AGE (2016) 38: 4
DOI 10.1007/s11357-015-9867-9

Age-related changes in neurochemical components and retinal projections of rat intergeniculate leaflet

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Received: 9 October 2015 / Accepted: 21 December 2015 / Published online: 30 December 2015

Abstract Aging leads to several anatomical and functional deficits in circadian timing system. In previous works, we observed morphological alterations with age in hypothalamic suprachiasmatic nuclei, one central component of this system. However, there are few data regarding aging effects on other central components of this system, such as thalamic intergeniculate leaflet (IGL). In this context, we studied possible age-related alterations in neurochemical components and retinal projections of rat IGL. For this goal, young (3 months), adult (13 months), and aged (23 months) Wistar rats were submitted to an intraocular injection of neural tracer, cholera toxin subunit b (CTb), 5 days before a tissue fixation process by paraformaldehyde perfusion. Optical density measurements and cell count were performed at digital pictures of brain tissue slices processed by immunostaining for glutamic acid decarboxylase (GAD), enkephalin (ENK), neuropeptide Y (NPY) and CTb, characteristic markers of IGL and its retinal terminals. We found a significant age-related loss in NPY immunoreactive neurons, but not in immunoreactivity to GAD and ENK. We also found a decline of retinal projections to IGL with age. We conclude aging impairs both a photic environmental clue afferent to IGL and a neurochemical expression which has an important modulatory circadian function, providing strong anatomical correlates to functional deficits of the aged biological clock.

Keywords Aging · Intergeniculate leaflet · Circadian timing system · Immunohistochemistry · Cholera toxin subunit b · Retinal projections

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Introduction

Aging is a multidimensional process which impairs many aspects of cognitive functions. This process is associated with a fragmentation of sleep-wake cycle (Pace-Schott and Spencer 2011) and increases in frequency of mood disorders (Magee and Carmin 2010; Wolkowitz et al. 2011). These functions are also regulated by the circadian timing system (CTS), an interconnected network of brain nuclei and peripheral tissues responsible for synchronization of anticipatory physiologic responses to environmental changes (see Dibner et al. 2010). In this context, several studies aim to understand the mechanisms underlying aging effects in this circadian clock in order to improve elderly health quality (Kondratova and Kondratov 2012; Farajnia et al. 2014; Fonseca Costa and Ripperger 2015).

Changes in aged circadian clock are described in many levels (see Engelberth et al. 2013). For instance, the aging process is associated with declines in biological rhythm amplitude and phase duration controlled by the clock (Satinoff et al. 1993) as well as fragmentation of locomotor activity rhythm (Valentinuzzi et al. 1997). In addition, aging leads to a reduction in central brain structures activation by light, as revealed by a decrease in expression of immediate early genes, c-Fos and nerve growth factor one A, after photic stimulation (Sutin et al. 1993). In general, an age-related disruption of CTS homeostatic function is linked to progression of neurodegenerative disorders and detrimental effects in sleep and memory (Kondratova and Kondratov 2012).

It should be noted that several age-related alterations of circadian rhythms may be due, or strongly influenced by, aging effects on central brain structures of CTS (Aujard et al. 2006). Among these nuclei of rodent circadian clock are hypothalamic suprachiasmatic nucleus (SCN) and intergeniculate leaflet (IGL) in lateral geniculate complex of thalamus. While SCN is a master pacemaker of circadian rhythms, IGL provides a major regulatory pathway to SCN function (for review see Morin 2013).

Anatomically IGL is a contralateral-predominant retinorecipient area of laminar shape, located between dorsal lateral geniculate (dLGN) and ventral lateral geniculate (vLGN) nuclei (Hickey and Spear 1976). Rat IGL is characterized by GABAergic neurons, revealed by glutamic acid decarboxylase (GAD) immunoreactivity (IR), and colocalized either with enkephalin (ENK) or neuropeptide Y (NPY), composing two neuron subpopulations in this structure (Moore and Card 1994). Interestingly, neural tracer studies using cholera toxin subunit b (CTb) reveal the retinal projections arising to NPY-IR cells are much more modest than those arising to ENK-IR ones, suggesting they have distinct functional roles (Juhl et al. 2007; Blasiak and Lewandowski 2013). Moreover, the main well-known function of IGL is to promote non-photic phase-shifting (e.g. such as behavioral arousal and food intake) due to NPY action in SCN, through geniculo-hypothalamic tract (GHT; Biello et al. 1994; Janik et al. 1995; Harrington 1997; Saderi et al. 2013). Additionally, IGL is thought to have an integrative function of photic and non-photic stimuli, since it also mediates the effects of constant light exposure to circadian system (Harrington 1997; Morin and Pace 2002; Gall et al. 2014).

Regarding morphological and neurochemical findings of aging effects on CTS, some studies report a sex-specific neuron number reduction in SCN of rats (Tsukahara et al. 2005), rhesus monkey (Roberts et al. 2012), and marmosets (Engelberth et al. 2014). Moreover, SCN have a reduced expression of two major neurochemical constituents, vasoactive intestinal polypeptide (Chee et al. 1988; Krajnak et al. 1998), and vasopressin (Roozendaal et al. 1987; Cayetanot et al. 2005), which accounts to a weakening of SCN interconnections, therefore affecting the circadian clock functionality (Engelberth et al. 2013). Current data, however, are focused solely on the aging effects on SCN, which brings a limitation to understand the whole process of aging in CTS (Yamazaki et al. 2002; Davidson et al. 2008). In view of this absence of data concerning effects of aging in IGL, we documented neurochemical and retinal afferent changes in IGL between young, adult, and aged rats based on GAD, ENK, NPY, and CTb immunoreactivity.

Methods

Animals

Male Wistar rats were divided into three groups: young ($n = 5$; 3 months old), adult ($n = 5$; 13 months old), and aged ($n = 5$; 23 months old). They were maintained at 22 °C, 50 % humidity in a 12:12 h light/dark (12:12 LD) cycle. Food and water were available ad libitum. All procedures were in accordance with Brazilian law number 11.794/2008 for animal experimental use. All experiments were approved by local ethics committee for animal use (CEUA-UFRN number 021/2014).

Intraocular tracer injection

Rats were anesthetized with a ketamine hydrochloride (80 mg/kg) and xylazin (20 mg/kg) intramuscular injection. Then, 18 μL of cholera toxin subunit b neural tracer (1 mg/mL; List Biological Laboratories, Campbell, CA) in a solution containing 10 % dimethylsulfoxide was injected into the vitreous chamber of one eye with a glass micropipette attached to a 10-μL Hamilton syringe. After a 5-day post-surgery period, animals were submitted to the following procedures.

Tissue fixation

All animals were submitted to an intracardiac paraformaldehyde perfusion between 5:30 and 6:30 p.m. They were deeply anesthetized with ketamine hydrochloride (80 mg/kg) and xylazin (20 mg/kg). Then, they were transcardially perfused with a 300-mL NaCl solution (0.9 %; 32 °C) followed by 300 mL paraformaldehyde (4 %) in a 0.1 M phosphate-buffered saline (PBS), pH 7.4. Following perfusion, the brains were removed, post-fixed with paraformaldehyde (4 %) overnight, and immersed in a solution containing 30 % sucrose in 0.1 M PBS, pH 7. 4 for 3 days. Brains were cut into six series of coronal sections (30 μm) collected at a 180-μm interval.

Immunohistochemistry

Four series of sections were incubated overnight with GAD, ENK, NPY, or CTb primary antibodies (see Table 1) in a dilution containing 2 % normal donkey serum with PBS (0.1 M) and 0.5 % Triton X-100. After rinsing, sections were incubated with biotinylated secondary antibodies (see Table 1) diluted with PBS (0.1 M) and 0.5 % Triton X-100. Then, sections were incubated in a 2 % avidin-biotin solution (ABC Elite kit, Vector Labs, Burlingame, CA, USA), with NaCl addition, for 120 min. The sections were placed with a 2.5 % solution of diaminobenzidine (DAB) diluted with PBS (0.1 M) for 5 min. The final reaction was performed by adding a 0.01 % H_2O_2 solution for 1 min, to reveal marked areas in brown colors resulting from DAB oxidation. After DAB revelation, sections were washed in PBS (0.1 M) four times and stored overnight at 4 °C. All sections were submitted simultaneously to these procedures in order to minimize background differences and ensure the same conditions to development of chromagen.

Sections were mounted in gelatinized slides, dried, dehydrated in graded ethanol solutions, cleared in xylene, and coverslipped with DPX embedding matrix. Sections in which the primary antibodies were omitted and replaced with normal serum from the same species served as controls. Under these conditions, staining was completely abolished.

Image analysis and statistics

After histochemical procedures, digital pictures of biological tissues were taken by a CCD camera (Nikon DXM-1200) connected to a light microscope (Olympus BX-41). To quantify GAD, ENK, NPY, and CTb immunoreactivities, five sections representing IGL from each brain were analyzed bilaterally using ImageJ 1.48v software, which performed optical density (OD) measurements based on gray levels of pixels. OD has been used in previous reports to quantify similar immunostainings in IGL and SCN (Saderi et al. 2013; Engelberth et al. 2014). For these measurements, the background was subtracted from the positive staining. Regarding NPY-stained sections, a cell count was also performed since well-defined NPY-IR cell bodies are observed in IGL. Quantitative data are expressed as mean ± standard error from the mean (SEM) and analyzed with one-way ANOVA followed by Tukey test for post-hoc comparisons. In all analyses, differences were considered significant at $p < 0.05$. Data analyses were performed using GraphPad Prism version 6.0.

Table 1 Characteristics of used antibodies

	Host and marked target	Source company	Catalog code	Lot number	Working dilution
Primary antibodies	Goat anti-GAD	Santa Cruz Biotechnology, Inc, Dallas, TX, USA	sc-7512	L0707	1:100
	Rabbit anti-ENK	Peninsula Laboratories Inc, San Carlos, CA, USA	T4290	010607-1	1:1000
	Rabbit anti-NPY	Sigma-Aldrich, St Louis, MO, USA	N9528	057K4869	1:1000
	Goat anti-CTb	List Biological Labs, Campbell, CA, USA	703	7032A	1:9000
Secondary antibodies	Donkey anti-goat	Jackson ImmunoResearch Labs, Westgrove, PA, USA	705-065-003	71434	1:1000
	Goat anti-rabbit		111-065-003	74532	1:1000

Results

Age-related alterations in neurochemical content of IGL

Visually, a densely stained plexus of GAD- and ENK-IR were observed, with no differences between age groups (Fig. 1a–f). This was confirmed by one-way ANOVA, which did not reveal a significant effect of age on changes in GAD [$F(2,12)=0.6420$; $p=0.5434$] and ENK [$F(2,12)=1.177$; $p=0.3414$] OD between young, adult, and aged groups (Fig. 1g, h).

We observed a reduction of NPY-IR between the age groups (Fig. 2a). One-way ANOVA showed a significant effect of age on alterations in cell counts [$F(2,12)=20.73$; $p=0.0001$] and OD [$F(2,12)=15.76$; $p=0.0004$] between age groups. The post hoc Tukey honest significant difference (HSD) multiple-comparison test revealed the mean cell count number (92 ± 6) and OD (0.152 ± 0.007) of the young group are higher than those of the adult group (59 ± 5 and 0.112 ± 0.011, respectively). The aged group have a reduced mean cell count number (47 ± 4) and OD (0.071 ± 0.013) when compared to both groups (Fig. 2b).

Age-related alterations in retinal input to IGL

The retinal projections to IGL are reduced with age, in both ipsi- and contralateral sites to the CTb tracer injection (Fig. 3a). One-way ANOVA showed a significant effect of aging on alterations in CTb OD, at the ipsilateral [$F(2,12)=4.315$; $p=0.038$] and contralateral [$F(2,12)=5.011$; $p=0.026$] IGL between the age groups. The post hoc Tukey HSD multiple-comparison test revealed the mean of the young group (ipsilateral, 0.169 ± 0.015; contralateral, 0.228 ± 0.018) and adult group (ipsilateral, 0.148 ± 0.017; contralateral, 0.205 ± 0.019) have no significant differences compared to each other, but they are significantly higher than the mean OD of the aged group (ipsilateral, 0.111 ± 0.006; contralateral, 0.157 ± 0.007; Fig. 3b).

Discussion

A central question regarding the aging process on a circadian system perspective is to understand the mutual antagonism between aging and circadian clock homeostatic function (Kondratova and Kondratov 2012). In the

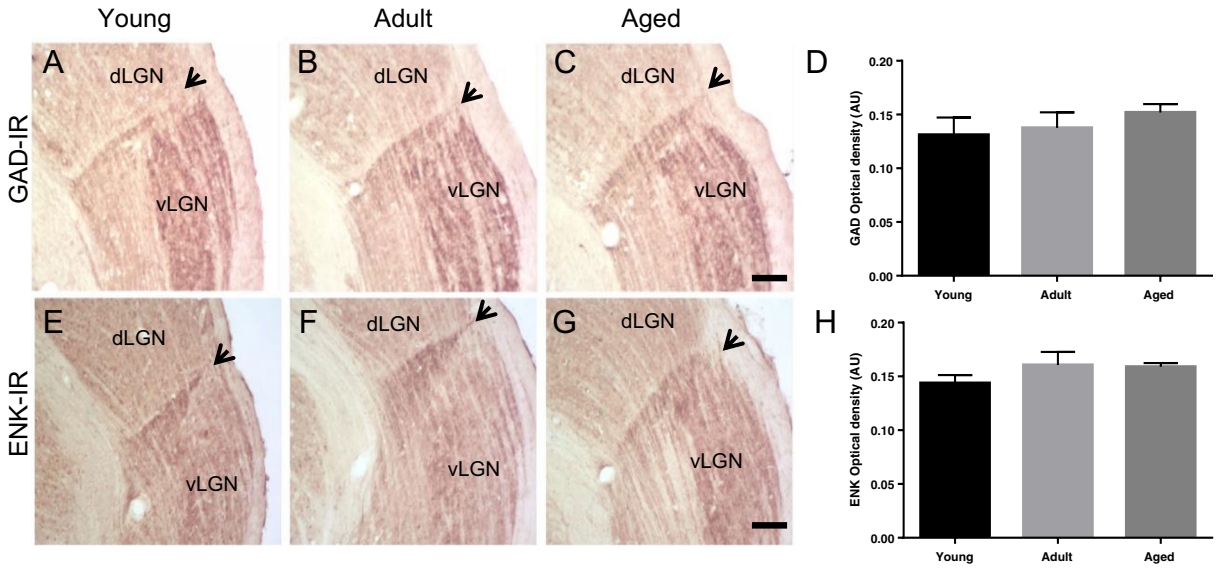

Fig. 1 Digital images of coronal sections, at thalamic level, of glutamic acid decarboxylase (*GAD*) (**a**, **b**, **c**) and enkephalin (*ENK*) (**e**, **f**, **g**) immunoreactivity (*IR*) in young, adult, and aged rats. *Black arrows* indicate intergeniculate leaflet (IGL), *stained area* between dorsal geniculate (*dLGN*) and ventral geniculate (*vLGN*) lateral nuclei. *Scale bar*, 100 μm. GAD-IR optical density (*OD*) measurements in arbitrary units (*A.U.*) of young ($n=5$), adult ($n=5$), and aged ($n=5$) animals (**d**). One-way ANOVA did not reveal a significant effect of age on changes in GAD-IR OD [$F(2,12)=0.6420$; $p=0.5434$] between age groups. ENK-IR OD measurements in A.U. of young ($n=5$), adult ($n=5$), and aged ($n=5$) animals (**h**). One-way ANOVA did not reveal a significant effect of age on changes in ENK-IR OD [$F(2,12)=1.177$; $p=0.3414$] between age groups

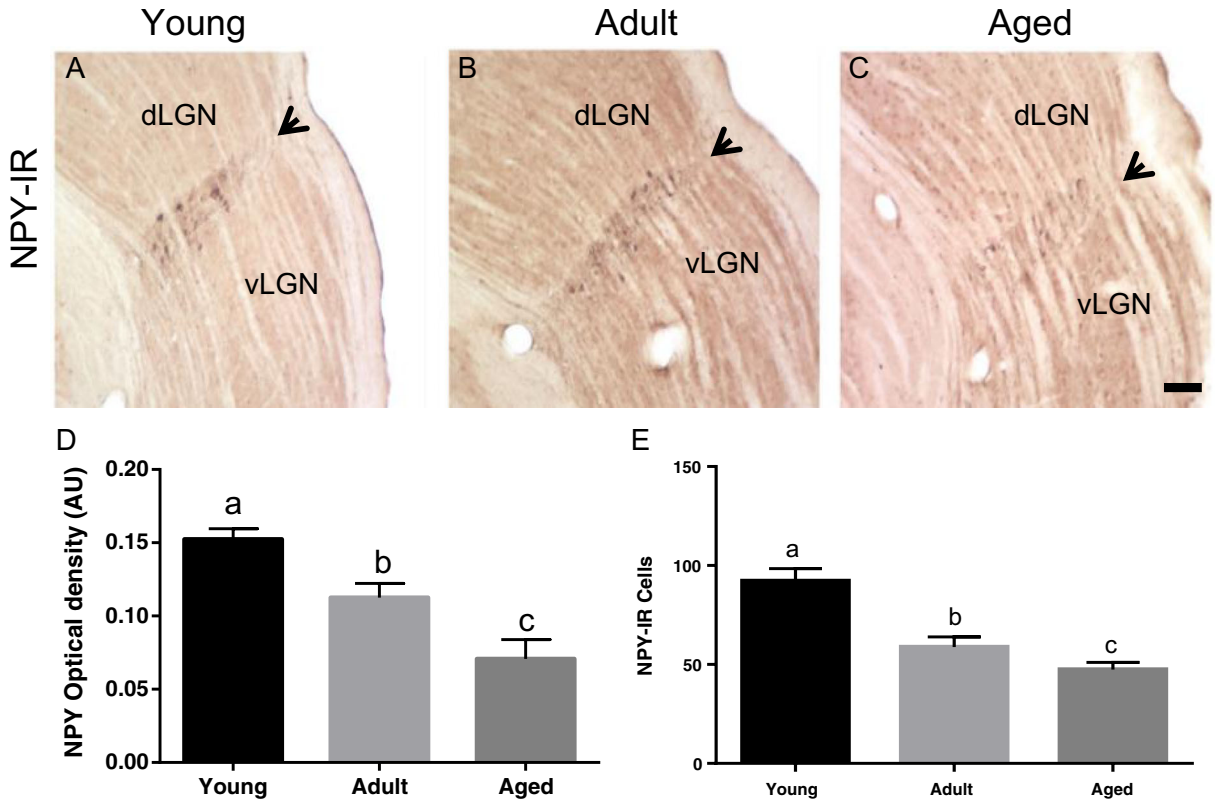

Fig. 2 Digital images of coronal sections, at thalamic level, of neuropeptide Y (*NPY*) immunoreactivity (*IR*) in young (**a**), adult (**b**), and aged (**c**) rats. *Black arrows* indicate intergeniculate leaflet (IGL), *stained area* between dorsal geniculate (*dLGN*) and ventral geniculate (*vLGN*) lateral nuclei. *Scale bar*, 100 μm. NPY-IR optical density (OD) in arbitrary units (A.U.) (**d**) and cell counts (**e**) of young ($n = 5$), adult ($n = 5$), and aged ($n = 5$) animals. One-way ANOVA shows a significant effect of aging in NPY-IR reduction in both adult and aged compared to young ones [counting: $F(2,12) = 20.73$; $p = 0.0001$; OD: $F(2,12) = 15.76$; $p = 0.0004$]

present study, we describe age-related neurochemical alterations in IGL—an important modulator of circadian rhythmicity—providing a new insight for further interventions in CTS to antagonize aging effects.

We found no age-related differences in both GAD and ENK immunoreactivities. Similarly, other brain nuclei, such as the CA3 and dentate gyrus in the hippocampus, have no GAD profile alterations in adult and aged animals (Shi et al. 2004). Also, ENK-IR of several brain areas such as the hypothalamus, frontal cortex, and hippocampus (Kowalski et al. 1992) remain unaltered between age groups. Furthermore, aged hamsters have a similar GAD-IR in SCN when compared to young ones (Duncan and Wheeler 1999), which points that our data is coherent in a CTS organization perspective. Taken together, these data indicate that any deficiency the aged IGL might have in ENKergic and GABAergic physiology would not be at a cellular level, which supports further molecular studies regarding age-related alterations in GABA and ENK receptors and synaptic terminals.

Aging leads to a NPY-IR decline in IGL. This indicates either an age-related loss in NPY cell subpopulation of IGL or a decline in NPY production and expression with age. Accordingly, aging is correlated with decreases in NPY gene expression, receptor subtypes, and cell number in the arcuate nucleus, cerebral cortex, and dentate gyrus (Gruenewald et al. 1994; Cha et al. 1996; Cadacio et al. 2003; Veyrat-Durebex et al. 2013). Considering IGL have two major neurochemical subpopulations, GABA-ENK and GABA-NPY (Moore and Card 1994), the former with no direct circadian function well established so far and the latter projecting to SCN promoting non-photic phase shifting, the observed reduction in NPY-IR suggests aging impairs the non-photic adjustments IGL provides to CTS.

This observation is reinforced by evidence of loss in circadian response to non-photic stimuli (Hughes and

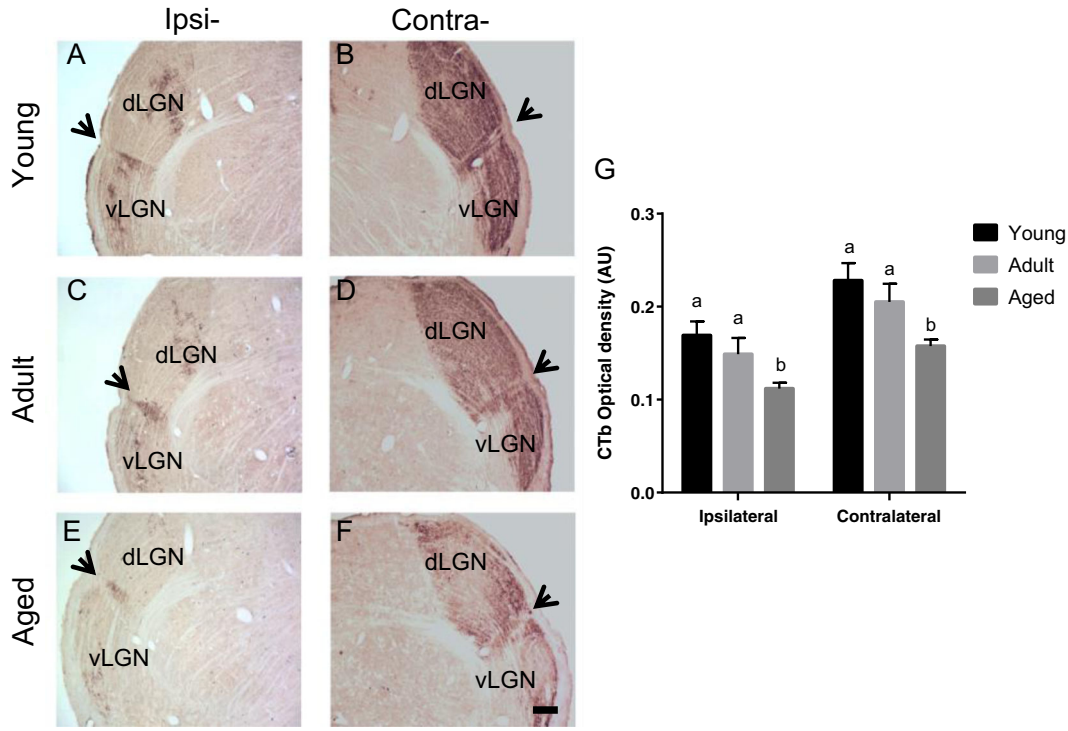

Fig. 3 Digital images of coronal sections, at thalamic level, of cholera toxin b (*CTb*) immunoreactivity (IR) in both ipsi- and contralateral sites to tracer injection in young (**a**, **b**), adult (**c**, **d**), and aged (**e**, **f**) rats. *Black arrows* indicate intergeniculate leaflet (IGL), *stained area* between dorsal geniculate (*dLGN*) and ventral geniculate (*vLGN*) lateral nuclei. *Scale bar* 200 μm. CTb-IR optical density measurements in arbitrary units (A.U.) (**g**). One-way ANOVA shows a significant effect of aging on CTb-IR reduction in aged animals compared to young ones in both ipsi- and contralateral sites [ipsilateral: $F(2,12) = 4.315$; $p = 0.038$; OD: $F(2,12) = 5.011$; $p = 0.026$]

Piggins 2012). For instance, non-photic phase-sifting induced by triazolam (Van reeth et al. 1992; 1993) and novel-wheel confinement (Mrosovsky and Biello 1994) are attenuated in old hamsters. In addition, locomotor rhythm fragmentation is observed in aged human and non-human primates (Czeisler et al. 1992; Zhdanova et al. 2011) and mice (Valentinuzzi et al. 1997; Farajnia et al. 2012). Hughes and Piggins (2012) state these alterations might be due to reduction in neurochemical mediators of circadian locomotor activity, such as NPY. Thus, our results corroborate this hypothesis.

Interestingly, locomotor activity rhythm is not simply an output of circadian function, once it provides a SCN feedback (Hughes and Piggins 2012). Therefore, age-related NPY reduction of IGL would account for locomotor activity fragmentation which jeopardizes the feedback provided to SCN, resulting in a loss of locomotor activity entrainment and establishing a disruptive loop effect (Farajnia et al. 2014). It is also noteworthy that phase advances caused by NPY administration in SCN at the middle of the day are equally observed in young and older mice indicating aged SCN do not lose its responsiveness to NPY (Biello 2009). For this reason, we suggest the reduction of NPY input to SCN, rather than the ability of NPY in activated SCN, is the main cause of age-related behavioral shortfalls influenced by IGL function in CTS.

Through CTb-IR analysis, we found a significant decline in IGL retinal afferents of aged rats. Correspondingly, Lupi et al. (2012) showed a reduction in CTb-IR and Fos expression after photic stimulation in both IGL and SCN of aged mice, indicating an age-related reduction in retinal input and light responsiveness of both nuclei. This impairment likely account for functional deficits of circadian system and may be caused by a deterioration of retinohypothalamic tract (RHT), since it forms both the retina-SCN and retina-IGL connections (Pickard 1985). Although aging diminishes the retinal input to circadian brain structures in mice and rats, Zhang et al. (1998) reported no reduction in retinal projections to SCN of elderly golden hamsters. Collectively, these data point the deleterious effect of

aging in retinal input to CTS occurs in a species-specific manner.

A question formerly addressed by Lupi et al. (2012) concerns the relative importance of retinal degeneration versus age in the circadian system. Comparing wild-type mice with ocular mutants lacking rods and cones, Lupi et al. (2012) showed a higher impact of the intrinsic aging effects, rather than degeneration of photoreceptors, on IGL and SCN activation. However, they did not study this relationship with melanopsin-based photosensitive retinal ganglion cells (pRGCs), which forms RHT (Gooley et al. 2001; Hattar et al. 2002). It should be noted that we used an albino rat animal model, which has an abnormally increased predominance of contralateral retinofugal projections (Fleming et al. 2006). Thus, the reduction in both ipsi- and contralateral projections to IGL indicates aging causes a general loss of pRGCs and therefore RHT. This idea is also supported by previous reports of age-related reduction in retinal ganglion cells in both albino and pigmented rat strains (Weisse 1995).

Moreover, Thankachan and Rusak (2005) characterized three classes of IGL neurons according to its activation by light and NPY presence. Among these classes, type I and type III neurons presented sustained activation by light and absence of NPY synthesis, differing in activation during darkness. Both types are likely involved in mediating IGL photic adjustments to SCN. In contrast, type II neurons presented large NPY amounts and heterogeneous response to illumination and mediate the IGL non-photic function (Thankachan and Rusak 2005). Since we found a decrease in both IGL retinal projections and NPY-IR, we believe these three types of IGL neurons are functionally disrupted within the aging process, type I and type III in its activation by light input and type II in NPY expression, which points the adjustments IGL provides to central clock, by integration of photic and non-photic clues, are hindered with aging. In a hodological perspective, two sources of retinal information to central pacemaker, a direct RHT and an indirect GHT, involved in entrainment to light-dark cycles (Moore and Lenn 1972; Pickard et al. 1987; Morin and Pace 2002) are impaired by the aging process. Based on our results, we summarize these age-related impairments in Fig. 4.

In summary, the present study is the first to demonstrate an age-related loss in NPY-IR and retinal afferents of rat IGL. Thus, IGL integrative function of photic and non-photic clues in circadian system is likely impaired in elderly rats providing strong anatomical correlates to functional deficits of aged circadian clock.

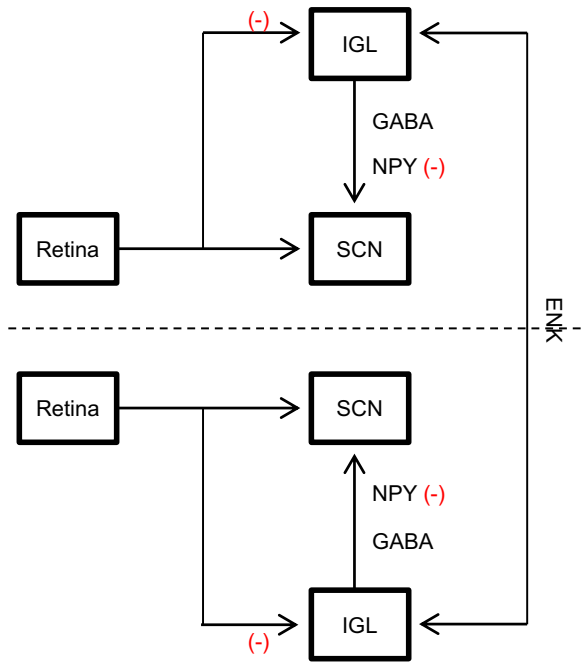

Fig. 4 Summary of age-related alterations in rat intergeniculate leaflet (IGL) connections with retina and suprachiasmatic nucleus (*SCN*). Although no age-related changes were observed in gamma-aminobutyric acid (*GABA*) and enkephalin (*ENK*) neurochemical content, the aging process is correlated with losses of retinal input and neuropeptide Y (NPY) in rat IGL, which are likely involved in functional deficits of the aged circadian timing system. *Minus sign* represents age-related impairments

Acknowledgments The study was supported by Brazilian funding agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Fundação de apoio à pesquisa no RN. The authors also would like to thank Dr. Ruthnaldo R.M. Lima, Dr. J. Ronaldo dos Santos, and Dr. Ramon H. Lima for the valuable comments during the manuscript preparation.

Compliance with ethical standards All procedures were in accordance with Brazilian law number 11.794/2008 for animal experimental use. All experiments were approved by local ethics committee for animal use (CEUA-UFRN number 021/2014).

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