Proenkephalin Mediates the Enduring Effects of Adolescent Cannabis Exposure Associated with Adult Opiate Vulnerability

Supplemental Information

Table S1. For each lentiviral (LV) construct, this table summarizes the corresponding adolescent treatment, the key structural elements, and the effects of the construct on *in vivo Penk* expression.

LV Vector	Adolescent Treatment	Key Vector Elements	*In vivo* Effect on *Penk* Expression
Penk	vehicle	mPGK1p-*Penk*-IRES-eGFP-WPRE	increase
GFP	vehicle	mPGK1p-eGFP-WPRE	none
miR *Penk*	THC	mPGK1p-emGFP-miR *Penk*-WPRE	decrease
GFP	THC	mPGK1p-emGFP-WPRE	none
miR ctrl	THC	mPGK1p-emGFP-miR Arab-WPRE	none

HIV, human immunodeficiency virus; LTR, long terminal repeat; mPGK1p, mouse phosphoglycerate kinase-1 promoter; eGFP, enhanced green fluorescent protein; WPRE, woodchuck post-transcriptional regulatory element; r*Penk,* 956 nucleotide fragment of the rat *Penk* coding region; IRES, encephalomyocarditis virus internal ribosome entry site; EmGFP, emerald GFP; miR Arabidoposis, miRNA targeting a non-vertebrate gene; miR *rPenk,* miRNA targeting nucleotides 709-729 of the rat *Penk* coding region.

Table S2. This table lists the *Penk* gene primer sequences used in the present study and their location relative to the *Penk* transcription start site (TSS).

Primer	Sequence (5'→3')	Target Region	Location Relative to *Penk* TSS
r*Penk*_DG_F	CGGCCAGGACAGCTTTCTCCAT	promotor	-1.5 kb
r*Penk*_DG_R	GACATGGGTTCTGGACTTGAGCTG	promotor	-1.5 kb
r*Penk*_Y_F	AACAACCCACCCACGGTGCC	promotor	-0.9 kb
r*Penk*_Y_R	GGAGGCTCCCCCACCAATCAGT	promotor	-0.9 kb
r*Penk*_B_F	GCCTCCTTCGGTTTGGGGCT	promotor	-0.6 kb
r*Penk*_B_R	AAGGCAAGTGTGAGGTGGCCG	promotor	-0.6 kb
r*Penk*_ex(4)1_F	AAGCCCGAGTTCCCTTGGGA	coding	+ 0.2 kb
r*Penk*_ex(4)1_R	TGGCTCCACGGGGTAAAGCTC	coding	+ 0.2 kb
r*Penk*_ex(4)2_F	AGCCACCAACAGGAAAGCACCA	coding	+ 0.4 kb
r*Penk*_ex(4)2_R	GCCCCCATAGCGCTTCTGCA	coding	+ 0.4 kb
r*Penk*_ex(4)3_F	AGCGCTTTGCTGAGTCTCTACCC	coding	+ 0.6 kb
r*Penk*_ex(4)3_R	AGGGCTTCAAAACCGCATAAAGCC	coding	+ 0.6 kb

Supplemental Methods and Materials

Animals

Reverse light-cycled 21-day-old male Long Evans rats (Taconic) were acclimatized to vivarium conditions. Rats were housed five to a cage and pseudo-randomly assigned to an adolescent treatment group (either Δ^9-tetrahydrocannabinol (THC) or vehicle) to ensure that treatments were mixed within each cage. All rats were housed singly following stereotaxic surgery. Behavioral testing was conducted during the dark phase of the light/dark cycle. For gene expression and chromatin immunoprecipitation (ChIP) experiments, rats were sacrificed either 24 hr or 30 days following the final adolescent treatment. All animal procedures were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Bronx VA Medical Center and the Mount Sinai School of Medicine.

Drugs

THC (50 mg/ml in ethanol solution; National Institute on Drug Abuse) was evaporated under nitrogen gas, dissolved in 0.9% NaCl with 0.3% Tween 80, and administered via intraperitoneal (IP) injection in a volume of 2 ml/kg as described previously (1). Heroin HCl (National Institute on Drug Abuse) was dissolved in 0.9% NaCl and self-administered intravenously at a volume of 85 µl/infusion. For stereotaxic and vascular cannulation surgeries, rats were anesthetized with isoflurane (2.5-4.5% in O_2) delivered via vaporizer (Stoelting).

Lentiviral Vectors

To generate the rat preproenkephalin lentivirus, a 956-bp fragment containing the rat *Penk* complementary DNA (cDNA; originally provided by S.L. Sabol, National Heart, Lung, and Blood Institute) (2) was cloned into a lentivirus transfer vector under control of the mouse phosphoglycerate kinase-1 promoter (mPGK1p). This transfer vector also contained an encephalomyocarditis virus internal ribosome entry site (IRES), enhanced green fluorescent protein (eGFP) gene and the woodchuck post-transcriptional regulatory element (WPRE). A control lentivirus (GFP) was constructed similarly, using a transfer vector with the mouse PGK-1 promoter driving expression of eGFP alone (no IRES). To generate the rat *Penk* microRNA (miRNA) lentivirus, miRNAs targeting the rat *Penk* gene were obtained from Invitrogen (Carlsbad, CA). Production and *in vitro* validation of the miRNA lentiviruses are provided in Figure S2. The rat *Penk* miRNA was subcloned into a lentivirus transfer vector containing the same promoter and regulatory elements as the *Penk* lentivirus. A control lentivirus was also similarly constructed with a miRNA known to target a sequence not found in vertebrate DNA (Invitrogen). Lentiviruses were produced by transfection of HEK293T cells with the individual transfer vectors, a packaging plasmid (psPAX2) and a plasmid encoding the vesicular stomatitis virus G glycoprotein (pMD2.G). Final titers of all lentiviruses were ~5-10 x 10^6 transducing units (tu)/µl. Purified virus stocks were stored at -80°C. In all cases, *in vivo* transgene expression was validated and nucleus accumbens (NAc) shell-specific expression confirmed via in situ hybridization histochemistry (ISHH).

Stereotaxic Surgery

Two weeks following the final THC or vehicle treatment, rats were anesthetized with isoflurane (2.5 - 4.5% in O_2). Twenty-six gauge beveled needles attached to 2.5 µl glass syringe barrels (Hamilton, Reno, NV) were used to stereotaxically infuse either 0.5 µl (*Penk* or GFP) or 1.0 µl (GFP, miR ctrl, miR *Penk*) purified lentivirus bilaterally into the NAc shell at a 10° angle from midline (relative to bregma: AP +1.7 mm; ML +2.3 mm; DV -6.8 mm ((from dura)) at a rate of 0.1 µl/min. Needles were kept in place in the NAc shell for 10 min to allow for sufficient diffusion of the virus in the surrounding tissue. Rats were allowed a two week recovery period before undergoing jugular vein cannulation surgery.

Agents:

Penk: mPGK1p-*Penk*-IRES-eGFP-WPRE

GFP: mPGK1p-eGFP-WPRE

miR *Penk*: mPGK1p-emGFP-miR *Penk*-WPRE

miR ctrl: mPGK1p-emGFP-miR Arab-WPRE

GFP: mPGK1p-emGFP-WPRE

Cannulation Surgery

Two weeks after stereotaxic surgery, rats were anesthetized with isoflurane (2.5-4.5% in O_2) and catheters (Brian Fromant, Cambridge, UK) were implanted into the jugular vein using a standard cannulation procedure (1, 3). On post-op days 1-3, rats received injections of carprofen (0.5 mg/kg, subcutaneous) and catheters were flushed with 10 U heparin and ampicillin (50 mg/kg) in sterile saline. Rats were allowed a one week recovery period prior to

initiation of self-administration experiments, which commenced no sooner than 21 days after stereotaxic viral infusion, a duration that is consistent with maximal viral-mediated transgene expression for the lentiviruses used in these experiments. Catheters were flushed with 30 U heparin before and after each self-administration session.

Heroin Self-Administration and Activity Monitoring

Drug self-administration experiments were conducted in standard operant chambers (29.5 cm x 32.5 cm x 23.5 cm) housed in sound-attenuating boxes (MED Associates Inc., Georgia, Vermont) with two retractable levers according to published protocols (1, 3). Depression of the drug-paired lever (defined as the active lever, AL) resulted in an intravenous heroin injection whereas depression of the inactive lever (IL) had no programmed consequence; all lever presses were recorded (MedPC IV, MED Associates Inc.). A single AL press resulted in a heroin injection (85 µl over 5 sec) and concurrent illumination of a stimulus light above the AL, followed by a 10s time-out period where both levers were retracted and the stimulus light turned off. At least 21 days following stereotaxic viral infusion, rats began self-administration training during the dark phase of the light/dark cycle. Rats were allowed to self-administer heroin (30 µg/kg/injection) under a fixed-ratio 1 (FR1) reinforcement schedule in daily 3 hr sessions. Rats were food-restricted (18-20 g food/day) during the acquisition phase that continued until stable baseline responding (<15% variation over three consecutive days) was established. Drug self-administration was continuous with no drug-free periods during the acquisition and maintenance phases. Following a 3-day maintenance period at 30 µg/kg/injection (FR-1), a between-session dose response test was conducted (30, 7.5, 100, 15,

and 60 µg/kg/injection heroin; one dose per day). Rats were then maintained in an abstinent (drug-free) state for 3 wk in their home cages. Cue-induced drug-seeking behavior was evaluated using a paradigm where AL resulted in illumination of the stimulus light, but no intravenous heroin administration. One week later, stress-induced drug-seeking behavior was evaluated following exposure to a mild stressor (24 hr food deprivation). Both reinstatement sessions were 1 hr. Locomotor activity was measured during each self-administration session by infrared beams fitted above the floor of each operant chamber. Rats were sacrificed 24 hr following the final drug-seeking test and brains were collected and sectioned as described below.

Penk Riboprobe Design and Validation

To detect *Penk* mRNA levels in the rat striatum, a single-stranded RNA probe (riboprobe) complementary to its single-stranded mRNA target *in situ* was polymerase chain reaction (PCR)-derived from a cDNA fragment of the preproenkephalin gene (bases 585-1140; Genbank accession: NM_017139). Primers tagged with sites for T7 and SP6 (lowercase italicized text below) resulted in generation of a cDNA template with 5' extensions containing promotor sequences recognized by SP6 and T7 polymerases (*Penk*_ForT7: *ctgtaatacgactcactatag*CAGCAGCAAACAGGATGAGA; *Penk*_RevSP6: *gggatttaggtgacactatag*GCTTCAAAACCGCATAAAGC). PCR product size was confirmed on a 1.5% agarose gel and the cDNA template was purified (illustra microspin S-200 HR columns, GE Healthcare, UK). Specificity of the SP6-transcribed (anti-sense) *Penk* riboprobe *in situ* was

verified via ISHH using test sections through the striatum. The T7-transcribed (sense) *Penk* riboprobe was run as a negative control.

In situ mRNA Hybridization Histochemistry

The ISHH procedure was similar to published protocols (1, 4). Briefly, coronal sections through the NAc were cut and slide mounted. The *Penk* riboprobe was transcribed in the presence of [^{35}S] uridine5'-(α-thio) triphosphate (Perkin Elmer), and the radiolabeled probe was applied to brain sections in a concentration of 2 $\times 10^3$ counts per minute/mm^2 of the cover slip area. Two or three adjacent sections from each animal were studied. Hybridization was carried out overnight at 55°C in a humidified chamber. After *in situ* hybridization, slides were exposed to an imaging plate (FUJIFILM) with ^{14}C standards for 28 hr before phosphoimaging. Densitometry readings were made of the NAc shell, NAc core, and caudate putamen using FUJI phosphoimage software (FLA-7000 V1.0.1) and optical density values were converted to disintegrations per minute (dpm/mg) by reference to co-exposed ^{14}C standards using FUJI analysis software (Multi Gauge V3.0). For each brain area measured, values obtained from duplicate or triplicate brain sections were averaged for each animal.

Gene Expression

Fresh-frozen bilateral 12-gauge NAc shell punches were homogenized and total RNA was isolated and purified using an RNA isolation kit (RNAqueous Small Scale Phenol-Free Total RNA Isolation Kit, Ambion, Austin, TX). First-strand cDNA was synthesized from 100 ng of total RNA using a cDNA synthesis kit (qScript cDNA SuperMix, Quanta BioSciences, Gaithersburg,

MD). Quantitative real-time PCR analysis was performed using Taqman-based probes (Applied Biosystems); *Penk* primers and probes were inventoried products of Assays-On-Demand from Applied Biosystems (inventoried assay ID Rn00567566_m1). Each reaction was run in triplicate, and eukaryotic 18S rRNA (product No. 4319413E; Applied Biosystems) was included in each multiplex PCR as an endogenous control. Real-time quantitative PCR and subsequent analysis were performed with a Roche Light Cycler 480 sequence detection system (Light Cycler 480 SW 1.5; Roche). Quantification of *Penk* gene expression in all samples was normalized to eukaryotic 18S rRNA and analyzed using the ΔΔCT method (5).

Chromatin Immunoprecipitation

Fresh NAc shell punches were formaldehyde cross-linked and prepared for ChIP as previously described (6, 7) with minor modifications. Briefly, two 12-gauge bilateral NAc shell punches per rat (three rats pooled per sample) were collected, cross-linked with 1% formaldehyde and quenched with 2M glycine. Sheep anti-rabbit/mouse (depending on precipitation antibody) IgG magnetic beads (Dynabeads M-280, Invitrogen) were prepared by incubating appropriate magnetic beads with either anti-H3K9me2 (mouse monoclonal ChIP grade, ab1220), anti-H3K9me3 (rabbit polyclonal ChIP grade, ab8898), anti-H3K36me3 (rabbit polyclonal ChIP grade, ab9050), or anti-H3K4me3 (rabbit polyclonal ChIP grade, ab8580) antibodies (Abcam, Cambridge, MA) overnight at 4°C under constant rotation in block solution (0.5% BSA in 1X PBS). Tissue sonication and chromatin shearing were carried out as previously described (6, 7). Following sonication, equal concentrations of chromatin were transferred to new tubes and ~5% of the final products was retained to function as 'input' controls. After

thorough washing and resuspension of the conjugated bead/antibody mixtures in block solution (0.5% BSA in 1X PBS), equal volumes of antibody/bead mixtures (~7.5 µg antibody/sample) were added to each chromatin sample and incubated for ~16 hours under constant rotation at 4°C. Samples were further washed in RIPA buffer (500 mM LiCl, 50 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 1% NP40, and 0.7% sodium deoxycholate in MilliQ H_2O) and 1X TE + 50 mM NaCl and reverse cross-linked with elution buffer (1% SDS, 50 mM Tris (pH 8.1) and 10 mM EDTA in MilliQ H_2O) at 65°C overnight before DNA purification using a DNA PCR purification kit (Qiagen, Valencia, CA). Normal rat IgG immunoprecipitations using a mouse polyclonal anti-IgG antibody (Abcam) were also carried out to control for appropriate enrichment of signal amplification. Following DNA purification, immunoprecipitated samples were subjected to qPCR using SYBR Green (Roche) and were normalized to their appropriate non-immunoprecipitated 'input' controls as previously described (6). Each reaction was run in triplicate and data were analyzed using the ΔΔCT method (5).

Statistics

Two-way analysis of variance (ANOVA) with repeated measures was used to determine group differences in the acquisition and maintenance of heroin self-administration behavior (treatment x self-administration session), dose-response (treatment x dose), and locomotor activity (treatment x self-administration session) followed by Tukey's honestly significant difference (HSD) test or two-tailed Student's *t* tests when appropriate. For the drug-seeking studies, one-way ANOVA was used. For analysis of *in situ* hybridization data, one-way ANOVAs were used for all comparisons between groups followed by Tukey's HSD tests when

appropriate. Two-tailed Student's *t* tests (for comparison of two groups) or one-way ANOVAs followed by Tukey's HSD test or Student's *t* tests when appropriate (for comparison of three groups) were used to determine the statistical significance of *Penk* gene expression and chromatin immunoprecipitation experiments. All values included in the figure legends represent mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). All statistical calculations were performed using JMP software.

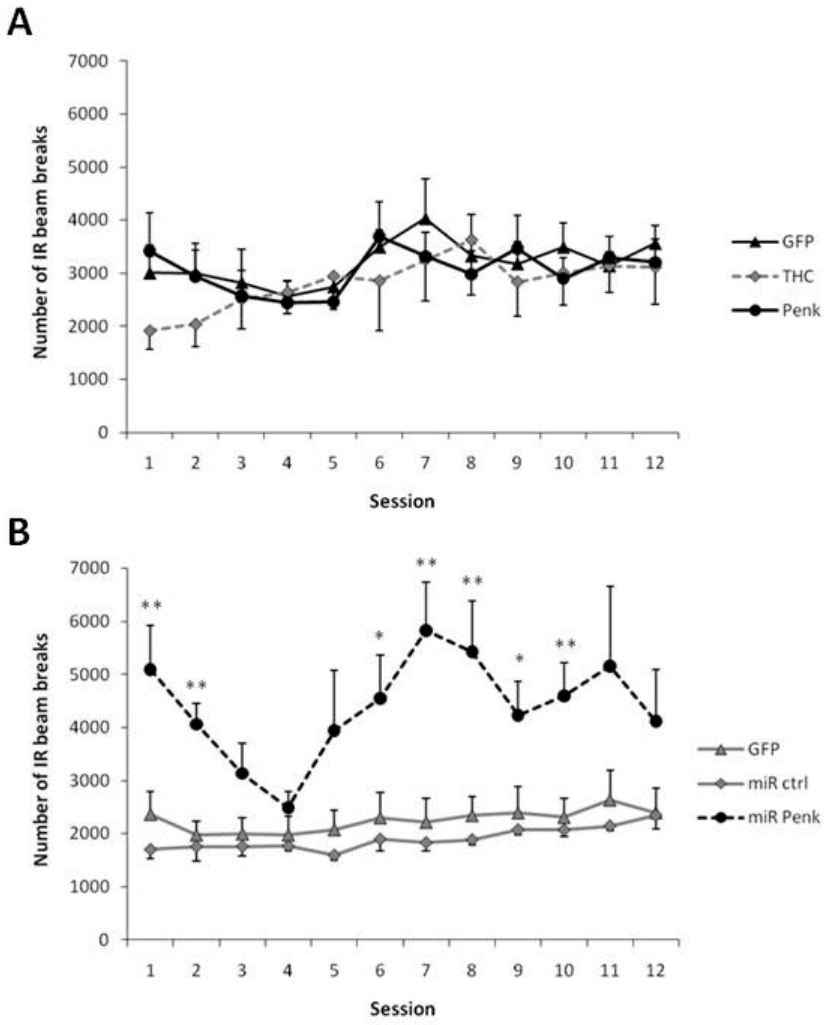

Figure S1. Number of infrared (IR) beam breaks during heroin self-administration in (**A**) *Penk*-infused, GFP-infused, or THC-exposed rats and (**B**) GFP-infused, miR ctrl-infused, or miR *Penk*-infused rats (n = 6-9/group). Data shown as mean ± SEM. *$p < 0.05$; **$p < 0.01$ compared to GFP-infused controls for each session. ctrl, control; GFP, green fluorescent protein; miR, microRNA; THC, Δ^9-tetrahydrocannabinol.

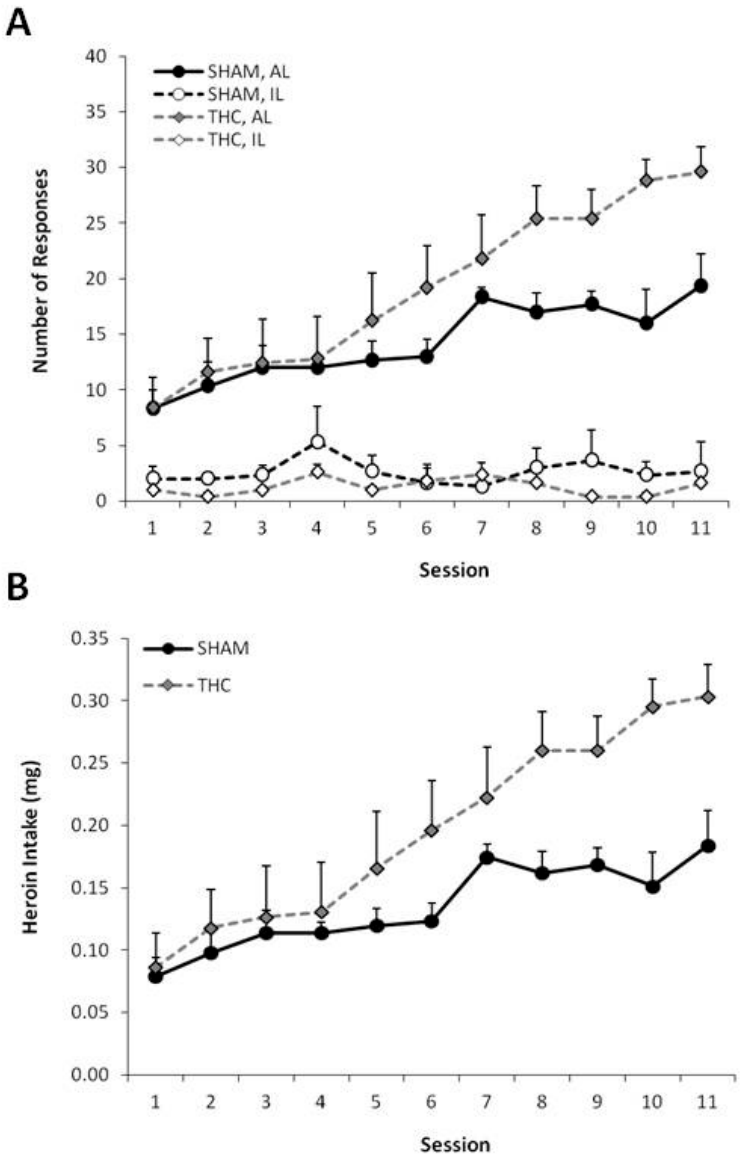

Figure S2. (**A**) Acquisition of heroin self-administration (FR-1, 30 µg/kg/injection). (**B**) Mean heroin intake in THC-exposed and vehicle-exposed surgical sham rats. *n* = 5-9/group. Data shown as mean ± SEM. AL, active lever; IL, inactive lever; THC, Δ^9-tetrahydrocannabinol.

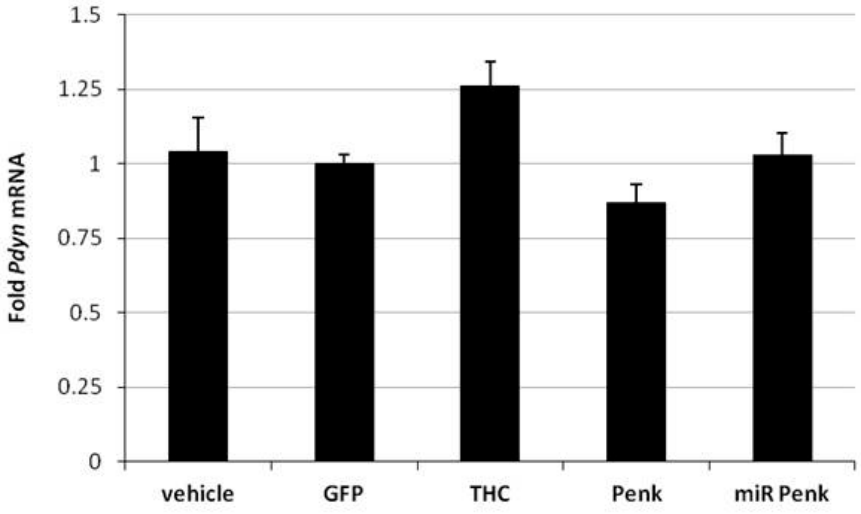

Figure S3. Nucleus accumbens shell *Pdyn* mRNA levels following heroin self-administration experiments in vehicle-exposed, GFP-infused, THC-exposed, *Penk*-infused, or miR *Penk*-infused rats (*n* = 5-6/group). See Figure S1 for abbreviations.

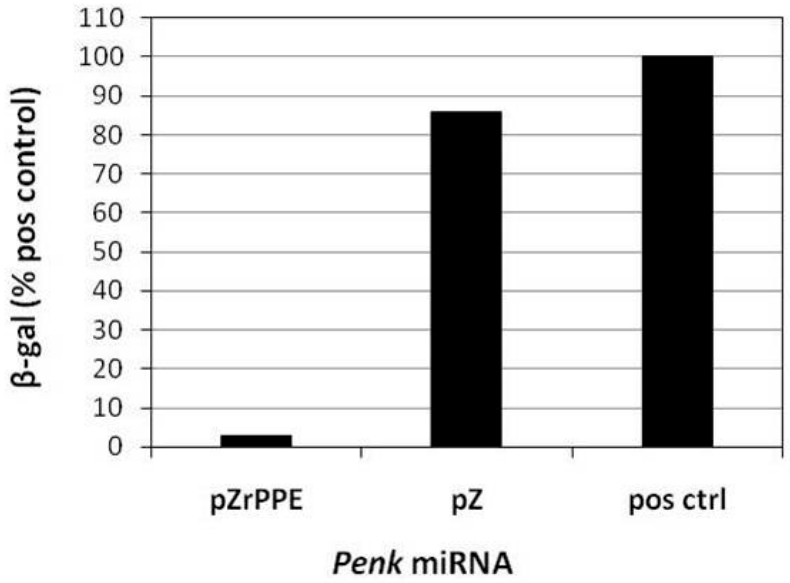

Figure S4. To generate the rat *Penk* microRNA (miRNA) lentivirus, miRNAs targeting the rat *Penk* gene were obtained from Invitrogen. Their activity and specificity were validated by co-transfection of HEK293T cells with a plasmid encoding emerald green fluorescent protein (EmGFP) and the candidate miRNA (pcDNA6.2-GW/EmGFP-miR, Invitrogen) and one encoding the rat *Penk* cDNA cloned 3' of the lacZ gene (pZrPPE: hCMV promoter-lacZ-r*Penk*-PA). A negative control and positive control miRNA (pmiRZ: hCMV promoter-miRNA lacZ-PA) were also included, and all miRNAs were tested against a plasmid encoding the lacZ gene only (pZ = hCMV promoter-lacZ). Two days after transfection, cell extracts were assayed for β-galactosidase activity. The rat *Penk* miRNA with the best activity, corresponding to nucleotides 709-729 of the *Penk* coding region, caused a 97% reduction of control β-galactosidase activity, but reduced β-galactosidase activity without the *Penk* cDNA by only 14% when tested at a concentration ratio of 6 μg miR plasmid to 1 μg target plasmid.

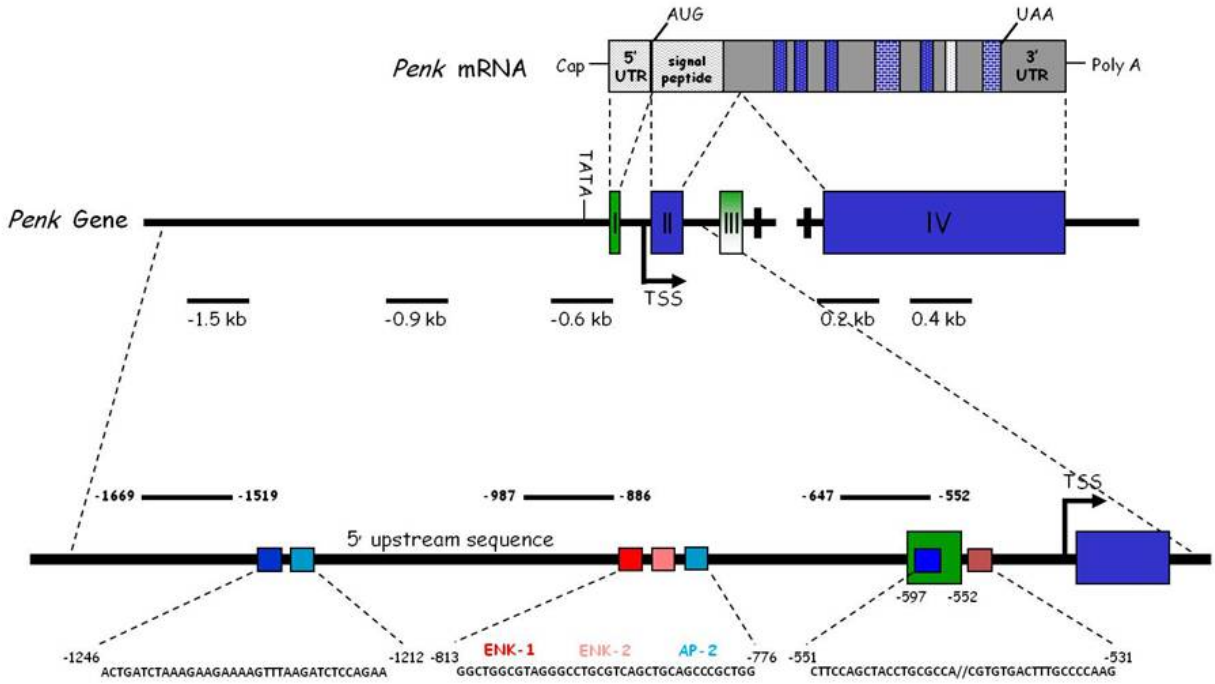

Figure S5. Schematic of the *Penk* gene promotor and coding region depicting conserved sequence elements that have been described in the 5' upstream region of *Penk* gene (8-12). Roman numerals depict the exons of the *Penk* gene (purple: coding exons, green: non-coding exons). TSS, transcription start site; ENK-1, binding site for ENKTF; ENK-2, binding site for ENKTF; AP-2, binding site for AP-2.

Supplemental References

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