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Reduced mitochondrial function but not mitochondrial mass in cerebellar granule cells of homozygous Ca(v) 2.1 mutant mice (leaner mice) at postnatal age 20

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ABSTRACT

Mutation in the CACNA1A gene coding for the a1A pore forming subunit of voltage-gated P/Q type (Ca_v 2.1) calcium ion channel is associated with several human neurological disorders like familial hemiplegic migraine, episodic ataxia type 2 and spinocerebellar ataxia 6. We study leaner mouse which carry a similar mutation in CACNA1A gene. These mice exhibit severe cerebellar granule cell (CGC) death which peaks at postnatal day (P) 20. Previously we have shown that CGC of leaner mice have altered intracellular calcium homeostasis at P20. Alteration of calcium homeostasis is known to cause mitochondrial dysfunction which can result in cell death. In this study we have investigated the mitochondrial function by measuring mitochondrial membrane potential ($\Delta\psi_m$) using TMRM dye. A reduced $\Delta\psi_m$ in leaner CGCs at P20 was observed as compared to age matched wild type CGCs. To confirm that reduced $\Delta\psi_m$ observed in leaner CGC is due to reduced mitochondrial function and not due to decreased mitochondrial mass, the latter was measured by quantifying mitochondrial specific phospholipid, cardiolipin in CGC and also by semi-quantifying mitochondrial specific protein, cytochrome C in whole cerebellum. Both the techniques demonstrated no change in mitochondrial mass in leaner CGC as compared to age matched wild type CGC confirming a reduced mitochondrial function in leaner CGC at P20. Further, generation of ROS was measured using CM-H2DCFDA dye. No change in generation of ROS was observed in leaner and wild type CGC at P20 indicating a mitochondrial specific and ROS independent mechanism of cell death in leaner CGCs.

INTRODUCTION

Leaner mice carry an autosomal recessive mutation in the CACNA1A gene coding for the a1A pore forming subunit of voltage-gated P/Q type (Cav 2.1) calcium ion channels [1]. Several autosomal dominant human neurological disorders are associated with mutations in the CACNA1A gene including; familial hemiplegic migraine, generalized epilepsy with ataxia, episodic ataxia type 2 and spinocerebellar ataxia type 6 [2, 3, 4, 5]. Cerebellar granule cells (CGCs) of homozygous leaner mice begin to exhibit apoptotic cell death after postnatal (P) day 10 and cell death peaks at P20 [6]. Apoptosis is an energy dependent, highly regulated process that allows for controlled dissolution of a cell without inducing an inflammatory response in the tissue surrounding the affected cell [7, 8]. Among the various pathways associated with apoptosis, one important pathway involves mitochondrial dysfunction. In neurons, mitochondria are involved in regulation of calcium ion homeostasis, alterations of which can severely affect the ability of mitochondria to function normally and lead to a cascade of events that eventually cause neuronal cell death [9].

Previously we have shown that calcium homeostasis is significantly altered in CGC of leaner mice at postnatal day 20 such that CGCs exhibited a nearly 40% reduction in depolarized induced calcium ion transients compared to CGCs of P20 wild type mice [10]. The purpose of this study is to investigate whether there is evidence of mitochondrial dysfunction in CGCs of leaner mice at this same time point, P20, which is the time of peak cell death. To address this question we measured mitochondrial membrane potential ($\Delta\psi_m$) in CGCs at P20. The $\Delta\psi_m$ is a major factor that ultimately controls cellular energy levels and is an excellent marker to assess mitochondrial function. Any observed change in $\Delta\psi_m$ could be due to an overall change in mitochondrial function or change in mitochondrial mass. The mitochondrial mass was estimated by quantifying the mitochondrial protein, cytochrome C in whole cerebellum and also by estimating the inner mitochondrial membrane phospholipid, cardiolipin content in CGCs of leaner mice. In addition, we measured the generation of reactive oxygen species (ROS) in the CGCs of both wild type and leaner mice.

MATERIALS AND METHODS

Wild type (+/+) and homozygous leaner (tg^{la}/tg^{la}) mice on an inbred C57BL/6 background were used. All mice were bred and housed at the Laboratory Animal Research and Resource facility at Texas A&M University. Details of leaner mouse management are described elsewhere [11]. Three male and three female wild type and homozygous leaner mice at postnatal age 20 days (P20) were used. Procedures for animal use were approved by the Texas A&M University Laboratory Animal Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

Mitochondrial membrane potential measurement

Cerebellar granule cells were acutely isolated and the protocol was validated by different experiments as described in detail elsewhere [11, 12]. Briefly, mice were anesthetized using isoflurane and killed by decapitation. The cerebellum was removed, chopped into small pieces and transferred to 50 ml chilled minimum essential medium (MEM; Life Technologies Inc., Grand Island, NY, USA) containing 1.5 U/ml protease (Sigma, Ronkonkoma, NY, USA) and 0.2% DNase (Sigma). The CGCs were centrifuged at 1000g for 10 min and resuspended in MEM. In all the experiments where fluoroprobes were used, appropriate positive and negative controls were used and mitochondrial depolarization using uncoupler was done as described elsewhere [11]. Autofluorescence was checked and background fluorescence was removed before data analysis. For all the experiments approximately 20 cells per animal were evaluated.

Isolated CGCs were loaded with tetramethyl rhodamine methyl ester (TMRM, Life Technologies, Inc.) at 150 nM made in 100% dimethyl sulfoxide (Sigma) and incubated at 37oC for 15 min. After incubation, the cells were kept in phenol red free media for imaging. Fluorescent images were acquired using a 40X oil objective on an Olympus 1X-70 microscope and a Hamamatsu ORCA-ER cooled charge-coupled device camera at excitation and emission of 555nm and 600nm, respectively. CGCs were illuminated minimally to

reduce the photo bleaching. Image analysis was done using the Simple PCI Imaging System (Compix Media, Inc., Irvine, CA, USA).

Mitochondrial mass measurement

Two different methods were used to estimate mitochondrial mass or volume in cerebellar granule cells of 20 day old wild type and leaner mice.

Quantification of Cardiolipin

Cellular cardiolipin content was estimated by visualization of CGCs stained with 10-N-Nonyl acridine orange (NAO, Life Technologies, Inc.), a dye that is $\Delta\psi_m$ independent and binds with high affinity to mitochondrial phospholipid cardiolipin [13]. CGCs were isolated and loaded with 10 µl of 50 µM NAO made in 100% ETOH to reach a final concentration of 500 nM and further incubated at RT for 25 min. Fluorescent images were acquired using a 20X objective on an Olympus 1X-70 microscope and a Hamamatsu ORCA-ER cooled charge-coupled device camera at excitation and emission of 488 nm and 520nm, respectively. CGC were illuminated minimally to reduce photo bleaching. Image analysis was carried out to determine the fluorescent emitted by CGC after subtracting background fluorescence as an index of cardiolipin content potential using Simple PCI Imaging System (Compix).

Cytochrome C expression

Total protein from 3 wild type and leaner mice cerebella was extracted by sonication in 0.5ml of protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) and one tablet of Complete, Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics Corp, Indianapolis, IN, USA) per 10 mL of M-PER. At the time of analysis, 30 µg of protein sample was denatured and loaded into a 4-20% gradient polyacrylamide gel. Protein was then transferred onto PVDF blotting membrane (BioRad, Hercules, CA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell system (BioRad). Membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween-20 for 2 hours at room temperature (RT) then incubated with monoclonal mouse cytochrome C antibody (1 µg/ml, BD Pharmingen, San Diego, CA, USA) overnight at 4ºC, followed by peroxidase labeled secondary antibody (1:20,000, Anti-Mouse IgG, Cell Signaling Technology, Danvers, MA) for 2 hours at RT. The immunoreactive bands were visualized by enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA). The chemiluminescence signal was obtained by capturing images using the FluorChem 8800 Imaging System (Alpha Innotech, San Leandro, CA, USA). After detecting immunoreactive bands, the gels and membranes were stained with Coomassie blue using GelCode blue stain (Pierce) to ensure equal protein loading and transfer. Densitometric analysis was performed using AlphaEaseFCTM Imaging system (Alpha Innotech) to determine the optical density of the immunoreactive bands. Gels were repeated at least three times and the integrated density value obtained was used as percent control for statistical analysis.

Reactive oxygen species (ROS) measurement

For determination of ROS we used Chloromethyl-dihydrodichlorofluorescein diacetate (CM-H2DCFDA) (Molecular Probes Inc), which is a redox-sensitive dye. It is membrane permeable and gets trapped in cells by binding of the chloromethyl group to cellular thiols. Subsequently the dye becomes fluorescent when oxidized by H_2O_2 and/or downstream free radical products of H_2O_2. Several dichlorofluorescein derivatives have been used for measuring intracellular ROS generation [12, 14]. Dichlorofluorescein (DCF) dyes have their own advantages and disadvantages. Potential problems associated with DCF dyes include leakage and photo-oxidation. To overcome these problems we used a derivative of DCF, CM-H2DCFDA, as it permits longer retention within the cell [15]. To reduce the photoreaction problem associated with DCF dyes we used very low concentration of dye. It has been shown previously that DCF dyes when used at concentration less than 1 µm shows minimal photoreaction [16].

CGCs were acutely dissociated as described above, plated onto chambered slides (VWR International, Inc.) and incubated in 95% O_2 and 5% CO_2 at 37°C for 25 min. The cells were loaded with CM-H2DCFDA at a concentration of 500 nmol and incubated in 95% O_2 and 5% CO_2 at 37°C for 8 min. At the end of loading period, cells were thoroughly washed to remove nonhydrolysed CM-H2DCFDA and subsequently cells were maintained

in phenol red free media (Sigma). Unstained cells were examined under green emission range to demonstrate any autofluorescence. As a second negative control fluorescence emitted by cell free mixture of phenol red free media and dye was also monitored. At a concentration of 500 nM negligible increase in fluorescence was observed in cell free mixture of buffer and dye showing minimal atmospheric and/or photo-oxidation (Data not shown). For positive control, oxidative activity was stimulated by adding 100 µM H_2O_2 to CM-H2DCFDA stained CGCs [17]. A 200% increase in fluorescence was observed following addition of 100 µM H_2O_2 (Data not shown). Sequential time course fluorescent image capturing was performed for 22.5 minutes using a 90 second interval with a 20X objective on an Olympus 1X-70 microscope and a Hamamatsu ORCA-ER cooled charge-coupled device camera at excitation and emission of 490 nm and 520 nm, respectively. Image capturing and ROS levels were analyzed using Simple PCI Version 5.0.0.1503 Compix Inc. and Imaging.

Statistics

Data are presented as means ± standard error of mean. All data were analyzed using Microsoft Excel and statistical software SPSS Version 12.0.1 for windows. For comparison of two groups T-test was employed using Microsoft Excel. The ROS data was analyzed using General Linear Model (GLM) - repeated measures analysis.

Figure 1:

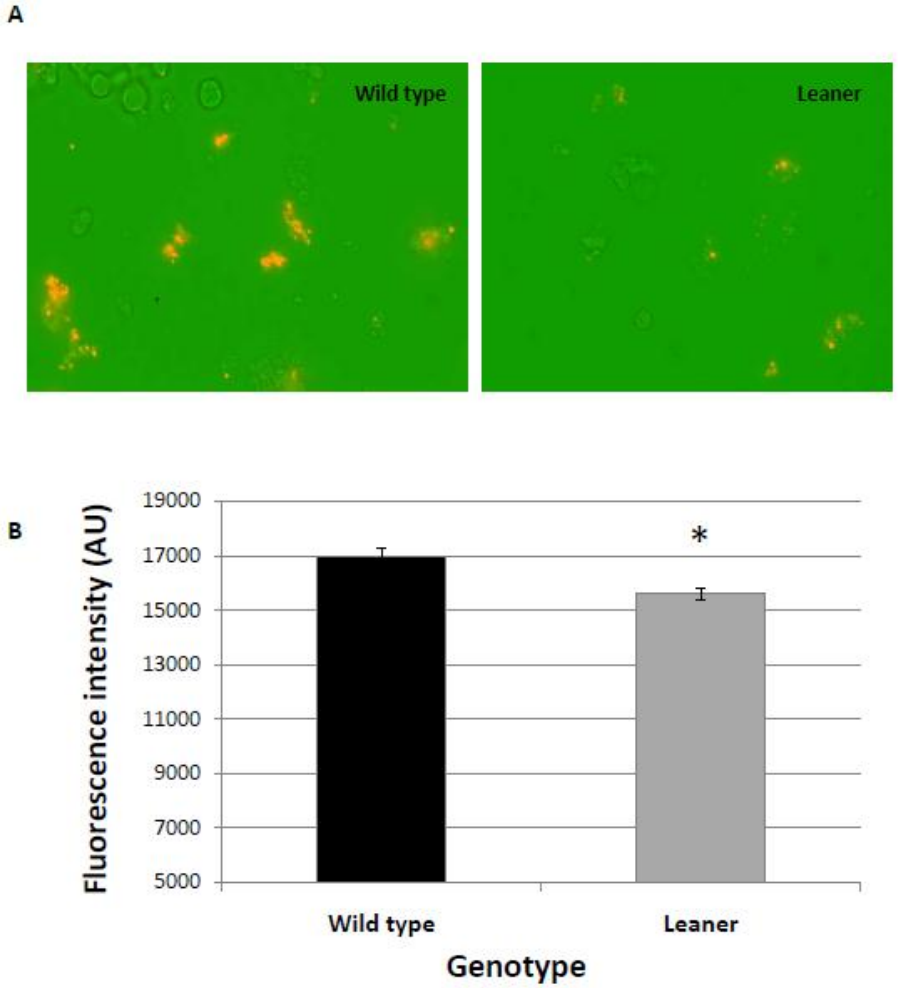

Figure 1. Average mitochondrial membrane potential of CGCs at P20. Photomicrographs in A show representative cerebellar granule cells stained with TMRM. Note that the fluorescence intensity of wild type CGC (wild type) is greater than leaner CGC (leaner), demonstrating higher MMP in wild type CGC. The graph in B indicates the mean ± SEM fluorescence intensity in arbitrary units (AU); A significantly reduced membrane potential (asterisk) was observed in leaner CGCs as compared to wild type CGCs ($P<0.001$; $n=6$).

RESULTS AND DISCUSSION

Studies have shown that mitochondrial impairment associated with alteration of calcium ion homeostasis can result in reduced ability to maintain stable resting intracellular calcium [18]. Previously our lab has shown that during postnatal development, CGCs of leaner mice experience reduced calcium ion buffering [19] and alteration of calcium ion homeostasis [10]. The objective of this study was to assess mitochondrial function in CGCs of leaner mice at the time of peak cell death, which is postnatal day 20. Mitochondrial membrane potential ($\Delta\psi_m$) was examined in individual CGCs isolated from leaner and wild type mice, using TMRM, which is a lipophilic cationic dye that accumulates in mitochondria in proportion to its membrane potential [20]. Only CGC showing a punctate pattern of staining (Fig 1A) were used for final analysis. Fluorescence intensity was measured from individual CGC after thresholding and subtracting background fluorescence.

Statistical analysis showed reduced $\Delta\psi_m$ in leaner CGCs compared to age matched wild type CGCs (Fig. 1B), indicating reduced mitochondrial activity in leaner CGCs. This result substantiated our previous finding that CGC death in leaner mice occurs through apoptosis via activation of mitochondria-associated genes and caspase-2 and -3 [6, 21].

Mitochondria have large conductance pores called mitochondrial transition pores (MTP), whose opening is considered to be a key event during apoptosis [22]. When MTP open, $\Delta\psi_m$ is decreased and in the process small, mitochondria-specific proteins such as cytochrome C, apoptosis inducing factor and/or Smac/Diablo, are released into the cytosol [23, 24]. These proteins in turn can act alone to trigger apoptosis or activate caspase proteases that lead to apoptosis [25]. However, the specific signaling molecules involved in leaner CGC death cascade are not yet known.

The reduced TMRM dye fluorescence intensity observed in leaner CGC as compared to wild type CGCs (with similar loading times and pH for both the groups) indicates a reduced $\Delta\psi_m$ in leaner CGCs or a reduced mitochondrial mass or volume in leaner CGCs. Various methods have been reported in the literature to assess mitochondrial mass [for review 20], each with its own advantages and disadvantages. In the first method used in this study, we measured mitochondrial mass by estimating its cardiolipin content, using the $\Delta\psi_m$ independent dye, NAO. Measuring mitochondrial mass using a $\Delta\psi_m$ independent dye such as Mitotracker green or Nonyl acridine orange is one of the most common approaches reported in the literature [20]. No overall significant difference in fluorescence intensity emitted by CGCs from leaner and wild type mice was observed (Fig. 2), indicating no difference in cardiolipin content. The drawback of using these probes is that they all exhibit variable sensitivity to $\Delta\psi_m$. Measurement of mitochondrial mass using NAO has been both supported [26, 27] and contradicted [28, 29] in the literature.

Figure 2:

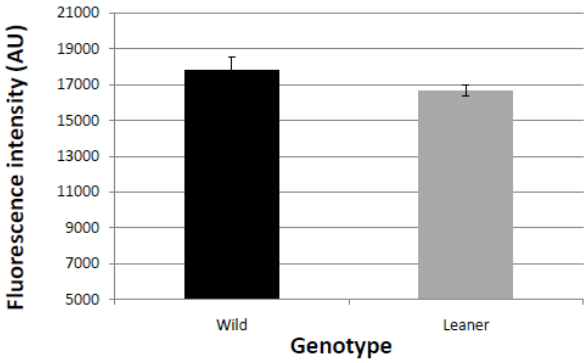

Figure 2. Mitochondrial phospholipids, cardiolipin content in cerebellar granule cells of wild type and leaner mice at P20. The graph indicates the mean ± SEM fluorescence intensity in arbitrary units (AU). No significant difference in cardiolipin content was observed between genotypes (n=6).

The second method used in this study was an indirect approach that measured expression of the key mitochondrial protein, cytochrome C, in whole cerebellum. Cytochrome C is a ubiquitous, heme-containing protein that normally resides in the space between the inner and outer mitochondrial membranes [30]. Since CGCs are the major cell type within the cerebellum, measurement of whole cerebellar cytochrome C expression by Western blotting would primarily reflect CGC cytochrome C expression. Western blot analysis done on total cerebellar protein revealed a 15 kDa band (Fig. 3A). Densitometric analysis performed on the immunoreactive bands did not reveal any significant difference in the optical density (Fig. 3B), indicating no difference in whole cerebellar cytochrome C expression of leaner and wild type mice. The experiment was run in triplicate and the same result obtained each

time. Hence, both experiments indicated no change in mitochondrial mass of CGCs from leaner and wild type mice.

Figure 3:

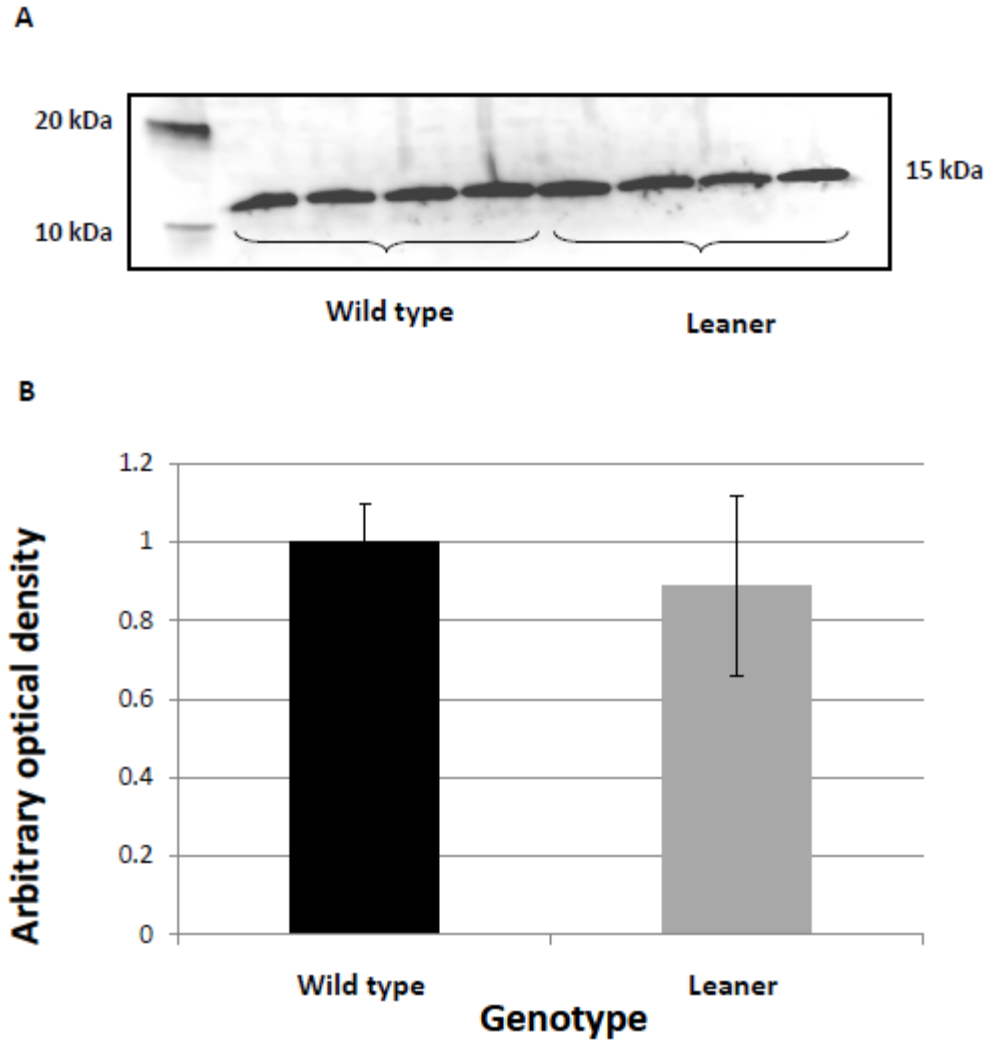

Figure 3. Cytochrome C protein expression in wild type and leaner mice whole cerebella. Panel A is a representative Western blot for cytochrome C, which is a 15-kDa protein. Panel B is graph of densitometry of the cytochome C protein expression. No significant difference in cytochrome C expression was observed between genotypes. Western blotting with densitometry was repeated three times with consistent results (n=3).

Generation of reactive oxygen species (ROS) is a part of normal cellular function. However, mitochondrial dysfunction may lead to increased generation of ROS and subsequent ROS mediated cell death. ROS mediated cell death is known to be involved in the pathogenetic mechanism of many neurodegenerative disorders including excitotoxicity, Alzheimer's disease and Parkinson's disease [31, 32, 33, 34, 35, 36]. We determined whether the mitochondrial stress demonstrated by loss of $\Delta\psi_m$ led to increased oxidative stress in leaner CGCs. A GLM Repeated measures test and the Tukey's HSD post hoc test revealed no significant difference in fluorescent intensity of CM-H2DCFDA dye in CGCs from two genotypes at all the time points (Fig. 4). Our results suggest a mitochondrial mediated but ROS independent cell death in CGCs of leaner mice. ROS have complex chemical and physiological properties in normal mitochondrial and cell functioning. It has been shown [37] that cell death in CGCs acutely exposed to Amyloid-β was independent of ROS generation. Vergun et al. [38] suggested that accumulation of ROS during toxic glutamate challenge in hippocampal neurons was not a prerequisite for altered calcium ion homeostasis

and/or the observed collapse of mitochondrial membrane potential. It also has been noted that polychlorinated biphenyls at doses that rapidly kill acutely isolated rat CGCs do not cause increased ROS generation [39]. Thus, it is the case that ROS exist in a state of dynamic equilibrium and excess ROS can result in fatal consequences for affected cells [40], but cell death also can take place independent of excess ROS.

Figure 4:

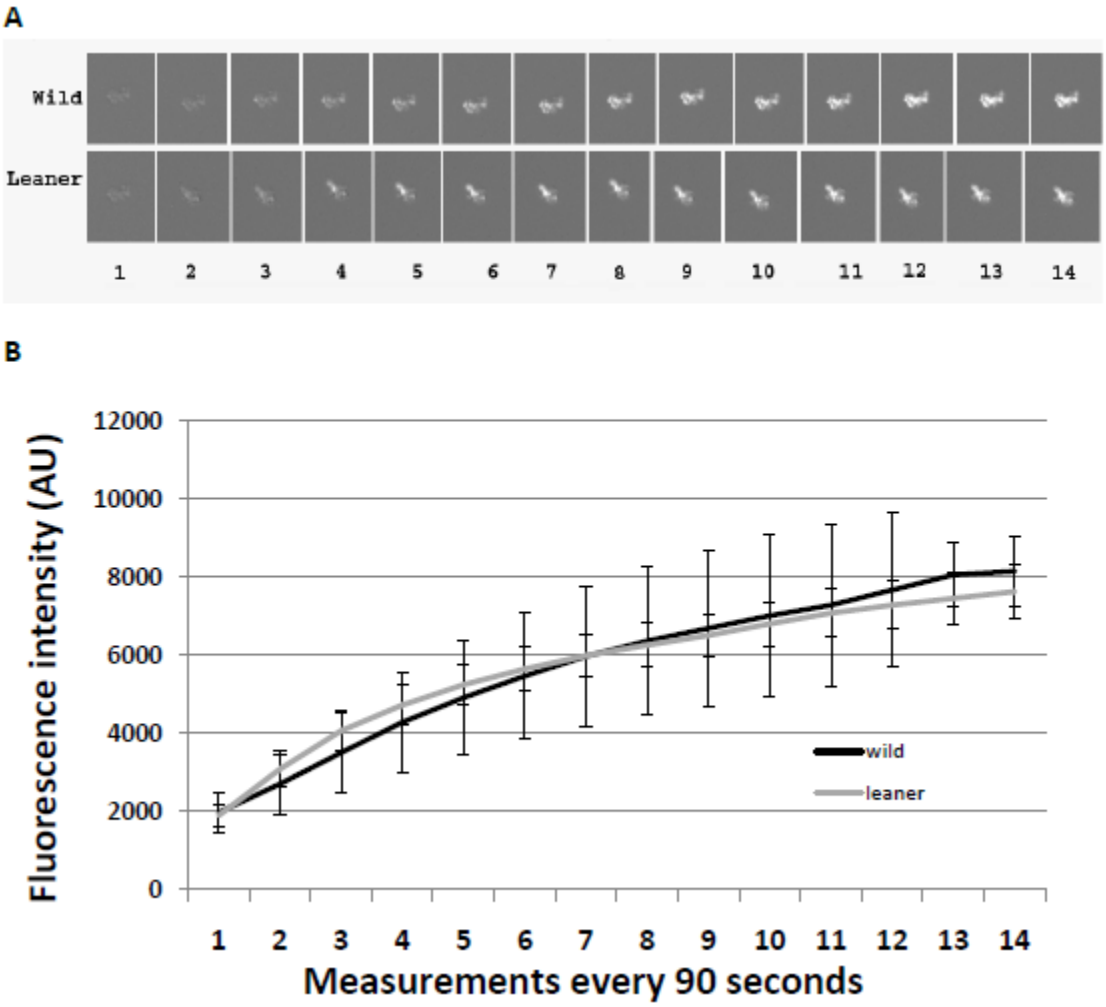

Figure 4. Measurement of reactive oxygen species (ROS) using CM-H2DCFDA dye in cerebellar granule cells at P20. Panel A shows photomicrographs of acutely isolated cerebellar granule cells from wild type and leaner mice loaded with CM-H2DCFDA dye. GLM – repeated measure analysis and Tukey's post hoc test indicated no significant difference in generation of ROS in CGCs of wild type and leaner mice at P20 (n=6).

In conclusion, we have shown that there is mitochondrial impairment in leaner CGCs at the time of peak cell death i: e P20, however no change in mitochondrial mass and generation of ROS was observed. This result further substantiates previous experiments in our lab, which suggest a mitochondrial mediated cerebellar granule cell death in leaner mice.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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