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

Doctoral thesis

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ABBREVIATIONS

ACTH – adrenocorticotropic hormone
A.D. – *anno domini*
AIDS – Acquired Immune Deficiency Syndrome
anti-HBc – antibody to hepatitis B core antigen
anti-HBs – antibody to hepatitis B surface antigen
B.C. – before Christ
BZD – benzodizepines
Cer – cerebellum
cDNA – complementary DNA
CN – caudate nucleus
CNS – central nervous system
DA – dopamine
DAD2 – dopamine D2 receptor
DAT – dopamine transporter
DNA – deoxyribonucleic acid
DOR – δ opioid receptor
DPM – disintegration per minute
DRD – drug-related death
GHB – gamma hydroxybutyrate
EtOH – ethanol
HBsAg – hepatitis B surface antigen
HBV – hepatitis B virus
HCV – hepatitis C virus
HIV – Human Immunodeficiency Virus
ISSH – *in situ* hybridization histochemistry
KOR – κ opioid receptor
LSD – lysergic acid diethylamide
M3G – morphine – 3 – glucuronide
M6G – morphine – 6 – glucuronide
MDA – methylendioxyamphetamine
MDMA – methylendimethoxyamphetamine
MEAP – Met-enkephalin-Arg6-Phe7
MOR – μ opioid receptor
mRNA – messenger RNA
MSH – melanocyte stimulating hormone
Nac – nucleus accumbens (ventral striatum)
NURR1 – orphan nuclear receptor
OD – overdose
OPMR1 – μ opioid receptor gene
PAG – periaqueductal grey
PBP – parabrachial pigmental nucleus
PCP – phencyclidine hydrochloride
PCR – polymerase chain reaction
PDYN – preprodynorphin
PENK – preproenkephalin

PMI – post-mortem interval
PN – paranigral nucleus
POMC – proopiomelanocortine
Put – putamen
RNA – ribonucleic acid
SN – substantia nigra
SNd – substantia nigra dorsal part
SNl – substantia nigra lateral part
SNv – substantia nigra ventral part
TH – tyrosine hydroxilase
THC – Δ-9-tetrahydrocannabinol
VTA – ventral tegmental area
6-MAM – 6-monoacetyl morphine
‰ – mg% (measure of ethanol concentration)

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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. One of the most grave consequence of heroin use is death caused by overdose. Another effect is an increase of certain infectious diseases (HIV, hepatitis), which has long-term impact on health that can shorten a person's life. As all drug related deaths in Hungary by law has to undergo forensic autopsy, the Department of Forensic Medicine at the Semmelweis University was a place of choice for performing studies concerning drug-related deaths. Our findings are the first detailed and well documented reports about Hungarian drug-related deaths after 1989.

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 affect 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. Special emphasis on heroin overdose (OD) related deaths and the risk factors for lethal heroin OD.
    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 reward function.
    c. To evaluate limbic neuronal systems related to opioid reward function.
    d. To assess the contribution of genetics to neurobiology and heroin abuse vulnerability.

BACKGROUND

The history of the poppy plant and opiate abuse (2-14)

Ancient history and Middle Ages

The poppy plant (*Papaver somniferum*) and its hypnotic qualities have been known since prehistoric ages. The word *papaver* is a Greek word meaning 'poppy'. *Somniferum* is a Latin word meaning 'I bring sleep'. According to some sources, Neanderthals may have used the opium poppy over thirty thousand years ago which was supported by archaeological evidence and fossilized poppy seeds. The first known written reference to the poppy and the use of opium appears in a Sumerian text dated around 4000 B.C., suggested by the fact that they had an ideogram for it. The opium poppy was known as *hul gil*, plant of joy.

The Egyptian Eber's papyrus from about 1552 B.C. is the oldest preserved medical document and it advises use of the condensed juice of the unripe seed pod as a sleep-inducing medicine to quiet children from excessive crying. Poppy was also apparently used in the treatment of abdominal tumors in this society. During this period poppy was also used for religious purposes and as a medicine.

In Europe *Papaver somniferum* has also been popular. Earliest historical evidence of the eating of poppy seeds in a form of fossil remains of poppy-seed cake and poppy-pods have been found in Neolithic Swiss lake-dwellings dating from 2500 B.C.

The word "opium" has been postulated to be of Greek origin, deriving from "*opos*" (juice) and "*opion*" (poppy juice). Opium likely came into Greece from Asia Minor and the ancient Greeks associated various divinities with opium, including Hypnos (sleep), Morpheus (dreams), Nyx (night) and Thanatos (the twin brother of Hypnos) (death). Old pictures often show Hypnos with poppy heads in his hands and adorning his head. The doorway to his realm was also surrounded with poppies. The poppy was also considered a plant of the Underworld.

Poppy juice in opium wine was mentioned in the writings of Hippocrates (460-377 B.C.), the Greek physician and father of medicine. He dismissed the magical attributes

of opium but acknowledged its usefulness as a narcotic and styptic in treating internal diseases, diseases of women and epidemics. The life-terminating properties of opium were well known, and opium and hemlock were a commonly used combination for the execution of condemned individuals.

The Arabs called the opium poppy "abou-el-noum" which literary translated as "father of the sleep", and during the seventh century A.D. when the Arabs ruled Egypt, the preparation became widely spread throughout the Arab Empire. The great Persian physician, poet, and philosopher Avicenna (Abu-Ali-Ibn-Sina) (10th century A.D.) wrote a famous thesis about opium, but later died of opium intoxication. As the cultivation of the opium poppy flourished, the product was exported to both Europe and India.

Arab traders brought the opium poppy to the China as early as the 9th century, but for the next almost 1000 years the substance was mainly used for the control of dysentery. Nevertheless, with the introduction of tobacco smoking from European sailors (15th century A.D.) Chinese people started to mix opium and tobacco in gradually increasing amounts and smoke it in special pipes. Finally many were smoking pure opium in the form of *chandu*, but it did not become widespread until the 19th century when approximately 25% of the Chinese population used opium.

16th – 18th century – repopulization of opium

In the 16th century Europe Paracelsus (1493-1541) repopulized opium introducing a preparation named Laudanum, a mixture of 10% opium in a hydroalcoholic extract (containing 10 milligrams of morphine per milliliter). Thomas Sydenham ("the English Hippocrates") (1624-1689) introduced opium into Britain and popularized tincture of laudanum as being useful in the treatment of plague. Thomas Dover, a pupil of Sydenham, invented Dover's Powder (opium, ipecac, licorice, and saltpeter). Other preparations included paregoric which is a camphorated tincture of opium, containing 0.4 milligrams of morphine per milliliter. Le Mort, professor of chemistry at the University of Leyden between 1702 and 1718 is credited with the invention of paregoric. Both laudanum and paregoric are still available by prescription in the United States.

Opiate use in Western Europe, but especially in Britain, increased rapidly in the 18th century. One reason could have been the very popular Brownian theory of disease at that time. In his very influential "Elements of Medicine" Dr. John Brown strongly recommended the excitatory effects of opium which in his view prevented the formation of many diseases. It must be also remembered, that India was part of the Commonwealth and the Indian opium production and trade belonged to Britain - it was the only European country (except for the Balkans) that had cheap and vast amounts of opium available at any times. Another reason could be the fact, that until the late 19th century laudanum could be bought at modest cost and without prescription in Europe and the United States. Another factor contributing to the ease of buying opium in Britain was the fact that the British "drug store" differed from the pharmacies in other parts of Europe. In most European countries, pharmacies were part of the public health system and were under strict regulations, which was not the case in England where drug stores even sold other products in addition to medicines.

The first description of the immense opium use among factory workers was given by Thomas de Quincey in his "Confessions of an English Opium-Eater". Initially a working class drug, laudanum was cheaper than a bottle of gin or wine, because it was treated as a medication for legal purposes and not taxed as an alcoholic beverage. Innumerable Victorian women were prescribed the drug for relief of menstrual cramps and vague aches and used it to achieve the pallid complexion associated with tuberculosis (frailty and paleness were particularly prized in females at the time). According to Virginia Berridge England's opium use in 1827 was around 7.5 tons which was doubled by 1833. An increase in opium consumption had to cause also an increase in opium related intoxications and death. Sadly, it was mostly evident in the youngest population of children below the age of 5. In those days mothers working for 16-18 hours a day in factories had to leave their children with so called "child-minders" who not only took care of a dozen children but worked also as washerwomen so they used opium containing syrups abundantly. The most popular products for children were for example: "Godfrey's Cordial", "Mrs Winslow's Soothing Syrup", "Street's Infant's Quietness" and "Atkinson's Infant's Preservative". There was also another product called "Chlorodyne" containing morphine, chlorophorm and cannabis tincture. It was commonly used in cholera cases. Nevertheless it should be remembered that these

products saved the lives of those people who would otherwise have died of dysenteries and other diseases caused by contaminated water and infected food.
Few sources are available as to the history of opium use in other European countries, but it seems, that although opium use as a medication slowly increased from the 16th century, until the middle of the 19th century, opium as a drug of abuse was not very popular or well documented.

Sertürner and isolation of morphine

In 1803 the first opium alkaloid was isolated by Friedrich Wilhelm Adam Sertürner a young drug clerk living in Einbeck, Germany. He called it morphine in honor of Morpheus, the Greek god of dreams. It was only in 1816 when he finally concluded a number of tests (many of which where conducted on himself, almost taking his life) and published a report about the chemical and pharmacologic properties of morphine. Codeine, the second major opium alkaloid, was isolated in 1832 by Robiquet.
Another important step was an invention of hypodermic syringe. Sir Christopher Wren (1656) succeeded in injecting morphine intravenously with a quill to which a small bladder was attached. However in was not until 1856 when hypodermic needle was independently reinvented by Charles Gabriel Pravaz (1791-1853), French surgeon, and Alexander Wood (1817-1884), Scottish physician. It was first used to inject morphine as a painkiller. By 1880 practically every American physician owned a syringe and the ability to administer morphine.

Synthesis of heroin

Heroin (diacetylmorphine) was first synthesized in 1874 by two English chemists G.H Beckett and C.P. Alder Wright. Nevertheless, real interest in the drug began after its subsequent analysis at the Bayer laboratories in Germany. The drug was put on the market in 1898 as a non-habit forming alternative to morphine. Incredibly, the extremely powerful addictive properties of heroin were not recognized until 1910.

Figure 1. Bayer heroin bottle produced from 1898-1910, marketed as a non-addictive morphine substitute and cough medicine for children. (15)

19th – 20th century

In the United States at the end of the 19th century opiates were the most commonly used medicines. Most of the American population used so called "patent medicines" which usually contained opium, morphine or cocaine without indicating this fact on the label of the product. On the other hand this action was not against the law. In these times the typical opiate user is a middle aged housewife.

During the second half of the 19th century the concern over addictive qualities grew both in Britain and United States. Slowly a consensus grew against commercial opium and its widespread availability which was shown by convening of international congresses first in Shanghai in 1909 by founding the International Opium Commission, then in Hague in 1912 and 1913. By 1914 thirty-four nations concurred in their belief that opium production and importation should be decreased. After World War I, the Commission next met in 1924, with sixty-two countries then participating. These Opium Conventions recommended various measures for the international control of the trade in opium the countries participating in it agreed to pass laws and regulations to limit the import, sale, distribution, export and use of all narcotic drugs to medical and scientific purposes. In the United States on December 17, 1914 the Harrison Narcotics Act was passed which aimed to reduce drug (especially cocaine but also heroin) abuse

and addiction. It required doctors, pharmacists and others who prescribed narcotics to register and pay a tax.

Between the World Wars drug addiction did not cause big problems in most European countries. The cases were morphine was abused were sporadic usually in doctors families or among artists. After the Second World War the situation remained stable for quite a long time – even at the beginning of the 60's the most often abused substances were medical forms of morphine, pethidine or methadone. In the second half of the 60's cannabis became one of the most popular drug of abuse in Western Europe. In the same time heroin was already produced in clandestine laboratories in South France and South Italy but the drug was smuggled by the Italian mafia to New York via Tangier. In the 80's heroin started to pour into Western Europe via the Balkan route, mainly through Yugoslavia, then Bulgaria; later also Hungary, Romania and Czechoslovakia became so called "transit countries". In the 80's in Eastern Europe, specifically in Poland, a new method of drug use has emerged – the production of so called "compote" from dried poppy pods. The addicts made the "compote" domestically with home-made equipment, which resulted in a production of a brownish liquid containing not only diacetyl morphine, monoacetyl morphine, morphine, codeine and other alkaloids but also a fair amount of impurities. This mixture was then applied intravenously. The "compote" did not remain a Polish specialty – it was readily accepted and adapted in Ukraine, Byelorussia and Russia where it is called "koknar". Due to the overwhelming popularity of "koknar" in the Soviet Union poppy production was totally banned in 1987; in Poland since 1985 poppy cultivation has been licensed, but it can be still produced for culinary purposes. Probably the popularity of intravenous "koknar" led to the high rate of HIV infections in the countries of the former Soviet Union.

In the USA after declaring the Harrison Narcotics Act there was an attempt to cure opiate addicted people in so called Narcotic Maintenance Clinics where addicts were often treated with morphine or cocaine. Later, doctors who prescribed addictive drugs to abusers were prosecuted. Narcotic addiction became a criminal offence which led to emergence of a criminal subculture. After the Second World War the Italian mafia took care of heroin trade in USA. They bought opium in Turkey or Iran, and transported it to the south of Europe where it was transformed to heroin. This procedure went on until

the beginning of 70's when the "French connection" was folded up by the French authorities.

Presently, the cultivation of the opium poppy is internationally regulated by the International Narcotics Control Board of the United Nations, with India being the only country that is significantly involved in legal opium production to meet world medicinal demands. Although opium is produced also in China and North Korea, this is reputed to be for exclusive domestic medical use.

Illicit opium trade is more commonly found in remote border areas of "the Golden Triangle" (Laos, Thailand, and Myanmar), "the Golden Crescent" (Afghanistan, Iran, Pakistan), India, Lebanon, and Mexico

János Kabay and his method of morphine isolation from the poppy (14)

Since Sertürner there were several attempts to extract morphine directly from the poppy pod with the omission of the opium stage. Most of these attempts were unsuccessful. In 1925 a young Hungarian pharmacists János Kabay developed a method of morphine extraction from the green, unripe poppy pods. In 1927 he founded a small chemical factory called "Alkaloida" where he produced morphine. Unfortunately his method was very expensive and troublesome: it could be performed only between the flowering and ripening of the poppy plant; also the poppy seeds, an important alimentary addition in Hungarian cuisine were lost during production. Thus Kabay in 1931 developed a new method of morphine extraction from the dried, used and until now useless poppy-straw which started a new era in morphine production worldwide. The method became more and more popular: the Hoffman LaRoche in Switzerland started production of morphine from the poppy pods; new factories in Poland and Czechoslovakia were opened based on Kabay's patent.

After the Second World War more and more countries decided to produce morphine from poppy straw instead of opium, thus the production of morphine derived from opium slowly decreased while morphine derived from poppy straw is still growing.

The work that Kabay started granted Hungary a unique position in morphine production – in 1957 Hungary was the sixth in the world production of raw morphine (7.9% of the

worlds production) and the first in the production of morphine from poppy straw (37% of the worlds production).

History of recreational drug abuse in Hungary (16,17)

According to Lévai Hungarian drug problem history can be divided into 5 main stages.
The first stage lasted from the mid 60's until 1970. During this period drug abuse was a sporadic phenomenon. It was during this time when the first drug related death was recorded (1969), and the police prepared the first records about drug-consuming groups.
The second stage occurred in the early seventies. In this time the modern psychosocial trends reached Hungary and more and more middle - class and lower - middle - class young people abused drugs at parties usually in combination with alcohol. The most commonly abused drug was Parkan containing trihexylphenidyl (medicine against Parkinson's disease). In this time the drug abuse phenomenon was restricted to Budapest.
The third stage lasted from 1973 to the early 80's. During this time drug abuse spread slowly in the country together with the phenomenon of vagrancy. As a consequence drug abuse became a mass phenomenon among young people. The most common way of abusing drugs was "glue sniffing". In the early eighties more and more codeine containing medicines were abused either via prescription forging or through burglaries at pharmacies or stealing from hospital wards.
The fourth stage, which began at the early 80's and lasted until 1989, saw the abuse spread even more: the abusers were younger then before and more women became involved in abusing drugs. The most popular were codeine preparations such as Hydrocodin used intravenously. Demand for "hard drugs" appeared. There were more and more attempts to produce mind altering substances for example by growing hemp plant or smuggling drugs of abuse. In the same time Hungary became the number one reserve route of drug trafficking from the Near and Far East to Western Europe.
The fifth stage started in 1989 with the change of regime. With the opening of the borders we can say that practically all the phenomena of drug abuse that existed in Western Europe are present in Hungary as well. The growing demand for drugs of abuse changed Hungary from a "transit country" to a "target country".

Forensic autopsies in Hungary

The Hungarian law (1997 CLIV act, 218 paragraph, section 3) enforces the medico-legal examination on several occasions. These include all deaths under unclear or suspicious circumstances and also:

- deaths as a result of violence (possible third party involvement)
- traffic or work related accidents
- other accidents or poisonings (this also includes accidental overdose deaths)
- suicidal deaths
- when there is a suspicion of medical malpractice
- death of a person of unknown identity
- deaths in custody.

The forensic autopsies in Hungary until the end of year 2006 were performed by forensic experts being employed either by the Forensic Departments of the Medical Universities in 4 big cities: Budapest (capitol), Debrecen, Szeged and Pécs, the Forensic Expertise Offices in 9 Hungarian cities or the police forces in the countryside or smaller towns.

In Hungary there are two types of medico-legal examinations: one is performed in the case of a suspicious or criminal death and involves two specialists; it is often called a "forensic autopsy". In the case of obviously non-criminal death (accidents, suicides etc) a so called "police autopsy" is performed by only one specialist.

Within the borders of the city of Budapest all medico-legal examinations are performed at the Department of Forensic Medicine, Semmelweis University. The number of autopsies performed yearly decreased since 1994 until 2000; later there is a slight increase in the total number of autopsies. The number of police autopsies gradually decreased since 1994 nevertheless there was a small noticeable increase in the past two years. Since 2000 there have been an increasing number of pathological autopsies. The number of forensic autopsies has stayed generally the same over the years, although there seems to be a slight decline over the past two years. (Fig. 3)

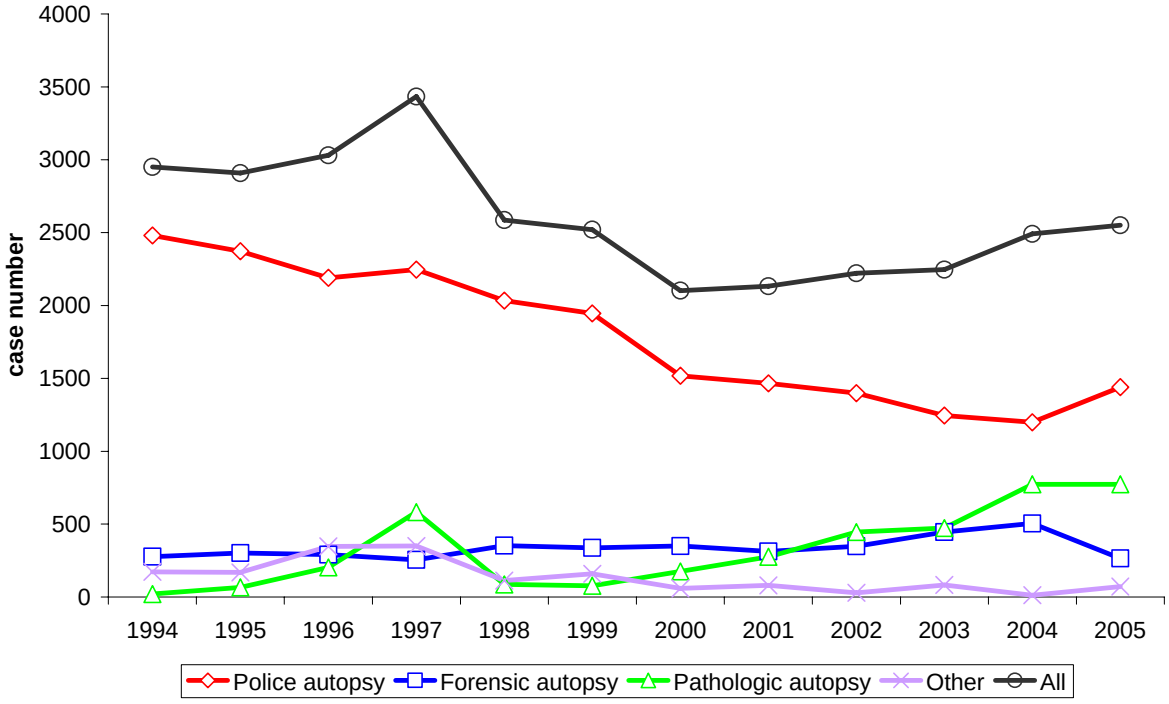


Figure 2. Number and type of autopsies at the Department of Forensic Medicine, Semmelweis University, Budapest, Hungary

Based on the previously mentioned criteria, all accidental overdose deaths must undergo obligatory medico-legal examination. This means that all deaths ***directly*** caused by drugs of abuse are sectioned, thoroughly examined and analyzed by highly qualified forensic experts. We can also distinguish a group of deaths connected ***indirectly*** to drugs of abuse which can be further subdivided into two main subgroups: one group includes violent deaths of known drug abusers (suicides, homicides, accidents etc.); the second group is formed by natural deaths of known drug abusers. A similar division of drug-related deaths was proposed previously (18).

Our project was based on all drug-related death cases (both direct and indirect) investigated at the Department of Forensic Medicine, Semmelweis University, Budapest, Hungary between 1994-2006. We used the decedent's medical history (if available from the family, hospital etc) and the circumstances and environment of the fatality (if available from the police) and the autopsy and laboratory findings to determine the cause of death. Toxicological analyses for blood and urine ethanol levels were performed at our Institute's Toxicology Laboratory. Toxicological analyses for

illicit drugs, prescription medications, pesticides and other organic compounds were performed at the National Department of Forensic Toxicology, Budapest, Hungary. According to Hungarian law, the toxicological analyses are supposed to be performed from a wide array of samples packed to plastic containers and properly labeled. The samples should include the following:

1. blood
2. urine
3. organs: liver, kidney, lung, brain, heart
4. stomach and content
5. bowels and content

In the case of intravenous injections the site of injection should be also analyzed for the presence of drugs. However, due to financial problems in the past, it was not always possible to perform toxicological analyses from all samples available. Thus in some cases we received results with only qualitative measurements from blood with quantitative measurements from urine whereas in other cases a whole array of toxicological results was available from practically all sent samples.

Inclusion criteria for the study were any documented links with illicit drug use either as a cause of death (accidental or suicidal overdose) or in the medical history of the subjects. We formed three subgroups within the drug-related deaths:

1. **Accidental overdose deaths**, which consisted of all cases with positive toxicology results for illicit drugs where there was no intent of self destruction. The group also includes butane and volatile solvent inhalators as well, even though the aforementioned substances are not illicit drugs. We also enrolled into this group those cases (n=28) that died in hospital after several hours / days after the intoxication and had negative toxicology result at autopsy, but the intoxication was confirmed in the hospital. There were also a few cases (n=13), where there was a known history of drug abuse together with characteristic circumstances and paraphernalia at death, characteristic autopsy finding and positive toxicology results of the substance found on the scene of death but negative toxicological examination from bodily fluids and organs. Most of these cases underwent far-gone decomposition which might be one of the reasons for

not detecting morphine metabolites, but nevertheless these cases were also included in the accidental overdose death group.

2. **Violent death** of known drug abusers consisted of all suicidal, homicidal and accidental deaths that occurred to people who were known drug-abusers, or who had positive toxicology results with an obvious, non-toxicological cause of death (e.g. hanging, jumping from heights, traffic accident etc.).

3. **Natural death** of known drug abusers contained cases where known drug addicts died because of natural causes; in some cases there was a positive toxicology, but the natural cause of death were overwhelmingly more important than the fact of the intoxication (e.g. cerebellar hemorrhage in leukemia with morphine positivity probably used to diminish pain).

Infectious diseases connected with intravenous heroin use

The connection between infectious diseases and drug use emerged with the invention of hypodermic needle and the subsequent practice to inject drugs of abuse. The oldest articles available on PubMed about the infectious complications of intravenous drug usage describe a number of infections which were the concern of doctors in the 60's and 70's. Most of these included (19):

1. Tetanus, a result of subcutaneous injections (skin-popping). A disease caused by *Clostridium* species with mortality close to 90%.
2. Malaria, which was connected to sharing of the injecting equipment by people infected by *Plasmodium vivax* (usually a veteran of the Vietnam War) with non-infected people. In the 1940's after several outbursts of malaria drug pushers concerned about their decreasing clientele from the epidemic of malaria sweeping the streets started to "cut" heroin with quinine to prevent further cases of malaria. As a result of this new practice, the malaria outbreak in New York was reasonably controlled. The use of quinine in illicit preparations continues today, because quinine's bitter taste prevents heroin buyers from being able to

judge the quality of heroin sold as well as adds to the "rush" of the heroin injection (20).

3. Infective bacterial endocarditis.
4. Skeletal bacterial infections (osteomyelitis) (21,22).
5. Recurrent skin infections due to using unsterile equipment or injections (cellulites, abscesses, bacteraemia, septicemia) (23).
6. Syphilis positivity concerning approximately 15% of female and 12% of male addicts.
7. Tuberculosis (24).

Acute hepatitis was estimated to be present in 10-15% of drug addicts while approximately 60% had biochemical abnormalities characteristic of chronic hepatitis. An article from 1984 (25) suggested a major role for non-A non-B virus infection (nowadays called hepatitis C) in the etiology of hepatitis in heroin abusers while others showed that HBV infection is responsible for liver disease in heroin addicts in the great majority of cases (26).

In 1981 the first article appeared describing pneumonia caused by *Pneumocystis Carinii* in drug abusers (27). In 1982 the acronym AIDS (Acquired Immune Deficiency Syndrome) was suggested for the new disease affecting in those times mainly homosexual people, intravenous drug users and hemophiliacs. Identification of the virus responsible for the disease occured in 1983 (28). From that time on numerous reports emerged about HIV situation among drug abusers. A study from 1989 comparing 92 published and unpublished reports about HIV infection in the USA showed that HIV seroprevalence among intravenous drug users in drug treatment programs ranged between 0-65% (29). According to a screening performed in England in 2000 out of 102 drug users in treatment, 3.7% were HIV seropositive, 20.4% were positive to HBc (hepatitis B) and 55.8% had antibodies to hepatitis C (30). In an Australian study from 2004 from 377 injecting drug users 36.6% tested HCV antibody positive and 28% had been exposed to HBV (31).

Users in Eastern Europe, especially the Newly Independent States (countries of the former Soviet Union), reported a large increase in illicit drug use during the second half

of the 1990s together with a boom in HIV infection and sexually transmitted diseases (STD) (32-35). Despite these alarming reports in Hungary injecting drug users are more at a risk of contracting hepatitis C then HIV (36-38) as there is a very low HIV prevalence within iv. drug users while the prevalence rate of HCV infection could reach 40% (39). According to the UNAIDS report in 2000 there was a 2.2% HIV prevalence among injecting drug users with an estimated number of 3200 people living with HIV infection in 2005 in Hungary (40).

Heroin use and overdose

Heroin (diacetyl morphine) is a semisynthetic opiate derivate and is synthesized from morphine by acetylation. It is one of the most potent drugs of addiction. It can be administered in a variety of ways:

- intravenous ("mainstreaming"),
- intramuscular injection ("muscling")
- subcutaneous injections ("skin popping"),
- inhaling the smoke through a straw ("chasing the dragon"),
- inhaling the powder through the nose ("tooting"),
- mixing with tobacco or marihuana and smoking as a cigarette.

How heroin is used usually depends on the concentration of the active substance. In the East where it is easier and cheaper to attain pure heroin it is usually smoked or snorted. In the USA or Europe, where heroin concentration is usually low (5-25%) only intravenous use can ensure the proper euphoric effect.

Definitions

Heroin overdose can be defined in a number of ways. The European Monitoring Center for Drugs and Drug Addiction (EMCDDA) has a definition (41) for drug-related death that "refers to those deaths that are caused directly by the consumption of drugs of abuse. These deaths occur generally shortly after the consumption of the substance(s). These cases are included into statistics when the death is due to a standard list of

specific drugs: opiates, cocaine, amphetamines and derivatives, cannabis, and hallucinogens". Since 2003 the "new DAWN" (Drug Abuse Warning Network) in the USA collects data about drug-related mortality which "includes deaths directly caused by drug use, misuse, or abuse, as well as deaths where the drug use, misuse, or abuse contributed to the death but did not cause it. Also included are deaths where a drug was simply implicated (presumed to be related to the death) and deaths where the drug's involvement was not well defined. Only recent drug use is considered, and the reason a decedent used the drug is irrelevant" (42). There are three main categories of DAWN cases, the third including all case types and manners of death when profiling deaths involving illicit drugs. These drugs include:

- Cocaine;
- Heroin;
- Marijuana;
- Major stimulants, which include amphetamines and methamphetamine;
- Club drugs, which include MDMA (Ecstasy), GHB, flunitrazepam (Rohypnol), and ketamine;
- Hallucinogens, which include LSD, PCP, and other hallucinogenic substances such as psilocybin; and
- Non-pharmaceutical inhalants.

In different forensic articles concerning heroin overdose from diverse parts of the world one can find a number of definitions for heroin overdose: "accidental death caused by respiratory depression following the administration of illicit opioids" (43), "any death resulting from the acute toxic action of a drug not legally available (and/or not prescribed for therapeutic purposes), which was voluntarily taken by the subject for recreational, hedonistic purposes (44)". According to some the term "heroin overdose" is a misnomer, as in a substantial proportion of cases, blood morphine levels alone cannot account for the fatal outcome of a heroin overdose (45).

Mechanism of heroin overdose

The differences in defining and approach to heroin overdose most probably arise from the fact that we still do not know what the exact mechanism of heroin overdose is. There are several hypotheses available:

- "True overdose" – the classical definition of heroin overdose where the death is a result of a good quality or high quantity that is in excess of the person's current tolerance to heroin. Although this seems the most logical explanation of heroin overdose there were several researchers reporting very low, practically non-toxic morphine levels in fatal overdoses.
- Contaminants – this theory argues that heroin overdose is a result of the presence of adulterants in illegal heroin (like quinine).
- Polydrug use theory – concomitant use of other central nervous system depressants (like ethanol, benzodiazepines or barbiturates) potentiates the respiratory depressant effects of heroin thus causing a fatal outcome even when morphine concentrations are low.

Although there are continuous efforts from the medical and forensic community to shed light on the mechanisms of fatal heroin overdose it still remains a mystery.

Effects of heroin

Heroin is a central nervous system depressant that relieves pain and induces sleep. Its use produces a dreamlike state of warmth and well-being. It may also cause a constriction of pupils, nausea, and respiratory depression. Its abuse is characterized by persistent craving for the drug, tolerance (the need for larger and larger doses to get the same results), and painful and dangerous withdrawal symptoms. Withdrawal symptoms include panic, nausea, muscle cramps, chills, and insomnia. Heroin has a much stronger effect then morphine due to the fact that it is more lipophilic and can pass the brain – blood barrier approximately 100x quicker.

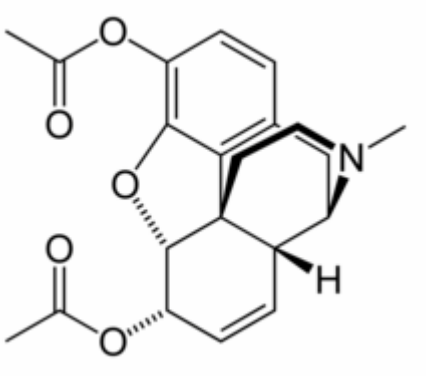


Figure 3. Structure of heroin

Toxicology of heroin

Heroin metabolizes in the human body very quickly, within minutes – first to 6-monoacetyl-morphine (6-MAM) then to morphine. 6-MAM has a half-life of 0.6 hours in the human body. The main metabolite of morphine is morphine – 3 – glucuronide (M3G), but morphine – 6 – glucuronide (M6G) received more scientific interest as it is also a μ receptor agonist and its antinociceptive properties are comparable to those of morphine (46,47). Within 24 hours 85% of morphine is excreted with urine as glucuronides. Only small amounts are excreted as unchanged morphine (2-10%) (48,49). Illicit heroin also contains variable amounts of codeine or acetylcodeine. Thus in the case of heroin overdoses codeine is often found in bodily fluids.

Concentrations of heroin metabolites after fatal overdoses are known to show large variations (50). Concentrations of free morphine in blood can range from 0.008 to 1.539 μg/ml (51), while in other studies the mean free morphine concentration was 0.36 ± 0.18 μg/ml (52). It is generally accepted that free morphine concentrations in blood above 0.1 μg/ml and the finding of pulmonary oedema during autopsy indicates opiate overdose (53). A diagnosis of a fatal heroin overdose can be further reinforced by the evaluation of the morphine/codeine ratio –if it is above 1 there is a high probability of heroin overdose, while values below entity indicate rather codeine intoxication(54). Another specific marker of heroin use is the presence of 6-MAM. It is formed during heroin metabolism and cannot be formed from morphine or codeine. However its short detection time (2-8 hours) limits the usefulness of 6-MAM in detecting heroin overdose,

its presence can be interpreted with confidence as a proof of previous heroin or 6-MAM usage (55).

Risk factors for fatal heroin overdose

The issue of heroin overdose and risk factors involved with it was addressed by several authors. One of the best review articles in this field by Darke and Hall(56), highlighted a number of these factors:

1. Demographic factors:
    a. Males
    b. Late 20's, early 30's
    c. Long-term dependent users
2. Polydrug use:
    a. Ethanol
    b. Benzodiazepines
    c. Tricyclic antidepressants
3. Tolerance: after imprisoning the risk of overdose is high; older addicts tend to take breaks from heroin use thus smaller amounts of drugs can be fatal for them
4. Route of administration: intravenous is still leading in fatal ODs, but non injecting routes of administration can also cause death.
5. Most OD cases are accidental; suicides are very rare. If a drug addict wishes to commit suicide it is usually a non-opioid overdose or other violent means.

There are also data suggesting that a different environment of injection can also increase the risk of fatal heroin overdose (57).

Neurobiology of opiate abuse

Opioid peptides and receptors

Morphine, the main active metabolite of heroin exerts its effects by activating μ opioid receptors. There are three main groups of opioid receptors in the human brain: μ, κ and

δ opioid receptors. They were independently discovered in the early 70's by three different research groups (58-60). The endogenous ligands that bind to these receptors create the family of opioid neuropeptides consisting of enkephalins, dynorphins and endorphins. They are derived from genetically distinct precursor genes: proenkephalin, prodynorphin and proopiomelanocortin (POMC) respectively. The μ and δ opioid receptors mediate euphoria (61) and conditioned place preference (62) whereas κ opioid receptors mediate dysphoria and conditioned place aversion (62).

Proenkephalin gives rise to 4 Met-enkephalins, 1 Leu enkephalin and 2 longer opioid peptides: Met-enkephalin-Arg-Phe and Met-enkephalin-Arg-Gly-Leu (63). Proenkephalin mRNA was found abundantly in brain regions associated with endocrine-reticular-motor continuum of the limbic system (hypothalamus, periaqueductal gray, various mesencephalic nuclei, bed nucleus of the stria terminalis, and ventral pallidum) (64) where also the presence of enkephalin peptides was confirmed (65). Enkephalins has high affinity to both δ and μ opioid receptors.

Prodynorphin gives rise to several peptides: dynorphin A, dynorphin B, α- and β-neoendorphine (66,67). Prodynorphin mRNA is widely expressed in brain regions traditionally included within the limbic system (e.g. amygdala, hippocampus, entorhinal cortex and cingulate cortex) as well as limbic-associated regions including the ventromedial prefrontal cortex and patch compartment of the neostriatum(64). Dynorphins have the highest affinity to κ opioid receptors.

Proopiomelanocortin (POMC) gives rise to only one opioid peptide (β-endorphin), while it also precursor to α, β and γ melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). β-endorphine has a high affinity to both μ and δ opioid receptors.

Opioid receptors belong to the superfamily of G-protein coupled receptors. Their location within the brain in the striatum, thalamus, hypothalamus, cerebral cortex, cerebellum and certain brainstem areas as well as the spinal cord suggests an important role in a broad range of functions and behaviors, such as sensory perception (particularly nociception), reinforcement and reward, neuroendocrine regulation, motor control, learning and memory. One of the main neurobiological substrates of addiction and abuse research is the mesocorticolimbic reward system, being responsible for a number of physiological and pathological conditions.

The reward pathway

The reward pathway (or mesocorticolimbic pathway) is a dopaminergic projection that arises from the ventral tegmental area in the midbrain and innervates various cortical and subcortical areas. The main projections include the nucleus accumbens (ventral striatum) and prefrontal cortices. The reward pathway mediates behavioral reinforcement that promotes activities important for the survival of the species such as sexual behavior, intake of food and water and nurturing behavior. The importance of the reward pathway was first recognized by Olds and Milner (68) who used intracranial electric stimulations in the brain to locate specific neuroanatomical sites which induced positive reinforcement. Later also substances with addictive potential were shown to activate this system, interestingly almost all caused an increase in dopamine release in the nucleus accumbens (69). The rewarding effects of opiates are strongly linked to the direct stimulation of μ and δ opioid receptors in the ventral striatum (nucleus accumbens), neurobiological substrates of drug reinforcement and natural rewards (70,71). An important role of the mesolimbic dopamine (DA) neurotransmission has also been strongly implicated in the development of opioid addiction, as with most drugs of abuse (72). Experimental studies have shown that stimulation of μ opioid receptors localized on GABAergic neurons in the midbrain ventral tegmental area (VTA) indirectly leads to enhanced activation of DA neurons (73) subsequently increasing DA levels in projection terminals such as the nucleus accumbens (74,75). Potentiation of DA levels in the nucleus accumbens are strongly associated with the reward saliency (76,77) and drug-seeking behavior (78,79). The VTA has a heterogenous anatomical and functional organization thus in addition to the mesolimbic neurons that innervate the nucleus accumbens, a different subpopulation of VTA DAergic neurons, namely mesocortical, project to the prefrontal cortex relevant for cognitive function and emotional regulation (80-82). Aside from the mesocorticolimbic pathway, abundant DA-containing cells are localized to basal ganglia neuronal populations of the substantia nigra that provide the main DAergic input to the dorsal striatum (caudate nucleus and putamen). Impairment of the nigrostriatal DA cells is characteristic of neurodegenerative disorders such as Parkinson's disease (83) and implicated in stimulus-response habit learning underlying compulsive drug use (84,85).

Dopaminergic markers

DA neurons are primarily characterized by expression of the DA transporter (DAT), a transmembrane protein that removes DA from the synapse into the presynaptic terminal providing one of the most important means by which the actions of synaptic (and extrasynaptic) DA are terminated in the brain. DAT is exclusively located on terminals of DA neurons and is abundant in efferent targets such as the dorsal striatum and nucleus accumbens. While reports have documented altered striatal DA and DAT levels in human methamphetamine and cocaine users, less attention has been focused on long-term effects of opiates (86-88). There is evidence from rodent models that would suggest decreased striatal DAT concentrations in association with chronic administration of opiates (89).

In addition to DAT, several other phenotypic markers are important for the regulation of DA function and thus relevant for addiction disorders. 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. A more recently identified presynaptic protein is α-synuclein, which is involved in the maintenance and transport of synaptic vesicles and has been implicated in synaptic plasticity (90). α-synuclein forms complexes with DAT, regulates the amount of DAT in the plasma membrane by modulating the shuttling of DAT between intracellular vesicular compartments and the plasma membrane (91), and also negatively effects DA uptake by the DAT (92). It has also been demonstrated that α-synuclein regulates DA biosynthesis by reducing the activity of TH (93). Dysfunction of α-synuclein has been highly implicated in Parkinson's disease, but only few studies have investigated its potential involvement in drug addiction. Of these, an overexpression of α-synuclein was observed in DA neurons of cocaine users (94).

Another relevant marker for midbrain DAergic function is Nurr1. This protein 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 (95). Nurr1 is abundant in the adult midbrain (96) and is suggested to be necessary for the regulation of genes that characterize DAergic phenotype such as TH (97) and DAT (98). Despite the important implications of Nurr1

for DA regulation, only few studies have examined Nurr1 in relation to drug abuse. Decreased Nurr1 transcription was documented in human cocaine abusers (96) as well as following chronic, but not acute or subchronic, cocaine administration in rats (99).

Periaqueductal grey (PAG)

The periaqueductal grey (PAG) or central grey of the midbrain is a midline structure surrounding the mesencephalic aqueduct. There are mainly enkephalinergic neurons in the PAG (64) though other opioid neuropeptides are also present in lower concentrations (100,101). An important function of the PAG is pain modulation. Stimulation of the PAG leads to inhibition of the nociceptive neurons in the dorsal horn of the spinal cord (102). Administration of an opiate antagonist attenuates this antinociceptive effect (103). The PAG is also involved in fear and anxiety behaviors as a critical component of the brain's fear circuit central the defensive fight or flight response (104). Electrical stimulation of the PAG in chronic pain patients revealed that such stimulation lead not only to antinociception but also induced unpleasant sensations which can be so aversive that the chronic pain patient refuses to stimulate the PAG (105).

The genetic influence on the neurobiology of heroin abuse

The vulnerability of addiction and also heroin abuse appears to have a very strong genetic load (106-108) however, our knowledge into the functional relevance of genes of risk is still only in its initial phase. The μ opioid receptor (MOR), which mediates the actions of enkephalin and β-endorphin peptides, is strongly linked to reward and reinforcement, thus polymorphism studies initially focused on the MOR gene (OPRM1). The most studied OPRM1 gene variant until now has been the A118G (encodes an Asp40Asp amino acid substitution) SNP in exon 1 of the human OPRM1; it is the most common mutation in the coding region and initial *in vitro* investigation suggested that it enhanced binding of β-endorphin and agonist-induced activation of G protein-coupled potassium channels (109). A clear functional relevance has recently

been documented between the 118G genotype and reduced OPRM1 expression at both the mRNA and protein level in the human brain (110) and increased basal cortisol level indicative of impaired stress response (111) . The distribution of the 118G allele varies greatly in relation to ethnicity (112,113). Investigations have found either no (112,114,115) or positive(109,116-118) associations of the A118G OPRM1 SNP with opioid dependence. In rather homogenous Caucasian populations (e.g., Swedish), a high percentage (≈ 65%-90%) of subjects carrying the 118G allele were found to be heroin abusers (118).

Factors affecting mRNA levels in post-mortem brain tissue

An important research strategy in recent years in attempts to understand the neurobiology of drug abuse has been the postmortem study of human substance abusers. There were initially reasonable concerns that such studies would be unfeasible due to factors at death or after death that would compromise the intactness of proteins, DNA or mRNA in human postmortem tissue. A number of studies have addressed these issues and several pre- and postmortem factors influencing the outcome of human postmortem experiments have now been recognized (119-121). Postmortem interval (PMI) was postulated to be one of the most crucial factors influencing the integrity of molecules(122), but today it is well recognized that PMI has only a modest impact (123-126).

Agonal state or the premortem phase has also been identified to contribute to the diversity of mRNA, protein or enzyme activity levels in postmortem human brain (126-131). Agonal state incorporates the events happening around the time of death and is very dependent on the cause and rapidity of death. According to several reports agonal state is closely related to brain pH (129,131-133). Based on the fact that the decrease in brain pH is most evident in prolonged periods of serious illness (e.g., pneumonia, cancer, cerebrovascular disease), pH has been proposed as a marker of protracted agonal period. Studies examining the association between brain pH, agonal state and mRNA levels have almost exclusively examined subjects with diverse neuropsychiatric conditions such as Alzheimer's disease, dementia, Parkinson's disease, major

depression or bipolar disorder, which often are associated with long agonal states. Very few studies to date have examined neuropsychiatric conditions with apparent short agonal periods.

MATERIALS AND METHODS

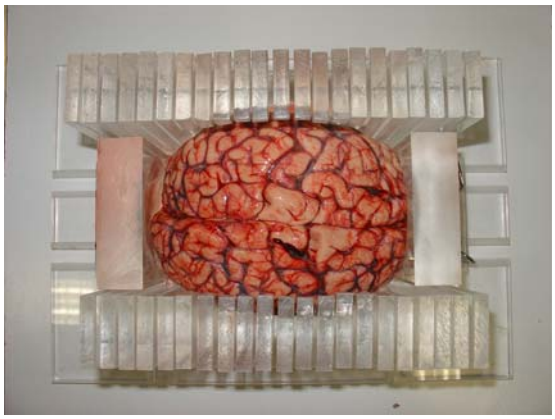
Post-mortem human brain specimens

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 and 2006, under the guidelines approved by the Semmelweis University Human Ethical Committee (Number of approval: 113/1995, 180/2001). The right hemisphere of the brain was cut into 2 cm coronal slabs (Fig. 4), the two hemispheres of the cerebellum were cut horizontally and the brainstem was cut into 1–2 cm blocks in a plane perpendicular to its long axis. Following this preparation the blocks were frozen in dry ice-cooled 2-methylbutane-isopentan (Fluka, 2.5L, 59075) and stored at -70°C. The left hemisphere was fixed for a minimum 6 weeks in 4% formaldehyde solution before dissection and paraffin embedding. Postmortem brain material was also obtained from the National Institute of Forensic Medicine (Karolinska Institutet, Stockholm, Sweden) under the guidelines approved by the Ethics Committee at Karolinska Institutet and the Swedish Board of Social Welfare. The number of cases used for each study is shown in Table 1. For detailed demographical data see the Method sections of the publications of interest.

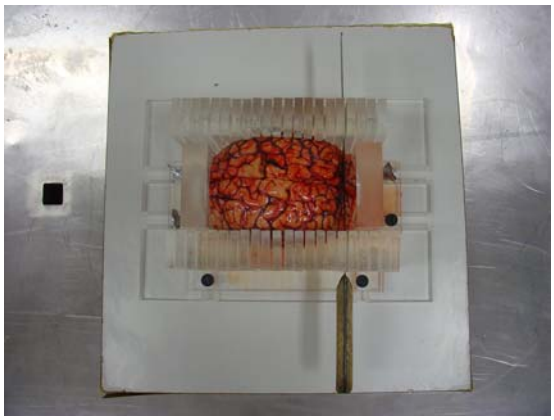
For the midbrain studies twenty micron thick cryosections (using a Jung-Frigocut 2800E cryostat; Leica, Heidelberg, Germany) were taken from brainstem blocks where specific brain regions were distinguishable (substantia nigra, superior cerebellar peduncle) and thaw-mounted onto Superfrost plus-glass (Brain Research Laboratories, Newton, MA). The brain sections were maintained at -30°C until later use. For striatal studies blocks of approximately 5x7 cm were cut over the rostral striatum. Twenty micron sections were taken from the striatal block using a Microm HM560 cryostat (Microm International GmbH, Walldorf, Germany), quickly mounted onto Superfrost plus-glass (Brain Research Laboratories, Newton, MA) and then kept at -30°C until analysis. Prior to sectioning, tissue punches were taken from the dorsal caudate nucleus

for peptide measurements. For immunohistochemistry, the formalin fixed tissue blocks from the striatum, where both the dorsal and nucleus accumbens were distinguishable, were paraffin embedded.

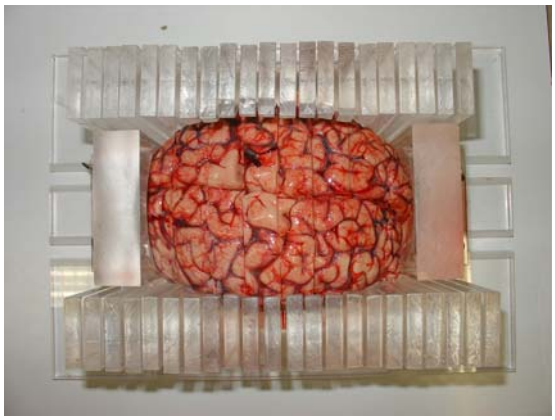
A. Brain cutting device



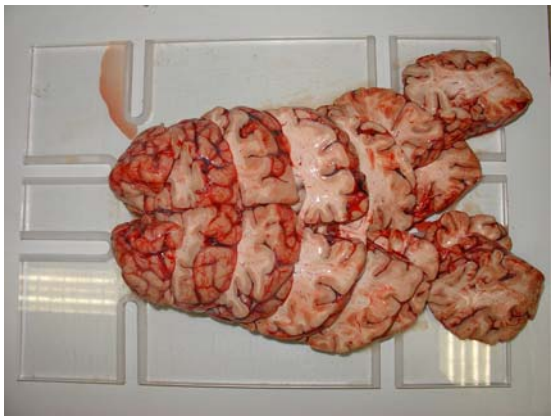
B. Positioning of the brain into the device



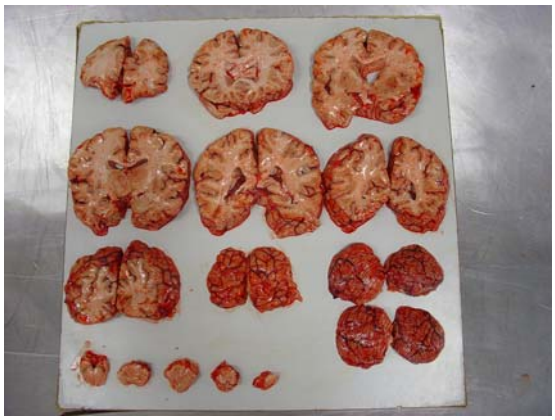
C. Cutting the brain to 2 cm slabs



D. Cut brain inside the device



E. Cut brain after the removal of the device



F.Brain slabs prepared for freezing

Figure 4. Preparation of the post-mortem human brain for freezing in dry-ice cooled isopenthane.

Table 1. Human brain tissue used in publications.

Neurobiological methods and regions of interest	Fresh frozen samples			Formalin fixed samples	
	Control	Heroin	Suicide	Control	Heroin
ISSH, GTPγS binding in the midbrain	10	22	-	-	-
Genotypization	26	39	-	-	-
ISSH, peptide measurements in the ventral striatum	19	34	-	-	-
pH measurement in the cerebellum	45	70	31	-	-
Immunohistochemistry in the ventral striatum	-	-	-	9	18

Serological examination (HIV, HCV, HBV, Syphilis)

From 1 January, 2000 before the autopsy blood was taken from the subclavian vein, centrifuged, and the serum was frozen at -40°C until further use. Due to hemolysis or decomposition there was no serum available in 93 cases from the total 219 cases registered after 2000.01.01. The serological examinations were performed at the Immunology Laboratory, Szt. László State Hospital, Budapest, Hungary. For the assessment of HIV infection we used the Organon Teknika Vironostika HIV Uniform Ag/ab ELISA kit (93 measurements), for the determination hepatitis C infection the Bioelisa HCV Biokit ELISA kit (93 measurements), for the presence of hepatitis B surface antigen the Organon Teknika Vironostika HBsAg Uniform II ELISA kit (87 measurements), for the presence of antibody to hepatitis B surface antigen the Organon Teknika Hepanostika anti-HBs ELISA kit (82 measurements) and for the presence of the antibody to hepatitis B core antigen the Organon Teknika anti-HBc Uniform ELISA kit (88 measurements), syphilis (90 measurements). There is a slight difference in the number of measurements, as there was not always enough serum to perform all analyses; the HIV and HCV status assessment being the key information we wanted to obtain.

When kits showed positive response for HIV, HCV or lues the subjects were regarded as being infected. In the case of hepatitis B different stages of the disease or the fact of being immunized against the infection was determined from different constellations of the main three antigens (anti-HBc, anti-HBs, HBsAg) as presented in Table 2.

Table 2. Different antibody and antigen constellations characterizing various stages and conditions of hepatitis B infection(134) and personal communication with dr. Eszter Újhelyi, Immunology Laboratory, Szt. László State Hospital, Budapest, Hungary.

Stages of hepatitis B infection	HBsAg	anti-HBc	anti-HBs
Not infected	negative	negative	negative
Acute or chronic hepatitis B infection	positive	positive	negative
Immune due to natural infection	negative	positive	positive
Immune due to vaccination	negative	negative	positive
Very recent hepatitis B infection	positive	negative	negative
Recovering from hepatitis B infection	negative	positive	negative

pH measurement of the brain

A piece of tissue (0.9g-1.4g) from frozen cerebellar cortex was homogenized in 10 volumes of double distilled water (pH has been adjusted to 7.0). The homogenates were centrifuged at maximum speed at 4°C for 20 minutes. The supernatants were subsequently maintained at room temperature for 15 minutes. The pH was measured in the supernatant in triplicates with a pH meter (PHM 92 LAB pH meter, Radiometer, Copenhagen) calibrated with two standards (pH 4 and 7). After each measurement the electrode was thoroughly rinsed with double distilled water. The final result used for statistical analysis is the mean of the three measurements.

Riboprobe preparation and in situ *hybridization histochemistry* – ISHH

DAT mRNA: synthesized from human cDNA BamH1/SacI 809 bp fragment subcloned into a pSP73 plasmid. The RNA probe for the DA D2 receptor was synthesized from a full length 1.58 kb cDNA fragment of the gene for the long form of the human

receptor(135) (provided by Dr O. Civelli), that was subcloned into a pBSKS plasmid vector. Alkaline hydrolysis was performed for the D2 receptor. The TH and α-synuclein probes were synthesized from a human cDNA library using nested PCRs (Table 3).
PENK was an EcoRI_Pvu 792-bp fragment complementary to the full coding region of the PENK human gene that was subcloned in a psp65 plasmid(136). The PDYNFL1 probe was synthesized from a pGEM3Z plasmid with the 5' region of the gene (GenBank accession no. NM_024411, bases -215/-72)(137). Templates for the PC2 and HERC1 probes were synthesized from a human cDNA library using nested PCRs (Table 3).

Table 3. PCR-derived DNA probe sequences and accession numbers.

Probe	Accession number	External primer	Internal primer
Tyrosine hydroxylase (TH)	NM_000360	5' – CGT GGA CAG CTT CTC AAT TTC CTC AT – 3' 5' – CAG CGC AGG AAG CTG ATT GCT GA - 3'	5' – GGG ATT TAG GTG ACA CTA TAG AAC CAG GCC AAT GTC CTG CGA GAA – 3' 5' - CTG TAA TAC GAC TCA CTA TAG GGG GAG CAC CTG GAG GCC TTT GCT T-3'
Orphan nuclear receptor (Nurr1)	NM_006186	5' – CCC AGC TTC AGT ACC TTT ATG GAC AA – 3' 5' – AGC TGA GAC GCG TGG CCG ATC T - 3'	5' – GAG ATT TAG GTG ACA CTA TAG AAG GAG ACT GGC GTT TTC CTC TGC T – 3' 5' - CTG TAA TAC GAC TCA CTA TAG GGC GAC GTC AAG CCA CCT TGC TTG TA-3'
α-synuclein	NM_000345	-	5' – GGG ATT TAG GTG ACA CTA TAG AAC ACA AAG ACC CTG CTA CCA T – 3' 5' – CTG TAA TAC GAC TCA CTA TAG GGG AAT TCT GGA AGA TAT GCC - 3'
Calbindin	NM_004929	5' – CTG ACG GAA GTG GTT ACC TGG AA – 3' 5' – GGT AGT AAC CTG GCC ATC TCA GTT- 3'	5' – CTG TAA TAC GAC TCA CTA TAG GGA GTT GGC TCA CGT ATT ACC – 3' 5' – GGG ATT TAG GTG ACA CTA TAG AAG GCC ATC TCA GTT AAT TC-3'
ProPC2	NM_003922	5' – ATC TGC TGA GCG ACC GGT CT – 3' 5' – GGT GGA GAT AGT CAA TCC CAT CA – 3'	5' – CTG TAA TAC GAC TCA CTA TAG GGC AAA GCT CGC CAA GTT GCA GCA – 3' 5' – GGG ATT TAG GTG ACA CTA TAG AAC CAG GCT TCA GCC ACA TTC AAA – 3'
proHERC1	NM_002594	5' – GGA GTT GCT GTT CTG TAT TCT AA – 3' 5' – CAG GCA TCA TCC AAC TTT GTC TT – 3'	5' – TCT GTA ATA CGA CTC ACT ATA GGG GAC CAC AGT TGC CAG ACT TTG AA – 3' 5' – GGG ATT TAG GTG ACA CTA TAG AAC ACC CAT TTC TAT AAG TGC ATC A – 3'

The validity of the PCR products was confirmed by DNA sequencing.
In vitro transcription was carried out in the presence of appropriate polymerases and [^{35}S]-αUTP (Amersham Biosciences, Europe) to radiolabel the probes. *In situ* hybridization was performed on duplicate sections as previously described(138).

Briefly, prior to hybridization, the brain sections were fixed with 4% paraformaldehyde in phosphate-buffered saline, incubated in 0.25% acetic anhydride in 0.1M triethanolamine/saline, dehydrated through a series of graded ethanol, and delipidated with chloroform. Cover-slipped sections were incubated with 0.21ml of hybridization cocktail at a final concentration of 20 x 10^3 cpm/ml overnight at 55ºC (65ºC for Nurr1 to reduce background). Hybridization was terminated by washes in a graded series of SSC solutions followed by dehydration in a graded series of ethanol. Riboprobe incubated slides were exposed to Kodak Biomax MR film for five to 15 days depending on the probe of interest with ^{14}C standards (American Radiolabelled Chemicals, St Louis, MO, USA), and developed (D19, Kodak). The specificity of the antisense probes was verified by their distinct anatomical distribution patterns and by the use of sense riboprobes, which did not show a specific hybridization signal. Brain sections used for analysis of the different DAergic and opioid gene mRNA expression levels were studied in separate experiments.

[^{35}S]GTPγS Autoradiography

The [^{35}S]GTPγS binding autoradiographic procedure was carried out as described previously (139) with minor modifications. Slides were incubated in assay buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) for 10 min at 25°C, and then were pre-treated with 2 mM of GDP in assay buffer for 15 min at 25°C. Sections were incubated in 0.04 nM of [^{35}S]GTPγS, 2 mM GDP, 0.1 mM DTT with 1 μM of MOR agonist, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO), or 0.5 μM U69-593 (KOR agonist) in assay buffer at 25° for 2 hours. Non-specific signal was assessed in present of 4.26 mM cold GTPγS. Basal activity was assessed in the absence of agonist. After incubation, slides were rinsed in cold Tris buffer (50 mM of Tris–HCl buffer, pH 7.4) for 2 min and rinsed in cold distilled water. The sections were thoroughly dried, and exposed to Amersham Hyper film with ^{14}C standards for 14-21 days.

Quantification of Autoradiograms

The autoradiograms from the *in situ* hybridization experiments or the [^{35}S]GTPγS binding were scanned using a ScanMaker III (Mikroteck Electronics, Düsseldorf, Germany) at a resolution of 300 dpi. Optical density values were measured from the digitalized images with an analysis software system (Scion Image; based on NIH Image for MacIntosh, Wayne Rasband National Institutes of Health, USA). 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 coexposed ^{14}C standards (ARC, St. Louis) using a Rodbard calibration curve (Scion Image).
In the midbrain, anatomical levels were identified in each case using TH expression pattern as a marker for the DAergic cell groups. Regional boundaries of the DA cell groups within the midbrain were determined according to published anatomical characteristics (140,141). Five cell groups were identified: limbic-related "dorsal tier" nuclei included the VTA (consisting of the paranigralis (PN) and nucleus parabrachialis pigmentosus (PBP)) and the substantia nigra pars dorsalis (SNd) and motor-related "ventral tier" nuclei included the substantia nigra pars ventralis and lateralis (SNv and SNl respectively)(Fig. 20). The subpopulations were distinguished by the co register with the distribution pattern of calbindin mRNA expression, which was high in "dorsal tier", but not in "ventral tier" neuronal populations (Fig. 20A). Background signal in the adjacent white matter was subtracted from the averaged values. Measurements from duplicates and right and left sides were averaged to one value per subject for each subregion.

Within the striatum measurements were taken from distinct regions of interest in the dorsal striatum (caudate and putamen) and NAc where it is dissociated into core and shell subdivisions (Figure 5). Distinction of motor and associative subregions of the caudate and putamen is based on the functional organization of the primate striatum (142). Because of the heterogeneous expression of PDYN in the dorsal striatum, in addition to measurements of the total area, separate analysis of expression in the patch (striosome; islands of high PDYN expression) and matrix compartments was also

performed by using threshold analysis. Background was subtracted from adjacent white matter areas, and the DPM/mg values from duplicate slides were averaged.

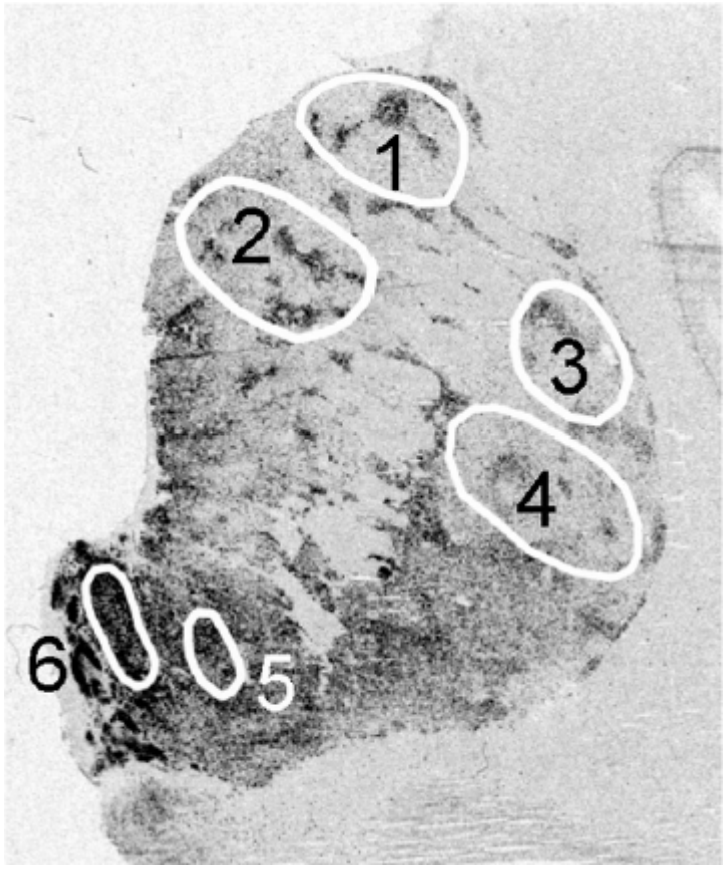


Figure 5. Outlines of striatal subregions used for optical density measurements. Representative autoradiogram of preprodynorphin (PDYN) mRNA expression in a control subject indicating the regions where measurements were taken. 1, motor caudate nucleus; 2, associative caudate nucleus; 3, motor putamen; 4, associative putamen; 5, core nucleus accumbens (NAc); 6, shell NAc. In the dorsal striatum, mRNA expression of preproenkaphalin (PENK), PC2, and HERC1 was determined only in the motor regions: 1, 2. Due to the heterogenous signal of PDYN mRNA expression in the dorsal striatum, additional measurements were taken in the associative regions: 3, 4.

Peptide Measurements

Opioid peptide concentrations from the caudate nucleus were determined by using radioimmunoassays for MEAP and dynorphin peptides (143). Radioimmunoassay (RIA). Briefly, extracted brain tissue material was run through SP-Sephadex ion exchange C-25 columns, and fractions containing Met-enkephalin-Arg-6-Phe-7 (MEAP), Leu-enkephalin-Arg, and dynorphin A and B were analyzed by RIA. All

antisera were raised in rabbits against the C-terminal end of the respective peptide. Crossreactivity of the MEAP (90:3DII) antiserum with Met-enkephalin, Met-enkephalin-Arg-6, Met-enkephalin-Arg-6Gly-7Leu-8, Leu-enkephalin, and Leu-Arg was <0.1%. Antidynorphin A antibodies demonstrated 100% molar crossreactivity with dynorphin A(9-17) and <0.1% molar crossreactivity with dynorphin B, dynorphin A(1-8), a-neoendorphin, Leu-enkephalin-Arg, and Big dynorphin. Antidynorphin B antiserum showed 100% molar crossreactivity with Big Dyn, 0.8% crossreactivity with Leu-morphine (29-aa C-terminally extended dynorphin), <0.1% crossreactivity with dynorphin A(1-17), dynorphin A(1-8), a-neoendorphin, and Leu-enkephalin.

Allelic Analysis of OPMR1

In order to study genotype, DNA was purified from cerebellar tissue of controls and heroin subjects (Table 1) using DNeasy columns (Qiagen, Valencia, CA). The OPMR1 A118G SNP was genotyped from PCR according to Gelernter et al.(112) 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.

Univariate analyses were carried out to obtain an overall estimate of the general characteristics of each individual variable on the brain pH levels. General Linear model

was used to determine the effect of group (control, suicide and heroin OD) and the impact of different variables (e.g., age, gender, PMI, storage time, respiratory distress) on brain pH. The variables with the weakest effect on the model were sequentially deleted and only those with a $p < 0.05$ association with brain pH were included in the final statistical model as covariates. Post-hoc group effects were evaluated by Tukey-Kramer analyses. Pearson correlation was used to determine the relationship between brain pH with blood/urine toxicological measurements. In all cases significance was set at $P < 0.05$ and trends considered for $P < 0.10$.

We calculated drug-related mortality rates caused by overdose death and also violent and natural death of drug abusers for each year from 1994 to 2006. The census population counts for Budapest for each year were obtained from the webpage of the Hungarian Central Statistical Office (144). All rates are expressed per 100.000 person-years.

RESULTS

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

There were 309 cases of drug-related death in Budapest, Hungary between 1994 and 2006. A majority (87%) of all decedents were male. 75% of all drug related death cases were people below 30 years of age. The majority of the cases had Hungarian citizenship; there were only 11 foreign decedents. 5% of the deceased were homeless and 13% had a domicile outside the capitol of Hungary, Budapest.

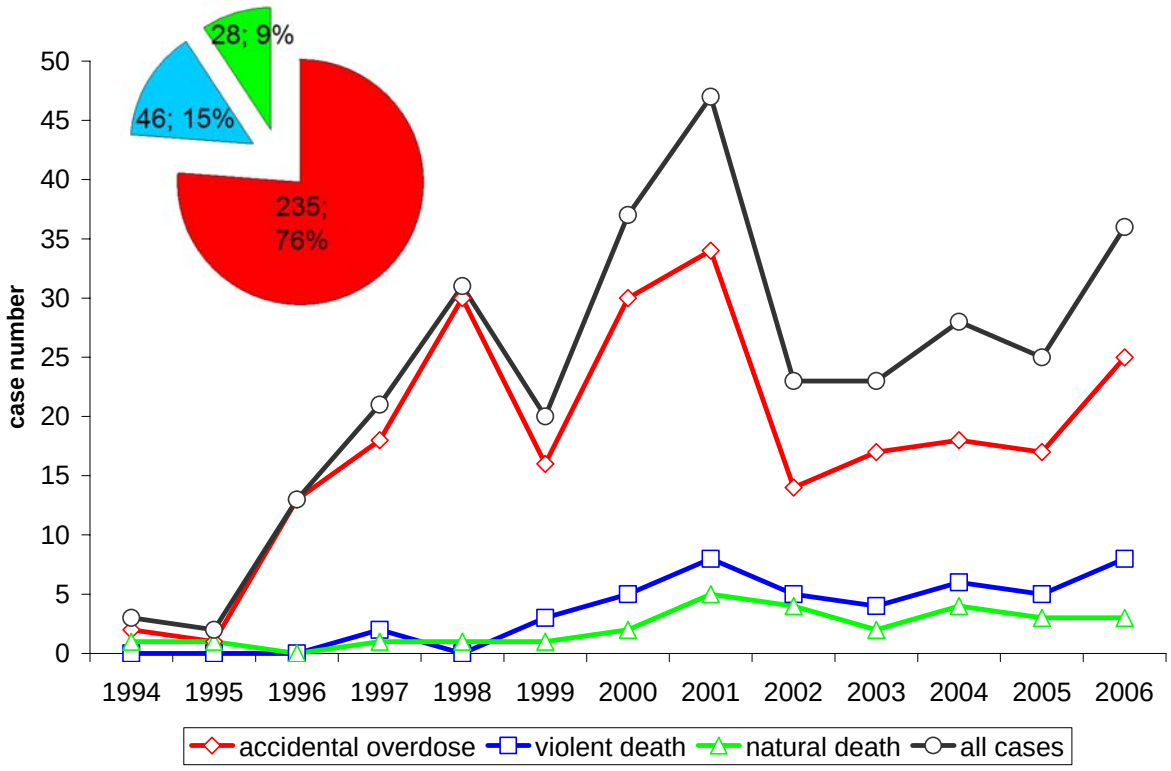


Figure 6. DRD in Budapest, Hungary between 1994-2006. The small pie chart shows the division of all cases of direct (accidental overdose) and indirect (violent and natural deaths of known drug users) DRDs. Color codes as in the line graph.

Violent deaths of known drug abusers

15% of the deceased drug users died of violent death that differed from accidental drug-overdose. The detailed causes of death, male / female ratio the mean age and ethanol concentrations are shown in Table 4.
Although violent deaths had the highest male / female ratio (lowest number of females) the higher number of males than females was also evident in each drug-related group. There were 4 homeless people and 7 with a domicile outside Budapest. 5 subjects died in hospital. We had available information about the estimated time of drug use only in 7 cases out of 47 and the mean time of usage was 4.92 ± 1.17 years with a range of 2.5-11 years of heroin use.

Natural deaths of known drug abusers

There were 27 cases when the forensic pathologists declared a natural cause of death in a known drug addict. The detailed demographical data are found in Table 4. The mean age is the highest from all drug-related mortality, but there is no statistical difference between the examined groups. There were three homeless people within this group and four cases with a domicile from outside Budapest. 11 of the decedents died in hospital. In the sudden heart and positive toxicology group (Table 4.) at toxicological examination we found drugs of abuse, but in too low concentrations to conclude an OD death or in untypical circumstances that made OD death improbable. The toxic agents found on autopsy were as follows: morphine (n=1), methadone (n=3), codeine (n=2), cocaine (n=1), amphetamine (n=1), volatile solvent (n=1) and THC (n=1). The preceding period of illegal drug use in this group could be estimated in 7 cases out of 27. In four cases the decedents were opiate users with a mean usage time of 7 ± 1.73 years (range 2 – 10 years). There was also one decedent who used cocaine for 10 years, another using amphetamine for 2 years and one using volatile solvents for the past 7 years. The four so called “other” causes of death were acute pancreatitis, epilepsy, toxic liver damage and a hematological disease respectively.

Table 4.
Subdivision of drug-related death cases in Budapest, Hungary between 1994-2006 with detailed causes of death.

Drug-related death type:	Case number	Male / Female	Mean age ± (range) years	Ethanol n; mean concentration ± SEM (range) in the blood and urine	Detailed cause of death
Accidental overdose deaths	235	205 / 30	25.9 ± 0.41 (15-57)	Blood: n = 47; 0.96 ± 0.1‰ (0.1 – 2.62) Urine: n = 31; 1.58 ± 0.17‰ (0.1 – 4.12)	Heroin OD: 203 Methadone: 4 Cocaine: 2 Amphetamine: 7 MDMA: 3 Butane inhalation[§]: 6 Volatile solvent inhalation[§]: 6 Halon[§]: 1 Not determined[†]: 2
Violent death of a known drug addict	47	42 / 5	27.08 ±1.01 (18-52)	Blood: n = 17; 1.79 ± 0.28‰ (0.3 – 4.13‰) Urine: n = 12; 2.38 ± 0.44‰ (0.23 – 5.17‰)	Suicide (25): • Heroin OD: 6 • Cocaine OD: 1 • Medicine OD: 9 • Falling from heights: 4 • Hanging: 4 • Vein cutting: 3 • Gunshot: 2 • Train: 2 Accident (14): • Alcohol intoxication: 4 • Medicine intoxication: 2 • Falling from heights: 1 • Electrocution: 1 • Drowning: 1 • CO intoxication: 1 • Traffic accident: 1 • Hypothermia: 1 • Train: 2 Homicide: 3
Natural death of a known drug addict	27	23 / 4	28.51 ± 1.9 (16-58)	Blood n = 5; 1.67 ± 0.36‰ (0.315 – 2.47‰) Urine n = 4; 2.63 ± 0.25‰ (2.08 – 3.11‰)	Sudden heart: 13 Sudden heart and positive toxicology: 10 Other: 4

§ - these substances are not illicit drugs, but nevertheless are substances with euphoric and addictive properties. For these reasons they will be discussed together with other drug-related death cases.
† - cases when material send for toxicology was destroyed. Most probably heroin OD cases.

Accidental overdose deaths

There were 235 cases of accidental drug overdose cases between 1994 and 2006 in Budapest, Hungary. The most common cause of death was intoxication with opiates and especially heroin. The detailed toxicological characteristic of each year is presented on Figure 7. Generally, it can be stated, that until 2001 heroin was the most common cause of drug overdoses.

Volatile solvent

Deaths due to volatile solvent inhalation were present in the Hungarian drug-related mortality since the mid – 1970's (17). In the examined population there were 6 cases of volatile solvent intoxications, all of them male with a mean age of 30 ± 3.5 years. Two of the decedents were homeless and all were Hungarian citizens.

Butane

In 2002 appeared the first death case related to inhalation of the gas used in lighters (butane). The butane inhalation deaths occurred among the very young, male population (range: 15-26 years, mean = 17 ± 1.7 years).

Cocaine

There were only 2 cases of cocaine abuse between 1994 and 2006. Both were male, one of the decedents aged 25 the other 35 years of age. The older person had a foreign citizenship. The blood examination revealed the presence of benzoylecgonine in one case (1.57 μg/ml) and the presence of methylecgonine ester in the other (0.01 μg/ml). Both are metabolites of cocaine. In the urine the following active components and metabolites were found:

- cocaine (71.6 and 0.013 μg/ml respectively)
- benzoylecgonine (68.9 and 0.016 μg/ml respectively)
- methylecgonine ester in one case (0.015 μg/ml)
- N-desmethyl-cocaine in one case (2.6 μg/ml)

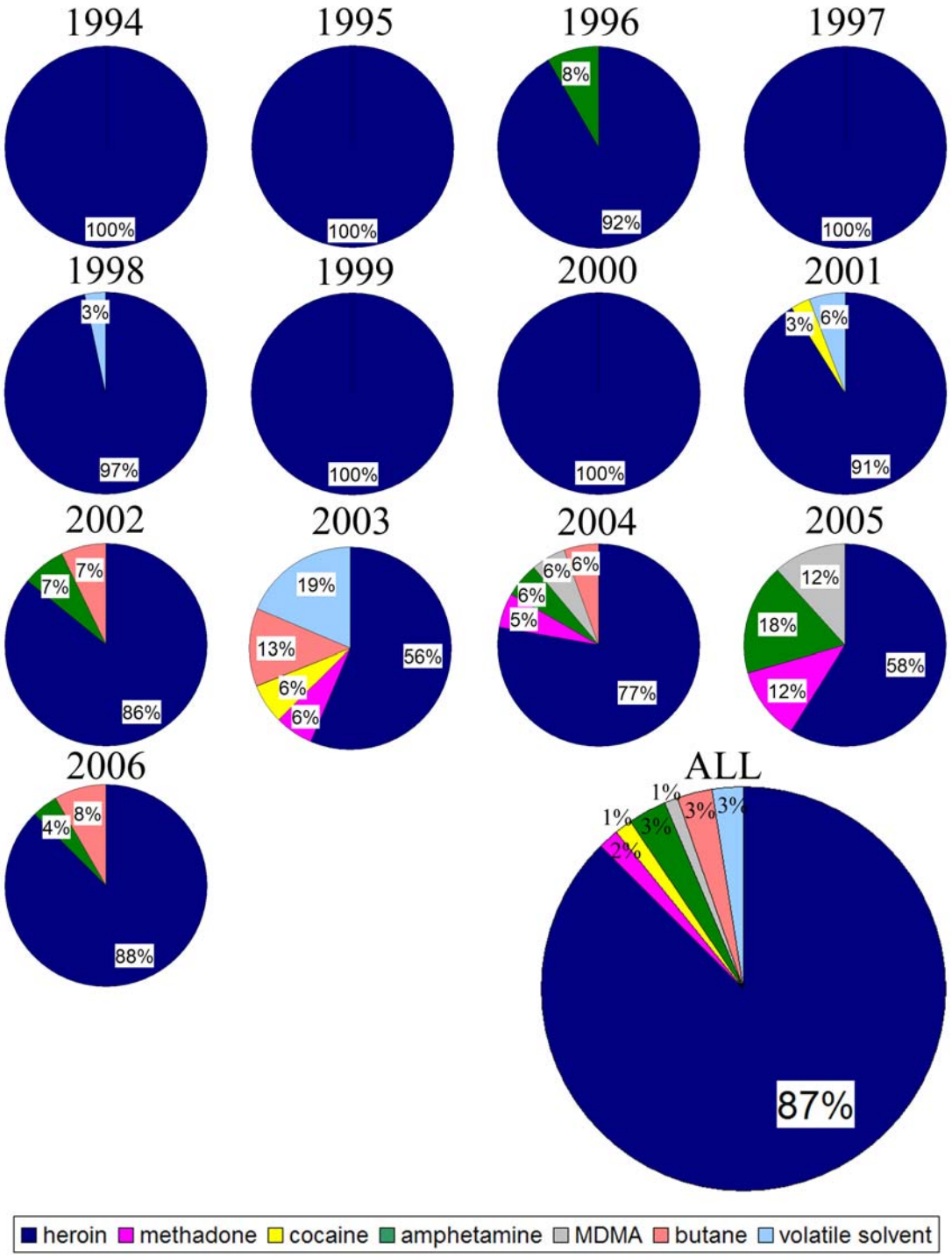


Figure 7. Yearly distribution of overdose deaths according to the drug of abuse between 1994-2006

Amphetamine

The first amphetamine overdose in our population appeared in 1996, but the remaining 6 cases happened after 2002. All of the decedents were male with a mean age of 24.8 ± 3.4 years and a range of 16-43 years. In four cases the death happened in a hospital, with a quantitative toxicological examination done in the hospital at admission. In the remaining cases mean blood amphetamine concentration was 1.085 ± 0.16 while in urine it was 8.87 ± 7.51 μg/ml.

MDMA

MDMA overdoses started in 2004, and since then there were 3 registered cases. All of the decedents were male with a mean age of 35.3 ± 0.88 years of age. The mean concentration of MDMA in blood was 34.37 ± 31.31 and the concentration of MDA was 0.3 ± 0.08 μg/ml. Urine concentrations were measured only in one case where the MDMA concentration was 21.17 μg/ml while the MDA concentration was 1.3 μg/ml.

Methadone

Methadone overdoses in Hungary were sparse, probably due to the low availability of methadone treatment (approximately 10% of all addiction treatments) which were primarily available in Budapest (145). The first methadone death was registered in 2003 and there were overall 4 cases of methadone overdose. Two of the decedents were female and the mean age was 25 ± 2.5 years of age. The mean blood methadone level was 12.45 ± 11.97. Two cases had additionally tramadol (synthetic opioid) in their blood as well which could have contributed to their death.

Heroin

There were 203 cases between 1994 and 2006 where the death was attributed to the overdose of heroin: 86.7% were males, and 77.3% were below 30 years of age. The detailed yearly age and gender distributions are presented on Figures 8 and 9 respectively.

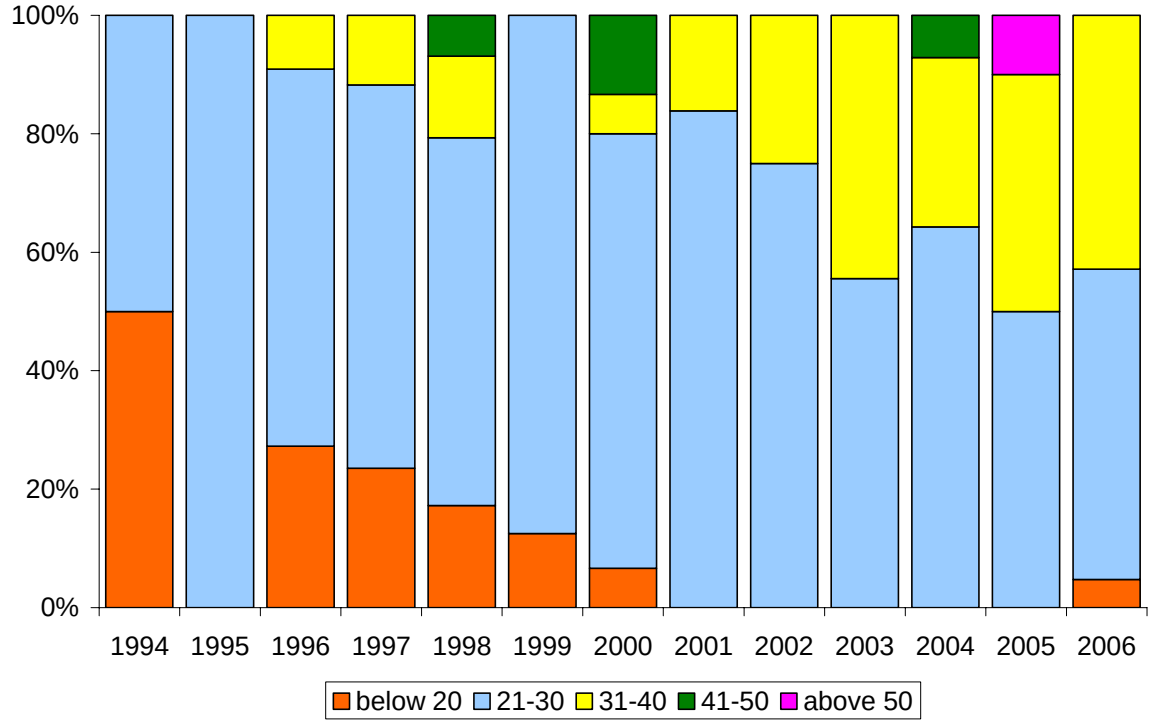


Figure 8. Yearly age distributions within heroin overdose deaths between 1994-2006.

The figure above shows a shift in the age of heroin OD victims – at the beginning there was a relatively high number of very young heroin OD victims (below 20 years of age), but slowly there were more victims above 30 years of age. The percentage of the victims between 20-30 years of age remained the same throughout the years. The gender distribution shows similar trends to the data published in international journals (43,45,56). 21 cases of heroin OD died in hospital. From the remaining 182 cases 55.5% died in familiar places (at home or at friends home) (Fig. 10). Most of the death cases happened on Thursday, and there was no significant difference whether the death occurred during a weekend or a weekday (Fig 11). Half of the decedents died in the same district where they lived; 8 of the heroin OD cases were homeless; 25 had a domicile outside Budapest. In 51 cases we had available information about the

approximate duration of drug abuse before death among the decedents. The average time of drug usage at death was 3.91 years ± 0.39 with a range of 0.3-13 years.

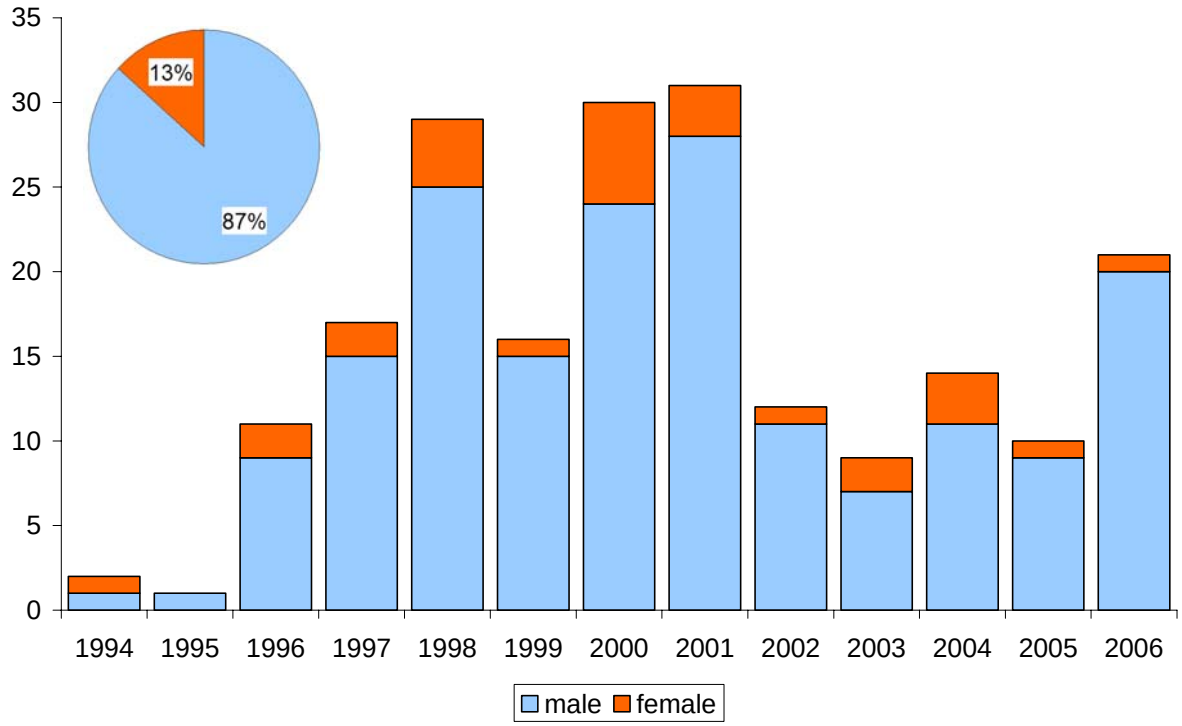


Figure 9. Yearly gender distribution within heroin overdose cases between 1994-2006.

There were also three cases of allegedly first time use. Information about abstinence preceding death was sparse – there were only 10 cases out of 203 where this information was available which ranged from 1.5 weeks to 20 months. All heroin OD cases were mapped by district number onto a map of Budapest showing the highest number of heroin OD cases in districts IV, VIII and XIII. There were practically no heroin OD cases in the residential areas on the outskirts of Budapest or in the business area of the city (V. district); there are only few cases in districts I, II, XII which are regions where the better situated population live; there is an accumulation of deaths in IV, VII and VIII which are districts with known social and economical problems (Fig. 12).

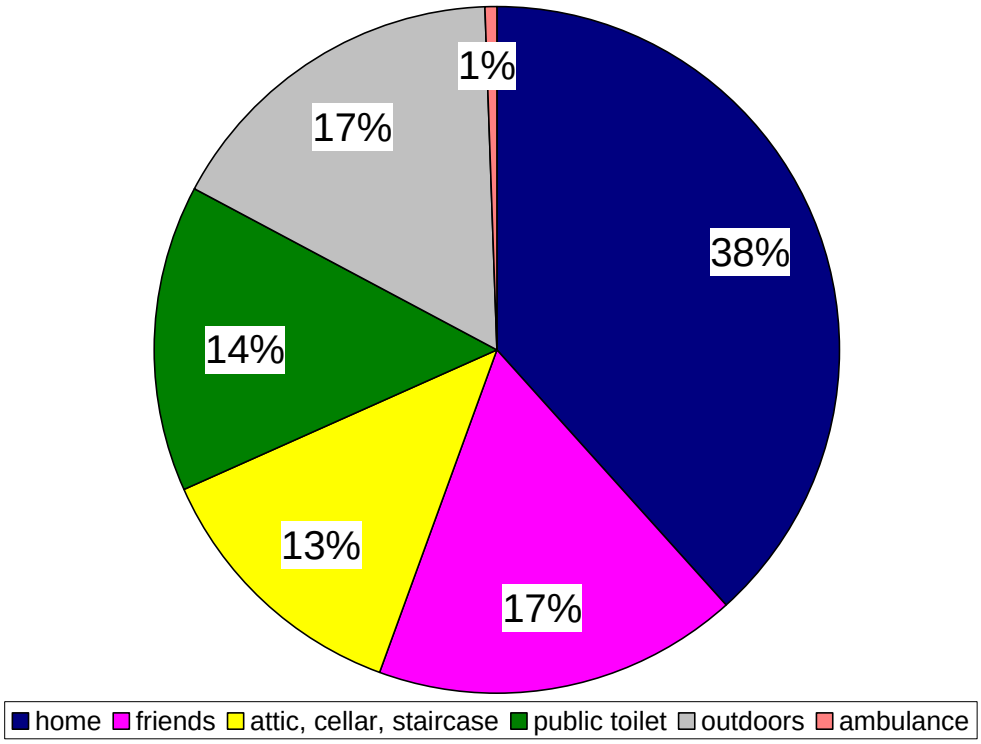


Figure 10. Occurrence of heroin OD deaths in different places.

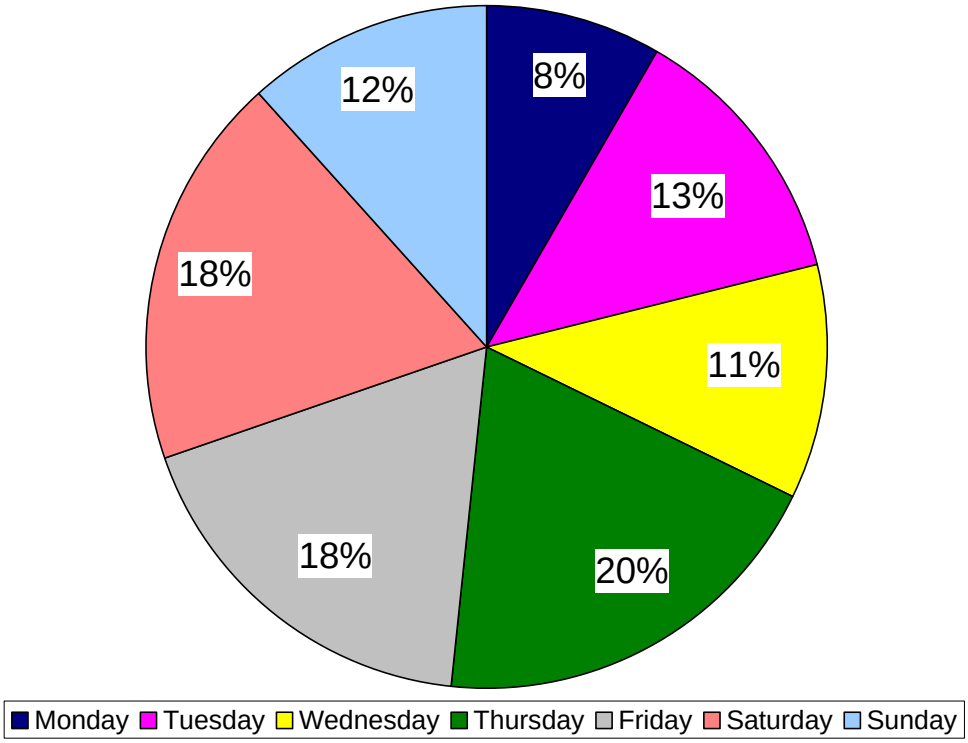


Figure 11. Heroin OD cases on different days of the week.

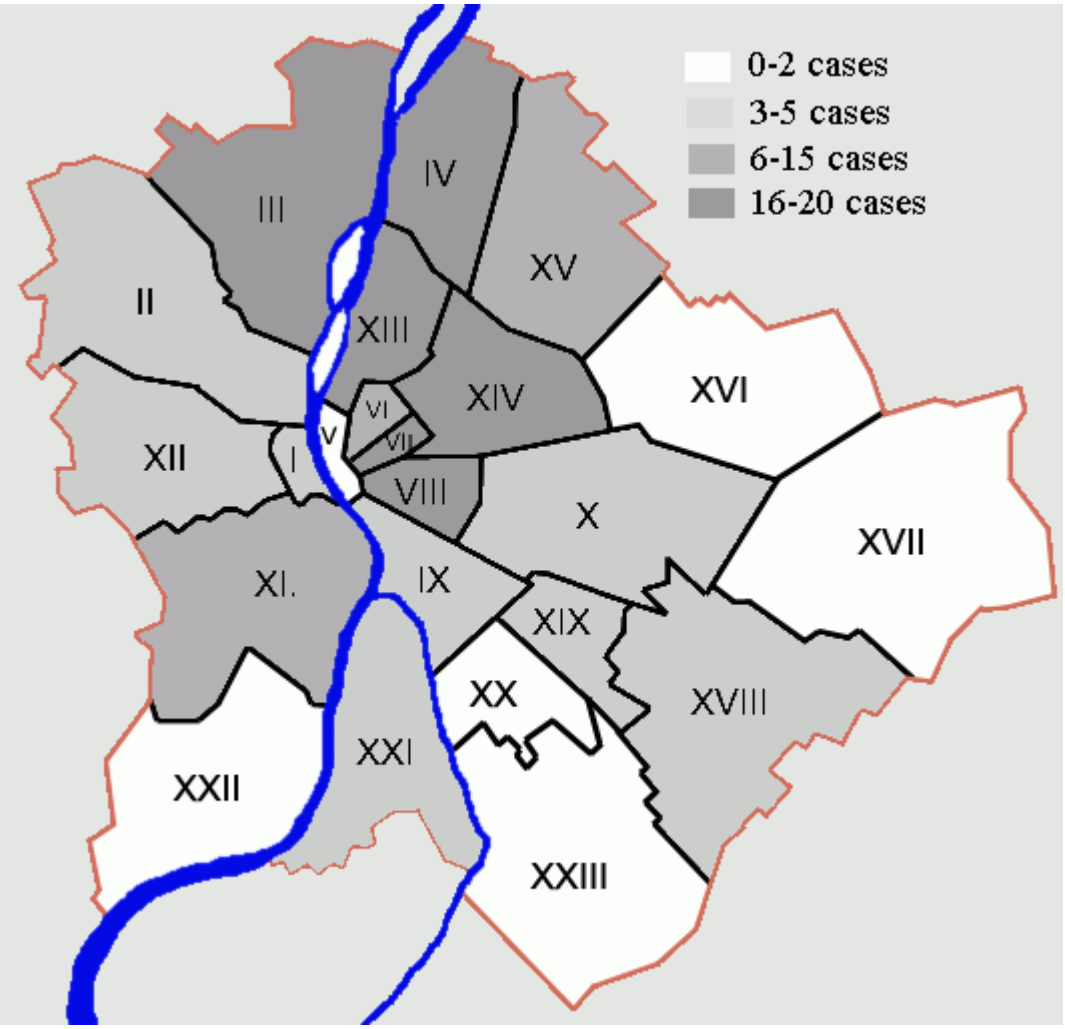


Figure 12. Heroin overdose deaths in the city of Budapest, January 1, 1994 to December 31, 2006.

Analyzing the toxicology results of the heroin OD deaths, we found that from the 203 cases there were 13 who had a negative toxicology results though they had positive substance abuse history, substance toxicology and a characteristic drug-related scene at death. Some of the latter cases were in the state of far-gone decomposition, which might have been the reason for the negative toxicology result. The remaining 190 cases had morphine or 6-monoacetyl morphine (6-MAM) (the metabolites of heroin) present in the blood, serum, liver or the kidney and in urine. Codeine was also detected in most of the cases, since acetyl codeine is often present in illegal heroin in high concentrations. The mean free morphine and codeine concentrations measured in different bodily fluids and organs are presented in Table 5.

Table 5. Concentrations of morphine and codeine in different organs and bodily fluids. Values given in μg/ml or μg/g.

	Blood	Serum	Urine	Liver	Kidney
Free morphine	1.15 ± 0.29	0.31 ± 0.05	4.69 ± 0.93	6.84 ± 3.5	3.37 ± 2.5
Free codeine	0.71 ± 0.15	0.28 ± 0.14	4.01 ± 1.82	8 ± 3.34	1.3 ± 1.2

In 11 cases where blood morphine concentrations were equal to zero, the serum still showed presence of morphine – thus we regard it is necessary to send both full blood and serum for toxicological examination if possible.

We also examined morphine to codeine concentration ratios in blood and urine which are regarded as a reliable means to determining whether the drug of abuse was heroin (ratio above entity) or codeine (ratio below entity). The mean morphine to codeine ratio in blood was 16.12 ± 9.35 while in the urine the mean morphine to codeine ratio was 44.01 ± 17.55.

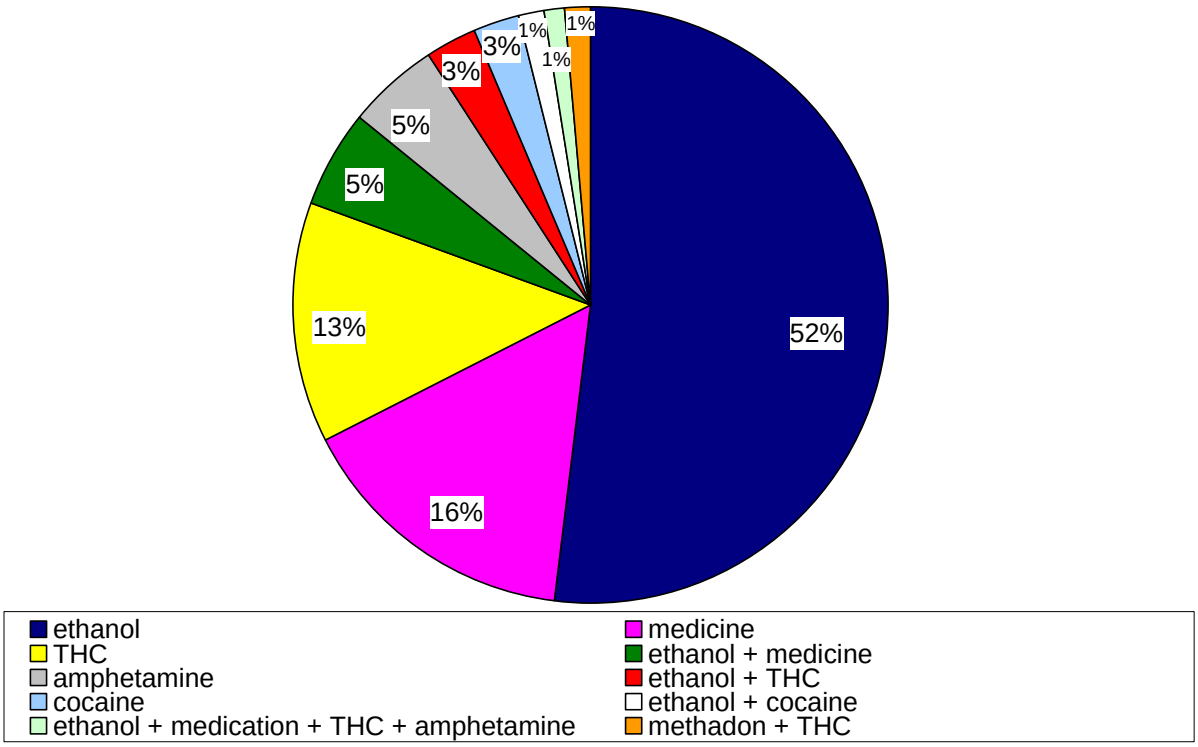


Figure 13. Other active substances present in heroin OD cases.

Taking into account the current theories about heroin overdose(56) 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 was present in most of the cases (n=40) with a mean concentration of blood ethanol of 1.14 ± 0.12 ‰, while ethanol concentration in urine was 1.71 ± 0.19 ‰. The next most common additional toxicological finding was the presence of legal prescription drugs (on figure 13 described as "medicine"). There were several types of drugs involved: benzodiazepines (n=5), barbiturates (n=3), antipsychotic medication (n=1), tramadol (n=1), barbiturate + gluthetimid (n=1), benzodiazepine + tramadol (n=1).

Multiple illegal drug use was not typical for this period of time but it was present: in 14 cases we found THC, in 5 cases amphetamine, in 3 cases cocaine and in one case methadone in addition to heroin metabolites, some of them in combinations (for details see Fig.13). When morphine and codeine concentrations in blood and urine were compared in "pure" heroin OD cases versus heroin OD cases where CNS depressant were present (ethanol and prescription medicines) we found that the mean concentrations (both morphine and codeine) in both bodily fluids were lower in the combined group (heroin + CNS depressant), but the difference was not statistically significant.

Infectious diseases among drug-related death cases

HIV

There was only one HIV infected person within the examined population (n=93). It was a 20 year old Hungarian female, who died of acute accidental heroin overdose. (Fig. 14)

HCV

28 cases out of 93 were positive for HCV. There were 3 females and 25 males infected with a mean age of 27.6 ± 1.39 years of age and range of 19-57. In 24 cases the cause of

death was acute accidental heroin overdose. 2 cases were suicides (hanging and heroin OD) and the other 2 accidental deaths (electrocution and ethanol overdose).

HBV

The hepatitis B status can be assessed with the help of three main markers: HBsAg, aHBc and aHBs. For details see Table 2. From 219 cases examined after 2000 there were 82 cases where all three markers were available, and thus the hepatitis B status could be assessed. There were 4 acute or chronic infections; 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; there were 39 not infected cases. Summing the cases that had ever been infected by HBV emerges a group of 12 people whose mean age is 28.41 ± 2.53 years with a range of 20-46 years. The majority of the cases are male (n=10) with only 2 females. Interestingly the only HIV infected person is also within this group and there are also 5 cases with HCV infection included. There are no lues infections in this group.

Syphilis (Lues)

From 90 examined cases we found 7 cases with positive results. There was one female and the remaining 6 subjects were males. The mean age was 22.5 ± 0.57 years with a range of 21-25. All of the infected people died of acute accidental heroin overdose.

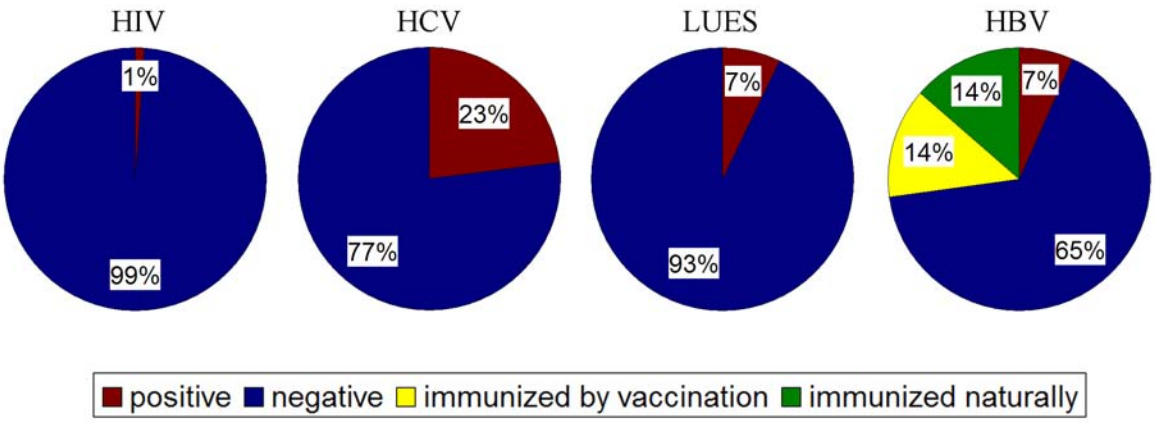


Figure 14. Percentage distribution of positive, negative and immunized cases within the examined population.

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

In the initial phase of our investigation of the molecular neurobiology of heroin abusers who died from OD, we noted significant variations in brain pH. The current study was thus carried out to determine the conditions in heroin OD that might contribute to alterations 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, we examined suicide subjects (in majority hanging with two cases of jumping from heights) with a documented rapid cause of death. We hypothesized that these were extremely fast manners of death that would not allow for prolonged terminal hypoxia. As such, the pH values should be the highest in the suicide victims.

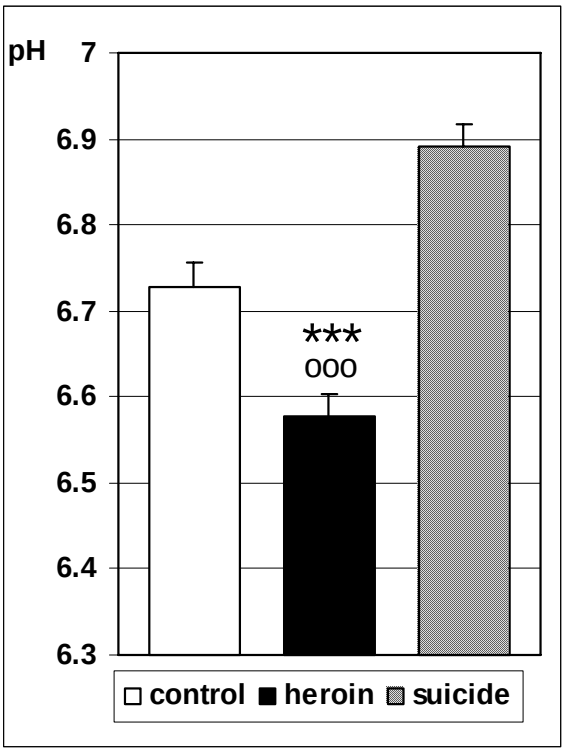


Figure 15. Means and standard errors of pH values measured in the brains of control, heroin overdose and suicide subjects. Overall group effect ($F_{6,144}$=23.85, p < 0.001) covaried for respiratory distress and alcohol toxicity *** = p < 0.001 vs. control; ooo = p < 0.01 vs. suicide

Figure 15 shows the mean pH values in three groups of subjects. The overall pH values from 146 subjects ranged between 6.06 and 7.16 (6.69 ± 0.019). There was an overall

significant main effect of group (ANCOVA $F_{6,144}$= 23.848, $p < 0.0001$; covaried for respiratory distress and ethanol toxicity as detailed below).

The distribution of pH values within each group is presented in Figure 16. In the control group, pH levels ranged between 6.15 and 6.98 (6.72 ± 0.028). The pH values in the heroin group (6.57 ± 0.025) contributed to the wide variation of the total population; the highest and lowest pH measurements were from this group. In the suicide group, the range of pH was between 6.41 and 7.08 (6.89 ± 0.024). Post-hoc statistical analyses showed that the heroin group had significantly lower pH values than the control and suicide groups ($p < 0.0003$). There was a non-significant trend ($p = 0.0639$) for brain pH to be higher in the suicide group as compared to controls. Based on the significant group differences in brain pH, all possibly contributing variables were examined separately in each group.

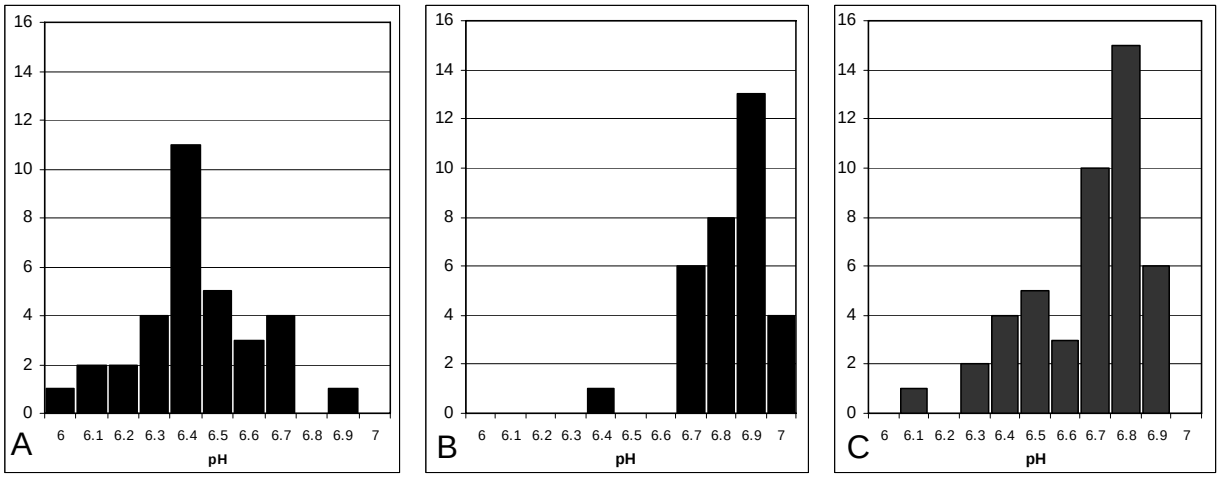


Figure 16. Distribution of pH values in control (A), heroin overdose (B) and suicide (C) subjects. The y axis represents the number of cases at each pH value.

Univariate analyses showed no significant effect of age, gender, PMI or brain storage time on the brain pH levels. However respiratory distress and ethanol toxicity were found to significantly influence the brain pH levels. Detailed description is provided below regarding the impact of these variables on brain pH.

Respiratory distress

The term “respiratory distress” was first used in connection with brain pH (124) 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: gross and microscopic evidence of vomit inhalation, gross and microscopic evidence of bronchopneumonia or other pneumonia, septic state, pulmonary embolia, suffocation and resuscitation. We compiled these informations from the autopsy reports and other available information sources blinded to the information regarding the pH values, conditions which might cause hypoxia, lactate acidosis and a consequential drop in pH were termed “respiratory distress”.

The presence or absence of respiratory distress was defined as “positive respiratory distress” or “negative respiratory distress” respectively. Positive respiratory distress correlated with lower pH values in comparison to negative respiratory distress when measured in all examined groups (negative respiratory distress, pH = 6.77 ± 0.018; positive respiratory distress, pH = 6.59 ± 0.03, $p < 0.0001$; Fig. 17.)

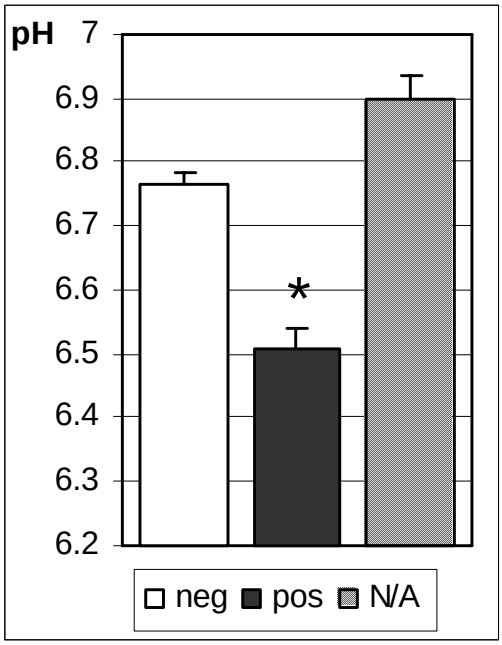


Figure 17. Mean brain pH values in correlation with respiratory distress. negative – negative respiratory distress (n=85), positive – positive respiratory distress (n=49), N/A – data not available regarding respiratory distress (n=12). * = $p < 0.05$ vs. control.

Evaluation of the respiratory distress in each group revealed that 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 (Fig. 18A.). In contrast, 16.6% of heroin subjects with no documentation of respiratory distress had low brain pH (Fig. 18B).

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). The lack of respiratory distress was associated with a significant increase of pH ($p = 0.0006$). There was also a group of 9 subjects with undefined respiratory distress with pH = 6.93 ± 0.037, values consistent with the negative respiratory distress group. Only two subjects belonged to the positive respiratory distress group; both were resuscitated previous to death and both had low pH values. (Fig. 18C.)

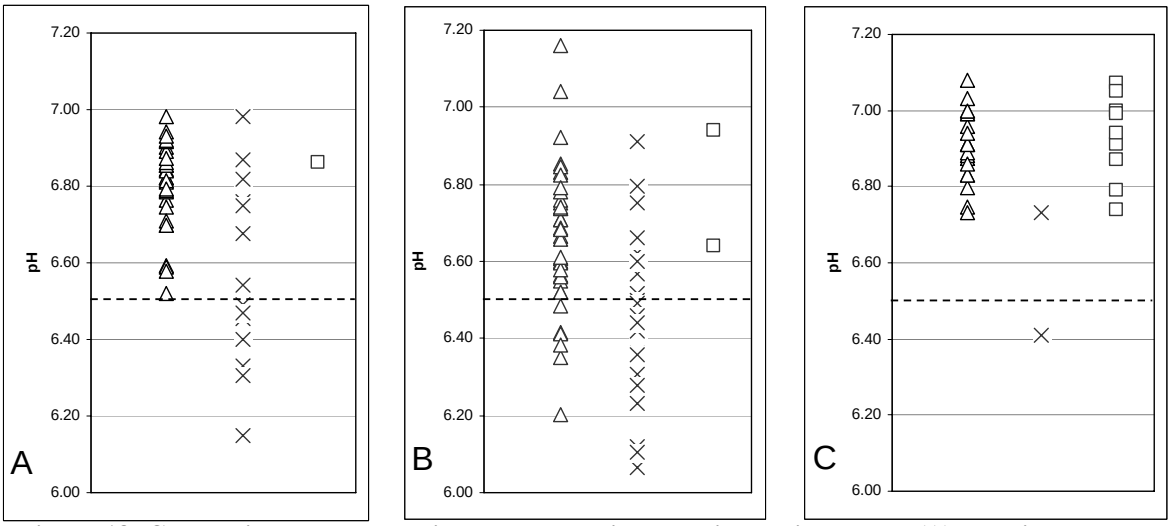


Figure 18. Correlation between brain pH and respiratory distress in control (A), heroin overdose (B) and suicide (C) groups. The triangles represent negative respiratory distress cases, the crosses represent positive respiratory distress cases and squares depict results with undetermined respiratory distress. The dotted line shows the average brain pH values in association with positive respiratory distress.

The effect of heroin metabolites on brain pH

Heroin metabolites were present only in the heroin group. The metabolites screened in association with autopsy were 6-monoacetylmorphine (6-MAM), morphine and codeine in blood and urine. As 6-MAM was present in the blood or urine in only a small number

of cases (n = 9), this opiate metabolite was not included as a parameter in the statistical analyses. There was no correlation between blood morphine concentration and brain pH. In the univariate analysis, urine morphine concentration had a significant relationship with brain pH ($p = 0.0260$). There was a weak, but significant correlation between brain pH and urine morphine concentration ($r = -0.3194$, $p = 0.0237$). This significance was however due to two subjects with high morphine values (34.49 ug/ml and 19.96 ug/ml). There was no significant correlation when the two subjects were excluded ($r = 0{,}0002$; $p = 0.9989$). No correlation was evident between brain pH and codeine concentration in the blood or urine.

The effect of ethanol on brain pH

The univariate analyses carried out on all the subjects showed that urine and blood ethanol levels had a significant effect on brain pH ($p = 0.0490$ for urine ethanol; $p = 0.0207$ for blood ethanol). In the heroin group, blood and urine ethanol concentrations had a significant effect on brain pH (for blood ETOH $p = 0.0022$, for urine ETOH $p = 0.0049$). As the concentration of ethanol in urine and blood were very highly correlated ($r = 0.9624$, $p < 0.0001$) and the significance for the blood ethanol was stronger than for urine ethanol, further statistical analyses were performed only with blood ethanol concentration levels. When the blood ethanol concentration was included as a covariant in the statistical model examining the relation between brain pH and the presence of respiratory distress it was revealed that the subjects with positive respiratory distress still had a significantly lower pH values than the subjects with negative respiratory distress ($p = 0.0003$, covaried for blood ethanol concentration $p = 0.0067$). When the negative and positive respiratory distress groups were analyzed separately, it was observed that the presence of ethanol in blood in the negative respiratory distress group was significantly associated with brain pH ($p = 0.0083$). Analyses of the heroin subjects showed significant correlations between brain pH and blood ($r = 0.4346$, $p = 0.0091$) as well as urine ($r = 0.4656$, $p = 0.0048$) ethanol levels in the negative respiratory distress group. There was no such association in the positive respiratory distress group with ethanol toxicity ($p = 0.6271$). (Fig. 19). Neither in the control nor in the suicide group was there any correlation between the blood or urine ethanol concentrations and brain pH.

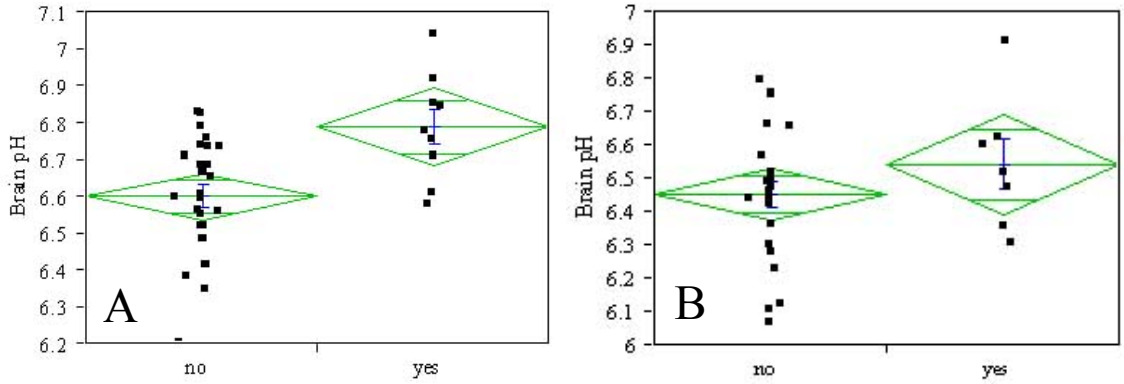


Figure 19. Correlation between the presence of ethanol and brain pH in negative (A) and positive (B) respiratory distress groups.

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 (146). Consistent with previous findings (147) the mRNA expression pattern for the dopamine markers were very similar with highest levels in the paranigral nucleus of the ventral tegmental area and in the ventral division of the substantia nigra. 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 (Fig. 20). For example in the brainstem, the paranigral nucleus showed a significant decrease of dopamine D2 receptor mRNA expression in correlation with reduced pH levels ($r = 0.466$, $p = 0.0094$) with a trend effect in the substantia nigra pars ventralis ($r = 0.34$, $p = 0.0569$). There was a similar positive correlation between pH levels and the dopamine transporter mRNA expression levels (paranigral nucleus: $r = 0.514$, $p = 0.0037$; substantia nigra pars ventralis: $r = 0.417$, $p = 0.022$). A similar pattern was also evident for the tyrosine hydroxylase expression levels. A significant decrease was also observed in the striatum for preproenkephalin mRNA expression in relation to pH levels; significance most

evident in the putamen and nucleus accumbens core (r = 0.409, p = 0.0086; r = 0.37, p = 0.0203).

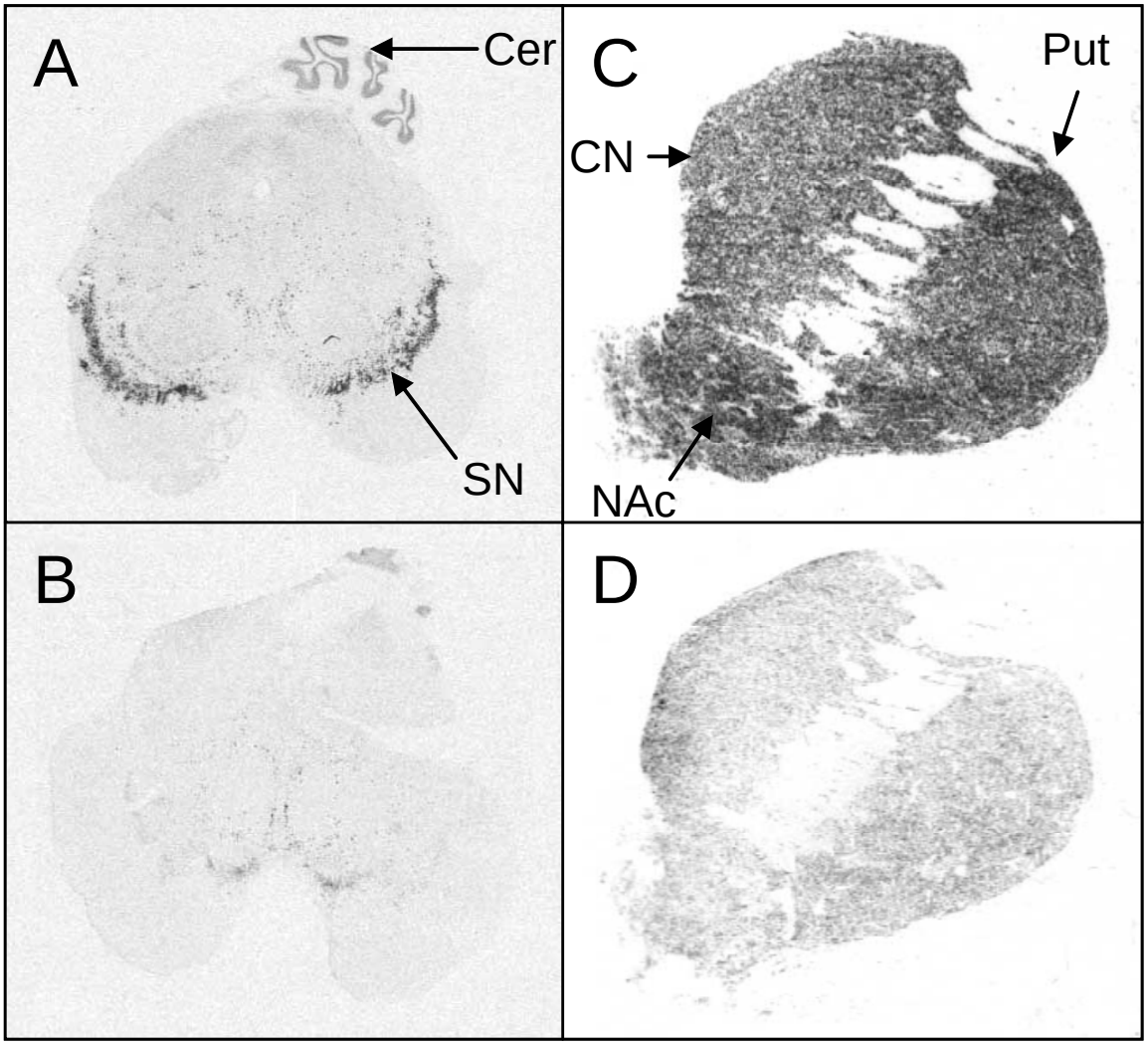


Figure 20. Autoradiogram images showing the mRNA expression of the dopamine D2 receptor in the human midbrain (A, B) and preproenkephalin in the striatum (C, D) in subjects with high (A, C) and low (B, D) brain pH. Cer, cerebellum; SN, substantia nigra; CN, caudate nucleus; Put, putamen; NAc, nucleus accumbens.

Alterations in the DAergic system in human heroin abusers

Given the strong implications of DA neurotransmission in addiction disorders and the relative paucity of data regarding the DA system in relation to opiate addiction, we examined phenotypic markers of the midbrain DAergic system in human heroin abusers. In order to address the issue of potential differences in mesocorticolimbic and nigrostriatal systems in relation to opiate use, gene expression of the DA markers were studied in subpopulations of the VTA and substantia nigra.

Midbrain – mRNA expression of DA-ergic markers in human heroin abusers

Using the described methodological conditions (p.35-37), all antisense riboprobes studied showed specific hybridization signals with very low background in the brainstem (Fig. 21). There was no specific hybridization signal in brain sections processed with the sense riboprobes. All the DA markers examined showed a typical expression pattern characteristic of the distribution of DAergic neurons. Consistent with previous studies, mRNA expression of the DAergic markers was highly abundant within the substantia nigra and VTA, most predominantly expressed in the paranigral nucleus (PN) followed by the substantia nigra ventral part (SNv) and substantia nigra lateral part (SNl). The mRNA expression levels were higher in the substantia nigra dorsal part (SNd) than in the parabrachial pigmental nucleus (PBP) for most markers with the exception of DAT where the PBP had higher expression.

The dpm/mg values measured in each subregion and for each gene expression marker are provided in Figures 22-26. In the case of DAT mRNA, expression levels were significantly lower in the PN of heroin subjects in comparison to controls ($F_{2, 22}$ = 7.4836, p = 0.0263, covaried for freezing time). A similar direction of change, but with strong trend effect, was also observed in the other dorsal tier subregions namely the PBP (p = 0.0515) and SNd (p = 0.0768). No significant group effect was detected in the ventral tier subnuclei (Fig. 22).

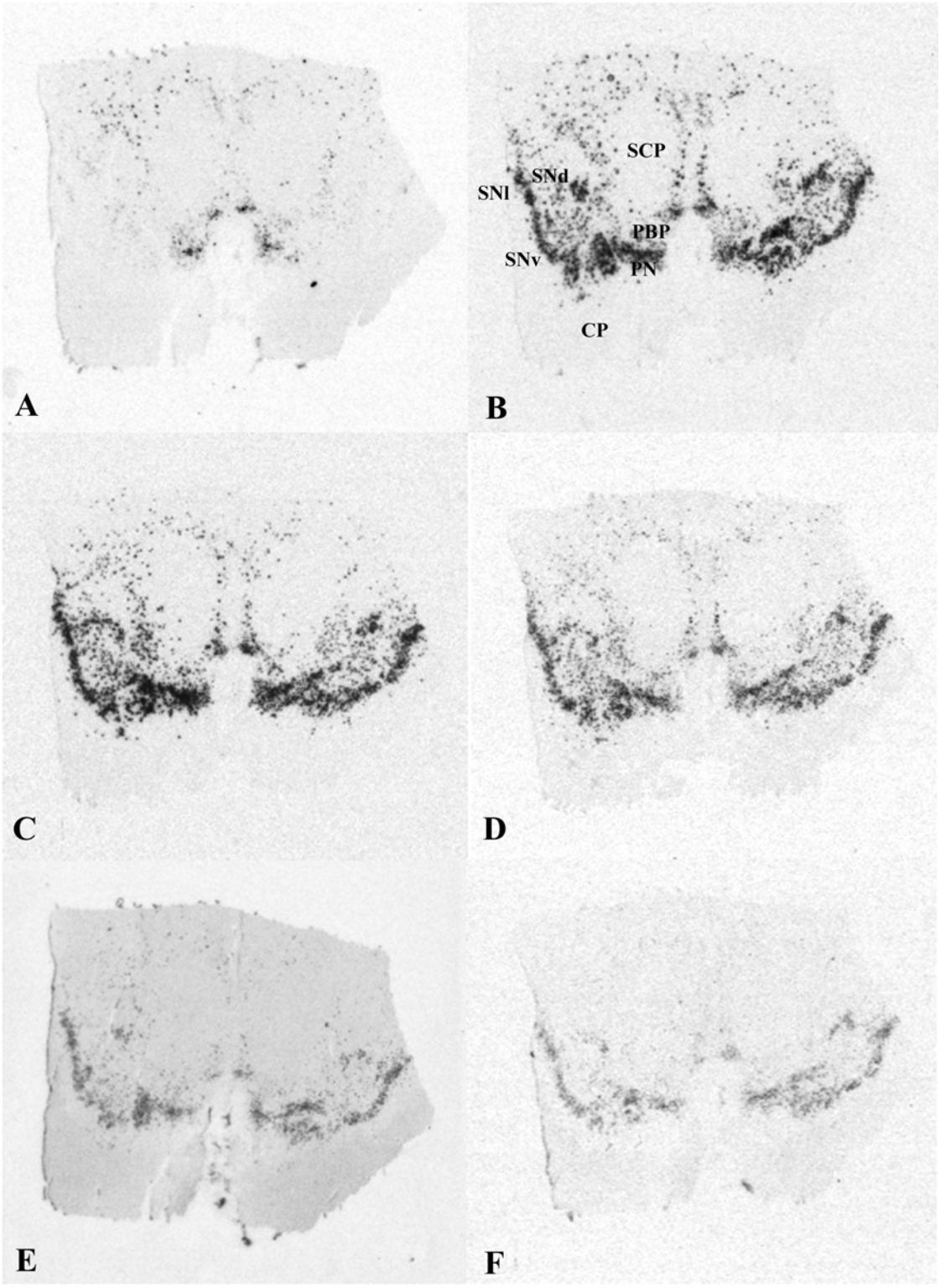


Figure 21. Autoradiograms showing the mRNA expression pattern and distribution of calbindin (A) as well as dopaminergic markers in the human midbrain for tyrosine hydroxylase (TH; B), dopamine transporter (DAT, B), DA D2 receptor (D), Nurr1 (E), and α-synuclein (F). PN – paranigral nucleus, PBP – parabrachial pigmental nucleus, SNv – substantia nigra ventral part, SNl – substantia nigra lateral part, SNd – substantia nigra dorsal part, SCP – superior cerebellar peduncle, CP – cerebral peduncle. Limbic-related "dorsal tier" nuclei include the PN, PBP, and SNd; motor-related "ventral tier" subpopulations include the SNv and SNl.

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 ($F_{2, 25} = 5.1965$, $p = 0.0494$, covaried for freezing time), SNd ($F_{1, 28} = 4.5344$, $p = 0.0425$) and SNl ($F_{2, 20} = 7.0989$, $p = 0.0035$, covaried for pH; Fig. 23).

mRNA expression levels of the DA D2 receptor did not differ between the heroin and control groups in any of the brainstem subregions studied (Fig. 24).

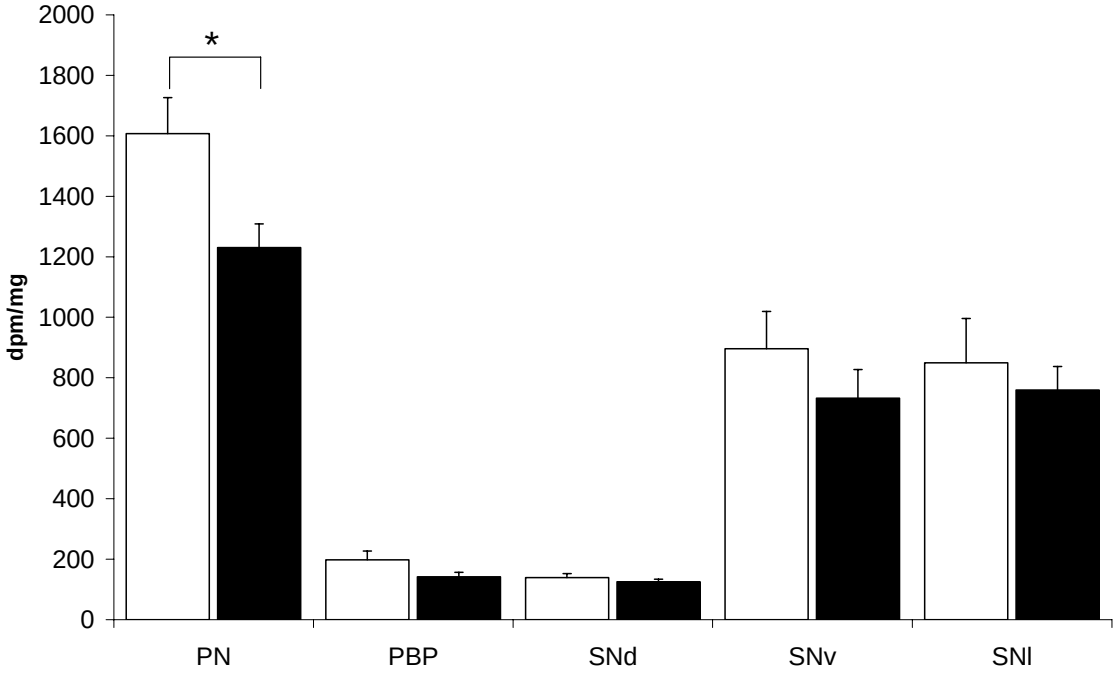


Figure 22. mRNA expression levels of DAT (PN covaried for freezing time), in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

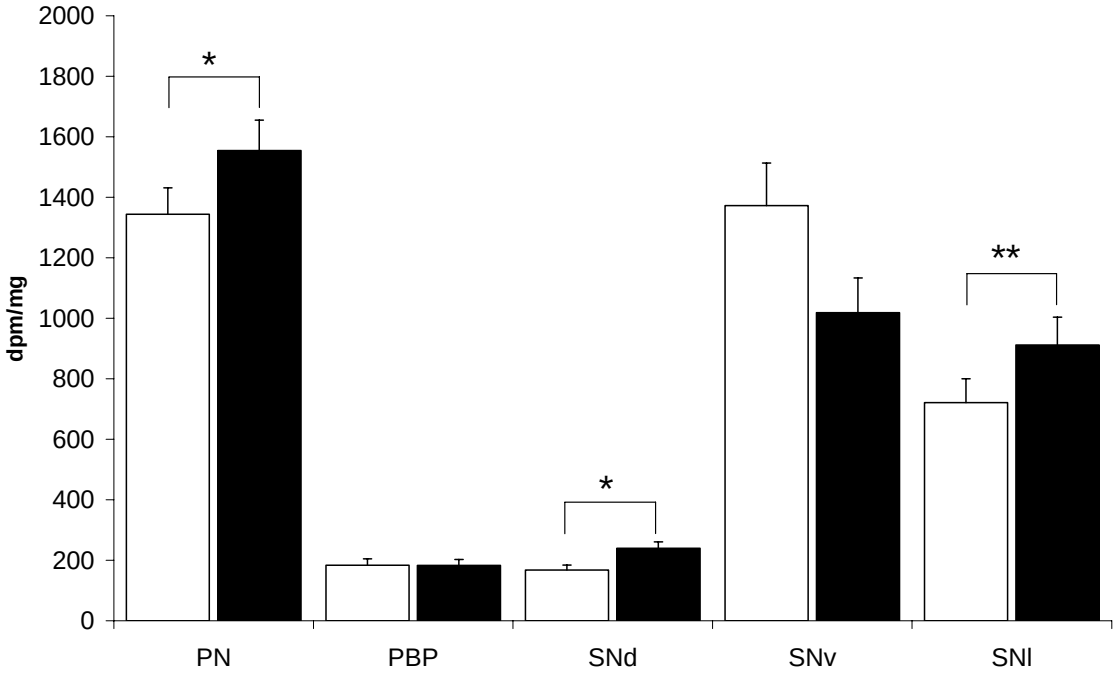


Figure 23. mRNA expression levels of TH (PN covaried for freezing time; SNd no covariates; SNl covaried for pH), in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

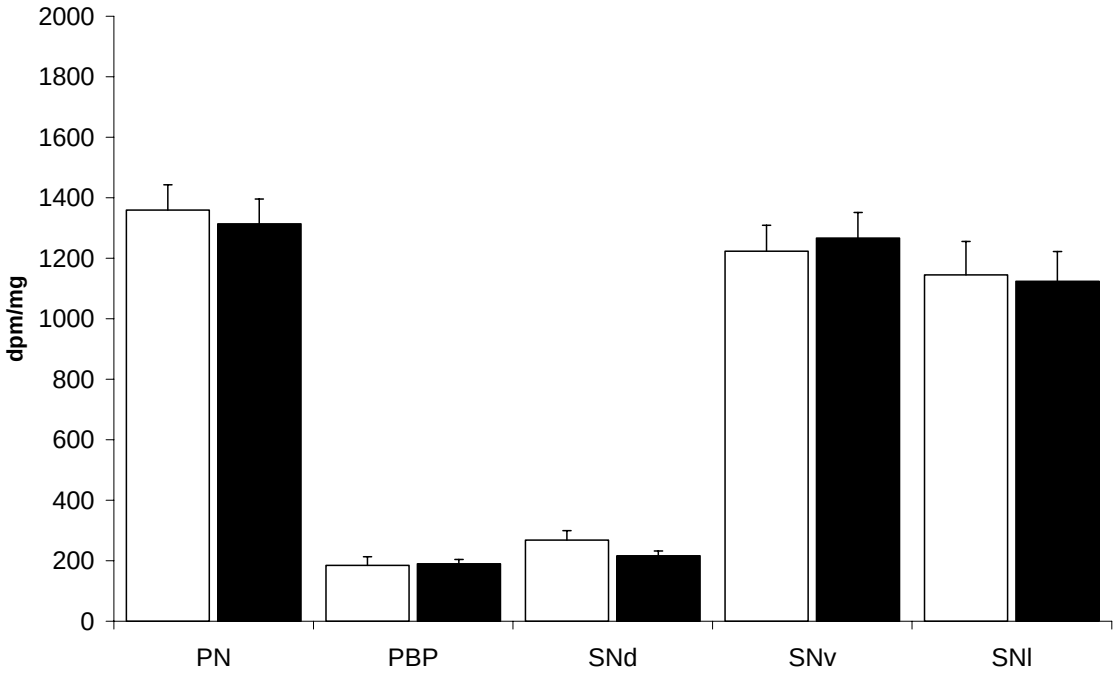


Figure 24. mRNA expression levels of dopamine D2 receptor, in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

Heroin related alterations of the Nurr1 gene expression were limited. Only the PN had significantly reduced mRNA levels ($F_{4, 24}$ = 13.711, p = 0.0089, covaried for age and ethanol presence) in the heroin subjects (Fig. 25). Age (p < 0.0001) markedly influenced the Nurr1 mRNA expression levels and there was a strong significant group x age interaction (p = 0.0012) such that Nurr1 mRNA levels were reduced to a greater extent in the PN of heroin subjects (r = -0.8268, p < 0.001) with increasing age than that observed for the control group (r = -0.6204, p = 0.0746, Fig 24). The Nurr1 decline with age was still evident in heroin subjects when examining only the youngest individuals under 30 years old (n = 14, r = -0.7099, p = 0.0044). No significant differences were detected for the Nurr1 mRNA expression between heroin and control subjects in the other midbrain DAergic subpopulations.

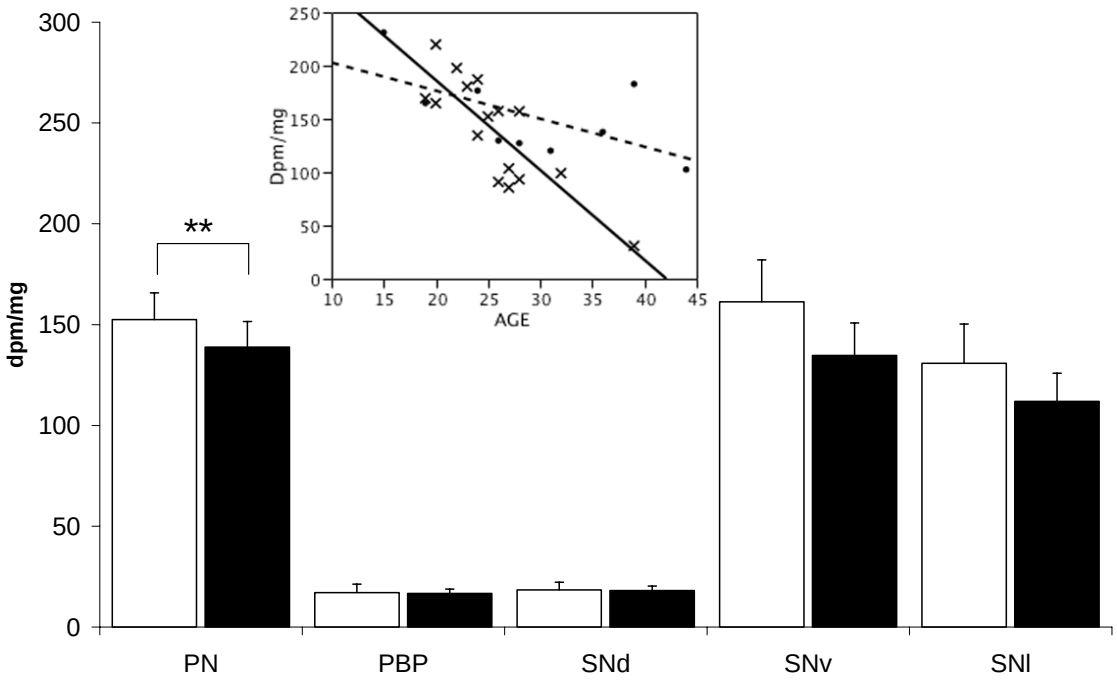


Figure 25. mRNA expression levels of Nurr1; PN covaried for age and ethanol toxicology; age x group interaction, in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001. Insert figure shows the correlation between Nurr1 dpm/mg values and age in the PN of control (dotted line, o; r = -0.08268, p < 0.001) and heroin (solid line, x; r = -0.6204, p = 0.0746) subjects.

Heroin abusers also showed discrete alterations of α-synuclein. Significant elevation of α-synuclein mRNA expression levels were observed in the PN ($F_{3, 22}$ = 6.3153, p = 0.0035, covaried for pH and sex) and SNv ($F_{2, 23}$ = 6.5692, p=0.0499, covaried for ethanol presence) (Fig. 26).

None of the expression levels of the DA-related mRNAs examined were significantly correlated to morphine toxicology.

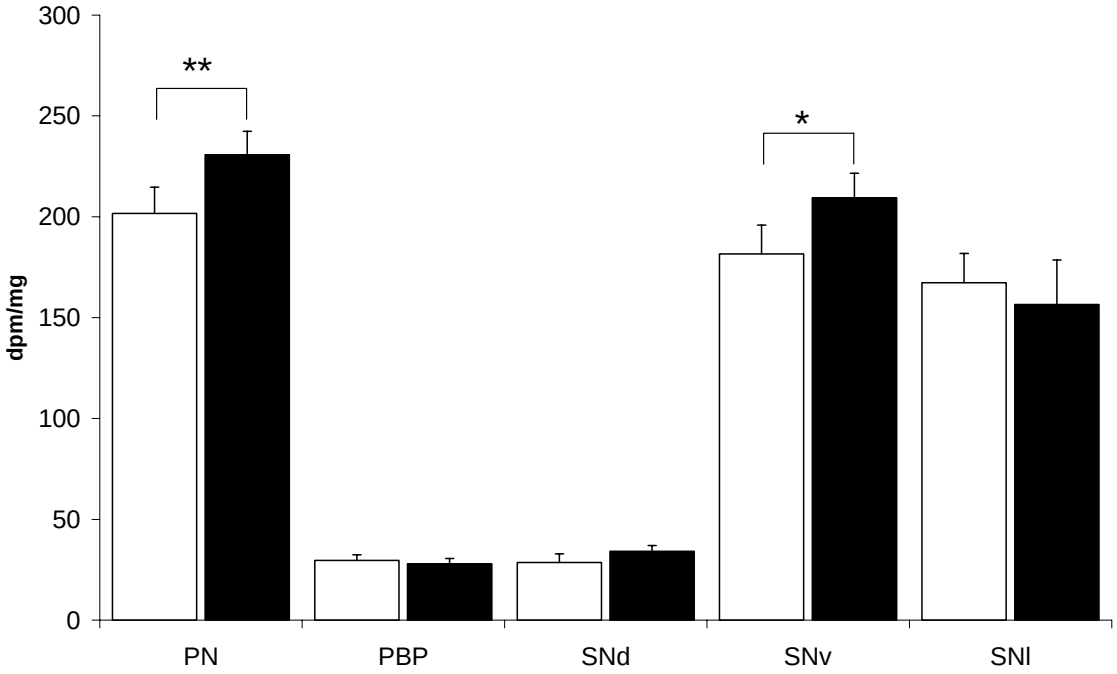


Figure 26. mRNA expression levels α-synuclein (PN covaried for pH; SNv covaried for ethanol toxicology) in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

Striatum - Striatal DAT immunoreactivity

We evaluated the density of DAT immunoreactivity in striatal subregions in a uniform population of heroin abusers and a control group to demonstrate selective vulnerability within limbic-related structures such as the ventral striatum (nucleus accumbens).

DAT immunoreactivity showed a characteristic heterogeneous distribution pattern in the striatum. In heroin subjects, the density of DAT immunoreactivity was significantly decreased by 55% in the nucleus accumbens as compared to controls (p = 0.002). No significant changes were observed in the caudate nucleus (p = 0.53) and putamen (p =

0.16; Figures 27 and 28). Examination of the different subregions of the nucleus accumbens showed significant reduction in both the core (42%) and shell (39%) ($p < 0.01$) of the heroin users. No demographic variable was found to influence the DAT density in any of the striatal subregions either when all cases or the individual groups were examined separately ($p > 0.1$ in all comparisons). However, there was a non-significant trend for an inverse correlation between DAT density in the nucleus accumbens and blood codeine levels in heroin subjects ($n = 13$, $r = -0.52$, $p = 0.06$).

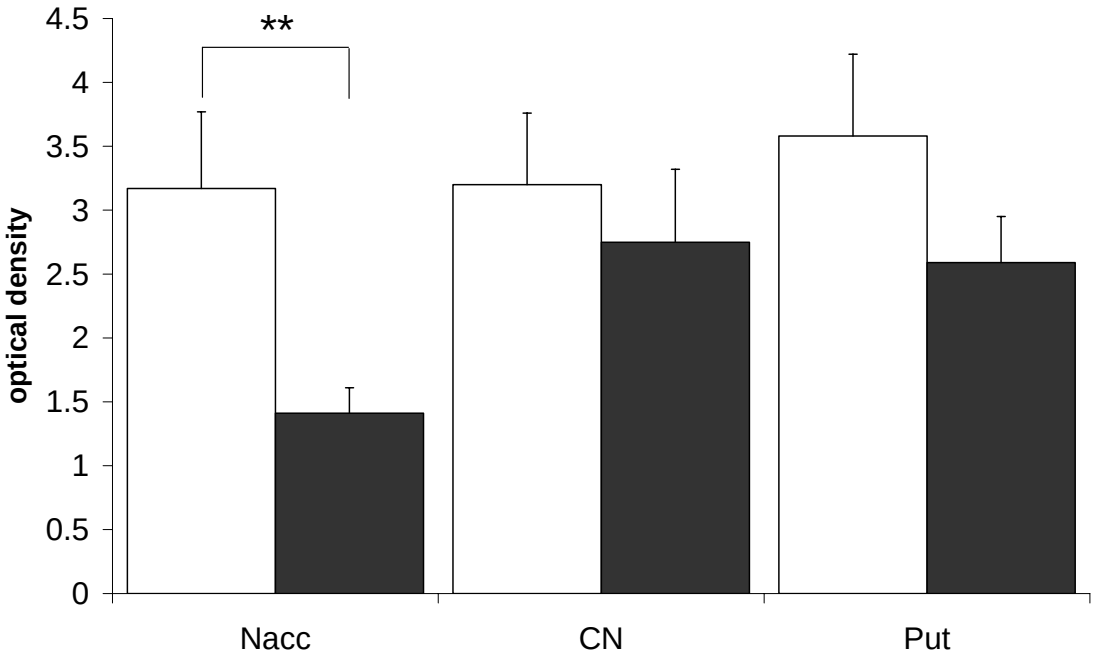


Figure 27. DAT protein density levels in subregions of the striatum in control (white) and heroin (black) groups. * $p < 0.05$; ** $p < 0.01$. Nacc – nucleus accumbens; CN – caudate nucleus; Put – putamen.

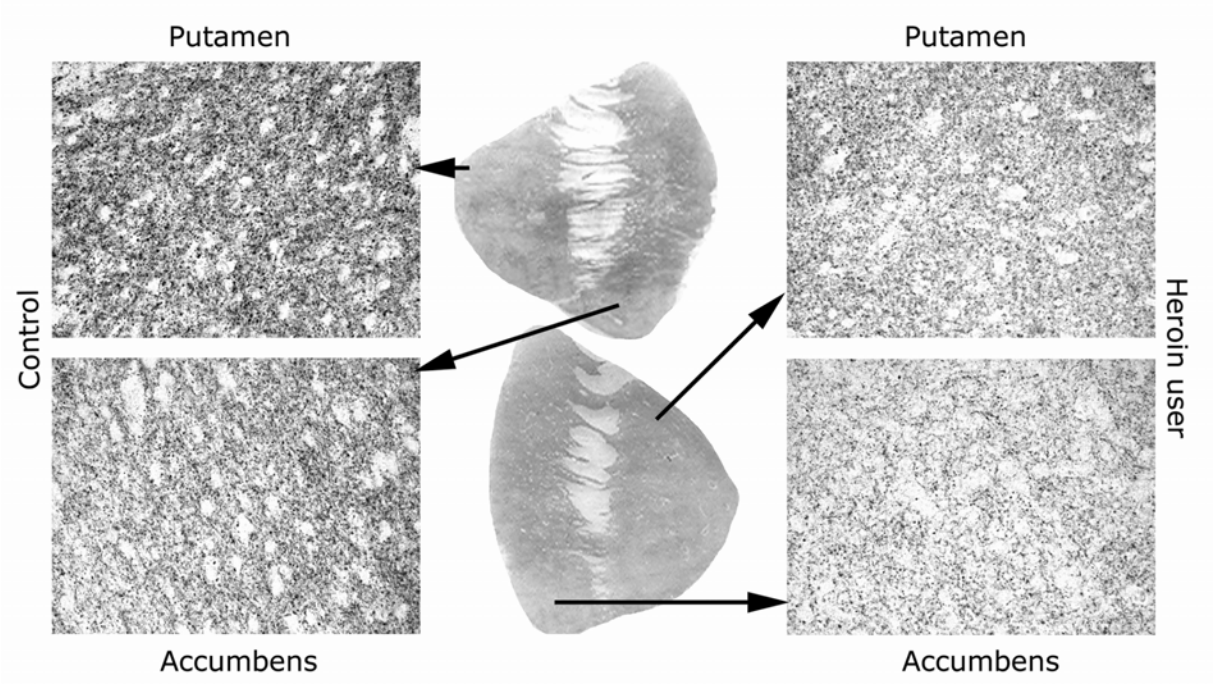


Figure 28. Immunohistochemistry for DAT in the basal ganglia of controls and heroin users. Arrows indicate enlarged area of putamen and nucleus accumbens (x400).

Alterations in the opioid system in human heroin abusers

Midbrain – GTPγS coupling after μ and κ opioid receptor stimulations in human heroin abusers

To see whether there are disturbances in the opioid system in human heroin abusers within the substantia nigra and the VTA, we performed [^{35}S]GTPγS autoradiography to detect the basal and opioid receptor stimulated levels of G-protein coupling in the midbrain (Fig.29).

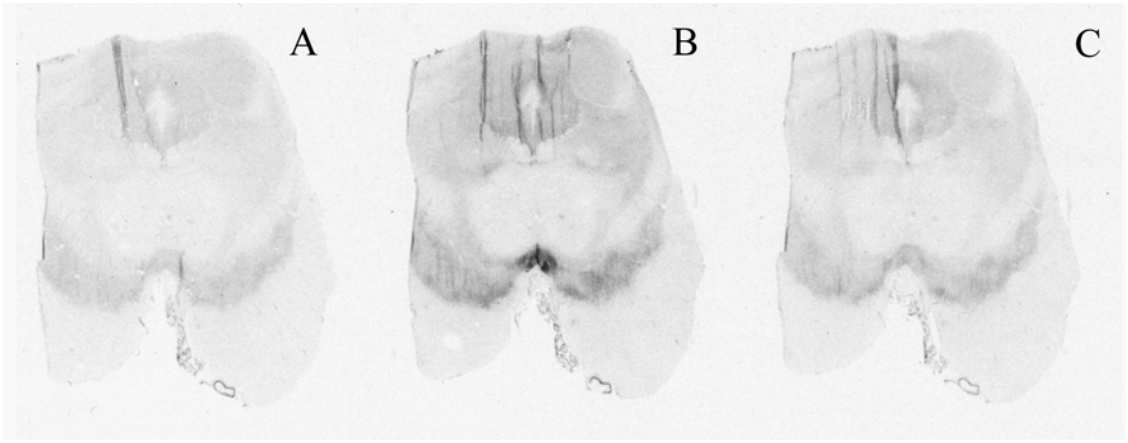


Figure 29. Representative film autoradiograms of coronal cryosections of the human midbrain showing the GTPγS coupling in basal (A), DAMGO stimulated (B) and U69-593 stimulated (C) sections of the human midbrain.

Basal levels of G-protein binding were significantly higher in the paranigral nucleus ($F_{2,26}$=13.266, p = 0.0062 covaried for pH) and periaqueductal gray (PAG: $F_{2,19}$=15.727, p = 0.047 covaried for pH) of heroin users with a trend effect in the parabrachial pigmental nucleus (p = 0.067, covaried for age) (Fig. 30).

DAMGO-stimulated [^{35}S]GTPγS binding revealed a strong significant induction of MOR coupling in the paranigral nucleus ($F_{1,30}$=11.9031, p = 0.0017) and in the PAG ($F_{1,19}$=29.4512, p <0.0001) in heroin users as compared to controls (Fig. 31). No other midbrain subregion studied was significantly altered.

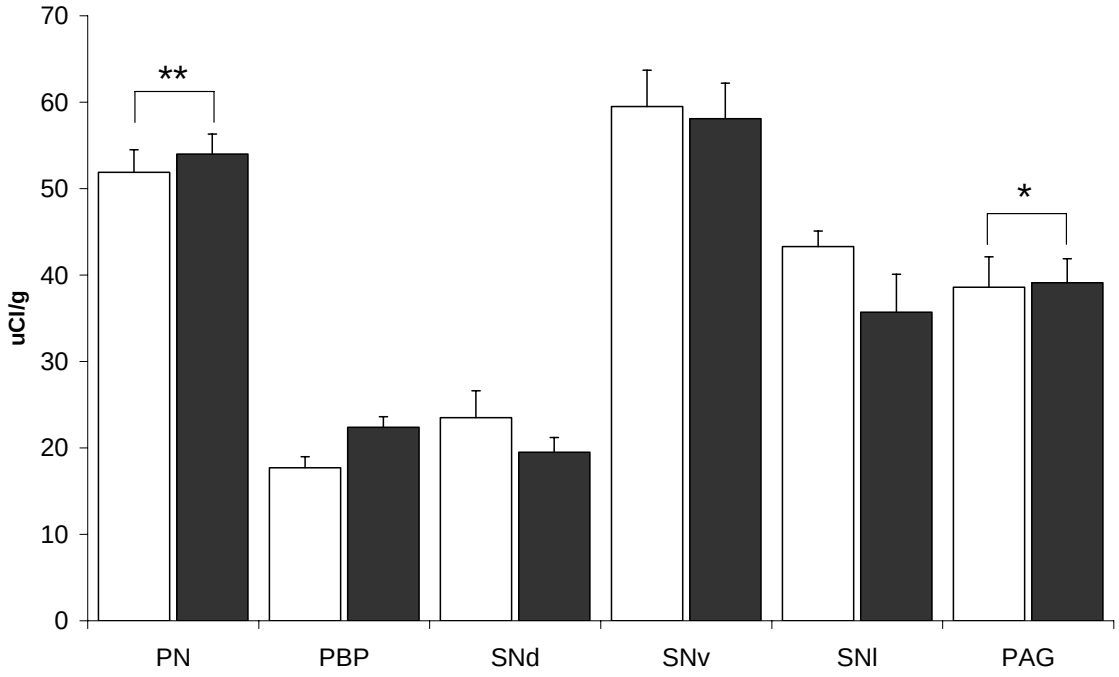


Figure 30. [^{35}S]GTPγS coupling (μCi/g) in the midbrain. Basal values in the absence of agonist stimulation; PN and PAG covaried for pH in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

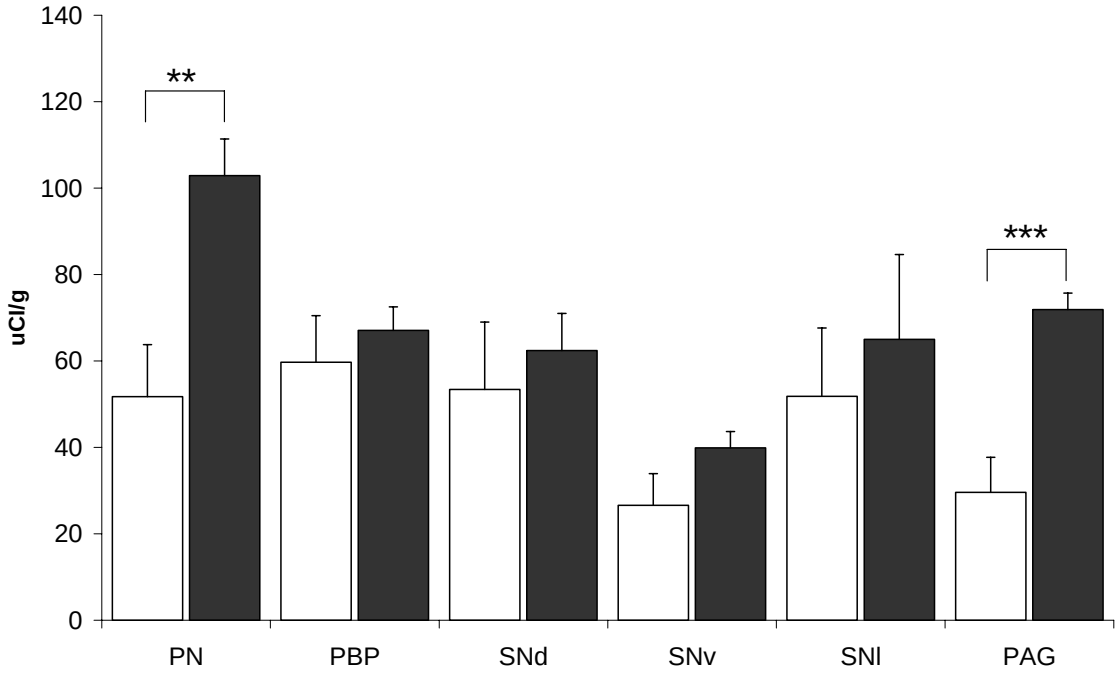


Figure 31. [^{35}S]GTPγS coupling (μCi/g) in the midbrain in the presence of DAMGO in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001.

U69-593-stimulated [^{35}S]GTPγS binding was performed to verify the efficiency ofcoupling of the KOR to G-protein in subpopulations of midbrain nuclei. [^{35}S]GTPγS-binding by U69-593 showed significant induction of MOR coupling in the paranigral nucleus ($F_{1,27}$ = 8.9853, p = 0.0059) and in the PAG ($F_{3,19}$=5.277, p = 0.03789, covaried for EtOH and age) in heroin users as compared to controls (Fig.32).
Morphine or codeine concentrations in blood or urine had no significant effect on GTPγS coupling in either of the experiments.

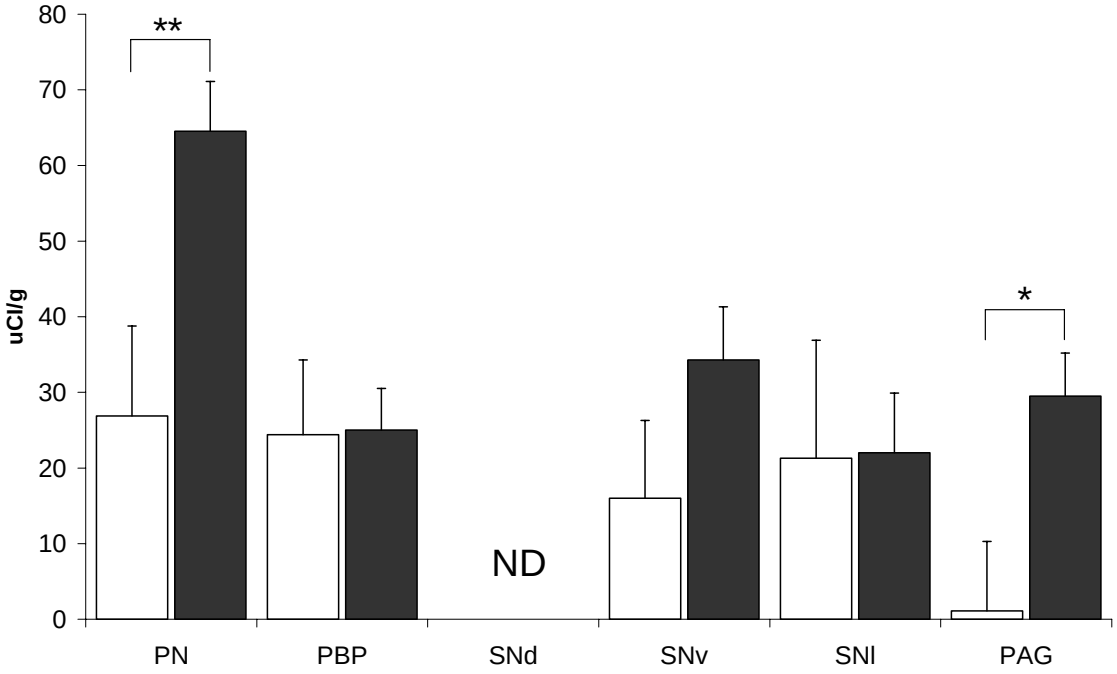


Figure 32. [^{35}S]GTPγS coupling (μCi/g) in the midbrain in the presence of U69-593 in control (white) and heroin (black) groups. * p < 0.05; ** p < 0.01; *** p < 0.001. In PAG covaried for EtOH and age.

Striatum

Opioid neuropeptide mRNA levels in the striatum in human heroin abusers

Striatal output neurons differentially express opioid neuropeptides prodynorphin and proenkephalin. Here we examine the disturbances in the striatal endogenous opioid neuropeptide system both on the gene expression (mRNA) and protein levels.

Preproenkephalin (PENK) mRNA expression

In normal subjects, PENK mRNA expression was highly abundant in the striatum with highest levels detected in the dorsal subregions (Fig. 20 C,D). Heroin subjects, as compared with controls, had reduced PENK mRNA levels throughout the dorsal and ventral striatum (Fig. 33).

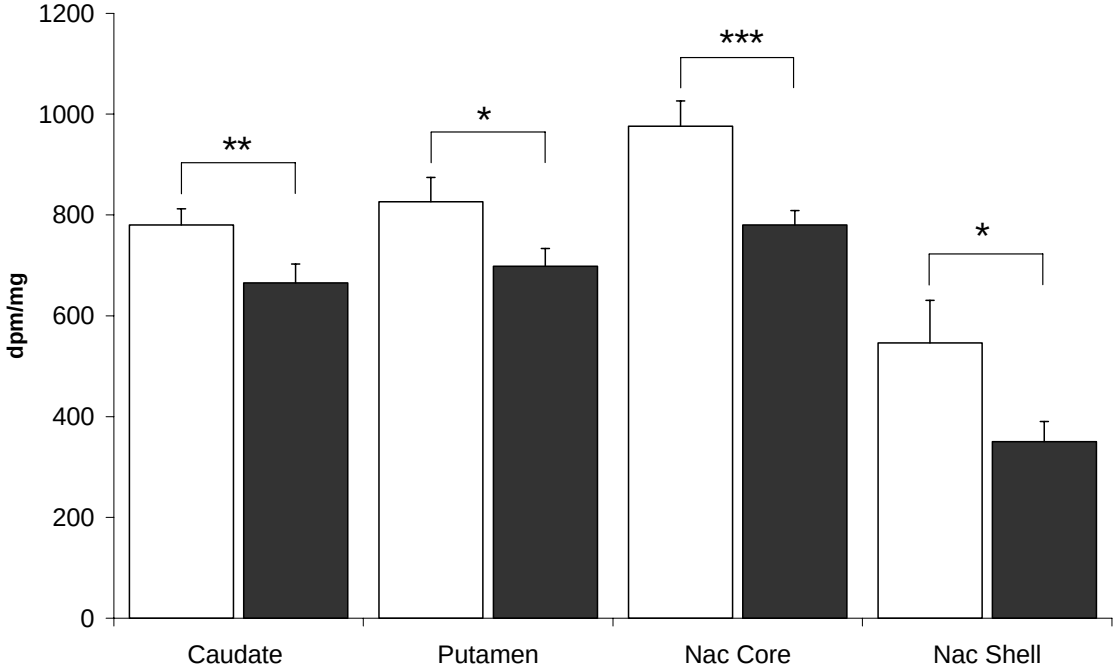


Figure 33. PENK mRNA expression in striatal regions of control (white) and heroin (black) subjects. Values are expressed as DPM per milligram (mean ± SEM). * p < 0.05; ** p < 0.01; *** p < 0.001.

mRNA levels were significantly lower in the caudate nucleus ($F_{2,46}$ = 5.678; P = 0.003, covariate age), putamen ($F_{3,46}$ = 5.602; P = 0.036, covariates age, pH), NAc core ($F_{1,39}$ = 13,418; P = 0.0007), and shell ($F_{1,39}$ = 4.445; P = 0.042). Age was negatively and pH positively correlated with mRNA expression levels; however, there was no significant age or pH x group interaction.

Preprodynorphin (PDYN) mRNA expression

The pattern of PDYN mRNA expression was most abundant in the limbic-related regions of the striatum, namely the NAc, in particular the shell subregion (Fig. 5), as well as the patch (striosome) compartment in the putamen and caudate nucleus(148). As compared with controls, heroin subjects had lower PDYN mRNA levels in the caudate nucleus (motor region, $F_{2,43}$ = 9.725, P = 0.0005, covariate age; associative region, $F_{2,43}$ = 9.425, P = 0.002, covariate age), putamen (motor region, $F_{1,45}$ = 9.075 P = 0.004; associative region, $F_{2,39}$ = 5.195; P = 0.067, covariate sex), and NAc core ($F_{1,36}$ = 6.010; P = 0.019). (Fig.34)

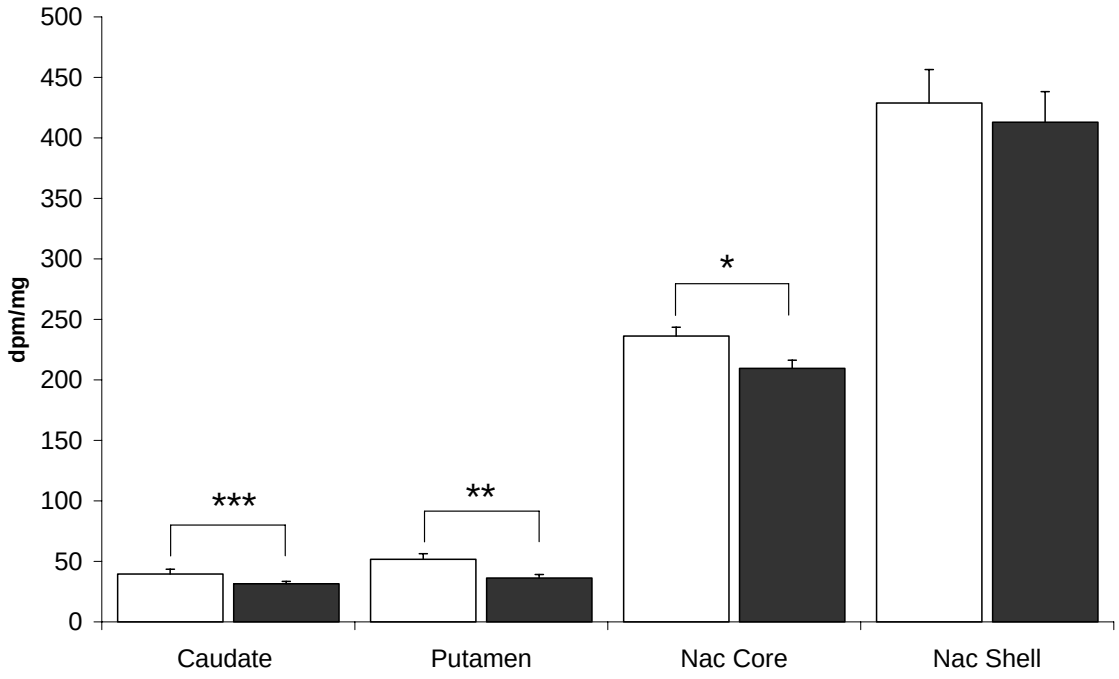


Figure 34. PDYN mRNA expression in striatal regions of control (white) and heroin (black) subjects. Values are expressed as DPM per milligram (mean ± SEM). * p < 0.05; ** p < 0.01; *** p < 0.001.

There was no PDYN mRNA alteration in the NAc shell. Assessment of the PDYN mRNA levels expressed in the patch and matrix compartments of the dorsal striatum revealed the same direction of change as that observed in the total area (data not shown). Age was negatively correlated to the PDYN expression levels, and there were no age x group interactions.

Opioid neuropeptide levels in the striatum in human heroin abusers: Met-enkephalin-Arg6-Phe7 (MEAP), dynorphin A, dynorphin B and Arg-Leu enkephalin.

To assess the relationship between gene expression and their peptide products, radioimmunoassay was used to measure opioid peptide levels in tissue taken from the caudate nucleus. Met-enkephalin-Arg6-Phe7 (MEAP) is specifically derived from the PENK gene. Despite significant alterations in PENK mRNA expression, there was no significant difference in MEAP peptide levels between all heroin and control subjects.

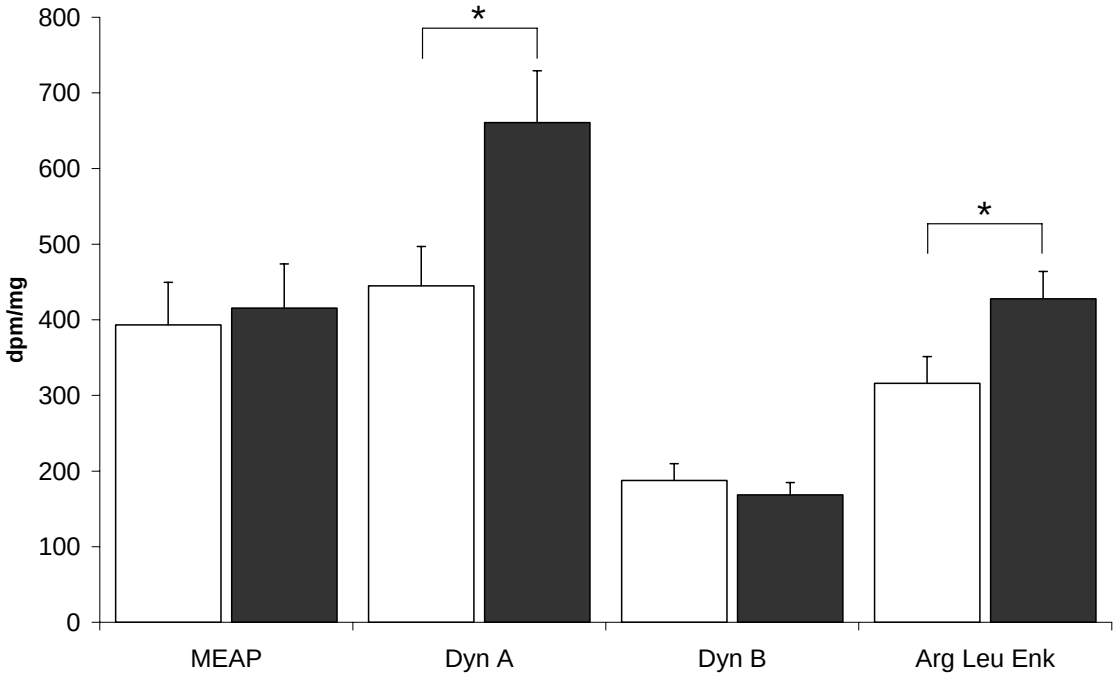


Figure 35. Enkephalin and dynorphin peptides levels in the caudate nucleus of control (white) and heroin (black) subjects. Values are expressed as fmol/mg protein (mean ± SEM).

Dynorphin A, dynorphin B, and Arg-Leu enkephalin are derived from the PDYN gene. Dynorphin A ($F_{1,37}$ = 6.903; P = 0.012) and Arg-Leu enkephalin ($F_{1,39}$ = 4.362; P = 0.043) were significantly increased in heroin subjects (Fig. 35).

Correlation between opioid peptides and respective mRNAs

Correlation analyses were carried out between the opioid peptide and respective mRNAs. In consideration that mRNA expression measurements of the caudate nucleus could be compromised because of the tissue taken from this region for peptide analysis, correlation analyses were performed with mRNA measurements obtained from the putamen, because this region was intact and representative of the dorsal striatum. Significant correlations were most evident in the control group. MEAP and PENK mRNA had a weak positive correlation of r = 0.550 (P = 0.051). Of the dynorphin-related peptides, significant correlations were apparent only for dynorphin B (motor region, r = 0.744, P = 0.006; associative region, r = 0.783, P = 0.003).

Genes related to intracellular processing in the striatum of heroin abusers

Prohormone convertase-2 (PC2) mRNA expression

One of the rate-limiting components of opioid peptide processing is the prohormone convertase-2 (PC2)(149,150). The mRNA levels of proPC2 were significantly increased ($F_{1,31}$ = 4.163, P = 0.050) in the NAc shell of the heroin group (Fig. 36). In contrast to the NAc shell, proPC2 mRNA levels were decreased in the putamen of the heroin subjects ($F_{2,42}$ = 13.749, P < 0.0001). There was no difference in proPC2 expression between control and heroin groups in the caudate nucleus and NAc core. Toxicological evaluation revealed that proPC2 mRNA expression had a strong positive correlation with 6-monoacetylmorphine (caudate nucleus, r = 0.933, P = 0.007; putamen, r = 0.852, P = 0.031).

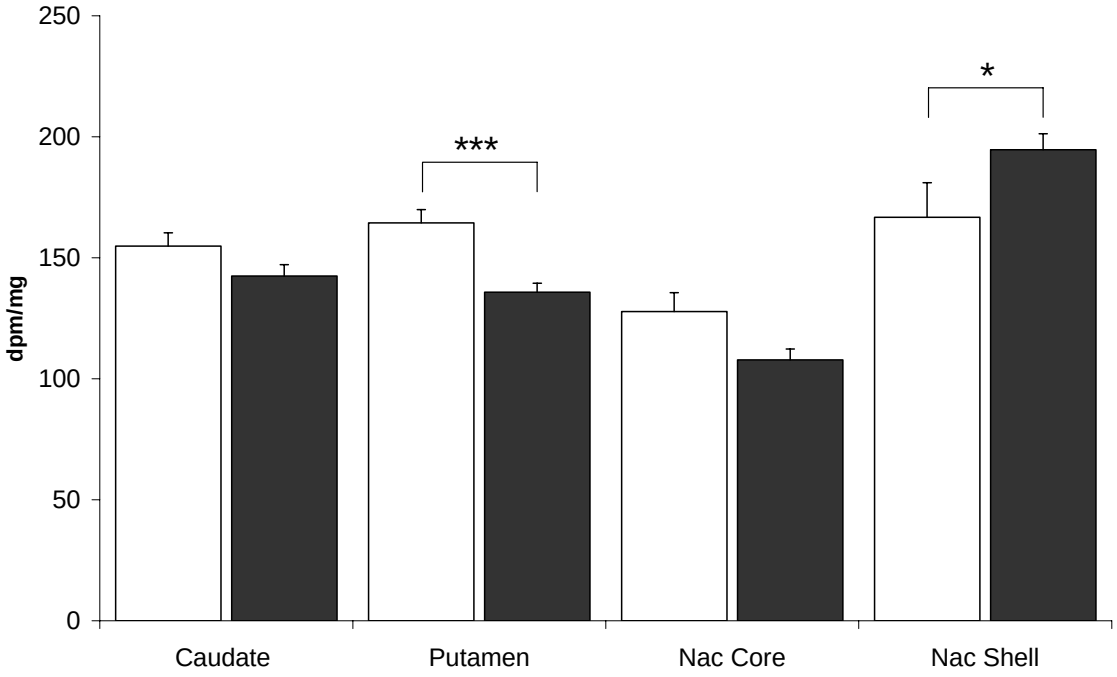


Figure 36. PC2 mRNA expression in striatal regions of control (white) and heroin (black) subjects. Values are expressed as DPM per milligram (mean ± SEM). * p < 0.05; ** p < 0.01; *** p < 0.001.

Pro-RCC1 domain protein (HERC1) mRNA expression

Agonist regulation of the MOR has been shown to be mediated via the ubiquitin/proteosomal system (151). E_3 ubiquitin ligases are critical enzymes in ubiquitination, and a pilot gene screening experiment showed a homologous to E6-AP C terminus (HECT) type of E_3 ubiquitin ligase HECT and RCC1 domain protein (HERC1), to be relevant to heroin exposure (our unpublished data). A significant group effect was detected for proHERC1 mRNA levels expressed in the caudate nucleus ($F_{1,43}$ = 4.356; P = 0.043) and putamen ($F_{1,43}$ = 7.659, P = 0.008; Fig. 37).

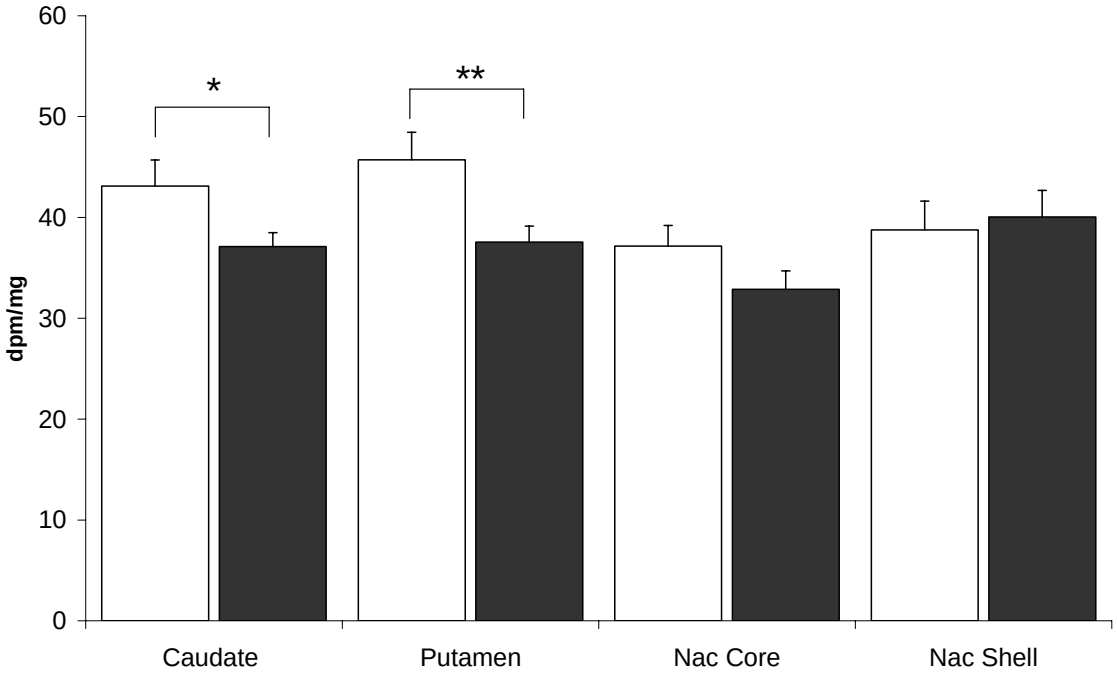


Figure 37. Pro-HERC mRNA expression in striatal regions of control (white) and heroin (black) subjects. Values are expressed as DPM per milligram (mean ± SEM). * p < 0.05; ** p < 0.01; *** p < 0.001.

A118G SNP OPRM1 genotype in association with heroin use.

In our main population of 65 subjects (Table 1, Methods section), the 118G allele was found to be in Hardy–Weinberg equilibrium. The finding of an overall allelic frequency of 11.5% for the G allele (Table 6) is in line with previous reports of 10–14% in populations of European descent(109,112,152,153). Subjects homozygous for the 118G are termed G/G, heterozygous subjects are termed A/G, and homozygous A118 subjects are termed A/A. There was an overall effect of genotype (likelihood ratio $\chi^2_{(1)} = 6.238$; $p = 0.044$). The A/G genotype was significantly more frequent in the heroin than in the control group $\chi^2_{(1)} = 6.153$, $p = 0.013$). 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 (Table 6). Thus, 91% of the A/G genotype individuals were heroin subjects. Only one control and one heroin subject were homozygous 118G carriers. To further substantiate the OPRM1 finding, an additional 53 subjects (14 controls and 39 heroin subjects) were genotyped, and the results of the total population corroborated the first

findings. The frequency of the A/G genotype was significantly higher in the heroin group ($\chi^2_{(1)} = 4.741$, p = 0.030), and 88% of all A/G genotype individuals were heroin subjects.

Table 6. Distribution of A118G single-nucleotide polymorphism (SNP) of the OPRM1 gene. Number (frequency).

	Controls (*n* = 26)	**Heroin (*n* = 39)**
Genotype		
A/A	24 (0.923)	28 (0.718)
A/G	1 (0.038)	10 (0.256)
G/G	1 (0.038)	1 (0.026)
Allele	(*n* = 52)	(*n* = 78)
A	49 (0.942)	66 (0.846)
G	3 (0.058)	12 (0.154)

OPMR1 genotype in relation to heroin-related effects on opioid neuropeptide mRNA expression.

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. The single heroin subject with a G/G genotype was included in the heroin A/G subgroup.

There was a significant influence of genotype on mRNA expression. Post hoc analysis revealed that PENK mRNA levels in the putamen (p = 0.008) and NAc shell (p = 0.045) of heroin users were decreased 20.4% and 41.4%, respectively, in subjects carrying the G allele as compared with the A/A subjects (Fig. 38). 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 (p = 0.024; Fig. 39).

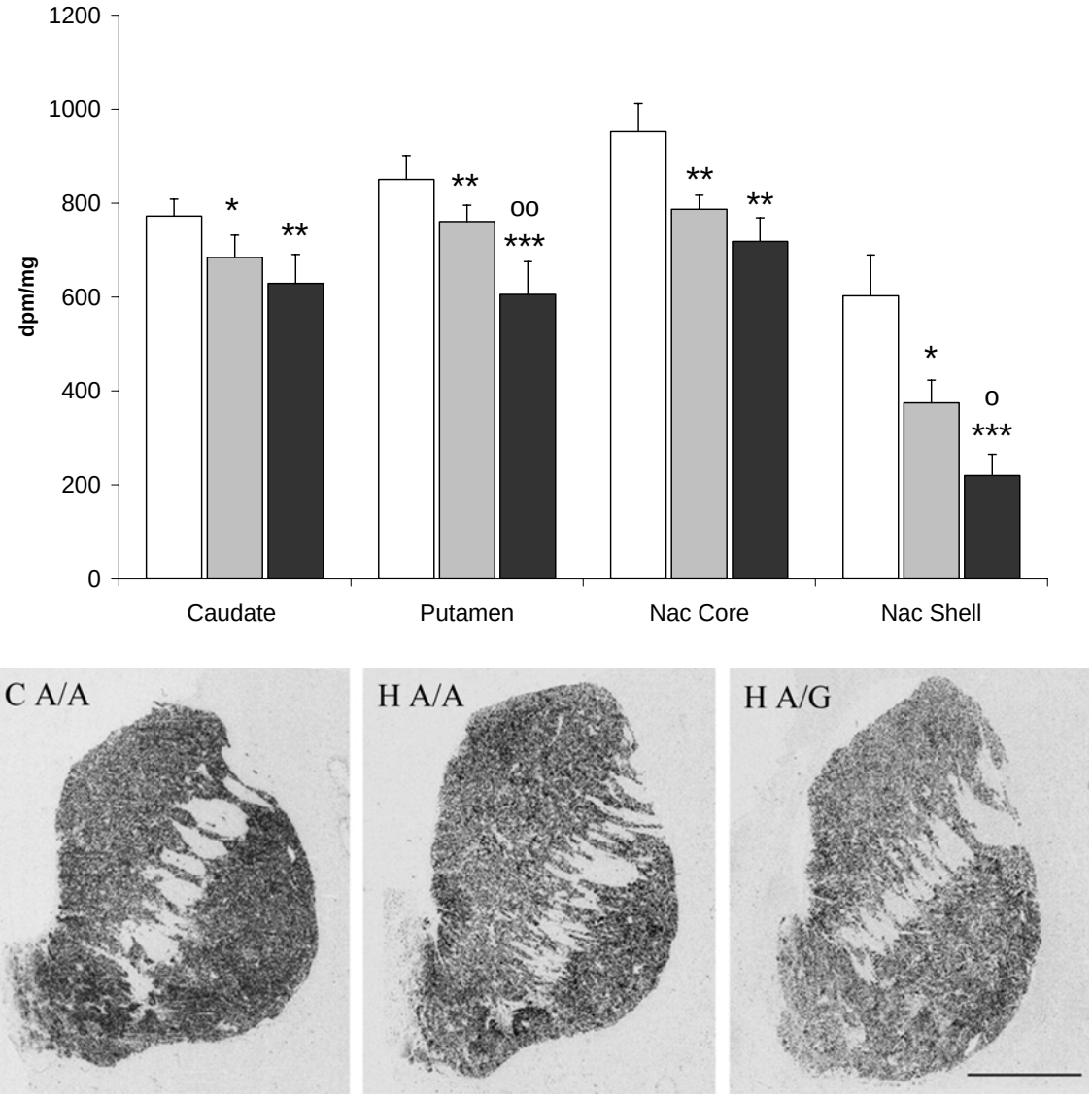


Fig 38. PENK mRNA expression in striatal regions of control and heroin subjects in connection to A118G OPMR1 polymorphism. White – control A/A, grey – heroin A/A, black – heroin A/G. * - difference from control; o – difference between heroin A/A and heroin A/G genotypes. *, o p < 0.05; **, oo p < 0.01; *, ooo p < 0.001. The lower part of the figure: representative autoradiograms of coronal cryosections hybridized with PENK antisense riboprobe. (Scale bar, 1 cm.)**

Toxicological measures of heroin metabolites in the urine and blood in the heroin subjects were analyzed for correlation to opioid gene expression. Urine and blood morphine levels were 1.75 ± 0.44 and 0.39 ± 0.08 μg/ml, respectively, with no significant difference between the A/G and A/A groups. A significant positive correlation was evident between PENK mRNA levels and urine morphine in the NAc (shell, $r = 0.652$, $p = 0.022$; core, $r = 0.881$, $p = 0.009$).

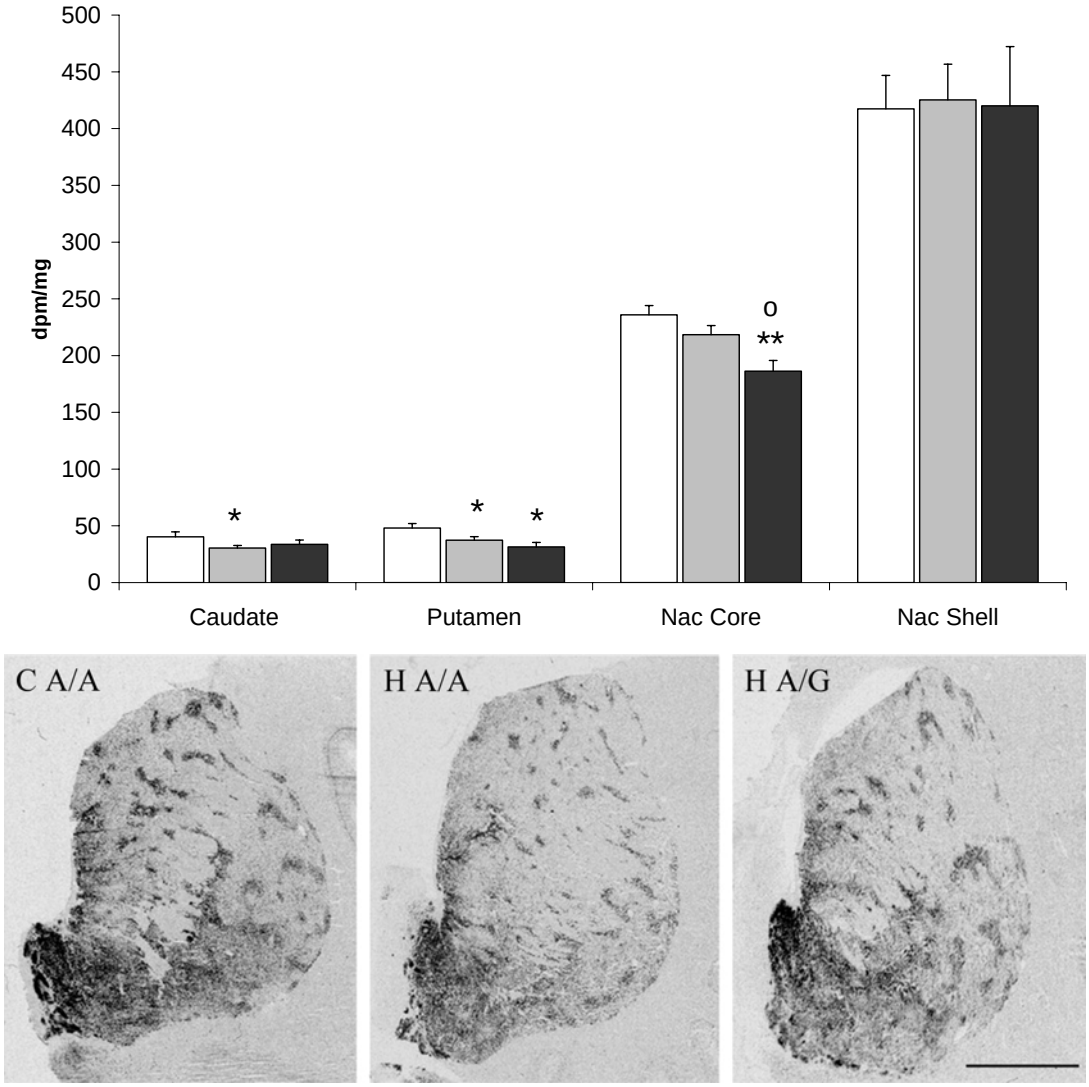


Figure 39. PDYN mRNA expression in striatal regions of control and heroin subjects in connection to A118G OPMR1 polymorphism. White – control A/A, grey – heroin A/A, black – heroin A/G. * - difference from control; o – difference between heroin A/A and heroin A/G genotypes. *, o p < 0.05; **, oo p < 0.01; *, ooo p < 0.001. The lower part of the figure: representative autoradiograms of coronal cryosections hybridized with PDYN antisense riboprobe. (Scale bar, 1 cm.)**

Urine morphine concentrations were negatively correlated with PDYN mRNA expression in the putamen (associative region, r = –0.631, p = 0.028; motor region, r = –0.569, p = 0.042). The significant correlations between toxicology and PENK and PDYN were contributed by the A/A subjects. The morphine/codeine concentration ratio was 16.2 ± 7.6 and 3.6 ± 1.2 μg/ml in the urine and blood, respectively; >1 morphine/codeine concentration ratio normally indicates heroin usage rather than other medication with codeine. Only a few subjects had positive toxicology for 6-

monoacetylmorphine, the rapid metabolite of heroin, and no significant correlation was found with the opioid neuropeptide mRNA expression levels.

OPMR1 genotype in relation to heroin-related effects on opioid neuropeptide levels.

MEAP: There were significant group effects in consideration of genotype ($F_{2,39}$ = 9.588, p = 0.0012; covariate age). Heroin subjects with A/G genotype had 2.3-fold higher levels of MEAP than heroin A/A individuals (p = 0.0002). Heroin subjects with A/G genotype had ≈2-fold higher levels of dynorphin A (heroin A/G vs. heroin A/A, p = 0.0002; heroin A/G vs. control, p = 0.0001) and Arg-Leu enkephalin (heroin A/G vs. heroin A/A; p = 0.0008, heroin A/G vs. control, p = 0.00005) than either A/A heroin or controls subjects. Heroin subjects with A/G genotype had significantly higher levels of dynorphin B than heroin A/A individuals (p = 0.001).

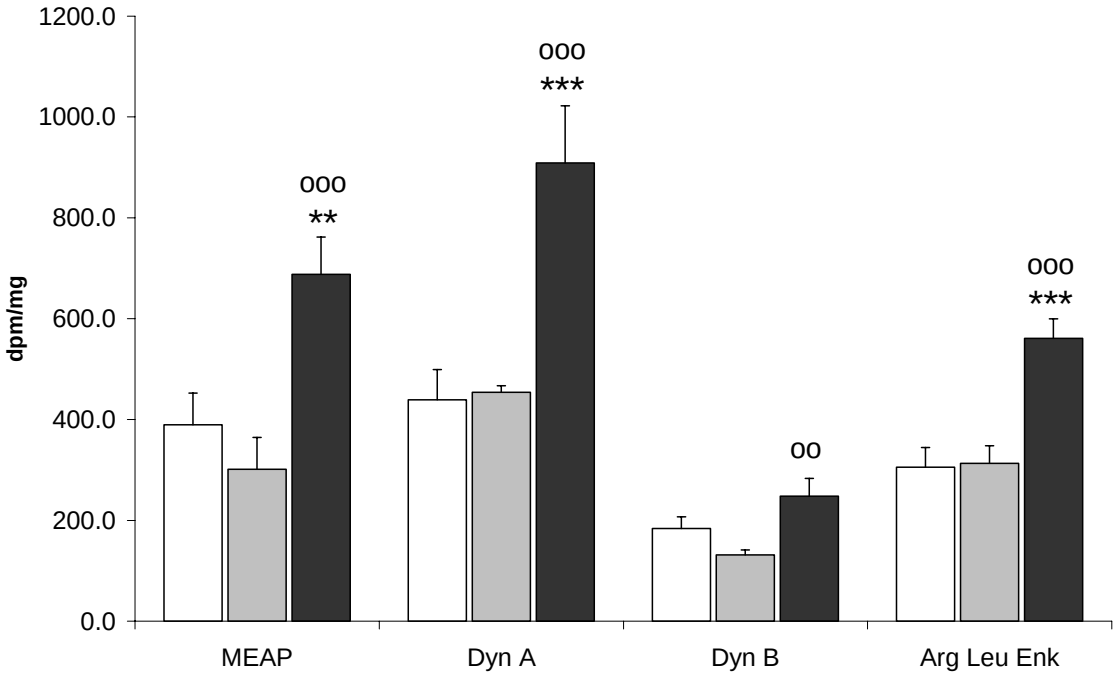


Figure 40. Enkephalin and dynorphin peptides levels in the caudate nucleus of control and heroin subjects. Values are expressed as fmol/mgprotein (mean± SEM). White – control A/A, grey – heroin A/A, black – heroin A/G. * - difference from control; o – difference between heroin A/A and heroin A/G genotypes. *, o p < 0.05; **, oo p < 0.01; *, ooo p < 0.001.**

There was a trend for increased dynorphin B levels in heroin subjects with the G allele vs. controls ($p = 0.061$), whereas the heroin subjects of genotype A/A showed a trend for decreased levels ($p = 0.070$) as compared with the control group. (Fig 40.) Peptide levels had no significant correlation to morphine or ethanol toxicology.

Genes related to intracellular processing in relation to heroin use and OPMR1 polymorphism.

Post hoc analysis in relation to genotype revealed that the increase was due to higher expression levels in the heroin A/A subjects. ProPC2 mRNA expression had a relative homogenous distribution in the striatum (Fig. 41), with the exception of some control and heroin subjects with strongly labeled cell clusters in the NAc and high-expressing patches in the putamen. In contrast to the NAc shell, proPC2 mRNA levels were decreased in the putamen of the heroin subjects ($F_{2,42} = 13.749$, $p < 0.0001$), and there was a trend for a stronger reduction in the A/G heroin subgroup as compared with the A/A heroin individuals ($p = 0.061$). Toxicological evaluation revealed that proPC2 mRNA expression had a strong positive correlation with 6-monoacetylmorphine (caudate nucleus, $r = 0.933$, $p = 0.007$; putamen, $r = 0.852$, $p = 0.031$). (Fig 41)

ProHERC1 mRNA had a homogenous expression throughout the human striatum (Fig. 42). ProHERC1 levels in the putamen were decreased to a greater extent in the A/G heroin subgroup as compared with the A/A heroin subjects ($p = 0.016$). Significantly lower HERC1 levels were also detected in the NAc shell of the heroin A/G subgroup as compared with the heroin A/A individuals ($p = 0.005$). Correlation analyses revealed that significant association between proHERC1 mRNA expression and blood morphine was predominantly contributed by the A/G subjects (caudate nucleus, $r = –0.99$, $p < 0.0001$; NAc shell, $r = –0.900$, $p = 0.037$). (Fig. 42)

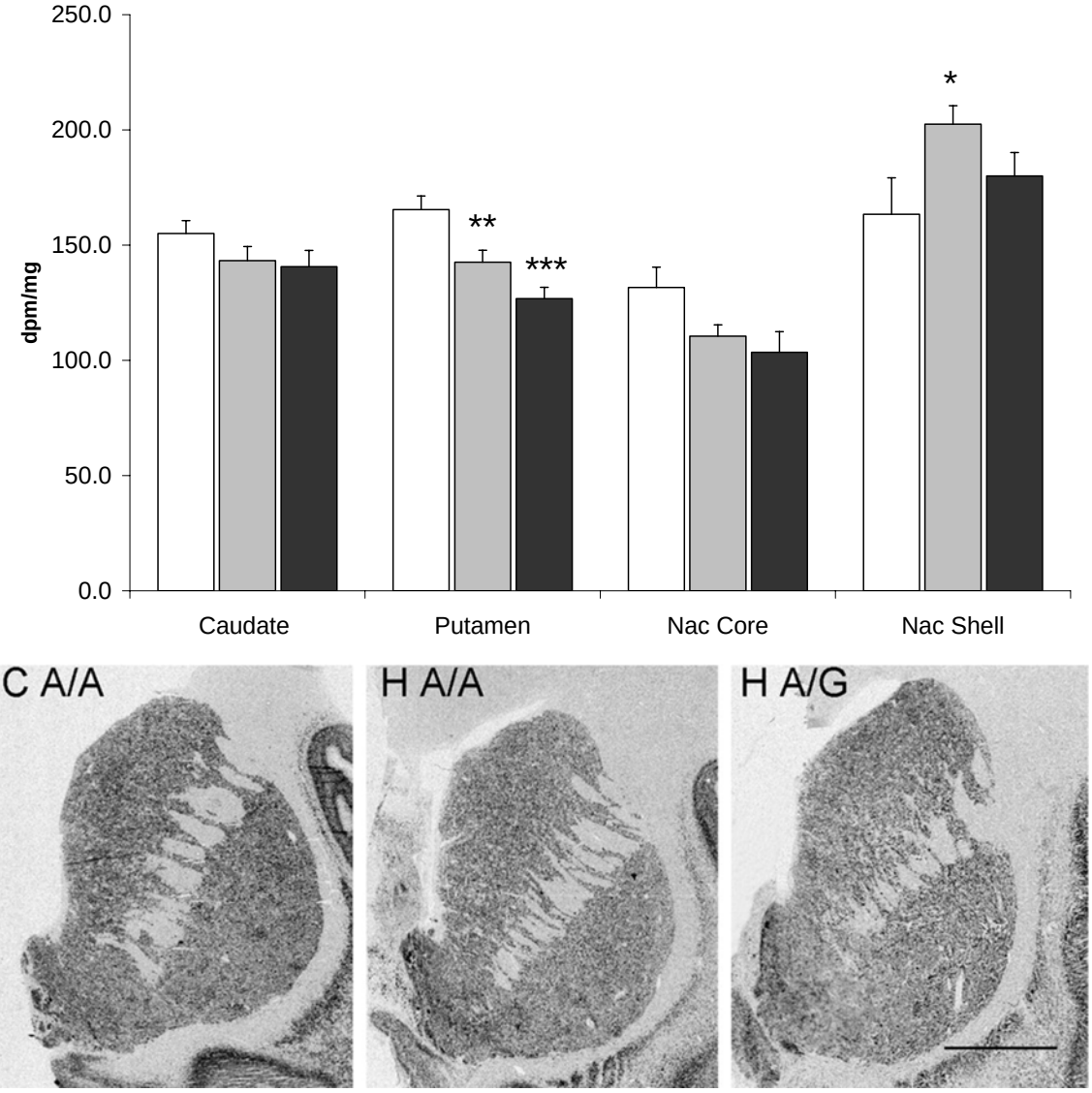


Figure 41. ProPC2 mRNA expression in striatal regions of control and heroin subjects in connection to A118G OPMR1 polymorphism. White – control A/A, grey – heroin A/A, black – heroin A/G. * - difference from control; o – difference between heroin A/A and heroin A/G genotypes. *, o p < 0.05; **, oo p < 0.01; *, ooo p < 0.001. The lower part of the figure: representative autoradiograms of coronal cryosections hybridized with PC2 antisense riboprobe. (Scale bar, 1 cm.)**

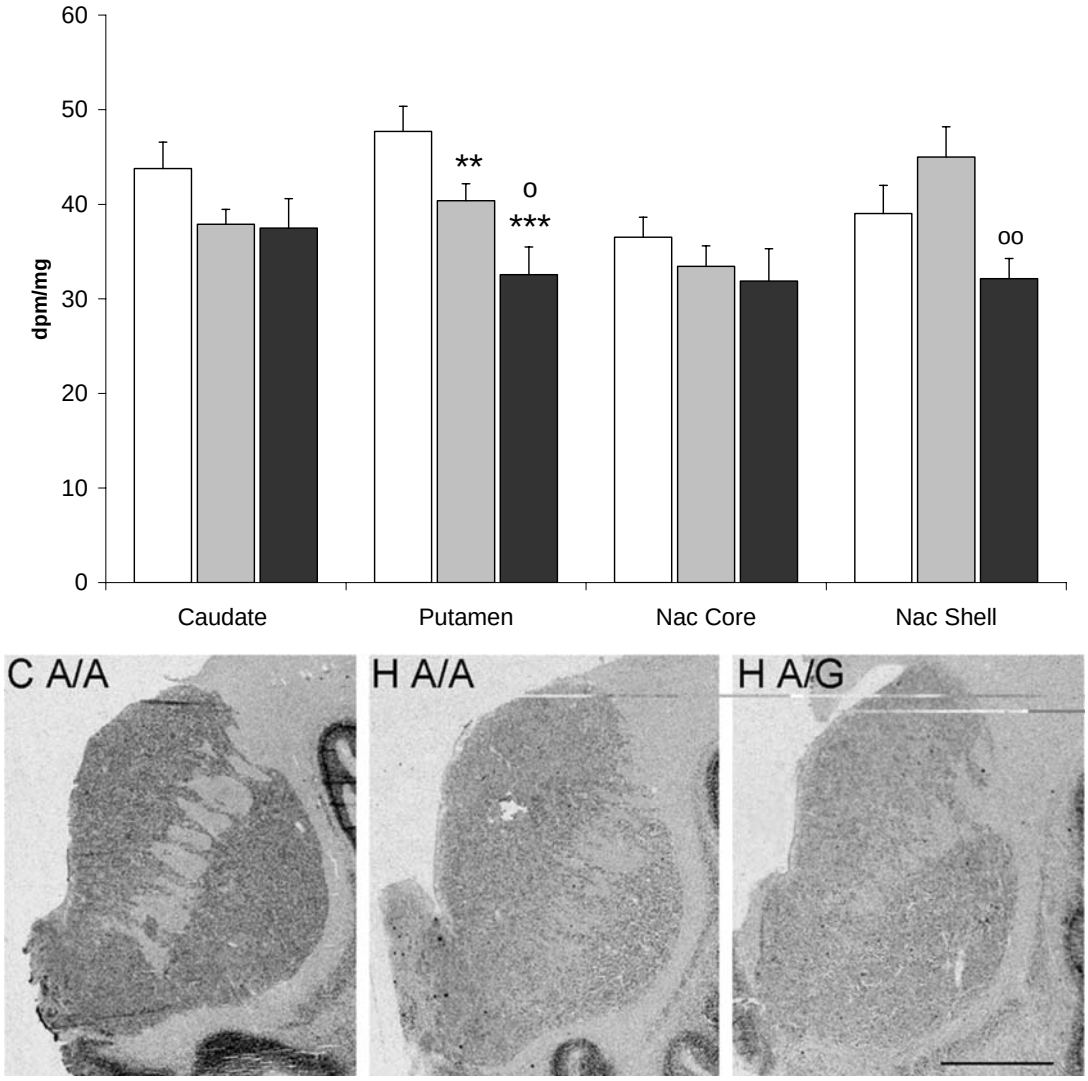


Figure 42. Pro-HERC mRNA expression in striatal regions of control and heroin subjects in connection to A118G OPMR1 polymorphism. White – control A/A, grey – heroin A/A, black – heroin A/G. * - difference from control; o – difference between heroin A/A and heroin A/G genotypes. *, o $p < 0.05$; **, oo $p < 0.01$; *, ooo $p < 0.001$. The lower part of the figure: representative autoradiograms of coronal cryosections hybridized with Pro-HERC antisense riboprobe. (Scale bar, 1 cm.)**

DISCUSSION

Patterns of drug-related death in Budapest, Hungary between 1994-2006

When analyzing epidemiological issues, it is usually preferable to use rates, which make our results comparable with results of other researchers describing different populations, cities, countries etc. For this reason I decided to present some already shown data (Fig. 6) about drug-related death cases as mortality rates, using the census data from the Hungarian Central Statistical Office about the number of citizens in Budapest (144) (Fig. 43).

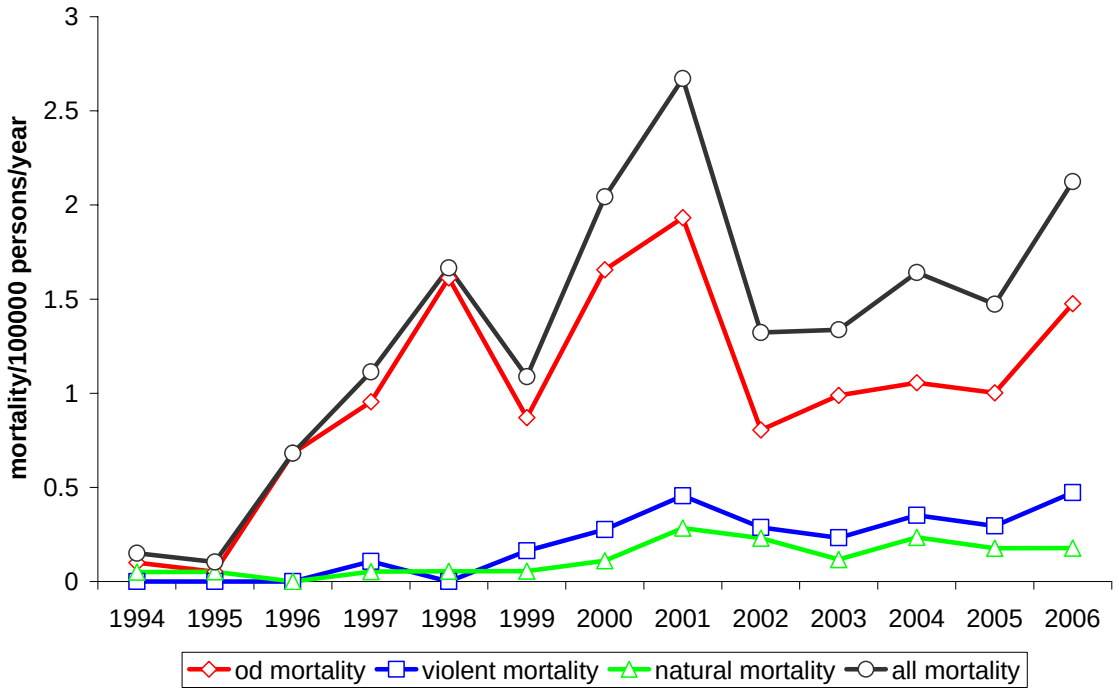


Figure 43. Mortality rates of drug-related death cases in Budapest, Hungary, between 1994-2006

In comparison to data from other countries (see Table 7.) the above presented numbers are within the lower ranges, but there are some interesting trends which are important to discuss. There has been a constant increase in DRD cases in Budapest with a sudden decrease in 2002. A similar trend has been reported from other European countries as

well (154). The years afterwards from 2002-2005 showed stagnation but alarmingly in 2006 the deaths caused by drugs of abuse seem to be again on the rise. The peak of DRDs in years 2000-2001 coincide with the beginning of our project aimed at understanding and analyzing the DRD, when we started to pay a very special attention to all death cases where drug use could have been possible. This might have also elevated the number of DRDs recorded in year 2000 in comparison to 1999. It is also from year 2000, when we can see an elevation in a number of violent and natural deaths of known drug abusers – most probably it is not a real increase, only before the beginning of our project these cases were coded according to their main cause of death (for example suicide or sudden cardiac death) and even though the fact that some might have been illicit drug abuser was noted in the autopsy report, it is practically impossible to identify these cases now. Most probably it is also the case for the DRDs during years 1994-1995, when forensic doctors were only getting familiar with the deaths caused by drugs of abuse.

Table 7. Mortality rates for opiate overdoses

Source	Region	Time span	Mortality rate/100000
Coffin et al. 2003(155)	New York, USA	1990-1998	5.06 – 10.09, maximum in 1993
Preti et al. 2002(44)	Italy	1984-2000	1 – 6 maximum in 1996
Degenhardt et al. 2005(156)	Australia	2001	3.46 in a population between 14-54 years of age
Fernandez et al. 2006(157)	Massachusetts, USA	1990-2003	1.4-8.8, maximum in 2003

Analyzing some other features of the examined population it can be stated that it resembles the populations characterized by other authors: the majority of the deceased are males (43,44,56,155), the majority of cases caused by intravenous heroin overdoses. However, the presented population has an age range generally between 20-30 years of age, which is a lower age span then the ones reported by others (43,155). Nevertheless, there is an interesting temporal shift (see Fig.8) that might at the end lead to age ranges similar to those in the international literature. At the beginning a large part of the DRD cases happened to very young people less than 20 years of age. Since then one can

observe a gradual decrease in the number of deceased below 20 years of age with a practically same amount of victims between 20-30 years of age and an increasing number of demised above 30 years of age. This trend might be a representation of the fact that fewer very young people started using drugs in the past few years. Another difference between international trends and our results is the history of drug use; according to some reports usually long-term (10 years of usage), dependent heroin users are at a greatest risk of the overdose deaths (56), while in our population the mean time of drug use was approximately 4 years. It has to be stated though that only for a small part of the studied population we had available data about the drug-abuse history (25%), and as illicit drug use in Hungary is a relatively new phenomenon there can be no long-term users yet. The fact, that police or family reports have not sufficient information about the history and patterns of drug use by the deceased can be in the future overcome in a several ways: firstly, we have already started hair collection and segmental hair analysis which can give us information about the drug-using habits of the decedents depending on their hair length (158,159). An another approach will hopefully be available through the Hungarian office of the EMCDDA, where in an online database information about drug addict patients in treatments and deceased drug abusers would converge – this would not give the coverage of all DRDs, but it would be more than what we have today. A third possibility is the analysis of bile morphine concentration; a bile morphine level greater than 40 μg/ml is said to be an indicator of chronic heroin usage according to one report (160).

When analyzing the drugs of abuse causing death between 1994 and 2006 (see Fig. 7) one sees that until 2001 there was practically only heroin related death registered in Budapest. In the more recent years the larger availability of stimulants (amphetamine, MDMA, cocaine) (161) resulted in a situation, when also other drugs appeared within our statistics. This was even enhanced by a new and dangerous trend among the youngest population who inhale the gas from lighters (butane). This is an alarming situation, as these substances are very cheap, easily accessible and can cause severe cardiac arrhythmias which very often lead to death.

Another difficulty encountered during the analysis of the toxicological data were the cases when the toxicology results were negative despite the known history of drug abuse, positive toxicology for substance found at the site of death and typical

circumstances and paraphernalia found at death. Most of these cases were in the state of far-gone decomposition, and most probably this was the reason for the consequent negative results. Since then, following foreign examples (162,163), in the cases of far-gone decomposition, in the absence of blood and urine, we secure the maggots found on the body. We have started this procedure in 2006, and we already have two cases where toxicology analyses proved to be positive for morphine.

The prevalence of infectious diseases within the examined population, although not very grave are nonetheless alarming. Our results show a much better situation then reports from surrounding countries, especially in the case of HIV (154). It is also interesting that the only person with HIV infection had also a previous HBV infection – hepatitis and HIV very often appear as co-morbidity in a number of studies describing intravenous drug users (164,165). Our data also show a lower prevalence of hepatitis B and C, in comparison to data from Western Europe (166-168) or USA (169,170) which was most probably caused by a shorter mean time of intravenous drug use in Hungary then in other countries. The 7% prevalence of syphilis seems to be a rather high number in comparison to other international studies reporting 0-5.7% of this sexually transmitted disease within drug using populations (171-173).

Infection with the HIV still remains a potential threat, and the presence of hepatitis B and especially C indicates that preventive measures are still necessary. The relatively high prevalence of syphilis is a serious problem, which should be addressed as soon as possible.

Neurobiology of heroin abuse

Impact of brain pH on molecular studies performed with brain tissue from human heroin abusers

One of the biggest challenges in our search for more information and knowledge about the neurobiology of heroin overdose deaths was the sparseness of information about certain factors, which might influence the mRNA expression, protein levels or G-protein activity within the post-mortem human brain. When extremely big differences in mRNA expression within the same group of subjects (heroin group, see Fig. 19. A, B)

were encountered it became obvious, that there must be another factor, next to demographic or toxicological circumstances that had to be responsible for the huge discrepancies. In our investigation we showed, that especially the heroin group had variable brain pH levels associated with different acute agonal states that in effect significantly impacted mRNA levels that are central to molecular postmortem studies of opiate abuse. We have also defined a number of agonal states that could influence the brain pH, thus we included them all under a term used first by Kingsbury et al – respiratory distress (174). There were some previously described long-term chronic conditions included in the respiratory distress group such as pneumonia or sepsis have already been accepted as factors contributing to hypoxia and thus pH reduction (119,126,132). We could now also identify respiratory distress factors such as vomit inhalation, resuscitation, pulmonary embolism, and suffocation that affected brain pH. Vomit inhalation, or aspiration of the acidic content of the stomach, is a very common complication when the coughing reflexes are diminished, as is the case in opiate overdose (175). It is also documented that morphine, next to its analgesic and euphoric features, has a pro-emetic quality that can induce vomit inhalation. According to forensic sources (176) a considerable number of heroin overdose subjects die of vomit inhalation which is consistent with the high percentage of respiratory distress detected in the current heroin population. In regards to the impact of artificial resuscitation on brain pH, it is well known that the ventilation and compression of the chest during this procedure is not entirely sufficient to ensure the proper oxygenation of the organs, and the initiation of anaerobic glycolysis under these conditions is likely to lead to acidosis (177). In the present study, the influence of resuscitation on brain pH was best evident in the suicide group which had a more homogenous cause of death; the only two suicide subjects with low pH values were attempted resuscitation cases. Pulmonary emboli, that blocks the pulmonary arteries, was also included in the respiratory distress category since it decreases the amount of blood being oxygenized in the lungs which can lead to hypoxia and further acidosis. In our study, cases of pulmonary embolism were recorded only in the control group. It was also not unexpected that conditions of suffocation contributed to lower brain pH as that clearly involves hypoxia leading to death. It is important though to distinguish suffocation (which involves a prolonged episode of hypoxia) from hanging where the mechanism of death may involve the compression of

the carotid arteries supplying the brain and the loss of consciousness in a matter of 5 to 10 seconds after which death usually occurs within 3 to 5 minutes (178). The fact that brain pH was very high in the suicide hanging cases confirms that rapid death was not a common feature of heroin overdose cases that were characterized by low pH.

A number of studies have already emphasized the importance of agonal state and brain pH on mRNA expression levels, primarily in long-term pathological cases (119,121,126,131,133). Our study confirmed that reduced mRNA expression contributes to low brain pH and not just due to chronic disease. Brain pH significantly reduced mRNA levels of drug abuse-related genes in heroin cases emphasizing the rapid impact of certain conditions around death on molecular events. Such events might contribute to some of the variability that is frequently evident in postmortem studies that do not relate to PMI or other demographic features of the subject. Of the drugs of abuse, heroin users may be most vulnerable to respiratory distress due to its effects to induce respiratory depression and emesis, thus attention needs to be taken when evaluating such cases in postmortem studies.

The alterations of the dopaminergic and opioid systems within the reward pathway of human heroin abusers

In our attempt to learn more knowledge about the neurobiology of heroin abuse we conducted a series of experiments within the regions known to be crucial for addiction – two major stations of the reward pathway, the ventral tegmental area and the ventral striatum (nucleus accumbens). To identify whether the alterations were limbic specific we also examined the motor and associative regions such as the substantia nigra and the dorsal striatum. Until now a relatively small number of reports were published in connection with research done on human post-mortem tissue in connection with heroin abuse and the markers of the dopamine or opiate systems (179-181). With our work we tried to fill in a gap in the general understanding of the neurobiology of human heroin abuse.

The major neurobiological alterations detected in the brains of human heroin abusers within the dopaminergic and opioid systems are found on Fig 44.

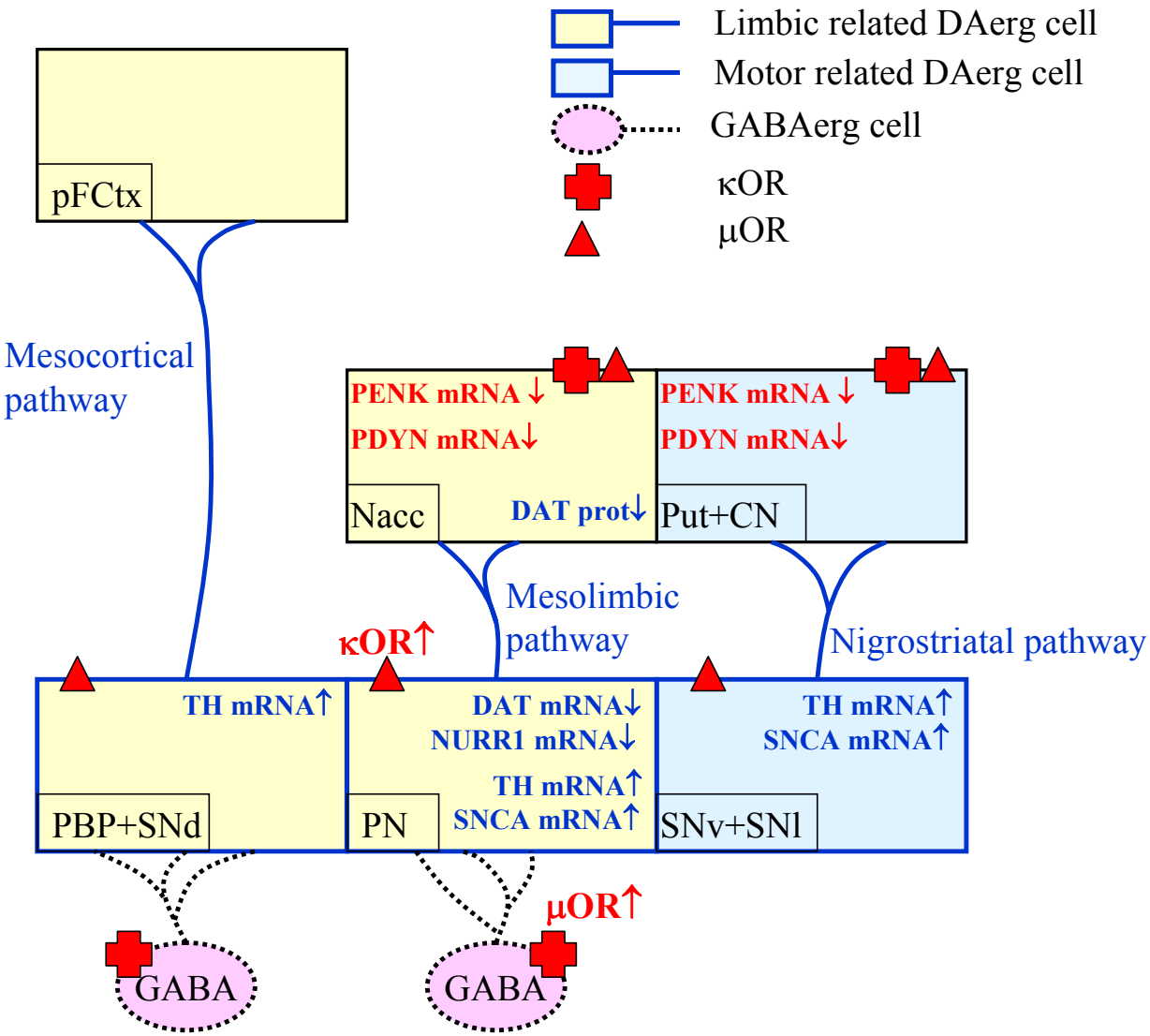


Figure 44. Schematic representation of the most important alteration found in neurobiological studies of human heroin abusers within the dopaminergic and opioid system.

Dopaminergic system

Our study provides direct evidence that discrete mesocorticolimbic DA neuronal populations are disturbed in association with chronic heroin use. Of the DA neuronal populations measured in the mesencephalon, the VTA PN subdivision was consistently altered for most of the dopaminergic markers examined – DAT, Nurr1, TH and α-synuclein. The distribution of mesocorticolimbic DA cells is segregated such that PN neurons predominantly comprise the mesolimbic pathway, whereas the PBP and SNd constitute the mesocortical circuit (182-184).

Another important fining of the study was the parallel decrease of DAT mRNA expression in the PN and protein levels in the projection terminals of the nucleus accumbens which might implicate a significant role of mesolimbic DAT in opiate abuse. In line with the specificity of DAT mRNA expression alterations observed in the brainstem, only the nucleus accumbens, both core and shell divisions, were significantly altered. Though there was a tendency for reduced levels in non-limbic areas, there were no significant alterations in the dorsal striatum for the DAT protein or for the corresponding mRNA expression levels in the SNv and SNl cell body regions.

Despite the strong link between impaired DA function and drug abuse, only one human postmortem study has evaluated the DA systems in heroin users (88). Kish et al. reported a non-significant trend for decreased DAT concentration in the nucleus accumbens of heroin users. A recent investigation with rhesus monkeys confirmed decreased DAT function after 12 days of morphine treatment that remained down-regulated during abstinence (185). Experimental rodent models have also shown that chronic, but not acute, morphine administration decreases DAT binding sites in the anterior basal forebrain, which included the nucleus accumbens, with no alterations in the dorsal striatum (89). However, using an eight-day morphine administration paradigm, DAT mRNA levels were reported to be unchanged in the midbrain of rats (186). The time course and chronicity of opiate effects on the DAT alterations are important considerations. In fact, the down-regulation currently observed in heroin abusers, both on the level of DAT gene expression and protein, appear to represent the chronic drug state rather than acute response to heroin use considering that there were no significant correlations between the DAT markers and morphine toxicology.

In contrast to reduced DAT, heroin use was associated with potentiated TH mRNA expression. These findings are compatible with experimental studies indicating that chronic but not acute morphine administration increases the levels of TH immunoreactivity. However, the morphine-induced alterations in the rodents were confined to the VTA (187), whereas heroin users in our population also had TH disturbances in the lateral division of the substantia nigra that innervates the dorsal striatum. Interestingly, it has been observed that the level of TH protein is increased in both the caudate nucleus and nucleus accumbens of human heroin abusers (88) which

would be in line with the increased mRNA expression detected in the VTA and SNl in our current population.

Nurr1 is a novel transcription factor essential for the development and maintenance of DA neurons and it has been linked with the transcription of DAT (188) and TH (97). Animal studies have shown that suppression of Nurr1 expression markedly decreases mRNA levels of DAT and TH (189). It could thus be hypothesized that decreased Nurr1 mRNA expression in the VTA PN contributes to the decreased DAT protein and mRNA levels that were evident in the heroin users. However, although down regulation of Nurr1 mRNA levels was coincident with diminished DAT transcription in our study, TH mRNA was increased in the heroin users and significant Nurr1 alterations were only detected in the PN, not in the other midbrain subdivisions in which TH mRNA expression was also up regulated. It should be noted that there are significant concerns regarding the relationship between Nurr1 and TH regulation based on animal model systems because the human TH promoter region has a low degree of homology with the rat and murine promoters (97,190), in contrast to the rodent, Nurr1 has minimal impact on the activation of the human TH gene (191), and Nurr1 expression does not correlate with TH mRNA expression in human neuronal cells (191).

Consistent with previous human and experimental studies (192,193) a strong age-related decrease of Nurr1 was also clear in our study. Interestingly, the use of heroin markedly accelerated the decrease of Nurr1 expression with increasing age though the subjects were relatively young (age range 19-39 years old). Some reports have suggested that the mesolimbic DA system is more vulnerable to aging than the nigrostriatal pathway (194), which could account for the exacerbated decrease of mesolimbic Nurr1 mRNA expression in subjects in which PN neuronal populations are already dysfunctional due to the opiate use. Decreased Nurr1 in heterogenous *Nurr1*-deficient animals is associated with age-related reduction of DA tissue levels in mesolimbic and mesocortical regions (195). No change (196) or decreased (197) DA levels have been reported for the dorsal striatum of these animals, discrepancies that may also depend in part on the age at which the animals were studied. Although acute opiate use may transiently increase mesolimbic DA, the current evidence of decreased DAT and increased TH in heroin abusers could reflect compensatory alterations in response to diminished DA concentrations associated with reduced Nurr1 by repeated opiate use.

The localized and marked age-related impairment of the Nurr1 in the PN in heroin abusers would strongly suggest that limbic function would be compromised as these individuals grown older. There is growing evidence that reduced Nurr1 may increase the vulnerability of DA neurons to stress and neurotoxins (192,196). *In vitro* models have documented that Nurr1 increases cell survival and confers resistance to oxidative stress (198).

In addition to Nurr1, α-synuclein is a recently described gene that appears to be a central regulator of the DA life cycle (199). The increased expression of α-synuclein mRNA detected in various brainstem DA populations of our heroin abusers is consistent with other human studies showing elevated α-synuclein mRNA and protein levels in cocaine abusers (94,200) as well as alcoholics (201,202). Elevated α-synuclein has also been linked with drug craving (202). Increased midbrain α-synuclein is characteristic of neurodegeneration of DA pigmented cells (203) especially of the substantia nigra in comparison to the VTA DA neurons, which appear to be resistant to the neurotoxic properties of α-synuclein (204) ; α-synuclein is a major component of Lewy bodies and dystrophic neurites that are pathological hallmarks of Parkinson's disease (205-207). In contrast to the current findings, chronic morphine treatment and withdrawal in mice lead to decreased α-synuclein mRNA expression in the VTA with no change in the substantia nigra (208). The decrease of α-synuclein in the rodent model appears to be associated with drug withdrawal. The subjects in our investigation had a history of heroin abuse and positive toxicology of recent heroin use prior to death thus the increased α-synuclein expression could indeed relate to the chronic drug use.

A perhaps surprising finding in the current study was the lack of heroin-induced alteration of the DA D2 receptor gene expression in either the limbic- or motor-related given the significant changes apparent for the other DAergic markers. Moreover, the DA D2 receptors has been highly implicated in the rewarding effects of opiates in transgenic animal models (209,210) and *in vivo* imaging human studies have reported diminished striatal DA D2 receptor binding in opiate abusers (211). The involvement of D2 receptors previously reported in relation to opiates most likely reflects D2 receptor sites on postsynaptic neurons in the striatum, whereas the present study focused on the mRNA expression of D2 receptors in DA cell bodies.

Opioid system

Midbrain

The largest changes of the opioid system within the midbrain occurred predominantly in the paranigral nucleus of the mesolimbic pathway and within the PAG, which is correlated with antinociception. The increase within the [^{35}S]GTPγS coupling occurred not only in basal levels but for both types of opioid receptor agonists: μ and κ. A previous study (179), found the density and affinity of μ opioid receptors unchanged in human heroin users vs. control groups. This finding does not contradict our results, as the [^{35}S]GTPγS coupling measures the activity of the receptor, not their number. Animal studies using a chronic morphine administration paradigm showed no significant changes in DAMGO stimulated [^{35}S]GTPγS binding either in the substantia nigra or in the PAG (212). Another study of the previous group, where the rats self-administered morphine showed a desensitization of DAMGO stimulated [^{35}S]GTPγS binding observed in the PAG with no significant changes within the VTA (213). Although we did not find previous studies that would in full support our results it should be emphasized that animal studies often do not mimic the human condition of chronic opiate dependence. Irrespective of the direction of changes our findings strongly emphasize that the mesolimbic paranigral subpopulation that showed greater activity and sensitivity of opioid G-protein receptor function.

Striatum

The most significant molecular alterations in the striatum were evident for the PENK mRNA in which down-regulation was apparent throughout the striatum in the heroin users, indicating widespread effects on limbic and motor functions. The PENK system, in contrast to PDYN, is associated with positive reinforcement (62), and the NAc shell is the striatal subregion most linked with the rewarding effects of drugs in animal studies (214). It is important to note that, despite the strong alterations of PENK mRNA

expression in both the dorsal and ventral striatum of heroin users, morphine toxicology was significantly associated only with expression levels in the NAc shell. This suggests a more dynamic response of the limbic PENK NAc populations to heroin intake that may relate to the rapid positive mood-altering effects of the drug.

Reduced PDYN mRNA was also detected in the NAc, but the alterations were confined to the NAc core. The NAc core is a central motor component of the limbic motive neurocircuit mediating, e.g., goal-directed behavior and impulse control (215). Similar to PENK, heroin related reduction of PDYN mRNA expression levels was also evident in the dorsal striatum. The down-regulation of the opioid neuropeptide genes in heroin users may directly reflect the drug stimulation of MORs coupled to inhibitory G proteins (216). Although this could be feasible for the PDYN transcript in light of the reduced mRNA expression with increased morphine concentrations, there was a positive correlation between morphine toxicology and PENK transcription. Thus, the reduction of PENK mRNA expression in the NAc would seem to reflect the long-term chronic state of heroin use. Multiple rat studies have reported similar reduced striatal PENK mRNA levels after repeated morphine exposure (217,218). The repeated presence of heroin in the brain is expected to function as ‘‘endogenous enkephalin’’ and lead to a counterbalance of decreased PENK expression. The opposing effects of acute heroin intake on the opioid neuropeptide transcription levels in our study indicate a differential regulation of PENK and PDYN striatal gene expression by heroin. However, MORs are situated on both PDYN and PENK medium spiny projection striatal neurons (219,220), suggesting that other neural systems such as dopamine, which differentially regulates striatal PDYN and PENK, might contribute to the differential opioid neuropeptide effects (221).

In contrast to the reduced transcription of the PDYN and PENK mRNAs, heroin subjects had elevated levels of some of the peptides derived from the opioid neuropeptide genes. Most of the striatal peptide levels measured are expected to arise from neuritis (222) and striatal axon collaterals of the PENK and PDYN projection neurons (142). Tissue levels of peptides reflect the balance of various factors, synthesis, processing, release, and degradation. Decreased release over time, in combination with reduced synthesis, could contribute to increased peptide tissue concentrations. Disturbance of peptide processing and degradation may also exist. The current proPC2

and HERC1 mRNA studies were performed to help elucidate the discrepancies between mRNA and peptide levels, as proPC2 is a rate limiting enzyme of opioid peptide processing and HERC1, part of the ubiquitin-proteosome pathway plays a prominent role in morphine-induced regulation of MOR (151). The neuronal systems underlying processing and degradation of the opioid peptides are complex and will require in-depth investigations.

The impact of the OPRM1 polymorphism on the opioid markers

Until now various investigations have evaluated the OPRM1 polymorphism in regard to drug abuse vulnerability (109,112,113,118,152,153). As compared with other studies, our results revealed a more profound (≈90%) association between heroin use and the A/G genotype. This could be due to the rather homogenous, although small, racial and ethnic population genotyped. Bart et al. (118) also found a high frequency (65%) of the 118G allele in Swedish heroin abusers from a relatively homogenous population. the present study clearly documents an impaired opioid neuropeptide system in heroin abusers that is exaggerated in A/G individuals. These alterations would most likely be linked to the downstream effects of MOR, because the A118G SNP directly influences the expression of MOR, at both the mRNA and protein levels (110), which is localized to PENK and PDYN-expressing striatal cells.

The most significant molecular alterations were evident for the PENK mRNA in which down-regulation was apparent throughout the striatum in the heroin users, indicating widespread effects on limbic and motor functions. Reduced PDYN mRNA was also detected in the NAc, but the alterations were confined to the NAc core and were present only in A/G subjects. When protein levels were checked with the genotype as a covariate, it turned out that in heroin subjects carrying the G allele had elevated levels of peptides derived from all opioid neuropeptide genes. The current proPC2 mRNA findings do not, provided an answer for the genotype differences in the peptide concentrations but were nevertheless intriguing, considering that the elevation of the proPC2 mRNA in the NAc shell was contributed only by A/A heroin subjects. Moreover, of the neural markers currently examined, the proPC2 gene expression

appeared most sensitive to the rapid effects of heroin intake, as evidenced by the strong correlation with 6-monoacetylmorphine proPC2 (and HERC1) mRNA expression, which was increased in the NAc but decreased in the putamen, clearly demonstrates subregional striatal differences in intracellular processing in heroin abusers. The observation that HERC1 expression in A/G individuals was positively correlated with morphine toxicology suggests an impact of genotype on the acute HERC1response. Genotype masked the overall heroin effect in the NAc shell, because a significant reduction of the proHERC1 mRNA was apparent only in A/G subjects. Based on the involvement of the ubiquitin-proteosome system with MOR function, the greater impairment of the proHERC1 mRNA expression in the A/G heroin users could relate to the dysfunction of MOR regulation that is a characteristic feature of 118G individuals (110).

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 male, between 20-30 years of age, with approximately 3 years of drug abuse history, practically all Hungarian citizens.

Heroin overdose deaths in 62% of cases were caused by heroin only, without any additional illegal or legal substances; in the remaining 38% ethanol was present in more then half of the subjects, with CNS depressants present in 16% and THC in 13%. We could find no statistically significant differences within the morphine or codeine concentrations in connection with pure heroin vs. polydrug use.

Theis 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 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 preproenkephalin 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. Heroin users had evident abnormal gene expression related to peptide convertase and ubiquitin-proteosome regulation.

SUMMARY

Heroin abuse is a widespread phenomenon which in Hungary, just as in other parts of the world, has been slowly but gradually increasing in the past 15 years. Our work was aimed at characterizing the population of drug-related death cases, with a special emphasis on the demographic and toxicological evaluation of the subjects. We analyzed 309 drug related death cases registered between 1994 and 2006 at the Department of Forensic Medicine at Semmelweis University, Budapest. We found that the number of drug-related deaths is slowly but constantly increasing; the majority of drug-related deaths were caused by accidental heroin overdoses whose victims were usually male, aged between 20-30 years of age, with an approximately 3.5 years of drug-use history, almost all Hungarian citizens. We also analyzed the prevalence of some infectious diseases within the population; HIV was very rare (1 case during 6 years) while hepatitis C and B and syphilis had a prevalence of 23%, 7% and 7% respectively.

In a subpopulation of the heroin overdose and control cases we collected human brain samples for further scientific assessment. In our attempt to understand the neurobiology of human heroin abuse, we examined the major brain regions linked to the "reward pathway": the ventral tegmental area (VTA) in the midbrain and the nucleus accumbens (Nacc) within the striatum. The systems most implicated in the neurobiology of heroin abuse are the dopaminergic and opioid systems; for this reasons we examined the major markers of these within the midbrain and the striatum.

As a result of our experiments, a consistent alteration of the VTA paranigral nucleus (PN) subdivision was revealed for most of the DAergic markers which strongly suggests that there is a more profound disturbance of the mesolimbic reward circuit in heroin-dependent individuals. The paralleled disturbance of dopamine transporter (DAT) mRNA and protein levels in the PN and Nacc, respectively, emphasizes the continuum of disturbances throughout the mesolimbic circuit.

In our population of subjects, 90% of individuals with an A118G polymorphism of the μ opioid receptor gene (OPMR1) were heroin abusers. Down-regulation of opioid neuropeptide genes detected in the heroin users were exaggerated in 118G subjects and were most prominent for preproenkephalin in the Nacc shell. Reduced opioid neuropeptide transcription was accompanied by increased dynorphin and enkephalin peptide concentrations exclusively in 118G heroin subjects, suggesting that the peptide processing is associated with the OPRM1 genotype. Overall, the findings from these studies emphasize a prominent mesolimbic disturbance in the brain of heroin abusers.

ÖSSZEFOGLALÁS

A heroin abúzus az elmúlt 15 évben növekvő tendenciát mutat szerte a világon, s így Magyarországon is. Munkánk során a kábítószerrel összefüggésben bekövetkezett halálesetek demográfiai, toxikológia és neurobiokémiai jellegzetességeit határoztuk meg. A Semmelweis Egyetem Igazságügyi Orvostani Intézetében 309 kábítószerrel kapcsolatos halálesetet vizsgáltunk 1994 és 2006 között. Megállapítható volt, hogy a kábítószerrel kapcsolatos halálesetek száma lassan de fokozatosan növekedett. A halálok az esetek túlnyomó részében, balesetszerű heroin túladagolás miatt következett be, mely az átlagosan 3,5 éve iv. heroint használó, 20-30 év közötti férfiakra volt a legjellemzőbb. A fertőző betegségek prevalenciáját vizsgálva megállapítottuk, hogy a HIV fertőzés nagyon ritka (1 eset 6 év alatt), azonban a C és B típusú májgyulladás valamint a lues prevalenciája elérte a 23%, 7% és 7%-ot.

A heroin túladagolásban elhaltak és kontroll csoportban humán agyminta-vétel történt további tudományos feldolgozás céljából. A humán heroin abúzus neurobiológiai hátterének jobb megismeréséhez, vizsgálatainkat a közti agy, ventrális tegmentális területe (VTA) és a törzsdúcok, a nucleus accumbens (Nacc) régiójában végeztük. Az eddigi ismereteink szerint, a heroin abuzus mechanizmusában az opiát és dopaminerg rendszereknek kiemelkedő jelentősége van, így ezen rendszerek markereinek eloszlását vizsgáltuk a köztiagy és striátum területeiben. Megállapítottuk, hogy a VTA-n belül fekvő paranigrális (PN) magban minden vizsgált DA-erg és opioid marker valamilyen formában károsodott, mely valószínűsíti, hogy a humán heroin használóknál a mezolimbikus rendszer súlyos zavara áll fenn. Ugyanakkor a dopamin transzporter (DAT) mRNS csökkenése a VTA PN magjában és a DAT protein szint csökkenése a Nacc-ben jelzi, hogy ezen zavar megtalálható a mezolimbikus pálya több terültén is.

A vizsgált populációban a μ opioid receptor gén (OPMR1) A118G polimorfizmusával jellemezhető személyek 90%-a heroin használó volt. A heroin használó csoportban megfigyelt opioid neuropeptid gének csökkent expressziója, legmarkánsabban a 118G esetekben jelent meg, a preproenkephalin vonatkozásában a Nacc-héj területén. A csökkent neuropeptid transzkripció kizárólagosan a 118G heroin alcsoportban járt dinorphin és enkephalin peptid-szint emelkedéssel, mely az OPRM1 genotípus hatását jelzi az opioid peptideknél. Összegezve, eredményeink hangsúlyozzák a kifejezett mezolimbikus eltérések jelenlétét humán heroin használókban.

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ABSTRACTS

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