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 family (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 path- way 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).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2012;122(2):507–518. doi:10.1172/JCI58470.
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 transfected 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/JCI58470DS1).

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 constituted by dopaminergic neurons projecting to striatum, and substantia nigra pars reticulata (SNR), 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–transfected 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 hydrophobic layer, but not in the hydrophilic layer, using PNKD antibodies (Figure 2B). Thus, PNKD-L appears to be membrane associated, though not a transmembrane protein integral to the membrane.

A mouse model harboring the PNKD mutations recapitulates the PNKD phenotype. Three lines of Pnkd mutant BAC transgenic mice (mut-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 d10 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 regions, the changes in Pnkd-KO mice may play an important role in the pathogenesis of PNKD.
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 D₁AR and D₂R 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 A₁ and adenosine A₂A receptors (A₁R and A₂AR), 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.

Pnkd mice have lower striatal dopamine release at rest, but an increased percentage of striatal dopamine release in response to challenge. To examine the pattern of nigrostriatal dopamine release, we performed in vivo microdialysis in alert, freely moving mice. Basal extracellular dopamine concentrations were assessed using the no net flux method to provide an unbiased estimate of transmitter concentration. The extracellular concentration of dopamine in Pnkd mice was less than 40% of that of control mice under basal conditions (P < 0.05; Figure 5A). In contrast, the extraction fraction was comparable in control and Pnkd mice, suggesting that the reduction in the basal extracellular dopamine concentrations in mutant mice is attributable to abnormalities in transmitter release (Figure 5B). Next, response
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.
The 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

**Figure 4**

PNKD mutant mice (mut-Tg, PNKD mice) recapitulate the PNKD phenotype and have altered dopamine signaling. (A) Quantifying activity of PNKD mice and WT littermates after caffeine injection (25 mg/kg, i.p.). Caffeine-induced hyperkinetic movements in mutant mice. (B) Activity ratings of PNKD mice and WT littermates after saline injection (10 ml/kg, i.p.). A slight increase in activity was observed in PNKD mice 30–50 minutes after saline injection. (C) The mut-Tg mice manifested profound dyskinesia after injection of ethanol (1.5 g/kg, 20% v/v, i.p.), but the same treatment failed to induce hyperkinetic movements in WT littermates. Activity ratings are expressed as mean ± SEM (n = 8 for each group). (D) There were no obvious differences in the dopamine levels in striatal extracts from mice of various genotypes at rest or after caffeine treatment. (E and F) Both DOPAC levels and DOPAC/dopamine ratios were significantly increased in PNKD mice (P < 0.01) after caffeine treatment, but decreased in WT and KO mice (P < 0.01). (G) The HVA levels at rest versus after caffeine treatment were significantly increased in PNKD mice (P < 0.05). All data are presented as mean ± SEM. *P < 0.05; **P < 0.01.
release was restored to normal levels in Pnkd slices after application of nomifensine. The spike width was significantly smaller and the half width (t1/2) was significantly larger in Pnkd than in WT controls (P < 0.05; Figure 6, D and E). The spike width returned to normal in the presence of nomifensine (Figure 6D), but the t1/2 did not (P < 0.01; 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 A1Rs and A2ARs are the major modulators of striatal signaling (20, 21). The A1Rs are enriched in striatonigral-striatoentopeduncular medium spiny neurons that constitute the direct output pathway expressing dopamine D1 receptors (D1Rs). A2ARs and dopamine D2 receptors (D2Rs) 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 A1R and A2AR to attacks in PNKD. Dyskinesias were induced in Pnkd mice approximately 20 minutes after i.p. injection of the selective A2AR agonist 8-(3-chlorostryl) caffeine (CSC) (5 mg/kg; Figure 7A) and lasted approximately 2 hours. The A1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (3 mg/kg) did not induce dyskinesias in Pnkd mice (Figure 7B). The A1R agonist 8-cyclopentyladenosine (CPA) (0.1 mg/kg) had no obvious effects on Pnkd mice or controls (Supplemental Figure 6A), and the A2AR agonist 2p-(2-carboxyethyl)phenethylamine-5′-N-ethylcarboxyamidoadenosine (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. D1R and D2R are major receptors in the regulation of striatal function, and antagonistic interactions between A1R-D1R and A2AR-D2R in the basal ganglia are important in motor control (13). Since D2Rs colocalize with A2ARs in neurons of the indirect pathway of the striatum, we hypothesized that the selective D2R 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 D1R 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 A₂ARs and D₂Rs in pathogenesis, as attacks can be triggered with a selective A₂AR antagonist and a selective D₂R 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 A₂A and D₂ 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 important role in primary dystonias. Dopas-responsive dystonia (DYT5) is caused by mutations of the GTP–cyclohydrolase 1 gene involved in catecholamine and serotonin biosynthesis or by mutations of TH (2, 33). In dt<sup>sz</sup> hamsters, an elevation of extracellular striatal dopamine levels has been observed during dystonic episodes (34). Interestingly, unlike dt<sup>sz</sup> hamsters, Pnkd mice have reduced extracellular dopamine in vivo, but, when challenged by stress, caffeine, or alcohol, there is a relative increase in dopamine compared with controls. Pnkd mice have normal dopamine content in striatal dopaminergic terminals and apparently normal dopamine 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 dopamine 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 akinnesia (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 nigrostriatal dopaminergic terminals. Alternatively, PNKD may participate in the modulation of striatal glutamatergic inputs projecting 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 glutamate 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 findings imply that dysfunction of the striatal dopamine signaling system plays a pivotal role in PNKD pathophysiology. Although expression levels of adenosine receptors and glutamate transporters 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 synapses on the medium spiny neurons of both direct and indirect pathways, and this balance is interrupted in models of Parkinson 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 Parkinson disease (46). Since the striatal dopamine signaling system may be upregulated, Pnkd mice may also display alterations of

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<sub>2</sub> 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<sub>1</sub> receptor antagonist DPCPX (3 mg/kg, i.p.). (C) There are significant hyperkinetic movements in Pnkd mice after injection of the dopamine D<sub>2</sub> 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<sub>1</sub> 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 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/ genome 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.

**Immunoblotting of transfected cells.** HEK293 cells were transfected with 2 μg DNA (PNKD-L fusion constructs), grown, fixed, and probed with appropriate antibodies using a standard protocol (see Supplemental Methods). Images were acquired using the Zeiss Pascal LSM5 confocal microscope in the UCSF microscopy core. Images were analyzed in Photoshop (Adobe Systems). Human embryonic kidney 293 (HEK293) cells were transfected with 2 μg DNA (PNKD-L fusion constructs), grown, and harvested 36 hours later, after transfection. HEK cells were sonicated in 500 μl of RIPA buffer with protease and phosphatase inhibitors; then the homogenates were used for Western blots incubated with PNKD antibodies and rabbit anti-GFP antibody (1:5000 dilution; Abcam). For the detergent phase partitioning experiments, the crude membrane fractions of transfected HEK293 cells were collected. TX-114 was added into crude membrane fractions and incubated; 2 layers (hydrophobic and hydrophilic) were collected as described (47, 48) and then were used in Western blots.

**Immunoblotting of mouse brain tissues.** Western blotting of whole-brain tissue homogenates obtained from WT, transgenic, and KO mice was performed using standard techniques detailed in the Supplemental Methods. Pnkd levels were normalized to α-tubulin using the average pixel intensity for immunoreactive bands in each lane, and the images were analyzed by NIH ImageJ software. Dorsal striata of mice (n = 8/genotype) were dissected and snap-frozen in liquid nitrogen, then sonicated in 300 μl of buffer with protease and phosphatase inhibitors. Blots were then incubated with antibodies raised against the following proteins: VGLUT1, Rah3A, synaptophysin, EAAT3, MAO-A, MAO-B, ADK, adenosine A1 receptor, COMT, synap sin-1, GAD65, GAD67, adenosine A2A receptor, TH, DAT, dopamine D1A and D2 receptors, and VMAT2 (details in Supplemental Methods). After detection, blots were stripped and reprobed with either anti-α-tubulin or anti–glyceraldehyde 3-phosphate dehydrogenase antibodies. Striaton protein levels were normalized, and data were analyzed using Student’s t test.

**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 0.1M TCA containing 10−2 M sodium acetate, 10−3 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 33°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, 10−2 M sodium chloride, 10−3 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 vivo 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 ml/kg, i.p.) and transferred to a novel cage. Six samples were collected after injection at 20-minute intervals. Then, 2 hours after

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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, after implantation, the probe was perfused with ACSF plus 250 μM ascorbic acid at 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 nM; 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 tested. The location of the probe within the striatum was confirmed histologically.

Samples were analyzed by HPLC (MD-150 column, 150 mm length; 3 mm 1D; 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 ml/min. Neurotransmitters and metabolites were identified by matching retention time to that of known standards. For no net flux microdialysis, data were subjected to a linear regression analysis of Cm – Cm versus Cm. The x-intercept provides an unbiased estimate of extracellular dopamine concentration. Data were analyzed using 2-factor ANOVA or Student’s t-test, where appropriate. Amperometry studies of Pnkd mice. Brain slice recordings were performed using standard protocols detailed in the Supplemental Methods. Details of the brain slice amperometric recordings have been described (50, 51). Local bath application of the dopamine reuptake blocker nomifensine (3 μM) for at least 30 minutes was used to access the contribution of reuptake in the evoked dopamine signal. Five signal pulses/slice were averaged into a grand mean, and the 2 groups (Pnkd mice vs. WT littermates) were compared with 1-way ANOVA of the mean. Pharmacology studies. To evaluate the response of mice to caffeine and ethanol, the triple stimulation technique was modified from Richter and Loscher (52). This procedure consists of (a) removing mice from home cages and placing them on a balance; (b) i.p. injection of saline or other drugs; and (c) placement of the animals into an empty 1000-ml glass beaker or empty cage (1 animal per beaker/cage). Mice were observed for 2 hours after stimulation. Drug effects were examined in Pnkd mice and WT littermates (8 mice/ genotype). Each group was used for 1 to 2 doses (with more than 1 week between doses). A control trial was undertaken with the triple stimulation technique, injecting the vehicle used for i.p. drug administration (predrug control). Two days later, the drug was administered in the same group of animals. As described for predrug controls, a control trial with vehicle was done 2 days after treatment (postdrug control). All trials were done between 1300 and 1700 hours. Test reagents were administered i.p. Ethanol was diluted and injected at 1.5 g/kg (20% w/v in 0.9% saline). The nonselective adenosine receptor antagonist caffeine (25 mg/kg), the D1 dopamine receptor agonist Chloro-APB hydrobromide (SKF 82958, 0.75 mg/kg), and the D2 dopamine receptor agonist quinpirole (2.5 mg/kg) were dissolved in 0.9% saline. The adenosine A1 receptor antagonist DPCPX (3 mg/kg), the A2a antagonist CSC (5 mg/kg), the adenosine A1 receptor agonist CPA (0.1 mg/kg), and the A2a receptor agonist CGS 21680 (0.5 mg/kg) were dissolved in 10% DMSO/10% Cremophor EL (Fisher Scientific). All solutions and suspensions were freshly prepared, and injection volumes were 10 ml/kg (10 μl/g body weight). The severity of the phenotype was rated by the following scoring system modified from Wang and McGinty (53): (a) asleep, inactive; (b) normal activity, grooming; (c) increased activity; (d) hyperactivity, running; (e) jerky movement and slow patterned movement (repetitive exploration); (f) fast patterned movement (repetitive exploration with hyperactivity); (g) stereotyped movements (repetitive sniffing/rearing in one location); (h) continuous gnawing, sniffing and/or licking; and (i) dyskinesia, seizures. The behavior of the mice was rated before the drug injection and every 5 minutes during the entire test period. Predrug and postdrug control trials were undertaken in the same animals 2 days before and 2 days after drug testing. All neuropharmacological testing was done in a blinded manner.

Statistics. All values are shown as mean ± SEM, and data were statistically analyzed by 1-way ANOVA (amperometry studies and pharmacology studies), 2-way ANOVA (studies of biogenic amines in striatum and in vivo microdialysis), or 2-tailed Student’s t test (Western blot analysis and in vivo microdialysis). P values of 0.05 or less were considered statistically significant.

Acknowledgments

We thank Anatol Kreitzer and Shu-Ting Lin for helpful discussions. This work was supported by grants from the Dystonia Medical Research Foundation, the Bachmann-Strassus Dystonia & Parkinson Foundation, NIH grant U54 RR19481, the Sandler Neurogenetics Fund, and P30 NS047243 (Tufts Center for Neuroscience Research). L.J. Ptácek is an investigator of the Howard Hughes Medical Institute.

Received for publication April 13, 2011, and accepted in revised form November 16, 2011.

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