Drug-related deaths in Budapest and the neurobiology of heroin abuse.

Doctoral thesis outline

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Budapest
2007.

INTRODUCTION

Drug use and especially heroin abuse is a widespread phenomenon which has been slowly but gradually increasing worldwide in the past 15 years [1]. The use of illegal substances became accelerated in Hungary after 1989, when the change of regime also generated the opening of the borders and easier access to illegal drugs of abuse. Hungary at the beginning was only a "transit" country, but soon became a "target" country and a growing demand for drugs of abuse appeared.

With an increase of drug-use, and especially intravenous heroin use, appeared all the consequences of it especially within the youngest Hungarian population.

The increase of drug use, and especially intravenous heroin use, brought many negative consequences particularly within the young Hungarian population. One of the gravest consequences of heroin use is death by overdose. Another effect is an increase of certain infectious diseases.

Both of the aforementioned factors can contribute to the mortality and morbidity rates within teenagers and young adults in Hungary. Autopsies are routinely performed at the Department of Forensic Medicine at the Semmelweis University for cases of so called "extraordinary death" such as suicide, homicides, accidents and also drug-related deaths. Nearly 70% of all drug-related deaths in Hungary are registered in Budapest and thus are examined in our Department. For this reason one of the national coordination centers dealing with drug-related death has been founded here. Thus, our research has been conducted with a special emphasis on epidemiological aspects and neurobiological changes within drug-related death cases with a focus on heroin abuse.

The neurobiology of heroin abuse and the mechanisms of all phenomena connected with heroin addiction such as tolerance, withdrawal or craving are still unclear. There are several animal models studying the effects of drugs of abuse on the brain, whose results gave us important insight into the mechanism of drug addiction in the rodent and primate brain, but there is still a need for more information about the direct effect of heroin on the ***human*** brain. A long-term collaboration with Professor Yasmin Hurd's laboratory, at the Department of Clinical Neuroscience at Karolinska University in Stockholm, enabled us to conduct a number of molecular and biochemical experiments on the midbrain and striatal regions of human heroin abusers focused on the two major neuronal components involved in opiate addiction: the dopamine and opioid systems. In order to expand our knowledge about the factors that could potentially affecting markers studied in the post-mortem human tissue we also examined the connection between agonal state, brain pH and mRNA expression of diverse dopaminergic and opioid markers.

SPECIFIC AIMS

1. To describe the drug-related death (DRD) population in Budapest, Hungary since 1994 until today at the Department of Forensic Medicine, Semmelweis University, Budapest, Hungary.
    a. To determine the pattern of drug use in regard to demographics and in-depth toxicological assessments.
    b. To determine what infectious diseases are characteristic for the population of deceased drug addicts as compared to the international trends
2. To study the neurobiology of human heroin abuse:

a. To determine postmortem factors that could impact neurobiological markers in the human brain.
b. To evaluate limbic neuronal systems related to dopamine and opioid reward function.
c. To assess the contribution of genetics to neurobiology and heroin abuse vulnerability.

METHODS

Post-mortem human brain specimens

Human brains from heroin overdose, suicide cases and normal control Caucasian subjects without head trauma were collected at autopsy within 24 hours after death at the Department of Forensic Medicine at Semmelweis University, Hungary, between 1996-2006, under the guidelines approved by the Semmelweis University Human Ethical Committee (TUKEB approval: 113/1995, 180/2001). The cut 2 cm slabs of the right hemisphere were frozen in dry ice-cooled 2-methylbutane-isopentan. Twenty micron thick cryosections were taken from brainstem blocks and striatal regions. The cryosections were then quickly mounted onto Superfrost plus-glass and then kept at -30°C until analysis. Prior to sectioning, tissue punches were taken from the dorsal caudate nucleus for peptide measurements.
The left hemisphere was fixed for a minimum 6 weeks in 4% formaldehyde solution before dissection and paraffin embedding. For immunohistochemistry, the formalin fixed tissue blocks from the striatum, where both the dorsal and nucleus accumbens were distinguishable, were paraffin embedded.
From each brain a piece of tissue (0.9g-1.4g) from frozen cerebellar cortex was homogenized in 10x volumes of double distilled water (pH has been adjusted to 7.0). The pH was measured in the supernatant in triplicates with a pH meter.

Serological examination (HIV, HCV, HBV, Syphilis)

From 1 January, 2000 before each autopsy blood was taken from the subclavian vein, centrifuged, and the serum was frozen at -40°C until further use. The serological examinations were performed at the Immunology Laboratory, Szt. László State Hospital, Budapest, Hungary. For the assessment of the infectious diseases we used kits of the Organon Teknika Vironostika.

In situ hybridization histochemistry (ISHH) and [^{35}S]GTPγS autoradiography

The DAT, DA D2, PENK, and PDYNFL1 mRNAs were synthesized from human cDNA subcloned into plasmids. Templates for the TH, α-synuclein, PC2 and HERC1 probes were synthesized from a human cDNA library using nested PCRs. With the help pf ISHH we measured the levels of mRNA expression within the regions of interest.
During [^{35}S]GTPγS coupling we incubated the slides in puffers containing either μ opioid receptor agonist, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO), or 0.5 μM U69-593 (κ opioid receptor agonist). Basal activity was assessed in the absence of agonist.
The slides were exposed to Kodak Biomax MR film for five to 21 days depending on the marker of interest with ^{14}C standards, and developed. Brain sections used for analysis of the different DAergic and opioid gene mRNA expression levels were studied in separate experiments.

Quantification of Autoradiograms

The autoradiograms from the *in situ* hybridization experiments or the [^{35}S]GTPγS binding were scanned at a resolution of 300 dpi. Optical density values were measured from the digitalized images with an analysis software system. The optical density values were then converted to disintegrations per minute (DPM) per milligram for in situ hybridization and μCi/mg for the [^{35}S]GTPγS binding by reference to co exposed ^{14}C standards using a Rodbard calibration curve.

Peptide Measurements

Opioid peptide concentrations from the caudate nucleus were determined by using radioimmunoassays for MEAP and dynorphin peptides.

Allelic Analysis of OPMR1

In order to study genotype, DNA was purified from cerebellar tissue of controls and heroin subjects. The OPMR1 A118G SNP was genotyped from PCR by using primers ArtUp (5' – CCG TCA GTA CCA TGG ACA GCA GCG GTG) and R2-D2 (5' – GTT CGG ACC GCA TGG GTC GGA CAG AT).

Statistical analysis

Statistical evaluations were carried out by using the JMP and STATISTICA software packages. Normal distribution of the data was analyzed by using Shapiro–Wilk's W test of normality. If not normally distributed, data sets were normalized using natural logarithm. General linear stepwise regression analysis was used to evaluate group differences and identify covariates: age, brain pH, postmortem interval (dichotomized <12 and <24 h), sex, blood ethanol, and brain freezer storage time. Statistical outliers were excluded. In publication IV post hoc analysis regarding heroin A/A and A/G genotype subgroups was performed by using Fisher's least square difference.

RESULTS

Drug-related death cases between 1994 - 2006 autopsied at the Department of Forensic Medicine, Semmelweis University, Budapest, Hungary

Heroin overdose

66% (203 cases)of all the drug-related death cases were attributed to the overdose of heroin. 86.7% were male, and 77.3% were below 30 years of age. 21 cases of heroin OD died in hospital. The death happened mostly at home, or in a nearby public place. In 51 cases we had available information about the approximate duration of drug abuse before death within the decedents. The average time of drug usage at death was 3.91 years ± 0.39 with a range of 0.3-13 years.

Taking into account the current theories about heroin overdose we also examined the possible involvement of ethanol, benzodiazepines or other legal and illegal drugs in the occurrence of heroin related deaths. In 78 cases (38,4% of all heroin OD cases) there were one or more additional active components present next to morphine, codeine or 6-MAM. Ethanol (n=40) and tranquilizers and sleeping medicines (n=10) were the mot common central nervous system depressants. (mean concentration of blood ethanol was 1.14 ± 0.12‰, while ethanol concentration in urine was 1.71 ± 0.19‰)

Infectious diseases

Since 2000 we found only 1 HIV positive person within the examined population; there were 28 cases positive for HCV (23%) and 7 cases with syphilis (7%). There were 4 acute or chronic infections of HBV (7%); there were 8 cases immunized against hepatitis B due to natural infection; equal amount (8 cases) were found to be immunized against hepatitis due to vaccination.

The relevance of brain pH measurement for the forensic and molecular studies.

In initial phases of our investigation of the molecular neurobiology of heroin abusers who died from OD, we noted significant variations in brain pH. In this study we tried to determine the conditions in heroin OD, which is considered a rapid cause of death that might contribute to alteration of brain pH and thus impact mRNA levels. To examine closer the interaction between short agonal state and brain pH, in addition to normal controls, with unknown agonal state, we examined suicide subjects with a documented rapid cause of death.

To understand better the agonal period of heroin OD cases we reintroduced the term "respiratory distress", which was first used in connection with brain pH to describe the state when the organism due to oxygen deprivation starts anaerobic glycolysis and the accumulating lactate causes low pH. Conditions included in the respiratory distress group were for example presence of vomit inhalation, pneumonia, septic state, suffocation or resuscitation. We compiled these information from the autopsy reports blinded to the information regarding the pH values.

Brain pH was measured in the population of subjects comprising of heroin OD cases (n=70), controls (n=45) and suicide (n=31). The overall pH values from 146 subjects ranged between 6.06 and 7.16 (6.69 ± 0.019). The heroin group had significantly lower pH values than the control and suicide groups. Statistical analyses showed no significant effect of age, gender, PMI or brain storage time on the brain pH levels. However respiratory distress was found to significantly influence the most the brain pH levels.

Positive respiratory distress correlated with lower pH values in comparison to negative respiratory distress when measured in all examined groups.

The majority of subjects contributing to the positive respiratory distress were within the control and heroin groups. In the control group, all subjects with low pH (< 6.5) were only present in the positive respiratory distress group. In the suicide group, there was a narrow range of brain pH in the negative respiratory distress group between 6.73–7.08 (6.91 ± 0.021). In the suicide group only two subjects belonged to the positive respiratory distress group; both were resuscitated previous to death and both had low pH values. The lack of respiratory distress was associated with a significant increase of pH.

Brain pH effects on mRNA levels

The mRNA levels of genes relevant to the neurobiology of drug abuse were examined in the brainstem and striatum of a subset of control and heroin subjects. The genes of interest were associated with the dopamine (e.g., tyrosine hydroxylase, dopamine D2 receptor, dopamine transporter) and opioid (preproenkephalin) systems that are strongly linked with the neurobiology of drug abuse. The results showed that subjects with low brain pH, irrespective if a control subject or heroin user, tended to have low mRNA expression levels especially evident in the high mRNA expressing regions.

Alterations in the dopaminergic and opioid systems of human heroin abusers

Dopaminergic system

The distribution of mesocorticolimbic DA cells is segregated such that paranigral nucleus (PN) neurons predominantly comprise the mesolimbic pathway, whereas the parabrachial pigmental nucleus (PBP) and dorsal part of substantia nigra (SNd) constitute the mesocortical circuit. The nigrostriatal pathway with motor related functions emerges from the ventral (SNv) and lateral part of substantia nigra (SNl). DA neurons can be characterized by a number of markers such as DA transporter (DAT), a transmembrane protein that removes DA from the synapse into the presynaptic terminal. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of DA (and other catecholamines) and the DA D2 receptor is an important negative autoregulator of DA cell firing. Another important marker is α-synuclein, which is a presynaptic protein involved in the maintenance and transport of synaptic vesicles and has been implicated in synaptic plasticity. Nurr1 is a member of the nuclear receptor superfamily of transcription factors expressed predominantly in developing DAergic neurons and is critical for the development and maintenance of DA cells. (Fig.1)
In the case of DAT mRNA, expression levels were significantly lower in the PN of heroin subjects in comparison to controls. A similar direction of change, but with strong trend effect, was also observed in the other dorsal tier subregions namely the PBP and SNd. No significant group effect was detected in the ventral tier subnuclei.
TH mRNA expression was significantly altered in three subregions. In all cases there was a significant increase in TH mRNA expression in the heroin as compared to control group. The significant changes were present within the PN, SNd and SNl.
mRNA expression levels of the DA D2 receptor did not differ between the heroin and control groups in any of the brainstem subregions studied.
Heroin related alterations of the Nurr1 gene expression were limited. Only the PN had significantly reduced mRNA levels in the heroin subjects. Age markedly influenced the Nurr1 mRNA expression levels and there was a strong significant group x age interaction such that Nurr1 mRNA levels were reduced to a greater extent in the PN of heroin subjects with increasing age than that observed for the control group.
Heroin abusers also showed discrete alterations of α-synuclein. Significant elevation of α-synuclein mRNA expression levels were observed in the PN and SNv.
None of the expression levels of the DA-related mRNAs examined were significantly correlated to morphine toxicology.

In heroin subjects, the density of DAT immunoreactivity was significantly decreased by 55% in the nucleus accumbens as compared to controls. No significant changes were observed in the caudate nucleus and putamen. Examination of the different subregions of the nucleus accumbens showed significant reduction in both the core (42%) and shell (39%) of the heroin users.

Opioid system

Within the midbrain the [^{35}S]GTPγS binding for both the basal or agonist stimulated levels revealed changes in the regions of PN and PAG. In each case we found an increase of [^{35}S]GTPγS coupling in heroin vs. control subjects.

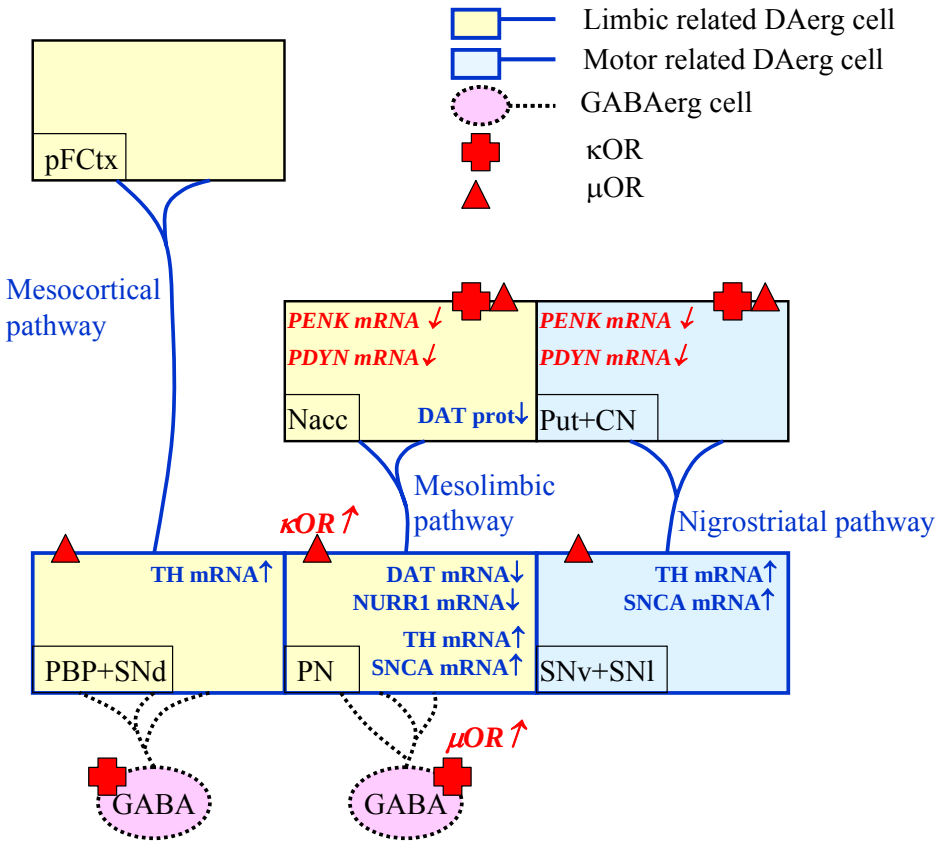

Figure 1. Schematic representation of the most important alterations found in neurobiological studies of human heroin abusers within the dopaminergic and opioid system. (α-syn – alpha synuclein, CN – caudate nucleus, DAT – dopamine transporter, Nacc – nucleus accumbens, PDYN – preprodynorphin, PENK – preproenkephalin, pFCtx – prefrontal cortex, PBP – parabrachial pigmental nucleus, PN – paranigral nucleus, PUT – putamen, SNd – substantia nigra pars dorsalis, SNl – substantia nigra pars lateralis, SNv – substantia nigra pars ventralis, TH - tyrosine hydroxylase)

In the striatum both preproenkephalin (PENK) and preprodynorphin (PDYN) showed a uniform decrease in mRNA expression in heroin abusers vs. control subjects. Only for PDYN the NAcc shell showed a non-significant decrease. The opioid neuropeptide levels from the caudate nucleus (CN) showed a significant increase for dynorphine A and Arg-Leu enkephalin, both derivates of the PDYN gene.

The impact of the genotype on the opioid markers

The μ opioid receptor (MOR), which mediates the actions of enkephalin and β-endorphin peptides, is strongly linked to reward and positive reinforcement, thus polymorphism studies initially focused on the MOR gene (OPRM1). The most studied OPRM1 gene variant until now has been the A118G in exon 1 of the human OPRM1.

In our studies the A/G genotype was significantly more frequent in the heroin than in the control group. The frequency of the A/G genotype among our control subjects was 3.8% (1 of 26), but it was 25.6% (10 of 39) among heroin individuals. Thus, 91% of the A/G genotype individuals were heroin subjects. To determine whether the A118G OPMR1 polymorphism influenced gene expression levels in heroin abusers, the measurements were also analyzed in consideration of genotype. Because of the small number (n = 2) of A/G and G/G genotypes in the control group, only control A/A subjects could be evaluated.

Post hoc analysis revealed that PENK mRNA levels in the putamen and NAc shell of heroin users were decreased 20.4% and 41.4%, respectively, in subjects carrying the G allele as compared with the A/A subjects. Analysis of the A118G polymorphism revealed genotype effects primarily on PDYN mRNA levels in the NAc core; the A/G heroin subgroup showed a 19.7% lower expression than the A/A heroin subjects.

Heroin subjects with A/G genotype had ≈2-fold higher levels of MEAP, dynorphine A and Arg-Leu enkephalin than either A/A heroin or controls subjects.

CONCLUSIONS

The number of drug-related deaths in Budapest has increased from 1994 to 2006.

Drug related death in Budapest, Hungary in great degree is due to opiate (heroin) overdose (65%). The majority of decedents were Hungarian males, between 20-30 years of age, with approximately 4 years of drug abuse history.

Heroin overdose deaths in 62% of cases were caused by heroin only, without any additional illegal or legal substances. In the remaining 38% ethanol or prescription medicines were the most common CNS depressants.
The infection rate within drug-related death cases in Budapest Hungary, was as follows: the prevalence of HIV was below 1%, with 23%, 7% and 7% prevalence for HCV, HBV and syphilis respectively.

Heroin OD cases with evidence of respiratory distress at the agonal state have reduced brain pH that significantly contributes to reduced mRNA levels of several genes relevant to the neurobiology of drug abuse.

There is a greater disturbance of mesolimbic DAergic circuits in association with the abuse of heroin in aspect of both the dopaminergic and opioid systems. Most changes were found within the subnucleus of the VTA – the paranigral nucleus, which showed decreased mRNA expression for DAT and NURR1, increased mRNA expression of TH and α-synuclein and increased μ and κ opioid receptor G-protein coupling.

Consistent with decrease of DAT mRNA in the paranigral nucleus DAT protein levels in the striatum were decreased exclusively within the nucleus accumbens – the forebrain target of the VTA mesolimbic reward pathway.

Our results provide a direct evidence of an apparent increased functional activity of μ-and κ- opioid receptors in the PAG in heroin abusers which would be linked to altered nociception in these subjects.

There is a profound (≈90%) association between heroin use and the 118G SNP of the OPRM1 genotype in our Caucasian population that has an apparent impact on striatal neuropeptide transcription. Individuals with the 118G SNP had a greater disturbance of PENK mRNA expression in the nucleus accumbens shell which might be linked to impaired drug reward.

Reduced opioid neuropeptide (PENK and PDYN) transcription is accompanied by increased dynorphin and enkephalin peptide concentrations exclusively in 118G heroin subjects, suggesting that the peptide processing is impaired in association with the OPRM1 genotype.

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ACKNOWLEDGEMENTS

The presented studies were performed at Semmelweis University, Department of Forensic Medicine in Budapest and at Karolinska Institute, Department of Clinical Neuroscience in Stockholm. My stay in Sweden was financially supported by a Guest Scholarship from the Swedish Institue, Hungarian State Eötvös Fellowship and Long-term Scholarship from the Hungarian Scholarship Board.

I wish to express my sincere gratitude to all, who contributed to these studies and helped me throughout theses years. All cannot be mentioned but you are in my thoughts. I would like to thank especially:

Prof. Eva Keller, my supervisor and friend, who introduced me to the forensic world, for her invaluable support and encouragement in my life both inside and outside work; for providing excellent working conditions in Hungary despite numerous financial problems and allowing me to spend all the time necessary to finish my studies at the Karolinska Institute. I am also eternally thankful for introducing me to Prof. Yasmin Hurd. ☺

Prof. Yasmin Hurd, my supervisor and friend, who embraced me with all her warmth upon arrival to her laboratory in Sweden. I thank you for showing me the way to become an independent scientist and for sharing your vast knowledge of the human brain. I am also thankful for guiding me in the art of scientific writing, basics of molecular biology and delights of sushi. I miss your laugh a lot, but fortunately we have Skype!

The former and present members of the Psychiatry Section at the Department of Clinical Neuroscience at Karolinska Institutet – thanks for accepting me with open hearts and creating the friendliest atmosphere I have ever been to. Some special thanks to some special people: Parisa Zarnegar for the laughs, “zrish poulo”, sharing your flat with me, travels on the 506 and a lot more; Katarina Drakenberg for the ice-skating, beautiful views in your garden and sharing your knowledge whenever necessary; Pani Olu, dziękuję za nieocenioną pomoc w laboratorium, rozmowy na tematy nie tylko naukowe, pyszne kolacje i receptę na “smörgåstårte”; Pernilla Fagergren for questioning and superb help in every scientific question; Maria Ellgren for being the nicest room-mate ever; Sabrina Spano for saffron cookies, room-sharing on Ischia and a wonderful day-trip; Xinyu Wang for all his help in my first steps in molecular biology; Andrej Nikoshkov for designing new probes and sharing his viewson different matters of life; Marita Signarsson for helping out in each administrative problem and just always being there for us; Babben

Berthelsson for her technical support; Pauline for spending an incredibly funny year together.

Some “outsiders” in our group: Nitya Jarayam-Lindström for always being kind, patient and very knowledgeable; Jenny Häggkvist for your company and travels from US; Anna Ökvist, I just like and value you a lot, I hope it will go ok for you in the US; Sofia Johansson for your valuable input into our discussions.

All the inhabitants of the corridor and the building I met during coffee-breaks and lunch breaks: members of the previous Hubin group: Katarina Varnäs, Håkan Hall, Monika Hellberg, Erik Jönsson, Diana Radu, Nils Lindefors, Ingrid Agartz and also Marianne Youseffi and Lisbeth Eriksson. Special thanks to Phong Truong from the PET group for all the dinners, talks and films and also other members of the PET group I met so often in the lab: Arsalan Amir, Magnus Schou, Jan Andersson, Sjoerd Finnema.

Dr. Henrik Druid from the Department of Forensic Medicine in Stockholm for the long-term friendship, introducing me to the mysteries of segmental-hair analysis and providing our Institute with a “brain cutting device” which helped our work enormously. I am also grateful for introducing me to your colleagues at the Department of Forensic Chemistry in Linköping, where I spent a week on a forensic toxicology course. I also would like to thank your coworkers Kanar Alkass and Joakim Strandberg for being friendly and helpful.

Dr. Jovan Rajs for his help in collecting brain samples at the Department of Forensic Medicine and his help in paper-revising.

To my parents, Ewa and Péter, and my brother, Niki-kot, for believing in me all these years against the odds. I love you all. Also to my late grandmothers – I miss you a lot.

Most important of all, Attila, my soul mate for all support, encouragement and that you came with me all the way to Stockholm.