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RESEARCH ARTICLE

Age-dependent differential expression of death-associated protein 6 (Daxx) in various peripheral tissues and different brain regions of C57BL/6 male mice

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Abstract Death-associated protein 6 (DAXX) is a ubiquitous protein implicated in various cellular processes such as apoptosis, tumorigenesis, development and transcription. The role of DAXX is however ambiguous and many contradictory results regarding its function in apoptosis upon various cellular stresses are described in the literature. In order to have a better understanding of the role of DAXX throughout the entire organism under physiological stress conditions, we have characterized the mRNA levels, protein expression and the proteolytic processing of DAXX in the normal aging process in peripheral organs and brain regions in C57BL/6 male mice. Overall, *Daxx* mRNA expression decreases with aging in the liver, kidney, heart, cortex and cerebellum. In contrast, an increase is observed in the striatum. The protein expression of DAXX and of its proteolytic fragments increases with aging in the kidney, heart and cortex. In liver and spleen, no changes are observed while in the striatum and cerebellum, certain forms increase and others decrease with age, suggesting that the functions of DAXX may be cell type dependent. This study provides important details regarding the expression

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and post-translational modifications of DAXX in aging in the entire organism and provides reference data for the deregulation observed in age-associated diseases.

Keywords Aging · DAXX · Caspase · Peripheral organs · Brain region · C57BL/6 mice

Introduction

Death-associated protein 6 (DAXX) is a ubiquitous protein that participates in various cellular processes including apoptosis, tumorigenesis, transcription and cell cycle regulation (Perlman et al. 2001; Roubille et al. 2007; Saeed et al. 2009; Salomoni et al. 2006; Salomoni and Khelifi 2006; Su et al. 2007; Tang et al. 2013; Torii et al. 1999; Wu et al. 2002; Zhao et al. 2004; Zobalova et al. 2008). DAXX was originally identified as a pro-apoptotic death receptor Fas/CD95 binding protein and described as a predominantly nuclear protein (Lindsay et al. 2009; Torii et al. 1999; Yang et al. 1997). It has been shown to translocate from the nucleus to the cytoplasm under conditions of stress and to activate the Jun N-terminal kinase (JNK) pathway (Song and Lee 2003). Thus, cellular localization is a crucial factor influencing the interaction between DAXX and Fas/CD95 (Salomoni and Khelifi 2006). The pro-apoptotic function of DAXX is not well understood and various studies reported contradictory findings on DAXX trafficking, interactions and functions (Lindsay et al. 2009; Niu et al. 2011; Salomoni and Khelifi 2006; Song and Lee 2003; Torii et al. 1999; Zobalova et al. 2008). As the loss of the DAXX-like protein reduces longevity in drosophila, and some functions of DAXX have been highlighted in senescence, the pro-apoptotic functions of DAXX may be implicated in the aging process (Bodai et al. 2007; Corpet et al. 2014; Pan et al. 2013).

DAXX is tightly regulated by post-translational modifications including phosphorylation, ubiquitination and sumoylation (Ecsedy et al. 2003; Fukuyo et al. 2009; Hofmann et al. 2003; Jang et al. 2002; Tang et al. 2013). All of these post-translational modifications and others have been proposed to explain the presence of the various molecular forms of DAXX observed on SDS-PAGE and confirmed by using several DAXX antibodies (See Supplementary Table 1). As the functions of DAXX in promoting cell death are closely related to DAXX intracellular localization, post-translational modifications may influence cell death and apoptotic pathways.

Proteolytic cleavage of DAXX may be another avenue for the regulation of DAXX function (Hollenbach et al. 2002, 1999; Lalioti et al. 2002, 2009; Michaelson et al. 1999; Michaelson and Leder 2003; Pineiro et al. 2012; Wu et al. 2002). The investigation for the presence of DAXX fragments is difficult as the majority of the immunoblots presented in the literature are cut and only show the full-length and/or hyperphosphorylated forms. However, fragments of DAXX were observed in analysis of some cell lines and tissues (Hollenbach et al. 2002, 1999, 2002, 2009; Michaelson et al. 1999; Michaelson and Leder 2003; Pineiro et al. 2012; Wu et al. 2002). Despite the presence of these fragments, little interest has been accorded to them and their possible function in apoptosis. Further support for a role of DAXX fragments in apoptosis originates from studies done using various DAXX deletion mutants. Transfection of these DAXX fragments has shown strikingly differential effects on the level of apoptosis induction in many cells lines (HeLa, HEK293 and L929) (Yang et al. 1997). Strong apoptotic effects were attributed to specific fragments, such as the deletion mutant DAXX 501–625 and DAXX C501. The DAXX C-terminal fragment has been used as a dominant negative form of DAXX (Song and Lee 2004; Yang et al. 1997).

Interestingly, caspase-6 has been identified as the first mammalian protease involved in the proteolysis of DAXX (Riechers et al. 2016). In various pathological conditions such as aging and neurodegenerative diseases, the increased activity of caspases and other proteases has been hypothesized to contribute to the production of proteolytic fragments and this may play a role in the apoptotic functions of DAXX (Albrecht et al. 2007; Graham et al. 2010, 2006, 2011; Rohn 2010; Roth 2001; Shalini et al. 2015; Zhang et al. 2002).

DAXX functions appear to be regulated in a cell-type dependent manner. In vitro stress conditions used previously have led to contradictory results regarding the effect on DAXX function(s). In order to use a physiological stress condition, and to have a better understanding of the role of DAXX throughout the entire organism, we here have characterized the mRNA levels, protein expression and the proteolytic

processing of DAXX in the normal aging process in peripheral organs (liver, kidney, heart, spleen) and brain regions (cortex, cerebellum, striatum and hippocampus) in C57BL6 male mice. These organs were chosen for their relevance in metabolic, inflammatory and neurological processes which are highly affected in the normal aging. This study provides important details regarding the expression and the post-translational modifications of DAXX in aging and new insights in its regulation observed in age-associated diseases.

Materials and methods

Animals

Peripherals organs and brain regions were collected as described previously from C57BL6 male mice and grouped according to age: 3–4 months (young), 12 months (adult), 23–28 months (old) and older than 30 months (very old) (Lessard-Beaudoin et al. 2015). The protocols for this study were approved by the animal care and ethics committee of University of Sherbrooke. The mice were anaesthetized (Isoflurane, Abbott) and euthanized by cervical dislocation. The upper part of the cranium was removed, and the brain was collected. The cortex, the cerebellum, the striata and the hippocampi were then dissected. In parallel, kidney, liver, heart and spleen were harvested. As estrogen will influence not only the reproductive process, but also various physiological processes such as metabolism, cardiovascular and renal functions, it would have been informative to include both sexes in the study and see the effect of gender on our results (Gangula et al. 2013; Wren 1992). However, due to financial constraints, only males were included in the study.

Western blot analysis

Peripheral organs and brain regions were homogenized and sonicated in lysis buffer (0.32 mM Sucrose, 20 mM Tris pH 7.2, 1 mM $MgCl_2$, 0.5 mM EDTA pH 7.2) containing protease inhibitors (Roche), 4.2 mM PefaBloc SC (Roche) and 10 μM Z-VAD-fmk (Enzo Lifes Sciences) and clarified by centrifugation at 13,000 rpm. The protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Pierce). Protein lysates (50 μg) were separated on a 7.5 % SDS-PAGE gel and transferred to a PVDF membrane (PerkinElmer). The membranes were probed with anti-DAXX (1:1000, ab105173, Abcam) or anti-actin (1:1000, MAB1501, Millipore) antibodies. Peroxidase activity was detected and densitometric values were obtained with the Odyssey Fc imaging system (Mandel) using Luminata Crescendo Western HRP substrate (Millipore). Quantification of β-actin or Coomassie staining was used to standardize the amount of protein in each lane depending on the protein stability in each organ and densitometric values obtained with the Odyssey Fc imaging system (Mandel). Relative density values were calculated by dividing the raw density of DAXX by the reference density level. All data are presented relative to the 3–4 months age-group data. A $n = 4$ by age-group was used for the protein expression analysis.

Real-time quantitative RT-PCR

Total RNA was extracted from tissues with the RNeasy mini kit (QIAGEN) and cDNAs were prepared using ProtoScript Reverse Transcriptase II (New England BioLabs). Quantification was done using Mx3005P QPCR Systems (Stratagene) with mouse-specific -actin primers (forward 5′-ACGGCCAGGTCATCACTATTG-3′; reverse 5′-CAAGAAGGAAGGCTGGAAAAGA-3′) and mouse-specific DAXX primers (forward 5′-CTCTCCAGGGTTCTGTCTCG-3′; reverse 5′-GGGATCTGTGGGAGGGTTAT-3′). Amplification of -actin was used to standardize the amount of sample RNA in the reaction. Gene-expression levels were measured using MxPro QPCR Software (Stratagene). Relative density values were calculated by dividing the raw Ct of DAXX by that of Ct β-actin. All data presented are presented relative to the 3–4 months age-group data. A $n = 5$ by age-group was used for the mRNA expression analysis.

Caspase cleavage assays

Recombinant caspases were diluted in a reaction buffer (100 mM Hepes pH 7.4, 200 mM NaCl, 0.2 % CHAPs, 2 mM EDTA, 20 % glycerol) at a concentration of 1000 nM and serially diluted. Final concentrations used for each caspase in the assay were 0, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 nM.

The concentrations of caspases used are similar to those chosen in a previous article using the same active site-titrated recombinant caspases (Scott et al. 2008). The diluted recombinant caspases and 50 µg of brain lysate were pre-heated separately at 37 °C for 30 min, were mixed and then incubated for 1 h at 37 °C. Immunoblotting was performed as described above.

Statistical analysis

Student *t* test, one-way ANOVA and the post hoc Tukey's multiple comparisons test were used for analysis between the age groups. The statistical significance was established at 0.05 ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). GraphPad Prism 6.0 software was used for all statistical analysis (GraphPad Software, Inc., La Jolla, CA).

Results

DAXX cleavage by caspase-3 and 6

Incubation of brain lysates with human recombinant caspases-3 or -6 both resulted in the cleavage of DAXX and the production of a ~47 kDa fragment, but with different efficacies (Fig. 1). Whereas relatively low concentration of caspase-6 was sufficient to produce a cleavage product, only the highest concentrations of caspase-3 generated such a fragment. The fragment was observed at a concentration starting at 62.5 and 250 nM for caspase-6 and caspase-3, respectively (Fig. 1). No cleavage of DAXX was generated by caspase-7.

General decrease of *Daxx* mRNA expression levels in peripheral organs and brain regions of aged mice

In order to have a better understanding of the role of DAXX throughout the entire organism, we used RT-PCR to determine *Daxx* mRNA expression. Using this approach, we observed an increase of *Daxx* mRNA expression in the liver and the kidney at 23–28 months of age (Fig. 2). In contrast, an age-dependent *Daxx* mRNA expression depletion over time was observed in the heart with aging (Fig. 2). No significant variation for *Daxx* mRNA expression levels was denoted in the spleen.

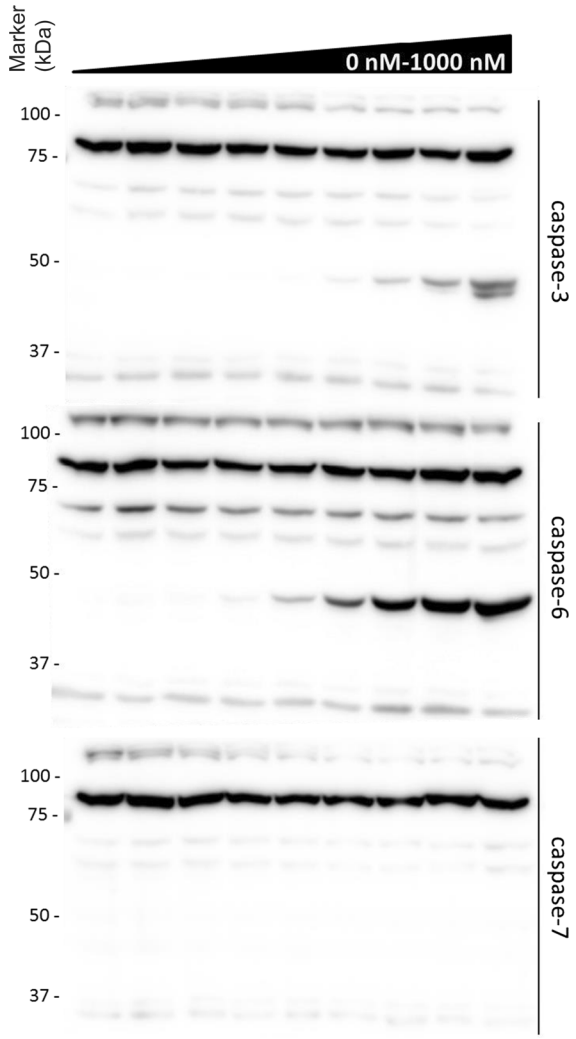

Fig. 1 DAXX protein is cleaved by caspases-3 and -6. Brain lysate was incubated with various concentrations of recombinant caspases 3, 6 and 7 for 1 h at 37 °C. The concentration of caspases from lane 1 to 9 are 0, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 nM respectively. Proteins were analyzed by immunoblotting. DAXX is cleaved by caspase 3 and 6 into ~47 kDa fragments however with different efficiencies (caspase-6 > caspase-3). No proteolytic fragments were observed with caspase-7

Daxx mRNA expression was decreased in the cortex and the cerebellum at 23–28 and 12 months of age, respectively (Fig. 3). In contrast, a significant increase (1.62-fold) in *Daxx* mRNA expression was observed at 23–28 months of age in the striatum (Fig. 3). No significant variation is observed in the hippocampus. Interestingly, within the same brain, the

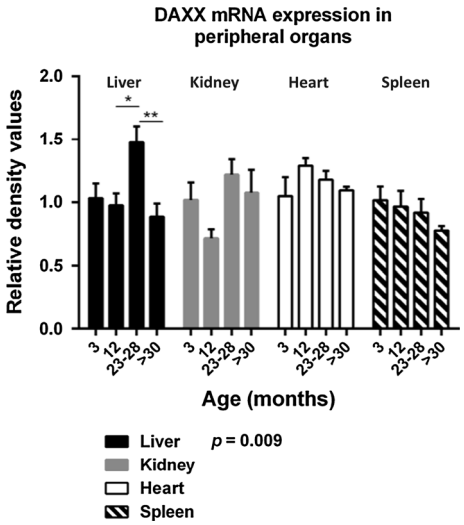

Fig. 2 *Daxx* mRNA expression decreases with aging in peripheral organs. mRNA expression of DAXX increases in the liver at 23–23 months of age and then decreases (ANOVA $p = 0.009$, post hoc: 12 vs. 23–28 months, $p < 0.05$; 23–28 vs. >30 months, $p < 0.01$). *Daxx* mRNA expression increase in the kidney (*t* test 12 vs. 23–28 months, $p < 0.01$). A decrease in *Daxx* mRNA expression is observed in the heart at >30 months of age (*t* test 12 vs. >30 months, $p < 0.05$). No significant variation is observed in the spleen with aging

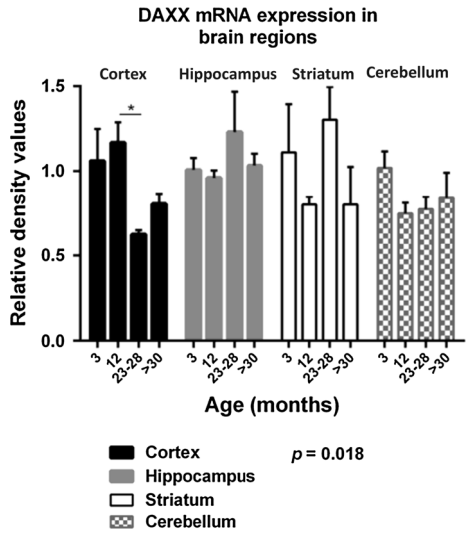

Fig. 3 Differential expression of *Daxx* mRNA in brain regions with aging. *Daxx* mRNA expression decreases with age in the cortex (ANOVA $p = 0.018$, post hoc: 12 vs. 23–28 months, $p < 0.05$). No significant variation is observed in the hippocampus. An increase in *Daxx* mRNA expression is observed at 23–28 months of age in the striatum (*t* test 12 vs. 23–28 months, $p < 0.05$). A decrease is observed in the cerebellum at 23–28 months of age (*t* test 3 vs. 12 months, $p < 0.05$)

different regions studied showed marked differences in temporal expression patterns of *Daxx* mRNA.

Overall increase of DAXX protein expression and cleavage in the aging kidney and heart

In spite of a decrease in *Daxx* mRNA expression observed at 12 months of age in the kidney, western blot analyses have revealed that all forms of DAXX, including full-length, hyperphosphorylated (pDAXX) and fragments of DAXX, were augmented in this tissue with age with the exception of the 50 kDa fragment which was decreased in older mice (23–28 months) (Fig. 4a). In the heart, a similar augmentation was observed for the full-length protein, and for the 50 and 30 kDa fragments in mice older than 30 months (Fig. 4b).

In contrast to the kidney and the heart, only slight variations of DAXX protein expression levels were denoted in the liver and spleen. In the liver, *Daxx* mRNA expression was significantly decreased at 23–28 months of age. At the protein level, the pDAXX and the 35 kDa fragment decrease with age (Fig. 4c). In contrast, at the same age, a significant 1.8-fold increase was observed for the 70 kDa form (Fig. 4c). In the spleen, only a trend increase of the 70 kDa form of DAXX was observed at >30 months (Fig. 4d).

Region-specific variations in DAXX protein expression and post-translational modification were observed in the aging brain

In contrast to the mRNA expression, all forms of DAXX including the full-length, pDAXX and the fragments were increased or tend to increase, with age in the cortex (Fig. 5a). According to the trend observed in the cerebellum, depletion in mRNA expression at 12 months of age, a decrease was also observed with aging in protein expression of the full-length DAXX and the 70 kDa fragment expression in this brain area (Fig. 5b). However, the decrease in protein expression observed at >30 months of age suggests that mRNA decay was not solely responsible for this decrease. In contrast to the 70 kDa fragment, the 30 kDa fragment was increased with aging (Fig. 5b). In the striatum, a decrease in expression of the pDAXX

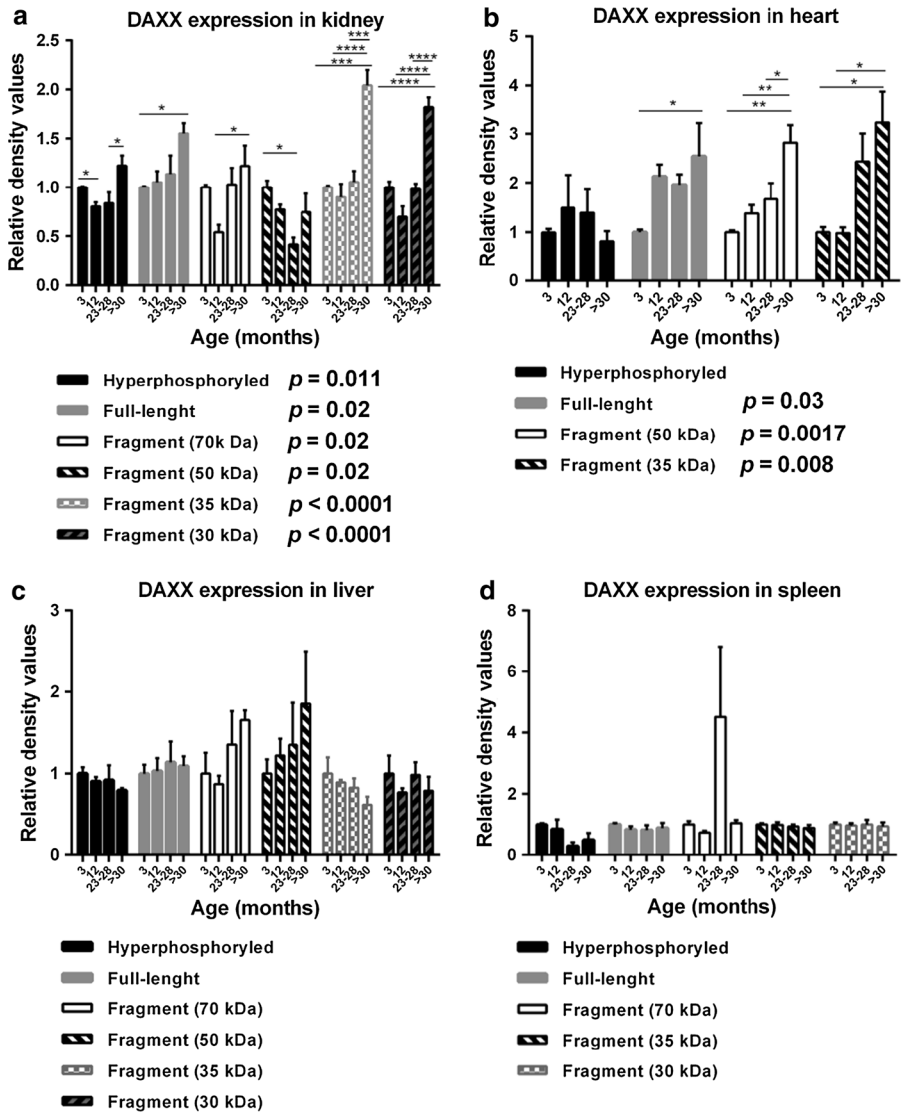

Fig. 4 Alteration in DAXX protein expression and fragment levels in heart and kidney with age. **a** An increase in all forms and fragments of DAXX, except for the 50 kDa fragment which decreases, is observed in the kidney with age (pDAXX: ANOVA $p = 0.011$; DAXX FL: ANOVA $p = 0.02$; fragment (70 kDa): ANOVA $p = 0.02$; fragment (50 kDa): ANOVA $p = 0.02$; fragment (35 kDa): ANOVA $p < 0.0001$; fragment (30 kDa): ANOVA $p < 0.0001$). **b** A global increase of the full-length protein and the fragments of DAXX is observed with aging in the heart (DAXX FL: ANOVA $p = 0.03$; fragment (50 kDa): ANOVA $p = 0.0017$; fragment (35 kDa): ANOVA $p = 0.008$). **c** The pDAXX and the 35 kDa fragment of DAXX decrease in the liver with aging while the 70 and 45 kDa fragments increase (pDAXX: *t* test, 3 vs. >30 months, $p < 0.05$; fragment (70 kDa): $p < 0.01$; fragment (45 kDa) 3 vs. 12 months, $p < 0.05$; 3 vs. 23–28 m, $p < 0.05$; 12 vs. >30 months, $p < 0.05$; fragment (35 kDa): *t* test, 12 vs. >30 months, $p < 0.05$). **d** In the spleen, only the 70 kDa fragment increase with the age (*t* test 12 vs. >30 months, $p < 0.05$). Licor quantification of Coomassie staining of the western blot was used as loading control. *pDaxx* hyperphosphorylated DAXX, *FL* full-length

and its 70 and 30 kDa fragments was denoted with aging, which may reflect a reduction in post-translational modifications (Fig. 5c). However, the full-length and the 50 kDa fragment of DAXX increased with aging in the striatum, which complements the increasing mRNA expression observed in this brain region (Fig. 5c). In addition to the absence of correlation between the protein and

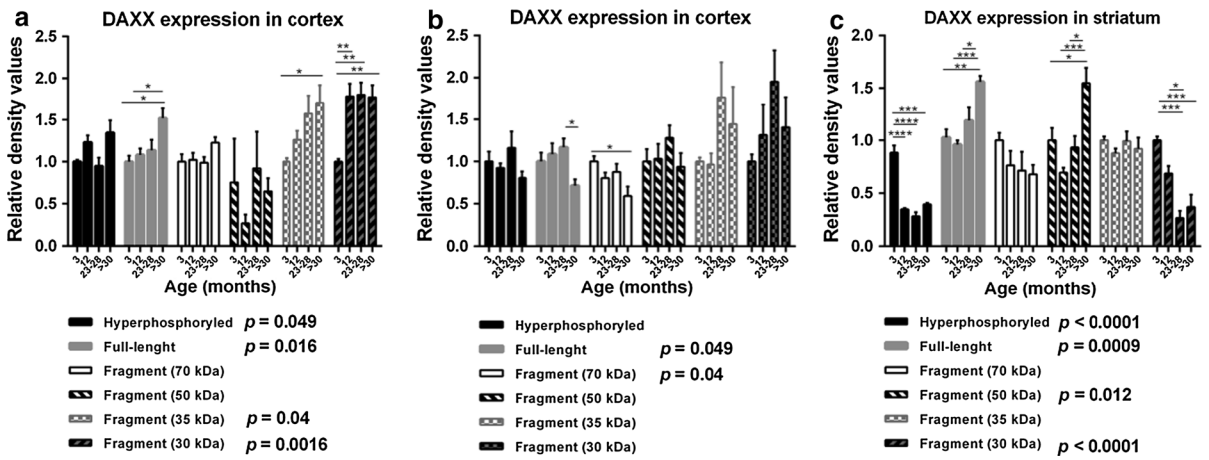

Fig. 5 DAXX protein expression varies in cortex, cerebellum and striatum with aging. **a** In the cortex, all forms and fragments of DAXX increase, or tend to increase with age (pDAXX: ANOVA $p = 0.04$; DAXX FL: ANOVA $p = 0.016$, post hoc: 3 vs. >30 months, $p < 0.05$; 12 vs. >30 months, $p < 0.05$; fragment (70 kDa): *t* test, 23–28 vs. >30 months, $p < 0.05$; fragment (50 kDa): *t* test, 12 vs. >30 months, $p < 0.05$; fragment (35 kDa): ANOVA $p = 0.03$, post hoc: 3 vs. >30 months, $p < 0.05$; fragment (30 kDa): ANOVA $p = 0.0016$, post hoc: 3 vs. 12 months, $p < 0.01$; 3 vs. 23–28 months, $p < 0.01$; 3 vs. >30 months, $p < 0.01$). **b** FL and the 70 kDa fragment of DAXX decreases while the 30 kDa fragment increase with aging in the cerebellum (DAXX FL: ANOVA $p = 0.049$, post hoc: 23–28 vs. >30 months, $p < 0.05$; fragment (70 kDa): ANOVA $p = 0.0354$, post hoc: 3 vs. >30 months, $p < 0.05$; fragment (30 kDa): *t* test, 3 vs. 23–28 months, $p < 0.01$). **c** The FL and the 50 kDa fragment of DAXX increases and the pDAXX, the 70 and 30 kDa fragment decreases or tends to decrease with the age in the striatum (pDAXX: ANOVA $p < 0.0001$, post hoc: 3 vs. 12 months, $p < 0.0001$; 3 vs. 23–28 months, $p < 0.0001$; 3 vs. >30 months, $p < 0.001$; DAXX FL: ANOVA $p = 0.0009$, post hoc: 3 vs. >30 months, $p < 0.01$; 12 vs. >30 months, $p < 0.001$; 23–28 vs. >30 months, $p < 0.05$; fragment (70 kDa): *t* test, 3 vs. >30 months, $p < 0.05$; fragment (50 kDa): ANOVA $p < 0.0001$, post hoc: 3 vs. >30 months, $p < 0.05$; 12 vs. >30 months, $p < 0.001$; 23–28 vs. >30 months, $p < 0.05$; fragment (30 kDa): ANOVA $p < 0.0001$, post hoc: 3 vs. 23–28 months, $p < 0.001$; 3 vs. >30 months, $p < 0.001$; 12 vs. 23–28 months, $p < 0.05$). *FL* full length, *pDAXX* hyperphosphorylated DAXX

mRNA expression levels, differential variation among all brain regions was also observed at the protein level.

Discussion

The function of DAXX throughout the organism is not well established. Various studies have reported contradictory results on its trafficking, interaction with partners, pro-apoptotic or anti-apoptotic effects, which all appear dependent on the stimuli or the cell line used for the experiment. Furthermore, the reliability of stimuli used on cell lines as well as the relevancy of cellular models to understand complex physiological processes are under debate (Lindsay et al. 2009). We therefore assessed DAXX expression throughout the peripheral organs (liver, kidney, heart and spleen) and brain regions (cortex, cerebellum, hippocampus, striatum) in the normal murine aging process (3–30 months of age). The organs were chosen for their relevance in inflammatory, metabolic and neurodegenerative processes. Overall, *Daxx* mRNA expression, protein levels and post-translational modifications (phosphorylation and proteolytic fragments) are organ specific and modulated by aging suggesting distinct roles for the various forms of DAXX. In light of our results, and the existing literature, each organ does not respond the same way to the aging process reflecting the metabolic and apoptotic particularities of each organ.

The increase in the number of TUNEL-positive cells in glomerular and corticotubular areas in the kidney observed in aged C57BL/6 mice is associated with an increase in reactive oxygen species (ROS) as well as a decrease in renal clearance and renal functions (Lim et al. 2012). An increase in the mRNA and protein expression of DAXX in the presence of oxidative stress have been reported previously, therefore, the global increase in DAXX forms observed in

the kidney may due to the increase in ROS previously observed with aging (Beckman and Ames 1998; Kim et al. 2005; Papa and Skulachev 1997; Ruiz-Torres et al. 1997). In our data, a similar increase was observed at the mRNA level and protein level in the aging kidney. In light of these results, the increase in DAXX protein expression we observed may be attributable to an increase in ROS and gene expression with aging rather than post-translational modifications affecting the half-life of the protein or a less efficient degradation mechanism with aging.

When considering only the pro-apoptotic functions of DAXX in the heart, increased expression of all forms of DAXX, as we have observed in our experiments, would be consistent with the ~30 % cardiomyocytes loss and the increase in cardiovascular issues observed with aging (Davis 2014; Sheydina et al. 2011). The pro-apoptotic functions of DAXX in cardiomyocytes are however contradicted by Zobalova et al. who observed an increase in peroxide-induced apoptosis when DAXX expression was suppressed (Zobalova et al. 2008). Others have also shown anti-apoptotic functions for DAXX. Indeed, the interaction of DAXX with mdm2 results in an increase in stability of mdm2 activity that leads to the degradation of p53 (Tang et al. 2006). Thus, the depletion of DAXX may promote apoptosis by the suppression of its anti-apoptotic function. Of note, numerous caspase substrates show both pro and anti-apoptotic functions, which may be explained by the specific roles of the full-length protein vs. their fragment in apoptosis (Chan et al. 2009; Levkau et al. 1999; Mazars et al. 2009). The global increase of DAXX protein expression levels with aging in kidney may imply post-translational modifications such as ASK1 phosphorylation of Ser172 and Ser184 and the poly-ubiquitination at Lys122, which are described to promote the accumulation of DAXX, to increase its half-life and to promote the production of specific fragments (Fukuyo et al. 2009). Moreover, In contrast to other cell types, hepatocytes have a long life span and rarely proliferate except in the case of injury or during liver regeneration. In response to methyl methanesulfonate treatment, the liver of old rats does not enter apoptosis as efficiently as the liver of their younger counterparts (Suh 2002). As a protein highly involved in cell cycle regulation and apoptosis, it is perhaps not surprising that DAXX expression in the liver did not present strong variations with aging (Drane et al. 2010; Giovinazzi et al. 2012; Gostissa et al. 2003; Kwan et al. 2013; Salomoni and Khelifi 2006; Tang et al. 2013, 2006, Zhao et al. 2004). Interestingly, the mRNA levels show an increase in the transcription of *Daxx* at 23–28 months of age in this tissue, but this does not translate into an increase in protein expression. The absence of variation in the full-length form in contrast to the mRNA may be the results of post-translational modifications of DAXX. As the full-length form is cleaved or degraded, new protein may be produced, refilling the protein pool upon degradation which stabilizes the full-length protein level. The slight increase in the 70 kDa form of DAXX may support this hypothesis. Moreover, the stable expression of DAXX may be important in the hepatocytes to preserve its anti-apoptotic functions in absence of cellular stress.

Apoptotic hallmarks, such as an increase in caspase expression, DNA fragmentation and decrease in the anti-apoptotic protein Bcl-2 were previously observed in aging rodent splenocytes. Surprisingly, in spite of these apoptotic signs, we only observed a slight variation in the 70 kDa molecular form of DAXX in this organ (Itzhaki et al. 2003; Zhang et al. 2002). Of note, we found considerable variation of this fragment level for the 23–28 months age-group. Interestingly, in a previous report we observed a large standard deviation in the spleen weight within this age group (Lessard-Beaudoin et al. 2015). Although no abnormality was apparent at the time of dissection, it cannot be ruled out that in this age-group a pathological state could be responsible for the results. In contrast to other cell types, it has been previously observed that when splenocytes were treated with concanavalin A or retinoic acid, DAXX translocates to the nucleus instead of the cytoplasm, as observed in other cell types (Zhong et al. 2000). In light of these results, DAXX may have a particular function in splenocytes. Moreover, there is a possibility that variations of DAXX with aging in this organ may be more attributable to a change of localization instead of a variation in mRNA or protein expression.

In the cortex, we observed a decrease in *Daxx* mRNA expression with aging that does not translate at the protein level, for which an increase is observed in all protein forms. This accumulation of the protein form of DAXX may be explained by a change in mRNA stability or a decrease in proteasome activity previously observed in the cortex with aging which

may increase the half-life of the protein (Baraibar and Friguet 2012). Another hypothesis is that some post-translational modifications of DAXX may increase with age in the cortex. As an example, the phosphorylation of DAXX by ASK1 and the ubiquitination at Lys122, which leads to protein stabilization of DAXX, may be deregulated in the aging cortex. Of note, the ASK1-MKK3/6-p38MAPK pathway, which is influenced by DAXX functions, is involved in Aβ-induced cell death and may be implicated in the tau phosphorylation pathway, two major components relevant in Alzheimer's disease (Song et al. 2014). The accumulation of pro-apoptotic proteins like DAXX and their fragments would be consistent with neuronal cell loss and the decrease in cerebral white matter integrity with age that is associated with several cognitive performance tasks (Bennett and Madden 2014). Interestingly, an increase in expression level of caspase-6, a protease recently implicated in DAXX processing, has previously been observed with aging in BALB/c and FVB mice which may result in increased cleavage of DAXX (Graham et al. 2010, 2012; Jiang et al. 2001).

Similar to the results in the cortex, *Daxx* mRNA expression decreases in the cerebellum with aging. At the protein level, there is a decrease in the full-length and the 70 kDa forms expression at 30 months of age. Previously, a ~2.5 % loss per decade of Purkinje cells in the cerebellum has been observed (Xu et al. 2000). However, others have demonstrated a stable number of Purkinje cells in most parts of the cerebellum, except in the anterior lobe, in which a loss of 40.9 % was observed with aging (Andersen et al. 2003; Zhang et al. 2010). Moreover, morphological effect of aging on specific parts of the cerebellum has been shown to correlate with certain motor and cognitive performance (Bernard and Seidler 2014). The variation in DAXX protein expression and its 70 kDa fragment at a very old age (>30 months) may reflect the age-related morphological changes in the cerebellum and play a role in the motor and cognitive deficits observed with advanced age.

In the striatum, an increase is observed at the mRNA level at 23–28 months of age, which may be responsible for the increase of the full-length protein at a later time-point. The strong decrease in pDAXX at 12 months of age may reflect deregulation in post-translational modifications such as ubiquitination, sumoylation or phosphorylation of DAXX with aging, which would contribute to the upper molecular weight bands of DAXX. The variations observed in DAXX fragments in the striatum indicate that DAXX cleavage may be regulated differentially in this tissue with the aging process as well. As strong apoptotic effects has been attributed to specific fragment of DAXX, all of these post-translational modifications combined with its increased expression, may take part in the increased age-associated apoptosis observed in the striatum by Ureshino et al. and corroborated by the increase in active caspase expression by Graham et al. (Graham et al. 2010; Ureshino et al. 2010).

In contrast to the increase in *Daxx* mRNA previously observed in aging and Alzheimer's disease, no variation in the mRNA expression of *Daxx* was observed in the hippocampus in our study (Lukiw 2004). However, in the previous study, only foetal and adulthood has been compared which may explain the difference with our results. A previous study noted increased level of enzymes involved in the mitochondria respiratory chain in the hippocampus with aging in BALB/c mice (Jiang et al. 2001). Without a change in *Daxx* mRNA in the hippocampus there is a possibility that DAXX protein expression may vary as a result of ROS formation, stimuli often related to the expression or modification of DAXX. However, the limited amount of hippocampus did not permit us to analyze the protein expression in this brain region.

Conclusion

A variety of cell lines and cellular stressors have been used to assess DAXX expression, modifications and investigate function, but few studies were performed on complex physiological processes in vivo. In this study, our results suggest distinct roles for the various forms of DAXX, reflecting the metabolic and apoptotic particularities of each organ during the aging process. Therefore, this study provides important details regarding the expression and the post-translational modifications of DAXX in the entire organism upon a physiological stress such as the normal aging process. In addition, these data may serve as a foundation for the study of DAXX regulation in age-associated diseases and its role throughout the organism.

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