# UC Davis
## UC Davis Previously Published Works

**Title**

Regional and age-related differences in GAD67 expression of parvalbumin- and calbindin-expressing neurons in the rhesus macaque auditory midbrain and brainstem

**Permalink**

https://escholarship.org/uc/item/1mb9m5v3

**Journal**

The Journal of Comparative Neurology, 522(18)

**ISSN**

1550-7149

**Authors**

Gray, DT
Engle, JR
Rudolph, ML
et al.

**Publication Date**

2014-12-15

**DOI**

10.1002/cne.23659

Peer reviewed

# Regional and age-related differences in GAD67 expression of parvalbumin and calbindin expressing neurons in the rhesus macaque auditory midbrain and brainstem

**DT Gray**[1,2], **JR Engle**[2], **ML Rudolph**[1], and **GH Recanzone**[1,3]

[1]Center for Neuroscience, University of California at Davis, One Shields Avenue, Davis, CA 95616

[2]Evelyn F. McKnight Brain Institute, University of Arizona, Tucson, AZ 85721

[3]Department of Neurobiology, Physiology and Behavior, University of California at Davis, One Shields Avenue, Davis, CA 95616

## Abstract

Neurons expressing the calcium binding proteins (CaBPs) parvalbumin (PV) and calbindin (CB) have shown age-related density changes throughout the ascending auditory system of both rodents and macaque monkeys. In the cerebral cortex, neurons expressing these CaBPs express markers of GABAergic neurotransmission, such as GAD67, and have well understood physiological response properties. Recent evidence suggests that in the rodent auditory brainstem, CaBP containing cells do not express GAD67. It is unknown whether PV and CB containing cells in subcortical auditory structures of macaques similarly do not express GAD67, and a better understanding of the neurotransmission of neurons expressing these proteins is necessary in understanding the age-related changes in their density throughout the macaque auditory system. This was investigated with immunofluorescent double-labeling techniques to co-register PV and CB expressing neurons with GAD67 in the superior olivary complex and the inferior colliculus of young and aged rhesus macaques. The proportions of GAD67 expressing PV and CB positive neurons were computed using unbiased sampling techniques. Our results indicate that between 42 and 62 percent of PV- and CB-positive neurons in the auditory brainstem and midbrain express GAD67, which is significantly less than in the cerebrum. In general, less PV+ neurons and more CB+ neurons expressed GAD67 as a function of age. These results demonstrate that the inhibitory molecular profile of PV and CB expressing neurons can change with age in subcortical auditory structures, and that these neurons are distinct from the well described inhibitory interneurons which express these proteins in the cerebral cortex.

## Introduction

The primate auditory system is characterized by two parallel, chemically defined processing pathways that can be segregated based on immunoreactivity to either parvalbumin (PV) or

Corresponding Author: Daniel T. Gray, University of Arizona, Tucson, AZ 85721 dtgray @email.arizona.edu.

calbindin (CB). The immunoreactivity to these calcium binding proteins (CaBPs) is sufficient to differentiate specific subdivisions of auditory nuclei, especially in midbrain, thalamic, and cortical structures (Jones, 2003; Parvizi and Damasio, 2003; Engle et al., 2014). The relative densities of neurons expressing these CaBPs and other calcium-dependent molecules change with age in auditory structures throughout the rodent auditory system (O'Neill et al., 1997; Zettel et al., 1997; Idrizbegovic 2003, 2004, 2006; Ouda et al., 2008, 2012), and similar observations have recently been made in the macaque monkey (Gray et al., 2013, 2014a,; Engle et al., 2014). These density changes occur in parallel with age-related cochlear pathologies that lead to decreased excitatory output from the sensory periphery (Schuknecht, 1955; Schuknecht, 1964; Schuknecht and Gacek, 1993; Nelson and Hinojosa, 2006; Fetoni et al., 2011; Engle et al., 2013), and changes in the balance of excitation and inhibition in auditory neurons which lead to over-excitable central auditory structures in aged animals (Caspary et al., 1995, 1999, 2005, 2006, 2008; Juarez-Salinas et al., 2010). Currently, the functional purpose of these density changes remains unknown.

PV- and CB-expressing neurons are chemically and physiologically well characterized in the cerebral cortex, striatum, and hippocampus where they have been shown to release gamma-Aminobutyric acid (GABA; Klausberger and Somogyi, 2008; Rudy et al., 2011; Bartos and Elgueta, 2012), and have fast spiking response properties which produce brief, non-adapting action potentials (Kawaguchi et al., 1987; Cauli et al., 1997; Kawaguchi and Kubota, 1997; Gibson et al., 1999; Ascoli et al., 2008; Xu and Callaway, 2009). Together, these characteristics make CaBP expressing interneurons powerful inhibitors in these cerebral structures. If these neurons behave analogously in the subcortical structures of the auditory system, one hypothesis explaining the function of their age-related density changes could be as an inhibitory compensatory mechanism combatting the hyperexcitability of the aged auditory system.

Few studies, however, have investigated the physiological properties of subcortical neurons expressing PV and CB, and those which do suggest that these neurons are not always inhibitory as in higher brain regions. For example, in the superior olivary complex (SOC) and cochlear nucleus of rodents, PV+ and CB+ neurons do not express markers of GABAergic neurotransmission, such as glutamic acid decarboxylase (GAD); however these markers are present in the majority of CaBP-expressing neurons in the inferior colliculus (IC; Fredrich and Reisch, 2009). This work has not been replicated in primates, therefore it is unknown whether CaBP-expressing neurons in the macaque auditory brainstem and midbrain are inhibitory (as in the cerebral cortex), not inhibitory (as in the rodent auditory brainstem), or a combination of the two.

Whether PV- and CB-expressing neurons are GABAergic in the macaque auditory system is critical information for understanding the functional consequences of the density changes of neurons expressing these proteins during natural aging. We therefore characterized the GABAergic molecular profiles of PV+ and CB+ neurons in the macaque SOC and IC using double-labeling immunofluorescent protocols with GAD67, an isoform which has been found previously in subcortical auditory structures (Burianova et al., 2009). We calculated the proportion of PV+ and CB+ neurons expressing GAD67 and compared these percentages between the auditory brainstem (SOC) and auditory midbrain (IC). Finally,

given the age-related changes in the density of PV+ and CB+ neurons in these auditory structures (Gray et al., 2014a; Engle et al., 2014), we segregated the animals into young and old age groups, and compared these proportions between age groups within these two auditory structures.

## Methods

The IC and SOC from eight rhesus macaques ranging in age from 12 to 28 years (corresponding to roughly 36 to 84 human years; Davis and Leathers, 1985) were quantified for GAD67 expression in PV+ and CB+ neurons. Table 2 describes the demographic information of all animals used in this analysis. No animal had a history of loud noise exposure, ear trauma, or ototoxic drug treatment. Other than one monkey (327 month old), none of the animals received auditory training prior to analysis. All procedures adhered to the National Institute of Health's guidelines, and were approved by the UC Davis Institutional Animal Care and Use Committee.

### Histological Processing and Antibody Characterization

Animals were euthanized with an overdose of sodium pentobarbital (60 mg/kg, i.v.), and transcardially perfused with a solution of 4% paraformaldehyde in 0.01% phosphate buffer saline (PBS; pH 7.4) followed by a mixture of 4% paraformaldehyde and 10% sucrose. Following perfusion, the brains were extracted and placed in a solution of 4% paraformaldehyde and 30% sucrose for cryoprotection. All brains were cut transversely at either 25 or 30 μm. Sections were stained for PV or CB immunohistochemistry along with GAD67 immunohistocemistry (see below). In total, at least 8 and up to 20 individual sections showing co-localization of both PV and CB with GAD67 were analyzed per animal, and this range depended on the size of the region studied. Adjacent sections were always processed for different combinations of CaBP and GAD67 or with other markers not presented in the current study, which allowed for a minimum spacing of 100-120 microns between sections processed for a given combination. This spacing was sufficient in sampling neurons from the majority of the rostro-caudal axis of both the IC and SOC in all animals.

Brain sections were blocked for 24 hours in 3% normal horse serum (Vector Labs; S-2000) and 0.25% Triton X (Sigma-Aldrich; × 100), followed by a 24 hour incubation in a solution containing a mixture of either the primary PV antibody (anti-PV in mouse, 1:2000; Sigma-Aldrich; p3088; RRID: AB_477329) or primary CB antibody (anti-CB in mouse, 1:2000; Sigma-Aldrich; c9848; RRID: AB_476894) along with the primary GAD67 antibody (anti-GAD1 in Rabbit; 1:1000, Sigma-Aldrich; SAB4300642; RRID: AB_11129033). Following this incubation the sections underwent three 15 minute washes in 0.01% PBS. The sections were then incubated for 48 hours in a solution containing two secondary fluorescent antibodies of different emission wavelengths, one specific to mouse IgG (Alexa Fluor 594; 1:250; Life Technologies; A21203; RRID: AB_10563558) and the other specific to rabbit IgG (Alexa Fluor 488; 1:250; Life Technologies; A21206; RRID: AB_10049650). Following three more 15-minute rinses in PBS the sections were mounted with a vectashield fluorescent mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector

Labs; H1200) and coverslipped. The information for all antibodies and chemicals used in these procedures is summarized in Table 3.

A variety of tests were used to confirm the isotype specificity of all antibodies used in the study. The Sigma immunoType™ Kit (Product Code ISO-1), and a double diffusion immunoassay using mouse monoclonal antibody isotyping reagents (Product Code ISO-2) confirmed isotype specificity of the anti-PV antibody as frog leg PV and the anti-CB antibody as purified bovine kidney CB-D-28k. Immunoblotting of the anti-GAD antibody against extracts from rat and mouse brain yielded a single band, confirming specificity to the GAD67 protein. Furthermore, exclusion of the primary antibody yielded only weak background signal in all animals and no punctuate cellular labeling, indicating positive signals resulted from the antibodies used, and not extraneous label.

### Identification of the inferior colliculus and superior olivary complex

The different sub-regions of the IC (central and external nucleus) and the superior olivary complex (medial, lateral and trapezoid regions) were defined using the DAPI counterstain on all CaBP stained sections. In accord with previous investigations, the IC was defined as being dorsal to the cuneiform nucleus and the nucleus of the lateral lemniscus, lateral to the periaquaductual gray, ventral and caudal to the superior colliculus, and caudal to the central tegmental field (Oliver, 2005). We differentiated the central nucleus (ICc) from the peripheral cortex (ICx) based on their relative immunoreactivity to PV and CB since the ICc and ICx of primates have previously been segregated based on the immunoreactivity to these molecules with the ICc being PV rich and the ICx being CB rich (Jones, 2003; Parviz and Damasio, 2003; Engle et al., 2014; Figure 1A, 1C). The three principle subdivisions of the SOC, the medial nucleus of the trapezoid body (MNTB), medial superior olive (MSO), and lateral superior olive (LSO), were segregated based upon the conventions of Strominger and Hurwitz (1976) and Irving and Harrison (1967). Briefly, the MSO was characterized as a vertically oriented cell rich region in the caudal pontine tegmentum surrounded by heavy staining of the neuropil. The LSO was characterized by a globular cell rich region located laterally and slightly dorsally to the MSO, and the MNTB was identified as a cell rich region with large perikarya lying medially and ventrally to the MSO (Figure 1B; see also Gray et al., 2014a).

### Data Analysis

Fluorescence microscopy was used to co-register CaBP immunoreactivity (PV or CB) with GAD67 immunoreactivity. Individual neurons were sampled by taking 20x or 40x images of the different subdivisions of the IC and SOC using an appropriate set of fluorescent filters. All images were line averaged to reduce background noise, and imported into Adobe Photoshop CS5 (Adobe Systems Inc.) where they were co-registered. Images of the counting fields were taken away from the anatomical borders of the regions of interest to ensure that all sampled neurons belonged to the appropriate subdivisions.

To assess the proportion of neurons co-expressing CaBPs and GAD67, the total number of neurons staining positively for PV or CB and GAD67 were counted by two observers blind to the identity of the animals. A CaBP immunopositive neuron was defined as containing

label and having a punctate, cellular-like morphology. Co-labelled neurons were identified in the overlaid images. More specifically, the PV and CB label filled entire cells, and the GAD67 label was found intracellularly in much smaller quantities within either the cytoplasm or nucleolus, which is in agreement with previous reports at this level of the auditory system (Ito et al., 2010). Neurons with minimal label in any filter were not considered co-expressing to minimize the effects of the weak background noise of these labels on the estimations of the proportion of co-expressing neurons (see below). Comparisons between the two observers showed no significant differences in their counts of all cell types (paired t-tests; all $p > 0.2$), and therefore their counts were combined for analysis. Proportions of co-localizing neurons ($P_C$) were calculated as follows:

$$P_C = \sum C / (\sum C + \sum N)$$

With C being the co-registered cells, and N being non-co-registered cells. These standards were used for all subdivisions of the SOC and IC. Furthermore, to ensure that our counting criteria and quantification metrics did not skew the data in any way, sections from the striatum underwent an identical analysis since PV and CB expressing neurons in these areas have been shown to primarily be GABAergic, and the results of this analysis confirm the appropriate sensitivity of this analysis (see Results).

## Statistical Analysis

Two statistical tests were performed, first at the level of individual neurons and second at the level of individual animals. In the first analysis neurons from all animals were grouped and categorized as olivary/collicular for the between region analysis and young/old for the aging analysis. A Mann–Whitney $U$ test for non-parametric populations was used to analyze whether the proportion of neurons that co-expressed GAD67 and PV/CB were significantly different between the IC and SOC, or between age groups. The age cutoff was 21.66 years (roughly 65 human years). A relatively stringent significance criterion of $p < 0.01$ was used in these analyses. The total number of neurons sampled in each comparison ranged between 272 and 498 neurons per group for the regional analysis, and between 51 to 202 neurons per group in the aging analysis. This grouping increased the sample size since each neuron was considered a data point that either did or did not express GAD67. Using this analysis gave more power to detect changes (given the relatively small number of animals) than the multi-way ANOVA described below, which uses each individual animal as a data point.

In the second analysis we sought to understand the data at the level of individual animals using a 2-way ANOVA with region (IC vs SOC) and age as factors. A significance criterion of $p < 0.05$ was used in this analysis since protection against the effects of multiple comparisons is built into the ANOVA model. Overall, this is a more stringent analysis than the Mann–Whitney $U$ test, especially given the much smaller sample size ($n = 8$ animals). Using both of these analyses together is useful in understanding the degree of inter-subject variability (reflected strongly in the ANOVA) for both regional and age-related differences (detected through the less stringent Mann–Whitney $U$ test).

Finally, to determine age-related density changes in PV+ and CB+ neurons (as opposed to the proportions described above), the density values from young and old animals underwent an unpaired t-test with a significance criterion of $p < 0.01$.

## Results

### Characterization of the inferior colliculus and superior olivary complex

We examined the IC and SOC in transverse histological sections from 8 rhesus macaques ranging in age from 12 through 28 years, which roughly corresponds to 36 through 84 human years (Davis and Leathers, 1985). Neurons from these sections were labelled with GAD67 antibodies along with either PV or CB antibodies (see Methods). All animals stained positively for all antibodies used; therefore both combinations of PV-GAD67 and CB-GAD67 were observed and analyzed. Borders of the IC and SOC (Figure 1A, 1B) were determined with the help of a DAPI counterstain (see methods).

### The proportion of PV+ and CB+ neurons expressing GAD67 differed in the IC and SOC

In rodents, the majority of PV+ and CB+ neurons in the IC express GAD67, whereas in the SOC this is not the case (Fredrich and Reisch, 2009). Since these observations have not been made in the macaque, at the first level of analysis we compared the proportion of sampled PV+ and CB+ neurons which expressed GAD67 in the IC and SOC (Figure 2A, 2B). We observed PV+ and CB+ neurons expressing GAD67 in both the IC and SOC, although to different proportions. In the IC, 57% of PV+ neurons sampled were also GAD67+, whereas only 42% expressed GAD67 in the SOC (Mann-Whitney U, $p < 0.01$; Figure 2D). Conversely, CB+ neurons in the IC and SOC did not differ in the proportion of the neurons expressing GAD67 (61% and 62%, respectively; Mann-Whitney U, $p > 0.05$; Figure 2D). These data suggest that at the level of individual neurons there are regional differences in the expression of GAD67 for populations of PV neurons, but not CB neurons. These observations held true when studied at the level of individual animals (2-way ANOVA; PV: $p = 0.03$; CB: $p > 0.05$), suggesting that there is little variability in these observations between monkeys.

As a control for the GAD67 specificity observed in the IC and SOC, we also sampled neurons from the striatum (Figure 2C), since the molecular profiles of PV+ and CB+ neurons in this region are well known (Carder et al., 1996; Parent et al., 1996; Prensa et al., 1998; Cicchetti et al., 2000; Rudy et al., 2011) and comprise separate chemically defined classes of GABAergic interneurons. Our results are consistent with these previous findings in that PV+ and CB+ neurons in the striatum expressed GAD67 in most neurons sampled. Quantification of these observation revealed that PV+ neurons expressed GAD67 in 83% of neurons sampled, and CB+ neurons expressed GAD67 in 92% of the neurons sampled (Figure 2, light grey bars). These proportions are significantly different from the proportion of PV+ and CB+ neurons expressing GAD67+ in the IC and SOC (Mann-Whitney U, $p < 0.01$; Figure 2D, asterisks). Again, a 2-way ANOVA yielded identical results (both PV and CB $p < 0.05$), indicating that these differences are also seen at the level of individual animals. Thus, our methodology yielded results consistent with that seen previously in the striatum, and the percentage of PV+ and CB+ neurons expressing GAD67 in the macaque

SOC and IC is significantly different than in the striatum. Together these data suggest that there exist subclasses of CaBP-expressing neurons in the auditory brainstem and midbrain that differ chemically and physiologically from cerebral neurons expressing the same CaBPs, and that these observations are consistent across animals.

### Age-related changes in PV+ and CB+ neurons expression

In order to determine whether there is an age-related change in the density of neurons expressing these CaBPs, as reported previously (Gray et al., 2013, 2014a, b; Engle et al., 2014), we quantified the age-related density changes of PV+ and CB+ neurons by counting the number of PV+ or CB+ neurons per section analyzed and compared these values across age groups. The results of the current study were consistent with prior studies in that PV expression was significantly increased in the MSO, LSO, and ICc with age (unpaired t-test, $p < 0.01$; Figure 3A), but not in the MNTB or ICx. Age-related increases in CB expression were noted only in the MSO (unpaired t-test, $p < 0.01$; Figure 3B), and to our knowledge this is the first report of age-related changes in the density of CB protein expressing neurons in the macaque auditory system.

### Age-related changes in the proportion of GAD67 expressing PV+ and CB+ neurons in the IC and SOC

Given that we observed age-related changes in the density of PV+ and CB+ neurons, the next level of analysis was to determine if there was also a difference in the percentage of these neurons that also expressed GAD67. The quantification of the proportion of these cells within the IC and SOC revealed changes in some, but not all subdivisions of these structures. PV and GAD67 showed no significant changes in co-expression in the ICc as a function of age (Mann- Whitney U; $p > 0.05$), but an increase with age in the ICx (Mann-Whitney U; $p < 0.01$; Figure 4A). The proportion of CB+ neurons in both the ICx and ICc expressed GAD67 equally with age (Mann-Whitney U; $p > 0.05$; Figure 4B). Changes were also noted in the SOC, although they differed from the changes seen in the IC. The proportion of PV+ neurons expressing GAD67 significantly decreased with age in the LSO (Mann-Whitney U; $p < 0.01$; Figure 4A), and showed a trend to decrease in the MSO (Mann-Whitney U; $p = 0.06$). The changes in CB and GAD67 co-expression with age were confined only to the MSO, where significant increases were noted (Mann-Whitney U; $p < 0.01$; Figure 4B).

Since one or more subdivisions showed either significant differences or trends towards increases or decreases in the co-expression of GAD67 and PV+/CB+ neurons as a function of age, we combined the values of all IC and SOC subdivisions. This analysis revealed that as a population there was no change in the co-expression of GAD67 with either PV+ or CB+ neurons in the IC with age, however in the SOC, PV+ neurons express GAD67 less often and CB+ neurons express GAD67 more often with age (Mann-Whitney U, $p < 0.01$; Figure 4C). Together this data suggests that, at the level of individual neurons, age-related changes in the inhibitory molecular profile in populations of PV+ and CB+ neurons exist in the SOC, but not the IC, and are different between CaBPs. In general, the populations of PV+ neurons became less inhibitory with age, whereas populations of CB+ neurons become more inhibitory with age, but these observations are not absolute (PV+ neurons in the ICx) nor

present in all auditory subdivisions. When this same data underwent a 2-way ANOVA, no statistically significant changes were observed indicating there is considerable inter-animal variation in these age effects which, given the small sample size, masks any age-related effects at the level of individual animals (Figure 4D). Together these data suggest that populations of PV and CB expressing neurons in the auditory brainstem change their expression of GAD67, but these changes do not necessarily occur in all aged animals.

## Discussion

### Age-related changes in PV and CB neuron density

Neurons of the central auditory system containing CaBPs have repeatedly been shown to change in density during the natural aging process of both rodents (O'Neill et al., 1997; Zettel et al., 1997; Idrizbegovic 2003, 2004, 2006; Ouda et al., 2008, 2012) and non-human primates (Gray et al., 2013, 2014a, b; Engle et al., 2014; Figure 3). The present study is the second report of age-related density changes in PV expressing neurons within the auditory system of the macaque, and the first report of age-related changes in CB expressing neurons. Although the density changes of CB expressing neurons were confined to the MSO, unlike the more widespread density changes of PV expressing neurons, it is clear that the number of neurons expressing both these CaBPs can change in aged auditory nuclei. Notably, all reported age-related changes in the density of CaBP expressing neurons have resulted with increases, whereas both increases and decreases have been shown in rodents. These observations suggest that there are cross-species differences in the expression of these neurons and in their density changes with age. The root of these inconsistencies may arise from cross-species differences in sound processing, or from differences in the distribution of inhibitory neurons throughout the auditory pathway. For example, the auditory thalamus of rodents does not contain inhibitory neurons, but the same structure in primates and felines do (Winer and LaRue, 1996; Bartlett and Smith, 1999). Also, the specifics of binaural sound processing can differ significantly between species with different hearing ranges (for reviews see Moore, 1991; Pollack et al., 2002; Grothe and Koch, 2011). Regardless of the exact cross-species differences, the results from rodent models cannot be completely generalized to primates, and vice versa.

What remains unclear, however, is the role of these changes in the aging process. This gap in understanding partially arises from our minimal knowledge of the physiological response properties of CaBP expressing neurons of subcortical structures. Most studies investigating the physiology of these neurons have occurred in the cerebral cortex, striatum, or hippocampal formation where most are GABAergic inhibitory neurons; therefore the first goal of the present study was to uncover the GABAergic profiles of populations of PV+ and CB+ neurons in the auditory midbrain and brainstem.

### Subcortical PV and CB cells differ from cerebral PV and CB neurons

The results of the current study revealed two important findings. First, as a population, PV+ neurons of the IC express GAD67 more often than in the SOC, a difference that is not seen with populations of CB+ neurons. Therefore, populations of CaBP expressing neurons can differ in their net GAD67 expression between the midbrain (IC) and brainstem (SOC), but

this is not the case with all classes of CaBP expressing neurons. Second, as a population, subcortical PV+ and CB+ neurons expressed GAD67 in significantly fewer cells than seen in populations of chemically analogous (with respect to CaBPs) neurons in the cerebrum (Figure 2). This observation suggests that the presence of PV and CB in these sub-cortical structures does not relate to a single specific physiological profile of neurons as it does in the cerebrum, but rather are likely expressed in neurons performing different cellular processes, some of which are likely affected during the aging process.

Together, these data suggest that CaBP expressing neurons of the subcortical auditory system cannot, as a population, be considered inhibitory through GABAergic neurotransmission. Caution should be taken, however when interpreting these results. It is possible that GAD67 expression is simply lower in SOC neurons when compared to IC neurons, bringing them below detection levels. Also, we cannot definitively rule out the possibility that these non-GAD67 expressing neurons are not inhibitory in these areas of the brain, for example, using a different neurotransmitter system. As an example, many subcortical auditory neurons use glycine as an inhibitory neurotransmitter instead of GABA. In order to investigate these possibilities, it would be informative for future studies to co-label these CaBP expressing neurons with other markers of GABA neurotransmission, such as the GABA vesicular transporter, GAT, or markers for other neurotransmitters. Alternatively, some neurons may express a different isoform of GAD, such as GAD65, which would elude the present analysis.

Despite the possibilities presented above, clearly a substantial proportion of both PV+ and CB+ neurons do express GAD67 in both the SOC and IC. These large values suggest that subclasses of CaBP expressing neurons contain GAD67, and may be differentiated from one another based upon independent morphological or chemical characteristics. The distribution of these subclasses of neurons may differ between regions of the auditory system, which would explain the IC-SOC differences in PV+ inhibitory cells. It is unknown what morphological cell types comprise the chemically defined neurons described in the current study. A better understanding of the morphological and chemical properties characterizing these CaBP containing, GAD67 expressing neurons would be useful in elucidating their different expression patterns observed in the IC and SOC.

### Functional Implications

Aged neurons of the auditory system of both rodents and non-human primates have increased spontaneous and driven firing rates in both subcortical and cortical structures (Caspary et al., 2005; 2006; Juarez-Salinas et al., 2010; Engle and Recanzone, 2012). These increases result with broader spectral and spatial tuning of auditory neurons, both deficits that correlate with decreases in psychophysical metrics of auditory perception (Engle and Recanzone, 2012). Aside from disrupting auditory perception, these age-related increases in neural activity put auditory neurons at risk for excitotoxicity. This toxicity occurs when relatively high levels of glutamate cause greater depolarizations and more frequent action potentials, increasing the influx of calcium which activates intracellular enzymes that in turn damage vital cellular components (Manev et al., 1989). Given the increased physiological activity in aged auditory neurons, mechanisms to prevent such excitotoxicity are likely

necessary for normal cell maintenance. An effective way to compensate for the increased intracellular calcium would be to increase the expression of CaBPs which act as calcium buffers.

The current data suggests that in the macaque, like in rodents (Fredrich et al., 2009), the presence of CaBP within subcortical auditory neurons does not necessarily relate to the neurotransmitters used by these neurons. Rather, they likely play different, non-electrophysiological roles in the aging process of the auditory system. One hypothesis explaining the age-related increases in these neurons could be that they function as a homeostatic response combatting the toxic effects of excitotoxicity. This idea is applicable to both rodent and non-human primate models since both experience age-related hyperexcitability in the auditory system (Caspary et al., 2006; Juarez-Salinas et al., 2010), both possess subcortical PV and CB expressing neurons which do not relate to a single class of neurotransmitter, and the basic intracellular physiological mechanisms (metabolic homeostasis, control of Ca++ levels, etc.) are very similar. Whether this hypothesis is true remains to be seen, however, these observations give credibility to such a preventative function for these cells.

## Summary and Conclusions

In the brainstem, the proportion of GAD67 expressing PV and CB cells changed in aged animals such that PV+ neuron populations were less likely to express GAD67, and CB+ populations were more likely to express GAD67, and this effect was not noted in the IC. Therefore, if the speculated compensatory effort of the auditory system to combat the hyperexcitability of neurons that occurs with age is true, neurons expressing CB are more likely to be responsible for this since the aged population of these cells expressed GAD67 more often. Conversely, PV neuron populations become less inhibitory with age, but these cells still increase in density, suggesting that the Ca++ buffering capacity of PV may be of importance to combat the excitotoxic effects from an intracellular level. Whether either of these two hypotheses are true cannot be determined from the present data. Regardless, this study suggests that the neurons of the IC and SOC are not equivalently affected by the aging process, which supports the hypothesis noted above that different morphological classes of neurons express PV or CB, and are distributed differently throughout the ascending auditory system. Taken together, the current data demonstrates that densities of GAD67 expressing PV and CB neurons change in the brainstem with age, which would clearly influence local and global circuit and network processing of auditory information between young and old animals. The functional consequences of these age-related changes at the brainstem and midbrain levels and how they relate to age-related hearing deficits defined at the perceptual level remain to be seen.

## Acknowledgments

# References

Ascoli GA, Alonso-Nanclares L, Anderson SA, Barrionuevo G, Benavides-Piccione R, Burkhalter A, Buzsaki G. Petilla terminology: Nomenclature of features of GABAergic interneurons of the cerebral cortex. Nat rev. 2008; 9:557–568.

Bartlett EL, Smith PH. Anatomic, intrinsic, and synaptic properties of dorsal and ventral división neurons in rat medial geniculate body. Jneurophysiol. 1999; 81:1999–2016.

Bartos M, Elgueata C. Functional characteristics of parvalbumin- and cholecystokinin- expressing basket cells. J Physiol. 2012; 590(Pt 4):669–681. [PubMed: 22250212]

Burianova J, Ouda L, Profant O, Syka J. Age-related changes in GAD levels in the central auditory system of the rat. Exp Gerontol. 2009; 44(3):161–9. [PubMed: 18930128]

Carder RK, Leclerc SS, Hendry SH. Regulation of calcium-binding protein immunoreactivity in GABA neurons of macaque primary visual cortex. Cereb Cortex. 1996; 2:271–287. [PubMed: 8670656]

Caspary DM, Milbrandt JC, Helfert RH. Central auditory aging: GABA changes in the Inferior colliculus. Exp Gerontol. 1995; 30:349–360.

Caspary DM, Holder TM, Hughes LF, Milbrandt JC, McKernan RM, Naritoku DK. Age-related changes in GABA(A) receptor subunit composition and function in rat auditory system. Neuroscience. 1999; 93:307–312. [PubMed: 10430494]

Caspary DM, Schatteman TA, Hughes LF. Age-related changes in the inhibitory response properties of dorsal cochlear nucleus output neurons: role of inhibitory inputs. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005; 25:10952–10959. [PubMed: 16306408]

Caspary DM, Hughes LF, Schatteman TA, Turner JG. Age-related changes in the response properties of cartwheel cells in rat dorsal cochlear nucleus. Hearing research. 2006; 216-217:207–215. [PubMed: 16644158]

Caspary DM, Ling L, Turner JG, Hughes LF. Inhibitory neurotransmission, plasticity and aging in the mammalian central auditory system. The Journal of experimental biology. 2008; 211:1781–1791. [PubMed: 18490394]

Cauli B, Audinat E, Lambolez B, Angulo MC, Ropert N, Tsuzuki K, Hestrin S. Molecular and physiological diversity of cortical nonpyramidal cells. J Neurosci. 1997; 17:3894–3906. [PubMed: 9133407]

Cicchitti F, Prensa L, Wu Y, Parent A. Chemical anatomy of striatal interneurons in normal individuals and in patients with Huntington's disease. Brain Res Rev. 2000; 34(1-2):80–101. [PubMed: 11086188]

Davis, RT.; Leathers, CW. Behavior and Pathology of Aging in Rhesus Monkeys. A.R Liss; New York: 1985.

Engle JR, Gray DT, Turner H, Udell J, Recanzone GH. Age- related neurochemical changes in the rhesus macaque inferior colliculus. Front Aging Neurosci Front Aging Neurosci. 2014; 6:73.10.3389/fnagi.2014.00073

Engle JR, Recanzone GH. Characterizing spatial tuning functions of neurons in the auditory cortex of young and aged monkeys: A new perspective on old data. Frontiers Aging Res. 2012; 4:36.10.3389/fnagi.2012.00036

Engle JR, Tinling S, Recanzone GH. Age-related hearing loss in rhesus monkeys is correlated with cochlear histopathologies. PloS One. 2013; 8(2):e550902.

Fetoni AR, Picciotti PM, Paludetti G, Troiani D. Pathogenesis of presbycusis in animal models: a review. Experimental gerontology. 2011; 46:413–425. [PubMed: 21211561]

Fredrich M, Reisch A. Neuronal subtype identity in the rat auditory brainstem as defined by molecular profile and axonal projection. Exp Brain res. 2009; 195:241–260. [PubMed: 19340418]

Gray DT, Rudolph M, Engle JR, Recanzone GH. Parvalbumin increases in the medial and lateral geniculate nuclei of aged rhesus macaques. Front Aging Neurosci. 2013; 8(5):69.10.3389/fnagi. 2013.00069 [PubMed: 24265617]

Gray DT, Engle JR, Recanzone GH. Age-related neurochemical changes in the rhesus macaque superior olivary complex. J Comp Neurol. 2014a; 522:573–591.10.1002/cne.23427 [PubMed: 25232570]

Gray DT, Engle JR, Recanzone GH. Age-related neurochemical changes in the rhesus macaque cochlear nucleus. J Comp Neurol. 2014b; 522:1527–1541.10.1002/cne.23479 [PubMed: 24127432]

Gibson JR, Beierlein M, Connors BW. Two networks of electrically coupled inhibitory neurons in neocortex. Nature. 1999; 402:75–79. [PubMed: 10573419]

Grothe B, Koch U. Dynamics of binaural processing in the mammalian sound localization pathway— the role of GABA(B) receptors. Hear Res. 2011; 279(1-2):43–50.10.1016/j.heares.2011.03.013 [PubMed: 21447375]

Idrizbegovic E, Bogdanovic N, Viberg A, Canlon B. Auditory peripheral influences on calcium binding protein immunoreactivity in the cochlear nucleus during aging in the C57BL/6J mouse. Hear Res. 2003; 179(1-2):33–42. [PubMed: 12742236]

Idrizbegovic E, Bogdanovic N, Willott JF, Canlon B. Age-related increases in calcium-binding protein immunoreactivity in the cochlear nucleus of hearing impaired C57BL/6J mice. Neurobiol Aging. 2004 Sep; 25(8):1085–93. [PubMed: 15212833]

Idrizbegovic E, Salman H, Niu X, Canlon B. Presbyacusis and calcium-binding protein immunoreactivity in the cochlear nucleus of BALB/c mice. Hear Res. 2006; 216-217:198–206. [PubMed: 16874908]

Irving R, Harrison JM. The superior olivary complex and audition: A comparitive study. J Comp Neurol. 1967; 130:77–86. [PubMed: 4962091]

Ito T, Bishop DC, Oliver DL. Two Classes of GABAergic Neurons in the Inferior Colliculus. J Neurosci. 2010; 29(44):13860. 2009 November 4. 10.1523/JNEUROSCI.3454-09.2009 [PubMed: 19889997]

Jones EG. Chemically defined parallel pathways in the monkey auditory system. Ann NY Acad Sci. 2003; 999:218–233. [PubMed: 14681146]

Juarez-Salinas DL, Engle JR, Navarro XO, Recanzone GH. Hierarchical and serial processing in the spatial auditory cortical pathway is degraded by natural aging. J Neurosci. 2010; 30(44):14795– 804. 2010 3. doi: 10.1523. [PubMed: 21048138]

Kawaguchi Y, Katsumaru H, Hosaka T, Heizmann CW, Hama K. Fast spiking cells in rat hippocampus (CA1 region) contain the calcium-binding protein parvalbumin. Brain Res. 1987; 416:369–374. [PubMed: 3304536]

Kawaguchi Y, Kubota Y. GABAergic cell subtypes and their synaptic connections in rat frontal cortex. Cereb Cortex. 1997; 7:476–486. [PubMed: 9276173]

Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science. 2008; 321:53–57. [PubMed: 18599766]

Manev H, Favaron M, Guidotti A, Costa E. Delayed increase of Ca2+ influx elicited by glutamate: role in neuronal death. Molecular Pharmacoloy. 1989; 36(1):106–112.

Moore DR. Anatomy and physiology of binaural hearing. Audiology. 1991; 30(3):125–34. 1991. [PubMed: 1953442]

Nelson EG, Hinojosa R. Presbycusis: a human temporal bone study of individuals with downward sloping audiometric patterns of hearing loss and review of the literature. The Laryngoscope. 2006; 116:1–12. [PubMed: 16946668]

Oliver, DL. Neuronal organization in the inferior colliculus. In: Winer, JA.; Schreiner, CE., editors. The Inferior Colliculus. New York: Springer- Verlang; 2005.

O'Neill WE, Zettel ML, Whittemore KR, Frisina RD. Calbindin D-28k immunoreactivity in the medial nucleus of the trapezoid body declines with age in C57BL/6, but not CBA/CaJ, mice. Hearing research. 1997; 112:158–166. [PubMed: 9367238]

Ouda L, Druga R, Syka J. Changes in parvalbumin immunoreactivity with aging in the central auditory system of the rat. Exp Gerontol. 2008; 43:782–789. [PubMed: 18486384]

Ouda L, Burianova J, Syka J. Age-related changes in calbindin and calretinin immunoreactivity in the central auditory system of the rat. Exp Geront. 2012; 47:497–506.

Parent A, Fortin M, Côté PY, Cicchetti F. Calcium-binding proteins in primate basal ganglia. Neurosci Res. 1996; 25(4):309–334. [PubMed: 8866512]

Rudy B, Fishell G, Lee S, Hjerling-Leffler J. Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. Devel Neurobio. 2011; 71:45–61.

Parvizi J, Damasio AR. Differential distribution of calbindin D28k and parvalbumin among functionally distinctive sets of structures in the macaque brainstem. The Journal of comparative neurology. 2003; 462(2):153–167. [PubMed: 12794740]

Pollak GD, Burger RM, Park TJ, Klug A, Bauer EE. Roles of inhibition for transforming binaural properties in the brainstem auditory system. Hear Res. 2002; 168(1-2):60–78. 2002 Jun. [PubMed: 12117510]

Prensa L, Giménez-Amaya JM, Parent A. Morphological features of neurons containing calcium-binding proteins in the human striatum. Journal of Comparitive Neurology. 1998; 390:552–563.

Schuknecht HF. Presbycusis. The Laryngoscope. 1955; 65:402–419. [PubMed: 14392966]

Schuknecht HF. Further Observations on the Pathology of Presbycusis. Archives of otolaryngology. 1964; 80:369–382. [PubMed: 14198699]

Schuknecht HF, Gacek MR. Cochlear pathology in presbycusis. The Annals of otology, rhinology, and laryngology. 1993; 102:1–16.

Strominger NL, Hurwitz JL. Anatomical aspects of the superior olivary complex. J Comp Neurol. 1976; 170:485–497. [PubMed: 826550]

Winer JA, Larue DT. Evolution of GABAergic circuitry in the mammalian medial geniculate body. ProcNatlAcadSciUSA. 1996; 93:3083–3087.

Xu X, Callaway EM. Laminar specificity of functional input to distinct types of inhibitory cortical neurons. J Neurosci. 2009; 29:70–85. [PubMed: 19129386]

Zettel ML, Frisina RD, Haider SE, O'Neill WE. Age-related changes in calbindin D-28k and calretinin immunoreactivity in the inferior colliculus of CBA/CaJ and C57Bl/6 mice. J Comp Neurol. 1997; 386:92–110. [PubMed: 9303527]
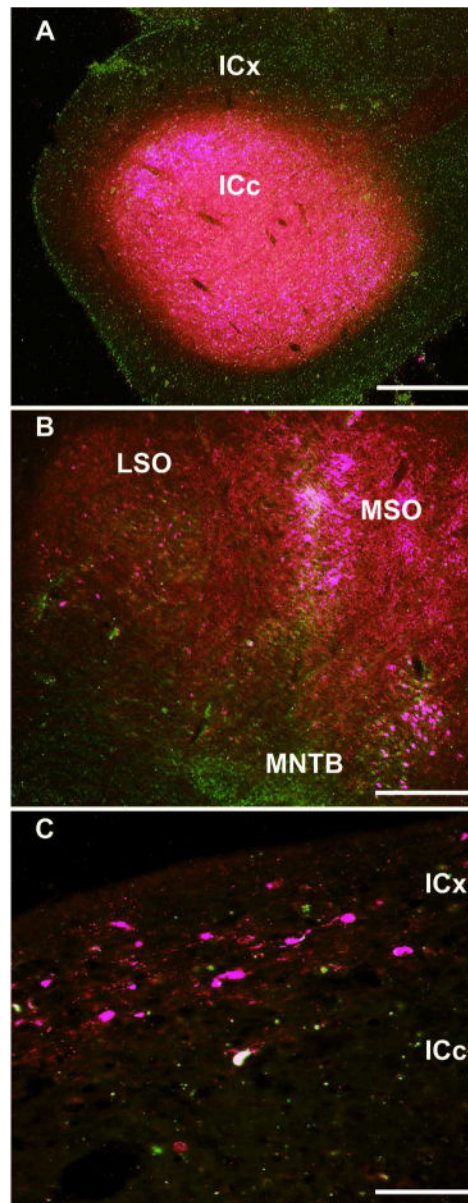
**Figure 1.**
Transverse sections of the auditory midbrain and brainstem. A) PV (magenta) and GAD67 (green) co-labelled section of the inferior colliculus. The two principle subdivisions, the core (ICc) and cortex region (ICx) are labelled. Scale bar = 500 μm. B) PV (magenta) and GAD67 (green) co-labelled section of the superior olivary complex. The medial superior olive (MSO), lateral superior olive (LSO), and medial nucleus of the trapezoid body (MNTB) are labelled. Scale bar = 500 μm. C) The CB (magenta)-rich Icx region, co-labelled with GAD67 (green) of the inferior colliculus. Scale bar = 250 μm.
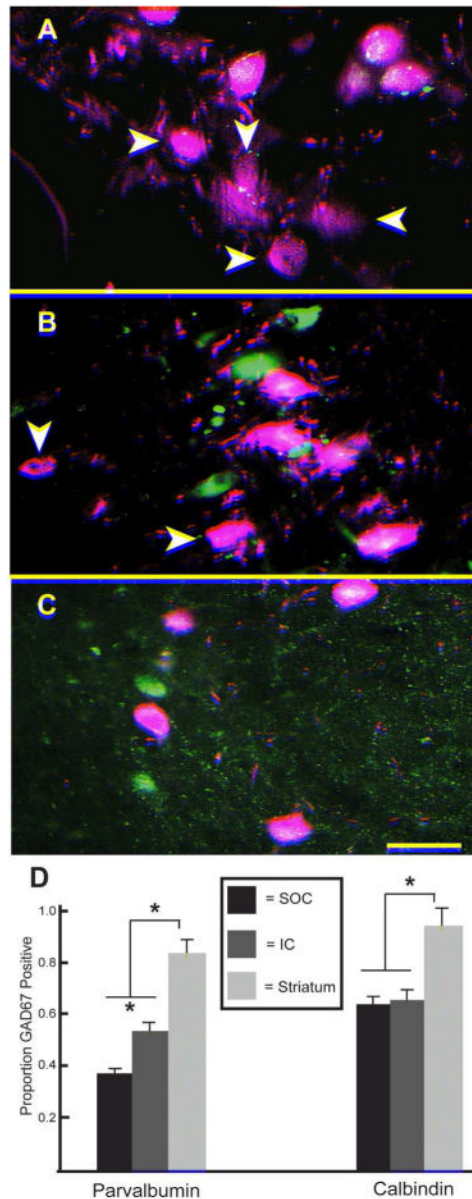
**Figure 2.**
Proportion of sampled PV positive and CB positive neurons expressing GAD67 in the IC and SOC. Neurons containing a significant amount of both PV (magenta) and GAD67 (green) resulted with white label in the co-registered images, and these neurons were considered GAD67 expressing PV positive neurons. The same analysis was carried out for CB positive neurons (data not shown).White arrows demarcate PV neurons that did not contain sufficient GAD67 label to be considered GAD67 positive. In the A) SOC, B) IC, and C) striatum there appeared to be differences in the proportion of PV and CB expressing neurons that expressed GAD67. Quantifications revealed that the proportion of GAD67 immunoreactive PV and CB positive neurons in the superior olivary complex (SOC), inferior colliculus (IC), and striatum indeed differed. D) In the SOC, 38 percent of PV-positive neurons expressed GAD67, whereas 52 percent were GAD67 positive in the IC.

Both of these percentages were significantly less than the 82 percent of PV-positive neurons expressing GAD67 in the striatum. Conversely, the GAD67 expression of CB-positive neurons did not differ between the SOC and IC, but both were significantly less than the 93 percent GAD67 expression of CB-positive neurons in the striatum.
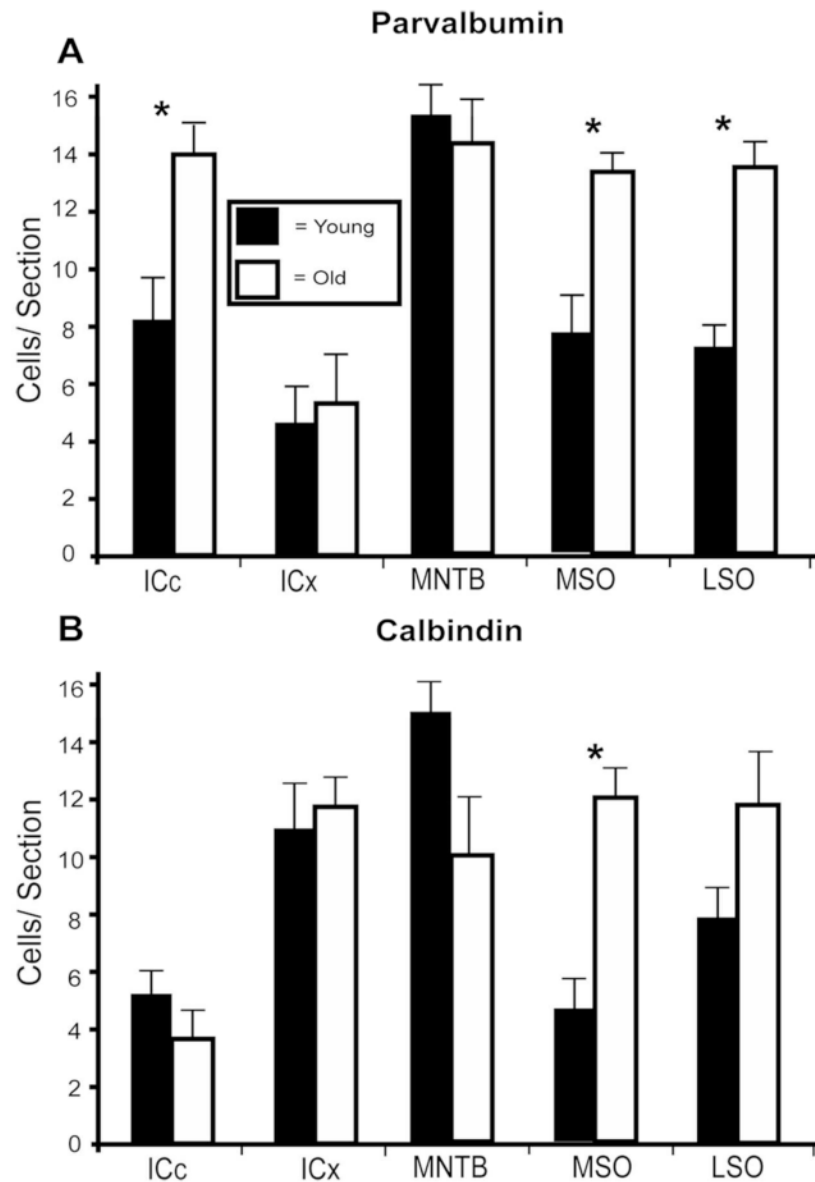
**Figure 3.**
Age-related changes in PV- and CB- expressing neurons. The average number of cells expressing PV (A) and CB (B) in the young animals (black bars) differed compared to old animals (open bars). Statistically significant differences in PV+ neurons were not seen in the medial nucleus of the trapezoid body (MNTB) or cortex region of the inferior colliculus (ICx), but were observed in the medial superior olive (MSO), lateral superior olive (LSO), and core region of the inferior colliculus (ICc). For CB+ neurons, only the MSO showed an age-related difference.
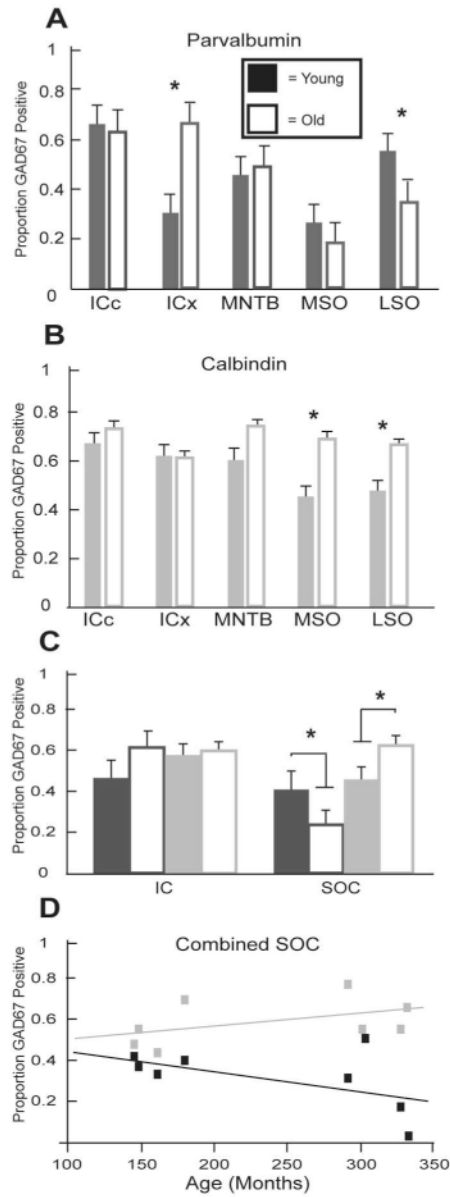
**Figure 4.**

Age-related changes in proportion of GAD67 expressing PVPV and CB- positive neurons in the inferior colliculus (IC) and superior olivary complex (SOC). A) The proportion of GAD67 expressing PV-positive neurons stayed constant with age in the ICc, but increased in the ICx. In the SOC, only the lateral superior olive (LSO) showed significant decreases in the proportion of PV positive neurons expressing GAD67. B) The proportion of GAD67 expressing CB-positive neurons stayed constant with age in the IC, and decreased only in the medial superior olive (MSO) of the SOC. C) When the subdivisions of the IC and SOC were combined and compared across age groups, the proportion GAD67 expressing PV- and CB-positive neurons remained constant, whereas significantly more PV-positive neurons and less CB-positive neurons expressed GAD67 in the SOC. D) Scatter plots of age against

the proportion of GAD67 expressing cells shows the considerable variability across animals in these age-related changes.

**Table 1**

## List of abbreviations

| | |
|---|---|
| PV | parvalbumin |
| CB | calbindin |
| CaBP | calcium binding protein |
| GABA | gamma-Aminobutyric acid |
| SOC | superior olivary complex |
| GAD | glutamic acid decarboxylase |
| IC | inferior colliculus |
| ICc | inferior colliculus, central nucleus |
| ICx | inferior colliculus, cortex |
| MSO | Medial superior olive |
| LSO | Lateral superior olive |
| MNTB | Medial nucleus of the trapezoid body |
| DAPI | 4′,6-diamidino-2-phenylindole |

**Table 2**

Demographics and methods used on monkeys.

| Age(mo) | Human Age(yr) | Gender | Thickness (μm) | Age Group |
|---|---|---|---|---|
| 146 | 36.5 | Male | 25 | Middle-aged |
| 148 | 37 | Male | 30 | Middle-aged |
| 161 | 40.25 | Female | 30 | Middle-Aged |
| 180 | 45 | Female | 25 | Middle-Aged |
| 291 | 72.75 | Male | 25 | Old |
| 302 | 75.5 | Male | 25 | Old |
| 327 | 81.75 | Male | 30 | Old |
| 331 | 82.75 | Female | 30 | Old |

**Table 3**

Information on the antibodies immunofluorescent reactions.

| Antibody/Chemical | Working Concentration (By Volume) | Immunogen Structure/Sequence | Manufacturer/Log #/ Other Informatiion |
|---|---|---|---|
| Anti-Parvalbumin | 0.05 % | PARV-19 hybridoma | Sigma-Aldrich, P3088; Monoclonal; RRIDAB_477329 Raised in Mouse |
| Anti-Calbindin | 0.05 % | CB-955 | Sigma-Aldrich, C9848; Monoclonal; RRID:AB_476894 Raised in Mouse |
| Anti-GAD1 | 0.1% | aa 17-21(A-D-P-N-T) | Sigma-Aldrich, SAB4300642; RRID: AB_11129033 Raised in Rabbit |
| Alexa Fluor 488 Donkey anti-Rabbit IgG | 0.4% | Rabbit IgG | Life Technologies; A-21203; RRID: AB_105635586 Raised in Donkey |
| Alexa Fluor 594 Donkey anti-Mouse IgG | 0.4% | Mouse IgG | Life Technologies; A-21206; RRID: AB_100496503 Raised in Donkey |
| Normal Horse Serum | 3% | N/A | Vector Labs; S-2000 |