeks Sacrificed 16-18 h after end of exposure Exposed in the light period	Quantita-tive microfluo-rimetry	Measured on slides for microsco-py	80 ppm: Increased CA depletions in PAFP and PAFM of hypothalamus Increased depletion of DA in anteromedial frontal cortex	von Euler et al., 1988 a

Appendix I C (Short-term exposure b, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Mouse CD-1 Male	Drinking water Concentrations 20, 100, or 500 mg/l 28 days	HPLC-ECD	Glowinski & Iversen, 1966	20 mg/l: Increased NA, DA, and DOPAC levels in hypothalamus Increased NA and 5-HT levels in midbrain 100 mg/l: Increased NA, DA, DOPAC, 5-HT, and 5-HIAA levels in hypothalamus Increased NA, 5-HT, VMA, 5-HIAA, and DOPAC levels in med. obl. Increased VMA, DA, and 5-HT levels in corpus striatum Increased HVA level in cerebellum Increased NA, VMA, and 5-HT levels in midbrain Increased 5-HT level in cerebral cortex 500 mg/l: Increased NA level in hypothalamus Increased 5-HT level in med. obl. Increased VMA, DA, HVA, and 5-HT levels in corpus striatum Increased NA, VMA, and 5-HT levels in midbrain Increased VMA in cerebral cortex	Hsieh et al., 1990b

Appendix I C (Short-term exposure b, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Mouse CD-1 Male 6 week old	Drinking water Concentrations 100, or 400 mg/l for 4 weeks	HPLC-ECD	Glowinski & Iversen, 1966	100 mg/l: Increased NA and DA levels in hypothalamus Increased NA, VMA, and HVA levels in corpus striatum Increased NA, 5-HIAA, and HVA levels in med. obl. Increased DA, HVA, DOPAC, and 5-HIAA levels in cerebral cortex Increased 5-HIAA level in midbrain 400 mg/ml: Increased NA, 5-HT, and 5-HIAA levels in hypothalamus Increased 5-HT level in corpus striatum Increased NA and VMA levels in med. obl. Increased DA and 5-HT levels in cerebral cortex Increased 5-HT level in midbrain	Hsieh et al., 1990a
Mouse CD-1 Male 6 weeks	Drinking water Concentrations 20, 100, or 500 mg/l for 28 days	HPLC-ECD	Glowinski & Iversen, 1966;	20 mg/l: Increased NA level in hypothalamus 100 mg/l: Increased NA level in hypothalamus 500 mg/l: Increased NA level in hypothalamus	Hsieh et al., 1991
Rat	Inhalation 30000 to 40000 ppm, 15 min/day for 30 days	HPLC-ECD		30000 to 40000 ppm: No effect on levels of CA's and indolamines in any examined part Increased K_d for 5-HT association to crude synaptic membranes	Castilla et al., 1993

Appendix I D

Long-term exposure.

Studies in which the animals were exposed for more than 30 days.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Female Young adult	Inhalation 1000 ppm, 21 h/day, 7 days/week for 9 weeks 12 of 21 h exposure was in the dark period	HPLC-ECD	Olson et al., 1983	1000 ppm: Increased NA and MHPG levels in cerebellar in oculo transplants Increased MHPG level in host cerebellum	Granholm et al., 1988
Rat Sprague-Dawley Male 12 months old	Inhalation 80 ppm, 6 h/day, 5 days/week for 3 months Sacrificed 16-18 h after end of exposure Exposed in the light period	Quantitative microfluorimetry Ligand bindings	Measured on slides for microscopy	80 ppm: Decreased CA level in PAFP of hypothalamus Affected VIP, neurotensin, etorphin and NPY bindings to many different discrete brain areas	von Euler et al., 1988 b

Appendix I D (Long-term exposure, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Decreased NA levels in hippocampus, hypothalamus, med. obl. Increased NA level in thalamus Increased DA levels in hemisphere, pons, thalamus, and whole brain Decreased DA level in med. obl. Decreased 5-HT level in hypothalamus 1500 ppm: Decreased NA level in hippocampus Increased NA level in pons Increased DA levels in hemisphere, hypothalamus, and whole brain Increased 5-HT levels in hemisphere, hippocampus, pons, and whole brain Decreased 5-HT level in hypothalamus	Ladefoged et al., 1991 **(I)**

Appendix II A

Cerebellum.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 220-240 g (6-7 weeks old)	*Single-exposure:* Inhalation 100, 300, or 1000 ppm, 8 h. Sacrificed immediately after end of exposure	Spectro-photo-meter	Glowinski & Iversen, 1966	1000 ppm: Increased 5-HT level	Rea et al., 1984
Rat	Inhalation 1500 or 10000 ppm, 30 min		Glowinski & Iversen, 1966	10000 ppm: Increased DA level	Kiriu et al., 1990

Appendix II A (Cerebellum, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Female Young adult	*Long-term exposure:* Inhalation 1000 ppm, 21 h/day, 7 days/week for 9 weeks 12 of 21 h exposure was in the dark period	HPLC-ECD	Olson et al., 1983	1000 ppm: Increased NA and MHPG levels in cerebellar in oculo transplants Increased MHPG level in host cerebellum	Granholm et al., 1988
Rat Wistar Male 3 months old at the beginning of exposure	Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: No effects 1500 ppm: No effects	Ladefoged et al., 1991 (I)

Appendix II B

Hemisphere.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Increased DA level 1500 ppm: Increased DA level Increased 5-HT level	Ladefoged et al., 1991 (I)

Appendix II C

Hippocampus.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat	*Single-exposure:* Inhalation 1500 or 10000 ppm, 30 min		Glowinski & Iversen, 1966	10000 ppm: Increased DA level	Kiriu et al., 1990
Rat Wistar Male 200-300 g (7-<12 weeks old)	*Short-term exposure a:* Inhalation 0.7%, 15 min/day for 14 days Sacrificed 3 h after end of exposure	5-HT binding studies	Glowinski & Iversen, 1966	Decreased B_{max} for 5-HT binding to hippocampal P2 membranes	Yamawaki et al., 1982
Rat Sprague-Dawley Male 8 weeks old	*Short-term exposure b:* I.p. inj. 100, or 200 mg/kg b.w./day for 14 days Sacrificed 6 or 18 h after last inj. Inj. in the beginning of the 12 h light period	HPLC-ECD	Glowinski & Iversen, 1966	200 mg/kg b.w.: a. 6 h after last inj.: Decreased 5-HT level b. 18 h after last inj.: Decreased 5-HT level	Arito et al., 1985

Appendix II C (Hippocampus, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Decreased NA level 1500 ppm: Decreased NA level Increased 5-HT level	Ladefoged et al., 1991 (I)

Appendix II D

Hypothalamus.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat	*Single-exposure:* Inhalation 1500 or 10000 ppm, 30 min		Glowinski & Iversen, 1966	1500 ppm: Decreased DA level 10000 ppm: Decreased DA level	Kiriu et al., 1990
Rat Sprague-Dawley Male 8 weeks old	*Short-term exposure b:* Inhalation 200, 400, or 800 ppm, 24 h/day for 30 days Exposed 12 h of 24 in the light period Sacrificed immediately after end of exposure	HPLC separation, fraction collection derivative formation and fluorimetry	Glowinski & Iversen, 1966	400 ppm: Decreased NA level	Honma et al., 1983

Appendix II D (Hypothalamus, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male weeks old	*Short-term exposure b:* I.p. inj. 100, or 200 mg/kg b.w./day for 14 days Sacrificed 6 or 18 h after last inj. Inj. in the beginning of the 12 h light period	HPLC-ECD	Glowinski & Iversen, 1966	200 mg/kg b.w.: a. 6 h after last inj.: Decreased 5-HIAA level Increased MHPG level b. 18 h after last inj.: Decreased 5-HIAA level Increased MHPG level Increased NA level	Arito et al., 1985
Rat Wistar Male 230 g (8 weeks old)	Inhalation 200, or 400 ppm, 24 h/day for 30 days Sacrificed immedia-tely after end of exposure	HPLC-FD	Glowinski & Iversen, 1966	400 ppm: Decreased NA level	Ikeda et al., 1986

Appendix II D (Hypothalamus, continued).

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Decreased NA level Decreased 5-HT level 1500 ppm: Increased DA level Decreased 5-HT level	Ladefoged et al., 1991 (**I**)

Appendix II E

Pons.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Increased DA level 1500 ppm: Increased NA level Increased 5-HT level	Ladefoged et al., 1991 (**I**)

Appendix II F

Thalamus.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communi-cation	500 ppm: Increased NA level Increased DA level	Ladefoged et al., 1991 (**I**)

Appendix II G

Med. obl.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 220-240 g (6-7 weeks old)	*Single-exposure:* Inhalation 100, 300, or 1000 ppm, 8 h Sacrificed immediately after end of exposure	Spectrophotometer	Glowinski & Iversen, 1966	1000 ppm: Increased NA level Increased 5-HT levels	Rea et al., 1984
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communication	500 ppm: Decreased NA level Decreased DA level	Ladefoged et al., 1991 **(I)**

Appendix II H

Whole brain.

Species Strain Sex Age at sacrifice	Toluene exposure	Amine analysis	Dissection procedure	Effects	Reference Remarks
Rat Sprague-Dawley Male 220-240 g (6-7 weeks old)	*Single-exposure:* Inhalation 100, 300, or 1000 ppm, 8 h. Sacrificed immediately after end of exposure	Spectrophotometer	Glowinski & Iversen, 1966	1000 ppm: Increased DA level	Rea et al., 1984
Rat	*Short-term exposure a:* Inhalation 0.7%, 15 min/day for 14 days Sacrificed 3 h after end of exposure		Glowinski & Iversen, 1966	Decreased B_{max} for 5-HT to P2 membranes from whole brain	Yamawaki et al., 1982
Rat Wistar Male 3 months old at the beginning of exposure	*Long-term exposure:* Inhalation 500, or 1500 ppm, 6 h/day 5 days/week for 6 months Sacrificed 4 months after end of exposure Exposed in the dark period	HPLC-ECD	Glowinski & Iversen, 1966; Edelfors, 1975; Edelfors, personal communication	500 ppm: Increased DA level 1500 ppm: Increased DA level Increased 5-HT level	Ladefoged et al., 1991 **(I)**

Appendix III A

Contents of aliphatic and cyclic alkanes in North European aromatic white spirit[a]

Number of C-atoms	Alkanes, w/w%	Monocyclic alkanes, w/w%	Dicyclic alkanes, w/w%
C_6	-	0.01	-
C_7	0.10	0.17	-
C_8	0.88	1.4	-
C_9	10	8.7	1.7
C_{10}	17	11	3.5
C_{11}	8.4	3.8	3.2
C_{12}	0.58	0.65	0.46
C_6-C_{12}	37	26	8.9
C_6-C_{12} in total	Alkanes, total 72% specified (+ 12% unspecified)		

a) Varnolene[R] (boiling range: 162-198°C), aromatic white spirit from the Danish market (Henriksen, 1980)

Adapted from IPCS, 1995.

Appendix III B

Contents of aromatic components of North European aromatic white spirit[a].

Number of C-atoms	Substance	w/w%	
C_6			
	benzene	0.001	
C_7			
	toluene	0.005	
C_8			
	ethylbenzene	0.2	
	o-xylene	0.34	
	m-xylene	0.49	
	p-xylene	0.22	
C_8-aromatic hydrocarbons, total			*1.3*
C_9			
	n-propylbenzene	0.97	
	isopropylbenzne (cumene)	0.21	
	1-methyl-2-ethylbenzene	0.60	
	1-methyl-3-ethylbenzene	1.2	
	1-methyl-4-ethylbenzene	0.66	
	1,2,3-trimethylbenzene	0.62	
	1,2,4-trimethylbenzene	2.1	
	1,3,5-trimethylbenzene	0.83	
	trans-1-propenylbenzene	0.40	
C_9-aromatic hydrocarbons, total			*7.6*
C_{10}			
	n-butylbenzene	0.97	
	isobutylbenzene	0.37	
	1-methyl-2-isopropylbenzene	0.06	
	1-methyl-3-isopropylbenzene	0.47	
	1-methyl-4-isopropylbenzene	0.62	
	1,2-diethylbenzene	0.13	
	1,3-diethylbenzene	0.25	
	1,4-diethylbenzene	0.13	
	1,2-dimethyl-3-ethylbenzene	0.08	
	1,2-dimethyl-4-ethylbenzene	0.25	
	1,3-dimethyl-4-ethylbenzene	0.26	
	1,3-dimethyl-5-ethylbenzene	0.38	
	1,4-dimethyl-2-ethylbenzene	0.28	
	1,2,3,4-tetramethylbenzene	0.16	
	1,2,3,5-tetramethylbenzene	0.14	
	1,2,4,5-tetramethylbenzene	0.34	
	tetralin	0.08	
C_{10}-aromatic hydrocarbons, total			*5.2*

C_{11}	
C_{11}-aromatic hydrocarbons, total	*1.2*
C_{12}	
C_{12}-aromatic hydrocarbons, total	*0.12*
C_6-C_{12}	
aromatic hydrocarbons, total	**15.4**

a) Varnolene[R] (boiling range: 162-198 °C), aromatic white spirit from the Danish market (Henriksen, 1980).

Adapted from IPCS, 1995.

Reference I

Pharmacology & Toxicology 1991, **68**, 384–390.

Irreversible Effects in Rats of Toluene (Inhalation) Exposure for Six Months

Ole Ladefoged[1], Poul Strange[2], Arne Møller[2], Henrik R. Lam[1]*, Grete Østergaard[1], Jens-Jørgen Larsen[1] and Peter Arlien-Søborg[2]

[1]Institute of Toxicology, National Food Agency, Søborg, and [2]Neurological Research Laboratory, Hvidovre University Hospital, Copenhagen, Denmark

(Received November 1, 1990; Accepted January 16, 1991)

Abstract: The irreversible CNS effects of six months' exposure to toluene (0, 500, and 1500 p.p.m.) in rats was studied applying a multi-disciplinary approach. After an exposure-free period, neurobehavioural, morphometric, pathological, and biochemical examinations were performed. No neurobehavioural or gross pathological changes were found. Morphometric measurements did not show loss of neurones. At 500 p.p.m. the mean nuclear volume and mean perikaryonal volume and the variation of the values of these parameters was increased in the exposed groups compared to the controls. Noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) levels were significantly changed in various brain regions. It is concluded that this investigation failed to reveal overt toluene-induced CNS-neurotoxicity, however, certain irreversible effects were found which further add to the accumulating evidence of the chronic CNS-neurotoxicity of toluene.

The neurotoxicity of toluene in humans has been discussed for many years. The acute effect is described under controlled conditions in exposure chambers with neuropsychiatric symptoms at 100 p.p.m. (Andersen *et al.* 1983), impairment of visual vigilance (Dick *et al.* 1984) and vestibular function (Hydén *et al.* 1983). At exposure levels at 150–300 p.p.m. a number of cognitive functions are disturbed (Gamberale & Hultengren 1972; Ogata *et al.* 1970; Echeverria *et al.* 1989). Epidemiological mainly cross-sectional studies have demonstrated acute subacute symptoms (Larsen & Leira 1988; Ørbæk & Nise 1989)) and impairment in neuropsychological functions (Matsushita *et al.* 1975; Elofsson *et al.* 1979; Iregren 1982). Some of these findings may, however, reflect the acute or subchronic narcotic effects caused by repeated exposure. A number of studies failed to disclose significant impairment in exposed groups compared to controls (Cherry *et al.* 1985; Juntunen *et al.* 1985). Several case stories describe a consistent pattern of neurological damage in individuals repeatedly inhaling toluene at very high concentrations because of its euphoric properties (WHO 1985; Spencer & Schaumburg 1985; Rosenberg *et al.* 1988; Fornazzari *et al.* 1983).

Groups of workers, occupationally exposed to toluene over a long period of time show evidence of neurological and psychological deficits reported as chronic toxic encephalopathy reflecting a basic change in personality developing gradually, and affecting energy, intellect, emotional life, and motivation. These findings are in accordance with those described and for which diagnostic criteria have been proposed in a report published by the WHO Nordic Council of Ministers in 1985.

In epidemiological cross-sectional studies the interpretation of results is confounded by the uncertainty of the nature of exposure, e.g. duration, levels of exposure, co-exposure with other neurotoxicants, and the differentiation between acute narcotic effects and true irreversible neurotoxicity. Hence, the experimental investigation in laboratory animal models is a necessary approach to clarify dose-response correlations, reversibility or irreversibility of neurotoxic effect, and mechanisms of solvent neurotoxicity.

Recommendations for future investigations of chronic toxic encephalopathy in an animal model include neurobehavioural, neurophysiological and neuropathological investigations complemented by biochemical studies (Proceedings of the Workshop on Neurobehavioral Effects of Solvents 1986). The acute effects of toluene have been thoroughly studied in mice and rats while the chronic effects are less well investigated.

The present study, taking advantage of a multi-disciplinary approach including neurobehavioural, morphometric, pathological, and biochemical parameters was undertaken to investigate the potential irreversible CNS-neurotoxic effects induced by toluene in rats. Specifically, care was taken to insert an exposure-free period of sufficiently long duration to ensure that all reversible effects had disappeared prior to data collection. The importance of this strategy in solvent investigations has only been appreciated by a few investigators (Haglid *et al.* 1981; Pryor *et al.* 1983; Naalsund 1986).

Materials and Methods

Chemicals. Animals were exposed to toluene purchased from E. Merck, GmbH, FRG. Prior to exposure the toluene was found to be >99.5% pure by gas liquid chromatography. All other chemicals used were of analytical grade.

Animals. One hundred and eight male rats (MOL:Wist), mean body weight 325 g, three months old, obtained from Møllegaard Breeding

* To whom correspondance should be directed.

Center Ltd., DK-4623 Ll. Skensved, Denmark, were used. They were housed in stainless steelwire cages, two animals/cage, conventionally in animals rooms with automatic control of temperature (22 ± 1°), relative humidity 55 ± 5%), air exchange (8 times/hr), and fluorescent light (9 p.m.–9 a.m.), with access to commercial pelleted diet (Altromin 1324, Brogården, Gentofte, Denmark) and acidified tap water in nipple bottles. During daily exposure the feed was removed. The animals were weighed once a week during the study.

Exposure. The animals were randomized into three groups each of 36 animals. One group served as control, a second group was exposed to 500 p.p.m. and a third to 1500 p.p.m. of toluene in the inhaled air for 6 hr/day, 5 days/week for 6 months followed by an exposure-free period of two months' duration prior to neurobehavioural data collection. The animals were killed four months after the end of exposure. The inhalation exposure conditions and the equipment used have previously been described by Ladefoged *et al.* (1990).

Behavioural tests. Motor activity was measured regularly during the entire trial period. Behavioural testing in passive avoidance test, Morris maze, and radial maze was conducted after the two-month exposure-free period.

Motor activity. The motor activity of ten pairs of rats from each group was recorded monthly before, during, and after exposure. The device consisted of an animal rack equipped with three horizontally placed photocells per cage for ambulation measurement, interfaced with an IBM XT computer. Each recording was made from Friday afternoon to Monday morning. Exploratory activity was measured in 6 periods of 10 min. each, after which the activity during several light-dark cycles was measured in 62 periods of 60 min.

Passive avoidance. Six pairs of rats from each group were tested as described by Walsh *et al.* (1984). The rats were placed in the illuminated compartment of a one-way shuttle box. After 60 sec. the guillotine door was opened. The rat's latency time (maximum 180 sec.) to enter the dark compartment was recorded. After the rat had entered the dark compartment the guillotine door was lowered and the rat given a foot shock in the range of 0.5–0.8 mA for 1.0 sec. The following day the retention of the passive avoidance response was tested.

Morris maze. Six pairs of rats from each treatment group were tested as described by Morris (1984) with minor modifications. The circular black swimming pool had a diameter of 220 cm and was filled with tap water of room temperature. The positions of the white rat on the black background was monitored and recorded by an overhead video camera connected to a computer with an image-analyzing program. The image-analyzing program delivered the following parameters: a) Escape latency (sec. from the trial was started until the rat reached the platform), b) route length, c) time spent in each pool quadrant, and d) procentual time spent in each quadrant.

The testing consisted of cued escape training, first place navigation task, transfer test, and second place navigation task. During cued escape training the rats were trained with the visible platform placed in the center of the NW quadrant. The rats were trained in blocks of four rats. Each block of rats were trained in four trials each day, using the four different start positions in a new random order every day. If a rat did not locate the platform within 60 sec., it was removed from the water and placed on the platform for 10 sec. Training of place navigation with the invisible platform continued in a similar fashion until a stable performance was established. In the transfer test, the platform was removed from the pool. The rats were then allowed 60 sec. of free swimming in a single trial. For the second place navigation task the platform location was changed to the SE quadrant, below the water surface.

Eight-arm radial maze. Six pairs of rats from each group were tested as described by Olton & Samuelson (1976) with minor modifications. The uncovered maze was made of transparent plastic. The rat's movements were registered by an IBM XT computer via signals from photocells placed at the entrance and at the end of each arm. A video camera mounted above the maze made monitoring possible. The maze was placed in a dark room where the only illumination came from a red lamp. Food-deprived rats were trained once every day, five days a week, for three weeks, with a cut-off time of 10 min. The maze was baited with chopped peanut. The number of correct choices in the first eight arm entries, the total number of visits required to obtain all eight rewards, and the time spent in the maze were recorded.

Morphometric measurements.

Macroscopic sampling and histological procedures. From each rat one hemisphere, chosen randomly between left and right, was embedded in agar and sliced coronally in 1.5 mm slices. Tissue rostrally to the rhinal fissure was cut away. Neocortical volume estimation was performed according to the Cavalieri principle. The slices were point-counted successively in a stereomicroscope (magnification × 10) using a square point grid spaced 1 × 1 mm printed on transparent plastic. In order to produce vertical sections the slices were cut into rods perpendicular to the pial surface. With the order preserved and starting randomly at the anterior slice every third rod of the approximately 25 were sampled. These rods were rotated systematically randomly along their longitudinal axis, aligned in adjacent grooves in an aluminum block and embedded in 3.5% agar. After cooling the agar rods were embedded in glycolmethacrylate (Historesin) and 35 μm thick sections were cut and stained i Wolbachs Giemsa dye for 20 min. For further details see Møller *et al.* (1990) and Strange *et al.* (1991).

Shrinkage. For estimation of shrinkage one slice from two animals in each group was examined before and after histological processing. The neocortical area of the cut surface facing frontally was estimated as described above for Cavalieri. A smaller-sized grid, though, was used to enhance precision. To obtain the linear shrinkage from the estimated shrinkage of area the square root was calculated.

Estimation of cell number. In the neocortical neurones of rats only one and always one nucleus is present. Thus the nucleus was chosen as sampling unit. With the neocortical tissue projected (magnification × 1620) on to a table with a printed counting frame, the fields of vision for measurements were sampled criss-crossing systematically random through the specimen. Countings were performed using the optical disector. In the optical disector advantage is taken from the fact that a microscope focuses on one plane. Using this method a three-dimensional probe for measurements is obtainable in only one section.

In each sampled field of vision, the disector is a box limited by the four sides of the counting frame on the table and two optical planes separated by a measurable distance (10 μm in this study) in the third dimension.

In each systematically chosen disector focus was initially put at the absolute top of the specimen, this being the surface of the section. The focus was then move 5 μm down from the surface to get away from the artificial surface. The disector was defined as the box limited by the area of the counting frame in this plane and a plane 10 μm further down into the tissue. Analogous to the "forbidden lines" in the conventional disector three of the six sides in the box are "forbidden". Moving the plane of focus down through the box all neurones having whole or part of their nucleus inside the counting frame and not touching the "forbidden lines" were sampled Q^-. For further details see Gundersen *et al.* (1988a & b).

Estimation of volumen. For estimation of volumen the nucleator was used. After counting Q^- neurones a l^3-ruler was applied with the center in the nucleolus of all Q^- neurones successively, measuring intercepts with both nucleus and cell membrane. The Q^- neurones are estimated from the number distribution thus the size estimations are also in the number distribution.

Pathological examinations. Twelve animals from each group were randomly selected for a thorough autopsy and the following organs were excised and weighed: liver, kidneys, adrenals, heart, spleen,

testis and brain. Samples from these organs were fixed in 10% buffered formalin, prepared for light microscopy and stained with haematoxylin-eosin. Samples from N. ischiadicus were stained with osmium-tetraoxide. Brains were not fixed but used for measuring biochemical parameters.

Biochemical analysis.

Sample preparation. Brains from twelve animals of each group were dissected into seven regions: cerebellum, hemisphere, hippocampus, hypothalamus, pons, thalamus and medulla oblongata according to the method described by Glowinsky & Iversen (1966) and Edelfors (1975, and personal communication). The samples were immediately transferred to icecold 0.32 M sucrose, weighed and thoroughly homogenized by an Ultra-Turrax T-25 at full speed using an ice-cooling jacket. Immediately after homogenization an aliquot of homogenate was deproteinized by the addition of icecold 0.2 M perchloric acid (1 + 1) and subsequent centrifugation. Supernatants were used directly for the determination of 5-hydroxytryptamine (5-HT) after addition of ω-N-methyl-5-HT as internal standard. Another aliquot of supernatant was added dihydroxy-benzylamine (DHBA) as internal standard for catecholamine determination carried out after aluminumoxide purification (Anton & Sayre 1962).

High pressure liquid chromatography – Electrochemical detection (HPLC-ED) analysis of biogenic amines. 75 µl perchloric acid extract was injected for 5-HT and ω-N-methyl-5-HT analysis, 75 µl aluminumoxide eluate for catecholamine analysis.

The instrumentation consisted of a Hewlett-Packard 1084B liquid chromatograph equipped with a 250 × 4 mm I.D. RP-18 Highbar, Supersphere LiChroCART (5 µm) analytical column protected by a 20 × 4 mm I.D. (5 µm) RP-18 guard column (both from E. Merck GmbH, FRG). Separation was achieved by use of an acetonitrile modified citrate/octenylsulfate buffer as described by Lin & Blank (1983). 5-HT, ω-N-methyl-5HT, noradrenaline (NA), DHBA and dopamine (DA) were detected by a Waters M 460 electrochemical detector applying an oxidation potential of +0.6 V.

Statistical analysis. Generally, groups of data were controlled for normal distribution and compared using two-sided, non-paired Student's t-test. The statistical analyses used on behavioural data are reported together with the results. Differences between groups are generally considered statistically significant at $P < 0.05$.

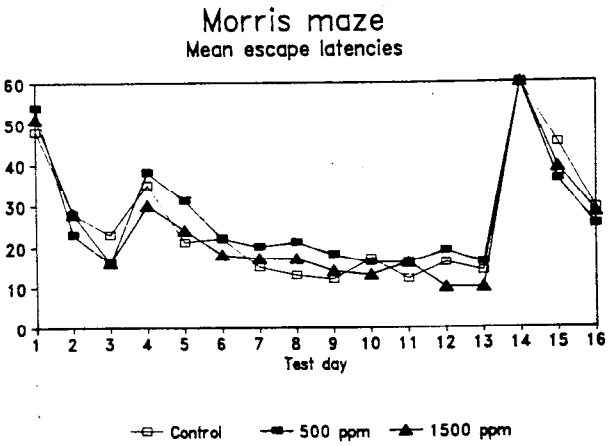

Fig. 2. Morris maze – mean escape latencies. The chronic effect of toluene exposure on behaviour in the Morris maze. Daily results are shown as means of trial blocks. Day 1–3: Cued escape training. Day 4–13: First place navigation task. Day 14: Transfer test. Day 15–16: Second place navigation task.

Results

Body weight. At the end of the study, the mean body weights had increased to 525 g. No differences were found between the three groups.

Behavioural tests.

Motor activity. The total activity counts are shown in fig. 1. The pattern of activity was the same in all groups. A general decline in activity was found throughout the 9-month observation period, with one (unexplained) peak occurring in weeke 10. The data (not shown) from individual week-ends divided into 'first hour' and 'remaining week-end' did not show consistent or dose-related differences in exploratory activity or in diurnal activity ($P > 0.05$, Student's t-test).

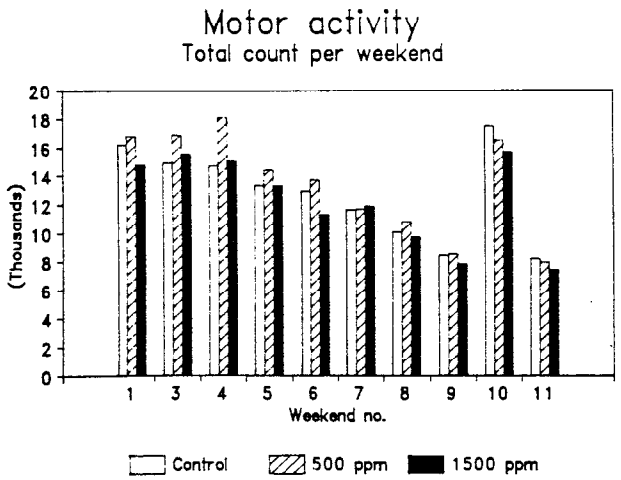

Fig. 1. Motor activity – total count per week-end. The effect of toluene exposure on motor activity. Total counts collected in the course of one week-end every month are shown. Data from week-end 2 were lost. The exposure period includes week-end 2–6.

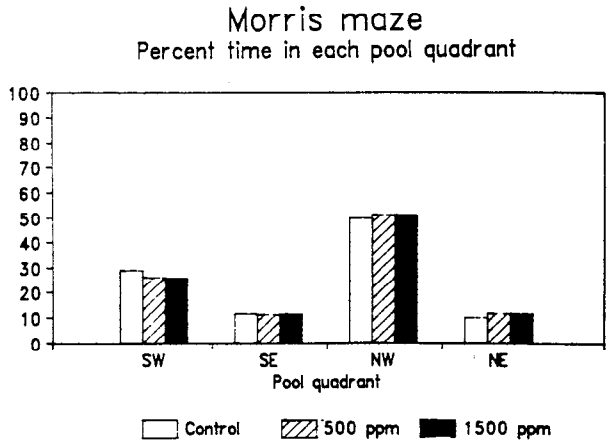

Fig. 3. Morris maze – percent time in each pool quadrant. The chronic effect of toluene exposure on spatial bias in the transfer test. The percentage of swimming time in each pool quadrant during one trial with no platform present is shown. The previous position of the platform was NW.

Passive avoidance. No differences were found between treated animals and controls ($P > 0.05$, Wilcoxon rank sum test, data not shown).

Morris maze. In the course of cued escape training all groups performed equally well showing unaffected motor function and motivation (fig. 2). In the first place navigation task an increase in escape latency of short duration was seen. In the transfer test (fig. 3) the percentage time spent in the NW quadrant was 50% in all treatment groups, which shows that the degree of learned spatial bias was not affected by treatment. In the second place navigation task the changing of platform position resulted in an increased escape latency which rapidly declined in the remaining trials.

Radial maze. The number of correct choices increased during the test period, with a simultaneous decrease in time required to perform the task ($P < 0.05$, Wilcoxon rank sum test). The treated groups did not differ from the controls (fig. 4).

Morphometric measurements.

The results are shown in table 1. The neocortical volume and the number of neurones were identical in exposed and non-exposed animals. The mean nuclear volume and the mean perikaryonal volume were significantly increased in the 500 p.p.m. group. The standard deviation on these parameters in both exposed groups were 2–3 times enlarged compared to the control values.

Pathological examinations.

No macroscopic pathological findings which could be attributed to the dosing with toluene were found. No dose dependent pathological changes were found. Relative weights of organs were calculated (weight of organ in g/ total b.wt. in g) and are shown in table 2. Statistically significant increases were found in spleen (500 p.p.m.), brain (500 p.p.m.), and kidney (1500 p.p.m.).

A toluene level dependent decrease in the weight of hippocampus was found, it was statistically significant at the 1500 p.p.m. level. The obtained weights were 0.1046 ± 0.0198 (control), 0.0970 ± 0.0163 (500 p.p.m.), and 0.0845 ± 0.0194 (1500 p.p.m.) as the mean $\pm$ S.D., $n = 12$.

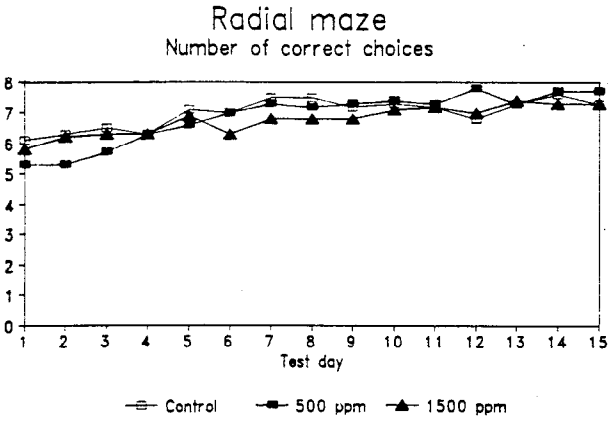

Fig. 4. Radial maze – number of correct choices. The chronic effect of toluene exposure on behaviour in the 8-arm radial maze. The number of correct choices made in one daily trial during three weeks is shown.

Table 1.

Irreversible effects on morphometric parameters in rat neocortex after six months of toluene exposure.

	Control	500 p.p.m.	1500 p.p.m.
Neocortical volume (mm^3)	242 ± 16	244 ± 25	233 ± 20
Number of neurones (10^6)	22.6 ± 2.8	20.5 ± 3.2	22.0 ± 4.8
Mean nuclear volume (μm^3)	297 ± 23	338 ± 58*	314 ± 66
Mean perikaryonal volume (μm^3)	786 ± 87	897 ± 141*	823 ± 145

All values are expressed as mean $\pm$ S.D. ($n = 12$).
* $P < 0.05$ between values from exposed and control rats by Student's t-test.

Biochemical analysis.

Regional amine concentrations are presented as the mean $\pm$ S.D. and are together with the calculated whole brain concentrations given in tables 3, 4, and 5.

Owing to chronic toluene exposure the concentrations of NA, DA, and 5-HT in various brain regions were significantly irreversibly changed as given in tables 3, 4, and 5.

Discussion

An important question for the evaluation of neurotoxicity is whether induced changes implicate toxicity. Mild functional changes may or may not be indications of tissue injury. On the other hand functional tests may not be sensitive enough to reveal brain damage owing to the ability of the brain to compensate for loss of neurones. This phenomenon is well known for Parkinson's disease where a striatal DA loss of 80–90 percent may precede symptoms (Calne *et al.* 1986) and probably a great part of dopaminergic nerve cells disappear before any symptoms develop.

The behavioural studies of higher cognitive functions were chosen on the basis of the results of investigations of

Table 2.

Relative organ weights of rats exposed to toluene for six months.

	Control	500 p.p.m.	1500 p.p.m.
Liver	2.73 ± 0.18	2.68 ± 0.11	2.64 ± 0.23
Spleen	0.158 ± 0.021	0.173 ± 0.022*	0.157 ± 0.025
Testes	0.710 ± 0.101	0.754 ± 0.061	0.768 ± 0.111
Heart	0.221 ± 0.014	0.226 ± 0.012	0.228 ± 0.019
Kidney	0.437 ± 0.052	0.437 ± 0.029	0.472 ± 0.044*
Adrenals	0.0094 ± 0.0016	0.0093 ± 0.0015	0.0095 ± 0.0011
Brain	0.387 ± 0.037	0.419 ± 0.035*	0.411 ± 0.048

All values are expressed as mean $\pm$ S.D. ($n = 12$).
* $P < 0.05$ between values from exposed and control rats by Student's t-test.

Table 3.

Regional brain noradrenaline concentration (nmol/g wet weight) in rats exposed to toluene for six months.

	Control	500 p.p.m.	1500 p.p.m.
Cerebellum	1.37±0.09	1.36±0.12	1.45±0.14
Hemisphere	2.17±0.26	2.25±0.22	2.25±0.16
Hippocampus	2.28±0.19	2.06±0.16*	2.15±0.14*
Hypothalamus	3.04±0.28	2.44±0.29*	2.95±0.32†
Pons	2.44±0.23	2.45±0.21	2.89±0.38*†
Thalamus	4.16±0.18	4.33±0.28*	3.79±0.89†
Medulla obl.	2.65±0.19	2.40±0.22*	2.71±0.24†
Total brain	2.49±0.14	2.49±0.11	2.49±0.11

All values are expressed as mean±S.D. (n=12).
* $P<0.05$ between values from exposed and control rats by Student's t-test.
† $P<0.05$ between values from rats exposed to 500 and 1500 p.p.m. by Student's t-test.

Table 5.

Regional brain 5-hydroxytryptamine concentration (nmol/g wet weight) in rats exposed to toluene for six months.

	Control	500 p.p.m.	1500 p.p.m.
Cerebellum	0.34±0.11	0.34±0.02	0.36±0.03
Hemisphere	2.26±0.22	2.29±0.18	2.46±0.25*†
Hippocampus	1.35±0.15	1.38±0.19	1.51±0.15*†
Hypothalamus	4.40±0.35	3.82±0.45*	3.70±0.39*
Pons	3.68±0.28	3.77±0.20	3.96±0.28*†
Thalamus	3.76±0.31	3.83±0.36	3.85±0.29
Medulla obl.	2.66±0.27	2.66±0.24	2.72±0.17
Total brain	2.48±0.14	2.50±0.16	2.63±0.14*†

All values are expressed as mean±S.D. (n=12).
* $P<0.05$ between values from exposed and control rats by Student's t-test.
† $P<0.05$ between values from rats exposed to 500 and 1500 p.p.m. by Student's t-test.

chronic exposure to toluene and other solvents in man. The predictability of animal models for the study of these effects of solvents in man has not been clarified and it might be questioned to what extent effects on higher cortical functions in man can be studied in animals.

The extensive literature on learning and memory and light and dark cycle regulation of the level of spontaneous activity indicates that these functions are comparable between species. These functions have been studied in several laboratories with short-term exposure and test of animals during or shortly after exposure.

Long-term studies including an exposure-free period for the study of irreversible effects of toluene or other aromatic solvents demonstrating behavioural changes have not been found in the literature. The behavioural procedures used in the present study have, however, been successfully applied to rats being studied for the impact of age and to rats being exposed to drugs or well-established CNS-neurotoxicants. Thus Rapp *et al.* (1987) found a significant change due to age in the performance of rats in the Morris water maze.

Table 4.

Regional brain dopamine concentration (nmol/g wet weight) in rats exposed to toluene for six months.

	Control	500 p.p.m.	1500 p.p.m
Cerebellum	ND	ND	ND
Hemisphere	11.5±0.8	12.6±0.8*	12.6±0.8*
Hippocampus	ND	ND	ND
Hypothalamus	3.92±0.43	4.12±0.59	4.52±0.92*
Pons	0.80±0.08	0.88±0.09*	0.81±0.30
Thalamus	10.1±1.4	12.0±1.3*	10.7±3.5
Medulla obl.	0.59±0.06	0.53±0.05*	0.56±0.04
Total brain	6.80±0.47	7.62±0.38*	7.55±0.52*

All values are expressed as mean±S.D. (n=12).
* $P<0.05$ between values from exposed and control rats by Student's t-test.
† $P<0.05$ between values from rats exposed to 500 and 1500 p.p.m. by Student's t-test.
ND: Not detected.

In the study by Gallagher *et al.* (1983) learning and memory of rats trained on an eight-arm radial maze were significantly improved by opiate antagonists. Studies on the significance of the different neuronal systems e.g. the GABA system or the cholinergic system in various brain regions by use of selective lesions or specific agonistic and antagonistic test drugs have with advantage measured rat behaviour in the step-through active or passive avoidance test, eight-arm radial maze and Morris water maze (Mactutus & Tilson 1984; Munoz *et al.* 1988; Chrobak *et al.* 1989; Fisher *et al.* 1989; Gower *et al.* 1989).

The present finding of unchanged exploratory and spontaneous motor activity and unchanged performance of rats exposed during 6 months to toluene followed by a toluene-free period of 2–3 months before testing in the passive avoidance test, Morris maze and eight-arm radial maze cannot be interpreted in a unambiguous manner. Toluene might have been without effect on the studied functions with the present exposure schedule, or an effect possibly being present during and shortly after the dosing period has not been irreversible. One of the purposes of this study was to detect behavioural changes after long-term exposure as such effects have been reported in man, and consequently the exposure-free interval was included. Under these conditions the methods applied were unable to disclose any behavioural changes.

Morphological changes of neurones have been demonstrated after repeated short-lasting exposure to 50 p.p.m. (Vazques-Nin *et al.* 1980). Changes in the hippocampus and frontal cerebral cortex were seen following exposure to 500 p.p.m. 16 hr/day, 5 days/week for 12 weeks (Naalsund 1986). Low-level toluene exposure of the rat has been shown to affect the volume and cell number of the granular cell layer in the developing hippocampal region (Slomianka *et al.* 1990).

In the present study no significant loss of cerebral cortical neurones was detected. On the other hand a significant increase in perikaryal and nuclear size was found at the low concentration level and a less pronounced increase at the

highest level. The variation of the values of nuclear and perikaryal volume was increased in both groups of exposed animals compared to the controls, reflecting that some neurones had increased and others decreased in size. Such an increase in variance has also been described at light microscopic inspection of brain sections from animals exposed to other solvents such as trichloroethylene (Baker & Tichy 1953) following acute exposure. In the present study an extended exposure-free interval of 4 months apparently did not result in normalization and may thus suggest an irreversible morphological effect.

In agreement with most other long-term studies we did not observe adverse effect on liver and kidney (Toxicological Profile for Toluene 1989). The small changes seen in relative weight of organs and the histopathological findings are most probably incidental. In a previous study we have found a slight but dose-dependent reduction in relative brain weight after oral exposure to toluene as measured on formalin perfusion fixated brains (Korbo *et al.* 1990). In an NTP study (1989) exposure for 14–15 weeks with 1250 p.p.m. toluene resulted in increased brain weight in mice and brain necrosis in rats. Over a period of two years no gross microscopic changes were observed in rats or mice exposed to toluene at concentrations up to 1200 p.p.m. (NTP 1989). However, the toluene level-dependent decreased weight of hippocampus was significant at 1500 p.p.m. in the inhaled air and deserves further investigations.

The rationale for the biochemical parameters chosen was the documentation of regional changes of CNS-neurotransmitter metabolism after acute, subchronic, and chronic exposures to inhaled toluene even in concentrations of 50–80 p.p.m. which are below TLVs (WHO 1985; Arito *et al.* 1985; Fuxe *et al.* 1987; von Euler *et al.* 1988a). In this study NA, DA and 5-HT levels were significantly and irreversibly changed in various brain regions due to exposures to 500 and 1500 p.p.m. (tables 3, 4, and 5). Except for the reduced concentrations of DA and 5-HT respectively in the medulla oblongata and hypothalamus, a tendency was suggested towards increased regional DA and 5-HT concentrations, indicating a general underlying mechanism e.g. reduced activity of transmitter degradating enzymes, enhanced activity of rate limiting transmitter synthesizing enzymes and or elevated precursor concentrations. These parameters are about to be investigated. The changed regional levels of NA, DA, and 5-HT suggest changes in activity of corresponding neurones either directly leading to neurotoxic effects or constituting mechanisms compensating effects induced on unknown targets. When accompanied by the previously reported toluene induced changes in receptor affinity (Celani *et al.* 1983; Fuxe *et al.* 1987) and membrane fluidity (von Euler *et al.* 1988b) regional changes in transmitter concentration may compromise normal CNS-function.

In conclusion, the techniques applied in this investigation failed to reveal overt toluene-induced CNS-neurotoxicity. However, significant though minor changes in the size of cerebral cortical neurones and their nuclei, and chronic changes in regional amine content indicate certain irreversible effects of toluene after long-term exposure. A biphasic response rather than dose-response correlation was observed. Supplementary morphometric and biochemical studies are in progress. Exposure schedule, sensitivity and choice of appropriate behavioural tests, target areas for morphometric measurements, and the applicability of morphometric and biochemical findings are important topics for future research. In our interpretation, the results of this study further adds to the accumulating evidence of the chronic CNS-neurotoxicity of toluene in rats.

Acknowledgements

The authors wish to express their gratitude to Morten Falding, Annette Landin, Vibeke Kegel, Vibeke Kjær, Bo Larsen, Karen Roswall, Hans-Jørgen Jensen, Lykke Steffensen and Susanne Primdahl for their skilled technical assistance in the conduction of the study.

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Reference II

SHORT COMMUNICATION, Pharmacology & Toxicology 1992, **70**, 394–396. **Short Communication**

Brain Concentrations of White Spirit Components and Neurotransmitters Following a Three Week Inhalation Exposure of Rats

Henrik Rye Lam[1], Agneta Löf[2] and Ole Ladefoged[1]

[1]Institute of Toxicology, National Food Agency, DK-2860 Søborg, Denmark and [2]Division of Work and Environmental Physiology, National Institute of Occupational Health, S-17184 Solna, Sweden

(Received November 11, 1991; Accepted February 20, 1992)

White spirit (Stoddard solvent) has been widely used as an organic solvent for many purposes. There are various products, all of which are complex hydrocarbon mixtures with numerous components (C7-C14), mainly aliphatic alkanes, mono- and dicyclic alkanes, and aromatic (C7-C14), hydrocarbons with a distillation range of 150–215°. The CNS neurotoxicity of white spirit in humans has been questioned for many years. As a consequence of epidemiological findings the use of white spirit as a solvent for paints for professional use was abandoned in Denmark in the late 1970's. The use declined significantly from around 1980 and onwards, however, it is still used in household products and for some industrial purposes.

Only a few well-controlled animal studies have been performed. The acute effects include dose related irritation of the eyes, the upper airways, and the lungs. Dysfunction of renal distal tubuli related to the C_8–C_{11} components has been reported (Viau *et al.* 1984). Chronic toxic effects are less investigated. CNS symptoms such as impaired coordination, tremor, and slowing of the pupillary reflex have been described. Only few neuropathological, neurochemical, and behavioural data are available.

Acute exposure of humans to white spirit in exposure chambers under controlled conditions have shown to affect visual reaction time, and to cause fatigue, headache and dizziness, a feeling of drunkenness, reduced vigilance and attention, and impaired short- and long-term cognitive functions (Stokholm & Cohr 1979a & b). Epidemiological investigations have demonstrated a significant increase in neuropsychiatric symptoms, reduced concentration ability, impaired learning, memory, and abstract thinking ability, as well as cerebral, mainly cortical atrophy. Neurophysiological and neuropsychological dysfunctions have been reported as a result of acute, subchronic, and chronic intoxications. The symptom complex is diagnosed as chronic toxic encephalopathy, provided that previous long-term exposure to organic solvents can be documented (WHO/Nordic Council of Ministers 1985).

The toxicokinetics of white spirit are extremely complex as more than 150 different components are involved. Also the analytical problems are huge, therefore, the application of markers for different fractions of white spirits has been proposed (Åstrand *et al.* 1975). Repeated exposure to white spirit resulted in accumulation in both adipose tissues and venous blood (Pedersen *et al.* 1984 & 1987).

It is important to determine the brain concentration after repeated administration to white spirit to evaluate the applicability of the rat for studies of the potential chronic neurotoxic effect. Hence, in the present study we focussed on the determination of the concentration of white spirit and its components in the rat brain. Furthermore, the possible effects on CNS neurotransmitter metabolism were studied.

Rats were exposed to white spirit (K-30) purchased from Shell. The relative mean molecular weight was 140 g/mol, 20 vol% were aromates, and the distillation interval at 760 mmHg was 148–200°. All other chemicals used were of analytical grade.

Male rats (Mol: WIST), 3 months of age, from Møllegaard Breeding Centre Ltd., DK-4623 Lille Skensved, Denmark, were housed conventionally in rooms with automatic control of temperature (22 ± 1°), relative humidity (55 ± 5%), air exchange (8 times/hr), and fluorescent light (21.00–9.00). One group served as control a second group was exposed to 400 p.p.m. (2.29 mg/l) and a third group to 800 p.p.m. (4.58 mg/l) of white spirit vapour in the inhaled air for 6 hr/

Table 1.

Whole rat brain concentration of white spirit and its components following exposure for 3 weeks. (mg/kg wet weight)

	Exposure concentration wt/vol.	
Component	400 p.p.m.	800 p.p.m.
Nonane	ND	1.66 ± 0.22
Decane	0.81 ± 0.11	2.79 ± 0.45
Undecane	ND	0.72 ± 0.24
Xylene	0.02 ± 0.04	0.23 ± 0.07
o-xylene + dodecane	0.12 ± 0.03	0.04 ± 0.04
1,3,5-trimethylbenzene	0.10 ± 0.09	0.08 ± 0.08
1,2,4-trimethylbenzene	ND	0.40 ± 0.03
1,2,3-trimethylbenzene	ND	0.07 ± 0.10
Aliphatic compounds	2.39 ± 1.01	8.65 ± 2.40
Aromatic compounds	0.73 ± 0.33	1.54 ± 0.52
Total white spirit	3.40 ± 0.88	10.2 ± 2.5

All values are expressed as mean ± S.D. (n = 5).

Table 2.

Effects on whole rat brain weight, protein, NA, DA, and 5-HT concentrations, and the esterase activities after exposure to white spirit for 3 weeks.

	Control	400 p.p.m.	800 p.p.m.
Weight of whole brain (in g)	2.034 ± 0.168	2.050 ± 0.073	2.092 ± 0.092
Protein concentration (mg/g wet weight)	111 ± 4	103 ± 7	109 ± 3
NA concentration (nmol/kg wet weight)	2.30 ± 0.19	2.63 ± 0.12*	2.98 ± 0.32*
DA concentration (nmol/kg wet weight)	4.89 ± 0.31	5.24 ± 0.32	5.94 ± 0.43*†
5-HT concentration (nmol/kg wet weight)	2.33 ± 0.18	2.57 ± 0.27	3.68 ± 0.14*
AChE activity (μU/kg wet weight)	53.3 ± 5.4	48.2 ± 2.3	52.0 ± 5.2
BuChE activity (μU/kg wet weight	15.1 ± 1.8	17.9 ± 3.2	16.7 ± 2.6

All values are expressed as mean ± S.D. (n = 5).

* $P < 0.05$ between values from control and exposed rats by Student's t-test.

† $P < 0.05$ between values from rats exposed for 400 and 800 p.p.m. by Student's t-test.

day, 5 days/week for three weeks. The inhalation exposure conditions and the equipment were as previously described (Ladefoged *et al.* 1990).

Immediately after the last 6 hr exposure period, five rats from each group were sacrificed by decapitation in CO_2/O_2 narcosis. Within 30 sec. the total brain was transferred into 9 vol. of 0.32 M ice-cold sucrose and homogenized at 0–4°. Samples of homogenate were used for the determination of protein, acetylcholine-esterase (AChE), and butyrylcholine-sterase (BuChE) activities. Protein was determined as described by Lowry *et al.* (1951) and modified by Hartree (1972), esterase activities by use of commercially available kits from Boehringer Mannheim.

Sample processing and noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) determinations by high pressure liquid chromatography with electrochemical detection were performed as described by Ladefoged *et al.* (1991).

Homogenate samples stored at −20° were transferred to gas-tight headspace vials (22.4 ml) and capped immediately with teflon lined membranes for analysis of aliphatic and aromatic compounds. The headspace air was analyzed automatically on a gas chromatograph (Perkin-Elmer 8700), equipped with a flame ionization detector and a polar column (Supelcovax 10, 30 m, 0.75 mm i.d.). The samples were thermostated for 30 min. at 37°. The injection temperature was 170° and the detector temperature was 290°. The initial column temperature was 45°; it was increased by 2°/min. to 90° and then by 25°/min. to 210°. Nitrogen at a flow of 5 ml/min. was used as carrier gas.

Groups of data were compared using two-sided, non-paired Student's t-test. Differences between groups were generally considered statistically significant at $P < 0.05$.

The whole brain concentrations of white spirit and its components are given in table 1. There was an obvious relationship between exposure levels and brain concentrations. Whole brain weight, protein, NA, DA, and 5-HT concentrations, and esterase activities are given in table 2. White spirit did not induce changes in brain weight, protein concentration, and esterase activities, whereas NA, DA, and 5-HT concentrations all increased significantly (table 2).

The accumulation of total white spirit in the brain was 3.40 and 10.2 mg/kg wet weight, compared to the exposure levels 2.29 and 4.58 mg/l. The concentrations in the rat brain of white spirit aromatic constituents increased proportionally with the exposure level (2.1 times), whereas that of the aliphates more than tripled (3.6 times), suggesting accumulation of this fraction during long term exposure to high concentrations. Our results agree with the findings in the human brain obtained by Pedersen *et al.* (1987), who found minimum and maximum steady-state contents of 0.6 and 5 mg/kg brain, respectively, at an exposure level at 100 p.p.m. of an aliphatic white spirit by application of a three compartment model on data obtained from blood and fat samples from human volunteers. The exposure to white spirit did not affect whole brain weight, protein concentration or AChE and BuChE activities. These findings, however, theoretically could conceal even marked regional or subcellular differences. The white spirit induced increased levels of the CNS neurotransmitters NA, DA, and 5-HT, which never published previously, suggest changes in the activities of corresponding neurones either directly causing neurotoxic effects or reflecting mechanisms compensating the effects induced on unknown targets.

The present results initiated a multidisciplinary 6 month's inhalation study including behavioural, morphometric, neuropathological, and brain regional and subcellular (synaptosomal) parameters. Furthermore, a three week inhalation exposure study was undertaken to investigate effects induced on synaptosomes. These studies are about to be completed.

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Reference III

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Effects of Six Months' White Spirit Inhalation Exposure in Adult and Old Rats

Grete Østergaard[1], Henrik R. Lam[1], Ole Ladefoged[1] and Peter Arlien-Søborg[2]

[1]Institute of Toxicology, National Food Agency, DK-2860 Søborg, and [2]Neurological Research Laboratory, Hvidovre University Hospital, DK-2650 Hvidovre, Denmark

(Received April 22, 1992; Accepted August 19, 1992)

Abstract: In two separate experiments in rats the irreversible effects of six months' exposure to white spirit (0, 400 p.p.m., and 800 p.p.m.) were studied. In one experiment the exposure started at the age of three months, in the other the rats were 15 months at the beginning of the exposure. After an exposure-free period of several months neurobehavioural, pathological, and neurochemical examinations were performed. A marked difference in motor activity between young and aged animals was found. A slight effect on kidney function was seen at 800 p.p.m. No macroscopic or histopathological changes related to dosing were found. The concentrations of noradrenaline, dopamine, and 5-hydroxytryptamine in various brain regions and in whole brain were irreversibly changed. In conclusion, the study revealed different changes within the CNS, but failed to demonstrate neurobehavioural white spirit-induced neurotoxicity.

White spirit (Stoddard solvent, mineral spirits) is widely used as a solvent in paints, printing inks, and varnishes. Various kinds of white spirit are produced as distillation fractions of crude oil. All are complex mixtures of straight and branched aliphatic, alkyl aromatic and naphthenic hydrocarbons with boiling points in the range of 150–215°.

Longitudinal epidemiological studies of occupationally solvent-exposed workers show that neuropsychiatric disorders are a frequent cause of early disability pension in this population compared with non-exposed controls (for review see WHO/Nordic Council of Ministers, 1985; Arlien-Søborg 1992; Arlien-Søborg *et al.* 1992). In most of the studies workers were exposed to mixtures of organic solvents, the principal component being white spirit. Mikkelsen (1980) and Mikkelsen *et al.* (1988) performed a cohort study of housepainters and bricklayers, and showed that signs of brain dysfunction increased significantly with the degree of solvent exposure in the group of painters. Ørbæk *et al.* (1985) examined workers from the paint industry and showed that signs of brain damage in the form of neurasthenic symptoms were significantly more common in the exposed workers, and that the severity of symptoms was dose-related. Identical findings were reported by Valciukas *et al.* (1985). Triebig (1988) performed two epidemiological studies in painters. The results of the two studies do not support the assumption of high neurotoxic risks in solvent-exposed workers.

The acute effects have mainly been studied in the rat. Dose-related irritation of eyes, upper airways, and lungs, and dysfunction of renal distal tubuli have been reported for the C_8–C_{11} components (Hass & Prior 1986). In acute toxicity experiments low aromatic white spirit produced a marked decrease in speed of responding in learned performance in doses of 200 p.p.m. (Kulig 1990). The results of three weeks' inhalation exposure to 400 and 800 p.p.m. demonstrated disposition of various white spirit components in the whole brain and significantly increased noradrenaline, dopamine, and 5-hydroxytryptamine concentrations (Lam *et al.* 1992).

Chronic effects have also been evaluated in the rat. In rats exposed for 12 months (8 hr/day–5 days/week) to 6500 mg/m^3 (=1135 p.p.m.) of aliphatic C_{10}–C_{12} white spirit, distal tubular dysfunction was found (Viau *et al.* 1984). Chronic exposure with low aromatic white spirit for 26 weeks (8 hr/day–5 days/week) in doses of 200, 400, and 800 p.p.m. did not produce lasting behavioural and histopathological nervous system effects (Kulig 1990). Aging of the rats might cause a reduced plasticity of the nervous system (Landfield *et al.* 1977) or an impaired defence against oxidative stress which has been proposed to be a common mediator of neurotoxicity (LeBel & Bondy 1991). It may be that old rats are more susceptible to neurotoxic insult by white spirit, since the aged brain is possibly less tolerant to toxicants. Therefore, a multi-disciplinary study on the neurotoxicity of white spirit in rats of different age was undertaken. It consisted of two separate experiments. The design of the first experiment was similar to the one used in our previous toluene study (Ladefoged *et al.* 1991). In the first experiment (referred to as the 'young rat experiment') adult rats, three months old at the start of the experiment, were used. In the second experiment – the 'old rat experiment' – aged rats, 1 year old at the start of the experiment, were used (a satellite non-exposed group from experiment 1).

Materials and Methods

Chemicals. White spirit was purchased from Shell, Denmark (Mineralsk terpentin K-30, Bp. 148–200°, 20 vol% aromatics). All other chemicals used were of analytical grade.

Animals. One hundred and fifty male rats (Mol:WIST), mean body weight 350 gram, three months old, obtained from Møllegaard

Breeding Centre Ltd., DK-4623 Ll. Skensved, Denmark, were used. One hundred and eight of the rats were used in the 'young rat experiment', and the remaining 42 were used in the 'old rat experiment', being 15 months old at the start of exposure. The rats were housed in stainless steelwire cages, 2 animals/cage, conventionally in animal rooms with automatic control of temperature (22° ± 1°), relative humidity (55% ± 5%), air exchange (8 times/hr), and fluorescent light (9 p.m.–9 a.m.), with access to commercial pelleted diet (Altromin 1324, Brogården, Gentofte, Denmark) and acidified tap water in nipple bottles. The rats were individually marked by ear clipping. During daily exposure the food was removed. The animals were weighed once a week during the study. In the 'young rat experiment', food and water consumption was measured during the last six weeks of exposure.

Exposure. In both experiments the rats were weight-randomized into three groups. In the 'young rat experiment', the group size was 36, and in the 'old rat experiment' the group size was 14. The exposure schedule was similar in the two experiments: One group was sham-exposed, a second group was exposed to 400 p.p.m. (2290 mg/m^3), and a third group received 800 p.p.m. (4580 mg/m^3) of white spirit in the inhaled air for 6 hr/day, 5 days/week for 6 months followed by an exposure-free period of two months' duration prior to neurobehavioural data collecting. The animals were sacrificed four months after end of the exposure. The inhalation exposure conditions and the equipment used have previously been described by Ladefoged *et al.* (1990).

Clinical chemistry. Two weeks after end of the exposure, 10 randomly selected animals from each group in the 'young rat experiment' were placed in metabolism cages for 24 hr urine collection. Urine was analysed for glucose, ketone bodies, haemoglobin, nitrite and protein. Furthermore, diuresis, density, and pH were determined (Ames Multistix 8 SG, Miles Laboratories Inc., U.S.A.).

The following parameters were measured in plasma: alkaline phosphatase (AP) (Boehringer Test-Combination® No. 415278), alanine amino transferase (ALAT) (Boehringer Test-Combination® no. 191345), urea (Chaney & Marbach 1962), creatinine (Merckotest® No. 3384), glucose (Boehringer Test-Combination® No. 263826), and protein by the biuret method.

Behavioural tests. In both experiments, motor activity was measured regularly during the entire trial period. After the two months exposure-free period all animals in the 'young rat experiment' were tested in a functional observational battery (FOB). The rats were then assigned randomly to three subgroups that were tested in passive avoidance test, Morris maze, and radial arm maze, respectively. In the 'old rat experiment', only FOB and Morris maze testing was carried out due to the limited number of animals. All old animals were tested in the two tests.

Motor activity. The motor activity was recorded monthly for one week-end before, during, and after the exposure. In the 'young rat experiment' ten pairs of rats from each group were tested, and in the 'old rat experiment' all rats were tested. The device and method were similar to the previously described procedure (Ladefoged *et al.* 1991). Activity counts were collected during the first hour after transfer from home to test cage (showing exploratory activity), and during several light/dark cycles (showing diurnal variations).

Functional observational battery (FOB) (Moser 1989). Briefly, the examination consisted of structured observations of the rats' behaviour in the home cage and in an open field, interactive tests involving handling, and physiologic measures. As the rats were identifiable by their ear clipping the observation was not done in a blind fashion.

Passive avoidance, eight-arm radial maze, and Morris maze. In each of these models six pairs of rats from each group in the 'young rat experiment' were tested. In the 'old rat experiment', all rats were tested in the Morris maze. The apparatuses used in the three models have previously been described in detail (Ladefoged *et al.* 1991).

In the passive avoidance test each rat was placed in the lit compartment of a one-way shuttle box. After 20 sec. the guillotine door was raised and the rat's latency to enter the dark compartment was recorded with a cut-off of 180 sec. Two hr later this procedure was repeated in a conditioning trial where, upon entry of the dark compartment, the rat received an electric foot shock (1 sec., 0.5–0.8 mA). The next day the rat was placed in the lit compartment. After 20 sec. the guillotine door was raised and the latency time to entry was recorded with a cut-off of 300 sec.

In the eight-arm radial maze test the animals were initially put on a one-week restricted food schedule which brought them down to 85% of their body weight. Subsequently the animals were trained daily in the maze using peanut chops for rewards. A training session lasted until all rewards had been eaten, or 10 min. The rats were trained in 14 sessions. On the 15th session the rats were removed from the maze to their home cage when they had eaten four rewards. They were kept here for 5 min and then put down in the central maze area to complete the trial session. Number of correct choices, number of errors (entry into a previously visited maze arm), total number of choices, and session duration were recorded.

The Morris maze testing consisted of cued escape training, first place navigation task, transfer test, and second place navigation test. During cued escape training the rats were trained with the visible platform placed in the centre of the southeast ('young rat experiment') or northeast ('old rat experiment') quadrant. The rats were trained in blocks of four rats. Each block of rats was trained in four trials each day, using the four different start positions in a new random order every day. If a rat did not locate the platform within 60 sec., it was removed from the water and placed on the platform for 10 sec. Training of place navigation with the invisible platform continued in a similar fashion until a stable performance was established. In the transfer test, the platform was removed from the pool. The rats were then allowed 60 sec. of free swimming in a single trial. For the second place navigation task the platform location was changed to the diagonally opposite position (northeast: 'young rat experiment', or southwest: 'old rat experiment'), below the water surface. In the 'young rat experiment' data from the transfer test were lost due to a technical error, so the trial was repeated with a regular training day of four trials inserted. In the 'old rat experiment', a second transfer test was conducted as the last trial. Data collected were: latency to escape onto the platform, length of route, and actual time and percentage time spent in each pool quadrant.

Pathological examinations. Twelve animals from each group in the 'young rat experiment' were randomly selected for a thorough autopsy. The following organs were excised and weighed: liver, kidneys, adrenals, heart, spleen, testes, and brain. Samples from these organs were fixated in 10% buffered formalin, prepared for light microscopy and stained with haematoxylin-eosin. Samples from N. ischiadicus were stained with osmium tetroxide. Brains were not fixated but used for measuring biochemical parameters.

In the 'old rat experiment' no organ weights were obtained because all animals were perfusion fixated for later morphometric analysis of brain tissue (investigation still in progress). Macroscopic examination was performed on all animals. Histopathology was done on changed tissue, and on the kidneys because clinical signs of kidney dysfunction was seen in the 'young rats'.

Neurochemical analysis. Sample preparation: Brains from seven randomly selected animals of each treatment group in the 'young rat experiment' were dissected into seven regions: cerebellum, cerebral hemisphere, hippocampus, hypothalamus, pons, thalamus and medulla oblongata according to the method described by Glowinsky & Iversen (1966) and Edelfors (1975, personal communication). The samples were immediately transferred to icecold 0.32 M sucrose, weighed and thoroughly homogenized by an Ultra-Turrax T-25 at

full speed using an ice-cooling jacket. Immediately after homogenization an aliquot of homogenate was deproteinized by the addition of icecold 0.2 M perchloric acid (1 + 1) and subsequent centrifugation. Supernatants were used directly for the determination of 5-hydroxytryptamine (5-HT) after addition of ω-N-methyl-5-HT as internal standard. Another aliquot of supernatant was added dihydroxybenzylamine (DHBA) as internal standard for noradrenaline (NA) and dopamine (DA) determinations carried out after aluminium oxide purification (Anton & Sayre 1962).

High pressure liquid chromatography – electrochemical detection (HPLC-ED) analysis of biogenic amines. Seventy-five µl perchloric acid extract was injected for 5-HT and ω-N-methyl-5-HT analysis, 75 µl aluminiumoxide eluate for catecholamine analysis.

The instrumentation consisted of a Hewlett-Packard 1084B liquid chromatograph equipped with a 250 × 4 mm I.D. RP-18 Highbar, Supersphere LiChroCART (5 µm) analytical column protected by a 20 × 4 mm I.D. (5 µm) RP-18 guard column (both from E. Merck GmbH, FRG). Separation was achieved by use of an acetonitrile modified citrate/octenylsulfate buffer as described by Lin & Blank (1983).

5-HT, ω-N-methyl-5HT, NA, DHBA and DA were detected by a Waters M 460 electrochemical detector applying an oxidation potential of +0.6 V.

Statistical analysis. The SAS PC-version software package (SAS Institute Inc., 1988) was used for all computations.

Body weight data, relative food and water consumption data, data from clinical-chemical and neurochemical measurement were analyzed by analysis of variance (PROC ANOVA) followed by Dunnett's test where indicated.

Data from passive avoidance test (escape latencies) were analyzed by nonparametric analysis of variance (PROC NPAR1WAY WILCOXON) where dosed groups were compared to control on each trial.

Data from radial arm maze and Morris maze were analyzed using a general linear model (PROC GLM).

Motor activity data were summed by cage for each data collection time point. Logarithms of sums were analysed by analysis of variance using a linear model (PROC GLM) followed by split-plot analysis.

The level of significance was set to 0.05.

Results

Clinical signs during exposure. During the daily exposure, the white spirit-exposed rats appeared to feel discomfort. Especially during the initial period the rats suffered from mucosal irritation with lacrimation and bloody discharge from the nose. The narcotic effect which was initially seen was gradually reduced.

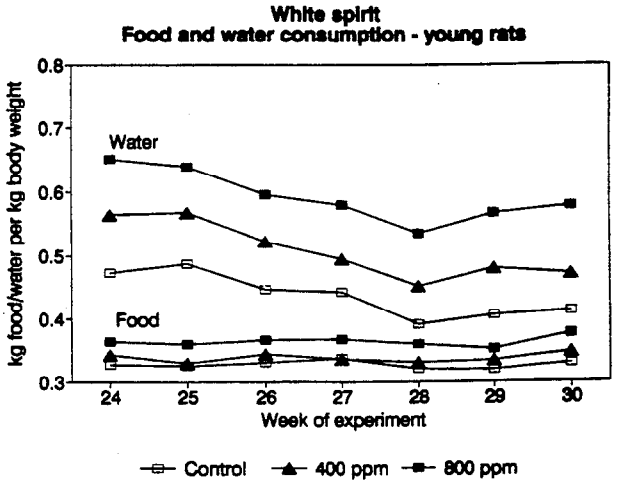

Fig. 1. The effect of white spirit exposure on relative food and water consumption measured during the last six weeks of exposure of the 'young rats'. In the 800 p.p.m. group both food and water consumption were significantly higher than in the control group. In the 400 p.p.m. group only water consumption was significantly higher than control values.

Survival. In the 'young rat experiment' all rats survived until the end of the experiment. In the 'old rat experiment' all rats survived until the end of the exposure period. However, three rats in the control group, three rats in the 400 p.p.m. group, and one rat in the 800 p.p.m. group were euthanized during the exposure-free period, before behavioural testing could be done. The clinical conditions that led to euthanasia were judged unrelated to exposure.

Body weight. The animals exposed to 800 p.p.m. white spirit had a lower body weight compared to the control group in both experiments during the exposure period. In the 'young rat experiment', but not in the 'old rat experiment', this was statistically significant. At sacrifice, there was no difference in body weights of the 'young rats' (control: 595 g ± 40, 400 p.p.m.: 619 g ± 66, 800 p.p.m.: 613 g ± 57).

Relative food and water consumption. ('young rat experiment', last six weeks of exposure, fig. 1) The animals exposed to 800 p.p.m. white spirit had a significantly higher

Table 1.

	Clinical chemical parameter					
Treatment	AP (u/l)	ALAT (u/l)	Urea (g/l)	Creatinine (µ/l)	Glucose (mM)	Protein (g/l)
0	93 ± 31	40 ± 22	4.57 ± 0.32	51.7 ± 6.2	5.87 ± 0.66	69.7 ± 2.4
400 p.p.m.	113 ± 15	23.9 ± 3.6*	5.67 ± 0.66*	62.5 ± 5.9*	6.14 ± 0.38	72.0 ± 3.1
800 p.p.m.	111 ± 22	19.8 ± 3.0*†	5.61 ± 0.63*	59.3 ± 6.3*	5.81 ± 0.40	71.5 ± 2.7

AP = alkaline phosphatase.
ALAT = alanine amino transferase.
All values are expressed as mean ± S.D. (n = 10).
* $P < 0.05$ between values from control and exposed rats by Dunnett's t-test.
† $P < 0.05$ between values from rats exposed to 400 and 800 p.p.m.

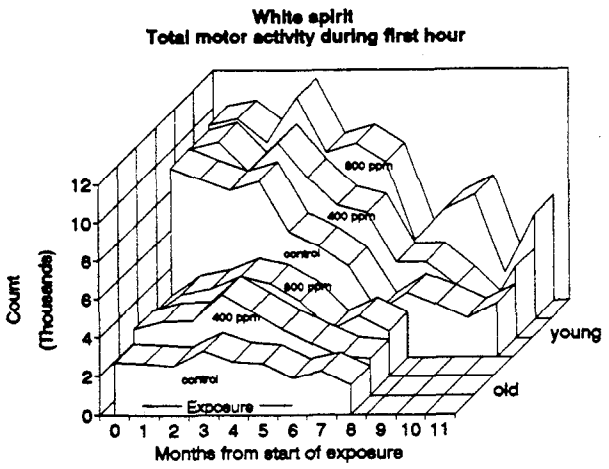

Fig. 2. The effect of white spirit exposure on motor activity. Total sums of exploratory activity counts of the first hour from one week-end per month.

food and water consumption compared to controls. The animals exposed to 400 p.p.m. had a significantly higher water (but not food) consumption compared to controls.

Clinical chemistry. There were no white spirit-induced effects on urine diuresis, density, pH, glucose, ketone bodies, haemoglobin, nitrite, or protein (results not given). As shown in table 1, the plasma ALAT activity was significantly and dose-dependently reduced. Plasma urea and creatinine was significantly increased.

Behavioural tests

Motor activity. The total sum of activity counts collected in the course of individual week-ends divided into exploratory activity during the first hour after transfer to the new cages (fig. 2), and activity during the remaining (major) part of the week-end (fig. 3) is shown. In the 'young rat experiment' there was a tendency towards higher motor activity in animals exposed to 800 p.p.m. compared to controls and to the animals exposed to 400 p.p.m. The difference was not statistically significant. The diurnal activity of the exposed animals did not appear to differ from control animals (data not shown).

Functional observational battery. In none of the experiments did the exposed animals differ from controls with respect to the examined parameters (data not shown). In the 'old rat experiment' it was observed that many animals exhibited abnormal mouth movements, i.e. chewing movements with open or closed mouth (with or without tongue protrusion) or teeth chattering (tremor of facial muscles). An attempt to quantitate this (5-min. observation in macrolon cage) showed that the phenomenon was equally present in all three groups with two thirds of the animals showing one or both symptoms during the observation period.

Passive avoidance. Latencies to enter the dark compartment on trial 1, 2, and 3 were compared using Wilcoxon Rank sum test. No differences between dose groups were found on any trial (data not shown).

Morris maze. Escape latencies are shown in fig. 4 as means of daily training trials. No differences between dose groups

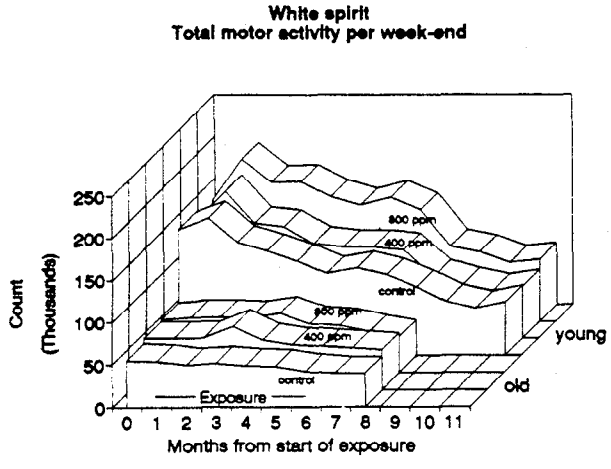

Fig. 3. The effect of white spirit exposure on motor activity. Total sums of activity counts for 62 hr from one week-end per month.

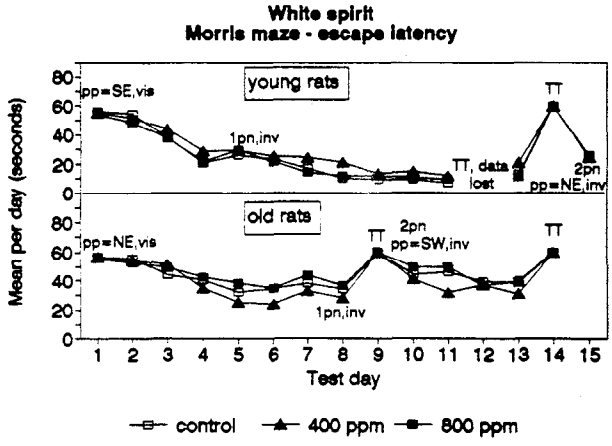

Fig. 4. Morris maze – mean escape latencies. The chronic effect of white spirit exposure on behaviour in the Morris maze. Daily results are shown as means of trial blocks. Data labels describe test situation and platform position.
Upper part of figure, 'young rats':
Day 1–4: pp=SE, vis – cued escape training, platform position southeast, visible. Day 5–11: 1 pn, inv – 1st place navigation task, same platform position, but invisible. Day 12: TT – transfer test, free swim with no platform present (data lost). Day 13: training day, platform position southeast, invisible. Day 14: TT – repeat of day 12. Day 15: 2 pn, pp=NE, inv – 2nd place navigation task, platform moved to northeast, invisible.
Lower part of figure, 'old rats':
Day 1–7: pp=NE, vis – cued escape training, platform position northeast, visible. Day 8: 1 pn, inv – 1st place navigation task, same platform position, but invisible. Day 9: TT – transfer test, free swim with no platform present. Day 10–13: 2 pn, pp=SW, inv – 2nd place navigation task, platform moved to southwest, invisible. Day 14: TT – transfer test, free swim with no platform present.

Table 2.

Regional brain noradrenaline concentration (nmol/g wet weight) in rats exposed to white spirit for six months.

	Control	400 p.p.m.	800 p.p.m.
Cerebellum	1.50±0.23	1.23±0.10*	1.33±0.16
Hemisphere	1.93±0.18	2.20±0.20*	2.29±0.20*
Hippocampus	1.53±0.26	1.49±0.23	2.26±0.40*†
Hypothalamus	2.99±0.58	2.37±0.66	2.62±0.30
Pons	2.49±0.46	2.60±0.48	3.08±0.51
Thalamus	3.81±0.49	3.67±0.47	3.90±0.53
Medulla oblongata	2.58±0.35	2.66±0.24	2.56±0.33
Whole brain	2.38±0.13	2.44±0.19	2.61±0.24

All values are expressed as mean±S.D. (n=7).
* $P<0.05$ between values from control and exposed rats.
† $P<0.05$ between values from rats exposed to 400 and 800 p.p.m.

Table 4.

Regional brain 5-hydroxytryptamine concentration (nmol/g wet weight) in rats exposed to white spirit for six months.

	Control	400 p.p.m.	800 p.p.m.
Cerebellum	0.25±0.03	0.13±0.02*	0.17±0.05*
Hemisphere	1.45±0.25	1.67±0.20	1.78±0.21*
Hippocampus	0.31±0.06	0.36±0.09	0.53±0.08*†
Hypothalamus	1.83±0.16	1.78±0.32	2.77±0.49*†
Pons	2.09±0.29	2.49±0.40	3.44±0.65*†
Thalamus	4.59±0.35	4.90±0.60	7.31±1.43*†
Medulla oblongata	3.73±0.49	3.19±0.43	4.71±0.46*†
Whole brain	2.41±0.24	2.46±0.20	3.62±0.82*†

All values are expressed as mean±SD values (n=7).
* $P<0.05$ between values from control and exposed rats.
† $P<0.05$ between values from rats exposed to 400 and 800 p.p.m.

were found in any of the two experiments.

Radial arm maze. No differences between the three groups were found (data not shown).

Pathological examinations. No macroscopic pathological changes which could be attributed to the dosing with white spirit were found in either experiment. Hyaline casts were found in the kidney tubuli in approximately half of the 'young rats', and in most of the 'old rats'. No relation to exposure could be shown.

Neurochemical analysis. There were no white spirit-induced effects on the weight of any brain region, absolute and relative total brain weights (data not shown) in the young rats. Regional neurotransmitter concentrations in the young rats are presented in tables 2, 3, and 4. Owing to white spirit exposure, the concentrations of NA, DA, and 5-HT in various brain regions and in the whole brain were significantly, irreversibly changed.

Discussion

The narcotic effect which was initally seen during exposure was gradually reduced as adaptation took place. This phenomenon has been reported by others (Carpenter *et al.* 1975).

Table 3.

Regional brain dopamine concentration (nmol/g wet weight) in rats exposed to white spirit for six months.

	Control	400 p.p.m.	800 p.p.m.
Cerebellum	ND	ND	ND
Hemisphere	7.67±0.61	9.19±0.92*	9.80±0.79*
Hippocampus	0.27±0.05	0.22±0.05	0.18±0.04*
Hypothalamus	3.39±0.42	2.88±0.49	3.78±0.50
Pons	0.57±0.08	0.53±0.11	0.53±0.09
Thalamus	8.87±0.69	10.1±0.37	10.7±1.50*
Medulla oblongata	0.40±0.05	0.43±0.07	0.43±0.08
Whole brain	4.85±0.47	5.80±0.26*	6.06±0.42*

All values are expressed as mean±S.D. (n=7).
* $P<0.05$ between values from control and exposed rats.
† $P<0.05$ between values from rats exposed to 400 and 800 p.p.m.
ND: Not detectable.

The decrease in body weight may be considered a sign of general malaise caused by the exposure. The age-related increase in the frequency of hyaline casts is in accordance with the pathological mechanism behind this lesion in male rats (Bernard & Lauwerys 1991). Plasma creatinine and urea was slightly increased, and relative food and water consumption was increased during the last six weeks of dosing in the young rats, consistent with compromised kidney function reported by Viau *et al.* (1984). There were no changes in urine clincal chemical parameters. The lack of consistency between clinical chemistry and histopathology may reflect a reversible nature of the lesions. The plasma ALAT activity was significantly, dose-dependently reduced. No explanation could be given.

The relevance of the behavioural parameters studied has previously been discussed (Ladefoged *et al.* 1991). Construct validity, i.e. the extent to which the endpoints are comparable in animals and humans can never be proved. However, learning and memory functions, and the diurnal regulation of motor activity exist in many species and are thought to be comparable functions. The tests used in this experiment are known to be able to identify known neurotoxicants. It is well known that age-related behavioural differences exist in laboratory animals. Impairment of spatial learning in aged rats has been demonstrated in the Morris maze (Rapp *et al.* 1987). Deficits in working memory in aged rats (deToledo-Morrell *et al.* 1988) and in aged gerbils (Carney *et al.* 1991) has been found in the radial arm maze test.

Consequently, if the effects caused by white spirit resemble the effects of aging it should be possible to detect them with these methods. In this experiment, the data for total motor activity clearly illustrate the difference in activity between young and old rats. Hence, an age-accelerating effect of white spirit on this endpoint presumably would have been detected. The lack of demonstrable behavioural effects like the results of the toluene experiment earlier reported (Ladefoged *et al.* 1991) may reflect the insensitivity of the rat model for this type of compound. On the other hand, compounds with simultaneous peripheral and central neurotoxic effect do produce observable neurobehavioural effects in the rat, e.g. hexanedione (Lam *et al.* 1991).

The rationale for the biochemical parameters chosen was the documentation of regional changes of CNS-neurotransmitter metabolism induced by other organic solvents, including aromatics, and the significantly increased concentrations of NA, DA, and 5-HT (whole brain, rat) owing to three weeks of inhalation exposure to white spirit at concentrations of 400 and 800 p.p.m. (Lam *et al.* 1992). These results were confirmed and extrapolated by the present study demonstrating significantly, irreversibly increased NA, DA, and 5-HT concentrations in the whole brain and in various regions. Except for the reduced concentrations of NA and 5-HT in the cerebellum and DA in the hippocampus, the general tendency was irreversibly increased regional NA, DA, and 5-HT concentrations. This suggests common underlying mechanisms of action, as also evidenced by results from toluene exposure. Toluene is not a component of the white spirit used in our studies (Lam *et al.* 1992), and due to the very complex composition of white spirit, it is impossible to pinpoint any responsible inducer for the present findings.

In conclusion, the techniques applied in this investigation failed to reveal neurobehavioural white spirit-induced CNS-neurotoxicity. Chronic changes in global and regional amine content indicate certain irreversible effects of white spirit. Supplementary neurochemical and morphometric studies are in progress to elucidate the mechanisms underlying the irreversible findings due to the white spirit exposures, which may compromise normal CNS-function.

Acknowledgements

The authors wish to express their gratitude to Morten Falding, Joan Gluver, Else-Marie Skov Jensen, Vibeke Kegel, Vibeke Kjær, Annette Landin and Karen Roswall for their skilled technical assistance in the conduction of the study.

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Reference IV

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Toxicology Letters 80 (1995) 39–48

Toxicology Letters

Three weeks' and six months' exposure to aromatic white spirit affect synaptosomal neurochemistry in rats

Henrik R. Lam*, Grete Østergaard, Ole Ladefoged

Institute of Toxicology, National Food Agency of Denmark, DK-2860 Søborg, Denmark

Received 14 June 1994; revision received 13 February 1995; accepted 13 February 1995

Abstract

The effects of 3 weeks' or 6 months' inhalation exposure of rats to aromatic white spirit 6 h/day, 5 days/week at 0, 400, or 800 ppm were studied. Synaptosomal neurochemistry was investigated as index of the in situ conditions in the presynaptic nerve terminal. In both studies, the relative and absolute yield of synaptosomal protein were significantly reduced in the two exposed groups. Both studies demonstrated increased synaptosomal noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) concentrations, high- affinity 5-HT uptake rate and uptake capacity. It is hypothesized that a reduced density and total number of synapses in situ may be functionally compensated by increased NA, DA, and 5-HT neurotransmitter release, or by increased activity of corresponding neurons. The increased synaptosomal 5-HT uptake rates and uptake capacities may explain the previously demonstrated increased global and regional neurotransmitter concentrations and the present finding of increased synaptosomal 5-HT concentrations. These changes are interpreted as an indication of toxic effect on the CNS function and are considered supportive of recent findings of electrophysiological changes and affected motor activity following 6 months' exposure to dearomatized white spirit followed by an exposure-free period.

Keywords: Organic solvents; Biogenic amines; Neurotoxicity; Stoddard solvent 64742-88-7

1. Introduction

White spirit is a widely used solvent in paints, printing inks, and varnishes. Various kinds are produced as distillation or chemically modified fractions of crude oil. All are complex mixtures defined on the basis of their content of aliphatic, aromatic, alkyl aromatic and naphthenic hydrocarbons. Boiling points are in the range of 140–215°C.

The principal route of occupational exposure is inhalation. White spirit is readily absorbed via the lungs, followed by distribution to various tissues a.o. the brain. Components of white spirit and their oxidized metabolites (alcohols, aldehydes, epoxides, carboxylic acids) are excreted by exhalation and by the urine. Other components and me-

* Corresponding author. Tel.: +45 3969 6600; Fax: +45 3966 0100.

0378-4274/95/$09.50
SSDI 0378-4274(95)03325-F

tabolites enter the intermediary metabolism. Short-term exposure of human subjects to white spirit under controlled conditions in exposure chambers results in several CNS disturbances [1,2].

Epidemiological studies of occupationally solvent-exposed workers show that neuropsychiatric disorders are a frequent cause of early disability pension in this population when compared with non-exposed controls [3–12]. As a consequence of epidemiological studies, white spirit was abandoned as a solvent for paints for professional purposes about 1980 in Denmark, resulting in a significantly reduced exposure. However, it is still widely used in household paints. White spirit and similar hydrocarbon mixtures (e.g. Stoddard solvent) are still in use in many other countries.

Experimental investigations in laboratory animals are necessary to disclose dose-response correlations, reversibility or irreversibility of CNS neurotoxic effects, and to study the mechanisms underlying the neurotoxicity.

In acute toxicity studies in rats, white spirit of low aromatic content produced a marked decrease in the speed of responding in learned performance when exposed to 200 ppm [13]. The results of 3 weeks' inhalation exposure to 400 and 800 ppm demonstrated disposition of various white spirit components in the whole brain and significantly increased noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) concentrations [14]. A study with dearomatized white spirit demonstrated affected indices of oxidative stress in the synaptosomal fraction of hippocampus and hemisphere of rats exposed for 3 weeks to 400 and 800 ppm [15]. Long-term exposure to low aromatic white spirit for 26 weeks (8 h/day, 5 days/week) at 200, 400, or 800 ppm did not produce lasting behavioural or histopathological nervous system effects [13]. In another study, dearomatized white spirit caused long-lasting effects in neurophysiological parameters (flash-evoked potential, somatosensory-evoked potential, auditory brainstem response) [16]. Irreversibly increased global and regional NA, DA, and 5-HT concentrations in the rat brain were induced by 6 months' inhalation exposure to aromatic white spirit at 400 and 800 ppm [17]. However, in this study it was not possible to demonstrate any accompanying irreversible neurobehavioural and/or neuropathological changes. No changes in motor activity, passive avoidance, Morris maze, and radial arm maze were demonstrated in rats tested 2–4 months after 6 months' exposure to aromatic white spirit [17].

Another solvent, toluene, was also found to cause irreversible effects on regional and global neurotransmitter concentrations [18].

The present investigation was undertaken to gain insight into the causes of the previously demonstrated changes in global and regional concentrations of NA, DA, and 5-HT [14,17], and to study mechanisms related to aromatic white spirit-induced CNS neurotoxicity. For this purpose, synaptosomal neurochemical parameters were measured as indices of function in the CNS presynaptic region in vivo. The investigation consisted of 2 separate studies: a short-term study with immediate sampling and a long-term study with insertion of an exposure-free interval before samples were taken, to ensure that observed effects were long-lasting [18].

2. Materials and methods

2.1. Chemicals

Aromatic white spirit (CAS no. 64742-88-7) was purchased from Shell, Denmark (Mineralsk terpentin K-30), b.p. 148–200°C, 20 vol.% aromatics. Toluene content was zero [14].

In the North European type of white spirit the aromatics include C_8 (1.3%), C_9 (7.6%), C_{10} (5.2%), C_{11}–C_{12} (1.3%). The principal aliphatic alkanes are C_7–C_{12} (37%), the principal monocyclic alkanes are C_6–C_{12} (26%), and the dicyclic alkane (C_9–C_{12}) content is 9%. All percentages are w/w [14].

5-Hydroxy[sidechain-2-^{14}C]tryptamine creatinine sulphate (Cat. no. CFA 170, specific activity 57 mCi/mmol) was from Amersham. All other chemicals were of analytical grade.

2.2. Animals

Male rats (Mol:WIST), mean body weight 350 g, 3 months old, obtained from Møllegaard Breeding Centre Ltd., DK-4623 Ll. Skensved, Denmark,

were used in both studies. The group size was 10 in the short-term study (total = 30), and 7 in the long-term study (total = 21). In both studies the rats were weight-randomized into 3 groups and individually marked by ear clipping.

The rats were housed in stainless steelwire cages, 2 animals/cage, conventionally in animal rooms with automatic control of temperature (22 ± 1°C), relative humidity (55 ± 5%), air exchange (8 times/h), and fluorescent light (21:00 to 09:00 h), with free access to commercial pelleted diet (Altromin 1324, Brogården, Gentofte, Denmark) and citric acid acidified tap water in nipple bottles (pH = 3.5). During the daily exposure the food was removed.

The exposure schedule was similar in the two studies: one group was sham-exposed and served as controls, a second group was exposed to 400 ppm (2290 mg/m^3), and a third group to 800 ppm (4580 mg/m^3) of white spirit in the inhaled air for 6 h/day, 5 days/week for either 3 weeks or 6 months. The inhalation exposure conditions and the equipment used have previously been described in detail [20].

Rats exposed for 3 weeks were killed 18–20 h after the last exposure at 4 months of age. In the long-term study exposure was followed by an exposure-free period of 4 months' duration prior to sacrifice at 13 months of age.

2.3. Preparation of synaptosomes

Rats were decapitated in CO_2/O_2-narcosis. Whole brain minus cerebellum was quickly transferred to 10 ml ice-cold 0.32 M sucrose and weighed and a 10% (w/v %) homogenate was made. Synaptosomes were prepared at 0–4°C by conventional homogenization, differential and discontinuous sucrose density-gradient centrifugation techniques [21].

The synaptosomal band was isolated, diluted in ice-cold Krebs-Ringer incubation buffer (1 + 1) and centrifuged at 10 000 × *g* for 15 min at 0–4°C. The synaptosomal sediment was washed by resuspension in 10 ml ice-cold incubation buffer and subsequent centrifugation. The final sediment was resuspended in 10 ml ice-cold incubation buffer. Immediately hereafter, samples were taken and prepared for protein, neurotransmitter, and esterase determinations. Samples were stored at −20°C until analysis.

The composition of the 'extracellular' Krebs-Ringer incubation buffer was: NaCl 132 mM, KCl 6 mM, $CaCl_2$ 0.75 mM, $MgSO_4$ 1.3 mM, NaH_2PO_4 1.3 mM, and glucose 10 mM.

2.4. Neurochemical analysis

All neurochemical parameters were expressed as absolute content in whole brain minus cerebellum and as the content relative to protein.

Synaptosomal protein. The yield of synaptosomal protein was determined by the method of Lowry et al. [22] as modified by Hartree [23] using bovine serum albumin as the standard.

Synaptosomal neurotransmitter concentrations. Sample preparation: immediately after the final resuspension, 4 vol. resuspended synaptosomes were deproteinized by the addition of 1 vol. ice-cold 0.5 M perchloric acid and subsequent centrifugation. One aliquot of supernatant was used directly for the determination of 5-HT, another for NA and DA determinations after conventional purification on aluminium oxide [24]. *N*-ω-Methyl-5-HT (Sigma, M 1514) was the internal standard for the 5-HT determinations. Dihydroxybenzylamine (DHBA, Sigma D 7012) was internal standard for NA and DA determinations.

Instrumentation. NA, DA, 5-HT were determined by HPLC with electrochemical detection (HPLC-ED). The analytical equipment consisted of a Hewlett-Packard 1084B liquid chromatograph equipped with a 250 × 4 mm I.D. RP-18 Highbar, Supersphere LiChroCART (5 μm) analytical column protected by a 20 × 4 mm I.D. (5 μm) RP-18 guard column (both from E. Merck GmbH, Germany). Separation was achieved at ambient temperature by use of an acetonitrile modified citrate/octenyl sulphate buffer [25]. The flow was 1.0 ml/min. The detector was a Waters M 460 electrochemical detector applying an oxidation potential of +0.6 V. A 75-μl sample was injected.

Synaptosomal 5-HT uptake. Resuspended synaptosomes were stored at 0°C until incubation which was performed within 30 min after resuspension in a metabolic shaker in the Krebs-Ringer buffer at 37°C. Preincubation with the MAO-inhibitors pargyline (Sigma P 8013) and

Table 1
Yield of synaptosomes after exposure to 0, 400, or 800 ppm white spirit for 3 weeks, or 6 months followed by a 4-month exposure-free period

	mg protein/g tissue (relative)		mg protein (absolute)	
	3 weeks	6 months	3 weeks	6 months
Control	18.5 ± 1.8	15.9 ± 1.3	26.2 ± 2.0	27.2 ± 3.8
400 ppm	15.8 ± 3.0*	14.5 ± 1.4	22.8 ± 3.4*	24.8 ± 3.0
800 ppm	15.3 ± 1.9*	13.8 ± 1.4*	21.5 ± 3.1*	22.8 ± 2.1*

All values are expressed as mean ± S.D.; $n = 10$ in the 3-week study and $n = 7$ in the 6-month study. *$P < 0.05$ between values from exposed and control rats.

clorgyline (Sigma M 3778) (final concentration 100 μM of each) lasted 10 min prior to the addition of [^{14}C]5-HT/5-HT to a final transmitter concentration of 100 nM. The final protein concentration was approximately 100 μg/ml, in a final incubation volume of 2000 μl. After 60 s uptake, samples of 250 μl were taken. After 15 min of uptake, a second 250-μl sample was taken. Immediately after each sampling, synaptosomes were trapped by rapid vacuum filtration on 0.45-μm disposable filters (Millipore, Cat No. HAWP01300) and washed by the addition of 2 × 1000 μl ice-cold incubation buffer. Filters were removed from Millipore filterholders (Cat No. SX00 013 00) and transferred to 4 ml Optifluor (Packard Instruments). Radioactivity was counted in a Packard Tri-Carb 460 CD Liquid Scintillation System with quench- and luminescence-corrections.

By this method, 2 synaptosomal 5-HT uptake parameters were measured:

(1) The 5-HT uptake rate as reflected by the uptake after 60 s of incubation.
(2) The maximum 5-HT uptake capacity as reflected by the steady-state 5-HT concentration attained after 15 min of incubation.

Synaptosomal cholinesterase activities. Resuspensions were treated with 0.1% (w/v) Triton X-100 for 30 min at ambient temperature and centrifuged. The resulting supernatant was used for cholinesterase activity determinations. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity were determined at 37°C on a Shimadzu UV 160 by the use of commercially available kits: Boehringer Mannheim GmbH Art. no. 124 117 and Boehringer Mannheim Art. no. 124 133, respectively.

2.5. Statistical analysis

Data were entered into Quattro Pro® spreadsheet version 2.0 where calculations as presented in Tables 1–4 were performed. The SAS PC-

Table 2
Relative neurotransmitter concentrations after exposure to 0, 400, or 800 ppm white spirit for 3 weeks, or 6 months followed by a 4-month exposure-free period

	pmol/mg protein					
	NA		DA		5-HT	
	3 weeks	6 months	3 weeks	6 months	3 weeks	6 months
Control	10.6 ± 1.3	13.6 ± 2.5	9.43 ± 2.25	6.88 ± 1.21	6.69 ± 0.89	8.53 ± 1.65
400 ppm	14.0 ± 2.7*	20.8 ± 3.3*	13.1 ± 2.2*	10.3 ± 2.1*	9.08 ± 1.13*	12.5 ± 1.9*
800 ppm	14.9 ± 3.0*	24.1 ± 3.6*	14.7 ± 3.3*	14.6 ± 2.9*	10.0 ± 2.0*	15.4 ± 3.3*

All values are expressed as mean ± S.D.; $n = 10$ in the 3-week study and $n = 7$ in the 6-month study. *$P < 0.05$ between values from exposed and control rats.

Table 3
Absolute neurotransmitter amounts after exposure to 0, 400, or 800 ppm white spirit for 3 weeks, or 6 months followed by a 4-month exposure-free period

	pmol					
	NA		DA		5-HT	
	3 weeks	6 months	3 weeks	6 months	3 weeks	6 months
Control	279 ± 42	376 ± 105	245 ± 52	190 ± 56	176 ± 32	229 ± 40
400 ppm	314 ± 50	514 ± 79*	297 ± 48	253 ± 48	208 ± 47	309 ± 48*
800 ppm	320 ± 79	550 ± 98*	312 ± 67*	333 ± 77*	215 ± 48	350 ± 76*

All values are expressed as mean ± S.D.; $n = 10$ in the 3-week study and $n = 7$ in the 6-month study. *$P < 0.05$ between values from exposed and control rats.

version software package [26] was used for all further computations. Data were analyzed by analysis of variance (PROC ANOVA/GLM) followed by Dunnett's test where indicated. The general level of significance was set to 0.05. Therefore, no individual P values are given.

3. Results

3.1. Clinical signs during exposure

Data regarding general condition, survival, body weight, clinical chemistry, and food and water consumption in the long-term study have been presented earlier [17]. The general toxicity in the short-term study was comparable with respect to clinical symptoms, i.e. symptoms of acute narcotic effect.

3.2. Brain weight

There were no white spirit-induced effects on the weight of whole brain minus cerebellum after 3 weeks' or 6 months' exposure (data not shown).

3.3. Neurochemical analysis

Synaptosomal protein. The relative and absolute yields of synaptosomal protein were statistically significantly reduced in the 2 exposed groups in both experiments (Table 1).

Synaptosomal neurotransmitter concentrations. The relative synaptosomal NA, DA, and 5-HT concentrations were statistically significantly increased in both experiments at both exposure levels (Table 2). The absolute amounts of synaptosomal NA were statistically significantly increased at both exposure levels in the long-term experiment (Table 3). The absolute amounts of synaptosomal DA were statistically significantly increased in the highest dose group in both experiments (Table 3). The absolute amounts of synaptosomal 5-HT were statistically significantly increased in the long-term experiment at both exposure levels (Table 3).

Synaptosomal 5-HT uptake. The relative and absolute 5-HT uptake rates and capacities were statistically significantly increased in both experi-

Table 4
Relative synaptosomal 5-HT compartments after exposure to 0, 400, or 800 ppm white spirit for 3 weeks, or 6 months followed by a 4-month exposure-free period

Percentage of absolute 5-HT uptake capacity relative to total 5-HT content			
	Control	400 ppm	800 ppm
3 weeks	68.3 ± 18.5	74.7 ± 27.5	91.5 ± 14.2*
6 months	59.7 ± 15.0	59.3 ± 11.3	60.4 ± 16.1

All values are expressed as mean ± S.D.; $n = 10$ in the 3-week study and $n = 7$ in the 6-month study. *$P < 0.05$ between values from exposed and control rats.

Table 5
Relative synaptosomal cholinesterase activities at 37°C after exposure to 0, 400, or 800 ppm white spirit for 3 weeks, or 6 months followed by a 4-month exposure-free period

	mU/mg protein			
	AChE		BuChE	
	3 weeks	6 months	3 weeks	6 months
Control	52.0 ± 11.0	76.4 ± 14.3	205 ± 22	230 ± 65
400 ppm	56.0 ± 7.3	91.8 ± 15.7	246 ± 50	298 ± 21
800 ppm	61.8 ± 23.3	97.2 ± 18.0	266 ± 41*	241 ± 64

All values are expressed as mean ± S.D.; $n = 10$ in the 3-week study and $n = 7$ in the 6-month study. *$P < 0.05$ between values from exposed and control rats.

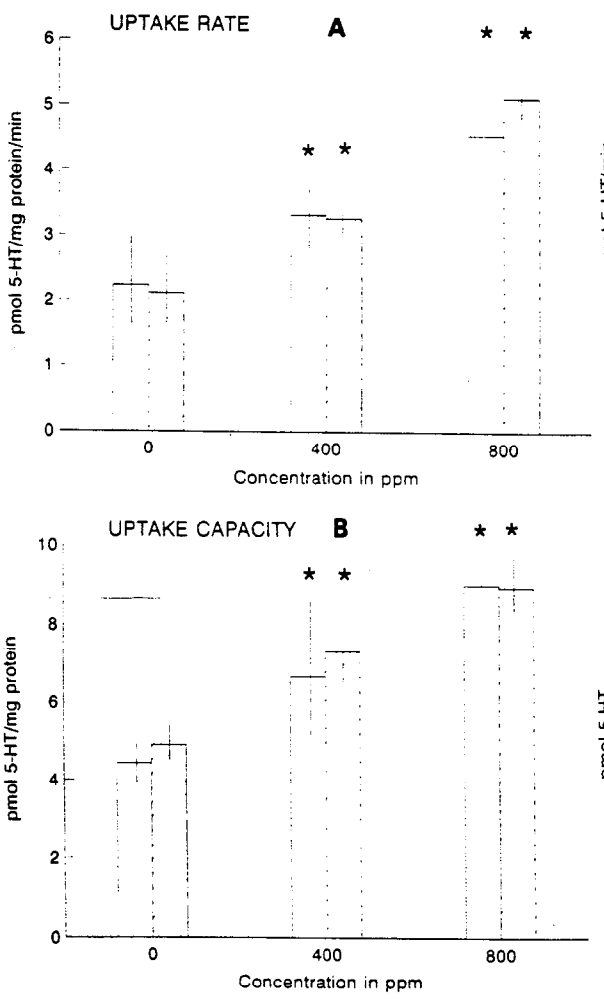

Fig. 1. The relative synaptosomal 5-HT uptake rate (A) and uptake capacity (B) in rats exposed to 0, 400 and 800 ppm for either 3 weeks (dotted) or 6 months (hatched). *Different from control; $P < 0.05$.

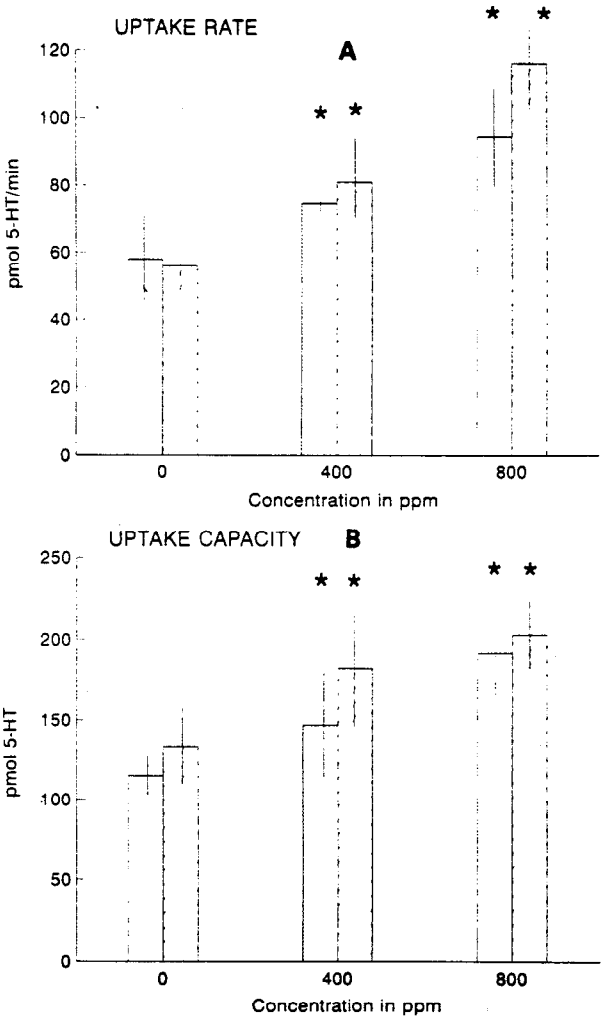

Fig. 2. The absolute synaptosomal 5-HT uptake rate (A) and uptake capacity (B) in rats exposed to 0, 400 and 800 ppm for either 3 weeks (dotted) or 6 months (hatched). *Different from control; $P < 0.05$.

ments at both exposure levels (Figs. 1A and B and 2A and B).

Synaptosomal 5-HT compartments. The relative synaptosomal 5-HT compartment (calculated as the percentage of absolute 5-HT uptake capacity relative to the total 5-HT content in the synaptosomal fraction of whole brain minus cerebellum) was significantly increased in the highest dosed group in the short-term experiment (Table 4). No changes were found in the long-term study.

Synaptosomal cholinesterase activities. The relative synaptosomal BuChE activity was statistically significantly increased at the highest exposure level in the short-term experiment (Table 5). There was no effect on the relative AChE activity due to the exposure (Table 5).

The absolute AChE or BuChE activities were unaffected in both experiments (data not shown).

4. Discussion

Two characteristic solvent-induced human CNS neurotoxicity syndromes exist. One is the CNS depressive effect (narcotic) induced by short-term exposure, the other is the irreversible encephalopathy which is a result of long-term exposure [3]. The present 2 studies attempt to simulate exposure situations believed to result in these 2 kinds of solvent neurotoxicity.

NA, DA, and 5-HT were chosen because of earlier reports on global and regional changes in the rat brain of these neurotransmitters induced by inhalation exposure of aromatic white spirit for 3 weeks or 6 months at concentrations of 400 and 800 ppm [14,17].

The possible effects on the cholinergic system were investigated by measurement of cholinesterase activities.

Synaptosomes constitute a well-established ex situ neurochemical research model for the presynaptic nerve terminal in situ. Isolated synaptosomes kept under ice-cold conditions from decapitation until sampling for neurochemical analysis have been proposed to represent a 'freezing' of the conditions in the presynaptic nerve terminal in situ at sacrifice [27].

The exposures to white spirit did not affect the weight of whole brain minus cerebellum in accordance with previous findings [14,17], whereas the relative (synapse density) and absolute (total synapse number) yields of synaptosomal protein were reduced in the 2 exposed groups (Table 1). Such effects of an organic solvent have not previously been published. It may be hypothesized that white spirit exposure for 3 weeks or for 6 months reduced the density and the total number of inter-neuron connections in situ, assuming that the synapse protein content is unchanged, and that the yield of synaptosomes in the homogenization and isolation procedures and the protein determination are uncompromized.

In both studies, the reduced yields of synaptosomal protein were accompanied by increased intrasynaptosomal NA, DA, and 5-HT contents (Tables 2 and 3). Such effects induced by organic solvents have not previously been reported. These phenomena suggest compensatory increased neurotransmitter release at nerve impulse transmission or increased activity of corresponding neurons.

In both studies, increased 5-HT uptake rates and 5-HT uptake capacities were found (Figs. 1 and 2). This could be the mechanism for the increased intrasynaptosomal 5-HT concentration and might explain the increased global and regional 5-HT concentrations reported previously. Similar effects might also be induced on high-affinity NA and DA uptake rates and capacities, which were not determined in this study [14,17]. Such increases could probably explain the increased global and regional NA and DA concentrations reported previously. No data about white spirit-induced effects on these NA and DA uptake parameters have been reported. The increased synaptosomal 5-HT uptake rate was in accordance with results in a study by Beving et al. [28] which demonstrated increased maximal 5-HT uptake rate of platelets isolated from occupationally exposed car painters. However, the increased 5-HT uptake capacities and contents are not in agreement with the results of a study in solvent-intoxicated workers, which showed unchanged 5-HT platelet concentrations [29]. Thus, the use of platelets as an alternative human model for the human presynaptic CNS nerve terminal needs further evaluation.

There was a statistically significant increase in the absolute 5-HT uptake capacity relative to the total endogenous 5-HT content due to 3 weeks of exposure to 800 ppm (Table 4). The endogenous 5-HT content was unaffected (Table 3). Synaptosomes spontaneously release 5-HT during incubation. Liberated endogenous 5-HT is substituted by labelled 5-HT during the incubation and a steady state is obtained after 15 min of incubation. Therefore, this finding (Table 4) suggests that there is an increased compartment of spontaneous releasable 5-HT ex situ due to 3 weeks of exposure to 800 ppm. The potassium stimulated synaptosomal 5-HT release was not measured.

If this effect can be extrapolated to the amount released by stimulation in situ, there is a great potential for affecting CNS function. The effect either reflects a dysfunction or a compensation.

A slight but statistically significant increase in the relative BuChE activity was found in the highest dosed group in the short-term experiment (Table 5). No effect on the relative AChE activity was revealed. The function of BuChE in CNS is not known. Therefore, at present it is not possible to interpret this finding in relation to neurotoxicity, so this data collection should be considered as an empirical finding. Anyway, the unchanged AChE activity indicates that there are no dramatic changes in the cholinergic system. However, no data of the choline acetyltransferase activity (CAT) were obtainable due to undetectable activity (data not shown).

The relation between dendritic length, dendritic spines, and synapses in neurotoxicity is very complex and the present knowledge does not allow a final interpretation of the results in terms of reduced synapse density [30]. A possible general mechanism for a reduced synapse density could be the induction of oxidative stress in the synapse region as reported previously for the dearomatized type of white spirit [15]. Ideally, a specific presynaptic marker should be measured to challenge the assumption that reduced protein yields imply reduced density and total number of synapses and not reduced protein content per synapse, and to test the possible compensatory mechanisms. Morphological examination could also be used to illuminate these questions.

The use of an occupationally relevant complex mixture of solvents in this study necessarily complicates the understanding of the mechanisms underlying the results obtained. The possibility of interaction of some of the constituents precludes the extrapolation of data obtained from single solvents to the evaluation of the hazard posed by exposure to a heterogeneous mixture of organic solvents such as white spirit. No data are available on the potential interactions of the constituents. An essential question for the assessment of neurotoxicity is whether the induced changes represent neurotoxic effect. No generally accepted neurochemical, electrophysiological, pathological, or behavioural parameter or test system is as yet established to completely document or predict CNS adversity or neurotoxicity. The behavioural tests that were performed in the long-term experiment did not reveal effects caused by exposure to aromatic white spirit [17].

The present findings of similar changes in yield of synaptosomal protein, and neurotransmitter concentrations after 3 weeks' and 6 months' exposure suggest that the changes induced after 3 weeks are a pattern of changes which partly may be an explanation for some of the symptoms seen in the chronic toxic encephalopathy.

We interpret these changes as an indication of toxic effect on the CNS function and consider that these results support our recent findings of electrophysiological changes and affected motor activity following 6 months' exposure to dearomatized white spirit followed by an exposure-free period [16]. Unfortunately, no electrophysiological measurements were performed when we conducted the aromatic white spirit experiment years ago [17].

In conclusion, this study demonstrates significantly reduced relative and absolute yields of synaptosomal protein due to 3 weeks' and 6 months' exposure to aromatic white spirit. Furthermore, both dosing regimes caused increased synaptosomal NA, DA, and 5-HT concentrations, high-affinity 5-HT uptake rate, and uptake capacity. We interpret these changes as a neurotoxic effect induced by white spirit exposure and suggest that the pattern of these induced changes after 3 weeks of exposure may contribute to the symptomatology of the chronic toxic encephalopathy.

Acknowledgements

The authors wish to express their gratitude to Morten Falding, Joan Gluver, Else-Marie Skov Jensen, Vibeke Kegel, Vibeke Kjaer, Annette Landin, Karen Roswall, and Lejf Burkal for skilled technical assistance in the conduction of the study.

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XIV. TABLES

Table I

Effects of 3 weeks of aromatic white spirit inhalation exposure on regional rat brain glial fibrillary acidic protein (GFAP) concentrations in young adult (5 months old at the beginning of exposure) and 'middle-aged' rats (14 months old at the beginning of exposure).

	µg GFAP/mg SDS-soluble protein		
Exposure	Control	400 ppm	800 ppm
Hippocampus, young adult	2.33±0.62	2.48±0.63	2.77±0.60
Hippocampus, 'middle-aged'	2.21±0.77	1.84±0.44	1.83±0.45
Hemisphere, young adult	2.59±0.59	2.51±0.53	2.45±0.90
Hemisphere, 'middle-aged'	2.37±0.70	2.71±0.64	2.89±0.75

All values are expressed as mean±S.D. n=12 for both the young adult and the 'middle-aged' rats.

Table II

Effects of 6 months inhalation exposure to aromatic white spirit on regional and calculated whole rat brain glial fibrillary acidic protein (GFAP) concentrations.

µg GFAP/mg SDS-soluble protein

Exposure	Control	400 ppm	800 ppm
Cerebellum	9.08±1.38	8.12±1.28	9.04±0.80
Hemisphere	3.33±0.66	3.45±0.46	2.90±0.22
Hippocampus	2.69±0.39	2.66±0.38	2.23±0.40
Hypothalamus	0.38±0.11	0.40±0.13	0.29±0.05
Pons	4.25±1.37	4.08±0.70	4.64±0.85
Thalamus	7.25±1.45	8.92±1.18	9.12±1.79
Med. obl.	12.2±0.76	13.3±1.25	13.7±2.32
Whole brain	6.06±0.75	6.82±0.77	6.74±0.83

All values are expressed as mean±S.D. n=7.