

Immunohistochemical study of doublecortin and nucleostemin in canine brain

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Abstract

Finding a marker of neural stem cells remains a medical research priority. It was reported that the proteins doublecortin and nucleostemin were related with stem/progenitor cells in central nervous system. The aim of the present immunohistochemical study was to evaluate the expression of these proteins and their pattern of distribution in canine brain, including age-related changes, and in non-nervous tissues. We found that doublecortin had a more specific expression pattern, related with neurogenesis and neuronal migration, while nucleostemin was expressed in most cells of almost every tissue studied. The immunolabeling of both proteins decreased with age. We may conclude that nucleostemin is not a specific marker of stem/progenitor cells in the dog. Doublecortin, however, is not an exclusive marker of neural stem cells, but also of neuronal precursors.

Introduction

In the central nervous system (CNS) of mammalian adult brain neurogenesis takes place mainly in two germinal zones: the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle wall.¹ In these germinal regions it has been demonstrated that neural stem cells (NSC), with characteristics of astrocytes, generate transit-amplifying cells which in turn divide to generate neuroblasts and glial cell precursors.^{2,3}

SVZ and SGZ astrocytes are a heterogeneous cellular population and it remains unclear whether only a subpopulation of them are NSC, or whether the neurogenic potential is latent in all astrocytes throughout the CNS, with inhibitory signals in the microenvironment preventing these cells from producing neurons.^{4,5} In this regard, the peculiar histo-

architecture of the NSC niche includes abundant cell-cell interactions, an enriched extracellular matrix, and extensive vascularity, with NSC and transit-amplifying cells contacting the vessels walls in place of astrocyte endfeet and pericytes.^{5,6} Currently, investigation of NSC is impeded by the lack of a specific marker that could allow identifying these cells and, consequently, regulating neurogenesis, as occurs after brain injury. Doublecortin (DCX) is a microtubule-associated protein⁷ implicated in neurogenesis and in the radial and tangential migration of neuroblasts during development of the brain.⁸ Some studies have shown its expression in mature neurons as well. Therefore it has been hypothesized that it could also play a role in structural plasticity such as neurite outgrowth and synaptogenesis.⁹⁻¹¹ With some differences among the investigated species, its expression has been reported in the germinal zones, neocortex, paleocortex, striatum, corpus callosum and septal nuclei.^{8,12-16}

Nucleostemin (NS) is a nucleolar protein discovered in 2002¹⁷ that belongs to the Y1qF/YawG GTPase family.¹⁸ It seems to be involved in cellular proliferation,¹⁹ maintenance of telomere length,²⁰ and ribosome biogenesis.²¹ In a pioneering study, Tsai and McKay, demonstrated that the protein was expressed in murine embryonic CNS NSC and that it was abruptly down-regulated during differentiation prior to terminal cell division.¹⁷ However, more recent investigations have shown that NS could be found even in neoplastic tissues, both rodent and human.²²⁻³⁰

To our knowledge, except for some studies to detect DCX conducted in canine hippocampus,³¹⁻³³ NS and DCX expression has not previously been described in canine tissues. Study of the protein tissue presence is the first step in the discovery of a putative stem cells marker, so the primary aim of this preliminary report was to detect these proteins by immunohistochemistry (IHC) in canine brain and to describe their distribution and age-related changes. Furthermore we evaluated expression of both markers in mature and proliferating non-nervous tissues.

Materials and Methods

Samples

Samples were obtained from the *Banc de Teixits Animals de Catalunya* and from the Veterinary Pathology Service of the *Universitat Autònoma de Barcelona* (Table 1).

For brain mapping we chose 3 dogs representing different stages of life: young (3 months), adult (5 years) and geriatric (17 years). These animals had not shown any neurological signs and the histopathological study

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Key words: nucleostemin, doublecortin, stem cells, dog brain, aging.

Contributions: EDN, collection of samples, processing, sectioning and immunohistochemical part of the work; PM-S, EB, MDP, helping in technical part; EDN, DF, evaluation of results and discussion; EDN, MP, work design, results discussion and manuscript writing.

Conflict of interests: the authors declare no conflict of interests.

Received for publication: 3 August 2012.

Accepted for publication: 18 December 2012.

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European Journal of Histochemistry 2013; 57:e9
doi:10.4081/ejh.2013.e9

of their brain had not revealed nervous lesions. Six representative transversal sections from frontal brain area to caudal medulla oblongata were selected (Tables 2 and 3). We also included other non-nervous samples and proliferating tissues such as testicle and skin samples with a process of a second intention healing showing epidermal regeneration and granulation tissue (Table 1).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections (3 µm) were deparaffinized with xylene, rehydrated in descending concentrations of ethanol, and boiled in bain-marie (96-98°C) for 20 min in citrate buffer (0.01M, pH 6.0). Endogenous peroxidase activity was suppressed with 3% H₂O₂ for 40 min. Slides were serum-blocked with normal rabbit (NS) and goat (DCX, betaIII-tubulin, NeuN, GFAP) serum for 1h. As primary antibodies we used a rabbit anti-doublecortin (Abcam, Ab18723, Cambridge, UK), diluted 1 to 1000, a goat anti-nucleostemin (R&D systems, AF1638, Abingdon, UK), diluted 1 to 100, a mouse anti-tubulin beta III isoform (Millipore, MAB1637, Darmstadt, Germany), diluted 1 to 200, a mouse anti-NeuN (Millipore, MAB377), diluted 1 to 100, and a rabbit anti-GFAP (Dako, Z0334, Denmark), diluted 1 to 3000. Immunohistochemistry was carried out with the Avidin-Biotin peroxidase Complex (ABC) method for

NS and EnVision™ for DCX, beta III-tubulin, NeuN and GFAP (Dako EnVision+ System-HRP, Denmark). As chromogen substrate 3, 3'-diaminobenzidine was used. Counterstaining was performed with hematoxylin. The negative control section from the same specimens was identically processed, replacing the primary antibody with irrelevant IgG of the same species class and concentration as the primary antibody.

Quantification and semi-quantification of data

Microscopic images were obtained with a Leica DFC 480 digital camera (Leica Microsystems Ltd., Heerbrugg, St. Gallen, Switzerland) coupled with a microscope (Leica DM6000B). For the reconstruction of the different anatomical areas, the photos were processed with the software Adobe® Photoshop® CS5.

DCX quantitative evaluation was conducted with the image processing program Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA), calculating the percentage of positive stained area for each region. The immunolabeling intensity was scored as negative (-), weak/moderate (I), or strong (II). For NS evaluation we considered the presence (+) or lack (-) of immunostaining and its intensity as weak/moderate (I) or strong (II).

Results

Doublecortin brain mapping

DCX immunostained cells (DCX⁺) appeared as irregular cells having a small cellular body and short extensions and showing a diffuse cytoplasmic granular staining (Figure 1A). DCX⁺ cells did not react against other neural markers as BetaIII-Tubulin, NeuN and GFAP (*data not shown*). The brain areas with DCX⁺ cells were found to be mainly concentrated in the cerebrum and the cerebellum. In the cerebrum the subventricular zone (SVZ) of lateral ventricles, the subgranular zone (SGZ), and granular layers of the *Gyrus dentatus* of the hippocampus showed the greatest number of DCX⁺ cells. The number of DCX⁺ cells decreased in cortical areas such as Layer II of the neocortex, where they showed a mature neuronal morphology (Figure 1B), and Layer II of the paleocortex (Figure 1C). Basal nuclei such as caudate and putamen nuclei also contained DCX⁺ cells. In the cerebellum the external germinal layer and cortical molecular layer showed the greatest number of DCX⁺ cells (Figure 1D), with their number decreasing at the level of the cortical granular layer. Scattered DCX⁺ cells were detected in thalamic nuclei. Considering the age of the dogs, the

Table 1. Animals and tissues included in the study.

Breed	Age	Sex	Sample
Maltese	3 months	Female	Brain
Miniature Schnauzer	5 years	Male	Brain
Mixed-breed	17 years	Male	Brain
Boxer	8 years	Female	Liver
Spanish Mastiff	3 years	Male	Skeletal muscle
Beagle	5 years	Male	Cardiac muscle Testicle
Beagle	8 years	Male	Granulation tissue

Table 2. Doublecortin expression and immunostain intensity in canine brain.

	Young dog	Adult dog	Geriatric dog
Cerebrum			
Frontal neocortex			
Molecular layer	Neg	Neg	Neg
Layer II	2.9% (I)	Neg	Neg
Layers III - VI	Neg	Neg	Neg
<i>Corpus callosum</i>	Neg	Neg	Neg
Corona radiata	Neg	Neg	Neg
Subventricular zone	7.82%* (II)	6.25%* (II)	1.19%* (II)
Basal nuclei			
Caudate nucleus	3.1% (I)	2% (II)	Neg
Putamen nucleus	1.7% (I)	Neg	Neg
Septal nuclei	2.1% (II-I)	6.2% (I)	Neg
Paleocortex			
Molecular layer	Neg	Neg	Neg
Layer II	17.6% (II)	0.8% (I)	Neg
Piriform lobe			
Molecular layer	Neg	Neg	Neg
Layer II	7.9% (I)	0.8% (I)	Neg
Archicortex			
Ammon's Horn	Neg	Neg	Neg
Dentate gyrus			
Subgranular layer	65.3% (I)	9.4% (II)	Neg
Granular layer	5.4% (I)	Neg	Ne
Hilus	3.9% (I)	Neg	Neg
Brain stem			
Thalamus	<5%	Neg	Neg
Midbrain	Neg	Neg	Neg
Pons	Neg	Neg	Neg
<i>Medulla oblongata</i>	Neg	Neg	Neg
Cerebellum			
Cortex			
Molecular layer	42.8% (II)	Neg	Neg
Purkinje cell layer	Neg	Neg	Neg
Granular layer	3.2% (II)	Neg	Neg
Golgi neurons	Neg	Neg	Neg
External germinal layer	100% (II)	n/a	n/a
Cerebellar nuclei	Neg	Neg	n/a
Cerebellar white matter	Neg	Neg	Neg

Neg, lack of immunostaining; (I), weak/moderate intensity; (II), strong intensity; n/a, not applicable. *Percentage of positive area compared to the total surface of subventricular zone.

number of DCX⁺ cells decreased drastically in adult and geriatric animals in all brain areas. In the adult dog, DCX⁺ cells showed a marked decrease compared to the young animal and were restricted to SVZ, SGZ (Figure 1E,F), layer II paleocortex, and caudate nuclei; unexpectedly, septal nuclei showed an increase in DCX⁺ cells. In the geriatric animal, only the SVZ maintained the presence of DCX⁺ cells.

In the SVZ of the young dog, clusters of DCX⁺ cells were observed, and we were unable to quantify the percentage of positive cells, so the portion of positive area compared to the total surface of this anatomical structure was calculated. This positivity decreased from the anterior to caudal and ventral parts of it. In the adult dog, below the SVZ, it was possible to identify clusters of DCX⁺ cells in direct contact with the vessel walls (Figure 1G).

Nucleostemin brain mapping

NS immunostained cells (NS⁺) showed clear nucleolar immunostaining. The study of NS expression did not include a quantitative analysis of positive cells because in all areas, except for the external germinal layer of the cerebellum of the young dog, almost all nucleoli appeared stained (Figure 1H).

Considering the age of the dogs, the number of NS⁺ cells decreased moderately or disappeared in adult and geriatric animals in all brain areas. Some structures of the medulla oblongata such as reticular formation and vestibular nuclei did not show any variation in NS⁺ cells. In the adult dog a similar number of NS⁺ cells, compared with the young dog, were restricted to neocortex, putamen, and cerebellar nuclei; other structures, such as paleocortex, caudate and septal nuclei, and cerebellar Purkinje and Golgi cells, showed a decrease in immunostaining. In the geriatric dog, a few scattered NS⁺ cells showing weak immunostaining were detected in the cerebrum and cerebellar Purkinje cells. In all dogs, some types of cells, such as cerebellar granules, did not express the protein.

Doublecortin and nucleostemin distribution in non-nervous tissues

No DCX immunolabeling was observed neither in mature non-nervous tissues nor proliferative tissues. Surprisingly, few DCX⁺ cells similar to Sertoli cells, were observed in the basal compartment of the seminiferous epithelium. We found NS⁺ cells in all adult canine non-proliferating tissue examined except for the skeletal muscle (skeletal muscle fibers and fibroblasts belonging to endomysium and perimysium). In the heart and liver we found positive nucleoli in cardiac myocytes and hepatocytes, respectively. In all tissues the nucleoli of endothelial cells were NS-positive. In the testicle we observed evident NS⁺ immunolabeling in the Sertoli and Leydig cells. Despite the

small size of the nucleoli, it was possible to discern NS expression in some spermatogonia and spermatocytes.

In second intention healing samples, the NS⁺ immunolabeling was found in every nucleolus of epidermal cells, fibroblasts/fibrocytes, adipocytes of hypodermis, and epithelial cells of sebaceous glands, sweat glands, and external root sheath of hair follicle. Inflammatory infiltrating cells present in this sample were also positive.

Discussion

In this preliminary study, DCX and NS immunoeexpression were evaluated in some canine tissues, focusing on nervous tissue along different ages to establish an age-related mapping. To our knowledge, previous studies have not been carried out on canine tissue for the two markers, except for DCX in the hippocampal region.³¹⁻³³

Doublecortin gave a more selective immunostaining but not limited to the neurogenic zones, as previously described in other species.⁸⁻¹¹ In our dogs, SVZ showed the strongest immunopositivity, which decreased with aging; DCX labeling decreased in this area caudally and ventrally. These results are

in agreement with previous results obtained in adult cat.⁸ In the adult dog, we noted how the DCX⁺ cells were organized around capillary vessels similar to the typical structure of stem cells vascular niche, where clusters of cells, neural stem cells and transit-amplifying cells are in direct contact with the vessel walls.^{5,6}

In the hippocampus of our dogs the decrease in immunostaining with age was evident and it was confined to the SGZ, coinciding with what was described earlier in dog.³¹⁻³³ The cerebellum of the young dog presented 100% of DCX⁺ cells in the external germinal layer, a transitory germinal zone made up of neuronal precursors that progressively migrate across the molecular layer into the inner granular layer where they differentiate into granule cells. Our results are in accordance with other previous studies carried out on young dogs.³⁴ In the adult and geriatric dogs this region was completely negative; after the disappearance of EGL, no neurogenic activity remained in the surface of the cerebellar cortex in the dog.

We found DCX immunolabeling in the neocortex of the young dog, especially in layer II, where the neuroblasts, migrating in the last steps of development, reside. These results are in agreement with other studies carried out in other animal species,^{8,13,16} but in contrast with previous findings,^{8,13} we observed this immunolabeling pattern only in the frontal

Table 3. Nucleostemin expression and immunostain intensity in canine brain.

	Young dog	Adult dog	Geriatric dog
Cerebrum			
Neocortex	+ (II)	+ (II)	+ (I)
<i>Corpus callosum</i>	+ (II)	-	-
Corona radiata	+ (II)	-	-
Subventricular zone	+ (II)	-	-
Ependymal layer	+ (II)	-	-
Basal nuclei			
Caudate nucleus	+ (II)	+ (I)	-
Putamen nucleus	+ (II)	+ (II-I)	-
Septal nuclei	+ (II)	+ (I)	+ (I)
Paleocortex			
Molecular layer	+ (II)	-	-
Layer II	+ (II)	+ (I)	+ (I)
Brain stem			
<i>Medulla oblongata</i>			
Reticular formation	+ (II)	+ (II)	+ (II-I)
Vestibular nucleus	+ (II)	+ (II)	+ (II-I)
Cerebellum			
Cortex			
Molecular layer	+ (II)	-	-
Purkinje cell layer	+ (II)	+ (I)	+ (I)
Granular layer	-	-	-
Golgi neurons	+ (II)	+ (I)	-
External germinal layer	-	n/a	n/a
Cerebellar nuclei	+ (II)	+ (II)	n/a
Cerebellar white matter	+ (II)	-	-

-/+, lack/presence of immunostaining; (I), weak/moderate intensity; (II), strong intensity; n/a, not applicable.

neocortex. These DCX⁺ cells showed a mature neuronal morphology and probably represented neuroblasts that were differentiating into GABAergic interneurons.¹³

Other brain structures such as basal ganglia, especially caudate nuclei, maintained DCX⁺ cells into adult age, including an increase in immunostaining. In the case of septal nuclei the increased number of DCX⁺ cells observed in the

adult dog was not expected, even if the intensity of their immunostaining was weak. In both cases these findings could indicate a late migration of neurons to this area.³⁵ In the young dog, we observed cells with very weak DCX immunostaining, scattered in several brain structures, including thalamic nuclei, that might represent migrating neuroblasts that were about to lose their immunolabeling.¹⁰

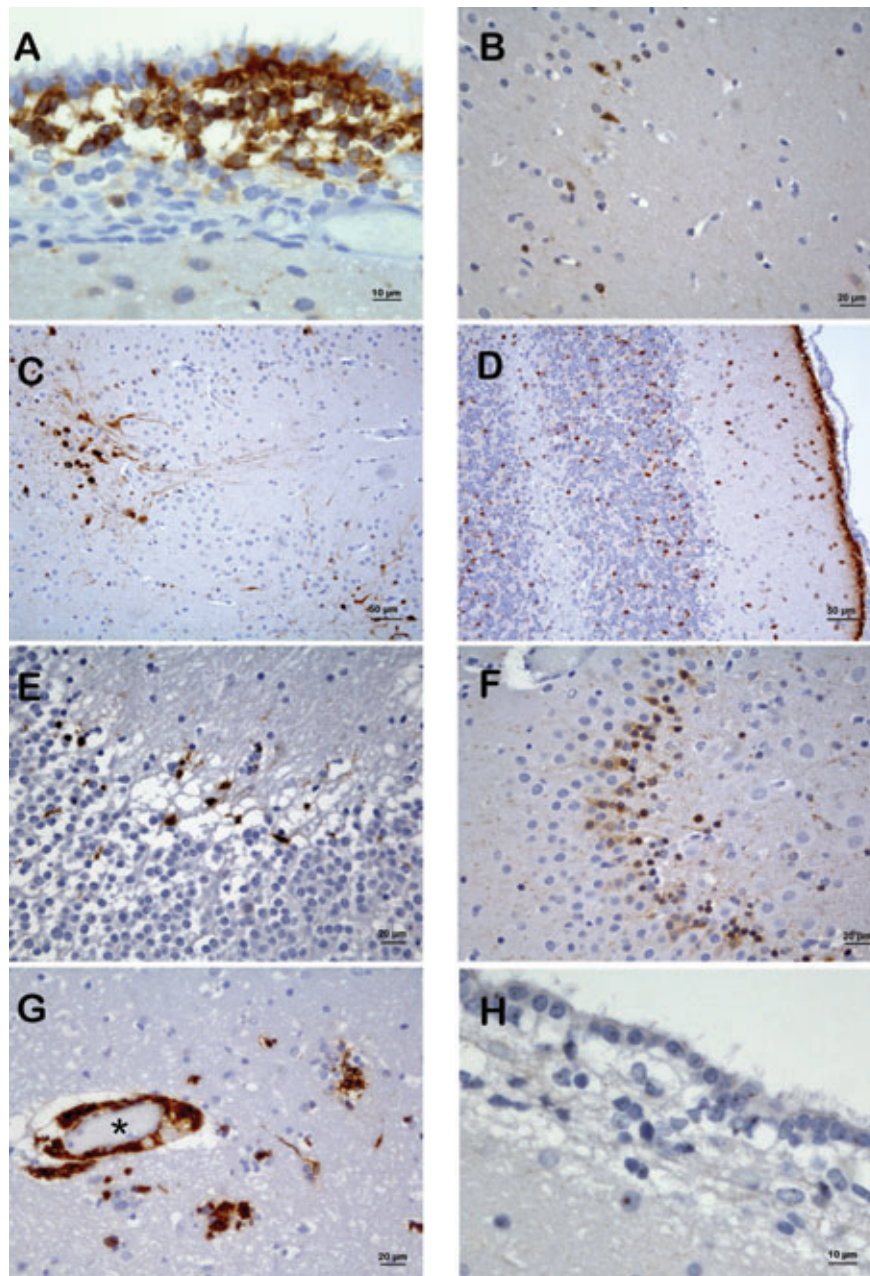


Figure 1. DCX (A-G) and NS (H) immunolabeling in brain regions. A) Magnification of DCX⁺ cells in the subventricular zone of young dog. B) Scattered neurons with mature morphology in layer II of frontal neocortex in young dog. C) DCX⁺ neurons in the paleocortex of young dog. D) Cerebellum in young dog with the 100% of EGL cells expressing DCX. E) Comparison of the dentate gyrus immunolabeling in adult dog and (F) young dog. G) Representative structure of stem cell vascular niche (*) below the SVZ in the adult dog. H) NS⁺ nucleoli in the subventricular zone of young dog.

In the case on nucleostemin we found an unexpected positivity in all brain areas in the young dog. The absence of positivity of cerebellar granules in all dogs is probably due to the inability to see the nucleoli of these neurons because of their extremely small size and the strong nuclear basophilia. Tsai and McKay¹⁷ detected NS⁺ cells in embryonic murine brain, but studies have not been performed in normal postnatal brain. NS⁺ cells decreased in number with age, with an irregular distribution along the brain. As adult neurons hold a well-developed rough endoplasmic reticulum, this positivity could be related to other functions of this protein, such as ribosome biogenesis.²¹ The physiologic reduction of protein biosynthesis with age could induce lowered expression of NS in adult and geriatric canine neurons.

In non-nervous tissue, the presence of few Sertoli-like cells DCX⁺ could be related with the presence of neural crest originated cells as described in human and rodent normal and tumoral cases.³⁶ Additional studies should be done on canine samples to determinate its specificity. We detected NS immunorexpression in the nucleoli of mature cells of other tissues, such as epithelial (epidermis and endothelia), glandular (sebaceous and sweat glands, hepatocytes and Leydig cells), and some muscle cells (cardiac myocytes and smooth cell fibers). Nucleoli of immature proliferating cells, such as epidermal basal cell layer, spermatogonia and spermatocytes, were also NS⁺. Our results are in agreement with those previously described in these tissues in other species (human, mouse and rat).^{17,23,24,37} The lack of immunostaining in canine skeletal muscle samples could be due to technical conditions, as in another published study, where, using low sensitivity techniques such as northern blot, the authors were unable to detect NS in this tissue, while with RT-PCR they could isolate its mRNA but only in small quantity.³⁸ Other NS⁺ proliferative cells detected by us on an adult canine skin biopsy with granulation tissue might be related to other recently described functions of NS, such as cellular proliferation¹⁹ and maintenance of telomere length.²⁰ Additionally, NS has been described in human and rodent neoplasms,²²⁻³⁰ as an indicator of nucleolar activity. Our findings could well corroborate all of these functions of NS in all studied canine tissues.

In conclusion, we can affirm that doublecortin expression is associated with neurogenesis and neuronal migration in canine brain, but it is not exclusive for neural stem cells. Our results showed that nucleostemin is not a specific marker of stem cells or progenitor cells in canine nervous tissue. It would be interesting to determine its role in cell proliferation and development of neoplasm. Additional studies should be carried out to clarify its role as a possible stem cell proliferation marker in canine neoplasia.

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