Synuclein expression in the lizard *Anolis carolinensis*

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Abstract

The synuclein (syn) family comprises three proteins: α-, β- and γ- syns. In humans they are involved in neurodegenerative diseases such as Parkinson's disease and in tumors.

Members of the syn family were sequenced in representative species of all vertebrates and the comparative analysis of amino acid sequences suggests that syns are evolutionar**ily** conserved, but information **about** their expression in vertebrate lineages **is** still scarce and completely lacking in reptiles.

In this study the expression of genes coding for α-, β- and γ- syns was analyzed in the green lizard *Anolis carolinensis* by semiquantitative RT-PCR and Western blot.

Results demonstrate good expression levels of the three syns in the lizard nervous system, similarly to human syns. This, together with the high identity between lizard and human syns, suggests that these proteins fulfill evolutionar**ily** conserved functions. However, differences between lizard and humans in the expression of syn variants (two different variants of γ-syn were detected in *A. carolinensis*) and differences in some amino acids in key positions for the regulation of protein conformation and affinity for lipid and metal ions also suggest these proteins may have acquired different functional specializations in the two lineages.

Keywords: synuclein, lizard, *Anolis carolinensis*, Parkinson's disease, RT-PCR.

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All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted and were approved by the Italian administrative order DM 70/96 of Italian Ministry of Health.

Abbreviations

aa	amino acid
Br	brain
Bs	brainstem
Ce	cerebellum
CNS	central nervous system
CT	C-terminal
Di	diencephalon
Ey	eye
He	heart
In	intestine
Li	liver
Lu	lung
Mu	muscle
MW	Molecular Weight
NAC	non-β amyloid component
NT	N-terminal
nt	nucleotide
NTr	N-terminal region
OT	midbrain tectum
pI	isoelectric point
PD	Parkinson's disease
R	rat brain
RT-PCR	reverse transcriptase-polymerase chain reaction
SC	spinal cord
syn	synuclein
Te	telencephalon

Introduction

The synuclein (syn) family comprises naturally unfolded proteins with a molecular weight (MW) of about 15-20 kDa that are specifically expressed in neurons of vertebrates (George et al. 1995; Jakes et al. 1994; Maroteaux and Scheller 1991). The first syn was discovered in the electric ray *Torpedo californica* (Maroteaux et al. 1988) but afterwards homologous proteins were identified in mammals (Akopian and Wood 1995; Jakes et al. 1994; Ji et al. 1997; Lavedan et al. 1998**a**; Tobe et al. 1992; Ueda et al. 1993) and in other vertebrates (George et al. 1995; Sun and Gitler 2008; Tiunova et al. 2000; Wang et al. 2011).

Synucleins in mammals are encoded by three genes that map on different chromosomes: *snca* coding for α-syn, *sncb* for β-syn and *sncg* for γ-syn (Campion et al. 1995; Lavedan et al. 1998a,b; Shibasaki et al. 1995; Spillantini et al. 1995). Human syn genes are composed of five coding exons of similar size (Lavedan 1998) and four different α-syn isoforms are produced by alternative splicing of *snca* (Beyer and Ariza 2013).

The biological functions of syns are still poorly understood. These proteins are involved in neurodegenerative diseases known as synucleinopathies (mainly α- and β- syns) and in some tumors (γ-syn). Synucleinopathies, **which** include Parkinson's disease (PD), dementia with Lewy bodies, PD dementia, multiple system atrophy, and neuroaxonal dystrophies (Dikiy and Eliezer 2012; Irwin et al. 2013; Luk and Lee 2014; Moore et al. 2005; Norris et al. 2004; Pfefferkorn et al. 2012), are characterized by the intracellular accumulation of proteinaceous bodies containing α-syn aggregates that lead to neurodegeneration (Spillantini 1999). α-syn is directly involved in PD since modifications of the *snca* gene such as single nucleotide polymorphisms (Maraganore et al. 2006; Pals et al. 2004; Pankratz et al. 2009; Rajput et al. 2009), missense mutations, duplications (Chartier-Harlin et al. 2004; Ibanez et al. 2004) and triplications (Singleton et al. 2003) have been associated with an increased risk for this disease. Moreover, α-syn post-translational modifications such as ubiquitination, nitration and phosphorylation (Giasson et al. 2000; Tofaris et al. 2003; Xilouri and Stefanis 2011) may promote the formation of the pathological inclusions.

Among synucleins, α-syn has been the most studied protein for its direct involvement in PD. The primary structure of human α-syn is commonly divided into N-terminal (NT) (1-60 residues), non-β amyloid component (NAC) (61-95 residues) and C-terminal (CT) (96-140 residues) regions. The N-terminus contains the amino acids involved in all the PD-related mutations (A30P, E46K, H50Q, G51D and A53T) identified so far (Appel-Cresswell et al. 2013; Conway et al. 1998; El-Agnaf et al. 1998; Hamilton 2004; Jensen et al. 1998; Kruger et al. 1998; Lesage et al. 2013; Mbefo et al. 2015; Narhi et al. 1999; Proukakis et al. 2013; Rospigliosi et al. 2009; Yonetani et al. 2009). NAC is a highly hydrophobic region **that is** directly involved in the formation of amyloid fibrils and characterized by the amino acid stretch GVTAVAQKTVE. **This sequence is** missing in β-syn and only partially conserved in γ-syn **and is** able to acquire a β-sheet structure when α-syn forms fibrils (Weinreb et al. 1996). The NT and NAC regions include the seven semi-conserved repeats of 11 residues containing the apolipoprotein lipid-binding motif [EGS]-K-T-K-[EQ]-[GQ]-V-XXXX that allows α-syn to acquire α-helix structure and bind to lipid membranes. Finally, the C-terminus is a negatively charged unstructured region proposed to act as a scaffold to recruit additional proteins to the membranes (Eliezer et al. 2001) and that contains low-affinity binding sites for metal ions such as Fe(II), Mn(II), Co(II) and Ni(II) (Binolfi et al. 2006; Lu et al. 2011). The binding of metals to syns can have important effects on the conformation and properties of the protein. For example, the binding of Cu(II) to α-syn induced the multimerization of the protein (Paik et al. 1999) and the incubation of α-syn with Mn(II) resulted in the formation of dityrosine cross-links (Uversky et al. 2001) that could lead to the initiation of α-syn fibrillization. The majority of α-syn post-translational modifications (truncation, phosphorylation, methionine oxidation, and nitration) occur within this region that contains phosphorylatable serines (S87 and S129) and tyrosines (Y125, Y133, and Y136).

In mammals, α-syn can adopt different secondary structures, folding in**to an** α-helix or β-sheet **structure** on the surrounding environment, and it is likely that the protein exists in multiple conformations in a living cell. In fact, evidence show**s** that wild-type α-syn contains little α-helix (3%) and β-sheet (23%), whereas the rest of the protein assumes a random conformation (Davidson et al. 1998; Weinreb et al. 1996). However, upon the binding of NT and NAC region to small phospholipid vesicles, the protein largely folds in an α-helical conformation (63–71% of the protein) (Davidson et al. 1998). On the other hand, α-syn in some conditions can change to an anti-parallel β-sheet conformation (El-Agnaf et al. 1998; Narhi et al. 1999; Weinreb et al. 1996) and readily assembles into filaments.

Evidence suggests that α-syn functions may be related to its ability to interact directly with membrane phospholipids (Iwai et al. 1995), proteins (Dunker et al. 2005) and metals (Lu et al. 2011). In particular α-syn has been implicated in vesicle trafficking during neurotransmitter release (Burre et al. 2010; Chandra et al. 2005), synaptic vesicle pool maintenance (Abeliovich et al. 2000), synaptic plasticity (Zarranz et al. 2004) and learning (George et al. 1995). On the other hand, γ-syn has been involved in tumor pathogenesis and it correlates with adverse outcomes in some cancers (Bruening et al. 2000; Hibi et al. 2009; Ji et al. 1997; Liu et al. 2010, 2012). Moreover, experimental studies showed that γ-syn over-expression stimulates cell proliferation (Jia et al. 1999) and increases the invasiveness of breast tumor cells (Jia et al. 1999).

According to the percentage of sequence identity with mammalian isoforms, non-mammalian syns were classified as α-syn, β-syn or γ-syn. However, there is a great variability in the number of non-mammalian isoforms due to the existence of additional genes encoding for β- and γ- syns in the vertebrate genome. In particular, four syn genes were described in *Xenopus laevis* and *Takifugu rubripes* due to the presence of two β-syn genes (*sncba* and *sncbb*) in the first (Wang et al. 2011) and two γ-syn genes (*sncga* and *sncgb*) in the latter (Yoshida et al. 2006). Moreover, the absence of *snca* was demonstrated in the genome of the zebrafish *Danio rerio* which consequently possesses only three syn genes (*sncb*, *sncga* and *sncgb*) (Milanese et al. 2012; Sun and Gitler 2008). Moreover, non mammalian syns were identified having scarce similarity to the three main isoforms, named by progressive numbers, i.e. "synuclein 3" of lamprey (Busch and Morgan 2012) or generically "synuclein-like" protein.

The genome sequencing of representative species from main vertebrate taxa has recently led to an increase in the number of available nucleotide (nt) and amino acid (aa) sequences for syns. These data suggested a still higher variability in syn isoforms of non-mammalian vertebrates due to the existence of β- and γ- syn variants named X1, X2 and X3. However, further analyses are needed to assess the actual expression of these isoforms in animal tissues (Toni and Cioni 2015).

The comparative analysis of syn sequences showed a high degree of conservation among vertebrates (George et al. 1995; Jakes et al. 1994; Maroteaux and Scheller 1991; Yuan and Zhao 2013) suggesting conserved physiological functions for these proteins. Among non mammalian vertebrates, the study of syn gene expression in both embryo (Chen et al. 2009; Sun and Gitler 2008; Tiunova et al. 2000; Wang et al. 2011), larval (Busch and Morgan 2012) and adult (Maroteaux et al. 1988; Yoshida et al. 2006) animals showed a different spatiotemporal expression pattern of syn isoforms with a prevalent expression of α- and β- syns in the central nervous system (CNS) and of γ-syns in the peripheral nervous system (Milanese et al. 2012; Tiunova et al. 2000; Wang et al. 2011). Among non mammalian vertebrates, syn gene expression was analyzed in the embryo of fish (*Danio rerio*, (Chen et al. 2009; Milanese et al. 2012)), amphibians (*Xenopus laevis*, (Wang et al. 2011)) and birds (*Gallus gallus* (Tiunova et al. 2000)) and these studies showed similarities in the embryonic expression pattern of syn genes suggesting that syns may have conserved functions in nervous system development (Milanese et al. 2012; Tiunova et al. 2000; Wang et al. 2011). To date no studies on syn expression in reptiles are available.

For this reason, we have studied the syn expression in the green lizard *Anolis carolinensis* **which** was chosen as **a** study model **because of** the availability of the whole genome sequence **of this species** and for the **ready availability** of specimens **from** local authorized providers. In this study, nt and aa syn sequences deduced from the lizard genome were analyzed and compared with human syn sequences. Moreover, gene and protein expression of lizard syns were evaluated in the CNS and non nervous organs by semi-quantitative RT-PCR and Western blotting. This work is part of a larger study started some years ago by our research group that aims to gain new information about syns in non-mammalian vertebrates, in order to obtain data useful to understand the evolution and the physiological functions of these proteins and to develop new models of PD, **as** alternative**s** to mammals (Toni and Cioni 2015; Vaccaro et al. 2015).

Materials and methods

Animals and sampling

12 adult individuals of *Anolis carolinensis* (Taxon 28377), obtained **from** local authorized providers (Tropicalia, Florence, Italy) were used. Animals were sacrificed by an intra-peritoneal injection of a lethal dose (20 mg) of sodium thiopental (Intervet, Italy) and several, nervous and non nervous, organs (brain, spinal cord, eye, muscle, heart, intestine, liver and lung) were quickly removed. Brains were further subdivided into the following samples: telencephalon, diencephalon, midbrain tectum, cerebellum and brainstem. For each brain region, tissues collected from 6 animals were pooled together in a single sample **in order to have sufficient biological material to perform the analysis**. One sample (derived from 6 animals) was successively analyzed by semi quantitative RT-PCR to evaluate syn gene expression and the other sample by Western blot to analyze syn protein expression. Tissues were kept in RNA Later (Ambion, Austin, TX) at -70°C until they were processed.

Sequence analysis

The nt and aa sequences of lizard syns were sought in the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein, RRID:nif-0000-03178). A total of 3 genes (*snca*, *sncb* and *sncg*), 5 nucleotide mRNA sequences and 4 deduced aa sequences defined by different Accession Number**s** were detected (Table 1). The comparative analysis of aa sequences was performed **using** the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). For human isoforms, the aa sequences of α- (Accession Number P37840.1), β- (AN AAB30860.1) and γ- (AN NP_003078.2) syns were used. The phosphorylation site predictions **were** performed using the NetPhosK 1.0 Server at http://www.cbs.dtu.dk/services/NetPhosK/ (Blom et al. 2004) and the secondary structure prediction**s** of lizard proteins were obtained by The PSIPRED Protein Sequence Analysis Workbench at http://bioinf.cs.ucl.ac.uk/psipred/ (Jones 1999).

RNA extraction and RT-PCR analysis

Total RNA was extracted from the samples previously collected using Trizol reagent (Invitrogen) for whole brain and organ samples and ReliaPrep™ RNA Tissue Miniprep System kit (Promega) for the brain regions according to the manufacturer's protocol. About one microgram total RNA of each sample was reverse transcribed using SuperScript® II Reverse Transcriptase kit (Invitrogen). After reverse transcription, 100 ng of each cDNA were used as template for polymerase chain reaction (PCR) amplifications. PCR was performed using Bioline Master Mix and specific primers for synucleins (α-, β-, and γ- syns) (Table 2) and glyceraldehyde3-phosphate dehydrogenase (GAPDH) (final concentration 1 μM). PCR conditions were initial denaturation at 94° C for 2 min; 30/35 cycles with the following profile, 94° C for 30 s, 60°C for 30 s, 72°C for 45 s; final extension at 72°C for 5 min. For each PCR reaction, 12.5μl was run on 1.6% agarose gel. GAPDH was used to normalize the intensity of the amplified bands. For the evaluation of X1 and X2 γ-syn expression, RT-PCR with primers X1 and X2 (Table 2) was carried out on 0.5 μg total RNA with the Access RT-PCR System (Promega) according to the manufacturer's instructions.

Antibodies

As no commercial antibodies against lizard syns were available, antibodies against mammalian proteins able to recognize lizard isoforms were sought in order to perform Western blot analysis. The characteristics of about 20 antibodies, whose datasheet clearly reported information on the recognized epitopes, were first evaluated to assay their putative ability to recognize and discriminate lizard syns. Among these, the following antibodies were tested in Western blot experiments on lizard sample. For α-syn immuno-detection two non commercial (2E3 and 3D5) and three commercial antibodies were tested: 1) 2E3 (RRID:AB_2315787, Department of Neurobiology, Xuanwu Hospital, Capital Medical University Cat# 2E3) directed against the epitope DMPVDPD (115-121 in human α-syn), 2) 3D5 (RRID:AB_2315791, Department of Neurobiology, Xuanwu Hospital,Capital Medical University Cat# 3D5), directed against the epitope QDYEP (amino acids 134-138 in human α-syn) (Yu et al., 2007) and previously used on carp brain (Vaccaro et al. 2015); 3) a rabbit polyclonal antibody against the 111-131 region of human α-syn (Millipore AB5038, RRID:AB_91648); 4) a mouse monoclonal antibody against the 115-122 region of human α-syn (ab27766, Abcam); 5) a mouse monoclonal antibody against the 15-123 region of rat α-syn (610787, BD Transduction Laboratories) that recognize**s** the epitope ATGFVKKDQ situated between residues 91 and 99 of rat α-syn (Perrin et al. 2003). For β-syn immunodetection, a mouse monoclonal antibody raised against amino acids 107-118 of rat β-syn (sc-136452, Santa Cruz) was tested.

The antibodies were tested by Western blot analysis on homogenates of lizard and rat brain (data not shown) and the two antibodies with the best performances in terms of labeling specificity, lower number of immuno-labeled bands and reduce**d or** absent background were used in the following analysis. The following antibodies were selected and used in subsequent experiments: the mouse monoclonal antibody against the 15-123 region of rat α-syn (610787, BD Transduction Laboratories) diluted at 1:500 and the mouse monoclonal antibody raised against amino acids 107-118 of rat β-syn (sc-136452, Santa Cruz) diluted 1:200.

A commercial β-actin antibody (Santa Cruz Biotechnology sc-47778, RRID:AB_626632) was used to normalize Western blot results. The anti mouse peroxidase-conjugated antibody diluted at 1:5000 (Sigma-Aldrich A9044, RRID:AB_25843) was used as secondary antibody.

Western blot experiments

Samples previously collected and constituted **from** tissues pooled from 6 animals were homogenized in a denaturing lysis buffer containing 30 mM Tris/HCl (pH 7.4), 1.5% sodium dodecyl sulfate (SDS, w/v), 8 mM EDTA (v/v) and 50 mM dithiothreitol (DTT, v/v) (Yang et al. 2014) and protease inhibitors (Roche, Germany); then the particulate matter was removed by centrifugation at 14000 g for 20 min. The protein concentration was determined by the Bradford assay. For SDS-PAGE analysis, proteins were denatured by boiling in Laemmli Sample Buffer for 5 min. Then 50 µg of protein was loaded in each lane and separated in 15 % SDS-polyacrylamide gels according to Laemmli (Laemmli 1970). After electrophoresis, gels were transferred to nitrocellulose paper (Hybond C+ Extra, GE Healthcare, UK) and membranes were stained with Ponceau S to confirm the transfer of proteins. The saturation step was performed by incubating membranes in 5% bovine serum albumin (BSA) in TBS-Tween for 2 hours at room temperature (Vaccaro et al. 2015).

Normalization was made against β-actin expression detected by β-actin antibody (Santa Cruz Biotechnology sc-47778, RRID:AB_626632) diluted at 1:3,000. Rat brain homogenates were used as positive controls and negative controls were performed by the omission of primary antibodies. Detection was done using the Westar µC Ultra enhanced chemiluminescent HRP substrate (Cyanagen, Italy) and Kodak X Omat LS films (Sigma-Aldrich, USA). The developed films were scanned as Tiff images in 8-bit gray scale format at setting of 300 dpi and the band intensities were measured by Image J software (U.S. National Institutes of Health, Maryland). In electrophoresis experiments, the "Amersham Full-Range Rainbow MW Markers (12–225 KDa)" were used.

Results

Synuclein genes and protein expression

The information on the nt and aa sequences of *Anolis carolinensis* syns present in the NCBI database consisted of sequences predicted by automated computational analysis of lizard genomic sequences annotated using the Gnomon gene prediction method. In the genome of *A. carolinensis* three different genes encoding syn proteins were identified: *snca* (Gene ID: 100 563 343), *sncb* (Gene ID: 100 559 889) and *sncg* (Gene ID: 100 561 651) (Table 1).

The *snca* gene is located on chromosome 5 [position NC_014780.1 (46219523…46294230)] and consists of six exons, the first of which is totally non-coding (Fig. 1). The predicted mRNA coding for α-syn (XM_003221301.2) consisted of 1693 nt with a 429 nt-long coding sequence (CDS) (662-1090 nt) (Fig. 1). The predicted α-syn aa sequence (XP_003221349.1) corresponded to a protein of 142 residues with a predicted MW of 14921 Da and an isoelectric point (pI) of 4.56.

The *sncb* gene (Gene ID: 100 559 889) consisted of six exons and its location has not yet been determined according to the present genome assembly (AnoCar2.0) (Fig. 2). In the NCBI database two different variants of the mRNA encoded by *sncb* gene were present: X1 (XM_008121117.1) and X2 (XM_003227782.2) variants of β-syn, constituted by 1453 and 1542 nt, respectively (Fig. 2 and Table 1). The differences between the two mRNA isoforms were restricted to the first non-coding exons and so the two β-syn mRNA variants had an identical CDS (296-697 nt in X1 and 385-786 nt in X2) and they coded for the same aa sequence (XP_008119324.1 for X1 and XP_003227830.1 for X2). The β-syn corresponded to a predicted protein of 133 aa with MW 14000 Da and pI 4.42.

The *sncg* gene (Gene ID: 100 561 651) consisted of five exons and its location has not yet been identified (Fig. 3). In the NCBI database, two different mRNA variants of γ-syn named X1 (XM_008114475.1) and X2

(XM_008114476.1) were present. X1 γ-syn consisted of 1336 nt with a CDS of 525 nt (400-924 nt) and coded for a predicted protein (XP_008112682.1) of 174 aa with MW 18600 Da and pI 4.74. The X2 γ-syn consisted of 1182 nt, had a CDS of 399 nucleotides (372-770 nt) and encoded for a predicted protein (XP_008112683.1) of 132 aa with MW 13814 Da and pI 4.46 (Fig. 3 and Table 1). The comparative analysis between the mRNA sequences of the two γ-syn variants showed that they are identical except for the insertion of a 126 nt sequence in the variant X1 caused by a different length of the fourth exon. Thus, variant X1 presents 42 additional amino acids following residue 126 in the CT region.

Sequence comparison and analysis

The comparative analysis performed among the aa sequences of lizard syns showed an overall identity of 66% between α- and β- syns, of 50% or 55% between α-syn and X1 or X2 γ-syns, and of 47% or 53% between β-syn and X1 or X2 γ-syns (Table 3). Among lizard syns the N-terminus (aa 1-60) was more conserved than the C-terminus and the identity in the first rose to values ranging from 78% to 81% whereas in the second it decreased to values ranging from 13 to 45% (Table 3 and Fig. 4a).

Comparative analyses were performed between lizard and human syns to evaluate the conservation of these proteins and to verify the presence in lizard syns of conserved domains or residues associated to specific functions in mammalian syns.

Lizard syns appeared well conserved when compared with the homologous human proteins (Fig. 4b-d)**.** The percentage of identity between lizard and human syns on the whole length was 86% between α-syns, 89% between β-syns and 68 and 69% between γ-syns (Table 3). In the N-terminus the identity rose to 88 and 90% respectively for α- and β- syns, and to 95% for both X1 and X2 γ-syns whereas in C-terminus the identity was 80% between α-syns, 89% between β-syns and 43 and 45% for X1 and X2 γ-syns. The identity of **the** NAC region betwe en lizard and human α-syns was 91%.

As in human α-syn some point mutations (A30P, E46K, H50Q, G51D, A53T) have been linked to PD, the conservation of these amino acids was evaluated in lizard α-syn (amino acids shown in red in Fig. 4b). In lizard α-syn alanine (A) at position 30 and glycine (G) at position 51 were conserved whereas conservative replacement of glutamic acid (E) at position 46 with aspartic acid (D) was observed (Fig. 4b). Interestingly, histidine-to-glutamine substitution (H50Q) and alanine-to-threonine substitution (A53T) were present in lizard α-syn (Fig. 4b).

In the NT and NAC regions of human syns, imperfect repeats of 11 amino acids containing the apolipoproteins lipid-binding motif ([EGS]-KTK-[EQ]-[GQ]-V-XXXX) are present. These repeats were preserved in lizard syns (Table 4 and Fig. 4b-d) in which seven imperfect repeats were present in α- and γ- syns and five repeats were present in β-syn (Table 4). The first 4 repeats were continuous in all isoforms and were separated from the following repeats by 3 (β-syn) or 4 (α-syn, X1 and X2 γ-syns) amino acids. However, only three repeats in α-syn (II, III and V) and two in β- and γ- syns (II and III) contained the complete apolipoproteins lipid-binding motif (underlined aa stretches in Fig. 4a and in Table 4).

The aa stretch GVTAVAQKTVE that in human α-syn is able to acquire a β-sheet structure when the protein aggregates in fibrils, was perfectly conserved in lizard α-syn but it was not present in β- and γ- syns, as in human β- and γ- syns (double underlined amino acids in Fig. 4a,b).

The C-terminus of human α-syn, an unstructured region characterized by the presence of negative charges, can bind metal ions and it is subjected to post-translational modifications, such as phosphorylation, that may affect protein

conformation and function. The negative charge distribution was generally preserved in lizard α-syn due to the presence of 5 residues of aspartic acid (D) and 10 residues of glutamic acid (E) (bold characters in Fig. 4b).

Interestingly both serines (S) located at position 87 and 129 in human α-syn (circled amino acid in Fig. 4b) that have been found to be relevant to PD were not conserved in lizard α-syn in which they were substituted by asparagine (N) and proline (P), respectively (Fig. 4b). On the contrary, other putative phosphorylation sites in human α-syn (Y125, Y133, and Y136) were perfectly conserved in the lizard protein (black arrowheads in Fig. 4b). Amino acids that in human α-syn are directly or indirectly involved in the binding of metal ions (Y39, Y125, Y133, Y136, M116 and M127) are conserved in the lizard isoform.

Secondary structure prediction confirmed that the C-terminus of lizard syns is mainly an unstructured region whereas the NT and NAC regions, where the semi-conserved repeats of 11 residues containing the apolipoproteins lipid-biding motif are located, may adopt α-helical conformations (Fig. 5).

Synuclein gene expression

The gene expression of the syn isoforms was evaluated in the major organs of the green lizard *A.carolinensis* by semi-quantitative RT-PCR (Fig. 6). The availability of the mRNA sequences coding for syn isoforms allowed the design of specific primers able to discriminate the expression of the different genes (Figs. 1-3).

α-, β- and γ- syns were expressed both in nervous and non-nervous organs and the most intense expression was detected in the brain (Fig. 6a). In the spinal cord α- and β- syns were also intensely expressed, whereas a low expression of γ-syn was detected. Variable levels of the three syn isoforms were also detected in the eye, heart, intestine, liver and lung. β-syn was substantially expressed in the muscle where α- and γ- syn expression was very low or absent.

The expression of γ-syn was further investigated by using primers able to discriminate X1 and X2 variants (Table 2 and Fig. 3) in order to evaluate whether both isoforms were expressed in the lizard. Interestingly, both X1 and X2 were expressed in the brain and spinal cord while in most of the other organs, the X2 isoform was the isoform predominantly expressed. Low or very low levels of X1 were detected in the eye, intestine and liver.

Syn expression was further investigated in selected brain regions where high levels of α-, β-, and γ- syns were detected in all the brain regions analyzed and interestingly both X1 and X2 γ-syns were detected in telencephalon, diencephalon, cerebellum and brainstem (Fig. 6b).

Antibody choice

As described in detail in the section “Materials and methods”, a preliminary search to identify commercial antibodies directed toward mammalian syns and able to recognize and discriminate the different isoforms of lizard syns was carried out on the basis of the sequence identity of relevant epitopes. Antibodies with the most suitable characteristics for this study were tested in preliminary Western blot experiments. Among them, the two antibodies with the best performances were chosen for subsequent analyzes: the α-syn (610787, BD Transduction Laboratories) and the β-syn (sc-136452, Santa Cruz) antibody. The first is a mouse monoclonal antibody recognizing the ATGFVKKDQ epitope (Perrin et al. 2003) that is present in both rat and lizard α-syns. The same antibody showed no cross reactivity with β- and γ- syns in Western blot experiments performed in α-syn null mouse (Perrin et al. 2003). For β-syn, a mouse monoclonal antibody raised against the epitope LIEPLMEPEGES corresponding to amino acids 107-118 of rat β-syn was used. This aa stretch is well conserved in lizard β-syn. This information suggests that the chosen antibodies can be

valuable tools to study the expression of α- and β- syns in lizard. On the contrary, because of the high identity among the NT region of lizard syns and of the low identity between the CT region of lizard and mammalian γ-syn, it has not been possible to identify antibodies specific for lizard γ-syn.

Synuclein protein expression

The protein expression of α- and β- syns was verified in the same organs by Western blot using antibodies directed against the mammalian isoforms (Fig. 7). The main purpose of these analyses was to confirm and corroborate the data obtained from semi-quantitative RT-PCR experiments demonstrating the effective expression of syn proteins in the brain regions and organs analyzed and to provide useful tools for future localization of these proteins by means of immunohistochemical methods.

The α-syn antibody revealed an intensely labeled band at about 14/16 kDa, corresponding to the predicted MW, in the brain and spinal cord while no bands were detected in the other organs analyzed (eye, muscle, heart, intestine, liver and lung) (Fig. 7b). Only longer film exposure times, leading to the signal saturation of the brain band, allowed detection of immuno-labeled bands in the eye, heart and intestine samples. Even at these exposure conditions, no defined bands were detected in the muscle and liver whereas in the lung a diffuse and undefined labeling that could correspond to a very low amount of protein or to a background of the film was detected.

The protein expression analysis of β-syn showed the presence of an immuno-labeled band at about 14/16 kDa only in brain, spinal cord and eye samples (Fig. 7c). Even at higher exposure time, very faint bands were observed in the heart, intestine and lung. Given the long exposure times and the weakness of the bands it is conceivable that this labeling could be non-specific.

The α- and β- syn expression was further investigated in the encephalic regions and results demonstrated that both proteins are expressed in telencephalon, midbrain tectum, diencephalon, cerebellum and brainstem (Fig. 7d, e).

The densitometric analysis of syn bands normalized on actin levels showed for both α- and β- syns a substantially similar expression pattern with the highest expression levels in the telencephalon. Other brain regions (diencephalon, midbrain tectum, brainstem, and cerebellum) showed lower expression levels with the lowest levels found in the spinal cord (about 10% for α-syn and 40% for β-syn with respect to telencephalon).

Discussion

In this study, syn gene and protein expression was studied in the CNS and non nervous organs of the green lizard *A. carolinensis* as a representative species for reptiles. Three genes encoding for α- (*snca*), β- (*sncb*) and γ- (*sncg*) syns were identified in the available genome of *A. carolinensis* (Genome assembly, AnoCar 2.0), as in bird and mammal**ian** genomes (Lavedan 1998). **In contrast**, three (*Danio rerio*) (Milanese et al. 2012; Sun and Gitler 2008) to four (*Takifugu rubripes)* (Yoshida et al. 2006), syn genes **have been** detected in teleost fish, and four in anurans (*Xenopus laevis*) (Wang et al. 2011). This variability in the number of syn genes in fish and amphibians has been attributed to the whole genome duplication **that** occurred in the ray-finned fish (Actinopterygia) about 230 million years ago (Amores et al. 1998; Jaillon et al. 2004) and to the pseudotetraploid condition of *X. laevis* (Wang et al. 2011).

In *A. carolinensis* the three syn genes consisted of five coding exons showing a similar organization to that previously described in fish (Yoshida et al. 2006) and humans (Lavedan 1998). The NT region of lizard syns is coded

by the first two exons, the NAC region by the third exon, and the unstructured and negatively charged C-terminus is coded by the fourth and fifth exons. Overall these data further confirm the high degree of conservation of syn genes among vertebrate species.

In the NCBI database, five different lizard syn mRNAs were present as two different mRNA variants, named X1 and X2, exist for both β- and γ- syns. X1 and X2 β-syns differed only in the non-CDS and so both encoded the same aa sequence, whereas the mRNAs of X1 and X2 γ-syns encoded two proteins of different size, as X1 was characterized by the insertion of 42 residues in the CT region. Our results demonstrate that X1 and X2 γ-syn isoforms are actually both expressed in the lizard with a different regional expression pattern, suggesting that the two γ-syn variants could fulfill different and specific functions. Thus, the expression of different syn variants is not a characteristic only of mammals, where alternative splicing events produce α-syn variants (Beyer et al. 2008; Campion et al. 1995; Ueda et al. 1994), but also occurs in reptiles, suggesting complex scenarios of action for syns also in this taxon.

The comparative analysis between the aa sequences of human and lizard syns showed a high level of identity throughout the entire sequence, confirming the high degree of conservation among vertebrate syns. This suggests that these proteins fulfill important and conserved physiological functions in vertebrates. However, the real physiological roles of syns have not yet been fully elucidated.

The aim of the present study was not to discover the physiological functions of lizard syns, but to analyze their gene and protein expression in the CNS and in the main organs. Nevertheless, the high identity between human and lizard syns and the presence of conserved regions suggest that most of syn features could be conserved in lizard, including the ability to acquire specific secondary structure and to bind lipid vesicles and metal ions. In fact, the aa stretch GVTAVAQKTVE was perfectly conserved in lizard α-syn, suggesting it **might** be able to fold in**to a** β-sheet structure and to aggregate forming fibrils, as may happen in humans. Moreover, the presence in *A. carolinensis* of perfectly conserved apolipoproteins lipid bi**n**ding motives suggests that these proteins could bind lipid membranes fulfilling related physiological functions such as vesicular trafficking and maintenance of synaptic vesicle pool. Furthermore, the conservation of some tyrosines (Y39, Y125, Y133 and Y136) and methionines (M116 and M127), suggests that human and lizard α-syns share the ability to bind metal ions.

However, despite the generally high sequence identity between human and lizard α-syn, small differences in the aa sequence were observed that can affect protein function and regulation. For example, in the NT region of lizard α-syn, the histidine (H) at position 50 is replaced by a glutamine (Q), and the alanine (A) at position 53 is replaced by a threonine (T). These changes correspond exactly to the H50Q (Appel-Cresswell et al. 2013; Proukakis et al. 2013) and A53T (Larsen et al. 2009) mutations that in human α-syn are linked to PD. **The H50Q was also detected in α-syn of the lobe-finned fish *Latimeria chalumnae* and of the emperor penguin *Aptenodytes forsteri* (Gruschus 2015) and this substitution** was described by Proukakis and co-workers (2013) as a mutation affecting copper coordination and enhancing the stability of the α-helix domain.

The A53T mutation alters human α-syn structure expanding the hydrophobic domain (from 11 to 30 aa), destabilizing the α-helical domain (Biere et al. 2000), and facilitating the folding of the protein in**to a** β-sheet structure required for the formation of oligomeric species (Biere et al. 2000; Conway et al. 1998; Giasson et al. 1999; Hashimoto et al. 1998). The presence of threonine (T) at position 53 has been described in α-syn of several fish (Vaccaro et al. 2015; **Gruschus 2015**), bird and mammalian species including some New World monkeys (Hamilton 2004; **Gruschus 2015**) suggesting that, on one side, the presence of T may not affect **the** life**span** of short-lived vertebrates and, on the other side, that long-lived animals may have adapted alternative mechanisms to minimize toxic accumulation of α-syn oligomers. On the basis of the presence of Q50 and T53 in lizard α-syn it could be speculated that this protein may be

more prone to form aggregates. However, further experiments are necessary to confirm this hypothesis. **Interestingly, a recent analysis of available vertebrate sequences of α-syn and glucocerebrosidase (GCase), including those of *Anolis carolinensis*, showed a correlation between PD-associated α-syn substitutions and residue substitutions in the GCase. This study suggested the co-evolution of the two proteins and provided evidence of altered α-syn/GCase interaction in PD pathology (Gruschus 2015).**

One difference between lizard and human α-syns was the replacement in *A. carolinensis* of serines at position 87 and 129 with asparagine and proline, respectively. These differences could have important effects on the regulation and functions of the protein as in humans the phosphorylation at S87 influences its binding to lipid membranes (Paleologou et al. 2010), and the phosphorylation at S129 regulates α-syn oligomerization, fibril formation, and neurotoxicity (Anderson et al. 2006; Fujiwara et al. 2002).

With regard to the binding of metal ions, the binding sites of Cu(II), Pb(II), Fe(II) identified by Lu et al. in the CT region of human α-syn (Lu et al. 2011) are not perfectly preserved in lizard, suggesting possible differences in the affinity and in the number of divalent cations bound by human and lizard proteins. In human α-syn the binding site for copper at the N-terminus involves M1 and D2 when the metal is bound as Cu(II), or M1 and M5 when it binds as Cu(I), in the latter case also M10 in β-syn participates in metal binding (De Ricco et al. 2015a). In human α-syn a role for H50 in Cu(II) coordination, acting as a molecular switch has recently been suggested (De Ricco et al. 2015b). In lizard α-syn, H50 is replaced by glutamine while in β-syn M10 is substituted by lysine, further suggesting that copper-binding properties will be different. On the basis of the detected differences we can assume that α-syn may be subjected to different regulation mechanisms in reptiles and mammals.

In adult specimens of the green lizard *A. carolinensis*, the three syn genes were expressed both in nervous and non-nervous organs suggesting a role for syns in the cell physiology of different tissues. syn gene and protein expression was particularly intense in the CNS where α-, β- and γ- syns were expressed in all the main encephalic regions, in the spinal cord and in the eye.

The three syn genes were also expressed in other organs such as heart, intestine, liver and lung, while in the skeletal muscle only β-syn mRNA was expressed at detectable levels. However, it should be emphasized that even in those organs in which discrete mRNA levels were detected, the protein expression levels of α- and β- syns were scarce or at the limit of detection. This suggests the presence of post-translational regulation systems in lizard organs.

Both X1 and X2 γ-syn mRNAs were expressed in the CNS, whereas X2 was the most expressed variant in non nervous organs. This suggests that X1 and X2 γ-syns may be specialized in fulfilling different roles in the lizard. The addition of 42 amino acids after residue 126 in the CT region in X1 γ-syn may affect the interactions of this protein with other molecules or its mechanisms of regulation. More focused experiments are needed to further investigate this issue.

The available data on syn expression in species belonging to the different vertebrate classes are still partial and limited. Depending on the species, the gene or protein expression was analyzed by using semi-quantitative methods (Northern blot, RT-PCR and Western blot) or by analyzing the tissue/cell distribution (*in situ* hybridization and immunohistochemistry). For this reason, a precise comparison of syn expression in vertebrate species cannot be performed. However, even considering these limits, differences in syn gene expression can be revealed comparing lizard and other vertebrate species. In the lizard, differently from what happens in humans (Lavedan 1998), α-syn was expressed in the liver, β-syn in the liver, lung and heart, and γ-syn in the lung. On the contrary, the three syn isoforms were expressed in human skeletal muscle, whereas in the lizard only β-syn was expressed in **skeletal tissue**. Such differences may result from the different sensitivity of the methods used (RT-PCR and Northern blot) or may reflect real differences due to different functional specializations of syns in the different species.

The expression of syn variants in the lizard might be restricted to specific cell types, as observed in mammals. For example, α-syn is mainly expressed in central catecholaminergic neurons, whereas β-syn is more localized in cholinergic neurons in the rat CNS (Li et al., 2002). Moreover, only few neuronal populations of the human nervous system are vulnerable to the neurodegenerative process in the idiopathic PD (Braak et al., 2003). Among non neuronal tissues, α-syn is expressed by enteric neurons but not in glial cells and smooth muscle (Paillusson et al., 2010). Alpha-syn is also specifically expressed in pancreatic β-cells (Geng et al., 2001) and in chromaffin cells (Larsen et al., 2006). Similar evidence has been found in teleosts. Indeed, the expression of α-syn is mainly localized to cholinergic neurons in the carp CNS (Vaccaro et al., 2015). Therefore, the expression of syn variants in specific cell types is expected in the lizard. *In situ* hybridization and immunohistochemical studies will contribute to compare cellular distribution of syn variants in lizard and other vertebrates.

The comparison of data recently obtained in carp (Toni and Cioni 2015; Vaccaro et al. 2015) with those of lizard shows that in both species α-syn protein is expressed in all brain regions. However, the relative expression levels among the encephalic regions is different, as in lizard the higher α-syn protein expression was detected in the telencephalon, while the same region in the carp showed a low expression. In this sense, the expression of α-syn in lizard most resembles that observed in mammals where higher **levels of** expression **have been detected** in the telencephalon and diencephalon than in the caudal brain regions (Li et al. 2002). At this regard, it may be worth mentioning that telencephalic regions of amniotes share a number of common organizational and functional features since comparative neuroanatomical studies have shown that most ascending sensory projections to the telencephalon described in mammals also occur in reptiles (Butler and Hodos, 20**0**5).

In conclusion, this paper analyzed for the first time the syn expression in the lizard *A. carolinensis*, **selected** as representative of reptiles. The results confirm that syns are evolutionarily conserved both in gene structure and in amino acid sequence. The presence in lizard syns of aa sequences that in humans are involved in the binding to lipid vesicles and to metal ions and in the formation of fibrillar aggregates suggests that these proteins may perform similar functions in reptiles and mammals. However, small differences in aa sequences, in the tissue expressional pattern and in the relative amount of proteins expressed in different tissues and brain regions, suggests that syns may have acquired functional specializations during reptile evolution.

Conflict of interest

The authors declare no conflict of interest.

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Tables

Synuclein	Gene ID	mRNA	Protein
α	100563343	XM_003221301.2	XP_003221349.1
β	100559889	XM_008121117.1(X1) XM_003227782.2(X2)	XP_008119324.1 (X1) XP_003227830.1 (X2)
γ	100561651	XM_008114475.1 (X1) XM_008114476.1(X2)	XP_008112682.1 (X1) XP_008112683.1 (X2)

Table 1 Gene, mRNA and amino acid sequence of lizard synucleins available at the NCBI database. ID and Accession number are indicated.

Primer name	Sequence (5'-3')
α-syn_F	TTCAAAGACCAGGGATGGAG
α-syn_R	CTTCATCCTGTTTGCCCAGT
β-syn_F	GGTCAAGAAAGATGAATTCCC
β-syn_R	TTCCGGCTCATATTCCTGAT
γ-syn_F	AGACATTGGCTACGCTGCAA
γ-syn_R	CCATGATGGCTTTGCTCTTG
GAPDH_F	TTTCAATGGCATGTCAAAGCA
GAPDH_R	GGACTCCACAACATAGTCTGCA
X1 γ-syn_F	GTCCAGAGGTAAAGCACAGTCA
X2 γ-syn_F	AAAGAACAGGCCAACCTCGT
X1/X2 γ-syn_R	GCTCTGGTGTCAGCTAACATTT

Table 2 Oligonucleotide sequences used as PCR primers for the analysis of synuclein transcripts.

A. carolinensis	B-syn	C	66																			
		NTr		81																		
		CTr				45																
	X1 γ-syn	C	50				47															
		NTr		80				78														
		CTr				13			22													
	X2 γ-syn	C	55				53			95												
		NTr		80				78			100											
		CTr				18			30			92										
H. sapiens	α-syn	C	86				67			50			55									
		NTr		88				88			82			82								
		NAC			91																	
		CTr				80			40			9			13							
	β-syn	C	66				89			52			59			67						
		NTr		85				90			87			87			90					
		CTr				40			89			24			31			36				
	γ-syn	C	52				51			68			69			55			58			
		NTr		75				73			95			95			77			82		
		CTr				7			29			43			45			11			32	
			C	NTr	NAC	CTr	C	NTr	CTr	C	NTr	CTr	C	NTr	CTr	C	NTr	CTr	C	NTr	CTr		
			α-syn				β-syn			X1 γ-syn			X2 γ-syn			α-syn			β-syn			
			A. carolinensis													*H. sapiens*						

Table 3 Determination of the percentage identity of human and lizard syn amino acid sequences. The Clustal Omega software at http://www.ebi.ac.uk/Tools/msa/clustalo/ was used. C: complete sequence; CTr: C-terminal region; NTr: N-terminal region; NAC: non-β amyloid component. Lizard α-syn (NTr=1-60 aa, NAC=61-95 aa, CTr= 96-142 aa), β-syn (NTr=1-59 aa, CTr=60-133), X1 γ-syn (NTr=1-60 aa, CTr=61-174) and X2 γ-syn (NTr=1-60 aa, CTr= 61-132aa). Human α-syn (NTr=1-60 aa, NAC= 61-95 aa, CTr= 96-140), β-syn (NTr=1-60 aa, CTr= 61-134 aa); γ-syn (NTr=1-60 aa, CTr= 61-127 aa).

	α synuclein	β synuclein	X1 and X2 γ synuclein
I	9 N**K**A**K**D**GV**VAAA 19	9 **SKAKEGV**AAAA 19	9 S**I**A**KEGV**VAAA 19
II	20 **EKTKQGV**AEAA 30	20 **EKTKEGV**AAAA 30	20 **EKTKQGV**TEAA 30
III	31 **GKTKEGV**LYVG 41	31 **EKTKEGV**LYVG 41	31 **EKTKEGV**MYVG 41
IV	42 **SKT**RD**GV**VQGV 52	42 **SKTREGV**AGVA 52	42 A**KTKEGV**VQSV 52
V	57 **EKTKEQV**SNVG 67	56 **EKTKEQ**ASQLG 66	57 **EKTKEQ**ANLVG 67
VI	68 **G**AVVT**GV**TAVA 78		68 **E**SVVAS**V**NTVA 78
VII	79 Q**KTVEG**AGNFA 89		79 KQ**TVEG**AETVV 89

Table 4 Repeats in lizard synucleins. The aa sequences of repeats I-VII of α-syn (residues 9-89), repeats I-V (residues 9-66) of β-syn, repeats I-VII (residues 9-97) of X1 and X2 γ-syns are shown. Conserved residues of the apolipoproteins lipid-biding motif [EGS]-K-T-K-[EQ]-[GQ]-V-XXXX are indicated in bold and repeats that contain a perfectly conserved lipid binding motif are underlined.

Figure captions

Fig. 1 Alpha synuclein of *Anolis carolinensis*: gene structure, mRNA and protein sequence

a organization of the lizard α-syn gene. Exons are represented by boxes and introns are shown as horizontal lines not drawn in scale (see **inset showing** the ratio line length/number of nucleotides). The non-coding regions are represented in black color whereas the coding regions of different exons are indicated by different colors (red, blue, green, orange and violet). **b** nt sequence of α-syn mRNA **(**XM_003221301.2). The regions against which the forward and reverse RT-PCR primers were designed are indicated by arrows. **c** deduced aa sequence of lizard α-syn (XP_003221349.1). The predicted MW and pI are indicated. In b and c, sequences coded by different exons are represented with the same color code used in the schematic drawing of the gene. The exon boundaries are indicated by square brackets.

Fig.2 Beta synuclein of *Anolis carolinensis*: gene structure, mRNA and protein sequences

a organization of the lizard β-syn gene. Exons are represented by boxes and introns are shown as horizontal lines (not drawn in scale). See **inset** of Fig. 1 **showing** the ratio line length/number of nucleotides. The non-coding regions are represented in black color whereas the coding regions of different exons are indicated by different colors (red, blue, green, orange and violet). **b** comparative analysis of the nt sequences of the two mRNA variants of lizard β-syn available in the NCBI database (X1, XM_008121117.1; X2, XM_003227782.2). Sequences were aligned with Clustal Omega. Asterisks indicate identity of nucleotides. The regions against which the forward and reverse RT-PCR primers were designed are indicated by arrows. **c** deduced aa sequence of lizard β-syn (XP_008119324.1; XP_003227830.1). As X1 and X2 β-syn mRNA differ only in the non-coding region, they code the same aa sequence. The predicted MW and pI are indicated. In b and c, sequences coded by different exons are indicated with the same color code used in the schematic drawing of the gene. The numbers refer to the nt and aa positions at the end of each line. The exon boundaries are indicated by square brackets.

Fig. 3 Gamma synuclein of *Anolis carolinensis*: gene structure, mRNA and protein sequences

a organization of the lizard γ-syn gene. Exons are represented by boxes and introns are shown as horizontal lines (not drawn in scale). See the **inset** of Fig. 1 **showing** the ratio line length/number of nucleotides. The non-coding regions are represented in black color whereas the coding regions of different exons are indicated by different colors (red, blue, green, orange and violet). **b** comparative alignment of the nt sequences of the two mRNA variants of lizard γ-syn available in the NCBI database (X1, XM_008114475.1; X2, XM_008114476.1). The regions against which the forward and reverse RT-PCR primers were designed are indicated by arrows: red ones for γ-syn and dotted ones for X1 (green) and X2 (purple) γ-syns. **c** comparative alignment of the deduced aa sequences of lizard X1 and X2 γ-syns (XP_008119324.1; XP_003227830.1). The predicted MW and pI are indicated. In b and c, sequences coded by different exons are indicated with the same color code used in the schematic drawing of the gene. Sequences were aligned with Clustal Omega. Asterisks indicate identity of amino acids; double dots indicate amino acids with the same polarity or size; dots indicate semi-conserved substitutions. The numbers refer to the nt and aa positions at the end of each line. The exon boundaries are indicated by square brackets.

Fig. 4 Comparative analysis of human and lizard synucleins

Amino acid sequence alignment among lizard syns (a) and between human and lizard α- (b), β- (c) and γ- (d) syns are reported. **a** The stretches of amino acids encoded by different exons are marked with different colors and the exon boundaries are indicated by vertical lines. Conserved repeats of the apolipoproteins lipid-biding motif [EGS]-K-T-K-[EQ]-[GQ]-V-XXXX are underlined (see Table 4 for details). The NAC region of α-syn is highlighted in gray. **b** black arrowheads indicate phosphorylatable tyrosines, arrows indicate methionines that represent binding sites for Mn(II) and other metals. The phosphorylatable serines are circled in yellow. N-terminal, C-terminal and NAC regions are indicated. Negative amino acids in the CT region are indicated in bold. The amino acids that in humans are involved in the pathological mutations linked to Parkinson's disease are shown in red. **a-d** The positions of the semi-conserved repeats are indicated by the roman numbers (I-VII) above the sequences. The aa stretch GVTAVAQKTVE, **which** is perfectly conserved between human and lizard α-syns and that is directly involved in the formation of human amyloid fibrils, is double underlined. Sequences were aligned with Clustal Omega. Asterisks indicate identity of amino acids; double dots indicate amino acids with the same polarity or size; dots indicate semi-conserved substitutions.

Fig. 5 Secondary structure prediction of lizard synucleins

Prediction of the secondary structure of α-, β-, X1 and X2 γ- syns in *Anolis carolinensis* using the PSIPRED Protein Sequence Analysis Workbench at http://bioinf.cs.ucl.ac.uk/psipred/ (Jones 1999). The position of the semi-conserved repeats is indicated by the roman numbers (I-VII) above the sequences. The NAC region of α-syn (61-95 residues) is underlined.

Fig. 6 Synuclein gene expression in the major organs of the lizard *Anolis carolinensis*

Semi-quantitative RT-PCR analysis of syn gene expression in the main organs (a) and encephalic regions (b) of the lizard *Anolis carolinensis*. For γ-syn detection, both general and specific primers able to discriminate X1 and X2 variants were used. Br: brain; Bs: brainstem; Ce: cerebellum; Di: diencephalon; Ey: eye; He: heart; In: intestine; Li: liver; Lu: lung; Mu: muscle; OT: midbrain tectum; SC: spinal cord; Te: telencephalon.

Fig. 7 Synuclein protein expression in the major organs of the lizard *Anolis carolinensis*

Western blot analysis of α- and β- syns in the main organs (a-c) and encephalic regions (d-f) of the lizard *Anolis carolinensis*. Red ponceau staining is shown in a, α-syn immunolabelling in b and d and β-syn immunolabelling in c and e. The densitometric analysis of immunolabeled bands normalized on actin levels is reported in f. Br: brain; Bs: brainstem; Ce: cerebellum; Di: diencephalon; Ey: eye; He: heart; In: intestine; Li: liver; Lu: lung; Mu: muscle; OT: midbrain tectum; R: rat brain; SC: spinal cord; Te: telencephalon.