

Opioid neuropeptide genotypes in relation to heroin abuse: Dopamine tone contributes to reversed mesolimbic proenkephalin expression

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Striatal enkephalin and dynorphin opioid systems mediate reward and negative affect, respectively, relevant to addiction disorders. We examined polymorphisms of proenkephalin (PENK) and prodynorphin (PDYN) genes in relation to heroin abuse and gene expression in the human striatum and the relevance of genetic dopaminergic tone, critical for drug reward and striatal function. Heroin abuse was significantly associated with *PENK* polymorphic 3' UTR dinucleotide (CA) repeats; 79% of subjects homozygous for the 79-bp allele were heroin abusers. Such individuals tended to express higher *PENK* mRNA than the 81-bp homozygotes, but *PENK* levels within the nucleus accumbens (NAc) shell were most strongly correlated to catecholamine-*O*-methyltransferase (*COMT*) genotype. Control Met/Met individuals expressed lower *PENK* mRNA than Val carriers, a pattern reversed in heroin users. Up-regulation of NAc *PENK* in Met/Met heroin abusers was accompanied by impaired tyrosine hydroxylase (TH) mRNA expression in mesolimbic dopamine neurons. In contrast to *PENK*, no association was detected between *PDYN* genotype (68-bp repeat element containing one to four copies of AP-1 binding sites in the promoter region) and heroin abuse, although there was a clear functional association with striatal *PDYN* mRNA expression: an increased number of inducible repeats (three and four) correlated with higher *PDYN* levels than adult or fetal subjects with noninducible (one and two) alleles. Moreover, *PDYN* expression was not related to *COMT* genotype. Altogether, the data suggest that dysfunction of the opioid reward system is significantly linked to opiate abuse vulnerability and that heroin use alters the apparent influence of heritable dopamine tone on mesolimbic *PENK* and TH function.

catecholamine-*O*-methyltransferase | drug abuse | mu opioid receptor | nucleus accumbens | prodynorphin

Endogenous opioid neuropeptides derived from proenkephalin (*PENK*) and prodynorphin (*PDYN*) genes are key neurobiological substrates of addiction disorders, because they are highly implicated in reward, mood regulation, stress response, and motor function (1–3). Addiction vulnerability is often linked to individual reward sensitivity, but self-medication of underlying negative mood states has also been speculated. Genetic variability of endogenous enkephalin and dynorphin opioid systems, which mediate reward and dysphoria, respectively, may therefore be related to opioid abuse that has a strong heritability (4, 5). We recently observed a significant association between heroin abuse and an A118G SNP of the gene encoding the mu opioid receptor (*MOR*), which mediates enkephalin's actions (6). In contrast to the *MOR* gene, which is widely examined in substance abuse genetic studies (6–8), very limited investigations have been directed to the *PENK* gene despite its apparent importance to reward and hedonic state. Only a dinucleotide (CA) repeat polymorphism in the *PENK* 3' UTR has been reported with potential relevance to opioid dependence (9).

Of the opioid neuropeptides, most genetic and molecular studies have been carried out on the *PDYN* gene. There is a polymorphic

68-bp repeat element of one to four copies that contains an AP-1 binding site in the *PDYN* promoter (10). *In vitro* evidence has implied that the polymorphism is functional with increased transcriptional activation with three or four, but not one or two, copies of the AP-1 repeats (10). Such allelic variations could influence gene expression and contribute to individual psychophysiological variability. Interestingly, increased numbers of the 68-bp repeats in the *PDYN* gene have recently been shown to be human-specific and driven by positive natural selection during evolution (11). Although several studies have implicated *PDYN* AP-1 polymorphism in substance abuse disorders, it is primarily in relation to psychostimulants (12, 13). There is still a large gap of knowledge regarding the relevance of the allelic variations of the *PDYN* promoter as related to the actual functional transcription of the *PDYN* mRNA in the human brain.

The present study directly examined *PENK* and *PDYN* polymorphisms in relation to heroin abuse and transcription levels in the postmortem human striatum. Given that striatal *PENK* and *PDYN* are localized to discrete medium spiny output pathways that are differentially regulated by prefrontal cortical activity and tonic dopamine levels (14), the impact of heritable prefrontal cortical dopamine tone to opioid neuropeptide mRNA expression was also explored by examining catecholamine-*O*-methyltransferase (*COMT*) polymorphism. *COMT* is a major enzyme for dopamine metabolism, particularly in the prefrontal cortex (15), and several human studies have linked polymorphism of the gene to cognition (16, 17) and downstream activity of midbrain dopamine function (18) and synthesis (19). As such, expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, was also examined.

Results

PENK, *PDYN*, and *COMT* genotypes were studied in our postmortem European Caucasian Hungarian and Swedish sample population (6) in relation to heroin abuse. Allele frequencies between the populations were similar, but the vast majority of subjects (90%) in this study were Hungarian. All genotypes conformed to Hardy-Weinberg equilibrium: $P > 0.5$.

***PENK* Genotype in Association with Heroin Abuse and *PENK* mRNA Expression.** DNA samples from control ($n = 46$) and heroin abuse ($n = 76$) individuals were amplified, and PCR products corresponding to *PENK* dinucleotide (CA) repeat alleles ranged from 77 (12 repeats) to 83 (15 repeats) bp in the population. Comparable with

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The authors declare no conflict of interest.

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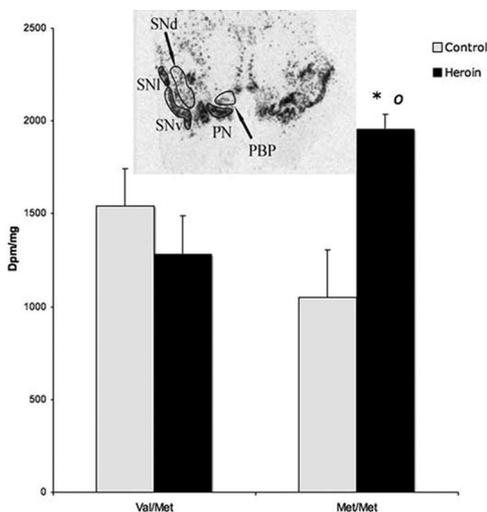


Fig. 3. Association of TH gene expression within the mesolimbic paragnalis nucleus (PN) with *COMT* genotypes in control (gray bars) and heroin users (black bars). Values are expressed as dpm/mg (mean \pm SEM). *, $P < 0.05$, heroin vs. control subjects; \circ , $P < 0.05$ difference between Val/Val and Met/Met genotypes in heroin users. (Inset) Autoradiogram of TH mRNA expression in the human midbrain. PBP, parabrachial pigmented nucleus; SNv, substantia nigra ventral part; SNl, substantia nigra lateral part; SNd, substantia nigra dorsal part; SCP, superior cerebellar peduncle; CP, cerebral peduncle.

COMT genotype, the fetal specimens were evaluated. There was an apparent difference in the population frequency of the *COMT* allelic distribution because very few Met homozygotes were present in the fetal specimens, which were predominantly (80%) of African-American descent. Nevertheless, there was a similar expression pattern to that observed in the adult control subjects such that individuals carrying the Met allele in the fetal specimens also expressed lower PENK mRNA levels than Val individuals in the NAc (68.75 ± 3.7 vs. 49.79 ± 3.0 for Val/Val and Val/Met groups, respectively; $P = 0.0004$, covaried for fetal weight), but no significant difference was found in the dorsal striatum.

To determine whether there were *COMT*-related downstream alterations of dopamine-related events, TH mRNA expression, which is regulated by synaptic dopamine levels (22, 23), was studied in the adult midbrain of the control and heroin users. Five cell midbrain neuronal subgroups were examined: paragnalis neurons (comprises the mesolimbic pathway), parabrachial pigmented nuclei and dorsal substantia nigra (constitutes the mesocortical circuit), and ventral and lateral substantia nigra (nigrostriatal pathway) (24). Midbrain samples were only available from 31 samples; because no Val/Val subjects were present in the controls, this genotype was excluded from the total analysis. *COMT* genotype midbrain samples were control Val/Met, $n = 6$; control Met/Met, $n = 4$; heroin Val/Met, $n = 10$; heroin Met/Met, $n = 4$. Consistent with the striatum, *COMT*-related association to TH mRNA expression was detected only in the mesolimbic subpopulation, namely the paragnalis nucleus (Fig. 3). In contrast to a tendency for decreased TH expression in normal Met/Met individuals, heroin subjects had a significant up-regulation of the gene ($P = 0.0221$).

PDYN Genotype in Association with Heroin Abuse and PDYN mRNA Expression. PCR analysis of DNA samples studied from control and heroin abuse subjects for the *PDYN* genotyping revealed bands of 272, 340, 408, and 476 bp corresponding to the presence of one to four 68-bp repeats in different *PDYN* alleles consistent with previous investigations (10, 12). Based on previous *in vitro* studies (10) the samples were subdivided into three groups according to copy number of inducible or noninducible alleles. The first group were

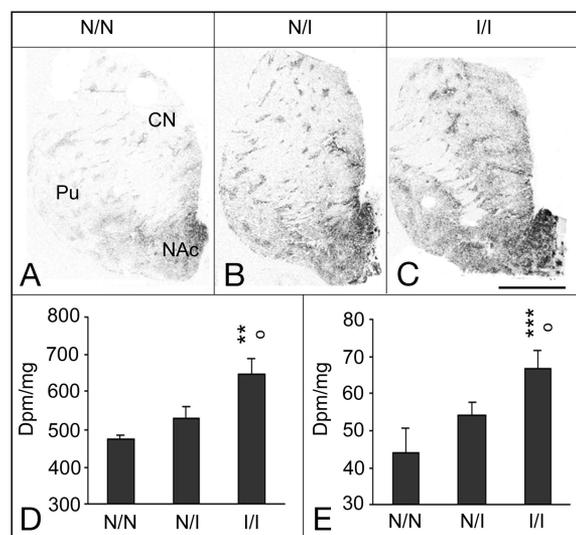


Fig. 4. Association of *PDYN* gene expression with *PDYN* genotypes in the adult striatum. (A–C) Representative autoradiograms for subjects with homozygous N/N genotype (A), heterozygous I/N genotype (B), and homozygous I/I genotype (C). (Scale bar: 1 cm.) (D and E) Semiquantification of *PDYN* mRNA expression levels in the shell NAc (D) and associative caudate nucleus (CN; E). Values are expressed as dpm/mg (mean \pm SEM). Pu, putamen; N, noninducible; I, inducible; N/N, subjects with one or two repeats; I/N, heterozygous subjects with one or two repeats and three or four repeats; I/I, homozygotes with three repeats and heterozygotes with three/four repeats. \circ , $P < 0.05$, difference between N/I and I/I. **, $P < 0.01$ and ***, $P < 0.001$, difference between N/N and I/I.

noninducible homozygotes (N/N), the second group, termed heterozygous (N/I), included noninducible (one or two) and inducible (three or four) repeats, and the third group was homozygous for inducible repeats (I/I). The frequency for the I alleles was 60% in the population. No significant difference was found in genotype distribution ($\chi^2 = 1.181$, $P = 0.7577$) and allele frequencies ($\chi^2 = 3.946$, $P = 0.6841$) between control and heroin users (SI Table 2).

PDYN genotype distribution in the postmortem subpopulation examined was similar to the large population: I/I ($n = 4$ control, 5 heroin), I/N ($n = 8$ control, 17 heroin), and N/N ($n = 9$ control, $n = 13$ heroin). Statistical analyses showed a general association between the presence of the inducible or noninducible allele and the level of *PDYN* mRNA expression in the adult (Fig. 4) and fetal (Fig. 5) striatum. In adult subjects, there was a significant main effect of *PDYN* genotype and drug group [the heroin users had previously been shown to have reduced striatal *PDYN* mRNA expression (6)]. Significant *PDYN* genotype effect was detected in the NAc shell ($F = 5.8269$, $P = 0.0059$; Fig. 4D) and caudate nucleus ($F = 8.9472$, $P = 0.0007$; Fig. 4E); age and brain pH were covariates that influenced the mRNA levels. Post hoc analysis revealed that inducible homozygous I/I subjects had significantly higher *PDYN* mRNA expression than the other genotypes (NAc shell: N/N vs. I/I, $P = 0.0039$; N/I vs. I/I, $P = 0.0103$; caudate nucleus: N/N vs. I/I, $P = 0.0009$; N/I vs. I/I, $P = 0.0115$; N/N vs. N/I, $P = 0.0972$; Fig. 4). The *PDYN* gene expression pattern is very heterogeneous in the striatum, and subregional analyses of the dorsal striatum showed generally comparable findings in the motor and associative subregions and in the patch and matrix compartments (data not shown). A significant genotype \times drug group interaction ($P = 0.0384$) was detected in the putamen because of reduction of *PDYN* mRNA levels in this subregion only being most evident in heterozygote heroin users. *PDYN* mRNA expression levels were significantly related to *PDYN* genotype in control subjects: NAc shell, $F = 6.379$, $P = 0.0153$; NAc core, $F = 9.6707$, $P = 0.0501$; caudate nucleus, $F = 7.109$, $P = 0.062$; putamen, $F = 17.243$, $P = 0.0001$. Although

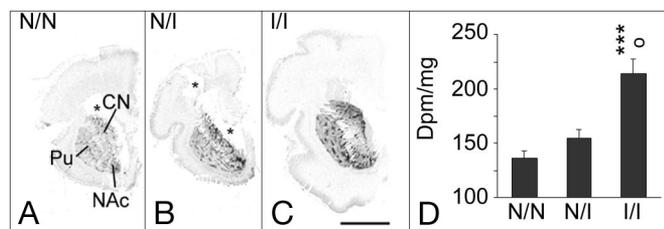


Fig. 5. Association of PDYN gene expression with PDYN genotypes in the human midgestational (week 17–21) fetal striatum. (A–C) Representative autoradiograms for subjects with homozygous N/N genotype (A), heterozygous I/N genotype (B), and homozygous I/I genotype (C). Pu, putamen; CN, caudate nucleus. (Scale bar: 1 cm.) (D) Semiquantification of PDYN mRNA expression levels in the fetal putamen. Values are expressed as dpm/mg (mean \pm SEM). N, noninducible; I, inducible; N/N, subjects with one or two repeats; I/N, heterozygous subjects with one or two repeats and three or four repeats; I/I, homozygotes with three repeats and heterozygotes with three/four repeats. \circ , $P < 0.05$, difference between N/I and I/I. $***$, $P < 0.001$, difference between N/N and I/I.

PDYN expression was lower in heroin users than controls (6), heroin subjects still tended to show an association between increased PDYN mRNA levels with increasing number of the AP-1 repeats in the NAc core ($F = 4.9129$, $P = 0.0261$), NAc shell ($F = 2.5387$, $P = 0.0999$), and caudate nucleus ($F = 5.283$, $P = 0.0031$). The potential influence of the PDYN genotype on PENK mRNA expression was also assessed. The results clearly showed no association between the PDYN genotype on striatal PENK mRNA expression levels ($P > 0.4$) for all striatal subregions examined (data not shown).

COMT genotype was not significantly related to PDYN mRNA expression levels in either the dorsal striatum or NAc although PDYN levels in Met/Met individuals tended to be lower than Val/Met in the dorsal striatum and NAc core, a pattern similar for both control and heroin subjects.

PDYN mRNA expression was also examined in the fetal samples to validate the associations to PDYN genotype in early life. Only a subpopulation was examined because of the influence of several variables on the PDYN mRNA levels in the fetal analysis. The same pattern between PDYN genotype (I, $n = 7$; I/N, $n = 7$; N/N, $n = 10$) and striatal mRNA expression was apparent in the fetal subjects as in the adults (Fig. 5), but significance was detected only in the putamen ($F = 10.5967$, $P = 0.0022$; N/N vs. I/I, $P = 0.0008$; N/I vs. I/I, $P = 0.0313$; Fig. 5D).

Discussion

The current study revealed distinct contributions of the PDYN and PENK polymorphisms to striatal function in the human brain and heroin abuse vulnerability with enhanced sensitivity of mesolimbic PENK transcription in relation to heritable dopamine tone that is dysregulated in Met/Met heroin users. In contrast to the cis-regulatory PDYN polymorphism, variations of the PENK 3'(CA) n repeat were significantly associated with heroin abuse. Approximately 80% of the 79/79 homozygous subjects in our study population were heroin abusers. These findings support a strong relationship between the PENK/MOR reward-related system and opioid abuse susceptibility considering that we also recently observed a significant association between heroin use and the A118G MOR gene (OPMRI) SNP in this European Caucasian population (6). There was no significant interaction between the PENK and OPMRI genotypes in the current population (data not shown), suggesting that each contributes independently to heroin abuse vulnerability, although impacting similar neurobiological networks.

The functional relevance of the 3' UTR is an active area of research where accumulating evidence has shown it to be an important structural component of the gene that can modulate e.g.,

gene expression, mRNA stability, and translation (25). The 3' UTR PENK dinucleotide repeat polymorphism appears to be of functional relevance to gene transcription as evidenced in the fetal brain where the 79 allele was associated with higher striatal PENK mRNA expression than the 81 homozygotes. Although a similar pattern was apparent in the adult brain, it was evident that other factors influenced the PENK mRNA levels. It is feasible that linkage disequilibrium with another as-yet-unidentified functional variant of the PENK or other genes more directly conferred the vulnerability to heroin abuse. Increased NAc PENK mRNA levels has been associated with elevated heroin self-administration (26), but enhanced NAc PENK transcription alone does not appear to directly correlate to opiate abuse vulnerability because 79/81 heterozygotes also had elevated PENK mRNA levels compared with 79 homozygous subjects, although the frequency of the 79/81 genotype was similar in control and heroin subjects. Further studies are required to fully reveal the specific association between the PENK gene, transcription and heroin abuse risk, and the contribution of other neurotransmitter systems to the apparent complex regulation of PENK.

An important finding was that heritable dopamine tone, as related to COMT polymorphism, influenced PENK mRNA levels within the NAc shell and depended on the history of heroin use. Based on the existing concept of the prefrontal cortical tonic-phasic dopamine regulation of the NAc (21) and anatomical organization (27), increased prefrontal cortex dopamine levels in Met/Met individuals would be associated with increased tonic, but decreased phasic, NAc dopamine levels. Increased tonic dopamine that acts primarily through D2 receptors specifically diminish the responsiveness of striatopallidal neurons (28). Our findings in control subjects are thus consistent with this concept given the strong PENK mRNA reduction in Met subjects and the opposite alterations in individuals carrying the Val allele who, based on the tonic-phasic model, would be predicted to have reduced tonic NAc dopamine levels. TH, which is well established to be regulated by synaptic dopamine levels particularly via D2 receptors (22, 23), was also associated with COMT genotype as observed (19); increased stimulation of D2 receptors in Met/Met individuals would predict feedback reduction of TH mRNA, a trend observed in control subjects. It should be noted, however, that most animal models have failed to detect significant association between COMT depletion and striatal dopamine levels (29). Such studies only examined the dorsal striatum where we also found no significant association with the opioid peptide mRNA levels. The alterations in relation to the COMT genotype were specifically within the ventral striatum in our population. That the apparent influence of COMT genotype on PENK was localized to the NAc shell is important because this striatal subregion is most linked with drug reward and the hedonic state mediated by both dopamine and enkephalinergic systems (30, 31). Moreover, COMT polymorphism was only significantly related to TH mRNA expression within the paranigralis dopamine neuronal populations that directly innervate the NAc underlining the continuum of disturbance along the mesolimbic circuit. Interestingly, *in vivo* imaging of MOR binding sites also revealed significant association with COMT genotype in the NAc, not dorsal striatum (32).

An opposite association pattern between COMT genotype and expression level was evident in heroin users as compared with control subjects. Heroin users generally express low striatal PENK transcription levels (6), but it was only evident in the Val/Met subjects, whereas Met homozygotes heroin users had increased PENK mRNA expression compared with their genotype controls. Although COMT genotype was not directly associated with heroin abuse and there was no interaction between the PENK and COMT genes, COMT polymorphism strongly influenced NAc PENK mRNA expression, emphasizing that critical networks of gene interaction contribute to striatal function. Previous *in vivo* studies

have observed an inverted u-shaped response curve regarding *COMT* such that increased dopamine after amphetamine administration improves cognitive function in Val subjects, but impairs cognition in Met individuals who already have an elevated dopamine tone (33). Such a compensatory switch in relation to the *COMT* genotype appears also relevant for *PENK* transcription and NAc function and for TH mRNA expression.

Another key aspect of the current study was providing evidence that tandem 68-bp repeats containing AP-1 binding sites in the *PDYN* promoter are associated with inducibility of the *PDYN* mRNA expression in the human striatum that primarily constitutes the major “direct” striatonigral output pathway. The fact that this association is evident in early stages of development in normal subjects and in the adult brain emphasizes the enduring individual nature of the *PDYN* transcriptional regulation despite expected diverse influences throughout life. Even heroin use in adult subjects, which generally attenuates striatal *PDYN* mRNA expression (6), failed to affect the association between *PDYN* allelic variation and mRNA levels expressed in the striatum, highlighting the important regulation of the *cis*-regulatory polymorphism on its transcription. Similar to our results, no association was detected between heroin abuse and *PDYN* polymorphism in larger sample size investigations in Caucasian populations (10, 34). Together, these findings strongly suggest that, in contrast to the *PENK* and *OPMRI* A118G polymorphism, genetic variants of the *PDYN* AP-1 promoter do not confer risk for vulnerability to heroin abuse in Caucasian populations. Ray *et al.* (34) did observe a weak association between *PDYN* polymorphism and opioid dependence in African-Americans, which underscores the potential relevance of ethnicity in regard to the *PDYN* genotype. Although the *PDYN* genotype was not associated with heroin abuse, *PDYN* plays an important role in various brain functions, and inherited features of this gene may be of critical importance in the individual predisposition to normal and pathological reactions of the CNS.

Limitations of this postmortem human investigation include the small sample size, lack of detailed medical and drug histories (although the subjects were documented heroin abusers), potential stratification even within this homogenous population, and the potential for haplotype rather than genotype analyses to provide stronger associations to the outcome variables. For example, *COMT* haplotype has been shown to influence *COMT* function to a greater extent than the *COMT* Val-158–Met genotype (35). Further studies in larger populations in which haplotype analyses can be better evaluated are clearly required to replicate and extend the current findings. Nevertheless, *COMT* genotype was significantly associated with specific neurobiological features that emphasize that genotype has important functional relevance. Moreover, although there were apparent ethnic differences in the allelic distribution of the *COMT* polymorphism, the neurobiological correlations to genotype were the same irrespective of population. In addition, the fact that the same general pattern of increasing *PDYN* transcript levels with increasing inducible alleles was evident in various striatal subregions irrespective of heroin use, and that a similar pattern was detected in fetal subjects, strongly suggests that the *PDYN* promoter polymorphism is functionally associated with *PDYN* transcription in the human striatum. The ability to detect significant polymorphic associations of heroin abuse with both the *PENK* and *OPMRI* genes, which are critical for the rewarding actions of opiates, implies that it is possible to dissociate systems linked with heroin vulnerability in the current population. The lack of association between the *PDYN* genotype and *PENK* mRNA expression and vice versa underscores the neurobiological specificity of the polymorphisms.

In summary, association of polymorphic disturbances within the enkephalin system, *PENK* and *OPMRI*, with heroin abuse provides strong evidence that impairment of enkephalin neurotransmission is tightly related to opiate abuse vulnerability. Although *COMT*

genotype on its own was not associated with heroin abuse, apparent mesolimbic dopamine tone specifically contributes to the *PENK* mRNA expression in the human NAc shell highly relevant to reward. The clear functional relevance of the *PDYN* promoter polymorphism in regulating the striatal *PDYN* mRNA expression suggests that striatal *PDYN* transcription is not linked with opiate abuse risk, but may have important significance to other neuropsychiatric conditions in which basal ganglia function or negative mood states are important features of the disorder.

Materials and Methods

Adult Human Brains. Human brain samples examined in this study were part of a heroin abuse postmortem collection that has been described (6). Briefly, brains from apparent heroin overdose and normal control adult Caucasian subjects without head trauma were collected at autopsy within 24 h after death at the Department of Forensic Medicine, Semmelweis University, and the National Institute of Forensic Medicine, Karolinska Institutet under local ethical approved guidelines. All cases were assessed for common drugs of abuse (including alcohol) and therapeutic agents. Subjects included in the heroin group died from apparent heroin overdose as verified by toxicology and had physical signs of heroin use such as needle track marks, and most had a history of heroin abuse as ascertained by medical records and police and/or family reports. The heroin group represented a unique drug abuse population given that they were predominant heroin users with no methadone treatment. The control group, predominant cause of death being myocardial infarction, had negative toxicology for opiates or other illicit drugs of abuse. Positive alcohol was evident in very few cases in which ethanol concentrations were similar to the limited alcohol-positive subjects identified in the heroin group. Nicotine toxicology was not conducted, but high tobacco use is frequent in the general population from which the subjects were examined. We studied 127 subjects: 46 controls (37 males, 9 females; average age 36.8 ± 1.98) and 81 heroin users (67 males, 14 females; average age 25.7 ± 0.61). Of these, 53 specimens were used for postmortem brain analyses (see ref. 6): controls, 16 male, 3 females, average age 23.4 ± 0.3 years, average brain pH 6.72 ± 0.05 ; heroin, 30 males, 4 females, average age 22.345 ± 0.33 , average brain pH 6.55 ± 0.04 . Immediately after autopsy, the brains were cut in 1.5-cm slabs, frozen, and kept at -70°C . Coronal 20- μm sections (Microm HM560; Microm International) were quickly mounted onto Superfrost plus-glass (Brain Research Laboratories) and then kept at -30°C . All procedures were carried out blinded to the subject group.

Fetal Brains. A total of 49 postmortem human fetal brain samples were studied from cases collected to evaluate developmental exposure to cannabis (36). The cannabis-exposed ($n = 24$) and nonexposed ($n = 25$) group were $\approx 80\%$ African-American: average age 20.2 ± 0.3 and 20.4 ± 0.3 weeks, male/female: 13/11 and 13/12, postmortem interval 7.9 ± 0.70 and 9.6 ± 0.75 h, respectively. Fetal brains were lightly fixed with 1% paraformaldehyde and frozen in isopentane at -40°C . Coronal sections (20 μm thick) were processed by using the same procedure as for the adult tissue.

Genotyping. DNA was purified from cerebellar tissue by using DNeasy columns (Qiagen). For *PDYN*, DNA (100 ng) was amplified by sense and antisense primers (5'-CTGTGTATGGAGAGGCTGAGT and 5'-GCGGTTAGGTAGATTGTGACAGATT), and PCR products were resolved on a 2.5% agarose gel. For *PENK*, the polymorphic area was amplified by sense and antisense primers (5'-TAATAAAGGAGC-CAGCTATG and 5'-ACATCTGATGTAATGCAAGT (6-FAM labeled) (37), and PCR products were analyzed by capillary electrophoresis DNA analyzer ABI3730 (Applied Biosystems). *COMT* genotypes were determined by NlaIII restriction of PCR products generated by DNA amplification with primers *Comt1* (5'-CTCATCAC-CATCGAGATCAA) and *Comt2* (5'-CCAGGTCTGACAACGGTCA) (32). Every analyzed set of PCR products included fragment size markers in the experiment, and genotyping results were always double-checked to provide genotyping accuracy.

In Situ Hybridization Histochemistry. The *PENK* riboprobe was an EcoRI/Pvu 792-bp fragment complementary to the full coding region of the *PENK* human gene (38). The *PDYN* probe contained the 5' region of the gene inserted (GenBank accession no. NM.024411, bases 215–72) (39). *In situ* hybridization is described in *SI Text*. Briefly, brain sections were hybridized with 20×10^3 cpm/ μl riboprobe solution. Optical density values measurements were taken from film images of the NAc and associative and motor divisions of the caudate nucleus and putamen based on the functional organization of the primate striatum (40).

Optical density values were converted to dpm/mg by reference to coexposed C¹⁴ standards (American Radiolabeled Chemicals).

Statistics. General linear stepwise regression analysis was used to evaluate genotype group differences with covariates: e.g., age, postmortem interval, brain pH, and sex. Other variables evaluated for adult subjects were ethanol toxicology, drug group (control vs. heroin) and brain freezer storage time; and for fetal specimens maternal drug use was evaluated. Post hoc analysis regarding genotype was performed by using Fisher's least-squares difference test. Association between genotypes and vulnerability to drug abuse and

Hardy-Weinberg equilibrium was analyzed by χ^2 tests. Significance was set at $P < 0.05$, and trends were considered for $P < 0.10$. See more detail in *SI Text*.

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