Dopamine dysregulation in a mouse model of paroxysmal non-kinesigenic dyskinesia

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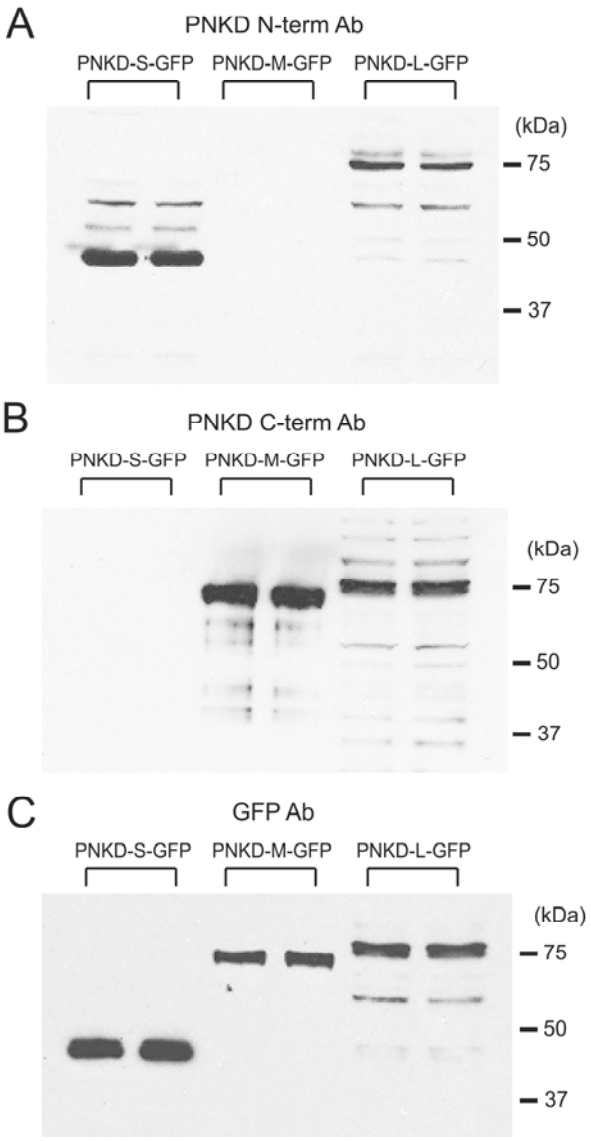
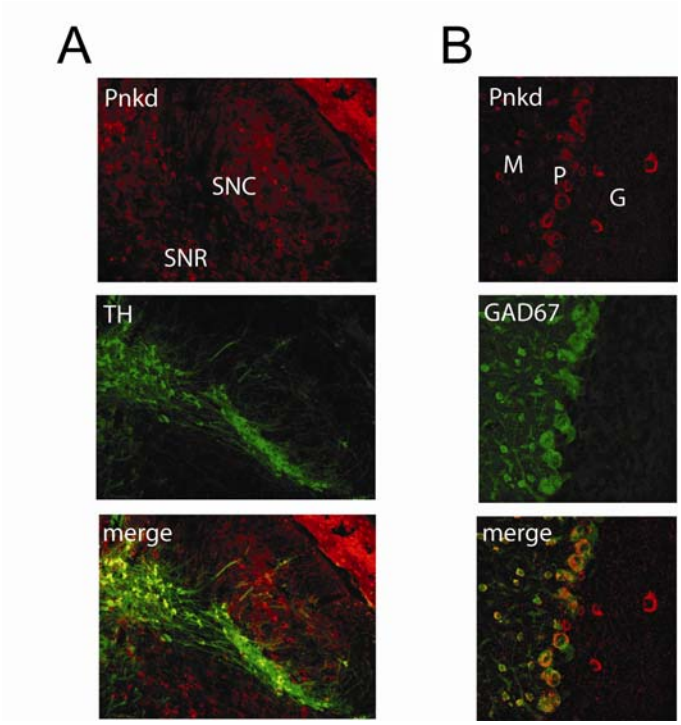
Supplemental Figure 1



Supplemental Figure 1

Western blot analyses of HEK293 cell extractions transfected with different isoforms of *PNKD-EGFP* by using PNKD antibodies and GFP antibody. PNKD N-terminal antibody can detect both PNKD-L-EGFP (~75 kDa) and PNKD-S-EGFP (~44 kDa), and PNKD C-terminal antibody can detect both PNKD-L-EGFP and PNKD-M-EGFP (~70 kDa). GFP antibody can positively identify all three EGFP-fusion proteins at similar sizes compared with PNKD antibodies.

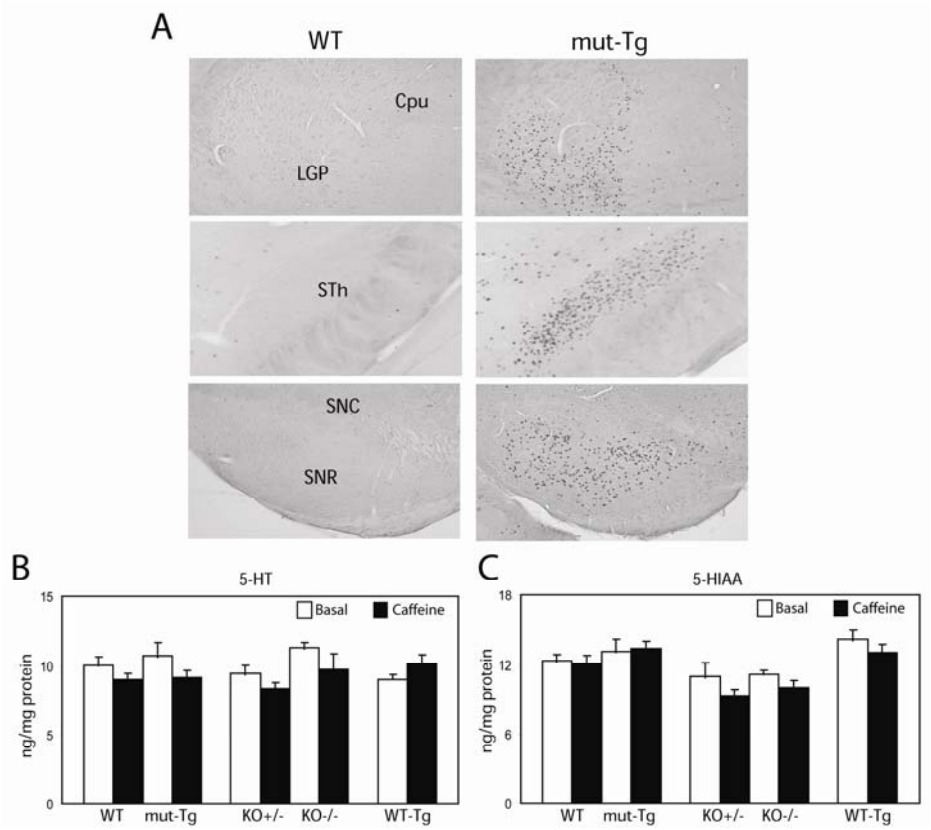
Supplemental Figure 2



Supplemental Figure 2

Pnkd immunochemistry in mouse brain. **(A)** Pnkd is expressed in substantia nigra pars reticulata (SNR), and substantia nigra pars compacta (SNC). In SNC, Pnkd is co-localized with the dopaminergic neurons which are positive for tyrosine hydroxylase (TH). Original magnification, x400. **(B)** In cerebellum, Pnkd is widely expressed in the Purkinje cells, and the molecular and granule cell layers. Purkinje cells and neurons in the molecular layer are co-labeled with anti-glutamate decarboxylase 67 (GAD67). Original magnification, x630.

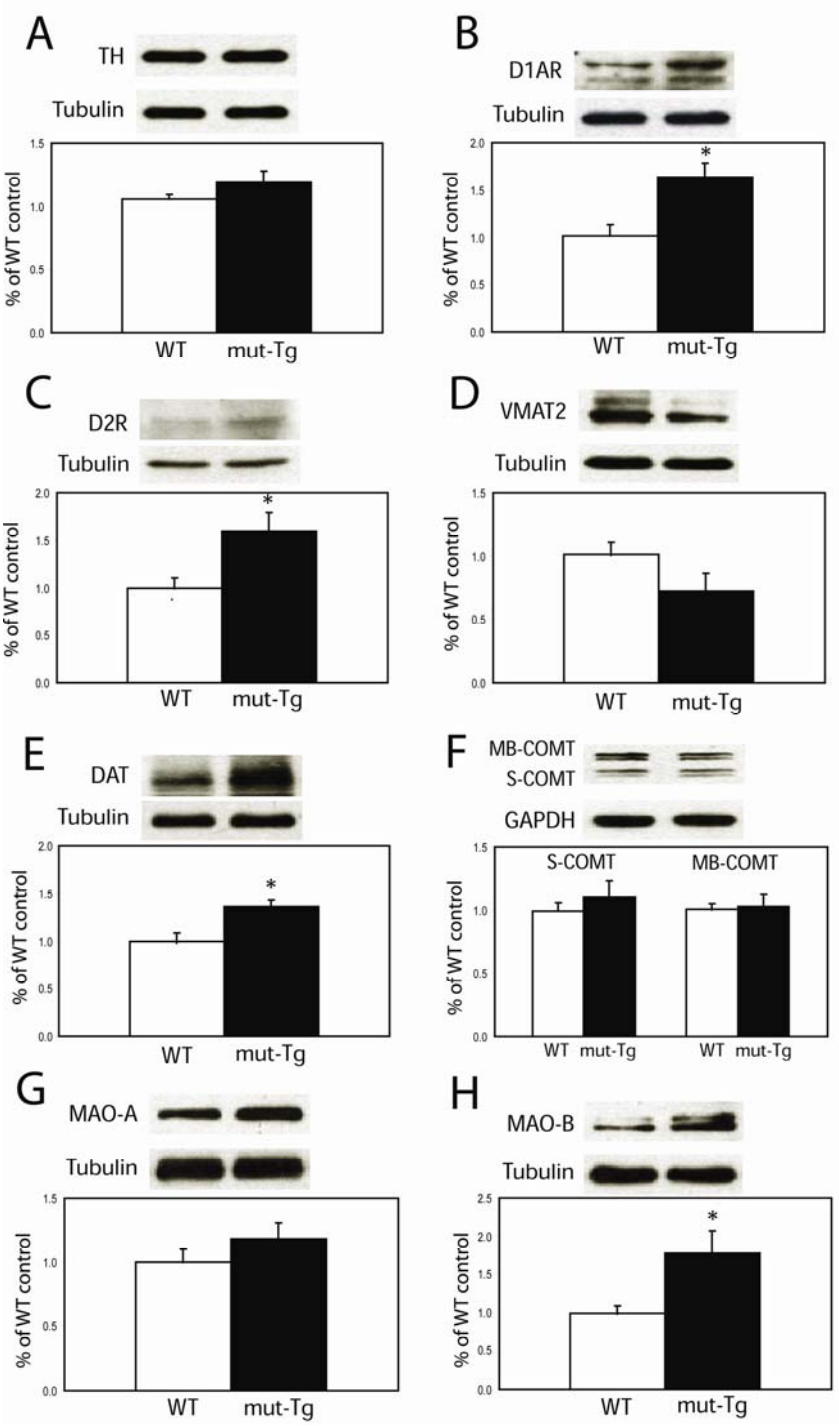
Supplemental Figure 3



Supplemental Figure 3

After caffeine treatment, strong c-Fos staining is detected in the basal ganglia of Pnkd (mut-Tg) mice vs. WT littermates but no obvious differences in 5-HT signaling pathways of PNKD mice or WT littermates were seen. **(A)** c-Fos immunohistochemistry in basal ganglia of Pnkd mice and WT littermates 2 hours after caffeine stimulation. Strong induction of c-Fos was detected in the lateral globus pallidus, subthalamic nucleus, and substantia nigra pars reticulata of Pnkd mice, but not in WT littermates. Abbreviations: **Cpu**, caudate putamen; **LGP**, lateral globus pallidus; **STh**, subthalamic nucleus; **SNR**, substantia nigra pars reticulata. Original magnification, x50. **(B, C)** There were no changes in the levels of serotonin (5-HT) or 5-hydroxyindoleacetic acid after caffeine treatment. All data are presented as mean ± SEM (n=10-12 mice for each group).

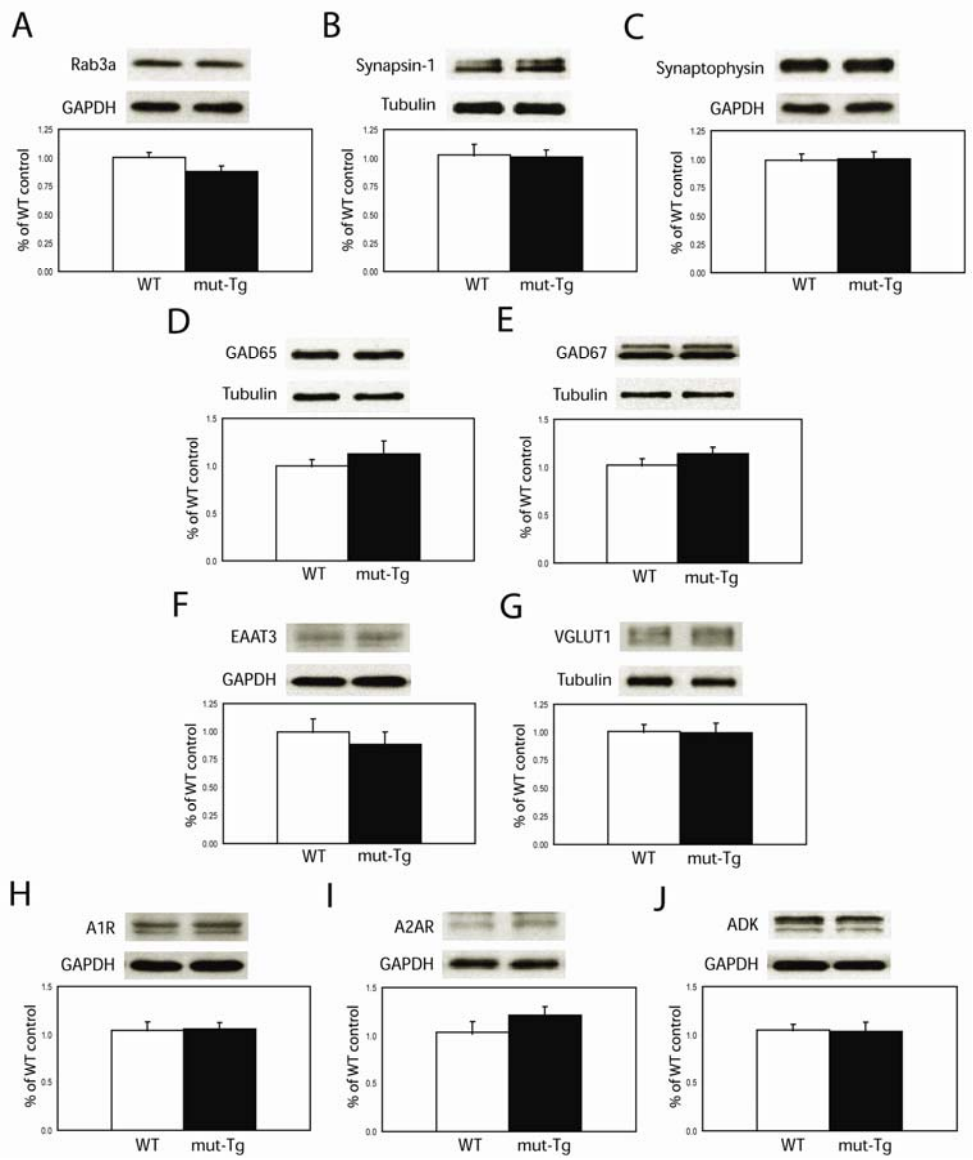
Supplemental Figure 4



Supplemental Figure 4

Western analysis shows no differences between Pnkd (mut-Tg) mice and WT littermates for a number of dopamine signaling and metabolism proteins, but the dopamine receptor, dopamine transporter, and monoamine oxidase B proteins are up-regulated in Pnkd mice. **(A)** No difference was found between genotypes for the expression levels of tyrosine hydroxylase (TH) in striatum of Pnkd mice and WT controls. **(B)** The protein levels of dopamine D_{1A} receptor (D1AR) is significantly higher in Pnkd mice compared with WT littermates ($P<0.05$). **(C)** The protein levels of dopamine D_2 receptor (D2R) is up-regulated in Pnkd mice compared with WT littermates ($P<0.05$) **(D)** The protein levels of vesicular monoamine transporter 2 (VMAT2) trended to being decreased in Pnkd mice vs. controls but the difference was not statistically significant. **(E)** The protein levels of dopamine transporter (DAT) is significantly higher in Pnkd mice compared with WT littermates ($P<0.05$). **(F, G, H)** There were no obvious differences in expression levels of two isoforms of catechol o-methyltransferase (COMT) and monoamine oxidase A (MAO-A) between genotypes, but the amount of monoamine oxidase B (MAO-B) is significantly increased in Pnkd mice ($P<0.05$). All data are given as mean ± SEM ($n = 8$ for each genotype).

Supplemental Figure 5

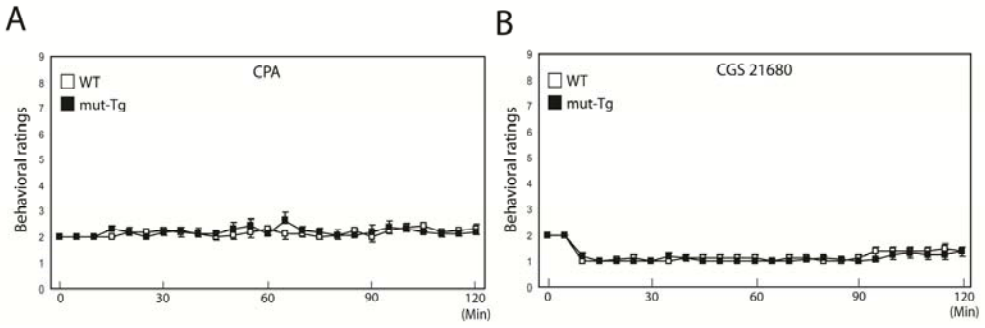


Supplemental Figure 5

Western analysis shows no differences between Pnkd (mut-Tg) mice and WT littermates for a number of striatal proteins. There were no differences in expression levels of **(A)** Rab3a, **(B)** synapsin-1, and **(C)** synaptophysin, **(D, E)** glutamate decarboxylase 65 and 67 (GAD65 and GAD67), **(F)** There were no differences in expression levels of excitatory amino acid transporter 3 (EAAT3), **(G)** vesicular glutamate transporter (VGLUT1), **(H, I)** adenosine A_1 and receptors (A_1R, $A_{2A}R$), and

(J) adenosine kinase (ADK) in mutant vs. control animals. All data are given as mean ± SEM (n = 8 for each genotype).

Supplemental Figure 6



Supplemental Figure 6

A_{1A} and A_2 Receptor Neuropharmacology. **(A)** No difference in activity ratings of Pnkd mice (mut-Tg) and WT littermates (WT) were observed after adenosine A_1 receptor agonist CPA (0.1 mg/kg, IP) or **(B)** adenosine A_2 receptor agonist CGS21680 (0.5 mg/kg, IP). Reduced activity was observed after treatment with CGS 21680, but no obvious difference is found between genotypes. These behavioral ratings are expressed as mean ± SEM (n = 8 for each group).

Figure legends of supplemental videos

Supplemental video 1.

Stress induced dyskinetic attacks in Pnkd mouse. In Pnkd (mut-Tg) mouse, stress by prolonged handling (>15-30 minutes) induced dyskinetic attacks.

Supplemental video 2.

Caffeine induced dyskinetic attacks in Pnkd mouse. In Pnkd (mut-Tg) mouse, caffeine (25 mg/kg) induced dyskinetic attacks ~15-20 minutes after treatment.

Supplemental video 3.

Ethanol induced dyskinetic attacks in Pnkd mouse. Ethanol (1.5 g/kg, 20% v/v) also induced dyskinetic attacks in the Pnkd (mut-Tg) mouse (right side) but not in WT littermate (left side) after **(A)** 15 minutes, and **(B)** 1 hour of injection.

Supplementary Methods

PNKD Antibodies. Polyclonal antibodies were developed (Covance, Berkeley, CA) using two synthesized oligopeptides corresponding to the N-terminus of PNKD-L and S (MAAVVAATALKGRGARNARVLRGC), and the C-terminus of PNKD-L and M (CDDYSRAQLLEELRRLKDMHKSK). Pre-immune bleeds and test bleeds were obtained individually from the supplier to monitor antibody titer and specificity in subsequent bleeds. Antibodies were affinity-purified using the oligopeptides coupled to activated CH-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). Antisera were added to the bead preparations, rotated, and incubated overnight at 4°C. Antiserum/bead mixtures were then poured into a 10 mL Poly-prep chromatography column (Bio-Rad, Hercules, CA), washed with four bead volumes of 1X phosphate-buffered saline (PBS) without calcium and magnesium (Gibco, Carlbad, CA), and eluted with 40 X 500μL fractions of 100mM glycine (pH 2.7) into microcentrifuge tubes containing 300μL 200mM Na_2HPO_4. Absorbance at 280nm was determined using the Coomassie PlusProteinAssay kit (Pierce Biotechnology, Rockford, IL) for each fraction with a DU 530 UV/Vis spectrophotometer (Beckman Coulter, Fullerton, CA). Affinity-purified antibodies were then used for Western blotting and immunohistochemistry experiments.

Generation of Pnkd transgenic mice. RP24-112K19 is a bacterial artificial chromosome (BAC) clone from the RP-24 mouse genomic library containing the entire *Pnkd* locus on a 183-kb genomic insert with 62kb upstream of the gene (Children's Hospital Oakland Research Institute). We constructed a rpsl/kan counter selection fragment. The primers were designed with 20 nucleotides for amplification of the rpsl/kan gene and an additional 50 nucleotides homologous to sequences flanking the region corresponding to the mutant region. This PCR product was transferred into the RP24-112K19 BAC by homologous recombination in *E. coli* strain DH10B already containing the plasmid pKD46 (1). The counter selection gene was removed by recombination using a synthetic oligonucleotide in the position carrying the Ala7Val and Ala9Val mutations as described elsewhere with modification (2). ET-cloning was then employed to introduce an internal ribosome entry site (IRES) followed by an enhanced red fluorescent protein gene (IRES/DsRed) into the 3' UTR of the *Pnkd* gene. All relevant segments generated by PCR and recombination were sequence confirmed. Purified BAC DNA (Nucleobond, BD) was subjected to Southern blotting to confirm correct homologous recombination. BAC DNA was digested with NotI restriction enzyme, run on a pulse field gel to identify the best samples to inject, and concentration of BAC DNA without linearization for injection

was adjusted to 1 ng/μL with microinjection buffer. WT transgenic mice were generated by microinjecting an unmodified BAC clone. The transgenic founders had a mixed C57BL/6XSJL F1 background and were backcrossed to C57/BL6 for >10 generations.

Generation of Pnkd knockout mice. To generate the targeting vector, a 2.6 kb short homologous arm upstream of *Pnkd* exon 5, was amplified with the LA PCR kit (Takara, Japan) using primers 5'-GCCCGGGCAGAAAGAGAGCGAAGTCAGAGAAG-3' and 5'-CTCGAGGAGCTCAGTACTGTCTGTAGGCCTCACATCTCTAGG-3'. A 6.5 kb long homologous arm, which is down stream of exon 9 was amplified using primers 5' CTCGAGGTGGTGAGTTTCTGCAAAAGAGAG-3' and 5'-ATCAGGTTTCCATGGTTTTGCTAT-3'. The fragments were inserted separately into the pMCIDT-A PGKNeo vector. About 1.5kb of genomic DNA, including exons 5 to 9, was replaced by the neomycin resistance gene. The targeting vector was linearized with *Not*I and used for electroporation into embryonic stem (ES) R1 cells. Culture of ES cells and isolation of homologous recombinants were performed according to standard protocols. Briefly, 10^7 R1 ES cells were transfected with 25 to 50 μg of the linearized vector. Cells were cultured under selection in G418. Resistant colonies were expanded in 96-well plates. Screening for homologous recombinants by PCR used amplification of external short arm area primer 5'-AGAGGCCACAGAAGGGAAGT-3' and vector side 5'GCCTCACATCTCTAGGATCTCTGT-3', and vector side 5'-GTTCTTCTGAGGGGATCAATTCTCTA-3' and external long arm area 5'-GATCCTTATAGGTCAAAGAGCAGGAG-3'. Positive ES clones were injected into C57BL/6J blastocysts and implanted in pseudopregnant females. Chimeric mice were screened using PCR and Southern blotting to detect recombinants and mated with C57BL/6 mice to produce F_1 heterozygous mice and heterozygotes identified by PCR. Confirmed *Pnkd* +/- F_1 mice were backcrossed to C57BL/6J mice or mated with siblings to generate null mutant mice.

Genotyping. PCR was performed on genomic DNA isolated from tail cuts of *Pnkd* transgenic, KO, and WT mice. PCR was performed using the following primer sets: 5'-TAGCGCTACCGGACTCAGAT-3' and 5'-AGGAACTGCTTCCTTCACGA-3' (mutant transgenic), 5'-AGTGCAGTCGTAAAAGTCAGAAC-3' and 5'-ACATACATGCAGGAAAACAACCA-3' (WT transgenic), and 5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3' and 5'-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-3' (KO mice). PCR was for 3 minutes at 94°C, 35 cycles of amplification (30s at 94°C, 30s at 60°C, and 60s at 72°C), followed by 3 minutes final extension at 72°C.

Immunoblotting. For Western blotting tissue homogenates obtained from *Pnkd* WT, transgenic and KO mice, protein concentrations were determined using a protein assay kit (Pierce, Rockhold, IL). Brain homogenates containing 20μg of protein were resolved on 10% polyacrylamide gels and electroblotted to nitrocellulose membrane using 50mM Tris-HCl buffer (pH 8.4). The blot was incubated with anti-PNKD antibody for 1 hour and detected using an ECL western blot chemiluminescent kit (GE Healthcare, Buckinghamshire, UK). Blots were stripped and reprobed with an anti-α-tubulin antibody (1:5000; Sigma, St. Louis, MO). Densitometry was performed using a Kodak 1-D system (Eastman Kodak, Rochester, NY). Pnkd levels were normalized to α-tubulin using the average pixel intensity for immunoreactive band in each lane and the images were analyzed by the NIH ImageJ software.

For the comparison of the levels of different proteins in striata of Pnkd mice and WT littermates, the dorsal striata of mice (n=8/genotype) were dissected and snap frozen in liquid nitrogen. Striata were sonicated in 300 μl of RIPA buffer (10mM Tris-HCl pH 7.2, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 1% SDS, 1% Deoxycholate) with protease inhibitor (Roche, Mannheim, Germany) and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Equal amounts of protein from the total striatal homogenate were used for the Western blot analysis as described above. Blots were then incubated with one of the following antibodies in 1:1000 dilution: anti-vesicular glutamate transporter 1 (VGLUT1), anti-Rab3A and anti-synaptophysin (Synaptic Systems, Goettingen, Germany); anti-excitatory amino acid transporter 3 (EAAT3), anti-monoamine oxidase A (MAO-A), and anti-monoamine oxidase B (MAO-B) (Santa Cruz biotechnology, Santa Cruz, CA); anti-adenosine kinase (ADK) (Abcam, Cambirdge, MA); anti-adenosine A_1 receptor (A_1R, EMD, Darmatadt, Germany); anti-catechol o-methyltransferase (COMT, BD biosciences, San Jose, CA); anti-synapsin-1, anti-glutamate decarboxylase 65 (GAD65), anti-glutamate decarboxylase 67 (GAD67), anti-adenosine A_{2A} receptor (A_{2A}R), anti-tyrosine hydroxylase (TH), anti-dopamine transporter (DAT), anti-dopamine D_{1A} receptor (D_{1A}R), anti-dopamine D_2 receptor (D_2R), and anti-vesicular monoamine transporter 2 (VMAT2) (Millipore, Billerica, MA), Detected was performed as described above, and were stripped and reprobed with either anti-α-tubulin or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Billerica, MA). Striata protein levels were normalized and data were analyzed using Student's *t* test.

RT-PCR. Total RNA was extracted from mouse brain with TRIzol (Invitrogen) according to the manufacturer's instructions, and then subjected to DNase treatment (Ambion). RNA (10μg) was reverse-transcribed using Superscript III (Invitrogen). The primer sequence for *Pnkd* were 5'-TGCTATTCTTCGCCTTCGTGCTG-3' and 5'-CCTGGCCCCAGAGCCTCCTGTAGT-3' (*Pnkd-M*), and

5’-GGGGTGGGACCCGAACATGGCGGC-3’ and 5’-CCTGGCCCCAGAGCCTCCTGTAGT-3’ (*Pnkd-L*). The following conditions were used for PCR: 3 minutes at 94°C, 35 cycles of amplification (30s at 94°C, 30s at 55°C, and 60s at 72°C), followed by 3 minutes final extension at 72°C.

Pnkd Immunohistochemistry in mice brain slides. Adult WT, and transgenic male mice were sacrificed, perfused, and brains/spinal cords were removed, fixed, postfixed, cryoprotected, and coronal sections were cut at 14 µm with a Leica CM1850 cryostat (Leica, Nussloch, Germany) and thaw-mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA). The sections were washed in 1 X PBST (PBS with 0.3% Triton X-100) for 5 minutes, then blocked by 10% normal goat serum-PBST (NGS-T) for 1 hour. Fluorescence immunohistochemistry staining was performed by incubating overnight in 2% NGS-T with PNKD N-terminal antibody (1:250 to 1:500 diluted) or PNKD C-terminal antibody (1:5000 diluted), and co-labeled with mouse monoclonal antibodies for detection of either neuron-specific nuclear protein (NeuN), glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), dopamine- and cyclic AMP-regulated neuronal phosphoprotein with molecular weight 32 kDa (DARPP-32), choline acetyltransferase (ChAT), parvalbumin, glutamic acid decarboxylase isoform 67 (GAD67), or tyrosine hydroxylase (TH) (Chemicon, Temecula, CA). After three washes with 2% NGS-T, sections were then incubated in 2% NGS-T with Cy^{TM}3-conjugated goat-anti rabbit IgG (diluted 1:300), alone or combined with Cy^{TM}2-conjugated goat-anti mouse IgG (1:50) (Jackson ImmunoResearch, West Grove, PA) for 1 hour, followed by washing three times in 1 X PBS-T in darkness. The sections were mounted under cover slides with Vectashield mounting medium (Vector Laboratories Inc. Burlingame, CA). Images were captured with a Zeiss Pascal LSM5 confocal microscope in the UCSF microscopy core and were imported into Photoshop (Adobe Systems, San Jose, CA) for analysis.

Immunostaining of transfected cells for PNKD-L localization. Human embryonic kidney (HEK)-293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal bovine serum (Hyclone, Logan, UT) and maintained at 37°C with 5% CO_2. After one day, the cells were split into 35mm culture dishes on coverslips pre-coated with poly-L-lysine (Sigma, St. Louis, MO). In parallel, HEK293 cells grown to 80-90% confluence were transfected with 2 µg DNA (*PNKD*-L, M, and S WT fusion constructs) using 20 µl of Polyfect reagent (Qiagen, Valencia, CA) using a standard protocol. Twelve hours post-transfection, cells were quick fixed in 4% paraformaldehyde and permeabilized with PBS/0.1% Triton or incubated in PBS and then stained with either PNKD N-terminal or C-terminal antibody followed by Cy^{TM}3-conjugated goat-anti rabbit

IgG (1:300) secondary antibody (Jackson ImmunoResearch, West Grove, PA) and mounted on Premium microscope slides (Fisher, Pittsburgh, PA) with Vectashield mounting medium (Vector, Burlingame, CA). Images were acquired using the Zeiss Pascal LSM5 confocal microscope in the UCSF microscopy core facility.

Mapping of c-Fos induction after a precipitation of a PNKD attack. Two hours after intraperitoneal (IP) injection of ethanol or caffeine, mice were perfused intracardially under deep tribromoethyl alcohol anesthesia with PBS followed by 4% paraformaldehyde in PBS. Following perfusion, brains were post-fixed and sliced into 30μM coronal sections using a vibratome. c-Fos immunohistochemistry was performed on free-floating brain slices. Brain sections were pretreated for 30 minutes 0.5 hour with 0.5% solution of H_2O_2 and blocked with 4% inactivated normal goat serum for 30 minutes in PBS/0.3% Triton X-100 prior to being rinsed with normal PBS. The primary antibody (Ab2, Oncogene Research Products) was diluted 1:1000 in PBS with 1% bovine serum alubumin (BSA)/0.3% Triton X-100 and incubated overnight at 4°C. Biotinylated goat anti-rabbit IgG (Oncogene Reseach Products) was diluted 1:400 in PBS with 1% BSA/0.3% Triton X-100. Sections were then incubated in avidin-biotin-horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories Inc.) for 1 hour. Labeling was revealed by exposure to 3,3'-diaminobenzidine (DAB) substrate (Peroxidase Substrate kit, Vector Laboratories Inc.) for 10-15 minutes. The sections were then mounted under cover slides with Vectashield mounting medium (Vector Laboratories Inc. Burlingame, CA) and images were captured with a Nikon Eclipse microscope with a CCD camera or a Leica DM5000B microscope with a SPOT RT camera (SPOT diagnostic Inc., Sterling Heights, MI) and were imported into Photoshop program for analysis.

References

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2. Narayanan, K., Williamson, R., Zhang, Y., Stewart, A.F., and Ioannou, P.A. 1999. Efficient and precise engineering of a 200 kb beta-globin human/bacterial artificial chromosome in E. coli DH10B using an inducible homologous recombination system. *Gene Ther* 6:442-447.