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Non-motor behavioural impairments in parkin-deficient mice

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Abstract

Mutations in the parkin gene are the major cause of early-onset familial Parkinson's disease (PD). We previously reported the generation and analysis of a knockout mouse carrying a deletion of exon 3 in the parkin gene. F1 hybrid *pa+/–* mice were backcrossed to wild-type C57Bl/6 for three more generations to establish a *pa–/–*(F4) mouse line. The appearance of tyrosine hydroxylase-positive neurons was normal in young and aged *pa–/–* (F4) animals. Loss of parkin function in mice did not enhance vulnerability of dopaminergic neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity. However, the *pa–/–* (F4) mice displayed impaired exploration and habituation to a new environment and exhibited thigmotaxis behaviour in the open field and Morris water maze. Abnormal anxiety-related behaviour of *pa–/–* (F4) mice was also observed in the light/dark exploration test paradigm. Dopamine metabolism was enhanced in the striatum of *pa–/–* (F4) mice, as revealed by increased homovanillic acid (HVA) content and a reduced ratio of dihydroxyphenylacetic acid (DOPAC)/HVA. The alterations found in the dopaminergic system could be responsible for the behavioural impairments of *pa–/–* (F4) mice. Consistent with a recent observation of cognitive dysfunction in parkin-linked patients with PD, our findings provide evidence of a physiological role of parkin in non-motor behaviour, possibly representing a disease stage that precedes dopaminergic neuron loss.

Introduction

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders. The major clinical features are bradykinesia, rigidity, resting tremor and postural instability (Fahn, 2003). In addition, neuropsychiatric, perceptual and cognitive deficits are increasingly recognized as non-motor manifestations of PD (Aarsland *et al.*, 1999; Kaasinen *et al.*, 2001; Houeto *et al.*, 2002; Pluck & Brown, 2002; Bodis-Wollner, 2003; Fahn, 2003). Reduced novelty seeking, anxiety and depression have been shown to precede motor manifestations, and are not attenuated by anti-parkinsonian therapy (Bodis-Wollner, 2003).

The neuropathological hallmarks of PD are characterized by a loss of dopaminergic neurons, primarily in the substantia nigra (SN) and locus coeruleus (LC), and by cytoplasmic eosinophilic inclusions termed Lewy bodies (LBs; Fahn, 2003). Even though several aspects of the biochemistry of dopaminergic neurons have been proposed to be responsible for the specific vulnerability, the mechanisms of PD pathogenesis are still widely unknown. Most cases of PD are sporadic and have been associated with a range of environmental risk factors (Di Monte, 2003; Chade *et al.*, 2006), including oxidative stress and reduced mitochondrial function (Dawson & Dawson, 2003; Tretter *et al.*, 2004; Lin & Beal, 2006). However, the identification of genes linked to familial forms of PD demonstrated the importance of genetic contributions to PD (Le & Appel, 2004; Schapira, 2006).

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A variety of autosomal recessive mutations in the parkin gene results in early-onset parkinsonism (AR-JP; Hattori *et al.*, 1998; Kitada *et al.*, 1998; Abbas *et al.*, 1999; Hayashi *et al.*, 2000; Lucking *et al.*, 2000), with symptoms often indistinguishable from sporadic PD (Lucking *et al.*, 2000). The majority of patients with mutations of the parkin gene display a severe loss of nigral dopaminergic neurons without the formation of LBs (Takahashi *et al.*, 1994; Mori *et al.*, 1998; Hayashi *et al.*, 2000; Farrer *et al.*, 2001; van de Warrenburg *et al.*, 2001). The age of onset of parkinsonism for parkin patients is extremely variable, even among patients who carry the same mutation and within the same family (Abbas *et al.*, 1999; Lucking *et al.*, 2000). This high variability in the age of onset suggests that the pathogenic impact of parkin mutations for parkinsonism is modulated by other factors.

The parkin gene encodes a protein of 465 amino acids that contains an N-terminal ubiquitin-like domain. A C-terminal domain resembling a RING finger E3 ubiquitin protein ligase binds and ubiquitinylates a variety of protein substrates (Kitada *et al.*, 1998; Imai *et al.*, 2000; Joazeiro & Weissman, 2000; Shimura *et al.*, 2000; Zhang *et al.*, 2000; Imai *et al.*, 2001; Yang *et al.*, 2003).

To investigate the pathogenic mechanism of PD and how mutations in parkin lead to dopaminergic neuron degeneration, we generated a parkin knockout mouse with an exon 3 deletion by gene targeting (Stichel *et al.*, 2007). Parallel to our investigation, four independently generated parkin-deficient knockout mice (*pa–/–*) have been reported (Goldberg *et al.*, 2003; Itier *et al.*, 2003; von Coelln *et al.*, 2004; Perez & Palmiter, 2005). However, the phenotypes of these four independent parkin-deficient mouse lines seem somewhat inconsistent. Deletion of parkin exon 3 in the first

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two knockout mice was reported to result in elevated extracellular dopamine (DA) levels (Goldberg *et al.*, 2003; Itier *et al.*, 2003). One mutant mouse displayed reduced synaptic excitability of neurons in the striatum (ST) and behavioural deficits related to the nigrostriatal pathway (Goldberg *et al.*, 2003), while the other *pa–/–* mouse exhibited deficits in amphetamine-induced DA release, in DA metabolism, glutamate neurotransmission and motor impairment (Itier *et al.*, 2003). The third knockout mouse, carrying an exon 7 deletion, displayed a reduced number of dopaminergic neurons in the LC and an accompanying deficit of norepinephrine (NE) in the olfactory bulb and the brain stem (von Coelln *et al.*, 2004). Exon 2 was deleted in the fourth parkin knockout mice. No evidence for nigrostriatal or noradrenergic dysfunction in this mouse line was found, in contrast to the previous three studies (Perez & Palmiter, 2005). These conspicuous phenotypic differences may be due to variations in the genetic background, as in all these studies the consequences of the parkin knockout were analysed with an F1 non-isogenic background (50% 129 and 50% C57BL/6). It is well known that locomotor activity and cognitive performance are polygenic traits varying widely between mouse inbred strains (Flint *et al.*, 1995; Kelly *et al.*, 1998; Holmes *et al.*, 2002). In addition, the susceptibility to neurotoxins, e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), differs vastly between strains. Particularly sensitive is the C57Bl/6 strain (Sonsalla & Heikkila, 1986; Sundstrom *et al.*, 1987; Hofele *et al.*, 2000).

We have previously reported the generation of the *pa–/–* (F4) mouse strain, which was backcrossed with C57BL/6 for three generations (F4-generation; Stichel *et al.*, 2007). Severe age- and region-dependent morphological alterations of mitochondria were found in neuronal somata of two double mutant mouse lines with the *pa–/–* (F4) and the α-Synuclein transgene (Stichel *et al.*, 2007). These alterations are accompanied by the reduced complex I capacity in the SN (Stichel *et al.*, 2007). In this study, we investigated the impact of the absence of parkin on general neurological function, such as motor ability, emotionality, learning, memory and neurochemistry. Here, we further report that loss of the parkin did not enhance vulnerability of dopaminergic neurons of the *pa–/–* (F4) mice to MPTP. However, *pa–/–* (F4) mutant mice exhibited abnormal behaviour in the open field and light–dark exploration, as well as spatial learning deficits in the Morris water maze. These observations suggest a physiological function of parkin in the regulation of some non-motor behaviours and spatial learning.

Materials and methods

Pa–/– (F4) mouse strain

The knockout mouse with an exon 3 deletion in the parkin gene has been generated by using R1 embryonic stem cells carrying the genetic background of 129SvJ, as described previously by Stichel *et al.* (2007), and was then backcrossed with wild-type C57BL/6J for three generations to establish *pa+/–* (F4) mice. At this stage, the genetic background of the mice was 94% derived from C57Bl/6 and 6% from 129SvJ. To expand the F4-generation and produce littermates with the appropriate genetic background as controls, *pa +/–* (F4) mice were crossed. For the experiments, the resulting *pa–/–* (F4) mice and their non-transgenic *pa+/+* (F4) littermates were used.

All animal experiments were conducted and approved using the German guidelines of the animal care and use committee of the state Nordrhein-Westfalen. The animals were housed in a 12 : 12 h light : dark cycle with free access to food and water. Ambient temperature was maintained at 24 °C.

MPTP and amphetamine treatment

Eighteen-month-old *pa–/–* (F4) mice and their littermates were treated with MPTP (Sigma, Munich, Germany) dissolved in sterile saline in an acute injection paradigm (15 mg/kg, i.p., four injections with a 2-h interval). After 4 weeks, the animals were killed either by decapitation for Western blot analysis or by transcardial perfusion with 4% paraformaldehyde for immunohistochemistry as described (Stichel *et al.*, 2000; Kühn *et al.*, 2003). *d*-Amphetamine sulphate was obtained from Sigma and dissolved in sterile saline. Three-month-old *pa–/–* mice and their littermates were placed onto the open field three times (30 min each). After the habituation, injections of 1.0 mg/kg amphetamine or saline were given i.p. to animals immediately before behavioural testing.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Kühn *et al.*, 2003). Frontal brain sections were incubated with antibodies specific for tyrosine hydroxylase (TH; 1 : 500, Chemicon, Hofheim, Germany), DA transporter (DAT; 1 : 5000, Chemicon), glial fibrillary acidic protein (GFAP; 1 : 250, DPC Biermann, Bad Nauheim, Germany) and phosphotyrosine PT66 (1 : 1000, Sigma). Following the first antiserum incubations, sections were treated with the corresponding biotinylated secondary antibody and ABC reagent. FluoroJade staining was performed as described (Kühn *et al.*, 2003). For the densitometric analysis of striatal TH-immunostaining, all sections of three brains for each genotype and treatment were processed simultaneously in a staining dish during the whole immunostaining procedure, and imaged with a microscope under identical parameters. The optical densities of striatal TH staining were measured by using a computerized image analysis system (Image-Plo Plus 3.0, Media Cybernetics, Bethesda, MD, USA).

Synaptosomal DA uptake

The *pa–/–* (F4) mice and their littermates were killed by decapitation. The synaptosomal fractions from the ST were prepared as described (Gordon-Weeks, 1987). Fifty microlitres of synaptosomal fraction was added to 450 μL of uptake solution (in mM: NaCl, 120; KCl, 5; $MgSO_4$, 1.2; K_2HPO_4, 1.2; $CaCl_2$, 1.25; ascorbic acid, 1; pargyline, 10 μM; [^{3}H]-DA, 31.4 nM, pH 7.4) with or without an inhibition (30 μM nomifensine, Sigma) in 24-well plates. For saturation analysis of DA uptake, the [^{3}H]-DA concentration was kept constant, and unlabelled DA was adjusted from 0 to 0.5 μM. The probes were incubated at 37 °C for 20 min. The incubation was terminated by rapid filtration using a cell harvester, filters were washed four times with H_2O, dried at 37 °C and counted in a scintillation counter. The difference between uptake without and with the DAT-specific blocker nomifensine was defined as specific uptake. The estimated K_m and V_{max} values were calculated by the iterative curve-fitting program of SigmaPlot (SPSS, Erkrath, Germany), and resulting values of four (aged 3 and 9 months) or three (aged 24 months) separate mice per group were averaged.

Neurochemical analysis

High-performance liquid chromatograph (HPLC) with electrochemical detection (ECD) was used to determine the concentration of NE, DA, dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT),

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5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA). *pa+/+* (F4) and *pa–/–* (F4) mice were decapitated, brains were quickly removed, and ST and SN were dissected. The tissue was weighed and sonicated in ice-cold buffered solution consisting of 0.1 N $HClO_4$ and 0.2 mM $NaHSO_4$ (20 μL/mg tissue). The homogenate was centrifuged (20 000 ***g***, 20 min, 4 °C) and the supernatant was filtered using a 0.2-μm Millipore-filter. A total volume of 20 μL, consisting of a mixture of 20 μL sample and 5 μL internal standard, was used for each measurement. The HPLC system consisted of a degasser (Dionex, Idstein, Germany), a high-pressure isocratic HPLC pump (K-1001, Kronlab GmbH, Sinsheim, Germany), a manual injector (Rheodyne, Rohnert Park, CA, USA) and a 3-μm nucleosil C-18 250-mm column (YMC, Dinslaken, Germany). ECD was performed with a VT-03 flow cell (Decade, Antec-Leyden, Netherlands). The mobile phase contained 14.14 g/L chloracetic acid, 4.66 g/L NaOH, 200 mg/L octylsulphate, 250 mg/L EDTA, 1.8% (v/v) THF and 7% (v/v) acetonitrile, adjusted to pH 3.0. The working potential of the electrochemical flow cell was adjusted to +0.7 V vs an Ag/AgCl reference electrode. The HPLC-ECD system was calibrated with external standards for each substance. Sample concentrations were corrected by using the internal standard. Data were integrated and analysed by the Chromeleon® software data system (Dionex) coupled with the software program DECADE Dialogue® (Antec-Leyden, Netherlands) to switch sensitivity of the detector simultaneously for the different amounts of transmitters and metabolites in the sample.

Behavioural testing

All behavioural tests were performed by investigators blind to the mouse genotypes. For rotarod performance, mice at different ages were initially trained to stay on the rod with a constant rotation speed of 10 ***g*** (TSE, Bad Homburg, Germany). After the initial training the mice were tested on the rod at a speed of 4 ***g***, which was accelerated to 40 ***g*** over 4 min. The testing was repeated four times on four consecutive days. The time between the start of the rotation and the time point at which the mice fell off the rod was monitored (a maximum cutoff time of 240 s). All scores for each mouse were used to build an average of the rotarod performance.

Locomotor activity was measured using a video-tracker, which tracked mice in four adjacent opaque Plexiglas enclosures (40 × 40 × 40 cm). A video camera, mounted above the enclosures, recorded the travel paths of all four mice (TSE). The open field of each enclosure was virtually divided by the computer into two separate regions: the centre region (20 × 20 cm in the midpoint of the open field) and the periphery. Mice were tested for 30 min per day on three separate test days. Their travel paths were recorded, and the horizontal path length (cm) and the time spent in the centre area were measured by the tracking software.

Spatial learning was assessed in a Morris water maze modified for use with mice. A circular water tank (diameter, 100 cm; height, 40 cm) was filled with water (23 °C) that had an opaque appearance through the addition of a small amount of milk. Four positions around the edge of the tank were arbitrarily designated, providing four alternative start positions and defining the division of the tank into four quadrants. A translucent Perspex escape platform (diameter 9 cm) was submerged 0.5 cm below the water surface and positioned at the midpoint of one of the four quadrants. A video camera was fixed above the centre of the swimming tank, and swimming trials were recorded. On the first day, mice were trained with a visible platform. The platform was made visible by the attachment of a flag. Starting on Day 2, mice were trained in two trials per day to swim to the submerged platform without the flag. The start position was pseudorandomized across the whole test. Mice were allowed up to 60 s to locate the escape platform, and their escape latencies were recorded. Mice that failed to locate the platform within the time limit were ascribed an escape latency of 60 s and were placed on the platform by hand. All mice were then allowed to stay on the platform for 30 s, before being removed and returned to the home cage. Training continued for 6 days. On Day 8, mice received a single probe trial, during which the escape platform was removed from the tank, and the swimming path of each mouse was recorded over 60 s while it searched for the missing platform. The search time in the trained quadrant (target quadrant, TQ) and the other three quadrants, and how often the mice swam over the former location of the platform were evaluated.

The light–dark exploration test was performed in a standard plastic rat cage divided into two compartments. One compartment contained two-thirds of the surface area, was transparent and without a lid, and illuminated by an overhead lamp. The remaining surface area was covered with a black Plexiglas lid. An opening in the divider panel allowed the mice to move from one compartment to the other. A transition counted when the mouse fully exited one compartment and entered the other compartment. The time spent in each area and the number of transitions were recorded over 10 min with mice aged 6 or 14 months.

Statistics

Statistical analysis was carried out using one- or two-way analysis of variance (ANOVA) followed by the *post hoc* Tukey test when significance (at the 0.05 level) was indicated. When comparisons were being made between two genotypes or two treatments, a Student's *t*- or *U*-test was performed, and $P < 0.05$ was considered significant. All data were analysed by using SIGMASTAT 3.1 (Systat Software, San Jose, CA, USA).

Results

Normal viability, body weight and neuroanatomy of DA neurons in pa–/– (F4) mice

The *pa–/–* (F4) mice appeared normal by all visual criteria, and did not show a reduced life span (data not shown). Their body weights were not significantly different from wild-type littermates at young and old ages (data not shown). Histological sections of the brain did not display any gross anatomical abnormality in *pa–/–* (F4) mice compared with their littermates (data not shown).

We examined DA neurons in *pa–/–* (F4) animals at different ages by TH- and DAT-immunostaining. Similar numbers and morphology of TH+ or DAT+ neurons were found in the SN and LC of *pa–/–* (F4) and *pa+/+* (F4) mice up to the age of 18 months (Fig. 1 for the age of 12 months; data not shown for 6 or 18 months, and DAT). The density of dopaminergic projections to the ST in *pa–/–* (F4) appeared morphologically indistinguishable from their littermates (Fig. 1). FluoroJade-positive cells were neither found in littermates nor in *pa–/–* (F4) mice. Furthermore, GFAP- and phosphotyrosine PT66-staining did not display enhanced numbers of reactive astrocytes or microglia in *pa–/–* (F4) mice (Fig. 1). These results demonstrated that loss of parkin protein does not result in the death of dopaminergic neurons in mouse SN or LC.

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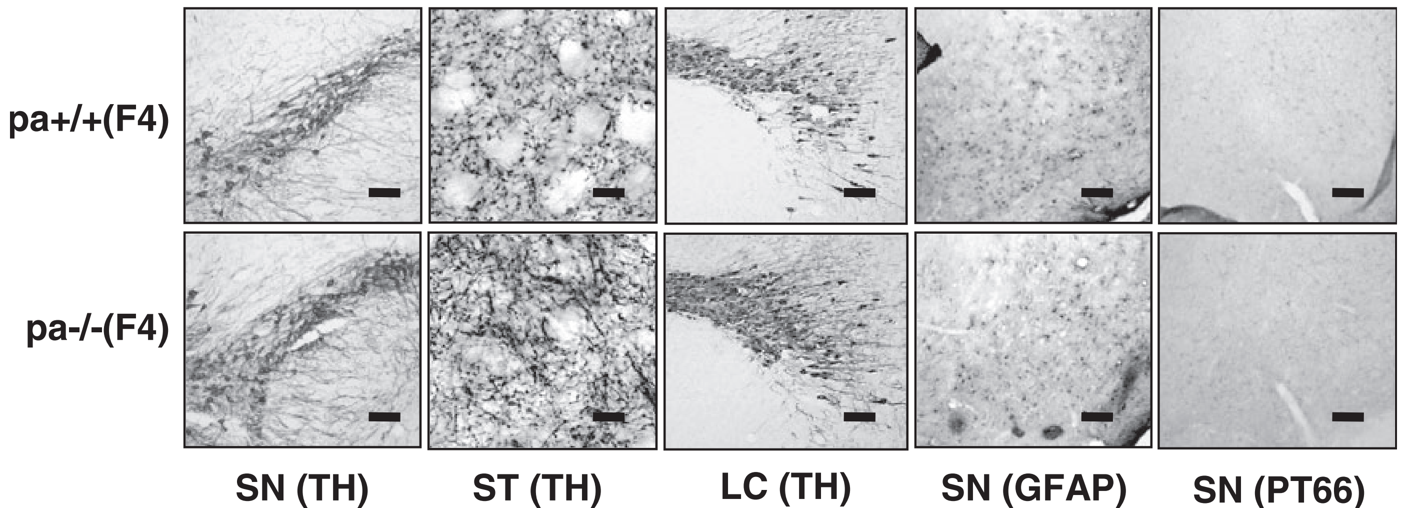

FIG. 1. Neuroanatomy of dopaminergic neurons and striatal synaptosomal [^{3}H]-DA uptake of *pa–/–* (F4) mice. Representative photomicrographs illustrating similar tyrosine hydroxylase (TH)-, glial fibrillary acidic protein (GFAP)- and PT66-immunoreactivities in the substantia nigra (SN), the striatum (ST) or the locus coeruleus (LC) of 12-month-old male *pa+/+* (F4) and age-matched male *pa–/–* (F4) mice (18 months old for LC). Scale bars, 200 μm (SN and LC), 20 μm (ST).

MPTP susceptibility of pa–/– *(F4) mice*

Administration of MPTP to primates or rodents decreases striatal DA levels, and leads to a loss of dopaminergic neurons in the SN (Speciale, 2002; Kühn *et al.*, 2003). To determine whether parkin dysfunction causes enhanced MPTP susceptibility of dopaminergic neurons in aged mice, 18-month-old male *pa–/–* (F4) and male wild-type controls were injected with MPTP or saline, and dopaminergic innervation of the ST was examined by TH-immunohistochemistry at 4 weeks after MPTP treatment. Mutant and wild-type mice treated with MPTP showed a strong reduction of TH+ axon density in the ST (two-way ANOVA: $F_{1,8} = 87.82$; $P < 0.001$; Fig. 2A and B). However, TH+ axon density of surviving dopaminergic axons in the ST was not significantly different between *pa–/–* (F4) mutant and wild-type mice treated with MPTP (two-way ANOVA: $F_{1,8} = 0.26$; $P = 0.624$; Fig. 2A and B). These results demonstrated that the loss of parkin function in mice does not appear to enhance the vulnerability of dopaminergic neurons to MPTP toxicity.

Normal motor skills in pa–/– *(F4) mice*

Another pathological feature of AR-JP is the severe impairment in motor behaviour. In order to explore whether the loss of parkin function had an impact on gross locomotor behaviour, 6-month-old mice of both genotypes were placed in the open field and their horizontal travel distances were recorded. This test was repeated when the animals reached the age of 12, 15, 18 and 21 months. As shown in Fig. 3A, the mutant mice and their littermates displayed similar locomotor activities during their whole lifespan.

The rotarod is widely employed to measure the ability to sustain complex coordinated movements over time. Six-month-old mice of both genotypes were tested in the rotarod task after a brief training session. The performance of *pa–/–* (F4) mice on the rotarod was not significantly different from that of their littermates (Fig. 3B). The *pa–/–* (F4) mice showed similar rotarod performance as littermates when they were re-exposed to the test every 3 months up to 21 months old (Fig. 3B). These results indicate that loss of parkin in mice does not result in deficient motor skills.

Non-motor behavioural impairments and spatial learning deficits in pa–/– *(F4) mice*

The involvement of DA neurotransmission in behavioural responses to novelty is suggested by several reports demonstrating that: (i) reward is related to enhanced DA activity; (ii) DA modulates exploratory behaviour in animals; and (iii) patients with PD report diminished responses to novelty (Aarsland *et al.*, 1999; Kaasinen *et al.*, 2001; Houeto *et al.*, 2002; Pluck & Brown, 2002; Bodis-Wollner, 2003; Fahn, 2003; Viggiano *et al.*, 2003). The open field test is thought to induce moderate anxiety by confronting rodents with a novel environment with no possibility of escape (Dulawa *et al.*, 1999). The naive wild-type mice exhibited locomotor habituation in the open field over time, showing reduced levels of activity on the second and third days compared with the first day (Fig. 4A). The *pa–/–* mutant mice showed similar locomotor activity to their littermates at Day 2 and 3, but the typical exploratory behaviour with higher locomotor activity in the new environment at the first day and subsequent habituation at the next days could not be detected during the test over the 3 days (Fig. 4A). The horizontal travel distance of *pa–/–* (F4) mice at the first test day was significantly less than wild-type mice (Fig. 4A). Over these three test days, *pa–/–* (F4) mice exhibited increased thigmotaxic behaviour relative to *pa+/+* (F4) mice, indicated by a trend to spend less time in the centre of the open field (Fig. 4B).

The amphetamine is a well-known anxiogenic drug, can induce DA release and thigmotaxic behaviour of mice in the open field (Ralph *et al.*, 2001). Consistent with this observation, 3-month-old *pa+/+* (F4) wild-type mice spent significantly less time in the centre after amphetamine treatment (Fig. 4C). In contrast to wild-type mice, 1 mg/kg amphetamine had no significant effect on time in the centre of age-matched male *pa–/–* (F4) mice (Fig. 4C), suggesting that the behavioural impairment in *pa–/–* mice may be related to a dysfunction of DA modulation.

We further examined a possible impairment of anxiety-related behaviour in *pa–/–* (F4) mice using a light/dark transition test, which is a frequently used test paradigm to assess anxiolytic and anxiogenic effects of drugs in rodents (Shimada *et al.*, 1995). Six- or 15-month old *pa–/–* (F4) mice spent significantly less time in the illuminated

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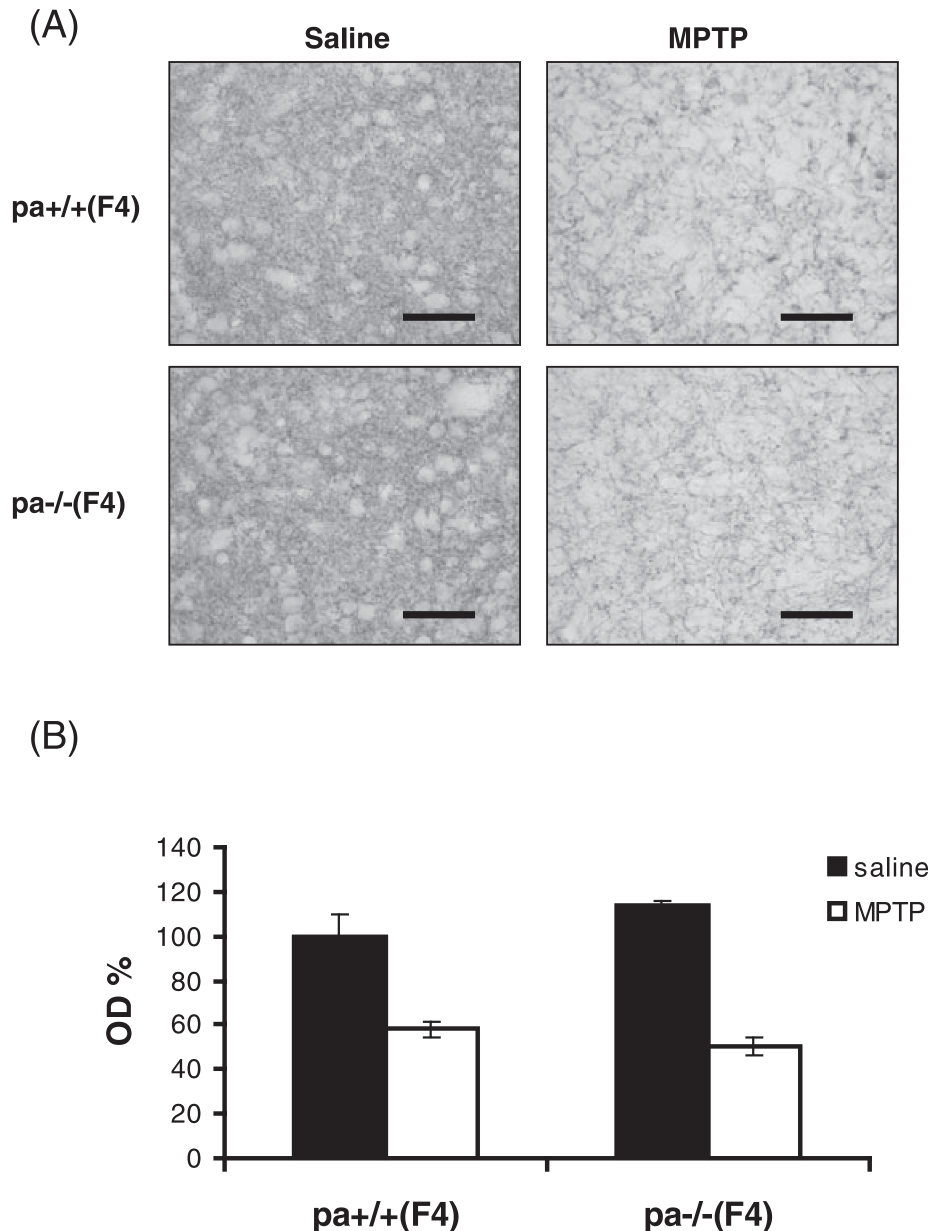

FIG. 2. Normal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) susceptibility of striatal TH-immunopositive axons in *pa–/–* (F4) mice. (A) Eighteen-month old *pa–/–* (F4) and non-transgenic littermate control mice (F4) were treated with four i.p. injections (saline or 15 mg/kg MPTP) at 2-h intervals. Dopaminergic innervation in the ST was analysed using TH-immunohistochemistry at 4 weeks after the MPTP treatment. The densities of TH+ axons were strongly reduced in the ST of MPTP-treated animals of both genotypes (Scale bar: 50 μm). (B) Optical density (OD) quantification of the intensity of immunostaining of TH-positive axons in the ST. Data are expressed as mean ± SEM in arbitrary units after normalization to the mean value of saline-treated non-transgenic littermate control mice. The reductions in the density of TH+ nerve terminals in the ST after MPTP treatment were not significantly different between both genotypes (two-way ANOVA: effect of genotype: $F_{1,8} = 0.26$; $P = 0.624$).

compartment during the 10-min test session than their littermates (Fig. 4D). Mutant mice also made significantly less transitions between the light/dark compartments (Fig. 4E). This finding is consistent with results obtained by the open field test paradigm, indicating that loss of parkin function in mice causes an impairment of exploratory behaviour and increased anxiety. This impairment in *pa–/–* (F4) mice does not seem to be age dependent, as the effects were equally visible in a 6-month-old and an independent 15-month-old group of mice (Fig. 4D and E).

Cognitive decline has frequently been described in patients with PD. To assess the cognitive state of *pa–/–* (F4) mutant mice, the Morris water maze test paradigm was used to examine spatial learning and memory. In learning to find the fixed hidden platform, mutant mice and their littermate controls showed marked improvements in escape latencies over the 7 days of training (Fig. 5A). However, *pa–/–* (F4) mice exhibited overall slightly, but significantly, longer latencies compared with their controls ($P < 0.01$, Fig. 5A). On the probe trail, mice of both genotypes exhibited evidence for spatial learning, spending more than average time in the platform quadrant (TQ; Fig. 5B). The two groups did not differ significantly (Fig. 5B). However, on a more stringent measurement of spatial navigation, i.e. counting the 'crossings', *pa–/–* (F4) mutant mice made significantly less crossings than their littermate control mice, and did not selectively cross the former platform site in

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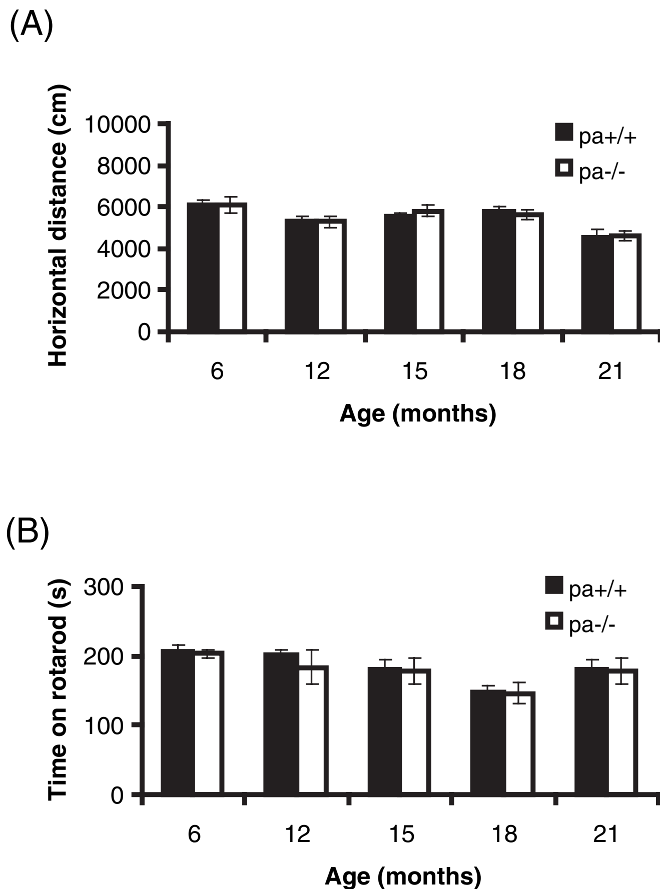

FIG. 3. *pa–/–* (F4) mice exhibit normal motor skills. (A) *pa–/–* (F4) mutant mice ($n = 8–12$) at different ages and their littermates ($n = 13$) exhibited similar levels of total horizontal distance travelled in 30 min in the open field. Average mean scores of tests on three consecutive days are shown ± SEM. Pair-wise comparisons of *pa–/–* (F4) mice and their *pa+/+* (F4) littermates within each age group show no significant differences (Student's *t-test*). (B) Rotarod performance is shown as the time that mice stayed on an accelerating rotating rod until they fell off. Data are mean scores ± SEM for *pa–/–* (F4) mutant mice ($n = 9–18$) and *pa+/+* (F4) littermate controls ($n = 12–13$) at the ages of 6, 12, 15, 18 and 21 months. Pair-wise comparisons of both genotypes for each age group show no significant impairment of the rotarod performance in the *pa–/–* (F4) animal (Student's *t*-test).

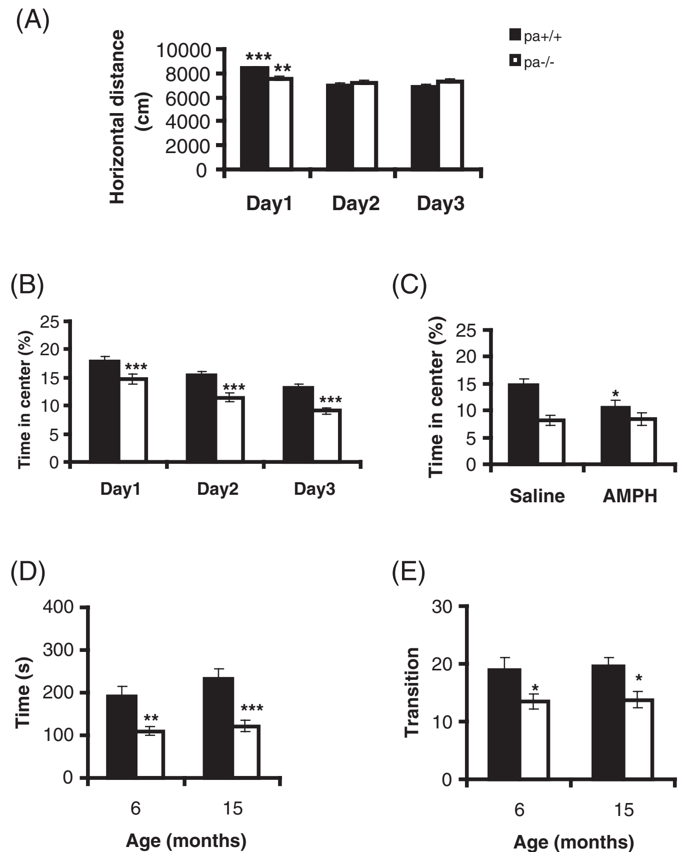

FIG. 4. Abnormal exploration and anxiety behaviour in the *pa–/–* (F4) mice. (A) Naive animals were exposed to the open field on three consecutive days and their locomotor activity, determined as total path length (cm), was monitored for 30 min. Data are mean ± SEM. Open bars, 3-month-old *pa–/–* (F4) ($n = 72$); and grey bars, wild-type *pa+/+* (F4) littermates ($n = 79$). Wild-type mice showed habituation over time (asterisks; $F_{2,234} = 21.14$; $P < 10^{-6}$), while *pa–/–* mutant mice did not exhibit exploration behaviour with higher locomotor activity on Day 1, and showed no habituation at the next days ($F_{2,213} = 0.759$; $P = 0.469$). A two-way ANOVA comparison revealed a genotype–day interaction ($F_{2,447} = 6.09$; $P < 0.01$). The locomotor activity of *pa–/–* (F4) mice at Day 1 was significantly lower than that of littermates (double asterisks: *post hoc* Tukey test; $P < 0.01$) and had no significant difference to that of their littermates at Days 2 and 3 (*post hoc* Tukey test). (B) In tests on three consecutive days, *pa–/–* (F4) mice spent reduced time in the centre, compared with *pa+/+* (F4) mice (asterisks: $F_{1,447} = 36.49$; $P < 0.001$). (C) Amphetamine (AMPH; 1 mg/kg) reduced the time in the centre for littermate mice ($n = 7$; asterisk; $P < 0.05$, Student's *t*-test), whereas no significant effect was seen with the age-matched *pa–/–* (F4) mutant mice ($n = 6, 9$; $P = 0.88$, Student's *t*-test). (D) Young and aged *pa–/–* (F4) mice (6 months: $n = 11$; 15 months: $n = 13$) spent significantly less time in the lighted compartment of the light/dark transition model during the 10-min test session relative to their male littermates (6 months: $n = 10$; 15 months: $n = 16$; asterisks; $P < 0.01$ for the age group of 6 months; $P < 0.001$ for the age group of 15 months, Student's *t*-test). (E) During the test, *pa–/–* (F4) mutant mice made significantly fewer transitions between the light/dark compartments than their controls (asterisks; $P < 0.05$ for the age group of 6 months; $P < 0.01$ for the age group of 15 months, Student's *t*-test). Data are mean ± SEM.

the TQ (Fig. 5C). The latter indicated an impairment in spatial learning.

An alternative way to present navigation accuracy is the proportion of time spent in the inner, middle and outer zones of the water maze. *pa–/–* (F4) mice spent significantly more time in the outer annuli and significantly less time in the middle than their wild-type littermates (Fig. 5D). This may indicate that *pa–/–* (F4) and control mice used different platform-searching strategies, with mutant mice swimming preferentially in the periphery of the water tank. This thigmotaxis behaviour of *pa–/–* (F4) mice in the water tank is consistent with the finding that *pa–/–* (F4) mice prefer to stay in the corners or near the edges rather than moving to the centre of the open field. This observation further strengthens our finding that loss of parkin protein causes an increased thigmotaxic behaviour.

Abnormal monoamine metabolism in pa–/– *(F4) mice*

According to the above results, *pa–/–* (F4) mice may exhibit deficits in habituation to the novel open field (Fig. 4A). Impaired response habituation in novel environments has also been found in DAT knockdown mutant mice (Zhuang *et al.*, 2001). Decreased levels of DAT protein and reduced DA uptake have also been reported in an independent parkin-deficient mouse line (Itier *et al.*, 2003). To determine whether the loss of parkin function may have an influence on striatal DAT function, we examined the K_m (inverse of transmitter affinity for transporter) and V_{max} (maximum uptake rate = transporter

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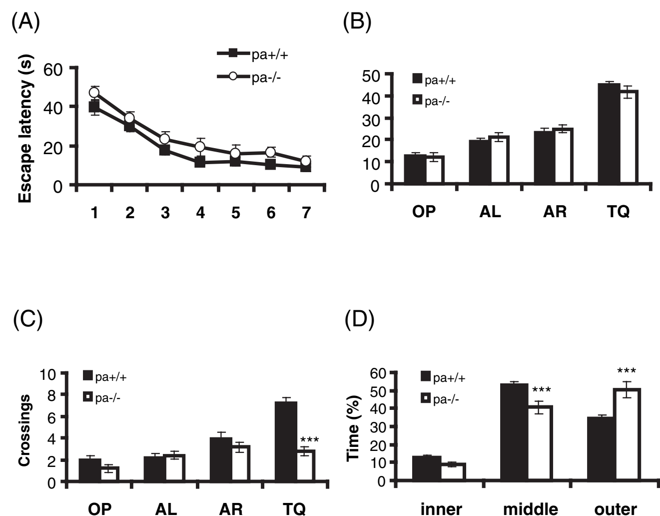

FIG. 5. Impairment of Morris water maze learning in *pa–/–* (F4) mice. (A) *pa–/–* (F4) mutant (n = 13) and wild-type *pa+/+* (F4) mice (n = 16) were tested in the hidden-platform version of the water maze. The average time required by the animals to reach the platform was plotted vs training day. The *pa–/–* (F4) null mutants were slightly impaired during training (two-way ANOVA: effect of genotype: $F_{1,195} = 11.08$; $P < 0.01$). (B) A probe test without a hidden platform was performed at the end of the training, and the search times in the quadrants are plotted (AL, adjacent left; AR, adjacent right; OP, opposite to the TQ; TQ, target quadrant). The *pa–/–* (F4) mutant mice searched selectively in the TQ vs other quadrants (one-way ANOVA: $F_{3,48} = 30.64$; $P < 0.001$). There was no difference in search time in TQ between both genotypes ($F_{1,108} = 0.002$; $P = 0.963$). (C) Mutant mice did, however, make significantly fewer crossings in the TQ site vs littermate controls (two-way ANOVA: $F_{1,108} = 18.34$; $P < 0.001$ followed by Tukey *post hoc* test, asterisks: $P < 0.001$), and did not selectively cross the TQ site (TQ site vs AR and AL sites: $P > 0.05$). (D) The search time is plotted for the search of the three different zones of the whole tank (inner, middle and outer zone). *pa–/–* (F4) mutant mice spent significantly more time in the outer zone and significantly less time in the middle zone than littermate control mice (genotype × zone: $F_{2,81} = 15.48$; $P < 0.001$, asterisks: $P < 0.001$; by *post hoc* Tukey test). Data are mean ± SEM.

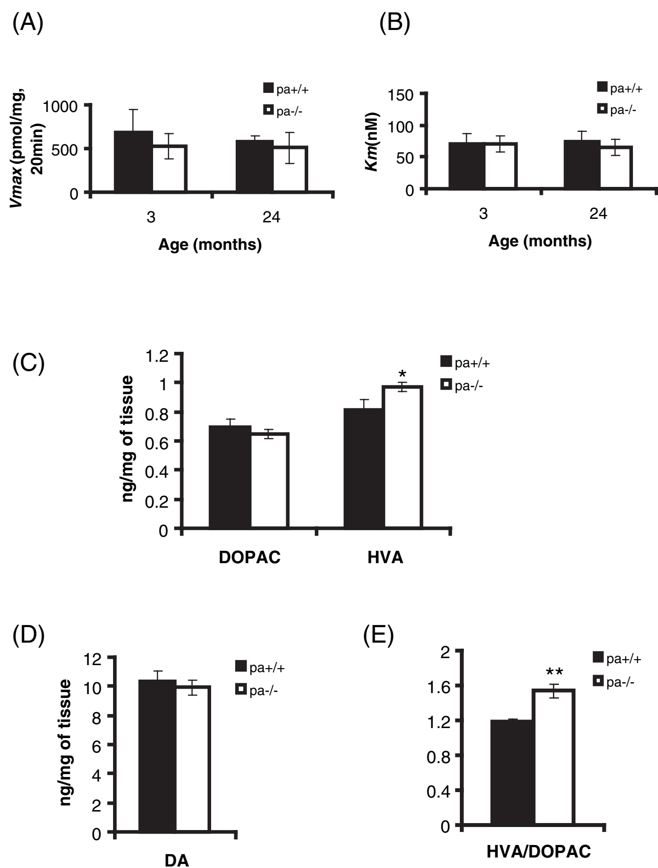

FIG. 6. Increased extracellular dopamine (DA) turnover in the striatum of *pa–/–* (F4) mice. (A and B) *pa–/–* (F4) mutant mice and their littermates exhibit similar V_{max} and K_m values for striatal synaptosomal dopamine uptake at the ages of 3 and 24 months (Student's *t*-test). (C–E) HPLC quantification of the striatal tissue content of DA, DOPAC and HVA. Results were calculated as ng/mg of fresh tissue and then expressed as a ratio. Values shown are means ± SEM of *pa–/–* (F4) (n=8) and litter mate control mice *pa+/+* (F4) (n=10). *$P < 0.05$, **$P < 0.01$, Student's *t*-test.

density) values of the DAT by measuring the [^{3}H]-DA uptake into striatal synaptosomes from 3-month-old *pa–/–* (F4) mice, compared with *pa+/+* (F4) littermates. Irrespective of the age of the animals, there was no significant difference in K_m and V_{max} values between *pa–/–* (F4) mutant and littermate control mice (Fig. 6A and B). These results suggest that parkin dysfunction has no influence on the DA reuptake activity in young and aged animals, and the behavioural impairments found in this study are not caused by DAT dysfunction.

We further assessed the levels of monoamines in *pa–/–* (F4) mice and their littermate controls in the ST by HPLC. The mean values of NE, DA and 5-HT in the ST were similar in both wild-type and mutant animals (data not shown). Analyses of the 5-HT metabolite 5-HIAA did not indicate any significant difference in 5-HT turnover between both genotypes (data not shown). However, analyses of the DA metabolites revealed increased mean values of HVA in the *pa–/–* (F4) ST (119%; Fig. 6C). Statistical analysis demonstrated that these increases are significant ($P < 0.05$, Student's *t*-test). The overall mean levels of DOPAC in *pa–/–* (F4) mice were slightly reduced, but the difference was statistically not significant (Fig. 6C). However, there was a significant difference in the ratio of HVA to DOPAC in *pa–/–* (F4) mice compared with non-transgenic littermates ($P < 0.01$, *U*-test). This may indicate an increased extraneuronal DA turnover in the mutant mice (Fig. 6E). Thus, the behavioural impairments of *pa–/–* (F4) mice could be caused by increased DA release in the ST.

Discussion

Since the identification of parkin mutations that cause PD, much information has been gathered about parkin's biochemical function. However, very few studies have directly assessed parkin function *in vivo*. We have used targeted mutagenesis to engineer a mouse strain carrying a deletion of exon 3 in the parkin gene that is analogous to the mutation found in some parkin-linked patients with PD.

Deletion of exon 7 of mouse parkin has been described to result in a reduced number of LC neurons (von Coelln *et al.*, 2004). However, loss of LC neurons was not observed in all three exon 3-deleted parkin mice, and has also not been reported in exon 2-deleted parkin knockout mice (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Perez & Palmiter, 2005). The reason for the discrepancy between the phenotype of the exon 7-deleted and the exon 2- or 3-deleted parkin mice is not known. In mouse brain, only one 3.3-kb parkin transcript has been found from exon 1 through to exon 12 (Kühn *et al.*, 2004). Transcription is under the control of a single promoter that is upstream of exon 1 of the mouse parkin gene (West & Maidment, 2004). There is no indication for the existence of a second promoter downstream of exon 3 of the parkin gene that could initiate a truncated parkin protein without the portion encoded by exons 1–3. Alternative splice variants without parkin exon 3 in mouse brain have been described in a

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previous study using reverse transcriptase-polymerase chain reaction (RT-PCR; Kühn *et al.*, 2004). von Coelln *et al.* (2004) speculate that these splice variants might still display intact parkin function. They argue that the occurrence of such splice forms may compensate the loss of the major 3.3-kb parkin transcript caused by the exon 3-deletion, and prevent neuronal degeneration in the LC. However, the existence of those parkin variants could neither be detected on the mRNA level by Northern blotting nor the protein level by Western blotting, neither in wild-type (Kühn *et al.*, 2004) nor in exon 3-deleted mouse brain (Goldberg *et al.*, 2003). It is known that very long heteronuclear (hn)RNA, such as that of parkin, is prone to splicing errors, and it must be taken into consideration that alternative splicings found by RT-PCR may simply be due to splicing errors. Therefore, a compensation of parkin function in exon 3-deleted mice by those splice variants is rather unlikely, and the deletion of exon 3 should result in the loss of parkin function. However, we cannot rigorously exclude the possibility that such splice variants exist at an extremely low level, and may partially compensate the loss of the major form of parkin in our knockout mice. Nevertheless, the analysis of the *quaking* mutant mice, which lack the entire parkin gene, did not show a loss of LC neurons either (Le Saux *et al.*, 2002). This renders it rather unlikely that the described loss of neurons in the LC is related to the exon 7-deletion in this mouse line.

Despite the phenotypic discrepancy in the LC, all five parkin-deficient mice did not exhibit degeneration of dopaminergic neurons (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Perez & Palmiter, 2005). In particular, dopaminergic neurons in the SN pars compacta were morphologically intact in all parkin knockouts, indicating that parkin is not essential for the survival of nigral dopaminergic neurons in mice. One reason why parkin-deficient mice do not induce degeneration of dopaminergic neurons may be a lack of appropriate environmental triggers. To investigate whether parkin dysfunction may cause enhanced susceptibility of dopaminergic neurons against neurotoxic insults, we challenged parkin-deficient mice with the neurotoxin MPTP that specifically triggers the degeneration of DA neurons and nerve terminals. No increased MPTP-induced degeneration of TH-positive axons in the ST was seen in parkin null mutants, indicating that DA neurons of parkin-deficient mice are not more sensitive to MPTP toxicity. Our result is consistent with a recent report showing that parkin dysfunction does not modify the susceptibility of DA neurons to acute methamphetamine or 6-hydroxydopamine neurotoxicity (Perez *et al.*, 2005). However, both studies cannot rule out the possibility that neurotoxins acting through different mechanisms could reveal an increased susceptibility of parkin-deficient mice to specific environmental factors.

The lack of degeneration of dopaminergic neurons and abnormal phenotypes such as a loss of body weight, reduced DAT function, nigrostriatal, cognitive, noradrenergic and mitochondrial dysfunction in parkin-deficient mice were previously reported for four independent mouse lines (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Palacino *et al.*, 2004; von Coelln *et al.*, 2004). However, many of the observations described in these studies were not reproduced in the study by Perez & Palmiter (2005). This phenomenon is continued by our study, where we did not reproduce the loss of body weight and the reduced function of DAT reported by previous studies (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Palacino *et al.*, 2004). Some of these discrepancies may be caused by experimental differences in different labs. A reason for such discrepancies may also be the higher portion of the 129-derived genetic background (50%) of the non-isogenic F1 generation used in all other studies and less 129-derived background (6%) in our parkin-deficient mice. Highly mixed genetic background may cause different phenotypes in parkin-deficient mouse lines. Findings are more likely to depend on the segregation of 129- vs C57BL/6-derived genes than in mice with reduced genetic heterogeneity. Even though the F4-generation used in our study is still not a genetically clean background (statistically, 6% of the 129-derived genetic background remains), it may already help to distinguish 'real' phenotypes caused by the parkin knockout from those that may be related to genetic differences between the knockout mice and the controls used. Therefore, we have as diligently as possible tried to overcome the potential shortcoming of the F4-background by using as controls mates of the exact same litters rather than a parallel breeding line. This study provides the first evidence for non-motor behavioural impairments in parkin-deficient mice. These deficits may not be attributed to motor dysfunction, as parkin mutant mice did not exhibit impairments in basic locomotion and general motor skills in the rotarod and in the open field tests.

Psychiatric complications, including depression, anxiety and psychosis, have been reported in patients with PD (Ishikawa & Tsuji, 1996; Hattori *et al.*, 1998; Khan *et al.*, 2003; Tretter *et al.*, 2004), with rates for depression ranging from 20 to 50% of the patients (Oertel *et al.*, 2001; Schrag *et al.*, 2001), anxiety disorders from 20 to 40% (Aarsland *et al.*, 1999), and psychosis from 15 to 20% (Aarsland *et al.*, 1999). The observed thigmotaxic behaviour of parkin-deficient mice in our studies could be caused by a number of factors, including the increased anxiety. The open field and light/dark transition tasks are unconditioned response tests, and widely used for description of fear- and anxiety-related behaviours in rodents, which have analogies to acute anxiety episodes in humans (Crawley, 2000). Therefore, the increased thigmotaxic behaviour and the anxiety-related impairments of parkin-deficient mice in our studies may reflect anxiety disorders associated with PD. Similar behavioural impairments have not been described for the other four parkin-deficient mouse lines (Goldberg *et al.*, 2003; Itier *et al.*, 2003; von Coelln *et al.*, 2004; Perez & Palmiter, 2005). The absence of these phenotypes could be a consequence of the mixed genetic backgrounds (50% 129 and 50% C57BL/6) in the mouse strains tested by these authors. It is well established that a great difference exists between the thigmotaxic behaviours of 129 and C57BL/6 strains (Ralph *et al.*, 2001). Because 129 mice display very strong thigmotaxic behaviour in the open field, it is conceivable that the high and variable portion of the 129 genome present in the F1-generation of parkin-deficient mice and their non-transgenic littermates may mask the moderate increase in thigmotaxis caused by the loss of parkin.

Cognitive deficits have recently been identified in parkin-linked patients with PD (Kaasinen *et al.*, 2001; Benbunan *et al.*, 2004). Parkin null mutant mice exhibited mild cognitive deficits, which became visible when stringent criteria were applied to mouse behaviour in the spatial learning test. The observed reduction in the number of crossings indicated a moderate impairment of spatial learning. Although a possible impairment of visual acuity in parkin-deficient mice cannot be ruled out by the present study, this possibility seems unlikely, as the mutant and wild-type mice spent equal time in the TQ indicating they could recognize spatial cues during the test. Lack of habituation and decreased exploratory behaviour of parkin-deficient mice in the open field test also indicated that mutant mice were less willing to explore and/or less able to remember what they had recently explored. This might reflect an abnormal learning ability and/or influence caused by increased anxiety or decreased exploratory behaviour of the mutant mice. The high *n*-size required to demonstrate the significant effect in the open field suggests a moderate impairment of habituation in parkin-deficient mice. A deficit in working memory was also observed in another exon 3-deleted F1-parkin mouse line

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(Itier *et al.*, 2003). The spatial learning in the water maze task was previously examined with an exon 2-deleted F1-parkin mouse line by measurement of the escape latency during the training (Perez & Palmiter, 2005). Although no deficit in the training could be found, the following probe trial in which crossings over the former platform location are counted and which represents the most rigorous test for the spatial learning was unfortunately not mentioned in the report by Perez & Palmiter (2005).

Interestingly, similar thigmotaxic behaviour in the water maze task was also observed in rats with striatal lesions (Devan *et al.*, 1999), suggesting that a striatal component may contribute to the impairment of thigmotaxic behaviour. Indeed, we found elevated levels of the DA metabolite HVA over DOPAC, indicating an increased extraneuronal DA turnover in the ST. This may contribute to the behavioural impairments of *pa–/–* (F4) mice in the water maze task. The observation that the extracellular turnover of DA is increased in the ST of *pa–/–* (F4) mice renders the question if parkin is associated with the regulation of DA release. Increased extracellular DA levels in the ST of parkin-deficient mice have also been reported in an independent study (Goldberg *et al.*, 2003). In addition, the *quaking* mutant mice show increased levels of DOPAC and HVA in the ST. Similar to our *pa–/–* (F4) mice, the DA content in the brains of these mice is unchanged, and the HVA/DOPAC ratio seems also increased (Nikulina *et al.*, 1994). These findings further strengthen the notion that abolition of parkin function causes increased DA release. This is consistent with our observation that the increased thigmotaxic behaviour of parkin-deficient mice in the open field task could be mimicked by the DA releaser amphetamine in wild-type control mice. Taken together, these results suggest that the behavioural changes reported here may be caused by a dysfunction of the dopaminergic system. We cannot, however, exclude the possibility that alterations in other neuronal circuits contribute to the observed behavioural deficits.

Despite the non-motor deficits in the parkin null mutant mice, a degeneration of dopaminergic neurons in the SN was not found in any of the parkin knockout mice described up to date. The variable age of onset in AR-JP, potential compensatory effects, or biological differences between human and mouse have been proposed as explanations for the absence of DA neuron degeneration in mice (Goldberg *et al.*, 2003; Itier *et al.*, 2003). However, mouse models of human neurodegenerative diseases are designed to replicate one or more components of the disease, rather than modelling all aspects of it. Behaviours involving striatal functions, such as exploration and habituation to a novel environment or the ability to learn a stimulus–response paradigm, may be useful in assessing striatal dopaminergic function. Because motor system organization differs in rodents and humans, the relevance of a particular phenotype is more related to its relationship to striatal dopaminergic function rather than its apparent similarity to a symptom of PD (Dauer & Przedborski, 2003).

In summary, our parkin null mutant mouse model displays non-motor behavioural impairments that are similar to behaviour seen in PD. The biochemical alterations observed in the brain dopaminergic system of *pa–/–* (F4) mice may be related to the behavioural impairment. Therefore, this mouse model may be useful to identify the early effects of a PD-causing mutation *in vivo*.

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Abbreviations

5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; AR-JP, autosomal recessive mutations in the parkin gene results in early-onset parkinsonism; ECD, electrochemical detection; DA, dopamine; DAT, dopamine transporter; DOPAC, dihydroxyphenylacetic acid; GFAP, glial fibrillary acidic protein; HPLC, high-perfomance liquid chromatograghy; HVA, homovanillic acid; LBs, Lewy bodies; LC, locus coeruleus; MPTP, 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine; NE, norepinephrine; *pa–/–* (F4), parkin knockout mouse strain etablished by three generations backcrossing the F1 hybrid *pa+/–* mouse with C57BL/6; PD, Parkinson's disease; RT-PCR, reverse transcriptase-polymerase chain reaction; SN, substantia nigra; ST, striatum; TH, tyrosine hydroxylase; TQ, target quadrant.

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