Neurotransmitter Receptor Imbalances in Motor Cortex and Basal Ganglia in Hepatic Encephalopathy

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Key Words

Human *post-mortem* brain tissue • Neurotransmitter receptors • Acetylcholine • Adenosine • Putamen

Abstract

Hepatic encephalopathy (HE) in chronic liver disease is characterized by neuropsychiatric and motor disturbances and associated with a net increase of inhibitory neurotransmission. Though many studies, mostly carried out in animal models, have linked dysfunctions of single neurotransmitter systems with the pathogenesis of HE, reports concerning neurotransmitter receptor alterations are controversial. Little is known about the situation in humans. We carried out a multireceptor assessment of HE-associated changes in neurotransmitter receptor densities and affinities in human *post-mortem* brain samples. Dissociation constants and densities of different binding sites for glutamate, GABA, acetylcholine, norepinephrine, serotonin, dopamine and adenosine were determined by *in vitro* binding assays and quantitative receptor autoradiography in

the motor cortex and putamen of HE and control brains. HE cases do not build a homogeneous group, but differ concerning direction and intensity of binding site density divergences from control values. The acetylcholine M₂ binding site dissociation constant was significantly higher in HE brains. Nicotinic acetylcholine and adenosine type 1 and 2A densities were significantly down-regulated in the putamen of HE brains. Our data suggest that neurotransmitter alterations are probably not the primary key factor responsible for the neuropsychiatric and motor disturbances associated with HE.

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Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome, which frequently occurs in acute or chronic liver disease. Its clinical manifestations range from minor sleep disturbances and changes in personality and motor activity, to overt deterioration of cognitive functions, motor

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coordination, decreased consciousness and coma [1]. At the molecular level, HE is associated with a shift in the balance between inhibitory and excitatory neurotransmission towards a net increase of inhibitory neurotransmission, which is generally attributed to either an increased GABAergic tone or to alterations of the glutamatergic system (for reviews see [2] and [3]). The pathophysiology of HE has also been attributed to a low-grade chronic cerebral oedema which is exacerbated in response to ammonia and accompanied by an increased production of reactive oxygen and nitrogen oxide species, which in turn trigger multiple protein and RNA modifications, thereby affecting brain function (for a comprehensive review see [1]).

Most of the experimental approaches have made use of animal models [4-23], and only few studies were carried out on *post-mortem* tissue from HE patients [23-31] or were based on *in vivo* imaging of HE patients [32-34]. Furthermore, for humans it has been noted for certain receptor types, that alterations described in *post-mortem* brains after acute liver failure could not be reproduced *in vivo* in the brain of cirrhosis patients with manifest hepatic encephalopathy [31, 34]. Such differences may be of biological importance and are indicative of stage specific individual molecular responses in patients which may also affect *post-mortem* findings.

A large body of evidence supports the hypotheses that ammonia toxicity in the brain is mediated by a dysregulation of the glutamatergic system, though results obtained from diverse animal models of chronic hyperammonemia revealed discrepant alterations of AMPA and kainate glutamate receptor densities [8, 13, 35, 36]. However, animal models featuring acute hyperammonemia show a consistent over-activation of NMDA receptors [5, 7, 18] and studies on human autopsy material also revealed significantly increased densities of this receptor type [24]. Ammonia has recently also been shown to induce selective RNA oxidation, since it affects astroglial messenger RNA (mRNA) coding for the glutamate/aspartate transporter, but not that coding for the GABA_B receptor subunit 1 [37].

Altered GABAergic neurotransmission was first implicated in the pathophysiology of HE based on the phenomenon of increased GABAergic tone in a rabbit model of fulminant hepatic failure [17]. However, the evidence gathered since then is not unequivocal. GABA concentrations were found to be unaltered [15, 26] or increased [17]. Likewise, GABA_A receptor densities were reported to be up-regulated [16, 29] in some studies, but were unaltered in others [9, 15, 24, 25, 28-30]. Additionally,

mRNA expression of the GABA transporter GAT-2 was increased in the cerebral cortex of the portacaval-shunted rat, whereas the genes for the GABA_{B1D} receptor and for the $\beta 2$ subunit of the GABA_A receptor were down-regulated [19]. Reports concerning GABA_A associated benzodiazepine (BZ) binding sites are also controversial, since they encompass unaltered [24, 25, 29, 30], down-regulated [27], or increased [4, 16] densities. Furthermore, it was recently shown, that a selective increase in the GABAergic tone occurs in the cerebellum and part of the ventromedial thalamus, though not in the cerebral cortex of hyperammonemic rats [38]. However, in the same series of experiments, a reduced cGMP response to the stimulation of GABA_A-receptors and a selective lack of responses when GABA_A receptors were antagonized were associated with hyperammonemia in the cerebral cortex [38], indicating that during this type of experimental hyperammonia either the GABA_A receptors are maximally blocked or that excitatory mechanisms related to glutamate receptors cannot be further stimulated in response to a lack of inhibitory input. A third alternative would be that precursors for NO synthesis and/or downstream cGMP production are at their limit under hyperammonia in the cerebral cortex. This would explain why the cortex is still able to respond with a reduction in cGMP productions but is not able to respond with increased levels in response to reduced inhibitory input when compared to controls. Therefore, one would expect disease related compensatory alterations of the cortical tissue in order to restore the physiological responsiveness observed in controls as it is known for most neuropathological conditions [39].

Although the GABAergic and glutamatergic hypotheses are the most widespread theories used to explain the increased inhibitory neurotransmission in HE patients, alterations of other neurotransmitter systems including that of the neuromodulator adenosine have also been associated with the pathogenesis of the disease (Table 1). Concentrations of norepinephrine and its neuronal metabolite 3,4-dihydroxyphenylglycol were decreased in most brain regions of acutely hepatectomised rats [6], although HE-coma was associated with increased extracellular norepinephrine concentrations and a loss of α_1 and β_1 binding sites in the frontal cortex and thalamus of rats with an experimentally induced liver failure [12]. Expression of the noradrenergic α_{1C} and α_2 receptor genes is up-regulated in the cerebral cortex of the portacaval-shunted rat [19]. Concerning the cholinergic system, it has been reported that acute ammonium acetate-induced encephalopathy resulted in

Transmitter	Alteration	Species
Glutamate	↑ Ca ²⁺ -dependent glutamate release	Rat [36]
	↓ glutamate uptake	Rat [36]
	glutamate/aspartate transporter mRNA oxidation	Rat [37]
	↓ AMPA receptor density	Rat [35]
	↔ AMPA receptor density	Human [24], rat [13]
	↑ kainate receptor density	Rat [13]
	↓ kainate receptor density	Human [24], rat [13, 35], rabbit [40], dog [8]
	↑ NMDA receptor density	Human [24]
	↔ NMDA receptor density	Human [29], Rat [35, 36], dog [8]
	MK-801 protected cultured astrocytes from ammonia toxicity	Rat [18]
	Memantine induced improvement in clinical grading	Rat [5]
	NMDA receptor activation	Rat [7, 66]
GABA	↔ GABA levels	Human [26], rat [36], dog [15]
	↓ GABA-transaminase activity	Rabbit [67]
	↔ Glutamate decarboxylase activity	Rabbit [67]
	↑ GABA transporter GAT-2 gene expression	Rat [19]
	↓ GABA _A receptor subunit β ₂ gene expression	Rat [19]
	↑ GABA _A receptor subunits α ₁ , β ₁ , γ ₂	Rat [68]
	↑ GABA _A receptor density	Human [29], rabbit [16]
	↔ GABA _A receptor density	Human [24, 25, 28-30], rat [9], dog [15]
	↓ GABA _B receptor subunit 1D gene expression	Rat [19]
	↔ GABA _B receptor subunit 1 gene expression	Rat [37]
	↓ GABA _B receptor density	Human [24], rat [21]
	↑ benzodiazepine binding site density	Human [69], Rat [4], rabbit [16]
	↓ benzodiazepine binding site density	Human [27]
	↔ benzodiazepine binding site density	Human [24, 25, 29, 30]
	↑ peripheral benzodiazepine binding site density	Human [31]
	↔ peripheral benzodiazepine binding site density	Human [34]
Acetylcholine	↑ acetylcholine levels	Tree shrew [10]
	↑ acetylcholinesterase levels	Human [23]
	↓ acetylcholinesterase levels	Rat [70]
	↔ choline acetyltransferase levels	Human [23]
	↓ Muscarine receptor density	Human [30]
	↔ Muscarine receptor density	Rabbit [40]
	↔ M ₁ receptor density	Human [24]
	↔ M ₂ receptor density	Human [24]
	↔ M ₃ receptor density	Human [24]
	↑ nicotinic α1 subunit gene expression	Rat [19]
	↔ Nicotinic receptor density	Human [24]
Norepinephrine	↓ norepinephrine levels	Rat [6]
	↑ norepinephrine levels	Rat [12]
	↑ α _{1C} gene expression	Rat [19]
	↓ α ₁ receptor density	Rat [12]
	↔ α ₁ receptor density	Human [24]
	↑ α ₂ gene expression	Rat [19]
	↔ α ₂ receptor density	Human [24]
	↓ β ₁ receptor density	Rat [12]
	↑ serotonin levels	Rat [11;12]
	↔ serotonin levels	Rat [71]
Serotonin	↓ serotonin transporter NTT gene expression	Rat [19]
	↓ serotonin transporter	Rat [11]
	↓ 5-HT _{1A} receptor density	Human [24, 72]
	↑ 5-HT ₂ receptor density	Human [72]
	↓ 5-HT ₂ receptor density	Rat [73]
	↔ 5-HT ₂ receptor density	Human [24;30]
	↑ 5-HT _{2b} gene expression	Rat [19]
	↓ dopamine levels	Rat [6]
Dopamine	↓ D ₄ receptor gene expression	Rat [19]
	↓ Dopamine receptor density	Rabbit [40]
	↓ D ₁ receptor density	Human [24]
	↔ D ₁ receptor density	Human [74]
	↓ D ₂ receptor density	Human [24, 74], rat [75]
	Adenosine modulates glutamate uptake	Rat [47]
Adenosine	↓ A ₁ receptor density	Human [24;32]
	↔ A ₁ receptor density	Human [30]
	↓ A _{2A} receptor density	Human [24]
	↓ A ₃ receptor gene expression	Rat [19]

Table 1. Overview of neurotransmitter-related alterations associated with HE. ↑: increase; ↓: decrease; ↔: no alteration.

Case	# 1	# 2	# 3	# 4	# 5	# 6	# 7
Clinical features							
Age (years) and gender (m/f)	68/m	45/f	36/m	69/m	63/m	57/m	57/f
alcoholic origin of disorder		x	x	x	x	x	x
TIPS		x	x				x
Hepatorenal syndrome	x	x	x	x	x	x	
Blood values							
Sodium [mmol/l]	131	122	138	-	120	158	126
Urea [mg/dl]	146	258	148	-	226	310	94
Creatinine [mg/dl]	2.6	2.9	1.5	-	7.9	5	2.4
Albumin [g/dl]	3.1	2.4	1.8	-	1.8	2	2.1
Bilirubin [mg/dl]	19	0.8	20	-	29	36.8	11.6
Leucocytes [per μ l]	-	30 000	22 900	-	2 500	27 300	15 800
CRP [mg/dl]	-	14.9	4.6	-	-	-	5.8
Prothrombin time [%]	-	37	10	-	13	29	26
Ammonia [μ mol/l]	-	97	154	-	148	86	48
Post-mortem time (h)	≤ 12	17	6	≤ 12	13.5	9	12

Table 2. Summary of comorbidities and results of blood tests carried out the day before death, as well as time elapsed between death and freezing of brain tissue. Cases 2 and 3 had participated in a study investigating changes of adenosine A1 receptor binding in cirrhotic patients by means of positron emission tomography [32].

significantly increased extracellular acetylcholine levels in the thalamus of tree shrews [10]. In a recent study García-Ayllón and colleagues [23] described increased acetylcholinesterase activity in cortex obtained from cirrhotic patients or from rats with liver failure. Studies on human autopsy material revealed significantly decreased muscarinic cholinergic binding site densities in the frontal cortex [30]. Additionally, expression of the gene for the $\alpha 1$ subunit of the nicotinic receptor is down-regulated in the cerebral cortex of the portacaval-shunted rat [19]. The serotonergic system has also been implicated in the pathophysiology of HE: i) mRNA expression of the serotonin transporter NTT was decreased in the cerebral cortex of the portacaval-shunted rat [19] and serotonin transporter sites were significantly reduced in cortical and subcortical brain regions of rats with severe encephalopathy resulting from acute liver failure [11], ii) expression of the 5-HT_{5B} receptor gene is up-regulated in the cerebral cortex of the portacaval-shunted rat [19], iii) the putamen of post-mortem brain tissue from patients who had died in hepatic coma presented significantly lower 5-HT_{1A} receptor densities than that of controls [24], and iv) transient increases of extracellular serotonin levels were observed in a rat model of acute liver failure [12]. Additionally, levels of dopamine and its precursor 3,4-dihydroxyphenylalanine were increased in most brain regions of hepatectomised rats [6]. Binding site densities of the D₁ and D₂ dopamine

receptors were down-regulated in the putamen of HE patients [24], expression of the D₄ receptor gene is down-regulated in the cerebral cortex of the portacaval-shunted rat [19], and K_D values of D₂ receptors were diminished in whole brain homogenates of a rabbit model of HE [40].

The neuromodulator adenosine has recently been proposed to further aggravate the excitatory/inhibitory imbalance at the synaptic cleft. Expression of the adenosine receptor A3 gene is down-regulated in the cerebral cortex of the portacaval-shunted rat [19]. We found lower adenosine A₁ cortical and striatal as well as lower A_{2A} striatal densities in post-mortem brain tissue from patients who had died in hepatic coma than in control brains [24]. This finding was corroborated in a recent positron emission tomography study which revealed that A₁ receptor densities were significantly down-regulated in a wide range of cortical regions and in the putamen of HE patients [32].

Previous studies mostly concentrated on single neurotransmitter systems and did not take a multifactorial approach to elucidate the molecular mechanisms involved in the disease. The latter is, however, a necessity in order to achieve an understanding as to whether (and which) excitatory, inhibitory and modulatory receptor systems are involved as well as due to the fact that the expression of a single neurotransmitter receptor type is influenced by alterations of other receptor types and neurotransmitter systems. Thus, synaptic signalling mediated by a given

Table 3. Summary of incubation conditions and concentration ranges for saturation analyses carried out on membrane suspensions. Adenosine A₁ receptors were labelled with both [³H]DPCPX and its analogue [³H]CPFPX (both in the presence of Gpp(NH)p, [43]).

Receptor	[³ H]-Ligand	Displacer	Incubation Buffer	Incubation time
mGluR2/3	LY 341,495 [0.1-15 nM]	L-Glutamate [1 mM]	10 mM K-Phosphate (pH7.6) + 100 mM KBr	60 min at 4°C
GABA _B	CGP 54626 [0.1-25 nM]	CGP 55845 [100 µM]	50 mM Tris-HCl (pH 7.2) + 2.5 mM CaCl ₂	60 min at 4°C
BZ	flumazenil [0.1-20 nM]	clonazepam [2 µM]	170 mM Tris-HCl (pH 7.4)	60 min at 22°C
M ₃	4-DAMP [0.1-10 nM]	atropinsulfate [100 µM]	50 mM Tris-HCl (pH7.4) + 1 mM PMSF + 1 mM EDTA	45 min at 22°C
Nicotinic	epibatidine [0.01-10 nM]	nicotine-di-hydrogen-tartrate [100 µM]	15 mM Hepes-Tris (pH7.5) + 120 mM NaCl + 5.4 mM KCl + 0.8 mM MgCl ₂ + 1.8 mM CaCl ₂	90 min at 22°C
α ₁	prazosin [0.01-2 nM]	phentolamine [100 µM]	50 mM Tris (pH7.4) + 10 mM MgCl ₂	30 min at 22°C
α _{2h}	UK-14,304 [0.01-30 nM]	phentolamine [100 µM]	50 mM Tris-HCl (pH7.7) + 100 µM MnCl ₂	60 min at 22°C
5-HT _{1A}	8-OH-DPAT [0.01-15 nM]	serotonin [100 µM]	50 mM Tris-HCl (pH7.4) +4 mM CaCl ₂ +0.01 % Ascorbic acid	15 min at 37°C
D ₂	racloprid [0.05-15 nM]	butaclamol [1 µM]	50 mM Tris-HCl (pH7.4) +4 mM CaCl ₂ +0.01 % Ascorbic acid	45 min at 22°C
A ₁	DPCPX [0.1-25 nM]	R-PIA [100 µM]	170 mM Tris-HCl (pH 7.4) + 2 Units/L adenosine-deaminase + 100 µM Gpp(NH)p	120 min at 22°C
A ₁	CPFPX [0.1-25 nM]	R-PIA [100 µM]	170 mM Tris-HCl (pH 7.4) + 2 Units/L adenosine-deaminase + 100 µM Gpp(NH)p	120 min at 22°C
A _{2A}	ZM 241 385 [0.1-25 nM]	Theophylline [5 µM]	TE Buffer (pH7.4) + 1 Unit/L adenosine-deaminase + 0.01% Triton X-100	120 min at 22°C

receptor can be altered concomitantly via alterations of other receptors and neurotransmitter systems. Therefore, for the first time, we compared the dissociation constants (K_D) and binding site densities of 19 receptors for 6 classical neurotransmitters and for the neuromodulator adenosine in the primary motor cortex and putamen of HE with values obtained from control brains.

Materials and Methods

Material

Post-mortem human brain tissue was obtained at autopsy from 7 control subjects and 7 patients suffering from liver cirrhosis and accompanying HE. The study was approved by the local ethics committee of the University Hospital, Heinrich-Heine-Universität, Düsseldorf, Germany. Controls (age 59±19; 2 females, 5 males; *post-mortem* time 10±4 hours) had no record of hepatic, neurological or psychiatric disorders and had given informed written consent and/or had been included in the body

donor program of the Department of Anatomy, University of Düsseldorf, Germany. Causes of death were a heart attack and carcinomas (bronchial [3 cases], rectal, breast, lymphoma). HE-patients were clinically diagnosed according to published criteria [41] at the Clinic for Gastroenterology, Hepatology and Infectiology of the Heinrich-Heine-University (cases 2, 3, 5-7) or at the Department of Internal Medicine of the University Hospital of Patras (cases 1 and 4). Comorbidities and results of blood tests carried out the day before death are summarised in Table 2. Informed written consent was given either by the patients or by their relatives.

Case 1 was diagnosed cirrhosis caused by a HBV infection twenty five years ago, but did not show overt signs of HE until four weeks before death. One week prior to death HE progressed to grade III, followed by grade IV during the last twenty hours.

Case 2 was diagnosed alcoholic, decompensated cirrhosis five years before death and a grade II HE two years later. Four weeks prior to death HE progressed to grade III followed by a further deterioration to grade IV during the last three days. This patient had been included in a recent PET study examining HE-related changes in adenosine A₁ binding site densities [32].

Table 4. Dissociation constants in nM ($K_D \pm$ s.d.) estimated in the cortex and putamen of control and HE brains. asterisks: significant differences in K_D between control and HE brains. *n.a.*: not analysed.

Receptor	[3 H]Ligand	Controls (n = 7)		Hepatic encephalopathy (n = 7)	
		cortex	putamen	cortex	putamen
AMPA	AMPA	7.9 $\pm$ 1.1	9.5 $\pm$ 3.9	7.8 $\pm$ 4.4	8.8 $\pm$ 2.3
Kainate	kainate	4.0 $\pm$ 1.3	4.7 $\pm$ 3.3	3.0 $\pm$ 0.6	2.7 $\pm$ 0.6
mGluR2/3	LY 341,495	9.0 $\pm$ 0.5	7.0 $\pm$ 0.6	9.8 $\pm$ 3.1	9.6 $\pm$ 3.0
GABA _A	muscimol	3.8 $\pm$ 1.0	4.6 $\pm$ 1.9	3.2 $\pm$ 1.0	2.3 $\pm$ 0.7
GABA _B	CGP 54626	3.1 $\pm$ 1.2	3.0 $\pm$ 1.3	2.2 $\pm$ 0.7	2.5 $\pm$ 1.6
BZ	flumazenil	3.2 $\pm$ 0.6	2.0 $\pm$ 0.5	2.7 $\pm$ 0.4	2.7 $\pm$ 1.4
M ₂ **	oxotremorine-M	2.5 $\pm$ 1.0	2.5 $\pm$ 2.0	7.6 $\pm$ 4.6	4.1 $\pm$ 2.8
M ₃	4-DAMP	1.1 $\pm$ 0.5	3.2 $\pm$ 2.0	1.9 $\pm$ 1.8	5.0 $\pm$ 2.1
Nicotinic	epibatidine	0.07 $\pm$ 0.03	0.06 $\pm$ 0.03	0.05 $\pm$ 0.02	0.06 $\pm$ 0.03
α_1	prazosin	0.2 $\pm$ 0.06	n.a.	0.2 $\pm$ 0.2	n.a.
α_{2h}	UK-14,304	5.4 $\pm$ 0.7	n.a.	3.2 $\pm$ 1.8	n.a.
5-HT _{1A}	8-OH-DPAT	1.4 $\pm$ 0.5	n.a.	2.5 $\pm$ 1.9	n.a.
D ₂	racloprid	n.a.	0.4 $\pm$ 0.2	n.a.	0.8 $\pm$ 0.3
A ₁	DPCPX	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	0.8 $\pm$ 0.3	0.8 $\pm$ 0.1
A ₁	CPFPX	1.9 $\pm$ 0.3	2.7 $\pm$ 1.7	1.6 $\pm$ 0.2	1.3 $\pm$ 0.4
A _{2A}	ZM 241 385	n.a.	0.5 $\pm$ 0.3	n.a.	0.5 $\pm$ 0.1

Case 3 had a history of alcohol consumption for more than twenty years and cirrhosis was diagnosed six years before death. During the three months prior to death, he slipped between grades I and II followed by approximately two weeks in grade III (with frequent comatose periods) and then lapsed into a steady grade IV. Sedation was necessary during the coma period due to artificial respiration. This patient had been included in a recent PET study examining HE-related changes in adenosine A₁ binding site densities [32].

Case 4 was classified as suffering from grade II HE approximately five months prior to death followed by approximately two weeks in grade III and a further two weeks in grade IV.

Case 5 was diagnosed alcoholic cirrhosis fifteen years before death and slipped between grades I and II for two months prior to death followed by three weeks in grades II-III before lapsing into a stabile grade IV which lasted four days.

Case 6 was diagnosed alcoholic cirrhosis eight years before death. He slipped between grades I and II approximately seven months prior to death followed by five weeks in grades II-III before lapsing into a stable grade IV which lasted four days.

Case 7 was diagnosed alcoholic cirrhosis two years prior to death. Shortly before death, HE progressed rapidly from minimal HE to grade II, followed by a week in grades II-III and two weeks in grade IV. Sedation was necessary during the coma period due to artificial respiration.

Saturation studies

We determined K_D values of mGluR2/3, GABA_B, BZ, M₃, nicotinic, 5-HT_{1A}, α_1 , α_2 , D₂, A₁ and A_{2A} binding sites in homogenate suspensions and a harvester. Since short wash steps (in the range of seconds) were not possible due to technical reasons, the K_D values of AMPA, kainate, GABA_A, M₂ binding sites were determined on cryosectioned pellets.

Cortex and putamen probes were homogenized separately for 1 min in 10 volumes of ice-cold 10 mM Tris-citrate buffer, pH 7.4 with a disperser (Micra; ART-Labortechnik, Germany). Homogenates were divided into 2 ml aliquots and centrifuged (Megafuge 1.0 R; Hereaus, Germany) 20 min at 20000xg and 4°C. The supernatant was discarded, the pellet (P1) resuspended in 2 ml ice cold buffer (Tris-citrate buffer, pH 7.4) and centrifuged 20 min at 20000xg and 4°C. The pellets (P2) were resuspended in 2 ml ice cold buffer (Tris-citrate buffer, pH 7.4) and centrifuged 20 min at 20000xg and 4°C. These two steps were repeated twice, the final supernatant was discarded and the P5 pellets were resuspended in 10 volumes of ice-cold buffer and stored at -80°C till further processing.

For homogenate suspensions saturation assays were performed with a Brandel Zellharvester M-48 (Brandel, UK) in triplicate according to the same basic protocol (Table 3): P5 pellets were thawed, resuspended and incubated in assay buffer with increasing concentrations of tritiated ligand in the presence of or without the displacer for the determination of non-specific or total binding, respectively. Incubations were terminated with 3x3 ml washes of ice-cold buffer over GF/B filters presoaked for 1 min in 0.03 % polyethylenimine. Filters were then soaked in 10 ml scintillation cocktail Ready Safe (Beckman-Coulter, Germany) for 48 h and retained radioactivity was determined using a β liquid scintillation counter (TriCarb, Packard, Germany).

For saturation assays carried out on cryosectioned pellets, P5 pellets were thawed, aliquoted (2000 μ l per cup) and centrifuged 10 min at 3000xg and 4°C. The pellet was frozen and cryosections (10 μ m, at -20°C; CM 3050; Leica) were processed for quantitative *in vitro* receptor autoradiography according to established protocols [42]. Concentration ranges of tritiated ligands were as follows: 1-100 nM [3 H]AMPA for AMPA binding sites, 0.1-50 nM [3 H]kainate for kainate binding sites, 1-70 nM [3 H]muscimol for GABA_A binding sites and

Table 5. Adenosine A₁ binding site densities (in fmol/mg protein) measured in the primary motor cortex and putamen of controls (n=7; mean ± s.d.) and in each of the examined HE cases. Binding sites were labelled with [³H]DPCPX or with [³H]CPFPX (both in the presence of Gpp(NH)p).

Region Ligand	Motor cortex		Putamen	
	[³ H]DPCPX	[³ H]CPFPX	[³ H]DPCPX	[³ H]CPFPX
control ± s.d.	1092 ± 135	1064 ± 14	1541 ± 108	1368 ± 119
Case 1	997	738	804	634
Case 2	985	947	979	924
Case 3	915	841	846	768
Case 4	1002	853	922	797
Case 5	1479	1070	1162	998
Case 6	1562	1280	1403	1255
Case 7	2386	1886	1749	1338

0.1–30 nM [³H]oxotremorine-M for M₂ binding sites. Displacers were quisqualate [10 μM], SYM 2081 [100 μM], GABA [10 μM] and atropine sulfate [100 μM] for the AMPA, kainate, GABA_A and M₂ binding protocols, respectively. Cryosections were subsequently co-exposed with plastic standards of known radioactivity concentrations against [³H]-sensitive imaging plates (Raytest, Germany) for 72 h at room temperature. Imaging plates were scanned with a Bioimaging System BAS 5000 (Raytest, Germany) and the ensuing autoradiographs were processed densitometrically with the AIDA software system (Raytest, Germany).

Protein concentration in the P5 pellets was determined the day of the saturation analysis with the Bradford test and bovine serum albumin (Sigma-Aldrich, Germany) as a standard. Binding data were evaluated with the Prism software (Graph Pad, USA) and the dissociation constant K_D was computed using a non-linear regression.

Tritiated ligands were purchased from PerkinElmer with the exception of [³H]CGP 54626 and [³H]ZM 241 385, which were purchased from Biotrend, and [³H]CPFPX, which was synthesized at the Institute of Neurosciences and Medicine – Nuclear Chemistry (INM-5), Research Center Jülich. Displacers were obtained from Sigma Aldrich with the exception of CGP 55845, LY 341,495 and SYM 2081 (Tocris Bioscience) as well as of L-glutamate (VWR GmbH) and carbachol, GABA, phentolamine, quisqualate and ZM 241 385 (Biotrend).

Quantitative *In vitro* receptor autoradiography

Serial coronal cryosections (20 μm) comprising the whole cross-section of unfixed brain slabs were prepared at -20°C using a large-scale cryostat microtome. Adjacent glass-mounted sections were processed for quantitative *in vitro* receptor autoradiography according to standard protocols [42;43] and receptor densities were determined by densitometry [42] in the primary motor cortex (averaged over all cortical layers) and putamen.

Statistical analysis

Statistical analysis of our two multireceptor datasets (binding site densities and K_D values) was carried out following the general strategy: we first performed an Omnibus test (ANOVA) involving the simultaneous comparison of all receptor types to determine whether control and HE groups differed significantly. Only in the case of a significant result of the ANOVA test, did we perform additional statistical testing using post hoc tests to reveal which receptor types differed

significantly between control and HE brains. The p values were not corrected for multiple comparisons. For all tests, significance level was set at p≤0.01. Following comparisons were carried out:

Differences between K_D values measured in the cortex and putamen of control brains were examined by calculating, for each brain and receptor type, the index

$$\frac{K_{D[cortex]}}{K_{D[striatum]}} - 1$$

and determining whether it differed significantly from 0 (expected value) with an ANOVA test followed by paired t-tests. The same procedure was followed for K_D values measured in HE brains.

Differences between K_D values of control and HE brains were examined with an ANOVA and subsequent t-tests.

Differences in binding site densities between HE and control brains were determined with an ANOVA followed by t-tests.

Additionally, we analysed the possible correlation between ammonia plasma concentrations (measured the day before death) and differences in binding site densities measured in the primary motor cortex and putamen of HE and control brains with a multiple linear regression analysis. Ammonia plasma concentrations constituted the dependent variable of the analysis. The independent variables were the density differences between HE and control brains in the two examined regions. Significance level was set at p≤0.01.

Results

Saturation studies

The K_D of AMPA, kainate, mGluR2/3, GABA_A, GABA_B, BZ, M₂, M₃, nicotinic and A₁ binding sites were determined in the motor cortex and the putamen of control and HE brains (Table 4). K_D values of α₁, α_{2h} and 5-HT_{1A} binding sites were determined in the cortex, and those of D₂ and A_{2A} binding sites in the putamen (Table 4). All examined binding sites were found to be saturable in both regions of control and HE homogenates. Scatchard plots of the binding data resulted in linear plots, suggesting a single high-affinity binding site for each of the examined

Fig. 1. Representative saturation curves and Scatchard plots of (a) [3 H]AMPA (AMPA receptor) and (b) [3 H]prazosin (α_1 receptor) binding to the motor cortex as well as of (c) [3 H]flumazenil (GABA_A associated benzodiazepine [BZ] binding site) and (d) [3 H]ZM241385 (A_{2A} receptor) binding to the putamen of HE and control brains. The Scatchard plots (insets) are consistent with ligand binding to single affinity sites. *B*: number of binding sites; *F*: free ligand concentration.

Fig. 2. Representative saturation curves of [3 H]oxotremorine-M binding to the motor cortex (a) and putamen (b) of HE and control brains. The Scatchard plots (c: motor cortex; d: putamen) are consistent with [3 H]oxotremorine-M binding to a single affinity site. The K_D obtained for [3 H]oxotremorine-M binding to the M_2 receptor was significantly higher in HE than in control brains. *B*: number of binding sites; *F*: free ligand concentration.

receptor types (Figures 1-2).

Control and HE brains only differ significantly in their affinities to [3 H]oxotremorine-M. The K_D obtained for [3 H]oxotremorine-M binding to the M_2 receptor was found to be significantly higher in HE than in control brains (Figure 2), thus reflecting a lower affinity of acetylcholine for this muscarinic receptor subtype in HE brains.

Binding site densities

Laminar distribution patterns of the examined receptor types were comparable in the primary motor cortex of control and HE brains (Figures 3-4). Some receptors are present in higher concentrations in the superficial cortical layers (e.g. AMPA, mGlu2/3, GABA_A, GABA_B, BZ, M_3 , α_1 , α_{2h} , 5-HT_{1A}), whereas for other types the opposite holds true (e.g., kainate, A_1). Yet a third group of receptors presented a relative homogeneous distribution throughout all cortical layers (e.g. NMDA,

Fig. 3. Exemplary sections through the motor cortex of control and HE brains. Neighbouring coronal sections through control and HE brains were labelled with (a) [^3H]kainate, (b) [^3H]MK801, (c) [^3H]muscimol, (d) [^3H] oxotremorine-M and (e) [^3H]8-OH-DPAT in order to visualize the kainate, NMDA, GABA_A, M₂ and 5-HT_{1A} binding sites, respectively. Colour scales code receptor densities in fmol/mg protein. Although some of the HE cases differ in their mean densities (averaged over all cortical layers) from the controls, the laminar distribution pattern is comparable between experimental groups.

Fig. 4. Adenosine A₁ receptor distribution as revealed by [^3H]DPCPX (a) or its analog [^3H]CPFPX (b) in the brains of controls and HE patients. Colour scales code receptor densities in fmol/mg protein.

M₁, M₂, 5-HT₂, D₁). Nicotinic receptors present a maximum in deep layer III. Cortical densities of D₂ and A_{2A} binding sites are practically undistinguishable from the background and were, therefore, not measured.

Distribution of the examined receptor types was also comparable in the putamen of control and HE brains, revealing its striosome/matrix dichotomy. The matrix compartment contains lower GABA_A, 5-HT_{1A}, D₁, but higher M₁, M₂ and D₂ binding site densities than the striosomes. The putamen contains significantly higher mGluR2/3, BZ, M₁, and A₁ binding site densities than the primary motor cortex.

Adenosine A₁ binding sites were labelled with [^3H]DPCPX or with its analogue [^3H]CPFPX. Binding of each of these two tritiated ligands to the A₁ receptor presented equivalent distribution patterns and mean densities in control and HE brains (Table 5, Figure 4).

Fig. 5. Relative receptor fingerprints of the primary motor cortex. The polar coordinate plots show the up- or down-regulation (in %) of receptors for glutamate (ionotropic AMPA, kainate, NMDA; metabotropic mGluR2/3), GABA (GABA_A, GABA_B, BZ), acetylcholine (muscarinic M₁, M₂, M₃; nicotinic [N]), norepinephrine (α_1 , α_{2h}), serotonin (5-HT_{1A}, 5-HT₂), dopamine (D₁) and adenosine (A₁, labelled with [³H]CPFPX) in each of the HE brains (bold black line) relative to the mean of controls (grey surfaces; dashed lines indicate s.d.). Note that not only the fingerprint shape differs considerably between cases, but that the axis scaling has been optimized for each case. Due to technical problems, the M₁ and nicotinic binding sites could not be measured in case 5, M₁ binding sites were not measured in case 6 either, nor BZ binding sites in case 7. Note, that cases 1-4 are similar in shape, as are cases 5-7. Whereas NMDA receptor densities are increased in cases 1-4, they are unchanged or even down-regulated in cases 5-7. GABA_A and GABA_B receptor densities are decreased in cases 1-4, but are unchanged or even up-regulated in cases 5-7. Furthermore, α_{2h} receptor densities are down-regulated in cases 1, 3 and 4, but are up-regulated in cases 5-7.

Both ligands are present in higher densities in the infragranular than in the supragranular cortical layers. Highest concentrations are reached in the CA1 region of the hippocampus, as clearly revealed by the colour coded autoradiographs. Motor and temporal association cortices have higher densities than the somatosensory cortex or the insula. Putamen and caudate nucleus contain comparable binding site concentrations, which are clearly higher than those present in the globus pallidus. Additionally, A₁ binding sites are heterogeneously

distributed throughout the thalamus, with highest densities in its anterior and dorsomedial nuclei.

For the primary motor cortex, the ANOVA (comprising all ligands) revealed that HE and control brains differ significantly in their binding site densities, although the post hoc tests did not reveal significances for any of the examined receptor types. This is due to the fact, that although HE and control brains differ as a whole (the motor cortex of HE brains tends to show higher binding site densities than that of controls), the alterations of a

Fig. 6. Relative receptor fingerprints of the putamen. The polar coordinate plots show the relative up- or down-regulation (in %) of receptors for glutamate (ionotropic AMPA, kainate, NMDA; metabotropic mGluR2/3), GABA (GABA_A, GABA_B, BZ), acetylcholine (muscarinic M₁, M₂, M₃; nicotinic [N]), norepinephrine (α_1 , α_{2h}), serotonin (5-HT_{1A}, 5-HT₂), dopamine (D₁, D₂) and adenosine (A₁, labelled with [³H]CPFPX, A_{2A}) in each of the HE brains (bold black line) relative to the mean of controls (grey surfaces; dashed lines indicate s.d.). Note that not only the fingerprint shape differs considerably between cases, but that the axis scaling has been optimized for each case. Due to technical problems, the M₁ and nicotinic binding sites could not be measured in case 5, M₁ binding sites were not measured in case 6 either, nor BZ binding sites in case 7. As described in figure 6 for the motor cortex the putamen fingerprints of cases 1-4 are similar in shape, as are those of cases 5-7. Again, NMDA receptor densities are increased in cases 1-4, but are unchanged or even down-regulated in cases 5-7. GABA_A and GABA_B receptor densities are also decreased in the putamen of cases 1-4, though they are unchanged or even up-regulated in cases 5-7. The α_{2h} receptor densities are down-regulated in cases 1-4, but are up-regulated in cases 5-7.

given receptor type, when examined individually, were not sufficient to reach the threshold significance level of $p \leq 0.01$. Figure 5 shows the relative changes (in %) of each of the cases to the mean control and clearly demonstrates the high interindividual variability present in the HE group. For example, AMPA binding sites were down-regulated (20-50%) in four of the examined cases (#1-4), unaltered in two (#5, 6), but 100% up-regulated in case 7.

For the putamen (Figure 6), the ANOVA test showed

that HE and control brains differ significantly in their binding site densities and subsequent t-tests revealed significant differences for the nicotinic, A₁ and A_{2A} binding sites. Nicotinic binding sites are down-regulated in cases 1-6 and unchanged in case 7; A₁ and A_{2A} binding sites are down-regulated in cases 1-5 and unchanged in cases 6-7. Despite this variability, the mean densities (averaged over all examined brains for each of the experimental groups) of these three receptor types are significantly lower in the putamen of HE brains than in that of controls.

The significant differences in A_1 binding site densities were found for the binding of both [3H]DPCPX and [3H]CPFPX. The putamen of HE cases also differs considerably concerning both the intensity and direction (up- or down-regulation) of density differences detected in comparison to controls. For example, AMPA binding sites were down-regulated (30-40%) in four of the examined cases (#1-4), unaltered in two (#5, 6), but 30% up-regulated in case 7 (Figure 6).

We examined the possible correlation between ammonia levels in plasma at the time of death and the alterations of binding site densities observed in the primary motor cortex and putamen of HE brains relative to the values measured in controls and found no significant connection between these parameters. Only variations in GABA $_A$ binding site densities showed a tendency ($p=0.047$) to correlate with ammonia levels, so that high GABA $_A$ densities were generally associated with low ammonia plasma levels.

Discussion

We determined for the first time HE-related alterations in K_D and binding site densities of 19 neurotransmitter receptors from 7 transmitter systems in the primary motor cortex and putamen of human *post-mortem* tissue. The HE cases form a heterogeneous group concerning the intensity and direction of receptor alterations they present. Mean binding site densities of nicotinic, A_1 and A_{2A} receptors were significantly decreased in the putamen of HE patients. Since the K_D values of these receptor types did not differ between the experimental groups, these density differences are due to alterations in binding site stoichiometry. Acetylcholine M_2 binding sites had a significantly higher K_D in HE brains, thus reflecting a lower affinity of acetylcholine for this receptor subtype in HE than in controls.

The HE cases do not form a homogeneous group as it is also known from other neuropathological conditions (Epilepsia). Rather, they differ considerably from each other in both the intensity and direction of receptor alterations. For a given receptor type we found higher, lower or unaltered densities in comparison to controls (Figures 5-6). This remarkable interindividual variability reflects the controversial results described in the literature, such as, for example, the higher [16, 29] or unaltered [9, 15, 24, 25, 28-30] GABA $_A$ receptor densities which have been associated with HE. This interindividual variability

could be due to the fact that the effects of different hyperammonemic situations on brain function and the mechanisms responsible for these effects vary depending on the concentrations of ammonia reached, the duration of the exposure to hyperammonemia, comorbidities, treatment modalities and the age of the patient [2]. This should not be surprising taking into account that when addressing certain cortical neuropathological conditions, even individual variations have to be considered among exactly staged individuals of one and the same breeding strain of experimental animals [39, 44].

The ubiquitous neuromodulator adenosine acts at four different G-protein-coupled receptors and exerts a neuroprotective function [45, 46]. Activation of the high affinity A_1 and A_{2A} receptors results in a modulation of neurotransmission at both pre- and postsynaptic levels [45, 47-49]. Activation of A_1 receptors results in a decreased neurotransmitter release, thus inducing neuronal hyperpolarization [50, 51]. The A_{2A} subtype has been shown to not only facilitate acetylcholine [51, 52] and glutamate release, but also activation of the NMDA receptors [53]. Activation of the A_{2A} receptors has also been shown to facilitate GABA release in the hippocampus [54], though they inhibit it in the rat striatum [52]. Additionally, adenosine receptors play a crucial role in the control of dopamine D_2 receptor function in the basal ganglia [55]. Furthermore, both adenosinergic receptor subtypes are known to interact with each other, whereby A_{2A} receptors can either inhibit or neutralize the consequences of A_1 receptor activation at the presynaptic level [48, 49]. All in all, and although the exact mechanisms through which adenosine controls neuronal function remain an open issue, it is widely accepted, that its main role is to assist maintaining homeostasis in biological systems [56], and this function is impaired in the brain of HE patients, since we found a significant down-regulation of both A_1 and A_{2A} binding sites in their putamen. This down-regulation could be the result of a compensatory neuroprotective mechanism, since A_1 receptor activation at the onset of neuronal injury attenuates brain damage, and A_{2A} receptors are not only up-regulated in noxious brain conditions, but their inactivation through antagonists has a neuroprotective effect (for a comprehensive review see [57]). The down-regulation of A_1 binding sites is in accordance with our previous results, which were also obtained from human *post-mortem* tissue with [3H]DPCPX [24] as well as with an *in vivo* study using positron emission tomography and [^{18}F]CPFPX [32]. Indeed, this

congruence isn't surprising, since our cases 2 and 3 are patients 6 and 1, respectively, of the study carried out by Boy and collaborators [32].

We were here able to confirm that binding of [³H]DPCPX (the prototypical A₁ receptor antagonist) and its analogue [³H]CPFPX to the adenosine A₁ receptors is not only comparable in rat [43] but also in human brains, since both ligands present comparable binding patterns and densities and did not differ in their affinities (Tables 4-5, Figure 4).

Acetylcholine modulates release of other neurotransmitters through activation of both muscarinic and nicotinic receptors. Activation of hippocampal nicotinic receptors mediates neuronal excitation [58] and activation of muscarinic receptors, in particular of the M₂ subtype, has been reported to suppress GABAergic synaptic transmission [59]. Our present data indicate that the brains of HE patients could be subject to a lower enhancement of neuronal excitation via nicotinic receptors, since we found their densities to be down-regulated in the putamen of HE brains. Additionally brains of HE patients could be subject to a lower inhibition of GABA release via muscarinic receptors, since i) we found the affinity of acetylcholine for the M₂ subtype to be lower than that of control brains ii) we found a down-regulation of A_{2A} receptors, the activation of which is known to facilitate acetylcholine release [51, 52], and iii) acetylcholine levels were reported to be considerably reduced in the brains of patients decreased from hepatic coma due to increased acetylcholinesterase activity [23]. Thus, the net increase of inhibitory neurotransmission which has been associated with HE (for reviews see [2] and [3]) could be effected by a decreased cholinergic neuromodulation. Other studies concerning HE-related alterations of cholinergic receptors are sparse and inconclusive, since they encompass contradictory results (up- and down-regulations, Table 1). Data reported in these studies was obtained mostly with subtype non-specific antagonists, thus hindering comparison of our present results with existing reports in the literature [30, 40]. We were able to reproduce our previous results concerning the densities of muscarinic receptor subtypes [24]. Although we previously described nicotinic receptor densities to be lower in HE than in control brains, this tendency did not reach the level of significance [24]. In the present study, probably due to the larger sample, we found nicotinic receptors to be significantly down-regulated in the putamen.

Astrocytic impairment due to cellular oedema and oxidative stress responses could be considered a hallmark of severe hyperammonia [18, 37, 60] and the glia derived

molecule kynurenic acid (KYNA) interacts with the high affinity binding sites of AMPA receptors [61] and antagonizes nicotinic cholinergic receptors as well as the glycine binding site of the NMDA receptor (for a review see [62]). Furthermore, the neurotoxin quinolinic acid, an additional product of the kynurenine pathway is a specific NMDA agonist with a high potential for free radical production [61]. KYNA synthesis [63, 64] and quinolic acid levels [65] are known to be altered in HE. Thus, differences in glial impairment and associated levels of KYNA or quinolinic acid may be an important link between ammonia induced glial impairment and the here observed alterations in binding to nicotinic ACh receptors and could also be responsible for the differences seen for NMDA binding sites among cases 1-4 and 5-7.

Concluding, our data offer new insights into the complex pathophysiological mechanisms of HE in humans. Receptor alterations associated with HE seem to be more complex than hitherto assumed, and can no longer be reconciled into a hypothesis featuring a single neurotransmitter system or its receptors. This complexity, which must be clarified by further studies, suggests that neurotransmitter alterations may not be the primary key culprits for the neuropsychiatric and motor disturbances associated with HE.

Abbreviations

4-DAMP (4-Diphenylacetoxy-N-methylpiperidine methiodide); 5-HT_{1A}, 5-HT₂ (serotonin receptors; subtypes 1A and 2); 8-OH-DPAT (8-hydroxy- 2-(di-n- propylamino) tetraline); α₁, α_{2h} (norepinephrine receptors; subtypes 1 and 2 (high affine binding site)); A₁, A_{2A}, A₃ (adenosine receptors; subtypes 1, 2A and 3); AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid); BZ (GABA_A associated benzodiazepine (binding sites)); CGP 54626 ([S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid); CGP 55845 ((2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl] (phenylmethyl)phosphinic acid); CPFPX (8-cyclopentyl-3-(3-fluoropropyl)-1-propyl-xanthine); CRP (*c*-reactive protein); D₁, D₂ (dopamine receptors; subtypes 1 and 2); DPCPX (8-Cyclopentyl-1,3-dipropylxanthine); EDTA (ethylene-diamine-tetraacetic acid); GABA_A, GABA_B (γ-aminobutyric acid receptors; subtypes A and B); Gpp(NH)p (5'-guanylimidodiphosphate); HE (hepatic encephalopathy); K_D (dissociation constant); M₁-M₃ (muscarinic

acetylcholine receptors; subtypes 1-3); mGluR2/3 (metabotropic glutamate receptors; subtypes 2 and 3); NMDA (*N*-methyl-D-aspartate); N (neuronal nicotinic acetylcholine receptors); PMSF (phenylmethylsulfonyl fluoride); R-PIA (R(-)-N⁶-(2-phenylisopropyl) adenosine); TIPS (transjugular intrahepatic portosystemic shunt).

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