Neuroscience 265 (2014) 323–331

DISSOCIATION OF DOUBLECORTIN EXPRESSION AND NEUROGENESIS IN UNIPOLAR BRUSH CELLS IN THE VESTIBULOCEREBELLUM AND DORSAL COCHLEAR NUCLEUS OF THE ADULT RAT

N. PAOLONE,[a] S. MANOHAR,[b] S. H. HAYES,[b] K. M. WONG,[a] R. J. SALVI[b] AND J. S. BAIZER[a]*

[a] *Department of Physiology and Biophysics, School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY 14214, USA*

[b] *Center for Hearing and Deafness, University at Buffalo, Buffalo, NY 14214, USA*

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 bromodeoxyuridine (BrdU), a thymidine analog, 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.

Key words: plasticity, auditory system, tinnitus, vestibular compensation, neurogenesis, BrdU.

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 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., 2011a,b).

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 analog 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

*Corresponding author. Tel: +1-716-829-3096; fax: +1-716-829-2344.
E-mail address: baizer@buffalo.edu (J. S. Baizer).
Abbreviations: BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; DCN, dorsal cochlear nucleus; DCX, doublecortin; GO, glucose oxidase; PBS, phosphate-buffered saline; tz, transition zone; UBCs, unipolar brush cells.

http://dx.doi.org/10.1016/j.neuroscience.2014.01.026
0306-4522/

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 libitum* access to water and standard laboratory rodent chow. They were housed individually and maintained on a 12-h light–dark cycle.

BrdU injections

We treated three 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.

Table 1. BrdU injection groups

Group	Animal #	Age (months)	# inj.	Survival	BrdU dose (mg/kg)
Group 1	401	3	1	1	150
	402	3			
	403	3			
Group 2	404	4	12	23	50
	405	4			
	406	4			
Group 3	727	5	7	1	50
	728	5			

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 0.1 M 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 post-fixed in 4% PFA, for 24 h–1 week.

The blocks were then cryoprotected in 15% and then 30% sucrose in PBS. Forty μm 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 μm 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 PBS. DNA was denatured by incubating the sections in 2N hydrochloric acid (2N HCl) for 30 min 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 the manufacturer's instructions. Immunoreactivity was visualized by incubating sections in a solution of 3,3′-diaminobenzidine (DAB, Sigma) with .0015–.003% H_2O_2 in 0.01 M 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);