Paroxysmal nonkinesigenic dyskinesia (PNKD) is an autosomal dominant episodic movement disorder. Patients have episodes that last 1 to 4 hours and are precipitated by alcohol, coffee, and stress. Previous research has shown that mutations in an uncharacterized gene on chromosome 2q33–q35 (which is termed PNKD) are responsible for PNKD. Here, we report the generation of antibodies specific for the PNKD protein and show that it is widely expressed in the mouse brain, exclusively in neurons. One PNKD isoform is a membrane-associated protein. Transgenic mice carrying mutations in the mouse Pnkd locus equivalent to those found in patients with PNKD recapitulated the human PNKD phenotype. Staining for c-fos demonstrated that administration of alcohol or caffeine induced neuronal activity in the basal ganglia in these mice. They also showed nigrostriatal neurotransmission deficits that were manifested by reduced extracellular dopamine levels in the striatum and a proportional increase of dopamine release in response to caffeine and ethanol treatment. These findings support the hypothesis that the PNKD protein functions to modulate striatal neurotransmitter release in response to stress and other precipitating factors.
Dopamine dysregulation in a mouse model of paroxysmal nonkinesigenic dyskinesia

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Paroxysmal nonkinesigenic dyskinesia (PNKD) is an autosomal dominant episodic movement disorder. Patients have episodes that last 1 to 4 hours and are precipitated by alcohol, coffee, and stress. Previous research has shown that mutations in an uncharacterized gene on chromosome 2q33–q35 (which is termed PNKD) are responsible for PNKD. Here, we report the generation of antibodies specific for the PNKD protein and show that it is widely expressed in the mouse brain, exclusively in neurons. One PNKD isoform is a membrane-associated protein. Transgenic mice carrying mutations in the mouse Pnkd locus equivalent to those found in patients with PNKD recapitulated the human PNKD phenotype. Staining for c-fos demonstrated that administration of alcohol or caffeine induced neuronal activity in the basal ganglia in these mice. They also showed nigrostriatal neurotransmission deficits that were manifested by reduced extracellular dopamine levels in the striatum and a proportional increase of dopamine release in response to caffeine and ethanol treatment. These findings support the hypothesis that the PNKD protein functions to modulate striatal neurotransmitter release in response to stress and other precipitating factors.

Introduction

The paroxysmal dyskinesias consist of clinically and genetically distinct phenotypes, including paroxysmal kinesigenic dyskinesia, paroxysmal exercise-induced dyskinesia, and paroxysmal nonkinesigenic dyskinesia (PNKD) (1, 2). PNKD is a highly penetrant autosomal dominant disorder in which individuals have 1- to 4-hour attacks consisting of dystonia and choreoathetosis (3). These attacks can be induced reliably by administration of caffeine or alcohol and frequently when patients are stressed. The causative gene was mapped to chromosome 2q33–q35 (4, 5), and mutations in the PNKD gene (formerly called MR-I) were subsequently identified in PNKD families (6–10).

The PNKD gene has at least 3 alternate splice forms, which encode proteins of 385, 361, and 142 amino acids. The long isoform of PNKD (PNKD-L) is specifically expressed in CNS, while the medium isoform (PNKD-M) and short isoform (PNKD-S) are ubiquitously expressed (7). Two missense mutations (Ala to Val) located at amino acids 7 or 9 of PNKD-L and PNKD-S were found in most patients, and a third mutation (Ala to Pro) at position 33 was reported in 1 patient (11). Both PNKD-L and PNKD-M have a putative catalytic domain that is homologous to hydroxyacylglutathione hydrolase (HAGH), a member of the zinc metallo-hydrolase enzyme family, which contains β-lactamase domains. HAGH functions in a pathway to detoxify methylglyoxal, a by-product of oxidative stress (12).

The normal role of PNKD in cells and the contribution of mutations to pathophysiology of PNKD are not known. Dyskinesia is seen with many genetic and acquired disorders of the brain. Theoretically, such hyperkinetic movements could have their genesis in the basal ganglia, the cerebellum, or even in the cortex. Having cloned the gene and shown by in situ hybridization that it is widely expressed, we were interested in probing the pathophysiology of this fascinating disorder.

In this study, we generated polyclonal antibodies specific for detecting PNKD isoforms. We also generated WT and mutant Pnkd-transgenic and Pnkd-KO mice in order to determine whether they can recapitulate human PNKD phenotypes. Together, these reagents provided a unique opportunity to begin addressing questions regarding the pathophysiology of PNKD. We specifically began by determining the expression pattern of the Pnkd gene and protein. Next, we set out to see whether attacks in mice could be precipitated by the same stimuli that cause attacks in human PNKD patients. Since alcohol and caffeine are known to be “dirty” drugs that act on many receptor systems in the brain, targeted neuropharmacological agents were used to test specific pathways through which they might be acting. Finally, the neurotransmitter systems and receptors involved in transducing the abnormal dyskinetic movements in PNKD and the brain region or regions involved were also investigated. Thus, these studies were aimed at a more systems-level understanding of the pathophysiology as opposed to the molecular or cellular basis of PNKD. Such understanding, along with more work aimed at the molecular basis of PNKD, will be necessary to ultimately develop better therapies for paroxysmal dyskinesias and, potentially, other movement disorders.

Results

Nomenclature. In this study, standard nomenclature for names of genes and proteins was used. In vitro experiments were performed in cells transfected with the human cDNA, and in vivo experiments were done in mice. PNKD represents the human gene name, while PNKD is the human protein name and the acronym for the disorder paroxysmal nonkinesigenic dyskinesia. Pnkd is the mouse gene name, and Pnkd is the name for the mouse protein. “Pnkd mice” is used to denote the animal model we created that harbored the PNKD phenotype (i.e., mice transgenic for a BAC harboring both the A7V- and A9V-encoding Pnkd mutations).

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Mapping Pnkd expression. In situ hybridization analysis previously showed that Pnkd-L mRNA is widely expressed in neurons of the CNS, but not in other tissues (7). We developed antibodies induced by oligopeptides from the N terminus (N-terminal antibody, expected to detect PNKD-L and -S) or C terminus (C-terminal antibody, expected to detect PNKD-M and -L) (Figure 1A). The C-terminal antibody detected 2 main bands (PNKD-L, ~47 kDa; PNKD-M, ~40 kDa), and the N-terminal antibody detected 2 bands (PNKD-L and PNKD-S, ~18 kDa) in mouse brain extracts (Figure 1B). We also tested the PNKD antibodies by detecting different isoforms of PNKD-EGFP transsected in human embryonic kidney 293 (HEK293) cells. In this heterologous expression system, the size of PNKD-L-EGFP, PNKD-M-EGFP, and PNKD-S-EGFP are approximately 75 kDa, approximately 70 kDA, and approximately 44 kDa, respectively (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI8470DS1).

Immunohistochemistry using these antibodies confirmed that Pnkd is expressed widely in the CNS, including striatum, substantia nigra, cerebellum, and spinal cord (Figure 1, C–F). Pnkd is expressed both in striatal medium spiny neurons and in interneurons (Figure 1C) and also in substantia nigra pars compacta (SNC), which is the output structure of the basal ganglia (Supplemental Figure 2A). In cerebellum, Pnkd is expressed in the granule cell and molecular layers and in Purkinje cells (Supplemental Figure 2B). Double immunohistochemical staining was performed with these antisera and markers specific for neurons or glia, and the results showed that Pnkd is expressed in neurons but not in oligodendrocytes or astrocytes (Figure 1, D–F). No difference in protein distribution was noted in Pnkd mutant mice versus littermate controls or Pnkd WT transgenic mice (data not shown).

There is one weak transmembrane segment prediction in PNKD-L (7). To determine whether PNKD-L is a transmembrane protein, we performed studies with permeabilized and nonpermeabilized PNKD-L-EGFP–transsected HEK293 cells. PNKD-L can be detected by both N- and C-terminal antibodies at the membrane in permeabilized cells, but not in nonpermeabilized cells (Figure 2A). We also prepared subcellular membrane fractions of HEK293 cells transfected with PNKD-L–EGFP, performed detergent partitioning with Triton X-114 (TX-114) onto subcellular membrane fractions, and showed that after TX-114 treatment, PNKD-L–EGFP can be detected in the hydrophilic layer, but not in the hydrophobic layer, using PNKD antibodies (Figure 2B). Thus, PNKD-L appears to be membrane associated, though not a transmembrane protein including the murine promoter and a large flanking region extending 62 kb upstream) and was expected to contain all of the cis-acting regulatory elements needed to recapitulate the expression pattern of the endogenous loci. In addition, we generated Pnkd WT BAC transgenic mice (WT-Tg, Pnkd mice) were generated by introducing both A7V- and A9V-encoding mutations into Pnkd (Figure 3A). The resulting BAC construct included the murine promoter and a large flanking region (extending 62 kb upstream) and was expected to contain all of the cis-acting regulatory elements needed to recapitulate the expression pattern of the endogenous loci. In addition, we generated Pnkd WT BAC transgenic mice (WT-Tg). Copy numbers of all lines were assessed by Southern blot; mut-Tg lines contained 1–2 copies, and the WT-Tg lines contained 2–3 copies (Figure 3B). Western blotting of mut-Tg and WT-Tg brain extracts showed that protein levels were consistent with the transgene copy number (Figure 3C). We also generated 2 lines of Pnkd-KO mice (Figure 3D). Both RT-PCR and Western blotting confirmed that they were true Pnkd nulls (Figure 3, E and F). Multiple lines of mut-Tg, WT-Tg, and KO mice were observed, and all were fertile, with normal growth, and had no gross phenotypic abnormalities. None of the lines showed neuropathological defects by Nissl staining (data not shown). The body weight of PNKD mice trended lower than that of WT littermates, but the difference was not statistically significant. However, in mut-Tg mice, we observed dyskinesia after stress, such as prolonged handling (15–30 minutes; see Supplemental Video 1). The dyskinesias included oral-facial movements (e.g., tongue protrusions) and stereotypic movements (repetitive sniffing and rearing in one location; see Supplemental Video 1). Dyskinesias were not seen in WT littermates, WT-Tg mice, or Pnkd-KO mice.

Mice were challenged with i.p. injection of caffeine (25 mg/kg) or ethanol (1.5 g/kg, 20% v/v). In mut-Tg mice, caffeine induced dyskinetic attacks approximately 10–15 minutes after treatment that persisted for 2 hours (Figure 4A and Supplemental Video 2). The severity of attacks in mut-Tg mice was significantly increased compared with that in predrug control or postdrug control using vehicle (saline, 10 ml/kg; Figure 4B). Behavioral effects of caffeine injection in WT littermates and WT-Tg mice were moderate hyperlocomotion during the first hour, but no dyskinesias were observed (Figure 4A). Ethanol also induced dyskinetic attacks in the mut-Tg mice (but not in WT littermates) beginning 10 minutes after injection and lasting 2–4 hours, and the attacks were more severe than those induced by caffeine. The mut-Tg mice suffered severe axial stiffness, with some abnormal movements of the limbs (Figure 4C and Supplemental Video 3, A and B). We did not observe any abnormal movements in the WT-Tg or KO mice after injection of caffeine or ethanol (data not shown). We examined multiple lines of mut-Tg, WT-Tg, and KO mice for i.p. injection of caffeine or ethanol, and the results showed no differences among genotypes. Unlike in diethylnitrosamine-treated hamsters, there were no obvious age-dependent differences in the expression of dyskinesias of mut-Tg mice.

Neuroanatomical characterization of Pnkd mice. Since Pnkd is widely expressed in brain, we performed immunohistochemical studies with anti–c-Fos antibody to identify neuronal populations activated during PNKD attacks. Under basal conditions, almost no c-Fos–reactive cells were found in the brains of mut-Tg mice or WT littermates. Induction of c-Fos was seen in globus pallidus, subthalamic nucleus, and substantia nigra reticulata of mut-Tg mice, but not WT littermates, after caffeine injection (Supplemental Figure 3A). Similar patterns of c-Fos–positive signal were also seen in mutant mice after an attack was provoked with ethanol (data not shown). Minimal c-Fos–positive immunostaining was seen in cortical areas, but was not different in mut-Tg mice versus littermates. There were no obvious changes of c-Fos expression in basal ganglia or other brain regions in either WT-Tg or KO mice (data not shown). Thus, basal ganglia neurons are activated specifically in mut-Tg mice after induction of attacks.

Neurochemical studies show alteration of striatal dopamine signaling system of Pnkd mice. Dopamine plays an important role in modulation of cortical and thalamic glutamatergic signal processing in the striatum, thus regulating movement (13–15). Therefore, we measured dopamine and its metabolites in the striatum by HPLC both before and after stimulating attacks. Pnkd and control mice had similar dopamine levels in striatum at rest (Figure 3D). After i.p. injection of caffeine (25 mg/kg), Pnkd mice had significantly higher levels of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and higher DOPAC/dopamine ratios than untreated animals (P < 0.01,
Figure 4, E and F) or WT littermates treated with caffeine ($P < 0.05$ for DOPAC, Figure 4E; $P < 0.01$ for DOPAC/dopamine ratio; Figure 4F). The DOPAC levels and the DOPAC/dopamine ratio were significantly decreased in *Pnkd*-KO mice after injection of caffeine ($P < 0.01$) or ethanol (data not shown), but did not change in WT-Tg mice (Figure 4, E and F). Homovanillic acid (HVA), the terminal metabolite of dopamine metabolism, was also significantly increased in Pnkd mice after caffeine stimulation ($P < 0.05$; Figure 4G), while the levels of serotonin (5-HT) and its metabolized product (5-hydroxyindoleacetic acid [5-HIAA]) were not different in Pnkd versus control or before versus after caffeine challenge (Supplemental Figure 3, B and C). No obvious changes of DOPAC, DOPAC/dopamine ratio, and HVA levels were found in the *Pnkd* WT-Tg mice (Figure 4, D–G). Thus, dysfunction of dopamine signaling is associated with PNKD pathophysiology. Since the striatum is the primary basal ganglia region receiving dopaminergic and glutamatergic inputs from other brain...
structures (16–18), it is possible that expression of proteins involved in dopamine, glutamate, and/or adenosine signaling is altered in PNKD. To test this, striatal extracts from Pnkd and control mice were run on Western blots and probed with antibodies to proteins important for striatal function.

Because striatal dopamine signaling in Pnkd mice is altered (Figure 4, E–G), we examined the expression levels of proteins involved in dopamine synthesis, signaling, reuptake, and metabolism. Tyrosine hydroxylase (TH), the rate-limiting enzyme in the generation of dopamine, was no different for Pnkd mice versus controls (Supplemental Figure 4A). But D_1AR and D_2R were both significantly upregulated in the striata of Pnkd mice (P < 0.05; Supplemental Figure 4, B and C). We noted a trend (although not statistically significant) of decreased expression of vesicular monoamine transporter 2 (VMAT2) in PNKD striata versus control (Supplemental Figure 4D). Dopamine transporter (DAT) (~55 kDa in size) expression was significantly higher in the Pnkd mice compared with WT littermates (P < 0.05; Supplemental Figure 4E), and Pnkd mice also showed significantly higher expression of another main band of DAT in Western blots (~85 kDa, data not shown). There were no obvious differences in the expression of soluble catechol-methyltransferase (S-COMT) or membrane-bound COMT (MB-COMT) (19) in Pnkd mice versus littermate controls (Supplemental Figure 4F). Immunoblotting did not reveal any changes in expression levels of monoamine oxidase A (MAO-A) among genotypes (Supplemental Figure 4G). However, the level of MAO-B expression was significantly higher in Pnkd mice compared with littermate controls (P < 0.05; Supplemental Figure 4H).

We also examined striatal levels of synaptic vesicle proteins Rab3a, synapsin-1, and synaptophysin. Expression of these proteins was not altered in mutant versus control mice (Supplemental Figure 5, A–C). We also examined striatal proteins involved in GABA synthesis and glutamate reuptake (GAD65 and GAD67, EAAT3, and VGLUT1) by Western blotting. Expression of these proteins was no different in Pnkd versus control mice (Supplemental Figure 5, D–G) suggesting that GABA synthesis in striatum and glutamate reuptake are normal in Pnkd mice. We next examined expression of adenosine A_1 and adenosine A_2A receptors (A_1R and A_2AR), and adenosine kinase (ADK), a key regulator of adenosine metabolism. These were all unchanged in Pnkd mice versus WT littermates (Supplemental Figure 5, H–J). These results indicate that the alteration of striatal dopamine signaling system of Pnkd mice may contribute to dyskinetic attacks of Pnkd mice.

**Figure 2**
PNKD-L is a membrane-associated protein. (A) Immunostaining studies of permeabilized and nonpermeabilized HEK293 cells indicate that PNKD-L is a membrane-associated protein. HEK293 cells are transfected with PNKD-EGFP followed by immunohistochemical staining using N- and C-terminal PNKD antibodies and Cy3-conjugated goat anti-rabbit IgG secondary antibody. Both PNKD N- and C-terminal antibodies detect transfected PNKD-L–EGFP in permeabilized HEK cells, but fail to detect it in nonpermeabilized HEK cells. Scale bars: 10 microns. (B) Detergent phase partitioning of HEK293 membrane fraction transfected with PNKD-L–EGFP. The PNKD-L–EGFP (~75 kDa) can be detected in crude membrane fraction by both PNKD antibodies, but after TX-114 treatment, the PNKD-L–EGFP can be detected in the hydrophilic layer, but not in the hydrophobic layer.
to stress and caffeine was assessed by conventional microdialysis. Consistent with the no net flux method, a significant reduction in extracellular dopamine concentrations in mut-Tg mice was seen both before and after challenge with stress and caffeine (Figure 5C). Two-factor ANOVA with repeated measures revealed a significant effect of genotype ($F_{1,15} = 10.8; P < 0.005$), but no effect of treatment ($F_{2,30} = 0.3; P < 0.5$). Interestingly, dopamine release in response to stress and caffeine (described as proportion of baseline release) was higher than in WT littermates (Figure 5D). These results indicate that abnormalities in dopamine release at rest and in response to different challenges are present in the striata of Pnkd mice.

**Figure 3**
Generation of Pnkd-transgenic and -KO mice. (A) 2 human mutations (A7V and A9V) were introduced into the BAC containing Pnkd. 62 kb of flanking sequence 5’ of the ATG start site is present in the BAC and is expected to contain all of the cis-acting regulatory elements leading to expression in a pattern faithful to the endogenous alleles. An internal ribosome entry site (IRES) followed by an enhanced red fluorescent protein gene (IRES/DsRed) was introduced into the 3’ UTR of the Pnkd gene. The original BAC without mutations was used for generation of Pnkd WT-Tg mice. (B) Southern blot analyses of mutant and WT-Tg mice. (C) Western blot analyses of brain extracts from mut-Tg and WT-Tg mice with C-terminal antibody. Tubulin was used as loading control. (D) Pnkd-KO mice. A short homologous arm upstream of exon 5 and a long homologous arm downstream of exon 9 were amplified and subcloned into the pMCIDT-A PGKNeo vector to replace 1.5 kb of genomic Pnkd DNA (including exons 5–9) with a neomycin resistance gene. (E) Genotype analyses of Pnkd-KO mice by RT-PCR. (F) Western blot analyses of mut-Tg and KO mice using C-terminal antibody. Tubulin was used as loading control. *Pnkd-L (~47 kDa); **Pnkd-M (~40 kDa).
Pnkd mice have deficits in nigrostriatal neurotransmission. We next performed carbon fiber amperometry studies to assess evoked release of dopamine in striatal slices from mutant and control mice. Electrically evoked release of dopamine was assessed by amperometry in striatal slices. The amperometry traces of striatal slices showed obvious differences between Pnkd mice and WT littermates (Figure 6A). Significantly lower dopamine signals were observed in PNKD mice versus controls (P < 0.01; Figure 6B). Application of the selective DAT inhibitor, nomifensine (3 μM/l for 30 minutes), did not significantly increase the dopamine signals in slices from mutant mice compared with slices from WT littermates (P < 0.01; Figure 6C). Dopamine release in response to electric stimulation was also significantly lower in slices from Pnkd mice versus WT littermates (P < 0.05; Figure 6D). Overall stimulated dopamine...
release was restored to normal levels in Pnkd slices after application of nomifensine. The spike width was significantly smaller and the half width ($t_{1/2}$) was significantly larger in Pnkd than in WT controls ($P < 0.05; \text{Figure 6}, \text{D and E}$). The spike width returned to normal in the presence of nomifensine (Figure 6D), but the $t_{1/2}$ did not ($P < 0.01; \text{Figure 6E}$). These results suggest that Pnkd mice feature significant deficits in nigrostriatal neurotransmission characterized by low levels of dopamine release and enhanced dopamine reuptake. The latter is compatible with DAT expression being significantly higher in the Pnkd mice compared with WT littermates (Supplemental Figure 4E).

Neuropharmacologic characterization of Pnkd mice. Striatal A$_1$R and A$_2$AR are the major modulators of striatal signaling (20, 21). The A$_1$Rs are enriched in striatonigral-striatoentopenduncular medium spiny neurons that constitute the direct output pathway expressing dopamine D$_1$ receptors (D$_1$Rs). A$_1$Rs and dopamine D$_2$ receptors (D$_2$Rs) are both expressed in striatopallidal medium spiny neurons that represent the indirect output pathway (22–24). Since caffeine is a nonselective adenosine receptor antagonist (25–27), we evaluated the contribution of A$_1$R and A$_2$AR to attacks in PNKD. Dyskinesias were induced in Pnkd mice approximately 20 minutes after i.p. injection of the selective A$_2$AR antagonist 8-(3-chlorostryl) caffeine (CSC) (5 mg/kg; Figure 7A) and lasted approximately 2 hours. The A$_1$R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (3 mg/kg) did not induce dyskinesias in Pnkd mice (Figure 7B). The A$_1$R agonist 8-cyclopentyladenosine (CPA) (0.1 mg/kg) had no obvious effects on Pnkd mice or controls (Supplemental Figure 6A), and the A$_2$AR agonist 2p-(2 carboxyethyl)phenethylamine-5’-N-ethylcarboxyaminoadenosine (CGS 21680) (0.5 mg/kg) led to a reduction of activity in both Pnkd mice and controls, with no difference between genotypes (Supplemental Figure 6B).

We speculated that dyskinesias induced by caffeine are caused by altered signaling between adenosine receptors and dopamine receptors in striatum. D$_1$R and D$_2$R are major receptors in the regulation of striatal function, and antagonistic interactions between A$_1$R-D$_1$R and A$_2$AR-D$_2$R in the basal ganglia are important in motor control (13). Since D$_2$Rs colocalize with A$_2$ARs in neurons of the indirect pathway of the striatum, we hypothesized that the selective D$_2$R agonist quinpirole would induce dyskinetic attacks in Pnkd mice. Mutant mice developed dyskinesias approximately 15 minutes after quinpirole treatment (2.5 mg/kg), but no phenotype was seen in controls (Figure 7C). The D$_1$R agonist SKF 82958 (0.75 mg/kg) increased the behavioral activity in all mice, without significant differences among genotypes (Figure 7D). Taken together, these results support a model of adenosine-dopamine signaling dysregulation in PNKD.
Discussion

PNKD is a rare disorder in which dyskinetic attacks can be induced by ingestion of caffeine or alcohol and frequently when patients are stressed (3). A transgenic Pnkd mouse model carrying both human mutations recapitulates the human phenotype. Pnkd-KO mice do not have a phenotype, arguing that the mutation of PNKD is a gain-of-function allele. It is possible that these PNKD mutations cause the disease by altering enzyme activity or specificity. Alternatively, they may not alter its enzymatic properties, but rather, cause altered trafficking and localization of the protein in neurons or through alterations of interactions between PNKD-L and other neuronal proteins. Finally, it is also possible that PNKD has evolved a novel function and is not an enzyme.

We presented in vivo evidence of increased dopamine turnover. Administration of caffeine has been shown to increase dopamine release accompanied by decreased DOPAC levels in rat striatum (28–30), similar to what we saw in WT control and Pnkd-KO mice. It is also known that acute ethanol administration can cause an increase of dopamine turnover as assessed by increased DOPAC in rat striatum, suggesting that both pre- and postsynaptic dopaminergic mechanisms may be involved in the mediation of some of the central effects of ethanol in striatum (31, 32). Increased c-Fos expression in basal ganglia of mutant versus control mice after caffeine administration shows that neurons in this part of brain are activated with the induction of attacks. Furthermore, Pnkd mice have significantly lower evoked dopamine release in real time and higher expression levels of dopamine receptors, DAT, and MAO-B in the striatum compared with controls. Taken together, these results suggest dysfunction of dopamine signaling in basal ganglia of Pnkd mice with induction of dyskinesias.

Neuropharmacological experiments implicate a role for A_2ARs and D_2Rs in pathogenesis, as attacks can be triggered with a selective A_2AR antagonist and a selective D_2R agonist. This, in turn, leads to dysfunction of antagonistic interactions between adenosine and dopamine receptors in modulation of motor outputs from striatum (22, 24). These results indicate strong involvement of the striatal indirect pathway in PNKD pathophysiology, but we cannot rule out the possibility that the direct pathway is also involved. Furthermore, though it is clear that the striatum is important for transducing abnormal dopamine signaling in PNKD, both A_2A and D_2 receptors are also expressed in other CNS regions. Besides, adenosine A1Rs are expressed presynaptically in CNS, including...
at glutamatergic terminals on medium spiny neurons. Thus, the
genesis of PNKD could be outside the striatum (e.g., cortex).

Alterations of dopaminergic function in striatum play an impor-
tant role in primary dystonias. Dopa-responsive dystonia (DYT5)
is caused by mutations of the GTP–cycohydrolase 1 gene involved in
catecholamine and serotonin biosynthesis or by mutations of TH
(2, 33). In drk hamsters, an elevation of extracellular striatal dopa-
mine levels has been observed during dystonic episodes (34). Inter-
estingly, unlike drk hamsters, Pnkd mice have reduced extracellular
dopamine in striatum in vivo, but, when challenged by stress,
caffeine, or alcohol, there is a relative increase in dopamine com-
pared with controls. Pnkd mice have normal dopamine content
in striatal dopaminergic terminals and apparently normal dopa-
nine production. But dopamine receptors are upregulated, and
when animals are stressed, striatal dopamine release is increased
in Pnkd mice and receptor sensitivity is increased due to low basal
extracellular dopamine levels. Excessive dopaminergic signaling
under these conditions may lead to abnormal neuronal activity in
Pnkd basal ganglia accompanied by significantly increased dopa-
mine turnover. Although, typically, less dopamine release might
be expected to yield less movement, dopamine loss occurring early
in development can result in abnormal and excessive movements.
This is true in humans with DYT5, where a developmental loss of
dopamine caused by mutations that reduce dopamine synthesis
causes dystonia (35, 36). It is also true for rodents, since dopamine
depletion by 6-hydroxydopamine (6-OHDA) in adult rats causes
akinesia (37–39). However, neonatal 6-OHDA treatment in rats
causes hyperactivity (40–42).

PNKD-L is a membrane-associated protein, and Pnkd mice
exhibit alterations of exocytosis, suggesting that PNKD may be
involved in modulation of neurotransmitter release at nigrostri-
trial dopaminergic terminals. Alternatively, PNKD may partici-
pate in the modulation of striatal glutamatergic inputs project-
ing from cerebral cortex and thalamus. Adenosine receptors and
dopamine receptors not only interact with each other, but also
cooperate with other signaling systems, such as metabotropic glu-
tamate receptors and cannabinoid receptors. These interactions
between different signaling systems are critical for modulation of
normal striatal function and plasticity (14, 20, 43, 44). These find-
ings imply that dysfunction of the striatal dopamine signaling
system plays a pivotal role in PNKD pathophysiology. Although
expression levels of adenosine receptors and glutamate transport-
ers are not different in striatum of Pnkd mice, we cannot rule out
the possibility of altered sensitivity of adenosine receptors and/or
glutamate release in glutamatergic terminals. Dopamine is critical
for the induction of bidirectional plasticity at glutamatergic syn-
apses on the medium spiny neurons of both direct and indirect
pathways, and this balance is interrupted in models of Parkin-
son disease that cause unidirectional changes in striatal synaptic
plasticity (45). Glutamatergic synapses onto the medium spiny
neurons of the indirect pathway show higher release probability
than glutamatergic synapses onto the medium spiny neurons of
the direct pathway, and they selectively express endocannabinoid-
mediated long-term depression that is absent in a model of Par-
kinson disease (46). Since the striatal dopamine signaling system
may be upregulated, Pnkd mice may also display alterations of

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**Figure 7**

Pharmacological studies of Pnkd mice. (A) There are significant hyperkinetic movements in Pnkd (mut-Tg) mice (versus littermate controls) after injection of the adenosine A_2 receptor antagonist CSC (5 mg/kg, i.p.). (B) No difference in activity ratings of Pnkd mice and WT littermates was observed after injection of the adenosine A_1 receptor antagonist DPCPX (3 mg/kg, i.p.). (C) There are significant hyperkinetic movements in Pnkd mice after injection of the dopamine D_2 receptor agonist quinpirole (2.5 mg/kg, i.p.). (D) Increased activity was observed in both Pnkd mice and WT controls after injection of SKF 82958 (D_1 receptor agonist, 0.75 mg/kg, i.p.), but no difference was found between genotypes. In all pharmacological studies, behavioral ratings are expressed as mean ± SEM (n = 8 for each group).
striatal synaptic plasticity under stress or with caffeine/ethanol treatment. In turn, transient alterations of neuronal activity in basal ganglia may occur during PNKD attacks. Further study will provide better understanding of the role of the PNKD protein in cellular and synaptic regulation and contribution of PNKD mutations to pathophysiology. Such work may allow development of better therapies for PNKD and potentially for other episodic disorders. Since PNKD is a novel gene participating in modulating striatal neurotransmitter release, further investigations both in vitro and in vivo will give new insights into understanding normal basal ganglia regulation of motor function and potentially have implications for developing an understanding other dystonias and striatal diseases.

Methods

Study approval. All animal studies were approved by the Animal Care and Use Committee at UCSF.

PNKD antibodies. Polyclonal antibodies were developed (Covance) using 2 synthesized oligopeptides corresponding to the N terminus of PNKD-L and -S and the C terminus of PNKD-L and -M. Preimmune bleeds and test bleeds were obtained individually from the supplier to monitor antibody titer and specificity in subsequent bleeds. Antibodies were affinity purified using standard procedures (see Supplemental Methods). Affinity-purified antibodies were then used for Western blotting and immunohistochemistry experiments (N-terminal antibody, 1:250 to 1:500 dilution; C-terminal antibody, 1:500 to 1:1000 dilution).

Generation of Pnkd-transgenic and -KO mice. Both mut-Tg and WT-Tg (as a dosage control) mice were generated (details in Supplemental Methods). The transgene copy number was estimated by Southern blotting. Tail DNA or control DNA spiked with BAC DNA representing 1, 3, 5, 10, and 30 copies/gene was digested with PstI, electrophoresed on 0.8% agarose, and transferred to a Hybond-N+ membrane. Blots were hybridized with a random primed 32P-dCTP-labeled probe amplified from the BAC clone. Primers and conditions for RT-PCR are detailed in the Supplemental Methods. Images were captured with a Nikon Eclipse microscope and CCD camera or a Leica DM5000B microscope with a SPOT RT camera (SPOT diagnostic Inc.) and imported into Photoshop for analysis. Neuroanatomical studies were conducted in a blinded manner.

Detection of biogenic amines in striatum by HPLC. Pnkd mice, WT littermates, Pnkd WT-Tg mice, and Pnkd-KO mice were sacrificed under basal conditions (10–12 mice per genotype) or 20 minutes after i.p. injection of caffeine (25 mg/kg; 10–12 mice for each genotype). Striata were quickly removed, frozen in liquid nitrogen, and stored at −80°C prior to HPLC. Dorsal striata were homogenized in 100–750 μl of 0.1 M TCA containing 10-2 M sodium acetate, 10-4 M EDTA, and 10.5% methanol (pH 3.8). Samples were spun in a microcentrifuge at 10,000 g for 20 minutes. The supernatant was removed and stored at −80°C, and the pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes, and supernatant samples were analyzed for biogenic monoamines and/or amino acids using a specific HPLC assay with an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 330° C. Then 20-μl samples of the supernatant were injected using a Water 717+ autosampler onto a Phenomenex Nucleosil (5 μl, 100A) C18 HPLC column (150 × 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1 M sodium acetate, 10-4 M EDTA, and 10.5% methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters S15 HPLC pump. HPLC control and data acquisition were managed by Millennium 32 software. Two-way ANOVA was used for analyzing the results.

In vitro microdialysis. Microdialysis was performed in alert, freely moving mice. After anesthesia with tribromoethanol and positioning in a stereotaxic frame (Stoelting), a microdialysis probe was implanted in the striatum (+0.6 AP, +1.7 ML, 4.5 DV) as previously described (49). After surgery, the probe was perfused continuously with artificial cerebrospinal fluid (ACSF: 147 mM NaCl, 3.5 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 1 mM NaH2PO4 and 25 mM NaHCO3, pH 7.0-7.4) at a flow rate of 0.6 μl/min. For conventional microdialysis, samples were collected 12–15 hours after surgery at 20-minute intervals, and 6 consecutive samples were collected as baseline. To determine the effect of stress on dopamine overflow, mice were then injected with saline (10 μl/kg, i.p.) and transferred to a novel cage. Six samples were collected after injection at 20-minute intervals. Then, 2 hours after
stress challenge, mice were injected with 25 mg/kg caffeine (10 ml/kg, s.c.) and 6 samples were collected at 20-minute intervals. For no net flux microdialysis, data were subjected to a bath application of the dopamine reuptake blocker nomifensine (3 μM) at least 30 minutes was used to access the contribution of reuptake in the process repeated until all dopamine concentrations were tested. The experiment was confirmed historically.

Samples were analyzed by HPLC (MDCL-150 column, 150 mm length; 3 mm I.D.; ESA) with a 5014B microdialysis cell. The mobile phase was composed of 1.7 mM 1-octanesulfonic acid sodium salt, 25 μM EDTA, 75 mM NaH2PO4, and 8% acetonitrile (pH 2.9) with a flow rate of 0.6 μl/min overnight. On the day of the experiment, the probe was perfused at the same rate with perfusate plus different concentrations of dopamine (0, 2, 10, or 20 μM; Cm) in pseudo-random order. After a 25-minute equilibration period, 3 samples were collected (Cm). The dopamine concentration in the perfusate was then switched and the process repeated until all dopamine concentrations were documented. The location of the probe within the striatum was confirmed historically.