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Nigrostriatal Dopaminergic Deficits and Hypokinesia Caused by Inactivation of the Familial Parkinsonism-Linked Gene *DJ-1*

Report

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Summary

The manifestations of Parkinson's disease are caused by reduced dopaminergic innervation of the striatum. Loss-of-function mutations in the *DJ-1* gene cause early-onset familial parkinsonism. To investigate a possible role for DJ-1 in the dopaminergic system, we generated a mouse model bearing a germline disruption of *DJ-1*. Although *DJ-1*$^{-/-}$ mice had normal numbers of dopaminergic neurons in the substantia nigra, evoked dopamine overflow in the striatum was markedly reduced, primarily as a result of increased reuptake. Nigral neurons lacking DJ-1 were less sensitive to the inhibitory effects of D2 autoreceptor stimulation. Corticostriatal long-term potentiation was normal in medium spiny neurons of *DJ-1*$^{-/-}$ mice, but long-term depression (LTD) was absent. The LTD deficit was reversed by treatment with D2 but not D1 receptor agonists. Furthermore, *DJ-1*$^{-/-}$ mice displayed hypoactivity in the open field. Collectively, our findings suggest an essential role for DJ-1 in dopaminergic physiology and D2 receptor-mediated functions.

Introduction

Parkinson's disease (PD) is an age-related movement disorder characterized clinically by bradykinesia, rigidity, resting tremor, and postural instability and neuropathologically by the selective loss of dopaminergic neurons and the presence of Lewy bodies (LB) in the substantia nigra (SN). Although most PD cases are sporadic, dominantly inherited mutations in *α-synuclein* and *LRRK2/dardarin* and recessively inherited mutations in *parkin*, *DJ-1*, and *PINK1* have been linked to familial forms of parkinsonism, which resemble idiopathic PD clinically. The parkinsonian features in idiopathic PD and familial parkinsonism are presumably caused by reduced dopaminergic input and loss of dopaminergic neurons in the pars compacta of the SN (SNpc). However, the pathogenic mechanisms underlying dopaminergic dysfunction and degeneration are unclear.

The first report linking *DJ-1* to autosomal-recessive parkinsonism on chromosome 1p36 (PARK7) identified a large deletion encompassing *DJ-1* exons 1 to 5 in a Dutch family and a substitution of a highly conserved leucine at position 166 with proline (L166P) in an Italian family (Bonifati et al., 2003). Subsequently, additional homozygous missense mutations and compound heterozygous frame-shift and splice site mutations have been identified in early-onset parkinsonian cases (Abou-Sleiman et al., 2003; Hague et al., 2003). The deletion mutation of exons 1 to 5 results in the absence of *DJ-1* mRNA and protein, while the L166P point mutation disrupts its normal dimer formation, leading to protein degradation (Miller et al., 2003; Olzmann et al., 2004; Wilson et al., 2003). Thus, mutations in *DJ-1* cause parkinsonism via a loss-of-function mechanism. Although DJ-1 is widely expressed in most tissues (Bonifati et al., 2003), it is unclear why the dopaminergic system is presumably most affected in patients bearing these loss-of-function mutations. The in vivo function of DJ-1, especially in the dopaminergic system, therefore, is highly relevant to the study of the pathogenic mechanism associated with *DJ-1* loss-of-function mutations.

To study the normal physiological role of DJ-1 in the dopaminergic system, we generated *DJ-1*-deficient mice. Real-time electrochemical measurements of acute striatal slices revealed significantly reduced evoked dopamine (DA) overflow in *DJ-1*$^{-/-}$ mice. The reduction in evoked DA overflow was primarily due to increased reuptake. Intracellular electrophysiological recordings showed that inhibitory responses of nigral neurons, such as hyperpolarization and blockade of action potentials, to DA or D2 receptor (D2R) agonists were decreased in *DJ-1*$^{-/-}$ mice, suggesting a partial impairment in D2R-mediated activities. While induction of corticostriatal long-term potentiation (LTP), which requires activation of D1 receptors, was normal in *DJ-1*$^{-/-}$ mice, induction of long-term depression (LTD), which requires activation of both D1 and D2 receptors, was impaired. The LTD deficit was restored by D2R agonists but not by D1R agonists. Consistent with these findings, behavioral analysis of *DJ-1*$^{-/-}$ mice demonstrated decreases in spontaneous activities in the open field.

Results

To generate mice that reproduce the genetic deletion of *DJ-1* in familial parkinsonism, we targeted exon 2,

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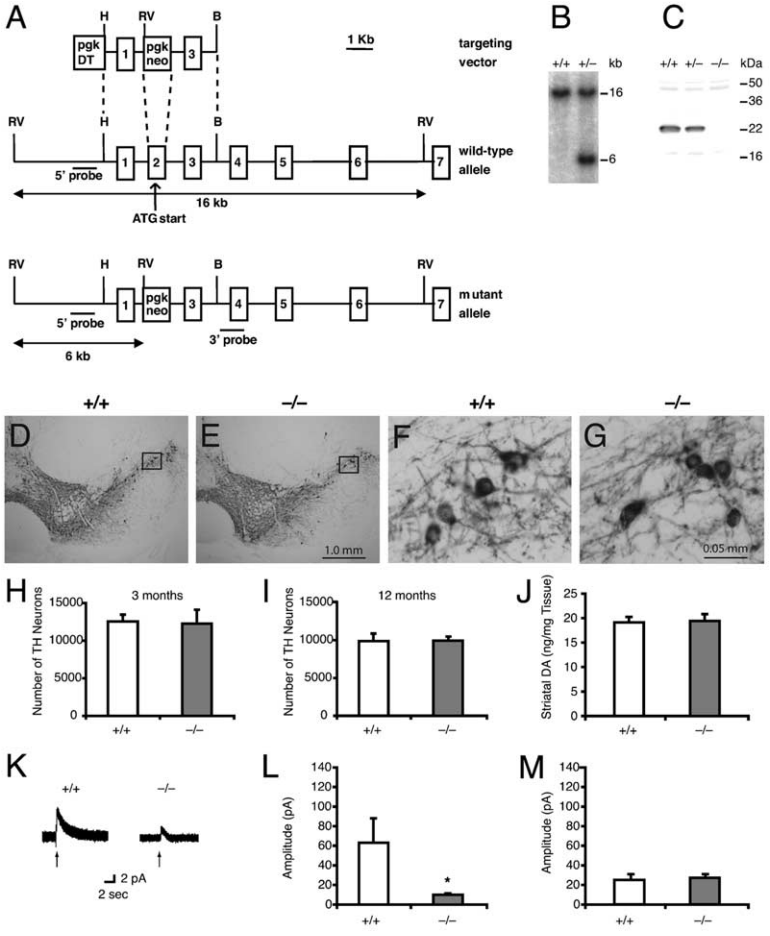

Figure 1. Reduced Evoked DA Overflow and Increased DA Reuptake in *DJ-1*$^{-/-}$ Mice

(A) Targeting strategy. (B) Southern analysis of F1 mice using the 5′ probe confirms the germline transmission of the mutant allele. The 16 kb and 6 kb EcoRV fragments correspond to the wild-type and targeted alleles, respectively. (C) Western analysis shows the reduction and the absence of DJ-1 protein in *DJ-1*$^{+/-}$ and *DJ-1*$^{-/-}$ mice, respectively. (D and E) Similar TH immunoreactivity in the SNpc of *DJ-1*$^{+/+}$ and *DJ-1*$^{-/-}$ mice. (F and G) Higher-power views of the boxed areas in (D) and (E). (H) Normal numbers of TH+ neurons in the SNpc of *DJ-1*$^{-/-}$ mice at 3 months of age (n = 6 per genotype; $p > 0.05$, t test). (I) Similar numbers of TH+ neurons in the SNpc of *DJ-1*$^{+/+}$ and *DJ-1*$^{-/-}$ mice at 12 months of age (n = 4 per genotype; $p > 0.05$, t test). (J) Normal striatal content of DA in *DJ-1*$^{-/-}$ mice (n = 4 per genotype; $p > 0.05$, t test). Data in (H)–(J) are presented as mean ± SEM. (K) Representative traces of the current caused by the electrochemical oxidation of extracellular dopamine by a carbon fiber electrode placed in the dorsal striatum of acute coronal slices from *DJ-1*$^{+/+}$ (left trace) and *DJ-1*$^{-/-}$ (right trace). Arrows indicate the time at which a single electrical pulse (0.5 mA for 2 ms) was delivered. (L) Reduced evoked DA overflow in *DJ-1*$^{-/-}$ mice. The mean amplitude of the current caused by evoked dopamine release is significantly smaller in *DJ-1*$^{-/-}$ ($10 \times 10^6 \pm 1 \times 10^6$) than wild-type mice ($63 \times 10^6 \pm 25 \times 10^6$; *$p < 0.05$, t test). Values are means ± SEM of 162 stimulations in 32 slices from 13 *DJ*$^{+/+}$ mice and 177 stimulations in 35 slices from 14 *DJ-1*$^{-/-}$ mice. (M) Increased dopamine reuptake in *DJ-1*$^{-/-}$ mice. Following a 30 min bath application of nomifensine (3 μM), the mean amplitude is similar in *DJ-1*$^{+/+}$ ($25 \times 10^6 \pm 6 \times 10^6$) and *DJ-1*$^{-/-}$ ($27 \times 10^6 \pm 4 \times 10^6$) mice ($p > 0.05$, t test). Values are mean ± SEM of 74 stimulations in 15 slices from 7 *DJ-1*$^{+/+}$ mice and 89 stimulations in 18 slices from 7 *DJ-1*$^{-/-}$ mice.

which contains the start codon (Figure 1A). Deletion of exon 2 would cause a reading frame-shift if aberrant initiation of translation were to occur at the only in-frame ATG in exon 1. Although downstream in-frame ATGs exist in exons 3, 5, 6, and 7, if aberrant initiation of translation were to occur at these sites, radically truncated proteins would be produced. Southern analysis of heterozygous F1 mice confirmed the germline transmission of the targeted allele (Figure 1B). Homozygous mutant mice were viable and grossly normal in appearance. Western analysis using a polyclonal DJ-1 antibody raised against a C-terminal peptide confirmed the absence and reduction of DJ-1 in homozygous and heterozygous mutant mice, respectively (Figure 1C). As this antibody recognizes the very C terminus of DJ-1 with high specificity and sensitivity, the absence of lower molecular weight immunoreactive bands indicated that no stable fragments of DJ-1 resulted from aberrant initiation of translation at downstream ATGs. We therefore concluded that deletion of exon 2 results in a null (–/–) allele, making *DJ-1*$^{-/-}$ mice excellent genetic models recapitulating the loss-of-function mutations in *DJ-1* linked to recessive parkinsonism.

Mating between *DJ-1*$^{-/-}$ mice yielded healthy litters of normal size, indicating normal reproductive functions. Histological analysis of various tissues of *DJ-1*$^{-/-}$ mice revealed no obvious abnormalities (data not shown). Immunohistochemical analysis of *DJ-1*$^{-/-}$ brains using antibodies specific for α-synuclein or ubiquitin, markers of Lewy bodies, showed no inclusions (data not shown). Immunohistochemical analysis using antibodies specific for tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis, showed no gross abnormalities in the morphology of dopaminergic cell bodies (Figures 1D–1G) or projections to the striatum (data not shown). We further counted the number of TH-immunoreactive neurons in the SNpc using stereological methods. No significant differences were observed in the number of nigral dopaminergic neurons in *DJ-1*$^{-/-}$ and wild-type littermates at the ages of 3 (n = 6 per genotype) and 12 (n = 4 per genotype) months (Figures 1H and 1I). Quantitative analysis following paraquat treatment (10 mg/kg, once per week for 3 weeks) of *DJ-1*$^{-/-}$ mice at the age of 3 months revealed similar numbers of dopaminergic neurons in the SNpc of *DJ-1*$^{-/-}$ mice and wild-type littermates (see Figure S1A in the Supplemental Data online), suggesting that dopaminergic neurons lacking DJ-1 are not more sensitive to paraquat at the dose tested.

We then used HPLC to quantify the total amount of

DA in dissected striata and found that levels of DA were unaltered in the striatum of *DJ-1*$^{-/-}$ mice at the age of 3 months (Figure 1J). Furthermore, the ratio of DA to its major metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was also unaltered, indicating normal turnover of DA in *DJ-1*$^{-/-}$ mice. To determine whether DA synthesis was affected in *DJ-1*$^{-/-}$ mice, we measured TH activity in vivo by HPLC quantification of the product of TH, L-DOPA, in the presence of NSD-1015, an inhibitor of DOPA decarboxylase capable of passing the blood-brain barrier. Similar levels of L-DOPA were found in *DJ-1*$^{-/-}$ (62.0 ± 4.9 ng/mg tissue) and wild-type mice (56.9 ± 2.5 ng/mg tissue; $p > 0.05$; n = 7 per genotype), suggesting normal TH activity in the absence of DJ-1. Consistent with this result, real-time RT-PCR analysis showed similar levels of *TH* mRNAs in *DJ-1*$^{-/-}$ and wild-type brains (Figure S1B).

To examine whether DJ-1 is required for normal dopaminergic neurotransmission, we measured evoked DA overflow. Acute striatal slices were prepared from *DJ-1*$^{-/-}$ and wild-type mice at 3 months of age. A carbon fiber recording electrode was placed in the dorsal striatum, which receives the majority of dopaminergic projections from the SNpc. A proximally placed bipolar electrode was used to deliver single pulses (0.5 mA, 2 ms) to stimulate release of synaptic vesicles. The current caused by the electrochemical oxidation of the released DA was measured with the recording electrode nearby. The current amplitude was markedly reduced in *DJ-1*$^{-/-}$ slices ($10 \times 10^6 \pm 1 \times 10^6$ pA; 177 stimulations in 35 slices from 14 mice) compared to wild-type slices ($63 \times 10^6 \pm 25 \times 10^6$ pA; 162 stimulations in 32 slices from 13 mice; $^*p < 0.05$, t test) (Figures 1K and 1L), indicating a marked decrease in evoked DA overflow in the absence of DJ-1. As evoked DA overflow is determined primarily by release and reuptake and DA reuptake is mediated through the dopamine transporter (DAT), we measured evoked DA overflow in the presence of the DA reuptake blocker nomifensine. Blockade of DA reuptake eliminated the reduction in evoked DA overflow in *DJ-1*$^{-/-}$ mice (Figure 1M), suggesting that increased DA reuptake primarily accounts for the reduced evoked DA overflow in *DJ-1*$^{-/-}$ mice. To determine whether increased DA reuptake could be simply explained by an upregulation of DAT expression, we performed real-time RT-PCR and radioligand binding assays and found that levels of *DAT* transcripts and proteins were similar in *DJ-1*$^{-/-}$ and control mice (n = 4 per genotype; Figure S1C).

The reduction in evoked DA overflow in the absence of DJ-1 prompted us to examine the responsiveness of nigral neurons to DA. Upon DA binding, somatodendritic D2 autoreceptors are activated and modulate conductance of potassium channels, resulting in hyperpolarization and blockade of action potentials. Intracellular recordings of nigral neurons from *DJ-1*$^{-/-}$ and wild-type mice at 1 month of age showed similar spontaneous, rhythmic firing activities (Figure 2A). In wild-type slices, brief bath application of DA (100 μM, 45 s) hyperpolarized the cell membrane and blocked firing activity (n = 6; Figure 2B). Upon washout, the resting membrane potential slowly recovered, and action potential discharge returned to control levels (Figure 2B). In *DJ-1*$^{-/-}$ slices, the response to DA (100 μM, 45 s) was much shorter (n = 7; Figure 2B). After DA washout, the membrane rapidly repolarized, and action potential firing resumed. Furthermore, DA reduced the frequency of action potential discharge less in *DJ-1*$^{-/-}$ than in wild-type mice (Figure 2B, insets). In control nigral neu-

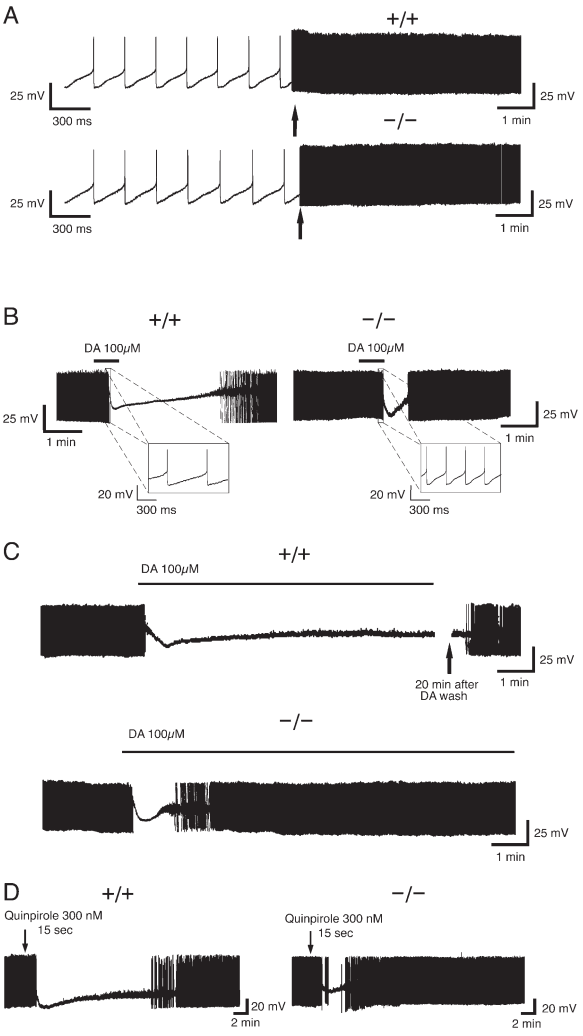

Figure 2. Reduced Responses to Dopamine or Quinpirole in *DJ-1*$^{-/-}$ Nigral Neurons

(A) Spontaneous, rhythmic action potentials recorded in dopaminergic neurons of the SNpc obtained from wild-type (+/+, upper trace) and *DJ-1*$^{-/-}$ mice (–/–, lower trace). Firing activity is shown at different chart speeds (arrow). (B) In slices from wild-type mice (left), brief DA application (100 μM, 45 s) hyperpolarizes the cell membrane and blocks the firing activity. Upon DA washout, the membrane slowly recovers, and action potential discharge returns to control levels. In dopaminergic neurons from *DJ-1*$^{-/-}$ mice (right), the response to DA (100 μM, 45 s) is significantly shorter. Insets show the marked difference in action potential frequency between the genotypes. (C) In wild-type mice, prolonged DA application (100 μM, 12 min) hyperpolarizes the cell and abolishes the firing activity. The membrane hyperpolarization persists throughout the DA application and returns to control levels after a long DA washout (20 min). The interruption as illustrated by the arrow indicates the DA washout. In nigral neurons from *DJ-1*$^{-/-}$ mice, an even longer perfusion with DA (100 μM, 14 min) caused a shorter response compared to wild-type littermates. (D) Quinpirole (300 nM, 15 s) causes a period of hyperpolarization and blocks the spontaneous firing activity of nigral neurons in wild-type mice (left trace), while the response of nigral neurons in *DJ-1*$^{-/-}$ mice is shorter (right trace).

rons, prolonged bath application of DA (100 μM, 12 min) hyperpolarized the cell and abolished the firing activity (n = 6; Figure 2C). The membrane hyperpolarization persisted throughout the DA application and returned to control levels after a long washout (20 min). In *DJ-1*$^{-/-}$ nigral neurons, an even longer perfusion with DA (14 min) caused a short-lasting response compared to controls (n = 5; Figure 2C). The membrane hyperpolarized but soon returned to resting levels and resumed firing activity. The inhibitory effect of DA on nigral neurons is mediated specifically by D2 autoreceptors, as it is completely blocked by D2R antagonists (Lacey et al., 1987) and is absent in *D2R*$^{-/-}$ mice (Mercuri et al., 1997). To confirm the impaired activation of D2 autoreceptors in the absence of DJ-1, we examined the effect of the D2R agonist quinpirole on firing activity of nigral neurons. As expected, brief application of quinpirole (300 nM, 15 s) caused a longer-lasting hyperpolarization of the cell membrane and blockade of the firing activity in wild-type mice (8.2 ± 3.1 min) than in *DJ-1*$^{-/-}$ mice (4.7 ± 1.4 min) (n = 4 per genotype; Figure 2D). Together, these data show that SNpc neurons lacking DJ-1 are less responsive to D2 autoreceptor stimulation, suggesting a partial impairment of D2 autoreceptor-mediated activities.

Because DA released from the nigrostriatal terminal modulates corticostriatal glutamatergic neurotransmission at medium spiny (MS) neurons in the striatum, we examined synaptic transmission and plasticity of MS neurons, which are the major target of dopaminergic projections from the SNpc. Intracellular recordings of MS neurons from corticostriatal slice preparations of *DJ-1*$^{-/-}$ and wild-type mice at the age of 1 month showed similar resting membrane potential and input resistance ($p > 0.05$). Striatal MS neurons were silent at rest and displayed membrane rectification and tonic firing activity during depolarizing current pulses (Figure 3A). Similarly, the current-voltage (I-V) relationship did not show significant differences between *DJ-1*$^{-/-}$ and wild-type mice (Figure 3B; n = 8; $p > 0.05$). The pharmacological profile of cortically-evoked excitatory postsynaptic potentials (EPSPs) was similar in wild-type (n = 5) and *DJ-1*$^{-/-}$ mice (n = 5). In the presence of physiological concentrations of external magnesium (1.2 mM), the AMPA glutamate receptor antagonist CNQX (10 μM) fully suppressed the EPSPs in both genotypes. Both genotypes showed a normal NMDA receptor-mediated component of the EPSP after removal of external magnesium, which relieves the voltage-dependent blockade of NMDA receptors (data not shown).

In wild-type mice, high-frequency stimulation of corticostriatal terminals, consisting of three trains of tetanic (100 Hz, 3 s) stimulation delivered at 20 s intervals, induced either LTP or LTD, depending upon the absence or the presence of 1.2 mM external magnesium, respectively (n = 6 neurons for LTP or LTD; Figure 3C). In *DJ-1*$^{-/-}$ mice, LTP was induced in MS neurons (n = 7), but LTD induction was absent in all cells examined (n = 8) (Figure 3C). Interestingly, when HFS was delivered in the presence of the D2R agonist quinpirole (3 μM), LTD induction was completely restored in *DJ-1*$^{-/-}$ neurons (n = 4) (Figure 3C). By contrast, LTD induction was not restored in any *DJ-1*$^{-/-}$ neurons (n = 6) when

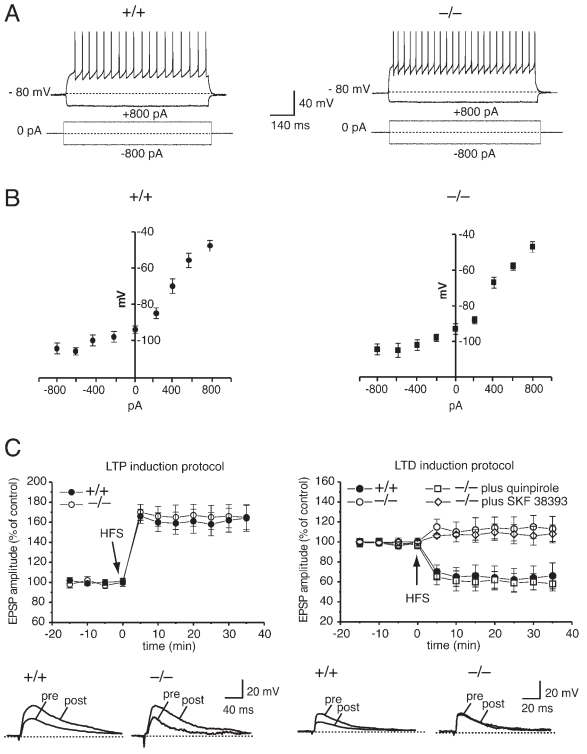

Figure 3. Reversal of Corticostriatal LTD Deficits by Quinpirole but Not SKF 38393

(A) Action potential discharge evoked by a depolarizing current step (800 pA, 700 ms) recorded in striatal medium spiny neurons from wild-type mice (left) is similar to the firing activity recorded from *DJ-1*$^{-/-}$ MS neurons (right). (B) Similar current-voltage (I-V) relationship of MS neurons in slices from wild-type (left) and *DJ-1*$^{-/-}$ mice (right) (n = 8; $p > 0.05$). Each point represents the mean ± SEM of more than four independent recordings. (C) In the absence of Mg^{2+} (LTP induction protocol), high-frequency stimulation of the corticostriatal pathway similarly induces LTP of cortically evoked excitatory postsynaptic potentials (EPSPs) in MS neurons of wild-type (n = 6 neurons; filled circles) and *DJ-1*$^{-/-}$ (n = 7; open circles) mice ($p > 0.05$). The superimposed traces below show EPSPs before (pre) and 30 min after (post) HFS in wild-type (left) and *DJ-1*$^{-/-}$ (right) mice. In the presence of Mg^{2+} (LTD induction protocol), HFS induces LTD in wild-type MS neurons (n = 6; filled circles), while LTD is absent in *DJ-1*$^{-/-}$ MS neurons (n = 8; open circles) ($p < 0.001$). LTD is restored in *DJ-1*$^{-/-}$ neurons when HFS is delivered in the presence of 3 μM quinpirole (open squares) (n = 4; $p < 0.003$ compared to *DJ-1*$^{-/-}$ neurons without quinpirole), but not in 10 μM SKF 38393 (open diamonds) (n = 6; $p > 0.05$ compared to *DJ-1*$^{-/-}$ neurons without quinpirole). The superimposed traces below show average EPSPs recorded before (pre)- and 30 min after (post)-HFS in wild-type (left) and *DJ-1*$^{-/-}$ mice (right). Data are presented as mean ± SEM.

HFS was delivered in the presence of 10 μM SKF 38393, a D1-like DA receptor agonist (Figure 3C). These results suggest that the LTD impairment is a consequence of reduced D2R-dependent activity, consistent with prior reports showing that corticostriatal LTD requires both D1 and D2 receptors, whereas corticostriatal LTP induction depends on the activation of D1 receptors but not D2 receptors (Calabresi et al., 1996).

Given the prominent abnormalities in dopaminergic neurotransmission caused by loss of DJ-1, we as-

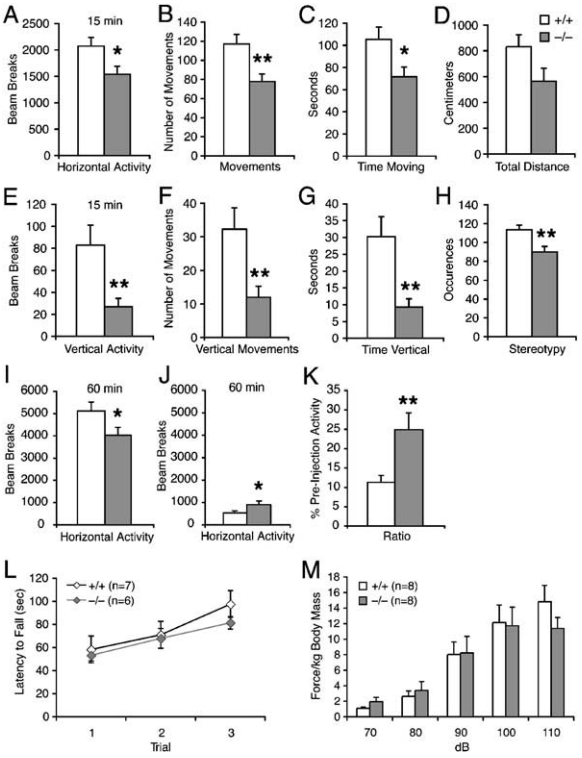

Figure 4. Reduced Spontaneous Activity of *DJ-1*$^{-/-}$ Mice

(A–H) Spontaneous activity of *DJ-1*$^{-/-}$ and wild-type mice in the open field (n = 18 per genotype). Two arrays measured horizontal movements (A–D), and one array measured vertical movements (rearing on hind legs) (E–G). Repeated sequential breakings of the same beam are scored as occurrences of stereotyped behaviors (scratching, grooming, etc.) (H). (I and J) Total open field activity during 60 min before (I) and 60 min after (J) injection of 1 mg/kg quinpirole (n = 18 per genotype). (K) The ratio of post-quinpirole activity to pre-quinpirole activity is significantly different between the genotypes ($p < 0.01$). (L) *DJ-1*$^{-/-}$ (n = 6) and wild-type (n = 7) mice show similar latencies to fall off an accelerating rotating rod ($p > 0.05$). The average time before falling off the rod is shown for each of three consecutive trials. (M) *DJ-1*$^{-/-}$ and wild-type mice exhibit similar acoustic startle responses at each dB tested (n = 8 per genotype, $p > 0.05$). Each mouse was tested ten times at each dB level, and the startle response was averaged and normalized to body mass. Body mass does not differ between the genotypes. Data in all panels are presented as mean ± SEM. Asterisk denotes statistical significance (*$p < 0.05$; **$p < 0.01$).

sessed the locomotor abilities of *DJ-1*$^{-/-}$ mice using a battery of well-established behavioral tests. Observation of spontaneous, voluntary movements during 15 min in the open field test revealed a marked reduction in the horizontal and vertical (rearing) activity of *DJ-1*$^{-/-}$ mice (n = 18) compared to wild-type littermates (n = 18) at age 3 months (Figures 4A–4H). *DJ-1*$^{-/-}$ mice also spent significantly less time moving and rearing and had fewer instances of stereotyped behavior. To examine the effect of D2 agonists, locomotor activity was monitored for 1 hr before (Figure 4I) and after (Figure 4J) administration of quinpirole (1 mg/kg), which has been established previously to decrease locomotor activity in the open field (Usiello et al., 2000; Wang et al., 2000). As expected, quinpirole suppressed locomotor activity in both genotypes (Figure 4J). Two-way ANOVA revealed that *DJ-1*$^{-/-}$ mice (n = 18) have a significantly diminished response to quinpirole compared to wild-type mice (n = 18; $p < 0.01$; Figure 4K). Involuntary movement was assessed using the rotarod and acoustic startle reflex paradigms. *DJ-1*$^{-/-}$ (n = 6) and wild-type littermate (n = 7) mice were similarly able to maintain their balance on the rotating rod before falling off during three independent trials (Figure 4L). Acoustic startle reflex, measured by the force with which the mouse jumped in response to a pulse of loud noise at varying intensities, was also similar in *DJ-1*$^{-/-}$ (n = 8) and wild-type (n = 8) mice (Figure 4M).

Discussion

Recessively inherited loss-of-function mutations in *parkin*, *DJ-1*, or *PINK1* cause early-onset parkinsonism. Thus, characterization of the in vivo functions of these gene products would provide insights into the pathogenic mechanism underlying familial and sporadic forms of parkinsonism (Shen and Cookson, 2004). Our previous studies of *parkin*$^{-/-}$ mice revealed that parkin is required for normal nigrostriatal and mitochondrial functions (Goldberg et al., 2003; Palacino et al., 2004). Here, in an effort to uncover the mechanism by which loss of *DJ-1* function leads to parkinsonism, we undertook a multidisciplinary analysis of the normal function of DJ-1 in the mouse brain. The results of this analysis reveal a link between DJ-1 and dopaminergic physiology in the nigrostriatal system. Furthermore, our findings identify an important role for DJ-1 in mediating the downstream effects of D2R activation in nigral neurons.

First, in the absence of DJ-1, evoked DA overflow is markedly reduced, suggesting altered dopaminergic neurotransmission. Dopamine overflow reflects the kinetic balance between DA release and reuptake, which are regulated by the D2 DA autoreceptors on presynaptic terminals. The elimination of the reduction in evoked DA overflow in the *DJ-1*$^{-/-}$ striatum by the reuptake blocker nomifensine suggests that enhanced DA reuptake primarily accounts for the reduced evoked DA overflow in the absence of DJ-1 (Figure 1). Interestingly, *D2R*$^{-/-}$ mice also exhibit reduced evoked DA overflow, as well as normalization of the DA overflow deficit by nomifensine (Schmitz et al., 2002). These observations suggest that DJ-1 is required for normal D2R-dependent regulation of DA reuptake in the presynaptic terminals of nigral neurons.

Second, the sensitivity of nigral neurons to DA is also reduced in *DJ-1*$^{-/-}$ mice (Figure 2). Somatodendritic D2-like autoreceptors regulate the membrane potential of nigral dopaminergic neurons through the modulation of the conductance of neuronal G protein-gated inward rectifying potassium channels (Schmitz et al., 2003). Upon administration of excess DA, D2 autoreceptors are activated, resulting in membrane hyperpolarization and suppression of firing activity (Lacey et al., 1987; Mercuri et al., 1990). This inhibitory effect of DA on SN neuron firing is absent in *D2R*$^{-/-}$ mice (Mercuri et al., 1997). In *DJ-1*$^{-/-}$ mice, this D2R-dependent hyperpolarization and suppression of action potentials is reduced

rather than abolished (Figure 2), suggesting partial blockade of D2 autoreceptor responses in the absence of DJ-1. The fact that the D2R agonist quinpirole is sufficient to reproduce the effects of DA on resting membrane potential and action potential firing in both control and *DJ-1*$^{-/-}$ nigral neurons further argues for the involvement of D2R-dependent mechanisms in this response. Thus, our data support an important role for DJ-1 in regulation of the excitability and firing of nigral dopaminergic neurons, which is an important determinant of the output of nigrostriatal projections.

Third, our electrophysiological analysis of the glutamatergic corticostriatal pathway showed normal induction of LTP but absence of LTD induction in *DJ-1*$^{-/-}$ mice (Figure 3). Dopamine released from nigrostriatal projections modulates corticostriatal glutamatergic neurotransmission at medium spiny neurons (Calabresi et al., 1996). LTP induction in striatal MS neurons is NMDA receptor dependent and requires D1 receptor activation, while LTD induction is NMDA receptor independent and requires activation of both D1R and D2R (Calabresi et al., 1996). Thus, this selective deficit in corticostriatal LTD also suggests an impairment of D2R-dependent responses in the absence of DJ-1, while the preservation of corticostriatal LTP indicates that D1R-mediated responses remain intact. Consistent with a partial blockade of D2R-dependent responses, the LTD deficit in the *DJ-1*$^{-/-}$ striatum is rescued by the D2R agonist quinpirole, but not by the D1R agonist SKF 38393 (Figure 3). It is as yet unclear whether the locus of this D2R-dependent effect on LTD in MS neurons is presynaptic or postsynaptic, since D2Rs have been localized to both postsynaptic sites on MS neurons and presynaptic nigrostriatal terminals.

Fourth, our behavioral analysis revealed that *DJ-1*$^{-/-}$ mice exhibit pronounced defects in locomotor activity (Figure 4), as shown by their generalized hypokinesia in the open field paradigm, which has been shown previously to be sensitive to alterations in dopaminergic neurotransmission (Baik et al., 1995). This reduction in spontaneous locomotor activity of *DJ-1*$^{-/-}$ mice may be due to reduced synaptic levels of DA, consistent with the observed reduction in evoked DA overflow. Dopamine-deficient mice and transgenic mice overexpressing DAT in dopaminergic neurons also exhibit hypoactivity in the open field in the absence of nigral cell loss (Donovan et al., 1999; Zhou and Palmiter, 1995). Moreover, reduced D2R-mediated signaling may contribute to the hypokinesia of *DJ-1*$^{-/-}$ mice, as reduced or absent D2R expression in *D2R*$^{+/-}$ or *D2R*$^{-/-}$ mice causes increasingly more severe reductions in spontaneous activity (Baik et al., 1995; Fowler et al., 2002; Jung et al., 1999; Kelly et al., 1998). Prior work has shown that quinpirole suppresses locomotor activity in the open field (Usiello et al., 2000; Wang et al., 2000). This effect is thought to be mediated by stimulation of D2 autoreceptors on nigral neurons and may therefore represent a behavioral manifestation of D2 autoreceptor-mediated inhibition of nigral neuron firing. Suppression of locomotor activity by quinpirole is reduced but not absent in *DJ-1*$^{-/-}$ mice, again suggesting a partial but not complete loss of D2R-dependent responses in the absence of DJ-1.

Although the pharmacological, behavioral, and electrophysiological phenotypes of *DJ-1*$^{-/-}$ mice strongly resemble those reported for *D2R*$^{-/-}$ mice, *DJ-1*$^{-/-}$ mice display normal *D2R* mRNA abundance and radioligand binding (Figure S1D). Furthermore, in contrast to *D2R*$^{-/-}$ mice, *DJ-1*$^{-/-}$ mice retain partial responsiveness to DA and the D2R agonist quinpirole, though this responsiveness is diminished relative to control mice. Collectively, these results point to a defect in D2R-mediated responses downstream of receptor activation in the absence of DJ-1, suggesting an important role for DJ-1 in regulation of D2R-dependent neuronal signaling mechanisms. The precise function of DJ-1 in neuronal responses downstream of D2R activation remains to be defined.

What is the relevance of the phenotypes observed in *DJ-1*$^{-/-}$ mice to parkinsonism? The clinical signs of primary parkinsonism reflect reduced dopaminergic innervation of the striatum as a consequence of nigral degeneration. Our study provides evidence that DJ-1 plays an essential role in normal dopaminergic physiology and D2 receptor-mediated functions. We did not observe a reduction in the number of SNpc dopamine neurons in *DJ-1*$^{-/-}$ mice at 3 and 12 months of age, but several parameters of nigral dopaminergic physiology are abnormal. It remains to be determined whether aged *DJ-1*$^{-/-}$ mice would exhibit substantial nigral degeneration. Taken together, these findings indicate that the nigrostriatal pathway is grossly intact but dysfunctional in *DJ-1*$^{-/-}$ mice. The spontaneous hypoactivity of *DJ-1*$^{-/-}$ mice resembles the hypokinesia of clinical parkinsonism and PD, and the partial defect in D2R-dependent responses in *DJ-1*$^{-/-}$ mice is consistent with the clinical efficacy of D2R agonists in parkinsonism and PD (Jenner, 2003). The association of loss-of-function mutations in DJ-1 with parkinsonism suggests that DJ-1 is required for the survival of nigral neurons. Our identification of a requirement for DJ-1 in D2R-mediated responses in nigral neurons therefore suggests that D2R-dependent mechanisms may play an important role in nigral neuron survival. Consistent with this possibility, two recent clinical trials in PD patients have provided evidence for neuroprotective effects associated with use of the D2R agonists pramipexole and ropinirole, which appear to retard loss of functional nigral projections to the striatum (Kitamura et al., 2003; Parkinson Study Group, 2002; Whone et al., 2002). Thus, the defects in D2R-mediated responses observed in *DJ-1*$^{-/-}$ mice may represent a mechanistic precursor to the overt nigral degeneration presumably present in affected individuals from *DJ-1* pedigrees. Moreover, the D2R-dependent pathway defined by DJ-1 may contribute to the pathogenesis of other forms of parkinsonism, such as sporadic PD, and may provide targets for novel therapeutic approaches.

Experimental Procedures

Generation of *DJ-1*$^{-/-}$ Mice

To target *DJ-1* exon 2, C57BL/6J genomic DNA BAC clone RP23-272N19 was obtained from Invitrogen. An 8.4 kb HindIII fragment encompassing *DJ-1* exons 1–5 was subcloned into pBluescript II. The 1.8 kb HindIII-BamHI fragment and the 2.1 kb NheI-BglII fragment surrounding exon 2 were used as the 5′ and 3′ homologous sequences in the targeting construct. Linearized targeting vector

was transfected by electroporation into MKV6.5 ES cells derived from B6/129 F1 mice (gift of R. Jaenisch). Desired homologous recombination events were confirmed by Southern analysis with the *neo* and the 5′ and 3′ probes. Two correctly targeted ES cells were injected into blastocysts. The resulting chimeric mice were mated with B6/129 F1 mice.

DA Neuron Count, Striatal Dopamine Measurement, Radioligand Binding Assay, and Real-Time RT-PCR

DA neuron count and striatal DA measurements were done exactly as previously described (Goldberg et al., 2003). DAT and D2R radioligand binding assays were performed using ^{3}H-WIN35428 and ^{3}H-N-methyl-spiperone, respectively, essentially as previously described (Goldberg et al., 2003). Real-time RT-PCR was carried out the same as previously described (Saura et al., 2004).

Carbon Fiber Electrode Amperometry

Dissected brains were placed on a Leica VT1000S vibratome in ice-cold oxygenated ACSF (in mM): 124 NaCl, 2.0 KCl, 1.25 KH_2PO_4, 2.0 $MgSO_4$, 25 $NaHCO_3$, 1.0 $CaCl_2$, and 11 glucose (pH 7.3). Coronal slices were cut 300 μm thick. After 1 hr recovery in ACSF, each slice was transferred into the recording chamber with perfusion of oxygenated ACSF set to 1 ml/min at 37°C. Disk carbon fiber electrodes of 5 μm in diameter with a freshly cut surface were placed in the dorsal striatum ~50 μm into the slice. A reference electrode (Ag/AgCl wire) was inserted into the ACSF bath. Voltage was set at +700 mV (Axopatch 200B, Axon Instr.). A bipolar electrode (diameter 0.005 inch: MS 303/3, Plastic1, Inc.) was placed 100–200 μm from the carbon fiber electrode to stimulate release of synaptic vesicles with a single rectangular 500 μA pulse of 2 ms duration (delivered by Isoflex stimulus isolator, AMPI Inc., triggered by Grass S88 Stimulator). Up to five single pulses were delivered per site with an interpulse interval of 5 min to allow for full recovery. Recordings were acquired at 50 kHz and digitally postfiltered at 1 kHz. The response of the amperometric electrode (increase from baseline) was monitored and quantified by Mini Analysis software (Synaptosoft, Inc.). The amplitude of each amperometric spike before and after a 30 min bath application of nomifensine (3 μM) was quantified in order to assess differences in dopamine reuptake. Electrodes were calibrated before and after use using background-subtracted voltammograms (five waves applied and averaged, 300 V/s, –400 to +1000 mV, in recording medium and medium with 10 μM dopamine).

Electrophysiology

Corticostriatal coronal and nigral horizontal slices (200–300 μm) were prepared and maintained as described previously (Mercuri et al., 1997). Brains were removed and slices were cut with a vibratome in Krebs solution (in mM: 26 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$). Each slice was transferred to a recording chamber mounted on the stage of an upright microscope, submerged in continuously flowing Krebs solution (2–3 ml/min). Sharp electrodes were filled with 2 M KCl (30–60 MΩ for striatal and 40–80 MΩ for nigral neurons). An Axoclamp 2B amplifier (Axon Instruments) was used, and traces were stored both on a high-gain chart recorder (Gould RS 3400) and on pClamp9 (Axon Instruments) running on a PC for offline analysis. Cell input resistance was measured by applying small electrotonic hyperpolarizing current steps. I-V curves were obtained by applying depolarizing and hyperpolarizing current steps (200 pA, 900 ms) of progressively higher amplitude and by measuring the resulting voltage at its steady state.

To evoke corticostriatal EPSPs, bipolar electrodes were located in the cortex in close proximity of the recording electrode. Test EPSPs were recorded at 0.1 Hz interval. Before tetanic stimulation (three trains: 3 s duration, 100 Hz frequency, at 20 s intervals), the intensity of synaptic stimulation was raised to suprathreshold levels. Quantitative data on EPSP changes were expressed as percentage of controls, representing the mean of at least four independent responses obtained during a stable period before the tetanus (10–15 min). The dopaminergic neurons of the SNpc were identified according to electrophysiological and pharmacological criteria (Lacey et al., 1987). Drugs were applied by dissolving them to the desired final concentration in the saline and by switching the perfusion from control saline to drug-containing saline. Values given in the figures and text are mean ± SEM of changes in the respective cell population. Student's t test was used to assess statistical significance.

Behavioral Tests

For the open field test, individual mice were placed in 42 × 42 cm acrylic animal cages for 15 min during which their horizontal and vertical movements were monitored by three arrays of 16 infrared light beam sensors (AccuScan Instruments) connected to a computer that recorded their position every millisecond. AccuScan VersaMax software was then used to calculate, in both the horizontal plane and along the vertical axis, the total number of movements, the distance traveled, the time spent moving, and the total number of infrared beam breaks for each mouse. To test the effect of quinpirole on open field activity, mice were tested for 1 hr before and after i.p. injection of 1 mg/kg quinpirole. The measurements of post-quinpirole activity began ~30 s after i.p. injections. The rotarod test was performed the same as previously described (Goldberg et al., 2003).

For the startle test, mice were weighed and placed into calibrated startle chambers (Med Associates) and allowed 5 min to acclimate with continuous 60 dB background white noise. Subsequently, every 30 s, mice were exposed to a 50 ms pulse of white noise of 70, 80, 90, 100, or 110 dB intensity and their responses measured by load cells. After two "dummy" pulses at 100 dB, each mouse was exposed to ten pulses at each dB intensity in pseudorandom order with a 30 s interval between pulses, and the average response at each dB was calculated. Startle response per kg body mass was analyzed by repeated-measures ANOVA.

Supplemental Data

Supplemental Data including supplemental figures can be found online at http://www.neuron.org/cgi/content/full/45/4/489/DC1/.

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