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Regulation of dopamine presynaptic markers and receptors in the striatum of *DJ-1* and *Pink1* knockout rats

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Abstract

Pathogenic autosomal recessive mutations in the *DJ-1* (*Park7*) or the PTEN-induced putative kinase 1 (*Pink1* or *PARK6*) genes are associated with familial Parkinson's disease (PD). It is not well known regarding the pathological mechanisms involving the *DJ-1* and *Pink1* mutations. Here we characterized *DJ-1* and *Pink1* knockout rats both through expression profiling and using quantitative autoradiography to measure the densities of the dopamine D_1, D_2, D_3 receptors, vesicular monoamine transporter type-2 (VMAT2) and dopamine transporter (DAT) in the striatum of transgenic rats and wild type controls. Expression profiling with a commercially available array of 84 genes known to be involved in PD indicated that only the target gene was significantly downregulated in each transgenic rat model. D_1 receptor, VMAT2, and DAT were measured using [^{3}H]SCH23390, [^{3}H]dihydrotetrabenazine, and [^{3}H]WIN35428, respectively. No significant changes were observed in the density of DAT in either model. Although the densities of VMAT2 and D_1 receptor were unchanged in *Pink1* knockout, but both were increased in *DJ-1* knockout rats. The densities of D_2 and D_3 receptors, determined by mathematical analysis of binding of radioligands [^{3}H]WC-10 and [^{3}H]raclopride, were significantly increased in both knockout models. These distinctive changes in the expression of dopamine presynaptic markers and receptors in the striatum may reflect different compensatory regulation of dopamine system in *DJ-1* versus *Pink1* knockout rat models of familial PD.

Keywords

DJ-1; Pink1; Parkinson's disease; dopamine receptors; autoradiography

1. Introduction

Parkinson's disease (PD) is the most common movement disorder, and is characterized by bradykinesia, rigidity, resting tremor and postural instability clinically. Significant loss of

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dopaminergic neurons in the pars compacta of the substantia nigra (SNpc) and formation of intraneuronal Lewy bodies (LBs) are the main neuropathological features in PD. Over 85% of PD cases are sporadic. Monogenic forms of this disease, resulting from pathogenic mutations in genes including *DJ-1* (*Park7*) and PTEN-induced putative kinase 1 (*Pink1* or *PARK6*), both of which are autosomal recessive, are associated with familial patients (less than 15% of all cases). Large exonic deletions or frame-shift truncations in *DJ-1* and *Pink1* are among the most frequent mutations found in these PD patients, suggesting a "loss of function" mechanism [1–3]. The mechanistic involvement of *DJ-1* and *Pink1* mutations in Parkinsonian etiology has not yet been elucidated.

DJ-1 and *Pink1* knockout mice have been previously used to study the monogenic form of PD. These animals manifest no significant dopaminergic neuronal loss [4–8]. In the *DJ-1* knockout mouse, although the evoked dopamine overflow is reduced [6], and dopamine release and reuptake are increased [5], the densities of dopamine transporter (DAT) [5, 6], vesicular monoamine transporter type-2 (VMAT2) [5] and dopamine D_2 like receptor [6] remain unchanged. One study in the *DJ-1* knockout mouse observed an increased DAT level in synaptosomes but not in cytoplasm [8]. In the *Pink1* knockout mouse, dopamine release was decreased, and the density of striatal dopamine D_1 and D_2 like receptors was unchanged [7]. The lack of dopaminergic neuronal loss in these mouse models makes them less than ideal as PD models. Severe dopamine depletion in the brain, measured using positron emission tomography (PET) or single photon emission computed tomography (SPECT) is found in *DJ-1* [9, 10] and *Pink1* patients [11, 12]. One postmortem neuropathological study showed neuronal loss in the SNpc in familial PD patients with *Pink1* mutations [11]. A *DJ-1*–nullizygous mouse was recently created which demonstrated progressive dopaminergic cell loss [13]. However, neither loss of striatal dopamine termini nor increase of striatal postsynaptic marker ΔFBJ was observed in this new model, indicating that both pre- and postsynaptic dopamine components are involved in compensatory regulation [13]. Unfortunately, the density of dopamine presynaptic markers and receptors was not determined in this study.

Because the rat has obvious advantages over the mouse for CNS studies, the Michael J Fox foundation provided funding to SAGE Labs to generate *DJ-1* and *Pink1* knockout rats as improved models of familial PD (See SAGE Labs website, http://www.sageresearchmodels.com/ and MJFF website, https://www.michaeljfox.org/). *DJ-1* and *Pink1* knockout rats demonstrate significant motor deficit and age-dependent neuronal loss in SNpc, consistent with human disease. In this study of post-mortem rat brains, we investigated the expression of an array of genes associated with PD and determined the absolute densities of dopamine D_2 and D_3 receptors using the D_3 receptor-specific ligand [^{3}H]**WC-10** and the D_2/D_3 ligand [^{3}H]raclopride [14]. We also measured the densities of dopamine D_1 receptor, DAT and VMAT2 in the striatum of *DJ-1* and *Pink1* knockout rats by quantitative autoradiography. We observed a distinct regulation of dopamine presynaptic markers and receptors in *DJ-1*and *PINK1* knockout rat model of PD.

2. Methods

DJ-1 and *Pink1* knockout rats were created using zinc finger nuclease (ZFN) technology [15] and maintained at SAGE Labs, monitored by SAGE's Institutional Animal Care and Use Committee (IACUC). The SAGE Labs IACUC closely oversees research and ensures that it is conducted in accordance with all provisions of the PHS Policy on Humane Care and Use of Laboratory Animals. Both transgenic rat lines are registered at the Rat Genome Database (RGD) under the following names: LE-Park7$^{em1Sage-/-}$ and LE-*Pink1*$^{em1Sage-/-}$.

2.1 RNA isolation

Whole rat brains were collected from knockout animals and wild type littermates at 10 or 15 weeks of age for each experimental group, snap-frozen and stored at −80°C. Frozen tissues were homogenized by mortar and pestle in liquid nitrogen, and immediately used for RNA isolation. Total RNA isolation was performed by using the TRizol reagent (a monophasic solution of phenol and guanidine isothiocyanate) following the manufacturer's instructions (Life Technologies, Carlsbad, CA). Isolated RNA was treated with RNAse-free DNase-I and purified on RNeasy columns (Qiagen, Germantown, MD).

2.2 PCR arrays

cDNA was prepared from 1 μg of total RNA using the RT^2 First Strand cDNA synthesis kit (Qiagen) and used as templates for amplification with the Rat Parkinson's Disease RT^2 Profiler PCR Array (Qiagen), following the manufacturer's protocols. In brief, 25 μl of the SYBR Green PCR cocktail was dispensed into each well of the 96-well PCR array using a multichannel pipettor. The SYBR Green PCR cocktail consisted of the 20 μl cDNA reaction, 1350 μl of 2× RT^2 qPCR Master Mix and 1330 μl molecular grade water for a final volume of 2.7 ml. The recipe provides excess volume to allow multiple pipetting. Real-time PCR detection was performed with a CFX96 instrument (Bio-Rad, Hercules, CA) under the following PCR conditions: 95°C, 10 min; 40 cycles of 95°C, 15 sec, 60°C, 1 min.

2.3 Brain samples and tissue processing

Whole brains were carefully harvested from three *DJ-1* knockout male rats at 8 months of age and three *PINK1* knockout male rats at 6 months of age as well as three age-matched wild-type control male rats for each group and immediately snap-frozen in a dry ice/ethanol bath to preserve shape and then stored at −80°C until further processing.

Brains were sectioned at 20 μm on a cryostat and thaw-mounted onto Fisher Superfrost Plus slides, with 6 sets taken from the rostral through caudal striatum. Slides were stored at –80°C until used for receptor autoradiography.

2.4 Radioligands and drug

[^{3}H]**WC-10** was custom synthesized by American Radiolabeled Chemicals (St Louis, Missouri, USA) by alkylation of the desmethyl precursor with [^{3}H]methyl iodide. The specific activity of the radioligand was 80 Ci/mmol. The synthesis of [^{3}H]**WC-10** has been previously described [16]. Chemical reagents and the standard compounds used in this study were purchased from Sigma (St. Louis, MO) and Tocris (Ellisville, MO). [^{3}H]Raclopride

(76 Ci/mmol), [^{3}H]SCH23390 (85 Ci/mmol) and [^{3}H]WIN35428 (76 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). [^{3}H]Dihydrotetrabenazine ([^{3}H]DTBZ) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA).

2.5 Quantitative autoradiography protocol

The quantitative autoradiography of the dopamine D_1, D_2, D_3 receptors, vesicular monoamine transporter type-2 (VMAT2) and dopamine transporter (DAT) in the striatum of the transgenic rat brains was performed using Beta Imager 2000Z Digital Beta Imaging System (Biospace, France),

A total of 6 brain sections in the adjacent slide were chosen for each animal. Using known neuroanatomical markers, bilateral regions of interest were drawn freehand along the border of the entire striatum of serial brain sections from each individual rat brain to define the representative binding density for the striatum (Fig. 1, 2g). Quantitative analysis was performed with the program Beta-Vision Plus (BioSpace, France), and the absolute densities of dopamine D_2 and D_3 receptors using the D_3-preferring radioligand [3H]**WC-10** and the D_2/D_3 ligand, [^{3}H]raclopride were calculated as previously described [14, 17].

2.8 Statistical analysis

The apparent binding density for each receptor-bound radioligand was calculated using the specific activity of the radioligand expressed as fmol/mg tissue, as previously described [14]. The experimenter was blinded to all conditions during the analysis. Comparison of receptor densities between knockout rats and age-matched controls was analyzed by Student's t-test.

3. Results

3.1 Confirmation of DJ-1 and Pink1 disruption

To confirm gene-specific knockout of *DJ-1* and *Pink1* in the transgenic rat models, total RNA from whole brain homogenates was analyzed for expression of PD-related genes using quantitative RT-PCR (see Methods). Compared to wild-type control rats, *DJ-1* expression was 6- to 11-fold lower in *DJ-1* knockout rats, and *Pink1* expression was reduced 40-fold (Table1). Only a few minor changes in mRNA levels of other genes analyzed were noted, and the observed differences were seen mostly in the older animals, such as upregulation of *Fjx1*, *NSF* and *synaptogyrin 3* in 15-week old *DJ-1* knockout rats. However, the magnitude of these changes was small (2.3-, 2-, and 2.3-fold, respectively). DAT and D_2 receptor expression did not change significantly based on mRNA levels. VMAT2 was slightly downregulated only in the 15-week *DJ-1* knockout rat brains. Interestingly, the serotonin 2A receptor (*Htr2a*) was slightly upregulated in both knockout rat models at both time points tested.

3.2 Quantitative analysis of dopamine D_1, D_2, D_3 receptors, DAT and VMAT2 densities in the striatum of DJ-1 knockout rats

To determine the density of dopamine receptors and DAT and VMAT2, target-specific radioligand binding was measured using quantitative radiography. *DJ-1* knockout rats were analyzed at eight months of age. Compared to wild type controls, the *DJ-1* knockout rats exhibited no significant change in DAT density in the striatum (Fig. 1a) and a 14% increase in VMAT2 density; this was statistically significant ($p = 0.05$, t-test, Fig. 1b). Additionally, the densities of all three dopamine receptors were significantly increased in *DJ-1* knockout rats in comparison with wild type controls: D_1 receptor, 6% increase, $p = 0.034$, t-test (Fig. 1c), D_2 receptor, 30% increase, $p = 0.012$, t-test (Fig. 1d), and D_3 receptor, 26% increase, $p = 0.047$, t-test (Fig. 1e).

3.3 Quantitative analysis of dopamine D_1, D_2, D_3 receptors, DAT and VMAT2 densities in the striatum of PINK1 knockout rats

In six months-old *Pink1* knockout rats, the densities of DAT, VMAT2 and D_1 receptor remained unchanged from those of wild type rats (Fig. 2a, b, c, respectively). However, the D_2 receptor demonstrated a 26% increase in density in the *Pink1* knockout rats over the wild type controls ($P=0.029$, t-test, Fig. 2d), while the D_3 receptor showed a 19% increase over wild type controls ($p=0.026$, t-test, Fig. 2e).

4. Discussion

Gene expression profiling using a commercial array of 84 genes involved in PD demonstrated that disruption of *DJ-1* and *Pink1* genes in the transgenic rat models led to significant downregulation of the specific target gene expression. A few other genes involved in PD were slightly up- or downregulated at the mRNA level in the brains of *DJ-1* and *Pink1* knockout rats, but no global disturbance in PD-related gene expression patterns resulted from ZFN-targeted knockout of either the *DJ-1* or *Pink1* gene.

Most of the non-target mRNA expression changes found was observed in older *DJ-1* knockout animals (15 weeks). The roles that these genes play in PD pathology are not yet clear. Four-jointed (Fj) protein regulates the development of the wing discs in drosophila, but its function in mammals is not known. It has been shown that loss of Fjx1, a mammalian ortholog of Fj, will increase number and branching of the neuronal dendrites [18]. N-Ethylmaleimide-sensitive factor (NSF) is involved in regulation of synaptic transmission [19]. A recent genetic study identified NSF as one of the candidate genes for study PD [20]. Synaptogyrin 3 is a synaptic vesicle protein, shown to interact with DAT and VMAT2, both functionally and physically [21]. Upregulation of 5HT2A receptor mRNA in both models is in agreement with the previous observations that loss of nigrostriatal cells causes an up-regulation of striatal 5-HT_{2A}R mRNA [22]. All the receptors and transporters analyzed in this study, except for D_1 receptor, were included in the PCR array. Yet, only VMAT2 showed slight decrease in the 15 week-old *DJ-1* rats. All the other genes showed no change in expression on the mRNA level.

The density of dopamine presynaptic markers DAT and VMAT2 were measured using quantitative autoradiography in the striatum of *Pink1* (at 6 months of age) and *DJ-1* (at 8

months of age) knockout rats; significant neuronal loss was found in the SNpc. Surprisingly DAT density remained unchanged in both rat models, while VMAT 2 density was found to be slightly but significantly increased in *DJ-1* knockout rats. Previously, DAT density in synaptosomes of *DJ-1* knockout mice, which lack dopaminergic neuronal loss, has been reported to be either unchanged [5, 6] or increased [8]. A new *DJ-1*–nullizygous mouse model was recently created which shows significant dopaminergic cell loss as early as 2 months of age but without clear loss of striatal dopamine termini by TH staining; this has been attributed to sprouting of neurites within the nigrostriatal pathway [13]. This report is in line with our findings showing no significant reduction of dopamine pre-synaptic markers although there was a significant neuronal loss in SNpc in *DJ-1* knockout rats. These observations seem to point to a compensatory mechanism in the presynaptic dopaminergic system of *DJ-1*-disrupted rodent brains. Consistent with these findings, SAGE has reported an increase in extracellular dopamine content without a corresponding change in its metabolites (DOPAC, HVA) (See SAGE Labs website, http://www.sageresearchmodels.com/ and MJFF website, https://www.michaeljfox.org/), indicating enhancement of dopamine release and reuptake in *DJ-1* knock out rat, which can be mediated by DAT and VMAT2. Similarly, increased dopamine release and reuptake was also observed in *DJ-1* knockout mice [5]. The changes in DAT and VMAT2 density in the *Pink1* knockout rats resemble those of the *DJ-1* knockout rats, implying that *DJ-1* and *Pink1* may function similarly in autosomal recessive PD. It is noteworthy that DAT binding has been reported to be decreased in familial PD patients with *DJ-1* or *Pink1* mutations [9, 11, 12]; this is in contrast to our current findings in the rat models. However, the changes in DAT and VMAT2 density in *DJ-1* and *Pink1* knockout rats may reflect a regulation of dopamine presynaptic markers in the preclinical or early stage of familial PD.

The density of dopamine D_2 receptor was increased in the striatal regions of both *DJ-1* and *Pink1* knockout rats, which is in agreement with previous findings in neurotoxin induced PD models [23–27] and PD patients [28, 29]. In *DJ-1*–nullizygous mice recently created by Rousseaux et al [13], an increase in murine osteosarcoma viral oncogene homolog B (ΔFosB), a striatal postsynaptic marker was observed; this has also been reported in PD patients and neurotoxin-induced animal models of PD, indicating that there is a similar compensatory response in *DJ-1* knockout mice. Our data suggests that the upregulation of striatal D_2 receptors possibly be one of the postsynaptic compensatory responses upon neuronal loss in SNpc in *DJ-1* and *Pink1* knockout rats.

The increased dopamine D_3 receptor density found in this study is in contrast to findings in 6-OHDA rats [30, 31] and MPTP treated monkey models of PD [32] as well as PD patients [33]. However increased striatal D_3 receptor mRNA has been reported in monkey [34] and cat [35] MPTP-induced PD models, reflecting post-synaptic regulation. The D_3 receptor was found to be increased in PD patients who responded well to L-dopa treatment [36]. It has been reported that BDNF from dopamine neurons is responsible for inducing normal expression of the dopamine D_3 receptor both during development and in adulthood [37], however its regulation in PD may also depend on the time course of disease and the severity of neuronal loss in SNpc; dopaminergic therapy may also affect D_3 receptor expression. Other factors including species differences, sample processing, selectivity of radioligands

and method can also influence the published D_3 receptor binding data. Nevertheless, the increase in D_3 receptor density in the *DJ-1* and *Pink1* knockout rats in this study may reflect a compensatory upregulation upon neuronal loss in SNpc. Familial PD patients with *DJ-1* or *Pink1* mutations are characterized by early onset but good response to L-dopa treatment [9, 11]. Elevation of D_3 receptors was positively correlated with L-dopa response in PD patients [36], although the relationship between this clinical observation and the regulation of D_2 and D_3 receptors needs to be further investigated.

In conclusion, this systematic measurement of dopamine presynaptic markers and receptors revealed alterations in dopaminergic regulation in *DJ-1* and *Pink1* knockout rats. To our knowledge, these are the first transgenic rat models of PD with dopaminergic neuron loss. Unchanged or elevated DAT and VMAT density may reflect compensatory regulation of dopamine presynaptic markers which can facilitate the turnover of extracellular dopamine; while the upregulation of dopamine D_2 and D_3 receptors suggests post-synaptic compensation for dopaminergic neuron loss caused by disruption of *DJ-1* and *Pink1* genes. These transgenic rat models of autosomal recessive PD can be potentially used for studying PD etiology as well as drug development.

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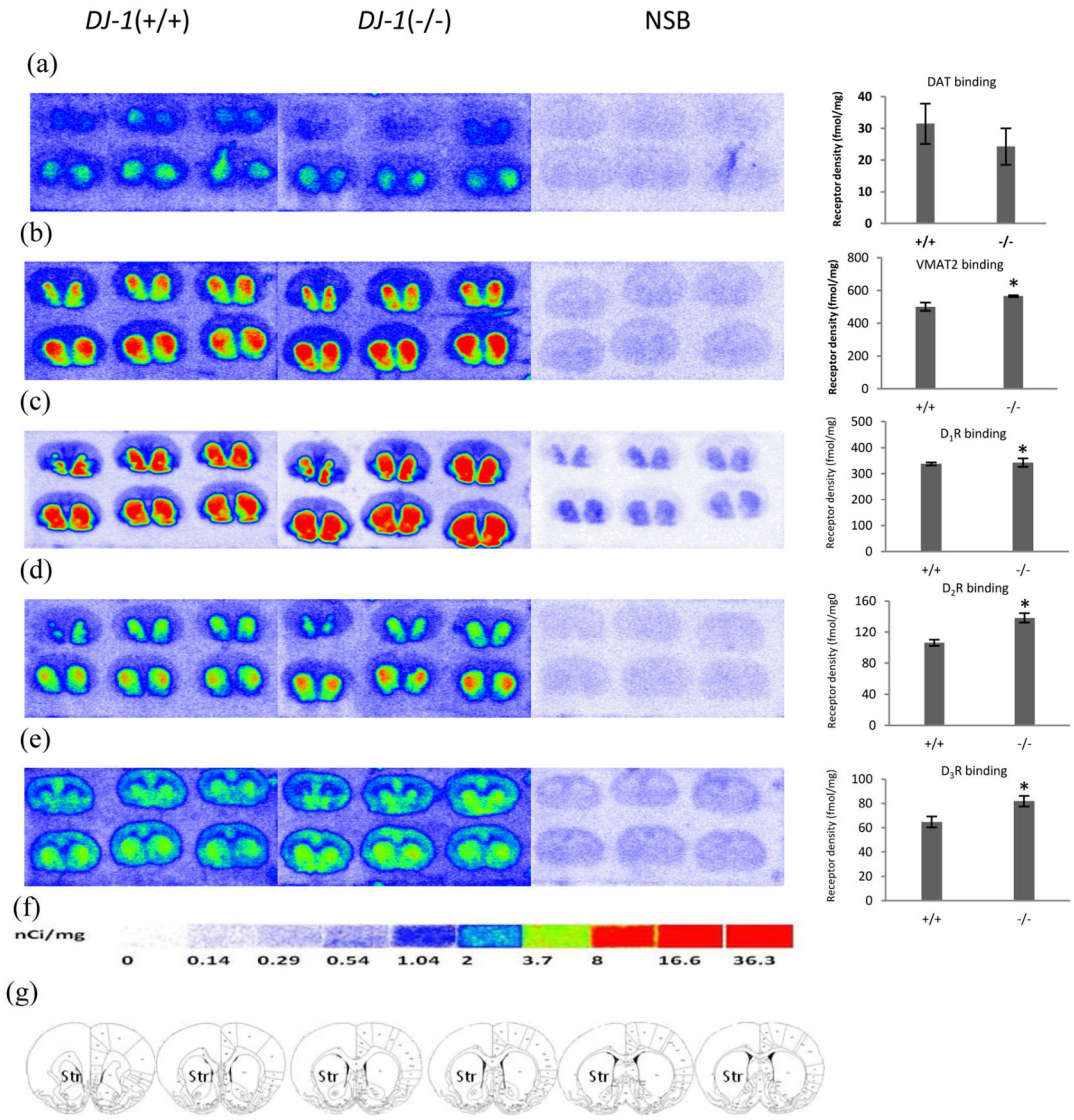

Figure 1. Quantitative autoradiographic analysis of DAT, DTBZ and dopamine receptors densities in the *DJ-1* gene knockout rat models of PD

Autoradiograms show binding of 8.5 nM [^{3}H]WIN35428 (a), 23.5nM [^{3}H]DTBZ (b), 3.8 nM [^{3}H]SCH23390 (c), 4.4 nM [^{3}H]raclopride (d) and 5.9 nM [^{3}H]**WC-10** (e) on multiple brain sections through the striatum in the *DJ-1* gene knockout and wild-type littermate rat. Nonspecific binding was determined in the presence of 1 μM nomifensine (for [^{3}H]WIN35428), 1 μM *S*(−)-tetrabenazine (for [^{3}H]DTBZ), 1 μM (+) butaclamol (for [^{3}H]SCH23390), 1 μM *S*(−)-eticlopride (for [^{3}H]raclopride and [^{3}H]**WC-10**). DAT binding did not significantly change in *DJ-1* gene knockout rat (a). The densities of VMAT2, dopamine D_1, D_2 and D_3 receptors were significantly increased in *DJ-1* gene knockout rats (b, c, d, e). [^{3}H]Microscale standards (ranging from 0 to 36.3 nCi/mg) were also counted (f). Schematic rat brain sections showing the rostral to caudal extent of the striatum, the region of interest in which DAT, VMAT2, D_1, D_2, and D_3 receptors were quantified, across a total

of six sections(g). NSB, nonspecific binding; Str, striatum. *p<0.05 for *DJ-1* knockout rat vs. control rat.

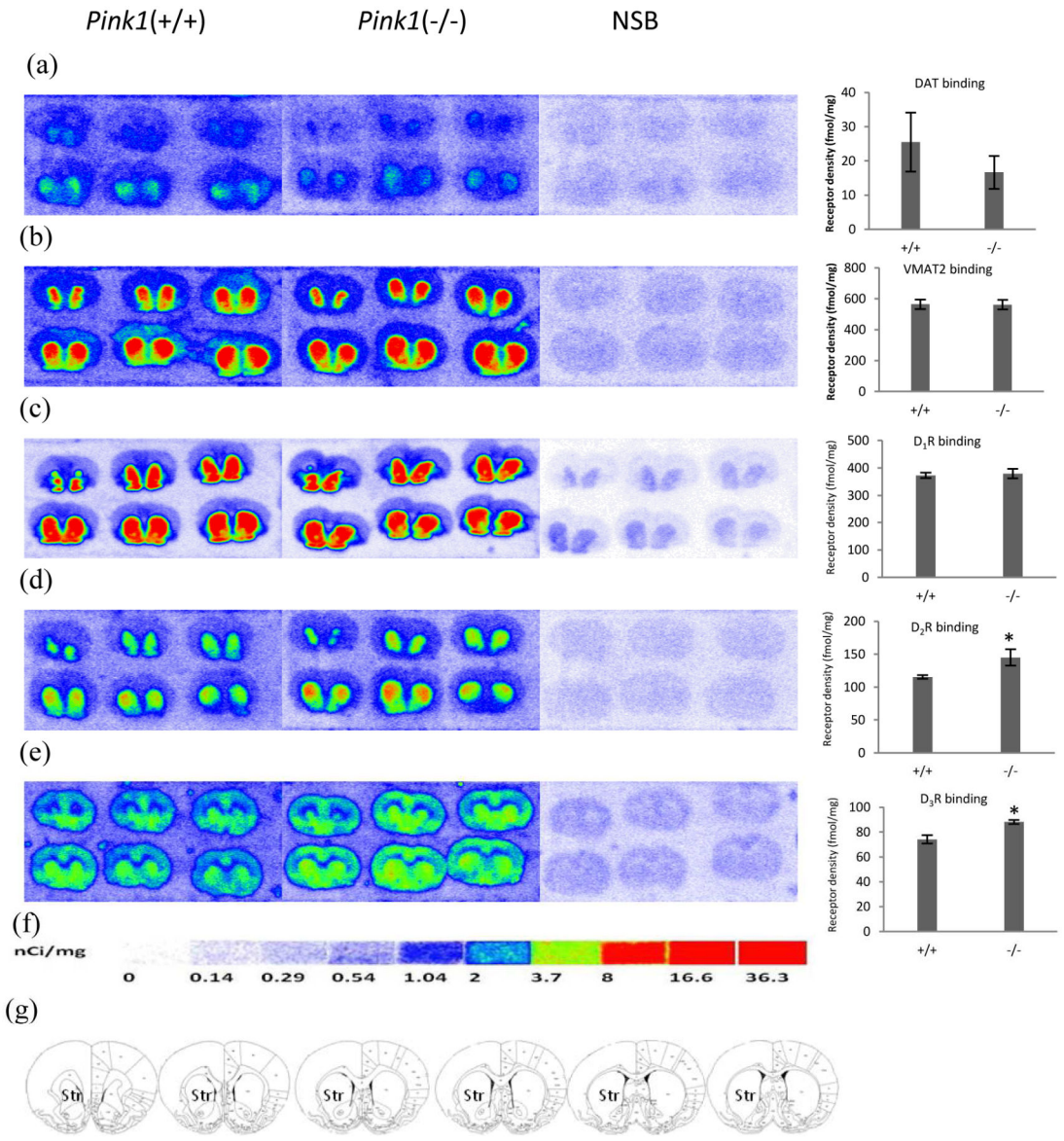

Figure 2. Quantitative autoradiographic analysis of DAT, DTBZ and dopamine receptors densities in the *PINK1* gene knockout rat models of PD

Autoradiograms show binding of 8.5 nM [^{3}H]WIN35428 (a), 23.5 nM [^{3}H]DTBZ (b), 3.8 nM [^{3}H]SCH23390 (c), 4.4 nM [^{3}H]raclopride(d) and 5.9 nM [^{3}H]**WC-10** (e) on multiple brain sections through the striatum in the *PINK1* gene knockout and wild-type littermate rat. Nonspecific binding was determined in presence of 1 μM nomifensine (for [^{3}H]WIN35428), 1 μM *S*(−)-tetrabenazine (for [^{3}H]DTBZ),1 μM (+) butaclamol (for [^{3}H]SCH23390), 1 μM *S*(−)-eticlopride (for [^{3}H]raclopride and [^{3}H]**WC-10**). DAT, VMAT2 and D_1 receptor binding did not change in *PINK1* gene knockout rat (a,b,c). The densities of dopamine D_2 and D_3 receptors were significant upregulated in *PINK1* gene knockout rats (d, e). [^{3}H]Microscale standards (ranging from 0 to 36.3 nCi/mg) were also counted (f). Schematic rat brain sections showing the rostral to caudal extent of the striatum, the region of interest in which DAT, VMAT2, D_1, D_2, and D_3 receptors were quantified, across a total of six

sections (g). NSB, nonspecific binding; Str, striatum. *p<0.05 for *Pink1* knockout rat vs. control rat.

Table 1

Fold changes in mRNA expression levels in PD KO rat brains as assessed by PCR arrays

Gene	GeneBank	Name	PARK7_KO 10 wks	PARK7_KO 15 wks	*Pink1*_KO 10 wks	*Pink1*_KO 15 wks
Park7	nm_057143	*DJ-1*	**−11.3**	**−6.2**		
Pink1	nm_001106694	PTEN induced putative kinase 1			**−41.4**	**−42.7**
Fjx1	nm_001108955	four jointed box 1		2.3		
Htr2a	nm_017254	serotonin 2A receptor	2.5	2.6	2.2	3.0
Nsf	nm_021748	N-ethylmaleimide- sensitive factor		2.0		
Slc18a2	nm_013031	vesicular monoamine transporter 2 (VMAT2)		−2.8		
Syngr3	nm_001106985	synaptogyrin 3		2.3		
Drd2	nm_012547	dopamine receptor D2	*−1.0*	*1.2*	*−1.1*	*1.1*
Slc6a3	nm_012694	dopamine transporter (DAT)	*−1.8*	*1.5*	*1.3*	*−1.0*