Molecular Medicine

Mouse Model for Protein Tyrosine Phosphatase D (*PTPRD*) Associations with Restless Leg Syndrome or Willis-Ekbom Disease and Addiction: Reduced Expression Alters Locomotion, Sleep Behaviors and Cocaine-Conditioned Place Preference

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The receptor type protein tyrosine phosphatase D (*PTPRD*) gene encodes a cell adhesion molecule likely to influence development and connections of addiction- , locomotion- and sleep-related brain circuits in which it is expressed. The *PTPRD* gene harbors genome-wide association signals in studies of restless leg syndrome (Willis-Ekbom disease (WED)/restless leg syndrome (RLS); $p < 10^{-8}$) and addiction-related phenotypes (clusters of nearby single nucleotide polymorphisms (SNPs) with $10^{-2} > p > 10^{-8}$ associations in several reports). We now report work that seeks (a) association between *PTPRD* genotypes and expression of its mRNA in postmortem human brains and (b) RLS-related, addiction-related and comparison behavioral phenotypes in hetero- and homozygous *PTPRD* knockout mice. We identify associations between *PTPRD* SNPs and levels of *PTPRD* mRNA in human brain samples that support validity of mouse models with altered *PTPRD* expression. Knockouts display less behaviorally defined sleep at the end of their active periods. Heterozygotes move more despite motor weakness/impersistence. Heterozygotes display shifted dose-response relationships for cocaine reward. They display greater preference for places paired with 5 mg/kg cocaine and less preference for places paired with 10 or 20 mg/kg. The combined data provide support for roles for common, level-of-expression PTPRD variation in locomotor, sleep and drug reward phenotypes relevant to RLS and addiction. Taken together, mouse and human results identify *PTPRD* as a novel therapeutic target for RLS and addiction phenotypes.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00017

INTRODUCTION

The gene that encodes the receptor type protein tyrosine phosphatase D (*PTPRD*) displays neurobiological features that make its variants attractive candidates to play roles in sleep, locomotor control and addiction. *PTPRD* encodes a single transmembrane domain protein whose expression is likely to alter the nature of connections between expressing neurons. Ventral midbrain neurons implicated in reward, locomotor control and sleep processes, likely dopaminergic, prominently express *PTPRD*, as do other neurons likely to use acetylcholine, norepinephrine, γ-aminobutyric acid (GABA) and glutamate as neurotransmitters (1). *PTPRD* can function as a homodimer (2) or may heterodimerize with partners that include the *SLITRK3* leucine-rich repeat-containing cell adhesion molecule (3). *PTPRD/SLITRK3* interactions are linked to organization and strength of GABAergic synapses (4,5). Although not yet documented, similar influences on the function of dopaminergic, cholinergic and other neurons that express *PTPRD* also appear likely.

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Submitted January 21, 2015; Accepted for publication July 10, 2015; Published Online (www.molmed.org) July 14, 2015.

PTPRD has been identified by associations that reach genome-wide ($p < 10^{-8}$) significance in studies of vulnerability to the common sensorimotor/sleep disorder restless leg syndrome (RLS) or Willis-Ekbom disease (WED) (6). There are associations with intronic single-nucleotide polymorphisms (SNPs) rs1975197 and/or rs4626664 (7,8). *PTPRD* has also been identified in several genome-wide association studies of addiction-related phenotypes that include extensive use of an addictive substance, vulnerability to develop dependence on an addictive substance and ability to quit smoking. The individually modest addiction association signals come from clusters of nearby SNPs with $10^{-2} > p > 10^{-8}$ associations that fail to survive conservative Bonferroni corrections (9–19).

These neurobiologic, genetic and genomic data nominate *PTPRD* for studies that seek (a) influences of common human allelic variation on *PTPRD* expression, (b) influences of variation in *PTPRD* expression on behavioral sleep and locomotor phenotypes that model features of RLS and (c) influences of variation in *PTPRD* expression on responses to rewarding addictive substances. We now report studies of *PTPRD* expression in human postmortem cerebral cortical samples. These studies identify associations between levels of *PTPRD* expression and *PTPRD* genomic markers that include the RLS/addiction-associated SNPs rs1975197 and rs4626664 as well as rs2381970. We discuss locomotor and sleep-related behavioral findings in *PTPRD* knockouts and the low likelihood that they represent only artifacts of the homozygotes' overall disabilities. In studies of drug reward, we use cocaine-conditioned place preference (CPP), a heavily validated mouse test for drug reward/reinforcement (20). Heterozygous knockouts display altered dose-dependent relationships for cocaine CPP, while failing to display potentially confounding alterations in performance on other tests. We note recently reported associations between *PTPRD* locus variation and differences in human psychostimulant and alcohol effects that enhance our confidence that *PTPRD* variation, and thus the neuronal properties and connections that *PTPRD* modulates, plays *bona fide* roles in addiction- and RLS-related phenotypes.

MATERIALS AND METHODS

Influences of *PTPRD* Haplotypes on Levels of Expression

Genetic *cis* influences on levels of *PTPRD* expression were sought in studies of *PTPRD* messenger ribonucleic acid (mRNA) and deoxyribonucleic acid (DNA) in rapidly frozen autopsy samples of frontal cortex of European American individuals who died without brain disease. We focused on this genetic background and this brain region, since this focus allowed us to assemble a large group of brain samples not possible using material from other brain regions or individuals with different racial/ethnic backgrounds. Causes of death were accidents/multiple trauma, cardiovascular disease and pulmonary embolisms (Supplementary Table S1). Average time to freezing was 14 h. Brains came from individuals who were 50.5% female and averaged age 42 years under protocols overseen by the Johns Hopkins (19% of samples) and University of Maryland (81% of samples) institutional review boards.

RNAs were prepared with the RNeasy lipid tissue mini-kits (Qiagen), with controls for mRNA quality based on intake ribosomal RNA bands on agarose gel electrophoresis and OD260/280 ratios (data not shown). Complementary DNA (cDNA) was synthesized with SuperScript III first-strand synthesis supermix (Invitrogen), and levels of mRNAs were assessed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using SybrGreen master mix (Applied Biosystems) and oligonucleotides PRPRD_EXON5F: GCAAGCAGTTCCTGGCCCGT, PRPRD_EXON6R: GGCACATTCTGCTGTGGTCTCAGG, PRPRD_EXON11F: CGTCTGGTGAACACCCGTGGGA, and PRPRD_EXON12R: GGAGCCGCAGCGAGTCTGTC that targeted the dominant long *PTPRD* mRNA isoform 1 (21) and the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ubiquitin C (UBC), according to the manufacturer's protocols (sequences available from authors on request). DNA was extracted from brain samples by using Qiagen kits (22) and was subjected to multiplexed genotyping using Sequenom panels (oligonucleotide sequences available on request). Each PCR contained 1 μL genomic DNA (2.5 ng/μL), 0.4 μL deoxynucleotide triphosphate (dNTP) mix (10 mmol/L), 0.5 μL AmpliTaq Gold PCR buffer (10×), 0.3 μL $MgCl_2$ (1.5 mmol/L), 0.2 μL each of forward and reverse primer (20 μmol/L each), 0.025 μL AmpliTaq Gold DNA polymerase (0.025 U/μL) (Applied Biosystems) and 2.38 μL double-distilled water (ddH_2O). Thermal cycles were (a) 10 min at 95°C; (b) 35 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C; and (c) a final 3 min at 72°C extension followed by 4°C. Unincorporated dNTPs were removed by 20-min 37°C incubation with shrimp alkaline phosphatase, enzymes were inactivated by 5-min incubation at 85°C and primer extension was performed with 0.2 μL of the appropriate combination of dNTP, dideoxynucleotide triphosphate (ddNTP) (2.25 mmol/L), 0.054 μL extension primer (100 μmol/L), 0.018 μL thermosequenase (32 U/μL) and 1.7 μL ddH_2O. Thermal cycles were (a) 94°C for 2 min; (b) 40 cycles of 5 s at 94°C, 5 s at 52°C and 5 s at 72°C; and (c) 4°C. Reactions were purified with SpectroClean resin (Sequenom), spotted in matrix on Sequenom arrays and subjected to MALDI-TOF mass spectrographic analyses with automatic allele detection and manual allele confirmation. A total of 34 SNPs with minor allele frequencies >0.05 were used for analyses. Expression and genotyping were successful in samples from 119 brains (Supplementary Table S1).

Mouse Models

PTPRD homozygous knockout, heterozygous knockout and littermate wild-

type animals were produced by heterozygote × heterozygote matings, genotyped and fed with food placed on the cage floors as described (23–25). Mice of both sexes were tested at 118 ± 49 d of age. All breeding, care and experimentation was approved by the NIDA-IRP Animal Care and Use Committee.

Mouse Behavioral Studies: Motor and Cocaine Effects and Learning

Motor: muscle strength/motor persistence test. Mice were timed for their ability to hold on to a wire screen (5-mm hardware cloth) mounted parallel about 20 cm above the countertop. The test was terminated at 120 s, or when the mouse fell.

Motor: coordination and learning. Coordination and learning were measured on an accelerating rotarod (a rotating cylinder on which the mice had to walk and maintain their balance to prevent falling). The animals were tested over 3 consecutive days with one test per day. The starting speed of the rotating cylinder was 4 rpm, and it gradually increased to 40 rpm over 5 min, which was the cutoff time.

Motor: behaviors at the sleep-onset period. These behaviors were evaluated by two independent observers blinded to genotype using videos taken in for the hour before and the hour after lights off for mice of each genotype. Mice were placed in a two-chambered 37 × 20 × 20 cm clear Plexiglas box 12 h before data collection to allow acclimation. One food pellet and one igloo were placed on the floor of each chamber; a water bottle with 10 mL water was taped to each side of the box. Video of the mice (Noldus wvbp-332) recorded at 0600–0800 (1 h with lights off and 1 h with lights on) was scored by two independent observers blinded to the genotypes and sexes of the mice. Times of sleep onset and wake were recorded. A period of wakefulness was defined as movement ≥3 s in length. A period of sleep was defined as ≥5 s without movement. "Twitches" and "non-twitch movements" during sleep periods were also recorded. "Twitches" were defined as sudden, rapid movements of the paws or entire body during sleep. "Non-twitch movements" were slower actions <3 s in length. Data were organized into 10-min intervals, and total numbers of sleep episodes per interval were recorded as "sleep bouts." A period of sleep overlapping from one 10-min interval to the next was counted as two bouts, one in each interval.

Cocaine effects: reward and CPP. Reward and CPP were assessed (26,27). Briefly, side preferences were assessed, four 20-min conditioning trials were conducted (two cocaine, two saline) and preferences were assessed again 24 h after the last conditioning session. Differences between times spent on the drug-paired side during the post- versus pretests were noted.

Cocaine effects: locomotion. Locomotion was recorded (a) during the 20-min pretest (in both halves of the 20 × 40–cm conditioning apparatus), conditioning (20 × 20–cm half of the apparatus) and test (20 × 40 cm) sessions and (b) in 42 × 42–cm sound-attenuated boxes to which the mice had not been previously exposed, for 120-min trials. Total distance traveled by mice that were untreated, saline treated and sampled before treatment, after one treatment and after the second treatment with cocaine was calculated from infrared beam breaks by an Optovarimax ATS System (27).

Memory and learning. Memory and learning were evaluated in Morris water maze testing (28). A black 90-cm-diameter pool filled with opaque 22°C water contained a 9-cm platform in the center of one quadrant that was visible for the first six trials and hidden 0.5 cm below the surface for subsequent trials. Mice received two trials, each lasting a maximum of 60 s, separated by a 15-s rest period on the platform. They were returned to home cages for 4 h and then subjected to an additional two-trial session. After acquisition, defined by an average latency to reach the platform of <10 s, the platform was removed and a 60-s probe trial was conducted. Probe trial data including the path of the subject in the pool and the time spent in each quadrant was analyzed with Ethovision software (Noldus). After probe trials, the platform was placed in the opposite quadrant to assess reversal learning by using the same procedure.

Statistical Analyses

Analyses of mouse data used PASW statistics 18 (SPSS) and *t* tests (Excel). Analyses of variance and covariance (ANOVA and ANCOVA) used between-subject factors of genotype and sex, age as cofactors, and within-subjects factors where appropriate, including time for locomotion and trial day for Morris water maze. Analyses of human association data and assessment of correlations between genotypes at nearby SNPs used PLINK (pngu.mgh.harvard.edu/~purcell/plink). Normalized gene expression levels found in human major allele homozygotes versus heterozygotes were compared by using *t* tests. Bonferroni corrections were performed (29) with observed 0.4 correlations (*r*) between SNPs.

All supplementary materials are available online at www.molmed.org.

RESULTS

Searches for *cis* Influences on Levels of *PTPRD* Expression

We identified associations between *PTPRD* genomic markers and levels of *PTPRD* mRNA expression. We detected *PTPRD* mRNA in triplicate assays of RNA extracted from cerebral cortical specimens from 119 individuals. Levels of expression, which are inversely related to C_T values, were 24.5 ± 0.24 (mean ± standard error of the mean [SEM] in samples of individuals from all genotypes) for *PTPRD*, 18.5 ± 0.18 for *GAPDH*, 23.08 ± 0.21 for *HPRT* and 20.17 ± 0.18 for *UBC*. Data from 34 SNPs that displayed minor allele frequencies >0.05 in these samples were analyzed. rs2381970 displayed the most robust association with levels of *PTPRD* mRNA expression ($p = 0.002$; exceeding Bonferroni-corrected threshold, $p < 0.006$).

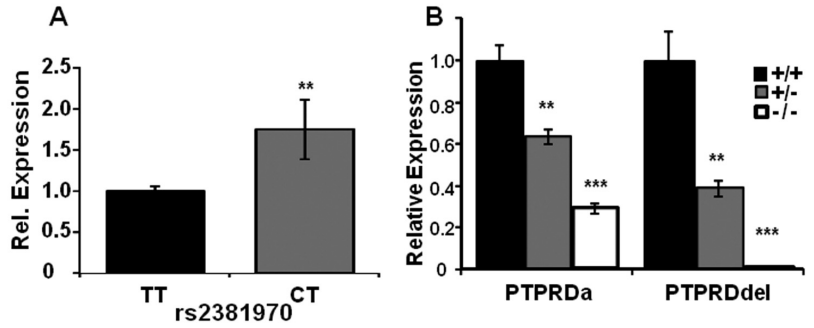

Figure 1. (A) Differential expression of *PTPRD* mRNA in cerebral cortex samples from individuals with different rs2381970 genotypes. Mean ± SEM of relative expression of *PTPRD* mRNA in cerebral cortical samples of individuals with major allele homozygote versus heterozygotes (n = 105 and 14, respectively). Triplicate RT-PCR assays. Relative expression was determined by the mean of two PTPRD amplimers in relation to the geometric mean of three control mRNAs from the same sample (see Materials and Methods). $^{**}p < 0.01$. SNPs and nominal *p* values were as follows: rs10115782/0.00298; rs10815873/0.587; rs2296094/0.00905; rs35929428/0.418; rs956154/0.134; rs1975197/0.0513; rs17584499/0.883; rs7850165/0.646; rs973117/0.172; rs10511520/0.681; rs2381970/0.00162; rs4626664/0.0396; rs10977684/0.173; rs914804/0.513; rs3949639/0.701; rs17829640/0.273; rs1412875/0.235; rs10977801/0.773; rs12337005/0.0589; rs639168/0.927; rs17183242/0.599; rs7862769/0.289; rs12350467/0.391; rs10115039/0.316; rs7470838/0.0383; rs9886850/0.850; rs12345015/0.975; rs649891/0.316; rs1322307/0.967; rs1322313/0.973; rs1923442/0.609; rs10756064/0.741; rs10809140/0.109; rs10738180/0.906. (B) Cerebral cortical *PTPRD* mRNA levels in wild-type, heterozygous and homozygous knockout mice. Relative expression was determined by RT-PCR with primers complementary to sequences of exons 5′ to the site of deletion in the knockout (PTPRDa) and with a primer pair complementary to sequences within the deleted exon (PTPRDdel). Bars show mean ± SEM, n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$.

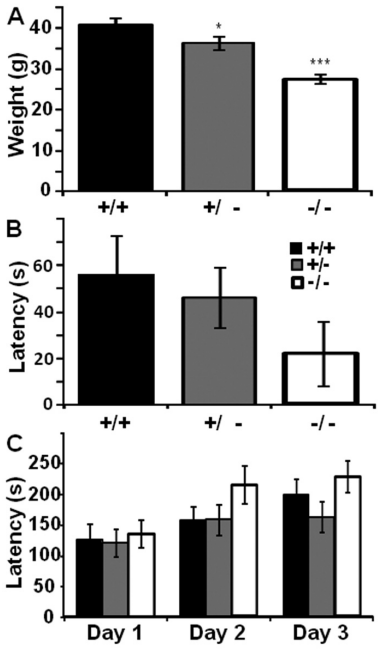

Figure 2. Initial characterization of *PTPRD* knockout mice. (A) Reduced *PTPRD* expression significantly decreased weight (ANCOVA effect of genotype, $p = 0.002$), with especial influence on homozygous knockouts. (B) Reduced *PTPRD* expression reduced strength/motor persistence in screen hang-time testing (effect of genotype, $p = 0.031$). Scheffé *post hoc* testing did not reveal any differences between heterozygous and wild-type mice ($p = 0.831$). (C) Reduced *PTPRD* expression did not significantly influence performance in rotarod testing (ANCOVA, $p = 0.600$). Data presented are mean ± SEM, n = 8–12/genotype. $^{*}p < 0.05$, $^{***}p < 0.001$.

rs2381970 CT heterozygotes displayed 172% of the normalized *PTPRD* mRNA levels displayed by homozygotes for the common T allele, although we identified no minor allele homozygotes (Figure 1A). rs2381970 lies near a RLS-associated SNP, rs4626664, that displays $p = 0.04$ association with PTPRD expression levels. Borderline nominal significance for this association ($p = 0.05$) was found for the other RLS-associated SNP rs1975197. Nearby *PTPRD* SNPs that also displayed nominally significant association include rs7470838 ($p = 0.04$), rs2296094 ($p = 0.01$) and rs10115782 ($p = 0.003$).

Mice

General characterization. There were significant influences of PTPRD genotype on weight (ANCOVA effect of genotype, $p = 0.002$; Figure 2A) and strength/motor persistence in screen hang times (ANCOVA effect of genotype, $p = 0.031$; Figure 2B). However, there was no significant effect on performance on the rotarod test of motor coordination and motor learning ($p = 0.60$; Figure 2C). Scheffé *post hoc* analyses of comparisons between animals of specific genotypes revealed that heterozygous mice were modestly but significantly lighter than their wild-type littermates ($p = 0.039$) while displaying normal strength in the screen hang test ($p = 0.831$). Homozygous *PTPRD* knockout mice survived at 27% the rate of their wild-type littermates and were about one-third lighter (Figure 2A; Scheffé *post hoc*, $p < 0.001$).

Heterozygous knockouts displayed 50–60% of the levels of cerebral cortical *PTPRD* mRNA displayed by wild-type mice (Figure 3). Homozygote knockouts expressed no detectable *PTPRD* mRNA that contained the exon deleted by the knockout construct and about 20% of wild-type levels of mRNA species that contained more 5′*PTPRD* exons (Figure 1B).

Locomotion

Locomotion during baseline exposures to the CPP apparatus. Mice were exposed to the CPP apparatus and allowed to explore both sides freely during each of two pretests (Figures 3A, B). ANCOVA of data from all the subjects submitted to the CPP test (n = 135) revealed highly significant effects of genotype on locomotion during both the first ($p = 1.46 \times 10^{-6}$) and second ($p = 5.92 \times$

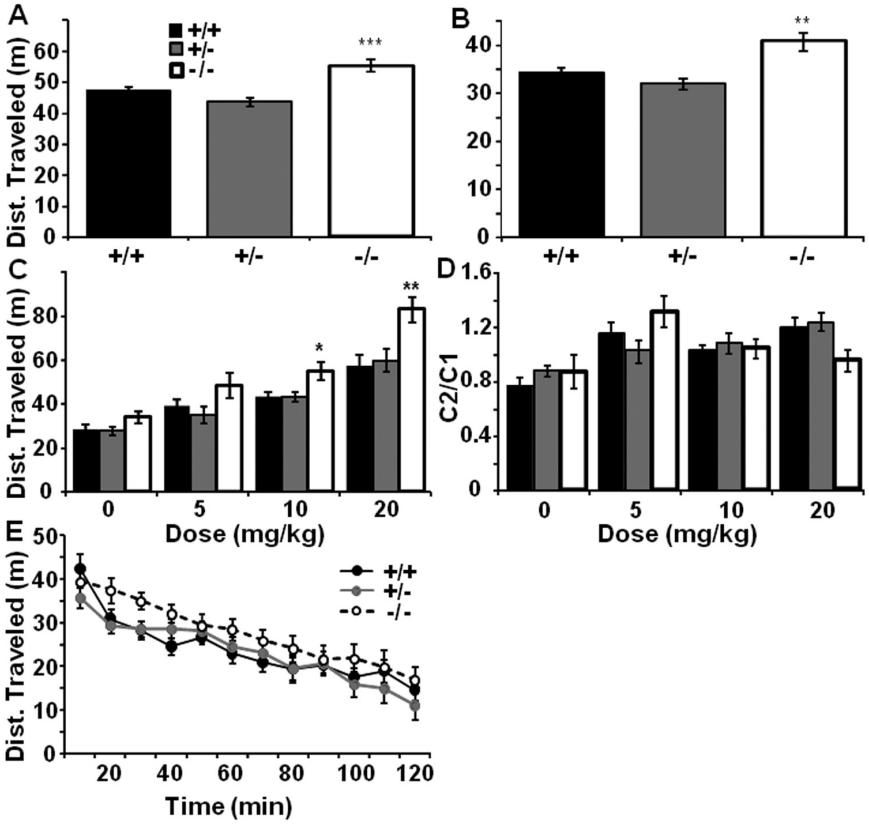

Figure 3. Locomotion in *PTPRD* homozygous knockouts, heterozygous knockout and wild-type mice in different settings. Values are mean ± SEM of the number of meters traveled. (A) During the first pretest, mice were exposed for the first time to the CPP apparatus and allowed to explore both sides (n = 31–51/genotype). (B) During the second pretest, mice were placed in the CPP apparatus and allowed to explore both sides (n = 31–51/genotype). (C) During the first exposure to cocaine, were confined to one side of the CPP apparatus and experienced effects of the cocaine doses shown (n = 10–16/genotype/dose for wild-type and heterozygotes, n = 4–5 for homozygotes). (D) Ratios between locomotion during first versus second exposures to differing cocaine doses (locomotor sensitization) in mice with PTPRD genotypes shown. Reduced *PTPRD* expression provided significantly different locomotor sensitization (repeated-measures ANOVA genotype × session × dose, $p = 0.048$). Scheffé *post hoc* testing revealed that differences between wild-type and homozygous knockouts were highly significant ($p < 0.001$), whereas heterozygotes were essentially indistinguishable from the wild-type mice ($p = 0.988$). n = 11–12 mice of each genotype for each dose. Gender displayed no significant main effect, interaction with genotype or interaction with dose (ANOVA, $p = 0.334$, 0.495 and 0.521, respectively). (E) During habituation after being placed into a larger, novel apparatus. Points represent mean ± SEM for meters traveled during each 10-min epoch of the 60 min after first exposure to a novel apparatus. n = 15–16 mice of each genotype. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

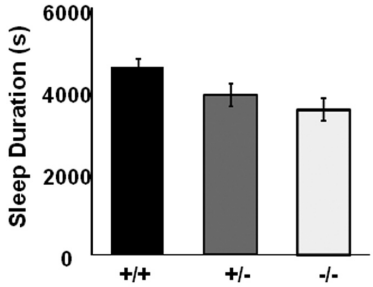

Figure 4. Length of sleep time (seconds) during the hour before and hour after lights on (beginning of subjective sleep period). Values are mean ± SEM of the mean number of seconds of sleep in mice of each genotype defined by each of two blinded observers. n = 8 mice of each genotype. There was 22% less sleep in homozygotes (ANOVA, $p = 0.037$).

10^{-5}) exposures to this environment. Scheffé *post hoc* analysis showed that homozygous knockouts demonstrated significantly greater locomotion ($p = 0.001$), although heterozygotes did not differ from their wild-type siblings ($p = 0.262$). There was no significant effect of sex ($p = 0.486$) or genotype × sex interaction ($p = 0.689$) during the first tests.

Locomotion during the first 20-min conditioning session. Locomotion during this time was assessed while mice were confined to 20 × 20 cm portions of the conditioned place preference boxes after receiving their first saline or cocaine injections (Figure 3C). ANCOVA of data from mice of all genotypes yielded significant differences between genotypes ($p = 4.5 \times 10^{-7}$), cocaine doses ($p = 6.6 \times 10^{-16}$) and a trend toward significance for genotype × dose interaction ($p = 0.071$). Scheffé *post hoc* testing confirmed that homozygous knockout mice displayed significantly greater locomotion after administration of all cocaine doses tested ($p = 1.15 \times 10^{-6}$).

Locomotion and habituation in a larger novel environment. Homozygous knockout mice also displayed numerically greater locomotion when assessed for 2 h in the novel, 42 × 42 cm novel apparatus during their diurnal inactive phases (Figure 3E). There was $p = 0.038$ nominal significance when data from homozygotes were compared with combined data from wild-type and heterozygous littermates. ANCOVA with age as a covariate found a significant effect of sex ($p = 0.033$). However, neither genotype ($p = 0.115$) nor genotype × sex interaction ($p = 0.409$) provided a significant influence.

Behaviors during the sleep onset period. These behaviors were evaluated similarly by two independent observers blinded to genotype ($r = 0.85$); analyses thus use their mean ratings. There were

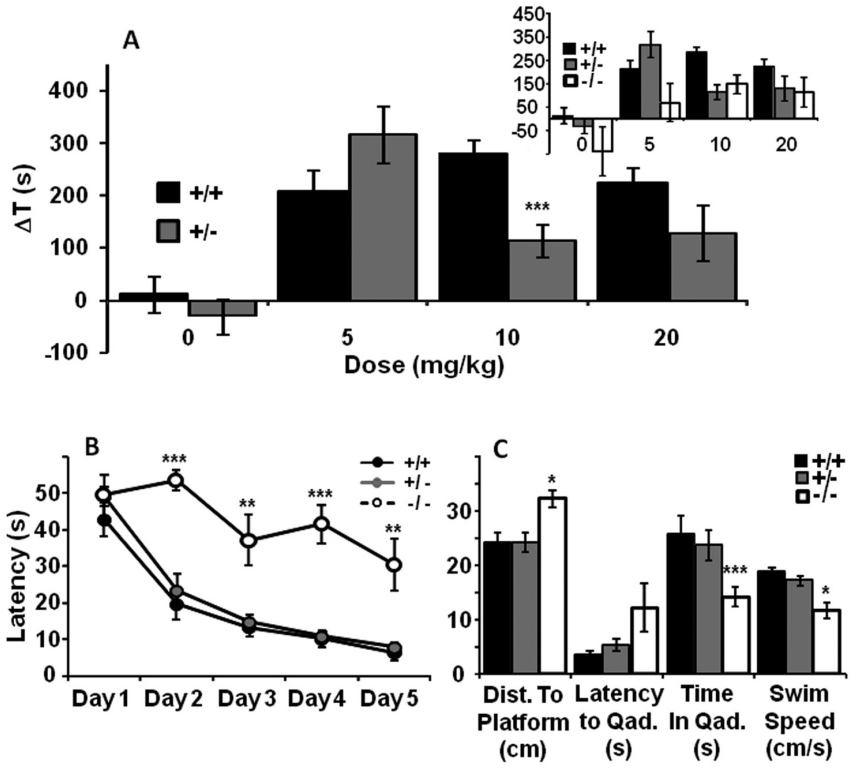

Figure 5. (A) *PTPRD* knockout provides leftward shift in the cocaine dose-response relationship for reward. Cocaine conditioned place preference in PTPRD heterozygous knockout and wild-type mice. Mean difference ± SEM in time spent on the cocaine-paired side (*y* axis) before and after conditioning with different doses of cocaine for wild-type and heterozygous knockout mice. n = 11–12 mice of each genotype for each dose. Data from male and female mice are combined, since gender displayed no significant main effect, interaction with genotype or interaction with dose (ANCOVA, $p = 0.096$, 0.672 and 0.072, respectively). Inset: Includes data from homozygous knockout mice, which display possibly confounding disabilities in mnemonic testing. ***$p < 0.001$. (B, C) Altered learning in the Morris Water maze task in homozygous, although not heterozygous, *PTPRD* knockouts. (B) Mean ± SEM of the latencies to reach Morris water maze platform for wild-type, heterozygous and homozygous PTPRD knockout mice (repeated-measures ANCOVA genotype × day, $p = 0.005$). Heterozygous knockout mice displayed no significant deficit in task acquisition (Scheffé *post hoc* test, $p = 0.694$), whereas homozygous mice displayed deficits as previously reported (25) (Scheffé *post hoc* test, $p < 0.001$). (C) Results of the probe trial confirmed learning deficits of the *PTPRD* knockout mice (ANCOVA, $p = 0.045$ for mean distance to platform, $p = 0.414$ for latency to quadrant, $p = 0.006$ for time in the target quadrant, and $p = 0.003$ for swimming speed). The homozygote knockouts were solely responsible for these differences; heterozygote mice did not differ from wild-type animals in any of the measures (Scheffé *post hoc* test, $p = 0.996$ for mean distance to platform, $p = 0.476$ for time in the target quadrant, and $p = 0.830$ for swimming speed). Data presented are mean ± SEM, n = 8–12/genotype, 1–2 females/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ΔT, Change in time spent on drug-paired side; Dist., distance; Qad., quadrant.

orderly influences of reducing PTPRD expression on (a) number of sleep bouts, 42% fewer in homozygous knockouts (ANOVA, $p = 0.001$); (b) total sleep duration, 22% less sleep in homozygotes (Figure 4; $p = 0.037$); and (c) sleep bout length, 38% longer in homozygotes ($p = 0.037$).

When the mice were observed to be asleep, the frequencies of twitch-like and slower movements did not differ significantly between mice of different genotypes (ANOVA, $p = 0.15$ and $p = 0.37$, respectively). However, differences in total length of sleep resulted in fewer total sleep-associated twitches and fewer slow limb movements in mice with reduced *PTPRD* expression (data not shown).

Cocaine-conditioned place preference. In wild-type mice, CPP was maximal at 10 mg/kg (Figure 5A), as observed in many other studies (30). By contrast, heterozygous knockouts displayed maximal CPP at 5 mg/kg doses. Comparisons of results from wild-type versus heterozygous knockout mice (ANCOVA) reveal significant effects of genotype ($p = 0.033$), dose ($p = 3.7 \times 10^{-7}$) and genotype × dose interactions ($p = 0.001$). We emphasize data from heterozygotes in the main part of Figure 5 because of the potential confounding motor influences in the homozygous knockouts. When we include mice of all genotypes, effects of genotype, dose, and genotype × dose interactions are each significant ($p = 0.001$, $p = 1 \times 10^{-9}$ and 0.025, respectively; ANCOVA).

One way to document these differences in dose-response relationships for CPP in heterozygous PTPRD knockout mice is to compare the times spent on the sides paired with 5 versus 10 mg/kg cocaine doses (compared to baseline values) in wild-type versus heterozygotes. A total of 10 mg/kg produced almost 2.5-fold greater place preference in wild-type mice than in heterozygotes ($p = 9.2 \times 10^{-5}$; *t* test). By contrast, 5 mg/kg produced about one-third less place preference in wild-type mice than in heterozygotes, a difference at the margin of statistical significance ($p = 0.054$, *t* test). Heterozygotes thus displayed apparent leftward shifts in the inverted U dose–response relationships typically noted for cocaine reward and reinforcement.

Locomotor sensitization. Locomotor sensitization was sought by comparing locomotion during the second versus first cocaine-conditioning sessions (Figure 3D). Significant differences between locomotion during the two sessions (repeated-measures ANCOVA, $p = 0.0005$)

were affected by cocaine dose (session × dose, $p = 0.0002$) and by genotype (genotype × session × dose, $p = 0.048$). Scheffé *post hoc* analyses ($p = 7.15 \times 10^{-6}$) document highly significant differences between wild-type and homozygotes and no significant differences for heterozygote/wild-type comparisons ($p = 0.988$).

Memory: Morris water maze. To provide a control for the possibility that the effects of reducing *PTPRD* expression on CPP might be confounded by widespread memory differences, we assessed the performance of these mice in the Morris water maze (28). Repeated-measures ANOVA showed that genotype influenced time taken to learn the location of the platform (Figure 5; genotype × day, $p = 0.005$) and time spent searching for the platform near its former location during probe trials (Figure 5C; effect of genotype, $p = 0.006$). There were significant effects of genotype on swimming speed ($p = 0.003$), but not on latencies to reach the target quadrant ($p = 0.414$). Scheffé *post hoc* analyses revealed that virtually all these differences were due to deficits in the homozygote knockouts. Homozygotes learned and swam more slowly ($p < 0.001$ and $p = 0.021$, respectively). During probe trials they searched further from the prior platform location, spending more time in the wrong quadrants ($p = 0.027$ and $p < 0.001$) as previously reported (25). On the other hand, heterozygous mice displayed no differences from wild-type mice in any Morris water maze parameter studied ($p = 0.694$ for learning curve, $p = 0.996$ for distance from platform, $p = 0.476$ for time in quadrant and $p = 0.830$ for swimming speed).

DISCUSSION

These data enhance our understanding of common human *PTPRD* allelic variation. They provide a mouse model for ways in which this variation can alter RLS and addiction-associated phenotypes. The data allow speculation about the paths through which *PTPRD* variation might influence RLS/WED and addiction vulnerability.

PTPRD SNPs (including rs1975197, rs4626664 and/or rs2381970) are associated with (a) differing levels of *PTPRD* mRNA expression in human brain samples, (b) RLS diagnoses and (c) addiction-related phenotypes. The relatively 5′ localization of these SNPs in the *PTPRD* gene is consistent with the 5′ localization of many, but not all, of the *PTPRD* association signals for RLS and addiction phenotypes. The >70% differences in levels of *PTPRD* mRNA identified in humans with different common *PTPRD* haplotypes suggest that we should consider data from both heterozygous and homozygous *PTPRD* knockout mice for which relative *PTPRD* expression levels bracket these common human interindividual differences. The contrastingly low frequencies of *PTPRD* missense variants identified in database searches (data not shown) and the locations of these missense SNPs in more 3′ *PTPRD* regions add support to the idea that variation in levels of *PTPRD* expression provides a major molecular source of common functional human individual *PTPRD* variation that contributes to RLS and addiction phenotypes. It will be interesting for future studies to test associations among rs2381970 alleles, RLS and other phenotypes that have displayed overall associations with *PTPRD*. Other brain regions that include cortex, caudate, thalamus, hippocampus, cerebellum and hypothalamus also display evidence for *PTPRD* mRNA expression (31,32). It will be interesting for future studies to seek influences of these haplotypes on expression in these brain regions.

The phases of associations of level-of-expression *PTPRD* SNPs with phenotypes of interest are also instructive. The rs4626664 and rs1975197 minor alleles that display association with RLS are associated with lower levels of expression of *PTPRD* mRNA. Results for rs2381970 support the same phase of association (for example, lower levels of expression of *PTPRD* mRNA are associated with the addiction risk allele; Supplementary Table S2 and Supplementary Figure S1) from the most positive studies of addiction. Available rs2381970 genotypes have allowed us to test association with substance dependence in (a) two samples for which there was nominally significant association ($p = 0.007$ for African American alcohol dependent vs. control and 0.026 for European American polysubstance dependent vs. control [13,16]), (b) two samples in which there were trends toward significance ($p = 0.08$ for African American polysubstance dependent vs. control and 0.06 for European alcohol dependent vs. control [13,33]) and (c) a single sample without association or trend toward association (European American alcohol dependent vs. control [16]). rs2381970 has also been genotyped in ways that allow assessment of its association with ability to quit smoking in (a) two samples with nominally significant association ($p = 0.018$ and 0.048) (14,18) and (b) two other samples without such association (10,19). Taken together, these data support use of mice with reduced *PTPRD* expression as models for common human *PTPRD* risk alleles of relevance for both RLS and addiction phenotypes.

Uetani *et al.* (25) constructed the *PTPRD* knockout mice studied here and characterized features of homozygous knockouts that include difficulties with feeding and with spatial learning/memory, but reported no data from heterozygotes. On the basis of human associations with addiction phenotypes, it is interesting that heterozygotes display significant differences in CPP. It is also interesting that these differences display specificity. Heterozygotes fail to display deficits in tests of memory or locomotion that would provide confounding explanations for altered CPP. There is no evidence that heterozygous knockout alters the conditioned locomotion that can be exerted by cocaine during conditioned place preference testing. There is no enhanced lethality or other physiological alteration noted with the lifelong reductions in *PTPRD* expression found in heterozygotes.

We would have more caution in interpreting motor and sleep-related results

from homozygous knockout mice if these mice displayed less locomotion and more sleep. However, the enhanced locomotion and reduced sleep actually observed in the homozygous knockout mice seem unlikely to be due to confounding influences.

The current results add to data that validate the behavioral assessments used here. While most existing validation of conditioned place preference as a model for human addiction comes from pharmacological experiments (20), the current concordant mouse/human data add to genetic validation of this test (34,35). The current data also support assessment of locomotor activity as modeling features of the motor restlessness found in human RLS, as does data from pharmacologic, iron modulation, brain lesion and other knockouts (6,36). Studies of behaviorally defined sleep, as reported here, also provide striking findings in knockout of the RLS risk gene *BTBD9* (36). Future work will be necessary to provide electroencephalographic support for the *PTPRD* findings. Nevertheless, assessments of sleep/wake patterns in the times before and after the onset of the lights on period in mice now provide an increasingly validated model for some of the RLS/WED sleep disturbances. Conceivably, the longer duration of sleep bouts found in the *PTPRD* knockouts might even reflect the increased sleep "pressure" that might accompany and provide a marker for sleep deficits in these mice.

PTPRD variant associations with RLS/WED and addiction vulnerability, phenotypes that are not documented to co-occur at greater than chance rates, raises interesting questions about nosology of complex and likely heterogeneous disorders with substantial genetic and environmental determinants. Conceivably, careful epidemiological studies will yet identify currently unappreciated comorbidity. If *PTPRD* variation provides an oligogenic influence on RLS vulnerability and a polygenic influence on addiction vulnerability, however, we might not expect epidemiologically discernable comorbidity to arise from variation in this single gene. Shared variation in many genes might be required to cause observable clinical comorbidity. Nevertheless, studies of the brain variation that comes from *PTPRD* variation may well reveal a pattern of brain pathway change that is shared between addiction and RLS, aiding understanding of shared pathophysiological elements that are likely to predispose to each of these common disorders.

CONCLUSION

PTPRD variation, implicated in RLS, in addiction and in rewarding and/or aversive responses to stimulants and alcohol by human molecular genetic studies, thus provides a pattern of altered behaviors in a mouse model that accords remarkably with human data for these phenotypes. The present results support the important roles that detailed patterns of neuronal connections (37), including those modulated by *PTPRD*, are likely to play in addiction and other complex brain phenotypes. These data add to support for *PTPRD* as a candidate druggable target for therapies for RLS and/or addictions (38).

ACKNOWLEDGMENTS

This work was supported by the NIH-IRP, NIDA, U.S. Department of Health and Human Services (HHS) (GR Uhl) and by New Mexico VA Health Care System (NMVAHCS) (GR Uhl). We are grateful for help from C Johnson, D Arking, D Naimen, J Bader and J Schroder; for access to brain samples from the University of Maryland Brain Tissue Bank; and for access to data for PTPRD from A Hart and A Palmer. All human and/or animal studies were approved by the appropriate institutional committees.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Cite this article as: Drgonova J, *et al*. (2015) Mouse model for protein tyrosine phosphatase D (*PTPRD*) associations with restless leg syndrome or Willis-Ekbom disease and addiction: reduced expression alters locomotion, sleep behaviors and cocaine-conditioned place preference. *Mol. Med*. 21:717–25.