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Age-related changes in rostral basal forebrain cholinergic and GABAergic projection neurons: Relationship with spatial impairment

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Abstract

Both cholinergic and GABAergic projections from the rostral basal forebrain have been implicated in hippocampal function and mnemonic abilities. While dysfunction of cholinergic neurons has been heavily implicated in age-related memory decline, significantly less is known regarding how age-related changes in co-distributed GABAergic projection neurons contribute to a decline in hippocampal-dependent spatial learning. In the current study, confocal stereology was used to quantify cholinergic (choline acetyltransferase (ChAT) immunopositive) neurons, GABAergic projection (glutamic decarboxylase 67 (GAD67) immunopositive) neurons, and total (NeuN immunopositive) neurons in the rostral basal forebrain of young and aged rats that were first characterized on a spatial learning task. ChAT immunopositive neurons were significantly but modestly reduced in aged rats. Although ChAT immunopositive neuron number was strongly correlated with spatial learning abilities among young rats, the reduction of ChAT immunopositive neurons was not associated with impaired spatial learning in aged rats. In contrast, the number of GAD67 immunopositive neurons was robustly and selectively elevated in aged rats that exhibited impaired spatial learning. Interestingly, the total number of rostral basal forebrain neurons was comparable in young and aged rats, regardless of their cognitive status. These data demonstrate differential effects of age on phenotypically distinct rostral basal forebrain projection neurons, and implicate dysregulated cholinergic and GABAergic septohippocampal circuitry in age-related mnemonic decline.

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Keywords

Watermaze; stereology; acetylcholine; GABA; inhibitory; spatial learning; hippocampus; NeuN; neuron number; memory; aging

1. Introduction

Explicit and spatial memory in humans is dependent upon the hippocampus and medial temporal lobe system, the function of which can decline precipitously with advanced age (Burke and Barnes, 2006; Della-Maggiore, et al., 2002; Squire, 2004; Wilson, et al., 2004). While aged individuals can exhibit learning and memory dysfunction similar to individuals with direct hippocampal damage (Gallagher and Rapp, 1997), neuron number in medial temporal lobe structures is stable across species during normal aging, even in subjects exhibiting profound mnemonic impairments (Calhoun, et al., 1998; Rapp, et al., 2002; Rapp and Gallagher, 1996; Rasmussen, et al., 1996; Shamy, et al., 2006; West, et al., 2004). Despite the absence of frank hippocampal neuron loss, there is clear evidence that aging can negatively impact the processing of hippocampal-dependent spatial information (e.g., Barnes, et al., 1997; Shen, et al., 1997; Tanila, et al., 1997) and that altered integrity of both cholinergic and GABAergic basal forebrain afferents to hippocampus may contribute to this functional decline (Gage, et al., 1984; Gallagher and Nicolle, 1993; Smith and Pang, 2005; Ypsilanti, et al., 2008).

Given their vulnerability to degeneration in Alzheimer's disease (AD), basal forebrain cholinergic neurons have been studied extensively within the context of aging. Some studies have reported that cholinergic basal forebrain neurons decline with age, supporting the notion that their degeneration mediates age-related spatial learning impairments (Altavista, et al., 1990; Armstrong, et al., 1993; Bartus, et al., 1982; de Lacalle, et al., 1996; Durkin, 1992; Fadda, et al., 2000; Fischer, et al., 1992; Fischer, 1989; Fragkouli, et al., 2005; Gustilo, et al., 1999). Notably, however, others have found no relationship between decline in cholinergic cell number and loss of cognitive abilities, and several recent studies have reported that cholinergic neuron number remains relatively stable at advanced ages (Lee, et al., 1994; McQuail, et al., 2011b; Ypsilanti, et al., 2008). The latter findings are consistent with those from studies showing that hippocampal-dependent spatial memory is largely spared following selective neurotoxic ablation of cholinergic neurons in rodents (Baxter, et al., 1995).

It is becoming increasingly clear that corticopetal basal forebrain GABAergic neurons influence hippocampal physiology and hippocampal-supported cognition (Freund and Antal, 1988; Kiss, et al., 1990a; Pang, et al., 2001) and that the combined influence of cholinergic and GABAergic afferents is important for optimal hippocampal function. For example, septohippocampal connectivity is critical for the generation of hippocampal theta rhythms, 3-12 Hz oscillations which are strongly implicated in successful spatial cognition, memory processes and sensorimotor integration (Bland and Colom, 1993; Bland and Oddie, 2001; Buzsaki, 2002; Colom, 2006; Rawlins, et al., 1979; Winson, 1978). Lesion studies have shown that both cholinergic and GABAergic afferents from rostral basal forebrain neurons are critically important for generating these oscillations (Yoder and Pang, 2005). In addition, pronounced spatial learning impairments are produced by disruption of both cholinergic and GABAergic input to the hippocampus but not by disruption of either projection system in isolation (Baxter, et al., 1995; Becker, 1980; Everitt and Robbins, 1997; McDonald, 1994; Pang, et al., 2001; Parent and Baxter, 2004).

Alterations in inhibitory circuitry occur in a variety of neurodegenerative and neuropsychiatric diseases such as epilepsy, depression, schizophrenia, and autism, many of which are associated with abnormal cognitive function (Briggs and Galanopoulou, 2011; Gonzalez-Burgos, et al., 2011; Lewis, et al., 2005; Pizzarelli and Cherubini, 2011). Indeed, GABA-mediated transmission appears crucial for processing information both within and between brain regions essential for mediating a variety of neurocognitive processes (Bartos, et al., 2007; Volk and Lewis, 2002). While inhibitory circuitry is increasingly the focus of mechanistic studies associated with neurodegenerative diseases, considerably less attention has been paid to the anatomical integrity of GABAergic inhibitory circuits in normal aging (McKinney, 2005).

Nevertheless, there is emerging evidence that GABAergic indices change in normal aging. For example, interneurons in both prefrontal cortex and hippocampus of aged rats degenerate or cease to express the GABA-synthesizing enzyme, GAD67 (Shetty and Turner, 1998; Stanley and Shetty, 2004; Stranahan, et al., 2011). Moreover, normal aging produces regionally specific changes in GABA(B) receptor expression and function (McQuail, et al., 2011a) and attenuated GABA(A) receptor activity and expression (Yu, et al., 2006). Evoked GABA release is also reportedly decreased in the CA1 subregion of the aged rat hippocampus (Stanley, et al., 2012). Finally, drugs targeting GABAergic signaling can improve cognitive functioning in both young and aged rats (Getova and Bowery, 2001; Getova and Bowery, 1998; Helm, et al., 2005; LaSarge, et al., 2009; Mondadori, et al., 1996a; Mondadori, et al., 1996b).

This study was designed to determine if changes in the integrity of hippocampal-targeting basal forebrain cholinergic and GABAergic neurons are associated with loss of spatial learning abilities in aging. Confocal and stereological methods were employed to determine cholinergic (ChAT immunopositive), GABAergic projection (GAD67 immunopositive) and total (NeuN immunopositive) neuron numbers in rostral basal forebrain of young and aged rats which were first characterized on a spatial learning task. The findings indicate that normal biological aging differentially impacts cholinergic and GABAergic projection neurons in rostral basal forebrain and suggest that alterations in inhibitory networks may be important contributors to age-related mnemonic dysfunction.

2. Methods

2.1. Subjects

Young adult (6 months; n=8) and aged (24 months; n=16) male F344 rats were obtained from the National Institute on Aging colony and housed in the vivarium in the Psychology Building at Texas A&M University for two weeks prior to the start of behavioral testing. This AALAC-accredited vivarium was maintained at a consistent 25 °C with a 12:12 hour light/dark cycle (lights on at 0800 hours). Rats had free access to food and water at all times. All rats in the study were screened for health problems including, but not limited to, cataracts, jaundice, food and water intake, and tumors. Sentinel rats, housed alongside the rats in this study, routinely tested negative for a range of pathogens. All animal procedures were conducted in accordance with approved institutional animal care procedures and National Institutes of Health guidelines.

2.2. Spatial learning assessment

Apparatus—Spatial learning abilities were assessed using the Morris water maze task as described previously (Bizon, et al., 2009; LaSarge, 2007). The water maze apparatus consisted of a white circular tank (183 cm in diameter with a wall height of 58 cm) filled with water (27°C) made opaque with the addition of non-toxic white tempera paint. A

retractable white escape platform (12 cm diameter, HVS Image, UK) was submerged 2 cm below the water's surface near the center of the southwest quadrant of the maze. Black curtains, to which white geometric shapes (extramaze cues) were affixed large, surrounded the maze. Data were acquired via a video camera mounted above the maze which was connected to a DVD recorder and computer with a video tracking system and Water 2020 software (HVS Image, Buckingham, UK).

Spatial reference memory (hidden platform) task—Rats' spatial learning abilities were tested according to methods developed by Gallagher and colleagues (Gallagher et al., 1993), with specific modifications for training F344 rats (Bizon, et al., 2009; LaSarge, 2007). Briefly, rats received three training trials/day with a 30s inter-trial interval, over eight consecutive days. On each trial, rats were placed into the water facing the wall of the maze at one of four equally spaced start positions (north, south, east, or west). The start positions were varied in a pseudo-random fashion, such that all rats started from each of the locations approximately the same number of times. Rats were allowed to search until they found the hidden platform or until 90 s elapsed, at which time rats were guided to the escape platform by the experimenter. Rats remained on the platform for 30 s and then were placed in a holding chamber for a 30 s inter-trial interval. Every sixth trial was a probe trial in which the platform was lowered to the bottom of the maze for the first 30 s of the trial, after which it was raised to allow the rats to escape.

Cued (visible platform) task—Following spatial reference memory training, rats were given a single session with six trials of cue training to assess sensorimotor abilities and motivation to escape. For cue training, rats were trained to escape to a visible platform (painted black and protruding 2 cm above the water's surface). Both the start position and platform location were varied on each trial, making the extra-maze cues explicitly irrelevant to the platform location. On each trial, rats were allowed to search for the platform for 90 s and then were allowed to remain there for 30 s before a 30 s inter-trial interval.

Behavioral and statistical analyses—Data files were created by the Water 2020 software and exported to SPSS (v. 16.0; Cary, NC) for analysis. Accuracy of performance on training and probe trials was assessed using a search error measure originally described by Gallagher et al. (1993). To calculate search error, the rat's distance from the platform location was sampled 10 times/s and these distances were averaged into 1 s bins. For training trials, cumulative search error was derived by summing these 1 s averages and then subtracting the optimal path between the start location and the platform location. For probe trials, a mean search error measure was derived by dividing cumulative search error by the 30 s duration of the probe trials. Training trial data were averaged into four blocks consisting of the five trials preceding each probe trial. Comparisons between groups on training trials were conducted using two-factor repeated measures ANOVA (age X training block) with Tukey's post hoc tests performed when warranted. In all statistical comparisons, p values ≤ 0.05 were considered significant.

To provide an overall measure of spatial learning ability for each rat, a "spatial learning index" (SLI) was calculated using mean search error from the interpolated probe trials as described previously (Bizon, et al., 2009; Gallagher, et al., 1993). Mean search error on probe trials was weighted and summed to provide the spatial learning index (Bizon, et al., 2009). For some comparisons of cell number, aged rats were subgrouped on the basis of their spatial learning index. This classification approach has been successfully used in prior studies to identify and investigate structural and signaling alterations in the hippocampus and related circuitry that are relevant to decline of spatial learning abilities in aged rats (Bizon, et al., 2001; Bizon, et al., 2004; Colombo, et al., 1997; Foster and Kumar, 2007; Nicolle, et al., 1999; Rapp and Gallagher, 1996). Aged rats that fell more than two times the

standard deviation outside of the mean spatial learning index calculated for young adult rats were classified as “aged spatially-impaired” (SLI > 275) and all other aged rats (which fell within the range of the young adult cohort) were classified as “aged spatially- unimpaired” (SLI < 275).

2.3. Immunofluorescent labeling of cholinergic, GABAergic projection and total rostral basal forebrain neurons

One week after completion of behavioral testing, rats were rapidly euthanized with an overdose of pentobarbital and perfused transcardially with ice cold 0.9% saline followed by 4% paraformaldehyde. Brains were removed from the skull, postfixed for 24h in perfusate and then cryoprotected in 20% sucrose in 0.1M phosphate buffer. Systematic uniform random sampling was achieved by exhaustively sectioning brains coronally on a calibrated freezing stage microtome (35 μ m) through the full rostro-caudal extent of the medial septum and vertical limb of the diagonal band of Broca (beginning just caudal to the olfactory bulbs and ending caudal to the crossing of the anterior commissure). A registered 1-in-4 series of sections was obtained for each animal and separate series (spaced at 140 μ m intervals) were randomly assigned for processing to detect rostral basal forebrain cholinergic (ChAT), GABAergic projection (GAD67) or total (NeuN) immunopositive neurons (Gundersen and Jensen, 1987). Sections were collected into cold 0.1M PBS and stored at 4°C until stained immunohistochemically.

For immunohistochemistry, free-floating sections were washed several times in 0.1M Tris-buffered saline (TBS; 100 mM Tris-HCL, 150 mM NaCl, pH 7.5), pre-incubated in a blocking solution containing 3% normal donkey serum (NDS) and 0.3% Triton X-100 in 0.1M TBS for 1 hour at room temperature and then incubated in blocking solution that contained rabbit anti-GAD67 (Bioworld, 1:500); goat anti-ChAT (Millipore AB144P, 1:1000) or mouse anti-NeuN (Millipore MAB377, 1:500) for 72 hours at 4°C. After primary incubation, sections were washed in 0.1M TBS, and incubated in 0.1M TBS containing 2% NDS and the appropriate Alexa 488-conjugated secondary antibodies (Molecular Probes, 1:300) for 2 hours at room temperature in the dark. The sections were washed in 0.1M TBS, and mounted onto Superfrost++ slides (Fisher Scientific). The sections were then coverslipped under ProLong Gold (Invitrogen), sealed with clear fingernail polish, and stored at 4°C until analysis.

2.4. Stereological counts of ChAT, GAD67, and NeuN immunopositive neurons

Delineation of the Rostral Basal Forebrain—The basal forebrain neurons that innervate the hippocampal formation are primarily localized within the medial septum (MS) and the vertical limb of the diagonal band of Broca (vDB; Dutar, et al., 1995; Lewis and Shute, 1967; McKinney, et al., 1983; Meibach and Siegel, 1977; Segal and Landis, 1974; Swanson and Cowan, 1979). As shown in Fig. 1, these are contiguous nuclei that emerge along the midline just caudal to the joining of the corpus callosum. The vDB is positioned ventral to the MS and these nuclei are bordered laterally by the lateral septum and the medial edge of the nucleus accumbens shell. The vDB extends ventrally to the medial intersection of the two hemispheres on the ventral edge of the tissue section. In more caudal planes, the rostral-most portion of the horizontal limb of the diagonal band of Broca (hDB) emerges. Contiguous with the vDB, the hDB extends laterally along the medial edge of the nucleus accumbens shell and is bordered ventrally by the ventral pallidum and olfactory tubercle. The caudal-most edge of the vDB is rostral to the joining of the anterior commissure. After the crossing of the anterior commissure, the hDB (sometimes also referred to as the magnocellular preoptic area in this plane) is a clearly defined nucleus located in the ventral and lateral basal forebrain. As such, the crossing of the anterior commissure is often used as a boundary between rostral basal forebrain and more caudal

neocortical-innervating basal forebrain nuclei (Colom, et al., 2005; McQuail, et al., 2011b; Peterson, et al., 1999; Ypsilanti, et al., 2008). In the current study, counts were obtained from equally spaced (140 μ m apart) sections throughout the entire rostro-caudal extent of the medial septum and vDB. Because there are not clear boundaries that allow the hDB to be reliably distinguished in the rostral basal forebrain, neurons within the hDB rostral to the crossing of the anterior commissure were also included in the population estimates. The rostral basal forebrain nuclei as a whole can be readily distinguished from surrounding structures (described above) within ChAT, GAD67 and NeuN immunolabeled material (See Figs. 2-4).

Estimation of neuron number using the optical fractionator—Starting at a randomly selected level within the first sampling interval, the optical fractionator method (Gundersen, 1986; Peterson, 1999; West, et al., 1991) was implemented using an Olympus Fluoview 300 confocal microscope, equipped with the appropriate filter sets, a CCD camera, and a computer-driven x, y, and z Ludl-motorized stage controlled with StereoInvestigator software (MBF Bioscience, Williston, VT). Regional boundaries for the rostral basal forebrain nuclei (shown in Fig. 1) were delineated at low power magnification (4X) in an evenly spaced series of immunolabeled sections. The series spanned the rostro-caudal extent of the MS and vDB, which are the basal forebrain nuclei that innervate the hippocampal formation (Dutar, et al., 1995; Lewis and Shute, 1967; McKinney, et al., 1983; Meibach and Siegel, 1977; Segal and Landis, 1974; Swanson and Cowan, 1979). This design yielded 7-8 sections for quantification (per 1-in-4 series) from each brain. The motorized stage of the microscope was moved in evenly spaced x-y intervals under the computer control, surveying the regions of interest in each section according to a systematic random sampling scheme (see Table 1 for sampling details). Using a 60X oil-immersion objective (with 1.4 numerical aperture), section thickness was measured at each sampling site and z-stacks (comprised of 1 μ m z-slices) were acquired through the full section thickness at the appropriate emission wavelengths. The mean section thickness was measured at 30.62 μ m (CV =0.13), indicating an approximate 12.5% tissue shrinkage in the z-plane. This degree of shrinkage is significantly less than that observed from immunohistological procedures that require dehydration and is consistent with other reports of immunofluorescent tissue processing (Hart and Terenghi, 2004; Prasad and Richfield, 2010). Quantification was performed offline on the acquired z-stacks and was confined to an optical disector 25 μ m in height which was positioned 3 μ m below the surface of the tissue. The top most nucleus associated with an immunopositive neuron was counted only when it first came into focus within the optical disector, provided it did not encroach on the exclusion lines of the counting frame (Gundersen, 1986; Sterio, 1984). The sufficiency of the guard zone was confirmed by plotting the distribution of the cells counted for each marker in the z-axis (Andersen and Gundersen, 1999; Dorph-Petersen, et al., 2009; Dorph-Petersen, et al., 2001). The number of cells counted was highly consistent across the disector height, indicating that the guard zone of 3 μ m was sufficient to minimize the effects of superficial damage associated with tissue sectioning (i.e., to avoid lost caps (Gundersen, 1986), Supplemental Fig. 1). Moreover, the distribution of cells along the z-axis did not differ between age- or cognitive- groups (Gardella, et al., 2003; Supplemental Fig. 1), confirming good antibody penetration throughout the full section thickness (see representative examples of cell positions within the disector in the orthogonal windows shown in Supplemental Fig. 1). The total number of ChAT, GAD67 and NeuN immunopositive cells in rostral basal forebrain was estimated using the optical fractionator method (West, et al., 1991) in which the product of the cells counted in a known, uniformly random sample of the region of interest is multiplied by the reciprocal of the sampling fraction. The shrinkage robust version of the optical fractionator based upon the number weighted mean section thickness was used (Dorph-Petersen et. al.,

2001). Additional details, including stereological sampling parameters are provided in Table 1.

The precision of the stereological estimates was determined by estimating the coefficients of error (CE) using methods described by Gundersen (1999). Equations used to generate the CEs are based on Gundersen's smoothness classification $m=1$, as the areas defined for the cell counts changed smoothly from the rostral entrance of the MS to the caudal conclusion of the vDB. These CEs (ranging from 0.04 to 0.06) were less than one half of the observed variation across subjects (CVs ranging from 0.16 to 0.2, Table 1), indicating that the sampling and counting parameters should be sufficiently precise to detect frank biologically-driven differences in neuronal population estimates among experimental groups (Boyce, et al., 2010; Dorph-Petersen, et al., 2001; Gundersen and Jensen, 1987; Gundersen and Osterby, 1981; West, 1999).

3. Results

3.1. Cognitive performance in young and aged rats

In the spatial water maze task, a comparison of performance on training trials using the cumulative search error measure (two-factor ANOVA; age X trial block) indicated that both young and aged rats improved performance over the course of training trials (main effect of training trial block, $F_{(3,66)}=35.8$, $p<0.0001$) but that aged rats were significantly impaired in finding the platform in comparison to young (main effect of age, $F_{(1,22)}=9.8$, $p<0.005$, Fig. 5A). Notably, these differences were not present on the very first training trial ($F_{(1,22)}=0.77$, n.s.), indicating that aged rats' initial search strategies were comparable to those of young rats. Performance on probe trials as assessed with the mean search error measure (two factor ANOVA; age X probe trial) revealed results similar to those on training trials, in that search for the platform became more accurate as training progressed (main effect of probe trial, $F_{(3,66)}=13.8$, $p<0.0001$) but aged rats were overall less proficient in their search than young rats (main effect of age, $F_{(1,22)}=7.34$, $p<0.05$). In contrast to the spatial task, there was no impairment in the ability of aged rats to locate the visible escape platform during cue (visible platform) training (one-factor ANOVA, $F_{(1,22)}=1.4$, n.s.), indicating intact sensorimotor and motivational processes.

In order to relate population estimates to cognitive abilities, mean search error during probe trials was used to calculate a Spatial Learning Index (SLI) for each rat as described above (Bizon, et al., 2009; Gallagher, et al., 1993). This measure, specifically designed to maximize individual differences in water maze performance within the context of aging, has been shown to correlate with age-related changes in numerous neurobiological substrates of spatial memory (Bizon, et al., 2001a; Bizon, et al., 2004; Colombo, et al., 1997; Nicolle, et al., 1999; Smith, et al., 2000). Figure 5B shows individual spatial learning indices of young and aged rats. For some analyses, aged rats were subgrouped based on their SLI such that aged rats performing more than two times the standard deviation of the mean SLI of the young group ($SLI > 275$) were classified as "aged spatially-impaired" ($n=8$) and all other aged rats ($SLI < 275$) were classified as "aged spatially-unimpaired" ($n=8$). A repeated measures ANOVA (training trial block X cognitive group) confirmed that cumulative search error across training blocks differed between these subgroups ($F_{(2,21)}=9.91$, $p<0.01$) and Tukey's post hoc analyses indicated that aged spatially-impaired rats performed significantly worse than both young and aged spatially-unimpaired rats ($ps<0.05$); however, performance of young and aged spatially-unimpaired rats did not differ (n.s.).

3.2. Distribution of ChAT , GAD67 and NeuN immunopositive neurons

Figs. 2-4 show low power photomicrographs of representative immunolabeling for ChAT (Fig. 2), GAD67 (Fig. 3) and NeuN (Fig. 4) in independent series of sections through the rostral basal forebrain of representative young, aged spatially-unimpaired and aged spatially-impaired rats. Both cholinergic (ChAT immunopositive; Fig. 2) and GABAergic projection (GAD67 immunopositive, Fig. 3) neurons were distributed heterogeneously throughout the rostral basal forebrain nuclei and represented clear subpopulations of those neurons immunolabeled for NeuN (Fig. 4). For each label and across age and cognitive groups, the rostral basal forebrain nuclei could be readily distinguished from bordering structures (including the lateral septum, nucleus accumbens, and olfactory tubercle).

High magnification immunofluorescent labeling of ChAT, GAD67 and NeuN immunopositive neurons is shown in Fig. 6. ChAT immunopositive cells (Fig 6A-6C) were robustly labeled with well-defined nuclei and tended to be polygonal and fusiform in shape. Overall, ChAT immunopositive neurons appeared larger but less densely distributed than GAD67 immunopositive cells (Fig 6D-6F) in the same region. In agreement with previous reports (Brashear, et al., 1986; Colom, 2006; Gritti, et al., 2006; Gritti, et al., 2003), the GAD67 immunopositive cells exhibited diverse morphologies (oval, fusiform or polygonal cell bodies were observed) and sizes (ranging from small oval cells to large multipolar cells). Labeling of ChAT and GAD67 was consistent with numerous neuroanatomical studies that have demonstrated that ChAT and GAD immunopositive neurons in the rostral basal forebrain are distinct nonoverlapping neuronal populations (Brashear, et al., 1986; Formaggio, et al., 2011; Gritti, et al., 1993; Kohler, et al., 1984; Semba, 2000). While the ChAT immunopositive cells were primarily clustered within the medial septum and along the ventral edge of the hDB, the GAD67 immunopositive cells were distributed more homogeneously throughout the rostral basal forebrain subfields. As expected, NeuN immunopositive cells (which include both ChAT and GAD67 immunopositive neurons as well as a variety of other projection and interneurons) had discernible nuclei, were located throughout the rostral basal forebrain, and exhibited diverse sizes and morphologies (Fig 6G-6I). No obvious morphological differences in ChAT, GAD67 or NeuN immunopositive cells were detected at either low or high magnification in sections obtained from rats that differed in age or cognitive status.

3.3. Age and cognitive comparisons of ChAT, GAD67, and NeuN immunopositive cell numbers in the rostral basal forebrain

ChAT immunopositive neurons—A comparison between young and aged rats yielded a significant but modest decline in the number of ChAT immunopositive cells in aged rats relative to young (-12.5% ; $t_{(22)}=2.00$, $p<0.05$; Fig. 7A). A comparison of ChAT immunopositive cells among young and aged rats sub-grouped based upon spatial learning ability indicated a similar trend toward decreased ChAT immunopositive cell number with age ($F_{(2,21)} 2.23$, $p=0.13$) but the magnitude of reduction in aged rats was comparable across aged spatially-unimpaired (-14%) and aged spatially-impaired (-12%) subgroups (Fig., 7B). In agreement with the observation that ChAT immunopositive cell number was reduced by a similar magnitude in both aged spatially-unimpaired and aged spatially-impaired rats, there was no reliable relationship between ChAT immunopositive cell number and spatial learning index among aged rats ($r=-0.10$, n.s.; Fig. 7D). Notably, however, a significant correlation between ChAT immunopositive cell number and spatial learning index was evident in young rats, such that higher ChAT immunopositive cell numbers were associated with better spatial learning performance ($r=-0.76$, $p<0.05$; Fig. 7C).

GAD67 immunopositive neurons—A very different pattern of results was obtained from estimates of total GABAergic projection (GAD67 immunopositive) neuron numbers in

the same subfields. As shown in Fig. 8A, GAD67 immunopositive cell number was greater in aged relative to young rats, although this difference was not statistically reliable ($t_{(21)} = 0.74$, n.s.). However, a one-factor ANOVA comparing GAD67 immunopositive cell number in young and aged rats sub-grouped on the basis of spatial learning ability revealed a highly significant difference among cognitive groups ($F_{(2,20)} = 16.2$, $p < .0001$; Fig. 8B). *Post-hoc* comparisons confirmed a robust statistically significant increase in the number of GAD67 immunopositive cells specifically in aged spatially-impaired rats relative to both young (+18%; $p < 0.005$) and aged spatially-unimpaired (+25%, $p < 0.0001$) rats. The number of GAD67 immunopositive cells did not differ significantly between young and aged spatially-unimpaired rats (n.s.). The strong relationship between elevated GAD67 immunopositive cell number and cognitive impairment was supported by a significant correlation among aged rats ($r = 0.60$, $p < 0.05$), such that higher numbers of GAD67 immunopositive cells were associated with higher spatial learning indices (i.e., worse learning; Fig. 8D). Among young rats the relationship between spatial learning ability and GAD67 immunopositive cell number was in the same direction but did not reach statistical significance ($r = 0.49$, $p = 0.22$; Fig. 8C).

NeuN immunopositive neurons—To investigate whether age- and cognition-related alterations in ChAT immunopositive and GAD67 immunopositive cell estimates contributed to an overall difference in neuron number in rostral basal forebrain, NeuN immunopositive cells were also quantified. As shown in Fig 9A, the mean estimated number of NeuN immunopositive cells was numerically lower in aged rats relative to young but this difference was not reliable ($t_{(1,21)} = 1.2$, n.s.). Likewise, no significant differences were evident using a one-factor ANOVA performed on young and aged rats subgrouped by cognitive ability ($F_{(2,20)} = 0.78$, n.s.; Fig. 9B). As expected based on these analyses, no reliable correlations were observed between NeuN cell number and spatial learning indices in young ($r = -0.13$, n.s.; Fig. 9C) or aged rats ($r = .26$; n.s., Fig. 9D).

4. Discussion

This study was designed to test the hypothesis that coordinated age-related alterations in rostral basal forebrain cholinergic and GABAergic neurons that project to the hippocampus contribute to hippocampal-dependent spatial learning abilities in aged rats. Indeed, age-dependent changes were evident in both cholinergic and GABAergic neuronal populations, but the direction of these changes and their relationships to cognitive abilities were distinct. A modest but significant decline in cholinergic (ChAT immunopositive) neuron number occurred in the rostral basal forebrain with age, although this reduction was not related to spatial learning ability in aged rats. However, there was a strong relationship between cholinergic cell number and spatial learning abilities in young rats. In contrast to findings with cholinergic neurons, a robust increase in the number of GABAergic projection (GAD67 immunopositive) neurons was observed selectively in the rostral basal forebrain of aged rats with impaired spatial abilities. These phenotypically-specific alterations were not reflected in the total number of rostral basal forebrain neurons, as NeuN immunopositive cell number was stable with age and across cognitive groups.

4.1. Cholinergic (ChAT immunopositive) neurons

Cholinergic signaling in the hippocampus and neocortex has been heavily implicated in cognitive functioning, including learning, memory, and attention (Deutsch, 1971; Dunnett and Fibiger, 1993; Everitt and Robbins, 1997; Fragkouli, et al., 2005; McKinney, 2005; Ormerod and Beninger, 2002; Sarter and Bruno, 1997; Sarter, et al., 2003; Schliebs and Arendt, 2006; Schliebs and Arendt, 2011; Woolf, 1997). A number of cholinergic indices decline with age, and the cholinergic system remains a primary target of drugs currently

used to treat age-related cognitive impairment (Giacobini, 2004; Lane, et al., 2004; Lane, et al., 2006; Nordberg, 2006). Indeed, degeneration of cholinergic neurons in the nucleus basalis of Meynert and concomitant depletion of acetylcholine in cortical targets is a hallmark of Alzheimer's disease (Whitehouse, et al., 1982), suggesting that degeneration of cholinergic neurons may be an important contributor to cognitive deficiencies observed in normal aging. However, across species, findings regarding cholinergic neuronal integrity at advanced ages have been mixed. A number of studies, employing a variety of counting techniques, report a decline in cholinergic neuron number in aging (Altavista, et al., 1990; Armstrong, et al., 1993; Bartus, et al., 1982; Baskerville, et al., 2006; Fischer, et al., 1992; Fischer, 1989; Lee, et al., 1994; Stroessner-Johnson, et al., 1992), although others report no or only a modest decline (Bigl, et al., 1987; McQuail, et al., 2011b; Ypsilanti, et al., 2008). While the current findings provide support for a significant loss of rostral basal forebrain cholinergic neurons in normal aging, it should be noted that this reduction was modest, even with the relatively large sample size used. Indeed, some of the reported differences in findings across studies comparing cholinergic neuron numbers between young and aged rats may be attributable to the numbers of subjects employed relative to the numbers necessary to achieve sufficient statistical power for detecting small-magnitude group differences.

In addition to the modest but significant decline in cholinergic neuron number with advancing age, there is considerable evidence that age-related decline in cholinergic neuronal function and signaling contributes to cognitive impairment. For example, age-associated dysregulation in calcium signaling in cholinergic neurons could affect a range of neuronal functions that include neurotransmitter release and synaptic plasticity (Disterhoft, et al., 1996; Foster, et al., 2001; Kumar, et al., 2009). Specifically, Murchison and Griffith (1998) reported increased intracellular calcium buffering in cholinergic basal forebrain neurons in aged rats, and more recently, that age-related calcium dysregulation in basal forebrain cholinergic neurons strongly predicts impaired hippocampal-dependent cognition using the same rat model employed here (Murchison, et al., 2009). Other evidence indicates that aging may disrupt cholinergic signaling at the receptor level. Specifically, muscarinic signaling in the hippocampus, a principal target of rostral basal forebrain projection neurons, is significantly altered in aged rats. Whereas muscarinic receptor-mediated phosphoinositide turnover is blunted in hippocampal CA1, CA3, and subiculum across aged rats (Nicolle, et al., 2001), G-protein coupling to muscarinic receptors in hippocampus is specifically associated with compromised hippocampal-dependent spatial learning (Zhang, et al., 2007). Together with the current data, these findings suggest that age-related alterations in cholinergic signaling not only result from the loss of acetylcholine-synthesizing neurons in basal forebrain but also reflect suboptimal functioning of these neurons and/or postsynaptic signaling associated with the cholinergic receptors in hippocampus and other cortical targets. This interpretation is consistent with the finding that cholinergic cell number strongly correlated with cognitive abilities in young but not aged rats (see Fig. 7). In young rats, the cellular machinery and environment is largely intact and functioning optimally, and, thus, the overall number of cholinergic neurons might provide an accurate index of acetylcholine signaling in target fields. In aged rats, however, disruption in cholinergic signaling at multiple levels might result in a functional decoupling, such that cell number is a less accurate predictor of overall cholinergic function.

4.2. GABAergic projection (GAD67 immunopositive) neurons

A major finding from the current work is the selective and significant increase in GABAergic projection neurons (indicated by greater numbers of GAD67 immunopositive cells) in the rostral basal forebrain of spatially-impaired rats. Notably, the 67 isoform of GAD is preferentially expressed by GABAergic neurons in rostral basal forebrain that project to cortical targets, whereas the 65 isoform is expressed by rostral basal forebrain

interneurons (Castaneda, et al., 2005). Very few prior studies have quantified rostral basal forebrain GABAergic neurons in aging, and to our knowledge, no other studies have quantified this specific GAD67 immunopositive neuronal population. Using a non-isoform-specific GAD antibody, Smith and Booze (1995) reported no age-related change in GAD immunopositive neurons in caudal neocortical projecting basal forebrain nuclei (Smith and Booze, 1995). The current findings are consistent with this previous report in that no significant difference in GABAergic projection neuron number was evident in rostral basal forebrain when age groups were compared in the absence of cognitive subgrouping. Together, these data indicate that GABAergic neurons do not degenerate with advancing age, an important finding for interpreting phenotypic and functional age-related alterations in basal forebrain. For example, the calcium binding protein parvalbumin is highly co-expressed in GABAergic projection neurons and reduced parvalbumin cell number has been reported in aging (Krzywkowski, et al., 1995). The present findings would support an interpretation that reduced parvalbumin cell number with age is indicative of cellular dysregulation of Ca²⁺ signaling akin to that described above for cholinergic neurons rather than overt neuronal loss (Murchison and Griffith, 1998). Additional characterization of age-related changes in the signaling properties and function of these GABAergic neurons represents an important direction of future work.

Importantly, the increase in GAD67 immunopositive cell number observed in aged spatially-impaired rats did not appear due to an overall increase in total neuron number in rostral basal forebrain, as stereological estimates of NeuN immunopositive cells in adjacent sections did not differ with age or as a function of cognitive ability. Although the specific mechanisms responsible for the elevation in GABAergic cell number observed in spatially-impaired aged rats remains an outstanding question, it seems plausible that this change might reflect an age-related upregulation of GAD67 protein expression in a subset of rostral basal forebrain neurons which might otherwise express only nominal levels of GAD67. Such an upregulation would increase the detectability of these neurons, which in turn would be reflected as an increase in the total number of GAD67 immunopositive neurons. Indeed, GAD67 expression can be regulated by a number of factors including activity, stress, oestrous cycle, and caloric restriction (Carta, et al., 2008; Cashion, et al., 2004; Cheng, et al., 2004; Liang, et al., 1996). Moreover, altered GAD expression has been reported in a number of brain disorders, including epilepsy, Parkinson's disease, and schizophrenia (Briggs and Galanopoulou, 2011; Lanoue, et al., 2010; Lewis, et al., 2005).

Alternatively, it is important to consider that the neurons in rostral basal forebrain are quite heterogeneous and the full extent of distinct neuronal subtypes in these fields remains undetermined and was not exhaustively evaluated in the current study. In addition to cholinergic and GABAergic projection neurons, other cell types include GABAergic interneurons and a recently identified subpopulation of hippocampal-projecting glutamatergic neurons (Colom, et al., 2005; Manseau, et al., 2005). As such it is possible that the failure to detect differences in numbers of NeuN immunopositive cells between age and cognitive groups reflects differential effects of age on multiple phenotypically distinct neuronal populations in this region. Notably, the rostral basal forebrain glutamatergic neurons share size and certain neurochemical characteristics with GABAergic projection neurons (Colom, et al., 2005; Manseau, et al., 2005) and some basal forebrain neurons have been identified that have the capacity to synthesize both GAD67 and phosphate-activated glutaminase (PAG), a mitochondrial enzyme used in the production of glutamate (Gritti, et al., 2006). In addition, RT-PCR studies have shown that mRNA for the glutamate vesicular transporter 2 (VGLUT2) may be coexpressed with ChAT and GAD67 mRNA in young and adult rats (Danik, et al., 2005). These findings make it intriguing to speculate that the present results represent a functional shift from excitatory to inhibitory signaling in a subpopulation of hippocampal-projecting neurons as has been shown in kindling models in which

stimulation induces the production of GAD67 in hippocampal granule cells (Gomez-Lira, et al., 2005; Sloviter, et al., 1996). It will be important in future studies to determine the effects of age on glutamatergic projections as well as on GABAergic interneuronal populations, in order to better understand how age-related changes in basal forebrain dynamics contribute to a loss of hippocampal-dependent learning and memory.

It is becoming increasingly clear that corticopetal basal forebrain GABAergic neurons influence neural transmission and cognitive functions linked to their terminal fields (Freund and Antal, 1988; Kiss, et al., 1990b; Pang, et al., 2001). GABAergic neurons are well-positioned to modulate cortical circuitry, both through their direct input to cortical structures and via the highly interconnected cholinergic and GABAergic neuronal networks within the rostral basal forebrain itself (Freund and Antal, 1988; Freund and Buzsaki, 1996). Neuronal tracing studies indicate that GABAergic hippocampal interneurons are a primary target of GABAergic afferents from rostral basal forebrain (Freund and Antal, 1988; Gulyas, et al., 1991). As such, an increase in the inhibitory influence from rostral basal forebrain would be expected to result in enhanced excitability of the hippocampal principal neurons. Indeed, many studies have reported hyperexcitability in the hippocampus with advanced age, and such alterations have been linked to hippocampal dysfunction and a decline in hippocampal-supported cognition (Dickerson, et al., 2005; Gallagher and Koh, 2011; Wilson, et al., 2005; Yassa, et al., 2010). The present findings support the hypothesis that age-related alterations in inhibitory signaling within septohippocampal circuitry might contribute to this reported shift in the inhibitory-excitatory dynamics of the aged hippocampal formation.

Overall, the findings from the current work add to the evidence that rostral basal forebrain systems are significantly altered in aging. In particular, these studies support a growing literature which indicates that inhibitory networks are particularly vulnerable to dysfunction in aging and that such dysfunction can profoundly impact cognition. Future work in which the present findings are extended to determine the innervation patterns of both inhibitory and excitatory afferents from rostral basal forebrain and that better characterize the intrinsic dynamics within basal forebrain of aged behaviorally characterized rats will help to further elucidate the role of this system in age-related cognitive decline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Rostral basal forebrain boundaries used for cell count estimations

Schematic illustrations modified from Paxinos and Watson (2007) showing the rostro-caudal boundaries (extending from 1.7 mm anterior to -0.26 mm posterior of Bregma) and the delineation of rostral basal forebrain nuclei (red outline) used in the current study. The basal forebrain neurons that innervate the hippocampal formation are primarily localized within the medial septum (MS) and vertical limb of the diagonal band of Broca (vDB). Just caudal to the joining of the corpus callosum, these contiguous nuclei emerge along the midline with the vDB situated ventral to the MS. These nuclei are bordered laterally by the lateral septum and in more caudal sections by the medial edge of the nucleus accumbens shell (AcbS). The vDB extends ventrally to the medial intersection of the two hemispheres on the ventral edge of the tissue. In more caudal planes, the rostral-most portion of the horizontal limb of the diagonal band of Broca (hDB) emerges. Contiguous with the vDB, the hDB is bordered laterally by the medial wall of the nucleus accumbens shell, and in more caudal sections, by the ventral pallidum and olfactory tubercle. The caudal-most edge of the vDB is just rostral to the joining of the anterior commissure (just prior to the Bregma -0.26 mm). At the crossing of the anterior commissure, the hDB (often referred to as magnocellular preoptic area in this plane of section) is located toward the ventral edge of the tissue and no basal forebrain nuclei remain along the midline. Because there are not clear boundaries reliably distinguishing the hDB from vDB in the rostral basal forebrain, immunopositive neurons localized within the hDB situated rostral to the crossing of the anterior commissure were included in the population estimates. Abbreviations: ac; anterior commissure; AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; cc, corpus callosum CPu; caudate putamen, hDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; LV, lateral ventricle; MS, medial septum; MCPO magnocellular preoptic area; Tu, olfactory tubercle, vDB, vertical limb of the diagonal band of Broca; 2n, optic nerve.

Fig. 2. Distribution of ChAT immunopositive cells in rostral basal forebrain

Low magnification photomicrographs of ChAT immunolabeling in coronal sections taken through the rostral basal forebrain (approximately 0.5 mm anterior to bregma) of representative young (A), aged spatially-unimpaired (B) and aged spatially-impaired (C) rats. Staining was robust in individual neurons localized to rostral basal forebrain nuclei including the medial septum (MS), vertical limb of the diagonal band of Broca (vDB) and horizontal limb of the diagonal band of Broca (hDB), and was also evident in scattered interneurons distributed throughout the caudate putamen (CPu), nucleus accumbens (Acb) and olfactory tubercle (Tu). The dense immunolabeling of the ChAT immunopositive neurons within the MS, vDB and hDB made these nuclei readily distinguishable from neighboring structures (including lateral septum, the medial wall of the nucleus accumbens shell and the olfactory tubercle) across age and cognitive groups. For orientation, white matter regions (i.e., corpus callosum and anterior commissure) and the lateral ventricle (LV) are labeled. Abbreviations: ac; anterior commissure; AcbS, nucleus accumbens shell; cc, corpus callosum CPu; caudate putamen; hDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; LV, lateral ventricle; MS, medial septum; Tu, olfactory tubercle, vDB, vertical limb of the diagonal band of Broca. Scale bar = 200 μ m.

Fig. 3. Distribution of GAD67 immunopositive cells in rostral basal forebrain

Low magnification photomicrographs of GAD67 immunolabeling in coronal sections taken through the rostral basal forebrain (approximately 0.5 mm anterior to bregma) of representative young (A), aged spatially-unimpaired (B) and aged spatially-impaired (C) rats. GAD67 labeling was robust throughout rostral basal forebrain nuclei, including medial septum and the vertical and horizontal limbs of diagonal band of Broca (vDB and hDB, respectively). Somewhat diffuse GAD67 immunolabeling was also observed in lateral septum (LS) and in nucleus accumbens (Acb), whereas scattered individual GAD67 immunopositive cells were observed in the caudate putamen (CPu) and olfactory tubercle (Tu). Across age and cognitive groups, dense labeling within basal forebrain nuclei allowed these regions to be readily delineated from surrounding nuclei. For orientation, white matter regions (i.e., corpus callosum and anterior commissure) and the lateral ventricle (LV) are labeled. Abbreviations: ac; anterior commissure; AcbS, nucleus accumbens shell; cc, corpus callosum; CPu; caudate putamen; hDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; LV, lateral ventricle; MS, medial septum; Tu, olfactory tubercle, vDB, vertical limb of the diagonal band of Broca. Scale bar = 200 μ m.

Fig. 4. Distribution of NeuN immunopositive cells in rostral basal forebrain

Low magnification photomicrographs of NeuN immunolabeling in coronal sections taken through the rostral basal forebrain (approximately 0.5 mm anterior to bregma) of representative young (A), aged spatially-unimpaired (B) and aged spatially-impaired (C) rats. NeuN expression is prominent in most neurons through rat forebrain, although differences in density and intensity of expression allow discrete nuclei to be clearly distinguished. NeuN immunolabeling is robust throughout the medial septum (MS) and the vertical and horizontal limbs of the diagonal band of Broca (vDB and hDB, respectively). It is also particularly prominent in lateral septum (LS) and the nucleus accumbens shell (AcbS), as well as in olfactory tubercle (Tu), allowing the boundaries of basal forebrain nuclei to be clearly delineated across age and cognitive groups. For orientation, white matter regions (i.e., corpus callosum and anterior commissure) and the lateral ventricle (LV) are labeled. Abbreviations: ac; anterior commissure; AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; cc, corpus callosum CPu; caudate putamen, hDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; LV, lateral ventricle; MS, medial septum; MCPO magnocellular preoptic area; Tu, olfactory tubercle, vDB, vertical limb of the diagonal band of Broca; 2n, optic nerve. Scale bar = 200 μ m.

Fig. 5. Spatial learning in young and aged rats

(A) Young and aged rats did not differ on the first training trial, and both groups improved over the course of training. As a group, aged rats were significantly impaired relative to young in learning to swim to a hidden (submerged) platform within the water maze. (B) Spatial learning index (SLI) scores were calculated from probe trial performance to provide an overall index of spatial learning ability for each subject. Note that there was considerable variability among aged rats, with many performing within the range of young (aged spatially-unimpaired rats; $SLI < 275$) and others performing more than two times the standard deviation of mean young rat performance, demonstrating impairment (aged spatially-impaired rats, $SLI > 275$). See text for statistical analysis.

Fig. 6. High magnification immunofluorescent labeling of ChAT, GAD67 and NeuN immunopositive neurons

Representative ChAT (A-C), GAD67 (D-F), and NeuN (G-I) immunopositive cells in young (left), aged spatially-unimpaired (middle) and aged spatially-impaired rats (right). Across ChAT, GAD67, and NeuN material, immunopositive cells were robustly labeled and contained a well-defined nucleus. Overall, ChAT immunopositive cells tended to be polygonal and fusiform shaped whereas GAD67 immunopositive cells exhibited diverse morphologies and sizes (ranging from small oval cells to large multipolar cells). The NeuN immunopositive cells (which would include both ChAT and GAD67 immunopositive neurons as well as a variety of other projection and interneurons) had discernable nuclei and exhibited diverse sizes and morphologies. No obvious morphological differences between ChAT, GAD67 or NeuN immunopositive cells were detected from rats that differed in age or cognitive status. Scale bar = 20 μ m.

Fig. 7. Cholinergic (ChAT immunopositive) cell number in the rostral basal forebrain of young and aged rats

(A) The number of ChAT immunopositive cells was modestly but significantly decreased in aged relative to young rats. (B) The reduction in ChAT immunopositive cells was of a similar magnitude (approximately -12.5%) in both aged spatially-unimpaired and aged spatially-impaired rats. (C) Scatter plot illustrating a strong relationship between ChAT immunopositive cell number and spatial learning index among young rats. (D) This relationship was not present among aged rats. See text for statistical analyses. $*p < 0.05$; Y= young; AU= aged spatially-unimpaired; AI= aged spatially-impaired.

Fig. 8. GABAergic (GAD67 immunopositive) cell number in the rostral basal forebrain of young and aged rats

(A) GAD67 immunopositive cell number was numerically but not significantly greater in aged rats in comparison to young. (B) A significant difference in GAD67 immunopositive cell number was evident between cognitive age groups, such that aged spatially-impaired rats exhibited a marked, reliable elevation in GAD67 immunopositive cells in comparison to both young and aged spatially-unimpaired rats. (C) Scatter plot shows a trending though nonsignificant relationship between GAD67 immunopositive cell number and spatial learning index in young rats such that higher numbers were associated with worse learning. (D) Scatterplot shows that the same relationship was observed among aged rats, but in this case, greater GAD67 immunopositive cell number was significantly associated with worse spatial learning ability. See text for statistical analyses. * $p<0.05$ relative to young and aged spatially-unimpaired; Y= young; AU= aged spatially-unimpaired; AI= aged spatially-impaired.

Fig. 9. Total (NeuN immunopositive) cell number in the rostral basal forebrain of young and aged rats

NeuN immunopositive cell number in the rostral basal forebrain did not differ as a function of age (A) or cognitive ability (B). Moreover, the total number of NeuN immunopositive cells did not predict spatial learning ability in either young (C) or aged (D) rats. See text for statistical analyses. Y= young; AU= aged spatially-unimpaired; AI= aged spatially-impaired.

Table 1

Sampling parameters used for estimating total number of rostral basal forebrain neurons.

Object	Sampling Grid ($\mu\text{m} \times \mu\text{m}$)	Counting Frame ($\mu\text{m} \times \mu\text{m}$)	Disector height (μm)	Average Object Counted $\pm$ SD	Average CE s	CV
ChAT+ cells	336 $\times$ 448	140 $\times$ 140	25	292 $\pm$ 42.89	0.05	0.16
GAD67+ cells	448 $\times$ 448	110 $\times$ 100	25	679 $\pm$ 101.14	0.06	0.20
NeuN+ cells	400 $\times$ 400	89 $\times$ 89	25	781 $\pm$ 175.33	0.04	0.16

Total cell numbers were estimated using the formula: $N(\text{Total number}) = 1/\text{ssf} (\text{section sampling fraction}) \times 1/\text{asf} (\text{area sampling fraction}) \times 1/\text{hsf} (\text{height sampling fraction}) \times \text{number of immunopositive cells counted}$. The total number of cells counted for each subject and the sampling grid and counting frame used to generate the asf for each label are provided above. The ssf is the current study equaled $\frac{1}{4}$ in every case. The hsf was calculated using the mean weighted thickness (with section thickness measured at each sampling site). SD=standard deviation; CE=coefficient of error, Gundersen's CE, $m=1$; CV=coefficient of variation ($N=24$ total rats for ChAT and $N=23$ for GAD67 and NeuN).

Table 2

Estimates of ChAT, GAD67 and NeuN immunopositive cells in rostral basal forebrain of young and aged behaviorally characterized rats.

	Young	Aged- Total	Aged-SU	Aged-SI
ChAT+ cells (SD, n)	21003.47 (3284.54, n=8)	18383.71 * (2626.83, n=16)	18113.89 (2515.56, n=8)	18653.53 (2879.54, n=8)
GAD+ cells (SD, n)	48586.17 (5564.82, n=8)	51118.00 (8721.43, n=15)	44459.45 (4408.62, n=8)	58727.78 ** (5317.29, n=7)
NeuN+ cells (SD, n)	140007.13 (19561.76, n=8)	128798.47 (21035.71, n=15)	124938.86 (22388.96, n=7)	132175.63 (20674.01, n=8)

*
p<0.05 relative to young

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p<0.001 relative to both young and aged unimpaired