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KATRIN SIKK

Manganese-ephedrone intoxication – pathogenesis of neurological damage and clinical symptomatology

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II. **Sikk K**, Taba P, Haldre S, Bergquist J, Nyholm D, Askmark H, Danfors T, Sorensen J, Thurfjell L, Raininko R, Eriksson R, Flink R, Farnstrand C, Aquilonius SM. Clinical, neuroimaging and neurophysiological features in addicts with manganese-ephedrone exposure. *Acta Neurol Scand*. 2010; 121:237–243.

III. **Sikk K**, Kõks S, Soomets U, Schalkwyk LC, Fernandes C, Haldre S, Aquilonius SM, Taba P. Peripheral blood RNA expression profiling in illicit methcathinone users reveals effect on immune system. *Front Genet*. 2011;2:42. doi: 10.3389/fgene.2011.00042.

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Applicant's contribution to these publications:

Paper I, II, IV: study design, finding patients, data collection, examination of patients, data analysis and writing the first draft of the manuscript to which other authors contributed.

Paper III: data collection, partial performing of gene analysis and writing the first draft of the manuscript to which other authors contributed.

ABBREVATIONS

ADL	activities of daily living
DAT	dopamine transporter
DBS	deep brain stimulation
DaTSCAN	tradename of ^{123}I Ioflupane manufactured by GE Healthcare
EDTA	ethylenediaminetetraacetic acid
FDG	fluorodeoxyglucose
GP	globus pallidus
HAND	HIV-associated neurocognitive disorders
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HY	Hoehn and Yahr Rating Scale
IBZM	iodobenzamide
L-dopa	levodopa
MDMA	3,4-methylene-dioxymethamphetamine
MMSE	Mini-Mental State Examination
Mn	manganese
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MRI	magnetic resonance imaging
OC	occipital cortex
PAS	sodium para-aminosalicylic acid
PD	Parkinson's disease
PDQ-39	Parkinson's Disease Quality of Life Questionnaire
PET	positron emission tomography
PSP	progressive supranuclear palsy
SBR	Striatal Binding Ratio
SD	standard deviation
SE	Schwab and England
SI	signal intensity
SN	substantia nigra
SPECT	single-photon emission computed tomography
T1	spin–lattice relaxation time
UPDRS	Unified Parkinson's Disease Rating Scale
VMAT2	vesicular monoamine transporter-2
WD	Wilson's disease
WM	white matter

I. INTRODUCTION

Parkinsonism is a hypokinetic movement disorder comprising symptoms of akinesia (slowness and fatiguing of movement) and rigidity which are often accompanied by tremor and postural instability. There are many causes of parkinsonian syndromes, among which Parkinson's disease (PD) is the most common. The age-adjusted prevalence of PD in Estonia was reported to be 152 per 100,000 population; the prevalence rate was higher in older age groups (1% among people over 60 years of age), but there were no cases younger than 40 years of age [Taba and Asser, 2002]. In addition to neurodegenerative diseases such as PD, drugs, structural lesions, cerebrovascular diseases, neuroinfections, and neurotoxins can induce parkinsonism [Cersosimo and Koller, 2006].

Since the late 1990s, several young adults with a parkinsonian syndrome of unknown etiology have been referred to neurologists in Estonia. It was determined that these patients had injected a home-made psychostimulant mixture derived from combining Sudafed (pseudoephedrine), a nasal decongestant, with potassium permanganate as the oxidant. In the reaction pseudoephedrine was converted into ephedrone (methcathinone), and the final mixture also contained high concentrations of manganese (Mn), a toxic by-product of the synthesis.

Mn is an essential element for biologic functions in humans, but excessive exposure to this compound can be toxic. Mn toxicity is well known to induce parkinsonism called manganism. However, overt clinical manganism is rarely seen in current clinical practice. Mn intoxication usually occurs via inhalation and results from chronic occupational exposure. In most cases, Mn homeostasis is effective and neurotoxicity is rare. Patients with prolonged and excessive exposure or those with compromised liver function are potentially more vulnerable to Mn toxicity [Jankovic, 2005].

Prior to this study, there were no reports of parkinsonsim in ephedrone abusers in the international literature. Although amphetamine-like drugs have toxic effects on dopaminergic receptors, parkinsonism is not a typical feature of psychostimulant abuse [Guilarte, 2001]. Addiction as a potential cause of manganism had not been mentioned previously.

Ephedrone abuse is an important cause of parkinsonism among young Estonian patients. We describe the typical clinical picture and natural history of the syndrome. Neuroimaging and laboratory studies were performed to find biomarkers of exposure and clarify the pathogenesis of the neurological damage. In our cohort, the syndrome resembles classic Mn intoxication and differs from idiopathic PD. In former ephedrone abusers there are no specific laboratory or imaging findings. Diagnosis depends on the typical clinical picture and previous drug abuse history.

Ephedrone abuse may cause permanent neurological damage and severe disabilty. The prognosis of the parkinsonian syndrome in the ephedrone users is poor, as there is no curative therapy. It is alarming that instructions to prepare this highly neurotoxic mixture are available on the Internet. There is considerable public health concern if intravenous ephedrone use spreads to larger populations.

2. LITERATURE REVIEW

Manganese is an essential trace metal. It is necessary for a multitude of functions, including skeletal system development, energy metabolism, activation of certain enzymes, nervous system function, and reproductive hormone function, and is an antioxidant that protects cells from damage due to free radicals. The most important source of Mn for the general population is diet, and the average intake of Mn from food ranges from 2 to 9 mg/d [Santamaria and Sulsky]. Under normal dietary consumption, systemic homeostasis of Mn is maintained via tight homeostatic control of both gastrointestinal absorption and biliary excretion. Exposure to high oral, parenteral or ambient air concentrations of Mn can result in elevations in tissue Mn levels [Aschner and Aschner, 2005].

2.1. Manganese neurotoxicity

Chronic Mn poisoning was first reported by James Couper in 1837 in five men who worked in a manganese ore-crushing plant in France [Couper, 1837]. Mn neurotoxicity was subsequently reported in miners [Cotzias et al., 1968; Mena et al., 1967; Rodier, 1955], smelters [Huang et al., 1989; Wang et al., 1989], welders [Koller et al., 2004; Tanaka and Lieben, 1969] and workers involved in the manufacture of dry-cell batteries [Emara et al., 1971]. Mn toxicity has been described in patients receiving long-term parental nutrition [Ejima et al., 1992], drinking contaminated water [Kondakis et al., 1989] and following potassium permanganate ingestion [Holzgraefe et al., 1986]. Manganism is also associated with chronic liver disease [Spahr et al., 1996], as the excretion of manganese is markedly impaired, with subsequent accumulation in the brain. Iron shares similar absorption mechanisms with essential divalent metals, particularly Mn. Blood Mn concentration is elevated in iron deficiency anemia patients [Kim et al., 2005]. Iron deficiency can be a risk factor for Mn accumulation in the central nervous system. Recently, inherited syndrome of hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia in cases without environmental Mn exposure has been reported [Tuschl et al., 2008] and the cause was identified as an autosomal recessive mutation in *SLC30A10* [Tuschl et al., 2012].

2.1.1. Clinical features and natural history

Manganism is characterized by symmetric bradykinesia, rigidity, postural instability, gait disturbance, difficulty walking backward, micrographia, masked face, hypophonia and dysphonia. Dystonia is a common and early feature, usually presents as facial grimacing, hand dystonia and/or a peculiar “cock gait”. Tremor is less common and tends to be action or postural. In early stages of manganese intoxication psychiatric symptoms including irritability, mania, uncontrollable laughter, compulsive or aggressive behaviour, hallucinations and

cognitive disorder have been described [Cersosimo and Koller, 2006; Guilarte, 2010]

Serial follow-up studies of previous chronic manganese intoxication patients were conducted in a small group of Taiwanese ferromanganese smelters. Their parkinsonian symptoms showed rapid progression during the initial 10 years, followed by a plateau during the following 10 years [Huang et al., 1998; Huang et al., 2007; Huang et al., 1993].

2.1.2. Treatment

Different effects of levodopa (L-dopa) therapy have been reported in patients with manganese-induced parkinsonism. Some patients had good response to L-dopa in earlier reports [Greenhouse, 1971; Mena et al., 1970], but a double-blind, placebo-controlled trial of L-dopa in Taiwanese smelters showed no benefit [Lu et al., 1994].

Chelating treatment with ethylenediaminetetraacetic acid (EDTA) reduced blood Mn levels in acutely poisoned patients and had therapeutic benefit if patients were removed from exposure at early stages of disease [Hernandez et al., 2006]. Another study showed also that the Mn concentration in blood decreased, however the clinical symptoms did not improve [Huang et al., 1989]. Sodium para-aminosalicylic acid (PAS), which is used to treat tuberculosis, has been found to be effective in a few patients with severe manganism, probably due to chelating properties [Ky et al., 1992].

2.1.3. Pathology and pathogensis

A limited number of autopsy studies have been performed in patients with chronic manganese intoxication. They demonstrate a consistent pattern characterized by neuronal loss and reactive gliosis in the globus pallidus (GP) (particularly the internal segment) and substantia nigra pars reticulata with sparing of the substantia nigra pars compacta and an absence of Lewy bodies. This stands in contrast to idiopathic PD, where neuronal loss in the substantia nigra pars compacta and the presence of Lewy bodies is a neuropathological hallmark of the disease. The putamen, caudate and subthalamic nucleus are affected to a lesser extent. Involvement of other regions, such as the cortex, thalamus, hypothalamus and red nucleus have also been reported [Bernheimer et al., 1973; Perl and Olanow, 2007; Yamada et al., 1986].

Recent studies in non-human primates provide neuropathological evidence of frontal cortex involvement in Mn neurotoxicity, including diffuse amyloid-β [Guilarte et al., 2008b] and α-synuclein aggregation [Verina et al., 2013]

Mn-induced neuronal toxicity is mediated by disruption of mitochondria initiating both apoptosis and necrotic cell death via formation of reactive oxygen species. Mn homeostasis in all tissues, including the central nervous system is maintained by several mechanisms including the divalent metal transporter

(DMT1), which is considered to be the major transporter for Mn. In the basal ganglia DMT1 levels are the highest in the CNS [Roth, 2009]. Mn exposure is not associated with dopamine neuron loss, but with the inability of dopaminergic neurons to release dopamine [Guilarte et al., 2008a]. Besides dysfunctional dopaminergic system alterations in the biology of other neurotransmitters, such as glutamate, γ-aminobutyric acid (GABA) and norepinephrine (NE) have been reported [Aschner et al., 2009].

2.1.4. Biomarkers of exposure

Many occupational studies have been conducted to determine whether Mn in the blood compartment (whole blood, plasma, or serum) can serve as biomarker of exposure. In general, these studies appear to suggest that blood Mn concentration serves as a reasonable indicator of exposure on a group basis; reflects recent, active exposure; and appears to be a modest indicator for distinguishing Mn exposed workers from control subjects at the individual level [Zheng et al., 2011].

Studies have attempted to use hair Mn concentration as a measure of cumulative or past exposure providing an average of hair growth period. The average value of Mn in hair tended to increase in workers with increased years of employment [Zheng et al., 2011]. Hair measurements have several limitations. In inhalational exposure there is a potential for external contamination. Mn hair concentration has a relatively great background variation [Bader et al., 1999].

Increased T1 MRI signal in the basal ganglia is a biologic marker of manganese accumulation in the brain tissue (see the next paragraph).

2.1.5. Neuroimaging

Mn has paramagnetic properties, causing shortening of the T1 relaxation time and signal intensity (SI) increase on T1-weighted magnetic resonance imaging (MRI). There are no alterations on T2 weighted images. Therefore MRI has been used to estimate Mn accumulation in brain tissue. Symmetric T1 hyperintensity in the basal ganglia, especially in globus pallidus were first reported in a patient with occupational Mn intoxication [Nelson et al., 1993]. Similar T1 hyperintensities have been observed in patients receiving total parenteral nutrition [Mirowitz et al., 1991] and in patients with liver cirrhosis [Krieger et al., 1995; Park et al., 2003]. Increased signal intensities on T1-weighted images reflect recent exposure to Mn and these MRI changes usually disappear within one year following the withdrawal from occupational exposure [Huang et al., 1998; Kim et al., 1999; Nelson et al., 1993]. Hyperintense globus pallidus is also present in most patients with advanced liver disease and is reversible 10 to 20 months after transplantation when liver function returns to normal [Pujol et al., 1993].

Direct T1 relaxation time measurement has been shown to be a better indicator of low tissue Mn level than visual assessment of SI or the use of a

pallidal index (signal intensity ratio of globus pallidus relative to the frontal white matter) in T1-weighted images [Choi et al., 2007].

Functional imaging studies in Mn-exposed humans with fluorodopa positron emission tomography (PET) [Kim et al., 1998; Shinotoh et al., 1997; Wolters et al., 1989] or single-photon emission computed tomography (SPECT) with a dopamine transporter (DAT) radioligand to access dopamine terminal integrity in striatum have been normal or with slightly reduced uptake [Huang et al., 2003]. Severe asymmetric reduction in striatal F-dopa uptake, reported in two welders with occupational Mn exposure [Racette et al., 2001] was probably due to coexisting idiopathic PD.

Workers with Mn-induced parkinsonism had normal iodobenzamide (IBZM) SPECT [Hernandez et al., 2006] or small decrease in D2 receptors measured by PET with raclopride [Shinotoh et al., 1997]. Markedly decreased D2 receptor density using methylspiperone PET was noted in a case with chronic manganism [Kessler et al., 2003].

New evidence from non-human primate studies indicates that besides the basal ganglia, also frontal white matter and cortical structures are susceptible to Mn-induced neurotoxicity [Guilarte et al., 2006].

Information about regional brain metabolism in manganism is limited. In two Mn intoxicated rhesus monkeys, no significant changes were found on fluorodeoxyglucose

(FDG) PET [Shinotoh et al., 1995], but four humans with mild manganism showed decreased cortical metabolism [Wolters et al., 1989].

2.1.6. Gene expression

Gene expression profiling using whole-genome microarray screening is a well-established technology to obtain a snapshot of the complex response variables (transcriptome) in biological systems. Expression profiling has great potential for examining molecular pathogenesis and biomarkers of disease. It is a relatively easy to use technology which gives a genome-wide overview of the molecular changes during pathological conditions. Peripheral blood gene expression has been used as a surrogate fingerprint of cerebral neurological diseases [Davies et al., 2009; Mohr and Liew, 2007; Sharp et al., 2006; Sullivan et al., 2006]. The resulting profile is a complex description of the molecular phenotype and could be used to analyze different acute and chronic conditions.

In a genome-wide study on primary human astrocytes, Mn-induced changes were reported in genes involved in inflammation (upregulated), DNA replication, and repair (downregulated) [Sengupta et al., 2007]. Gene expression studies in the frontal cortex of non-human exposed to Mn showed gene expression changes mainly associated with apoptosis, cholesterol metabolism and transport, axonal/vesicular transport, inflammation/immune response, cell cycle/ DNA repair and biosynthesis, and protein folding and degradation [Guilarte et al., 2008b].

2.2. Drug abuse and movement disorders

2.2.1 MPTP

Historically, intravenous use of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), also known as a "synthetic heroin", has caused a severe parkinsonian syndrome with rigidity, hypokinesia, gait disorder, and hallucinations within some weeks after starting injections of the drug [Ballard et al., 1985; Langston et al., 1983]. In [^{18}F]fluorodopa PET, there was a reduction of dopaminergic function in the caudate and putamen in MPTP users and L-dopa therapy was effective [Snow et al., 2000]. Discovering of the ability of MPTP to produce selective nigral cell degeneration and parkinsonism in humans has probably been the greatest advance in experimental models of PD [Jenner, 2008].

2.2.2 Amphetamine-like psychostimulants

Amphetamine and the amphetamine-like psychostimulants methamphetamine, cathinone and methcathinone (Fig. 1) bind to dopamine, noradrenaline, and to a lesser extent serotonin transporters located on neuronal cell membranes. Transporters pump these drugs into the neuron where they are taken up by vesicular monoamine transporters. Amphetamines disrupt the proton gradient which normally keeps monoamines within the vesicle causing monamines leaving the vesicle and accumulate in the cytoplasm where they are reverse-transported out of the cell through the same transporters that pumped amphetamines into the cell [Fleckenstein et al., 2007]. In addition to increasing their release, amphetamines also decrease monoamine reuptake and enzyme degradation. The net result is that amphetamine-like psychostimulants cause a rapid and sustained increase in the extracellular concentrations of monoamines [Suzuki et al., 1980].

With repeated use in both humans and experimental animal models, amphetamines deplete the brain's stores of dopamine and damage dopamine and serotonin nerve terminals. Increases in intra and extracellular concentrations of dopamine set off a cascade of events including oxidative stress, neuroinflammation, and excitatory neurotoxicity – the net result of which is neurotoxicity [Yamamoto et al., 2010].

Cathinone is the principal active constituent present in the leaves of the khat shrub (*Catha edulis*). The stimulant effects have led khat to be known as a 'natural amphetamine'. Chewing the leaves of the khat plant is common in certain countries of East Africa and the Arabian peninsula. Synthetic derivatives have been abused for their amphetamine-like stimulant effects, most notably methylone, methcathinone (ephedrone), and 4-methlymethcathinone (mephedrone). Methcathinone was first synthesized in 1928. It was used in the Soviet Union during the 1930s and 1940s as an anti-depressant. It has been used as a recreational drug in the Soviet Union from the 1970s and subsequently in the USA in the early 1990s. In 1994, methcathinone was added to Schedule I of the UN Convention of Psychotropic Substances [Kelly, 2011].

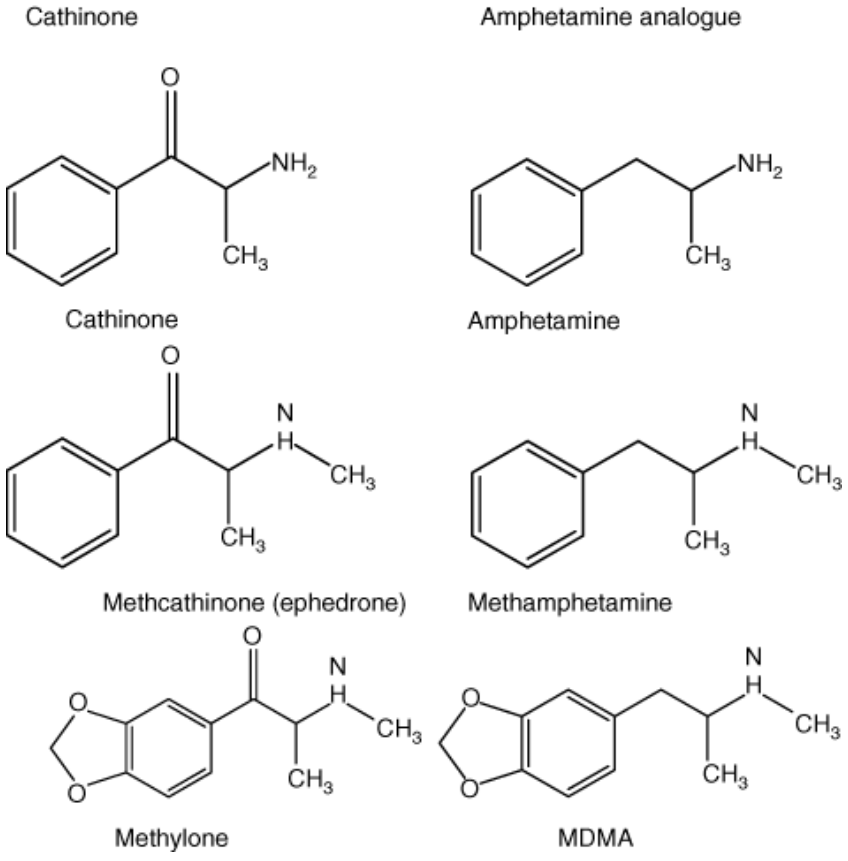


Figure 1. Amphetamine and cathinone derivates

Prolonged release of central monoamines and activation of the sympathetic nervous system can cause acute neurologic complications like cerebral hemorrhage and agitation [Schep et al., 2010].

Chronic methamphetamine abuse is associated with deficits in neuropsychological testing, especially in episodic memory and executive function [Scott et al., 2007]. Methamphetamine abusers also suffer from mental illnesses with anxiety, depression and psychosis [Darke et al., 2008].

A potential complication of amphetamine-induced damage to the dopaminergic nervous system is the development of dyskinesias and choreoathetoid movements [Rhee et al., 1988]. The symptoms usually disappear within a week when the drug is discontinued, but may remain for years [Lundh and Tunving, 1981].

Similarly to PD patients, amphetamine users may develop punding (non-goal directed repetitive activity) [Schiorring, 1981]. Though several animal and human studies have shown that amphetamines cause alterations in striatal dopaminergic neurotransmission, parkinsonism is not a feature of amphetamine

abuse [Guilarte, 2001]. There are several hypotheses to explain the discrepancy. Methamphetamine abuse causes alterations in dopaminergic nerve terminals, not in the cell bodies. Methamphetamine users have greater dopamine reductions in caudate compared to the putamen, a pattern opposite to that of PD [Moszczynska et al., 2004]. Another explanation is that damaged dopaminergic nerve terminals recover with abstinence [Volkow et al., 2001a] or the reduced dopamine transporter levels are a compensatory response to increased levels of extracellular dopamine. Vesicular monoamine transporter-2 (VMAT2), which is known to be resistant to drug-compensatory regulation, is not reduced in abstinent methamphetamine abusers [Johanson et al., 2006]. However, some studies have shown an increased risk for developing PD in amphetamine abusers [Callaghan et al., 2010; Christine et al., 2010].

2.2.3 Ecstasy

Ecstasy is the colloquial name given by its users to 3,4-methylene-dioxymethamphetamine (MDMA). It is a ring-substituted amphetamine derivative that is also related to the hallucinogenic compound mescaline. MDMA binds to all three of the monoamine presynaptic transporters with highest affinity for the serotonin transporter. Chronic ecstasy use causes depletion of serotonin, which has subtle but important long-term effects on cognition and mood [Morton, 2005]. A few cases of parkinsonism have been reported in ecstasy users [Kuniyoshi and Jankovic, 2003; Mintzer et al., 1999; O'Suilleabhain and Giller, 2003], but in these cases MDMA use was not toxicologically verified. It is possible that the young-onset parkinsonism and drug exposure were just coincidental [Kish, 2003].

2.2.4 Cocaine

Cocaine is derived from the leaves of the coca plant. It enhances dopaminergic, noradrenergic, and serotonergic neurotransmission by blocking reuptake of these monoamines. Neurological complications are more common with the smokable alkaloidal cocaine "crack", as it allows much higher doses than with snorted cocaine hydrochloride. Stereotyped hyperkinetic movements ("crack dancing") are well recognized by cocaine users and there are occasional reports of acute reversible parkinsonism in the acute phase of intoxication [Bartzokis et al., 1999; Daras et al., 1994]. Acute dystonia and chorea following cocaine use usually last from minutes to a few days [Brust, 2010], but a case with persistent dyskinesias after 20 months of abstinence has been described [Weiner et al., 2001]. The use of potassium permanganate in the processing of coca-leaf extraction, can also lead to manganese intoxication [Ensing, 1985].

2.3 Manganese-Ephedrone abuse

It is difficult to estimate the scope of ephedrone abuse and the prevalence of parkinsonism among drug addicts. There are approximately 13,800 intravenous drug users in Estonia, 10,000 of them living in Tallinn and its neighbouring areas, and 2,500 in the Northern-Eastern part of Estonia [Uusküla et al., 2007]. In a survey of risk behaviours in these areas, 700 intravenous drug users in Tallinn and Eastern Estonia were interviewed, yielding the following results: 11% of the participants reported ephedrone use in their history, 1.3% were current users, and in 0.9% ephedrone was the main drug of abuse [Lõhmus et al., 2008]. Based on these findings, it could be estimated that there could be approximately 100 persons injecting ephedrone as the main drug of abuse in Estonia. Ephedrone abuse is a common cause of parkinsonism among Estonian patients younger than 40 years of age.

The first cases of "ephedrone encephalopathy" were described in Russian literature 25 years ago [Lukacher et al., 1987]. Parkinsonian syndrome in ephedrone users had not been depicted in the international literature before this study. Although both Mn and ephedrone (similarly to other psychostimulants) are toxic to the dopaminergic nervous system, parkinsonism has been described only in association with Mn intoxication. Aims of the present study were to characterize the clinical picture and course of the syndrome in intravenous ephedrone users in Estonia, and to confirm the presumed neurotoxicity of Mn in the pathogenesis.

Following our first report [Sikk et al., 2007], several cases of parkinsonian syndrome in ephedrone users have been reported in Eastern Europe as well as among immigrants from Western Europe and Canada. Clinical characteristics and MRI data from the case reports are summarized in Table 1 [Sikk et al., 2011]. The first neurological symptoms – usually gait disturbance and slurred speech – have occurred anywhere from only a few months after the beginning of ephedrone injections [Sanotsky et al., 2007] up to 17 years later [Stepens et al., 2008], but generally they have emerged within the first few years. The most commonly reported neurological findings were postural instability with retro-pulsion and falls, gait disturbance, hypomimia, limb and face dystonia, dysarthria, hypophonia, and symmetric bradykinesia. Less frequently described symptoms included limb and axial rigidity, gait freezing, postural and resting tremor, micrographia, apraxia of eyelid opening, some slowing of vertical saccades, pathological laughter, palilalia, and primitive reflexes. Alhough hyperactive deep tendon reflexes were commonly found, pathological reflexes were mentioned only in one case [Colosimo and Guidi, 2009]. Few cases had oromandibular dyskinesia, blepharospasm [Varlibas et al., 2009], myoclonus [Levin, 2005], or restriction of vertical saccadic eye movements [de Bie et al., 2007].

Table 1. Summary of clinical and MRI data from different reports

Publication Country	No. of the subjects	Age (y)	Time to the symptoms	Initial symptoms	MRI findings	Treatment	Outcome
[Levin, 2005] Russia	21	15–36 (21±5.8)	3–14 (6.8±4.9) months	Disorders of speech (33%) and gait (29%), fatigue (29%), bradykinesia (14%), affective symptoms (19%)	T1 bilateral symmetric SI in GPi, SNr – 18 (86%); no correlation with duration of usage, dosage or severity of symptoms	EDTA, L-dopa, clonazepam, amantadine	Spontaneous regression of the symptoms – 29%, worsening – 33% even after 4 y abstinence
[de Bie et al., 2007] Canada (Azerbaijan*)	1	36	Not exactly reported, possibly 4 –16 months	Decrease of libido; sleepiness, slowness	Symmetric SI increase in GP, SN dentate nucleus, and pontine tegmentum	Pramipexole, selegiline, L-dopa	No improvement
[Sanotsky et al., 2007] Ukraine	6	23–45	2 months – 1 y	Speech and gait disorder – 1, plus bradyphrenia or depression – 2, slowness – 2, hyperthermia – 1	Striking bilateral SI increase in lentiform nucleus, SN, dentate nucleus	EDTA, cerebrolysin, amantadine, L-dopa	Mild to moderate improvement – 4, no improvement – 2
[Meral et al., 2007] Turkey	2	21 and 32	Unknown, 4 months	Bradykinesia, gait and speech disorders	Bilateral SI increase in GP, after withdrawal of injections improvement of MRI in case 2	Not reported	No improvement
[Stepens et al., 2008] Latvia	23	37.5±6.5	5.8 ± 4.5 y	Gait disturbance –20 (87%), hypophonia – 3 (13%)	T1 SI increase in GP – all 10 active users, in SN – 9; former users had lesser degrees of change (SI increase in GP – 11, SN – 2, anterior midbrain – 3)	L-dopa in 3 patients, EDTA in 1 patient	No substantial improvement in 13 subjects after with-drawal for 2 – 6 y.
[Selikhova et al., 2008] Ukraine	13	18–46 (29.9)	8.5 ± 3.2 months	Loss of balance – 7, slurred speech – 4, mood disorders – 2	T1 SI increase mostly in GP, – all; other frequently involved – STN, SNr, putamen	L-dopa, amantadine, EDTA.	Significant residual deficit; delayed progression in some cases

Publication Country	No. of the subjects	Age (y)	Time to the symptoms	Initial symptoms	MRI findings	Treatment	Outcome
[Colosimo and Guidi, 2009] Italy (Ukraine)	1	28	2 y	Gait and speech disturbance, mental slowness, generalized fatigue	Repeated MRI normal	L-dopa, dopamine agonists, anticholiergics	No significant change 2 y after withdrawal
[Yildirim et al., 2009] Turkey	1	29	9.5 y since start of abuse, 5 y abstinence	Gate disturbance, mood disorders	Normal	Piracetam, carbamazepine, fluoxetine, L-dopa	Gradual worsening over time
[Varlibas et al., 2009] Turkey	3	15–19	2–6 y	Postural instability, face and limb dystonias, tremor, dysphonia, dysarthria, bradykinesia	Bilateral symmetric SI increase in dentate nucleus, white matter of cerebellum, GP and putamen	EDTA, PAS, L-dopa	No improvement
[Iqbal et al., 2012] Irland (Eastern Europe)	1	30	2 y	Gait disturbance	T1 SI increase in GPi, repeated MRI 2 y later normal	L-dopa	No improvement 2 y after withdrawal
[Koksal et al., 2012] Turkey	7	19–31	7 – 35 months	Impaired speech followed by gait disturbance and bradykinesia	Bilateral T1 SI increase in basal ganglia, brainstem and dentate nuclei	L-dopa	No improvement

EDTA – ethylenediaminetetraacetic acid, GP – globus pallidus, GPi – internal part of globus pallidus, NCh – head of caudate nucleus, PAS – para–aminosalicylic acid, SI – signal intensity, SN – substantia nigra, SNr – reticular part of substantia nigra, STN – subthalamic nucleus, y – years, *country of origin, in immigrant cases

The scores of the Mini Mental State Examination were normal. More extensive neuropsychological testing showed mild executive cognitive impairment, including bradyphrenia, attenuated attention, reduced working capacity, decreased phonetic verbal fluency, and tendency for impulsiveness [Colosimo and Guidi, 2009; Djamshidian et al., 2012; Levin, 2005; Selikhova et al., 2008; Yildirim et al., 2009].

Generally, the syndrome is unresponsive to L-dopa and other antiparkinsonian medication, and there are no other effective treatments that could continuously improve parkinsonian or dystonic symptoms in the ephedrone users. Even when mild improvement has been observed, it has been short-term, and the condition may worsen progressively despite of discontinuation of the drug injections.

MRI of the brain in active ephedrone users showed symmetrical hyperintensity on T1-weighted images in the globus pallidus and substantia nigra pars reticulata. Less frequently involved structures were subthalamic nucleus, substantia innominata, putamen, caudate, anterior midbrain, pontine tegmentum, and dentate nucleus.

3. AIMS OF THE STUDY

1. To characterize the clinical syndrome in intravenous ephedrone users: assess clinical severity, and quality of life (Papers I and IV).
2. To assess the course and prognosis of the neurological syndrome in ephedrone users (Paper IV).
3. To find biomarkers of exposure (Paper IV).
4. To describe findings from MRI and functional neuroimaging (Papers II and IV).
5. To explore the molecular pathogenesis of the syndrome by performing gene expression analysis (Paper III).

4. MATERIAL AND METHODS

4.1. Study population

Subjects were identified from the neurology and psychiatry departments of Estonian hospitals, from addiction rehabilitation centers, and from information provided by other drug addicts. Written informed consent was obtained from all subjects. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu. Patient involvement in different studies in summarized in Table 2.

4.2. Clinical examination

Patients underwent general neurological examination and most of the cases were video-taped. Detailed information about co-morbidities, drug use history and appearance of first symptoms was gathered. Disease severity was scored by the Unified Parkinson's Disease Rating Scale (UPDRS); Hoehn-Yahr (HY) Rating Scale; and Schwab and England (SE) Activities of Daily Living (ADL) Scale [Fahn S et al., 1987].

UPDRS is the most widely used standardized scale to assess parkinsonism. It is designed to monitor PD disability and impairment. Parts I (mentation, behavior and mood), II (ADL) and III (motor examination) contain 44 questions each measured on a 5-point scale (0–4). Total UPDRS score is the combined sum of parts I, II, and III: 0 (not affected) to 176 (most severely affected). Part IV (complications of therapy) was not performed.

HY scale provides a global assessment of disease severity, based on clinical findings and functional disability. The scale includes stages from 0 (no signs) to 5 (wheelchair bound or bedridden unless aided) to indicate the relative level of disability.

SE ADL scale is a means of assessing a person's ability to perform daily activities in terms of speed and independence through a percentage, with 100% indicating total independence, falling to 0%, which indicates a state of complete dependence.

To evaluate health related quality of life, Parkinson's Disease Quality of Life Questionnaire (PDQ-39) was completed by the patients [Peto et al., 1995]. It comprises 39 questions, relating to eight key areas of health and daily activities, including both motor and non-motor symptoms. It is scored on a scale of 0 to 100, with lower scores indicating better health and high scores more severe symptoms.

In order to examine cognitive function, the Mini-Mental State Examination (MMSE) was used [Folstein et al., 1975]. Scores of 25–30 out of 30 are considered normal.

A standardized acute L-dopa challenge test with 200/50 mg levodopa/ carbidopa was performed in 4 former users to evaluate treatment response after one hour.

Table 2. Patient involvement in different studies

			I	II	III	IV				
Patient	UPDRS	HIV	n=4	L-dopa challenge test MRI* DAT SPECT FDG PET n=4	Gene expression n=20	Follow-up n=24	MRI n=11	IBZM SPECT n=8	Plasma Mn n=26	Hair Mn n=17
1	26	pos	x	x						
2	77	NP	x							
3	25	pos	x		x	x	x	x	x	
4	59	neg	x	x	x	x	x	x	x	x
5	42	NP								
6	83	NP								
7	52	NP								
8	47	NP								
9	69	pos			x	x			x	x
10	35	pos			x	x	x	x	x	x
11	68	neg		x	x	x	x	x	x	x
12	51	neg		x		x	x	x	x	x
13	19	NP							x	
14	50	NP								
15	47	NP								
16	23	pos			x	x	x	x	x	x
17	27	pos			x	x			x	x
18	44	neg			x				x	x
19	17	pos			x	x			x	x
20	12	pos			x	x			x	x
21	45	pos			x	x			x	x

			I	II	III	IV				
Patient	UPDRS	HIV		L-dopa challenge test MRI* DAT SPECT FDG PET	Gene expression	Follow-up	MRI	IBZM SPECT	Plasma Mn	Hair Mn
			n=4	n=4	n=20	n=24	n=11	n=8	n=26	n=17
22	79	neg			x	x	x	x	x	
23	47	NP				x				x
24	50	pos			x	x			x	x
25	19	pos			x	x			x	x
26	13	pos			x	x			x	x
27	0	pos			x				x	x
28	26	pos			x		x	x	x	x
29	42	neg			x	x	x		x	
30	53	pos			x	x			x	
31	61	NP			x	x			x	
32	77	pos				x			x	
33	40	neg				x			x	
34	68	neg				x			x	
35	42	neg				x	x		x	
36	52	neg								
37	16	neg				x	x			
38	41	neg								

Roman numerals (I–IV) refer to number of original publication, UPDRS – Unified Parkinson's Disease Rating Scale, MRI* – MRI with T1 relaxation time measurements, NP – not performed, n – number of patients

4.3. Follow-up

From among the 38 subjects seen during the first examination, two had died by the time of the next contact, one had moved abroad, one declined further participation, and 10 were not traceable because they no longer had the same phone number or address. Final follow-up evaluations of 24 subjects were carried out 21±15 months after the initial examination, including 12 former (26±18 months) and 12 active users (15±7 months). Three of the 12 former users were defined as active users during the first study, other 9 had been drug free for 3.8±2.4 years at baseline.

4.4. Laboratory synthesis of the mixture

Laboratory synthesis of the mixture and Mn concentration measurements were performed at the Department of Physical and Analytical Chemistry of Uppsala University.

According to the information provided by the interviewed patients, the following recipe is regularly used in the preparation of ephedrone: a package (12 tablets) of the nasal decongestant Sudafed, containing 60 mg pseudo-ephedrine hydrochloride, 1–2 mL 30% acetic acid and "a point of the knife" of potassium permanganate were poured into 60–100 mL of boiling water. The mixture was left to cool down for about 10 minutes and filtered through a cotton pad. The solution was used for 8–20 mL intravenous injections at irregular intervals many times a day.

To evaluate the chemical reactions and yields of the reported procedure, the following synthetic and analytical investigations were performed: to 1 tablet Sudafed (Pseudoephedrine hydrochloride [(+)-(1S,2S)-2-methylamino-1-phenylpropan-1-ol, C10H15NO, CAS 90-82-4, Mw 165.22] 60mg, GlaxoSmithKline Greenford, Middlesex, Great Britain), 0.145 g KMnO4 (VWR International AB, Stockholm, Sweden), 5 mL H2O at 100° C and 0.125 mL 30% acetic acid (p.a., Merck, Darmstadt, Germany) was added and the solution was mixed (vortexed) until the tablet was fully dissolved. The solution was left to cool for 10 min before it was filtered through a filter paper (Munktell 3, Stora Kopparberg Filter Products, Grycksbo, Sweden).

Synthesized ephedrone was qualitatively analysed using electrospray mass spectrometry (ESI-MS) using a QTRAPTM linear ion trap mass spectrometer (Applied Biosystems, MDS SCIEX, Toronto, Canada) equipped with a pneumatically assisted Turbospray ionization interface. The software Analyst 1.4.1 (MDS Sciex, Concord, ON, Canada) was utilized for data acquisition and evaluation. The experiments were performed by direct infusion at 10 uL/min, employing enhanced mass scan (EMS) configuration.

The manganese (Mw 54.9) content of the final synthetic mixture was analyzed using a SpectroCirosCCD Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) (Spectro Analytical Instruments GmbH&Co,

Kleve, Germany). Analytical standards for Mn were prepared from certified single-element stock solutions and used for calibration.

4.5. Laboratory tests

4.5.1. Virology, hepatic function, iron levels

Subjects (n=28) were tested for Human immunodeficency virus (HIV) (anti-HIV antibodies), Hepatitis C virus (HCV) (anti-HCV antibodies) and Hepatitis B virus (HBV) (HBsAg and anti-HBc antibodies). For evaluation of hepatic function bilirubin and alanine transaminase levels were measured (n=28). For detecting iron deficiency serum ferritin, iron and transferrin levels were measured (n= 25).

4.5.2. Plasma and hair manganese concentration

Plasma (n=26) and hair (pooled pubic+scalp) (n=17) Mn concentration was analyzed using a SpectroCirosCCD Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Spectro Analytical Instruments GmbH&Co, Kleve, Germany). Hair samples from 21 age- and sex-matched Estonian inhabitants (from the Ida-Viru county) were also examined.

4.6. Statistical analysis

Data analyses were performed with Stata software version 10. Continuous data were expressed as mean and standard deviation (SD). Differences between study groups were compared with unpaired t-tests, for single variable determination upon repeated evaluation paired t-test was applied. Bivariate relationships were assessed with Pearson's correlation coefficient. All p-values were two-tailed; p-values <0.05 were considered statistically significant.

4.7. Neuroimaging

MRI with T1 relaxation time measurements, DAT SPECT and FDG PET were performed in 4 former ephedrone users (abstinence for at least 4 years) at Uppsala University Hospital, MRI was evaluated by neuroradiologist Raili Raininko, DAT SPECT and FDG PET by Torsten Danfors. IBZM SPECT (n=8) and MRI (n=11, including active users) were performed in North Estonian Medical Center, IBZM SPECT was evaluated by Malle Paris and MRI by Äli Roose.

4.7.1. MRI with T1 relaxation time measurements

MRI was performed with a Philips Gyroscan Intera MR imager operating at 1.5 T. All slices were 5 mm thick. T1-weighted spin echo images were obtained in sagittal (TR/TE 501/12 ms), axial (TR/TE 599/13 ms) and coronal (TR/TE 500/19 ms) planes and T2-weighted fast SE (FSE) images in axial (TR/TE 5075/100 ms) and coronal (TR/TE 6099/100 ms) planes. Diffusion-weighted images were obtained in an axial plane with a FLAIR sequence (TR/TE/TI 10000/140/2000 ms) using b values of 0 and 1000 s/mm^2. Apparent diffusion coefficient (ADC) maps were calculated. The images were evaluated visually by an experienced neuroradiologist.

Signal intensities (SI) were measured in the T2-weighted axial images in the lateral globus pallidus, anterior putamen, caput nuclei caudati, thalamus and frontal white matter bilaterally. Cerebrospinal fluid (CSF) in the anterior part of the frontal horns was used as an SI reference. The SI of the white matter was also measured in the centrum semiovale bilaterally by using CSF on the frontal convexity in the same slice as an SI reference. The SI measurements were repeated two weeks apart and the means were used in the calculations.

For T1 relaxation time measurements, an axial 5-mm-thick slice was obtained through the basal ganglia using a field of view 250 x 150 mm and an in-plane resolution of 0.98 x 1.42 mm. A Lock-Looker technique was used. The 180 degree pulses were repeated every 3400 ms with 40 alphapulses of 6 degrees at 25 ms spacing between the alphapulses. A monoexponential fit was performed to the real signal in every pixel to determine the T1 value in each pixel. The fitting algorithm included corrections for noise and imperfect 180 degree pulses. The T1 relaxation times were measured by using a NICE (Nordic NeuroLab AS, Oslo, Norway) software in the lateral globus pallidus, anterior putamen, caudate head and frontal white matter bilaterally. The measurements were repeated in the globi pallidi, except in one control, by another observer and the means of the two measurements were used in analysis.

Four healthy age- and sex-matched volunteers were examined as controls. In the measurements, the values beyond 2 SD were considered pathological.

4.7.2. DAT SPECT

SPECT imaging took place 3.5 to 4 hours after intravenous injection of ^{123}I Ioflupane (185 MBq, DaTSCAN GE Healthcare, Amersham, UK). Before the administration, the patients received iodide preparation to minimize radiation exposure to the thyroid gland. A Siemens E.CAM (2001) with a low energy high resolution (LEHR) collimator and software eSoft 6.0 were used. For all acquisitions 120 projections, 360 degrees rotation, 30 s per projection and a matrix size of 256 x 256 was used.

Data were reconstructed with the HERMES software v4.5-B (HERMES iterative reconstruction, HOSEM) (Hermes Medical Solutions, Nuclear Diagnostics AB, Stockholm).

Further image processing was performed using an in-house developed software according to the following: First each scan was spatially normalized to fit a normal template defined in Montreal Neurological Institute (MNI) space using a fully automated method. Then a volume of interest (VOI) template was applied to the data and counts in regions corresponding to the head of the caudate nucleus and the anterior and posterior putamen were extracted. Striatal Binding Ratios (SBRs) were computed by normalising the count data in the Striatal VOIs against the mean value in a reference region located in the occipital and temporal lobe. In addition, the ratio between putaminal and caudate uptake was computed.

SPECT images were visually classified by experienced nuclear medicine physicians as normal or abnormal. A normal pattern was defined as a symmetric bilateral intense uptake in both the caudate nucleus and putamen. An abnormal pattern as either an asymmetrical uptake with reduced uptake in putamen in one hemisphere; or symmetrically reduced putamen activity in both hemispheres; or a reduced uptake in both the caudate nucleus and the putamen. The latter resulting in a significant reduction in contrast and the visualisation of background activity throughout the rest of the image.

4.7.3. FDG PET

Patients fasted overnight before FDG PET scanning. All scans were performed with the subject's eyes open and in a dimly lit room with minimal auditory stimulation. All four subjects were scanned using a Siemens ECAT EXACT H+ scanner after an i.v. injection of 3 MBq/kg (184-227 MBq) of [^{18}F] FDG. A 15-min emission scan was acquired 30 min after injection. Image data were analysed by a voxel-by-voxel method. All four scans were performed within 2 weeks.

All PET scans were spatially normalised to Montreal Neurological Institute (MNI) space. This was done by registering each PET scan to the MNI PET template using an automated method for inter-individual registration (4). The same method was used to spatially normalise FDG scans from 20 healthy controls (mean age 69 y). Mean and SD of the spatially normalised control scans were then computed on a voxel-by-voxel basis to define an FDG normal data base. A computer program was implemented allowing for voxel-based comparison of individual PET scans with the FDG normal data base. This comparison was made using Z-scores, i.e., by taking the subject's voxel value and subtracting the mean value in the normal data base and then dividing by the SD for the corresponding voxel and repeating this for all voxels. The Z-scores show by how many SDs the subject's voxel value differs from the mean. The result of this comparison was expressed as Z-score images; one image volume for negative Z-scores showing hypometabolism and one with positive Z-scores showing hypermetabolism. In the subsequent analysis we used a threshold on volume and degree of deviation and only hyper- and hypometabolic regions with a volume larger than 0.3 mL and degree of deviation of 3 SD or more were

considered. Moreover, due to anatomical variations, hyper- and hypometabolic regions located in the cortical or subcortical areas were excluded. Additionally, three persons randomly selected from the normal database were evaluated as described above.

4.7.4. MRI

MRI examination was performed in 11 subjects, 4 subjects had repeated MRI scans.

Brain MRI was performed using GE 1.5 T Signa HDx system according to standard evaluation protocol using 8 channel neurovascular head coil with slice thickness of 5 mm and interval of 1 mm on T1-weighted sequence in sagital, coronal and axial planes, T2-weighted sequence in axial plane and FLAIR sequence in coronal plane, DWI images with b-value 1000s/mm2.

In addition to general MRI description, signal hyperintensity on T1-weighted sequence in different anatomical structures was graded in four categories: 0 – normal, 1 – mildly hyperintense, 2 – moderately hyperintense, and 3 – severely hyperintense (signal intensity agrees with fatty marrow tissue signal intensity).

4.7.5. IBZM SPECT

Single-photon emission computed tomography (SPECT) with iodine-123 iodobenzamide (IBZM), a ligand for D2 receptors was performed in 8 cases using a dual head gamma camera (SPET/CT INFINIA, GE Healthcare) with a high resolution collimator. Images were acquired in a step and shoot mode with 120 equally spaced projections over 360°, taking 40 s per step and using 128×128 matrix size, with a zoom of 1.0. The energy window was centered on 159 keV. Image reconstruction was performed using a Butterworth filter, and attenuation correction was performed using Chang coefficient. Transversal slices were reconstructed parallel to the cantomental plane. The individual ^{123}I-IBZM occupancy regions of interest (ROIs) were drawn over the striatum and the occipital cortex, serving as a reference region. The activity ratios of striatal to occipital cortex uptake (S/OC) were used as a semiquantitative measure of the relative density of striatal D2 receptors. S/OC ratios previously obtained from a control group of 8 patients with idiopatic Parkinson´s disease (IPD) with a mean age of 59 years were used for comparison.

4.8. Gene expression

Blood samples from 20 subjects and 20 age- and sex-matched healthy controls were collected into Tempus tubes (Applied Biosystems, Foster City, USA). Blood was frozen and stored until further processing. RNA extraction from whole blood was performed according to the manufacturer’s protocol (PN 4379228C). After RNA extraction, alpha and beta globin mRNA was depleted

with GlobinClear Whole Blood Globin Reduction kit (Ambion, Austin, USA). The quality of RNA was analyzed with a Bioanalzer 2100 (Agilent, Santa Clara, USA) and gene expression profiling was performed with GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, USA) containing probes for all exons of 28,869 genes. We analyzed all the genes on the array without any filtering for presence/absence cells.

In order to label the RNA we used the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling Assay (Affymetrix, Santa Clara, USA), which is designed to generate amplified and biotinylated sense-strand targets from the entire expressed genome without bias. Briefly, double-stranded cDNA was synthesized from 300 ng of total RNA by reverse transcription using random hexamers tagged with a T7 promotor primer sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7 RNA polymerase producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. This DNA was fragmented with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1). DNA was labelled by terminal deoxynucleotidyl transferase (TdT) and hybridization was performed according to the manufacturer's protocol. The arrays were subsequently washed, stained with phycoerythrin streptavidin and scanned according to standard Affymetrix protocol. Images were processed using the Affymetrix Expression Console and the MAS 5.0 algorithm was used to measure quality control parameters.

The normalized, background subtracted and modelled expression (Robust Multi-array Analysis, RMA) data (GEO accession number GSE28686) were further analyzed using a linear model combined with Bayesian moderation for standard errors implemented in the Bioconductor *limma* package of the statistical software R (http://www.r-project.org/) [Ihaka and Gentleman, 1996; Smyth, 2004]. False Discovery Rate (FDR) was used to address multiple testing corrections [Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003]. Moderated model calculates B value, which is the log-odd that the gene is differentially expressed. B-statistics of zero corresponds to a 50–50 chance that the gene is differentially expressed and B-statistics is automatically adjusted for multiple testing.

Eight genes with significant differences and with potentially interesting functions from the gene expression profiling data were further analyzed by means of real-time PCR (RT-PCR). These genes were: C15orf26, GPR15, IGFR1, SNRPN, IFI44L, IFI44, IFI27, and IFNG. RNA was converted into cDNA using the High Capacity cDNA Synthesis kit from Applied Biosystems. TaqMan assays and Gene Expression Master mix was used for the RT-PCR reaction in the SDS 7900 HT system (Applied Biosystems). Sample (20 controls and 20 methcathinone users in four replicates) comparisons were made using Welch's t-test.

To define the functional networks of the differentially expressed genes, data were analyzed by Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). A data set containing Affymetrix probeset identifiers and corresponding fold change (log2) values was uploaded. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base to generate the list of focus genes. Networks of these focus genes were then algorithmically generated based on inter-gene relationships manually curated in the Ingenuity database from published studies. Ingenuity Pathways Analysis calculates a significance score for each network. The score is generated using a p-value calculation, and is displayed as the negative logarithm of that p-value. This score indicates the likelihood that the assembly of a set of focus genes into a network could be explained by chance alone (e.g. score of 2 indicates that there is a 1 in 100 chance that the focus genes are together in a network by a random chance). In whole-genome gene expression studies scores >8 ($p<0,000000001$) are considered statistically significant.

5. RESULTS

5.1. Study population

During the period of 2006–2012, we studied 38 intravenous ephedrone users (31 men and 7 women) aged between 18 and 58 years (mean age 33 years). Majority of the subjects (76%; n=29) came from the Ida-Viru county, with additional 7 (18%) subjects from Tallinn and the Harju county, and 2 cases from Tartu. All except one study participant (an Estonian) were Russian speakers. The educational level was 9 years or less in 66%, 10 to 12 years in 32% (vocational or secondary education), and one subject had higher education. The rate of unemployment was very high (95%), and in 70% the main income was disability allowance. Ten subjects were currently married and in 8 cases both spouses were ephedrone users. Most of the subjects were single (58%) or divorced (16%). More than half of the subjects lived alone (n=20), 10 with their mothers, and 8 had children in their family. At the time of the baseline study, two subjects were in prison, one was homeless, and 12 (32%) lived in a dormitory.

5.2. Clinical examination

8–20 ml of ephedrone solution was injected 2 to 30 times per day. As the total injected volume and Mn amount in the solution differed between subjects, duration of exposure gives the best estimation of the total exposure. The average duration of ephedrone use was 4.6±3.9 years (0.25 to 13 years). According to the subjects exposure history they were defined as a active (n=20) or former users (n=18) (reported abstinence more than one year previously). Former users had been drug free 4.9±2.2 years. Ephedrone was the main drug of abuse, 55% had also used or tried amphetamine and 63% opiates (heroin, fentanyl and poppy liquid). The mean age of starting the ephedrone injections was 25 years with a range of 14 to 41 years.

Based on the history given by the subjects, the first neurological symptoms appeared on average 2.8±3.1 years (0.5 to 12 years) after the beginning of ephedrone use. The presenting feature was gait disturbance in 14 (37%) cases, speech disturbance in 10 (26%) and loss of balance in 9 (24%).

The average total UPDRS score was 43.3±20.8; within this the mean score of part III (Motor Examination) was 28.3±14.7. The total UPDRS score was ≤ 25 in 20%, 25–49 in 40%, and ≥ 50 in 40% of the patients. The mean value of HY staging was 2.9±0.9; the biggest group of patients (n=19, 50%) were categorised as stage III (Figure 2), indicating bilateral disease with postural instability and moderate effect on ADL.

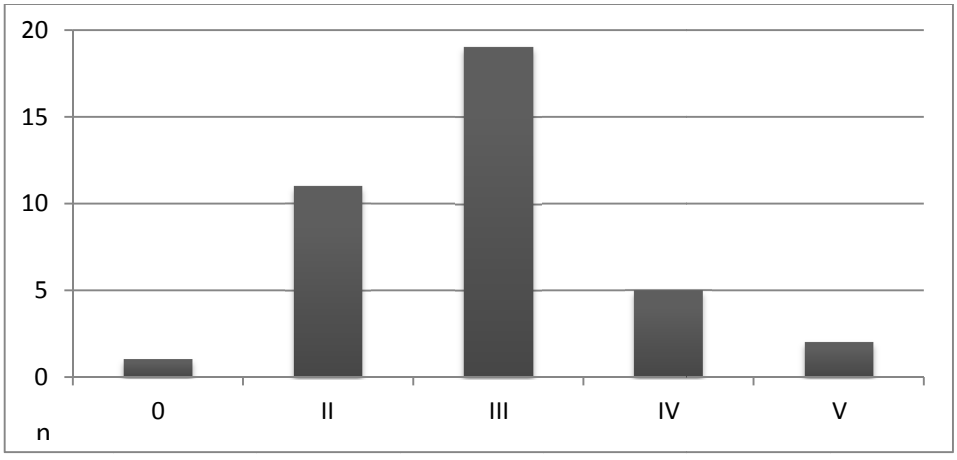


Figure 2. Distribution of the patients by Hoehn-Yahr stages

The average scores of UPDRS part III motor domains are given in Figure 3. Besides the signs accounted in UPDRS, 16 (42%) subjects had some dystonic features, most often in feet presenting as a cock-like gait. Some had dystonic smile and dystonic hand postures, severe generalized dystonia was seen in 3 cases. Less frequent findings were palilalia (n=3) and apraxia of eyelid opening (n=2). Freezing and start hesitation were seen in only one subject. Brisk tendon reflexes in legs was a common finding, though plantar responses were normal.

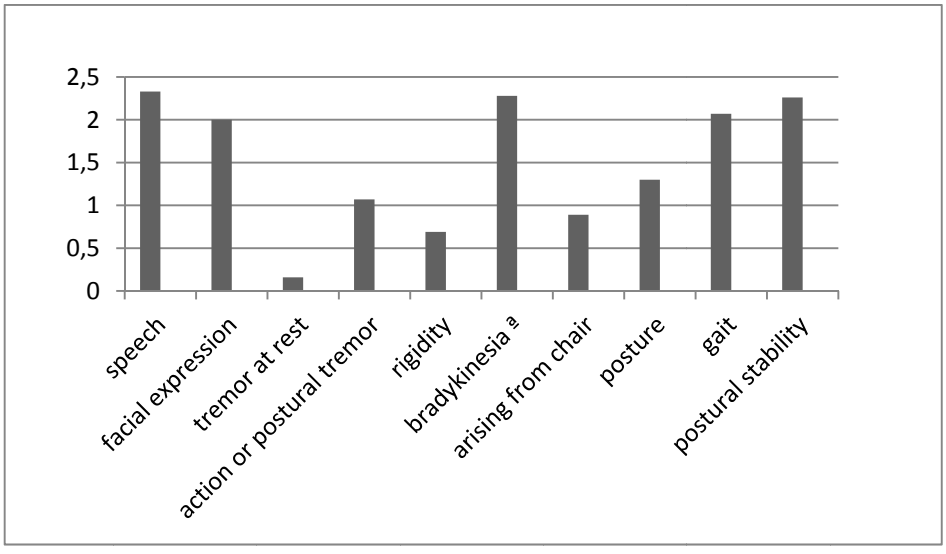


Figure 3. The average score of UPDRS part III motor domains, each domain is measured on a 5-point scale (0–4). [a]Sum of items 23–26 and 31

The average score of SE activities of daily living was 76.2±14.5%, indicating not complete independence; more difficulty with some chores; two to three times as long in some; must spend a large part of the day with chores.

Mean score in health related quality of life scale PDQ-39 was 43.4±17.5% and the most affected domains were mobility, communication, stigma and emotional well-being, the latter was not correlated with the UPDRS score (Figure 4).

Symptom severity measured by UPDRS, HY and SE was not influenced by the duration of exposure, but a positive correlation was observed between the duration of exposure and PDQ-39 (worse quality of life with longer exposure) ($r=0.41$; $p=0.014$). Worse scores in PDQ-39 were also correlated with worse results in UPDRS ($r=0.61$; $p<0.001$), HY ($r=0.58$; $p<0.001$) and SE ($r=–0.56$; $p<0.001$). HIV status did not affect PDQ-39 scores.

MMSE cognitive function average score was 28.4±2.0 (min 20). Two patients scored below 25, indicating cognitive impairment.

L-dopa challenge test showed no objective or subjective improvement.

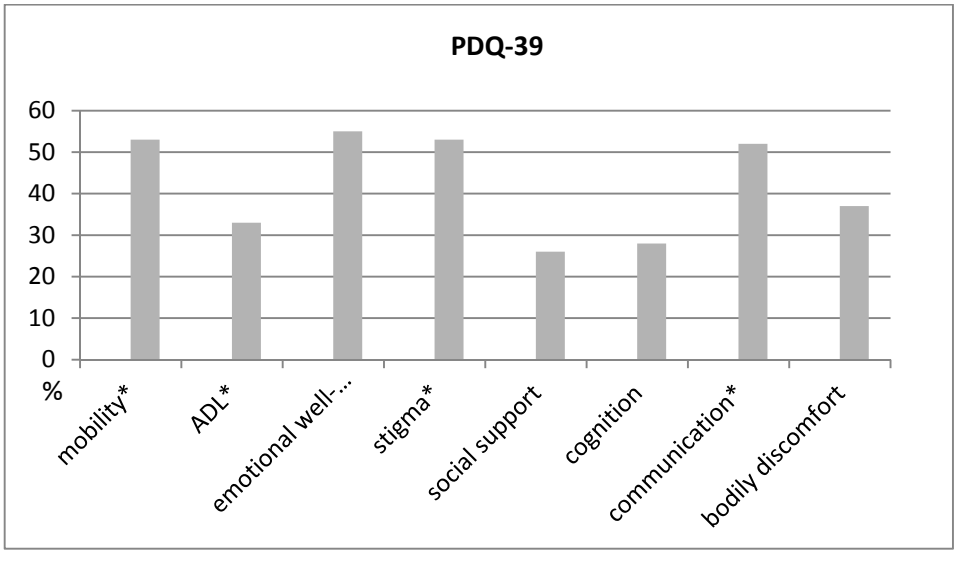


Figure 4. The average score of PDQ-39 domains. *Correlation with UPDRS score ($p<0.05$)

5.3. Follow-up

Results of follow-up examination are given in Table 3. Worsening of HY stage and SE scores reached statistical significance. The changes in all follow-up rating scales exept MMSE were significantly bigger if the interval between two evaluations was longer than a year. The changes in rating scales between former and active users were not different, but active users had a shorter follow-up time.

Table 3. Baseline and follow-up mean scores of assessment scales (±SD) in all ephedrone users and in active and former users separately

	ALL (n=24)			Active (n=12)			Former (n=12)		
	Baseline	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**
UPDRS total	43.3 (±20.9)	45.5 (±20.4)	0.488	36.6 (±22.1)	38.8 (±14.7)	0.680	50.1 (±17.9)	52.2 (±23.6)	0.548
UPDRS motor	27.3 (±15.5)	28.6 (±16.2)	0.558	21.5 (±14.6)	23.5 (±9.6)	0.600	33.1 (±14.7)	33.7 (±20.0)	0.814
Hoehn and Yahr	2.9 (±0.8)	3.3 (±0.9)	0.026*	2.6 (±0.6)	3.0 (±0.8)	0.220	3.1 (±0.8)	3.5 (±1.0)	0.054
Schwab and England	76.5 (±14.1)	68.3 (±18.4)	0.006*	82.1 (±12.3)	74.2 (±18.3)	0.124	70.9 (±14.0)	62.5 (±17.3)	0.009*
PDQ-39	43.8 (±15.8)	46.2 (±14.2)	0.457	45.5 (±19.2)	45.4 (±14.2)	0.990	42.0 (±12.0)	46.9 (±14.8)	0.013*
MMSE	28.3 (±2.3)	28.9 (±1.25)	0.009	27.7 (±2.7)	28.4 (±1.4)	0.169	28.9 (±1.7)	29.4 (±0.8)	0.339

UPDRS – Unified Parkinson's Disease Rating Scale, PDQ-39 – Parkinson's Disease Quality of Life Questionnaire, MMSE – Mini Mental State Examination; *$p<0.05$

5.4. Laboratory synthesis of the mixture

Based on the relative signal intensities of the M+H+ (m/z 164.2 for ephedrone and m/z 166.2 for pseudoephedrine, see Figure 5.) it can be evaluated that the ephedrone yield of the non-optimized reaction is approximately 44%. So, about 56% of pseudoephedrine is not converted.

The mixture was found to contain 595 ppm Mn (equals to 0.6 g/L or 10.8 mM). In tap water (from Uppsala, Sweden) the normal Mn concentration is <0.1 ppb (<0.1 ug/L).

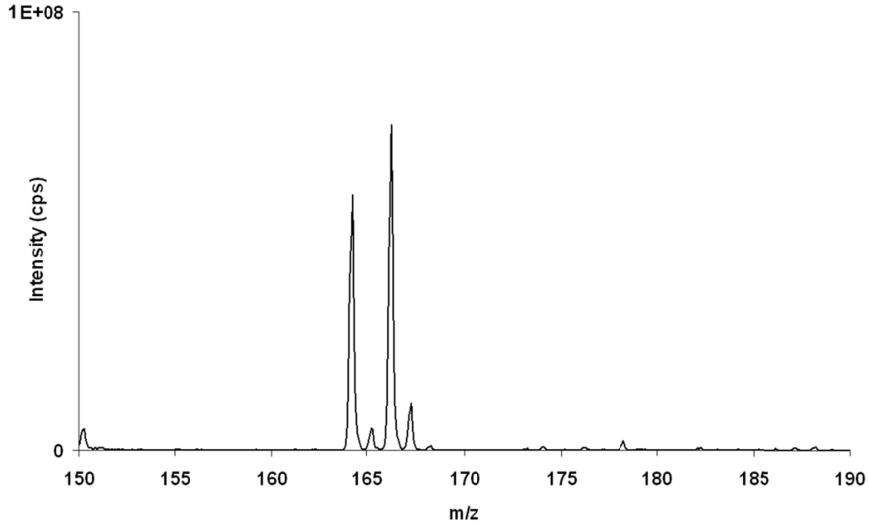


Figure 5. Direct infusion electrospray ionization mass spectrum of the synthesized drug and its precursor (M+H+. m/z 164.2 for ephedrone and m/z 166.2 for pseudoephedrine)

5.5. Laboratory tests

5.5.1. Virology, hepatic function, iron levels

All tested subjects (n=28) were positive for HCV, 18 (64%) were positive for HBV (HbsAg and/or anti-HBc positive) and 16 (57%) were positive for HIV. Bilirubin was mildly increased in 3 cases. Alanine transaminase levels were mildly elevated in 9 and moderately in 5 cases (more than 3 times times the upper limit). One subject had iron deficiency (low serum iron and ferritin, high transferrin).

5.5.2. Plasma and hair manganese concentration

The subjects had significantly higher mean level of Mn in hair (2.9±3.8 ppm) than controls (0.82±1.02 ppm), p=0.02. The concentration of Mn in former

users and in controls was not different (1.08±0.4, p=0.56) (Fig. 6). Samples in former users were collected on average 4.6±2.9 years after discontinuation of ephedrone use. At the individual level there was a large variablity in hair Mn level, in the control group the highest value was 4.5 ppm. The concentration of Mn in a former user with 8 years of abstinence and normal MRI was 3.7 ppm. On the other hand, an active user who had high plasma Mn concentration (19.3 ppb), had low Mn level in hair (0.81 ppm).

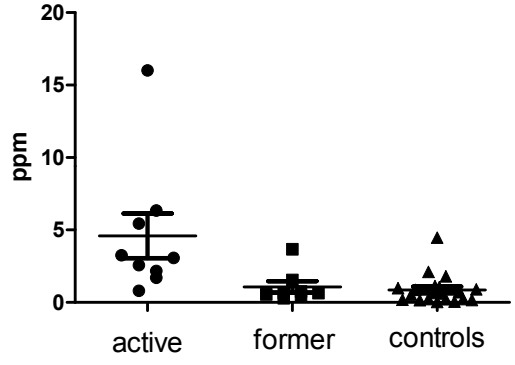


Figure 6. Mn concentration in hair in active and former users, and controls

Plasma Mn concentrations were significantly higher (11.5±6.2 ppb) in active than in former users (5.6±1.8 ppb), p=0.006. The average plasma Mn concentration in subjects (n=5) who reported ephedrone use on the same day when the samples were taken was 18.3±5.5 ppb and in the other active users 8.1±2.7, p=0.0003. The difference in plasma Mn levels between active users who did not report ephedrone use on the same day and former users was much smaller (p=0.02) (Fig. 7).

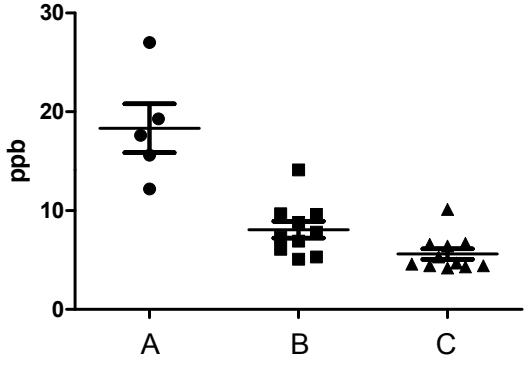


Figure 7. Mn concentration in plasma. A – active, ephedrone abuse on the same day; B – other active; C – former

5.6. Neuroimaging

5.6.1. MRI with T1 relaxation time measurements

Visual evaluation did not reveal pathological SI in any patient. Age and gender did not affect the T1 relaxation times or T2 SI in the healthy controls and SDs were small. In frontal white matter, both the SI on the T2-weighted images and the T1 relaxation times were similar in patients and controls. In the basal ganglia, some values were beyond two SD of the control values (Table 4).

Table 4. MRI measurement values beyond 2 SD of the healthy controls

Patient	Change in T1 relaxation times			Change in SI on T2-weighted images		
	Globus pallidus	Putamen	Caudate head	Globus pallidus	Putamen	Caudate head
1	↑ (R,L)	↑ (R)	↑ (L)	0	↑ (L)	0
2	↓ (R,L)	↓ (R,L)	↓ (R,L)	0	0	↑ (R,L)
3	↓ (R,L)	↓ (R,L)	↓ (R)	0	↓ (R)	0
4	0	0	0	0	0	0

R – right, L – left, 0 – no change beyond 2 SD, ↑ – > +2 SD, ↓ – < –2 SD, SI – Signal intensity

5.6.2. DAT SPECT

All four patients were visually classified as having a normal pattern of ^{123}I Ioflupane uptake. Also, the striatal binding ratio and the ratio between putamen and caudate uptake were estimated as normal (Table 5).

Table 5. Striatal dopamine transporter binding ratio

Patient	SBR* Caudate (R/L)	SBR* Putamen (R/L)	Putamen/CaudateRatio (R/L)
1	2.54/2.45	2.38/2.30	0.94/0.94
2	2.97/3.19	2.59/2.51	0.87/0.79
3	2.34/2.28	2.05/1.94	0.88/0.85
4	2.19/2.28	1.79/1.75	0.82/0.77

* Striatal Binding Ratios (SBRs) defined as: (uptake in region – non specific uptake)/ non specific uptake

5.6.3. FDG PET

All four patients showed a widespread, but not uniform, pathological pattern of FDG uptake with changes mainly located to the central part of the brain, including the basal ganglia, thalami, and the surrounding white matter (Fig. 8). Some of the areas were situated in the transition between grey and white matter. All but one of the pathological areas showed a decreased uptake of FDG. The only area of increased uptake was located in the white matter above the right ventricle (centrum semiovale, patient 3). No areas of significant increased FDG uptake were found in the basal ganglia and thalami (Table 6). None of the subjects selected from the database had any significant areas of pathological uptake.

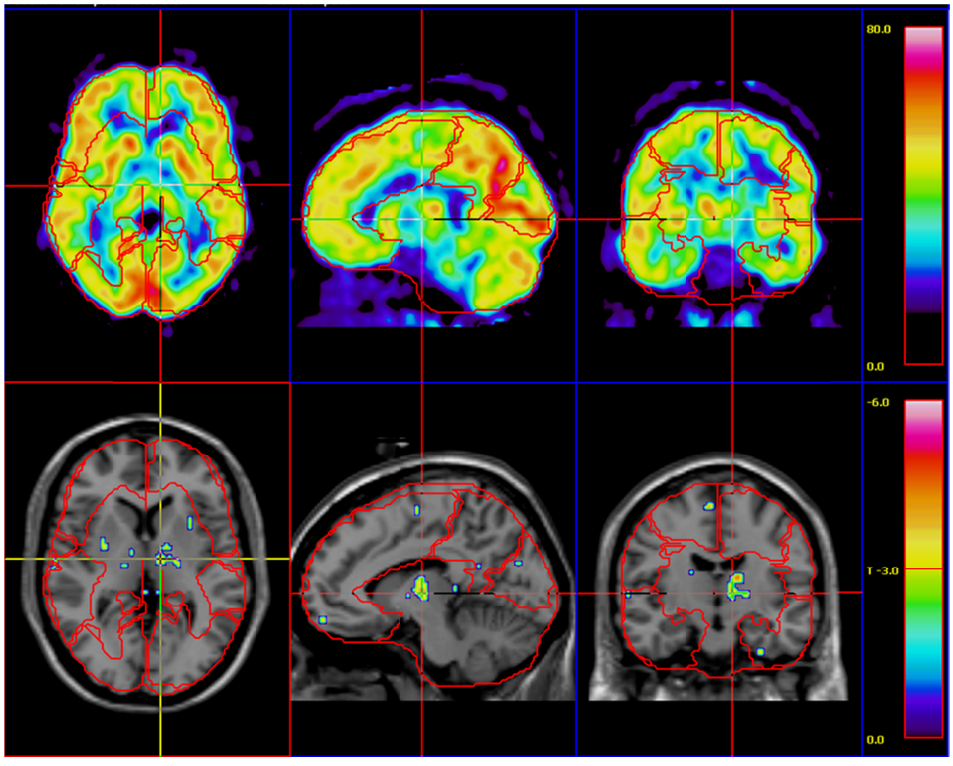


Figure 8. FDG PET in Patient 3. Decreased uptake in the left thalamus

Table 6. Regional increases and decreases in FDG uptake

Patient	Decrease	Increase
1	Putamen (R). Thalamus (R.L). Pons	
2	Internal capsule (R). Putamen (R)	
3	Thalamus (L). Putamen (R)	Centrum semiovale (R)
4	Putamen (R). Internal capsule (L). Centrum semiovale (R. L)	

R – right, L – left

5.6.4. MRI

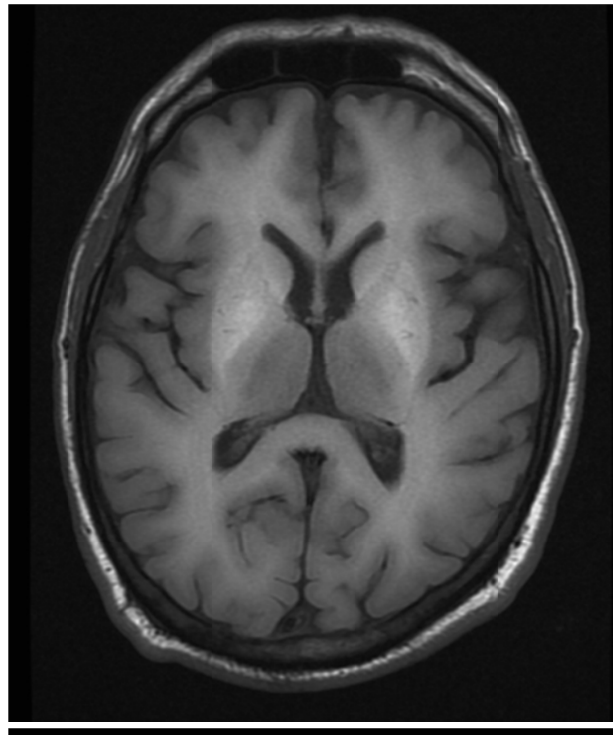
All active users had quite similar findings on MRI T1 weighted images: symmetric hyperintensity in globus pallidus, substantia nigra, periaquaductal grey matter and cerebral pedunculi, less in putamen, caudate nucleus, dentate nucleus and white matter (Table 7, Figure 9). No signal alterations were noted on T2-weighted, DWI and FLAIR images. No other abnormalities were seen on MRI.

Table 7. MRI findings and plasma Mn concentrations in active and former ephedrone abusers

	Duration of cessation	**Mn in plasma**							
Case	**(years)**	**(ppb)**	**PAGM**	**SN**	**CP**	**GP**	**NC**	**P**	**WM**	**ND**
1	4	NP	0	0	0	0	0	0	0	0
2	8	4.6	0	0	0	0	0	0	0	0
3	8	4.7	0	0	0	0	0	0	0	0
4	8	4.4	0	0	0	0	0	0	0	0
5	2.5	10.9	3	2	2	3	1	2	2	2
6	active	7.1	3	3	3	3	1	2	2	2
7	active	6.9	3	3	3	3	1	2	2	2
8	active	NP	3	2	1	3	0	0	2	1
	3	5.3	0	0	0	0	0	0	0	0
9	active	7.3	0	1	0	2	0	0	0	0
	3.5	NP	0	0	0	0	0	0	0	0
10	active	NP	3	0	3	3	0	0	1	2
	active	NP	2	0	2	2	0	0	0	1
11	active	NP	0	0	1	1	0	0	0	0
	2.5	NP	0	0	0	2	0	0	0	0

PAGM – periaquaductal grey matter, SN – substantia nigra, CP – cerebral peduncles, GP – globus pallidus, NC – nucleus caudatus, P – putamen, WM – White Matter, ND – nucleus dentatus, NP – not performed, 0 – normal, 1 – mildly hyperintense, 2 – moderately hyperintense and 3 – severely hyperintense (signal intensity agrees with fatty marrow tissue signal intensity)

A



B

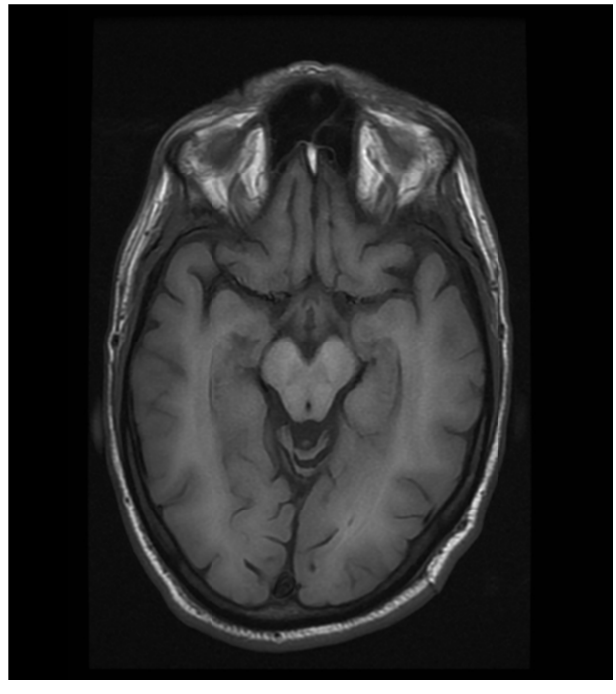


Figure 9. MRI T1-weighted axial images. A – increased signal in globus pallidus and B – substantia nigra

5.6.5. IBZM SPECT

IBZM SPECT showed normal symmetric tracer uptake in striatum. The average S/OC ratio in in subjects (1.85±0.2) (Table 8) was not lower than in PD patients (1.69±0.07), indicating preserved striatal dopamine D2 receptors.

Table 8. Binding ratios for ^{123}I-IBZM

Patient	dex		sin		
	Caudate/ OC	Putamen/ OC	Caudate/ OC	Putamen/ OC	Striatum total/OC
1.	1.84	1.67	1.76	1.57	1.71
2.	1.79	1.89	1.86	1.81	1.84
3.	1.58	1.60	1.56	1.64	1.60
4.	1.69	1.9	1.69	1.73	1.76
5.	2.12	2.06	1.86	2.08	2.03
6.	2.05	2.37	1.89	2.18	2.13
7.	1.73	1.57	1.60	1.55	1.61
8.	1.81	1.77	1.72	1.78	1.77

OC – Occipital cortex

5.7. Gene expression

Comparison of the blood RNA samples isolated from methcathinone users and healthy controls revealed distinct gene expression profiles. Statistically significant differential expression was observed for 326 genes, with the p-values less than 0.05 (FDR adjusted for multiple testing, Table 9). The magnitudes of expression signal differences (fold change or logFC) between these two groups were generally low: 17 genes were up-regulated in the methcathinone user more than 1.1-fold and only one gene was down-regulated more than 1.1-fold. However, the B-statistics values for the list of genes were very high, suggesting real biological differences between these groups. A heatmap (Figure 10) from unsupervised hierarchical clustering indicates clear clustering of samples into drug users and non-users. However, in the middle of the heatmap, some overlap and mixture between groups occurs. These samples are from methcathinone users with HIV negative status. Therefore, HIV status has a significant impact on the gene expression profile and is a strong confounding factor.

A functional annotation analysis was performed to define gene networks within the observed expression profiles. Relationships among the 326 genes significantly different in the methcathinone users compared with the control

groups included two major networks. The first, (with the highest score of 47; score indicates the –log of enrichment p-value) contained genes annotated with cell death, antigen presentation, or neurological disease functions (Table 10A, Figure 11). A second network (score 42) was related to immunological disease, cellular movement, or cardiovascular disease. Enrichment or activation of these networks suggests that methcathinone users have changes in the genetic pathways related to the function of the nervous and immune systems.

Table 9 includes many genes that are related to immune response, and may be differentially expressed compared with healthy controls because most of drug users were HIV positive. We therefore compared the subgroup of HIV negative drug users with the healthy control group. In this comparison, no genes were significantly different at FDR less than 5%, but 93 genes were different at or below a 30% FDR threshold. Annotation analysis for this dataset of HIV negative drug users indicated up-regulation (23 genes, score 54) in a network of genes with functions including hematological disease, cellular assembly and organization, or cell cycle (Table 10B). In another subgroup analysis we compared HIV positive subjects according to their user status, current versus discontinued. This comparison should indicate the difference caused by active drug abuse (Table 10C). In this case, both groups were HIV and HCV positive, so infection status was balanced. The most significantly enriched network in this comparison included genes in the Genetic Disorder, Immunological Disease, or Cellular Movement categories (25 genes, enrichment score 46). This network supports the involvement of the immune system in the drug abuse induced pathologies.

To further verify changes found with the GeneChip experiment, we performed quantitative real-time PCR and analyzed gene expression levels of eight selected genes. For seven genes, statistically significant differential expression between 20 healthy controls and 20 methcathinone users was seen in the expected direction of change (C15orf26 $p<0.0001$, GPR15 $p<0.0001$, IGFR1 $p=0.0002$, SNRPN $p=0.038$, IFI44L $p=0.0264$, IFI27 $p=0.0450$, IFNG $p<0.0001$). These results support the validity of our microarray gene expression analysis.

Table 9. Differential expression analysis of the blood RNA suggests significant differences between study groups

Probeset ID	Gene Symbol	logFC	AveExpr	adj. P-val	B-value	Gene Title
8081214	GPR15	1.6	8.2	1.11E-04	10.199	G protein-coupled receptor 15
8046488	CDCA7	0.6	6.5	1.11E-04	9.901	cell division cycle associated 7
7917283	MCOLN2	1.0	7.5	4.31E-04	8.329	mucolipin 2
7964787	IFNG	1.1	7.1	0.001	7.697	Interferon, gamma
8081799	TIGIT	1.1	8.4	0.001	7.607	T cell immunoreceptor with Ig and ITIM domains
8038861	SIGLEC6	0.3	7.3	0.001	6.485	sialic acid binding Ig-like lectin 6
8165653	ND1	0.4	13.2	0.001	6.463	NADH dehydrogenase, subunit 1 (complex I)
8175365	---	0.5	6.5	0.001	6.337	---
8165674	ND3	0.3	13.0	0.002	6.065	NADH dehydrogenase, subunit 3 (complex I)
8165648	C7orf11	0.5	12.5	0.002	6.027	chromosome 7 open reading frame 11
7946563	---	0.6	11.3	0.002	5.917	---
7898793	C1QA	0.5	6.0	0.002	5.834	complement component 1, q subcomponent. A chain
7903358	VCAM1	0.8	6.0	0.002	5.722	vascular cell adhesion molecule 1
8149979	C8orf80	0.5	7.3	0.002	5.684	chromosome 8 open reading frame 80
8169249	MID2	0.5	6.7	0.002	5.367	midline 2
7983365	TRIM69	0.5	5.9	0.003	5.237	tripartite motif-containing 69
8044154	CD8B	0.9	9.1	0.003	5.172	CD8b molecule
7944739	CRTAM	1.0	7.6	0.003	4.949	cytotoxic and regulatory T cell molecule
8053584	CD8A	0.9	9.9	0.003	4.873	CD8a molecule
8109639	PTTG1	0.5	7.8	0.003	4.751	pituitary tumor-transforming 1

Probeset ID	Gene Symbol	logFC	AveExpr	adj. P-val	B-value	Gene Title
7902660	WDR63	0.6	5.3	0.003	4.749	WD repeat domain 63
8019842	TYMS	0.8	6.7	0.003	4.682	thymidylate synthetase
7948420	FABP5	1.0	6.5	0.003	4.662	fatty acid binding protein 5 (psoriasis-associated)
8102643	CCNA2	0.6	6.8	0.003	4.653	cyclin A2
7917576	GBP5	1.0	11.1	0.004	4.466	guanylate binding protein 5
8002975	CDYL2	0.3	7.6	0.004	4.465	chromodomain protein. Y-like 2
7930577	CASP7	0.5	8.1	0.004	4.455	caspase 7, apoptosis-related cysteine peptidase
8151334	MSC	0.6	7.0	0.004	4.428	musculin (activated B-cell factor-1)
7898535	---	0.3	3.9	0.004	4.367	---
8018352	SLC25A19	0.3	6.7	0.004	4.307	solute carrier family 25 member 19
8053690	IGKC	1.0	9.4	0.004	4.291	immunoglobulin kappa constant
7981962	SNRPN	0.6	7.1	0.004	4.159	small nuclear ribonucleoprotein polypeptide N
7898805	C1QB	0.5	6.8	0.005	4.040	complement component 1, q subcomponent. B chain
7940028	SERPING1	1.4	8.7	0.005	3.978	serpin peptidase inhibitor, clade G (C1 inhibitor). member 1
7986359	IGF1R	0.7	8.6	0.005	3.914	insulin-like growth factor 1 receptor
8116130	FAM153B	-0.6	6.9	0.005	3.898	family with sequence similarity 153, member B
8043480	IGKV1OR15-118	0.9	10.9	0.005	3.863	immunoglobulin kappa variable 1/OR15-118 pseudogene

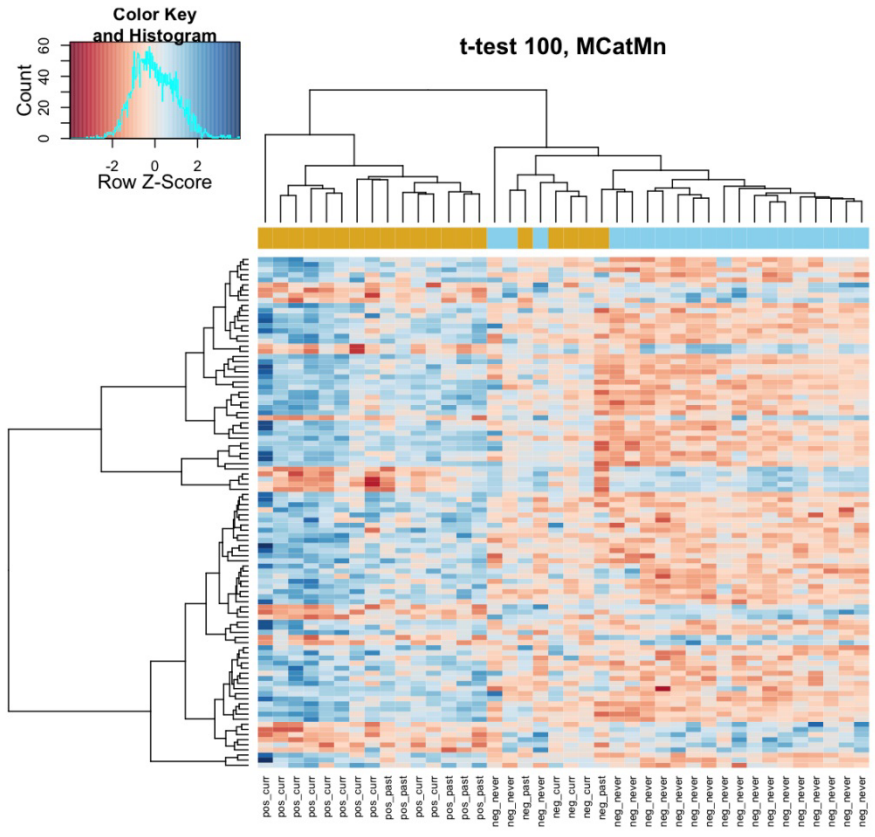


Figure 10. Methcathinone users group distinctly on the hierarchical clustering heatmap of blood gene expression levels. The top 100 genes from the decreasing ordered list of moderated t-values were clustered according to similarity in their gene expression patterns. Signals are scaled to Z-scores of the rows. The colored bar above the heatmap indicates the grouping variable – goldenrod for methcathinone user, blue for controls. Column labels at the bottom combine HIV status (positive or negative) with the drug user status (current, past, never)

Table 10. Genetic networks significantly changed. Score indicates the –log of enrichment p-value

Molecules in Network	Score	Focus Molecules	Top Functions
A. Genetic networks siginficantly changed in methcathinone users.			
BAK1,CD3,CHI3L2,ENG,EOMES,FBXO6,GBP5,GBP6,GBP7,GBP4 (includes EG:115361),GCH1,Growth hormone,HLA-DR,IFI27,IFI30,IFI44L,IFIT3,IFNG, KLF10,MT1F	47	28	Cell Death, Antigen Presentation, Neurological Disease
AGER,C2,C1q,C1QA,C1QB,CCR5,CD80,CD81,CD40LG,Complement component 1,Creb,CXCR6 (includes EG:10663),ERK,IFI44,IFITM3,IFN& alpha;/&beta	42	24	Immunological Disease, Cellular Movement, Cardiovascular Disease
B. Genetic networks siginficantly changed in HIV-negative methcathinone users, compared to controls.			
Actin,Adaptor protein 2,ANK1,Ap1,Ap2 alpha,AP2A1,Calmodulin,Caspase,CKS2,Clathrin,CLU,DAB2,EGF,EIF2AK1,EPB41,F Actin,FKBP8,GNAS,GUK1,HDGF,HSPB1,IgG,MPRIP,NFE2,NFkB (complex),NRGN	54	23	Hematological Disease, Cellular Assembly and Organization, Cell Cycle
AGTR1B,ANKH,BAT3,betaestradiol,C14ORF45,CA2,CDKN1B,CKS2,CTSA,CUL2,CUL4A,DGKD (includes EG:8527),GRINA,GYPA,HNF4A,MIR135A1, MIRLET7C,MMD,NBL1,NEU1,PHOSPHO1,progesterone	31	15	Amino Acid Metabolism, Cancer, Cell Morphology
Genetic networks siginficantly changed in HIV-positive methcathinone users, compared by user status.			
AICDA,CARD8,CCNH,CD40,CD226,CXCL5,DDR1,EIF2AK1,EMP1,ERN1,GYPA,IFIT1L,IFN Beta,Iga,Ige,IgG,IGHA1,IGJ,IGLL1,Igm,IL1,IL12 (complex),IL1RAP,Interferon alpha,IRAK1BP1,KLF3,MAP4K5,NFkB (complex),NLRP1,RIOK3,Sapk,THBD,TNFSF4,XCL1,ZNF675	46	25	Genetic Disorder, Immunological Disease, Cellular Movement
14-3-3,Adaptor protein 2,Ap1,ARHGEF12,CAMK2D,CCR3,CDC25A,CREG1,CSPG4,DAB2,DENND4A,DLEU2,E2f,ERK,GAB1,hCG,HDLBP,Histone h3,ITGB3,LDLRAP1,LRRFIP1,Mapk,MAX,MYO10,ORC1L,PDGF BB,PGRMC1,Pkc(s),Ras,RGS13,RICS,SKP1,STON2,TFDP2,YBX1	43	24	Cellular Assembly and Organization, Cancer, Cell Cycle

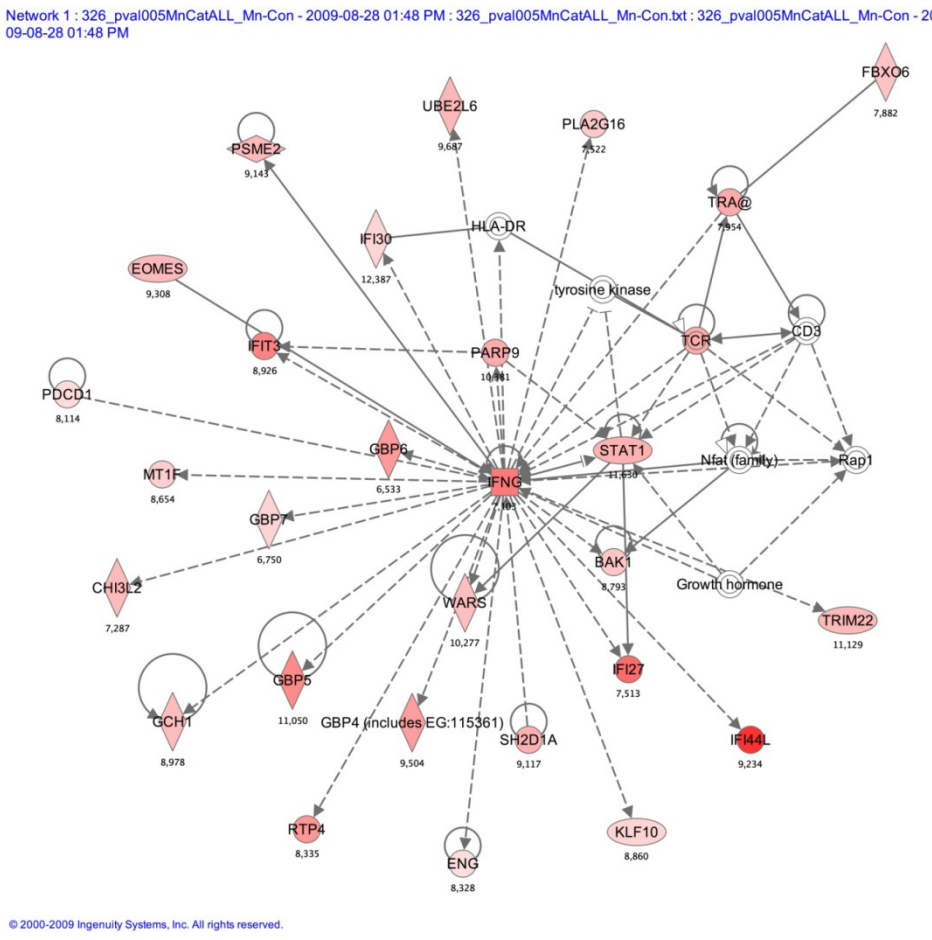


Figure 11. Annotation enrichment analysis indicated that a network including "cell death, antigen presentation, neurological disease" gene functions is significantly enriched in the subjects with methcathinone abuse (score 47, score is –log (p-value)). Red nodes designate up-regulated genes and the number indicates log2 fold change (0 is for equal expression). Uncolored nodes are genes in this network that were not in our list of differentially expressed genes

6. DISCUSSION

6.1. Clinical examination and laboratory tests

Because cases of manganism appeared following long-term total parenteral nutrition [Nagatomo et al., 1999], a recommendation of 0.1 mg as the maximal intravenous daily supplementation of manganese serves as the guideline today [Bertinet et al., 2000]. It is interesting to compare this recommended load to that administered in the cases of the present study. A daily manganese load of 0.18 g can be roughly calculated from the concentration of 0.6 g/L obtained in the chemical analysis and an average daily injection volume of 0.3 L. This means that the subjects were exposed to an extreme manganese load – almost 2,000 times higher than recommended.

Manganism has been described in a variety of occupational and non-occupational settings, but overt clinical manganism is rarely seen in current clinical practice. In our cohort, the syndrome was similar to parkinsonism reported in other ephedrone users and resembled classic Mn intoxication. The induced extrapyramidal syndrome tends to be symmetrical with bradykinesia, dystonias, early postural, gait, and speech impairment, and differs from the classical inital symptoms of PD. Rest tremor is rarely present and rigidity is mild.

The disability in the ephedrone abusers is mainly caused by postural and gait impairment and dystonias in a relatively early phase of the disease. In UPDRS, only a few items in the motor score are related to gait and postural impairment, and there are more items for evaluation of tremor, which is not a characteristic sign for parkinsonism in ephedrone users. Combining UPDRS with HY staging, Schwab and England ADL rating scale, and PD Quality of Life Questionnaire provides better assessment of the syndrome severity.

Symmetrical parkinsonism, usually without tremor, early falls, dysarthria, and dystonia are also characteristic symptoms of progressive supranuclear palsy (PSP) [Steele et al., 1964]. Vertical supranuclear-gaze palsy, a cardinal feature of PSP, was absent in our patients.

Progressive hepatolenticular degeneration, or Wilson's disease (WD), a rare genetic disorder of copper metabolism with a wide range of clinical presentations, including dystonia and parkinsonism, is another possible differential diagnosis. In tested study patients, copper and ceruloplasmin levels were normal. MRI in patients with WD has shown widespread lesions in the putamen, globus pallidus, caudate, thalamus, midbrain, pons, and cerebellum. Opposite to Mn intoxication, these lesions show high-signal intensity on T2 weighted images and low-intensity on T1 scan [Ala et al., 2007].

The quality of life is negatively influenced by disease severity, disability, and duration of exposure. The social situation is challenging in most abusers. In our patients, treatment with chelating agents was not suitable because Mn exposure in former users had stopped several years earlier and others continued ephedrone use, despite the substance abuse counselling. L-dopa, the mainstay treatment for PD, was not effective. Unresponsiveness to L-dopa has also been

confirmed in chronic occupational Mn intoxication [Lu et al., 1994]. We did not try L-dopa therapy in all of the subjects, becuse using prescribed dopamine enhancing drugs in HIV positive individuals could induce viral replication in the central nervous system [Gaskill et al., 2009].

The outcome of the neurological deficit after the cessation of ephedrone abuse is individual. Mild to moderate improvements have been observed in some cases [Levin, 2005; Sanotsky et al., 2007], but several cases progressions after withdrawal have been described [Colosimo and Guidi, 2009; Selikhova et al., 2008; Yildirim et al., 2009].

The present study is the largest follow-up study of ephedrone users. No improvement was noted in active or former users, and some assessment scales indicated statistically significant worsening. In active users the worsening was not significant, but the follow-up time of about one year may be insufficient for revealing a slow progression. Former users had stopped ephedrone use on average four years before the baseline study. Despite the long duration of exposure cessation, their disabilty was still worsening. These findings indicate the possibilty that Mn exposure may trigger a chronic neurodegenerative process. In a previous longitudinal study in four smelters with chronic Mn intoxication, a plateu occured after the initial ten years of disease progression [Huang et al., 2007].

Parkinsonism is a common complication of an HIV infection. Parkinsonian features usually develop due to HIV-associated dementia or underlying opportunistic infections, but also may occur due to the HIV infection [Tse et al., 2004]. Among the tested patients, 16 were HIV positive and 12 HIV negative. No MRI findings suggestive of opportunistic infections were found. Only one HIV positive patient had moderate cognitive impairment and in follow-up the score was surprisingly 25. There was no difference between the UPDRS scores of HIV positive and HIV negative patients. All of the tested patients were HCV positive, and a few of them had moderately increased alanine transaminase levels. Overt clinical signs or symptoms of hepatic cirrhosis were not noted. HIV and HCV infections do not cause the parkinsonian syndrome in ephedrone users, but in some cases possible additive effect on the developement of the syndrome cannot be ruled out.

Mn is rapidly eliminated from plasma and subjects with exposure on the same day had significantly higher plasma concentrations than other active users. Hair Mn level remains higher for a longer period. In a Ukrainian study, patients who had been off ephedrone for over 2.5 years had some elevation in pubic hair Mn levels [Selikhova et al., 2008]. In our series, hair Mn concentration in former users was not different from controls, but the subjects had been drug free on average for 4.6 years. There is a large variability in mean values of hair Mn. At an individual level Mn concentrations are not reliable biomarkers of exposure, due to fast elimination from plasma and large variability in hair.

Ephedrone users develop a distinctive extrapyramidal syndrome that is irreversible after the cessation of exposure, and there is a trend of worsening. Subjects are exposed to a high load of Mn and the clinical syndrome is similar

to classical manganism. The toxic role of Mn in the psychostimulant mixture is stressed by the fact that when during preparation potassium permanganate is substituted with sodium dichromate, sulfuric acid, acetone or toluene, the drug users avoid the extrapyramidal syndrome [Emerson and Cisek, 1993].

Ephedrone addiction is an alarming new cause of toxic parkinsonism. Ephedrone is a widespread recreational drug because it is relatively cheap and easy to prepare. The described cases could be just "the tip of the iceberg". In the most extreme cases, only a few months of ephedrone abuse could lead to severe and progressing disability.

The prognosis of the parkinsonian syndrome in the ephedrone users is poor as there is no curative therapy. The addicts seem to be unaware of the long-term side effects and deterioration. Though presently reported mainly in Eastern Europe, a global spread might occur, and the diagnosis needs to be considered within the social risk groups anywhere.

6.2. Neuroimaging

Shortened T1 relaxation in two of the patients may be a sign of some remaining Mn, although the cessation of exposure was many years before and the plasma values of Mn were normal. Increased SI on T2-weighted images is a common finding in many pathological conditions e.g. in gliosis or demyelination. Decrease of SI on T2-weighted images is more uncommon. Most often shortening or lengthening of T1 and T2 relaxation times occurs simultaneously and is a non-specific sign of tissue damage or alteration. This might explain the changes in patient 1 and 3 (Table 4). In patient 2, the changes were in opposite directions. It is possible that Mn shortens the T1 relaxation time, but tissue injury may increase the T2 SI. Although the number of patients is small, we have shown that there are some changes in the basal ganglia. This is in accordance with the few human neuropathological investigations which show clear evidence that the basal ganglia are primary targets for Mn neurotoxicity [Yamada et al., 1986].

Despite the neurological damage, cases which had stopped ephedrone abuse 0.5 to 2 years before had normal MRI signals [Iqbal et al., 2012; Yildirim et al., 2009]. However, in some studies, MRI signal change was still visible 3 to 5 years after the cessation of ephedrone use [Selikhova et al., 2008; Stepens et al., 2008]. The localization of T1 hyperintense signal in our study was similar to previous reports, but increased signal in white matter has not been described before. Widespread white matter abnormalities in ephedrone users were previously demonstrated with diffusion tensor imaging [Stepens et al., 2010]. We divided the subjects into former and active users according to the information provided. However, some cases admitted continuing abuse after the MRI results were revealed. High signal in globus pallidus on MRI T1-weighted images in some reported former users (Table 7, case 5 and 11) might be due to continuing ephedrone abuse or to impaired liver function. Alanine transaminase

levels were moderately elevated in both cases. In summary, high MRI signal intensities on T1-weighted images reflect recent exposure to Mn, but normal findings do not exclude former exposure. High signal intensities several years after cessation of exposure should awake a suspicion of active abuse, unless other susceptibility factors like chronic liver disease are present.

Besides Mn intoxication, another cause of pallidal T1 shortening is non-ketotic hyperglycemia. Patients typically present with chorea and hemiballismus. Follow-up neuroimaging usually shows the resolution of findings. The mechanism for the signal abnormalities is unkown. Depostition of proteins, myelin breakdown products, blood, or calcium or other minerals have been proposed [Lai et al., 1996].

Lasting reduction of DAT density has been shown in abstinent ephedrone and methamphetamine abusers without overt neurological or psychiatric illness by means of PET [McCann et al., 1998]. However, later studies in former methamphetamine abusers revealed significant recovery of DAT levels with protracted abstinence, suggesting that the changes were adaptive rather than neurotoxic [Volkow et al., 2001a]. Our normal DaTSCAN results indicate that the presynaptic neurons in the nigrostriatal pathway are intact in ephedrone abusers after prolonged abstinence. DAT SPECT studies in other ephedrone users have confirmed the normal integrity of the nigrostriatal dopaminergic terminals [Colosimo and Guidi, 2009; Selikhova et al., 2008]. Occupationally exposed workers with Mn-induced parkinsonism and patients with chronic liver disease had also normal DAT levels in the striatum [Huang, 2007; Kim et al., 2007], whereas there is asymmetric loss of DAT in PD [Kagi et al., 2010]. Instead, there could be an impairment of dopamine release as shown in studies with non-human primates chronically exposed to Mn [Guilarte et al., 2008; Guilarte et al., 2006]. This theory might explain why our patients reported progressively weaker psychostimulatory effect of ephedrone, acting as a dopamine releaser. A recent study demonstrated that Mn promoted internalization of the cell surface DAT into intracellular compartments, which may account for the decrease in dopamine uptake and release. When Mn and dopamine were added simultaneously to the media, cell toxicity was remarkably similar to that produced by Mn alone. Preincubation of dopamine prior to the addition of Mn resulted in cell death which was essentially additive with that produced independently by the two agents [Roth et al., 2013]. Previous observations have demonstrated that DAT is required for, and dopamine is an essential mediator of, methamphetamine induced striatal dopaminergic neurotoxicity [Fumagalli et al., 1998]. Mn exposure could be protective against ephedrone induced alterations in dopaminergic nerve terminals.

Untreated PD patients have normal or elevated levels of D2 receptors in the striatum [Brooks et al., 1992]. In atypical parkinsonian syndromes, D2 receptor bindings are usually decreased [Koch et al., 2007; Vlaar et al., 2008]. The present study shows no decrease in the level of postsynaptic D2 receptors in the striatum.

In typical idiopathic PD, glucose metabolism in the putamen and globus pallidus is preserved or raised, whereas it is reduced in most atypical parkinsonian cases [Brooks, 2010]. Detoxified methamphetamine abusers had higher metabolism in the parietal cortex and lower metabolism in the striatum and thalamus [Volkow et al., 2001b]. With protracted abstinence, there was significant recovery of the thalamic, but no improvement in striatal metabolism [Wang et al., 2004]. A single study in humans with mild manganism showed decreased cortical metabolism [Wolters et al., 1989]. The results of our PET study showed a widespread non-uniform pathological subcortical affection of FDG uptake. Whether these changes are mainly manganese induced, as the clinical syndrome, or correspond to the effect of long-term ephedrone abuse cannot be concluded.

Evidence from our neuroimaging studies support the hypothesis that accumulation of Mn in the brain is not associated with the degeneration of dopaminergic neurons as in PD, instead the dopaminergic system could be dysfunctional.

6.3. Gene expression

In this study, we examined changes in the gene expression profiles in peripheral blood induced by methcathinone abuse. All but one of the subjects had a clinically relevant extrapyramidal syndrome. RNA expression patterns of drug users clearly differed from those of healthy controls. Also, RT-PCR results confirmed differences between these two groups. The overlap between controls and HIV negative methcathinone users in the middle of the heatmap (Figure 10) suggested that most of the hierarchical clustering was driven by HIV infection status. During recent years several microarray studies of HIV induced alterations in host cell gene expression have been performed using *in vitro* infected primary cells, cell lines, or tissue samples [Giri et al., 2006]. However, few *in vivo* studies in HIV infected humans have evaluated expression profiles of tissue samples [Everall et al., 2005; Masliah et al., 2004] or peripheral blood mononuclear cells [Kottilil et al., 2009; Monaco et al., 2009; Motomura et al., 2004]. Several of the same apoptosis, antigen presentation and immune response genes (IFIT3, IFI27, IFI44, IFNG, NFAT, STAT1, TCR, CCR5, ERK, IFITM3, LY6E, IFNG, NFkB, OAS1, PLSCR1) described in previous HIV microarray studies were upregulated in our analysis. Thus, peripheral blood can be used for finding gene expression alterations in HIV infected humans. Besides cell death and antigen presentation, the genetic network with the highest score was also related to neurological disease. HIV infection can lead to a syndrome of neurological dysfunction termed HIV-associated neurocognitive disorders (HAND). Multiplication of HIV infected macrophages/microglia could result in the release of HIV proteins such as gp120 and Tat, which can impair DAT function, leading to increased levels of dopamine. Accumulated synaptic dopamine can activate adjacent microglia, which may result in increased HIV replication and icreased production of inflammatory mediators. HIV proteins

and inflammatory mediators can induce apoptosis of adjacent dopamineric neurons [Purohit et al., 2011]. The use of psychostimulants is a major comorbidity in the development of HAND. These drugs further increase extracellular dopamine, oxydative stress, and permeability of the blood-brain barrier, as well as exacerebrate the neurotoxic effects of gp120 [Silverstein et al., 2012].

Minor subgroup analysis of HIV negative users who were all positive for HCV indicated that genes related to cell cycle, cellular growth and proliferation, cancer, and cellular development were upregulated compared with healthy controls. The same pathways have been shown to be involved in HCV induced hepatic changes like cirrhosis, dysplasia, or hepatocellular carcinoma [De Giorgi et al., 2009; Wurmbach et al., 2007]. The presence of HCV co-infection among HIV-infected individuals increases neurologic disease burden and risk of death [Vivithanaporn et al., 2012]. The mechanism by which HCV contributes to that is not well defined, but there is evidence to suggest that HCV does invade the brain where it may exacerbate the neurotoxic inflammatory response [Laskus et al., 2005].

As the aim of our study was to analyze the drug abuse effect, we decided to stratify our group of subjects. We analyzed only HIV and HCV positive subjects according to their injection status (past versus current) and a clinical measure of the severity of the neurological syndrome UPDRS. The most significantly enriched network after comparing HIV positive current and past users included genes annotated with functions in Genetic Disorder, Immunological Disease, or Cellular Movement categories (25 genes, enrichment score 46). This network confirms the involvement of the immune system in drug abuse induced pathologies. However, as both comparison groups had neurological symptoms, we can't draw any conclusions on the potential causes of the extrapyramidal syndrome. On the other hand, injection status seems to influence the immune system and this is one factor involved in the development of a neurodegenerative syndrome [Frank-Cannon et al., 2009]. Psychostimulant abuse has been shown to compromise immunological status [Everall et al., 2005]. Chronic Mn exposure has similar effects on the immune system [Guilarte et al., 2008b; Sengupta et al., 2007]. Thus, both of these factors have potential to induce neurodegeneration.

The UPDRS score reflects the clinical severity of the extrapyramidal syndrome. No significant correlations were found between the duration of Mn-methcathinone use and UPDRS, or between blood RNA profiles and UPDRS.

There are several limitations to our study. The main problem is related to the sample organization. Our sample contains many confounding factors – HIV and HCV infection, different duration of methcathinone use, concomitant consumption of other drugs, alcohol, and tobacco. We have not analyzed the clinical status of the HIV and HCV infections. In addition, several drug abusers were undernourished, which makes identification of a relevant control group even more complicated. To overcome these limitations, we have applied complex bioinformatics analytical tools in relatively large samples (N=20 in both groups) that allowed us to perform some cohort stratification.

6.4. Future prospects

Though parkinsonism is not a feature of ephedrone abuse, it remains possible that the induced profound depletion of catecholamines could increase the vulnerability to extrapyramidal damage. To clarify the pathogenetic mechanisms underlying combined Mn and ephedrone toxicity experimental animal studies are being carried out. Very valuable would be an autopsy neuropathological study in humans.

For cases with severe generalized dystonia deep brain stimulation (DBS) could be a treatment option. DBS has been used successfully in various forms of dystonia, especially in the treatment of primary generalized dystonias [Volkmann et al., 2012], but improvement in secondary dystonias has been shown as well [Katsakiori et al., 2009].

Longer follow-up periods in former ephedrone users are necessary to provide information about the duration of progression or reversibility.

Ephderone users comprise a very important study cohort to understand the molecular mechanisms by which chronic exposure to Mn alters neurochemical pathways in the brain. Measuring the in vivo dopamine release using amphetamine-induced PET in ephedrone users could provide further information about Mn neurotoxicity, but there are ethical problems in giving amphetamine to the former users and difficulties in making arrangements with the current users.

Transcranial sonography can detect trace metal accumulation in deep brain structures with higher sensitivity than conventional MRI, as elevated trace metal content leads to an increased echogenicity. Hyperechogenicicity of the globus pallidus was detected in two patients with welding-related parkinsonism with normal MRI. Substantia nigra hyperechogenicity, characteristic for idiopathic PD, was absent in those patients [Walter et al., 2008]. Whether or not transcranial sonography of the former ephedrone users can be useful for detecting Mn accumulation and can differentiate it from PD, needs to be confirmed in future studies.

For further gene expression studies within similar populations, more balanced control samples are needed (e.g. drug-free HIV positive subjects, or comparison with other drug abusers). It would be useful to evaluate CD4 counts in HIV positive subjects to assess the immune system. Body mass index and plasma albumin measuring are simple means to diagnose malnutrition.

7. CONCLUSIONS

1. Ephedrone users develop a distinctive extrapyramidal syndrome that resembles classic Mn intoxication. The most prevalent symptoms are symmetrical bradykinesia, dystonias, early postural, gait, and speech impairment. Unlike what is seen in patients with idiopatic PD, rest tremor is rarely present and rigidity is mild; levodopa therapy is not effective.

2. Most of subjects have a disability that interferes with their activities of daily living and impairs the quality of life. Syndrome severity was not influenced by the duration of exposure; i.e., a severe motor impairment could develop only after a short time of ephedrone abuse. The syndrome is irreversible after cessation of exposure and there is a trend of worsening.

3. The injected solution contains high concentration of Mn. At an individual level, Mn concentrations are not reliable biomarkers of exposure, due to fast elimination from plasma and large variability in hair. High MRI signal intensities on T1-weighted images reflect recent exposure to Mn; normal findings do not exclude former exposure. High signal intensities several years after cessation of exposure should awake a suspicion of active abuse, unless other susceptibility factors like chronic liver disease are present.

4. MRI showed that Mn accumulates in globus pallidus, substantia nigra, periaquaductal grey matter, and cerebral pedunculi, and less in putamen, caudate nucleus, dentate nucleus and white matter. Functional imaging results indicate that the pre- and postsynaptic neurons in the nigrostriatal pathway are intact in ephedrone abusers. FDG PET demonstrated decreased brain metabolism mainly in the central part of the brain, including the basal ganglia, thalami, and the surrounding white matter.

5. Ephedrone users and healthy controls have distinct gene expression profiles, with most of the differences driven by HIV and HCV infection status. Comparing active and former HIV and HCV positive ephedrone users revealed involvement of the immune system and this is one factor involved in the development of a neurodegenerative syndrome.

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9. SUMMARY IN ESTONIAN

Mangaani ja efedrooni intoksikatsioonist tingitud neuroloogilise kahjustuse patogenees ning kliiniline sümptomaatika

Parkinonistlike sündroomide põhjuseid on palju, Parkinsoni tõbi on nendest kõige sagedasem. Parkinsoni tõve standarditud levimus Eestis Tartu populatsiooniuuringu andmetel oli 152 juhtu 100 000 elaniku kohta, sealjuures vanusespetsiifiline levimuskordaja alla 39-aastaste vanuserühmas oli 0 [Taba ja Asser, 2002]. Parkinsonistlikud sündroomid võivad tekkida ka teistel põhjustel: neurodegeneratiivsete haiguste, ravimite, aju koldeliste kahjustuste, vaskulaarsete haiguste, infektsioonide ja toksiinide tõttu [Cersosimo ja Koller, 2006]. Mangaanimürgistusega seotud parkinsonismi kirjeldati esimest korda juba 1837. aastal mangaanikaevanduse töölistel [Couper, 1837]. Kirjanduses kõige rohkem rõhutatud manganismi põhjuseks ongi mangaanikaevandustes töötamine [Mena jt, 1967] ja ka keevituselektroodide kasutamine [Koller jt, 2004]. Suu kaudu manustatuna ja inhaleerituna jõuab kesknärvisüsteemi ainult väike kogus mangaani, mistõttu neurotoksilisus on küllaltki harv ja tekib pikaaegse ekspositsiooni järel või kaasuva maksapuudulikkuse korral [Jankovic, 2005]. On mõned näited sümptomite arengust täielikul parenteraalsel toitmisel olnud patsientidel [Ejima jt, 1992].

Alates 1990. aastate lõpust on Eesti neuroloogide poole pöördunud parkinsonistliku sündroomiga noori patsiente. Esimeste haigete pöördumisel oli keeruline tuvastada haiguse tekkepõhjust, kuid hiljem selgus, et need patsiendid olid eelnevalt mingi aja jooksul intravenoosselt manustanud n-ö kodumeetodil valmistatud pseudoefedriini, kaaliumpermanganaadi, äädikhappe ja keeva vee segu. Pseudoefedriini sisaldavad mitmed nohu sümptomaatiliseks raviks näidustatud käsimüügipreparaadid. Kaaliumpermanganaat on tugev oksüdeerija. Reaktsiooni tulemusel tekib pseudoefedriinist efedroon ehk metkatinoon. Efedroon on psühhostimulant, mille toime on sarnane metamfetamiiniga, kuid enamasti vähem intensiivne. Toksilise kõrvalproduktina sisaldab valmis narkootiline segu ka mangaani.

Enne uuringu alustamist puudusid rahvusvahelises kirjanduses viited, et efedrooni tarvitamine on üks võimalik parkinsonismi tekkepõhjus. Kuigi psühhostimulandid mõjuvad dopaminergilistele neuronitele toksiliselt, ei arene tarvitajatel parkinsonistlikku sündroomi [Guilarte, 2001]. Samuti ei olnud varem kirjeldatud narkootikumide tarvitamist manganismi võimaliku põhjusena.

Eestis oli 2007. aastal Tallinna ning Ida-Virumaa süstivate narkomaanide seas korraldatud uuringu andmetel 700 küsitletu hulgas 11% (n = 76) neid, kes olid tunnistanud efedroonisegu tarvitamist, kuid peamiseks narkootikumiks oli efedroon vähem kui 1%-l (n = 6) [Lõhmus jt, 2008]. Tervise Arengu Instituudi hinnangu kohaselt on Eestis *ca* 13 800 süstivat narkomaani. Seega võiks isikuid, kes peamise narkootikumina süstivad efedrooni, olla Eestis umbes sadakond.

Uurimistöö eesmärgid

1. Kirjeldada efedrooni süstijatel esinevat neuroloogilist sündroomi ning hinnata spetsiaalsete skaalade abil parkinsonistlikku sündroomi, haiguse raskusastet ja elukvaliteeti.
2. Uurida efedrooni tarvitajatel neuroloogilise sündroomi kulgu.
3. Leida segu tarvitamise kindlakstegemiseks iseloomulikud biomarkerid.
4. Kirjeldada muutusi magnetresonantstomograafilises (MRT) ja funktsionaalsetes radioloogilistes uuringutes.
5. Teostada perifeerse vere geenikspressiooni analüüs, et selgitada neuroloogilise kahjustuse molekulaarset patogeneesi.

Uuritavad ja meetodid

Uuringu kiitis heaks Tartu Ülikooli inimuuringute eetika komitee. Kõik uuringus osalejad allkirjastasid teadliku nõusoleku vormi. Uuritavate kontaktandmed saadi Eesti suuremate haiglate neuroloogia ja psühhiaatria osakondade arstidelt, narkomaania rehabilitatsiooni keskustest ja efedrooni tarvitajatelt.

Haigeid intervjueeriti ning hinnati nende kliinilist neuroloogilist leidu, enamiku uuritavate neuroloogilist sündroomi ka filmiti. Hinnati parkinsonistlikku staatust, haiguse raskusastet, elukvaliteeti ja kognitiivset funktsiooni. Selleks kasutati järgmisi rahvusvaheliselt tunnustatud skaalasid: Parkinsoni tõve hindamise ühtlustatud skaala (UPDRS), Hoehni-Yahri skaala (HY), Schwabi-Englandi (SE) igapäevaste tegevuste skaala [Fahn S jt, 1987], Parkinsoni tõve elukvaliteedi küsimustik (PDQ-39) [Peto jt, 1995] ning vaimse seisundi miniuuring (MMSE) [Folstein jt, 1975].

UPDRS koosneb 31 osast, millest iga alajaotus annab 0 kuni 4 punkti, lähtudes sümptomi raskusest (0 – normaalne, 4 – väga väljendunud). Kogu testi maksimumskoor on 176 punkti. HY skaalaga hinnatakse haiguse raskusastet, võttes aluseks kliinilise leiu ja funktsionaalse võimekuse. HY skaala sisaldab staadiume 0 (leiuta) kuni 5 (ratastoolis või voodis; abitu). SE igapäevaste tegevuste skaala abil hinnatakse inimese iseseisvust protsentuaalselt 0 kuni 100%-ni (100% – täiesti iseseisev, ei tunneta mingeid raskusi; 0% – säilinud ainult vegetatiivsed funktsioonid). PDQ-39 on samuti protsentuaalne skaala, milles suurim tulemus näitab kehvimat hinnangut elukvaliteedile. MMSE maksimaalne tulemus on 30 punkti, normiks peetakse vahemikku 25 kuni 30. Levodopa raviefekti hindamiseks tehti neljale uuritavale levodopa (200 mg) ja karbidopa (50 mg) standarditud provokatsioonitest, mille tulemust hinnati ühe tunni möödumisel.

Korduvalt testiti 24 patsienti, keskmiselt 21 ± 15 kuud pärast esimest hindamist, sh 12 endist ja 12 narkootilise segu aktiivset tarvitajat.

Narkootilise segu süntees ja keemiline analüüs tehti Uppsala Ülikooli analüütilise keemia osakonnas. Sünteesitud efedrooni analüüsiti kvalitatiivselt elektropihustus-ionisatsiooni-mass-spektromeetriga. Mangaani (Mn 54,9) sisaldust lõpplahuses mõõdeti induktiivse plasma aatomemissioon-spektromeetriga.

Plasma (n = 26) ning karvade (juukse- + kubemekarvade) (n = 17) Mn-kontsentratsiooni analüüsiti induktiivse plasma aatomemissioon-spektromeetriga. Kontrolliks analüüsiti soo ja vanuse poolest sobitatud 21 Ida-Virumaa elaniku karvu.

Uppsala Ülikooli neuroteaduste osakonnas tehti MRT koos T1-signaali relaksatsiooniaja mõõtmisega; ioflupaaniga (^{123}I Ioflupane) üksikfooton-emissioon-kompuutertomograafiline (SPECT) uuring ning positronemissioon-tomograafiline uuring 2-desoksü-fluoro-D-glükoosiga (FDG-PET) neljal patsiendil, kes polnud efedrooni tarvitanud vähemalt neli aastat.

IBZMiga (^{123}I-iodobensamiid) SPECT (n = 8) ja MRT uuringud (n = 11, sh aktiivsed kasutajad) tehti Põhja-Eesti Regionaalhaiglas.

20 patsiendil ja 20 kontrollisikul eraldati RNA, tehti globiini redutseerimine ja hinnati geenieksprессiooni analüsaatoriga Affymetrix GeneChip. Kontrollidega võrreldes erinevalt avaldunud geenidest valiti välja 8 geeni, mille tulemused kinnitati kvantitatiivselt reaalaja PCRiga.

Uurimistöö tulemused ja arutelu

Ajavahemikul 2006–2012 uuriti kokku 38 patsienti, neist 31 meest ja 7 naist, kelle keskmine vanus oli 33 aastat (vahemikus 18–58 aastat). Põhiliselt olid patsiendid pärit Ida-Virumaalt (n = 29, 76%), väiksem osa Tallinnast ja Harjumaalt (n = 7, 18%), kaks Tartust. Peamiselt olid efedrooni tarvitajate hulgas vene rahvuse esindajad (97%), üks uuritav oli eestlane. Enamik patsiente oli koolis käinud kuni 9. klassini ehk oli põhiharidusega või alla selle (n = 25; 66%), 6 patsiendil oli keskharidus, 6-l kutseharidus ja ühel patsiendil kõrgharidus. 95% patsientidest olid töötud, 70%-l oli vormistatud invaliidsuspension. Abielus oli 10 patsienti, nendest 8 juhul olid mõlemad partnerid uuritavad. Suurem osa patsientidest olid vallalised (58%) või lahutatud (16%). Uurimise ajal elas sotsiaalmajas 32% patsientidest, kaks olid vanglas ja ühel kindel elukoht puudus. Ligikaudu pooled efedrooni tarvitajatest elasid üksi (46%), neljandik elas koos vanema(te)ga ja neljandik koos abikaasaga, viiendikul kuulus(id) perekonda ka laps(ed).

Patsiendid jagati neilt saadud anamneesi alusel kas aktiivseteks (n = 20) või endisteks (n = 18) efedrooni tarvitajateks (lõpetasid segu tarvitamise vähemalt üks aasta varem, keskmiselt olid nad lõpetanud selle 4,9 ± 2,2 a varem). Intravenoosselt olid nad süstinud korraga 8 kuni 20 ml paar kuni mitukümmend korda päevas. Segu komponentide vahekord (eelkõige kaaliumpermanganaadi osa) erines patsienditi veidi. Efedroon oli peamine tarvitatav narkootikum. Teisi stimulante (peamiselt amfetamiini) olid proovinud pooled narkomaanid ning 63% olid tarvitanud ka opiaaate (heroiini, fentanüüli ja moonivedelikku). Vanus efedrooni tarvitamise alustamisel oli 25 aastat (vahemikus 14 kuni 41 aastat). Kõige parema ülevaate mangaani võimalikust üldekspositsioonist andis efedrooni kasutamise kestus, mis oli keskmiselt 4,6 ± 3,9 aastat (0,25 kuni 13). Esimesed neuroloogilised kaebused tekkisid 2,8 ± 3,1 aastat (mõne kuu kuni 12

aasta jooksul) pärast tarvitamise alustamist. Esmassümptomiks märgiti 37% juhul kõnnaku muutus, 26%-l kõne muutus ja 24%-l tasakaaluhäire.

Uuritavate UPDRS-skoor oli keskmiselt 43 ± 21 punkti, millest UPDRSi III osa (motoorika) andis kokku keskmiselt 28 ± 15 punkti. UPDRSi III osa alusel olid peamisteks sümptomiteks jäsemete sümmeetriline bradükineesia (keskmine skoor 2,29 skaalal 0–4), posturaalsed (2,26), kõnnaku- (2,07) ja kõnehäired (2,33). Rahutreemorit enamasti ei ilmnenud (0,16) ja rigiidsust oli harva (0,69). Lisaks esines sageli jäsemete düstooniat, mida UPDRSi III-ga ei hinnata.

Kliinilise raskusastme järgi klassifitseerusid pooled patsientidest HY skaala alusel III staadiumisse (kerge kuni mõõdukas kahepoolne haigus; mõningane posturaalne ebastabiilsus; füüsiliselt sõltumatu). SE skaalal oli keskmine tulemus 76 ± 15% (mitte täiesti iseseisev; mõnede tegevustega rohkem raskusi; ajakulu on kaks kuni korda suurem kui tavaliselt; suur osa päevast kulub igapäevastele kodustele töödele). Elukvaliteedi küsimustiku PDQ-39 keskmine skoor oli 43 ± 18%. Kõige halvem oli hinnang elukvaliteedile füüsilise võimekuse (53%), emotsionaalse heaolu (55%), stigma (53%) ja suhtlemise (52%) alaosades. Elukvaliteet sõltus UPDRSi, HY ja SE skaala tulemustest. Inimese immuunpuudulikkuse viirusega (HIV) nakatunute elukvaliteet ei olnud halvem võrreldes HIVga mittenakatunutega. Seost süstimise kestuse ja kliinilise leiu raskuse (mõõdetuna UPDRSi, HY ja SE skaalaga) vahel ei olnud, kuid esines tarvitamise kestuse ja elukvaliteedi seos ($r = 0,41$; $p = 0,014$). MMSE keskmine tulemus oli 28,4 ± 2,0. Kahe uuritava punktisumma jäi alla 25, mis viitab kognitiivsele defitsiidile. Standarditud levodopatest ei näidanud objektiivset ega subjektiivset paranemist.

Korduvalt testimisel oli kõikide skaalade keskmine muutus patsiendi kohta halvenenud, HY ($p = 0,026$) ja SE ($p = 0,006$) skaala puhul oli halvenemine statistiliselt oluline. Kõikide skaalade muutus, v.a MMSE, oli oluliselt suurem, kui kahe testimise vahe oli pikem. Aktiivsete tarvitajate skaalade muutus ei olnud suurem kui endistel tarvitajatel, kuid aeg korduva testimiseni oli neil lühem.

Ligikaudu pool pseudoefedriinist (44%) oksüdeerus efedrooniks. Segu mangaanisisaldus oli 595 ppm ehk 0,6 g/l. Ravijuhendi järgi ei tohiks täielikult parenteraalsel toitmisel olevate patsientide maksimaalne päevane Mn-annus ületada 0,1 mg [Bertinet jt, 2000]. Uuritavad süstisid päevas umbkaudu 0,3 l lahust, seega kokku *ca* 180 mg mangaani, mida on soovituslikust päevaannusest ligi 2000 korda enam.

Patsiendid, kellele oli võimalik teha vereanalüüs viirusmarkerite määramiseks ($n = 28$), olid kõik nakatunud C-hepatiidiga ning 64% neist oli nakatunud B-hepatiidiga (HbsAg ja/või anti-HBc marker olid positiivsed) ja 57% olid HIV-positiivsed. Ühelgi uuritaval ei esinenud transaminaaside aktiivsuse väljendunud suurenemist ega maksatsirroosi kliinilisi sümptomeid. Rauavaegus (raua ja ferritiini sisalduse vähenemine ning transferriinisisalduse suurenemine) esines ühel patsiendil.

Uuritavate Mn-kontsentratsioon karvades (2,9 ± 3,8 ppm) oli oluliselt suurem kui kontrollidel (0,82 ± 1,02 ppm), $p = 0,02$. Endiste tarvitajate ja

kontrollide vahel erinevust ei olnud (1,08 ± 0,4; p = 0,56) ning see oli tõenäoliselt tingitud sellest, et endised tarvitajad olid süstimise lõpetanud keskmiselt juba 4,6 aastat enne proovide kogumist. Individuaalselt hinnatuna oli karvade Mn-kontsentratsioon väga erinev, kontrollrühma suurim väärtus oli 4,5 ppm. Samas oli ühe suure plasmakontsentratsiooniga aktiivse tarvitaja Mn-tase karvades madal (0,81 ppm).

Aktiivsete tarvitajate plasma Mn-kontsentratsioon (11,5 ± 6,2 ppb) oli oluliselt suurem kui endistel tarvitajatel (5,6 ± 1,8 ppb); p = 0,006. Samal päeval efedrooni süstinud aktiivsete tarvitajate (n = 5) keskmine plasma Mn-kontsentratsioon oli 18,3 ± 5,5 ppb ja teistel aktiivsetel tarvitajatel 8,1 ± 2,7; p = 0,0003. Erinevus analüüside võtmisega samal päeval mittesüstinud aktiivsete tarvitajate ja endiste tarvitajate vahel oli ka oluline, kuid palju väiksem (p = 0,02).

MRT T1- ja T2-signaaliga relaksatsiooniajad basaaltuumades erinesid kolmel patsiendil kontrollidest enam kui 2 standardhälvet. See võib viidata vähesele Mn-jäägile, kuid sobib ka mittespetsiifilisele koekahjustusele või muutusele. Üksikud neuropatoloogilised uuringud inimestel Mn-mürgistuse korral on samuti näidanud, et peamiselt on kahjustatud basaaltuumad [Yamada jt, 1986].

Dopamiini transportijate (DAT) ioflupaaniga SPECT-uuringu alusel ei esinenud uuritavatel presünaptilist dopaminergilist kahjustust. Ka mangaanimürgistusega seotud parkinsonismi korral töölistel või kroonilise maksahaigusega patsientidel on DAT-tase olnud *striatum*'is normis [Huang, 2007; Kim jt, 2007]. Parkinsoni tõve korral on DAT-tase asümmeetriliselt alanenud ja dopamiini 2. tüüpi (D2) resteptorite tase *striatum*'is normis [Kagi jt, 2010]. Atüüpilise parkinsonismi puhul on enamasti *striatum*'i D2-retseptorites märkaine kogunemine alanenud [Koch jt, 2007; Vlaar jt, 2008]. Efedrooni tarvitajatel tuli IBZMiga SPECT-uuringul nähtavale normaalne sümmeetriline märkaine kogunemine *striatum*'ite projektsioonis.

FDG-PET-uuring viitas laialdasele, kuid mittespetsiifilisele aju metabolismi aeglustumisele peamiselt basaaltuumades, talamustes ja ümbritsevas valgeaines. Pole võimalik järeldada, kas muutused on tingitud mangaani või efedrooni kahjustusest. Püsivat *striatum*'i metabolismi aeglustumist on täheldatud ka endistel metamfetamiini tarvitajatel [Wang jt, 2004].

Kõigil aktiivsetel kasutajatel oli MRT-leid sarnane: sümmeetriline T1-signaali hüperintensiivsus peamiselt *pallidum*'is, *substantia nigra*'s, ajuveejuha ümbruses hallaines ja ajujalakestes, vähem *putamen*'is, *nucleus caudatus*'es, *nucleus dentatus*'es ja valgeaines. Enamikul endistel tarvitajatel oli signaali intensiivsus normaliseerunud.

Analüüsitud 28 869 geenist oli 326 geeni avaldumus patsientidel ja kontrollrühmas statistiliselt erinev. Tulemusi analüüsides selgus, et enamik erinevustest oli seotud patsientide HIV-positiivsusega. Seetõttu võrreldi järgnevas analüüsis ainult HIV-negatiivseid patsiente (n = 5) kontrollidega. Selgus, et erinevalt oli avaldunud 93 geeni. Kasutades analüütilist tarkvara Ingenuity Pathway Analysis (IPA), leiti, et aktiveeritud olid geneetilised rajad, mis on seotud hematoloogiliste haigustega, rakutsükli reguleerimisega ja aminohapete metabolismiga. Järgnevalt võrreldi omavahel aktiivseid ja endisi HIV-positiivseid tarvitajaid

(selles võrdluses olid mõlema rühma uuritavad HIV- ja C-hepatiidi viiruse suhtes (HCV) positiivsed ehk infektsiooni staatus oli balansseeritud). Selles võrdluses tuli esile geneetiline rada, mis on seotud geneetiliste häirete, immunoloogiliste haiguste ja rakulise liikumisega. Et tuvastada, millised muutused on olulised kliinilise sündroomi tekkes, rakendati HIV-positiivsete geenieksprессiooni väärtuste ja UPDRS-skoori suhtes lineaarset regressiooni. Mitmete geenide väljendumismuster oli seotud kliinilise raskusastmega, kuid kahjuks puudusid paljude geenide kohta selged annotatsioonid, mistõttu ei olnud vastavaid geene võimalik leida ja ka statistiline erinevus polnud eriti suur.

Uurimistöö järeldused

1. Efedrooni tarvitajatel tekib parkinsonistlik sündroom, mis sarnaneb mangaani klassikalise intoksikatsiooniga. Enam väljendunud sümptomid on sümmeetriline bradükineesia, düstooniad, varased kõnnaku-, posturaalsed ja kõnehäired. Idiopaatilisest Parkinsoni tõvest erineb kliiniline pilt eelkõige enamasti rahutreemori ja rigiidsuse harva esinemise ning L-dopa raviefekti puudumise tõttu. Enamikul efedrooni tarvitajatel on puue, mis segab igapäevaeluga toimetulekut ja halvendab elukvaliteeti.
2. Puudub seos efedrooni kasutamise kestuse ja haiguse kliinilise raskuse vahel ehk ka siis, kui narkootikumi tarvitada väga lühikese perioodi jooksul, võib tarvitamise järel tekkida raske motoorne defitsiit. Kahjustus on pöördumatu ja haiguse progresseerumise tendents esineb ka pärast tarvitamise lõpetamist.
3. Süstitava segu mangaanisisaldus on suur, uuritavad on eksponeeritud mangaanisisalduse soovituslikust normist ligi 2000 korda suurematele väärtustele. Individuaalsel tasemel on efedrooni tarvitamist raske kindlaks teha. Veres on mangaani metabolism kiire ja enamasti plasma mangaaniväärtused normaliseeruvad ruttu. Juuksekarvades püsib suur mangaanikontsentratsioon kauem, kuid esineb suur normiväärtuste variaablus. MRT T1-signaali intensiivsuse suurenemine *pallidum*'is viitab mangaaniekspositsioonile, normileid ei välista varasemat kokkupuudet. MRT T1-signaali hüperintensiivsus aastaid pärast efedrooni tarvitamise lõppu võib viidata narkootikumi tarvitamise jätkumisele, kui ei kaasne teisi mangaaniladestumist soodustavaid tegureid, näiteks maksapuudulikkust.
4. MRT-uuringute tulemused näitavad, et mangaan ladestub peamiselt *pallidum*'is, *substantia nigra*'s, ajuveejuha ümbruses hallaines ja ajujalakestes. Pre- ja postsünaptilises dopaminergilistes retseptorites olulist defekti ei ole, võimalik, et kahjustatud on dopamiini vabanemine. FDG-PET-uuringu tulemused näitavad aju ainevahetuse aeglustumist peamiselt basaaltuumades, talamuses ning neid ümbritsevas valgeaines.
5. Efedrooni tarvitajate ja kontrollrühma geenieksprессiooni profiil on erinev. See tuleneb peamiselt uuritavatel kaasuvatest HIV- ja HCV-infektsioonidest. Võrreldes aktiivseid ja endiseid tarvitajaid (mõlemad rühmad ka HIV- ja HCV-positiivsed), oli praegustel tarvitajatel immuunsüsteem aktiveerunud. Immuunsüsteemil on oluline roll ka neurodegeneratiivsete haiguste tekkes.

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PUBLICATIONS

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