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ORIGINAL PAPER

Postmortem proteomic analysis in human amygdala of drug addicts: possible impact of tubulin on drug-abusing behavior

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Abstract Besides the ventral tegmental area and the nucleus accumbens as the most investigated brain reward structures, several reports about the relation between volume and activity of the amygdala and drug-seeking behavior have emphasized the central role of the amygdala in the etiology of addiction. Considering its proposed important role and the limited number of human protein expression studies with amygdala in drug addiction, we performed a human postmortem proteomic analysis of amygdala tissue obtained from 8 opiate addicts and 7 control individuals. Results were validated by Western blot in an independent postmortem replication sample from 12 opiate addicts compared to 12 controls and 12 suicide victims, as a second "control sample". Applying 2D-electrophoresis and MALDI–TOF–MS analysis, we detected alterations of beta-tubulin expression and decreased levels of the heat-shock protein HSP60 in drug addicts. Western blot analysis in the additional sample demonstrated significantly increased alpha- and beta-tubulin concentrations in the amygdala of drug abusers versus controls ($P = 0.021$, 0.029) and to suicide victims ($P = 0.006$, 0.002). Our results suggest that cytoskeletal alterations in the amygdala determined by tubulin seem to be involved in the pathophysiology of drug addiction, probably via a relation to neurotransmission and cellular signaling. Moreover, the loss of neuroprotection against stressors by chaperons as HSP60 might also contribute to structural alteration in the brain of drug addicts. Although further studies have to confirm our results, this might be a possible pathway that may increase our understanding of drug addiction.

Keywords Drug · Addiction · Amygdala · Human · Proteomics · Tubulin

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Introduction

Drug addiction, a severe, chronically relapsing neuro-psychiatric disorder can be characterized according to the definition of Koob and LeMoal by the compulsion to seek and take drugs, the loss of control in limiting the intake and by the occurrence of a negative emotional state reflecting a withdrawal syndrome when there is no access to the drug [1]. There is no doubt today that the origin of addiction is multifactorial including genetic, neurobiological and psychosocial correlates [2, 3].

A key element in the neurobiology of drug addiction is the reward system in the brain, a collection of brain structures involving mesolimbic and mesocortical pathways such as the amygdala, hippocampus and ventral striatum and its neuroadaptation during the development of the disorder [4]. After acute administration almost all drugs of abuse activate the mesolimbic dopaminergic system primarily in the ventral tegmental area (VTA) and the nucleus accumbens, structures that project finally also to the amygdala and the frontal cortex [5, 6]. These effects are

additionally mediated by the serotonergic-, opioid- and γ-aminobutyric acid (GABA) systems by direct and indirect effects in these brain areas [7–9]. By contrast, chronic drug exposure leads to a decrease in function of the same neuronal networks with a decline in neurotransmitter concentrations and receptor density [10]. Besides the alterations of several neurotransmitter systems, an increased sensitivity of receptor-meditated signal transduction mechanisms could also be observed in the nucleus accumbens, including activation of several enzymes and transcription factors during drug abuse [11]. These mechanisms seem to represent long-term neuroadaptative changes on the genomic level, associated with the vulnerability to relapse.

Besides the VTA and the nucleus accumbens as the most investigated brain reward structures, several reports have emphasized the central role of the amygdala in the etiology of addiction. The amygdala is the key area for emotional learning and in this way a crucial factor for affective conditioning (e.g. reward) and decision-making [12–14]. In an animal model of relapse after chronic cocaine self–administration, amygdala lesions attenuated the drug-seeking behavior in rats [15]. Structural abnormalities of the amygdala have been shown in cocaine-dependent subjects, which displayed amygdala volume reductions that were connected to baseline measures of drug craving [16]. Reduced amygdala volumes were also reported in alcohol-dependent patients [17]. Recent studies have demonstrated that the activation of the brain stress systems by the action of the corticotrophin-releasing factor (CRF) and norepinephrine in the extended amygdala seems also to be a key component in the pathophysiology of drug addiction [18].

However, although these interesting findings suggest that multiple pathways in different brain regions are involved in the pathophysiology of drug addiction, the precise cellular and molecular mechanisms, respectively their interactions that predispose to drug abuse/dependence and neurotoxicity in the central nervous system (CNS) are largely unknown. This highlights the importance of new comprehensive technologies to study the neuropathology of addiction in more detail. One method of choice is the proteomic approach that allows the analysis of the global protein expression in defined brain regions and its comparison between physiological and altered states without any prior hypothesis [19, 20].

Considering the proposed important role of the amygdala in addictive behavior and the negligible number of human protein expression studies with amygdala in drug addiction, we performed a human postmortem proteomic analysis of amygdala tissue obtained from 8 opiate addicted individuals and 7 control individuals. The global protein expression was determined by two-dimensional (2D) gel electrophoresis and subsequent analysis using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI–TOF–MS). Proteins were identified by matching peptide mass fingerprints against the mass spectrometry protein sequence human IPI data base. Significant results were validated by Western blot analysis in an independent postmortem replication sample from 12 drug addicts compared to 12 control individuals and 12 suicide victims, as a second "control sample".

Materials and methods

Subjects

Two independent postmortem samples were included in the present study: one sample for the proteomic analysis (original sample) and a second sample for the Western blot analysis (replication sample). Tissue probes were provided by the Institute for Legal Medicine of the Ludwig-Maximilians-University Munich. Subject demographics are presented in Table 1. The original sample consisted of 8 individuals who died in consequence of opiate dependency (6 men, 2 women, mean age 29.7 ± 9.9 years) and 7 control individuals (5 men, 2 women, mean age 36.0 ± 13.4 years) who died suddenly from diseases not directly involving the CNS as heart failure ($N = 2$), aortic aneurysm ($N = 2$), accidentally CO-intoxication ($N = 2$) or accident ($N = 1$). For the replication sample, brain specimens were derived from 12 drug addicts (9 men, 3 women, mean age 29.9 ± 9.7 years), 12 control individuals (8 men, 4 women, mean age 39.4 ± 13.6 years) and 12 suicide victims (9 men, 3 women, mean age 40.6 ± 14.9 years) as a second "control sample". Ten of the suicide victims have committed violent suicides, as e.g. hanging ($N = 5$), jumping from height ($N = 1$), cutting ($N = 3$), electric shock ($N = 1$) and 2 were employing so called soft suicide (intoxication). Causes of death of the control individuals were the following: heart failure ($N = 3$), homicide ($N = 3$), accident ($N = 4$), aortic aneurysm ($N = 2$). All opiate addicts were chronic abusers who died either from an acute overdose after a period of abstinence or from polytoxicomania. In every case, a toxicological analysis was performed on blood, urine and stomach content.

The clinical, medical data sheets of the control individuals were available at the Institute for Legal Medicine of the Ludwig-Maximilians-University Munich and did not give any hint on lifetime psychiatric or neurological disorders. According to the medical records, there was no history of psychopharmacological medication, alcohol or drug abuse. Toxicological reports of the included controls and suicide victims were not available in every case. All

Table 1 Demographic characteristics of the included individuals

Subject no.	Sex	Age (years)	PMI (h)	Cause of death	Toxicology	Axis I
Original sample						
OD subjects						
OD 1	M	18	7	Intoxication	Methadone, benzodiazepine, doxepine	Drug abuse
OD 2	M	44	5	Intoxication	Benzodiazepine, codeine, alcohol	Drug abuse
OD 3	F	20	16	Intoxication	Morphine methadone, cocaine, heroin	Drug abuse
OD 4	F	21	12	Homicide	Morphine, benzodiazepine	Drug abuse
OD 5	M	41	17	Intoxication	Methadone, benzodiazepine, doxepin	Drug abuse
OD 6	M	35	16	Intoxication	Morphine, benzodiazepine, doxepine	Drug abuse
OD 7	M	26	6	Intoxication	Methadone	Drug abuse
OD 8	M	33	15	Accident	Morphine, benzodiazepine	Drug abuse
Mean (range)		29.7 ± 9.9 (18–44)	11.7 ± 5.0 (5–17)			
CON subjects						
CON 1	F	47	14	Heart failure	NA	None
CON 2	M	39	17	Heart failure	NA	None
CON 3	M	50	9	Aortic aneurysm	NA	None
CON 4	M	15	16	Aortic aneurysm	NA	None
CON 5	M	31	11	CO_2-Intoxication	NA	None
CON 6	M	23	12	CO_2-Intoxication	NA	None
CON 7	F	47	9	Accident	NA	None
Mean (range)		36.0 ± 13.4 (15–50)	12.6 ± 3.2 (9–17)			
Replication sample						
OD subjects						
OD 9	M	35	11	Intoxication	Methadone, benzodiazepine	Drug abuse
OD 10	M	33	9	Intoxication	Methadone, benzodiazepine	Drug abuse
OD 11	M	27	7	Intoxication	Methadone, benzodiazepine	Drug abuse
OD 12	M	27	19	Intoxication	Morphine, codeine	Drug abuse
OD 13	M	39	4	Intoxication	Morphine, methadone, benzodiazepine	Drug abuse
OD 14	F	20	7	Intoxication	Morphine, alcohol	Drug abuse
OD 15	M	22	27	Intoxication	Morphine	Drug abuse
OD 16	F	54	5	Intoxication	Cocaine, benzodiazepine	Drug abuse
OD 17	M	25	21	Intoxication	Morphine, alcohol	Drug abuse
OD 18	M	26	15	Intoxication	Morphine, methadone	Drug abuse
OD 19	F	19	28	Intoxication	Methadone, benzodiazepine	Drug abuse
OD 20	M	32	37	Pulmonary embolism	Morphine, benzodiazepine	Drug abuse
Mean (range)		29.9 ± 9.7 (19–54)	15.8 ± 10.6 (4–37)			
SUV subjects						
SUV 1	F	47	21	CO-Intoxication	NA	Bulimia, depression
SUV 2	M	57	12	Hanging	NA	None
SUV 3	M	42	28	Hanging	NA	Depression
SUV 4	M	23	19	CO-Intoxication	NA	History of suicidal behavior

Table 1 continued

Subject no.	Sex	Age (years)	PMI (h)	Cause of death	Toxicology	Axis I
SUV 5	F	55	12	Electric shock	NA	None
SUV 6	F	19	16	Hanging	NA	Borderline personality
SUV 7	M	38	21	Cutting	NA	Depression
SUV 8	M	33	5	Cutting	NA	Depression
SUV 9	M	47	22	Fall from height	NA	Schizophrenia
SUV 10	M	67	17	Cutting	NA	Depression
SUV 11	M	22	20	Hanging	NA	None
SUV 12	M	37	11	Hanging	NA	Alcohol abuse
Mean (range)		40.6 ± 14.9 (19–67)	17.0 ± 6.2 (5–28)			
CON subjects						
CON 8	M	19	38	Accident	NA	None
CON 9	M	49	5	Heart failure	NA	None
CON 10	M	35	4	Accident	NA	None
CON 11	M	59	10	Aortic aneurysm	NA	None
CON 12	F	32	15	Homicide	NA	None
CON 13	M	36	27	Heart failure	NA	None
CON 14	M	49	22	Heart failure	NA	None
CON 15	F	30	10	Homicide	NA	None
CON 16	M	65	6	Homicide	Codeine, paracetamol	None
CON 17	F	30	17	Aortic aneurysm	NA	None
CON 18	M	41	7	Accident	NA	None
CON 19	F	28	29	Accident	Alcohol	None
Mean (range)		34.7 ± 16.0 (12–59)	11.5 ± 4.0 (3–17)			

OD opiate dependency, *CON* control, *SUV* suicide victim, *PMI* postmortem interval, *M* male, *F* female, *NA* not assessed, respectively not available, *CO* carbon monoxide

individuals were Caucasians from the same geographical region in southern Germany.

The project was performed under the approved guidelines of the local ethical committee of the Ludwig-Maximilians-University Munich and according to the declaration of Helsinki.

Tissue collection

The unfixed brain specimens were obtained 3–38 h after death during routine autopsy. The postmortem interval, defined as the time of death to time of freezing the brain tissue, was 12.0 ± 4.9 h in the original sample and 16.2 ± 9.3 h in the replication sample (no significant differences). Sections were taken from the amygdala. After dissection, the samples were immediately frozen and stored at −80°C.

Sample preparation

Approximately 0.3 g of each tissue sample was thawed at room temperature and homogenized using an Ultrathorax-homogenizer for 30 s in 3.5 µl/mg sample buffer (9 M urea, 1% Dithiothreitol (DTT)), 1.5 Immobiline pH gradient (IPG) buffer (pH 3–10, non-linear), 2% CHAPS (all reagents from GE Healthcare, Munich, Germany). After centrifugation at 7,000 g for 30 s, the supernatant was aliquoted and stored at −80°C until use. Protein content was determined using Bradford Protein Assay (Biorad, Munich, Germany). As the accuracy of this assay could have been influenced when measuring in urea, the results were validated using a second method that was compatible to urea. Therefore, in a series of samples, we performed a nephelometric quantification of benzethonium chloride precipitated proteins. Absorption spectra of the dilution

series were measured on a Hitachi 912 automated analyzer (505 nm) (Hitachi, Irvine, CA, USA). It could be shown that the linearity of the results of the Bradford Protein Assay was not significantly disturbed by urea.

Two-dimensional gel electrophoresis

The 2D electrophoresis was performed individually. For the first dimension, Immobiline Dry Strips (24 cm, pH 3–10, non-linear, GE Healthcare, Munich, Germany) were rehydrated in brain tissue homogenate corresponding to 100 µg protein and sample buffer (see above) to a total volume of 450 µl. After rehydration for 14 h, isoelectric focusing (IEF) was performed at 20°C using GE Healthcare Bioscience System (IPG-Phor, Universal Strip Holder) according to the following conditions: 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h (step-and hold), 6,000 V for 1 h (gradient) and 6,000 V up to 43 kVh (step-and hold). Prior to the second dimension, IPG-strips were equilibrated according to the method of Gorg et al. [21]. SDS–PAGE was conducted with 15% gels at 25°C with a maximum of 18 W/gel constant power. Protein spots were visualized with silver staining according to a slightly modified protocol of Shevchenko [22], which keeps the proteins compatible for MALDI–TOF–MS analysis.

Image analysis

Two-dimensional gels were digitized with a flat bed scanner (UMAX, Willich, Germany) and LabScan software (GE Healthcare, Munich, Germany). Spot editing, detection, quantification and matching were performed using PD Quest software (Biorad, Munich, Germany). Spot intensities were quantified and compared between drug addicts and controls. Differences between both groups were evaluated statistically by PD Quest applying the student *t*-test and the criteria of a minimum of two-fold difference in spot intensities.

In-gel protein digestion

Sample preparation for MALDI–TOF–MS analysis was performed according to the method of Tebbe et al. [23]. Spots of interest were excised, transferred to filter microtiter plates (Multiscreen Durapor MAHV N45, Millipore, Bedford, MA, USA) and destained. After removing the destaining solution, gel pieces were treated with 50% acetonitrile and 50 mM NH_4HCO_3 each for 10 min. This procedure was repeated, followed by enzymatic digestion with 0.1 µg/spot modified trypsin (Promega, Madison, WI, USA) at 37°C overnight. Digested peptides were eluated in three steps with water, 50% acetonitrile and finally with 50% acetonitrile, 0.1% TFA contained. Eluates were frozen in liquid nitrogen and dried in a SpeedVac centrifuge.

MALDI–TOF–MS and database analysis

For MALDI–TOF–MS analysis, dried samples were dissolved in 33% acetonitrile, 0.1% TFA. Using Bruker's MAP pipetting robot, 0.5 µl of each sample was mixed automatically with 0.5 µl of an a-cyano-4-hydroxy-cinnamic acid in 40% acetonitrile, 0.1% TFA on a ground steel MALDI target. MALDI–TOF–mass spectra were measured using a Reflex III spectrometer (Bruker Daltonics, Bremen, Germany). Peptide mass fingerprint data from MALDI–TOF–MS were matched against IPI database (IPI _Human, Vers. 3.69) using MASCOT search engine (Vers. 2.2.06, Matrix Science, London, UK, http://www.matrixscience.com) [24] (for search parameters see Table 2). The validity of the results for protein identification was measured applying the probability-based Mowse score of Mascot. The score is defined $-10*\log(P)$, where P is the probability that the observed match is a random event ($P < 0.05$). The magnitude of the score basically depends on size of the database, number of peptides and size of the protein. In this study, the Mascot score was 63. Furthermore, all measurements were repeated threefold in order to ascertain the protein identifications.

Table 2 Mass spectrometry parameters of the identified proteins; search parameters were as follows: peptide mass tolerance (±200 ppm), fixed modifications (carbamidomethyl), mass values (monoisotopic), peptide charge state (1+), maximum of missed cleavage (1), protein mass (unrestricted)

Spot Nr.	Protein	Mowse score	Accession number[a]	Molecular weight (kDa)	Isoelectirc point (pI)	Fold-change[b]	Number of mass values searched	Number of mass values matched
001	Human chaperonin GroEL precursor (HSP60)	179	P10809	61.19	5.24	−10.5	18	13
002	Human tubulin beta-4 chain TUBB4	198	P04350	50.01	4.78	−2.1	30	16

[a] According to UniProtKB/Swiss-Prot

[b] between mean spot intensities (OD/CON)

Western blotting

The protein concentrations were measured with spectral photometry. Ten micrograms of each sample was loaded and run on 10% polyacrylamide gels. After semi dry blot transfer to Immobilon P PVDC membranes (Millipore, Schwalbach, Germany), blots were blocked with 5% milk, 5% fetal calf serum, and 0.05% Tween-20 in 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5 (PBS-T) for 45 min and incubated for 90 min at room temperature with anti-beta-tubulin (diluted 1:1,000), respectively anti-alpha-tubulin (1.3000) (Abcam, Cambridge, UK) The labeling was visualized after incubation with a horseradish peroxidase-conjugated secondary antibody (diluted 1:3,000) for 60 min at room temperature (Sigma, Munich, Germany) using an immunoblot assay kit and a SDS–PAGE length standard (Biorad, Munich, Germany) according to the manufacturer's instruction. The densitometric evaluation of the signal intensities was done on a gel-documentation system using the associated analysis software (Gel Doc XR System PC, The Discovery Series™ Quantity One 1-D Analysis Software; Biorad Laboratories, Munich, Germany).

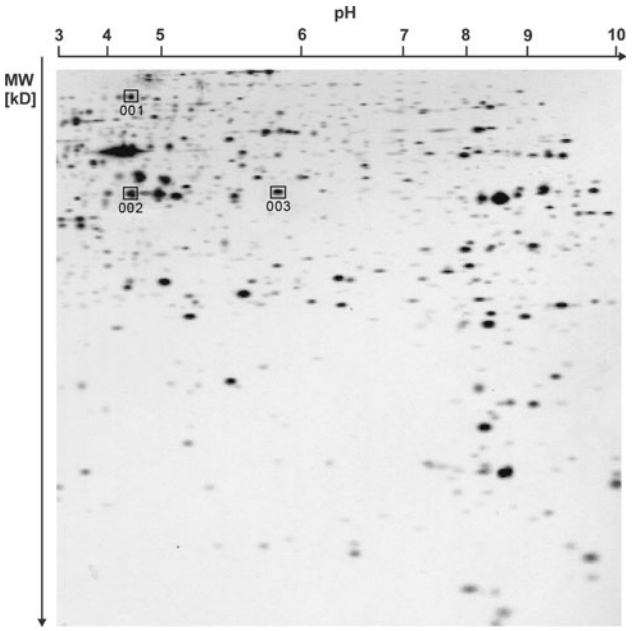

Fig. 1 2D-gel of the amygdala from one opiate overdose victim. Samples were resolved on non-linear ph 3–10 Immobiline dry strips followed by separation on a 15% SDS–PAGE gel in the second dimension. *Boxes indicate* positions and numbers of differentially expressed *spots* between drug addicts and controls

Statistical analysis

All statistical analyses were performed with SPSS for Windows (Version 15.0; SPSS; Chicago, IL).

All dependent variables were examined by the Kolmogorov–Smirnov test on normality. Stepwise linear regression analyses (pin = 0.05, pout = 0.10) with the independent variables gender, age and postmortem interval (PMI) were performed for all dependent variables in all groups. Correlations analyses between variables and age, as well as PMI, were performed with the Pearson's test. Analyses of covariance (ANCOVA) were conducted to test the diagnostic group differences concerning the tubulin protein expression levels determined by Western blot. The intervening factor gender, as well as the covariates age and PMI, was added to the analyses to test for significant influences on the initial regression analysis.

Results

Individual two-dimensional gel electrophoresis (2D) was performed with amygdala tissue of 8 individuals who died in consequence of opiate dependency (OD) and 7 control individuals (CON) (named as original sample in Table 1).

To investigate the influence of confounding factors, a comprehensive statistical evaluation was performed in all patient and control groups. Due to the small sample sizes, the power for the Kolmogorov–Smirnov test was low. However, the results suggested a normal distribution of the data and analysis by parametric tests. One-way analysis of variance (ANOVA) was conducted to evaluate group differences concerning age, gender and postmortem interval (PMI). Additionally stepwise linear regression analysis with the independent variables gender, age and PMI were performed for all dependent variables. Taken together, there were no statistical significant differences between the investigated groups concerning gender, age and postmortem interval. Furthermore, we could not observe any correlation between independent and dependent variables determined by the Pearson's test in all investigated groups.

The image analysis of the 2D-gels detected about 1,000 protein spots per gel. A mean of 690 spots (69% of detected spots per gel) could be matched against the master gel. Three spots were differentially expressed in the amygdala between drug addicts and controls (Fig. 1).

In general, the spot intensities of all three spots were decreased in the OD sample. In detail, spot 001 could be observed in one OD probe, whereas 5 probes of the control group expressed this spot ($P = 0.05$, $F = 4.79$, one-way ANOVA). Spots 002 and 003 were detectable in all amygdala samples of both groups with higher mean intensities in the CON probes ($P = 0.023$, $F = 7.21$ and $P = 0.05$, $F = 4.96$, one-way ANOVA). We conducted analysis of covariance (ANCOVA) to test the diagnostic group differences and to control statistically for the

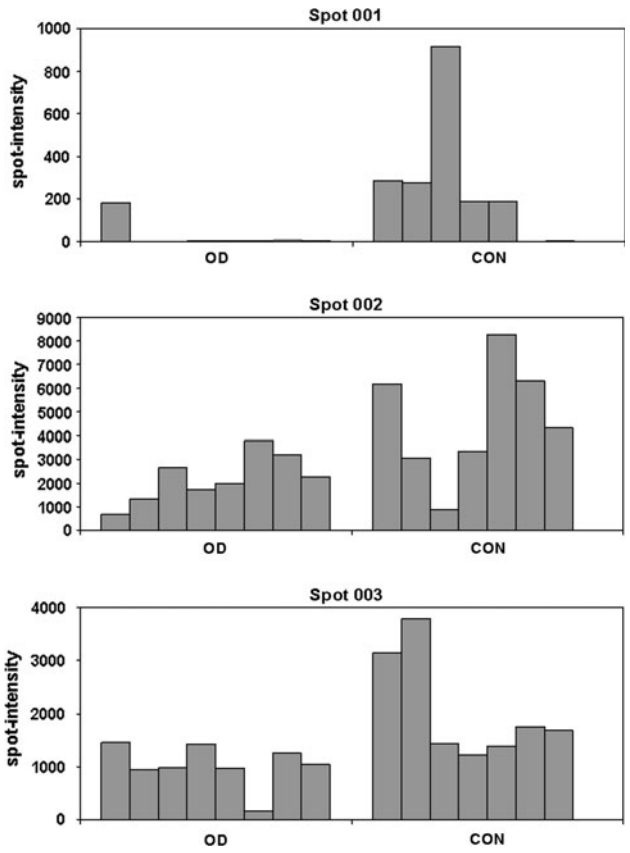

Fig. 2 Distribution of spot intensities in the amygdala of controls (*CON*) compared to opiate-dependent individuals (*OD*). Optical densities were digitized and evaluated with PD Quest software. Statistical analysis was performed by ANCOVA. OD versus CON: spot 001: $P = 0.05$, $F = 4.79$; spots 002: $P = 0.023$, $F = 7.21$; spot 003, $P = 0.05$, $F = 4.96$

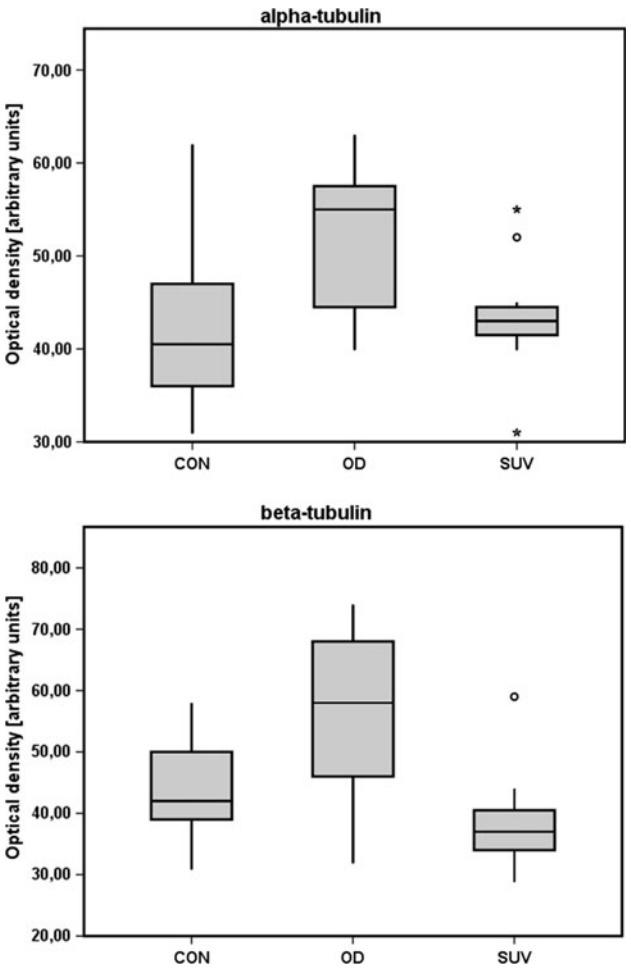

Fig. 3 Graphical presentation of the densitometric evaluation of the alpha- and beta-tubulin Western blot. Data are presented as box-plot for opiate-dependent individuals (*OD*), control individuals (*CON*) and suicide victims (*SUV*). Statistical analysis was performed by ANCOVA

potential effects of age, gender and PMI. No significant influence of the covariates could be observed. Figure 2 gives an overview about the significant differences of the single spot intensities for each sample.

The spots of interest were analyzed using MALDI–TOF–MS (Table 2). Subsequent matching against IPI database allowed the identification of spots 001 and 002. Spot 001, which was present in the amygdala of one drug addict and five control individuals, could be identified as human chaperonin GroEL (HSP60) precursor (accession number P10809, 61.19 kDa). Spot 002 emerged at a Mowse score of 198 as human tubulin beta-4 chain (TUBB4, accession number P04350, 50.01 kDa). Spot 003 could not be identified, even after repeated measurements.

Due to some recent publications about altered protein expression of cytoskeletal components in the brain of humans and animals after drug exposure [25–30], we focused on the tubulin beta-4 chain spot and performed Western blot analysis in a second independent postmortem sample of amygdala tissue (named as replication sample in Table 1) to evaluate the 2D-findings in more detail. With regard to the heterodimerization of alpha- and beta-tubulin to the microtubules, we decided to investigate not only the beta form of tubulin but also the alpha isoform applying commercially available antibodies against both human tubulin isoforms. Antibodies were selected according to their ability to cover the different tubulin alpha- and beta isoforms. The tubulin expression was investigated in specimens from 12 drug addicts compared to 12 suicide victims and 12 control individuals.

Western blot analysis revealed clear bands of the predicted size of ~50 kDa for both tubulin isoforms (data not shown).

As demonstrated in Fig. 3, drug addicts show a significant higher tubulin expression of both isoforms in the amygdala compared to controls (alpha-tubulin: $P = 0.021$, $F = 6.38$; beta-tubulin: $P = 0.029$, $F = 5.61$, ANCOVA), as well as to suicide victims (alpha-tubulin: $P = 0.006$,

$F = 9.42$; beta-tubulin $P = 0.002$, $F = 13.30$, ANCOVA). We found no differences in the tubulin expression between control individuals and suicide victims for both isoforms. As to the original sample, there were no statistical significant differences between the three groups concerning gender, age and PMI.

Discussion

In a postmortem proteomic study, we compared the protein pattern in the amygdala of 8 opiate-dependent drug abusers and 7 control individuals applying 2D-electrophoresis followed by MALDI–TOF–MS analysis and IPI protein database screening.

Our proteomic results could demonstrate a significantly decreased expression of the heat-shock protein HSP60 and of the beta-4 tubulin expression in the amygdala of drug addicts compared to control individuals.

To analyze the tubulin beta-4 chain finding in more detail, we subsequently carried out Western blot analysis in a second independent postmortem sample of 12 drug addicts compared to 12 suicide victims and 12 controls. Due to the heterodimerization of alpha- and beta-tubulin to microtubules, we included also alpha-tubulin into the analysis. Our findings clearly demonstrated that not only the beta-tubulin expression was significantly elevated in the amygdala of drug addicts compared to healthy controls ($P = 0.029$) and suicide victims ($P = 0.002$) but also the alpha-tubulin expression compared to the other groups ($P = 0.021$ and $P = 0.006$).

Surprisingly, we could not confirm the decreased tubulin beta-4 chain expression in drug addicts from the 2D-electrophoresis in the Western blot analysis, but observed the contrary effect. Several reasons might be responsible for this phenomenon. A study by Verdier-Pinard et al. (2008) has shown that the impact of tubulin proteomics is still limited by the absence of adequate methods to clearly detect and differentiate the numerous tubulin isoforms [31]. The IPI protein database analysis of spot 002 suggests not only tubulin beta-4 chain by the highest Mowse score but also other tubulin isoforms (beta-2C, beta-2A, beta-2B, beta-7) with lower scores, but still over the threshold. In the Western blot analysis, we used antibodies that compass the numerous alpha- and beta isoforms. Thus, the Western blot results might rather represent an overall effect, than a specific finding as obtained by the 2D electrophoresis. A further explanation might be the possibility that the opposite beta-tubulin expression patterns are the result of drug effects on protein expression, in dependence of a chronic abuse or an acute overdose. The opiate addicts of the present study were chronic abusers who died either from an acute overdose after a period of abstinence or from polytoxicomania. Therefore, we can unfortunately not clearly distinguish between chronic and acute effects.

Structural alterations of the amygdala seem to play a key role in the pathophysiology of drug addiction, as investigations in humans have demonstrated that opiate and alcohol abusers present significant amygdala volume reductions, which are associated with abnormal patterns of activation during cognitive memory tasks, inhibition and decision-making [16, 17, 32].

Our findings of an increased expression of the cytoskeletal alpha- and beta-tubulin proteins, as well as decreased HSP60 levels in drug addicts might support this hypothesis, as discussed in the following.

Tubulin is a 100-kDa heterodimer composed of an alpha- and beta-isoform, which are equivalent in size and structure [31]. The tubulin heterodimers polymerize to microtubules, a structural component of neurons. Being a cytoskeletal element, tubulin is involved in numerous processes such as cell division, migration, transport mechanisms and contributes to the formation, maintenance and function of synapses, axons and dendrits, as well as to signal transduction mechanisms [33–36].

Drugs of abuse produce widespread adaptation effects, which are believed to underlie the long-lasting behavioral phenotypes that characterize drug dependence and finally addiction. In this context, it has been proposed that changes in neurotropic factors (e.g. brain derived neurotrophic factor: BDNF) or related neurotrophins, and in components of their signaling pathways (e.g. kinases as protein kinase A: PKA, phophatidylinositol kinase 3: PIK3, mitogen-activated protein kinase: MAPK or transcription factors as nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) and cAMP response element-binding protein (CREB) alter the function of neurons within the VTA-nucleus accumbens circuit and other reward regions [37]. For example, chronic stimulant administration increases branching of dendrites and the number of dendritic spines and dynamically increases levels of BDNF in several brain reward regions, whereas chronic opiate administration decreases dendritic branching and spines as well as BDNF levels in some of the same regions [38].

In vitro and in vivo studies could clearly demonstrate a relation between tubulin and BDNF. Elevations in the BDNF concentration lead also to a proportional increase of tubulin in sensory neurons of the rat and in neuronal cell cultures [39, 40].

Moreover, it is well known that tubulin form high-affinity complexes with certain G-proteins and phosphatidylinositol 4,5-bisphosphate and can thereby activate Gs-, Gq- and phosphatidyl 3-kinase (PI3 K) pathways [41–43]. Several proteins in these signal cascades, as extracellular signal-regulated kinase (ERK), phospholipase C (PLC) or

PI3 K have been shown to be increased following drug administration in numerous brain regions [37].

Based on these observations, one might hypothesize from our findings that drug induced structural alterations in the amygdala determined by increased alpha- and beta-tubulin concentrations might be a source for subsequent disturbances of signal transduction pathways. Such structural variations might be initially, followed by alterations of neurotransmission and signaling mechanisms, which might finally lead by still unknown mechanisms to the described volume reductions in the amygdala, as a final common pathway in addiction. It has to be mentioned that we did not find any differentially expressed signal- and/or neurotransmission proteins in the amygdala of drug addicts, thus direct causal evidence that structural changes via tubulin is the cause for altered cell signaling or disturbed signal transduction remains lacking.

Several other human and animal proteomic studies support this hypothesis by the identification of altered expression patterns of structural proteins in different brain regions. The proteomic analysis of tyrosine phosphorylation of frontal cortical proteins in rat brains after morphine infusion for 72 h yielded increased concentrations of several phosphorylated cytoskeletal proteins, including alpha- and beta-tubulin isoforms [25]. A proteomic approach by Matsumoto et al. reported about the unpublished observation that 18% of the identified proteins in the amygdala of methamphetamine-sensitized rats were cytoskeletal [26]. In a whole-cell proteomic investigation into neuroblastoma cells several cytoskeleton proteins were differentially regulated after long-term exposure to morphine [27]. Our results are in concordance with a recent report by Tannu et al., who performed a human postmortem proteomic study albeit in a different brain region of 10 cocaine overdose victims and 10 control individuals applying 2D-electrophoresis and MALDI–TOF–MS analysis, wherein beta-tubulin was significantly elevated in the nucleus accumbens of the drug addicts [28]. However, in animal studies the alpha-tubulin concentration was found to be decreased both in the nucleus accumbens and in the striatum of rats, which were treated with morphine, respectively cocaine for 5 days [29, 30]. One reason for this discrepancy could be the analysis of different species.

Besides the tubulin alterations, we found a down regulation of the human chaperonin GroEL (HSP60) in the amygdala of drug addicts. HSP60, a member of the heat-shock protein family, is a mitochondrial chaperonin that is involved in the transport and refolding of proteins from the cytoplasm into the mitochondrial matrix. The molecular chaperone system is considered to be a defense mechanism against proteotoxic stresses at the cellular level. It is activated by heat and assists in folding linear amino acid chains into their respective three-dimensional structure [44]. Interestingly, heat-shock proteins are not only induced by heat but also by various other environmental stressors as e.g. oxidative stress.

There is growing evidence that opioids and psychostimulants induce oxidative stress, which will finally promote the neurotoxic effects of drugs of abuse [45]. To date only a limited number of studies investigated the relationship between drug effects and brain HSP60 levels. Hayase et al. examined the effects of 4 day cocaine treatment on the HSP distribution in the rat hippocampus [46]. They found an early enhancement of HSP60, whereas attenuation was observed after 24 h. Two furthers studies by Salminen et al. observed no HSP60 alterations in mouse liver after a single dose of cocaine or amphetamine [47, 48]. From these data, one might suggest that short-time drug abuse increases the HSP60 concentration, whereas chronic conditions rather attenuate HSP60.

The results of our study support this hypothesis. We detected decreased spot intensities of HSP60 in drug addicts. Assuming that the majority of the included opiate addicts died after a period of chronic drug abuse, it might be probable that after an initial HSP60 increase the protein concentrations finally diminish during the long-term abuse by a yet unknown mechanism. This down regulation might result in the loss of neuroprotection, a higher rate of neurotoxicity and as a common final pathway in structural alterations of the amygdala.

A limiting factor in conducting postmortem research is the quality of the tissue. The traditionally described confounding factors are agonal factors, the postmortem interval and the tissue pH. The postmortem intervals of the brain samples were relatively short (12.0 ± 6.6 h for the original sample and 16.2 ± 9.3 h for the replication sample). We performed comprehensive statistical evaluations to analyze not only the influence of postmortem variables but also of further covariates as age and gender. We could not detect significant differences between the investigated groups concerning gender, age and postmortem intervals. No information about the agonal states was available and unfortunately the pH levels of the brain tissues were not determined during the routine autopsies. Considering the results of Stan et al. (2006), who found no correlation between agonal state, postmortem interval or tissue pH of 100 postmortem cases and the protein concentration, these factors seem to be of minor importance [49]. Moreover, we included suicide victims as a "second control group" in the Western blot tests. It has to be mentioned that the phenotype suicide represents a heterogeneous group with different psychiatric diagnosis. Thus, the results have to be interpreted with caution.

In summary, this is the first proteomic analysis of the human amygdala in relation to opiate addiction to our knowledge. The present postmortem study demonstrated

increased concentrations of alpha- and beta-tubulin and a diminished expression of the heat-shock protein HSP60 in opiate-dependent drug victims compared to control individuals. These data suggest that cytoskeletal alterations determined by tubulin seem to be involved in the pathophysiology of drug addiction, probably via a relation to neurotransmission and cellular signaling. Moreover, the loss of neuroprotection against stressors by chaperons as HSP60 might contribute to structural alteration in the brain of drug addicts. Further studies are needed to uncover the function and effects of proteins that are altered during the administration of drugs of abuse and to elucidate the role of these proteins in the development of addictive behavior.

Conflict of interest None.

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