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Increased densities of nitric oxide synthase expressing neurons in the temporal cortex and the hypothalamic paraventricular nucleus of polytoxicomanic heroin overdose victims: Possible implications for heroin neurotoxicity

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ABSTRACT

Heroin is one of the most dangerous drugs of abuse, which may exert various neurotoxic actions on the brain (such as gray matter loss, neuronal apoptosis, mitochondrial dysfunction, synaptic defects, depression of adult neurogenesis, as well as development of spongiform leucoencephalopathy). Some of these toxic effects are probably mediated by the gas nitric oxide (NO). We studied by morphometric analysis the numerical density of neurons expressing neuronal nitric oxide synthase (nNOS) in cortical and hypothalamic areas of eight heroin overdose victims and nine matched controls. Heroin addicts showed significantly increased numerical densities of nNOS immunoreactive cells in the right temporal cortex and the left paraventricular nucleus. Remarkably, in heroin abusers, but not in controls, we observed not only immunostained interneurons, but also cortical pyramidal cells. Given that increased cellular expression of nNOS was accompanied by elevated NO generation in brains of heroin addicts, these elevated levels of NO might have contributed to some of the known toxic effects of heroin (for example, reduced adult neurogenesis, mitochondrial pathology or disturbances in synaptic functioning).

Introduction

Heroin (alias diamorphine, diacetylmorphine, or morphine diacetate) is an opioid analgesic and one of the most dangerous drugs of abuse. Chemically, it is the 3,6-diacetyl ester of morphine. Heroin is converted to morphine inside the body. It may thus be regarded as a pro-drug of morphine. Frequent and regular administration of this drug is associated with the development of tolerance and physical dependence (Sawynok, 1986). Heroin addiction is characterized by a compulsive drive to take the drug despite serious consequences. As with other drugs of abuse, heroin in many ways interacts with different brain systems, profoundly affecting both affective and cognitive processes (reviewed in Li and Sinha, 2008). The core neuroanatomical substrate for escalated heroin intake is the so-called extended amygdala, which receives afferents from limbic and olfactory cortices and projects to the hypothalamus and midbrain, thus linking the basal forebrain to the classical reward systems of the lateral hypothalamus via the medial forebrain bundle reward system (reviewed in Koob et al., 2004). The main target of heroin in the brain is the μ-opiate receptor. Various neurotransmitter and neuromodulator systems, such as dopamine, glutamate, GABA, serotonin, noradrenaline and certain hypothalamic neuropeptides, are known to be implicated in heroin addiction (Economidou et al., 2011; Kreek et al., 2012; Urban and Martinez, 2012; Wu et al., 2012; Yang et al., 2012, and others). Interestingly, clinical and experimental evidence has accumulated over the years to show that heroin is a clinically relevant neurotoxin, which is involved in gray matter loss, neuronal apoptosis, mitochondrial dysfunction, synaptic defects, depression of adult neurogenesis, and the development of spongiform leucoencephalopathy (Pearson et al., 1976; Wolters et al., 1982; Salach et al., 1984; Ren et al., 1998; Eisch et al., 2000; Nath et al., 2002; Jia et al., 2005; Schlaepfer et al., 2006; Cunha-Oliveira et al., 2007; Tramullas et al., 2008; Lai et al., 2011; Qui et al., 2013; Tan et al., 2013). The mechanisms leading to the diverse poisonous effects of heroin are complex and not yet fully understood.

Abbreviations: CRF, corticotropin releasing factor; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase (65/67); GS, glutamine synthetase; HIV, human immunodeficiency virus; NADPH, nicotinamide adenine dinucleotide phosphate; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PVN, paraventricular nucleus; SON, supraoptic nucleus; TC, temporal cortex; VF, volume shrinkage factor.

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Moreover, with regard to studies with humans it should be emphasized that heroin users are often polytoxicomanic (i.e. taking several other drugs in addition to heroin). Since these substances (alcohol, cannabis, cocaine, codeine, benzodiazepines and others) may have their own harmful effects to the brain, it is sometimes difficult to determine whether or not the observed pathological alterations are merely due to heroin, to other drugs, or a combination of both (see Discussion Section). Some of heroin's identified actions, however, undoubtedly involve the gas nitric oxide (NO), increased amounts of which are formed in the brain under the influence of chronic heroin/morphine intake (Bhargava and Cao, 1997; Kolesnikov et al., 1997; Zang et al., 1999; Cuéllar et al., 2000; Zhang and Wang, 2003; Yoo et al., 2006) most probably as the result of drug-dependent NMDA receptor activation (reviewed in Kuljis et al., 2002; Yoo et al., 2006; Kreek et al., 2012). NO acts as an important signaling molecule. NO and its metabolites are known to be involved in a plethora of cell physiological and pathological events, including those which are pathophysiologically related to heroin neurotoxicology such as reduced adult neurogenesis (Packer et al., 2003; Volobouéva and Giffard, 2011; Carreira et al., 2012), mitochondrial pathology (Mahadik and Mukherjee, 1996; Erusalimsky and Moncada, 2007; Volobouéva and Giffard, 2011) and compromised synaptic synthesis and re-modeling (Sunico et al., 2005; Cho et al., 2009). Heroin/morphine induced, elevated brain NO levels are thought to mainly result from increased activity of inducible nitric oxide synthase (iNOS; Zang et al., 1999; Lysle and How, 2000; Lanier et al., 2002; Szczytkowski and Lysle, 2007, 2010; Dyuizen and Lamash, 2009), but findings from different laboratories show that neuronal nitric oxide synthase (nNOS) activity may also be involved (Kolesnikov et al., 1997; Cuéllar et al., 2000; Özek et al., 2003; Zhang and Wang, 2003; Yoo et al., 2006). Reported strong up-regulation of nNOS in different brain areas of mice after repeated morphine exposure (Cuéllar et al., 2000; Yoo et al., 2006), as well as increased histochemical staining for NADPH diaphorase in cortical neurons of heroin addicts (Kuljis et al., 2002), prompted us to look at the cellular expression of nNOS in different brain regions of heroin victims and to estimate the numerical densities of immunostained cells. In this study we show that in brains of polytoxicomanic heroin overdose victims the neuronal expression of nNOS is significantly increased in some, but not all, brain regions, which might be a contributing factor to heroin neurotoxicity.

Materials and methods

Human subject characteristics

All brains were from the Magdeburg Brain Bank. Sampling of the human brain material and preservation were done in accordance with the Declaration of Helsinki (1964), German law and approval by the local Ethics commission. Analysis included eight chronic heroin abusers who died from a drug overdose (seven males and one female, aged 29.4 ± 3.8 years; post-mortem interval 22.5 ± 11 h) and nine controls (i.e. non-drug using subjects; five males and four females; aged 30.8 ± 5.4 years; post-mortem interval 38.4 ± 13 h, see Tables 1 and 2). An ANOVA was performed employing these parameters, which did not reveal significant differences among the groups concerning age and post-mortem delay. In addition to heroin, all but one drug abusers had a history of taking other legal and/or illegal substances, including morphine glucuronides, cannabis, alcohol, cocaine, barbiturates, benzodiazepines and hallucinogens (see Table 2). All patients were matched for age, gender and post-mortem delay. The matching processes were done prior to all analyses. Information for clinical diagnosis was obtained by the careful study of clinical records and by structured interviews with people who either lived with, or had frequent contact with, the subjects before death.

Qualitative neuropathological changes due to neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Pick's disease), tumors, inflammatory, vascular or traumatic processes were ruled out by an experienced neuropathologist or legal medical officer. None of the heroin addicts was HIV-positive.

Tissue processing and histology

All brains were obtained from pathologists or medical examination officers. Tissue preparation was performed as previously described (Bernstein et al., 1998). Brains were removed and fixed *in toto* in 8% phosphate-buffered formaldehyde for at least two months. Frontal and occipital poles were separated by coronal cuts anterior to the genu and posterior to the splenium of the corpus callosum. After embedding all parts of the brains in paraffin wax, serial, whole brain coronal sections of the middle block were cut on a large-scale microtome (Balzers, Liechtenstein) at 20 μm and mounted. Every 50th section was stained for anatomical orientation and morphometric investigations (see below) with a combined cell and fiber staining according to Nissl (cresyl violet) and Heidenhain-Woelcke procedures (Bernstein et al., 1999). Volume shrinkage was determined for each brain before and after dehydration and embedding of tissue. Volume shrinkage factors were calculated using the formula: $VF = (A1/A2)^{3/2}$ (VF = volume shrinkage factor; A1 = cross-sectional area before processing of tissue; A2 = cross sectional area after processing of tissue). Mean shrinkage factor was 2.18 for control brains and 2.15 for heroin addicts.

Immunolocalization of neuronal nitric oxide synthase (nNOS)

Mounted sections were deparaffinized and hydrated. To detect nNOS immunoreactivity we first carried out antigen demasking of the dewaxed sections by boiling them for 4 min in 10 mM citrate buffer (pH 6.0). After pre-incubation with methanol/H_2O_2 to inactivate endogenous peroxidases, the samples were washed with phosphate-buffered saline. A polyclonal rabbit nNOS antiserum (Eurodiagnostica AB, Stockholm, Sweden) by Western blotting and immunocytochemistry was added at a 1:1000 dilution and the samples were incubated for 48 h at 7 °C. Further immunocytochemical protocols involved being incubated with goat anti-rabbit IgG serum (Amersham, Freiburg, Germany) and the application of the avidin-biotin technique (Amersham, Freiburg, Germany). The chromogen 3,3′-diaminobenzidine (DAB) was used to visualize the reaction product. The color reaction was enhanced by adding nickel ammonium sulphate solution to the diaminobenzidine, as described previously (Bernstein et al., 1999). The procedure yielded a dark purplish-blue to dark-blue color reaction product. The specificity of the nNOS polyclonal antibody serum was tested by the manufacturer (Eurodiagnostica AB, Stockholm, Sweden) as well as by Western blotting and immunocytochemistry (Bernstein et al., 2000; Bielau et al., 2012). Additional controls involved replacement of the primary antiserum either by a buffer or with normal serum. Specificity of the primary antiserum was further demonstrated by immunoabsorption with a blocking peptide (SA-227, Biozol, Eching, Germany) or with the highly purified nNOS (rat, recombinant, Biozol) as described earlier (Bernstein et al., 2001). Sections without the specific primary antiserum (or with pre-absorbed antiserum) did not show any specific immunostaining (Fig. 1D).

In order to better characterize nNOS expressing cell populations we used two cellular markers for comparison: GAD (marker of GABAergic neurons) and glutamine synthetase (marker of a majority of astroglial cells).