



# Mitochondrial and calcium perturbations in rat CNS neurons induce calpain-cleavage of Parkin: Phosphatase inhibition stabilizes pSer<sup>65</sup>Parkin reducing its calpain-cleavage

Hu Wang<sup>1</sup>, Fanny Cheung<sup>1</sup>, Anna C. Stoll, Patricia Rockwell, Maria E. Figueiredo-Pereira\*

Department of Biological Sciences, Hunter College and Graduate Center, City University of New York, NY 10065, USA

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## ABSTRACT

Mitochondrial impairment and calcium (Ca<sup>++</sup>) dyshomeostasis are associated with Parkinson's disease (PD). When intracellular ATP levels are lowered, Ca<sup>++</sup>-ATPase pumps are impaired causing cytoplasmic Ca<sup>++</sup> to be elevated and calpain activation. Little is known about the effect of calpain activation on Parkin integrity. To address this gap, we examined the effects of mitochondrial inhibitors [oligomycin (Oligo), antimycin and rotenone] on endogenous Parkin integrity in rat midbrain and cerebral cortical cultures. All drugs induced calpain-cleavage of Parkin to ~36.9/43.6 kDa fragments. In contrast, treatment with the proinflammatory prostaglandin J2 (PGJ2) and the proteasome inhibitor epoxomicin induced caspase-cleavage of Parkin to fragments of a different size, previously shown by others to be triggered by apoptosis. Calpain-cleaved Parkin was enriched in neuronal mitochondrial fractions. Pre-treatment with the phosphatase inhibitor okadaic acid prior to Oligo-treatment, stabilized full-length Parkin phosphorylated at Ser<sup>65</sup>, and reduced calpain-cleavage of Parkin. Treatment with the Ca<sup>++</sup> ionophore A23187, which facilitates Ca<sup>++</sup> transport across the plasma membrane, mimicked the effect of Oligo by inducing calpain-cleavage of Parkin. Removing extracellular Ca<sup>++</sup> from the media prevented oligomycin- and ionophore-induced calpain-cleavage of Parkin. Computational analysis predicted that calpain-cleavage of Parkin liberates its UbL domain. The phosphagen cyclocreatine moderately mitigated Parkin cleavage by calpain. Moreover, the pituitary adenylate cyclase activating peptide (PACAP27), which stimulates cAMP production, prevented caspase but not calpain-cleavage of Parkin. Overall, our data support a link between Parkin phosphorylation and its cleavage by calpain. This mechanism reflects the impact of mitochondrial impairment and Ca<sup>++</sup>-dyshomeostasis on Parkin integrity and could influence PD pathogenesis.

## 1. Introduction

Parkinson's disease (PD) is a multifactorial disorder [16] in which mitochondrial impairment [24,32] and calcium dyshomeostasis [12,92] play a central causative role. Understanding how mitochondrial impairment and calcium dyshomeostasis contribute to the loss of

neurons in the nigrostriatal dopaminergic pathway is important for developing neuroprotective therapies that slow or prevent neurodegeneration in PD. To this end, we focused on Parkin, an E3 ubiquitin ligase that is important for proteostasis, mitochondrial homeostasis, and survival of neurons that degenerate in PD. Parkin provides neuroprotection by (a) mediating proteasomal-degradation of cytoplasmic

**Abbreviations:**  $\Delta\Psi_m$ , mitochondrial membrane potential; Ca<sup>++</sup>, calcium; C3I, caspase III inhibitor (Z-DEVD-FMK); CCr, cyclocreatine; CHO, Chinese hamster ovary; CPT, calpeptin (Z-Leu-Nle-CHO); DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; Epox, epoxomicin; IBR, in between ring domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OA, okadaic acid; Oligo, oligomycin; PACAP, pituitary adenylate cyclase-activating peptide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PD, Parkinson's disease; PGJ2 and J2, prostaglandin J2; RING, real important novel gene domain; ROS, reactive oxygen species; Rpn, regulatory particle non-ATPase; Rpt, regulatory particle triple-A ATPase; SDS, sodium dodecyl sulfate; s.e., standard error; Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Tom20, translocase of the outer membrane; Ub, ubiquitin; Ub-conjugate, ubiquitin conjugate; UbLD, ubiquitin like domain; UPP, ubiquitin/proteasome pathway; VDAC, voltage-dependent anion channel; VTA, ventral tegmental area

\* Corresponding author at: Department of Biological Sciences, Hunter College and Graduate Center, City University of New York, 695 Park Ave., New York, NY 10065, USA.

E-mail address: [pereira@genectr.hunter.cuny.edu](mailto:pereira@genectr.hunter.cuny.edu) (M.E. Figueiredo-Pereira).

<sup>1</sup> These authors contributed equally to the work.

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proteins, (b) targeting some mitochondrial proteins for proteasomal degradation, and (c) regulating mitochondrial turnover via mitophagy. Mutations in the *parkin* gene (PARK2) are the most common genetic link to PD [17,45]. These mutations act in a loss-of-function manner that impairs the ability of Parkin to ubiquitinate its substrates.

Disruption of Parkin integrity can also account for the loss of its function. Parkin can be cleaved by several proteases. During apoptosis caspase-1, caspase-3 and caspase-8 cleave Parkin at Asp126 resulting in Parkin inactivation [39]. The serine protease HtrA2/Omi, which is located in the mitochondrial intermembrane space [89], cleaves Parkin between the RING1 and IBR domains also inactivating Parkin [64]. Although Parkin fragments are detected in the substantia nigra of PD patients and Lewy bodies from diseased tissue, the fragments remain to be characterized [75,78]. In addition, upon ischemia in mice, Parkin was shown to be rapidly catabolized to unspecified fragments [56]. Importantly, Parkin cleavage by calpain has not been investigated.

To address this gap, we compared the effects of oligomycin, epoxomicin and prostaglandin J2 (PGJ2) on Parkin integrity in rat midbrain and cerebral cortical cultures. Oligomycin (Oligo) is a macrolide antibiotic which binds to a polypeptide in the F0 baseplate and blocks ATP synthesis by the F0/F1 mitochondrial ATP synthase [52]. Epoxomicin (Epo) is a specific and irreversible inhibitor of the proteasome. Epo forms a covalent adduct with the amino terminal Thr of the 20S proteasome catalytic subunits, generating irreversible morpholino adducts [28,55]. PGJ2 is a product of the cyclooxygenase pathway [86]. In rodents, brain levels of PGJ2 are highly induced in vivo upon stroke (cerebral ischemia) [50,51,76] and traumatic brain injury (TBI) [33,46], reaching concentrations [76] that are neurotoxic. Stroke and TBI increase the long-term risk for PD [3,9,37,72,85]. Recently we were able to mimic in mice and rats various aspects of PD pathology, including neurodegeneration, gliosis, and motor impairment by micro-infusing PGJ2 into their substantia nigra [14,67,79]. Together, these studies suggest a role for PGJ2 in PD [25].

When intracellular ATP levels are lowered such as upon Oligo-treatment,  $Ca^{++}$ -ATPase pumps are impaired causing cytoplasmic  $Ca^{++}$  to be elevated leading to calpain activation [8]. Therefore, we also investigated the effect of the calcium ionophore A23187 on Parkin integrity. A23187 increases intracellular calcium levels. The ionophore works mostly by forming stable complexes with divalent cations, which are then able to cross the plasma membrane leading to an increase in the intracellular levels of calcium [18], thus inducing calpain activation [13].

The potential of the pituitary adenylate cyclase activating peptide (PACAP27) was assessed to prevent Parkin cleavage induced by Oligo, Epo and PGJ2. PACAP27 is an endogenous bioactive peptide that is a member of the vasoactive intestinal polypeptide (VIP)-secretin-growth hormone-releasing hormone-glucagon superfamily [31]. PACAP was shown to have neuroprotective effects in several in vivo and in vitro models of neurodegenerative disorders including PD [69]. The neuroprotective effects of PACAP are mediated, in part, by the cAMP/PKA pathway that is known to modulate protein degradation via the UPP [35].

Taking into consideration the structure of Parkin [70,84,90], our data support the notion that upon mitochondrial impairment or calcium dyshomeostasis or under pro-apoptotic conditions, calpain or caspase-cleavage of Parkin removes its N-terminal ubiquitin-like domain (Ubl). Cleavage of the Ubl domain of Parkin may prevent its interaction with the 26S proteasome and favor its mitochondrial recruitment to promote mitophagy. Moreover, full-length Parkin phosphorylated at Ser<sup>65</sup> is less vulnerable to calpain cleavage, while other Parkin phosphorylation sites remain to be assessed. In conclusion, Parkin phosphorylation/dephosphorylation cycle impacts Parkin integrity. Fully understanding its complex regulation and influence on the diverse functions of Parkin, is critical for promoting the survival of neurons that degenerate in PD.

## 2. Materials and methods

### 2.1. Materials

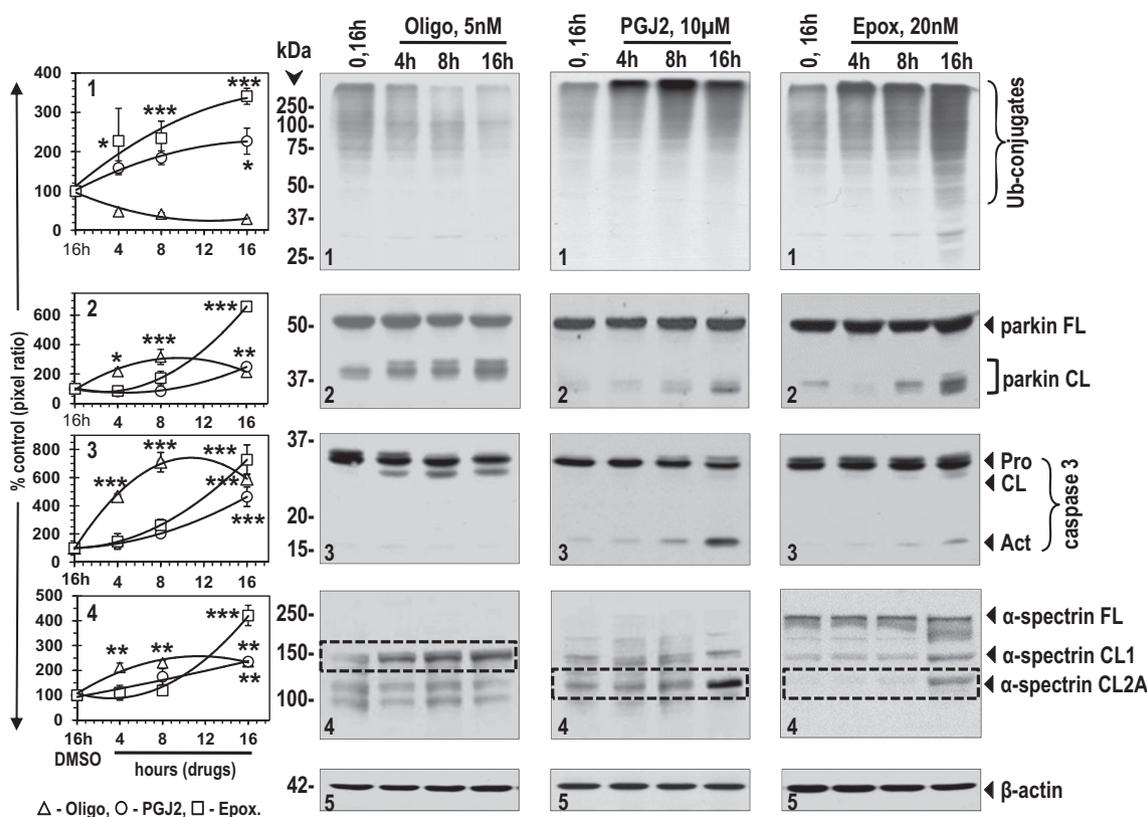
**Inhibitors:** oligomycin A (5 nM), antimycin A (5 nM), and rotenone (20 nM to 50 nM) were from Sigma-Aldrich (St. Louis, MO); epoxomicin (20 nM, Peptides International Inc., Louisville, KY); calpeptin (20  $\mu$ M, Z-Leu-Nle-CHO, Calbiochem/EMD Bioscience, Gibbstown, NJ); caspase-3 inhibitor (2  $\mu$ M, Z-DEVD-FMK) and the proteasome substrate Suc-LLVY-AMC (BACHEM Bioscience Inc., King of Prussia, PA); PGJ2 (10  $\mu$ M) and okadaic acid (400 nM) were from Cayman Chemical, Ann Arbor, MI). **Other drugs:** ionophore A23187 (20  $\mu$ M) from Calbiochem, Gibbstown, NJ; PACAP27 (100 nM, BACHEM Bioscience Inc., King of Prussia, PA) and cyclocreatine (5 mM, Sigma, St. Louis, MO). **Primary antibodies:** rabbit polyclonal anti-Parkin (1:1000, cat#2132) and anti-caspase-3 (1:1000, cat#9662), as well as mouse monoclonal anti-Parkin (Park8, 1:1000 cat#4211) from Cell Signaling Technology, Danvers, MA; rabbit polyclonal anti-ubiquitinated proteins (1:1500, cat# Z0458) from Dako North America, Carpinteria, CA; rabbit polyclonal anti- $\beta$ 5 (1:5000, cat# PW8895) and mouse monoclonal anti-Rpt6 (1:2000, cat# PW9265) from ENZO Life Sciences, Inc., Farmingdale, NY; mouse monoclonal anti- $\beta$ -actin (1:10,000, cat# A-2228, from Sigma, St. Louis, MO); mouse monoclonal anti-spectrin  $\alpha$  chain (1:5000, clone AA6, cat# MAB1622) from Millipore, Billerica, MA; mouse monoclonal anti- $\beta$ III-tubulin (1:10,000, cat# MMS-435P) from Covance, Oakland, CA; rabbit polyclonal anti-HSP90 (1:2000, cat# PA3-013) and anti-pSer<sup>262</sup>-Tau (1:20,000, cat#44-750G) from Thermo Fisher Scientific, Rockford, IL; mouse monoclonal Tau C5 (1:50,000; detects all Tau isoforms; epitope, residues 210–241), courtesy of Dr. L. Binder (Northwestern University, Chicago, IL); rabbit polyclonal TOM20 antibody (1:1000, cat# sc-11,415) from Santa Cruz biotechnology Inc., Santa Cruz, CA; mouse monoclonal fibrillarin antibody (1:1000, cat# ab4566) from Abcam, Cambridge, MA; rabbit polyclonal VDAC antibody (1:1000, cat# PC548) from Calbiochem, Gibbstown, NJ. The anti-pSer<sup>65</sup>-Parkin rabbit monoclonal antibody was raised by Epitomics (Burlingame, CA) in collaboration with the Michael J Fox Foundation (1:10,000), cat# MJF-17-42-4). **Secondary antibodies** with HRP conjugate (1:10,000) from Bio-Rad Laboratories, Hercules, CA.

### 2.2. Primary neuronal cultures

These studies included two different types of primary neuronal cultures obtained from Sprague Dawley rat embryonic (E18, both sexes) midbrain and cerebral cortical neurons. Midbrain isolation was carried-out as described in [68]. Ventral midbrain and cerebral cortical cultures were prepared as we described in [36], details in [60]. Cultures were maintained in Neurobasal media supplemented with 2% B27 and 0.5 mM L-Glutamax (all from Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Half of the medium was changed every 4 days. Experiments were run upon 8–11 DIV. According to manufacturer's specifications, Neurobasal medium contains several proprietary factors that ensure a mostly pure (> 95%) neuronal culture; glial growth is inhibited without a need for the anti-mitotic agent arabinofuranosyl cytidine [7,61].

### 2.3. Culture treatments

Neurons were treated (30 min to 16 h) with DMSO or with the different drugs in DMSO added directly to DMEM (cat# 11960-044) without serum, supplemented with 0.5 mM L-GlutaMAX and 1 mM sodium pyruvate (all from Invitrogen). In some experiments, cells were treated in Calcium-free DMEM (cat# 21068-028, Invitrogen). The final DMSO concentration in the medium was 0.5%.



**Fig. 1.** Effects of oligomycin (Oligo), prostaglandin J2 (PGJ2), and epoxomicin (Epo) on ubiquitinated proteins, and on Parkin, caspase-3 and spectrin cleavages. Rat midbrain neuronal cultures were treated as indicated. Total lysates were analyzed by western blotting (50  $\mu$ g of protein/lane) probed with the respective antibodies to detect: Ub-proteins (panels 1); full-length (FL) and cleaved (CL) Parkin (panels 2); full-length (Pro), cleaved inactive (CL), and cleaved active (Act) caspase-3 (panels 3); full-length (FL), calpain-cleaved (CL1) and caspase-cleaved (CL2)  $\alpha$ -spectrin (panels 4);  $\beta$ -actin (loading control, panels 5). Molecular mass markers in kDa are shown on the left of the blots. Graphs (far left, polynomial order 2) show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for Ub-proteins (1), cleaved Parkin (2), cleaved inactive (for Oligo) and cleaved active (for PGJ2 and Epo) caspase-3 (3), or calpain-cleaved (for Oligo) and caspase-cleaved (for PGJ2 and Epo)  $\alpha$ -spectrin (4), over  $\beta$ -actin (5) for each condition compared to Oligo control (100%). Values are means  $\pm$  s.e. from at least three experiments. Asterisks identify values that are significantly different from Oligo control (one-way ANOVA, Dunnett's multiple comparison test, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001).  $\Delta$  - Oligo,  $\circ$  - PGJ2,  $\square$  - Epo.

#### 2.4. Mitochondrial fractionation

Mitochondrial fractionation was carried-out with a mitochondrial isolation kit cat# 89874 (Pierce, Rockford, IL) following the manufacturer's specifications. Four fractions resulted from this procedure: total lysate, crude nuclear, cytosolic and enriched mitochondrial fractions. Protein concentration was determined with the bicinchoninic acid assay (BCA) kit (Pierce, Rock., IL) for this assay and all others.

#### 2.5. ATP assay

Steady state ATP content was measured with a kit using the sensitive luciferin/luciferase system (Molecular Probes, Carlsbad, CA) and the Luminoskan Ascent microplate luminometer (Thermo Electron Corporation, Waltham, MA) as previously described [36]. ATP levels were normalized for protein concentration.

#### 2.6. Cell viability assay

Cells were treated under various conditions for 1 h to 16 h. Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, [93]) assay as previously described [36].

#### 2.7. Western blotting [details in [60]]

After treatment, cells were rinsed twice with PBS and harvested by gently scraping into hot (100  $^{\circ}$ C) SDS buffer (0.01 M Tris-EDTA, pH 7.5 and 1% SDS) to make sure all intracellular proteins were included. Samples were subjected to a 5 min boil at 100  $^{\circ}$ C followed by brief sonication. After determination of the protein concentration the following was added to each sample (final concentrations):  $\beta$ -mercaptoethanol (4%), bromophenol blue (0.005%), and glycerol (4%). Following SDS-PAGE on 6%, 8%, 10% or 12% polyacrylamide gels, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were probed with the respective antibodies and antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantitative analysis of protein detection was done by densitometry and image analysis with the ImageJ program (Rasband, W.S., ImageJ, U.S. NIH, Maryland, <http://rsb.info.nih.gov/ij/>, 1997–2006).

#### 2.8. In gel proteasome activity and levels [details in [60]]

Upon treatment with vehicle (control, DMSO) or the respective drugs, cells were washed twice with PBS and harvested for the in gel assay as described in [60]. The native gels loaded with 30  $\mu$ g protein/lane, were run at 150 V for 120 min. The in gel proteasome activity was detected by incubating the native gel on a rocker for 10 min at 37  $^{\circ}$ C with 15 ml of 300  $\mu$ M Suc-LLVY-AMC followed by exposure to UV light

(360 nm). Gels were photographed with a NIKON Cool Pix 8700 camera with a 3–4219 fluorescent green filter (Peca Products, Inc.). Proteins on the native gels were transferred (110 mA) for 2 h onto PVDF membranes. Immunoblotting was carried-out for detection of the 20S and 26S proteasomes with the anti-Rpt6 and anti- $\beta$ 5 antibodies, which react with subunits of the 19S or the 20S particles, respectively, thus detecting 26S and 20S proteasomes. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

### 2.9. Caspase-3 and calpain activation

Cell lysates were analyzed by standard western blotting with the anti-caspase-3 antibody to detect caspase-3 cleavage that is indicative of apoptosis. Calpain activation was assessed with the anti- $\alpha$ -spectrin antibody. Calpain cleavage of  $\alpha$ -spectrin generates a 150 kDa doublet, while caspase cleavage generates a 120 kDa fragment.

### 2.10. Statistical analysis

Statistical significance was estimated using one or two-way ANOVA (Dunnett's or Tukey's multiple comparison test) or the Student's *t*-test with the Prism 7 program (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Oligo, PGJ2, and Epox induce Parkin cleavage in neuronal cultures

We compared the effects of Oligo, PGJ2 and Epox on Parkin integrity. As shown in Fig. 1 (panels 2), all three drugs induce the cleavage of Parkin in a time-dependent manner. However, the pattern of cleaved Parkin differs among the three drugs. While the size of the Parkin fragment(s) induced by PGJ2 and Epox are below 37 kDa, the ones induced by Oligo are above 37 kDa and appear as a doublet.

The three drugs also differed in relation to their effects on the levels of ubiquitinated proteins, and on activation of caspase-3 and calpain. Oligo induced (1) a decrease in the basal levels of ubiquitinated (Ub) proteins (Fig. 1, panel 1), (2) calpain activation, indicated by generation of the 150 kDa calpain-specific fragments (Fig. 1, panel 4,  $\alpha$ -spectrin CL1), and (3) cleavage of caspase-3 to a fragment (~29 kDa) that reflects caspase-3 inactivation (Fig. 1, panel 3). In contrast, PGJ2 and Epox induced (1) an increase in Ub-proteins (Fig. 1, panels 1), (2) caspase activation, confirmed by generation of the 17 kDa fragment corresponding to activated caspase-3 (Fig. 1, panels 3), and (3) formation of the caspase-dependent 120 kDa spectrin fragment. Note that full-length spectrin is only visible in the Epox blot (Fig. 1, panel 4). This blot was exposed for a longer period of time to show the time-dependent formation of the spectrin 120 kDa fragment, detected in the last lane.

Together, these results clearly indicate that PGJ2 and Epox diverge from Oligo on their impact (1) on the function of the ubiquitin/proteasome pathway (UPP), in particular on Parkin cleavage and Ub-protein levels, and (2) on cell death pathways represented by caspase-3 (apoptosis) or calpain (necrosis) activation.

### 3.2. Oligo, PGJ2, and Epox induce calpain and caspase-cleavage of Parkin

It is known that Parkin is a substrate for caspases, as it is cleaved by caspase-1, caspase-3 and caspase-8 at Asp126, leading to its inactivation in Parkin overexpressing CHO and SY5Y cells [39]. We now report that in rat midbrain neuronal cultures PGJ2 and Epox induce caspase-3 mediated Parkin cleavage. This is confirmed by inhibition of Parkin-cleavage under these conditions with a caspase-3 specific inhibitor (Z-DEVD-FMK, C3I, Fig. 2B, panel 1) but not by the calpain specific inhibitor calpeptin (CPT, Fig. 2A, panel 1). As expected, caspase-mediated cleavage of  $\alpha$ -spectrin ( $\alpha$ -spectrin CL2) induced by PGJ2 and Epox, was lessened by the caspase inhibitor.

We also established for the first time to our knowledge, that Parkin is cleaved by calpain upon mitochondrial impairment with Oligo. This cleavage of Parkin is blocked by the calpain inhibitor (CPT, Fig. 2A, panel 1), and is refractory to the caspase inhibitor (C3I, Fig. 2B, panel 1). Calpain-cleaved Parkin migrates at a size that differs from the caspase-cleaved fragments triggered by PGJ2 and Epox (Fig. 2A and B, panels 1). Activation of calpain and caspase and the specificity of the inhibitors for these two proteases are confirmed by the pattern of  $\alpha$ -spectrin cleavage shown in Fig. 2A and B, panels 2. It is clear that Oligo induced calpain-cleavage of  $\alpha$ -spectrin to a 150 kDa fragment ( $\alpha$ -spectrin CL1).

Based on semi-quantitative analysis of the cleaved Parkin doublet compared to full-length Parkin, we estimate that at least  $43.6 \pm 4.9\%$  of full-length Parkin is cleaved upon treatment with 5 nM Oligo for 16 h ( $n = 9$ ).

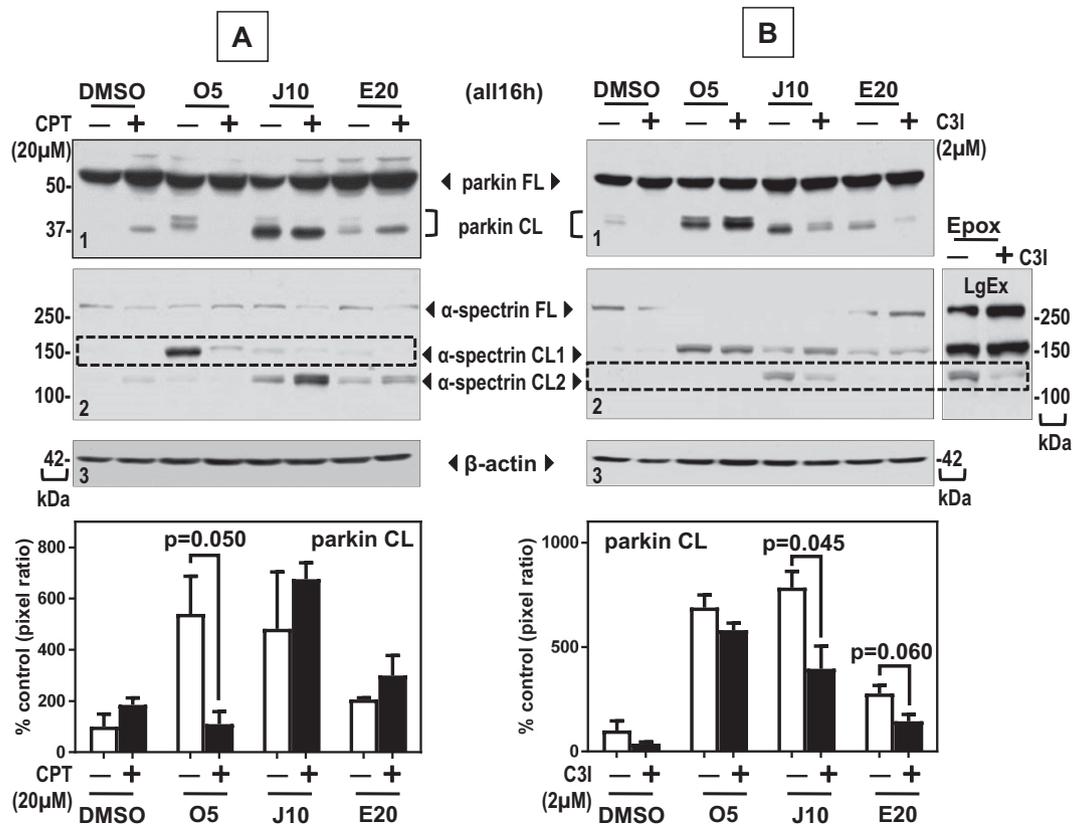
### 3.3. The effect of Oligo on Parkin is mimicked by other mitochondrial inhibitors

To test if other mitochondrial inhibitors recapitulate the effect of Oligo on Parkin, rat midbrain neuronal cultures were treated with two different mitochondrial toxins that affect different elements of the electron transport chain: (1) antimycin A (A5) that binds to the quinone reduction site of complex III (ubiquinol-cytochrome *c* oxidoreductase), inhibiting the reduction of cytochrome *c*, and (2) rotenone (R) that binds to ND1 and inhibits NADH-ubiquinone reductase activity of complex I [19]. Rotenone is particularly relevant to PD, as its effects in *in vivo* models mimic some of the pathological features of PD [4]. We showed previously with rat cortical neurons that oligomycin, antimycin and rotenone reduced the mitochondrial membrane potential ( $\Delta\Psi_m$ ) and increased ROS in addition to decreasing ATP levels [36].

Like Oligo, the two other mitochondrial inhibitors induced calpain-dependent cleavage of Parkin (Fig. 3A, panels 1 and 2). Panel 2 (short exposure) compares the loss of full-length Parkin (parkin FL) caused by each of the drugs. Antimycin and rotenone, like Oligo, also triggered cleavage of caspase-3 to an inactive form (Fig. 3A, panel 3 and 4), and cleavage of  $\alpha$ -spectrin (Fig. 3A, panel 5). From the three mitochondrial drugs tested, rotenone caused the weakest changes as it required the highest concentrations to generate detectable levels of calpain-cleaved Parkin by western blot analysis (Fig. 3B). In summary, we show that three mitochondrial toxins that inhibit oxidative phosphorylation at different steps, lead to the generation of calpain-cleaved forms of Parkin. These data support that calpain rather than caspase-3 functions under conditions where ATP levels are limiting. The effect of PGJ2 (J10 in A and J2 in B) are included for comparison.

### 3.4. Calpain-cleaved Parkin is enriched in neuronal mitochondrial fractions

We investigated the subcellular localization of cleaved Parkin in rat cerebral cortical cultures treated with Oligo (O5, 5 nM), PGJ2 (J10, 10  $\mu$ M), and Epox (E20, 20 nM). We performed these studies with cortical cultures instead of midbrain cultures, because this study requires large numbers of cells that are easily prepared from rat E18 brains. As shown in Fig. 4 (panels 1 & 2), the cortical neurons respond to the three drugs in a manner that is similar to the midbrain cultures. It is clear that full-length (FL) Parkin is detected in nuclear, mitochondrial and cytosolic fractions regardless of cellular conditions, as previously reported in [88]. In contrast, most of the calpain-cleaved Parkin (induced by Oligo) fractionates with mitochondria, while caspase-cleaved Parkin (induced by PGJ2 and Epox) is distributed between the nuclear and mitochondrial fractions (Fig. 4, panels 1 & 2). Subcellular markers are: nuclear – fibrillarin (Fig. 4, panels 3 & 4), a nucleolar protein that is regulated by the UPP [23]; mitochondrial – Tom20 (Fig. 4, panels 5 & 6), a peripheral member of the TOM complex that is the primary receptor for import of mitochondrial precursor proteins [29], and VDAC (Fig. 4, panels 7 & 8), the voltage-dependent anion channel located on



**Fig. 2.** Effects of the calpain inhibitor calpeptin (A, CPT, Z-Leu-Nle-CHO) and the caspase-3 inhibitor (B, C3I, Z-DEVD-FMK) on Parkin and spectrin cleavages induced by oligomycin (O, 5 nM), PGJ2 (J2, 10  $\mu$ M), and epoxomicin (E, 20 nM). Rat midbrain neuronal cultures were pre-treated for 30 min with CPT (A) or C3I (B) and then for 16 h with Oligo, PGJ2 or Epox as indicated. Total lysates were analyzed by western blotting (50  $\mu$ g of protein/lane) probed with the respective antibodies to detect: full-length (FL) and cleaved (CL) Parkin (panels 1); full-length (FL), calpain-cleaved (CL1) and caspase-cleaved (CL2)  $\alpha$ -spectrin (panels 2);  $\beta$ -actin (loading control, panels 3). Molecular mass markers in kDa are shown on the left (A) and right (B) of the blots. Graphs show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved Parkin over  $\beta$ -actin for each condition compared to DMSO control (no inhibitor, 100%). Values are means  $\pm$  s.e. from three experiments. Significant difference was assessed for each drug pair ( $\pm$  inhibitor, multiple *t*-test, Holm-Sidak method with  $\alpha = 0.05$ ). Only *P* values (above brackets) close to significant difference are shown. LgEx, long exposure.

the mitochondrial outer membrane [80]; and cytosolic – Hsp90 (Fig. 4, panels 9 & 10) a well-established chaperone [5] also localized to mitochondria [1].

### 3.5. Phospho-Parkin is less vulnerable to calpain-cleavage

Studies with HEK293 and SH-SY5Y cells demonstrated that the turnover of phospho-Parkin is rapid, as treatment with the phosphatase inhibitor okadaic acid (OA) is necessary to stabilize phospho-Parkin [91]. We confirmed with the rat neuronal cultures that OA stabilizes a Parkin form with a slightly higher molecular weight than full-length Parkin (pSer<sup>65</sup> parkin FL, Fig. 5, anti-parkin panel, lanes b and d, dashed box). As seen in Fig. 5 (anti-pSer<sup>65</sup> parkin panel, lanes b and d, dashed box), we further established that this higher molecular weight form of Parkin is detected with an antibody developed specifically for pSer<sup>65</sup> Parkin (pSer<sup>65</sup> parkin FL). This antibody was raised against a synthetic peptide corresponding to residues surrounding Ser<sup>65</sup> of human Parkin [43]. Other potential Parkin phosphorylation sites remain to be assessed. Our data support a transient phospho/dephospho cycle for Parkin FL (Fig. 5, compare lanes a/c with b/d).

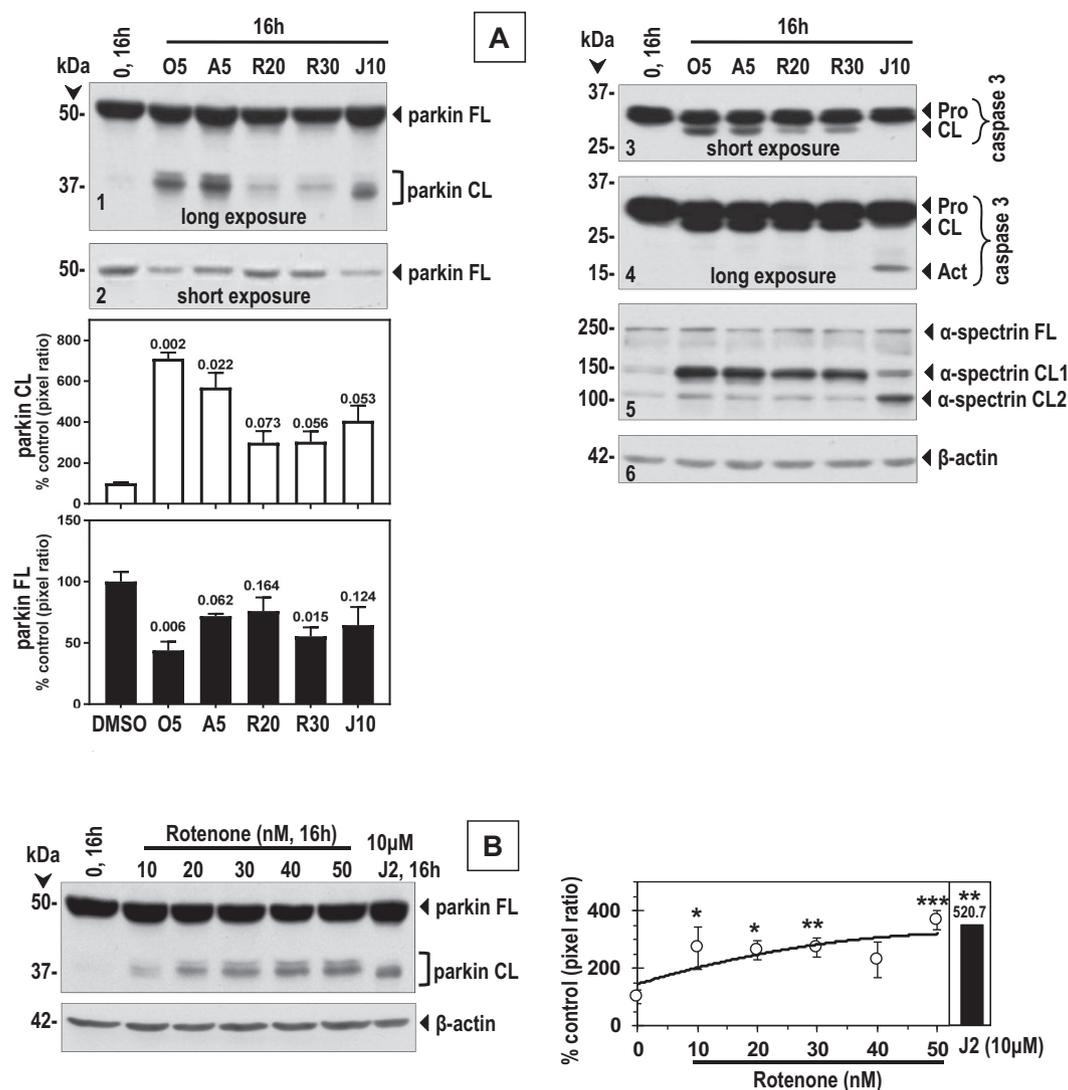
Importantly, we also show that pre-treatment with the phosphatase inhibitor OA decreased the susceptibility of full-length Parkin (parkin FL) to calpain-cleavage by as much as 56% (Fig. 5, anti-parkin panel, compare parkin CL in lanes c and d, and the graph). Moreover, it is clear that the calpain-cleaved forms of Parkin (parkin CL) are not detected with the anti-pSer<sup>65</sup> parkin antibody, suggesting that the UbL domain containing the Ser<sup>65</sup> of Parkin was removed (Fig. 5, panel 2). The

potential calpain-mediated cleavage sites on Parkin are addressed below under “discussion”.

We previously demonstrated that Oligo-treatment of neurons induces calpain-cleavage of other substrates, in particular the microtubule associated protein tau [36]. In the current study, we evaluated the levels of total and pSer<sup>262</sup>tau for comparison with Parkin, as well as cell morphology in neuronal cultures pre-treated with OA prior to Oligo. As expected Oligo-treatment induced cleavage of tau to several fragments (Fig. 5B). OA promoted tau phosphorylation at Ser 262 (Fig. 5B), which accumulates in neurofibrillary tangles in Alzheimer's disease (AD) brains [48]. Furthermore, OA and Oligo-treatment separately or in combination altered cell morphology, in particular the neuronal processes, suggesting cytoskeletal disruption (Fig. 5C).

### 3.6. Calcium dyshomeostasis also induces calpain cleavage of endogenous neuronal Parkin

Treatment with the Ca<sup>++</sup> ionophore A23187 (ION, 20  $\mu$ M, 1 h) or Oligo-treatment (5 nM, 16 h) induced similar calpain-dependent Parkin cleavage to a doublet with a molecular mass > 37 kDa (Fig. 6A, panel 1). Calpain-activation is depicted by spectrin cleavage to the 150 kDa fragment (Fig. 6A, panel 3). Therefore, we established for the first time to our knowledge, that Parkin is cleaved by calpain upon treatment with the ionophore A23187, which disrupts calcium homeostasis. Calpain-cleaved Parkin was also detected with the mouse monoclonal anti-Parkin (Park8) antibody from Cell Signaling (not shown). The ionophore-induced cleavage of Parkin was blocked by the calpain inhibitor



**Fig. 3.** Effects of oligomycin (O, 5 nM), antimycin (A, 5 nM) and rotenone (R, 10 nM to 50 nM) on Parkin, caspase-3 and spectrin cleavages. PGJ2 (J, 10 μM) is included for comparison. Rat midbrain neuronal cultures were treated for 16 h as indicated (in A and B). Total lysates were analyzed by western blotting (50 μg of protein/lane) probed with the respective antibodies to detect: full-length (FL) and cleaved (CL) Parkin (panel 1, long exposure; panel 2, short exposure); full-length (Pro), cleaved inactive (CL, panel 3, short exposure), and cleaved active (Act, panel 4, long exposure) caspase-3; full-length (FL), calpain-cleaved (CL1) and caspase-cleaved (CL2) α-spectrin (panel 5); β-actin (loading control, panel 6). Molecular mass markers in kDa are shown on the left of the blots. Graphs show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved (CL) and full-length (FL) Parkin over β-actin for each condition compared to DMSO control (100%). (A) Values are means ± s.e. from three experiments. Respective P values shown above each bar (unpaired *t*-test with Welch's correction). (B) Values and means ± s.e. from three experiments. Asterisks identify values that are significantly different from control (one-way ANOVA, Dunnett's multiple comparison test, \*\**p* < 0.01, \*\*\**p* < 0.001). ○ - Rotenone.

calpeptin (CPT, Fig. 6B, panels 1), as was spectrin cleavage (Fig. 6B, panels 3).

We also addressed whether Oligo- and ION-induced calpain-cleavage of Parkin and spectrin are Ca<sup>++</sup>-dependent. We incubated (1 h) neurons in Ca<sup>++</sup> free medium prior to exposure to Oligo (6 h 30 min) or ION (1 h) in the same Ca<sup>++</sup> free medium (Fig. 6C and D, respectively). This protocol was chosen in part since the calcium chelator BAPTA was toxic to neurons under our incubation conditions. To prevent excessive toxicity, the Oligo-treatment was shortened to 6 h 30 min under Ca<sup>++</sup> free conditions. We show that calpain-cleavage of Parkin (Fig. 6C and D, panels 1) or spectrin (Fig. 6C and D, panels 3) are largely eliminated under Ca<sup>++</sup> free conditions.

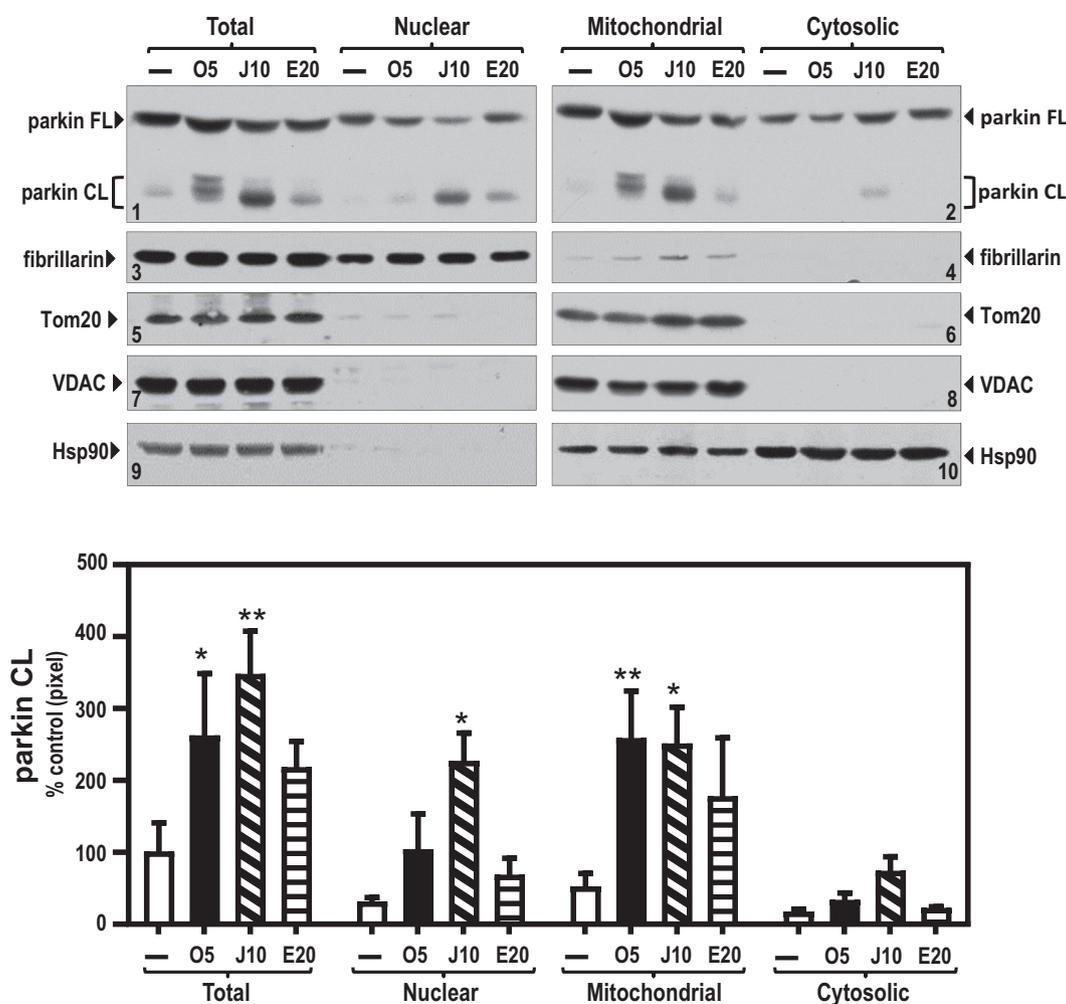
Chronic neurodegenerative disorders like PD, are linked to mitochondrial dysfunction, disruption of calcium homeostasis, and accumulation/aggregation of ubiquitinated (Ub) proteins [62]. We compared the effect of the mitochondrial inhibitor Oligo (5 nM, 16 h) with the ionophore A23187 (ION, 20 μM, 1 h) on Ub-protein levels in the

cortical neurons. Both drugs decreased the basal levels of Ub-protein detected under control conditions (Fig. 6A, panel 4).

### 3.7. Effects of Oligo, PGJ2 and Epox on the proteasome

These current studies are a continuation of our previous findings with cortical neurons [36] showing that Oligo, antimycin or rotenone trigger 26S proteasome disassembly with a rise in 20S proteasomes.

Therefore, we assessed with the native in-gel assay, the effects of the three drugs on proteasome activity and levels in the midbrain neurons as described in [36]. The in-gel assay detects the three assembled forms of the proteasome: 26S proteasomes with either two regulatory caps [26S(2)] or one cap [26S(1)], and the 20S core particle alone (20S). The three drugs decreased 26S proteasome activity with Epox being the most efficient of the three (Fig. 7, panels 1 & 6). Oligo (*p* < 0.001) and PGJ2 (did not reach statistical significance) increased 20S proteasome activity (Fig. 7, panels 2 & 6). Oligo (O, 5 nM) and PGJ2 (J, 10 μM, did



**Fig. 4.** Subcellular localization of full-length (FL) and cleaved (CL) Parkin upon treatment with oligomycin (O, 5 nM), PGJ2 (J2, 10  $\mu$ M), and epoxomicin (E, 20 nM). Rat cerebral cortical neurons were treated for 16 h with Oligo, PGJ2 or Epox as indicated. Upon subcellular fractionation of the total lysates, 40  $\mu$ g of protein from the total lysates, as well as nuclear, mitochondrial and cytosolic fractions were analyzed by western blotting probed for full-length (FL) and cleaved (CL) Parkin (panels 1 & 2). Subcellular fractions were validated with the following markers: nuclear (fibrillarlin, panels 3 & 4), mitochondrial (Tom20, panels 5 & 6, VDAC, panels 7 & 8) and cytosolic (Hsp90, panels 9 & 10). Graphs show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved Parkin over the respective fraction markers for each condition, compared to DMSO control (for each fraction, 100%). Values are means  $\pm$  s.e. from three experiments. Asterisks identify values that are significantly different from control (two-way ANOVA with  $\alpha = 0.05$ , Dunnett's multiple comparison test, \* $p < 0.05$ , \*\* $p < 0.01$ ).

not reach statistical significance) also decreased the levels of the 26S proteasome while increasing those of the 20S proteasome (Fig. 7, panels 3, 4 & 7). Epox however, did not increase 20S proteasome levels. In conclusion, Oligo affects proteasome activity in midbrain cultures replicating the Oligo-effect on cortical neurons.

Previously, we also showed that the mitochondrial inhibitors induce a reduction in basal levels of ubiquitinated proteins due in part to inhibition of the E1A ubiquitin activating enzyme [36]. It is thus clear that mitochondrial impairment slows down the ubiquitin/proteasome pathway by: 1) blocking its first step, i.e. preventing ubiquitin activation by the E1 enzyme, which is ATP-dependent; 2) provoking the demise of 26S proteasomes in neurons caused by 26S proteasome disassembly. This latter event seems to be linked to selective processing of the proteasome Rpn10 subunit by calpain [36].

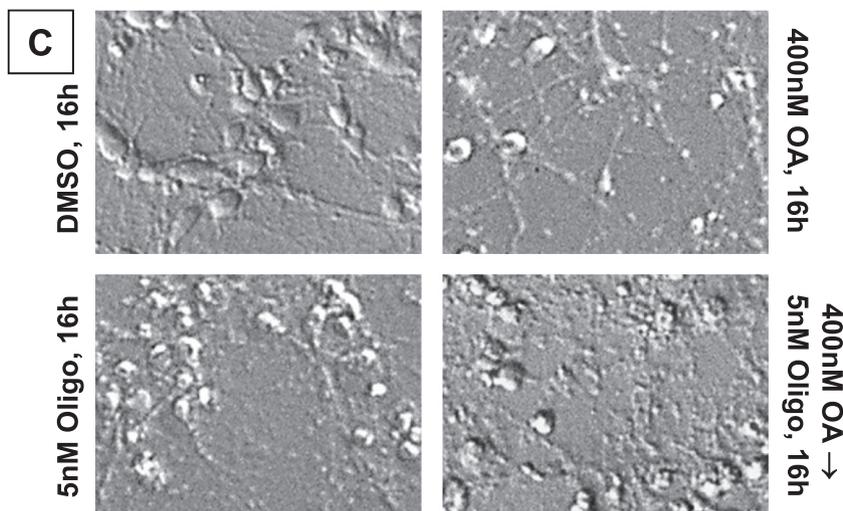
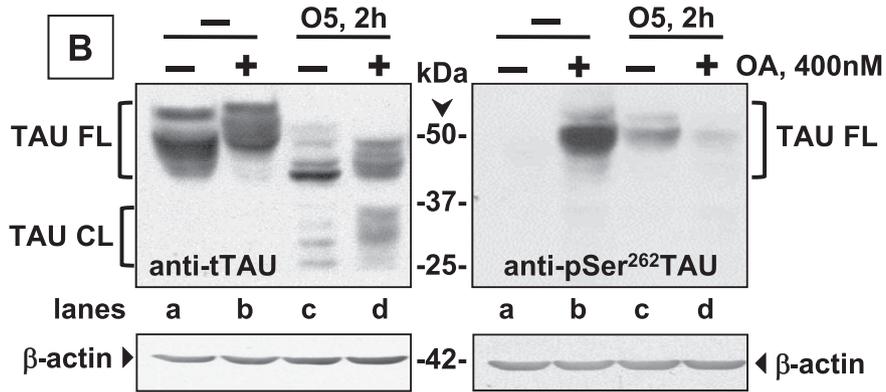
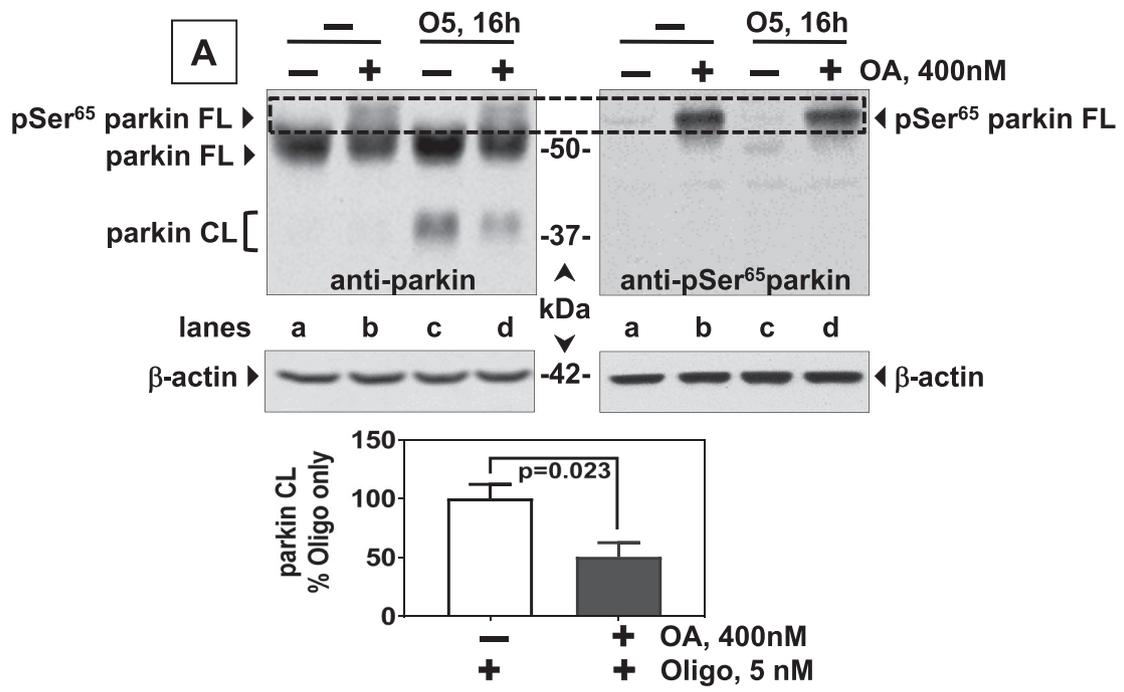
### 3.8. Comparison of the effects of Oligo, PGJ2 and Epox on cell viability and ATP levels

Since the three drugs act via different mechanisms, we compared their effects on cell viability and ATP levels. At the concentrations and times tested, Oligo (5 nM) was the most neurotoxic, decreasing cell

viability by  $\sim$ 50%, followed by PGJ2 (10  $\mu$ M,  $\sim$ 20% decrease) and Epox (20 nM)  $<$  4% (not significant), as shown in Fig. 8A. Oligo was the only drug that decreased intracellular ATP levels, causing  $\sim$ 60% depletion (Fig. 8B). Moreover, we previously demonstrated that Oligo-treatment of rat cortical neurons elevated ROS production and decreased  $\Delta\Psi_m$  [36], further supporting that Oligo-treatment impairs mitochondrial function. We proposed then that the decline in  $\Delta\Psi_m$  caused by long-term oligomycin treatment is consistent with the overall loss of metabolic activity [36].

### 3.9. PACAP27 and cyclocreatine mitigate some of the effects of PGJ2 and Oligo, respectively

cAMP-signaling is known to be neuroprotective [35]. In particular, we previously established that raising intracellular cAMP levels with the lipophilic peptide PACAP27 prevents the cleavage of TAU, a microtubule associated protein, and caspase-3 activation both induced by PGJ2 in rat cerebral cortical neuronal cultures [57]. We now demonstrate in rat midbrain cultures (Fig. 9A) that PACAP27 mitigates PGJ2-induced caspase-dependent Parkin cleavage (panel 1), caspase activation (panel 4), and loss in cell viability (panel 5). However, PACAP27



(caption on next page)

**Fig. 5.** Effect of the phosphatase inhibitor okadaic acid (OA) on Parkin. Rat cerebral cortical neurons were pre-treated for 30 min with OA (400 nM) and then with Oligo (O, 5 nM for 16 h or 2 h) as indicated. Total lysates were analyzed by western blotting (50  $\mu$ g of protein/lane) probed in (A) for full-length (FL) and cleaved (CL) Parkin (anti-parkin panel), parkin phosphorylated at Ser<sup>65</sup> (anti-pSer<sup>65</sup> parkin panel), and  $\beta$ -actin (loading control, actin panels), and additional in (B) for total tau (tTAU) and pSer<sup>262</sup>-TAU. Molecular mass markers in kDa are shown in the middle. Full-length parkin phosphorylated at Ser<sup>65</sup>, pSer<sup>65</sup> parkin FL. Dashed box highlights pSer<sup>65</sup> parkin FL. Graphs in (A) show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved Parkin in cells treated with OA followed by Oligo, (black bar), compared to Oligo only (white bar, 100%). Values are means  $\pm$  s.e. from three experiments. The P value shows significant difference (below bracket, one tailed t-test). (C) depicts the morphology of the cortical neurons treated under the specified conditions. DIC images (20 $\times$ ) obtained with a LeicaDMI-4000B inverted microscope (Leica Microsystems, Buffalo Grove, IL).

did not cause a decline in the levels of ubiquitinated proteins raised by PGJ2 (Fig. 9A, panel 3).

PACAP27 failed to abolish the Oligo effects that we analyzed in midbrain cultures (data not shown), thus we tested another protective approach. Since Oligo inhibits ATP synthesis by mitochondria, we decided to increase ATP production via a different pathway, i.e. the phosphagen system. We did not consider activating glycolysis, since this approach can increase lactic acid production leading to pH changes. The phosphagen system involves phosphagen kinases, such as creatine kinase, that function to temporally buffer ATP levels in cells, including neurons, that display high and variable rates of aerobic energy production [22]. To activate the creatine phosphate/creatine kinase (CP/CK) phosphagen system we treated rat midbrain cultures with cyclocreatine, as this compound is phosphorylated and dephosphorylated by CKs at the same rate as creatine, but has the advantage of crossing membranes due to its relatively planar structure [47].

We show (Fig. 9B) that cyclocreatine (CCr, 5 mM) mitigated the Oligo-induced ATP depletion (panel 6) and calpain-dependent cleavage of Parkin (panel 1) and  $\alpha$ -spectrin (panel 4), but did not affect the levels of ubiquitinated proteins (panel 3). Changes in cell viability are not apparent (panel 5), due to the short time of the treatment. In Fig. 9, the incubation times with drug differ based on the optimal times needed to see drug effects. In this regard, the effects of CCr is short lived, thus, the 4 h incubation time in Oligo treated cells.

#### 4. Discussion

In this study we characterize the vulnerability of Parkin integrity to pathological conditions associated with the development of PD. In particular, we address the effects of mitochondrial impairment and calcium dyshomeostasis on endogenous Parkin integrity in rat midbrain and cerebral cortical cultures. Disruption of Parkin integrity via its cleavage by different proteases, blocks its ubiquitination activity [39,64,89], just like Parkin mutations associated with familial PD.

We demonstrate that calpain-mediated Parkin cleavage is induced by three mitochondrial toxins, Oligo, antimycin and rotenone, and by the Ca<sup>++</sup> ionophore A23187, which disrupts calcium homeostasis. Our previous studies [36], showed that Oligo, antimycin and rotenone induce a significant decline in ATP levels and  $\Delta\Psi_m$ , as well as increase ROS production in rat cerebral cortical neurons. Oligo most efficiently depleted ATP levels, followed by antimycin, and rotenone being the least effective. Since Oligo induced the strongest Parkin cleavage and rotenone the weakest, these studies support the notion that changes in ATP levels induced by the three mitochondrial inhibitors correlate with the levels of calpain-mediated Parkin cleavage. Calpain was shown to cleave other proteins highly relevant to PD and other neurodegenerative conditions. Some of these proteins are  $\alpha$ -synuclein that becomes aggregation prone upon calpain cleavage [20], 26S proteasome subunit Rpn10 (full-length Rpn10 binds Parkin), Tau and caspase 3 (calpain cleaves caspase 3 to an inactive form) [36], as well as huntingtin [26].

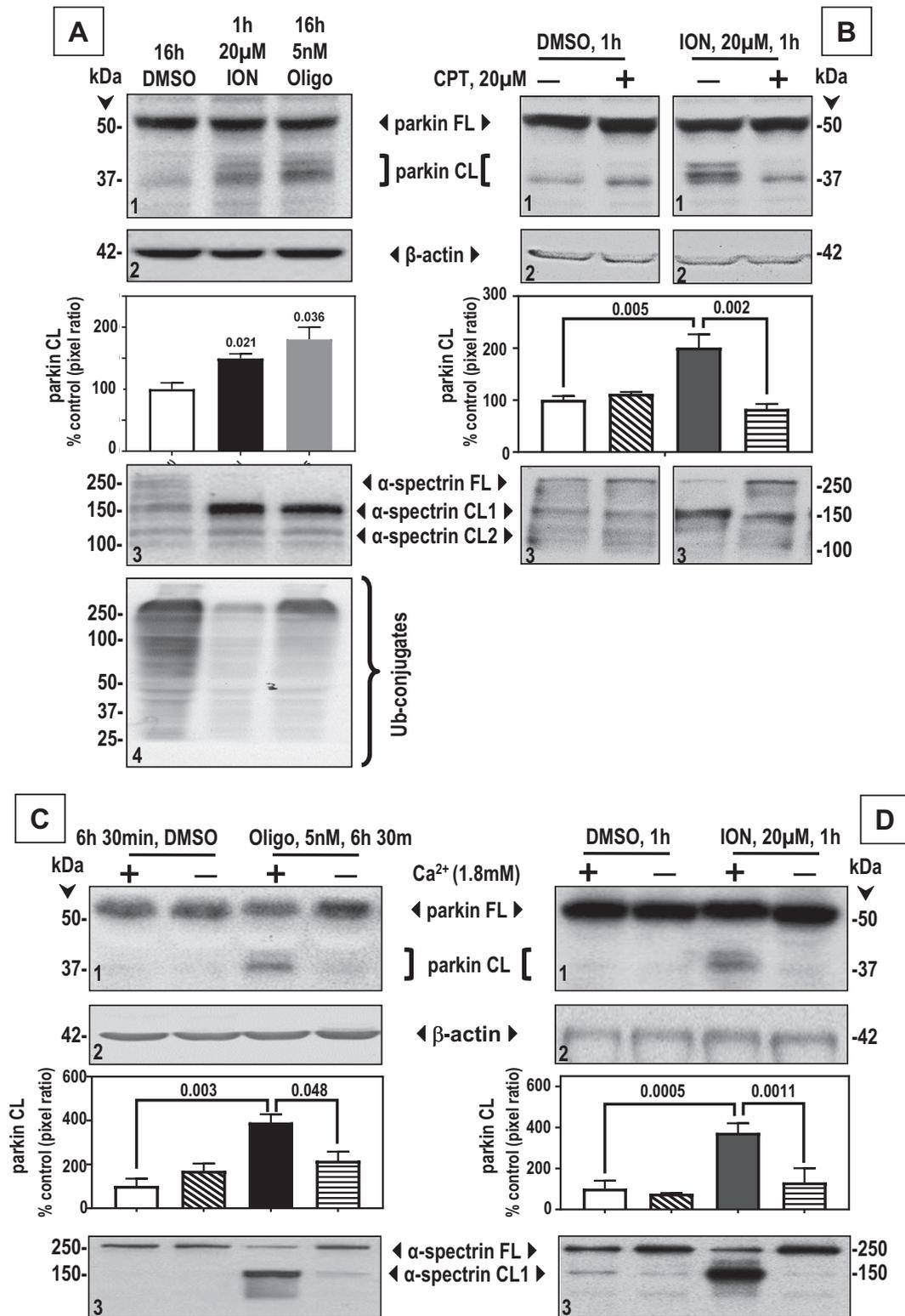
Although a considerable amount of full-length Parkin is not cleaved, we speculate that it may only take a small fraction of Parkin cleavage by calpain to perturb mitochondrial function. In support of this notion, our subcellular fractionation studies show that cleaved Parkin is localized to the mitochondrial fraction in response to Oligo. We base our premise on previous demonstrations that: 1) Parkin translocates to the

mitochondria in response to the mitochondrial stressor CCCP in mouse cortical neurons expressing PINK1 [38], and that 2) Parkin was localized in the mitochondrial fractions of human iPSC-derived neurons and in fibroblasts from PD patients [34]. While our results show that Parkin is partially cleaved in the mitochondrial fraction, it raises the possibility that Parkin function may be sufficiently compromised in response to calpain to affect mitochondrial homeostasis. It is known that Parkin is inactivated by caspase cleavage after Asp 126. Since we predict that calpain-cleavage occurs in the same region, it is possible that calpain would also inactivate Parkin function. Furthermore, we previously showed [36] that mitochondrial inhibitors induce a reduction in ubiquitinated proteins due in part to inhibition of the E1A ubiquitin activating enzyme, as well as 26S proteasome disassembly with a rise in 20S proteasomes. Together these effects could lead to a toxic accumulation of Parkin substrates and neuronal cell death. Further studies are needed to show whether calpain-cleavage of Parkin perturbs the role of Parkin in maintaining mitochondrial quality control.

Calpain activation is Ca<sup>++</sup>-dependent. Disruption of Ca<sup>++</sup> homeostasis is linked to mitochondrial impairment [11] and is implicated in the pathogenesis of PD [92]. Ca<sup>++</sup> concentration in the cytoplasm is 20,000-fold lower than in the extracellular space [83]. While extracellular Ca<sup>++</sup> is in the millimolar range, cytosolic Ca<sup>++</sup> is usually less than one micromolar, except during a Ca<sup>++</sup> signaling event. Na<sup>+</sup>/Ca<sup>++</sup> exchangers and Ca<sup>++</sup>-ATPase pumps in the plasma and ER membranes maintain this low concentration by transporting Ca<sup>++</sup> away from the cytoplasm, either out of the cell or into the ER [74]. The dopaminergic neurons (DA) of the substantia nigra pars compacta (SNpc) are particularly sensitive to Ca<sup>++</sup> dyshomeostasis, because of two main reasons [71,82]. Firstly, DA neurons of the SNpc have intrinsically low Ca<sup>++</sup> buffering capacity, compared to DA neurons of the ventral tegmental area (VTA), which express high levels of calbindin, a Ca<sup>++</sup> buffering protein [71,82]. Secondly, DA neurons of the SNpc are autonomously active in the absence of synaptic input, by engaging L-type Ca<sup>++</sup> channels, which cause significant increases in Ca<sup>++</sup> levels [71,82]. DA neurons of the VTA also exhibit pacemaker activity, but have significantly lower density of the L-type Ca<sup>++</sup> channels. Taking these factors into consideration, it is clear that DA neurons of the SNpc are highly vulnerable to Ca<sup>++</sup> dyshomeostasis, such as caused in our studies by Oligo or ION-treatments. We clearly demonstrate that Oligo- or ION-treatment induce Parkin cleavage in a calcium-dependent manner, thus disrupting Parkin integrity.

Calpains do not have a strict cleavage specificity, although some preferences for particular amino acid sequences, secondary structure (disordered regions) and PEST regions were suggested [63,81]. To find a putative calpain cleavage site on Parkin, we used two computational programs [<http://ccd.biocuckoo.org/>] [53] and [<http://calpain.org>] [21]. These programs predict two calpain cleavage sites (P1 position) in human/rat full-length Parkin (Fig. 10): (1) Gln71 within the following sequence in human: *IVHIVQ-RPWRK* and in rat: *IVHIVQ-RPQRK*, with a predicted molecular weight of 43.6, and located within the UbL domain. (2) Ala134 within the following sequence in human: *KDSPPA-GSPAG* and in rat: *SDSEAA-RGPAA*, with a predicted molecular weight of 36.9, and located within the linker region between the UbL and RING0 domains.

From all reported Parkin alternative splicing variants [reviewed in [15]], transcript variant 3 (TV3) lacks exons 3–5 that encode for a.a.



**Fig. 6.** Effects of Oligo ( $\pm$  Ca<sup>++</sup>) and the Ca<sup>++</sup> ionophore A23187 (ION,  $\pm$  Ca<sup>++</sup>) on Parkin and spectrin cleavage, and on ubiquitinated protein levels. Rat cerebral cortical neurons were treated as indicated. Total lysates were analyzed by western blotting [50  $\mu$ g of protein/lane] probed with the respective antibodies to detect: full-length (FL) and cleaved (CL) Parkin (panels 1);  $\beta$ -actin (loading control, panels 2); full-length (FL), calpain-cleaved (CL1) and caspase-cleaved (CL2)  $\alpha$ -spectrin (panels 3); and in (A) Ub-proteins (panel 4). In (B) cells were pre-treated for 30-min with the calpain inhibitor calpeptin (CPT, Z-Leu-Nle-CHO) prior to the Ca<sup>++</sup> ionophore A23187 (1 h). In (C) and (D) cells were respectively treated with Oligo (6 h 30 min) or A23187 (1 h), in the presence and absence of Ca<sup>++</sup> (1.8 mM) in the media. Molecular mass markers in kDa are shown on the right and left of the blots. Graphs show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved (CL) Parkin over  $\beta$ -actin for each condition compared to DMSO control (100%). Values are means  $\pm$  s.e. from three experiments for all panels. In A, the respective *P* values are shown above each bar (unpaired *t*-test with Welch's correction). In B, C and D only *P* values close to significant difference are shown (above brackets, one-way ANOVA, Tukey's multiple comparisons test).

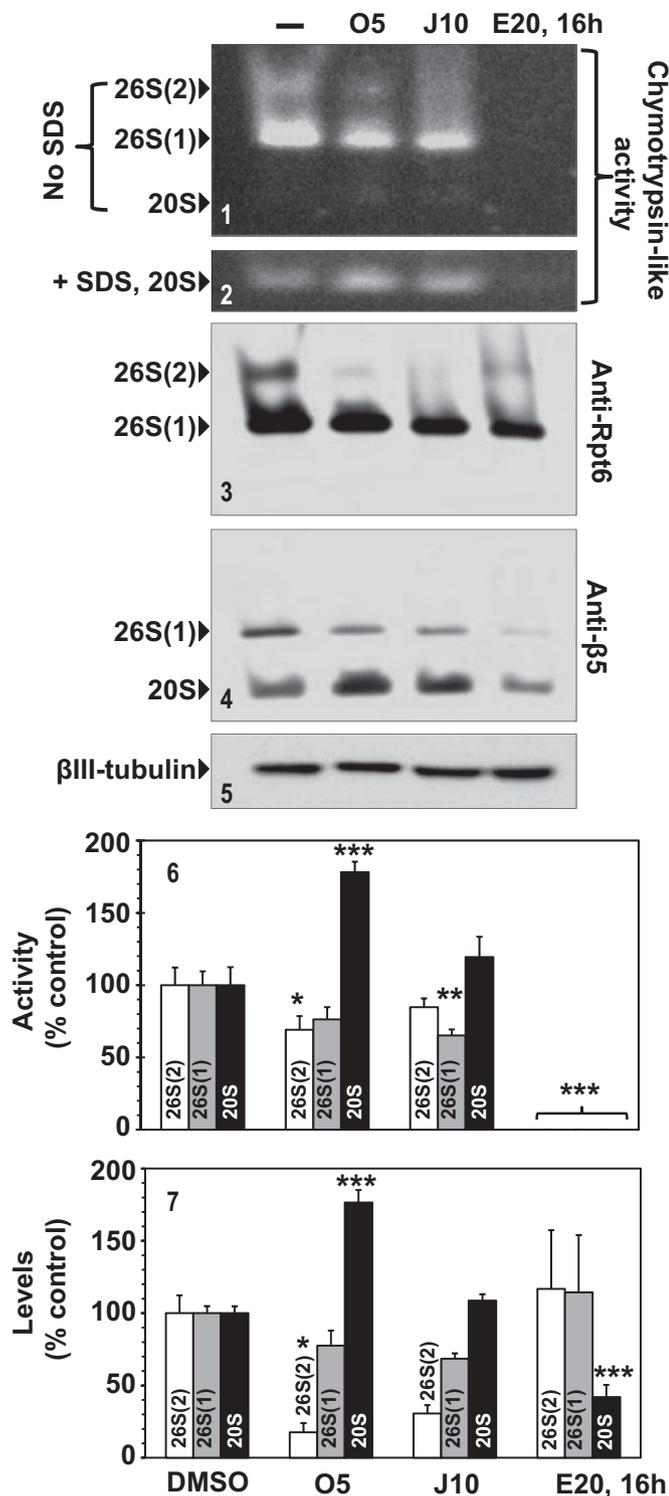


Fig. 7. Effects of oligomycin (O, 5 nM), PGJ2 (J, 10 μM), and epoxomicin (E, 20 nM) on proteasome activity and levels. Rat midbrain neuronal cultures were treated for 16 h with Oligo, PGJ2, and Epox for 16 h. Cleared lysates (25 μg/sample) were subjected to non-denaturing gel electrophoresis as described under “Materials and Methods”. The chymotrypsin-like activity of fully assembled 26S [two caps 26S(2) and one cap 26S(1)] and 20S proteasomes (all indicated in the middle by arrows) was assessed with Suc-LLVY-AMC by the in-gel assay (panel 1). To improve detection of 20S proteasome activity, 0.04% SDS was added to the reaction buffer (panel 2). Proteasome levels were detected by immunoblotting with anti-Rpt6 (panel 3), anti-β5 (panel 4), and anti-βIII-tubulin (panel 5, loading control) antibodies. Proteasome chymotrypsin-like activity (panel 6) and levels (panel 7) were semi-quantified by densitometry (values in graphs). Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values are means ± s.e. from at least five experiments. Asterisks identify values that are significantly different from control (one-way ANOVA, Dunnett’s multiple comparison test, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

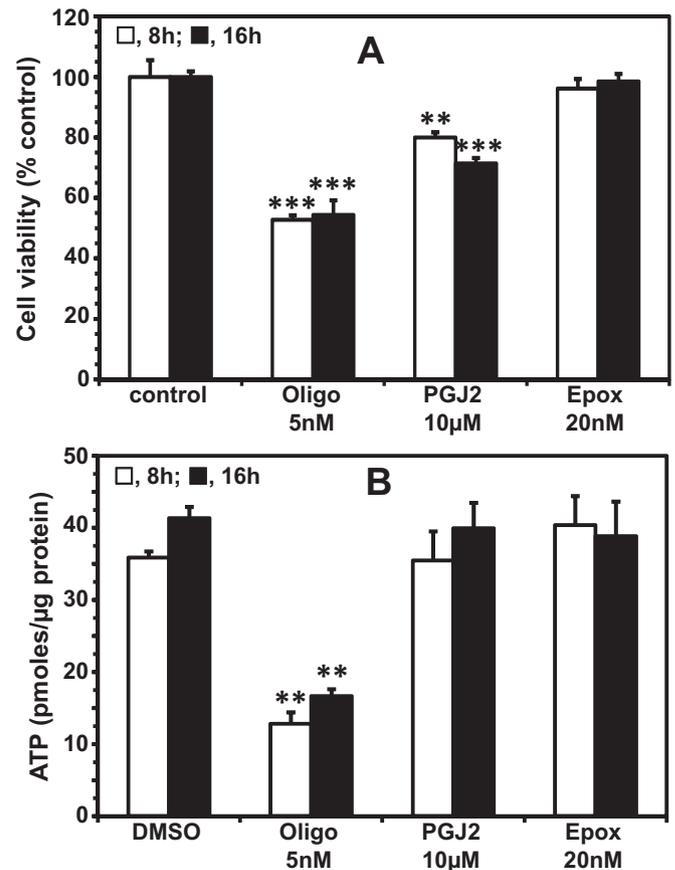
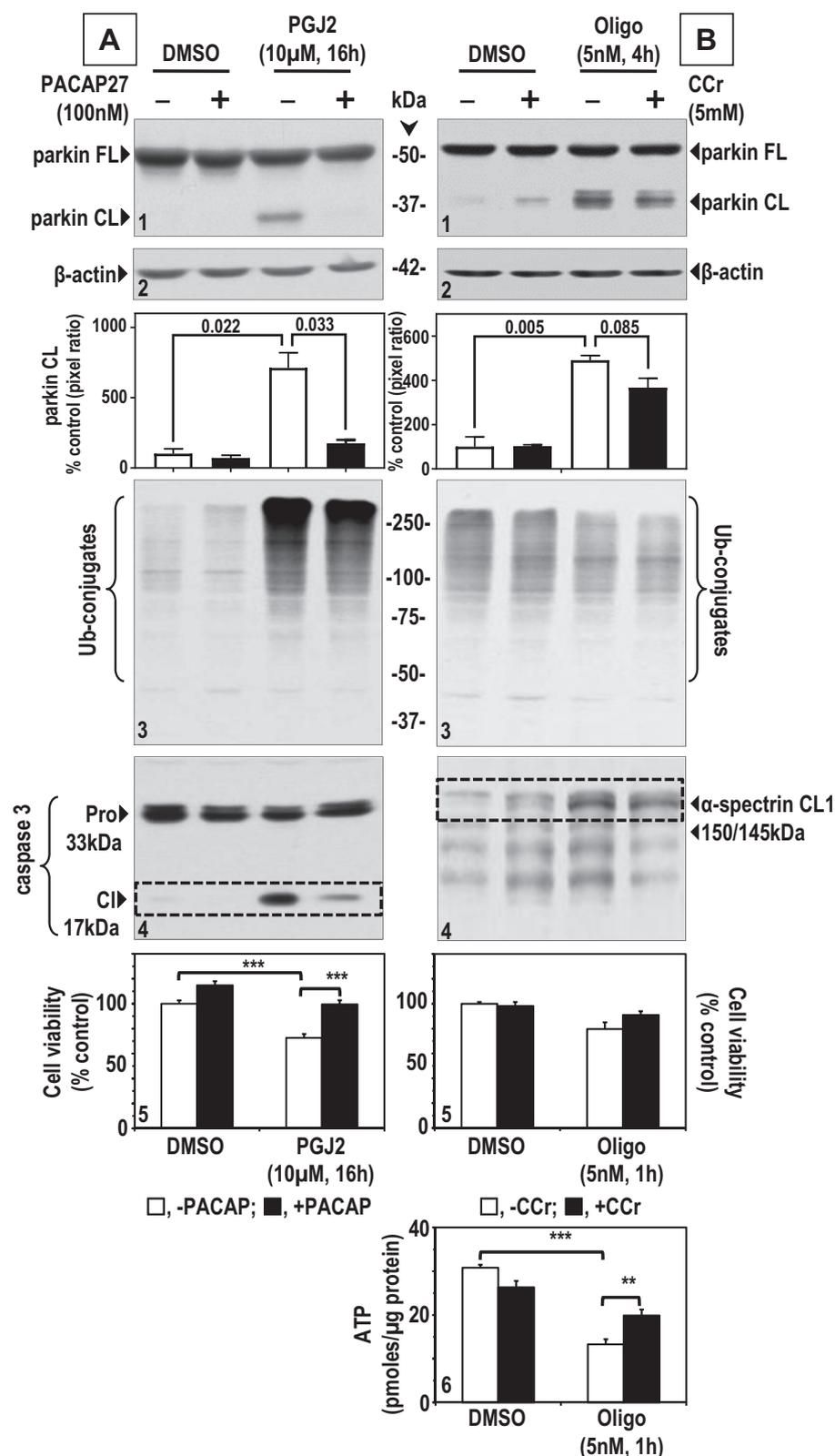


Fig. 8. Effects of oligomycin (O, 5 nM), PGJ2 (J, 10 μM), and epoxomicin (E, 20 nM) on cell viability (A) and ATP levels (B). Rat midbrain neuronal cultures were treated as indicated. Cell viability (A) was assessed with the MTT assay. Total (B) ATP steady state levels (pmoles/μg of protein) were assessed with the luciferin/luciferase system. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means ± s.e. from at least three experiments per group. Asterisks identify values that are significantly different from control (one-way ANOVA, Tukey’s multiple comparisons test, \*\**p* < 0.01; \*\*\**p* < 0.001).

58–206 of full-length Parkin [59]. TV3 has a predicted molecular weight of ~36 kDa [15] that is similar to the calpain-cleaved forms of Parkin. However, since the TV3 form of Parkin is not detected in cells treated with calpain inhibitors, and TV3 lacks the calpain (predicted) cleavage sites, we conclude that the Parkin fragments detected in our studies are cleavage products of full-length Parkin.

In our current studies with rat primary neuronal cultures, we determined that the phosphatase inhibitor okadaic acid stabilized a form of full-length phospho-Parkin. Phospho-Parkin was thus less vulnerable

to calpain-cleavage, as the levels of cleaved Parkin were decreased with okadaic acid pre-treatment. One of the calpain-cleavage sites on Parkin that we predicted, Gln71, is in close proximity to one of the Parkin phosphorylation sites, Ser<sup>65</sup> (Fig. 10). Both sites, Gln71 and Ser<sup>65</sup>, are located within the UbL domain of Parkin. We found that calpain-cleaved Parkin was not detected with an antibody specific for pSer<sup>65</sup> within the UbL domain of Parkin. Only full-length phospho-Parkin was

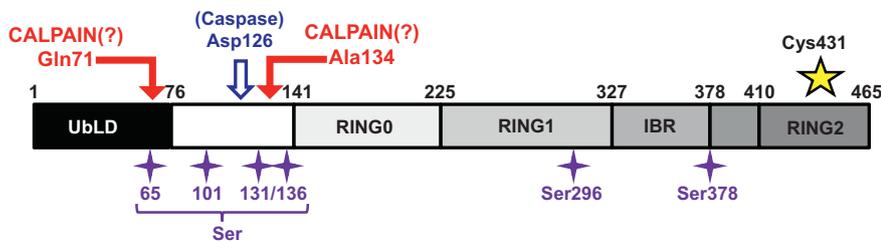


**Fig. 9.** PACAP27 and cyclocreatine diminish the effects of PGJ2 or oligomycin (Oligo), respectively. Rat midbrain cultures were treated with DMSO (control, vehicle), or: (A) PGJ2 (10 μM, 16 h) in conjunction with water (control, vehicle) or PACAP27 (100 nM); in (B) Oligo [5 nM, 1 h (panels 5 & 6) or 4 h (panels 1–4)] in conjunction with cyclocreatine (CCr, 5 mM in medium). For the 4 h Oligo treatment CCr was co-added and then added again 2 h post-Oligo. Total lysates were analyzed by western blotting (50 μg of protein/lane) probed for full-length (FL) and cleaved (CL) Parkin (panels 1); β-actin (loading control, panels 2). Ub-proteins (panels 3); full-length (Pro) and cleaved active (CL) caspase-3 (panels 4A); full-length (FL), and calpain-cleaved (CL1) α-spectrin (panels 4B). Molecular mass markers in kDa are shown in the middle. Graphs show semi-quantification of the respective signals by densitometry. Data represent the percentage of the pixel ratio for cleaved (CL) Parkin over β-actin for each condition compared to DMSO control (100%). Values are means ± s.e. from three experiments. Relevant P values shown (above brackets, unpaired t-test with Welch's correction). Cell viability (panels 5) was assessed with the MTT assay. Percentages represent the ratio between the data for each condition and control (100%). ATP steady state levels (pmoles/μg of protein, panel 6) were measured with the luciferin/luciferase system. Values indicate means ± s.e. from at least 4 determinations. Asterisks identify values that are significantly different (two-tailed t-test) as indicated, with \*\*p < 0.01, \*\*\*p < 0.001.

detected with this antibody. These data suggest that the UbL domain of Parkin is removed upon calpain cleavage. Others showed that PINK1 phosphorylates Parkin at Ser<sup>65</sup> [43,77], and that this is necessary for maximal Parkin E3-ligase activation [42]. Based on these findings, we propose that stabilization of Parkin phosphorylation at Ser<sup>65</sup> by phosphatase inhibition attenuates calpain-mediated cleavage of activated Parkin. This phosphorylation-cleavage crosstalk could be a mechanism

to prevent long-lasting activation of Parkin.

Studies by others established that Parkin is constitutively phosphorylated at the N- and C-termini, but its phospho/dephospho cycle is rapid as phosphatase inhibitors are necessary to detect phospho-Parkin [91]. Besides Ser65, two other Parkin phosphorylation sites (Ser131 and Ser136, Fig. 10), were identified in Parkin overexpressing HEK293 and SH-SY5Y cells upon treatment with okadaic acid [91]. The second



**Fig. 10.** Model for Parkin structure and domains showing the potential calpain-mediated cleavage sites. C431, catalytic cysteine; purple stars, Ser phosphorylation sites; Asp126, caspase-cleavage site; Gln71 and Ala134, predicted calpain cleavage sites. UbLD, ubiquitin like domain; RING, real important novel gene domains; IBR, in between ring domain. Modified from [10] and based on [70,84,90].

calpain-cleavage site we predicted on Parkin, Ala134, is in close proximity to Ser131 and Ser136 (Fig. 10). This suggests that phosphorylation at Ser131 and Ser136, may also protect Parkin from calpain cleavage. Furthermore, since okadaic acid exhibits selectivity for PP2A over PP1, the two most abundant serine/threonine phosphatases in mammalian cells [6], it is possible that PP2A activity diminishes Parkin phosphorylation, just as it does for tau hyperphosphorylation [27]. We show herein that okadaic acid treatment increases the levels of pSer<sup>262</sup>tau, shown to accumulate in tangles in AD brains, and to have a strong disruptive effect on microtubules [48]. Alternatively, at the concentration tested (400 nM) okadaic acid could inhibit both PP2A and PP1.

Parkin activation seems to be a multistep event regulated by phosphorylation, cysteine modification, and ligand binding to the UbL domain [84]. By mediating protein ubiquitination and by binding to proteasomes, Parkin is able to respond promptly to specific cellular proteolytic requirements involving the UPP. As expected, subcellular fractionation of the neuronal cultures showed that FL-Parkin is located in the cytosolic and mitochondrial fractions. FL-Parkin was also detected in the nuclear fraction. The translocation of Parkin to the nucleus, induced by a range of DNA damaging agents, is thought to play an important role in DNA repair and protect against genotoxicity [40]. This is relevant to PD, as oxidative stress induced DNA damage is a potential pathogenic mechanism implicated in parkinsonism [58,66].

In contrast to FL-Parkin, calpain-cleaved Parkin generated upon mitochondrial dysfunction induced by Oligo-treatment, was present only in the mitochondrial fraction. Calpains are located in the cytoplasm, and more recently were found to also be present in mitochondria [41]. The intracellular location where Parkin is cleaved by calpain and how PINK1 may affect this process remains to be established. Studies by others showed that Parkin associates and regulates the 26S proteasome via binding of the Parkin UbL domain to the Rpn10 subunit of the 26S proteasome [73,87]. Moreover, it is conceivable that the UbL domain alone retains activity and negatively affects proteasome-mediated degradation by competing with other factors for Rpn10 binding [39]. In addition, the UbL domain of Parkin seems to delay the translocation of Parkin to mitochondria [30]. Together, all of these findings support the notion that calpain cleavage liberates the UbL domain of Parkin, thus blocking the Parkin/Rpn10 interaction and freeing Parkin to facilitate mitophagy. This is particularly relevant under conditions of ATP depletion when protein ubiquitination is limited by E1 inhibition as we previously demonstrated [36]. Interestingly, the Arg to Pro mutation at position 42 of the UbL domain of Parkin, identified in patients with autosomal-recessive juvenile parkinsonism (ARJP), disrupts the Rpn10-Parkin interaction [73]. Thus, disruption of the Rpn10-Parkin interaction due to mutations in Parkin [87] or to its calpain-cleavage, as we demonstrate here, could mediate disruption of Parkin-26S proteasome interactions, and have an impact on PD pathology.

Based on our data, PGJ2 does not mitigate ATP levels, and increases the levels of ubiquitinated proteins and caspase-mediated Parkin cleavage. PGJ2 is a product of inflammation postulated to activate the Fas/FasL death signaling pathway [44] and caspase-8 [57], thus linking inflammation to caspase-mediated loss of Parkin activity and accumulation of its substrates. These effects are replicated by proteasome inhibition with Epox. In contrast, Oligo significantly reduced

mitochondrial production of ATP and protein ubiquitination, and leads to calpain-mediated Parkin cleavage. Both of these conditions are associated with PD pathogenesis, since both caspase and calpain activation are linked to PD [65]. Two studies report detection of Parkin processed to a ~41/42 kDa form in human brains of a control [75] and a PD patient [78], but there is no discussion of the possible cleavage mechanisms.

Since cAMP-signaling is considered to be neuroprotective [35], we tested the effects of raising intracellular cAMP levels with the lipophilic peptide PACAP27 as a potential therapeutic strategy for PD that can target Parkin. PACAP27 mitigated the loss of cell viability, caspase-3 activation, and caspase-cleavage of Parkin induced by PGJ2 in rat midbrain cultures. In contrast, calpain-cleavage of Parkin induced by Oligo, was diminished by cyclocreatine. Interestingly, cyclocreatine was used *in vivo* to improve cognition in mice with creatine transporter deficiency [47], and in other neurodegenerative conditions including PD [2].

In conclusion, our data suggest that Parkin undergoes calcium-dependent calpain-cleavage that could potentially affect its intracellular localization and function. Phosphorylated Parkin is less sensitive to cleavage by calpain. Moreover, cleaved Parkin localizes to mitochondria in response to mitochondrial and proteasomal impairment, and the product of inflammation PGJ2. This is highly significant to PD as Parkin dysfunction is associated with familial as well as sporadic forms of PD. Recent studies underline the impact of calcium in PD-neurodegeneration, showing that C-terminal calcium binding to  $\alpha$ -synuclein modulates synaptic vesicle interaction, and that an imbalance in calcium can cause synaptic vesicle clustering [49]. Based on these studies, it was proposed that excessive levels of calcium in the brain may trigger the formation of toxic protein clumps typical of PD [54]. Moreover, ongoing clinical trials in PD patients are evaluating the clinical benefit of calcium channel blockers [54]. Since PD is associated with multifaceted pathological mechanisms, we propose that therapeutic approaches should include targeting necrosis (calpain activation) and apoptosis (caspase activation) to effectively treat (stop/delay) the progression of neurodegeneration in PD.

#### Conflict of interest

None.

#### Transparency Document

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monoclonal antibody.

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