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INVITED SHORT COMMUNICATION FOR THE NEUROCHEMISTRY INTERNATIONAL SPECIAL ISSUE ON "MITOCHONDRIA IN THE NERVOUS SYSTEM: FROM HEALTH TO DISEASE"

Mitochondrial respiratory chain disorganization in Parkinson's disease-relevant PINK1 and DJ1 mutants

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Keywords: PINK1; DJ1; human; mouse; neurons; Parkinson's disease; mitochondria; complexes

Abbreviations: AO, with antioxidants; BNGE, blue native gel electrophoresis; CI, complex I; CIII, complex III; CIV, complex IV; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; MRC, mitochondrial respiratory chain; mROS, mitochondrial reactive oxygen species; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; Scaf1, supercomplex assembly factor 1; SC, supercomplex

ABSTRACT

Brain mitochondrial complex I (CI) damage is associated with the loss of the dopaminergic neurons of the *Substantia Nigra* in Parkinson's Disease (PD) patients. However, whether CI inhibition is associated with any alteration of the mitochondrial respiratory chain (MRC) organization in PD patients is unknown. To address this issue, here we analyzed the MRC by blue native gel electrophoresis (BNGE) followed by western blotting, in mitochondria purified from fibroblasts of patients harboring PD-relevant *Pink1* mutations. We found a decrease in free CI, and in free *versus* supercomplexes (SCs)-assembled CI in PD; however, free complex III (CIII) was only modestly affected, whereas its free *versus* SCs-assembled forms decreased. Interestingly, complex IV (CIV) was considerably lost in the PD samples. These results were largely confirmed in mitochondria isolated from cultured neurons from *Pink1*$^{-/-}$ mice, and in cultured neurons and forebrain samples from the PD-related *Dj1*$^{-/-}$ mice. Thus, besides CI damage, the MRC undergoes a profound structural remodeling in PD likely responsible for the energetic inefficiency and mitochondrial reactive oxygen species (mROS) over-production observed in this disease.

INTRODUCTION

The MRC complexes can be organized into SCs in a process that has been proposed to regulate electron transfer efficiency (Bianchi et al., 2004) and mROS production (Bianchi et al. 2004, Lapuente-Brun et al. 2013). Recently, we reported that the extent of assembly of CI (NADH-ubiquinone oxidoreductase) with CIII (ubiquinol-cytochrome *c* oxidoreducatase)-containing SCs differs between neurons and astrocytes (Lopez-Fabuel et al. 2016, 2017), determining differences in mitochondrial metabolism and mROS generation between these cells. Thus, in neurons CI is predominantly assembled into I-III SCs, whereas in astrocytes the abundance of free CI is higher (Lopez-Fabuel et al. 2016). Therefore, it can be hypothesized that changes in the normal MRC organization under certain brain pathological situations might impact on energy efficiency, mROS and neuronal survival.

It is well known that mitochondrial CI is damaged in the *Substantia Nigra* of PD patients (Schapira et al. 1989, 1990). Mutations in PTEN (phosphatase and tensin homologue)-induced kinase 1 (PINK1), a mitochondrial Ser/Thr kinase, cause an autosomal recessive form of PD (Valente et al. 2004). By phosphorylating PARKIN, PINK1 regulates the turnover of damaged mitocondria (Clark et al. 2006, Park et al. 2006). Interestingly, in PINK1 knockout (*Pink1*$^{-/-}$) mice it has been described that CI subunit NDUFA10 undergoes PINK1-mediated Ser^{250} phosphorylation to support CI-mediated ubiquione reductive activity (Morais et al. 2009, 2014). Furthermore, in *Pink1*$^{-/-}$ flies, a loss in CI assembly has been observed along with a loss in CIV (cytochrome *c* oxidase) activity (Liu et al. 2011). However, whether these changes alter the MRC supercomplex organization in PD patients is unknown. Here, we addressed this issue and found that the MRC in fibroblasts from PD patients harboring *Pink1* mutations, and in primary neurons from *Pink1*$^{-/-}$ mice, was disorganized. These changes were confirmed in primary neurons and brain tissue from the PD-relevant *Dj1*$^{-/-}$ mice.

MATERIALS AND METHODS

Human Samples

Skin samples were obtained from subjects expressing mutated forms of PINK1 (samples PDP1, PDP3 and PDP7) diagnosed at the Hospital Universitario Insular de Gran Canaria (La Palma de Gran Canaria, Spain), Hospital San Cecilio (Granada, Spain) and from age-matched healthy individuals (samples FH1103, FH0819 and

FH0821) at the Hospital Donostia and Onkologikoa (San Sebastian, Spain) (Azkona et al. 2016). PD patients presented an early-onset, typical parkinsonian syndrome, characteristic of PINK1-associated PD (Samaranch et al. 2010). Demographic data are provided in Azkona et al. (2016). Dermal fibroblasts were cultivated as described previously (Lopez de Maturana et al. 2014) in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose and 10% (vol/vol) of fetal calf serum. Fibroblasts were used at passage 12-23.

Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Ethical Committee on the Use of Human Subjects in Research in Euskadi, Spain. All subjects gave informed consent for the study using forms approved by the ethical committees on the Use of Human Subjects in Research at Hospital Universitario Insular de Gran Canaria, La Palmas de Gran Canaria; Hospital Donostia and Onkologikoa, San Sebastián; and Hospital San Cecilio, Granada, respectively.

Ethical statement regarding the use of animals. Mice were bred at the Animal Experimentation Unit of the University of Salamanca, and all protocols were approved by the Bioethics Committee of the University of Salamanca in accordance with the Spanish legislation (RD53/2013). PINK1 knockout (*Pink1*$^{-/-}$) mice (Requejo-Aguilar et al. 2014) and DJ1 knockout (*Dj1*$^{-/-}$) mice (Requejo-Aguilar et al. 2015) were bred under a C57Bl/6 background under heterozygosis. *Pink1*$^{-/-}$, *Dj1*$^{-/-}$ and their corresponding wild type (*Pink1*$^{+/+}$ or *Dj1*$^{+/+}$) siblings were used as controls.

Neurons in primary culture. PINK1-, DJ1-knockout, or wild type mice cortical primary neurons (Requejo-Aguilar et al. 2014, 2015) were prepared from 15.5 days-old embryos, seeded at 2.0 x 10^5 cells/cm^2 in plastic plates coated with poly-D-lysine (10 μg/ml) and incubated in Neurobasal (Life Technologies) supplemented with 2 mM of glutamine and 2% (v/v) B27-with antioxidants (AO) supplement. Cells were incubated at 37 °C in a humidified 5% (v/v) CO_2-containing atmosphere. At 72 hours after plating, medium was replaced, and cells used at day 7.

Mitochondria isolation and solubilization. Mitochondria were obtained according to a previously published protocol (Acin-Perez et al. 2008). Briefly, cells (12-100 millions)

were collected, and cell pellets frozen at -80 ºC. Forebrain tissue or cell pellets (neurons or dermal fibroblasts) were homogenized (10 strokes) in a glass-teflon Potter-Elvehjem, in Buffer A (sacarose 83 mM; MOPS 10 mM; pH 7.2). The same volume of Buffer B (sacarose 250 mM; MOPS 30 mM) was added to the sample, and the homogenate was centrifuged (1000 g, 5 minutes) to remove unbroken cells and nuclei. Centrifugation of the supernatant was then performed (12000 g, 2 minutes) to obtain the mitochondrial fraction, which was washed in Buffer C (sacarose 320 mM; EDTA 1 mM; Tris-HCl 10 mM; pH 7.4). Mitochondria were suspended in Buffer D (6-aminohexanoic acid 1M; Bis-Tris-HCl 50 mM; pH 7.0). Solubilization of mitochondria was performed with digitonin at 4 g/g (5 minutes in ice). After a 30 minutes centrifugation at 13000 g, the supernatant was collected.

Blue native gel electrophoresis (BNGE) and western blotting. For the assessment of mitochondrial complexes organization, digitonin solubilized mitochondria (5-20 μg) were loaded in NativePAGE Novex 3-12% gels (Life Technologies). After electrophoresis, in-gel NADH dehydrogenase activity was evaluated (Diaz et al. 2009), or the resolved proteins were transferred electrophoretically to nitrocellulose membranes (Amersham protran premium 0.45 nitrocellulose, Amersham). Membranes were blocked with 5% (w/v) low-fat milk in 20 mM Tris, 150 mM NaCl, and 0.1% (w/v) Tween 20, pH 7.5, for 1 h. Subsequently of blocking, membranes were immunoblotted with NDUFS1 (a CI subunit) (1/500 by vol; sc-50132; Santa Cruz Biotechnologies), UQCRC2 (a CIII subunit) (1/1000 by vol; ab14745; Abcam) or MT-CO1 (a CIV subunit) (1/1000 by vol; ab14705; Abcam) primary antibody overnight at 4ºC. After incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1/10,000 by vol; 170-6516; BioRad) or rabbit anti-goat IgG (1/10,000 by vol; ab6741; Abcam), membranes were immediately incubated with the enhanced chemiluminescence kit WesternBright ECL (Advansta), before exposure to Fuji Medical X-Ray film (Fujifilm), and the autoradiograms scanned. In all cases, at least three biologically independent replicates, or human samples, were performed, though only one representative western blot is shown in the article. The protein abundances of the protein bands were measured by densitometry on the films using ImageJ 1.48u4 software (National Institutes of Health, USA); the ratio of free *versus* SC-embedded CI or CIII, and free CIV *versus* free CIII band intensities were calculated against control samples (control or WT).

Protein determinations. Protein samples were quantified by the bicinchoninic acid protein assay kit (Thermo) following the manufacturer's instructions, using bovine serum albumin as a standard.

Statistical Analysis. All measurements were carried out at least in three independent culture preparations or animals, and the results were expressed as the mean values ± SEM. The statistical analysis of the results was performed by the Student's *t* test using the SPSS software. In all cases, $p<0.05$ was considered significant.

RESULTS

In order to investigate the MRC organization in PD, mitochondria purified from fibroblasts of patients harboring PD-relevant *Pink1* mutations, and age-matched control subjects (Azkona et al. 2016), were analyzed by BNGE followed by western blotting. We had previously shown that these cells showed increased glycolytic rate (Azkona et al. 2016), which is often a feature of damaged mitochondria. As shown in Fig. 1A, we observed a decrease in free CI, and in free *versus* SCs-assembled CI in the PD human fibroblasts. Free complex III (CIII) was only modestly affected, whereas the free *versus* SCs-assembled CIII was decreased (Fig. 1A). Interestingly, CIV protein was considerably lower in the human PD fibroblasts (Fig. 1A). To confirm these results in neural samples, we performed neurons in primary cultures from the *Pink1*$^{-/-}$ mice, in which we also previously described increased glycolytic rate (Requejo-Aguilar et al. 2014). As shown in Fig. 1B, the lower free *versus* SC-assembled CI and CIII ratios, and the CIV loss, were confirmed in *Pink1*$^{-/-}$ primary neurons. To further confirm that these changes in the MRC are conserved in other relevant PD genes, we next analyzed the MRC organization in primary neurons and in the forebrain of mice lacking DJ1 (*Dj1*$^{-/-}$), a gene whose mutations cause loss of mitochondrial integrity (Wang et al. 2012) and contribute to the development of autosomal recessive early-onset PD (Bonifati et al. 2003). As shown in Fig. 2A and B, we confirmed, in neurons and brain, respectively, the lower free *versus* SC-assembled CI and CIII ratios, and the CIV loss in the *Dj1*$^{-/-}$ mice model of PD.

DISCUSSION

Here, we show that in samples from PD-relevant *Pink1* mutant patients, as well as in *Pink1*$^{-/-}$ and *Dj1*$^{-/-}$ neural tissue, CI is disassembled from SCs and partially lost. Interestingly, the loss in CIV is even more dramatic than the loss in CI, which might contrast with the generalized view that damage to CI, not to CIV, is a hallmark of PD (Schapira et al. 1989). However, it should be considered that the human PD samples used for this study are skin-derived fibroblasts, not the human PD *Substantia Nigra* samples in which the CI deficiency was first uncovered (Schapira et al. 1989). Given that neurons strongly depend on OXPHOS for survival (Almeida et al. 2004), it will be interesting to explore this issue in the future in dermal fibroblasts-derived differentiated neurons from patients harboring different *Pink1* and *Dj1* mutations. It is also worth noting that, on our hands, the decrease in CIII was rather modest or negligible. Whether this mitochondrial complex is particularly reluctant to protein degradation is unknown; nevertheless, given that CIII is known to stabilize CI (Acín-Pérez et al. 2004), the higher stability of CIII in PD may be important at maintaining traces of intact and, maybe active, CI in this disease. The MRC in PD mitochondria would therefore still accept NAD-linked electrons that would be used for superoxide generation at CI, rather than being terminally transported to O_2 because of the dramatic loss of CIV that we herein describe. If so, these observations may contribute explaining the higher mROS and oxidative stress associated with this disorder (Perry et al. 1982). Whilst such a mechanism could explain the previously reported ATP reductions in PD (Davey et al. 1998; Pogson et al. 2014), whether complex V (ATP synthase) and/or other complexes of the MRC, such as complex II, are also affected remains to be determined.

Humans, but not C57Bl/6 mice, express active Scaf1, a CIII-CIV assembly factor (Lapuente-Brun et al. 2013). Recently, we have shown that the ability of mitochondria to assemble I-III$_2$-IV SCs impacts on CI activity (Lopez-Fabuel et al. 2017). Intriguingly, here we did not observe any III-IV-containing SC band in the human samples analyzed; whether tissue-specific differences, or inter-individual variations, in the ability to assemble CIII with CIV explains this observation is unknown. Nevertheless, CIV is virtually lost in PD (our data), an observation that could be explained by the reported decrease in cardiolipin concentration in PD (Vos et al. 2017; McKenzie et al. 2006; Althoff et al. 2011). Interestingly, it is known that CIV loss triggers oxidative damage and partial degradation of critical CI subunits (Cogliati et al.

2016; Guarás et al. 2016). Therefore, it is tempting to speculate the possibility that CIV loss in PD would be an important contributor factor in CI deficiency associated with PD (Perry et al. 1982; Schapira et al. 1989, 2012).

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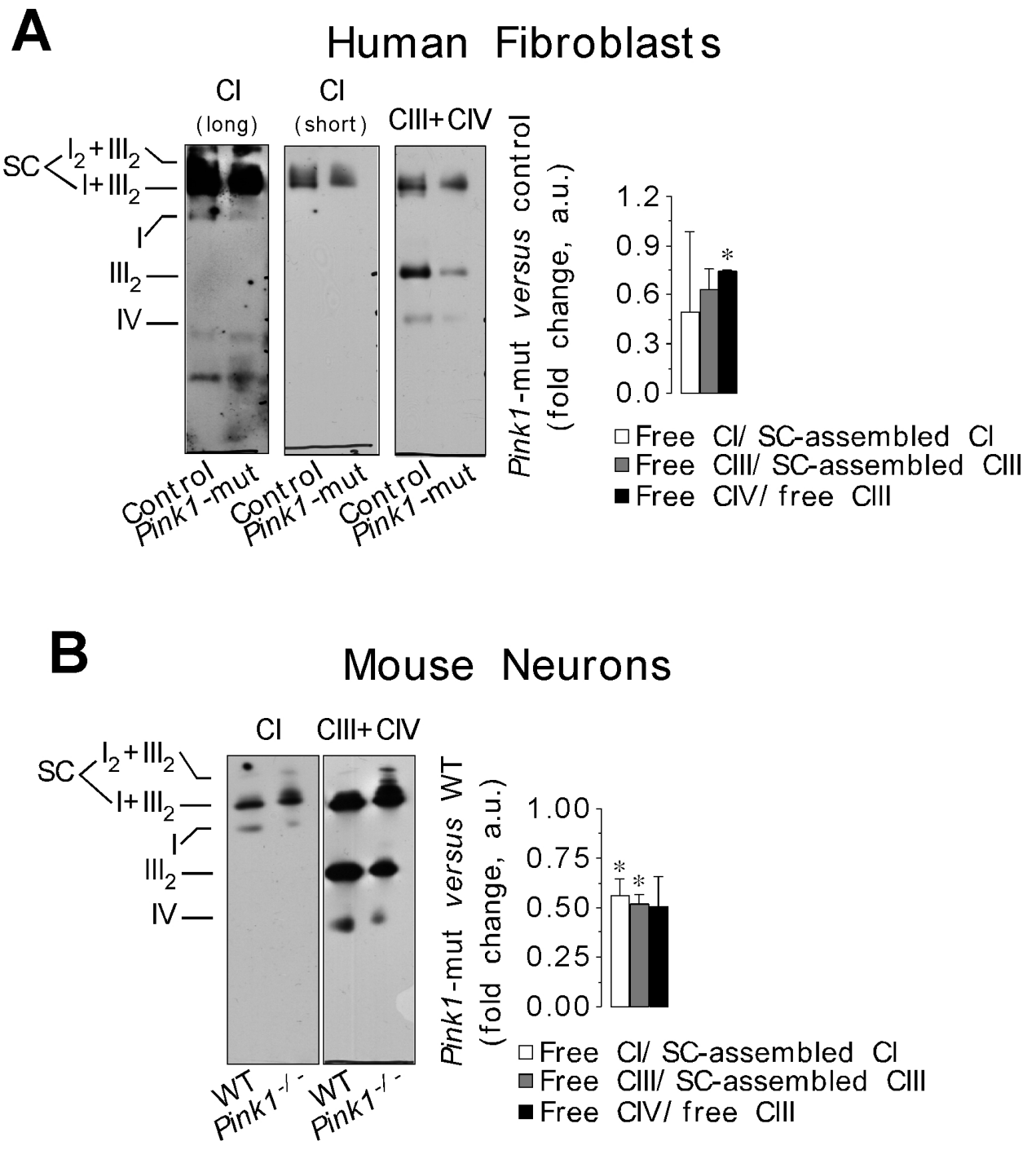
FIGURE LEGENDS

Fig. 1. Mitochondrial electron transport chain organization in *Pink1* mutated human samples and in *Pink1*$^{-/-}$ mice neurons. Digitonin-solubilized isolated mitochondria from either **(a)** control and *Pink1* mutated human fibroblasts, or **(b)** mouse WT and *Pink1*$^{-/-}$ neurons, were subjected to blue-native gel electrophoresis (BNGE) followed by western blotting against a CI, CIII and CIV subunits. Long and short time exposure times are shown for the CI subunit in fibroblasts. At least three biologically independent replicates, or human samples, were performed, though only one representative western blot is shown. Averages obtained from the quantification of the amount of CI (free *versus* SCs-assembled), CIII (free *versus* SCs-assembled) and CIV (*versus* free CIII) were calculated and shown. Given that the quantification was expressed as the ratios within the same samples, no loading control is necessary. Data are the mean values ± SEM of the fold changes *versus* the corresponding controls or WT, from three independent culture preparations. **p*<0.05 (Student's *t* test).

Fig. 2. Mitochondrial electron transport chain organization in *DJ1*$^{-/-}$ *mice neurons and forebrain*. Digitonin-solubilized isolated mitochondria from either **(a)** WT and *DJ1*$^{-/-}$ mouse neurons, or **(b)** WT and *DJ1*$^{-/-}$ mouse forebrain, were subjected to blue-native gel electrophoresis (BNGE) followed by western blotting against a CI, CIII and CIV subunits. An in gel activity assay for complex I (IGA-CI) was shown for mouse forebrain. At least three biologically independent replicates, or human samples, were performed, though only one representative western blot is shown. Averages obtained from the quantification of the amount of CI (free *versus* assembled into supercomplexes), CIII (free *versus* assembled into supercomplexes) and CIV (*versus* free CIII) were shown. Given that the quantification was expressed as the ratios within the same samples, no loading control is necessary. Data are the mean values ± SEM of the fold changes *versus* the corresponding WT, from three independent culture preparations or animals. **p*<0.05 (Student's *t* test).


A
Human Fibroblasts
CI
(long)
CI
(short)
CIII+CIV
SC
I2+III2
I+III2
I
III2
IV
Control
Pink1-mut
Control
Pink1-mut
Control
Pink1-mut
Pink1-mut versus control
(fold change, a.u.)
1.2
0.9
0.6
0.3
0.0
*
Free CI/ SC-assembled CI
Free CIII/ SC-assembled CIII
Free CIV/ free CIII
B
Mouse Neurons
CI
CIII+CIV
SC
I2+III2
I+III2
I
III2
IV
WT
Pink1-/-
WT
Pink1-/-
Pink1-mut versus WT
(fold change, a.u.)
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0.25
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Free CIII/ SC-assembled CIII
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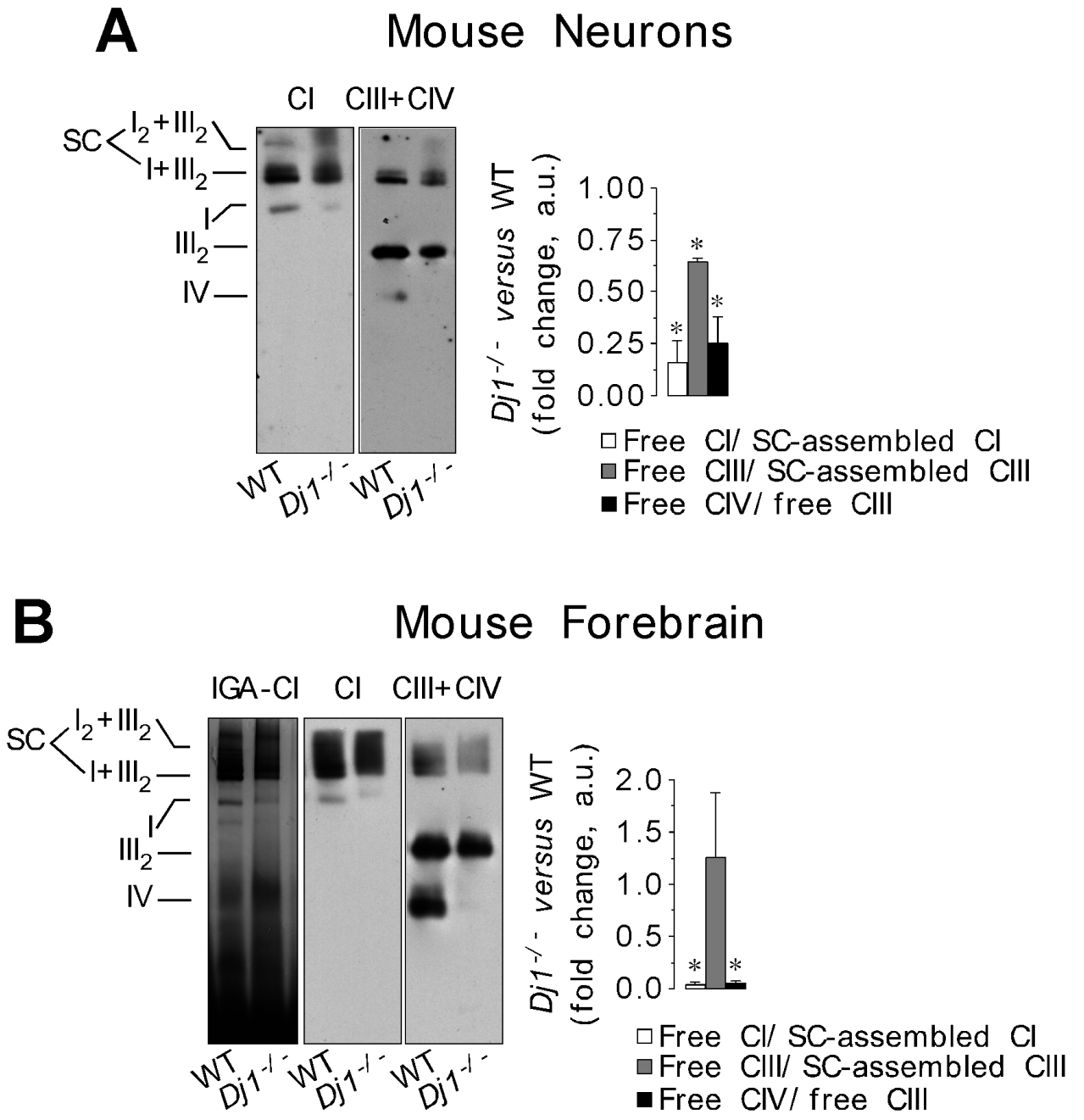
A
Mouse Neurons
CI
CIII+CIV
SC
I2+III2
I+III2
I
III2
IV
WT
Dj1-/-
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Dj1-/-
Dj1-/- versus WT
(fold change, a.u.)
1.00
0.75
0.50
0.25
0.00
Free CI/ SC-assembled CI
Free CIII/ SC-assembled CIII
Free CIV/ free CIII
B
Mouse Forebrain
IGA-CI
CI
CIII+CIV
SC
I2+III2
I+III2
I
III2
IV
WT
Dj1-/-
WT
Dj1-/-
WT
Dj1-/-
Dj1-/- versus WT
(fold change, a.u.)
2.0
1.5
1.0
0.5
0.0
Free CI/ SC-assembled CI
Free CIII/ SC-assembled CIII
Free CIV/ free CIII