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Transcriptional and behavioral interaction between 22q11.2 orthologs modulates schizophrenia-related phenotypes in mice

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Microdeletions of 22q11.2 represent one of the highest known genetic risk factors for schizophrenia. It is likely that more than one gene contributes to the marked risk associated with this locus. Two of the candidate risk genes encode the enzymes proline dehydrogenase (PRODH) and catechol-*O*-methyltransferase (COMT), which modulate the levels of a putative neuromodulator (L-proline) and the neurotransmitter dopamine, respectively. Mice that model the state of PRODH deficiency observed in humans with schizophrenia show increased neurotransmitter release at glutamatergic synapses as well as deficits in associative learning and response to psychomimetic drugs. Transcriptional profiling and pharmacological manipulations identified a transcriptional and behavioral interaction between the *Prodh* and *Comt* genes that is likely to represent a homeostatic response to enhanced dopaminergic signaling in the frontal cortex. This interaction modulates a number of schizophrenia-related phenotypes, providing a framework for understanding the high disease risk associated with this locus, the expression of the phenotype, or both.

An increased frequency of microdeletions at the 22q11.2 locus has been found in individuals with schizophrenia[1]. Two independent systematic approaches[2–4] and several candidate gene studies have identified candidate schizophrenia susceptibility genes from the 22q11.2 region. Both systematic approaches, using different methodologies to analyze almost all the genes in this locus, provided convergent evidence for an involvement of the gene encoding the mitochondrial enzyme proline dehydrogenase (*PRODH*). A previous study[2] provided evidence that a haplotypic variant of the gene is preferentially transmitted in individuals with schizophrenia, a finding recently replicated in a large-scale family study[5]. The earlier study[2] also found that incidences of rare variants of *PRODH* affecting highly conserved amino acids and causing drastic reduction in enzymatic activity[6] are elevated to various degrees in individuals with schizophrenia—a finding since replicated in an independent set of studies[4,7]. Other genes in the 22q11.2 region, including the *ZDHHC8* gene and the gene encoding catechol-*O*-methyltransferase (COMT), have also been implicated by systematic[3,8] and candidate gene approaches[9,10], respectively. Taken together, these studies suggest that 22q11.2 microdeletion-associated schizophrenia may have the characteristics of a contiguous gene syndrome, in which more than one gene contributes to the marked increase in disease risk. Given the limitations of genetic association studies and the restricted knowledge of the functional impact of human genetic variation, we used studies in animal models as a powerful means for examining the function and genetic interactions of candidate schizophrenia susceptibility genes from this locus.

RESULTS

Establishment of a genetic mouse model

Previously[11], in the Pro/Re hyperprolinemic mouse strain, we identified a mutation in the mouse ortholog of the human *PRODH* gene that introduces a premature termination (E453X) and results in a reduction in enzymatic activity. Preliminary analysis indicated that these *Prodh*-knockdown mice have regional neurochemical alterations in the brain accompanied by a deficit in sensorimotor gating, similar to that seen in individuals with schizophrenia and other neuropsychiatric disorders[11]. To minimize the influence of genetic background, we introduced the E453X mutation into the 129/SvEv strain through backcrossing for ten

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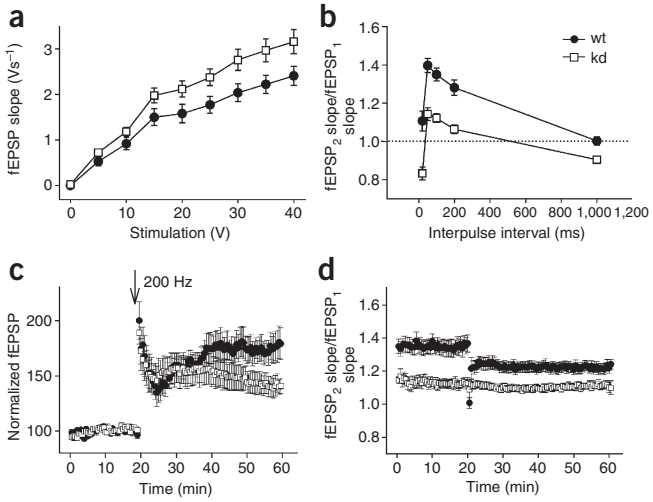

Figure 1 Electrophysiological characterization of Prodh-deficient mice. (**a**) Mean fEPSP slope measured as a function of stimulation intensity in slices from Prodh-deficient mice ($n = 15$ slices) and their wild-type littermates ($n = 15$ slices). (**b**) Paired-pulse ratio ($fEPSP_2$ slope to $fEPSP_1$ slope) versus the interpulse interval length for slices from Prodh-deficient mice ($n = 50$ slices) and wild-type littermates ($n = 35$ slices). (**c**,**d**) Mean fEPSP slope (**c**) and paired-pulse ratio with a 50-ms interpulse interval (**d**) as a function of time before and after tetanization at 200 Hz (arrow) for slices from Prodh-deficient mice (open squares, $n = 11$ slices) and wild-type littermates (closed circles, $n = 7$ slices). All data show mean ± s.e.m. wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice.

generations. Measurements of serum proline levels confirmed the previously described increase in L-proline levels in mice homozygous for the mutation. Serum levels of L-proline in the homozygous mutant mice range from 300 to 600 μmol liter^{-1} as compared to less than 100 μmol liter^{-1} in their wild-type littermates[11]. These levels are comparable to those observed in some individuals with the 22q11.2 microdeletion and in heterozygous carriers of *PRODH* rare variants but are well below the levels observed in individuals with hyperprolinemia type I, a rare condition often accompanied by epilepsy and mental retardation[4]. Therefore, both in terms of the nature of the mutation and the ensuing increase in L-proline levels, homozygous *Prodh*-knockdown mice represent an accurate animal model not only of a susceptibility gene but also of a susceptibility allele.

Prodh deficiency alters glutamatergic transmission

Evidence that L-proline is selectively accumulated by a brain-specific, high-affinity transporter localized exclusively in a subset of glutamatergic synapses suggests that L-proline may modulate transmission at glutamate synapses[12]. High concentrations of exogenous L-proline can activate NMDA and AMPA receptors[13], and more physiological concentrations potentiate excitatory transmission at hippocampal synapses between CA3 and CA1 pyramidal neurons[14]. Nevertheless, there is no evidence that PRODH affects basic glutamate-mediated synaptic transmission. To examine the role of PRODH, we compared the synaptic input-output relation at the well-characterized glutamatergic CA3–CA1 synapses in acute hippocampal slices from Prodh-deficient mice and their wild-type littermates. Recordings of the extracellular field EPSP (fEPSP) showed that the loss of Prodh caused a significant enhancement in synaptic transmission over a wide range of stimulus intensities (**Fig. 1a**), similar to results previously reported with exogenous application of L-proline[14]. However, unlike the results observed with acute application of L-proline, where there was no enhancement in presynaptic function, an analysis of paired-pulse stimulation suggested that glutamate release is increased in Prodh-deficient mice. Thus, we found that the paired-pulse ratio (PPR) measured at all interpulse intervals ranging from 20 ms to 1,000 ms was significantly decreased in the mutant mice compared to their wild-type littermates (**Fig. 1b**). Notably, paired pulses with a 20-ms interval yielded facilitation in wild-type mice but depression in the Prodh-deficient mice. The decrease in PPR is often a hallmark of an increase in the probability of transmitter release during the first pulse, which can lead to depression of release during the second pulse or to an occlusion of the normal increase in the probability of release.

It has recently been shown that long-term potentiation (LTP) at CA3–CA1 hippocampal synapses induced by 200-Hz or theta-burst stimulation protocols enhances presynaptic function by increasing the probability of transmitter release[15]. We therefore explored the effect of Prodh deficiency on the induction of 200-Hz LTP at CA3–CA1 synapses. In wild-type mice, 200-Hz tetanic stimulation enhanced the fEPSP to180.3 ± 14.8% (mean ± s.e.m.; $n = 7$) of its initial level, when measured 40 min after tetanization (**Fig. 1c**). In contrast, the magnitude of 200-Hz LTP in Prodh-deficient mice was only 139.9 ± 6.6% ($n = 11$), significantly lower than that in their wild-type littermates ($P < 0.05$, Kolmogorov-Smirnov test).

In support of the view that 200-Hz LTP enhances presynaptic function, we found that the increase in the fEPSP in wild-type mice was accompanied by a significant decrease in the PPR (measured at a 50-ms interpulse interval; **Fig. 1d**). Thus, 40 min after induction of LTP, the PPR decreased to 90.5 ± 1.4% ($n = 7$) of its initial value (1.35 ± 0.03, $n = 7$; $P < 0.001$, paired t-test). In contrast, induction of 200-Hz LTP caused no change in the PPR in Prodh-deficient mice. Before induction of LTP, the PPR in Prodh-deficient mice was 1.13 ± 0.02 (50-ms interval, $n = 11$), which was significantly lower than that in their wild-type littermates ($P < 0.001$, Kolmogorov-Smirnov test). Forty minutes after the induction of LTP in Prodh-deficient mice, the PPR remained at 99.0 ± 2.6% of its initial level ($n = 11$; $P > 0.05$, paired t-test).

In summary, Prodh deficiency seems to increase the initial probability of glutamate release at CA3–CA1 synapses that leads to a decrease in the dynamic range of plastic presynaptic modifications at glutamatergic synapses, inhibiting paired-pulse facilitation and LTP.

Behavioral analysis

Mice treated chronically with NMDA receptor antagonists, such as dizocilpine (MK801) and phencyclidine (PCP), represent one of the most widely used pharmacological models of schizophrenia. NMDA receptor antagonists increase glutamate release[16], suggesting that this class of drugs may act primarily via secondary activation of non-NMDA receptor glutamatergic neurotransmission[16]. It has been shown that persistent glutamatergic dysfunction in these models leads to secondary dysregulation of frontal dopaminergic neurotransmission and hypersensitivity to the locomotor effects of amphetamine[17], as well as to performance decrement in some cognitive tasks[18–20]. We asked whether a similar pattern of deficits develop as a result of Prodh deficiency and the ensuing increase in glutamate release.

Response to psychomimetic drugs

We evaluated baseline locomotor activity and the locomotor response to two psychomimetic drugs, MK801 (an NMDA receptor antagonist) and D-amphetamine (which causes dopamine release), by monitoring drug-induced locomotor responses. In an open-field test, we detected strong deficits in baseline locomotor activity of Prodh-deficient mice compared to that of their wild-type littermates (**Fig. 2a**). In the

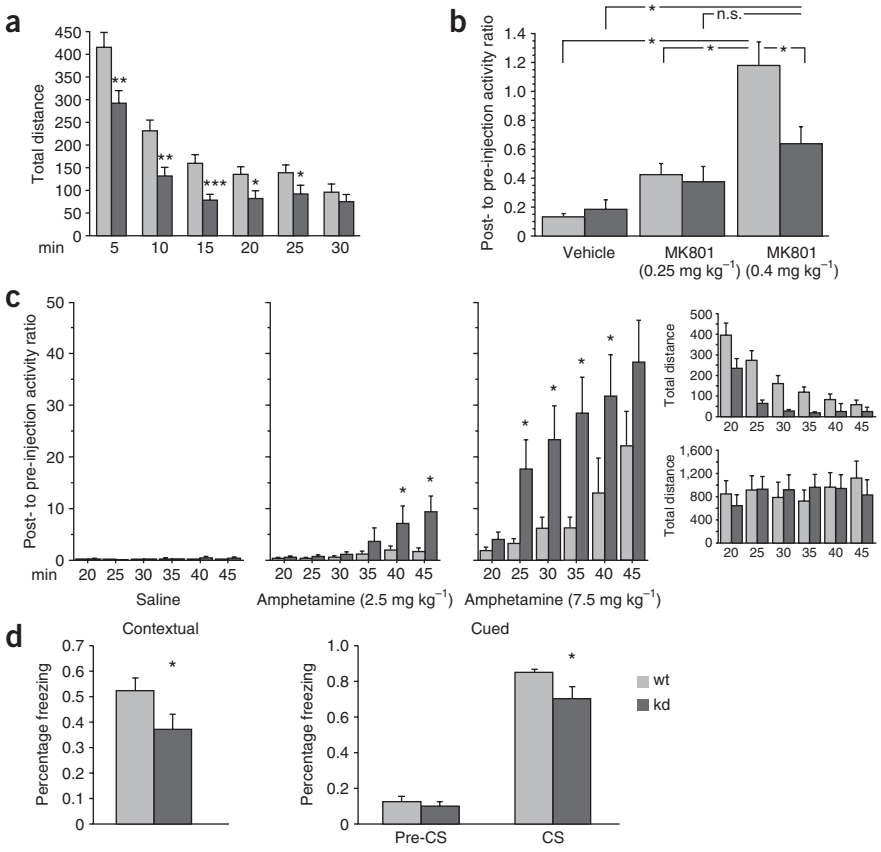

Figure 2 Behavioral characterization of Prodh-deficient mice. (**a**) Total distance traveled is significantly decreased in Prodh-deficient mice (overall $P = 0.0006$). The figure shows the time course of the effect of the mutation (E453X) over 30 min, grouped in six 5-min intervals. Results are from an open-field test probing spontaneous exploratory behavior under mildly stressful conditions (novel environment and light). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**b**) Locomotor-activating effect of MK801 on Prodh-deficient mice and wild-type littermate control mice. Mice were injected i.p. with vehicle, drug at a low dose (0.25 mg per kg body weight) or drug at a high dose (0.4 mg per kg body weight). $^*P < 0.05$. (**c**) D-Amphetamine–induced activity in Prodh-deficient mice and wild-type littermate control mice. *y*-axis represents ratios of post-treatment to pre-treatment values of the effect of the mutation over 5-min intervals (counting from the time of D-amphetamine administration, 15 min before the animals were placed in the open-field apparatus). Mice were injected i.p. with vehicle or with drug at 2.5 or 7.5 mg per kg body weight. Far right, the total distance traveled (absolute values) by Prodh-deficient mice and wild-type littermate control mice over the 30-min period, before (top) and after (bottom) administration of 7.5 mg of D-amphetamine per kg body weight. $^*P < 0.05$. (**d**) Associative learning and memory in Prodh-deficient mice. Results from both contextual and cued tests are shown. All data show mean ± s.e.m. wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice. $^*P < 0.05$.

presence of a difference in baseline locomotor activity, we used the ratio of the activity during the half-hour after injection of a test drug to the activity during the half-hour before injection as an index of a drug's locomotor activating effects.

Consistent with previous reports[16], both in wild-type control and mutant mice, the administration of MK801 or D-amphetamine stimulated locomotor activity in a dose-dependent manner, as compared to saline administration which did not stimulate activity (**Fig. 2b,c**). However, at the higher drug dosage, Prodh-deficient mice seemed to be less responsive to MK801 than their wild-type littermates were (**Fig. 2b**). Such a response may indicate an effect of the increase in basal glutamate release that occludes any additional effects of MK801 on glutamate efflux, a developmental desensitization of the MK801-modulated glutamatergic pathways[21], or both. By contrast, Prodh deficiency led to a significant potentiation of D-amphetamine–induced locomotor activity, especially at the dose of 7.5 mg per kg body weight (**Fig. 2c**). This pattern of enhanced amphetamine-induced locomotor activity is similar to the one described after persistent glutamatergic dysfunction in pharmacological models[17] and is reminiscent of the increased susceptibility to the disorganizing effects of D-amphetamine[22] observed in individuals with schizophrenia.

Cognitive tasks

First, we used the Pavlovian conditioned-fear protocol to assess associative learning and memory in Prodh-deficient mice. In the contextual fear-conditioning test—which is both amygdala and hippocampus dependent—Prodh-deficient mice froze significantly less than did wild-type mice when, 24 h after training, they were returned to the context in which they had received the shock ($P < 0.05$; **Fig. 2d**). In the cued version of the test—which requires the amygdala but not the hippocampus[23]—tone-dependent freezing 48 h after training was significantly reduced in the Prodh-deficient mice compared to wild-type littermate control mice ($P < 0.05$; **Fig. 2d**). In contrast, no significant differences ($P > 0.05$) were found between groups in the absence of the tone.

We also used the T-maze delayed-alternation task to examine whether low Prodh activity was associated with changes in spatial working memory, which is thought to be modulated by the frontal regions of the mouse neocortex[24]. Prodh-deficient mice learned the 5-s–delay T-maze task during five consecutive training days and performed as well as their wild-type littermates. Mutants also did not show any difference in their T-maze working memory test (data not shown; also see below). Because the pattern of the behavioral profile of the mutant mice is probably determined by the primary synaptic deficit as well as by the emergence of brain region–specific compensations, normal working memory performance may indicate the emergence of cortical compensations in the Prodh-deficient mice.

Expression profiling in the frontal cortex

The effect of a mutation on synaptic transmission and associated pathways, as well as the development of any compensatory changes, can be reflected at the level of transcriptome[25]. We reasoned that transcriptional profiling in the brain of a mouse model for a schizophrenia susceptibility allele might provide an unbiased evaluation of the transcriptional programs altered by the disruption of the gene; such alterations would reflect either causal downstream effects of the mutation or reactive changes. We focused on the frontal cortex, on the basis of clues provided by our initial behavioral analysis as well as of numerous studies suggesting functional and structural pathology of this brain region in schizophrenia.

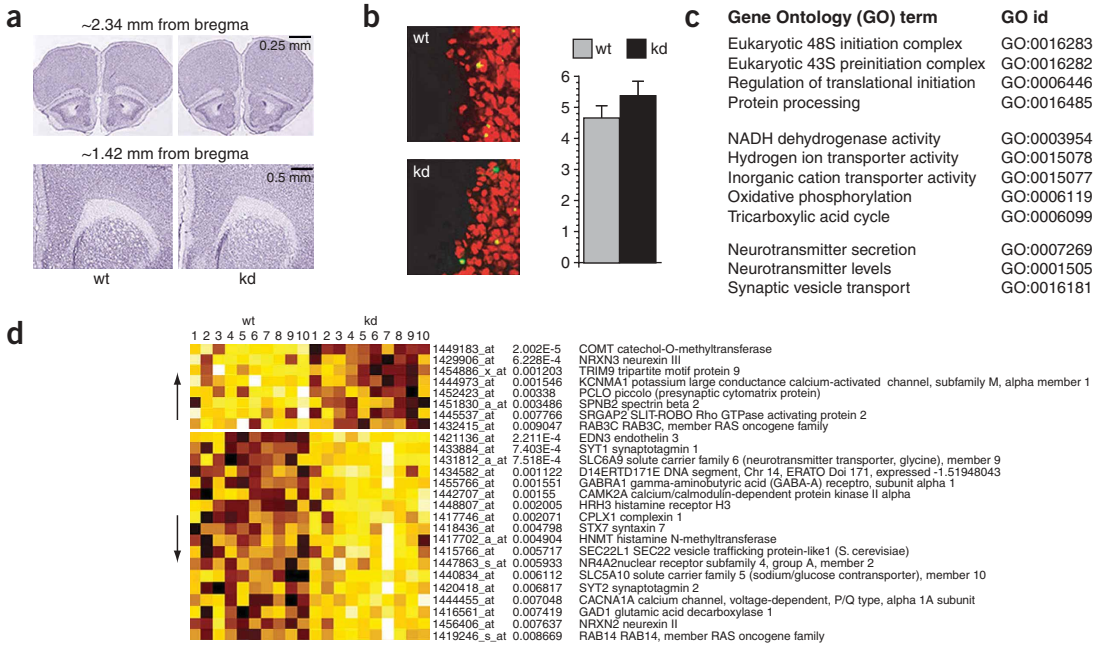

Figure 3 RNA expression profiling in the frontal cortex of Prodh-deficient mice. (**a**) Representative Nissl-stained coronal section, from Prodh-deficient mice and wild-type littermate control mice, of frontal cortex localized to approximately 2.34 mm rostral to bregma and medial prefrontal cortex localized to approximately 1.42 mm rostral to bregma. (**b**) TUNEL assay in the frontal cortex of postnatal day 8 (P8) Prodh-deficient mice (E453X) and wild-type littermate control mice. Graphs represent mean ± s.e.m. of positive cells per section. (**c**) Top-scoring Gene Ontology (GO) terms listed with the corresponding *P*-value and GO identification numbers. This approach provides a statistical measure of the significance of the co-occurrence of genes with related function. (**d**) Matrix visualizing Affymetrix microarray data of the top-scoring, differentially expressed 'neurotransmitter release and regulation' genes. Genes are ranked in order of increasing template-match *P*-value and are listed separately as up- or down-regulated. Each gene is visualized as a row of colored squares, with one square for each sample. The color indicates the relative expression of the gene, with lighter colors indicating lower levels of expression. Annotations for each gene and the *t*-test *P*-value are shown at the right of the figure. The *Comt* gene shows an upregulation of about 70% ($P = 2.0 \times 10^{-5}$). wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice.

In control experiments in our mouse model, histological analysis (**Fig. 3a**), cell counting (data not shown) and cell death assessment in the developing cortex (**Fig. 3b**) did not reveal any gross anatomical abnormalities. We used ten independent RNA samples from each genotype to compare and contrast gene expression profiles in the frontal cortex of 8-week-old Prodh-deficient mice. We used *t*-tests on Affymetrix GeneChip data analyzed by Robust Multi-array Average (RMA)[26,27] to identify genes whose expression differed between mutant mice and their wild-type littermate controls. We observed changes in the levels of a few hundred transcripts in response to the mutation (false discovery rate < 0.05; M.P., P.P., M.K. and J.A.G., unpublished data). For the purpose of this study, we clustered the annotated genes into groups by biological function and applied a statistical analysis using Ermine[28]. On the basis of strict criteria, expression of 12 sets of genes (Gene Ontology (GO) terms) was significantly altered (**Fig. 3c**), implicating only three major processes: (i) translational initiation and protein processing, (ii) mitochondrial function and metabolism and (iii) presynaptic neurotransmitter release and regulation. Most notably, one of the top upregulated genes in the entire dataset and the top upregulated gene in the third group was *Comt* ($P = 0.00002$) (**Fig. 3d**), which also maps within the 22q11.2 locus and is a candidate schizophrenia susceptibility gene. We prioritized *Comt* for further investigation because our expression analysis suggested a previously unsuspected interaction between *Prodh* and *Comt* that could, in principle, modulate the risk associated with the 22q11.2-related psychiatric phenotypes, their expression or both.

Confirmation of *Comt* upregulation

Quantitative reverse transcription polymerase chain reaction (RT-PCR) experiments confirmed the upregulation of *Comt* mRNA in an independent cohort of frontal cortices (~25% increase, $P = 0.004$; **Fig. 4a**). Western blots in the frontal cortices of adult Prodh-deficient mice and wild-type littermate control mice indicated a highly reproducible increase ($P = 0.0001$) in the levels of both the soluble and the membrane-associated form of the Comt protein (**Fig. 4b**). In rodent cortex, Comt is primarily localized in large pyramidal neurons[29]. Non-quantitative immunocytochemical analysis using an antibody to Comt confirmed both the cellular distribution pattern as well as the increase in the Comt protein levels observed in the western blots (**Fig. 4c**). Developmental analysis following the time course of change in Comt levels suggested that the observed increase was established at around the third week of life (~30% increase, $P = 0.01$; **Fig. 4b**), a period of active synapse formation and stabilization in the frontal cortex that coincides with a robust postnatal peak in *Prodh* gene expression and a two- to threefold decrease (compared to adult levels) in synaptic L-proline uptake[30,31]. Degradation by Comt is likely to be a key mechanism for regulating the synaptic action of dopamine in the frontal cortex, where the dopamine transporter is expressed at very low levels, but not in the striatum, where the dopamine transporter is abundantly expressed. Consistent with this view, immunoblot analysis did not reveal a significant increase of Comt in the striatum of Prodh-deficient mice (**Fig. 4b**); therefore, the upregulation we observed selectively in the frontal cortex, a major recipient of dopaminergic input, may signify a response to increased local dopaminergic

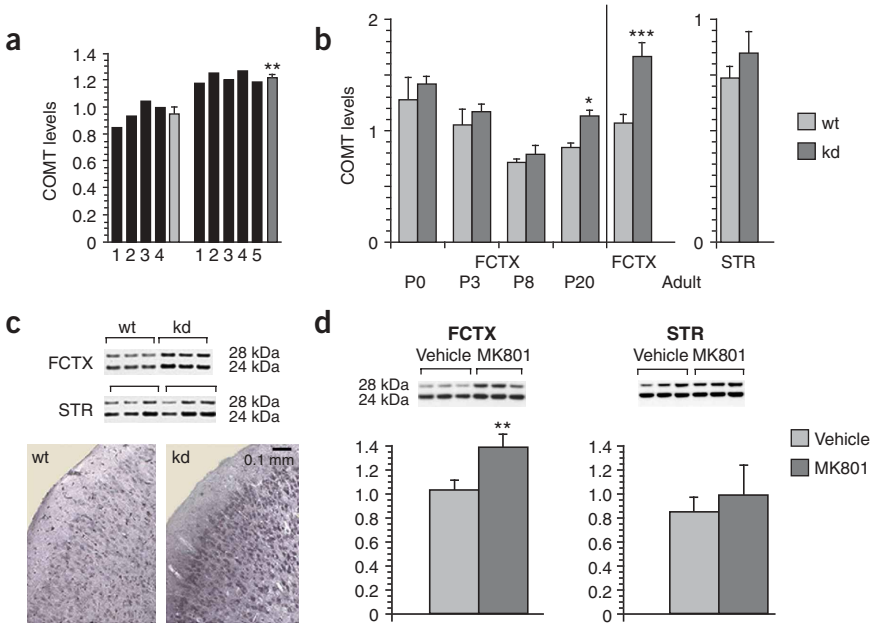

Figure 4 Validation of RNA expression profiling of *Comt* in Prodh-deficient mice. (**a**) Validation of RNA expression profiling of *Comt* using Taqman real-time RT-PCR. *Comt* shows an upregulation of about 25% ($P = 0.004$). Data (threshold cycle) are standardized to β-actin and shown both as values for each of the four or five independent experiments (black bars) and as mean ± s.e.m. of all experiments performed for each genotype (gray bars). $^{**}P < 0.01$. (**b**) COMT levels in the frontal cortex during left, early postnatal development and right, in the adult frontal cortex and striatum of Prodh-deficient mice ($n = 20$) and wild-type littermate controls ($n = 10$). COMT levels are also statistically unchanged in the striatum ($P = 0.083$). We observed small increases in this brain region occasionally, but not reproducibly between experiments. Mean ± s.e.m. optical densities are shown, reflecting the normalized Comt levels. $^{*}P < 0.05$; $^{**}P < 0.001$. (**c**) Representative immunoblots and immunostained sections from the frontal cortex and striatum of Prodh-deficient mice and wild-type littermate control mice. (**d**) Effect of subchronic administration of the non-competitive NMDA antagonist MK801 on *Comt* expression in frontal cortex and striatum of 129/SvEv wild-type mice. Data were standardized by β-actin and are given as mean ± s.e.m. of normalized optical densities for 10 mice per group. wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice; FCTX, frontal cortex; STR, striatum.

hypersensitivity. Notably, subchronic treatment of 129/SvEv wild-type mice with MK801 (one daily injection of 0.25 mg per kg body weight for 2 weeks), followed by a comparison of Comt levels in the frontal cortex and striatum of treated and untreated animals, revealed a regionally selective pattern of Comt expression which phenocopied that of the Prodh-deficient mice. That is, Comt levels increased in frontal cortex (by roughly 35%, $P = 0.01$) but not in striatum ($P = 0.4$; **Fig. 4d**). This observation, along with the finding that upregulation of Comt levels (unique among all 22q11 orthologs) is part of a coordinated change in expression of genes regulating neurotransmitter action, strongly suggests that it is the effect of Prodh deficiency on synaptic transmission that underlies the increase of cortical Comt levels.

Dopaminergic dysregulation in the frontal cortex

We asked whether the observed upregulation of Comt levels signifies a more generalized dysregulation of cortical dopamine turnover and transmission. First, we determined levels of both total dopamine and extracellular dopamine in both cortex and striatum. We did not observe any significant sustained differences in basal total dopamine levels in either the frontal cortex or striatum of Prodh-deficient mice as compared to their wild-type littermates (levels of 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine and hydroxytryptamine (5-HT) were also unchanged; **Fig. 5a**). We also monitored extracellular levels of dopamine using *in vivo* microdialysis. Baseline levels did not differ between the two genotypes in either cortex or striatum. After acute systemic D-amphetamine administration (2.5 and 7.5 mg per kg body weight intraperitoneally (i.p.)), extracellular dopamine increased from the baseline levels in both genotypes and both brain regions (**Fig. 5b**,**c**). Notably, Prodh deficiency significantly ($P < 0.05$) potentiated cortical (**Fig. 5b**), but not striatal (**Fig. 5c**), dopamine overflow induced by administration of 7.5 mg of D-amphetamine per kg body weight; this was in good correlation with the response pattern observed in locomotor assays.

Second, we examined whether, in addition to Comt upregulation, other changes that would also result in dampening of dopaminergic transmission develop in the frontal cortex of Prodh-deficient mice. We analyzed the levels of dopamine receptors and of several signaling molecules shown to participate in or modulate dopamine action[32–34] (**Fig. 6**). We assessed protein levels in western blots from cortical extracts (dopamine receptor D1 (DRD1) was assayed by quantitative RT-PCR because antibodies with acceptable mouse specificity in western blots were not available). We observed significant changes, reproducible in three experiments, in the levels of the following: (i) DRD1 (~35% downregulation, $P = 0.02$); (ii) the dopamine- and cAMP-regulated phosphoprotein DARPP-32, also known as PPP1R1B (~30% downregulation, $P = 0.02$); and (iii) protein phosphatase 3, also known as calcineurin (all three catalytic subunits, PPP3CA, PPP3CB and PPP3CC; about 33% upregulation, $P = 0.001$). Because the antibody to calcineurin recognizes all three catalytic subunits, we performed a follow-up analysis with subunit-specific antibodies. A specific, quantifiable signal was observed only for PPP3CC (calcineurin gamma catalytic subunit), revealing a significant increase ($P = 0.03$) and suggesting that an increase in the levels of this subunit accounts, at least partially, for the signal observed with the non-discriminant antibody. Notably, dopamine acting through DRD1 results in a phosphorylation of DARPP-32 mediated by protein kinase A (PKA), causing inhibition of phosphatase 1. Calcineurin acts as the principal mediator of the dephosphorylation-dependent inactivation of DARPP-32 (ref. 32; see also **Fig. 6**). Because DARPP-32 phosphorylation is enhanced by DRD1 activation and inhibited by calcineurin, the net effect of the changes we observed in the cortex of Prodh-deficient mice—namely, an increase in COMT and calcineurin and a decrease in DRD1 and DARPP-32—should result in a synergistic decrease in dopamine-mediated protein phosphorylation and signaling. Notably, a chronic MK801 treatment protocol was previously shown to result in a similar decrease of DRD1 levels in the cortex of rats[35]. By contrast, levels of dopamine receptor D2 (DRD2, which is predominantly localized in subcortical areas), as well as of downstream DRD2 effectors[33,34], remained statistically unchanged ($P > 0.05$) (**Fig. 6**).

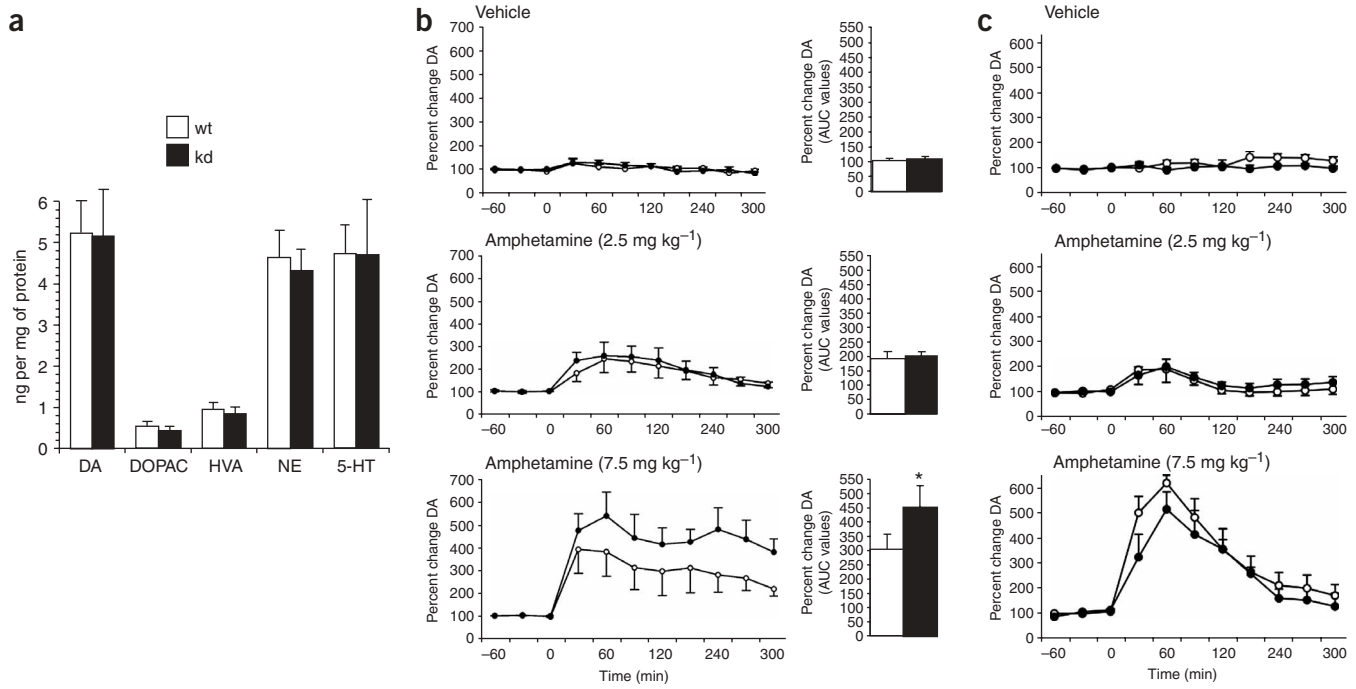

Figure 5 Dopaminergic dysregulation in the frontal cortex of Prodh-deficient mice. (**a**) HPLC analysis of dopamine (DA), DOPAC, HVA, norepinephrine (NE) and 5-HT (ng per mg of protein) in the frontal cortex of Prodh-deficient mice and wild-type littermate control mice (mean ± s.e.m., $n = 5$). (**b**,**c**) Monitoring the extracellular levels of dopamine using *in vivo* microdialysis. Microdialysis probes were placed in both prefrontal cortex (**b**) and dorsal striatum (**c**) and dopamine levels were measured at baseline after administration of saline as well as after acute systemic administration of D-amphetamine (at 2.5 and 7.5 mg per kg body weight i.p.) (mean ± s.e.m.). wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice; AUC, area under curve. $^*P < 0.05$.

Behavioral interaction between *Prodh* and *Comt*

We next extended our molecular studies to behavioral and pharmacological analysis to ask whether the epistatic interaction between *Prodh* and *Comt*, which we observed at the transcriptional level, correlates with epistatic interactions at the behavioral level. If Comt upregulation develops as a homeostatic response to, rather than primary cause of, local dopaminergic hypersensitivity, inhibition of Comt activity should exaggerate rather than attenuate observed behavioral deficits and may induce additional deficits. It was not practical to address this issue by generating double heterozygous and double homozygous mouse mutants for both *Prodh* and *Comt* because of the physical proximity of the two genes and the fact that heterozygous *Prodh* mutant mice have near normal L-proline levels. Instead we used tolcapone, a reversible Comt inhibitor that crosses the blood-brain barrier[36], to examine the effects of reduced Comt activity in three schizophrenia-related mouse behaviors that are critically influenced by the level and pattern of cortical dopamine transmission: namely, sensitivity to the locomotor effects of D-amphetamine[37,38], working memory as assayed by the T-maze test[39] and sensorimotor gating as assayed by the PPI test[40].

First, we compared the effect of tolcapone on the locomotor effects of low-dose D-amphetamine (which does not stimulate locomotor activity when injected alone) in wild-type and homozygous *Prodh*-knockdown mice. We used locomotor activity as a convenient behavioral trait to assess because of the good correlation between D-amphetamine–induced cortical dopamine release and response in locomotor assays and because of several previous rodent studies demonstrating that locomotor activity is profoundly influenced by the level and pattern of cortical dopamine transmission[17,41]. Prodh-deficient mice and wild-type littermate control mice received injections of saline, tolcapone (30 mg per kg body weight), D-amphetamine (1 mg per kg body weight) or both drugs together. Although the locomotor stimulating effect (as compared to the saline vehicle) of individual administration of tolcapone or low-dose D-amphetamine was not significantly different in either genotype, tolcapone significantly potentiated the effect of low-dose D-amphetamine in Prodh-deficient mice ($P = 0.04$), but not in wild-type littermate mice (**Fig. 7a**).

Second, we compared working memory performance in a delayed-alternation test (T-maze), a cognitive task critically dependent on synaptically released dopamine in prefrontal cortex, in the wild-type and homozygous *Prodh*-knockdown mice. As mentioned previously, at baseline, Prodh-deficient mice do not have deficits in their spatial working memory, probably owing to the emergence of compensation. One day after they reached the testing criteria (training accuracy >70% for two consecutive days), mice were challenged with vehicle, the DRD1 agonist SKF-38393 (5 mg per kg body weight subcutaneously), tolcapone (30 mg per kg body weight i.p.) or a combination of SKF38393 and tolcapone. After the administration of either tolcapone alone or combined tolcapone-SKF-38393 injections, we found a significant difference in working memory performance between the two genotypes. Specifically, under conditions of Comt inhibition, mutant but not wild-type mice consistently made more working memory errors in the T-maze than did vehicle-treated controls ($P < 0.05$) (**Fig. 7b**). SKF38393 had no effect on the error rate in our assay on either genotype.

Third, we compared the efficiency of sensorimotor gating as assayed by the PPI test. Prodh-deficient mice and wild-type littermate control mice were treated with saline or tolcapone (30 mg per kg body weight), 20 min before testing (**Fig. 7c**). Under conditions of Comt inhibition, the mutant mice consistently demonstrated lower PPI as compared to vehicle-treated mutants ($P < 0.05$). No such effect was observed in wild-type control mice (**Fig. 7c**).

In summary, Comt inhibition had minimal or no effect on all three tested behaviors in wild-type mice. However, it invariably either exaggerated or induced behavioral deficits in Prodh-deficient mice. This pattern of non-additivity in the behavioral contributions of these two genes is a hallmark of epistasis, providing strong evidence of an interaction between the two genes.

DISCUSSION

The genetic complexity of common psychiatric disorders has been repeatedly inferred from the pattern of inheritance and researchers' inability to identify consistent linkage signals. Epistasis among susceptibility genes lies at the core of this complexity but its biological basis remains elusive. In this context, perhaps the most important insight provided by our analysis is the clear demonstration of epistatic interaction between the *Prodh* and *Comt* genes at the level of transcription and behavior, which is likely to represent a homeostatic response to enhanced dopaminergic signaling in the frontal cortex that emerges as a result of Prodh deficiency. The fact that interaction between these two genes modulates a number of schizophrenia-related phenotypes in mice provides a framework for understanding the high risk for schizophrenia associated with microdeletions of the 22q11.2 locus, which encompasses both the *PRODH* and *COMT* genes. If COMT upregulation is indeed one of the mechanisms used to control cortical dopaminergic hypersensitivity, then individuals with schizophrenia who have a 22q11.2 deletion are at a particular disadvantage because they are deficient for both genes and perhaps unable to compensate efficiently, through COMT, for the cortical dopaminergic hyperactivity induced by PRODH deficiency. Therefore, as already implied by genetic association studies, 22q11.2-associated schizophrenia may have the characteristics of a contiguous gene syndrome: deficiency in more than one gene contributes to the condition by impairing both synaptic function and the development of compensatory response. Such synergistic interaction among two physically linked genes could in principle lead to the high disease risk associated with this locus, modulate the expression of the phenotype or both. Similar patterns of genetic interactions among unlinked loci that produce impaired synaptic function or impaired development of homeostatic response may also account for the epistatic component of the genetic risk of psychiatric disorders in general. Identification, in the brains of Prodh-deficient mice, of the upregulation of genes encoding calcineurin subunits—including *PPP3CC*, a strong candidate schizophrenia susceptibility gene[42]—supports this idea; it also suggests that variation in the *PPP3CC* gene may increase the risk of the disease by impairing compensation for an overactive dopaminergic transmission caused by other primary deficits, including PRODH deficiency.

Although the emergence of cortical dopaminergic dysregulation lies at the basis of the observed epistasis, its cause has yet to be determined. One possibility, supported by the results presented here, is that in early postnatal life, PRODH deficiency affects the state of cortical dopaminergic transmission and signaling indirectly, through interference with

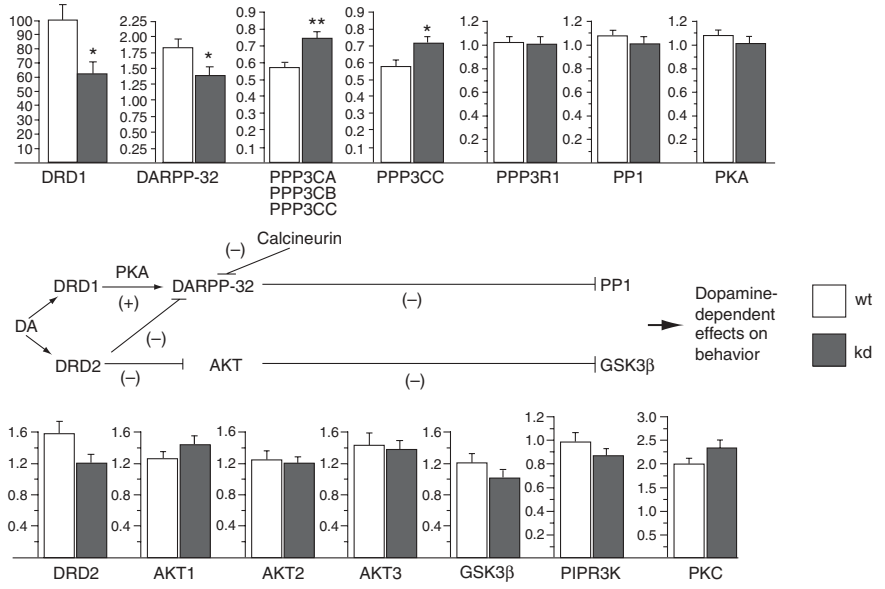

Figure 6 Dopamine-related signaling molecules in the frontal cortex of Prodh-deficient mice. The figure shows levels of dopamine receptors as well as of several signaling molecules that participate in or modulate dopamine action. Relations among some of these molecules are summarized in the diagram. Data were normalized to β-actin and represent mean ± s.e.m. of normalized optical densities for between 18 and 20 mice per group and three independent experiments. DRD1 levels were compared using TaqMan real-time RT-PCR in ten mutant and ten wild-type mice in three independent experiments. Data were standardized by β-actin and given as mean ± s.e.m. wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice. $*P < 0.05$; $**P < 0.01$.

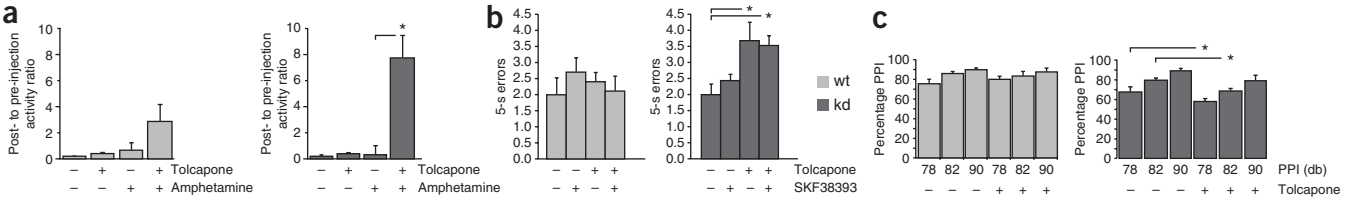

Figure 7 Epistatic interaction between Prodh and Comt at the behavioral level. (**a**) Effect of tolcapone on D-amphetamine–induced locomotor activity. (**b**) Effect of tolcapone on working memory performance in a delayed-alternation test (T-maze). Effect of SKF38393, a DRD1 agonist, was also assayed as a control. There were no tolcapone-induced effects in latency and time to complete the task. In control experiments, we found that tolcapone treatment had no effect on locomotor activity of either genotype (data not shown). Results for 20-s inter-run delays are not shown because the accuracy of T-maze choices were close to chance for this delay interval (the number of 20-s delay errors in ten trials was 4.243 ± 0.553 for wild-type mice and 4.571 ± 0.528 for Prodh-deficient mice), suggesting mouse-strain–specific difficulties in mnemonic maintenance. (**c**) Effect of tolcapone on sensorimotor gating assayed by the PPI test. Mice received injections of saline or tolcapone 20 min before the test. All data are mean ± s.e.m. wt, wild-type littermate control mice; kd, homozygous *Prodh*-knockdown mice. $*P < 0.05$.

glutamatergic pathways. This possibility is also supported by extensive literature on pharmacological rodent models of schizophrenia in which chronic administration of drugs such as MK801 or PCP that dysregulate glutamatergic transmission results in a sustained hyper-responsivity of dopaminergic neurotransmission[17,43]. Indeed, the pattern of enhanced amphetamine-induced cortical dopamine release and locomotor activity is very similar to the one observed after chronic treatment with PCP in rodents[17]; notably, the behavioral effects of such drugs are also modulated by COMT levels and are exaggerated in Comt-null mice (M.P., M.K. and J.A.G., unpublished data). Of course, we cannot formally exclude the possibility that PRODH deficiency directly affects dopaminergic transmission (either within the frontal cortex or in other brain areas that supply dopaminergic afferents); neither can we ignore the possibility that the emergence of cortical dopaminergic dysregulation may be indirectly related to prominent changes in cortical expression of genes regulating protein synthesis or processing and mitochondrial function.

It is also unclear how a deficiency in Prodh causes an enhancement of glutamatergic synaptic transmission. This enhancement is probably due to an increase in probability of glutamate release given the observed change in paired-pulse facilitation, a marker of presynaptic function. Furthermore, we found that 200-Hz LTP at these synapses is significantly decreased. As this form of LTP is due in part to an increase in glutamate release[15], the reduction in LTP may be explained by an occlusion of this presynaptic component by the enhanced release resulting from the loss of Prodh activity. As our experiments were performed in the absence of any blockers of GABA receptors, it is also possible that a reduction in inhibitory synaptic transmission may contribute to the enhancement in the EPSP. However, the finding that the proline transporter is localized to a subset of glutamatergic terminals[12] and is not present in GABAergic synaptic terminals suggests that the primary change may involve glutamatergic synapses.

Despite the insights into 22q11.2-associated schizophrenia provided by the present analysis, several issues remain unresolved. What percent of the risk or what aspects of the phenotype in individuals with schizophrenia can be explained by this interaction? Do additional genes in the region that may contribute to the risk exert their effects by modulating this interaction? Or do they act independently of *PRODH*? These questions notwithstanding, our model offers testable predictions that can now be examined in individuals with 22q11.2 microdeletions.

METHODS

All animal procedures have been performed according to protocols approved by appropriate Animal Care and Use Committees established by Columbia University, Rockefeller University and the University of Utrecht, under federal and state regulations.

Prodh-deficient mice. The Prodh-deficient mice have been described in detail elsewhere[11].

Electrophysiology. Hippocampal slices were prepared from Prodh-deficient and wild-type male mice, between 8 and 13 weeks of age. All experiments were performed in a blind manner as described previously[15] and as detailed in **Supplementary Methods**.

RNA isolation and probe preparation for the microarray hybridization. We dissected a total of 20 frontal cortices from male littermate mice 8 weeks of age: 10 were wild-type mice and 10 were homozygous *Prodh*-knockdown mutants; these were processed using standard protocols recommended by Affymetrix (http://www.affymetrix.com/products/arrays/specific/mgu74.affx). For hybridization, cRNA was fragmented and exposed to Affymetrix Mouse genome 430 2.0 array set chips (which probe expression of 39,000 full-length genes and expressed sequence tag (EST) clusters from the Unigene database maintained by the National Center for Biotechnology Information). After hybridization, microarrays were washed and scanned (Agilent).

Microarray data analysis. Initial microarray images were analyzed with Affymetrix Microarray Suite version 5 to extract intensity values for each probe. Expression values for each probe set were then calculated using the RMA[27] approach, run using default settings. Standard Student's *t*-tests were used to identify genes showing group-dependent expression changes. We used the method described in a previous study[44] to determine the false discovery rate at different *P*-value thresholds for gene selection.

Quantitative real-time RT-PCR. Real-time RT-PCR analysis was performed on an ABI Prism 7900 sequence-detection system (PE Applied Biosystems) as detailed in **Supplementary Methods**.

TUNEL. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling assay was performed 8 d after birth, in triplicate, as detailed in **Supplementary Methods**.

Gross brain morphology. Morphometric analysis was performed on four male mice 10 to 16 weeks of age as detailed in **Supplementary Methods**.

Protein extraction and western blots. We prepared protein extracts from frontal cortex and striatum of male mice 6 to 8 weeks of age of both genotypes (20 *Prodh*-mutant and 18 wild-type littermate control mice). In assays involving chronic MK801 treatment, mice received daily injections with vehicle or drug (MK801 at 0.25 mg per kg body weight) for 14 d and were killed 1 h after the last drug treatment. Extract preparation and western blot assays were performed as detailed in **Supplementary Methods**.

HPLC analysis of total content of neurotransmitters and their metabolites. Five pairs of mice were analyzed for tissue content of dopamine, DOPAC, HVA, norepinephrine and 5-HT, as detailed in **Supplementary Methods**.

***In vivo* microdialysis.** Forty male Prodh-deficient mice were tested together with wild-type littermate controls. Copper microdialysis probes (Microbiotech) were placed in both the dorsal striatum and prefrontal cortex according to the stereotaxic atlas of the mouse brain[45]. Microdialysis was performed as detailed in **Supplementary Methods**.

Behavioral testing procedures. Behavioral testing was performed as described previously[8,11] and as detailed in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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