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Dissociation of doublecortin expression and neurogenesis in unipolar brush cells in the vestibulocerebellum and dorsal cochlear nucleus of the adult rat

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Abstract

We have previously shown expression of the protein doublecortin (DCX) in unipolar brush cells (UBCs) in the dorsal cochlear nucleus and vestibulocerebellum of the adult rat. We also saw DCX-immunoreactive elements with the appearance of neuroblasts around the fourth ventricle. Expression of DCX is seen in newborn and migrating neurons and hence considered a correlate of neurogenesis. There were two interpretations of the expression of DCX in UBCs. One possibility is that there might be adult neurogenesis of this cell population. Adult neurogenesis is now well-established, but only for the dentate gyrus of the hippocampus and the subventricular zone. The other possibility is that there is prolonged expression of DCX in adult UBCs that may signal a unique role in plasticity of these neurons. We tested the neurogenesis hypothesis by systemic injections of BrdU, a thymidine analogue, followed by immunohistochemistry to examine the numbers and locations of dividing cells. We used several different injection paradigms, varying the dose of BrdU, the number of injections and the survival time to assess the possibility of neuronal birth and migration. We saw BrdU-labeled cells in the cerebellum and brainstem; cell division in these regions was confirmed by immunohistochemistry for the protein Ki67. However, neither the numbers nor the distribution of labeled nuclei support the idea of adult neurogenesis and migration of UBCs. The function of DCX expression in UBC's in the adult remains to be understood.

Introduction

We have described the expression of the protein doublecortin (DCX) in unipolar brush cells (UBCs) of the vestibulocerebellum and dorsal cochlear nucleus (DCN) in the adult rat (reviews in Manohar et al., 2012). The distribution of the DCX-ir UBCs was similar to the

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overall distribution of UBCs in the rat cerebellum and DCN as shown by Mugnaini (Floris et al., 1994, Sekerkova et al., 2007, Diño and Mugnaini, 2008). This was an intriguing finding since DCX expression has been seen primarily in newborn and migrating neurons, and is usually considered an indicator of neurogenesis (Francis et al., 1999, Gleeson et al., 1999, Brown et al., 2003, Tanaka et al., 2004, Couillard-Despres et al., 2005). Further, we saw DCX-ir profiles around the fourth ventricle; these profiles resembled neuroblasts, suggesting a neurogenic zone around the fourth ventricle (see Fig. 12 in Manohar et al. 2012). The idea of adult neurogenesis in the brainstem is supported by several studies that showed evidence of “reactive neurogenesis” in the brainstem following vestibular damage (Dutheil et al., 2009, Dutheil et al., 2011b, a).

The idea of adult neurogenesis of neurons in the DCN, vestibular brainstem or cerebellum, however, does not align with the many studies that have established only two sites of adult neurogenesis in the normal rodent, the dentate gyrus of the hippocampus and the subventricular zone (Bayer, 1982, Bayer et al., 1982, Gould and Cameron, 1996, Cameron and McKay, 2001, Dayer et al., 2003, Ming and Song, 2005, Gould, 2007).

In order to investigate the possibility of adult neurogenesis of UBCs we turned to another technique, the systemic injection of the thymidine analogue bromodeoxyuridine, BrdU, to label dividing cells (Leuner et al., 2009). Our hypothesis, based on the pattern of label with DCX, was that neurons destined to become UBCs were born around the fourth ventricle and then migrated to the vestibulocerebellum or DCN, the regions in which we had seen DCX-ir UBCs. To test the migration hypothesis, we used several different delays between the injections of BrdU and the day of sacrifice. If the migration hypothesis were correct, the distribution of BrdU labeled cells should change with the delay between injections and sacrifice. The hypothesis predicted that there would be BrdU-labeled neurons around the ventricle at short delays and that these would be displaced to the regions in which DCX-ir UBCs were found with longer delays. We also looked for neurons double-labeled with DCX and BrdU, as would be expected if the DCX-ir UBCs included adult-born neurons. Our results, however, do not support the hypothesis that there is adult neurogenesis of UBCs.

Experimental Procedures

Animals

We used adult (age 3-5 months) male, albino SASCO Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA). We followed the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University at Buffalo. All animals had ad lib. access to water and standard laboratory rodent chow. They were housed individually and maintained on a 12 hour light-dark cycle.

BrdU injections

We treated 3 groups of rats with injections of BrdU (i.p.). We used three different injection protocols designed to answer three major questions: the locus of newborn cells, the

possibility of migration of newborn cells and the survival of newborn cells. Group 1: One injection of 150 mg/kg BrdU, sacrifice 1 day after the injection (animals 401, 402, 403). This protocol should show labeled cells near the locus of cell division. Group 2: Twelve days of once daily injections of 50 mg/kg BrdU, sacrifice 23 days after the last BrdU injection (animals 404, 405, 406). This protocol allows time for migration of newborn cells so that a different distribution of labeled cells would be expected compared to group 1 if the migration hypothesis is correct. The delay however, is also long enough to allow for cell death/BrdU dilution should those be occurring. Group 3: Seven days of once daily injections of 50 mg/kg BrdU, sacrifice 1 day after the last injection (animals 727, 728). Our hypothesis was that this group should show labeled cells around as well as slightly displaced from the fourth ventricle. These groups are summarized in Table 1.

Tissue preparation

Rats were deeply anesthetized with 86 mg/kg i.p. of Fatal-plus (Vortech, Pharmaceutical Ltd, Dearborn, MI) and perfused through the heart with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed, divided into blocks just rostral to the cerebellum in the coronal plane and postfixed in 4% PFA, for 24 hours-1 week.

The blocks were then cryoprotected in 15% and then 30% sucrose in PBS. Forty μ m coronal sections were cut on a cryostat; every section was collected. Sections were stored in tissue culture plates in a cryoprotectant solution (30% ethylene glycol and 30% glycerol in phosphate buffer) at -20° C.

Immunohistochemistry

All tissue-processing was done on free-floating sections.

BrdU-DAB visualization—For each animal, we processed a set of sections about 600 μ m apart from the caudal medulla beginning approximately at Bregma -12.80 (Paxinos and Watson, 1986) to about Bregma -9.8 rostrally. This region includes the DCN and the part of the vestibulocerebellum in which DCX-ir UBCs were seen (Manohar et al., 2012). Sections were removed from the cryoprotection solution and rinsed in phosphate buffered saline (PBS). DNA was denatured by incubating the sections in 2N hydrochloric acid (2N HCl) for 30 minutes at 50° C. Sections were rinsed again and incubated in a blocking solution of 1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 1.5% normal horse serum (NHS, Vector Laboratories, Burlingame CA), and 0.2% TritonX-100 (TX; Sigma, St. Louis, MO) in PBS for 30 min. The primary antibody, mouse anti-BrdU (Becton Dickinson, Catalogue #347580, 1:200) was added to the blocking solution and sections incubated at 4° C overnight on a tissue rocker. Further processing was with a Vector Elite ABC kit (mouse IgG), according to manufacturer's instructions. Immunoreactivity was visualized by incubating sections in a solution of 3,3'-diaminobenzidine (DAB, Sigma) with .0015-.003% H₂O₂ in 0.01M PBS. Sections were mounted on Fisher "Superfrost" slides (Fisher Scientific, Pittsburgh, PA), dried at room temperature overnight, dehydrated in ethanol, cleared in HistoSol (National Diagnostics, Atlanta, Georgia) and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

DCX single label, DAB-GO visualization—We used the same protocols described in the earlier report (Manohar et al., 2012). Sections were removed from the cryoprotectant, rinsed in PBS, and nonspecific-binding was blocked by incubation in a solution of PBS, 1% BSA, 1.5% normal rabbit serum (Vector Laboratories), 0.2% TritonX-100. Sections were then incubated overnight on a rocker at 4° C in the blocking solution with the addition of the primary antibody, goat anti-DCX (Santa Cruz Biotechnology, SCBT, # sc-8066, 1:500). Sections were then rinsed and incubated in the appropriate biotinylated secondary antibody (Vector Laboratories, following manufacturer's instructions); further processing was with the Vector ABC method using a Vectastain kit. Immunoreactivity was visualized using the glucose oxidase (GO) modification of the diaminobenzidine (DAB) method (Shu et al., 1988).

BrdU single-label immunofluorescence—For single-label immunofluorescence, sections were blocked and incubated overnight in the primary antibody, then rinsed and incubated in a fluorescent secondary antibody, donkey anti-mouse Alexa 488, (Molecular Probes, Life Technologies, Grand Island, NY) 2 µg/ml, with 1% BSA, 0.2% TX in 0.1 M PBS for 1-2 hours in the dark. Sections were rinsed, mounted on Fisher Scientific "Superfrost" slides and coverslipped with Vector Laboratories Vectashield HardSet mounting medium.

BrdU and DCX, double-label immunofluorescence—We used double-label immunofluorescence to see if BrdU-ir cells also expressed DCX. We used both sequential and simultaneous incubation protocols in different experiments. For the simultaneous incubations, after denaturing DNA and blocking, both primary antibodies (mouse anti-BrdU, 1:200, and goat anti-DCX, 1:500, SCBT, #sc-8066) were added to the blocking solution and the sections incubated overnight in that solution. They were then rinsed and incubated in a cocktail containing two secondary fluorescent antibodies (donkey anti-mouse Alexa 488, 2 µg/ml and donkey anti-goat Alexa 594, or Alexa 568, 2 µg/ml). For the sequential incubations, on the first day after denaturation and blocking sections were incubated overnight in the first antibody. The next day they were rinsed and incubated in the appropriate secondary antibody. Sections were rinsed again and incubated in the next primary antibody overnight and then with the appropriate secondary antibody and finally mounted and coverslipped as above.

Ki67 single label, DAB-GO visualization—Sections were removed from the cryoprotectant, rinsed in PBS, and nonspecific-binding was blocked by incubation in a solution of PBS, 1% BSA, 1.5% normal goat serum (Vector Laboratories), and 1.0% TritonX-100. Sections were then incubated overnight on a rocker at 4° C in the blocking solution with the addition of the Ki67 primary antibody, 1:2000 (Novocastra rabbit polyclonal antibody to Ki67, code NCL-Ki67p). Sections were then rinsed and incubated in anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame CA, following manufacturer's instructions); further processing was with the Vector ABC method using a Vectastain kit. Immunoreactivity was visualized using the glucose oxidase (GO) modification of the diaminobenzidine (DAB) method (Shu et al., 1988).

Data analysis and photography

The DAB and DAB-GO sections were examined with a Leitz Dialux 20 light microscope. Digital images were captured using a SPOT Insight Color Mosaic camera (1200 x 1600 pixels) mounted on either the Leitz microscope or on a Wild Makroskop M420 microscope. Contrast and brightness of images were adjusted with Adobe Photoshop. In order to compare the distributions of BrdU, Ki67 and DCX-labeled cells we used the MDPlot system (Accustage, Shoreview, MN) to plot the distributions of labeled cells. Microscope stage position encoders were mounted on a Leitz Dialux 20 microscope. Using MDPlot software, we drew an outline of the brainstem and cerebellum and plotted the locations of labeled cells. An image of an outline drawing is shown in Figure 1. Plots showing both the section outlines and the locations of labeled cells are shown in Figures 2, 3A, 7A and 7B. For the BrdU and Ki67 plots, one dot represents one labeled nucleus; examples of labeled nuclei are shown in Figs. 1 (BrdU) and 6 (Ki67). For the DCX plot, one dot represents one labeled UBC soma. Examples of DCX-ir UBCs are shown in Figure 3, B, C, D. The immunofluorescent sections were examined with a Zeiss Axioimager fluorescence microscope and images captured with Zeiss software. All images were exported as tiff files and adjustment of brightness and contrast and assembling of plates was done with Adobe Photoshop software (Adobe Systems Inc., San Jose CA).

Results

The results are based on eight rats in three different groups (Table 1). In all cases, there were BrdU-labeled cells scattered throughout the brainstem and cerebellum. The number of such cells, however, varied dramatically with the injection paradigm. Figure 1 shows photomicrographs of examples of BrdU-labeled cells on an outline drawing of a section from Rat 728 (Group 3, 7 injections on 7 consecutive days, survival 24 hours after the last injection). The numbers 1-5 on the outline drawing indicate the locations of the cells shown in the five different insets. The BrdU-labeled profiles were round or oval and often found in pairs. In the cerebellar cortex many were found very close to the outer edge of the cerebellum (in the molecular layer, examples in panels 1 and 5). Figure 2 shows the distribution of all BrdU-ir profiles on outline drawings of the cerebellum and brainstem on sections from 3 animals, one from each group. The sections are all from about the same Bregma level, and allow comparison of the different numbers of labeled cells resulting from the different injection paradigms. Figure 2A shows the total distribution of all BrdU-labeled cells on a section from Rat 728 (Group 3, 7 injections, 1 day survival time; this is the same section for which examples of BrdU-ir cells are shown in Figure 1). Note that the labeled cells are scattered throughout the cerebellum and brainstem and not preferentially located along the ventricle. Many are located along the ventral border of the brainstem. Animals in this group had the greatest density of labeled cells at all rostrocaudal levels. Figure 2B shows a plot of all labeled cells on a section at about the same level from Rat 401 (Group 1, single injection, one day survival time). There are many fewer cells than the section shown in Fig. 2A, but the overall distribution is similar. Finally, Figure 2C shows that there are only a few labeled cells in Rat 404, (Group 2, 12 injections, 23 day survival time).

These results did not support the hypothesis that DCX-ir UBCs are newly-born neurons. In order to be sure that such DCX-ir neurons were detectable in the BrdU-injected animals, we also processed sections from these animals for DCX immunoreactivity. Figure 3 shows DCX-ir neurons on a section from rat 406 (Group 2, 12 injections, 23 day survival). Figure 3A shows a plot of the distribution of DCX-ir UBCs in a section containing the dorsal cochlear nucleus (DCN) and transition zone (tz), the region between the flocculus and paraflocculus in which there were many DCX-ir neurons (Manohar et al., 2012). Figure 3B is a photomicrograph of the tz and DCN on the left side of the section. Figure 3C is a higher magnification image of the tz on the contralateral side of the brain; at this magnification many labeled cells clearly resemble UBCs with a stained soma and a single dendritic stalk ending in a “brush”. Labeled elements that appear to be astrocytes (arrowhead) are also scattered through the tissue (only UBCs were plotted in Fig. 3A). Figure 3D shows DCX-ir elements in the ventricle near cerebellar lobule 1. Sections processed for BrdU-ir from this case were similar to the one illustrated in Fig. 2C, with very few BrdU-ir cells. These data show that there is robust DCX expression in UBCs in animals that had undergone BrdU injections.

There are many different protocols for immunohistochemical detection of BrdU and the results from different protocols may be very different (Leuner et al., 2009). In order to confirm that our immunohistochemistry protocols were adequate for labeling dividing cells, we also processed sections from the dentate gyrus of the hippocampus and the subventricular zone, confirmed sites of adult neurogenesis (Gould, 2007). Figure 4 shows sections through the dentate gyrus (Fig. 4A) of Rat 402 (Group 1, 1 injection, 1 day survival time) and the subventricular zone of Rat 403 (Group 1; Fig. 4B). BrdU-ir cells (arrows) are seen in both of these neurogenic zones. Figure 5 shows a section through the dentate gyrus of the hippocampus that was double-labeled for BrdU and DCX. Figure 5A shows a green BrdU-ir profile, Figure 5B shows that the same element is DCX-ir, and the colocalization of the two proteins is confirmed in the merged image (Fig. 5C). These results reassured us first that if new neurons were being born around the fourth ventricle, our methods would have been able to detect them, and second, we also would have been able to detect colocalization of DCX and BrdU if that were occurring in the cerebellum and DCN.

Our data with BrdU showed labeled cells scattered throughout the cerebellum and brainstem. In order to confirm the presence of dividing cells in the cerebellum and brainstem we used an additional technique, immunohistochemistry for the protein Ki67. Ki67 is expressed during all phases of the cell cycle but not in resting cells (Scholzen and Gerdes, 2000) and has been shown to be a marker for neurogenesis in the hippocampus (Kee et al., 2002). We saw scattered Ki67-ir cells in the cerebellum and brainstem. Figure 6A shows a low power photomicrograph of the cerebellar cortex of rat 401 with scattered Ki67-ir nuclei (examples at arrows). The panels on the right show higher magnification images of three cells (1, 2, 3). Figure 6B shows scattered Ki67 nuclei in the DCN (examples at arrows); those examples are shown at higher magnification in the three panels on the right (cells 4, 5, 6). Figure 7A shows the total distribution of Ki67-ir cells on the section illustrated in Figure 6; Figure 7B shows the distribution of Ki67-ir cells on a section at about the same Bregma level from a second animal, Rat 728.

DISCUSSION

We consistently found BrdU-ir cells in the brainstem and cerebellum of the adult rat. The numbers of these cells varied with the number of BrdU injections and the delay between those injections and sacrifice of the animals. In animals with a single BrdU injection and a one day survival time (Group 1, Figure 2B) there were a few labeled cells. In contrast, in the animals in Group 3 (7 injections for 7 days, survival one day after the last injection) there were many more labeled cells (Fig. 2A) and almost none in Group 2 (12 injections over 12 days, survival time of 23 days, Fig. 2C).

The results from the Ki67 immunohistochemistry are consistent with the BrdU results, with scattered labeled cells found in brainstem and cerebellum. These data argue strongly against our hypothesis of neurogenesis of UBCs occurring around the fourth ventricle, since the Ki67-ir cells must be located at the site of cell division. What might the labeled cells be? In brain, these markers have been used to establish adult neurogenesis as well as gliogenesis in the neurogenic zones of the dentate gyrus and subventricular zone (reviews in Gritti et al., 2002, Kempermann et al., 2004, Steiner et al., 2004, Zhao et al., 2008). However, there are data that suggest that neural stem cells/neural progenitor cells in the forebrain may exist outside of the established neurogenic zones, for example in the leptomeninges, or in the outer layers of the cerebral cortex (Bifari et al., 2009, Nakagomi et al., 2009, Ohira et al., 2010, Nakagomi et al., 2011). These cells divide in response to injury (Nakagomi et al., 2009, Ohira et al., 2010, Nakagomi et al., 2011); reactive astrocytes in cortex may also proliferate in response to injury (Shimada et al., 2012). Similar cell populations in the brainstem and cerebellum could be the source of the reactive neurogenesis seen with vestibular damage (Tighilet et al., 2007, Dutheil et al., 2009, Dutheil et al., 2011b), and might be the source of the BrdU/Ki67-ir cells we see. However it is also possible that some or all of the cells we are labeling are non-neuronal. There are several possibilities. Angiogenesis continues in the adult, and both endothelial cells and pericytes have been shown to proliferate (Sumpio et al., 2002, Winkler et al., 2011, Walchli et al., 2013). In fact, some of the labeled profiles we see are in close proximity to blood vessels (e.g. Cell 6 in Fig. 7B). Activated microglia can proliferate in the adult (Nakajima and Kohsaka, 2001, Dheen et al., 2007). Finally, there is also the possibility of proliferation of ependymal cells (Batiz et al., 2011). The labeled cells that we see could be any or a combination of all of these cell types.

There were different numbers of BrdU-labeled cells in animals sacrificed at different times after the last BrdU treatment. This could reflect limited survival time of the newborn cells. Many newborn cells originating in the dentate gyrus or subventricular zone die within days or weeks of their birth (Gould and McEwen, 1993, Gould and Cameron, 1996, Biebl et al., 2000, Winner et al., 2002, Biebl et al., 2005). The decrease in the numbers of labeled cells with longer delays could also occur if these are cells that continue to divide with resulting dilution of BrdU below the level of resolution of the visualization technique (Ganusov and De Boer, 2012).

The distribution and numbers of the BrdU-ir and Ki67-ir cells, however, clearly do not support the hypothesis there is adult neurogenesis of UBCs. The presence of DCX-ir profiles

near the fourth ventricle (Manohar et al., 2012 and in Fig. 3C) led to the hypothesis that new neurons were generated around the fourth ventricle. However, we did not see dense clusters of either BrdU-ir or Ki67 neurons along the fourth ventricle in any of the animals. We had also proposed that these neurons migrated to their final sites in the DCN and vestibulocerebellum. If that were true we would have predicted few or no BrdU-ir around the ventricle in Group 2 (12 injections and a 23 day survival time), and many in the vestibulocerebellum and DCN. Instead, there were far fewer labeled profiles overall.

The results suggest that DCX expression in UBCs is not limited to the developmental period but persists into, and perhaps continues for the entire duration, of adult life. DCX expression in the adult has been reported for other cell populations, for example in mature neurons in regions of the adult rodent piriform cortex, striatum and corpus callosum (Nacher et al., 2001, Klempin et al., 2011).

What role does DCX expression play in the life of mature neurons? One suggestion is that DCX expression can underlie plasticity in the adult neurons as well as the mechanisms of migration in the developing brain. Nacher et al. (2001) suggested that DCX might be important for functions like neurite outgrowth or synaptogenesis, phenomena that continue in the adult. One well-known form of plasticity involving the auditory system and possibly the cerebellum as well, is tinnitus, the perception of phantom sounds, often, but not always, secondary to damage to receptor cells (Jastreboff and Sasaki, 1986, Jastreboff et al., 1988, Jastreboff and Sasaki, 1994, Kaltenbach, 2006b, a, 2007, Kaltenbach, 2011). Increased DCX expression in rats with behavioral evidence of tinnitus has been reported in a recent study (Bauer et al., 2013).

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Highlights

- Does expression of DCX in adult unipolar brush cells reflect neurogenesis?
- We made BrdU injections at different doses and delays
- We found BrdU cells in cerebellum and brainstem
- Their numbers and distribution argue against neurogenesis of UBCs
- DCX may have a role in synaptic plasticity in UBCs

Figure 1. Examples of BrdU-labeled cells on a section from Rat 728, the section is at approximately Bregma -10.5. In the center is an outline drawing of the section made with MDplot software. Scale bar 2 mm. The numbers 1-5 indicate the locations of the BrdU-ir cells shown in the five insets. Panels 1-5, photomicrographs of BrdU-ir cells. Scale bars in insets 50 μ m.

Figure 2.

Densities of labeled cells as a function of injection protocol plotted on outline drawings of the brainstem and cerebellum. Each dot represents one labeled profile. All sections are at approximately Bregma -10.5. A. Rat 728. B. Rat 401. C. Rat 404. Scale bar 2 mm.

Figure 3.

DCX expression in vestibulocerebellum and DCN of Rat 406, (Group 2, 12 injections, 23 day survival). A. Plot of the distribution of labeled UBCs. Each dot represents a single cell. The letters B, C, and D indicate the sites of the photomicrographs in the 3 panels. B. Photomicrograph showing DCX-ir UBCs (example at arrow) in the DCN and transition zone (tz) on the left. Scale bar 100 μ m. C. Higher magnification image of the labeled UBCs in the tz on the right. The arrowhead indicates a labeled cell in the molecular layer. Scale bar 100 μ m. D. Labeled profiles along the ventricle near cerebellar lobule 1 (arrow). Scale bar 100 μ m.

Figure 4.

A. BrdU-ir profiles (arrow) in the dentate gyrus (dg) of the hippocampus of Rat 402 (Group 1). B. BrdU-ir profiles (arrow) in the subventricular zone of Rat 403 (Group 1).

Abbreviations dg, dentate gyrus; lv, lateral ventricle. Scale bar 50 μ m.

Figure 5.

Hippocampal neuron in Rat 727 that colocalizes BrdU (A) and DCX (B). C. The merged image shows BrdU in the nucleus and DCX in the cytoplasm and apical dendrite. Scale bar 20 μ m.

Figure 6.

A. Ki67- labeled cells in the cerebellar cortex. The arrows indicate three examples that are shown at higher magnification in the three panels on the right. Scale bar 100 μ m. Abbreviations P, Purkinje cell layer; ml, molecular layer. B. Ki67 labeled cells (examples at arrows) in the dorsal cochlear nucleus. Scale bar 100 μ m. The panels on the right show higher magnification images. Scale bar 10 μ m for cells 1-6.

Figure 7.

Distributions of Ki67-labeled cells on MDPlot outline drawings of the brainstem and cerebellum. Each dot represents one labeled profile. A. Section from Rat 401. B. Section from Rat 728. Scale bar 2 mm.

Table 1

BrdU injection groups.

Group	animal #	age	# inj.	survival	BrdU dose
Group 1	401	3 mo	1	1	150 mg/kg
	402	3 mo			
	403	3 mo			
Group 2	404	4 mo	12	23	50 mg/kg
	405	4 mo			
	406	4 mo			
Group 3	727	5 mo	7	1	50 mg/kg
	728	5 mo			