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Effect of Experimental Hepatic Encephalopathy on Striatal and Cortical Glutamatergic Modulation of Dopaminergic Neurotransmission

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ACADEMIC DISSERTATION

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CONTENTS

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II. Borkowska, H.D., Oja, S.S., Hilgier, W., Saransaari, P. and Albrecht, J. (2000) Effect of acute hepatic encephalopathy on [^{3}H]dopamine release from rat cerebral cortex and striatum in vitro: role of Ca^{2+}. Acta Neurobiol. Exp. 60:1-7.

III. Borkowska, H.D., Oja, S.S., Oja, O.S., Saransaari, P., Hilgier, W. and Albrecht, J. (1999) N-Methyl-D-aspartate-evoked changes in the striatal extracellular levels of dopamine and its metabolites in vivo in rats with acute hepatic encephalopathy. Neurosci. Lett. 268:151-154.

IV. Borkowska, H.D., Albrecht, J., Saransaari, P. and Oja, S.S. (1998) Ionotropic glutamate receptors and dopamine release in the frontal cortex in experimental hepatic encephalopathy. Proc. West. Pharmacol. Soc. 41:107-109.

V. Saransaari, P, Oja, S.S., Borkowska, H.D., Koistinaho, J., Hilgier, W. and Albrecht, J. (1997) Effects of thioacetamide-induced hepatic failure on the N-methyl-D-aspartate receptor complex in the rat cerebral cortex, striatum and hippocampus: binding of different ligands and expression of receptor subunit mRNAs. Mol. Chem. Neuropath. 32:179-193.

VI. Oja, S.S., Borkowska, H.D., Albrecht, J. and Saransaari, P. (1996) Kainate and AMPA receptors in the rat brain in thioacetamide-induced hepatic encephalopathy. Proc. West. Pharmacol. Soc. 39:15-17.

ABBREVIATIONS

AMPA	2-amino-3-hydroxy-5-methyl-4-isoxalepropionate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
cGMP	cyclic guanosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DOPAC	dihydroxyphenylacetate
EDTA	ethylenediaminetetra-acetate
EEG	electroencephalography
GABA	γ-aminobutyric acid
HE	hepatic encephalopathy
HEPES	N-hydroxyethylpiperazine-N'-2-ethanesulfonate
HPLC	high-pressure liquid chromatography
HVA	homovanillate
L-AP4	L-2-aminophosphonobutyrate
MAP-2	microtubule-associated protein-2
mGluR	metabolic glutamate receptor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
TAA	thioacetamide
TCP	N-1[-(2-thienyl)cyclohexyl]piperidine

ABSTRACT

Acute or chronic hepatic failure leads to a syndrome called hepatic encephalopathy (HE), associated with ammonia overload. Clinical observations and animal experiments have provided general biochemical and morphological characteristics of HE but the pathomechanism of this disease remains unclear. Advanced HE is accompanied by extrapyramidal symptoms, including rigidity and tremor, which are thought to reflect depression of the striatal dopaminergic system. On the other hand, the dopaminergic system is known to be controlled by glutamatergic inputs. The aim here was to examine the effect of acute experimental HE provoked in rats by administration of a hepatotoxin, thioacetamide, on the interactions of glutamatergic and dopaminergic transmission.

The release of exogenous preloaded [^{3}H]dopamine from brain slices was assayed in a superfusion system *in vitro*. Experimental HE increased the high-K^+-induced, Ca^{2+}-dependent dopamine release from cerebral cortical and striatal slices and synaptosomes. In the absence of Ca^{2+}, experimental HE reduced the high-K^+ stimulated dopamine release only in frontal cortical slices. In striatal slices, experimental HE reduced the N-methyl-D-aspartate (NMDA)- and kainate-stimulated dopamine release, while in frontal cortical slices only the kainate-stimulated release was increased. Stimulation of dopamine release by NMDA was completely blocked by the NMDA receptor antagonist dizocilpine in preparations derived from both control and experimental HE rats. The kainate-stimulated release was blocked completely in striatal slices by the potent kainate and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), while in frontal cortical slices the inhibitory effect was only partial. Application of CNQX and dizocilpine together blocked the stimulatory effect of kainate in the frontal cortex. HE did not affect the release stimulated by AMPA.

The release of endogenous dopamine and dopamine metabolites was studied *in vivo.* Experimental HE lowered the concentration of the dopamine metabolite homovanillic acid (HVA) in the effluents. NMDA and high-K^+ increased the extracellular dopamine level and lowered the level of HVA and that of another dopamine metabolite, dihydroxyphenylacetic acid (DOPAC), in both control and experimental HE rats. Experimental HE reduced the inhibitory effect of NMDA on the level of HVA by 45% and slightly attenuated the stimulatory effect of NMDA on the level of dopamine. Experimental HE did not significantly reduce the high-K^+ stimulated dopamine release.

The functional properties of different classes of glutamate receptors were studied in experiments in which the binding of specific radioligands to the striatal and frontal cortical receptors was determined under different experimental conditions *in vitro*. In striatal membranes, experimental HE reduced the B_{max} of ^{3}H-labeled N-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) binding, kainate- and NMDA-displaceable [^{3}H]glutamate binding and the K_D of NMDA-displaceable [^{3}H]glutamate binding. Also in striatal membranes, experimental HE increased the K_D of [^{3}H]TCP binding. In frontal cortical membranes experimental HE increased the B_{max} of NMDA-, AMPA- and glycine-displaceable [^{3}H]glutamate binding and the K_D of NMDA-displaceable [^{3}H]glutamate binding.

The results show that experimental HE affects the cerebral dopaminergic system and these changes in functions partly reflect its altered glutamatergic modulation.

INTRODUCTION

Hepatic encephalopathy (HE), a neuropsychiatric syndrome arising from acute or chronic liver dysfunction and related to an increase in blood and brain ammonia, is characterized by extrapyramidal symptoms including rigidity and tremor. Despite substantial advances in knowledge of the neurological and biochemical changes in this disorder, the pathogenesis of HE remains unclear. One possible mechanism responsible for HE might be reflected in the fact that an imbalance between the excitatory amino acid glutamate and the inhibitory catecholamine serotonin appears to predominate in HE. However, the major symptoms of HE, motor abnormalities, are thought to reflect depression of the striatal dopaminergic system, i.e., a substantial loss of the D_2 class of striatal dopamine receptors, an increased striatal content of dopamine metabolites and an enhanced activity of the dopamine-catabolizing enzyme monoamine oxidase B. In the healthy brain, the striatal release of dopamine is modulated by the excitatory glutamatergic input. Dopamine release is stimulated mainly by activation of the N-methyl-D-aspartate (NMDA) class of glutamate receptors, but also by activation of kainate and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. Disturbances in glutamate metabolism and transport and receptor interactions appear to play an important role in the pathomechanisms of HE.

The hypothesis of this study was that the extrapyramidal symptoms of HE are associated with impaired glutamatergic modulation of striatal dopamine release. In order to elucidate the role of striatal dopamine in the motor dysfunctions in HE, a detailed analysis was undertaken of the effects of HE on those elements in the glutamatergic systems which may modulate dopaminergic neurotransmission, particularly in the striatum.

REVIEW OF THE LITERATURE

1. Hepatic encephalopathy

Encephalopathy is to be distinguished from encephalitis as a brain dysfunction or damage resulting from exposure to toxic or infective agents without the involvement of an inflammatory process. The neurological symptoms resulting from degeneration of liver cells are defined as hepatic encephalopathy, due to acute liver failure or chronic liver disease. The liver is the largest organ in the body, exerting many complex functions such as filtering and destruction of bacteria, detoxification of drugs, alcohol or environmental poisons and regulation of defenses against septic challenge [Ikeda et al. 1992, Butterworth 1996]. The mechanisms underlying liver damage include chemical, immunological and mechanical injuries. Injury-related apoptosis is likewise an important potential mechanism.

2. Symptoms of hepatic encephalopathy

The connection between yellow liver injury and clinical neurologic symptoms was first described by Frerichs in 1861 [Diemer 1978]. Since then, the pathogenic mechanisms responsible for HE have been increasingly widely studied but would seem to remain unclear. Neurotoxins in blood serum, for example aromatic amino acids, free fatty acids, mercaptan and, above all, ammonia have been attributed the main role [Butterworth et al. 1987, Zieve 1987, Weissenborn 1992, Hawkins and Mans 1994, Jones and Basile 1997].

2.1. Clinical manifestations

The clinical manifestations of HE are divided into four stages [Zieve 1987, Jones and Weissenborn 1997, Jalan and Hayes 1997, Albrecht and Jones 1999, Shakil et al. 1999]. In the least severe stage I, euphoria or depression manifests itself. There may emerge mild confusion, untidiness, slowing of mental functions, irritability and disorders in sleep rhythm and speech. Patients usually exhibit a normal electroencephalographic pattern and have only a slight tremor. As HE progresses to stage II, the earlier alterations are accentuated. In addition there occur lethargy, drowsiness, inappropriate behavior, intermittent disorientation, lack of sphincter control and often a slowing of the electroencephalogram. The "flapping tremor", asterivis, is also observed, although it is not specific for HE [Adams and Foley 1949]. Characteristic of stage III is more or less continuous sleep (though the patient can be awakened), incoherent speech, persistent disorientation, amnesia, pronounced confusion and tremor which is noticed if the patient is able to cooperate. At this stage, the encephalogram is always irregular. In stage IV, patients are comatose, may or may not respond to painful stimuli and tremor is usually not testable.

HE, like many other liver diseases, is associated with fetor hepaticus [Muting and Sommer 1979], hypothermia [Gayed 1987, Margolis 1979], hyperventilation [Strauss et al. 1998] and lack of appetite (leading to a loss of muscle mass). In addition, the composition of the plasma is altered. The level of prolactin [Langer et al. 1981, McClain et al. 1981, Bauer et al. 1983, Masala et al. 1985, Bianchi et al. 1992] and the peripheral elimination

rate of growth hormone [Muggeo et al. 1979, Langer et al. 1981, Bauer et al. 1982] are increased, both possibly as a result of abnormalities in the dopaminergic system.

2.2. Neurotoxins and brain cell destruction

2.2.1. Role of ammonia in HE

HE impairs the functions of the blood-brain barrier, which favors the penetration of neurotoxins into the central nervous system. The level of ammonia in peripheral blood has been noted to be elevated in the majority of human patients with hepatic failure or compromised portal blood flow, in particular in those with disturbed consciousness [Gabuzda and Davidson 1969, Ehrlich et al. 1980, Rossle et al. 1984] and also in animal HE models [Zeneroli et al. 1991, de Knegt et al. 1993, Matsushita et al. 1999]. The ammonia concentration in the brain has been found to be increased from 0.05-0.1 mM in control animals to 1-5 mM in animal models of acute and chronic HE [Vogels et al. 1997a, Butterworth 1998]. The increased ammonia level finally induces coma in patients whose immunological system is not strong and in some animal models [Ehrlich et al. 1980, Rossle et al. 1984, Dejong et al. 1993, Kanamori et al. 1996, Mullen et al. 1997].

Source and elimination of ammonia in a healthy organism

Ammonia is continuously produced in tissues by amino acid metabolism. A part of it arises from the action of intestinal bacteria on nitrogen-containing substrates derived from nutritional proteins and from urea in the alimentary secretions. The ammonia produced is absorbed into portal blood. Its concentration in portal venous blood in healthy subjects varies from 300 to 600 μM. Ammonia metabolism in the hepatic cells reduces the content in the blood draining from the liver to 20 to 60 μM. In the liver, ammonia metabolism shows regional heterogeneity. In periportal hepatocytes, ammonia, generated from glutamine by phosphate-activated glutaminase, is converted to urea, which is subsequently excreted by the kidneys into the urine. In perivenous hepatocytes, ammonia is either incorporated by glutamine synthase to glutamate to form glutamine, or to oxo acids, e.g. oxaloacetate and 2-oxoglutarate, forming aspartate and glutamate [Häussinger et al. 1990].

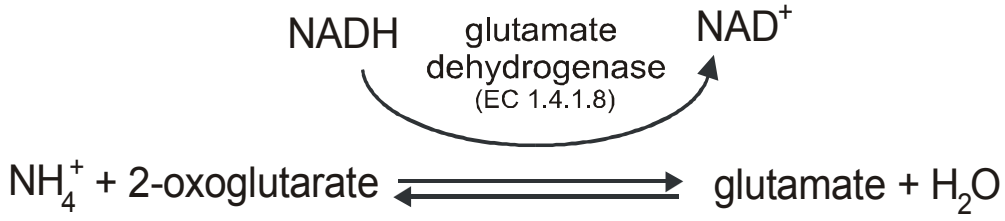


Fig.1. Reducing amination of 2-oxoglutarate.

Influence of ammonia on the brain during liver failure

By and large, ammonia penetrates into all organs in HE when the concentration in the arterial blood is high. Although the brain is normally partly protected from this neurotoxin, increased passage of ammonia across the blood-brain barrier has been noted in cirrhotic patients [Butterworth et al. 1995a, Larsen 2001]. Such subjects are hypersensitive to high-protein diets or gastrointestinal bleeding (both of which increase blood ammonia) and show neurological dysfunction proportional to the ammonia concentration in the blood [Butterworth 1993, Butterworth 1996]. This may in some instances be exacerbated, as noted in experimental HE [Lockwood et al. 1980] and HE [Lockwood et al. 1991], by increased ammonia penetration across the blood-brain barrier. In the brain, the major route of ammonia metabolism is its incorporation into glutamate to form glutamine. A portion of ammonia is metabolized via reductive amination of 2-oxoglutarate to glutamate. Both reactions form the two legs of the glutamate and glutamine cycle, which is compartmented between astrocytes and neurons, with glutamine synthesis occurring almost exclusively in astrocytes [Wastergaard et al. 1995]. Most recent evidence implicates glutamate transamination to alanine as an important step in the intercellular transfer of ammonia [Waagepetersen et al. 2000]. It was surmised forty years ago that significant urea synthesis also occurs in the brain [Sporn et al. 1959), but this would seem in fact to be only marginal [McIlwain and Bachelard 1971].

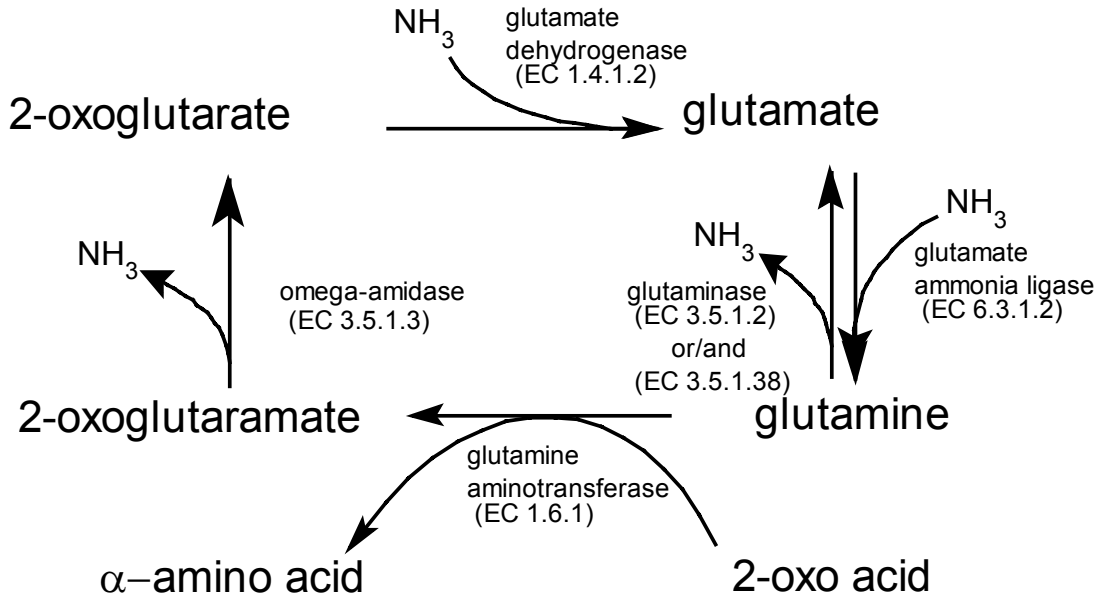


Fig. 2. Ammonia metabolism in the brain [modified from McIlwain & Bachelard 1971].

Mechanisms of ammonia toxicity

Hyperammonemia alters brain functions by both direct and indirect mechanisms [Butterworth 1993]. Ammonia disturbs both postsynaptic inhibition and excitatory neurotransmission in the brain and spinal cord, first by blocking chloride efflux from postsynaptic neurons [Raabe 1989] and second by a reversed depression of synaptic transmission (e.g., from Schaffer collaterals to CA1 neurons in the hippocampus) [Raabe 1989, Fan et al. 1990]. It can inhibit the neuronal or astrocytic uptake of glutamate or affect the postsynaptic glutamate receptors [Butterworth 1993]. In addition, the firing of

glutamatergic neurons in the CA1 region of the hippocampus evoked by iontophoretically applied glutamate is abolished by ammonia *in vivo*. This finding has given impetus to the proposal that ammonia exerts direct postsynaptic actions [Fan 1990, Szerb and Butterworth 1992]. HE can also affect neurotransmission by altering the functions of the systems transporting neutral amino acids across the blood-brain barrier [Hawkins and Mans 1994]. Hyperammonemia can also affect the substrate levels for the synthesis of the amine neurotransmitters dopamine, noradrenaline, serotonin and histamine [Basile et al. 1991a, Hawkins and Mans 1994]. Ammonia-induced changes in the glutamate-glutamine cycle enzymes, including glutamine synthetase (EC 6.3.1.2), have obvious implications for the functioning of the glutamatergic system. Hyperammonemia can also cause cerebral energy failure by affecting the tricarboxylic acid cycle, increasing the activity of 2-oxoglutarate dehydrogenase (EC 1.5.1.7) [Therrien et al. 1991, Yao et al. 1987], elevating the lactate concentration [Hindefelt et al. 1977, Therrien et al. 1991] or radically enhancing glutamine synthesis [Schenker et al. 1967]. A high level of ammonia can also reduce the brain adenosine triphosphate (ATP) content and cause coma [Hindfelt et al. 1977]. Ammonia toxicity alters the transfer of water and electrolytes through neuronal cell membranes [Cordoba and Blei 1996] and alleviates brain damage caused by other factors [Basile et al. 1991a]. Other factors involved in indirect ammonia toxicity in the brain, recently seriously considered, pertain to the levels of glutamine and 2-oxoglutarate [Duffy and Plum 1975, Watanabe et al. 1984, Hawkins and Mans 1994].

Morphological observations on the brain have indicated degenerative **astroglial changes**. HE foments cell proliferation, and causes hypertrophy, slight destruction of the form of klasmatodendrosis, chromatin margination and enlarged nuclei [Hilgier et al. 1983, Norenberg et al. 1991, Butterworth 1998, Albrecht and Jones 1999, Matsushita et al. 1999]. Acute HE pathology is connected to general astrocytic swelling rather than to the Alzheimer II -type astrocytosis characteristic of chronic liver disease [Butterworth 1998, Blei and Larsen 1999, Matsushita et al. 1999]. An ammonia-dependent high level of extracellular glutamate (see below for details), resulting in NMDA-mediated neurotoxicity, and a high concentration of glutamine [Rothman and Onley 1987, Rao et al. 1995b, Laubenberger et al. 1997, Butterworth 1998, Hilgier et al. 1999] lead to insufficient cellular osmoregulation and increased intracellular levels of water, sodium and chloride [Choi 1985, Traber et al. 1987, Miñana et al. 1997, Vogels et al. 1997a]. This process finally leads to swelling of astrocytes, which increases intracranial pressure and causes brain edema and death due to brain stem herniation [Traber et al. 1987, Kato et al 1992, Vogels et al. 1997a].

In summary, ammonia at different concentrations gives rise to a number of symptoms such as clonic convulsions, dementia and coma. However, ammonia cannot be the sole factor underlying HE symptoms. Other neurotoxins and the failure of neurotransmission also play essential roles. In particular, in some human patients [Philips et al. 1952, Fischer 1974] and animal models [Hilgier and Mossakowski 1979] HE has not been accompanied by hyperammonemia.

2.2.2. Toxic role of glutamine

Several lines of evidence implicate glutamine as a mediator of ammonia neurotoxicity in general, and a major contributor to astrocytic swelling, and subsequently to the increase in the water content of the whole brain seen in particular in acute hyperammonemic conditions. Glutamine synthesis from ammonia and glutamine occurs in

a reaction catalyzed by glutamine synthetase, which is an astrocyte-specific enzyme. Studies with cultured astrocytes have revealed that ammonia controls the rate of glutamine synthetase-mediated reaction. Accordingly, an increased accumulation of glutamine in the brain is the rule in a whole spectrum of conditions associated with hyperammonemia, and interventions aimed at reducing brain glutamine content have been shown to ameliorate neurological symptoms. Treatment of hyperammonemic rats with moderate (nontoxic) doses of the glutamine synthetase inhibitor L-methionine-D,L-sulfoximine has been shown to normalize cerebral glutamine content, to prevent an increase in the brain water content, and to reduce cerebrovascular CO_2 responsiveness and astrocytic swelling. In addition, inhibition of glutamine accumulation by methionine sulfoximine has counteracted ammonia-induced cerebral oxygen consumption and glucose utilization [Albrecht and Jones 1999, and references there].

The mechanism underlying glutamine neuro(glio)toxicity remains largely unknown. In the simplest terms, the toxic effects could be tentatively ascribed to an action of glutamine as an osmolyte whose excessive accumulation will drive an excess of osmotically obligated water to different cellular and subcellular compartments. Indeed, a recent study from our laboratory [Ziemińska et al. 2000] has indicated that mitochondrial swelling and the subsequent activation of mitochondrial permeability transition may be potential mechanisms by which glutamine induces metabolism disturbances in the brain in hyperammonemic conditions.

2.2.3. Toxic role of manganese

Manganese is another compound which presumably plays an important toxic role in HE [Butterworth 1996]. Manganese does not normally damage the brain because the trace amount of this metal ion in the blood is bound to plasma proteins, which impedes its permeation across the blood-brain barrier [Rabin et al. 1993]. Natural and experimental HE increases the manganese concentration in the serum [Versieck et al. 1974, Spahr et al. 1996] and globus pallidus [Kulisevky et al. 1992, Butterworth et al. 1995a, Weissenborn et al. 1995] by hampering the removal of this metal from the organism through the hepatobiliary route [Hazell and Butterworth 1999] and by facilitating its penetration through the blood-brain barrier due to a decrease in the binding of manganese to plasma proteins [Butterworth et al. 1995a]. A high manganese level affects the glutamatergic transmitter systems by lowering glutamate uptake into astrocytes [Hazell and Norenberg 1997] and by inhibiting the cerebral energy metabolism due to an increase in the activity of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) [Hazell et al. 1999]. Manganese may also play a role in the proliferation of Alzheimer II cells by reason of the marked capacity of astrocytes to accumulate manganese [Pentschew et al. 1963, Ashner et al. 1992]. Chronic manganese intoxication causes degeneration of the basal ganglia [Yamada et al. 1986] and selectively affects the dopaminergic transmitter systems. The dopamine concentration is reduced [Bird et al. 1984], dopamine is depleted from storage vesicles in presynaptic neurons [Lista et al. 1986] and the release of dopamine from nerve endings [Drapeau and Nachshen 1984] and the activity of monoamine oxidase are increased [Leung et al. 1981, Subhash and Padmashree 1991]. This indicates that manganese, together with ammonia, participates in the genesis of HE-induced brain disorders in HE.

2.2.4. Mercaptans, phenols and fatty acids

High concentrations of mercaptans, phenols and fatty acids are characteristic of human encephalopathy patients and animals with liver disease [Zieve and Nicoloff 1975, Brunner and Siehoff 1976, Teyshenne et al. 1976, Doizaki and Zieve 1977, Rabinowitz et al. 1978]. Each of them may induce coma or cerebral edema, with impaired central nervous system functions [Basile et al. 1991a, Weissenborn 1992]. Simultaneously and synergistically acting they make for a dramatic increase in mortality [Zieve 1984].

2.3. Dysfunction of neurotransmission

HE is characterized by disturbances in several neurotransmitter systems in the brain. In the following, special emphasis is laid on alterations in dopaminergic and glutamatergic neurotransmission. In this section, a brief overview of a number of other systems is also provided.

An increased concentration of aromatic amino acids (phenylalanine, tyrosine and tryptophan) [Hirayama 1971, Fisher et al. 1975, James et al. 1978, James et al. 1979, Mans et al. 1982] or probably even more important, a disproportion of them in relation to the branched-chain amino acids (leucine, isoleucine and valine) [Soeter and Fisher 1976, Cascino et al. 1982] may precipitate hepatic encephalopathy by providing **false neurotransmitters** (phenylethanolamine, octopamine, tyramine) [Fisher and Baldessarini 1971, Albrecht 1984b, Colombo et al. 1996]. These false transmitters compete for the uptake of dopamine into neurons or foment its release from storage vesicles [Riordan and Williams 1997].

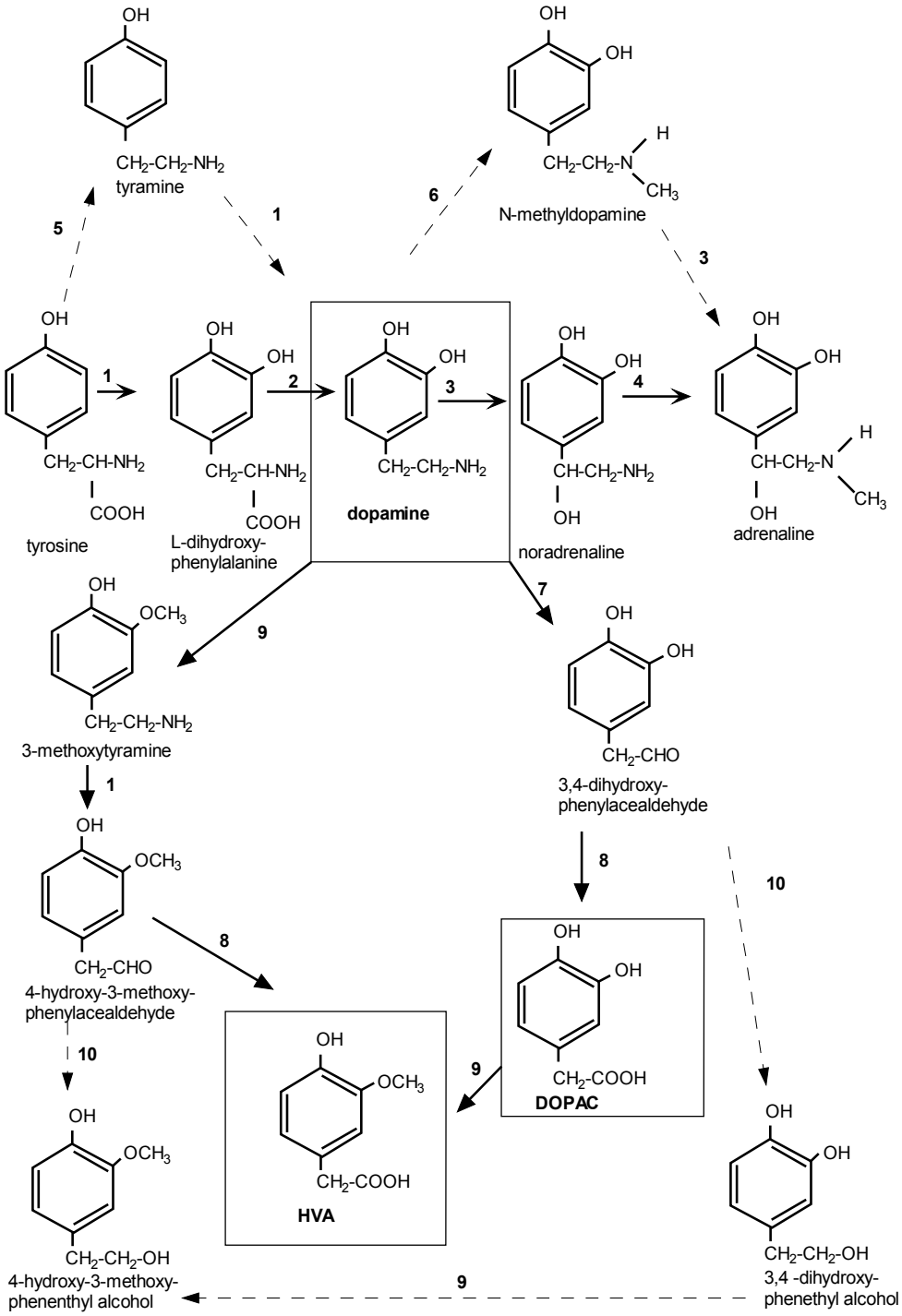
It has been surmised that one reason for the impairment of motor functions and the lowered level of consciousness in HE is increased **GABA**ergic tone [Anderson 1984, Shafer et al. 1984, Basile et al. 1991b,]. Elevated levels of γ-aminobutyric acid (GABA) and/or those of ammonia in the brain lead to alterations in the functions of postsynaptic $GABA_A$ receptors [Albrecht and Jones 1999, Hazell and Butterworth 1999]. In addition to this, an elevated concentration of endogenous benzodiazepines may activate the GABA/benzodiazepine receptor complex [Ferenci et al. 1984a, Mullen et al. 1990, Gitlin 1996]. However, no clear correlation obtains between the level of benzodiazepine receptor ligands in the blood and the clinical response to benzodiazepine antagonists in HE patients [Butterworth et al. 1995b].

Some neuropsychiatric symptoms, e.g. the altered sleep pattern, may result from modifications of inhibitory **serotoninergic** neurotransmission. In HE the concentrations of tryptophan, serotonin and its metabolite 5-hydroxyindoloacetate are increased in the brain of human patients [Bergonzi et al. 1978, Baldy-Moulinier et al. 1981, Borg et al. 1982, Bergeron et al. 1989] and experimental animals [Colombo et al. 1996, Yurdaydin et al. 1996, Butterworth 1998, Wikell et al. 1998, Yurdaydin et al. 1990]. Binding of ligands to postsynaptic serotonin receptors is increased in human patients [Rao and Butterworth 1994] and hyperammonemic mice [Robinson et al. 1992]. Studies with serotonin receptor antagonists have also implicated hypoactivity of the serotoninergic system in the pathogenesis of HE.

2.3.1. Dopamine

Dopamine was long considered to be only a precursor of noradrenaline. Since the pioneering studies of Blaschko (1957), however, dopamine has also been recognized as an important neurotransmitter of its own in the mammalian brain. It is synthesized mainly in presynaptic parts of a neuron, but possibly also in axons. Dopamine is stored in vesicles in nerve endings at a concentration of about 0.1 M (the level is 10-1000 times higher than that in the cytosol). It may also be synthesized in dendrites and stored there in the vesicles and endoplasmic reticulum [Elsworth and Roth 1997]. In dopaminergic neurons L-tyrosine is first hydroxylated by the rate-limiting enzyme tyrosine 3-monooxygenase (EC 1.14.16.2) to L-3,4-dihydroxyphenylalanine, which is then decarboxylated to L-dopamine by aromatic-L-amino-acid decarboxylase (EC 4.1.1.28) (Fig. 3). There is another, albeit less important pathway, from tyrosine to dopamine through tyramine. Depolarizing stimuli evoke dopamine release mainly from the vesicular pool [Kelly 1993]. Newly synthesized dopamine is also released from the cytoplasmic pool [McMillen and Shore 1980]. The biosynthesis and release of dopamine are both modulated by presynaptic autoreceptors. Both processes are enhanced by autoreceptor antagonists [Elsworth and Roth 1997]. Extracellularly released dopamine is taken up predominantly into synaptic terminals by the Na^+- and Cl^--dependent dopamine transporter [Reith et al. 1997, Drago et al. 1998, Kuhar 1998]. It has also been suggested that neighboring glial cells and non-dopaminergic neurons participate in the re-uptake and subsequent metabolism of extracellular dopamine [Elsworth and Roth 1997]. Intraneuronal dopamine is metabolized mainly in presynaptic neurons by amine oxidase (EC 1.4.3.4) to 3,4-dihydroxyphenylacealdehyde and then by aldehyde oxidase (EC 1.2.3.1) to 3,4-dihydroxyphenylacetate (DOPAC). Catechol O-methyl transferase (EC 2.1.1.6) converts DOPAC to homovanillate (HVA) [Kopin 1985, Elsworth and Roth 1997]. Extraneuronally dopamine is also metabolized to HVA by catechol O-methyl transferase (EC 2.1.1.6) through 3-methoxytyramine [Westerink and Spaan 1982, Elsworth and Roth 1997].

Dopamine is known to control a wide range of physiological functions. For instance, it participates in motor coordination [Clark and White 1987, Jackson and Westlind-Danielsson 1994, Missale et al. 1998, Emilien et al. 1999]. Dysfunction of the dopaminergic system plays a dominant role in Parkinson's disease due to lesions in the nigrostriatal system, with a loss of dopaminergic neurons. Furthermore, Huntington's chorea is associated with a dominance of the dopaminergic over the acetylcholinergic system. Dopamine regulates the functions of other neurotransmitter (glutamate, GABA, acetylcholine, noradrenaline) systems [Grace et al. 1998, Johnson 1998, Missale et al. 1998, Tepper et al. 1998, Emilien et al. 1999], and the activity of dopaminergic neurons can also be regulated by the above neurotransmitters [Grace et al. 1998]. In addition to such central effects, dopamine also evokes peripheral responses; for instance, dopamine acting on the adrenergic systems elevates diastolic blood pressure and has emotional effects [Missale et al. 1998]. The dopaminergic system can affect endocrinological control [Missale et al. 1998] by regulating the secretion of pituitary hormones, e.g, dihydroxyphenylalanine-stimulated secretion of gonadotropic and growth hormones and output of prolactin. Moreover, dopamine regulates fat and carbohydrate metabolism, water and mineral balance and excites the vomiting center. It stimulates cyclic adenosine monophosphate (cAMP) synthesis (by activating adenylate cyclase), has a calming action, elevates body temperature, dilates blood vessels in the brain, kidneys, heart and intestines, increases stroke and minute volumes of the heart and potentiates peripheral catecholamine release [Missale et al. 1998, Emilien et al. 1999].



1 = Tyrosine 3-monooxygenase (EC 1.14.16.2), 2 = aromatic-L-amino-acid decarboxylase (EC 4.1.1.28), 3 = dopamine-β-monooxygenase (EC 1.14.17.1), 4 = phenylethanolamine N-methyltransferase (EC 2.1.1.28), 5 = tyrosine decarboxylase (EC 4.1.1.25), 6 = amine N-methyltransferase (EC 2.1.1.49), 7 = amine oxidase (EC 1.4.3.4), 8 = aldehyde oxidase (EC 1.2.3.1), 9 = catechol O-methyltransferase (EC 2.1.1.6, and 10 = alcohol dehydrogenase (EC 1.1.1.1).

Fig. 3. Biosynthesis and catabolism of dopamine. Modified from Kostowski and Pużyński [1986].

There are three main central dopaminergic systems (Fig. 4): (1) The nigrostriatal pathway, joining the substantia nigra (A9 region) to the striatum, which is involved in extrapyramidal motor functions. (2) The mesolimbic/mesocortical pathway from the ventral tegmental area (A10 region) to the cortical structures, being responsible for the cognitive functions, motivation and indirectly for motor activity [Alexander et al. 1990, Missale et al. 1998, Emilien et al. 1999]. (3) The tubular/infundibular pathway from the hypothalamus (A12) to the hypophysis, regulating neuroendocrine functions [Missale et al. 1998 Emilien et al. 1999]. In addition to these general pathways, dopaminergic neurons join the frontal cortex with the septum, striatum and nucleus accumbens [Hantraye 1998].

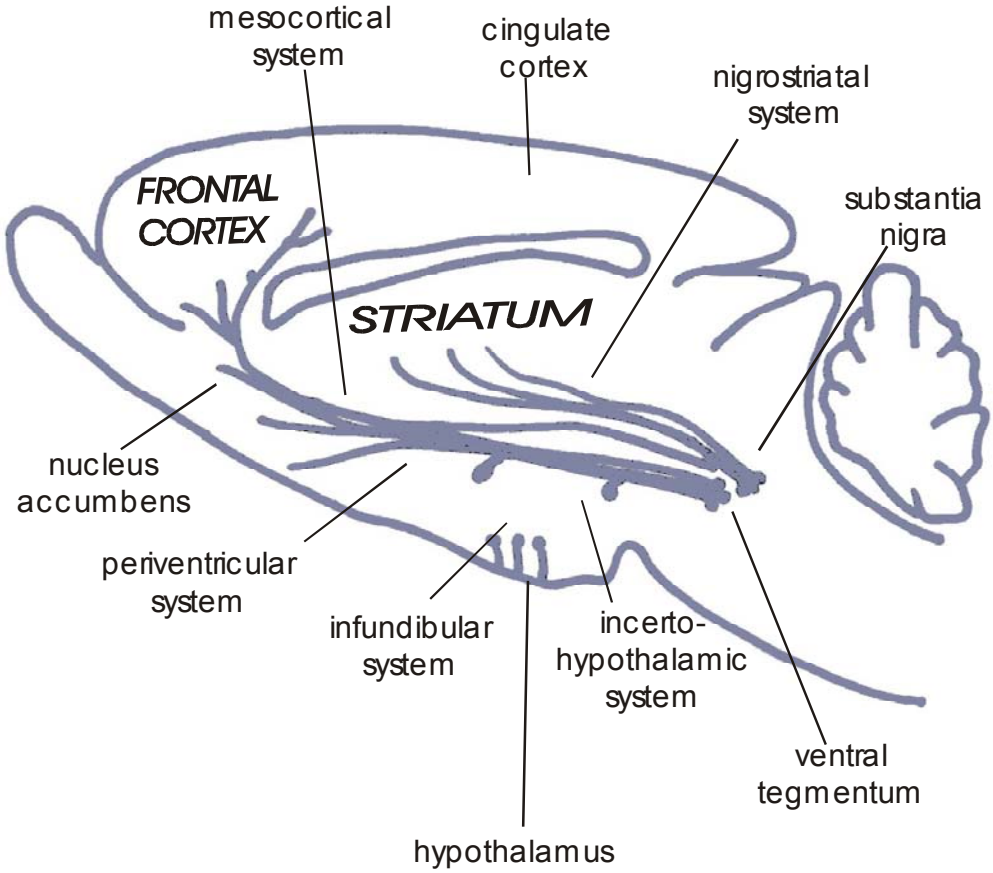


Fig. 4. Dopaminergic pathways in the brain. Modified from Kostowski and Pużyński [1986].

Six types of receptors mediate the physiological effects of dopamine. They relate to the seven transmembrane domains of G-protein-coupled receptors, containing three extracellular and two intracellular loops [Probst et al. 1992]. Two receptors belong to the D_1 subfamily, types D_1 and D_5. The other four, D_{2S} (a short form), D_{2L} (a long form with 29 additional amino acids, located in the region where the receptor is coupled to G-proteins), D_3 and D_4, belong to the D_2 subfamily [Missale et al. 1998, Palermo-Neto 1998, Emilien et al. 1999, Sibley 1999]. The D_1 receptors predominate in the direct inhibitory pathways, while the D_2 receptors are located predominantly in the indirect excitatory pathways [Palermo-Neto 1997, Grace et al. 1998, Emilien et al. 1999]. The D_1-like receptors abound in the striatum and nucleus accumbens (D_1 type), hypothalamus and hippocampus (D_5 type). The D_{2L} receptors are located mainly in the striatum and

substantia nigra, rather as postsynaptic receptors. In the striatum, hypothalamus and mesencephalon the D_{2S} receptors act mainly as autoreceptors in the dopaminergic pathways. The D_4 receptors are found in the frontal cortex and midbrain and the D_3 type in the olfactory tubercle and hypothalamus, with a few in the nucleus accumbens [Khan et al. 1998, Emilien et al. 1999].

The dopamine receptors in the central nervous system are mainly involved in the control of locomotion, cognition, the emotions and neuroendocrine secretion [Missale et al. 1998, Emilien et al. 1999]. The functions of the D_1 and D_2 receptor subfamilies differ markedly. The D_1-like receptors are most universal [Dearry et al. 1990]. The dopamine autoreceptors belong however to the D_2 subfamily. They are found in most brain regions containing dopamine neurons. The activation of autoreceptors by dopamine released from synaptic terminals and dendrites is an important mechanism regulating the functions of dopaminergic neurons [Emilien et al. 1999]. The exact role of the autoreceptors depends on their localization in the brain and on the cell region in question. For example, the mesencephalic autoreceptors modulate membrane excitability and dopamine synthesis and release, whereas the autoreceptors in the frontal cortex only modulate dopamine release [Palermo-Neto 1997]. Otherwise, in the somatodendric region impulse-regulating autoreceptors inhibit the firing rates of dopamine neurons [Cragg and Greenfield 1997, Elsworth and Roth 1997]. The autoreceptors at nerve endings inhibit the release and/or synthesis of dopamine [Emilien et al. 1999]. The D_2 receptors at dopamine and other neurons are located both pre- and postsynaptically [Sesack et al. 1994]. The localization of D_1 receptors depends on the region: in the substantia nigra they are located preferentially presynaptically, in the caudate nucleus and putamen, again, often postsynaptically [Emilien et al. 1999]. In some striatal regions the D_1 receptors are located preferentially in neurons co-expressing substance P [Gerfen et al. 1990]. The D_1-like receptors with the short third intracellular loop activate adenylate cyclase (EC 4.6.1.4), increasing the intracellular concentration of cAMP [Onali et al. 1981, Palermo-Neto 1997, Missale et al. 1998, Emilien et al. 1999] They modulate both directly and indirectly the level of intracellular Ca^{2+} and inhibit K^+ currents in striatal neurons [Cote et al. 1981, Onali et al. 1981, Kitai and Surmeier 1993, Missale et al. 1998].

The D_2-like receptors, which interact with G_i-proteins, have a long third intracellular loop [Kitai and Surmeier 1993, Missale et al. 1998, Emilien et al. 1999]. They inhibit adenylate cyclase and Ca^{2+} channels but activate K^+ channels [Carlsson 1993, Civelli et al. 1993, Ginrich and Caron 1993, O'Dowd 1993, Palermo-Neto 1997]. Dopamine acts upon vital-related stimuli in the nucleus accumbens and on motivational-relevant stimuli in the frontal cortex [Di Chiara et al. 1998]. The agonists of D_1 and D_2 receptors inhibit and activate, respectively, the activity of Na^+/H^+ exchangers which are responsible for the regulation of intracellular pH and cell volume [Yun et al. 1995]. The dopamine receptors also influence the activity of plasma membrane ion pumps (dopamine inhibits Na^+/K^+-ATPase (EC 3.6.1.37)). Furthermore, D_1 receptors are involved in the memory processes [Grace et al. 1998] and D_3 receptors play a role in yawning, hypothermia and penile erection [Emilien et al. 1999].

The striatal D_1, D_2 and D_3 receptors are particularly involved in the control of locomotion. Locomotor activity is inhibited by the D_2 autoreceptors, which lower the level of dopamine, and by D_3 receptors located postsynaptically in the nucleus accumbens. Otherwise, the activation of postsynaptic D_2-like receptors slightly increases locomotion [Daly and Waddington 1992, Jackson and Westlind-Danielsson 1994, Diaz et al. 1995]. The D_1 receptors stimulate locomotion by means of an interaction with D_2 receptors rather than acting alone [Gershanik et al. 1983, Dreher and Jackson 1988, Carlsson 1993, Jackson and

Westlind-Danielsson 1994]. The effects of the dopaminergic systems on motor functions are connected with dopaminergic-glutamatergic interactions in the nigrostriatal pathway. Konradi [Konradi 1998] has shown that stimulation of D_1 receptors mediates c-fos expression, which is dependent on the NMDA receptor function as shown by inhibition of the glutamate agonist dizocilpine. This comprises a direct modulation not affected by tetrodotoxin [Emilien et al. 1999]. Such experiments suggest that dopamine acting at the D_1-like receptors potentiates the postsynaptic glutamate responses mediated by the NMDA receptor, while D_2 agonists cause non-NMDA-mediated glutamatergic excitation of striatal neurons [Carlsson 1993, Adriani et al. 1998, Cepeda and Levine 1998, Grace et al. 1998].

Effects of HE on the dopaminergic system

HE increases the activity of monoamine oxidase in the frontal cortex and caudate nucleus [Rao et al. 1993] and in animal models has been seen to alter the metabolism of aromatic amino acids, which causes changes in the brain content of dopamine, DOPAC and HVA [Murakami et al. 1992]. In cirrhotic patients, the level of HVA increases but that of dopamine is not changed [Bergeron et al. 1989]. In most experimental models, HE has not influenced the dopamine levels either in the whole brain or in most brain regions [Yurdaydin et al. 1990, Murakami et al. 1992, Bergqvist et al. 1995, Hadesman et al. 1995, Colombo et al. 1996, Wikell et al. 1998]. An increase in dopamine has been noted in the hippocampus and frontal cortex in the thioacetamide (TAA) model of HE [Yurdaydin et al. 1990], whereas a decrease has been observed in the striatum of dogs with portal systemic encephalopathy [Zeneroli et al. 1991].

The level of HVA is increased in the frontal cortex, caudate nucleus and basal ganglia of cirrhotic patients [Bergeron et al. 1989] and in many brain regions of rats with experimental portacaval-anastamosis-induced HE [Bergeron et al. 1995]. Only in TAA-treated rats is the HVA concentration not changed in the whole brain [Murakami et al. 1992] or in the striatum [Yurdaydin et al. 1990], but is decreased in the neocortex of chronic HE (liver shunt) rats [Bergqvist et al. 1995].

The most controversial experimental results are related to the DOPAC concentration in the rat brain. Experimental HE has increased the level of DOPAC in the whole brain of acute ischemic HE rats [Murakami et al. 1992] and in most brain regions studied in chronic HE rats [Hadesman et al. 1995], but the level has also been noted to be decreased in the neocortex of chronic HE rats [Bergqvist et al. 1995] as well as in many other brain regions [Bergeron et al. 1995]. In some experiments with acute TAA-induced HE, no significant changes have been seen in the DOPAC levels in the striatum, hypothalamus, substantia nigra and brain stem [Yurdaydin et al. 1990].

In addition to all the above discrepancies, HE has caused a loss of D_2 receptors in human patients (in the pallidus/putamen but not in the nucleus accumbens and frontal cortex) [Mousseau et al. 1993] and in animal models [Zeneroli et al. 1991, Waśkiewicz et al. 2001].

2.3.2. Glutamate receptors

L-Glutamate and L-aspartate are excitatory amino acids. L-Glutamate was first recognized as such on the basis of its convulsive, depolarizing and exciting effects on central neurons. It is responsible for about 75% of excitatory neurotransmission [Danysz et al. 1995]. In mammalian excitatory synapses glutamate released from nerve endings activates different types of glutamate receptors at the postsynaptic membrane [Greenamyre and Young 1989, Watkins et al. 1990, Dong et al. 1997, Dunach et al. 1999, and references therein].

There are also glutamate receptors located presynaptically in the olfactory tract [Collins et al. 1983], hippocampus [Errington et al. 1987] and cerebellar granule cells [Olson et al. 1987]. Glutamate mediates learning and memory and regulates neuronal outgrowth and synaptogenesis [Lipton and Kater 1989, Monaghan et al. 1989, Gasic and Hollman 1992, Récasens et al. 1992, Olney 1993, Dong et al. 1997]. The glutamatergic pathways are numerous in the brain. Those from the cerebral cortex to the striatum seem to be involved in regulation of the activity of striatal output neurons and in this manner control the motor behavior related to the extrapyramidal system [Danysz et al. 1995, Nakanishi et al. 1998]. Otherwise, glutamate in excess is likely to cause Ca^{2+}-dependent neuronal damage. This process is involved in the pathogenesis of many neurodegenerative diseases, e.g., ischemia, hypoglycemia and epilepsy, but also in that of HE [Moroni et al. 1983, Olney 1989, Lipton and Rosenberg 1994, Nakanishi et al. 1998].

The glutamate receptor family consists of ionotropic receptors with glutamate-gated cation channels and metabotropic receptors modulating the production of second messengers via G proteins [Nakanishi et al. 1998, Ziff 1999]. The receptors are divided into six classes based on differences in the relative potencies of their agonists. Three ionotropic receptor classes equipped with integral ion channels are (1) NMDA, (2) AMPA and (3) kainate receptors, the latter two being collectively known as non-NMDA receptors [Monaghan 1989, Watkins et al. 1990, Récasens et al. 1992]. The three metabotropic receptor classes consist of the following metabotropic glutamate receptor (mGluR) types: (1) phosphoinositol-linked mGluR1 and 5, (2) cAMP-linked mGluR2 and 3 and (3) L-2-aminophosphonobutyrate (L-AP4) -activated (c-AMP- or cyclic guanosine monophosphate (cGMP) -linked) mGlu4, 6 and 7 [Conn and Desai 1991, Nakanishi 1992, Pin and Duvoisin 1995, Nakanishi et al. 1998]. This classification renders discussion of the receptors meaningful. However, the receptor agonists are usually not strictly specific to one class only, since they often act, for instance at both NMDA and non-NMDA receptors, and their responses to agonists are generally only partially blocked by the specific receptor antagonists [Watkins et al. 1990]. For example, kainate activates AMPA receptors to a small degree. Quisqualate, frequently used as an agonist of the AMPA sites, also markedly activates NMDA receptors and some metabotropic receptors [Janáky 1994]. The receptor subunits exhibit different physiological properties and pharmacological profiles [Monyer et al. 1992, McBain and Mayer 1994] and are variably distributed in the brain. The subunit composition of glutamate receptors in the cell may be different with different subsynaptical localization. This kind of arrangement allows specific targeting of neurotransmitter receptors, which is essential for correct neurotransmission [Froechner 1993, Danysz et al. 1995, Ehlers 1996, Ottersen and Landsend 1997]. NMDA and non-NMDA receptors are found on both neurons and astrocytes [Monaghan et al. 1989, Muller et al. 1992, Gallo and Russel 1995, Porter and McCarthy 1995]. The striatal NMDA, AMPA and kainate receptors are mainly postsynaptically located. A small population of kainate receptors may also lie presynaptically at corticostriatal terminals acting as autoreceptors [Biziere and Coyle 1979, Henke and Cuénod 1979, Seeburg 1993a]. They may be responsible for limbic seizures [Lothman and Collins 1981] or cell death [Sucher and Lipton 1991].

The NMDA receptor has been particularly thoroughly explored because its activation is connected to two essential processes: learning and neuronal death. The receptor contains a cation channel and inside and outside binding sites for glutamate/aspartate/NMDA, glycine, polyamines, Zn^{2+}, Mg^{2+}, and the channel blockers dizocilpine, phencyclidine and ketamine [Wroblewski and Danysz 1989, Nakanishi 1992, Scatton 1993, McBain and Mayer 1994, Nakanishi et al. 1998,]. Glycine is a co-agonist at the strychnine-insensitive site. It is necessary for the activation of the ion channel [Johnson and Ascher 1987, Wroblewski and

Danysz 1989, McBain and Mayer 1994, Danysz et al. 1995]. This channel allows the inward passage of Ca^{2+} and Na^{+} and the outward passage of K^{+}. It is use-dependently (in the open state) and voltage-dependently controlled by Mg^{2+}. When the Mg^{2+} blockade is alleviated by depolarization of the postsynaptic membrane (e.g. by a neighboring AMPA receptor), glutamate, with the cooperation of glycine, opens the channel and allows cations to pass [Mayer et al. 1984, Kwiatkowska 1991, Danysz et al. 1995, Obrenovitch and Urenjak 1997, Ottersen and Landsend 1997]. Phencyclidine, dizocilpine or ketamine block the receptor in a use-dependent manner by binding to a site inside the ion channel [Fagg 1987, Foster and Wong 1987, Parsons et al. 1993, Rogawski 1993]. The NMDA receptors are also inhibited by alcohols [Lovinger et al. 1990], oxygen-free radicals, multifaceted thiol group(s), low pH [Aizerman et al. 1989, Levy et al. 1990, Traynelis and Cull-Candy 1991, Lipton 1993, Sullivan et al. 1994], gangliosides and some tricyclic antidepressants [Reynolds and Miller 1988].

The NMDA receptors consist of the subunit families NMDA-preferring NMDAR1 and quinolinate-preferring NMDAR2. The NMDAR1 subfamily comprises eight functional isoforms (NMDAR1A-H) [Hollmann and Heinemann 1994, McBain and Mayer 1994] essential for channel activity [Hollman and Heinemann 1994, McBain and Mayer 1994, Dunah et al. 1999,], while NMDAR2 consists of four functional isoforms (NMDAR2A-D). It modulates the functional properties of the associated ion channels [Meguro et al. 1992, Monyer et al. 1992, Hollmann and Heinemann 1994, McBain and Mayer 1994, Dunah et al. 1999]. The receptors, composed of different subunits, exhibit different Mg^{2+} sensitivities and physiological and pharmacological profiles, and are differently distributed throughout the brain [Monyer et al. 1992, Hollman and Heinemann 1994, McBain and Mayer 1994]. The NMDA receptors are usually recognized as a slow component in the repetition of activity generated predominantly by non-NMDA receptors [Watkins et al. 1990].

The AMPA receptor probably mediates all forms of fast glutamatergic neurotransmission [Danysz et al. 1995]. This receptor class consists of four subunits, GluR1 to GluR4 [Seeburg 1993b, Hollmann and Heineman 1994, Danysz et al. 1995], of which GluR2 seems to be the dominant [Hollmann et al. 1991, Verdoorn et al. 1991, Hollmann et al. 1993]. There are two alternative variants of the subunits: flip and flop, which differ in 9 to 11 amino acids located in a segment between the transmembrane domains 3 and 4 [Seeburg 1993a]. They have different possibilities to fulfil their tasks. For example, the subunits GluR1-3 of the flip form are five times more sensitive to glutamate and AMPA than those of the flop form [Sommer et al. 1990, Monyer et al. 1991]. Their expression changes during development, only the flip form being expressed in the prenatal brain while later both forms are present. The channels are highly permeable to Na^{+} or K^{+} and much less so to Ca^{2+}, and only in the presence of GluR2 [Sommer et al. 1991, Danysz et al. 1995, Pellegrini-Giampietro et al. 1997]. The Ca^{2+} permeability is under strong control especially in the adult [Janáky 1994]. Receptor activation increases the inflow and outflow of ions, which is the basis of fast depolarization. The AMPA receptors affect the activation of NMDA receptors and thus participate in long-term potentiation [Seeburg 1993b, Hollmann and Heinemann 1994].

The distinction between the kainate and AMPA receptor classes is based on (1) differences in the sensitivity of spinal cord C-fibers to kainate and quisqualate and (2) different responses to activation by agonists (even though kainate crossreacts with AMPA receptors) [Watkins et al. 1990, Linden 1994, Bettler and Mulle 1995, Danysz et al. 1995, Nicoll and Malenka 1995]. The kainate receptors consist of two subunit families, GluR5-7 and KA1-2. GluR5 and 6 are only activated by K^{+} and are less sensitive to activation than the KA1 and 2 receptors [Egebjerg and Heinemann 1993]. The kainate receptors possess sites

with low and high affinities for the ligand [Coyle 1983]. The low-affinity binding site is equivalent to the high-affinity AMPA receptor [Collingridge and Lester 1989]. The high-affinity kainate receptors are coupled to a Na^+-K^+ ionophore, but their cation selectivity is quite different from that of the AMPA receptors [Seeburg 1993a]. They regulate the Ca^{2+} influx which modulates synaptic neurotransmission [Köhler et al. 1993]. Activation of the GluR6 channel is Ca^{2+}-dependent, while kainate may activate voltage-dependent channels [Williams and Johnson 1989, Egebjerg and Heinemann 1993].

The metabotropic glutamate receptors are coupled to G-proteins. They modulate intracellular signal transduction. In contrast to the ionotropic receptors, the metabotropic receptors are located both pre- and postsynaptically. Some of them may exist presynaptically only in the concrete fiber systems [Ottersen and Landsend 1997]. The mGluR7 receptors are operative in the presynaptical part in hippocampal pyramidal cell axons [Shigemoto et al. 1996], whereas mGluR2 receptors act mainly in the presynaptical area of hippocampal mossy fibers [Yokoi et al. 1996]. They are mainly autoreceptors modulating glutamate release [Sánchez-Prieto et al. 1996]. In contrast to the postsynaptic receptors, the presynaptic receptors are activated only by a high extracellular level of glutamate [Scanziani et al. 1997]. Moreover, the mGluR4 receptors are found presynaptically in the hippocampal but not in glutamatergic neurons [Bradley et al. 1996]. The physiological role of metabotropic glutamate receptors is not yet clear. The receptors activated by L-AP4 may influence amino-acid-induced transmission and by acting on glutamate autoreceptors reduce transmitter release. The phosphoinositol-linked receptors are activated by glutamate, quisqualate and ibotenate, but not by NMDA, AMPA or kainate, nor are they blocked by the antagonists of ionotropic glutamate receptors but are sensitive to toxins. They may participate in developmental plasticity [Watkins et al. 1990, and references therein].

Effects of HE on the glutamatergic system

In HE, glutamate may be responsible for neuronal excitation, but in uncomplicated liver failure a decrease in glutamate is associated with neuronal inhibition [Albrecht and Jones 1999]. The total concentration of glutamate in the whole brain and in different brain regions is decreased in HE patients [Record et al. 1976, Lavoie et al. 1987] and in animal models of HE [Zimmermann et al. 1989, Swain et al. 1992a, Swain et al. 1992b, Hilgier et al. 1999]. Very seldom has the brain glutamate level remained unchanged [Albrecht and Hilgier 1986, Zieve 1987]. Extracellular glutamate is elevated in several brain regions in animal HE models [Hamberger and Nyström 1984, Therrien and Butterworth 1991, Bosman et al. 1992, Swain et al. 1992a, Swain et al. 1992b, de Knegt et al. 1994, Michalak et al. 1996, Butterworth et al. 1997,] and in the cerebrospinal fluid of human patients [Watanabe et al. 1984]. Portacaval-shunted rats show only a slightly increased extracellular glutamate level in the striatum, whole cerebral cortex and frontal cortex [Tossman et al. 1983, Tossman et al. 1987, Rao et al. 1995b]. The increase in extracellular glutamate probably results from a decrease in glutamate re-uptake by cerebral astrocytes [Albrecht et al. 1988, Suzuki et al. 1992, Bender and Norenberg 1996], associated with diminished expression of the astrocytic glutamate transporter [Knecht et al. 1997, Norenberg et al. 1997; for discussion see also Michalak and Butterworth 1997].

In experimental HE the high level of glutamate and the decreased concentration of the antagonist of the ionotropic glutamate receptor, kynurenate [Saran et al. 1998], lead to overactivation of NMDA receptors, with a consequent increase in intracellular Ca^{2+}, and cause cell swelling (see above for details) [Vogels et al. 1997a]. Stimulation of the NMDA receptors foments the generation of nitric oxide [Kosenko et al. 1994] and degradation of microtubule-associated protein-2 (MAP-2). The important role of NMDA receptors in HE

pathogenesis is corroborated by the findings that administration of NMDA antagonists alleviates clinical symptoms, restores the extracellular glutamate concentration to normal and also normalizes electroencephalography (EEG) activity [Vogels et al. 1997b].

Evidence regarding the NMDA receptor activity in different animal HE models has been contradictory. The binding of ligands to glutamate agonist sites has been found to be decreased [Ferenci et al. 1984b, Peterson et al. 1990, Rao et al. 1991, Michalak et al. 1996,] or unchanged [Watanabe et al. 1988, Zimmermann 1989, Maddison et al. 1991, de Knegt et al. 1993]. Dizocilpine binding has been decreased in the cerebral cortex of patients with liver cirrhosis [Dodd 1992] and in many brain regions of hyperammonemic and chronic animal models of HE [Peterson et al. 1990, Maddison et al. 1991, de Kneght et al. 1993, Marcaida et al. 1995, Miñana et al. 1995], but has remained unchanged in rats with acute liver filure [Michalak and Butterworth 1997]. An attenuation of the activity of AMPA and kainate receptors has been noted in animals with acute liver failure, portal systemic encephalopathy and portacaval shunts [Ferenci et al. 1984b, Fan et al. 1990, Maddison et al. 1991, Rao et al. 1991, Lombardi et al. 1994, Michalak and Butterworth 1997].

Hyperammonia inhibits the glutamate - nitric oxide - cGMP pathway. Hermenegildo and associates [1998] have shown that this occurs stepwise. (1) At the first step, hyperammonia has only a small effect on NO synthesis, which is induced by NMDA receptor activation and dependent on the NO synthase activators Ca^{2+} and calmodulin. (2) However, cGMP formation induced by NMDA and NO-generating agents is strongly reduced. The down-regulation of NMDA receptors is thought to be involved in memory deficits, since the above-mentioned pathway participates in long-term potentiation [Hermenegildo et al. 1998].

Electrophysiological studies have demonstrated that ammonia diminishes the responsiveness of glutamate receptors to glutamate. This finding further witnesses that ammonia can also attenuate excitatory neurotransmission [Albrecht and Jones 1999].

3. Animal models of hepatic encephalopathy

A suitable animal model is essential for research into human diseases. More than ten years ago Mullen and McCullough [1989] suggested the outlines for a satisfactorily standardized animal model for HE and described its characteristics. The animals should exhibit typical symptoms of liver disease, such as reversible behavioral changes, hyperammonemia, amino acid alterations, alleviation of symptoms by lactulose and protein withdrawal, appearance of Alzheimer II astrocytes and increase in T 1 (longitudinal relaxation time constants influencing the amount of signal in magnetic resonance imaging) intensity in the globus pallidus. The model should moreover meet five further criteria [Terblanche and Hickman 1991]: (1) The most important is a selective liver damage eventually leading to death and reminiscent of that seen in human patients. (2) The liver failure should be reversible, allowing the animals to survive after cessation of the noxious treatment. For instance, a totally devascularized and destroyed liver or a complete hepatectomy do not fulfil this criterion. (3) The experimental duration should be long enough for adequate treatment to be initiated after the insult. (4) In order to save animals and money the experiments should be so planned that every animal should provide answers to many questions. (5) The methods should be minimally dangerous to laboratory personnel.

At present, surgical and hepatotoxic drug models are mainly used, even through these models do not fully meet all the above criteria. In one surgical model, the blood is shunted from the portal vein directly to the caudal vena cava, bypassing the liver. The procedure is

not ideal, since it can be reversed only by liver transplantation, the type of coma is different from that seen in human patients, and there are no damaged or dead liver cells such as are usually noted in HE [Giges et al. 1953]. In another surgical model, complete graded devascularization of the liver is generated by the portacaval shunt and permanent ligation of the hepatic artery. This is irreversible and seldom used by reason of many defects. The graded devascularization of the liver with a portacaval shunt and temporary ligature of the hepatic artery is the most appropriate surgical model.

The first drug generally used in hepatotoxic models was carbon tetrachloride, administered intra-arterially, intraportally, orally or intragastrically [van Leenhoff et al. 1974, Mourelle et al. 1988]. Its toxic effect on the liver is connected with the metabolism of hepatocytes. Toxicity is oxygen-free-radical- and lipid-peroxydation-dependent [Mourelle et al. 1988]. However, hepatocyte injury is histologically quite different from than clinically seen in human patients. Morever, an equal amount of carbon tetrachloride which is metabolized in the liver is also distributed to neighboring organs and damages them [Terblanche and Hickman 1991]. An intravenous injection of galactosamine hydrochloride to animals has effects similar to those of fulminant hepatic encephalopathy in humans [Blitzer et al. 1978]. There are many objections to the use of this model, however, owing to differences in the responses between different strains of rabbits, the expensive costs of the drug (in particular when large animals are used) and difficulties in generating hepatic encephalopathy in rats [Mullen et al. 1986]. In several laboratories acetaminophen (paracetamol) has been used to induce fulminant hepatic failure [Clark et al. 1973, Miller et al. 1976, Francavilla et al. 1988].

A combination of surgical and drug models has also been used, with fairly satisfactory results [Shibayama 1986]. Rats infected with Long Evans virus are mainly used in Japan. They spontaneously develop hepatic encephalopathy [Tekeichi et al. 1988]. Administration of ammonium acetate was formerly one of the animal models of HE, but is now regarded as a model of acute ammonia intoxication (with different clinical characteristics to those of HE). It is now mainly used to check whether or not the HE symptoms are associated with a high level and/or altered metabolism of ammonia [Chamuleau 1996, and references therein].

About 20 years ago our laboratory in the Medical Research Centre in Warsaw began to use a model based on repeated i.p. injections of TAA to rats [Hilgier 1983, Hilgier et al. 1983, Albrecht 1984b]. This treatment induced liver damage resembling liver cirrhosis in humans, where typically the cells around the intrahepatic bile ducts are destroyed, contain abundant fat and have smaller nuclei than in controls [Butterworth 1998, Matsushita et al. 1999]. Blood parameters typically manifest liver dysfuntion: bilirubin and some liver enzymes are increased [Norton et al. 1997, Bruck et al. 1999]. Astrocytic damage and swelling are similar to those observed in the brains of HE patients [Hilgier and Olson 1994, Matsushita et al. 1999]. The rats exhibit characteristic abnormalities in motor functions and reflexes [Yurdaydin et al. 1996]. They are at HE stage three or four [Widler et al. 1993, McArdle et al. 1996] and show increased mortality [Matsushita et al. 1999]. The animals hyperventilate in the same manner as human patients [McArdle et al. 1996] and the level of ammonia in the blood and brain [Hilgier and Albrecht 1984, Yurdaydin et al. 1995, McArdle et al. 1996] and the levels of met-enkephalin and β-endorphin in plasma [Yurdaydin et al. 1995] are increased. At more advanced stages, TAA-induced HE is also associated with severe disturbances of the cerebral blood flow [Larsen et al. 1994, and references therein].

The abnormalities in neurotransmission are characterized by a decrease in $GABA_B$ binding sites in the cerebral cortex [Oja et al. 1993], whereas there are no changes in the $GABA_A$ receptors [Zimmerman et al. 1989, Oja et al. 1993]. Since $GABA_B$ receptors negatively modulate glutamate release, their loss is thought to contribute to the increased

glutamate release and subsequent overactivation of NMDA receptors. TAA-induced HE is also associated with the presence in the brain of increased levels of central benzodiazepine receptor ligands with agonist properties, which are thought to contribute to GABAergic tone [Basile et al. 1991b]. This is in agreement with the findings in the brains of human patients who have died of acute HE induced by paracetamol [Baudouin et al. 1995, Morton and Arana 1999]. TAA-induced HE has been seen to enhance the spontaneous and ammonium chloride-stimulated release of GABA from cerebral cortical slices [Wysmyk et al. 1992] and to reduce the concentration of GABA in the cerebral cortical, hippocampal and striatal tissues [Zimmerman et al. 1989]. TAA treatment of mice has also been found to increase the brain concentration of benzodiazepine receptor ligands and subsequent accumulation of pregnenolone-derived neurosteroids, which are positive modulators of the $GABA_A$ receptor complex [Itzhak et al. 1995].

TAA-induced HE affects the brain glutamatergic systems. However, differences in experimental design (number of TAA injections, dosage of the drug and interval after the last injection and measurements) have yielded different results. Experimental HE has increased the total glutamate content in the whole rat brain [Hilgier and Olson 1994] and the extracellular glutamate level in the striatum [Hilgier et al. 1999] and hippocampus [McArdle et al. 1996]. On the other hand, total tissue glutamate measured by ^{1}H-nuclear magnetic resonance spectroscopy has been unchanged in the animal HE model in the striatum, hippocampus, occipital cortex, cerebellum and brain stem and diminished only in the frontal cortex [Peeling et al. 1993]. A decreased glutamate level has also been observed in the striatum, cerebral cortex and hippocampus [Zimmermann et al. 1989]. Experimental HE has in rats increased the activity of both glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.8) [Faff-Michalak and Albrecht 1993] and 2-oxoglutarate uptake [Albrecht et al. 1997] but reduced the glutamate uptake [Albrecht et al. 1997] and 2-oxoglutarate concentration in the brain [Oppong et al. 1995].

In TAA-treated rats, alterations have likewise been noted in the dopaminergic systems. Experimental HE increases the level of dopamine and lowers that of noradrenaline in the frontal cortex and hippocampus. No changes have been found in the dopamine, DOPAC, HVA or noradrenaline level in the striatum [Yurdaydin et al. 1990]. In the striatum activation of D2 receptor has been significantly inhibited [Waśkiewicz et al. 2001].

It has been suggested that TAA and its metabolites found in some brain regions may cause complications when this method is used [Peeling et al. 1993], but the traces of TAA and metabolites found by the investigators in question could result from the very high amounts of TAA injected (3 times 600 mg/kg). In our method, the rats received 300 mg/kg of TAA daily and no TAA or its metabolites have been detected in the brain [Hilgier et al. 1985].

AIMS OF THE STUDY

The present aims were the following:

1. To study in an animal model the effect of HE on [^{3}H]dopamine release from striatal and frontal cortical slices stimulated by the ionotropic glutamate receptor agonists NMDA, kainate and AMPA and by the generic depolarizing stimulus, high-K^+.

2. To assess the effects of experimental HE on [^{3}H]dopamine release from striatal and frontal cortical slices stimulated by high-K^+ in the presence and absence of Ca^{2+}.

3. To evaluate the influence of HE on the basal extracellular levels of dopamine and its metabolites HVA and DOPAC, and on their release stimulated by NMDA and high-K^+ *in vivo*.

4. To analyze how HE-induced alterations in the NMDA-, kainate- and AMPA-displaceable binding of glutamate to its receptors could affect the dopaminergic systems.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley (in *in vivo* experiments) and Wistar (in *in vitro* experiments) rats weighing 180-200 g were used for the investigation (altogether 460 animals). The animals were kept under standard conditions (light from 7 am to 7 pm, temperature 22°C, humidity 23%, and given food and water ad libitum). In order to generate HE, the rats were given three i.p. injections of 300 mg/kg TAA at 24 h intervals (900 mg/kg per rat) and used for studies 24 h after the last injection. The treatment induced liver damage resembling liver cirrhosis in humans, where typically the cells around the intrahepatic bile ducts are destroyed, contain abundant fat and have smaller nuclei than in controls. The astrocytic damage and swelling were similar to those observed in the brains of HE patients, and levels of ammonia in the blood and brain were increased [Hilgier and Albrecht 1984, Hilgier and Olson 1994]. The rats exhibited characteristic abnormalities in motor functions and reflexes, being precomatose at HE stage three. The control rats received no treatment. All experiments were approved by the University committee for the use of experimental animals.

2. [^{3}H]Dopamine release

The rats were decapitated and the brains rapidly excised and cooled on ice.

Slices. Two middle striatal slices and the first frontal cortical slice 0.5 mm thick were manually cut, preincubated with 0.26 TBq/l [^{3}H]dopamine in 2.5 ml of Krebs-Ringer-Hepes medium (concentrations in mM: NaCl 126, $MgSO_4$ 1.29, NaH_2PO_4 1.29, KCl 5, $CaCl_2$ 0.8, N-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES) 15, D-glucose 10, nialamide 4, ascorbic acid 20, NaOH 11.74, pH 7.4) for 20 min at 37°C under O_2 and then rinsed with 5 ml of Krebs-Ringer-Hepes medium without dopamine. Mg^{2+}-free medium was used in the NMDA and kainate experiments. The freely floating shaken slices were superfused in 0.5 ml of medium at a rate of 0.25 ml/min at 37°C under O_2 [Korpi and Oja 1984]. The first 20-min superfusate was discarded, whereafter 0.5-ml fractions were collected at 2 min intervals. At 28 min, the superfusion medium was changed into stimulation medium containing NMDA, kainate, AMPA or K^+ ("stimulated release") or left unchanged ("basal release"). After superfusion the slices were extracted overnight in 1 M NaOH. The superfusate samples and the extracts were then counted for radioactivity. In the absence of Ca^{2+} the medium was supplemented with ethylenediaminetetra-acetate (EDTA) (0.5 mM) and choline chloride (0.8 mM).

Synaptosomes. The synaptosomal fractions, designated S2, were prepared as described by Girbe and associates [1994] with minor modifications. The frontal cortex or striatum were homogenized in 10 volumes of ice-cold 0.32 M sucrose with pargyline (0.1 mM) and centrifuged (1000g/10 min/2°C). One ml of supernatant (crude synaptosomal fraction) was then preincubated with 0.23 TBq/l [^{3}H]dopamine in 8.6 ml Krebs-Ringer-HEPES medium (10 min/37°C under O_2) and centrifuged (2500g/10 min). The pellet (S2), suspended in 2.5 ml of Krebs-Ringer-HEPES solution, was transferred to superfusion chambers and superfused in a Brandel machine (0.25 ml/min, 40 min). At 27 min, the medium was changed into stimulation medium or left

unchanged. In the absence of Ca^{2+} the medium was supplemented with 1.7 mM $MgSO_4$. The superfused samples and NaOH extracts of S2 pellets were counted for radioactivity.

Calculations. The results were plotted as fractional release (see I for details). The integrated area between the basal line and the curve delineating stimulated release in the interval of 28-40 min for the slices and 27-35 min for the S2 fraction was taken as a measure of the stimulation. Statistical evaluation of the differences was carried out by Student's t-test.

3. Extracellular levels of dopamine and its metabolites

Microdialysis procedure. The experiments were conducted according to Lillrank and colleagues [1994] with minor modifications. The rats were anesthetized with 4% halothane in air within 2 min and then maintained in anesthesia during the experiments with a 1% halothane-air mixture. They were fixed in a Kopf stereotaxic instrument and body temperature was kept at 37.4°C by a heating pad controlled by a rectal thermometer (CMA 150, CMA Microdialysis, Stockholm, Sweden). Microdialysis probes of concentric design with a 2 x 0.5 mm dialyzing membrane (CMA 12, CMA Microdialysis, Stockholm, Sweden) were implanted in the caudatus-putamen (coordinates: AP = +1.2 mm, L = +3.5 mm, D = -5.5 mm, relative to the bregma and brain surface [Paxinos and Watson 1984]). The probes were perfused at 2.5 µl/min with artificial cerebrospinal fluid solution (155 mM Na^+, 132.73 Cl^-, 1.1 mM Ca^{2+}, 2.87 mM K^+, 0.83 mM Mg^{2+}, pH 7), and perfusate samples were collected every 20 min starting 1 hour after implantation of the probe. The location of the microdialysis probes in the brain was verified post mortem with cryostat microtome sections.

Assay methods. Dopamine, DOPAC and HVA were separated by high-pressure liquid chromatography (HPLC) with electrochemical detection. A reverse-phase column (MD-150/RP-C18, 150 x 3 mm, ESA, Inc.) was perfused with a mobile phase containing 75 mM NaH_2PO_4 x H_2O, 1.4 mM 1-octanesulfonic acid, 20 mM EDTA and 0.01% triethylamine in acetonitrile (pH 3.0, adjusted with ortho-phosphoric acid).

Calculations. The results were plotted as pmoles per 30 µl of dialysis sample. The presence of significant differences in the experimental data was evaluated by two-way analysis of variance using BMDP software (BMDP Software, Inc, Los Angeles, CA) and 2V program for repeated samples in time series. Differences between the experimental groups in the levels of dopamine and its metabolites were assessed by non-parametric Mann-Whitney *U* test.

4. Binding experiments

NMDA-, kainate- and AMPA-displaceable [^{3}H]glutamate binding. Synaptic plasma membrane fractions were prepared from the striatum and frontal cortex [Jones et al. 1989]. Tissue samples were homogenized in 20 volumes of 0.32 M sucrose and centrifuged twice (1000g/10 min and 20000g/20 min). The P_2 fraction obtained was suspended in 20 volumes of ice-cold water and centrifuged (8000g/20 min). The resulting pellet was similarly washed once again and frozen overnight at -20°C. On the day of binding assays the pellet was thawed, washed and centrifuged twice in incubation buffers (48000g/20 min) and used for experiments. Fifty mM Tris-acetate and Na^+-free

50 mM HEPES buffers, pH 7.4, were used for [^{3}H]glutamate and [^{3}H]glycine binding, respectively.

The NMDA-, kainate- and AMPA-displaceable bindings of [^{3}H]glutamate to synaptic membranes were determined by the method of Larder and McLennan [1983] with minor modifications [Varga et al. 1994]. The incubation of synaptic membranes (about 0.3 mg protein per assay) at 0°C for 20 min with [^{3}H]glutamate was stopped by filtration (Whatman GF/C glass filter) with ice-cold buffer. Nonradioactive NMDA (0.3 mM), kainate and AMPA (both 0.1 mM) were used as displacers to define NMDA-, kainate- and AMPA-displaceable bindings, respectively. The bindings were assessed within a ligand concentration range of 1-200 nM.

[^{3}H]glycine binding. Binding was measured as in Saransaari and Oja [1993a]. In these assays synaptic membranes (about 0.3 mg protein) were incubated in 0.5 ml buffer with [^{3}H]glycine (4°C/20 min). Nonspecific binding was determined in the presence of 1 mM glycine. The incubation was stopped by rapid filtration on GF/C glass fiber filters washed twiced with 5 ml of ice-cold buffer

[^{3}H]dizocilpine binding. The binding of [^{3}H]dizocilpine to striatal and frontal cortical membranes was carried out as described by Procter and associates [1991] with slight modifications [Saransaari and Oja 1995]. The tissue samples were homogenized in 9 volumes of 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 20000g for 25 min. The resulting pellets were suspended in 75 volumes of buffer and centrifuged at 48000g for 25 min. This procedure was then repeated three times, but using ice-cold water instead of buffer. The last pellets were stored overnight at -70°C. On the day of binding experiments the thawed pellets were washed three times in 75 volumes of buffer and centrifuged at 48000g for 25 min.

The resulting synaptic membranes (about 0.3 mg protein per assay) were incubated in buffer with [^{3}H]dizocilpine (25°C/45 min). Nonspecific binding was determined in the presence of 25 mM dizocilpine. The incubation was rapidly stopped by filtration on Whatman GF/C glass filters previously soaked in a 0.01% solution of polyethyleneimine (to diminish binding to the filters) and the filters were then washed twice with 5 ml of ice cold buffer.

N-[1-(2-thienyl)cyclohexyl][^{3}H]piperidine ([^{3}H]TCP) binding. Binding of [^{3}H]TCP was done as described in Saransaari and Oja [1993b] with slight modifications. Striatal and frontal cortical samples homogenized in 10 volumes of 0.32 M sucrose were first centrifuged (1000g/20 min) and the supernatant then recentrifuged at 17500g for 20 min. The resulting pellet, suspended in 50 volumes of ice-cold water, was incubated at 37°C for 30 min and then centrifuged at 32000g for 20 min. This procedure was repeated once. The pellet, resuspended in 50 volumes of 10 mM HEPES buffer (pH 7.5) and centrifuged (32000g/20 min), was frozen at -70°C and on the day of binding experiments thawed, washed twice with 30 volumes of buffer and centrifuged (32000g/ 20 min).

The membrane preparations (about 0.3 mg protein per assay) were incubated (23°C/ 45 min) in 0.5 ml of HEPES buffer with [^{3}H]TCP. Nonspecific binding was determined in the presence of 30 mM phencyclidine. The incubation was rapidly stopped by filtration on GF/C glass fiber filters presoaked in 0.01% poly-L-lysine. The filters were then washed twice in 5 ml of ice cold buffer.

Assays and calculations. The radioactivity trapped in the filters was measured by liquid scintillation spectrometry. The parameters were estimated by nonlinear regression analysis using Marquardt's algorithm in iterative optimization. The statistical significance of differences was estimated by Student's t-test.

In situ hybridization. Striatal and cortical brain sections frozen at –70°C in physiological saline were cut and collected as described by Schalling and associates (1988) with slight modifications. Oligonucleotide probes (see details in V) were synthesized with an Applied Biosystems DNA synthesizer. After hybridization with 20 MBq/l ^{35}S -labeled probes (18 h/40^{o}C), sections were rinsed in sodium chloride-sodium citrate solution (pH 7.0), dehydrated, and exposed to Kodak XAR-5 film for 18 days.

RESULTS

1. Effects of experimental HE on [^{3}H]dopamine release *in vitro*

HE did not significantly alter the basal release of [^{3}H]dopamine from any of the structures and preparations studied *in vitro* [I, II]. NMDA (0.1 mM) evoked [^{3}H]dopamine release from striatal and frontal cortical slices from control and HE rats, the release being maximal after the 6-min stimulation. It was only slightly attenuated during the further 12 min of stimulation. Dizocilpine (0.1 mM), a non-competitive NMDA receptor antagonist, blocked the stimulatory effects of NMDA. Experimental HE reduced the NMDA-dependent dopamine release from striatal slices by 46% [I, Table 1].

Kainate at the 0.1-mM concentration did not affect [^{3}H]dopamine release from either preparation. One mM kainate caused a long-lasting increase in [^{3}H]dopamine release in striatal and frontal cortical slices from control and experimental HE rats. In HE rats, the kainate-dependent [^{3}H]dopamine release was increased by 35% in frontal cortical slices and lowered by 21% in striatal slices [Table 1]. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), a specific kainate receptor antagonist, only partly inhibited the stimulatory effect of kainate in frontal cortical slices from both control (by 56%) and experimental HE rats (by 82%). It attenuated dopamine release to the control level in experimental HE rats. In control and experimental HE rats the effect of kainate was totally inhibited by a combination of CNQX (0.1 mM) and dizocilpine (0.1 mM) [I, IV, Table 1]. In striatal slices, CNQX (0.1 mM) totally blocked the stimulatory effect of kainate. AMPA (0.5 mM) slightly increased [^{3}H]dopamine release from frontal cortical slices but not from striatal slices from control rats. The stimulatory effect of AMPA in frontal cortical slices was not influenced by HE [I].

High-K^+ (50 mM) increased [^{3}H]dopamine release from striatal and frontal cortical slices and synaptosomes in both the presence and absence of Ca^{2+} in control and experimental HE rats [I, II]. In slices, dopamine release did not level off during stimulation, except for striatal slices from HE rats in the presence of Ca^{2+}. In synaptosomes, in the presence of Ca^{2+} high-K^+ evoked a marked [^{3}H]dopamine release which subsequently leveled off. In the absence of Ca^{2+}, the onset of [^{3}H]dopamine release was more gradual, with no decline during the stimulus. The absence of Ca^{2+} in the superfusion medium reduced the stimulated release of [^{3}H]dopamine in all preparations except for striatal slices, where the release remained unchanged. TAA-evoked HE in the presence of Ca^{2+} enhanced K^+-stimulated [^{3}H]dopamine release from striatal slices and synaptosomes by 20% and 24%, respectively, and from frontal cortical slices and synaptosomes by 34% and 36%, respectively [Table 2]. Experimental HE reduced the Ca^{2+}-dependent [^{3}H]dopamine release from frontal cortical synaptosomes by 15%. Furthermore, HE in the present model invariably enhanced the difference between the magnitudes of [^{3}H]dopamine release in Ca^{2+}-containing and Ca^{2+}-free media.

TABLE 1. Effect of 0.1 mM NMDA, 1 mM kainate and 0.5 mM AMPA on [^{3}H]dopamine release from striatal and frontal cortical slices from control rats and rats with TAA-induced HE. Inhibition of stimulation by the responding receptor agonists 0.1 mM dizocilpine and 0.1 mM CNQX

Region	Stimulus	Stimulated release (integrated area)		
		Control	HE	% change
Striatum	NMDA	22.7 ± 1.4 (23)	1 2.3 ± 1.4* (19)	- 46
	NMDA + dizocilpine	nd	nd	
	Kainate	22.2 ± 0.6 (10)	16.0 ± 1.3* (8)	-21
	Kainate + CNQX	nd	nd	
	Kainate + CNQX + dizocilpine	nd	nd	
	AMPA	nd	nd	
Frontal cortex	NMDA	28.4 ± 3.05 (15)	32.9 ± 3.9 (16)	ns
	NMDA + dizocilpine	nd	nd	
	Kainate	27.3 ± 2.0 (10)	37.0 ± 1.0* (8)	+ 35
	Kainate + CNQX	11.9 ± 2.9 (7)	6.8 ± 1.8 (7)	ns
	Kainate + CNQX + dizocilpine	nd	nd	
	AMPA	8.1 ± 1.5 (16)	11.6 ± 1.6 (12)	ns

Results are mean values ± SEM. Number of independent experiments in parentheses. Differences HE vs control: *P<0.01; ns = statistically not signifcant, nd = not detectable.

2. Extracellular levels of striatal dopamine and its metabolites

Experimental HE lowered the basal level of HVA in microdialysates by 27%, while the concentrations of dopamine and DOPAC were unchanged. The level of dopamine in control rats was increased 2.8 ± 1.1-fold (mean ± SEM) and 12.9 ± 2.2-fold by NMDA (10 mM) and high-K^+ (50 mM), respectively. After withdrawal of both stimuli the dopamine concentration returned to the basal level. Experimental HE reduced the effects of both stimuli by 70%. In control rats, NMDA lowered the levels of DOPAC and HVA by 77% and 71%, respectively. The concentrations remained reduced after cessation of treatment. Experimental HE attenuated the decrease in HVA to 45%. In addition, high-K^+ diminished the microdialysate levels of dopamine metabolites.

After cessation of stimuli the concentration of DOPAC returned totally and that of HVA partially to the basal level. Experimental HE did not affect the accumulation of dopamine metabolites [III].

TABLE 2. Effect of 50 mM K^+ on [^{3}H]dopamine release from striatal and frontal cortical slices and synaptosomes from control and HE rats

Region	Ca^{2+}	Preparation	Stimulated release (integrated area)		
			Control	HE	% change
Striatum	+	slices	157.4 ± 6.4 (15)	188.1 ± 7.3 (14)*	+20
	-		144.4 ± 10.5 (6)	136.7 ± 9.2 (8)	ns
	% change ↓		ns	-27	
	+	synaptosomes	36.9 ± 1.1 (10)	45.7 ± 2.3 (12)*	+24
	-		28.1 ± 0.7 (16)	27.3 ± 1.5 (11)	ns
	% change ↓		-24	-40	
Frontal cortex	+	slices	85.6 ± 3.9 (19)	114.6 ± 6.2 (14)*	+34
	-		34.7 ± 2.9 (6)	39.2 ± 5.1 (7)	ns
	% change ↓		-60	-66	
	+	synaptosomes	27.6 ± 1.3 (14)	37.5 ± 1.4 (16)*	+36
	-		18.6 ± 0.8 (12)	15.9 ± 0.4 (16)*	-15
	% change ↓		-33	-58	

Results are mean values ± SEM. Number of independent experiments in parentheses. Differences HE vs control: *P<0.01, ns = statistically not significant.

3. Ionotropic glutamate receptors

Experimental HE did not affect the binding of [^{3}H]dizocilpine or [^{3}H]glycine and the AMPA-displaceable binding of [^{3}H]glutamate to striatal membranes, or the binding of [^{3}H]dizocilpine or [^{3}H]TCP and the kainate-displaceable binding of [^{3}H]glutamate to frontal cortical membranes [IV-VI].

TABLE 3. Binding of [^{3}H]TCP, [^{3}H]glycine, [^{3}H]dizocilpine and [^{3}H]glutamate (NMDA-, kainate- and AMPA-displaceable binding) to striatal and frontal cortical membranes from control and HE rats

	B_{max} (μmol/kg protein)		K_D (nM)	
	control	HE	control	HE
Striatum				
[^{3}H]TCP	2.12 ± 0.16	1.16 ± 0.06*	50.2 ± 6.2	81.3 ± 6.5*
[^{3}H]glycine	1.29 ± 0.09	1.20 ± 0.09	274.1 ± 25.0	220.3 ± 22.5
[^{3}H]dizocilpine	2.46 ± 0.06	2.37 ± 0.09	16.0 ± 0.7	15.1 ± 1.2
[^{3}H]glutamate/kainate	0.30 ± 0.04	0.17 ± 0.01*	37.1 ± 5.9	33.7 ± 6.1
[^{3}H]glutamate/AMPA	0.31 ± 0.04	0.26 ± 0.03	69.3 ± 17.7	86.4 ± 22.6
[^{3}H]glutamate/NMDA	0.65 ± 0.03	0.50 ± 0.02*	111.7 ± 8.7	84.6 ± 5.9*
Frontal cortex				
[^{3}H]TCP	2.25 ± 0.37	2.38 ± 0.29	65.0 ± 6.7	82.3 ± 8.7
[^{3}H]glycine	0.65 ± 0.18	1.17 ± 0.15*	81.3 ± 52.3	242.4 ± 22.4
[^{3}H]dizocilpine	2.70 ± 0.09	2.56 ± 0.06	10.6 ± 0.72	11 5 ± 0.6
[^{3}H]glutamate/kainate	0.19 ± 0.02	0.21 ± 0.03	87.9 ± 13.4	99.3 ± 22.0
[^{3}H]glutamate/AMPA	0.28 ± 0.05	0.35 ± 0.06*	177.8 ± 36.7	205.3 ± 37.2
[^{3}H]glutamate/NMDA	1.15 ± 0.04	1.55 ± 0.08*	129.4 ± 10.2	285.9 ± 26.3*

Results are mean values ± SEM. Differences HE vs control: *P< 0.05.

In the striatum, experimental HE diminished the B_{max} of [^{3}H]TCP binding by 45%, of kainate-displaceable glutamate binding by 43% and of NMDA-displaceable glutamate binding by 33% [Table 3]. The K_D of NMDA-displaceable glutamate binding decreased by 24% in striatal membranes. At the same time, the K_D of [^{3}H]TCP binding increased by 62% in striatal membranes and the B_{max} of [^{3}H]glycine binding by 80%, and the B_{max} of NMDA- and AMPA-displaceable [^{3}H]glutamate bindings by 35% and 25%, respectively, in frontal cortical membranes [Table 3]. The K_D of NMDA-displaceable [^{3}H]glutamate binding in frontal cortical membranes was increased by 121% [IV-VI]. [^{3}H]Dizocilpine binding was increased in striatal membranes from control and TAA–evoked HE rats by pre-treatment of the membranes with a protein kinase C activator (phorbol ester), ATP and Ca^{2+} [V].

HE in the present model did not visibly alter the expression or distribution of any NMDA receptor mRNA transcript [I]. The expression of NR1 and NR2B transcripts was found to be slight in the striatum.

DISCUSSION

In general terms, the results of the present study are in accord with the hypothesis that motor disturbances in HE may be associated with alterations in dopaminergic neurotransmission and that altered glutamatergic control of dopamine functions may be a contributory factor.

Methodological considerations

Applicability of the animal model used

The TAA animal model was used to induce acute HE because it induces liver damage similar to liver cirrhosis in humans (see details in chapter 3). As opposed to surgical and toxic models used in other laboratories, but not accepted for the present study, the TAA model guarantees at least a partial reversibility of the symptoms. This model possesses, moreover, genuine hepatotoxic component which is missing in surgical models. Other hepatotoxic models used so far have a number of disadvantages. The carbon tetrachloride model causes severe toxic damage to other organs, often exceeding hepatotoxicity in their severity [Terblance and Hickman 1991]. In turn, the galactosamine model, though adequate in terms of its comparability to human HE, is very expensive and applicable to rabbits rather than to rats [Blitzer et al. 1978, Mullen et al. 1986].

Dopamine release

The *in vitro* dopamine release was analyzed using radioactive [^{3}H]dopamine. This is a common and useful method. To be sure that the released dopamine, and not the release of its metabolites is measured, these compounds were separated by thin-layer chromatography. Analyses showed that more than 75% of radioactivity was recovered in dopamine and, which is likewise important, that this percentage was not changed by the treatments. In order to avoid error resulting from possible differences between the different sets of experiments, tissues from experimental HE and control rats were always analyzed at the same time. The experiments were performed in two different laboratories, but the same recipes and experimental protocols were used, rendering the results comparable.

An *in vivo* technique allows monitoring of the basal and stimulated extracellular levels of neurotransmitters. Microdialysis was conducted following the method and procedures already tested by Lillrank [1994] on similar laboratory rats. Some minor modifications were necessary in view of the overall condition of animals with experimental HE. The amount of halothane used during the experiments had to be reduced to a minimum adequate to maintain proper anesthesis. The „waiting time„ before the first sample had to be as short as possible to keep the HE animals alive up to the end of experiments. The enzymatic breakdown of dopamine is fast. In order to minimize it, the samples were collected in tubes placed on ice in darkness and as soon as possible mixed with perchloric acid. Chromatographic separations were accomplished immediately using a well-established technique based on HPLC with electrochemical detection. It should however, be borne in mind that the extracellular level of dopamine is also affected by halothane anesthesia and tissue damage resulting from probe implantation.

Binding experiments

Well-established standard ligand-binding methods were used here to study the effects of experimental HE on the properties of NMDA and non-NMDA receptors in synaptic plasma membrane preparations. To minimize experimental variation, measurements on the experimental groups to be compared were also here invariably done at the same time. These pharmacological binding experiments, using ligands and antagonists specific for different ionotropic glutamate receptors, characterize changes at the level of receptor classes. They do not however differentiate between the different subunits comprising the receptors. The NMDA receptors appeared to be most markedly affected by HE in the present studies. With an eye to obtaining better insight into the changes in the NMDA receptors, in situ hybridization experiments were therefore undertaken on the expression of receptor subunits but yielded negative results.

Effects of experimental HE on NMDA-stimulated [^{3}H]dopamine release

The NMDA-dependent dopamine release in rats with TAA-induced HE was significantly decreased in striatal slices *in vitr*o and in striatal microdialysates *in viv*o. The NMDA receptors in the striatum are responsible for an array of neurotransmission-related events such as phasic increases in dopamine release underlying conditioned emotional responses [Saulskaya and Marsden 1995], and the long-term enhancement of dopamine release [Jedema and Moghaddam 1996] and motor responses due to sensory stimulation [Wheeler et al. 1995]. Alterations in the glutamatergic control of dopamine release could trigger disturbances in the above-mentioned events and contribute to HE symptoms.

HE in the TAA model reduced dopamine release in the striatum, but not in the frontal cortex. In contrast to the pattern in the striatum, NMDA binding was also moderately increased by experimental HE in the frontal cortex. This increase in ligand binding to NMDA receptors was not reflected as an increase in dopamine release, which suggests that factors other than NMDA receptor activation are engaged in control of the release [I, V]. It is noteworthy in this context that HE in the TAA model increases GABA release in the cerebral cortex [Wysmyk et al. 1992]. In the case of the striatum, the NMDA modulation of dopamine release may be guided through the striatal cholinergic [Carter et al. 1988] or GABAergic systems [Chaudieu et al. 1994, Krebs et al. 1994].

Effects of experimental HE on kainate- and AMPA-dependent [^{3}H]dopamine release

Non-NMDA receptor agonists are weaker stimulants of dopamine release *in vitro* than NMDA [Clow and Jhamandas 1989, Jacobs and Cox 1992, Krebs et al. 1994]. Kainate induces dopamine release directly or indirectly through the NMDA receptors [Carter et al. 1988]. We noted the same phenomenon but only in the frontal cortex. Since the kainate-induced dopamine release was only partly blocked by the specific inhibitor of the kainate/AMPA receptors, CNQX, and completely only by CNQX and dizocilpine when applied together, a part of the release could be due to the interactions of kainate with the NMDA receptors. Experimental HE increased dopamine release in the frontal cortex, although kainate binding to glutamate receptors was not altered [VI]. Interestingly, CNQX totally abolished the difference in dopamine release between HE model and control rats. Since CNQX is a specific antagonist of the glycine modulatory site [Mead and Stephens 1999] and experimental HE increases the activity of the glycine site in the NMDA receptors [V], this site is likely to play a role in the kainate-stimulated dopamine release.

The GluR1 and GluR4 subunits are structural elements of the AMPA-preferring receptors [Seeburg 1993b, Hollmann and Heineman 1994, Danysz et al. 1995]. Since the B_{max} for the AMPA-displaceable binding of glutamate was significantly increased in experimental HE [VI], this can partly explain why the kainate-stimulated dopamine release was also increased. The AMPA-preferring receptors are also involved in the kainate-stimulated neurotransmitter release [Bettler and Mulle 1995, Danysz et al. 1995]. In this light, the absence of effects of HE on the AMPA-induced dopamine release [VI] is puzzling. It is conceivable that the low sensitivity of the present *in vitro* system to AMPA masked the effect of TAA-evoked HE.

The dopaminergic D_2 receptors affect non-NMDA-receptor-mediated glutamatergic excitation of striatal neurons (see chapter 2.3.1, pp. 24-25). HE in the present model led to a loss of D_2 receptor activity in the striatum [Waśkiewicz et al. 2001], and this loss may have contributed to the decrease in the kainate-stimulated dopamine release from striatal slices.

Effects of experimental HE on K^+-stimulated [^{3}H]dopamine release

The high-K^+-stimulated release of dopamine *in vitro* is thought to reflect the *in vivo* release evoked by depolarization [Ochi et al. 1995]. In the TAA model, HE caused a transient increase in dopamine release from striatal slices, which was most prominent at the onset of stimulation and then rapidly declined. The mechanism underlying this effect is unknown, but several possibilities may be envisaged. One is related to the secondary effect of high-K^+-induced depolarization, i.e., to the extracellular accumulation of neurotransmitters which, acting at their own receptors, could trigger dopamine release. A major neurotransmitter candidate for this is glutamate, which evokes dopamine release acting via NMDA or AMPA/kainate receptors. However, the involvement of HE in the increase in K^+-induced dopamine release via this mechanism is not very likely. HE in the present model reduced the K^+-induced release of the radiolabeled glutamate analog, D-aspartate, from hippocampal slices and the effect occurs instantly after induction of K^+ [Hilgier et al. 1991]. The attenuated K^+-evoked release of glutamate may have contributed to the rapid cessation of stimulation of dopamine release. The animal HE model enhanced the K^+-induced dopamine release from frontal cortical slices even more promptly than from striatal slices and the stimulatory effect did not level off. Glutamate, acting at NMDA and AMPA receptors [Roberts and Anderson 1979, Desce et al. 1992, Ochi et al. 1995] at the beginning of stimulation, may facilitate dopamine release in experimental HE rats more than in controls [Moghaddam et al. 1990]. Glutamate release is also stimulated by K^+, but this effect begins somewhat more slowly and is inhibited by experimental HE. It may lead to a prompt decrease in dopamine release [I].

Disturbances in the activity of Na/K-ATPase may affect the dopamine level. A low ATPase activity increases dopamine synthesis [Connor and Kuczenski 1986] and facilitates opening of the voltage-gated sodium channels, provoking an increase in the tetrodotoxin-sensitive and Ca^{2+}-dependent exocytotic release of dopamine [Fairbrother et al. 1990]. An attenuation of ATPase also diminishes dopamine uptake, which in turn increases the extracellular dopamine level. The effect on the Ca^{2+}-independent, carrier-mediated release of cytoplasmic dopamine is, however, only minor [Milusheva et al. 1996] or insignificant [Fairbrother et al. 1990].

Dopamine release can be mediated by or coupled to the synthesis of nitric oxide, an intercellular messenger in the brain [Zhu and Luo 1992, Lonart et al. 1993, Ohno et al. 1995, Stewart et al. 1996]. The activity of nitric oxide synthase (EC 1.14.13.39)

increases in a chronic HE model [Rao et al. 1995a] and in patients with liver cirrhosis [Genesca et al. 1999]. Nitric oxide activates NMDA receptors but no other glutamate receptor class [West and Galloway 1997, Lu et al. 1998]. On the other hand, the generation of nitric oxide is induced by NMDA receptor activation [Garthwaite et al. 1988, Kosenko et al. 1994, Hermenegildo et al. 1998]. Whether the NMDA-nitric oxide pathway is activated in the TAA model of HE remains to be seen.

The experimental HE-induced increase in the high K^+-evoked release of dopamine was greater in frontal cortical than in striatal slices and synaptosomes [I]. This difference could reflect activation of the glycine site in the NMDA receptor [Martinez Fong et al. 1992], noted in the frontal cortex but not in the striatum [V]. The data taken together would indicate that HE directly stimulated dopamine exocytosis in both structures.

In order to differentiate between the vesicular high-K^+-evoked and Ca^{2+}-dependent exocytosis and the cytoplasmic Ca^{2+}-independent and carrier-mediated release of dopamine, the release from both slices and synaptosomes was measured in the presence and absence of Ca^{2+}. In both types of preparations and structures effects of experimental HE were noted only in the presence of Ca^{2+}. This bespeaks the involvement of an exocytotic mechanism. The implication of nitric oxide as a mediator of the enhanced dopamine release is also an alternative. Nitric oxide synthesis increases in HE models [Rao et al. 1995a] and could trigger dopamine release *in vivo* and *in vitro*. We tend to dismiss this possibility, however, in view of the absence of the HE effect on the spontaneous dopamine release, which could have been predominantly affected (see discussion in II). Experimental HE did not affect the Ca^{2+}-independent K^+-stimulated dopamine release except in the frontal cortex, where dopamine release decreased.

Effects of experimental HE on the basal extracellular level and evoked release of dopamine and dopamine metabolites *in vivo*

The unaltered basal levels of dopamine and DOPAC in experimental HE are in keeping with the results reported by Yurdaydin and colleagues [1990] in a slightly different TAA model of HE. In that study, however, there was no significant decrease in HVA such as was seen in the present study. The difference may possibly arise from differences in the experimental HE models and the measuring methods or from differences in the parts of the striatum used for analyses. The striatum is both structurally and functionally heterogeneous [Whitton et al. 1994]. In that study HE did not significantly depress the NMDA-induced elevation of extracellular dopamine, which is in apparent contrast to the present *in vitro* results. The reasons for this discrepancy are not clear. The intrinsic difference between the *in vivo* and *in vitro* preparations is the absence in the latter of the control by the neuronal inputs from other brain regions. A possibility to be taken into account is that *in vivo* the frontal cortical inputs stimulate striatal dopamine release which surpasses the direct inhibition. This interpretation appears plausible in the light of the increase in NMDA and glycine binding in the frontal cortex [V]. It does not take into consideration, however, the potential control by other neurotransmitter systems.

The results *in vivo* and *in vitro* were also dissimilar in the case of the K^+-induced dopamine release. The release was stimulated *in vitro* but tended to decrease *in vivo*. High K^+ may promote dopamine release i*n vivo* by means of a number of mechanisms: (1) Potassium ions may directly induce exocytosis due to depolarization of nerve endings. (2) These may increase the release of glutamate, which would trigger dopamine exocytosis by interacting with ionotropic glutamate receptors (see the previous section).

Of these, mechanism (2) and control by D_2 receptors are likely to be involved in the changes induced by HE. Treatment with TAA leads to a decrease in the K^+-induced glutamate release in the striatum [unpublished results] and causes a substantial loss of D_2 receptors [Waśkiewicz et al. 2001].

The effects of D_2 receptor loss on dopamine release may be complex. D_2 receptor activation increases K^+ conductance in neuronal membranes [Lacey et al. 1987] and may inhibit the opening of Ca^{2+} channels [Vallar and Meldolesi 1989]. It has been estimated that 30-40% of dopamine D_2 receptors are located on corticostriatal afferents [Schwarcz et al. 1978]. This mechanism is not operative in striatal slices *in vitro*.

The infusion of NMDA or KCl reduced the DOPAC level in the striatum in control and experimental HE rats to the same degree. This would indicate that experimental HE does not affect dopamine breakdown to DOPAC. HE diminished both the basal and NMDA-stimulated accumulation of the other dopamine metabolite HVA. The HVA level depends, among other factors, on the functional status of dopamine-metabolizing enzymes. Of these, the activity of monoamine oxidase B is known to be increased in the caudate-putamen and likewise in other brain structures in chronic HE patients [Rao et al. 1993] and in congenitally hyperammonemic mice [Rao et al. 1994]. It is not settled, however, whether this is also the case in the present HE model.

CONCLUSIONS

1. Experimental HE inhibits NMDA-induced dopamine release from striatal slices. The inhibition is explicable by compromised functions of the ionophore in the NMDA receptor complex. The animal HE model does not affect dopamine release from frontal cortical slices.

Kainate stimulates dopamine release from striatal slices by means of kainate receptor activation. Experimental HE reduces the kainate-dependent dopamine release. This effect may stem from a decrease in the B_{max} for kainate binding and indirectly from a loss of dopamine D_2 receptors. On the other hand, in the frontal cortex kainate stimulates dopamine release both directly or indirectly due to an interaction of kainate with the NMDA and AMPA receptors. The kainate-dependent dopamine release can be stimulated by activation of the glycine site in the NMDA receptors and by activation of AMPA receptors, since the binding of ligands to them significantly increases in HE.

No correlation was noted between the experimental HE-evoked changes in the ligand binding to AMPA receptors and the stimulation of dopamine release.

2. Experimental HE enhanced the high-K^+-evoked dopamine release from striatal and frontal cortical slices. Hypothetically, this effect may stem from changes in the glutamate level, a decrease in Na/K-ATPase activity and an increase in the activity of nitric oxide synthase. Only in the frontal cortex may alterations in the properties of the agonistic NMDA and co-agonistic glycine sites in the NMDA receptors stimulate dopamine release.

3. Experimental HE affects differently dopamine release from the vesicular and cytoplasmic pools. HE increases the evoked vesicular dopamine release from striatal and frontal cortical slices and synaptosomes in the presence of extracellular Ca^{2+} but less readily alters the carrier-mediated, Ca^{2+}-independent release of cytoplasmic dopamine. This indicates that HE affects the exocytotic release mechanisms.

4. NMDA- and high-K^+-stimulated dopamine release is not significantly decreased in experimental HE *in vivo*. This is at variance with the results obtained *in vitro*. The discrepancy is explicable by mechanisms not operative in slices. HE alters the function of the corticostriatal glutamatergic pathways (NMDA-induced release), leading simultaneously to a decrease in the activity of D_2 receptors and glutamate release (high-K^+-induced release). Hypothetically, the decrease in the basal level of the dopamine metabolite HVA in experimental HE may result from an increase in the activity of monoamine oxidase. The breakdown of dopamine to DOPAC is not affected by HE.

In general, the present results demonstrate that experimental HE-evoked changes in the stimulated release of dopamine and its metabolites, thought to be involved in extrapyramidal symptoms (rigidity and tremor), are associated with changes in the glutamatergic regulation of dopaminergic neurotransmission. This opens the field for future attempts to treat HE symptoms using pharmacological manipulations at the glutamate receptor sites.

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