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Mitochondrial complex I activity is conditioned by supercomplex I-III_2-IV assembly in brain cells: relevance for Parkinson's disease

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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; MAO, minus antioxidants; 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

The assembly of complex I (CI) with complexes III (CIII) and IV (CIV) of the mitochondrial respiratory chain (MRC) to configure I-III- or I-III-IV-containing supercomplexes (SCs) regulates mitochondrial energy efficiency and reactive oxygen species (mROS) production. However, whether the occurrence of SCs impacts on CI specific activity remains unknown to our knowledge. To investigate this issue, here we determined CI activity in primary neurons and astrocytes, cultured under identical antioxidants-free medium, from two mouse strains (C57Bl/6 and CBA) and Wistar rat, i.e. three rodent species with or without the ability to assemble CIV into SCs. We found that CI activity was 6- or 1.8-fold higher in astrocytes than in neurons, respectively, from rat or CBA mouse, which can form I-III_2-IV SC; however, CI activity was similar in the cells from C57Bl/6 mouse, which does not form I-III_2-IV SC. Interestingly, CII-III activity, which was comparable in neurons and astrocytes from mice, was about 50% lower in astrocytes when compared with neurons from rat, a difference that was abolished by antioxidants- or serum-containing media. CIV and citrate synthase activities were similar under all conditions studied. Interestingly, in rat astrocytes, CI abundance in I-III_2-IV SC was negligible when compared with its abundance in I-III-containing SCs. Thus, CIV-containing SCs formation may determine CI specific activity in astrocytes, which is important to understand the mechanism for CI deficiency observed in Parkinson's disease.

INTRODUCTION

The MRC complexes can be organized into SCs in a process that has been proposed to regulate electron transfer efficiency [1], ROS production [1-3] and mitochondrial cristae shape [4-6]. Recently, we reported that the extent of assembly of CI with CIII-containing SCs differs between neurons and astrocytes of the C57Bl/6 mouse [7], 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 [7]. This leads neuronal mitochondrial respiratory chain to show higher energetic efficiency and less mitochondrial ROS production than astrocytes [7].

In an earlier study, we reported that the specific activity of CI in Wistar rat cultured astrocytes is 6-times higher than that in neurons [8], a difference that does not occur in C57Bl/6 mice or Sprague-Dawley rats [9]. Furthermore, the specific activity of CI in rat (Wistar or Sprague-Dawley) astrocytes is 6-times higher than that in C57Bl/6 mice [9]. However, the mechanism explaining such differences in the specific CI activities in neurons *versus* astrocytes, and in astrocytes across these species remains elusive. Given the importance of CI activity for the pathology of several neurodegenerative diseases, here we addressed this issue and found that the specific activity of CI is associated with the ability of rodent species or strains to assemble CIV into I-III_2-IV SC.

MATERIALS AND METHODS

Ethical use of animals. Wistar rats, C57BL/6 and CBA mice were bred at the Animal Experimentation Unit of the University of Salamanca. All protocols were approved by the Bioethics Committee of the University of Salamanca in accordance with the Spanish legislation (Law 6/2013).

Primary cell cultures. Primary cultures of rat or mice cortical neurons [10] were prepared from fetal Wistar rats of 16.5 days of gestation, and 15.5 days in C57BL/6 or CBA mice, seeded at 2.0 x 10^5 cells/cm^2 in different size 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. When indicated, the B27-minus antioxidants (MAO) supplement (lacking vitamin E, vitamin E acetate, superoxide dismutase, catalase and glutathione) was used instead of the B27-

AO supplement during the last 24 h. Cells were incubated at 37 °C in a humidified 5% (v/v) CO_2-containing atmosphere. At 72 hours after plating, medium was replaced. Cells were used at day 7. Astrocytes in primary culture were obtained from 0-24 h old neonates, and cell suspension seeded at the ratio of 3-4 brains per 175 cm^2 plastic flask, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal serum (FCS) [11]. To detach non-astrocytic cells, after one week *in vitro*, the flasks were shaken at 180 rpm overnight. The supernatant was discarded, and the attached, astrocyte-enriched cells, were reseeded at $0.5\text{-}1\cdot10^5$ cells/cm^2 in different size plates in DMEM with 10% FCS. Twenty-four hours before the experiments, the medium was changed to Neurobasal (Life Technologies) supplemented with 2 mM of glutamine and 2% (v/v) B27-MAO supplement. When indicated, the B27-AO supplement was used instead of the B27-MAO supplement. Astrocytes were used for experiments on day 14.

Activity of mitochondrial complexes. Cells were collected and suspended in phosphate buffer (PB; 0.1 M KH_2PO_4 pH 7.0). After three cycles of freeze/thawing, to ensure cellular disruption, CI, CII-III, CIV and citrate synthase activities were determined. Rotenone-sensitive NADH-ubiquinone oxidorreductase activity (CI) [12] was measured in KH_2PO_4 (20 mM, pH 7.2) in the presence of 8 mM $MgCl_2$, 2.5 mg/ml BSA, 0.15 mM NADH and 1 mM KCN. Changes in absorbance at 340 nm (30 ºC) (ε=6.81 $mM^{-1}cm^{-1}$) were recorded after the addition of 50 µM of ubiquinone and 10 µM of rotenone. CII-III (succinate-cytochrome *c* oxidorreductase) activity [13] was determined in the presence of 100 mM phosphate buffer, plus 0.6 mM EDTA(K^+), 2 mM KCN and 200 µM of cytochrome *c*. Changes in absorbance were recorded (550 nm; 30 ºC) (ε=19.2 $mM^{-1}cm^{-1}$) after the addition of 20 mM of succinate and 10 µM of antimycin A. For CIV (cytochrome *c* oxidase) activity, the first rate constant of cytochrome *c* oxidation was determined [14] in the presence of 10 mM phosphate buffer and 50 µM of reduced cytochrome *c*; absorbance was recorded every minute at 550 nm, 30ºC (ε=19.2 $mM^{-1}cm^{-1}$). Citrate synthase activity [15] was measured in the presence of 93 mM of Tris-HCl, 0.1 % (v/v) triton X-100, 0.2 mM acetyl-CoA, 0.2 mM DTNB; the reaction was started with 0.2 mM of oxaloacetate, and the absorbance was recorded at 412 nm (30 ºC) (ε=13.6 $mM^{-1}cm^{-1}$).

Mitochondria isolation and solubilization. Mitochondria were obtained according to a previously published protocol [16]. Briefly, cells (12-100 millions) were collected, cell pellets frozen at -80 ºC and 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). For the assessment of mitochondrial complexes organization, digitonin solubilized mitochondria (10-50 µg) were loaded in NativePAGE Novex 3-12% gels (Life Technologies). After electrophoresis, in-gel NADH dehydrogenase activity was evaluated [17]. After identification of individual CI and CI-containing SCs bands according to the NADH dehydrogenase activity, a second dimension (2D) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed to identify the NDUFV1 subunit of CI. Thus, individual CI or CI-containing SCs bands were excised from the gel and denatured in 1% (v/v) SDS (containing 1 % (v/v) β-mercaptoethanol) during 1 hour. The proteins contained in the gel slices were separated electrophoretically, followed by Western blotting against a NDUFV1 specific antibody.

Western blotting. The gel slices-containing mitochondrial proteins, obtained as in the previous section, were subjected to sodium docedyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis on a 10% (v/v) acrylamide gel (MiniProtean, Bio-Rad) including PageRuler Plus Prestained Protein Ladder (Thermo). 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 NDUFV1 primary antibody (1/500 by vol; AV48312; Sigma) overnight at 4ºC. After incubation with

horseradish peroxidase-conjugated goat anti-rabbit IgG (1/10,000 by vol; sc2030; Santa Cruz Biotechnologies), 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. Three biologically independent replicates were performed, though only one representative western blot is shown in the article. The protein abundances of the western blots were measured by densitometry of the bands on the films using ImageJ 1.48u4 software (National Institutes of Health, USA), and the resulting values were normalized by the intensity of the bands in neurons in each region and used for the statistical analysis.

Protein determinations. Protein samples were quantified by the bicinconic 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, and the results were expressed as the mean values ± SEM. To compare two groups of values, the statistical analysis of the results was performed by the Student's *t* test using the SPSS software. For multiple values comparisons, we used one-way analysis of variance (ANOVA) followed by Bonferroni test. In all cases, $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

To investigate whether CI activity is conditioned by the occurrence of SCs in brain cells, we first determined CI specific activity in neurons and astrocytes from rodent species known to have different abilities to assemble CIV into SCs [2]. As shown in **Fig. 1a**, CI activity was similar in neurons and astrocytes from C57BL/6 mice cultured under identical conditions, i.e. using the defined Neurobasal® medium with the B27 minus antioxidants supplement (B27-MAO). This lack of intercellular difference in CI activity confirms our previous results observed in neurons cultured with Neurobasal® with B27 with antioxidant supplement (B27-AO), and astrocytes cultured with DMEM with 10% FCS, of the C57BL/6 mice [7].

Since C57BL/6 mice express a short (inactive) form of the III-IV SC assembly factor 1 (SCAF1) [2], the ability to form CIV-containing SCs is impaired in this mouse strain

[18]. In good agreement with this, mitochondrial proteins isolated from C57BL/6 mouse astrocytes, subjected to BNGE, confirmed the lack of I-III_2-IV SC (**Fig. 2a**). However, Wistar rat expresses the long (active) form of SCAF1 [2, 18], which we herein confirmed as judged by the occurrence of I-III_2-IV SC in the BNGE astrocytic mitochondrial proteins from this species (**Fig. 2a**). This led us to interrogate whether the occurrence of I-III_2-IV SC affected CI activity. As shown in **Fig. 1a**, CI activity in rat neurons was very similar to that observed in the C57BL/6 mouse neurons; however, CI activity in rat astrocytes was 6-fold higher than in neurons. This difference in CI activity between neurons and astrocytes from rats confirms previous works in which cells were cultured under different culture media composition (DMEM plus 10% FCS) [8, 9]. To further confirm this, we next analyzed CI activity in a mouse strain that harbors active SCAF1, such as CBA [2]. As shown in **Fig. 2a**, BNGE in astrocytic mitochondrial proteins from the CBA mice revealed the occurrence of the I-III_2-IV SC. Interestingly, CI specific activity was about 1.8-fold higher in astrocytes when compared with neurons from the CBA mice (**Fig. 1a**). These results confirm that the higher CI activity in astrocytes when compared with neurons is associated with the occurrence of I-III_2-IV SC. However, whether rat neurons and astrocytes differ in their abilities to form these SC that explain the different CI activities is unknown. As shown in **Fig. 3a**, BNGE of mitochondrial proteins from rat neurons expressed the I-III_2-IV SC, as did the astrocytes. Nevertheless, the total abundance of SC is higher in neurons (**Fig. 3**), as occurred in C57BL/6 mice [7]. These results are compatible with the notion that the difference in CI activity between neurons and astrocytes is associated with the species-specific ability to form I-III_2-IV SC.

Next, we aimed to understand how the formation of I-III_2-IV SC would influence CI activity specifically in astrocytes. We hypothesized whether the differences in CI activity between neurons and astrocytes in rats and CBA mice might be consequence of cell-specific differences in CIII abundance. In fact, CIII abundance is much lower in astrocytes than in neurons [7]. To assess whether this affected CIII activity, we measured CII-III activity -which is limited by CIII. As shown in **Fig. 1b**, CII-III activity was similar in neurons and in astrocytes of the C57BL/6 mice, confirming our data in the same cells cultured in a different culture media [7]. However, CII-III activity in astrocytes was 50% of that in neurons from the rats (**Fig. 1b**), which suggests that rat astrocytes express even lesser abundance of CIII than neurons, when compared with

C57BL/6 mouse. It should be mentioned that a major difference in the astrocytic MRC organization between rat and C57BL/6 mouse relies on their different abilities to form CIV-containing SC (**Fig. 2a**). Interestingly, the relative abundance of CI bound to SCs in rat neurons is much higher than in rat astrocytes (**Fig. 3b**), a fact that is consistent with the higher CIII abundance in neurons [7]. Altogether, these results strongly suggest that the higher CI activity in astrocytes, when compared with neurons, in Wistar rats –and, maybe, in Sprague-Dawley rats and humans [9]– is due to CIII assembly with CIV, thus sparing a limited amount of CIII to bind CI. Thus, the abundance of free CI -and therefore, CI activity- in rat astrocytes, would be higher than in other species that lack the ability to form I-III_2-IV SC. Intriguingly, CBA astrocytes, which also express higher CI activity when compared with neurons (**Fig. 1a**), show no differences in CII-III between these cells (**Fig. 1b**). However, it should be noticed that the extent of the difference in CI activity between astrocytes and neurons (1.8-fold) is considerably lower than the difference observed in rat (6-fold). This likely indicates that CIII abundance in the rat is considerably lower than in CBA mouse, thus sparing a higher fraction of free CI in the former species.

Next, we aimed to understand why CII-III activity was lower in astrocytes from the rat when compared with those from the C57BL/6 mouse (**Fig. 1b**). To assess this, we cultured the rat cells in the presence of the B27-containing antioxidant supplement as an attempt to mimic the antioxidants-containing conditions of the C57BL/6 mice cultured cells [7]. As shown in **Fig. 4a**, the presence of antioxidants abolished the difference in CII-III activity between rat neurons and astrocytes. Furthermore, given that previous data performed in Wistar rat neurons and astrocytes cultured in DMEM plus 10% FCS reported no differences in CII-III activities between these cells [8, 9] , whereas here we observed decreased CII-III in astrocytes (**Fig. 1b**), we investigated whether the medium composition was responsible for this apparent controversy. The results depicted in **Fig. 4a** reveal that culturing astrocytes with DMEM plus 10% FCS abolished the differences in CII-III activity between neurons and astrocytes. Since the presence of either antioxidants or FCS (which contain antioxidants) increased CII-III activity in rat astrocytes (**Fig. 4a**), we suggest that ROS would be a putative responsible factor for the decreased CII-III activity in these cells; however, to clarify this issue further investigation would be required. Finally, we observed no differences in the activities of CIV or citrate synthase under any of the circumstances analyzed (**Figs. 1c, 1d and 4b**).

In conclusion, here we show that the ability to assemble I-III_2-IV SC can impact on CI activity. This is particularly important for the rat astrocytes, which show features consistent with very low CIII abundance that is sequestered by CIV in SC, thus decreasing spared CIII to bind with CI. This would explain the atypical high CI activity previously observed in rat astrocytes [8, 9]. Whether a similar mechanism operates in astrocytes from other species expressing SCAF1, such as humans [2], is an interesting suggestion worth investigating in the future. Given the occurrence of thresholds for CI inhibition [19, 20], our results may contribute explaining the higher vulnerability of mouse *versus* rat astrocytes to the parkinsonian-like toxin and CI inhibitor, 1-methyl-4-phenylpyridinium (MPP^+) [19]. If so, human inter-individual variations in efficiently assembling CIII and CIV might impact on CI activity, hence contribute to explaining the differences in the CI damage vulnerability associated with neurodegenerative disorders, in particular sporadic Parkinson's disease [21-23].

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FIGURE LEGENDS

Fig. 1. Specific activities of the mitochondrial respiratory chain complexes in primary neurons and astrocytes from mouse and rat. The specific activities of the mitochondrial respiratory chain complexes I (**a**), II-III (**b**), IV (**c**) and citrate synthase (**d**) were determined spectrophotometrically in the C57BL/6 mouse, Wistar rat and CBA mouse cell homogenates as described in Materials and Methods. Data are the mean values ± S.E.M. from three independent culture preparations. *p<0.05 (Student's *t* test).

Fig. 2. Free and supercomplexes-bound complex I abundance in primary astrocytes from mouse and rat. (**a**) Digitonin-solubilized isolated mitochondria from astrocytes were subjected to blue-native gel electrophoresis (BNGE) followed by in-gel CI activity assay. CI occurs both free and part of I-III_2, I_2-III_2 and I-III_2-IV supercomplexes. (**b**) Gel slices corresponding to the rat free CI and to each supercomplex-containing CI were excised from the blue native gels and subjected to polyacrylamide gel electrophoresis (PAGE) followed by western blotting against NDUFV1 (a CI subunit); given the different protein content of each slice, no loading control is provided.

Fig. 3. Free and supercomplexes-bound complex I abundance in primary neurons and astrocytes from Wistar rat. (**a**) Digitonin-solubilized isolated mitochondria from astrocytes were subjected to BNGE followed by in-gel CI activity assay. CI occurs both free and part of I-III_2, I_2-III_2 and I-III_2-IV supercomplexes (SCs). (**b**) Gel slices corresponding to the free CI and to SC-containing CI were excised from the blue native gels and subjected to polyacrylamide gel electrophoresis (PAGE) followed by western blotting against NDUFV1 (a CI subunit); given the different protein content of each slice, no loading control is provided.

Fig. 4. Specific activities of the mitochondrial complex II-III and citrate synthase in primary neurons and astrocytes from Wistar rat. The specific activities of the mitochondrial respiratory chain complex II-III (**a**) and citrate synthase (**b**) were determined spectrophotometrically in the cell homogenates as described in Materials and Methods. Data are the mean values ± S.E.M. from three independent culture preparations. n.s., not significant (ANOVA post-hoc Bonferroni).

Figure 1

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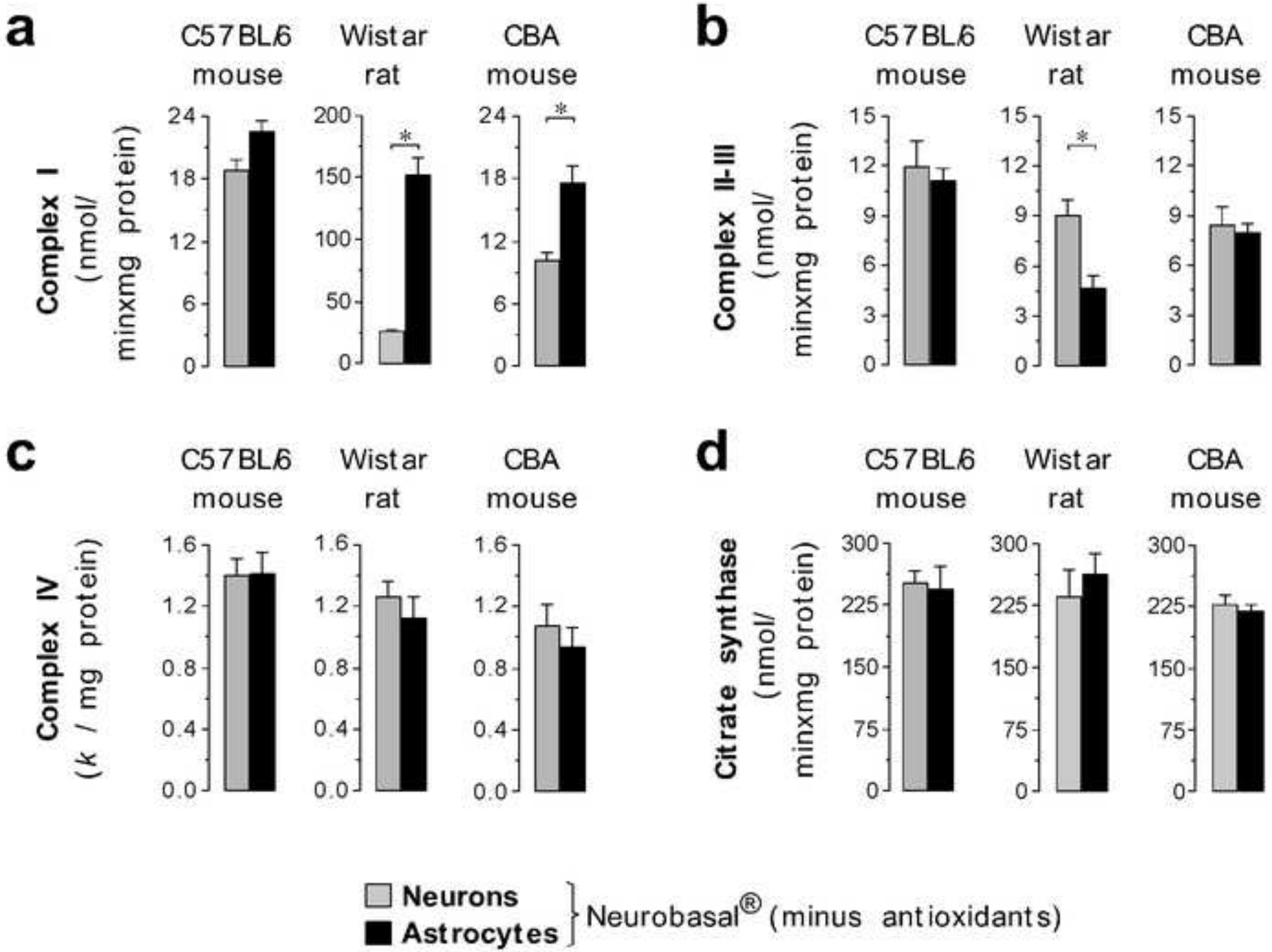

Figure 2

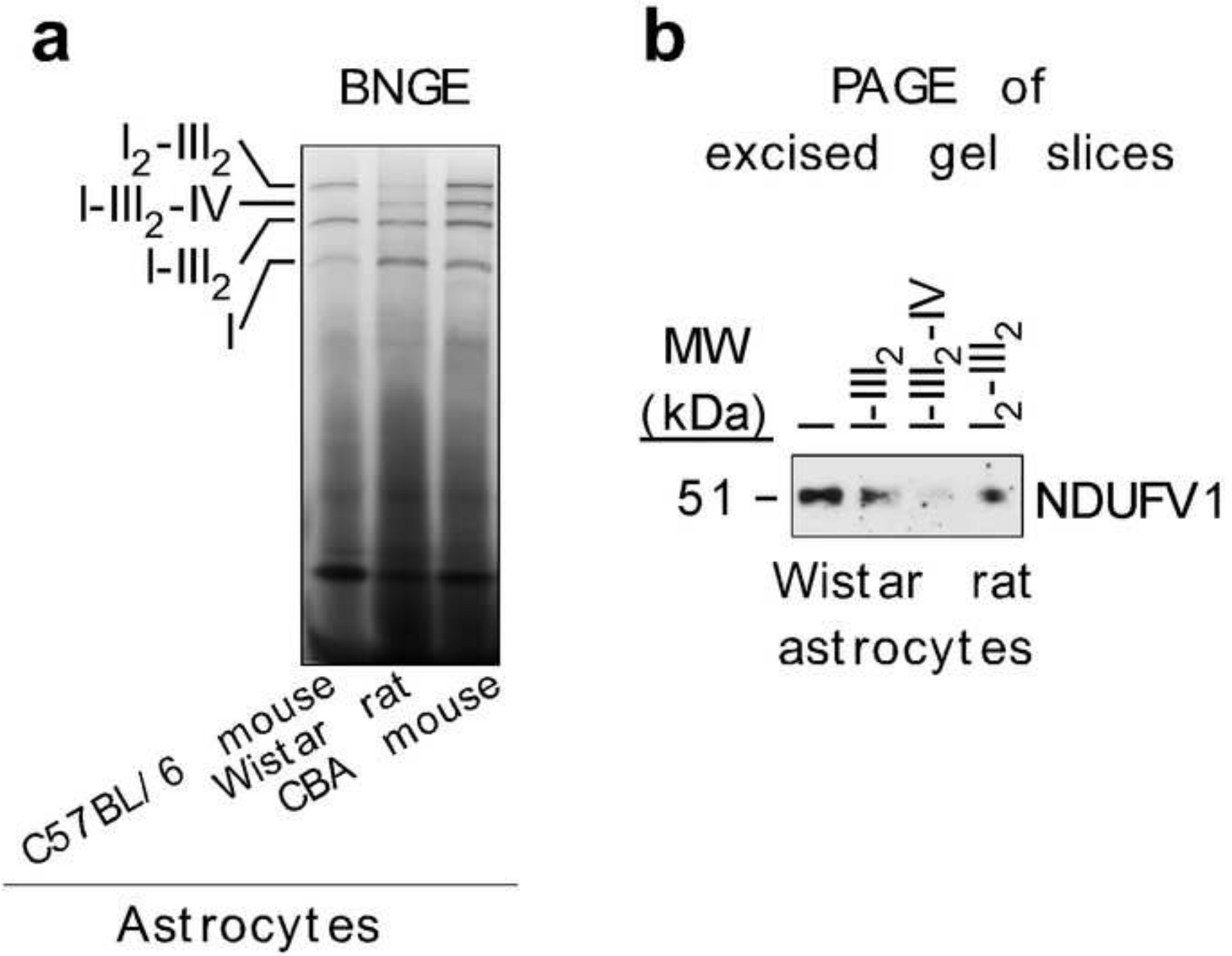

Figure 3

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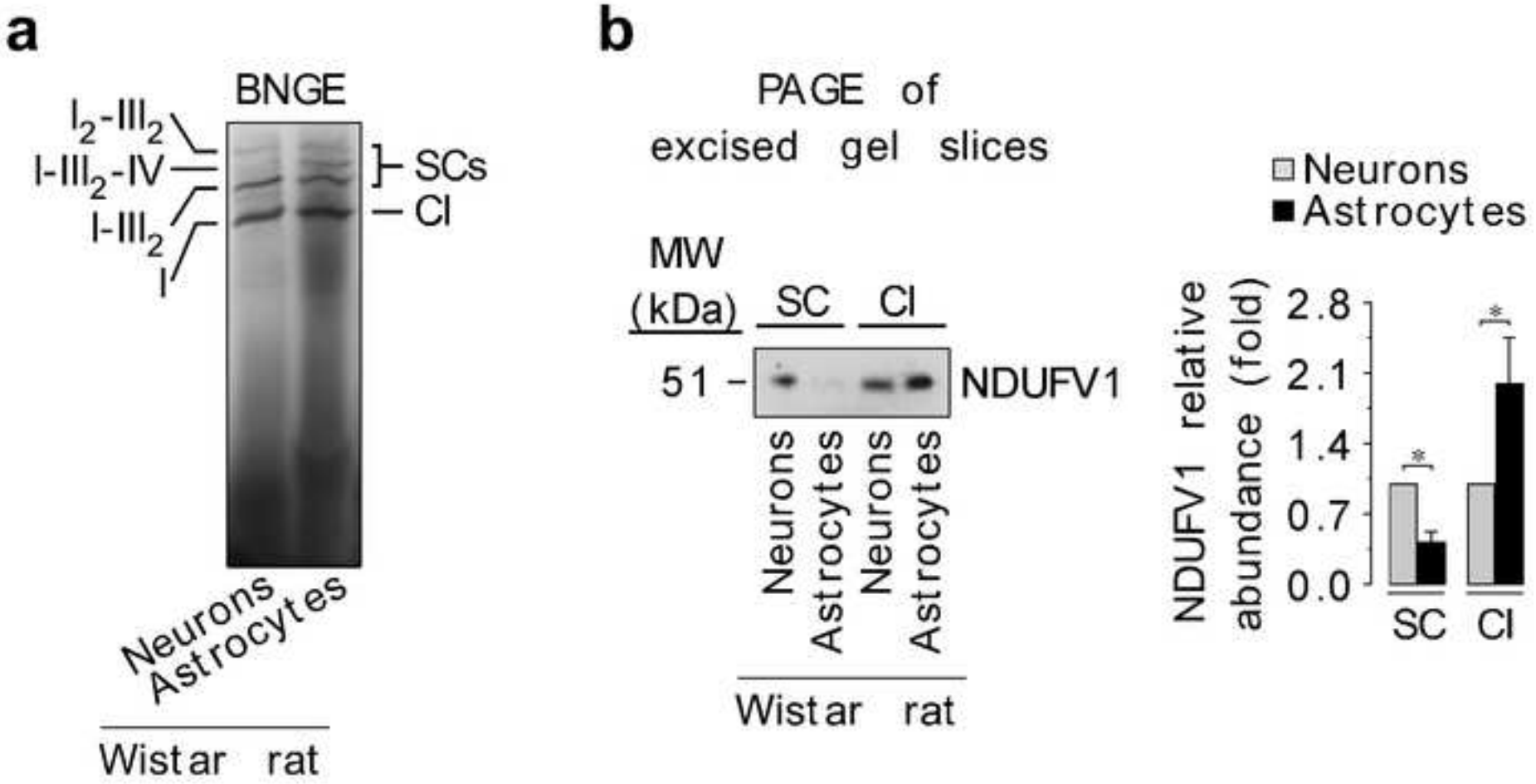

Figure 4

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