Metabolic Brain Disease (2019) 34:705–713
https://doi.org/10.1007/s11011-019-0386-8

ORIGINAL ARTICLE

# Evaluation of neuroglobin and cytoglobin expression in adult rats exposed to silver nanoparticles during prepubescence

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Received: 6 July 2018 / Accepted: 20 January 2019 / Published online: 30 January 2019


## Abstract
Silver nanoparticles (AgNPs) are clusters of silver atoms with diameters that range from 1 to 100 nm. Due to the various shapes and large surface areas, AgNPs have been employed in the food and textile industries and medical fields. Therefore, because of the widespread use of these compounds, the aim of this study was to evaluate the effect of AgNP exposure on the gene and protein expression levels of Neuroglobin (Ngb) and Cytoglobin (Cygb), in the rat cortex, hippocampus and cerebellum. Post-natal day (PND) 21 male Wistar rats were randomly divided into three groups. One group received 15 μg/kg body weight of AgNP by gavage another group received 30 μg/kg and the control group that received saline, from PND23 to PND58. On PND102 the animals were euthanized and the cortex, hippocampus and cerebellum were isolated and evaluated for gene and protein expression levels of Nbg and Cygb. The results demonstrated that the 30 μg/kg AgNP group displayed increased gene and protein expression of Cygb in the cortex. In the Hippocampus, AgNP exposure did not modulate gene or protein expression levels of Ngb and Cygb. In cerebellum the Ngb gene and protein expression was increased with both doses of AgNP. AgNP exposure during prepubescence can modulate the gene and protein expression levels of Ngb and Cygb in adulthood. Furthermore, the observed modulation was specific to the cerebellum, and cortex, and was dose dependent.

**Keywords** Silver nanoparticle · Neuroglobin · Cytoglobin · Brain

## Introduction

Silver nanoparticles (AgNPs) are clusters of silver atoms that exhibit a range of sizes between 1 and 100 nm (Mathur et al. 2017) and because of their antimycotic and antibacterial properties (Juan et al. 2010; Kim et al. 2007; Salomoni et al. 2017; Vazquez-Garcia et al. 2016) are used in physical, biological, and pharmaceutical sciences (Dos Santos et al. 2014).

For example, these compounds are commonly used in the production of disinfectants and anti-odor sprays (Yu et al. 2013), as well as in industrial drying, mechanical grinding, mixing and packaging processes (Wright et al. 1999). In addition, clothing, respirators, household water filters, contraceptives, antibacterial sprays, cosmetics, detergents, dietary supplements, socks and shoes are among the products that exploit the anti-bacterial activity of AgNPs (Marambio-Jones and Hoe 2010), thus the silver nanomaterial has become commonly used in consumer products (Quadros and Marr 2010; Rejewski 2009). As a consequence of their widespread use, there is a considerable risk for AgNPs contaminating the environment and being consumed by living organisms.

Previous studies have demonstrated that AgNPs can potentially accumulate in the liver, spleen, testis, muscle, lung, bladder, blood and brain (Fathi et al. 2018; Lankveld et al. 2010; Loeschner et al. 2011; Martin et al. 2018; van der Zande et al. 2012). Furthermore, chronic exposure to AgNPs has been

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shown to modulate the gonadal axis, consequently promoting sperm morphology alterations and impacting sexual behavior (Mathias et al. 2015). Additionally, AgNP accumulation in the brain has been shown to augment the expression of genes and proteins directly linked to amyloid beta deposition (Huang et al. 2015), neuronal degeneration and astrocyte swelling (Xu et al. 2015).

Neuroglobin (Ngb) and Cytoglobin (Cygb) are heme proteins that carry $O_2$, and share a great deal of sequence and structural similarities with hemoglobin (Burmester et al. 2004; Fordel et al. 2004; Moens and Dewilde 2000). In rats and mice, Ngb is primarily expressed in brain neurons; however, it does not appear to be expressed in glial cells (Cardinale et al. 2018; Fordel et al. 2004; Van Acker et al. 2018). This protein has the ability to increase intracellular ATP reserves in response to the addition of $H_2O_2$, which is essential to cytoskeleton preservation and cell viability (Antao et al. 2010). On the other hand, Cygb is found in all tissues (Burmester et al. 2002), and the over-expression of this protein protects the brain from ischemia and/or hypoxia, thus affording protection against neuronal damage (Khan et al. 2006; Ou et al. 2018).

Previous studies have demonstrated that AgNPs promote oxidative stress, through the generation of reactive oxygen species (Gonzalez-Carter et al. 2017) and free radicals (Dabrowska-Bouta et al. 2018; Rahman et al. 2009), reduce mitochondrial function (Amiri et al. 2018; Hussain et al. 2005) and increase intracellular calcium.

Previously, our laboratory demonstrated that Ngb and Cygb are modulated in different animal studies, such as: hyper- and hypothyroidism (Oliveira et al. 2015), secondhand cigarette smoke exposure (Tae et al. 2017), Bisphenol A exposure (da Conceicao et al. 2017) and exercise (Maglione et al. 2018). The purpose of the present study was to evaluate the effects of daily oral AgNP exposure, during the prepubertal period, on the gene and protein expression Ngb and Cygb in adulthoold. The results provided evidence that a relationship exists between AgNPs exposure and the modulation of Ngb and Cygb expression in different regions of the brain.

## Materials and methods

### Animals

Male Wistar rats were kept in size-controlled litters until after weaning, at post-natal day 21 (PND21). They were maintained in polypropylene cages (43 cm × 43 cm × 20 cm) with a 5 cm layer of wood shavings. The animals were provided with standard chow (Nuvilab CR-1, Nuvital, PR, Colombo, Brazil) and water ad libitum, and were maintained under a 12:12 h dark/light cycle in a temperature-controlled room (23 ± 1 °C).

### Study design

All of the PND21 animals were randomly divided into one of three groups. One group received 15 μg/kg body weight (BW) of AgNP, another group received 30 μg/kg BW of AgNP and the control group received saline containing no AgNP, from PND23 to PND 58. On PND102, the rats were anesthetized with ketamine and xylazin and euthanized by decapitation. The AgNP suspension utilized in this study contained nanoparticles that were 60 nm in diameter (reference number 730815, Sigma-Aldrich Co., Seelze, Germany). Dynamic light scattering (BIC 90 plus – Brookhaven Instruments Corp., Holtsville, NY) was used to assess the dilution stability, by measuring the mean particle size and polydispersity index, at a scattering angle of 90° and a temperature of 25 °C.

The stock AgNPs were diluted in an aqueous suspension and administered daily, in the morning, by gavage with dosing volume of 0.25 mL/100 g of BW. The AgNP doses were calculated based on the experimental toxicological values of no observable adverse effect level (NOAEL, 30 μg/kg) and lowest observable adverse effect level (LOAEL, 125 μg/kg) in rats, as previously reported (Kim et al., 2010). Thus, the AgNP doses of 15 and 30 μg/kg corresponded to values that were 2000- and 1000-fold lower than the NOAEL and 8330- and 4170-fold lower than the LOAEL, respectively. All of the experimental procedures were performed in accordance with the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Research at the Universidade Estadual do Centro-Oeste (protocol number 016/2012) and by the Ethics Committee for Animal Research at the Universidade Federal de São Paulo (protocol number 5097101316).

### RNA isolation and quantitative real-time PCR

Immediately following decapitation, the cortex, hippocampus and cerebellum were rapidly removed, frozen in liquid nitrogen and maintained at −70 °C until use. Total RNA was isolated using the TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer's recommended protocol. RNA concentrations were determined spectrophotometrically by measuring the absorbance at 260 nm. First-strand cDNAs were synthesized using the MML-V reverse transcriptase (Invitrogen, UK). Quantitative real time PCR (qRT-PCR) was performed using the SYBR Green real-time PCR assay (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The following oligonucleotide primers were used to quantify Ngb and Cygb gene expression:

Ngb forward: 5′- CTCTGGAACATGGCACTGTC-3′;
Ngb reverse: 5′-CCAGGAATTCTGGAGAGGAG-3′;
Cygb forward: 5’-GCACACCCTCCTTAGCTTTC-3′;
Cygb reverse: 5’-GCAGGGCTAGTTCCTTTCTG-3’

Cyclophilin A forward: 5 -GGATTCATGTGCCA GGGTGG-3;

Cyclophilin A reverse: 5’-CCATGCTTGCCATC CAGCC-3′.

The following reaction conditions were employed: 10 min at 95 °C followed by 45 cycles of 20s at 95 °C, 20s at 58 °C and 20s at 72 °C. Gene expression was determined using the $2^{\Delta\Delta Ct}$ method and all values were expressed using cyclophilin A mRNA as the control housekeeping gene (Dussault and Pouliot 2006).

## Western blot

For Ngb and Cygb protein expression, the brain tissues were homogenized with a Polytron (Polytron Aggregate, PT 2100 – Kinematica – Swiss) and the appropriate buffer (0.3 M sucrose; 0.1 M KCl; 20 mM Tris–HCl, pH 7.0). To remove the nuclear fraction and plasma membrane, the homogenate was centrifuged at 1000×g for 10 min and then the supernatant was centrifuged at 12,000×g for 40 min (Eppendorf Centrifuge – 5415R – Hamburg – Germany). The protein content of the supernatant (cytosolic fraction) was determined using the Bradford method (Bradford 1976). Laemmli sample buffer was added to aliquots of the supernatants corresponding to 30 μg of total cytosolic protein. The samples were then heated in a dry bath at 100 °C for 10 min, and resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins from the gel were then transferred to nitrocellulose membranes (Biotech, Little Chalfont). To reduce nonspecific protein binding, the membranes were blocked for 2 h in blocking buffer (1% nonfat dry milk, 10 mMTris, 150 mM NaCl and 0.02% Tween 20). The blots were then incubated with anti-neuroglobin and anti-cytoglobin antibody at a ratio of 1:100 *v*/v (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing away unbound primary antibody, the blots were then incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer at room temperature for 1 h, and washed three times with 1× PBS containing 0.1% Tween, for 10 min each. Followed by standard chemiluminescence detection, and normalization using anti-actinin antibody (Santa Cruz Biotechnology, Santa Cruz,CA, USA), diluted in blocking buffer and incubated overnight at 4 °C and washed three time with PBS 1× Tween 0.1% for 10 min each time.

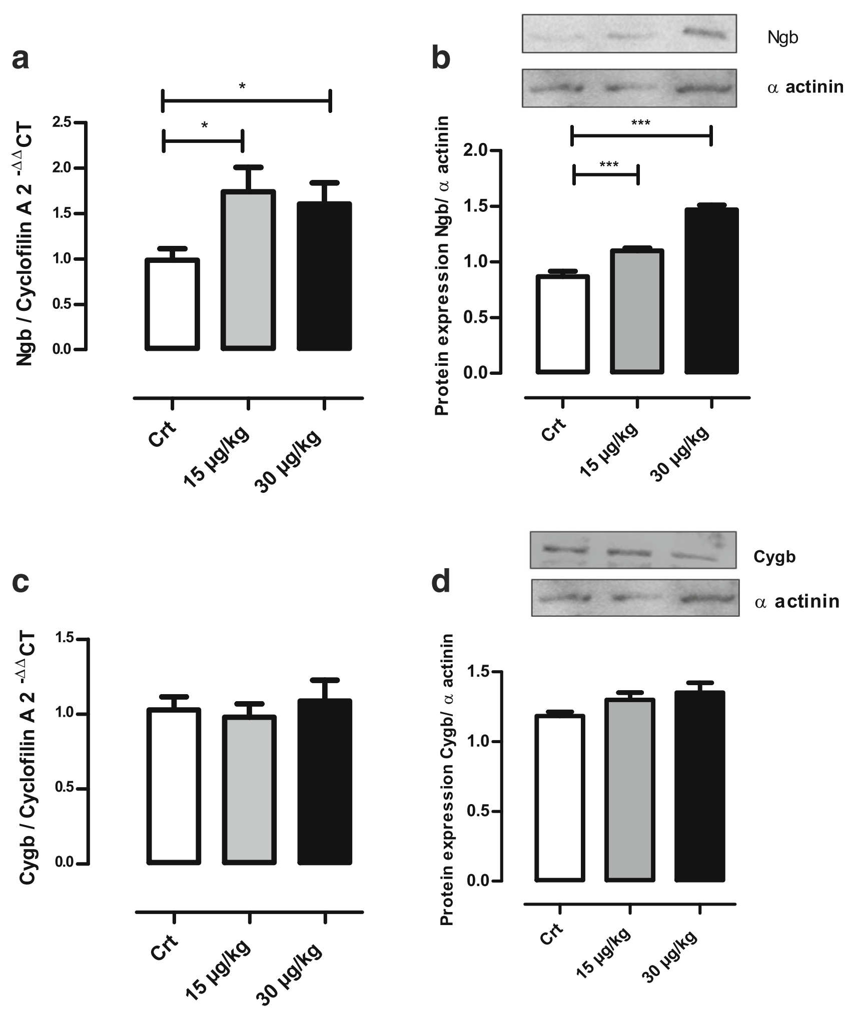


**Fig. 1** Gene and protein expression of neuroglobin (Ngb) and cytoglobin (Cygb) in the cerebellum: Ngb gene expression (**a**), Ngb protein expression (**b**), Cygb gene expression (**c**), Cygb protein expression (**d**). The control group is represented by white bars, the 15 μg/kg group is represented by gray bars and the 30 μg/kg group is represented by black bars. The significance among groups was determined using a one-way ANOVA test followed by the Newman-Keuls post hoc test (*$P < 0.05$, **$P < 0.01$ ***$P < 0.001$) $N = 8–9$

## Statistical analysis

All results are presented as the mean ± standard error (SE). The assumption of normal data distribution was assessed with the Shapiro-Wilk test, followed by a one-way analysis of variance (one way ANOVA) and Newman-Keuls post hoc test, using the Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant when $P < 0.05$.

## Results

### Effect of AgNPs exposure on the gene and protein expression of neuroglobin and cytoglobin in the cerebellum

The effect of AgNP exposure on the gene and protein expression of Ngb and Cygb in the cerebellum is shown in the Fig. 1. With regard to gene expression, AgNP exposure significantly upregulated Ngb mRNA levels in the 15 μg/kg (1.738 ± 0.711) and 30 μg/kg (1.607 ± 0.609) groups, when compared to the control group (0.985 ± 0.3781) (Fig. 1a). Additionally, Ngb protein expression was also significantly elevated in both treatment groups, 15 μg/kg (1.382 ± 0.24) and 30 μg/kg (1.467 ± 0.103), when compared to controls (0.816 ± 0.116). As shown in Fig. 1c-d, AgNP treatment did not alter Cygb gene or protein expression levels in the cerebellum.

### Effect of AgNPs exposure on the gene and protein expression of neuroglobin and cytoglobin in the hippocampus

There were no significant changes in Nbg gene or protein expression observed in rats exposed to AgNP (Fig. 2a, b). However, significant increases in Cygb gene and protein expression were observed in the 30 μg/kg group (1.550 ± 0.170; 1.551 ± 0.672), when compared to the control group (1.05 ± 0.117; 0.979 ± 0.213) (Fig. 2c, d).

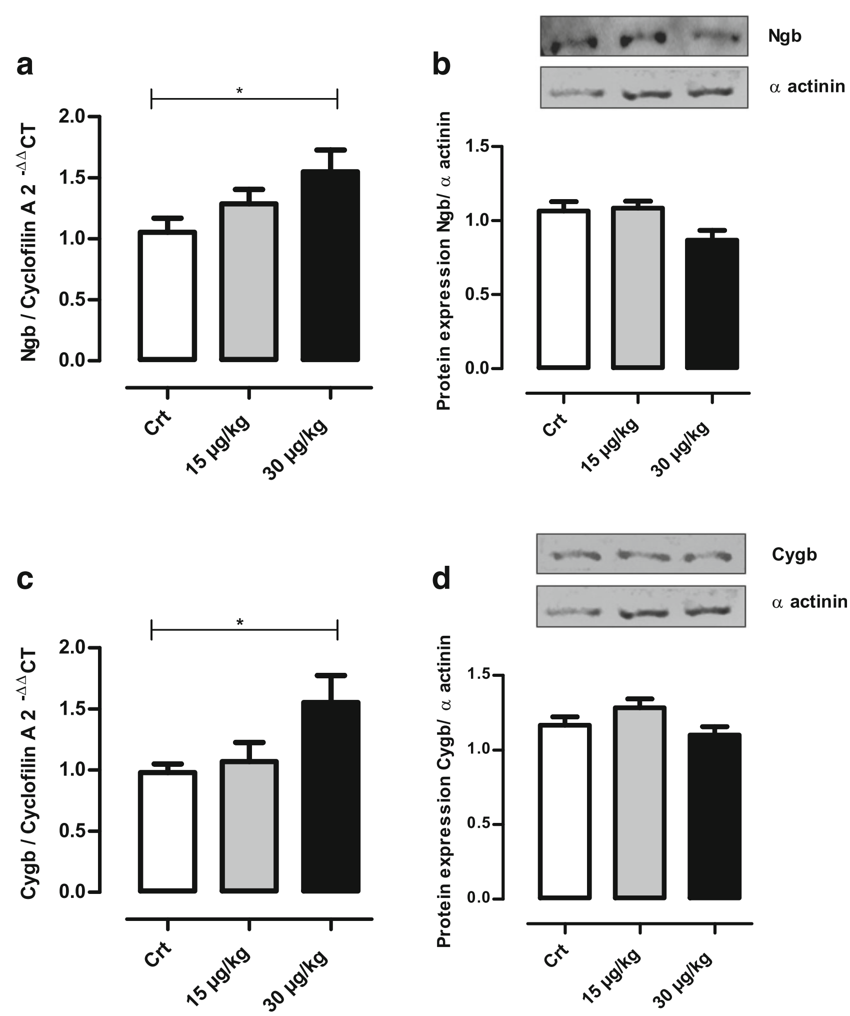


**Fig. 2** Gene and protein expression of neuroglobin (Ngb) and cytoglobin (Cygb) in the hippocampus: Ngb gene expression (**a**), Ngb protein expression (**b**), Cygb gene expression (**c**), Cygb protein expression (**d**). The control group is represented by white bars, the 15 μg/kg group is represented by gray bars and the 30 μg/kg group is represented by black bars. The significance among groups was determined using a one-way ANOVA test followed by the Newman-Keuls post hoc test (*P < 0.05, **P < 0.01 ***P < 0.001) N = 8–9

### Effect of AgNPs exposure on the gene and protein expression of neuroglobin and cytoglobin in the cortex

In the cortex, there were no detectable changes in the gene or protein expression of Ngb, at either AgNP dose (Fig. 3a, b). However, the 30 μg/kg (1.500 ± 0.089) dose induced a significant increase in the gene and protein expression of Cygb (Fig. 3c, d), when compared to controls (1.167 ± 0.066) Fig .4.

## Discussion

Previous studies have demonstrated that AgNP exposure can induce oxidative stress in the brain (Krawczynska et al. 2015; Skalska et al. 2016), since these chemicals can cross the blood brain barrier and accumulate in the brain (Dan et al. 2018; Dziendzikowska et al. 2012). The central nervous system (CNS) is particularly sensitive to oxidative damage, since it has high mitochondrial energetic activity, which is dependent on oxygen, low levels of antioxidant enzymes and high concentrations of free iron and polyunsaturated lipids. Furthermore, the brain is also capable of producing ROS, such: as hydrogen peroxide, superoxide, hydroxyl radicals and nitric oxide (Hu et al. 2013; Ter-Minassian 2006).

In the CNS, globin proteins participate in the transport and facilitate the diffusion of intracellular oxygen, by binding to $O_2$ and delivering it to the mitochondria of metabolically active neurons (Cai et al. 2011; Li et al. 2011; Oleksiewicz et al. 2011). This function, of the globin proteins, is critical for maintaining neural integrity (Brunori and Vallone 2007), and also contributes to ROS detoxification, which can contribute to the onset and progression of neurodegenerative diseases (Ahmed et al. 2008; Fordel et al. 2006).

Perhaps the most important finding was that AgNP exposure, during the prepubertal period, alters the gene and protein expression levels of Ngb and Cygb in adulthood, with different responses detected, depending on AgNP dose and the region of the brain evaluated. Previous studies investigating the influence of AgNPs in the brain also demonstrated this AgNP-mediated differential gene expression, in different regions of the brain (Rahman et al. 2009 and Xu et al. 2015). The study of Rahman, through the use of array techniques, showed that the expression of genes directly related to

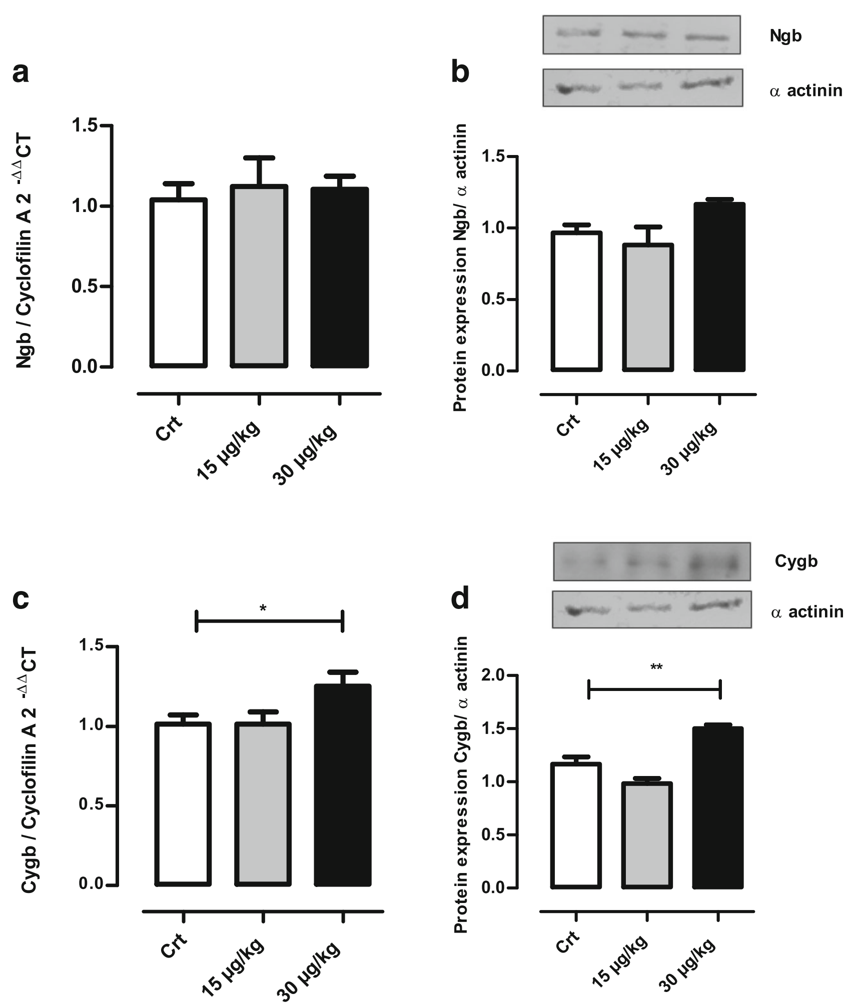


**Fig. 3** Gene and protein expression of neuroglobin (Ngb) and cytoglobin (Cygb) in the cortex: Ngb gene expression (**a**), Ngb protein expression (**b**), Cygb gene expression (**c**), Cygb protein expression (**d**).The control group is represented by white bars, the 15 μg/kg group is represented by gray bars and the 30 μg/kg group is represented by black bars. The significance among groups was determined using a one-way ANOVA test followed by the Newman-Keuls post hoc test (*$P < 0.05$, **$P < 0.01$ ***$P < 0.001$) N = 8–9

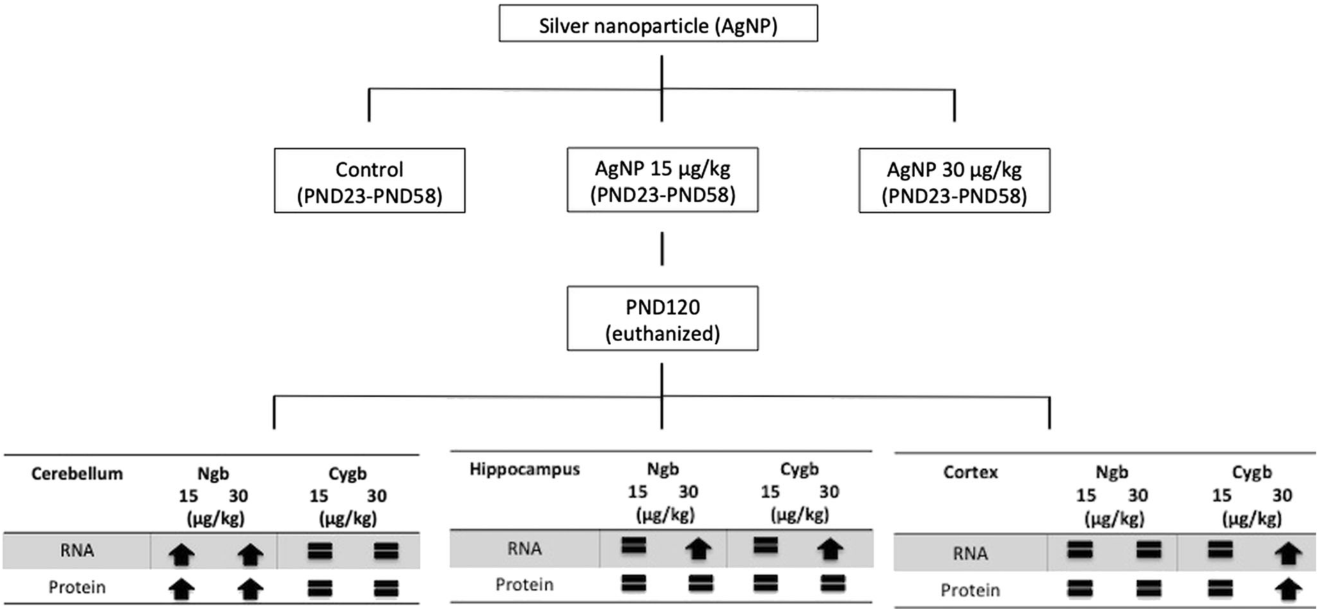


**Fig. 4** Diagram of results

oxidative stress were altered following AgNP exposure, which suggests that AgNPs can promote neurotoxicity and apoptosis (Rahman et al. 2009). In addition, the study of Xu et al. (2015) demonstrated that AgNP exposure can result in the accumulation of these chemicals in the brain, thus promoting neurodegeneration and astrocyte swelling, which are processes related to inflammation (Xu et al. 2015).

With regard to the cortex, the results showed that AgNP exposure induced a significant increase in Cygb gene and protein expression, in the 30 μg/kg group. In this region of the rodent brain, it has been reported that both Ngb and Cygb are well expressed (Hundahl et al. 2010), but not in humans (Hundahl et al. 2013). It should also be pointed out that rats exposed to secondhand cigarette smoke also presented a significant upregulation in Cygb gene and protein expression (Tae et al. 2017). Taken together, there appears to be a potential link between hypoxia (low oxygen) and augmented Cygb expression (Khan et al. 2006). Indeed, Fordel et al. (2004) found that Cygb expression increased in the rat brain, under hypoxic conditions, and Guo et al. (2006) observed that Cygb expression is downregulated in Hif1 alpha knockout mice (Fordel et al. 2004; Guo et al. 2006).

In the cerebellum, there was a significant increase in Ngb gene and protein expression observed with both AgNP doses. In fact, it was previously reported that AgNPs modulate the expression of genes directly involved neurodegeneration, motor neuron disorders and immune function, thus providing further evidence for AgNPs eliciting potentially immunotoxic and neurotoxic effects (Lee et al. 2010). It is also plausible that the upregulation of Ngb functions to promote neuron survival (Burmester et al. 2007).

In hippocampus, there were significant increases in Cygb and Ngb gene expression levels, but there were no observable increases in protein expression. Discrepancies, like these, can often be explained by post transcriptional and/or post translational regulation of protein expression and is indicative of protein degradation (Vogel et al. 2010).

In all of the brain regions studied there was a significant increase in the expression of Ngb and/or Cygb, at level of the gene and in some cases protein, as well. It is likely that this upregulation is directly related increased ROS generation, induced by AgNP exposure. Under such conditions, it is plausible that Ngb and Cygb are involved with supplying oxygen to the respiratory chain, sustaining ATP production and detoxifying ROS and nitric oxide (NO), thus protecting neurons from irreversible damage (Brunori and Vallone 2007; Fordel et al. 2007; Greenberg et al. 2008). Indeed, previous studies have shown that both Ngb and Cygb play a role in neuroprotection (Sun et al. 2001; Sun et al. 2003). ROS formation $H_2O_2$, $OH.^-$, and $O_2$ in different areas of the brain suggests that both areas are highly vulnerable to oxidative stress (Renis et al. 1996), which can result in neuronal cell damage and/or death. Therefore, the observed upregulation, in different regions of the brain, may be associated with AgNP bioaccumulation and/or ROS production.

In Summary, our results demonstrated that AgNP exposure during the prepuberal period upregulated Ngb and Cygb gene and protein expression in adulthood. However, this modulation occurs in an anatomically specific way and was also found to be dose dependent. In addition, based on the brain regions evaluated, the cerebellum and the cortex appear to be the most susceptible areas affected, since these tissues presented increased gene and protein expression of Ngb, whereas the gene expression of both globins was modulated in the hippocampus.

**Acknowledgements** We thank the Coordenação de Aperfeicoamento de Pessoal de Nivel Superior (CAPES) for fincancial support through the grant to MRDS (23038009864/2013-98) and the scholarships to RRC, JSS, and KCO. We would also like to thank the Sao Paulo Research Foundation (FAPESP) for supporting this work through the research grant to MIC (2013/26851-7) and the scholarships to JSS (FAPESP 18952-21-7).

## Compliance with ethical standards

**Conflict of interest** We wish to confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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