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Age-related loss of orexin/hypocretin neurons

Brice A. Kessler, **Emily M. Stanley**, **Danielle Frederick-Duus**, and **Jim Fadel***
University of South Carolina School of Medicine, Columbia, SC 29208 USA

Brice A. Kessler: brice.kessler@nih.gov; Emily M. Stanley: emily.stanley@uscmed.sc.edu; Danielle Frederick-Duus: dani.frederick-duus@uscmed.sc.edu; Jim Fadel: jim.fadel@uscmed.sc.edu

Abstract

Aging is associated with many physiological alterations—such as changes in sleep patterns, metabolism and food intake—suggestive of hypothalamic dysfunction, but the effects of senescence on specific hypothalamic nuclei and neuronal groups that mediate these alterations is unclear. The lateral hypothalamus and contiguous perifornical area (LH/PFA) contains several populations of neurons, including those that express the neuropeptides orexin (hypocretin) or melanin-concentrating hormone (MCH). Collectively, orexin and MCH neurons influence many integrative homeostatic processes related to wakefulness and energy balance. Here, we determined the effect of aging on numbers of orexin and MCH neurons in young adult (3–4 months) and old (26–28 months) Fisher 344/Brown Norway F1 hybrid rats. Aged rats exhibited a loss of greater than 40% of orexin-immunoreactive neurons in both the medial and lateral (relative to the fornix) sectors of the LH/PFA. MCH-immunoreactive neurons were also lost in aged rats, primarily in the medial LH/PFA. Neuronal loss in this area was not global as no change in cells immunoreactive for the pan-neuronal marker, NeuN, was observed in aged rats. Combined with other reports of altered receptor expression or behavioral responses to exogenously-administered neuropeptide, these data suggest that compromised orexin (and, perhaps, MCH) function is an important mediator of age-related homeostatic disturbances of hypothalamic origin. The orexin system may represent a crucial substrate linking homeostatic and cognitive dysfunction in aging, as well as a novel therapeutic target for pharmacological or genetic restoration approaches to preventing or ameliorating these disturbances.

Keywords

orexin; hypocretin; melanin concentrating hormone; hypothalamus; aging; energy balance

1. Introduction

Aging is associated with a variety of physiological alterations of peripheral or central origin. Some of these, such as changes in sleep patterns, metabolism and food intake are suggestive of hypothalamic dysfunction, but relatively few studies have documented how aging, *per se*, impacts specific hypothalamic nuclei and neuronal groups.

*To whom correspondence should be addressed: Jim R. Fadel, Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, 6439 Garners Ferry Road, Columbia, SC 29208, 803-733-3245 (office), 803-733-1523 (FAX), jim.fadel@uscmed.sc.edu.

Neurons that express the neuropeptides orexin A and B (hypocretin 1 and 2) were discovered in the late 1990's (de Lecea et al., 1998, Sakurai et al., 1998) and are located exclusively in the lateral hypothalamus and contiguous perifornical area (LH/PFA). Although limited in number, these neurons send axonal projections to a wide variety of telencephalic, diencephalic, brain stem and spinal cord regions (Peyron et al., 1998). Orexin neurons appear to play a crucial role in sleep architecture and stabilization of state-dependent behavior (Mochizuki et al., 2004, Zeitzer et al., 2006), and their spontaneous or induced deficiency is associated with both human and animal narcolepsy (Chemelli et al., 1999, Nishino et al., 2000, Thannickal et al., 2000). Orexins also modulate food intake, consistent with their responsiveness to circulating factors indicative of metabolic status, such as glucose and leptin (Sakurai, 1999, Sweet et al., 1999, Burdakov et al., 2005). Collectively, the broad range of functions influenced by the orexin system has led to the description of these neurons as "physiological integrators" (de Lecea et al., 2002). The anatomical substrates underlying the functional diversity of the orexin system may stem, in part, from medial-to-lateral differences across the LH/PFA. Specifically, more medially-located orexin neurons have been implicated in stress and arousal whereas the lateral bank of these cells may play a preferential role in responses to natural or pharmacological rewards (Harris and Aston-Jones, 2006). Previous studies have suggested an age-related decline in orexin function, either at the expression or receptor level (Matsumura et al., 2002, Terao et al., 2002, Porkka-Heiskanen et al., 2004), but there has been no systematic investigation of the effect of age on different sectors of orexin neurons and other neurons found in the same hypothalamic region.

The LH/PFA is a functionally and phenotypically heterogeneous zone. Neurons expressing another neuropeptide, melanin concentrating hormone (MCH), are extensively co-distributed (but not colocalized) with orexins in this area (Broberger et al., 1998, Kilduff and de Lecea, 2001) and continue into more dorsal regions that include specific nuclei such as the zona incerta (Swanson et al., 2005, Hahn, 2010). MCH neurons have been implicated in some of the same homeostatic phenomena, such as energy balance and sleep regulation, as orexins (Peyron et al., 2009, Pissios, 2009), but there has been little investigation into how the MCH system is altered with aging.

Here, we used a natural animal model of aging (old Fisher 344/Brown Norway rats) to investigate the effect of age on numbers of orexin and MCH neurons of the LH/PFA. The results indicate a preferential loss of orexin neurons with age, and suggest a novel target for the development of therapeutic approaches to the treatment of age-related changes in homeostatic function.

2. Experimental procedures

2.1 Animals

Ten young adult (3–4 months) and ten aged (26–28 months) male Fisher 344/Brown Norway F1 hybrid rats (National Institutes of Aging Colony, Baltimore, Maryland, USA) were pair housed on a 12 hr light/dark cycle (lights on at 07:00 hr) and handled daily, with *ad libitum* access to both food and water. This strain of rats was chosen because they have reduced susceptibility to many of the non-neurological (e.g. intraperitoneal tumors) age-related complications seen in other strains, even well into the 3rd year of life (Lipman et al., 1996). All animal care and use procedures were carried out in accordance with protocols written under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

2.2 Immunohistochemistry

Approximately one week after arrival, the animals were anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Immediately after perfusion, brains were removed and post-fixed in 4% paraformaldehyde. After 24 hours, brains were transferred to a 30% sucrose/0.1 M phosphate buffer cryoprotecting solution for several days. Coronal sections (45 μm) were taken from the hypothalamus using a cryostat and collected in five wells, each containing a one-in-five serial collection of tissue (i.e., every serial section was 225 μm apart) representing the rostral-caudal extent of the hypothalamus.

Using procedures similar to those previously described (Frederick-Duus et al., 2007) we performed single labeling of hypothalamic tissue for orexin A, and double-labeling for orexin A plus MCH or orexin A plus the pan-neuronal marker, NeuN. Immunohistochemistry was performed on free-floating sections from the one-in-five series of hypothalamic tissue. All sections were incubated with a rabbit anti-OxA antibody (1:2000; 48 hours at 4° C; Calbiochem; La Jolla, CA) followed by a biotinylated donkey anti-rabbit IgG secondary antibody (1:1000; 1.5 hours at room temperature; Jackson Immunoresearch Laboratories, Inc.; West Grove, PA) and horseradish peroxidase-conjugated streptavidin (1:1600; 1 hour; Jackson Immunoresearch Laboratories, Inc.]. Orexin A-immunoreactive (OxA-IR) neurons were visualized by developing sections with hydrogen peroxide in a nickel-cobalt-enhanced diaminobenzidine solution, yielding a blue-black reaction product in OxA-IR neurons.

For double label immunohistochemistry, sections were processed for orexin labeling as described above, then incubated in either chicken anti-MCH (1:3000; BMA; Augst, Switzerland) or mouse anti-NeuN (1:1000; Millipore; Billerica, MA) for 48 hours at 4°C, followed by unlabeled donkey anti-chicken or unlabeled donkey anti-mouse secondary antibodies (1:200, 2 hours at room temperature; Jackson Immunoresearch Inc.) and chicken or mouse PAP (1:500; 1.5 hours at room temperature; Covance; Berkeley, CA). MCH- or NeuN-immunoreactive (MCH-IR; NeuN-IR) neurons were visualized by developing the sections with hydrogen peroxide in plain (i.e. without nickel-cobalt) diaminobenzidine solution to yield a brown immunopositive reaction product. Sections were mounted on slides with gelatin, dehydrated, delipidated and coverslipped prior to microscopic analysis. All immunohistochemical staining procedures included sets of tissue from both young and aged animals run in parallel.

2.3 Cell counts

Cell counts were performed using Stereo Investigator Software (version 7.00.3; Microbrightfield Inc., Williston, VT) and a Nikon Eclipse 80i microscope equipped with a CX9000 camera. Serial sections were organized, and four areas were isolated as closed contours in the lateral hypothalamus/perifornical area of individual sections. These areas included 1) left hemisphere, lateral to the fornix, 2) left hemisphere, medial to the fornix, 3) right hemisphere, medial to the fornix, and 4) right hemisphere, lateral to the fornix (Figure 1). The optical fractionator probe was used to move automatically between counting frames in each area. The counting frame was 275 μm^2. Sections were cut at 45 μm on the cryostat, but were typically 28 μm after processing for immunohistochemistry, mounting, dehydration and coverslipping. The top of the cell below the surface of the tissue was used as the counting landmark. Cells that fell outside the counting frame were not counted. If a cell was more than 50% outside the boundary, it was not considered inside the counting frame. To determine the total number of positive cells within the four areas dividing the LH/PFA of the animals, all segments were summed and multiplied by five. Counts were performed in a rostral to caudal direction, beginning with the first appearance of an immunoreactive cell.

2.4 Statistical analysis

All cell counts were analyzed using an independent samples t-test to determine significant ($p < 0.05$) main effects of age. Statistical analyses were performed using SPSS for Windows (v. 17.0, SPSS Inc; Chicago, IL).

3. Results

3.1 Descriptive aspects of orexin and MCH labeling

The distribution and morphology of OxA- and MCH-IR neurons were consistent with previous reports (Broberger et al., 1998). In short, blue-black cytoplasmic staining was observed in OxA-IR cells in both medial and lateral subpopulations of the two age groups. Orexin immunoreactivity was limited to a band of neurons extending from the lateral hypothalamus proper (adjacent to the ventromedial extent of the internal capsule), arching over the fornix into the dorsomedial hypothalamus (Figure 2). Subpopulations of orexin neurons were identified by a sagittal line through the fornix to delineate medial and lateral populations (Figure 1). Substantial co-distribution (but not co-localization) of MCH-IR neurons with OxA-IR neurons was observed within the hypothalamus (Figure 3), but the MCH population tended to extend further in the dorsal dimension. No orexin or MCH cell body labeling was observed in any other cortical, limbic or diencephalic structures contained in the sections of interest.

3.2 Aging results in a loss of orexin neurons

Dense perifornical populations of OxA-IR neurons were found in the young animals, while aged animals displayed comparatively sparse OxA-IR neuron distribution (Figure 2). Significant reductions in the total number of OxA-IR cells were observed in all areas counted (Figure 2C). The total number of OxA-IR cells throughout the lateral hypothalamus were reduced in aged animals (2323 ± 271) compared to young animals (3998 ± 459; $t_{18} = 3.144$; $p = 0.006$). There was significant loss of cells medial to the fornix in aged animals (928 ± 164) compared to young animals (1635 ± 247; $t_{18} = 2.385$; $p = 0.028$), as well as significant loss of cells lateral to the fornix in aged animals (1407 ± 146) compared to young animals (2339 ± 226; $t_{18} = 3.462$; $p = 0.003$).

3.3 Aging results in a region-selective loss of MCH neurons

Immunohistochemistry revealed MCH neuronal staining and distribution consistent with previous studies, with light brown cytoplasmic staining of MCH-IR cells confined bilaterally to the LH/PFA (Fadel et al., 2002, Frederick-Duus et al., 2007). A second, dark blue-black label for OxA cells was used to identify codistribution of MCH and OxA cells. As with orexin neurons, MCH-IR subpopulations were identified using the fornix to delineate medial and lateral sectors. Aged animals displayed fewer MCH-IR cells throughout the lateral hypothalamic zone (Figure 3). The total number of MCH-IR cells decreased significantly in aged animals (5668 ± 289) compared to young animals (8025 ± 933; $t_{18} = 2.414$; $p = 0.027$).

Additionally, there was significant loss ($P<0.05$) of cells medial to the fornix in aged animals (2034 ± 238) compared to young animals (3289 ± 485; $t_{18} = 2.326$; $p = 0.032$). There was a strong trend toward a reduction of MCH-IR cells lateral to the fornix in aged animals (3635 ± 486) compared to young animals (4736 ± 502; $t_{18} = 2.097$; $p = 0.051$).

3.4 Age-related loss of lateral hypothalamic neurons is not global

NeuN immunohistochemistry revealed dense light brown cytoplasmic staining within the LH/PFA. Double-labeling for orexin (blue-black) resulted in excellent contrast that allowed

NeuN-IR cells to be counted within the area defined by orexin immunoreactivity (Figure 4). There were no significant differences in the numbers of NeuN-IR cells between young and aged animals (Figure 4), with the total number of NeuN-IR in aged animals (241380 ± 77984) virtually the same as in young animals (245250 ± 11781; $t_{18} = 0.274$; $p = 0.787$). Additionally, there were not any significant age-related differences in NeuN-IR cells in either the medial or lateral sectors of the LH/PFA.

Although the blue-black immunoprecipitate in OxA-IR neurons made concomitant visualization of NeuN-IR difficult on a single-cell level, it was assumed for counting purposes that all OxA-IR neurons were also NeuN-positive. Hypothalamic NeuN-IR subpopulations were identified using the fornix to delineate medial and lateral cells.

4. Discussion

The present study used anatomical approaches in young adult and aged rats to demonstrate an age-related loss of hypothalamic orexin neurons. The extent of orexin cell loss exceeded 40%, and was observed in both medial and lateral sectors of the LH/PFA. Assessment of non-orexin neurons indicated that at least one other cell population in this area, those expressing melanin-concentrating hormone, also was reduced in aging. The extent of this reduction (<30%) was less robust than that seen with orexin, and was significant in medial and total sectors of the LH/PFA, but not the lateral sector. Finally, cell loss in the LH/PFA with aging was not global, as staining with the pan-neuronal marker, NeuN, revealed virtually identical numbers of neurons in young and aged rats.

Previous studies have estimated the total number of orexin neurons in the rat brain to be as few as 1128 (Peyron et al., 1998) or as many as 3445 (Harrison et al., 1999). However, the authors of the lower figure subsequently recalculated the total number of these cells to be 4092 (Kilduff and Peyron, 2000), well within the margin of error of our estimate of 3998 ± 485 orexin neurons in young adult rats. Combined with the classic distribution and morphology of orexin neurons that we observed, these numbers bolster the reliability of our cell counting methods and the validity of the age-related loss that we report. While our estimate of the total number of MCH neurons in young rats (about 8000) was somewhat lower than prior reports (Bittencourt et al., 1992, Hanriot et al., 2007), this may be due to differences in rat strain or primary antibody source.

These findings complement previous reports of age-related alterations in the orexin system in rats and other species, including decreased orexin innervations of target regions (Zhang et al., 2002, Downs et al., 2007), decreased expression of the peptide or its receptors (Terao et al., 2002, Porkka-Heiskanen et al., 2004), diminished behavioral responses to centrally-administered orexin (Takano et al., 2004, Kotz et al., 2005) and altered patterns of orexin release across the diurnal cycle (Desarnaud et al., 2004). In addition, a recent report has documented an age-related decline in orexin neuron number in rats (Sawai et al., 2010), but did not extend this finding to other cell types in this area or to functionally heterogeneous (medial vs. lateral) sectors of the LH/PFA. While the literature on age-related changes in the MCH system is sparse, our data are consistent with at least one report of decreased MCH gene expression in hypothalamic tissue obtained from 24 month old rats (Kappeler et al., 2003).

Our NeuN data indicate that age-related cell loss is not a global hypothalamic phenomenon. However, declines in neuronal numbers have been reported for other non-hypothalamic neuromodulatory systems involved in arousal or energy balance, although the consistency and degree of such decline suggests that the orexin system is more heavily impacted in aging. Some have reported loss of brainstem dopamine and noradrenergic cells, for example,

on the order of 15–30% (Sturrock and Rao, 1985, Sanchez et al., 2008) while others have shown no age-related decline (Goldman and Coleman, 1981, Cruz-Muros et al., 2007). Although studies on age-related loss of other neuronal populations specifically involved in feeding and energy balance are sparse, the available data suggest that the magnitude of orexin degeneration suggests a prominent role for this system in age-related alterations in homeostatic functions.

We have characterized our findings as indicative of neuronal loss in the hypothalamus. An alternative possibility, however, is that orexin (or MCH) neurons are preserved in aging but undergo a “phenotypic silencing” such that these neurons remain intact, but no longer express detectable levels of neuropeptides. Supportive of this notion, we did not observe any quantitative change in NeuN-IR cells in the LH/PFA of aged animals. If, indeed, the cells that normally express orexin are preserved in aging, it raises the possibility of “phenotypic rescue” by up-regulating or reinstating orexin expression using genetic approaches such as virus-mediated gene transfer. Our preliminary work in this area supports the feasibility of such an approach. Regardless of how the cell loss vs. phenotypic silencing debate is resolved, the present data are clearly corroborative of studies indicating age-related dysfunction of the orexin system, and further point to this system as a potential therapeutic target for the treatment of age-related alterations in homeostasis.

If orexin neurons are lost in aging, it is not clear what pathophysiological mechanisms underlie this phenomenon. One possibility is glutamate-mediated excitotoxicity secondary to undefined alterations in cellular energy metabolism or neurotrophic support, which are widely-speculated to contribute to age- or disease-related neurodegenerative processes in general (Beal, 1992, Mattson, 2008). Glutamatergic inputs modulate the activity of orexin neurons, which have a comparatively depolarized resting membrane potential, making them spontaneously active (Li et al., 2002, Eggermann et al., 2003). Indeed, orexin neurons appear to be particularly sensitive to NMDA-elicited neurotoxicity, at least in vitro (Katsuki and Akaike, 2004). It is important to note, however, that this enhanced susceptibility may depend in part on the specific excitotoxin employed, as MCH neurons appear to be more greatly affected by quinolinate (Obukuro et al., 2010). In either case, both orexin and MCH cell populations may be subject to excitotoxicity-mediated neurodegeneration in aging.

Another possible contributor to senescence-related orexin cell loss or phenotypic silencing is dysregulation of peripheral cues that normally govern orexin neuron activity. Orexin neurons are responsive to several circulating signals indicative of energy balance, including leptin and glucose. In general, elevated levels of these factors are indicative of positive energy balance and result in diminished orexin neuron activity (Cai et al., 1999, Hakansson et al., 1999, Burdakov et al., 2006). Accordingly, at least in vitro, inhibition of neural activity decreases the number of orexin-immunoreactive neurons, albeit without apparent cell death (Michinaga et al., 2010). Thus, alterations in body composition and glucose homeostasis could contribute to age-related reductions in orexin activity, expression or cell number.

The apparent cell loss that we observed was of similar magnitude across the entire (potentially functionally-heterogeneous) medial-lateral gradient of orexin cells, suggesting that age-related deficits may be manifest across the whole spectrum of physiological phenomena in which this neuropeptide system has been implicated, including wakefulness/arousal, feeding/energy balance, and cognition. Sleep disturbances such as excessive daytime sleepiness and sleep fragmentation are more common in the elderly (Wolkove et al., 2007). While age-related alterations in sleep architecture do not typically rise to the level of narcolepsy, they are at least reminiscent of some of the abnormalities of this disorder, which is clearly associated with orexin cell loss (Nishino et al., 2000, Thannickal et al., 2000). Old

animals do not invariably become anorexic under standard lab conditions, but they frequently exhibit diminished responses to homeostatic challenge. Senescent terminal weight loss has been reported in F344 rats (Black et al., 2003), and even prior to near-terminal age, older rats fail to appropriately increase food intake following food deprivation (Wolden-Hanson, 2006).

Functional diversity within the MCH neuronal system has not been characterized to the same extent as the orexin system. However, detailed mapping studies using in situ hybridization (Swanson et al., 2005) or immunohistochemistry (Hahn, 2010) have mapped out the localization of orexin and MCH neurons in a manner beyond the more simple medial-lateral variation. Importantly, these maps allow for further specificity in delineating functionally-heterogeneous subpopulations of both of these cell groups. According to this parcellation scheme, the “medial” sector of MCH neurons that appeared to be most prominently impacted in aging primarily included cells found in the posterior hypothalamic nucleus, the dorsomedial/juxtadorsomedial region, and the medial portion of the zona incerta. A series of elegant tract-tracing studies by Bittencourt and colleagues revealed MCH projections arising from the medial portions of the zona incerta project to areas such as the medial septum, dorsal periaqueductal grey and medial mammillary nucleus, suggesting an integrative role for these neurons in arousal related to energy balance or pain processing (Elias and Bittencourt, 1997, Bittencourt and Elias, 1998, Casatti et al., 2002).

The LH/PFA in general, and the orexin system in particular, may contribute to a mechanistic link between homeostatic function and cognition. In addition to the aforementioned role of orexins in physiologic regulation, there is increasing evidence that orexins modulate cognitive function. Anatomically, orexins activate regions such as the prefrontal cortex and basal forebrain cholinergic system that are strongly implicated both in normal cognitive function and in age-related cognitive decline (Espana et al., 2001, Fadel et al., 2005, Lambe et al., 2005, Fadel and Frederick-Duus, 2008, Arrigoni et al., 2010). Furthermore, administration of an orexin-1 receptor antagonist into the basal forebrain impairs performance in an attentional task in rats (Boschen et al., 2009, Fadel and Burk, 2010). Finally, in humans, attentional deficits have been reported in (presumably orexin deficient) narcoleptic patients even during periods of normal wakefulness (Rieger et al., 2003, Naumann et al., 2006). While remaining subject to verification at multiple levels, these findings collectively support the hypothesis that orexin dysfunction contributes to age-related changes in both homeostatic and cognitive function.

In conclusion, we have shown an age-related loss of orexin and MCH neurons in the rat in the absence of global loss of neurons in the lateral hypothalamus. The role of these neurons in integrating physiological and cognitive function suggests that their deficiency is pathologically relevant to age-related deficits in these domains.

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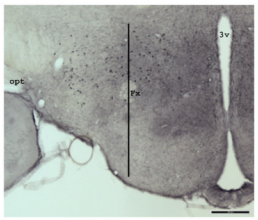

Figure 1.

Low power photomicrograph showing one hemisphere of the rat hypothalamus with characteristic orexin neuron distribution within the LH/PFA. The black line demarcates areas medial and lateral to the fornix (Fx). *3v*, third ventricle; *opt*, optic tract. Scale bar = approximately 1,000 μm.

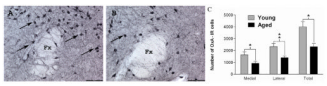

Figure 2.

Age-related reduction in orexin neurons. **A.** Representative labeling of OxA-IR neurons (black cell bodies indicated by arrows) in the LH/PFA of a young rat. **B.** Representative labeling of OxA-IR cells in the LH/PFA of an aged rat. Note the sparser distribution of orexin neurons in both lateral and medial to the fornix (Fx). **C.** Aging was associated with a significant reduction of OxA-IR neurons in both medial and lateral sectors of the LH/PFA, as well as overall. *P < 0.05; **P < 0.01. Scale bars = approximately 100 μm for both A and B.

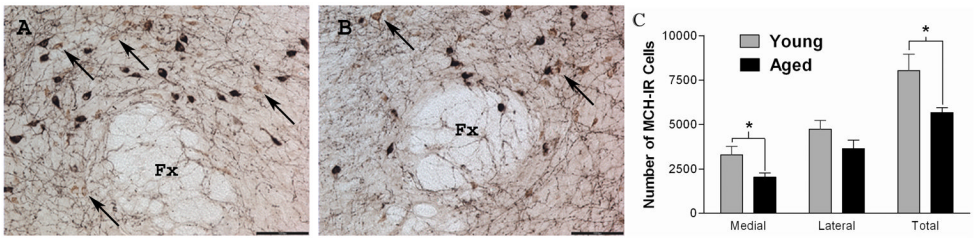

Figure 3.

Age-related reduction in MCH neurons. **A.** Representative labeling of MCH-IR neurons (brown cell bodies indicated by arrows) in the LH/PFA of a young rat double-labeled for orexin (black cell bodies), showing co-distribution (but not colocalization) of these cell populations. **B.** Representative labeling of MCH-IR cells in the LH/PFA of an aged rat. MCH-IR was sparser, particularly in the medial (left of the fornix) portion of the LH/PFA. **C.** Aging was associated with a significant reduction of MCHA-IR neurons in the medial sector of the LH/PFA, as well as overall, but the lateral sector appeared to be relatively spared. *$P < 0.05$. Scale bars = approximately 100 μm for both A and B.

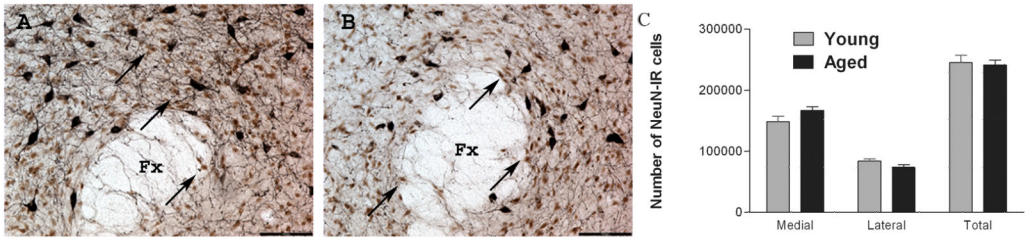

Figure 4.

Total LH/PFA neuronal number in aged rats. **A.** Representative labeling of cells using the pan-neuronal marker, NeuN (brown cell bodies indicated by arrows) in the LH/PFA of a young rat double-labeled for orexin (black cell bodies). **B.** Representative labeling of NeuN-IR cells in the LH/PFA of an aged rat. **C.** Aging was not associated with any change in the number of NeuN-IR cells in the LH/PFA. Scale bars = approximately 100 μm for both A and B.