Loss of PINK1 Function Affects Development and Results in Neurodegeneration in Zebrafish

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Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder in the Western world. PTEN (phosphatase/tensin homolog on chromosome 10)-induced putative kinase 1 (PINK1), a putative kinase that is mutated in autosomal recessive forms of PD, is also implicated in sporadic cases of the disease. Although the mutations appear to result in a loss of function, the roles of this protein and the pathways involved in PINK1 PD are poorly understood. Here, we generated a vertebrate model of PINK1 insufficiency using morpholino oligonucleotide knockdown in zebrafish (*Danio rerio*). PINK1 knockdown results in a severe developmental phenotype that is rescued by wild-type human PINK1 mRNA. Morphants display a moderate decrease in the numbers of central dopaminergic neurons and alterations of mitochondrial function, including increases in caspase-3 activity and reactive oxygen species (ROS) levels. When the morphants were exposed to several drugs with antioxidant properties, ROS levels were normalized and the associated phenotype improved. In addition, GSK3 β -related mechanisms can account for some of the effects of PINK1 knockdown, as morphant fish show elevated GSK3 β activity and their phenotype is partially abrogated by GSK3 β inhibitors, such as LiCl and SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)1H-pyrrole-2,5-dione]. This provides new insights into the biology of PINK1 and a possible therapeutic avenue for further investigation.

Key words: Parkinson's disease; zebrafish; neurodegeneration; mitochondria; ROS; PINK1

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder in the Western world. To date six genes with pathogenic mutations have been identified as contributing to Mendelian forms of PD. These include two autosomal dominant genes, α -synuclein (*SNCA*) and leucine-rich repeat kinase 2 (*LRRK2*), and three autosomal recessive genes, *parkin*, *DJ-1*, and phosphatase/tensin homolog on chromosome 10 (*PTEN*)-induced putative kinase 1 (*PINK1*) (Valente et al., 2004; Klein and Schlossmacher, 2006). An apparently dominant mutation in a sixth gene, ubiquitin C-terminal hydrolase L1 (*UCHL1*), has only been found in one small family and its relevance to familial PD is still debated (Healy et al., 2006). *PINK1* mutations are the second most common cause of autosomal recessive PD after *parkin*

(Li et al., 2005), and *PINK1* is also implicated in sporadic cases of the disease (Valente et al., 2004). *PINK1* is a highly conserved 581 aa protein that is widely expressed in the human brain and localizes to mitochondrial membranes via an N-terminal targeting motif (Gandhi et al., 2006). It shares homology with calmodulin-dependent protein kinase 1, contains a catalytic serine–threonine kinase domain, and has autophosphorylation activity *in vitro* (Sim et al., 2006). PD-causing mutations appear to result in loss of function (Sim et al., 2006).

Recent studies of *PINK1* in *Drosophila melanogaster* suggest its importance for mitochondrial function. Loss-of-function mutants of *PINK1* exhibit male sterility, muscle and dopaminergic neuron degeneration, and increased sensitivity to stressors (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). *PINK1* mutant flies have decreased expression of *parkin* and have phenotypes similar to flies with loss of *parkin* function. Because overexpression of *parkin* rescues phenotypes caused by *PINK1* deficiency, but not vice versa, it has been proposed that *parkin* acts downstream of *PINK1*, also suggesting that understanding *PINK1* function may be relevant for PD caused by either *parkin* or *PINK1* mutations. The molecular targets of *PINK1* kinase activity are poorly studied. Recently, the phosphorylation of mitochondrial molecular chaperone TRAP1 was attributed to *PINK1* (Pridgeon et al., 2007), further strengthening the mitochondrial relevance of *PINK1*.

Materials and Methods

Zebrafish strain husbandry and in situ hybridization. Wild-type zebrafish of the AB strain were maintained at 28.5°C under standard conditions

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(Westerfield, 2000) in compliance with Animal Welfare legislation. Embryos were collected after natural spawning, staged as described previously (Kimmel et al., 1995), and raised in embryo medium (Westerfield, 2000). Whole-mount *in situ* hybridization was done as described previously (Jowett and Lettice, 1994). Antisense digoxigenin-labeled RNA probes were generated using cDNAs of *pink1*, *parkin*, *reelin* (Costagli et al., 2002), *fezl* (Levkowitz et al., 2003), and neurogenin-1 (*ngn-1*) (Blader et al., 1997). All husbandry and experimental procedures were performed in accordance with the Animals (Scientific Procedures) Act.

In silico identification of zebrafish PINK1 orthologue. To identify the zebrafish *PINK1* orthologue, a basic local alignment search tool (BLAST) search of the Ensembl zebrafish cDNA database (http://www.ensembl.org/Danio_rerio/index.html) was performed using the human *PINK1* amino acid sequence (ENSG00000158828). Based on the obtained sequences, primers were designed to amplify the complete sequence of the zebrafish gene. mRNA, isolated from different age embryos, was reverse transcribed with the First-Strand Synthesis Kit (Invitrogen). PCR products were analyzed by agarose gel electrophoresis and sequenced.

Morpholino and mRNA injections and drug treatment. Morpholino oligonucleotides (MOs) (ATG targeting, 5'-GCT GAG AAC ATG CTT TAC TGA CAT T-3'; 5'-untranslated region (UTR) targeting, 5'-ATA TTG ACT ATG AGA GGA AAT CTG A-3'; ATG-targeting five-mispair control, 5'-GCT CAC AAC ATC CTT TAG TGA GAT T-3'; 5'-UTR targeting five-mispair control, 5'-ATA TTC AGT ATC AGA GCA AAT GTG A-3') targeting either the ATG or 5'-UTR region of the zebrafish *PINK1* gene were obtained from Gene Tools and dissolved in 1× Danieau medium [containing (in mM) 58 NaCl, 0.7 KCl, 0.4 MgSO₄, 0.6 Ca(NO₃), 2.5 HEPES, pH 7.6]. One-cell stage embryos were injected with a combination of ATG and 5'-UTR morpholinos at 7, 9, and 13 ng each. Five-mispair control morpholinos were used to determine the concentrations of injected morpholino that produced phenotypes that were distinct from morpholino toxicity. Additional controls to monitor the quality of the injection technique included injections of a standard control morpholino (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'), which targets a splice site in the human β -globin gene (negative control), and a morpholino targeting the zebrafish chordin gene (5'-ATC CAC AGC AGC CCC TCC ATC ATC C-3') (positive control) (all from Gene Tools).

Capped mRNAs were synthesized from cDNAs cloned into pcDNA/V5-DEST, encoding human full-length wild-type *PINK1* gene using the T7 mMessage mMachine kit (Ambion). Sites corresponding to the clinically relevant mutations A168P and W347X were altered using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Sixty-five picograms of mRNA were coinjected together with *PINK1* MOs into embryos at the one-cell stage.

For the pharmacological rescue experiments, LiCl (0.5–50 mM; Sigma-Aldrich), 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763; 1–10 μ M; Tocris Bioscience), reduced glutathione (GSH; 100 μ M) and *N*-acetyl-cysteine (NAC; 100 μ M) were dissolved in DMSO or water and were introduced into the embryonic media 6 h after morpholino injection. The final concentration of DMSO was 0.2% in the water. Fish were analyzed 24 and 48 h after injection.

For quantification of the rescue results, data were gathered from 15–20 embryos at each stage and age. The normal phenotype was expressed as 100%, and the changes in the morphology were scored and calculated accordingly. Data from at least three experiments were pooled for statistical analyses.

We used five-mispair control MOs as the “control state” in the present study unless indicated otherwise. Generally, five-mispair control MO at the concentration used did not produce any phenotype different from naive fish.

Immunohistochemistry and PAGE. Immunohistochemistry for acetylated tubulin (Sigma-Aldrich), tyrosine-hydroxylase, serotonin (5-HT) (Immunostar), and PINK1 (Cayman Chemicals) was performed as described previously (Kaslin and Panula, 2001). Images were taken using an Olympus SZX12 or BX51 fluorescence microscope equipped with digital camera, and processed using AnalySIS^D 5.0 software (Soft Imaging System). No adjustment of brightness or contrast was made after image acquisition. For Western blotting, embryos of 24–48 h postfertilization

(hpf) were dechorionated, deyolked (Link et al., 2006), homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors, and processed for SDS-PAGE. Immunoreactive bands were detected with ECL reagent (GE Healthcare Life Sciences) and chemiluminescent signal was visualized by the exposing membrane to ECL Hyperfilm (GE Healthcare Life Sciences). Films were scanned using a desktop scanner HP ScanJet 3800, and densitometric analysis of blots was performed using ImageJ software (National Institutes of Health). The background intensity of the film was subtracted from the band intensity. At least three separate experiments were analyzed, and band intensities were normalized to the loading control band intensity. Quantification of tyrosine hydroxylase (TH)-positive neurons was done in 24 and 48 hpf whole-mount, deyolked, flat-mounted embryos by counting all fluorescent cell bodies in the brain using a 40× objective. The number of neurons in control five-mispair MO-injected embryos was presented as 100%, and the changes in the *PINK1* MO group was calculated accordingly. Each experiment analyzed at least seven embryos in each group, and data from at least four experiments were pooled for the statistical analyses.

Detection of cell death. Apoptotic/necrotic cells were detected by incubation of live 24- to 48-hpf-old embryos in acridine orange solution (5 μ g/ml) (Furutani-Seiki et al., 1996). Cells loaded with the dye were visualized using a tetramethylrhodamine isothiocyanate filter on the Olympus SZX12 fluorescent microscope. To detect the activity of caspase-3 in zebrafish embryos, fish were homogenized in ice-cold RIPA buffer and the homogenate was mixed with an equal volume of 0.39 mM acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Sigma-Aldrich), a selective caspase-3 substrate, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). The excitation and emission wavelengths of AMC (360 and 460 nm, respectively) were detected using a Victor³ plate reader (PerkinElmer Life And Analytical Sciences). The reactions were normalized with total protein concentrations. Embryos treated with UV light (400 μ J/cm², 0.1 s) for 6 h before the assay served as a positive control of apoptosis-induced caspase-3 activation. Incubation with 100 μ M pan-caspase-3 inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD; R&D Systems) was used as a negative control.

Reactive oxygen species (ROS) accumulation, assessed by the levels of the oxidized form of the cell-permeant ROS indicator acetyl ester of 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Invitrogen), was detected in live fish as follows. Injected embryos were dechorionated, several groups of three embryos were put in the wells of a 96-well plate, and excess medium was removed using a fire-polished glass Pasteur pipette. Embryos were incubated in 1 μ g/ml CM-H₂DCFDA for up to 2 h, and the fluorescence was measured with a Victor³ plate reader (PerkinElmer Life And Analytical Sciences) (excitation 485 nm, emission 560 nm) after every 10 min. The spontaneous oxidation of CM-H₂DCFDA was monitored in the wells without fish, and the levels were low and did not change with time. H₂O₂ solution served as a positive control.

Isolation of mitochondria. Crude mitochondrial and cytoplasmic fractions were isolated using a Mitoiso 1 isolation kit (Sigma-Aldrich). Briefly, embryos 80–100 24 hpf were collected, dechorionated, and homogenized in the supplied extraction buffer (10 mM HEPES buffer, pH 7.5, containing 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 2 mg/ml BSA). Homogenates were spun at 600 × g in 4°C, and supernatants were then spun at 11,000 × g for 10 min in 4°C. Supernatants, representing cytoplasmic fractions, were removed and stored in ice. Pellets were resuspended in extraction buffer, and the centrifugations were repeated. The resulting pellets were resuspended in storage buffer [10 mM HEPES buffer, pH 7.5, containing (in mM) 250 sucrose, 1 ATP, 0.08 ADP, 5 sodium succinate, 2 K₂HPO₄, and 1 DTT], and the protein concentration was assayed using the Bradford method. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) uptake, an indicator of mitochondrial $\Delta\psi$ membrane potential, was then assessed by incubation adjusted by protein levels. The preparations were incubated for 10 min in the dark, and fluorescence was detected using a Victor³ plate reader (PerkinElmer Life And Analytical Sciences) (excitation, 490 nm; emission, 525 and 590 nm). The ratio of 590/530 nm (orange/green)

Figure 1. Characterization of zebrafish *PINK1*. **A**, RT-PCR analysis of *PINK1* mRNA expression at different hours after fertilization. **B**, *In situ* hybridization showing expression of zebrafish *PINK1* mRNA in 24 and 48 hpf embryos. Right, Negative control staining with sense probe. **C**, Immunohistochemical analysis of *PINK1* protein (green) and TH (red) in adult zebrafish brain (*i–iv*). *PINK1* protein is localized ubiquitously with the tendency to be enriched along ventricular surfaces. In some areas (hypothalamus), *PINK1* protein is colocalized with tyrosine hydroxylase. **i**, Posterior hypothalamus; **ii**, optic tectum; **iii**, areas around diencephalic ventricle; **iv**, posterior tuberculum (*PINK1* protein, red; TH, green). **D**, The *PINK1* antibody used (Cayman Chemicals, catalog #10006283) detects bands of ~66 and ~33 kDa in mouse liver mitochondria preparations. Similarly sized bands are seen in the wild-type zebrafish, and these disappear in *PINK1* MO-treated fish. The trend in this gel is representative of at least five replicate experiments. **E**, *PINK1* morphants at 24 hpf after injection of 7 ng of MOs show long-tail, short-tail, and no-tail phenotypes. Five-mismatch MO control zebrafish look the same as uninjected siblings. **F**, Phenotype of *PINK1* morphants at 48 hpf after injection of 7 ng of MO. Enlarged brain ventricle, slim tail-yolk extension, and curved spines are indicated by arrows in mildly (middle) and severely (right) affected morphants. Scale bars: **B**, **E**, **F**, 100 μ m; **C**, 50 μ m.

was considered as an indicator of mitochondrial $\Delta\psi$ membrane potential. Incubation with 1 μ g/ml valinomycin resulted in decrease of JC-1 orange/green fluorescence, and was used as a positive control. The purity of the resulting preparation was assessed, using actin as a control for

cytoplasm, and porin as a mitochondrial loading control (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Statistics. Pooled estimates for the changes in tail phenotype, resulting from perturbations assessed in multiple experiments, were calculated as

Figure 2. Morphological characterization of PINK1-deficient fish. **A, B**, Acetylated tubulin immunostaining in MO-injected (**A**) and five-mispair MO control (**B**) embryos at 48 hpf. The decrease in staining in the tectal area is indicated by an arrow. **C, D**, TH and 5-HT staining in representative MO-injected (**C**) and five-mispair MO control (**D**) embryos at 48 dpf. The diencephalic cluster of dopaminergic cells is outlined. **E**, *In situ* hybridization of PINK1 MO-injected and control embryos at 48 hpf. The increase in *fezl* and *ngn-1* expression in morphants is indicated by arrows. **F, G**, Immunohistochemistry with antibody 40.2D6 (islet-1) in the trunk of control (**F**) and MO-injected (**G**) fish, showing decreased staining in MO fish. **H, I**, Acetylated tubulin staining in the trunk of control (**H**) and MO-injected (**I**) fish. Changes in the morphology (e.g., decrease in neuron numbers) of spinal neurons are indicated by arrows. RB, Sensory Rohon-Beard neuron; MN, primary motoneuron. Scale bars, 100 μ m.

odds ratios with 95% confidence intervals. We used this method frequently in the past to allow analysis of data from multiple independent experiments (Wyttenbach et al., 2001, 2002; Carmichael et al., 2002). Odds ratios and *p* values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software. In other experiments, two-way ANOVA and *t* test (GraphPad Prism 4) were used when applicable.

Results

Identification of zebrafish *PINK1*

Here, we inhibited translation of *PINK1* in zebrafish with MOs to examine its roles in vertebrate development. To identify the ze-

brafish gene, we performed BLAST searches with the human *PINK1* protein sequence (NP_115785) in the Ensembl zebrafish database and identified putative zebrafish *PINK1* transcripts, which were confirmed by PCR cloning. Zebrafish *PINK1* (NM_001008628) encodes a 574 aa protein with a predicted molecular mass of 64 kDa and is 54% identical to the human *PINK1* protein (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Analysis of the zebrafish primary sequence revealed a putative mitochondrial targeting domain at the N terminus of the protein with a possible cleavage site at amino acid 88 (MitoProt) (Claros and Vincens, 1996) and a putative

Figure 3. Rescue of *PINK1* MO phenotype with full-length human *PINK1* mRNA. **A**, Rescue of the overall MO phenotype at 48 hpf by coinjected full-length human *PINK1* mRNA. The phenotypes assessed (curved back, small tail–yolk extension) are indicated with arrows. Scale bar, 100 μ m. **B**, Coinjection of MOs with human full-length mRNA partially restores PINK1 levels in 24 hpf embryos. **C**, Coinjection of mutant human mRNA did not produce a rescue of PINK1-deficient phenotype. The bars represent changes in the assessed phenotype relative to the MO-induced phenotype in three independent experiments. Error bars indicate SEM.

Ser-Thr protein kinase active site at amino acids 346–358, which is highly similar to human PINK1 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Using RT-PCR, zebrafish *PINK1* mRNA was detected as early as 2.5 hpf and throughout development up to 144 hpf (Fig. 1A). *In situ* hybridization detected *PINK1* mRNA that was distributed fairly uniformly in zebrafish larvae at 24 and 48 hpf, with increased intensity in the nervous system and muscle (Fig. 1B). Polyclonal antibodies raised against human PINK1 peptide detected signals within the paraventricular regions of the adult zebrafish brain, including the hypothalamus and the deep layers of the optic tectum (Fig. 1C). In some areas (e.g., the hypothalamus), we observed overlapping signals for PINK1 and TH, suggesting the presence of PINK1 in some dopaminergic neurons. We detected two PINK1-positive bands in the whole zebrafish lysates: a smaller one, ~33 kDa, and a larger one, ~66 kDa. Injection of *PINK1* MOs led to a dose-dependent decrease of the intensity of the PINK1 bands from 24 hpf fish (Fig. 1D). We observed these bands in both cytoplasmic and mitochondrial fractions of whole 24 hpf embryos homogenates (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). PINK1 bands approximating the size of the smaller fragment we observed in zebrafish have been reported recently in human cell lines (Lin and Kan, 2008).

Phenotype of PINK1 knockdown

To examine morphological changes caused by *PINK1* deficiency, embryos were injected with MOs directed against the 5'-UTR and the ATG start codon of zebrafish *PINK1* (7 ng of each ATG and UTR MO in combination). Using otherwise identical MOs with 5 bp substitutions (control MO), no morphological abnormalities were observed up to 6 d postfertilization (dpf). MOs that block zebrafish PINK1 translation did not affect gastrulation but

resulted in three distinct phenotypic groups at 24 hpf (Fig. 1E). The first group had dark, nontransparent heads but properly developed trunks/tails [“long-tail” group, $13.8 \pm 2.71\%$ (SD) of embryos]; the second group had dark, nontransparent heads, small eyes, and short, curved tails with misshapen somites (‘‘short-tail’’ group, $79 \pm 3.5\%$ of embryos); and the third group appeared to have halted head/tail development (‘‘no-tail’’ group, $6.6 \pm 2.8\%$ of embryos). Injection of higher doses of MO (>10 ng) resulted in an increase in the size of no-tail group and disappearance of the long-tail group. At 48 hpf, the predominant phenotype (with 7 ng of MO) was in the trunk/tail: MO-injected fish had various degrees of ventral curvature of the spine with misshapen tail ends (Fig. 1F). In addition, they had enlarged brain ventricles, small heads, and slim yolk extensions and occasionally manifested cardiac edema. These embryos failed to swim away when touched on the tail (impaired escape response). By 72 hpf, the most severely affected larvae died, whereas surviving larvae showed some recovery. Note that the effects of MOs decrease with time after injection, with most activity being lost by ~5 d. In general,

MO-induced translational inhibition is most efficient during the first 2–3 d of development (Sumanas and Larson, 2002).

Injection of *PINK1* MO resulted in structural alterations in the axonal scaffold of the larval nervous system: commissures were less prominent, sometimes the tegmental commissure failed to develop and the paramedial descending axonal tract in the hindbrain and spinal cord were disorganized (Fig. 2A,B). The number of TH-positive neurons in the diencephalon of MO-injected zebrafish at 48 hpf was reduced to $62.7 \pm 6.05\%$ SEM of control levels 100% ($p = 0.012$; $n = 3$), whereas serotonergic (5-HT) neurons were not affected (Fig. 2C,D, supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Several *in situ* hybridization probes were used to examine neuron development (Fig. 2E). Expression of *reelin*, a general neuronal marker (Costagli et al., 2002), was decreased in *PINK1* morphants. In accordance with the *Drosophila* *PINK1* knock-out data (Yang et al., 2006), *parkin* expression was also reduced in the brain of *PINK1* morphants. However, *fezl* mRNA, one of the factors required for the development of the dopaminergic system in zebrafish, was selectively increased in the posterior diencephalon of *PINK1* morphants. Neurogenin-1, a downstream target of *fezl* (Jeong et al., 2006), was similarly upregulated in *PINK1* morphants (Fig. 2E). In the spinal cord, *PINK1* morphants showed decreased islet-1 and acetylated tubulin staining, suggesting loss of peripheral neurons (Abdelilah et al., 1996; Devine and Key, 2003) (Fig. 2F–I).

To test whether the zebrafish *PINK1* knockdown phenotype was relevant to human *PINK1* function, experiments were performed to ascertain whether the MO phenotype could be rescued by wild-type human *PINK1* mRNA. mRNA (65 pg, which by itself did not cause an obvious external phenotype) was injected together with the MOs and the phenotype scored at 48 hpf (Fig. 3A). The mRNA mostly rescued the *PINK1* MO phenotype as

changes of the shape of tail yolk, curvature of the spine/tail and loss of escape response were reversed. Although 71.7% ($\pm 6.6\%$, SEM) of the MO treated fish had the slim tail yolk/no tail yolk phenotype, this was reduced to 35.8% ($\pm 7.3\%$, SEM; $p < 0.001$; $n = 6$) with coinjection of the human *PINK1* mRNA with the MO. Likewise, the curved spine phenotype in the MO fish ($82.4 \pm 6.3\%$) was improved to 41.8% ($\pm 14.2\%$, SEM; $p < 0.001$; $n = 4$) by coinjection of the human *PINK1* mRNA, and the escape response in the MO fish ($30.9 \pm 8.5\%$, SEM) was improved to 55.3% ($\pm 7.2\%$, SEM; $p < 0.001$; $n = 7$) by coinjection of the human *PINK1* mRNA. In addition, overexpression of human *PINK1* mRNA partially restored decreased levels of PINK1 protein, confirming the specificity of the used antibodies (Fig. 3B, supplemental Fig. 5A, available at www.jneurosci.org as supplemental material). The injection of mRNA into five-mispair control MO-injected embryos at this concentration did not produce obvious changes in the normal phenotype.

Several mutations of *PINK1* that alter its activity have been described in humans (Valente et al., 2004; Silvestri et al., 2005). We introduced two such mutations, A168P and W437X, into the human full-length *PINK1* mRNA. These mutated mRNAs did not rescue the phenotypes observed with *PINK1* MO in the same way that we observed with the wild-type *PINK1* mRNA (Fig. 3C).

PINK1 knockdown influences AKT-GSK3 β axis

The tail phenotype of *PINK1* MO-injected zebrafish at 24 hpf (short tail) looked remarkably similar to *wnt* pathway mutants (e.g., *pipe-tail*) (Rauch et al., 1997). Furthermore, *fezl*, the expression of which is upregulated in our *PINK1* morphants, is also induced in zebrafish embryos expressing the *wnt* pathway inhibitor DKK1 (Hashimoto et al., 2000a). Accordingly, we hypothesized that *PINK1* knockdown enhanced GSK3 β activity (a consequence of *wnt* inhibition). Indeed, *PINK1* morphants showed a decrease in the amount of inactive Serine 9 (Ser9)-phosphorylated forms of GSK3 β (Fig. 4A). Active GSK3 β phosphorylates β -catenin, enhancing its degradation. Consistent with predictions, active β -catenin levels were decreased in *PINK1* morphants (Fig. 4B).

To test whether enhanced GSK3 β activity contributed to the phenotypes we observed in *PINK1* morphants, we treated them with nonspecific (LiCl) and specific (SB216763) GSK-3 β inhibitors. After performing preliminary concentration–response curves with LiCl (supplemental Fig. 5B, available at www.jneurosci.org as supplemental material), we used 50 mM for a series of independent replicate experiments. Both compounds were able to rescue $\sim 20\%$ of short tail morphants at 24 hpf (Fig. 4C). Moreover, treatment with LiCl partially restored decreased levels of active β -catenin in *PINK1* MO embryos (Fig. 4D). However, both treatments failed to reverse the dark-head phenotype.

AKT is an important regulator of GSK3 β activity as it phosphorylates GSK3 β on serine 9, resulting in inhibition of the enzyme. AKT activity, assessed by the amounts of its phosphorylated serine 473 form, was decreased in whole lysates of 24 hpf *PINK1* morphants (Fig. 4E). As expected, LiCl, which rescued tail phenotype of *PINK1* morphants and restored Ser9 GSK3 β levels, did not affect decreased levels of Ser473 AKT.

Loss of PINK1 leads to cell death and accumulation of ROS

PINK1 morphants exhibited dark, nontransparent heads as early as 24 hpf. Later, at 48 and 72 hpf, the heads of MO-injected fish were notably smaller, nontransparent, and occasionally lacking the hindbrain–midbrain boundary. These phenotypes have been attributed to ongoing neurodegeneration (Furutani-Seiki et al.,

Figure 4. Rescue of *PINK1* MO phenotype via modulation of AKT/GSK3 β pathway. **A**, Decrease of Ser9-phosphorylated GSK3 β in lysates of 24 hpf *PINK1* MO embryos. Because this antibody detects both Ser9 GSK3 β (47 kDa) and GSK3 α (51 kDa), the lower band (GSK3 β) was used for quantification. Blots obtained from three independent experiments were used for quantification and the levels of Ser9 GSK3 β were normalized to the corresponding levels of the total GSK3 β . Ser9 GSK3 β phosphorylation was reduced by the morpholino to 27.6% ($\pm 16.56\%$, SEM) of the control level (set to 100%). $p = 0.01$ as analyzed by *t* test. **B**, Decrease of the active form of β -catenin in 24 hpf embryos injected with 7 ng of *PINK1* MO. Active β -catenin bands were quantified as a function of the actin loading control in three independent experiments. Active β -catenin levels were reduced by the morpholino to 40.2% ($\pm 11.1\%$, SEM) of the control levels (set to 100%). $p < 0.01$ as analyzed by *t* test. **C**, Rescue of the short tail phenotype in 24 hpf *PINK1* MO-injected embryos by nonspecific (50 mM of LiCl) and specific (10 μ M of SB216763) GSK3 β inhibitors. Bars represent three individual experiments, with 15–20 fish in each group per experiment. * $p < 0.05$, ** $p < 0.01$, as analyzed by unconditional logistical regression analysis. **D**, Recovery of active β -catenin levels in *PINK1* MO-injected embryos after treatment with 50 mM LiCl. The numbers under the blots are the ratios between the active β -catenin band intensities and actin loading control band intensities in relation to the control values. Independent replicate of the experiment could be seen in Figure 6C. **E**, The activity of AKT is decreased in *PINK1* MO-injected embryos at 24 hpf. Ser473 AKT bands were quantified as a function of total AKT in three independent experiments. *** $p < 0.01$, as analyzed by *t* test. Error bars indicate SEM.

1996). Here, the lack of PINK1 protein led to an increase of acridine orange accumulation in the brain and throughout the body at 24 hpf (Fig. 5A). Because acridine orange accumulation *in vivo* is indicative of the cell death, we examined the activity of caspase-3, which was increased in the whole fish lysates already at 24 hpf (Fig. 5B) above the levels seen in the positive control group (UV-exposed embryos). Exposure to 50 mM LiCl for 18 h led to the partial decrease of caspase-3 activity in 1 dpf *PINK1* MO-injected embryos (Fig. 5C). Administration of the pan-caspase inhibitor zVAD did not rescue the tail phenotype like LiCl did (data not shown), suggesting that although GSK3 β overactivity does contribute to the caspase-3 activation, the effect of GSK3 β inhibitors on the tail phenotype in *PINK1* MO is not related to the caspase-3 activity.

To assess the integrity of the mitochondrial outer membrane,

Figure 5. Increase of apoptosis in *PINK1* MO-injected fish. **A**, Acridine orange staining of MO-injected embryos. Stained cells (fluorescent) represent dead cells. The arrows show increased acridine orange accumulation in brain and spine of short-tail *PINK1* MO-injected embryos. **B**, Caspase-3 activity is increased in MO-injected fish at 24 hpf. Values represent means of three samples, each of which is the total lysate of 15 fish. **C**, LiCl (50 mM) rescues the increased levels of caspase-3 in 24 hpf *PINK1* MO-injected embryos. Values represent means of three samples, each of which is the total lysate of 15 fish. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, as analyzed by one-way ANOVA with Bonferroni *post hoc* test. **D**, The mitochondrial $\Delta\psi$ membrane potential, represented as the ratio 590/530 nm (orange/green) of JC-1, is decreased in *PINK1* morphants. LiCl (50 mM) does not change decreased levels of JC-1 in MO embryos. Bars represent three independent experiments, each of which uses the total lysate of 80–100 24 hpf embryos. Scale bars, 100 μ m. Error bars indicate SEM.

mitochondria were isolated from ~ 100 embryos at 24 hpf and incubated with JC-1. MO-injected embryos displayed an $\sim 30\%$ decreased JC-1 orange/green ratio in the mitochondrial fraction, indicating a lower mitochondrial potential ($\Delta\psi$) (Fig. 5D). Inhibition of GSK3 β with LiCl did not improve mitochondrial potential in *PINK1* MO embryos. *PINK1* morphants also had increased levels of ROS: 24 hpf MO-injected embryos had increased levels of ROS, as detected by accumulation of oxidized CM-H₂DCFDA probe *in vivo* (Fig. 6A). Intracellular oxidation of CM-H₂DCFDA can be caused by various reactive species, including H₂O₂, peroxy products, peroxy radicals, and singlet oxygen. The increased ROS levels caused by the *PINK1* MO were mostly normalized by 50 mM LiCl or antioxidant drugs (100 μ M of either GSH or NAC) (Fig. 6A). In addition, these antioxidant drugs resulted in a small improvement in the tail phenotype (Fig. 6B), and the treated fish looked generally less severely affected by the loss of *PINK1*. As expected, LiCl rescued the decrease in active catenin levels in the *PINK1* MO-treated fish, whereas GSH and NAC did not (Fig. 6C).

Discussion

Here, we describe a vertebrate model of PINK1 insufficiency. Decreased PINK1 activity in embryonic zebrafish resulted in a general developmental delay, aggravated by a severe mispatterning of axonal scaffolds and reduction of neuronal populations, in the dopaminergic system but also in other areas (e.g., spinal mo-

tor neurons). We observed a relatively modest decrease of TH-positive cells in the brains of 2 dpf *PINK1* MO-injected embryos, $\sim 30\%$ from the control. It is difficult to assess whether this decrease is attributable to the developmental retardation of dopaminergic neurons, or to the degeneration of already developed neurons. However, neurodegeneration and increased apoptosis (as judged by acridine orange staining and elevated caspase-3 activity) does occur as a result of *PINK1* knockdown, and it is thus likely that this contributes significantly, at least in part, to the neuronal loss observed.

We found that *PINK1* knockdown was associated with elevation of GSK3 β activity, which contributes to at least some aspects of the knockdown phenotype, because a degree of rescue was seen with GSK3 β inhibitors. Our data are compatible with the elevation of GSK3 β activity attributable to the inhibition of AKT via dephosphorylation at Ser9. AKT is one of main contributors to the phosphorylation of GSK on Ser9, producing the inhibition of the enzyme.

The question remains how the loss of function of mitochondrially located PINK1 would affect the functional activity of cytoplasmic proteins, such as GSK3 β and AKT, and further studies will be required to elucidate the molecular events linking PINK1 to AKT/GSK3 β . However, GSK3 β has been identified within mitochondria (Hoshi et al., 1996), as well as in the cytoplasm. Because the LiCl rescue is restricted to the tail/trunk organization

and development, it is possible that the apparently pleiotropic phenotypes seen in the *PINK1* knock-out fish are related to mitochondrial GSK3 β and general mitochondrial dysfunction. For instance, zebrafish morphants with specific inner mitochondria membrane pathologies display severe tail/trunk anomalies that are similar to those we saw with *PINK1* knock-out (Guo et al., 2004), and *PINK1* RNA is enriched in mitochondria rich tissues such as muscles and brain (Unoki and Nakamura, 2001). The roles of PINK1 in mitochondria that have been elucidated to date are compatible with the pleiotropic phenotype model we proposed. Recently, it was shown that the activation of HtrA2/Omi and consequent protection of mitochondria from stress is PINK1-dependent (Plun-Favreau et al., 2007). In addition, TRAP1 and *parkin* have been shown to operate downstream of PINK1 (Clark et al., 2006; Park et al., 2006; Pridgeon et al., 2007). All of these proteins are involved in the protection of the cell from various insults, further strengthening the idea of PINK1 being the important neuroprotective mitochondrial protein.

We detected two PINK1-immunopositive bands in the lysates of whole zebrafish embryos. The intensity of both bands was decreased after MO injection and increased after overexpression of wild-type human *PINK1* mRNA, supporting the specificity of the observed signals. Whereas the full-length zebrafish PINK1 has predicted molecular mass of 64 kDa and is most likely represented by the bigger band in our experiments, the nature of the

Figure 6. ROS levels are increased in PINK1 MO-injected fish. **A**, Accumulation of the CM-H₂DCFDA dye, as the indicator of ROS levels, in live 24 hpf PINK1 MO-injected embryos. The increased levels of ROS are decreased using 100 μ M GSH or 100 μ M NAC, compounds with antioxidant qualities. Values represent means of three samples, each of which is the total lysate of 15 fish. **B**, Quantification of the short-tail phenotype at 24 hpf in embryos from **A** representing three independent experiments, each of which is the quantification of 15–20 24 hpf embryos. * $p < 0.05$, one-tailed t test. Error bars indicate SEM. **C**, Detection of active catenin in PINK1 MO-injected fish, treated with 50 mM LiCl, 100 μ M GSH, and 100 μ M NAC. Gel is representative of data from two independent experiments.

smaller band (~ 30 kDa) is not clear. The truncation of N terminus of the protein could contribute to the generation of such a fragment (as our antibodies detect C terminus of the protein). Also, in experiments in which films had long exposures, we observed another band (e.g., 50 kDa) (supplemental Fig. 5A, available at www.jneurosci.org as supplemental material), which could represent another splice variant. This sized PINK1 band has been described in the human brain (Gandhi et al., 2006).

Consistent with loss of PINK1 causing increased GSK3 β activity, we observed an increase in the expression of *fezl* (a gene regulated by the wnt/GSK3 β pathway) (Hashimoto et al., 2000b) and its downstream target neurogenin-1 in our PINK1 morphants. Our PINK1 morphants had decreased numbers of dopaminergic neurons, whereas neurogenin-1 overexpression in otherwise normal zebrafish leads to an increase in this neuronal population (Jeong et al., 2006). Thus, it is likely that the phenotype mediated by GSK3 β activity in the brain is independent of neurogenin-1 and is mediated by other pathways regulated by this broad-acting enzyme. We observed no obvious benefit of LiCl treatment on the neurodegeneration in the brain of mor-

phants, in contrast to the rescue seen with the tail/trunk phenotype. These findings raise the possibility that PINK1 deficiency may selectively affect mitochondrial function and consequently, the development of mitochondria-rich tissues.

We observed that zebrafish PINK1 morphants had increased levels of ROS as early as at 24 hpf. This could be caused by mitochondrial malfunction, as the mitochondrial membrane potential, assessed by accumulation of JC-1, was decreased in PINK1 morphants. However, we cannot exclude the possibility that increased ROS levels result from activation of cell death pathways in these fish. We have attempted to test whether the GSK3 β and ROS pathology seen in the PINK1 morphants were related. The antioxidants used did not affect GSK3 β activity. However, LiCl did reduce ROS and caspase-3 levels while not affecting the decrease of mitochondrial membrane potential, suggesting that at least some of the ROS accumulation may be downstream of GSK3 β in this model. Furthermore, LiCl failed to rescue the TH $^{+}$ neuronal deficit in our morphants (data not shown). This suggests that GSK3 β overactivity is related to PINK1 function in peripheral tissues, and the decrease in TH $^{+}$ neurons is primarily attributable to factors distinct from GSK3 β .

In our study, we obtained similar rescue of the PINK1 knock-down phenotype with a “specific” GSK3 β inhibitor or with lithium, which inhibits this enzyme but also has other effects. Although it is very likely that the effects we observed are caused by GSK3 β inhibition, they are probably not caused by a complete inhibition of enzyme activity. The inhibition of GSK3 α and β activity results in severe apoptosis in the CNS, accompanied with abnormalities in body axis formation and heart development (Lee et al., 2007). These data indicate that the complete inhibition of this enzyme results in very severe consequences. [It would not really be informative to try to test whether Akt overexpression had similar effects to lithium, as Akt has protective effects independent of GSK3 β signaling and has diverse signaling consequences (in addition to GSK3).]

The reduction of PINK1 levels in zebrafish also led to a decreased expression of *parkin* (Fig. 2F). In future studies it will be interesting to test whether overexpression of *parkin* can rescue aspects of the PINK1 knockdown phenotype in a similar way to what was described previously in *Drosophila* (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). However, such studies will be a major undertaking, as the expression patterns of zebrafish *parkin* are uncharacterized, and as it is not clear whether zebrafish *parkin* is functionally orthologous to human *parkin*, knockdown and overexpression experiments will be required for robust interpretations.

In conclusion, decreasing levels of PINK1 protein produces significant developmental and neurochemical alterations in embryonic zebrafish. These alterations are clearly associated with mitochondrial alteration, leading to the activation of caspases and accumulation of ROS and with activation of GSK3 β . These data lead to insights into normal PINK1 function, which is believed to be lost in PD cases with mutations in this gene (Sim et al., 2006). Our data raise the speculative possibility that drugs like lithium chloride and more specific GSK3 β inhibitors, as well as antioxidants, may be of value in some forms of PD, particularly in familial cases caused by PINK1 mutations.

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