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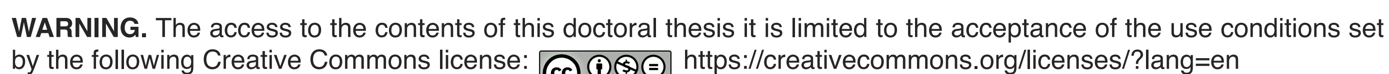

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THE CANCER STEM CELL HYPOTHESIS IN SPONTANEOUS CANINE GLIOMAS: FROM TUMORS TO NEUROGENESIS IN THE ADULT DOG

FACULTAT DE VETERINÀRIA DE BARCELONA
Departament de Medicina i Cirurgia Animals

Memòria presentada per
Francisco Fernández Flores

Per optar al grau de
Doctor en Medicina i Cirurgia Animals

Bellaterra, 23 de setembre de 2016

Tesi doctoral dirigida per
Martí Pumarola i Batlle
Carles Arús Caralto

UNIVERSITAT AUTÒNOMA
DE BARCELONA

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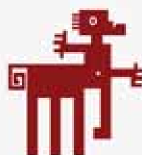

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Departament de Medicina i Cirurgia Animals

FRANCISCO FERNÁNDEZ FLORES com a autor de la memòria titulada "The cancer stem cell hypothesis in spontaneous canine gliomas: from tumors to neurogenesis in the adult dog" dirigida per MARTÍ PUMAROLA I BATLLE I CARLES ARÚS CARALTO confirma la seva presentació a Bellaterra, el 23 de setembre de 2016.

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I

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FAN CONSTAR

que la memòria titulada "The cancer stem cell hypothesis in spontaneous canine gliomas: from tumors to neurogenesis in the adult dog" presentada per Francisco Fernández Flores per optar al títol de Doctor en Medicina i Cirurgia Animals, s'ha realitzat sota la nostra direcció i, en considerar-la conclosa, autoritzem la seva presentació per ser jutjada pel Tribunal de tesi corresponent

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Dr. Carles Arús Caralto

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SUMMARY

1. Summary

Cancer stem cells (CSCs) can originate both for the transformation of normal stem cells as progenitors or from more differentiated cells that have acquired capacity for self-renewal. The hypothesis of the CSCs proposes that a specific subpopulation of cells is responsible for maintaining the tumor. This hypothesis has been demonstrated in a wide variety of tumors, including gliomas. Nestin and CD133 are used as markers for the detection and study of neural stem cells. In the present study, a total of 20 canine brain tumors were diagnosed as gliomas, according to the international classification of tumors of the central nervous system in humans. They were studied by immunohistochemistry (IHC) using Nestin and CD133 as markers of neural precursors; Neu-N, doublecortin (DCx) and βIII tubulin as neuronal markers; glial fibrillary acidic protein (GFAP), vimentin, S-100 protein and protein Olig2 as glial markers and Ki67 as a marker for cell proliferation. These tumors were neuropathologically classified as: oligodendogliomas (grade II), anaplastic oligodendrogliomas (grade III), mixed glioma (grade II) and glioblastomas (grade IV). IHC evaluation showed more positivity for Nestin and CD133 in high-grade gliomas (HGGs). Olig2 was expressed in most tumors, whereas positivity for GFAP was higher in mixed glioma and glioblastomas. Differentiated neuron markers were negative, while in some high grade gliomas DCX positivity was detected. Our results agree with the hypothesis of the CSCs, confirming the presence of undifferentiated neural precursors in canine

gliomas, increasing their number in HGGs. Subsequently, the potential for expansion *in vitro* of cells extracted from areas of the center and periphery of canine gliomas were evaluated by neurosphere assay in order to assess the ability of proliferation, expansion and survival of the neural progenitors. Neurospheres of all samples corresponding to the center of the tumor were obtained indicating the existence of neural precursors in tumors of all grades of canine glioma. Furthermore, the amount of cells capable of proliferate was increased in HGGs, indicating their possible influence of these neural precursors in malignant gliomas canine behavior. The neural precursors isolated from all samples were able to differentiate into three neural lines indicating their multipotentiality. These results demonstrated the involvement of neural progenitors in canine gliomas, according to the second criteria for demonstration of the hypothesis of CSCs. In parallel neural stem cells from normal postnatal adult dog brain phases were studied. The cytoarchytecture of its main niche it was studied in the adult brain. This corresponds to the subventricular zone (SVZ) which has been widely described in human and murine species. By histological, immunohistochemical, ultrastructural and cell culture studies, the presence of multipotent neural cells in SVZ were confirmed, residing in neurogenic niches structurally and cellularlly similar to those described in other mammalian species. Moreover, the architecture of these components is similar to that described in humans, probably related with complexity similar brain structures, which could be related to adult neurogenesis related processes. Finally, chains of migratory neuroblasts associated with vascular structures outside the SVZ indicating

a high migration capability and adaptability of resident neural stem cells in the brain of adult and comparable with similar events described in other mammalian species including human species. The results of the studies performed in this thesis conclude that the dog can be a relevant animal model in future related to the identification of new therapeutic targets in the field of comparative oncology studies, as well as a model to consider in studies and the progress of studies related to neurogenesis in the adult individual.

RESUMEN

2. Resumen

Las células madre cancerosas (CSCs) se pueden originar tanto por la transformación de las células madre normales como a partir de progenitores o células diferenciadas que han adquirido capacidad de auto-renovación. La hipótesis de las CSCs propone que una subpoblación específica de células madre cancerosas es la responsable de mantener el tumor. Esta hipótesis se ha demostrado en una amplia variedad de tumores, incluidos los gliomas. Nestina y CD133 se utilizan como marcadores para la detección de las células madre neurales. En el presente estudio, un total de 20 tumores caninos fueron diagnosticados histopatológicamente como gliomas, de acuerdo con la clasificación internacional de neoplasias del sistema nervioso central en humanos. Posteriormente fueron estudiados mediante inmunohistoquímica (IHC) usando Nestina y CD133 como marcadores de precursores neurales; Neu-N, Doblecortina (DCx) y βIII tubulina como marcadores neuronales; proteína ácida glial fibrilar (GFAP), Vimentina, proteína S-100 y proteína Olig2 como marcadores neurogliales y Ki67 como marcador de proliferación celular. Estos tumores se caracterizaron neuropatologicamente como: oligodendogliomas (grado II), oligodendrogliomas anaplásicos (grado III), glioma mixto (grado II) y Glioblastomas (grado IV). La evaluación IHC reveló una positividad mayor para Nestina y CD133 en tumores de alto grado. Olig2 fue expresada en la mayoría de los tumores, mientras que la positividad para GFAP fue mayor en el glioma mixto y los glioblastomas. Los marcadores de neurona

diferenciada fueron negativos, mientras que en algunos gliomas de alto grado se detectó positividad para DCX. Nuestros resultados están de acuerdo con la hipótesis de las CSCs, confirmando la presencia de precursores neurales indiferenciados en los gliomas caninos sobretodo en los tumores de grado más alto. Posteriormente, el potencial de expansión in vitro de células extraídas de áreas del centro y periferia de gliomas caninos fueron cultivadas *in vitro* con la finalidad de evaluar la capacidad de proliferación, expansión y superviviencia de los progenitores neurales en los tumores gliales caninos. Se obtuvieron neuroesferas de todas las muestras correspondientes al centro del tumor indicando la existencia de precursores neurales en tumores de todos los grados de glioma canino. Además, la cantidad de células capaces de proliferar se vio incrementada en los tumores de mayor grado, indicando la posible influencia de estos precursores neurales en el comportamiento maligno de los gliomas caninos. Los precursores neurales aislados de todas las muestras con capacidad de expansión fueron capaces de diferenciar en las tres líneas neurales indicando su multipotencialidad. Estos resultados demostraron la participación de progenitores neurales en los gliomas caninos, de acuerdo con el segundo criterio de demostración de la hipótesis de las CSCs. En paralelo se estudiaron las células madre neurales en fases post-natales del cerebro de perro adulto. Para ello se estudió la citoarquitectura de su principal nicho en el encéfalo adulto. Este nicho corresponde a la zona subventricular (SVZ) anterior la cual ha sido ampliamente descrita en humanos y especies murinas. Mediante el estudio histológico, immunohistoquímico, ultraestructural y cultivo celular, se confirmo la presencia de células neurales multipotentes en la

SVZ canina en nichos neurogénicos estructural y celularmente similares a los descritos en otras especies. Además, la arquitectura de estos componentes es similar a la descrita en humanos, probablemente relacionado con una complejidad similar de las estructuras encefálicas, lo cual podría tener relación con procesos relacionados con la neurogénesis adulta. Por último, se identificaron cadenas de neuroblastos migradores asociados a estructuras vasculares fuera de la SVZ indicando la elevada capacidad de migración y adaptabilidad de las células madre neurales residentes en el encéfalo y comparable con eventos similares descritos en otras especies de mamíferos incluyendo la especie humana. Los resultados de los estudios llevados a cabo en esta tesis doctoral concluyen que el perro puede ser un modelo animal relevante en futuros estudios relacionados con la identificación de nuevas dianas terapéuticas en el campo de la oncología, así como un modelo a considerar en el estudios y el avance de estudios relacionados con la neurogénesis en el individuo adulto.

INTRODUCTION

3. Introduction

The present doctoral thesis began with interest in the study of oncogenic processes that could be related with the formation of gliomas both in humans and in dogs.

Currently, gliomas in humans represent a group of diseases that cause serious and even incurable problems in cases where the malignancy grade is high or the tumor involves structures and functions of the nervous system that are essential to vital function in the adult individual.

The incidence of gliomas in dogs is associated with a greater predisposition of the brachycephalic breeds, which are increasingly popular as pets. In addition, diagnostic neuroimaging techniques have been significantly improved in veterinary medicine and this has led to increased detection of gliomas *in vivo*, increasing access to cases of canine gliomas. Consequently, over the years the number of diagnosed gliomas in the neuropathology veterinary services has increased. The increased availability of samples has allowed us to study the incidence, morphology, and subtypes of gliomas in the canine species.

The objective of this study is to evaluate the spontaneous canine glioma as a model to identify tumor stem cells, in order to detect new diagnostic and therapeutic targets that could be helpful for future treatment of neural tumors in humans and pets.

The use of a spontaneous tumor model implies a low capability to control the sampling. Once our study was presented to the Neurology and Neurosurgery (SNN) Service of the *Fundació Hospital Clínic Veterinary* (FHCV) of the *Universitat Autònoma de Barcelona.* The owners of the animals with gliomas were informed about the possibility of participating in our study by donating the cadaver of their pet for scientific purposes. The approach was widely accepted and useful cases for our study immediately began to appear.

Therefore, this study began focusing on the pathological features of gliomas in dogs. Samples were collected in parallel from control animals in order to make comparisons and to validate the results obtained with samples of glioma. However, the complete comparitive study of control animals and animals with glioma was not carried out until near the end of this study.

Therefore, both the literature review and the results obtained in every study began focused on the neuropathological characteristics of canine gliomas. The correlative study developed in normal dog brain enabled us to understand the possible relationship between neurogenesis in the adult dog and the origin of tumors.

In the following pages we will explain how the biological features of spontaneous gliomas led to the study of adult neurogenesis in the dog.

3.1 Gliomas

3.1.1 Definition and classification

Gliomas are a group of neuroepithelial tumors of the central nervous system (CNS) that includes tumors from the astrocytic, oligodendroglial, ependymal, and choroidal plexus cell lineages (Perry and Wesseling, 2016).

The World Health Organization (WHO) classifies human gliomas based on their proposed relationship to specific glial lineage according to their hematoxylin-eosin (HE), immunohistochemical (IHC), and their ultrastructural features (Louis et al., 2007). The malignancy grade is determined based on specific pathological features, including cellular anaplasia, nuclear atypia, mitotic activity, vascular proliferation, necrosis, proliferative potential, clinical course, and treatment outcome (Louis et al., 2007).

Neuropathologically, gliomas are ranked from grade I to grade IV, with grades I and II considered as low-grade glioma (LGG) and grades III and IV as high-grade glioma (HGG) (Louis et al., 2007).

Although the same malignancy grades were maintained, the classification was recently updated (Louis et al., 2016) (Table 1) and now includes a group of diffuse tumors in which astroglial and oligodendroglial tumors with different malignancy grades are grouped.

Table 1. WHO international classification of astrocytic and oligodendroglial tumors of the CNS

2007 WHO Classification	I	II	III	IV	2016 WHO Classification	I	II	III	IV
Astrocytic tumors					**Diffuse astrocytic and oligodendroglia tumors**				
Subependimal giant cell astrocytoma	*				Diffuse astrocytoma		*		
Pylocytic astrocytoma	*				Anaplastic astrocytoma			*	
Pilomixoid astrocytoma		*			Glioblastoma				*
Diffuse astrocytoma		*			Diffuse midline glioma				*
Pleomorphic Xantoastrocytoma		*			Oligodendroglioma		*		
Anaplastic astrocytoma			*		Anaplastic Oligodendroglioma			*	
Glioblastoma				*	**Other astrocytic tumors**				
Giant cell glioblastoma				*	Pylocytic astrocytoma	*			
Gliosarcoma				*	Subependimal giant cell astrocytoma	*			
Oligodendroglial tumors				*	Pleomorphic Xantoastrocytoma		*		
Oligodendroglioma		*			Anaplastic pleomorphic xantoastrocytoma			*	
Anaplastic oligodendroglioma			*						
Oligoastrocytic tumors									
Oligoastrocytoma		*							
Anaplastic Oligoastrocytoma			*						

The heterogeneity within gliomas in terms of histology, grade, clinical outcomes, and genomics increases the complexity of risk factor research in this tumor type (Ostrom et al., 2015). The WHO classification of human tumors of the CNS has been updated, introducing their molecular characterization (Louis et al., 2016). These new concepts have greatly expanded the use of immunohistochemistry (IHC) in terms of the diagnostic, prognostic and predictive assistance in gliomas.

In veterinary medicine, the classification of tumors of the CNS is not as well developed and defined (Koestner A, 1999) despite its subsequent updating (Valentine, 2001). Canine gliomas share the same histopathological features as human, which allows for the use of similar diagnosis and grading criteria. Recent veterinary publications on canine gliomas preferentially use the human WHO classification to grade canine tumor samples (Bentley et al., 2013; Boudreau et al., 2015; Higgins et al., 2010; York et al., 2012; Young et al., 2011). Of course, the same classification scheme must be adopted in order to compare human and canine gliomas.

3.1.2 Epidemiological aspects

The incidence rate of intracranial tumors in humans is 21 cases per 100,000 (Bash, 2015) and represents approximately 2% of the total of tumors. Similarly, canine intracranial tumors have an estimated incidence of approximately 14–20 cases per 100,000 (Dobson et al., 2002; Moore et al., 1996) representing 0.9% of total tumors (Snyder et al., 2006) (Table 2).

Gliomas are the most common type of primary brain tumor in humans with a prevalence of 81% (Ostrom et al., 2014); in dogs represents the second type of primary brain tumor with a 32% prevalence (Marc Vandevelde, Robert J.Higgins, 2012). Apart from gliomas, the most common intracranial tumors are meningiomas with an estimated incidence of 13-25% in humans and 43% in dogs (Marc Vandevelde, Robert J.Higgins, 2012), although some variations has been reported between epidemiological studies (Snyder et al., 2006; Song et al., 2013a).

In humans, astrocytic tumors represent 65% of all gliomas. Oligodendroglial tumors are less common and represent the second subtype with a prevalence of 18.3%. In contrast, oligodendroglial tumors are the most commonly occurring subtypes in dogs representing 28% of neuroectodermal tumours and 14% of primary CNS tumors overall (Snyder et al., 2006). The second subtype is astrocytoma representing 17% of all canine primary intracranial neoplasias (Snyder et al., 2006; Vandevelde et al., 1985) and about 10% of all CNS tumors.

Glioblastoma multiforme (GBM) is the most common histological subtype of astroglial tumors in humans. It represents 15–20% of all primary brain tumors and 50% of all gliomas. In dogs, GBM represents 5% of all astrocytic tumors, 3% of all primary CNS tumors and 12% of all neuroectodermal tumors (Snyder et al., 2006), although recent studies report a lower incidence (Song et al., 2013b).

Table 2. Comparative available epidemiological data on the incidence of intracranial tumors in humans and dogs.

	Human	Dog
Intracranial tumors	21 cases per 100,000	14– 20 cases per 100,000
Glioma (% brain tumors)	81%	32%
Meningioma (% brain tumors)	13-25%	30-45%
Oligodendroglial (% gliomas)	10% (3-12%)	28% (20%-40%)
Astrocytic (% gliomas)	65-70%	17% (10%-20%)
Glioblastoma		
(% brain tumors)	15-20%	3%
(% glial tumors)	40-50%	12%

In general terms, gliomas mostly affect brachycephalic breeds. Astrocytomas are commonly observed in Boston terriers and Boxers; the latter breed also presents the highest incidence of canine GBM (Stoica et al., 2011).

Frontal, parietal and temporal lobes are the main localizations of oligodendrogliomas in the canine brain which often grow around the white and gray matter near the lateral ventricles (Snyder et al., 2006). Most of the astrocytomas in dogs affect the prosencephalon and nearly

one third have been documented in the cerebellum (Snyder et al., 2006).

It has been established that 20 years old is the age of epidemiological risk of developing glioma in humans (Ostrom et al., 2014) but the incidence of histologic types of glioma are different in adults versus that observed in children. While in children pilocytic astrocytoma is the most common subtype with 37% of prevalence, Oligodendroglioma (WHO grade II) and oligoastrocytomas (WHO grade II) are most common in those 35- to 44-year-olds. Anaplastic astrocytoma (WHO grade III) and GBM are the most commonly diagnosed tumors in 75- to 84-year-olds (Ostrom et al., 2015).

No differences between ages have been studied in animals but it has been established that the highest incidence of brain tumors among domestic dogs is 8.6 years old and above (Marc Vandevelde, Robert J.Higgins, 2012).

3.2 Stem cells and tumor development

3.2.1 Introduction

Traditionally, it had been assumed that solid tumors only originated from the neoplastic transformation of mature somatic cells. The advance in the research about stem cells (SCs) in terms of their localization, regulation, and biological properties resulted in the conclusion that stem cell and cancer development share several features (Pardal et al., 2003; Reya et al., 2001; Shackleton, 2010).

The main features that define SCs are:

1. Self-renewal activity: SCs can yield a high amount of identical undifferentiated cells during long time periods or during the whole life of the individual.
2. Undifferentiated: SCs do not usually have tissue-specific differentiated structures which allow them to perform tissue-specific functions.
3. Capability to differentiate: SCs can give rise to partially or fully differentiated cells.

The uncontrolled self-renewal activity and the lack of full differentiation activity observed in neoplastic cells suggest the involvement of stem cells in the development of neoplastic diseases (Reya et al., 2001). According with this concept, initation and promotion stages of malignancy could occur in SCs rather than in

somatic cells. and consequently they might have indefinite proliferative potential that drive the formation and growth of tumors.

3.2.2 Adult stem cells

SCs appear during early development and remain functional in tissues till later stages. These primordial cells are defined as embryonic stem cells (ESCs) and are localized in the embryo, as part of the inner cell mass (Can, 2008). These cells are mainly pluripotent and will give raise to the three germinal layers: ectodermal, mesodermal, and endodermal. ESCs are responsible for forming the primordial organs of the organism.

As these primary organs grow, homeostatic signaling stops the cell proliferation rate of these undifferentiated cells. Some of these cells start to differentiate and a pool of more specialized adult stem cells (ASCs) remains in the adult tissues. These cells are relatively quiescent and are only activated to regulate tissue homeostasis (Rezza et al., 2014). They have a long life span and replace the cells of their progeny that die during adult development. Thus, the stem cell properties of these cells are maintained during the whole life of the organism (Duan et al., 2008). Over the lifespan, multiple derived cells are produced, and these cells are hierarchically organized. This accounts for the wide heterogenicity of the stem cell plate present in adult tissues (Morokoff et al., 2015)

In adult organs ASCs have a specific localization provided by a specific microenvironment that determines their behavior. This localization is

called the 'niche' and it has a particular cytoarchitecture that meets requirements to maintain the stem properties (Rezza et al., 2014).

3.2.3 Neural stem cells

Neural stem cells (NSCs) are self-renewing multipotent ASCs in the CNS capable of producing cells which can differentiate into the 3 major cell types including neuronal, astroglial, and oligodendlial precursor cells (Fig. 3) (Kornblum, 2007). They increase their proliferative activity in response to exogenous stimuli from their environment (Grégoire et al., 2015).

These cells represent a memory of embryonic neuroepithelial cells arising from the embryonic phases, allowing continuous turnover of mature neural cells in the adult brain (J. C. Conover and Allen, 2002; Doetsch and Alvarez-Buylla, 1996; Galli et al., 2003)

NSCs are primary cells that by asymmetric cell division yield two daughter cells: a non-specialized cell which replaces the mother, and a specialized cell that becomes a transit amplifying cell (Winquist et al., 2014). The direct non-SC progeny of a neural stem cell is the neural progenitor cell (NPC) which has a lineage-specific differentiation capability: neuronal, oligodendroglial, or astrocytic (Emsley et al., 2009).

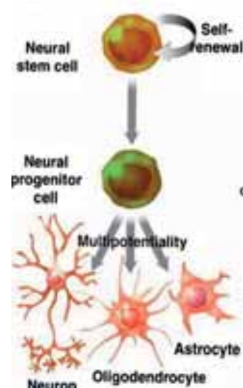

Figure 1. Hierarchy of NSCs. (Ortega-Perez et al., 2007)

The progeny of the neural stem cell is the neural progenitor cell, which differentiates into lineage-restricted progenitor cells. These give rise to oligodendrocytes, astrocytes, and neurons.

Although neural progenitors are more highly differentiated than NSCs, it is sometimes difficult to distinguish between these two cell developmental stages. Hence, the term neural precursor cell was introduced, which combines NSCs and NPCs. Their co-existence implies that the cell is undifferentiated and proliferating (Emsley et al., 2009).

NSCs have the ability to migrate through diffusible signals as well as extracellular matrix (ECM) signals (Cayre et al., 2009).

These signaling pathways are all critical in allowing NSCs to maintain their tracking ability (Suh et al., 2009). They can navigate to a tumor mass as well as follow migratory cancer cells when they invade surrounding tissue (Cayre et al., 2009).

In addition to secreted signals, migratory glioma cells modify the ECM as they invade the normal brain parenchyma. These modifications leave a specifically altered trail of molecules that serves as a path for migratory neural progenitor cells. Such molecules include tenascin, fibronectin, laminin, and different types of collagen (Faissner and Reinhard, 2015)

3.2.4 Cancer stem cell hypothesis

Previous studies in both hematopoietic and solid malignancies (Bruce and Van Der Gaag, 1963; Huntly and Gilliland, 2005) demonstrated that only a small percentage of cells isolated from a tumor were clonogenic. These studies suggested that cancer arises from tissue-resident ASCs as a result of the accumulation of genetic mutations along their lives (Rahman et al., 2011; Shih et al., 2002)

The cancer stem cell (CSC) hypothesis postulates that a subpopulation with unique self-renewal ability that differentiates into non-self-renewing cells within the tumor is responsible for maintaining tumor growth in a manner like that of stem cells in normal tissues (Ailles and Weissman, 2007; Reya et al., 2001; Xu et al., 2009). This population of cells corresponds to cancer stem cells (CSCs) and can originate from the transformation of adult stem cells in their niches, but they can also come from restricted progenitors as well as from more differentiated cells that have acquired self-renewing capacity (Reya et al., 2001; Ailles and Weissman, 2007; Visvader and Lindeman, 2008; Xu et al., 2009; Blacking et al., 2012) (Fig. 2).

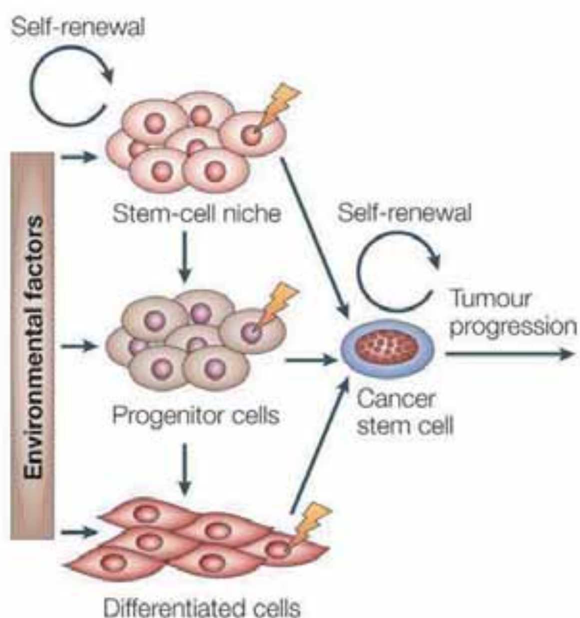

Figure 2. Origin of CSCs (Bjerkvig et al., 2005)

According with the CSC hypothesis, the cells that maintain the tumor growth may arise from:

1. A stem cell undergoing a mutation.

2. A progenitor/differentiated cell undergoing several mutations, thus allowing them to acquire the self-renewal ability.

3. Fusion of a cancer cell with a normal stem cell, thereby equipping the former with self-renewal capability

In all scenarios, the resulting CSC has lost normal growth regulation and progress into malignancy.

This phenomenon can be observed both between tumors (inter-tumor heterogeneity) and within individual tumors (intra-tumor heterogeneity). Spontaneous tumors frequently display substantial intra-tumor and inter-tumor heterogeneity made evident by different phenotypic features (Albini et al., 2015). This heterogeneity has been explained as follows: neoplastic cells possess asynchronous activation of the tumorigenic activity, at a low frequency together with genetic and epigenetic changes that influence the tumor microenvironment and the possible hierarchical organization in cancers (Albini et al., 2015; Reya et al., 2001; Shibata and Shen, 2013).

The CSC hypothesis attributes this heterogeneity to the neoplastic transformation of a minority population of cells with the ability to instigate and sustain tumor growth. These characteristics can be explained by 2 models of tumor growth (Dick, 2009)(Fig. 3):

1. The stochastic model, which assumes that the entire neoplastic cell population possesses the same tumorigenic potential. Variability within the neoplastic cell population results in the heterogeneity of the tumor. Intrinsic genomic factors such as the level of transcription factors and altered signaling pathways, together with the microenvironmental variability and the immune response, increase cell plasticity. This results in a heterogeneous neoplastic population with different grades of differentiation.

2. The hierarchal model, which proposes that a small proportion of cells are responsible for the malignant tumor growth resulting in an organization that resembles the tissue of origin.

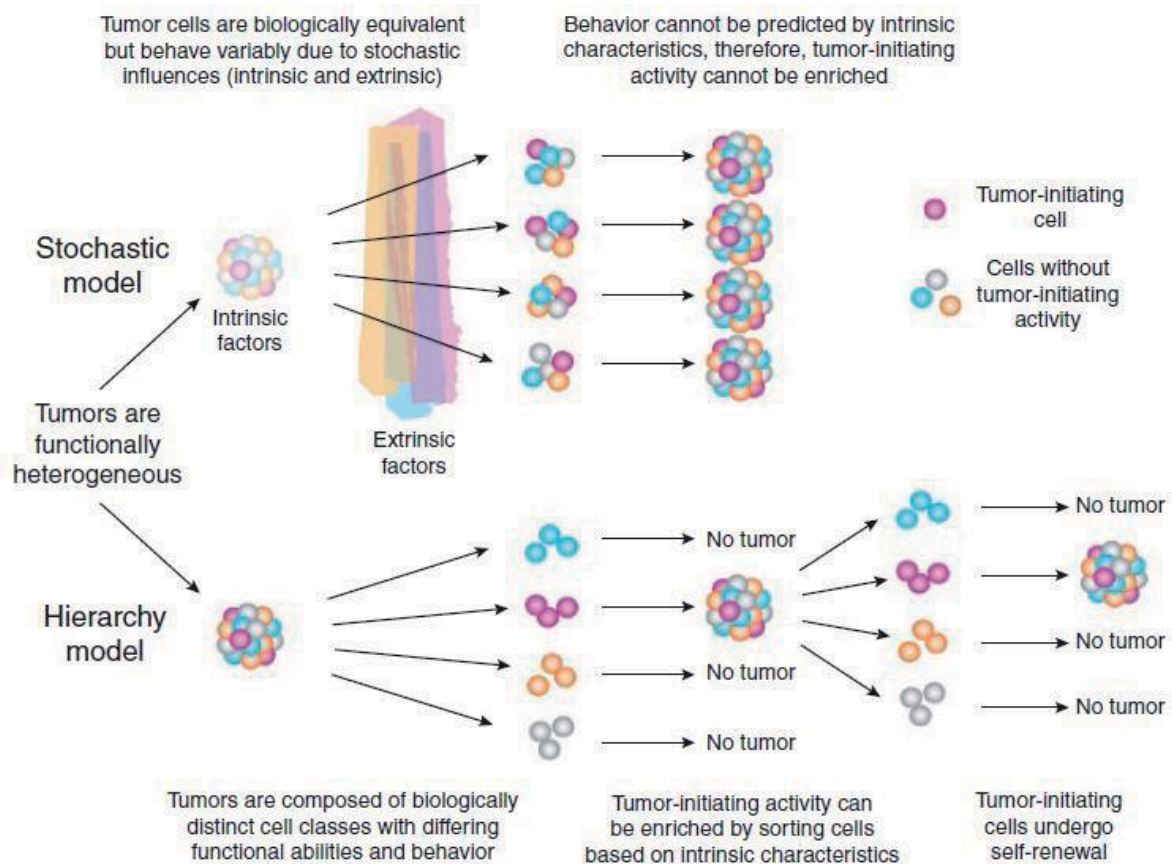

Figure 3 (Dick, 2009)

Stochastic model (above): Heterogeneity arises through the acquisition of additional mutations (genetic/epigenetic) under the influence of intrinsic and extrinsic factors such as genetic instability, the environment, and drug therapy.

Hierarchical model (below): Heterogenicity arises through successive differentiation steps, giving rise to all of the different cell types within the tumor, and also possibly self-renewing to produce other CSCs. CSCs are thus biologically distinct from the majority of tumor cells and sustain tumor growth and progression. These inherent properties may be predicted, allowing the identification and prospective isolation of tumorigenic cells.

The hierarchal model of growth is the basis of the CSC hypothesis and postulates that the CSCs populations appear to be at the apex (Dell'Albani, 2008) and are also capable of initiating and maintaining cancer.

These models are not mutually exclusive and can act together to determine tumor histopathology and behavior. Given their inherent ability to self-renew, adult stem cells are believed to represent excellent targets for oncogenic transformation. However, in many cases cancer can also arise from cell types that are not stem cells, which thereby acquire the property of self-renewal upon transformation (Shibata and Shen, 2013). In addition, extrinsic mechanisms may be involved in generating tumor heterogeneity, because interactions between tumor cells and the stromal micro-environment are a crucial determinant of malignant growth.

This model provides a possible explanation for therapeutic resistance and the eventual tumor relapse in many cancer types (Dean et al., 2005)

3.2.5 Characterization of neural progenitors

The study of NSCs involves taking into account their stem nature and the fact that different various stages may simultaneously appear in the same individual and localization (Thanemozhi et al., 2012). The characterization of NSCs is important to fulfill the requirements of CSC hypothesis. A neoplastic cell must show two essential stem cell properties: unlimited self-renewal capacity and ability to give rise to

all cell types within a tumor (Das et al., 2008; Wan et al., 2010). There are 3 types of studies that can be performed in order to identify these cells.

3.2.5.1 Stem markers

To date, regardless of the technique utilized, there is no single specific marker to identify a stem cell. In IHC different stem markers have been used to identify candidate NSCs in normal brain tissue and in tumors of the nervous system.

3.2.5.1.1 Nestin

Molecular features

Nestin is a type VI intermediate filament formed by 1600 amino acids and showing a molecular weight of 198kDa (Gilyarov 2008). It is structurally similar to the type IV filaments class IV (neurofilaments and α-internexin). Nestin forms heterodimers with class III intermediate filaments and internexins in the cytoskeleton of neuroblasts during embryonic development (Gilyarov 2008).

Functions

When the cell starts to differentiate, other filaments as neurofilaments (class IV) in neurons or GFAP (class III) in astrocytes are inducted and determine the differentiated cell type (Dell'Albani 2008) while nestin is down-regulated. Assays in nestin knockout mice have suggested its relevant role in the self-renewal activity of NSCs (Park et al., 2010). Althought little is known about its function, nestin is expressed in the majority of mitotically active CNS progenitors (Hendrickson et al.,

2011). These characteristics has led to widely consider nestin as a marker for SCs.

In adult individuals nestin has been detected in the subventricular zone (SVZ) of the lateral ventricles and in cells that display processes similar to the radial glia (Sakurada et al., 2008). Moreover, it has also been identified in late precursor cells of the subgranular zone (SGZ) of the dentate gyrus (DG), showing co-expression with markers of undifferentiated neurons such as PSA-NCAM (Gilyarov 2008). More recently, it has also been described that in human meninges nestin expression still persisted in a small population of newborn meningeal cells and decreases in later stages of development (Yay et al., 2014).

Moreover, in pathological conditions (trauma, excitotoxicity, epilepsy, inflammation, etc.) nestin can also be expressed by activated astroglia in the brain or in ependymal cells (Gilyarov, 2008). Furthermore, it has been also observed in proliferative vascular endothelium cells, suggesting its role as an angiogenic marker (Ernst and Christie, 2005; Matsuda et al., 2013).

Nestin positivity has been also identified outside the CNS (Gilyarov, 2008) in organs as retina (Matas et al., 2015), striated muscles and heart (Kishaba et al., 2010), skin and annexes, tooth, liver, pancreas, kidneys, adrenal and testicular glands and in the stroma of the intestine, lung, uterine cervix and endometrium (Kishaba et al., 2010). The diversity in nestin expression in different organs indicates the pluripotency of those nestin^{+} cells.

Nestin in tumors

Nestin expression has been observed in several neural tumors, especially those with high-grade or poorly differentiated (Schiffer, Manazza & Tamagno 2006) and especially in neurocytomas, neuroblastomas, astrocytomas, ependymomas, medulloblastomas, and peripheral nerve sheath tumors (PNST) (Gilyarov, 2008; Neradil and Veselska, 2015; Schiffer et al., 2006). In glial tumors increased expression related with lack of differentiation has been observed in GBM (Kitai et al., 2010; Schiffer et al., 2006) while variable expression has also been observed in ependymomas (Milde et al., 2012). It is considered that the reexpression of nestin in gliomas is an indicator of dedifferentiation (Dell'Albani, 2008) and its expression can delimitate the tumor infiltration to the brain parenchyma (ref). Thus, in some reports it has been acclaimed as a marker of prognosis in glial tumors (Arai et al., 2012; Chinnaiyan et al., 2008).

Nestin has also been reported in canine neuroepithelial tumors, showing a remarkable positivity in medulloblastoma, peripheral nervous tumors, and high grade astrocytomas (Ide et al., 2010).

3.2.5.1.2 CD133

Molecular features

CD133 (prominin-1) is one, but not the only, transmembrane glycoprotein observed both in neuroepithelial stem cells and human and mouse hematopoietic stem cells (Dell'Albani, 2008; Singh et al., 2004).

Functions

CD133 has been related with polarization, migration, and interaction between stem cells and the extracellular matrix (Dell'Albani, 2008). In the normal CNS, studies have primarily focused on characterization of in stem cell compartments, and interestingly, CD133 is also expressed by radial glia and neuroepithelial cells (Pfenninger et al. 2007) but its expression in other cell types and their lineage is not well understood (Holmberg Olausson et al., 2014). Moreover it is a marker that is also expressed at the level of Bowman's capsule in renal tubules and trophoblasts of human placentas (Christensen, Schroder & Kristensen 2008).

CD133 in tumors

It has been observed that $CD133^+$ cells isolated from nerve tumors exhibit properties of stem cells, and can initiate and direct *in vivo* tumor progression (Ailles, Weissman 2007, Singh et al. 2004, Dell'Albani 2008, Singh et al. 2004). Furthermore, these $CD133^+$ cells are resistant to drugs and toxins, activating their DNA repair capacity and resistance to apoptosis, radiation, and chemotherapy (Dell'Albani 2008). These cells are also related to overexpression of chemokine receptor CXCR4 on the cell surface, which may regulate the direction of cell migration and thus tumor invasion activity (Dell'Albani 2008). Consequently, CD133 has been used in neural and nervous system tumors to identify the stem cell niche (Christensen, Schroder & Kristensen 2008). On the basis of CD133 expression, CSCs and adult NSCs can be isolated from tumors by cell sorting (Singh et al. 2004a; Galli et al. 2004). The identification of this population suggests that

these cells represent the tumor-initiating fraction of human gliomas (Singh et al. 2004b), which has led to the establishment of the CSC hypothesis in brain tumors.

Given the variability of results obtained across these studies that have used these markers, a combination of Nestin and CD133 has been proposed to ensure greater reliability in identifying NSCs (Ailles, Weissman 2007, Singh et al. 2004, Dell'Albani 2008), especially for the study of nervous system tumors (Das, Srikanth & Kessler 2008).

3.2.5.1.3 Olig2

Molecular features

Oligodendrocyte lineage transcription factor 2 (Olig2) is a member of a family of basic helix–loop–helix transcription factors (TFs) that includes two other members: Olig1 and Olig3.

Functions

It plays a significant role in the formation of multipotent NPCs including their development into oligodendrocyte precursor cells (Tsigelny et al., 2016). In the adult individual, the oligodendroglial progenitor cells generate new oligodendrocytes and their migration is limited to specific locations (Menn et al. 2006). At early embryonic stages, one key role of Olig2 is to maintain progenitor cells in a replication-competent state (Ligon et al., 2007). Olig2^{+} cells are highly proliferative and form mature myelinating oligodendrocytes(Menn et al., 2006). Moreover it can also be expressed by immature astrocytes at early stages but not in those with a neuronal lineage. Therefore, Olig2 expression is sufficient to promote glial identity (Menn et al. 2006).

Olig2 in tumors

Olig2 has been proposed as one of the most significant GBM stem cell marker (Trépant et al., 2015). Moreover its use in other CNS neoplasms has been reported. While positive staining for Olig2 does not rule out a possible astrocytic origin of gliomas, a negative staining for Olig2 rules out an oligodendroglial origin (Preusser et al., 2007), suggesting its potential utility in the diagnosis of glial tumors (Ligon et al., 2004)

In veterinary medicine it has been proposed as a useful marker for neural neoplasia (Marc Vandevelde, Robert J.Higgins, 2012; Rissi et al., 2015).

3.2.5.1.4 Doublecortin and βIII tubulin

Molecular features

Doublecortin (DCX) is a microtubule-associated stabilizing protein that is expressed in neural precursor cells while actively dividing. It continues to be expressed as neuroblasts migrate and begin differentiation into neurons (Couillard-Despres et al., 2005).

Functions

DCX plays a central role in the regulation of microtubule dynamics and stability throughout morphogenesis (Gleeson et al., 1999). In addition, DCX deletion *in vivo* results in branching defects in the dynamics of migrating cells. This causes DCX knockout cells that produce more, but less stable, processes. These results suggest DCX plays an important

role in migration and maintaining bipolar morphology during the migration of neuroblasts (Koizumi et al., 2006).
In adult individuals, neurons do not express DCX, so its expression is considered as a marker of neurogenic activity, mostly related with brain areas where continuous neurogenesis occurs (Couillard-Despres et al., 2005).

In canines DCX expression in the adult brain has been reported, showing strong and greater expression in young dogs, in the subventricular zone, hippocampus, cortical areas, and brainstem with a more restricted distribution in older dogs (De Nevi et al., 2013)

DCX in tumors

The expression of DCX in neuroepithelial tumors has been reported in medulloblastomas, and oligodendroglial and astrocytic tumors, showing greater intensity in those tumors with a higher malignancy grade (Daou et al., 2005).

In canines, the expression of DCX has been observed in undifferentiated neuroepithelial tumors including medulloblastomas and neuroblastomas (Ide et al., 2010). Its expression has also been reported in other brain tumors such as meningiomas, especially in those with a higher malignancy grade, such as anaplastic meningiomas (Ide et al., 2011). Lack of expression has been reported in oligodendrogliomas (Ide et al., 2010).

Neuron-specific class III β-tubulin can be detected in immature neurons starting and persisting throughout adulthood. Tuj1-positive cells can be colabeled with DCX-positive and PSA-NCA-positive cells.

(Katsetos et al., 2003). Furthermore, it has been reported to be detected in basket cells in SGZ.

3.2.5.1.5 Vimentin and S100β

Vimentin is a primary intermediate filament protein, which has the property of glial cells (Lebkuechner et al., 2015), and is mainly exhibited in the radial-glia and immature astrocytes of early brain development and then vanishes at the end of gestation. Simultaneously, GFAP presents in the astroglia cells instead of vimentin (Lebkuechner et al., 2015). However, Seri's research also suggests that vimentin is expressed in both radial-glia and horizontal cells in SGZ.

S100β, also called calcium binding protein β, is a member of the S100 family, which is anchored at the cytoplasm and nucleus and participates in the procedure of cell cycle and differentiation (Holland et al., 2010). It can be detected in a subgroup of specific post-mitotic astrocytes. The expression of S100β discriminates a cohort of cells losing their NSCs potential from the GFAP$^+$ cells and indicates a more mature stage. Given the above characteristics, we may conclude that GFAP$^+$ and nestin$^+$ progenitor cells are negative for S100β.

3.2.5.2. The neurosphere assay

The neurosphere assay represents one of the gold standards in the study of NSCs. This system was developed and first reported in 1992,

as the observation of a high proliferative activity of normal neural stem cells cultured with epidermal growth factor (EGF). A small proportion of stem and progenitor populations from the embryonic rat striatal cells proliferated as clonal, spheroid clusters (neurospheres) whereas most (>99%) cells died (Rahman et al., 2011) (Reynolds et al., 1992; Reynolds and Weiss, 1992).

The utility of the neurosphere assay is the ability to reproduce the functional criteria of NSCs (Pavon et al., 2014):

- Capability for self-renewal with the ability to form colonies.
- Expression of an extensive proliferative ability.
- Multipotential cells able to differentiate.

In the case of nervous tissue, cells are dissociated into a single cell suspension under serum-free conditions and in the presence of cytokines such as EGF and basic fibroblast growth factor (bFGF). Under these conditions, the only cells that survive are those that divide in response to mitogenic stimulation (Shaker et al., 2015). This culture system allows a small population of growth factor-responsive cells to enter a period of active proliferation in which they form clusters of undifferentiated cells referred to as neurospheres (Shaker et al., 2015). These cells can be (i) dissociated to form numerous secondary spheres or (ii) induced to differentiate, generating the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes (Fig. 4).

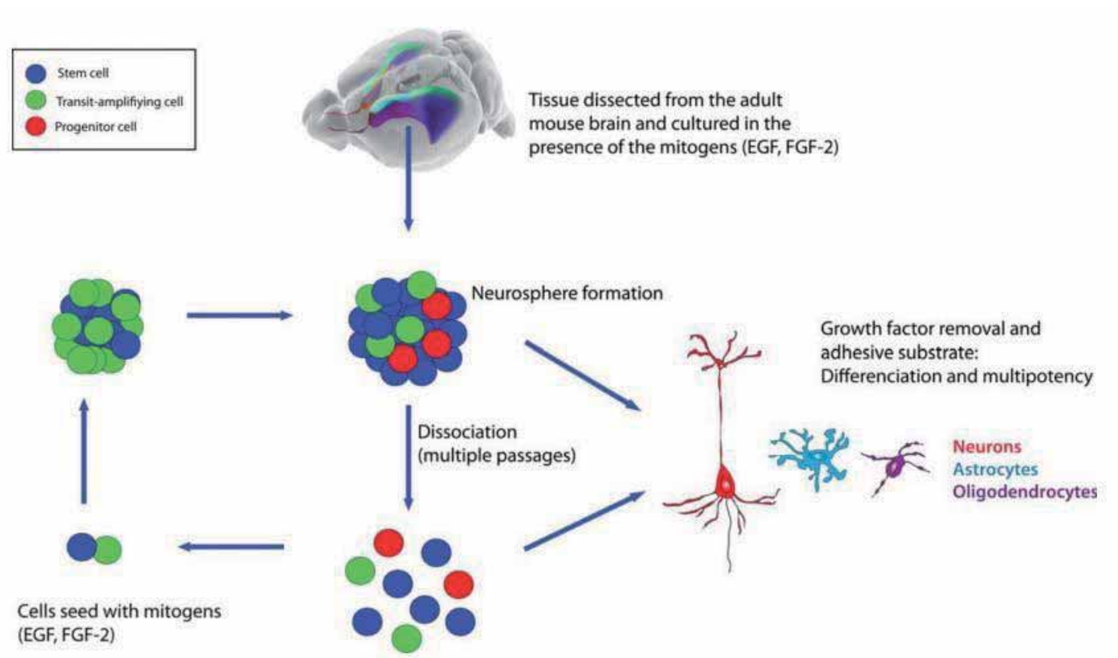

Figure 4 (Shaker et al., 2015)**.** Representation of the neurosphere assay. After the disstiation of the tissue graphs, primary neurospheres are obtained. Disaggregation and re-culture of these cells allows the formation of secondary spheres and selection of only those cells with proliferative potential. Finally, multipotent cells will have the ability to differentiate into the three neural cell lineages.

The neurosphere assay allows for the study of clinical applications of therapeutic molecules in the neurogenic process to increase understanding of the function of deleted or over-expressed genes that could regulate the proliferation and self-renewal of neural precursors (Wan et al., 2010).

Primary neurospheres can be dissociated and re-cultured in similar cell culture conditions. The obtaining of secondary neurospheres is indicative of the proliferative capacity of these cells (Gil-Perotín et al., 2013). Moreover, the reproduction of new neurospheres allows for selection of those cells with stem phenotype, as differentiated cells that are unable to form secondary neurospheres (Wan et al., 2010).

The interpretation of the results obtained in the neurosphere assay has to take in consideration that cells of mixed fate in differentiation can

exist in the same primary cell culture. Thus, assuming the clonal capacity of the neurosphere-forming cells, NSCs and NPC must be admixed in secondary neurospheres (Gil-Perotín et al., 2013).

4.5.3 Neurosphere assay in neoplastic disease

The appearance of the CSC hypothesis resulted in modification of the traditional concept that glial tumors originated from differentiated glial cells. To meet the CSC hypothesis, a cancer cell must show the cardinal stem cell properties of unlimited self-renewal capacity and the ability to give rise to all heterogenous cell types within a tumor (Blacking et al., 2011, Das, Srikanth and Kessler, 2008, Wan et al., 2010).

The obtaining of free-floating spheres from solid tumors indicates that stem cells and progenitors exist among the neoplastic cell population as they exhibit stem-like properties when cultured under specific conditions (Dontu et al., 2003).

Phenotypic and behavioral similarities between gliomas and adult NSCs raise the possibility that stem or progenitor cells can give rise to gliomas. This has been described in human GBM (Gilbert et al., 2009) and in one case of a canine GBM (Stoica et al., 2009).

Neurosphere cultures of GBM cells not only demonstrate the presence of self-renewing, multipotent precursors within GBM, but also show that they can generate GBM (in other words, acting like tumor stem cells) in immunodeficient mice (Kim et al. 1996). The capacity of stem cells for disseminating like tumor cells has been used to distribute cytokines to tumors for therapy (Ehtesham et al. 2002).

To date, using assays adapted from those used in the study of human tumors, such as tumorsphere formation and the demonstration of tumorigenicity in immunosuppressed mice, a wide variety of canine tumors have been evaluated for the CSC hypothesis: osteosarcoma (Wilson et al., 2008), lung carcinoma (Nemoto et al., 2011), hepatic carcinoma (Cogliati et al., 2010), melanoma (Blacking et al., 2012), mammary carcinoma (Blacking et al., 2012; Ferletta et al., 2011; Cocola et al., 2009; Penzo et al., 2009; Michishita et al., 2011), B-cell lymphoma (Blacking et al., 2012), and haemangiosarcoma (Blacking et al., 2012). Many researchers have investigated the presence of CSCs in canine mammary tumors (Cocola et al., 2009; Michishita et al., 2011; Penzo et al., 2009) and sarcoma cell lines (Fujii et al., n.d.; Wilson et al., 2008).

3.2.5.4 Ultrastructural studies

Electronic microscopy has been extensively used for the description of the cellular component and cytoarchitecture of different tissues. It is important to consider that the ultrastructural appearance of cells accurately reflects their physiological state. For example, neurons with a high metabolic activity have large nucleoli with a reticulated structural configuration, whereas neuronal cell types with low protein synthesis activity, such as cerebellar granule cells, show micronucleoli with a ring-shaped configuration (Ponti et al., 2008).

Ultrastructural studies on a wide spectrum of animal species have led to the conclusion that the presence of neural progenitors in some germinating areas in the adult brain can be identified, and these cells

show some characteristic traits. Studies performed in the adult mouse brain have established the cellular components that make up the germinating niches (F Doetsch et al., 1997). The description of chain migrations of neuronal precursors by ultrastructure has been reported since the first studies in the adult brain of the mouse (Luskin, 1998).

Later studies have established the characteristics and organization of these components in other mammalian species (Bonfanti et al., 2006; Gil-Perotin et al., 2009; Sawamoto et al., 2011; Takamori et al., 2014). Thanks to these studies, comparative aspects of adult neurogenesis in mammals could be revealed, establishing that there are some common cytological features of the niches that are maintained throughout the mammalian species.

Thanks to this technique, the cytoarchitecture of the neurogenic areas in the adult brain has been adequately studied (see 3.3 Adult Neurogenesis paragraph of this study).

3.3 Adult neurogenesis

3.3.1 Introduction

Traditionally it was considered that in the adult individual, CNS was unable to regenerate neurons that died over the course of life. However, in the late 1960s Altman and Das discovered, using autoradiography techniques in rat brain, the presence of cells labeled with tritiated thymidine ([3H] tymidine), demonstrating a proliferation capacity in some groups of encephalic cells (Altman, 1962; Altman and Das, 1965a, 1965b; Altman and Malis, 1962).

Later, more sophisticated studies were developed with the use of thymidine analogues such as bromodeoxyuridine (BrdU). The results allowed defining the existence of subpopulations responsible for the regeneration of neural cells residing in the brain of adult individuals in several mammalian species (Altman and Das, 1965b; Das and Altman, 1971; Gould et al., 1997) and several species of invertebrates (Macagno, 1980; Palka, 1986).

These new-formed neural cells migrate towards areas of the adult brain, mainly to the olfactory bulb (Alvarez-Buylla et al., 1998; Eriksson et al., 1998; Gould et al., 1999).

Thus, neurogenesis in the adult nervous tissue has been defined as an active process that includes the proliferation and determination of adult neural progenitor cells, and their differentiation, migration, displacement and integration into existing nervous systems (Gould, 2007) under physiological or pathological conditions (Lindsey and Tropepe, 2006).

In the adult mammalian brain a memory of embryonic neurogenic areas results in the continuity of neural regeneration (Conover & Allen, 2002; Doetsch & Alvarez-Buylla, 1996; Galli, Gritti, Bonfanti, & Vescovi, 2003).

These areas are defined as neurogenic, containing niches that correspond to local tissue microenvironments that maintain and regulate the NSCs in the adult brain(Tavazoie et al., 2008).

3.3.2 The neurogenic niche

The isolation of cells with the capacity to proliferate *in vitro* obtained from specific areas of the adult brain indicates the existence of neurogenic niches. They provide optimal environments for the maintaining and proliferation of neural precursors, giving rise to the development of neural cells (Ninkovic and Götz, 2007).

Neural precursors have been isolated from several encephalic areas such as the striatum (Ernst et al., 2014), amygdala (Bernier et al., 2002), substantia nigra (Cassidy et al., 2003), and piriform cortex (Shapiro et al., 2007)

Nonetheless, it is widely accepted that the adult mammalian brain contains two main germinating neurogenic niches: the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG) of the hippocampus. We have focused our attention on the SVZ.

3.3.2.1 Subventricular zone (SVZ) of lateral ventricles (LV)

The subventricular zone (SVZ) is localized in the lateral wall of lateral ventricles. It is able to generate inhibitory interneurons that migrate to the olfactory bulb (Lois et al., 1996; Shapiro et al., 2009). In embryonic stages, this area corresponds to the ventricular zone (VZ) formed by neuroepithelial cells along the primordial ventricles (Fig. 5). The embryonic VZ consists of two functional regions, the dorsal neocortical and the ventral ganglionic VZ. Whereas the first one generates the layered cortex, the latter gives rise to interneurons, astrocytes and oligodendrocytes, each presenting a lineage into which a neural stem cell can differentiate (J C Conover and Allen, 2002)

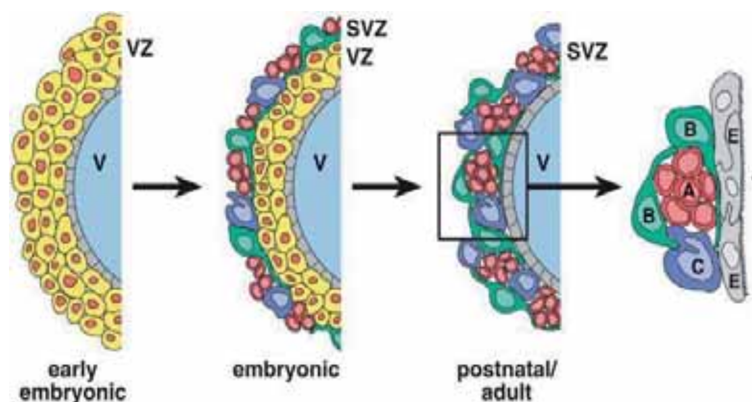

Figure 5 (Tramontin et al., 2003). Development of the SVZ. Undifferentiated embryonic stem cells (ES) around the neural tube progressively differentiate and migrate to their final destination. The result is the final organization of niches with more differentiated heterogeneous cells forming perivascular niches in post-natal periods.

The adult neurogenic niche has been widely studied in rodents at the ultrastructural level. These studies have allowed us to determine the cellular components that make up the SVZ niche.

3.3.2.2 Cellular components of the SVZ

The SVZ in adults is found under or subjacent to a layer of ependymal cells that separates it from the ventricle cavity. The cellular components correspond to a subpopulation of astrocytic and migratory cells. These cells, organized in a niche, correspond to (da Silva et al., 2012; Fiona Doetsch et al., 1997; Gage, 2000; Moore et al., 1978) and can be distinghised by their cell morphology and theyr immunophenotype (Table 3):

- Type A cells correspond to migrating neuroblasts and proliferating, migrating neural precursor cells. They are considered to be the cell population which performs chain migration from the SVZ to the olfactory bulb.

- Type B cells represent the astrocyte-like stem cells of the SVZ. They can be divided into B1 and B2 cells. B1 cells display cilium in the apical surface that extends into the ventricle cavity, which has been related to their proliferative activity. B2 cells are non-neurogenic astrocytes that are located at the underlying striatal parenchyma and do not make contact with the ventricle.

- Type C cells are the so-called *transient amplifying progenitor cells.* They show the strongest proliferative activity among the cells in the SVZ.

- Type E cells are ependymal cells that are form an ependymal layer lining the lateral ventricles.

Table 3. Differential stem cell markers expressed by cell components of the niche

E cells	B1 cells	B2 cells	C cells	A cells
VIM	GFAP	GFAP	Nestin	Nestin
S100	Nestin	VIM		DCX
Nestin	CD133	Nestin		βIII
	VIM			Ki67

3.3.2.3 Dynamic of the stem cell niche

In the adult SVZ, B, C and A cells interact and are responsible for the proliferation of the cellular components in the niche. Typically, B1 astrocytes divide asymmetrically, maintaining their population and giving rise to the highly proliferative type C cells. These B cells are relatively quiescent but are believed to be the precursors of the rapidly expanding population of C cells (Seri et al., 2001; Uylla, 1999), characterized by a short cell cycle time. Type C cells are considered to be the precursor cells of type A cells, and are not found in the rostral migratory stream (RMS)(Alvarez-Buylla and Lim, 2004) (Fig.6).

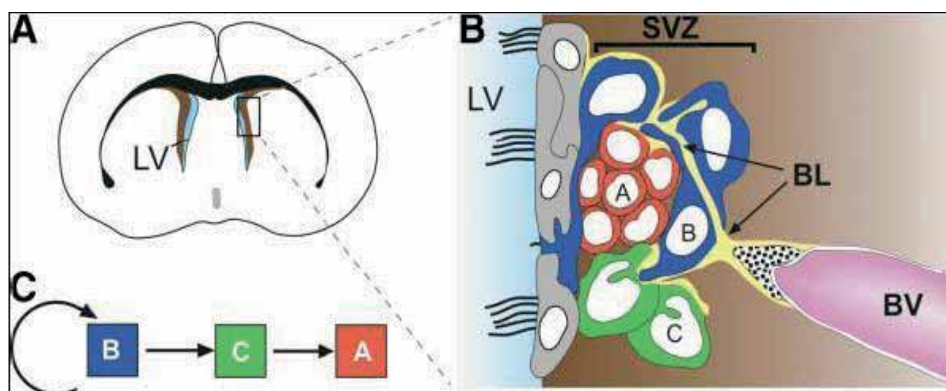

Figure 6 (Alvarez-buylla and Lim, 2004). Representation of the different cell types included in the niche and their progression from B neural progenitor cell and C proliferative transient cells to A migratory cells. Different IHC markers are associated with their different features so can be used in order to discriminate between the different cell types.

3.3.3 Cytoarchitecture of the mammalian SVZ

Although the cellular components seem to be conserved across species, different organizations of SVZ are described in different mammalian species, including humans.

Mouse SVZ

The mouse SVZ is characterized by the formation of large chains of neuroblasts that are surrounded by astrocytes. The B2 astrocytes are similar to B1 astrocytes but have a greater number of intermediate filaments and do not have contact with the ventricle. Type E cells exhibit multiple cilia and microvilli on their apical surface (Fiona Doetsch et al., 1997; Mirzadeh et al., 2008).

Neuroblasts migrate away from the SVZ, where they form large cellular chains, ensheathed by processes of B2 astrocytes and the gliotubes that SVZ-derived cells use to migrate toward the OB (Cayre et al., 2009; Doetsch and Alvarez-Buylla, 1996). Gliotubes of astrocytes converge in the anterior dorsal horn of the SVZ and initiate the RMS, which extends rostrally into the central region of the OB, where neuroblasts become mature neurons (Sawamoto et al., 2011).

Human SVZ

In humans the SVZ cytoarchitecture is significantly different from that in rodents. In contrast to rodents, human SVZ astrocytes are accumulated in a ribbon that is not adjacent to the ependymal layer (Barbaro et al., 2004). Instead, a gap layer that is largely devoid of cells

separates the astrocytic ribbon from the ependymal cells (Quiñones-Hinojosa et al., 2006). Adult human SVZ is composed largely of astrocytes with a large number of intermediate filaments and ependymal cells; there has been no description of type C cells. Some astrocytes are found to extend across the gap layer to contact the ventricular surface, similar to B1 astrocytes described in rodents (Quiñones-Hinojosa et al., 2006). During human brain development, the RMS has been described in the fetal brain as being where a rostral extension of neuroblasts is evident. Here, neuroblasts do not form chains but exhibit a migratory phenotype (Sawamoto et al., 2011).

Bovine SVZ

The lateral ventricles of bovines are divided into an anterior horn, central body, and temporal and occipital horns. The morphological and immunohistochemical study of all these regions has led to the subdivision of SVZ into three wall types that are distributed along the lateral ventricles (Rodriguez-Perez et al., 2003).

Type 1 wall is located at the level of interventricular septum and corpus callosum, and the most caudal lateral ventricular system. Neurogenic charge of this area corresponds to the lowest potential for creating new neural precursors.

Type 2 wall is the area corresponding to the striatum subventricular zone and is located at the central body of the ventricle.

Type 3 wall is the area covering the rostral anterior horn. It is the area with the greatest evidence of neurogenesis.

The presence of nestin^{+} cells indicates that the extensions of precursor cells may possibly be found in this layer. However, in the subependymal layer the higher proportion of precursor cells shows a gradual emergence of βIII and decreasing proportion of nestin marker positivity.

Rabbit SVZ

In contrast with the SVZ described in other mammalian species, rabbits contain groups of migratory cell chains within the mature brain parenchyma and they has been described as "parenchymal chains" (Luzzati et al., 2003). Ultrastructural studies had been allowed to distinguish between a ventricular SVZ, with a similar cellular arrangment observed in other mammal species and a abventricular SVZ mostly composed by those parenchymal chains and a loose glial meshworh (Ponti et al., 2006). The detailed descriptions of these chains has allowed to differereniate between anterior and posterior chains and have been establishe that anterior chains are related with blood vessels, indicating that blood vessels can constitute a way of migration of newformed neural cells in order to reach encephalic areas outside the SVZ (Ponti et al., 2006). These results also allow to hypothetisize that the migratory pathways depends on the different substrates with wich migratory cells may interact, an issue that might be important in understanding differences in the adaptation of persistent germinative layers to different mammalian brain anatomies (Ponti et al., 2006).

3.4. Dog as an animal model

The use of new animal models able to reproduce pathological or physiological processes analogous to humans is gaining importance in research. One of the reasons to work with new animal species is the lack of the ability to reproduce human diseases using traditional murine models. This has been attributed to the differences that exist between the murine and normal human and disease development processes (Chen et al., 2013). These considerations have led to the assumption that the availability of the observations to reach outstanding results largely depends on their capacity to be extrapolated to humans (Chen et al., 2013).

Preclinical steps in the development of new therapeutic strategies are needed in order to predict the response and adverse reactions of new treatments (Conn, 2008). In these phases accurate animal models that can reproduce the diseases in a way similar to humans become more important.

Although rodent species are well described, murine models are often induced in the disease under artificial laboratory conditions and frequently show differences from the pathogenesis in humans (Hansen and Khanna, 2004; Qazi et al., 2014). Thus, these animal models used in cancer research present three main obstacles (Conn, 2008; DN Louis DJ Brat, DW Ellison, 2008):

- The histopathological features of the tumors do not always recapitulate what is observed in their human counterparts.

- The growth of human tumor xenografts in immunosuppressed animals will give little insight into the influence of the immune system on the pathogenesis of cancer.
- Prediction of therapeutic response is poor in these genetically engineered models.

In contrast, the canine species presents some characteristic traits that make it favorable as an animal model and which may yield new results that bring approximate the human model. Some of these features correspond to physiological species-associated features of canines while others involve similarities in the development of diseases, especially in neoplastic conditions (Malik et al., 2012).

1. The canine nervous system is much closer in size to that of humans than that of rodents, alleviating some concerns regarding scalability and technical limitations such as cellular migration. Although smaller in size, the dog's brain is very similar histopathologically to the human brain due to their closer evolutionary relationship (Pang and Argyle, 2009; Schneider et al., 2008; Vaags et al., 2009).

2. Dog presents a large median life span that has been reported in beagles to reach 12-14 years (Albert et al., 1994). Some models of equivalences have been described between beagle and human aging; a 1-year-old animal is equivalent to a 5.5-7 years-old human (Albert et al., 1994). More complex models indicate that 5- to 9-year-old animals represent people from 40 to 69 years old, providing a more representative model (Patronek et al., 1997). Thus, the dog has been used to study neurological and neuropathological age-related aspects for comparison with humans (Head, 2011; Vite and Head, 2014). Some

authors have described the development of behavior compatible with cognitive impairment. Neuropathological examination has revealed the presence of encephalic beta-amyloid protein deposits, suggesting the canine as a valuable model of Alzheimer disease (Borràs et al., 1999; Cotman et al., 2002; Milward et al., 1997).

3. The genome sequence and gene expression profile have been studied in dog (Lindblad-Toh et al., 2005). This has allowed analysis of protein-coding genes showing a close relationship between dogs and humans. Other studies have demonstrated by the use of microarrays the up-regulation of genes related with apoptosis, cell signaling, signal transduction, cell development, cellular trafficking, protein processing, and immune function, together with downregulation related with ATP synthesis, metabolicsm, subsets of cellular trafficking, development, protein processing, and production of BDNF factor (Swanson et al., 2004). These results are consistent with those reported in humans, indicating the involvement of similar gene families in these processes (Swanson et al., 2009).

4. There are over 400 behaviorally and morphologically characterized dog breeds. This confers a diverse genetic background on the canine species. Most of these breeds have been created under strong artificial selection, resulting in relatively inbred populations. For this reason, certain breeds harbor a higher or lower incidence of certain diseases (Switonski, 2014).

5. Spontaneous tumors are a major cause of non-age-related death in domestic dogs; many of these mirror those seen in humans in terms of disease course, histology, biological behavior, and response to

treatment. Canine spontaneous tumors share morphological, biological, and molecular characteristics with human tumors, presenting tumor imitation and progression in a similar way to humans. In many respects, spontaneous cancer in the dog is a more representative model of human disease than experimentally-induced tumors in laboratory rodents. Specifically, new classification methods are in the same line as in human gliomas; studies have aimed at defining genes that have abnormal expression in canine gliomas such as VEGFR-1, VEGFR-2, EGFR, PDGFR and c-MET, which lend support to the purported similarities with human gliomas (Dickinson et al., 2006; Higgins et al., 2010)

Comparative oncology can provide insights into cancer biology and may improve understanding of disease in humans. The continued adaptation and use of investigative techniques in the canine system could be mutually beneficial as it could aid in the development of new therapies for both dogs and humans (Bentley et al., 2013; Schiffman and Breen, 2015). In fact most of the treatment modalities used have been adapted successfully to treat canine patients.

In this doctoral thesis we have studied NSCs in normal canine brain, in the neurogenic area of SVZ, together with their presence in canine glial tumors. All our findings have been compared with those obtained in other animal models and in humans. We have added data to support the usefulness of dogs as an animal model to study human neural development and diseases.

OBJECTIVES

4. Objectives

The main objective of this study was to provide insights into the identification of new cellular targets that could be useful for future lines of research in canine neurohistology and neuropathology.

To reach this objective, we decided to use canine spontaneous glioma as a model of study.

The specific objectives of this doctoral thesis are:

1. To describe the main histological and immunohistochemical features of canine glioma, and compare their morphological aspects with those described in the literature, including human gliomas.

2. To obtain data of relevance in order to contribute to the evaluation of the cancer stem cell hypothesis in spontaneous canine glioma. Using immunohistochemical and cell culture studies we will try to identify cellular components with stem properties. An intrinsic primary objective was to standardize these cells immunohistochemically and use *in vitro* techniques in the canine species. Additionally, a secondary objective was to evaluate the relationship of these components with the pathogenesis of canine glioma, according to their malignancy grade.

3. In parallel, our aim was also to investigate and confirm neurogenesis in normal adult canine brain, especially in the subventricular zone of the lateral ventricles. The same morphological and *in vivo* techniques used for the glioma studies should be applied.

4. A final global objective was to evaluate the usefulness of the dog as a potential animal model for the study of human gliomas and other neural diseases.

MATERIAL, METHODS AND RESULTS

Study 1. Presence of neural progenitors in spontaneous canine gliomas: A histopathological and immunohistochemical study of 20 cases

Fernández F, Deviers A, Dally C, Mogicato G, Delverdier M et al. (2015) Presence of neural progenitors in spontaneous canine gliomas: a histopathological and immunohistochemical study of 20 cases. *The Veterinary Journal*. 209: 125-132

Introduction

Gliomas are the most common brain tumors in humans without effective treatments. The dog seems to be an accurate animal model to study the human gliomas. Our aim in this study was to evaluate by histology and immunohistochemical (IHC) techniques the cell lineages present in canine glioma and compare them with human samples. The cancer stem cell (CSC) hypothesis was evaluated in these canine samples

Material and Methods

Twenty spontaneous canine gliomas were morphologically graded. Cell lineages were evaluated with IHC. The presence of neural progenitors was assessed with nestin and CD133 markers. Neuronal lineage cells were assessed with DCX, βIII, and NeuN markers. Glial lineage cells were assessed with Olig2, GFAP, S100, and VIM markers. Microglial cells were assessed with Iba-1 marker. Proliferative index was assessed with Ki-67 marker.

Results

$Nestin^{+}/CD133^{+}$ neural precursors correlated with the malignancy of canine gliomas. $GFAP^{+}/VIM^{+}$ astroglial precursors predominated in astrocytomas and were more common in high grade gliomas, while $Olig2^{+}/DCX^{-}$ oligodendroglial precursors were predominant in low grade gliomas. DCX^{+} neuronal precursors represented a small fraction of cells in canine gliomas.

Conclusion

The spontaneous nature of canine gliomas together with the presence of neural progenitors and similarities in their lineage commitment with human published results supports the use of the dog as a good animal model to investigate the CSC hypothesis in gliomas.

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Presence of neural progenitors in spontaneous canine gliomas: A histopathological and immunohistochemical study of 20 cases

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ABSTRACT

Gliomas are the most common primary brain tumours in humans and are associated with a poor prognosis. An accurate animal model of human glioma tumorigenesis is needed to test new treatment strategies. Dogs represent a promising model because they develop spontaneous diffusely-infiltrating gliomas. This study investigated whether spontaneous canine gliomas contain cancer stem cells previously identified in all grades of human gliomas.

Twenty spontaneous cases of canine gliomas were graded according to the human WHO classification. The expression of different markers of lineage differentiation was evaluated with immunohistochemistry as follows: nestin and CD133 for neural stem cells, doublecortin for neuronal progenitor cells, Olig2 for glial progenitor cells, glial fibrillary acidic protein, vimentin and S-100 for mature glial cells, and NeuN and βIII-tubulin for mature neurons. Gliomas were characterised as follows: five grade II (oligodendrogliomas); nine grade III (seven anaplastic oligodendrogliomas, one anaplastic astrocytoma, one anaplastic oligoastrocytoma); six grade IV (glioblastomas).

Immunohistochemical evaluation revealed that (1) nestin and CD133 were expressed in all grades of gliomas with a higher proportion of positive cells in high-grade gliomas; (2) the expression of S-100 protein and Olig2 did not differ substantially between astrocytic and oligodendroglial tumours, and (3) all gliomas were negative for mature neuron markers. The results demonstrated the presence of undifferentiated neural progenitors in all grades of spontaneous canine gliomas, confirming the relevance of this animal model for further studies on cancer stem cells.

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Introduction

Gliomas are the most common primary brain tumours in adult people, representing 81% of all malignant brain tumours (Ostrom et al., 2014). Diffusely infiltrating gliomas are classified as low-grade (grade II) and high-grade (grades III and IV) according to their degree of malignancy (Louis et al., 2007). They are associated with a poor prognosis largely because of their widespread invasiveness and resistance to multimodal treatments.

Recent studies have indicated that all grades of human gliomas contain putative cancer stem cells (CSCs), a small subpopulation of cells thought to be responsible for initiating and maintaining cancer growth through their ability to self-renew (Reya et al., 2001; Singh et al., 2004; Beier et al., 2007; Rebetz et al., 2008). In agreement with the cancer stem cell hypothesis, gliomas are hierarchically organised: self-sustaining CSCs at the apex have the potential to differentiate into astrocytic, oligodendroglial, and neuronal lineages, and give rise to malignant progenitors, lineage-restricted precursors, and differentiated cells (Singh et al., 2004). It is also suspected that CSCs are

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responsible for resistance to chemo- and/or radiotherapy leading to local relapse after treatment (Bao et al., 2006; Eyler and Rich, 2008). Immunohistochemically, CSCs show expression of markers such as nestin and prominin-1 (CD133) (Singh et al., 2003; Galli et al., 2004; Dell'Albani, 2008).

Efforts are currently being made to decipher the oncogenic mechanisms of these tumours in order to identify new therapeutic targets and improve response to treatment. Although rodent glioma models have been used in preclinical glioma research for over 30 years (Das et al., 2008), their reliability has increasingly been questioned (Sughrue et al., 2009). In contrast, the dog presents an attractive model because of its close evolutionary relationship with humans, its greater brain size compared to rodent models, and the spontaneous nature of gliomas, which are the second most frequent intracranial tumours in this species, with a prevalence of 32% (Chen et al., 2013).

The CSC hypothesis has been investigated in a wide variety of canine tumours (Wilson et al., 2008; Cocola et al., 2009; Fujii et al., 2009; Penzo et al., 2009; Cogliati et al., 2010; Ferletta et al., 2011; Michishita et al., 2011; Nemoto et al., 2011; Blacking et al., 2012; He et al., 2014). However, only one case of canine glioblastoma containing CSCs has been published (Stoica et al., 2009).

Evaluation of lineage commitment of tumour cells in human infiltrating gliomas has demonstrated that low-grade glioma cells are reminiscent of glial-progenitors, while high-grade glioma cells maintain glial progenitor-like features and additionally exhibit enhanced expression of neural precursors (Rebetz et al., 2008). So far, the lineage commitment and differentiation blockage of tumour cells in spontaneous canine gliomas have not been investigated or compared with those aspects of human infiltrating gliomas.

The aim of the present study was to investigate the expression of markers of glial and neuronal lineage differentiation hierarchy in 20 spontaneous canine infiltrating gliomas and to determine whether canine tumours show a lineage commitment similar to their human counterparts.

Material and methods

Case selection

Twenty canine gliomas were retrospectively selected from the databases (2008–2012) of the Veterinary Neuropathology group of the Universitat Autònoma de Barcelona (UAB, Spain) and the Laboratoire d'Anatomie Pathologique Vétérinaire du Sud-Ouest (LAPVSO, France). All of the samples were obtained during necropsy performed immediately following a presumptive diagnosis of glioma. The diagnosis was made by a Board-certified neuroradiologist (SA, CF, LC, KG) based on clinical criteria, magnetic resonance imaging features, and cerebrospinal fluid analysis. All owners gave their written consent for necropsy and histopathological analysis.

Histology and morphological diagnosis

Representative tissue samples were fixed in 10% formalin, processed into 5 µm paraffin-embedded sections, and stained with haematoxylin and eosin (HE) for microscopic evaluation. Gliomas were evaluated by six experts (FF, AD, CD, MD, DF and MP) in a blind study, according to the criteria defined by the World Health Organisation (WHO) for human tumours of the central nervous system (Louis et al., 2007). This classification has been updated more recently compared with the WHO animal grading system (Koestner et al., 1999), and recent veterinary publications have used the human WHO scheme to characterise canine gliomas (Higgins et al., 2010; Young et al., 2011; York et al., 2012; Bentley et al., 2013).

Morphological diagnosis was a two-step process: (1) identification of tumour phenotype and (2) grading. Diagnosis of oligodendroglial tumours relied on the recognition of neoplastic cells with well-defined membranes, cytoplasm clearing, and round and hyperchromatic nuclei, typically organised in a 'honeycomb' pattern. Astrocytic tumours were identified by elongated neoplastic cells with scant eosinophilic cytoplasm, organised in a loosely structured matrix. In oligoastrocytoma, two neoplastic cell populations with astrocytic and oligodendroglial phenotypes, respectively, were intermingled.

High-grade gliomas were distinguishable from low-grade gliomas by an increased degree of cytonuclear atypia (all high-grade gliomas) and an increased frequency of necrosis and/or endoluminal proliferation of endothelial cells leading to glomeruloid-like vessels (all high-grade gliomas except anaplastic astrocytoma). The mitotic index (i.e. number of mitoses per 10 high-power fields) was calculated for each sample but no threshold value was used in the grading scheme. Typical pseudopalisading of neoplastic cells around necrotic foci was a pathognomic feature of glioblastoma. Additional features observed were the growth pattern (relationship with the surrounding tissue), mucinous secretion, and inflammation.

Immunohistochemistry

The immunohistochemical (IHC) markers we used were characteristic of glial and neuronal lineage differentiation hierarchy: nestin and CD133 as stem cell markers; Olig2 protein and doublecortin (DCx) as glial and neuronal progenitor cell markers, respectively; glial fibrillary acidic protein (GFAP) and vimentin (VIM) as mature astrocyte markers; S-100 protein as a mature oligodendroglial and astrocytic marker; and NeuN protein (NeuN) and βIII-tubulin as mature neuron markers. The Iba1 microglial marker was used for the evaluation of the inflammatory-associated response. The nuclear antigen Ki-67 was used as a marker of cellular proliferative activity.

Sections 5-µm thick were mounted on capillary glass slides, deparaffinised, and rinsed with water. The primary antibodies used are summarised in Table 1. When antigen retrieval was necessary, sections were heated for 20 min in a water-bath or

Table 1
Immunohistochemical markers used for the study of canine gliomas: main features.

	Antibody name	Company	Dilution	Pretreatment
Nestin	Rabbit anti-nestin polyclonal antibody	Abcam ab5968	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
CD133	Rabbit anti-CD133 polyclonal antibody	Abcam 19898	1:200	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
DCx	Rabbit anti-doublecortin polyclonal antibody	Abcam ab18723	1:1000	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
ßIII	Mouse anti-ßIII tubulin monoclonal antibody	Chemicon MAB1637	1:200	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
NeuN	Mouse anti-neuronal nuclei monoclonal antibody	Chemicon MAB377	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
Olig2	Rabbit anti-Olig2 polyclonal antibody	Chemicon AB9610	1:100	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
GFAP	Rabbit anti-glial fibrillary acidic protein polyclonal antibody	Dako Z0334	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
S100	Rabbit anti-S100 polyclonal antibody	Dako Z0311	1:1000	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
VIM	Mouse anti-vimentin clone V9 monoclonal antibody	Dako M0725	1:200	Without pretreatment
Ki-67	Mouse anti-Ki-67 antigen monoclonal antibody	Dako M7240	1:100	Citrate buffer 10 mM pH 6.0, 4 min PC + 30 min RT
Iba1	Goat anti-Iba1 polyclonal antibody	Abcam ab5076	1:600	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT

RT, room temperature; PC, pressure cooker.

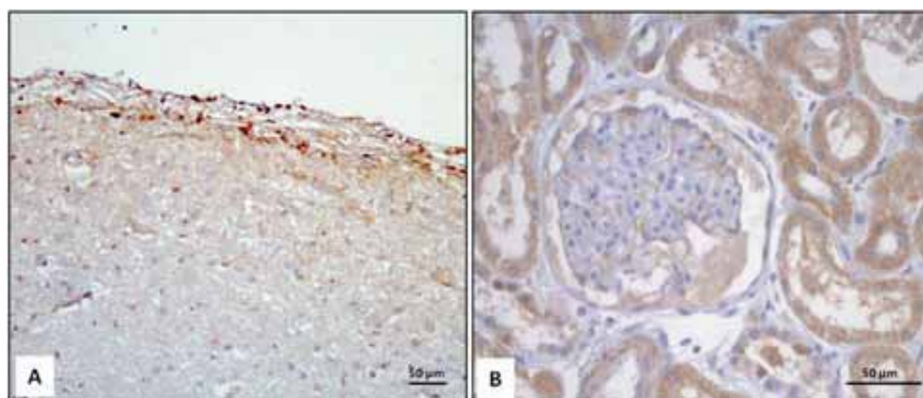

Fig. 1. Immunohistochemical evaluation of markers of neural stem cells in control tissues. (A) Presence of nestin⁺ cells located in the subventricular zone of an adult dog. (B) Expression of CD133 protein by the epithelial tubular cells of the kidney of adult dog.

4 min in a pressure cooker with 10 mM citrate buffer pH 6.0, cooled for 30 min at room temperature, and rinsed in phosphate-buffer saline (PBS) (Table 1). Previously, sections were treated 35 min with 3% peroxide to block endogenous peroxidase activity. Non-specific binding was blocked by normal goat or rabbit serum 30% diluted with PBS for 1 h. Samples were incubated overnight with primary antibodies at 4 °C (nestin, Olig2 and VIM) or 40 min at room temperature (all other antibodies tested). Sections were rinsed with PBS and incubated for 40 min with a labelled polymer according to the manufacturer's instructions (mouse kit K4007 or rabbit kit K011, Dakocytomation). For Iba1, the incubation was performed using a secondary goat anti-rabbit antibody and then with a standard ABC Peroxidase staining kit (Thermo Scientific, kit number 32020) diluted with PBS, for 1 h at room temperature. Staining was completed by a 10 min incubation with 3,3′-diaminobenzidine (DAB) and counterstaining in haematoxylin for 3 s. The positive control used for GFAP, Olig2, S-100, VIM, DCx, NeuN, and βIII-tubulin was normal canine brain tissue including grey and white matter. The positive control for nestin was brain tissue of the same canine brains, including the subventricular zone in order to verify cell specificity of this marker (Fig. 1A). Positive control for CD133 was adult canine healthy kidney tissue, because it is expressed by tubular epithelial cells (Fig. 1B). In all experiments, negative controls were obtained by omitting the primary antibody.

Evaluation of immunohistochemical data

A semiquantitative, four-class, proportion score was defined as the percentage of positive tumour cells labelled by the different markers: 0, <5% positive cells; 1, 5–30% positive cells; 2, 30–60% positive cells; 3, 60–90% positive cells; 4, >90% positive cells. Ten high-power fields were evaluated in each case. The proliferation index was estimated by counting Ki-67 stained and unstained cells in 10 high-power fields (approximatively 2000 cells) and then expressed as a percentage. Only nuclear staining was considered positive, and areas with the most positivity were chosen for evaluation.

Results

Clinical features

Breed, sex, age, and clinical signs of all dogs included in the study are summarised in Table 2. Eighty percent of the cases were Boxers

Table 2
Clinical data of dogs included in the study. M, male; F, female. Age expressed in years.

Case	Breed	Sex	Age	Neurological signs	Tumour localisation
1	Boxer	F	8	Head pressing, gait abnormality, right circling, depressed mental status, absent left proprioceptive reactions	Right prosencephalon
2	Boxer	F	9	Clusters of generalised tonic–clonic seizures, depressed mental status, right circling	Right prosencephalon
3	French bulldog	M	4	Generalised tonic–clonic seizures, autonomous signs, depressed mental status, vestibular signs, absent right proprioceptive reactions	Left prosencephalon
4	Boxer	M	10.5	Right blindness, absent menace response (right eye), decreased right proprioceptive reactions, mild cerebellar ataxia	Left prosencephalon
5	French bulldog	M	8	Seizures, behavioural changes, myosis	Right prosencephalon
6	Boxer	F	8	Seizures	Left prosencephalon
7	Bulldog	M	6	Left proprioceptive deficits, seizures	Right prosencephalon
8	French bulldog	F	5	Behavioural changes, depressed mental status, left circling, absent right proprioceptive reactions	Left prosencephalon
9	Boxer	F	13	Generalised tonic–clonic seizures, right circling, decreased right proprioceptive reactions, right facial hemispasm and hyperesthesia	Right prosencephalon/VII cranial nerve nucleus
10	Boxer	F	7	Weight loss, head tilted to the right side, left walking from 10 days	Left mesencephalon
11	Boxer	M	10	Status epilepticus, generalised tonic–clonic seizures.	Left prosencephalon
12	Boxer	F	12	Ataxia, circling, recumbency, head tilted to the left side	Left prosencephalon/mesencephalon
13	French Bulldog	M	13	Depressed mental status, tremors, epileptiform seizures	Right prosencephalon
14	French bulldog	F	4	Depressed mental status, right hemiparesis, absent menace response (right eye), absent right proprioceptive reactions	Left prosencephalon
15	French bulldog	M	4	Behavioural changes, generalised tonic–clonic seizures	Left prosencephalon
16	Boxer	F	10	Spinal ataxia of forelimbs, left circling, absent menace response (right eye), bilateral blindness, right proprioceptive deficit	Left prosencephalon
17	Jack Russell	M	3	Seizures	Left prosencephalon
18	Golden retriever	M	7	Right circling, behavioural changes	Right prosencephalon
19	Cross breed	F	11	Balance disorder, vestibular ataxia, left proprioceptive deficit, nystagmus	Right rhombencephalon
20	Scottish terrier	F	10	Behavioural changes, seizures	Right prosencephalon

Table 3
Histopathological diagnoses and features in glial tumours.

Case number	Diagnosis	GR	ATYP	MI	MSEC	NEC	VASC	INF	RNT
1	Oligodendroglioma	HC	Low	6	Yes	No	CB	No	Compressive
2	Oligodendroglioma	HC	Low	4	Yes	No	CB/G	Yes	Infiltrative
3	Oligodendroglioma	HC	Low	10	Yes	No	CB/G	No	Infiltrative
4	Oligodendroglioma	HC	Low	7	Yes	Yes	CB/G	No	Infiltrative
5	Oligodendroglioma	HC	Low	8	No	No	G	Yes	Infiltrative
6	Anaplastic oligodendroglioma	S	Moderate	9	Yes	Yes	G	No	Compressive + infiltrative areas
7	Anaplastic oligodendroglioma	S	Moderate	16	Yes	No	G	No	Infiltrative
8	Anaplastic oligodendroglioma	S	Moderate	7	Yes	No	G/CB	No	Compressive
9	Anaplastic oligodendroglioma	S	Moderate	2	No	No	CB/H	Yes	Infiltrative
10	Anaplastic oligodendroglioma	S	Moderate	12	No	Yes	CB	No	Compressive + infiltrative areas
11	Anaplastic oligodendroglioma	S	High	15	No	No	CB/H	No	Infiltrative
12	Anaplastic oligodendroglioma	S	Moderate	25	No	No	CB	No	Infiltrative
13	Anaplastic astrocytoma	S	High	10	No	No	CB/H	No	Infiltrative
14	Anaplastic oligoastrocytoma	S/HC	High	10	No	No	CB/H	Yes	Infiltrative
15	Glioblastoma	S	High	12	Yes	Yes	G	No	Infiltrative
16	Glioblastoma	S	High	15	No	No	CB	No	Compressive
17	Glioblastoma	S	High	2	No	Yes	G	No	Compressive
18	Glioblastoma	S	High	2	No	Yes	G	No	Compressive + infiltrative areas
19	Glioblastoma	S	High	17	No	Yes	H	Yes	Compressive + infiltrative areas
20	Glioblastoma	S	High	28	Yes	Yes	H	No	Infiltrative

GR, growth; ATYP, cytonuclear atypia; MI, mitotic index; MSEC, mucinous secretion; NEC, necrosis; VASC, vascular features; INF, inflammation; RNT, relation with surrounding nervous tissue; HC, honeycomb; S, solid growth; CB, capillary branching; H, haemorrhages; G, glomeruloid vessels.

and French bulldogs and most tumours were located in the prosencephalon (18/20, 90%). The mean age of affected dogs was 8 years (range 3–13 years). Both sexes were equally affected (9 males and 11 females).

Histological characterisation of canine gliomas

Tumours were microscopically characterised as follows: five grade-II gliomas (all oligodendrogliomas); nine grade-III gliomas (seven anaplastic oligodendrogliomas, one anaplastic astrocytoma, and one anaplastic oligoastrocytoma), and six grade-IV astrocytomas (all glioblastomas). The histological features of each tumour are summarised in Table 3 and representative images are displayed in Fig. 2.

Immunohistochemical features of canine gliomas

Expected patterns of immunostaining for each marker of lineage differentiation hierarchy were observed in positive controls. In canine healthy brain tissue, nestin$^+$ cells were found in the subventricular zone adjacent to the lateral ventricles (Fig. 1A), a neurogenic niche that has been previously described in dogs (Lim et al., 2012; Walton et al., 2013).

Proportion scores for markers of neuronal and glial lineage differentiation hierarchy are summarised in Table 4. Grade II-gliomas demonstrated low proportion scores for the stem cell markers nestin and CD133 (median 1 for both markers), but higher positivity was observed in the majority of high-grade gliomas, especially in glioblastomas for which the median proportion score for nestin was 3 (range 1–4) (Figs. 3A, B). With the markers of neuronal progenitor cells, expression of DCx was lacking in more than half of the cases (11/20). Samples with DCx expression were represented by low-grade (4/5) and high-grade (5/15) gliomas. Irrespective of the grade or morphological type of tumour, DCx expression was generally low, with 7/9 DCx$^+$ samples exhibiting a proportion score of 1. In contrast, expression of Olig2 was detected at high levels in almost all cases (18/20). The pattern of immunoreactivity for Olig2 appeared to be the same regardless of the morphological diagnosis or grade of malignancy. Indeed, the median proportion score was equal to 3 for oligodendroglial (range: 2–4) and astrocytic (range:

Table 4
Immunohistochemical features (proportion score) for the different glial tumours.

Case	Morphologic diagnosis	Nestin	CD133	DCx	βIII tubulin	NeuN	Olig2	GFAP	S100	VIM
1	Oligodendroglioma	1	0	1	0	0	3	1	1	1
2	Oligodendroglioma	1	1	1	0	0	3	1	3	2
3	Oligodendroglioma	1	1	3	0	0	3	1	1	1
4	Oligodendroglioma	1	0	1	0	0	3	1	1	2
5	Oligodendroglioma	1	1	0	0	0	4	0	4	0
6	Anaplastic oligodendroglioma	1	0	0	0	0	3	0	4	1
7	Anaplastic oligodendroglioma	2	0	0	0	0	4	0	0	0
8	Anaplastic oligodendroglioma	0	1	1	0	0	2	1	4	1
9	Anaplastic oligodendroglioma	1	1	0	0	0	3	1	1	2
10	Anaplastic oligodendroglioma	3	1	0	0	0	3	0	0	0
11	Anaplastic oligodendroglioma	1	1	1	0	0	4	1	4	1
12	Anaplastic oligodendroglioma	3	3	0	0	0	3	0	2	3
13	Anaplastic astrocytoma	3	3	0	0	0	0	3	3	0
14	Anaplastic oligoastrocytoma	1	3	1	0	0	2	3	1	2
15	Glioblastoma	4	1	2	0	0	4	2	1	2
16	Glioblastoma	1	0	1	0	0	4	4	1	4
17	Glioblastoma	3	3	0	0	0	3	3	2	3
18	Glioblastoma	1	1	0	0	0	3	3	3	2
19	Glioblastoma	3	3	0	0	0	0	0	2	1

Correspondence between the proportion score and the percentage of positively stained tumour cells: 0, <5%; 1, 5–30%; 2, 30–60%; 3, 60–90%; 4, >90%.

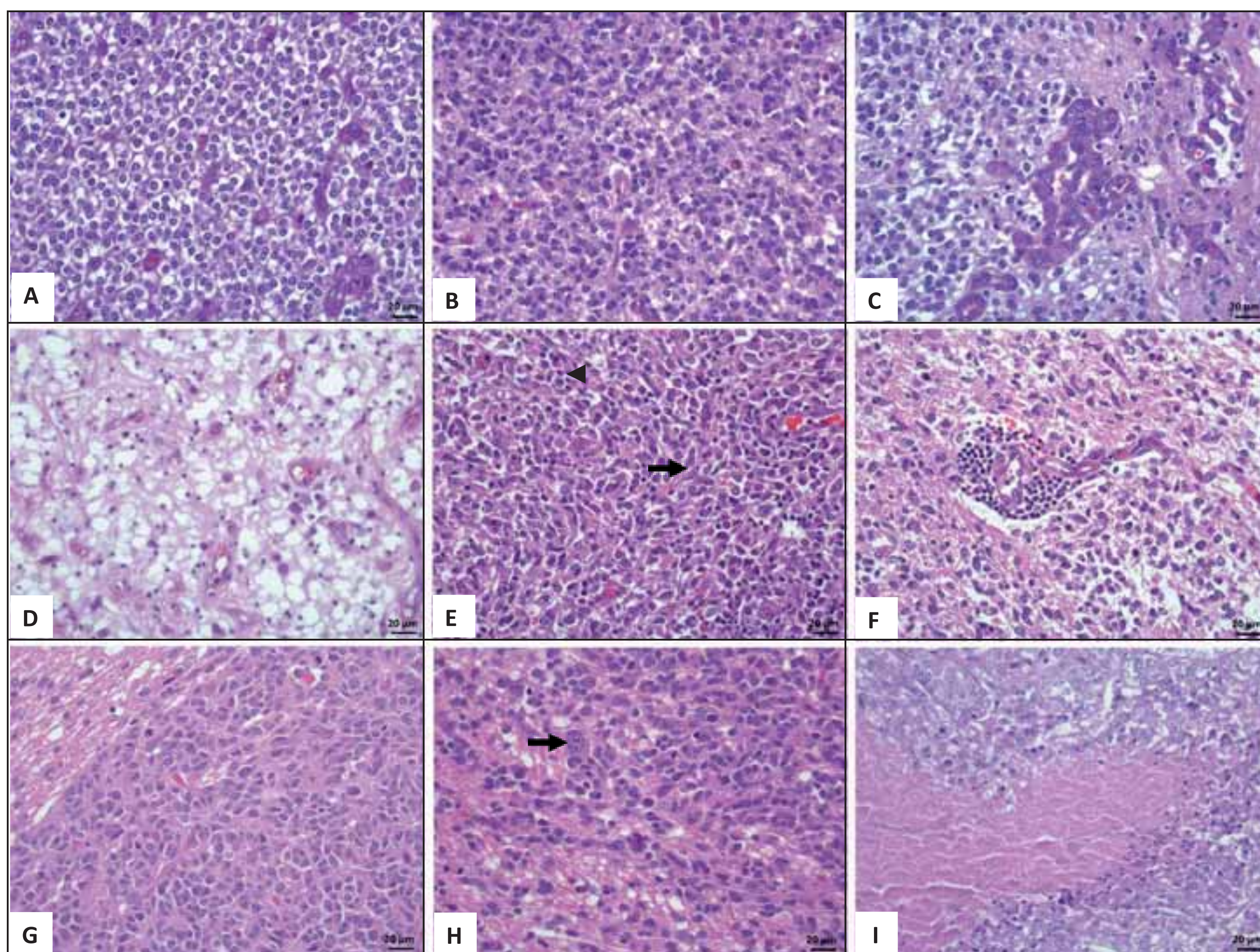

Fig. 2. Histopathological features of canine gliomas. (A, B) Honeycomb and solid growth patterns displayed by oligodendrogliomas and anaplastic oligodendrogliomas, respectively. (C) Glomeruloid-like vessels found in the margin of anaplastic oligodendrogliomas. (D) Anaplastic astrocytoma showing a diffuse infiltration of pleomorphic cells and numerous blood vessels. (E) Anaplastic oligoastrocytoma showing two cell populations: one represented by round cells with poorly defined eosinophilic cytoplasm (arrowhead, oligodendrocytes) and a second by elongated cells with an ill-defined eosinophilic cytoplasm (arrow, astrocytes). (F) Perivascular non-proliferative cuffs in anaplastic oligoastrocytoma that evidence an inflammatory associated response. (G, H) Compressive and infiltrative growth patterns displayed by glioblastomas. Note the giant nucleus frequently observed in this grade of tumour. (I) Typical pseudopalisading of neoplastic cells around a necrotic focus observed in most glioblastomas.

0–4) tumours, and also considering low-grade (range: 3–4) and high-grade (range: 0–4) gliomas (Table 4).

Distinctly different patterns of immunoreactivity for GFAP and VIM were evident between oligodendroglial and astrocytic tumours. Oligodendroglial tumours, irrespective of the grade of malignancy, had a low proportion score (range 0–1; median 1 for GFAP and range 0–3; median 1 for VIM; Fig. 4B), while astrocytic tumours showed a high proportion score with strong immunostaining (range 0–4; median 3 for GFAP and range 0–4; median 2 for VIM). Regarding S-100 protein expression, similar proportion scores were found for oligodendroglial (range 0–4; median 1.5) and astrocytic tumours (range 1–3; median 2). The inflammatory response was evaluated using the microglial marker Iba1, demonstrating a high level of expression corresponding to macrophages in perivascular cuffs, especially in the anaplastic oligoastrocytoma (Fig. 2F).

Nine of the tumour samples were found to be negative to Ki-67 immunostaining. In these tumours, cells assumed to be activated (endothelial cells in glomeruloid-like vessels) were not labelled, so the tumours were not considered when calculating the proliferation index. In tumour samples for which Ki-67 immunostaining was successful (cases 1–5, 8, 9, 14–16), mean proliferation index increased from low-grade to high-grade gliomas (6.5% in grade II gliomas, 8.5% in grade III gliomas and 9.5% in grade IV gliomas; Fig. 3G). These results were in agreement with the increase in mean mitotic index from low-grade to high-grade gliomas (Table 3).

Discussion

In this study, we investigated for the first time the IHC expression of characteristic markers of glial and neuronal lineage differentiation in 20 canine spontaneous gliomas. The study achieved two objectives: (1) gaining greater insight into the morphological and IHC characteristics of infiltrating canine gliomas (including lineage commitment and differentiation blockage of tumour cells), and (2) providing data regarding the potential of canine tumours for use as a relevant animal model for human glioma studies.

In order to investigate the similarities of phenotypic features of spontaneous canine and human gliomas, the human WHO classification was used to classify the canine gliomas. The tumours included in the study consisted of gliomas of differing phenotype

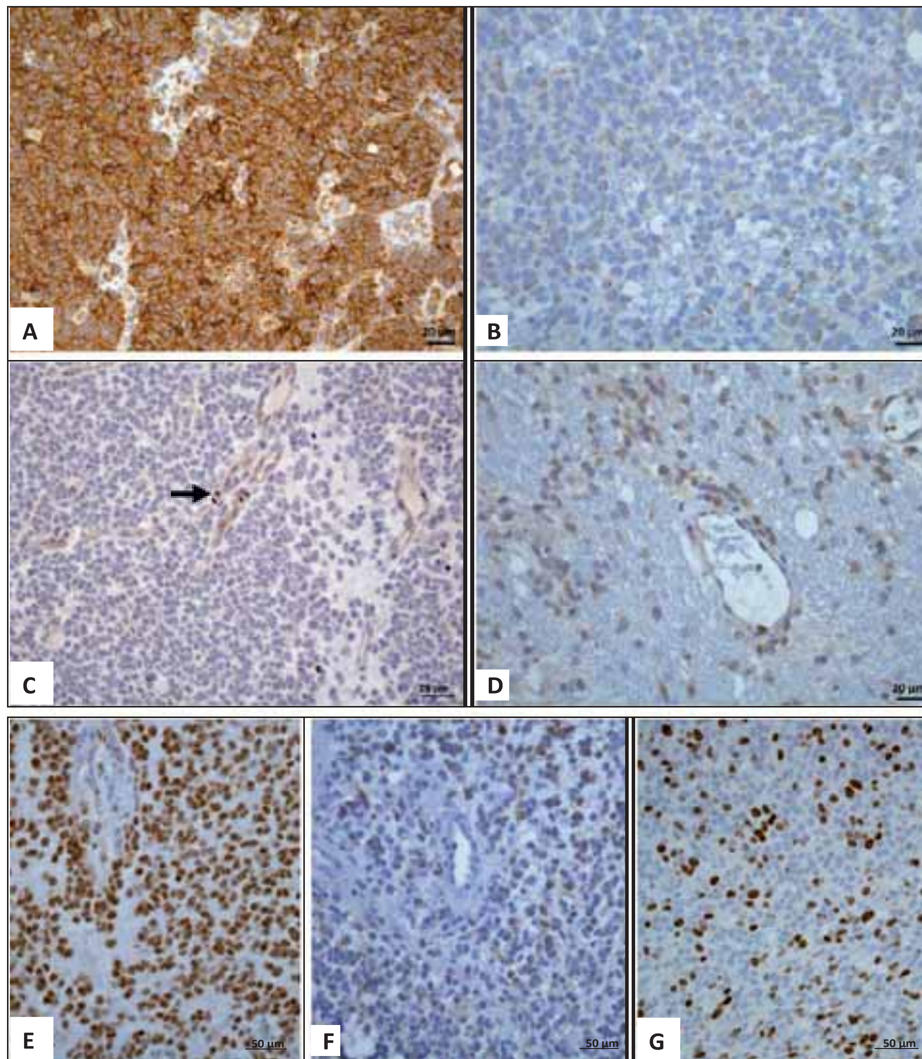

Fig. 3. Immunohistochemical features of glioblastomas. (A) A high proportion score and strong, diffuse cytoplasmic nestin immunolabelling were observed for this marker in case 15. (B) A lower proportion score and a weaker intensity with a fibrillary pattern could also be observed, as in case 16. (C, D) CD133 immunoreactivity observed in both neoplastic and endothelial cells, but with a weaker intensity in the latter. Note the evident presence of CD133+ cells in case 15 (arrow). (E) Representative example of neoplastic cells exhibiting obvious Olig2 nuclear expression. (F) Cytoplasmic expression of DCx by tumour cells in case 16. (G) Ki-67 immunohistochemistry revealed a high proliferation index in glioblastomas.

and grade of malignancy: grade II and III oligodendrogliomas, grade III and IV astrocytomas, and a grade III oligoastrocytoma. The analysis of the expression of multiple cell markers characteristic of glial and neuronal genesis hierarchy in these various samples allowed us to assess the lineage commitment of canine glioma cells (Singh et al., 2004; Dell'Albani, 2008).

In order to evaluate whether CSCs were present in the different tumour samples, the glycoprotein CD133 and the intermediate filament proteins nestin were used as markers of 'stemness'. Nestin and CD133 immunolabelling were compared, and if both were expressed, this was taken to indicate the presence of undifferentiated progenitors. Nestin$^+$CD133$^+$ progenitors typify a less differentiated subpopulation compared to nestin$^+$CD133$^-$ cells (Zeppernick et al., 2008). Combining these two markers also allowed exclusion of CD133$^+$ cells that are not CSCs (epithelial cells or activated endothelial cells for example) and non-specific CD133 labelling (secondary to tissue disturbances due to the pre-treatment step of the IHC process). A better approach would have been to perform double labelling, either by associating nestin with CD133 to ensure that positive cells were actual undifferentiated cells, or associating one of these stem cell markers with EGFRvIII in order to confirm the neoplastic nature of the progenitors. Unfortunately, the small amount of available tissue for each glioma sample prevented us from carrying out such an analysis.

Expression of both nestin and CD133 was found in the different types and grades of canine gliomas investigated, while the proportion of samples exhibiting putative CSCs was equivalent for grade II gliomas and high-grade gliomas. This observation is in line with studies of human gliomas showing that the proportion of CD133$^+$ cells in the tumour increases with the degree of malignancy and is correlated with the prognosis of patients (Beier et al., 2007; Rebetz et al., 2008; Zeppernick et al., 2008; Zhang et al., 2008). Unfortunately, in our study this trend was not found to be statistically significant, perhaps due to the heterogeneous representation of glioma grades (5 low-grade vs. 15 high-grade) and the limited number of cases. Further investigations using a greater number of cases will be necessary to confirm or reject the present findings.

The lack of immunoreactivity to βIII-tubulin and NeuN indicates that canine glioma cells do not show neuronal differentiation. This finding is in contrast with IHC features of high-grade human gliomas in which the expression of βIII-tubulin along with other markers of neurons such as neuron specific enolase (NSE) has been

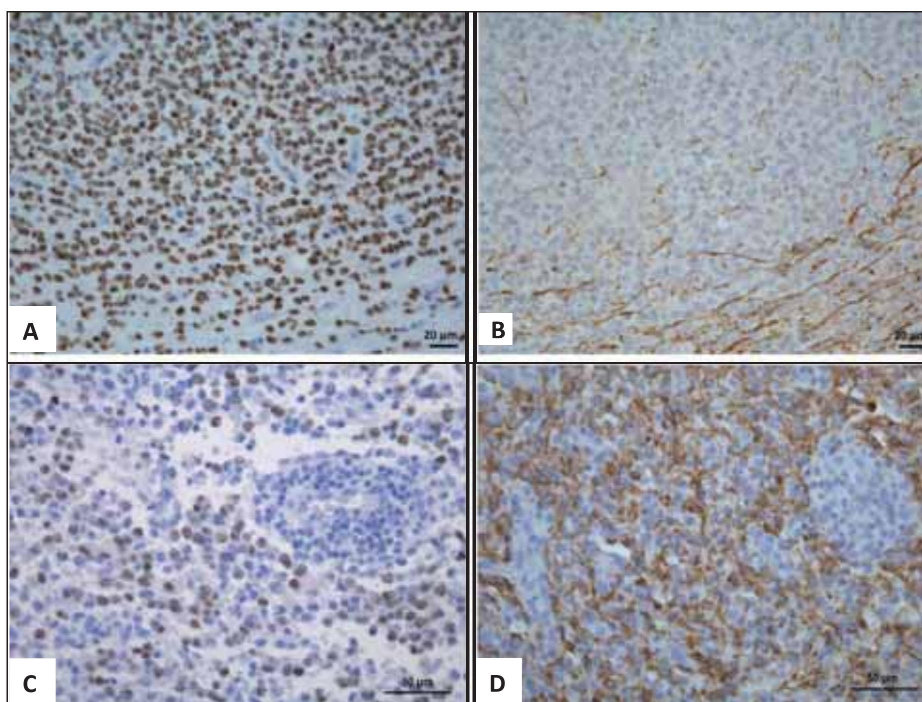

Fig. 4. Immunohistochemical features of oligodendroglial tumours and anaplastic oligodendroglioma. (A) Olig2 positivity in most of the nuclei of oligodendrogliomas. (B) Lack of expression of GFAP by neoplastic oligodendrocytes but positivity of entrapped reactive astrocytes. (C, D) Anaplastic oligoastrocytoma showed both strong Olig2 and GFAP positivity, in the nuclei of neoplastic oligodendrocytes and the cytoplasm of neoplastic astrocytes, respectively.

reported (Katsetos et al., 2007; Rebetz et al., 2008). Further evaluation of canine glioma cells, either with IHC or Western-blotting, and with different neuronal markers including NSE, will be necessary to confirm this observation.

Two markers were used to identify lineage-restricted progenitors: the transcription factor Olig2 expressed by oligodendrocyte precursor cells and the microtubule-associated protein DCx detected in neuronal precursor cells. Regardless of glioma type or grade, expression of DCx was weak or absent while the proportion score for Olig2 was high. As with human gliomas, our results suggest that both astrocytic and oligodendroglial tumours are endowed with oligodendrocyte lineage differentiation potential (Ligon et al., 2004; Rebetz et al., 2008; Rhee et al., 2009).

As expected, astrocytic tumours showed high median proportion scores for GFAP and VIM whereas median proportion scores for these markers were low in oligodendroglial tumours. The co-expression of GFAP and VIM detected in oligodendroglial tumours can be attributed to entrapped reactive astrocytes in the tumour, while GFAP expression without VIM expression suggests the presence of immature neoplastic oligodendroglial cells. As described in human gliomas, both the astrocytic and oligodendroglial tumours in our study showed similar immunoreactivity to the S-100 protein, which demonstrates the limited usefulness of this marker in the classification of spontaneous infiltrating canine gliomas.

The proliferation index tended to increase with the grade of glioma malignancy. Although this finding needs to be confirmed in a larger cohort of canine tumour samples, the higher proliferative potential of neoplastic cells in high-grade gliomas of our study, confirmed by the Ki-67 marker, is in agreement with previously published reports in humans (Hu et al., 2013).

Taken together, the lack of expression of neuronal markers and the scarcity of expression of DCx, combined with the diffuse expression of Olig2 and the presence of nestin and CD133 positive cells, suggest that tumour-initiating cells demonstrate a glial progenitor-like phenotype in canine gliomas. This finding is partially in agreement with the description of lineage commitment of glioma cells in human patients. Glial progenitor-like phenotype has been described for all human gliomas but high-grade gliomas show additional expression of markers for neuronal lineage differentiation (Rebetz et al., 2008).

Owing to the heterogeneous representation of the various glioma types and the limited number of cases in our study, a statistical analysis was not appropriate. Despite the absence of statistically significant results, this proof of concept study shows the feasibility of detecting progenitor cell markers with IHC in spontaneous canine tumour samples and the presence of these cells in all grades of diffuse gliomas. The results are a further step in demonstrating the value of spontaneous models in comparative oncology. New therapeutic strategies specifically targeting CSCs are being developed (Cho et al., 2013) and the dog could serve as a translational model for such treatments. Additional studies with a larger cohort will be necessary to allow rigorous statistical analysis of pre-planned outcome measures. We plan to extend our investigation using a greater number of canine glioma samples, currently included in a multicentre study, to evaluate IHC as well as biological characteristics of primary culture cells (capacity for neurosphere formation, multi-lineage differentiation, and tumour initiation in immunocompromised xenografted rodents).

Conclusions

To the best of our knowledge, this is the first study to evaluate lineage commitment of tumour cells in spontaneous canine gliomas. Our results suggest that, as in human infiltrating gliomas, canine glioma cells are reminiscent of glial-progenitor cells with enhanced nestin and CD133 expression in high-grade compared to low-grade gliomas. Similarities of histological and immunohistochemical features between human and canine gliomas lend support to use of the dog as a relevant animal model for preclinical evaluation of new treatment strategies.

Conflict of interest statement

Royal Canin provided financial support for this study but played no role in the study design, in the collection, analysis and interpretation of data, or in the manuscript writing or submission for publication. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Study 2. Spontaneously arising canine glioma as a potential model for human glioma

Herranz, C., Fernández, F., Martín-Ibáñez, R., Blasco, E., Crespo, E., De la Fuente, C., Añor, S., Rabanal, R. M., Canals, J. M., & Pumarola, M. (2016). Spontaneously arising canine glioma as a potential model for human glioma. *Journal of Comparative Pathology*. 154:169-179.

Introduction

GBM represents the most common glial tumor in humans. While murine animal models fail in the development of neuropathological features of human gliomas, the dog shows spontaneous gliomas equivalent to humans. The main objective of this work was to confirm the cancer stem cell hypothesis correlating the presence of neural precursors and their potential to generate neurospheres and posterior differentiation in different neural lineages, with the malignancy grade of canine gliomas.

Material and Methods

Histological and immunohistochemical studies were carried as in Study 1. Fresh samples of center and periphery gliomas and contralateral SVZ and cerebral parenchyma were taken. Cell culture techniques in neurobasal medium with EGF and FGF factors were applied in all samples. Neurospheres were expanded, passed, differentiated, and cryopreserved. Neuronal, astrocytic, and oligodendrocytic cell lineages were applied with differentiation techniques.

Results

Histological and immunohistochemical results were equivalent to Study 1. Neurospheres were obtained from all tumors. The number of expanded neurospheres was higher in HGG than in LGG. Neurospheres from 3 of 5 tumors reached pass 6. All neurospheres collected from tumors and SVZ showed capability to expand in the three neural lineages.

Conclusions

The amount of multipotent neural progenitors able to form neurospheres was related with the greater expression of neural progenitor markers in HGG in canine gliomas. Samples obtained from the central part of HGG produced better results than those from the periphery. These results were similar to those published on human gliomas and indicate that spontaneous canine glioma is a good animal model for the study of the CSC hypothesis.

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NEOPLASTIC DISEASE

Spontaneously Arising Canine Glioma as a Potential Model for Human Glioma

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Summary

Human gliomas are malignant brain tumours that carry a poor prognosis and are composed of a heterogeneous population of cells. There is a paucity of animal models available for study of these tumours and most have been created by genetic modification. Spontaneously arising canine gliomas may provide a model for the characterization of the human tumours. The present study shows that canine gliomas form a range of immunohistochemical patterns that are similar to those described for human gliomas. The *in-vitro* sphere assay was used to analyze the expansion and differentiation potential of glioma cells taken from the periphery and centre of canine tumours. Samples from the subventricular zone (SVZ) and contralateral parenchyma were used as positive and negative controls, respectively. The expansion potential for all of these samples was low and cells from only three cultures were expanded for six passages. These three cultures were derived from high-grade gliomas and the cells had been cryopreserved. Most of the cells obtained from the centre of the tumours formed spheres and were expanded, in contrast to samples taken from the periphery of the tumours. Spheres were also formed and expanded from two areas of apparently unaffected brain parenchyma. The neurogenic SVZ contralateral samples also contained progenitor proliferating cells, since all of them were expanded for three to five passages. Differentiation analysis showed that all cultured spheres were multipotential and able to differentiate towards both neurons and glial cells. Spontaneously arising canine gliomas might therefore constitute an animal model for further characterization of these tumours.

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Keywords: animal model; dog; glioma; neurosphere

Introduction

Glioblastoma is the most common primary human brain tumour and it is among the more malignant and difficult to treat of these cancers (Furnari *et al.*, 2007). Its incidence is 2e3 cases per 100,000 people, and the median survival time in treated patients is 12 months (Das *et al.*, 2011). The infiltrative nature of the tumour is a major determinant of its aggressiveness (Rao, 2003). Many efforts are directed to the study and understanding of this devastating malignancy in order to develop effective treatments that may increase the life span of these patients.

Rodents are frequently used to model human gliomas; however, such models are limited by the inbred nature of many rodent strains, differences in life span, size of the animals and lack of spontaneous

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development of the disease (Lim et al., 2012). The canine nervous system is much closer in size to that of man, alleviating some concerns regarding scalability and technical limitations such as cellular migration. In addition, dogs have a high incidence of spontaneously arising intracranial tumours (14.5 cases per 100,000 dogs; Stoica et al., 2009). The magnetic resonance imaging (MRI) features of some canine brain tumours have been reported to be similar to those of man (Rodenas et al., 2011; Young et al., 2011). In addition, although smaller in size, the canine brain is microscopically very similar to the human brain (Candolfi et al., 2007; Schneider et al., 2008; Pang and Argyle, 2009; Chen et al., 2013) and canine gliomas are similar to the human equivalents (Candolfi et al., 2007; Vandevelde et al., 2012; Fernández et al., 2015).

The aim of the present study was to further characterize aspects of spontaneously arising canine glioma in order to determine whether this tumour provides an appropriate animal model for investigations of new therapeutic options for human glioma.

Materials and Methods

Case Material

Gliomas from five dogs were studied, including two oligodendrogliomas, one anaplastic oligodendroglioma, one mixed glioma and one glioblastoma. Dogs were evaluated by a certified neurologist from the Neurology and Neurosurgery Service of the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona, Barcelona, Spain, and all had neurological signs consistent with a focal brain lesion. Based on MRI of the brain and cerebrospinal fluid analysis, a presumptive diagnosis of glioma was reached in all cases. Breed, sex, age and neurological signs are summarized in Table 1. A healthy, 1-year-old male Beagle dog was used as control. All owners gave their written consent for necropsy examination. All experimental procedures were approved by the Ethics Committee for Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

Sample Collection

Complete post-mortem examinations were performed immediately after death. Serial transverse sections of the brain were made based on MRI images and samples were taken from the centre and periphery of the tumour, contralateral cerebral tissue and the contralateral subventricular zone (SVZ) (Fig. 1). Each sample was approximately 8 mm^3. For cell cultures, tissue samples were immersed in control medium (CM) consisting of neurobasal medium containing 100 U/ml penicillin, 100 mg/ml streptomycin and 0.3 mg/ml glutamine (Life Technologies, Madrid, Spain) and preserved in ice until processed.

Histological and Immunohistochemical Evaluation

Samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 mm) were stained with haematoxylin and eosin (HE). Classification and grading of the tumours was performed according to the criteria defined by the World Health Organization (WHO) for human central nervous system (CNS) tumours (Louis et al., 2007). The microscopical features used for tumour classification were: growth pattern, type and morphology of neoplastic cells, grade of anisokaryosis, presence of mitosis, mucinous secretion, presence of necrosis, vascular features and relationship between tumour and adjacent nervous tissue (i.e. compression or infiltration). Cell markers used for immunohistochemistry (IHC) are shown in Table 2. Anti-mouse (catalogue number K4007; Dako, Glostrup, Denmark) and anti-rabbit (catalogue number K011, Dako) EnVision™ kits were used to label the primary antibodies. In all cases a 3, 3^0 diaminobenzidine-based detection kit was used

Table 1
Clinical data from dogs included in the study

Case number	Breed	Sex	Age (years)	Clinical signs	Location of the tumour
1	Boxer	F	8	Head pressing, compulsive gait, right circling, depressed mental status, absent proprioceptive reactions in left forelimb and left hindlimb, cervical hyperaesthesia	Right prosencephalon
2	Boxer	F	9	Clusters of generalized toniceclonic seizures every 2 weeks, depressed mental status, right circling, cervical hyperaesthesia	Right prosencephalon
3	Boxer	M	10	Status epilepticus, generalized toniceclonic seizures	Prosencephalon
4	French bulldog	F	4	Depressed mental status, right hemiparesis, absent menace response in right eye, absent proprioceptive reactions in right forelimb and right hindlimb	Left prosencephalon
5	French bulldog	M	4	Behavioural changes, generalized toniceclonic seizures	Prosencephalon

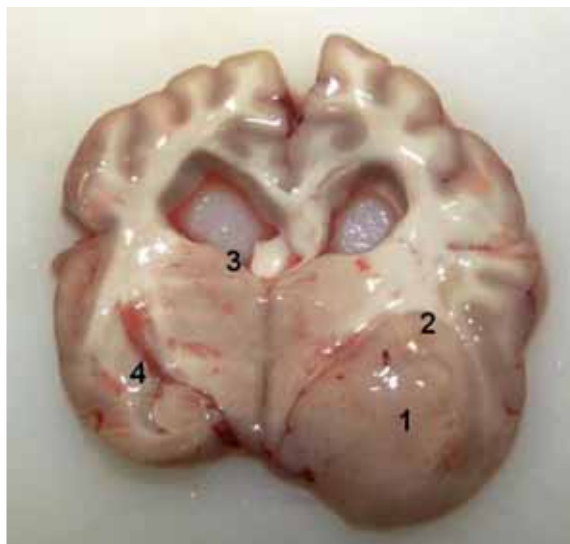

Fig. 1. Sample collection for cell culture analysis. Transverse brain section of a grade II oligodendroglioma (case 2). Four samples were taken from each case for cell culture analysis. Numbers indicate the brain areas from which samples were dissected: (1) centre, (2) periphery, (3) contralateral SVZ and (4) cerebral parenchyma (4).

(Dako). Sections were counterstained with haematoxylin. The percentage of cells positive for each marker was determined by two neuropathologists in a blinded fashion by counting the number of labelled cells in five randomly-selected ×400 fields. The proliferation index (PI) was estimated by counting the number of Ki67 labelled and unlabelled cells in ten ×400 fields (approximately 2,000 cells) and was expressed as a percentage. Only nuclear labelling was considered positive and areas with the greatest positivity were chosen for evaluation.

Cell Culture

One sample from each brain tumour and area was processed for cell culture expansion. Conditions for primary tissue culture were adapted from Stoica *et al.* (2009). Samples were cut into small pieces and subsequently dissociated mechanically in CM. The resulting cell suspension was washed twice with phosphate buffered saline (PBS), filtered through a 40 mm filter and resuspended in complete media (CTM) consisting of CM with 2% B27 supplement, 20 ng/ml epidermal growth factor (EGF; Life Technologies) and 20 ng/ml fibroblast growth factor (FGF; Sigma Aldrich Qu´ımica SA, Madrid, Spain). Floating sphere cultures were obtained by seeding 10^4 cells/cm^2 in CTM. Cells were expanded in T75 flasks (TPP, Techno Plastic Products AG, Trasadingen, Switzerland) at 37°C in 5% CO_2. These cultures were considered as passage 0 (P0). Every 3e4 days, one half of the medium was changed. The spheres tended to adhere to the plastic wall of the flasks, so the expansion methodology was improved using ultralow attachment surface culture flasks (Corning, New York, New York, USA).

After 10e15 days, spheres were collected, centrifuged, incubated with Accutase 1X (PAA Laboratories Gmb, Pasching, Austria) for 5e10 min at 37°C and dissociated into a single cell suspension by vigorous pipetting. Cell viability was evaluated by trypan blue exclusion and 10^4 cells/cm^2 were seeded into fresh CTM. The same procedure was repeated for several passages up to a maximum of six (P6). Cultures that could be expanded for more than five passages were considered to contain bona fide glioma stem cells that could be cryopreserved.

Cryopreservation of Cultured Cells

Tumour sphere cultures that reached P6 were cryopreserved. Spheres were dissociated with Accutase as described above and cultured in CTM for 2 days. They were then resuspended in cold freezing media (CTM + 5% dimethyl sulphoxide; Sigma). Vials containing the spheres were frozen at —80°C and subsequently transferred to a liquid N_2 tank.

Cell Culture Differentiation

A protocol described by Martin-Ibanez *et al.* (2010) was used for differentiation studies. Briefly, dissociated spheres were disaggregated using Accutase and seeded at a cell density of 12.5 × 10^3 cells/cm^2 onto coverslips that were precoated with Matrigel (BD Biosciences, Madrid, Spain). Cells were allowed to differentiate for 3 days in differentiation media (DM) 1, which consisted of CM supplemented with 2% B27 and 20 ng/ml FGF. The culture medium was then changed to DM2, which consisted of CM supplemented with 2% B27 and 2% fetal bovine serum (FBS; Life Technologies), for the last 3 days. Under these conditions glioma cells were able to differentiate spontaneously towards the three neural lineages, neurons, astrocytes and oligodendrocytes, as described previously for mouse neural stem cells (Martin-Ibanez *et al.*, 2010).

Immunocytochemistry

Differentiated cell cultures were characterized by immunocytochemistry according to the protocol described by Martin-Ibanez *et al.* (2010). The panel of primary antisera employed is shown in Table 2. Cy3-conjugated donkey anti-rabbit IgG (1 in 500 dilution) and Cy3-conjugated donkey anti-mouse IgG (1 in 500 dilution), both from Jackson

Table 2
Primary antibodies used for immunohistochemistry and immunocytochemistry

Marker	*Antibody name*	*Manufacturer and catalogue number*	*Dilution*	*Pretreatment*
Nestin	Rabbit polyclonal to nestin (neural stem cell marker)	Abcam, Cambridge, UK; ab5968	1 in 500	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
DCx	Rabbit polyclonal to CD133 stem cell marker	Abcam, ab18723	1 in 1,000	Citrate buffer 0.01 M pH 6.0, 20 min water bath 96e98°C + 30 min room temperature
bIII (Tuj1)	Mouse monoclonal anti-tubulin bIII isoform	Merck Millipore, Barcelona, Spain; MAB1637	1 in 200	Citrate buffer 0.01 M pH 6.0, 20 min water bath 96e98°C + 30 min room temperature
bIII (Tuj1)	Mouse monoclonal anti-tubulin bIII isoform	Sigma Aldrich, Madrid, Spain; T8660	1 in 200	Used for immunocytochemistry (without pretreatment)
NeuN	Mouse anti-neuronal nuclei	Merck Millipore, MAB377	1 in 500	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
Olig2	Rabbit Olig2 polyclonal antibody	Merck Millipore, AB 9610	1 in 100	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature. Also used for immunocytochemistry without pretreatment
Glial fibrillary acidic protein	Rabbit anti-bovine glial fibrillary acidic protein	Dako, Glostrup, Denmark; Z0334	1 in 500	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature and used for immunocytochemistry with no pretreatment
S100	Rabbit anti-S100	Dako, Z0311	1 in 1,000	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
Vimentin	Mouse monoclonal anti -vimentin clone V9	Dako, M0725	1 in 200	No pretreatment
Iba1	Goat polyclonal to Iba 1	Abcam, ab5076	1 in 600	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
Ki67	Mouse anti-human Ki67	Dako, M7240	1 in 100	Citrate buffer 0.01 M pH 6.0, pressure cooker 4 min + 30 min room temperature

Immunoresearch Laboratories (Newmarket, UK) were used as secondary reagents. Cell cultures were counterstained with 4^{0}, 6-diamidino-2-phenylindole dihydrochloride (Sigma). Fluorescent photomicrographs were taken on a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems, Barcelona, Spain).

Results

Characterization of Canine Gliomas

All affected dogs were brachycephalic and 4e10 years of age. Younger dogs developed the highest grade malignant tumours and no sex predilection was observed. Neurological signs correlated with the imaging findings (Fig. 2AeD) and post-mortem localization of tumours. All tumours were located in the cranial fossa and affected mainly the ventral areas of one or both cerebral hemispheres (Figs. 1 and 2A). All tumours, especially those of high grade, showed invasion of the lateral ventricles (Figs. 2BeD). Two tumours were oligodendrogliomas (grade II), one was an anaplastic oligodendroglioma (grade III), one a mixed glioma (grade III) and one a glioblastoma (grade IV).

The immunohistochemical analysis is summarized in Table 3. The neural progenitor marker nestin was very mildly expressed by most tumours, except for the glioblastoma. Similarly, immature neuronal markers, such as DCX, were very mildly expressed by all tumours. However, increased DCX expression was detected in high-grade gliomas, specifically in the glioblastoma (Fig. 2K; Table 3). No expression of bIII-tubulin was found for any of the gliomas (Fig. 2G). Glial fibrillary acidic protein (GFAP)

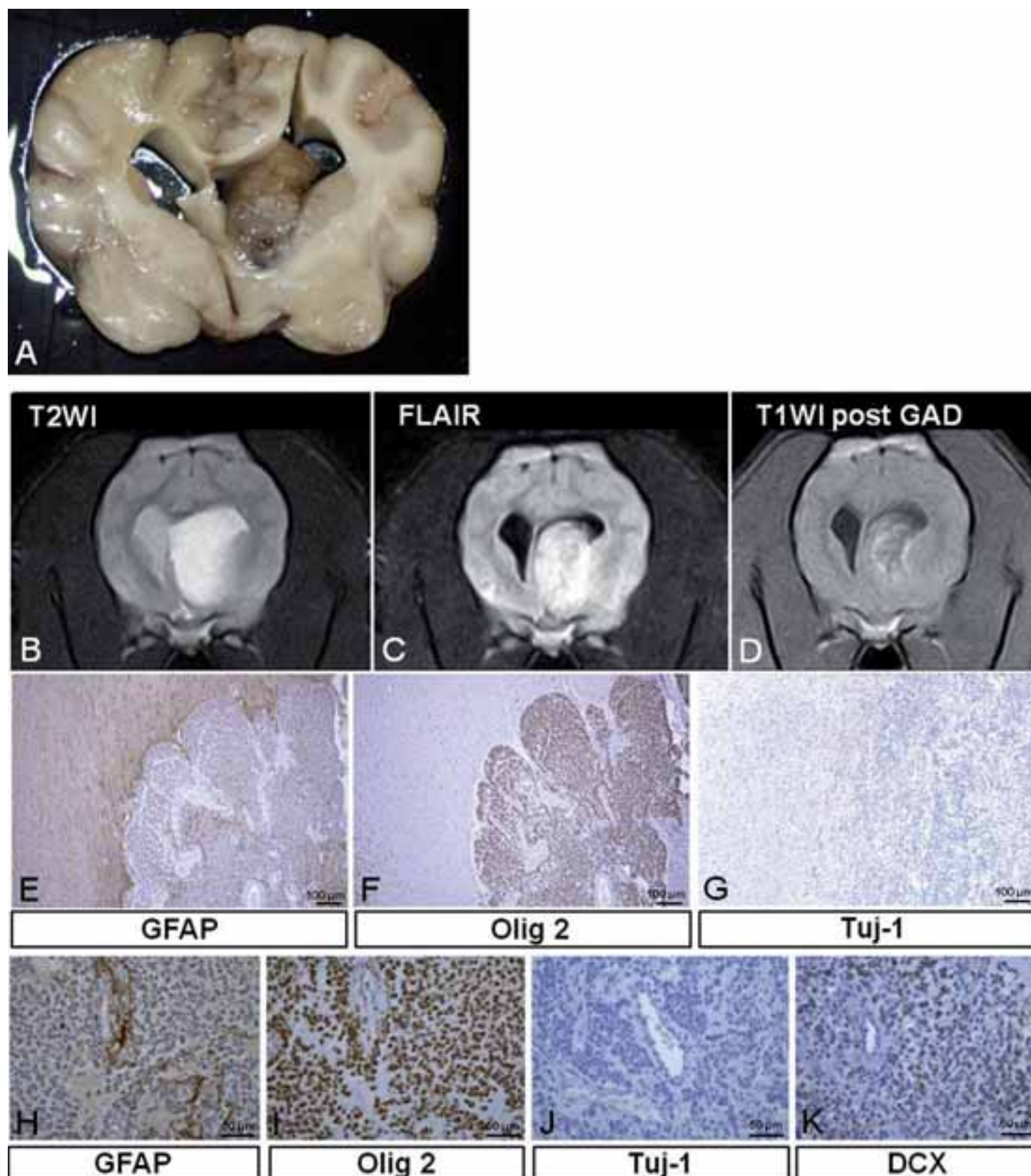

Fig. 2. *In-vivo* characterization of a canine glioblastoma (case 5). (A) Transverse brain section showing the presence of the tumour in the caudate nucleus invading the lumen of the lateral ventricle. (BeD) Brain magnetic resonance images at the level of the caudate nuclei. (B) A hyperintense mass lesion on (C) T2-weighted and (D) FLAIR images with heterogeneous peripheral contrast enhancement on post-contrast T1-weighted images, consistent with a glioblastoma. (EeK) Immunohistochemical analysis of tumour sections revealed that this glioblastoma is characterized by (E, H) low to moderate levels of GFAP expression confined to reactive astrocytes surrounding vessels (low and high magnification, respectively). (F, I) In addition, the tumour also has high expression of Olig2 (low and high magnification, respectively). (G, J) No expression of bIII-tubulin was found (Tuj1; low and high magnification, respectively). (K) The tumour does have moderate expression of the immature neuron marker doublecortin (DCX).

and vimentin were similarly expressed by all tumours, except for the mixed glioma, which had increased GFAP labelling in reactive astrocytes surrounding vessels. S100 protein was highly expressed by both low- and high-grade oligodendrogliomas, but very low levels were found in the mixed glioma and glioblastoma (Table 3). Olig2 was highly expressed by all oligodendrogliomas (Table 3), but the mixed glioma showed lower Olig2 expression. In this tumour, the highest level of Iba1 expression was observed in cells of the perivascular cuffs.

The PI ranged from 7 T 3.13 to 25 T 11.13 in the grade II oligodendrogliomas and from 25 T 11.13 to 45 T 10.05 in the grade III anaplastic

Table 3
Immunohistochemical features of the canine glial tumours

Case	*Diagnosis*	*Nestin*	*DCx*	b*III*	*NeuN*	*Olig2*	*Glial fibrillary acidic protein*	*S100*	*Vimentin*	*Iba1*
1	Oligodendroglioma grade II	+	+	—	—	+++	+	+	+	+
2	Oligodendroglioma grade II	+	+	—	—	+++	+	+++	++	+
3	Anaplastic oligodendroglioma grade III	+	+	—	—	++++	+	++++	+	+
4	Mixed glioma grade III	+	+	—	—	++	+++	+	++	+++
5	Glioblastoma grade IV	++++	++	—	—	++++	++	+	++	+

Percentage of positive cells: —, #5%; +, 5e25%; ++, 25e50%; +++, 50e85%; ++++, $85%.
DCx, doublecortin; bIII, bIII tubulin.

oligodendroglioma. The highest PI was found in the mixed glioma (160 T 12.47). The glioblastoma had an intermediate PI of 60 T 6.84.

Expansion and Differentiation Potential of Canine Tumors

Two of the main characteristics of glioma forming cells are their ability to expand and the potential to differentiate towards the three neural lineages. In order to study whether the cells obtained from the five canine brain gliomas were able to form spheres, expand and differentiate, we characterized them *in vitro*. For each glioma the centre and periphery of the tumour was analyzed in order to study the differences between the cells within these two areas. In addition, the contralateral parenchyma and the SVZ were examined as negative and positive controls, respectively.

The ability to form spheres and their expansion potential was studied by growing cells in suspension (Figs. 3AeD). The SVZ has been shown to be an adult stem cell niche for other species (Alvarez-Buylla and Garcia-Verdugo, 2002), so a sample from this area of control normal canine brain was studied initially as a positive control. SVZ cells from an adult dog were able to form spheres and expand (Fig. 3A) and cells obtained from the contralateral SVZ were also able to expand as spheres (Table 4).

Samples from the center and periphery of the tumour were also studied. Cells isolated from the centre of the tumours (for all four types of glioma) could form spheres and be expanded, but with some differences between them (Figs. 3BeD; Table 4). Grade II oligodendroglioma-derived cells (case 1) were only able to be expanded for four passages, indicating that these may have been cells or progenitors with low proliferation capacity. It was not possible to expand cells from case 2, having the same type of glioma. However, cells from the centre of the grade III oligodendroglioma were expanded for six passages, although the expansion was minor and the culture was lost. Cells derived from high-grade gliomas, both mixed glioma and glioblastoma, were readily expanded for six passages and sufficient cells were obtained for cryopreservation (Table 4).

None of the samples obtained from the tumour periphery formed spheres, except for one from a mixed glioma (case 4) that could be expanded for four passages (Table 4).

Samples from the contralateral cerebral parenchyma were used as non-proliferative controls. Sphere formation was not observed for samples from cases 1 and 3; however, samples from cases 2 and 4 were expanded and reached passages five and six, respectively (Table 4 and Fig. 3E).

Most such derived cells showed a low proliferation rate. From a total of 11 samples processed *in vitro* for cell expansion studies, five attached to the culture flask after P3 (Table 4) and so to avoid sphere attachment non-adherent culture flasks were used.

Finally, the differentiation potential of the glioma derived-expanded spheres was characterized. Differentiated spheres were obtained from the five glioma cases and from the control dog SVZ. After 6 days of differentiation, all samples differentiated into the three neural lineages (Fig. 4). Similar to spheres derived from the control dog SVZ (Figs. 4AeC), cells derived from all tumours maintained their differentiation potential following *in-vitro* expansion for several passages, independently of the type of glioma from which they were derived (Figs. 4CeL).

Discussion

The results of the present study suggest that the dog may provide a useful animal model for human

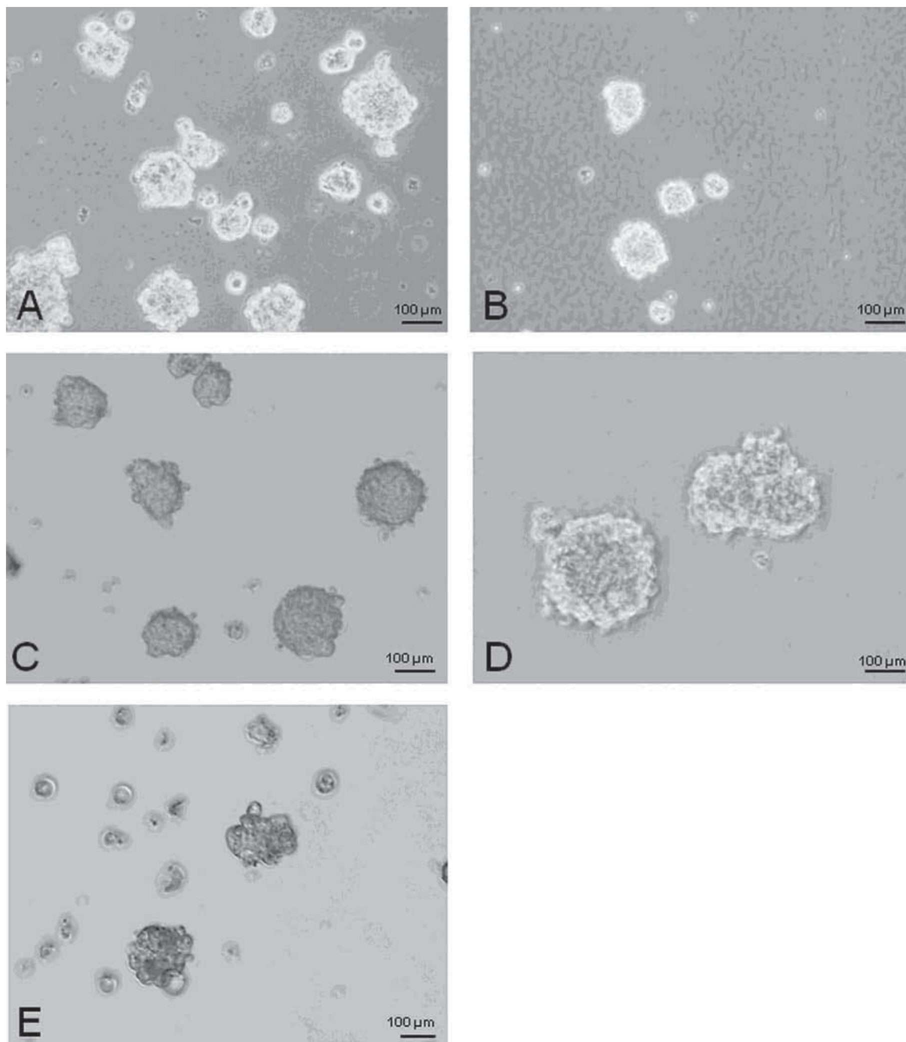

Fig. 3. Sphere formation and expansion potential of cultures obtained from five gliomas. Representative pictures show the ability of gliomas to divide and grow in suspension forming cell aggregates called spheres. (A) Neurospheres derived from a non-affected dog SVZ. (BeD) Glioma-derived spheres from high-grade gliomas expanded up to passage six (B, case 3; C, case 4; D, case 5). In some cases, spheres from contralateral cerebral tissue were obtained. Representative image of spheres obtained from contralateral cerebral tissue (E, case 4).

glioma. Spontaneously arising canine gliomas have similar microscopical characteristics to human gliomas with respect to morphology, infiltration, the pattern of expression of immunohistochemical markers and their labelling intensity according to the grade of malignancy. Three of the five gliomas were oligodendrogliomas, which reflects a higher incidence than astrocytomas in our referral hospital population, as has been described previously described (Song *et al.*, 2013). Cells derived from grade II and III oligodendrogliomas, mixed glioma and glioblastoma were able to form spheres, proving the presence of progenitors with proliferative capacity. There appeared to be a correlation between tumour grade and the ability to form spheres. Glioma-derived spheres were multipotential since they could be differentiated to form cells from the three neural lineages, as described for these tumours from man and other species (Dell'Albani, 2008; Zhang *et al.*, 2008; Stoica *et al.*, 2009).

Tumours were classified and graded according to the latest human WHO scheme and were well correlated with human gliomas (Louis *et al.*, 2007). Grade II and III oligodendrogliomas displayed high levels of Olig2 expression, which indicates the expression of oligodendroglial and neural precursor cell proteins, as has been described previously for the human tumours (Rhee *et al.*, 2009). Grade II and III tumours displayed low nestin expression, but DCX protein was observed in a few neoplastic cells, also indicating the

Table 4
Expansion potential of all samples dissected from the ftve cases of glioma

Case	Tumour		Contralateral	
	Centre	Periphery	Cerebral parenchyma	Subventricular zone
1	Yes P4 (attached)	No	No	Yes P3 (attached)
2	No	No	Yes P5	Yes P4
3	Yes P6	No	No	Yes P5 (attached)
4	Yes P6 (cryopreserved)	Yes P4 (attached)	Yes P6 (cryopreserved)	Yes P4 (attached)
5	Yes P6 (cryopreserved)	x	x	x

For each case (1e5) and sample (tumour, periphery, contralateral cerebral parenchyma and subventricular zone) it is indicated whether it was possible (Yes) or no (No) to expand the culture. If the culture was successfully expanded, the number of passages that the culture reached is indicated (P3, P4 or P6: passages 3, 4 or 6, respectively). In addition, cultures that attached to the culture flasks during expansion and were lost are indicated, as are those that were cryopreserved at passage 6. Boxes with 'x' indicate the inability to obtain these samples for cell expansion studies.

co-expression of neuronal progenitor cell proteins. Glioblastoma cells displayed the highest expression of Olig2 and nestin, but also had high levels of GFAP, vimentin and DCX. Previous reports suggested that high levels of nestin expression were directly related to the presence of progenitor cells in glial tumors (Dell'Albani, 2008; Zhang *et al.*, 2008; Stoica *et al.*, 2009). Our results show that markers associated previously with proliferative progenitors in the human tumours identify neural precursor cells in the canine counterparts (Fernández *et al.*, 2015), supporting the concept that glioblastomas are composed of undifferentiated self-renewing progenitor cells (Louis *et al.*, 2007). The immunohistochemical findings suggest that the presence of immature neural cells increases with tumour grade, as described for the human tumours (Phillips *et al.*, 2006; Mangiola *et al.*, 2008; Zeppernick *et al.*, 2008; Gilbert and Ross, 2009).

It has been shown previously that tumour-forming cells are able to floating spheres, have a high percentage of G0/G1 phase cells, a higher colony-forming ability and increased capability to develop into multiple lineages (Qiang *et al.*, 2009). To date, only one case of sphere formation and neural differentiation has been described from a canine glioblastoma (Stoica *et al.*, 2009). The results of the present study confirm that spheres derived from canine glioblastomas can be expanded and differentiated *in vitro*. In addition, cells derived from other glioma types, including low-grade oligodendrogliomas, were also able to form spheres and differentiate. Interestingly, high-grade glioma- and glioblastoma-derived spheres grew more easily than those from other gliomas. Taking into account that the PI is higher for these types of gliomas, we hypothesize that their proliferative progenitors contribute to the increased ability to form spheres and expand. However, a larger sample size would be necessary in order to establish a clear correlation between glioma grade, number of proliferating cells and ability to form spheres. Nevertheless, the results of this study are in agreement with previously published results, which also suggest that self-renewal, potential as measured by Ki67 expression in spheres, correlates with tumour grade (Mao *et al.*, 2007). The results presented here provide a new *in-vitro* model to study tumour cells obtained from an animal that spontaneously develops gliomas (Stoica *et al.*, 2009), in a similar manner to man (Qiang *et al.*, 2009; Tabatabai and Weller, 2011; Knights *et al.*, 2012). In addition, our results show that spheres obtained from different types of glioma retain the differentiation potential for neural phenotypes. This behaviour is similar to that of non-neoplastic stem cells and it is also a feature of human glioblastomas (Gilbert and Ross, 2009).

Another interesting observation was the difference between cells from the periphery and centre of the tumours. No morphological changes were observed in any tumour between these two areas, except for glioblastoma, where necrosis occurred mainly in the centre of the tumour. However, while most samples obtained from the centre of tumours could be expanded as floating spheres, only one sample from the periphery was able to do this. This sample was from the mixed glioma, which was the tumour with the highest PI. Therefore, we hypothesize that some of the neoplastic cells could have infiltrated the periphery. These results suggest that, regardless of tumour type, the tumour center contains most of the proliferative cells, while the tumour periphery contains few or none. These results are in agreement with those from studies of human tumours, in which cells obtained from the centre of the tumour show a higher ratio of continuously self-renewing cells compared with cells derived from the tumour periphery (Das *et al.*, 2008). This particular feature has been associated with the high capacity of progenitor cells from the centre of the tumour to adapt to a hypoxic environment (Singh *et al.*, 2014).

Spheres were also obtained from non-neurogenic areas of the cerebral parenchyma in two dogs. This

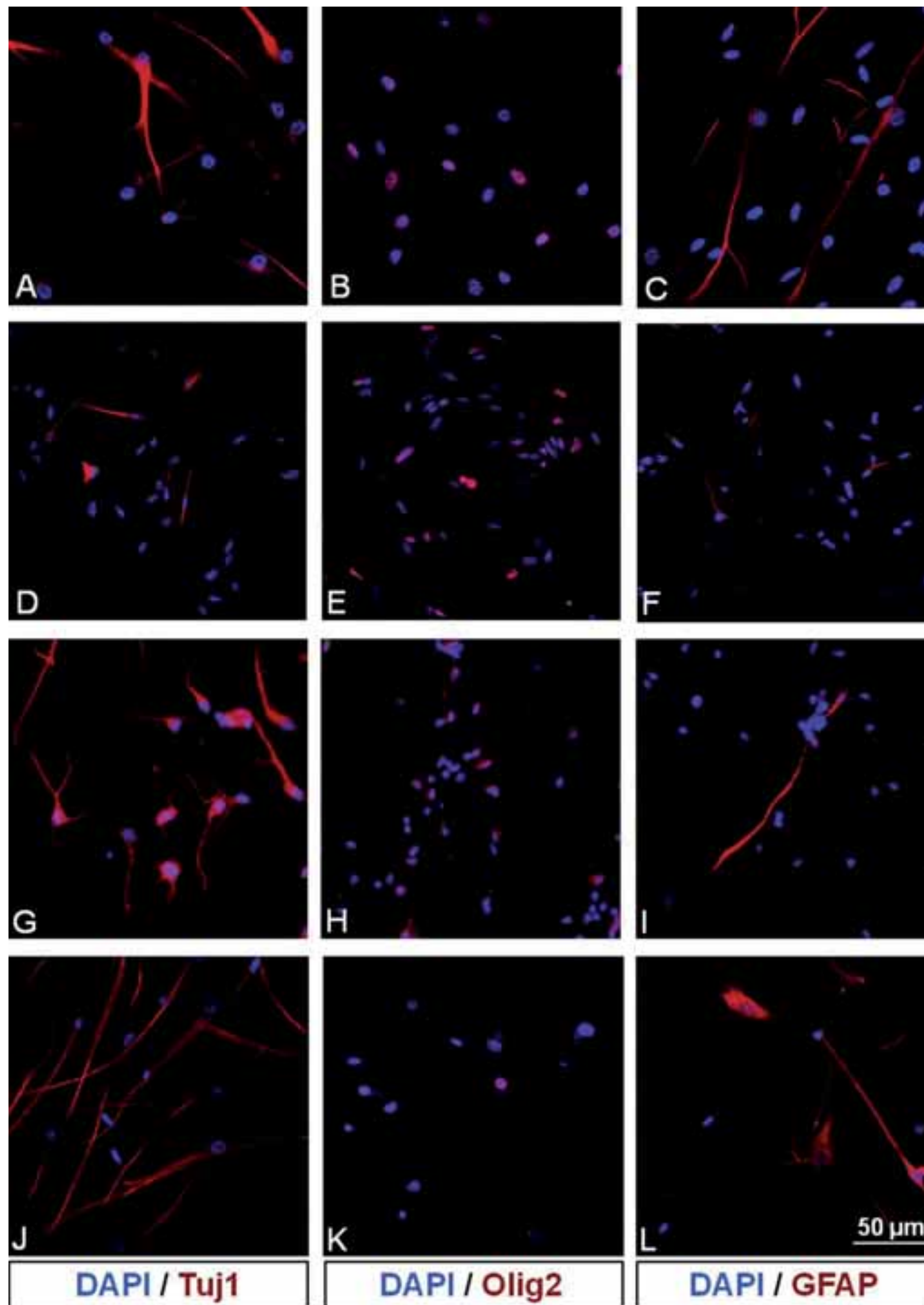

Fig. 4. Expanded spheres obtained from the SVZ of a control dog, mixed glioma, glioblastoma and contralateral brain parenchyma from a grade III oligodendroglioma are multipotent under differentiation conditions. (AeC) Immunocytochemical analysis of differentiated cultures obtained from the SVZ of a control dog. (DeF) Mixed glioma (case 4). (GeI) Glioblastoma (case 5). (JeL) Contralateral brain parenchyma (case 2). Images show the ability to generate bIII-tubulin-positive neurons (Tuj-1), Olig2-positive oligodendrocytes and glial fibrillary acidic protein (GFAP)-positive astrocytes. All images are representative and counterstained with nuclear marker 4^0, 6-diamidino-2-phenylindole (DAPI).

could be related to initiation of secondary tumours due to the presence of metastatic tumour cells close to blood vessels outside the primary tumour. Tumour cells migrate to other areas using the vascular niche located around blood vessels (Christensen *et al.*, 2008; Kazanis *et al.*, 2008). The vascular niche is essential for survival and expansion of adult stem cells in the SVZ (Conover and Notti, 2008)

and tumour proliferative cells (Christensen *et al.*, 2008).

In most of the cases, cells derived from the contralateral SVZ formed spheres, leading to the hypothesis that this zone may act as a reservoir of stem cells in the adult canine brain, as it has been described for man and other species (Gould, 2007; Walton and Wolfe, 2008; Zhang *et al.*, 2008). Ongoing studies in healthy dogs will further characterize the adult canine SVZ and its derived spheres.

This study has shown that different canine glioma types are analogous to their human counterparts and that tumour cells behave similarly in terms of sphere formation, expansion and differentiation potential. Additional studies with a larger number of samples are necessary for rigorous statistical analyses. Nevertheless, the dog may provide a suitable animal model for the study of human gliomas.

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Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Study 3. Characterization of the canine rostral subventricular zone: morphological, immunohistochemical, ultrastructural and neurosphere assay studies.

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Introduction

The mammalian subventricular zone (SVZ) presents the higher neurogenic potential in the adult brain. In rodents, the SVZ if mainly formed by chains of neuroblasts. In humans is organized in layers and neuroblasts do not form chains. Neurospheres have been obtained from the adult SVZ of rodents, primates, humans and pigs. The aim of this study was to describe the cytoarchytecture of the canine SVZ, asses its neurogenic potential, and to compare the results obtained with those previously described in other mammals.

Material and Methods

We have studied by histology, immunohistochemistry (IHC), ultrastructuraly, and using *in vitro* techniques the morphology, cellular components and neurogenic potential of the canine SVZ. Different age groups of animals were selected and grouped in young, adult-young, middle-age and aged animals.

Results

The histological and ultrastructural features indicate that the canine SVZ is organized in layers as in humans, but including migratory chains as in rodents. The neural precursors were identified in niches in the subependymal layer. Their number decline with age. Adult dogs contain neural progenitors capable to expand and differentiate. Adult dogs present perivascular migratory chains of neural precursors outside the SVZ.

Conclusions

The presence of migratory chains and their capacity to proliferate and differentiate in vitro may allow to use the dog in comparative and preclinical studies. The age-related decline of neural progenitors in the SVZ together with the presence of migratory chains in adults indicates their capacity to forms perivascular niches and could be related compensatory cortical neuronal loss in the adult dog. The similarity between the canine and human SVZ indicates that the dog could represent better the human adult neurogenic processes compared with rodents.

Characterization of the canine rostral subventricular zone: morphological, immunohistochemical, ultrastructural, and neurosphere assay studies.

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Introduction

The subventricular zone (SVZ) of the adult brain contains the largest capacity for constitutive regeneration of new neural cells in mammals (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002; Sohn et al., 2015). It represents one of the main neurogenic areas of the adult brain together with the subgranular zone of the hippocampus (Alvarez-Buylla and Garcia-Verdugo, 2002; Gould, 2007). Adult neurogenesis has been extensively described as an active process that includes the processes related with the proliferation, maintenance, differentiation, and migration of newly formed neural cells to their final destination (Ming and Song, 2005). Adult neurogenic areas derive from a reminiscent embryonic neuroepithelial cells from embryonic phases of the brain development, allowing continuous turnover of mature neural cells in the adult brain (Conover & Allen, 2002; Doetsch & Alvarez-Buylla, 1996; Galli, Gritti, Bonfanti, & Vescovi, 2003) from specific reservoirs of neural stem cells (NSCs) (Bernier et al., 2002; Steindler and Pincus, 2002). The maintaining of the stem properties of NSCs has been related with a specific environment provided by the structural interactions from different glial and extracellular matrix (ECM) components together with the release of local diffusible factors (Moore et al., 1978; Tavazoie et al., 2008). These areas have been described as neurogenic niches were the stem status is regulated or allowed to differentiate in adult neural cells. In the SVZ these cells appear to maintain a low proliferation index during the lifetime of the adult individual in order to generate new neural cells mainly destined to migrate through the rostral migratory stream (RMS) to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996; Cayre et al., 2009).

The components of these niches have been studied by ultrastructure principally in rodents (Bernier et al., 2000; Weickert et al., 2000; Rezza et al., 2014). The organization of the SVZ niche is based on four major cell types: ependymal cells (E cells), SVZ astrocytes (B1 and B2 cells), transient amplifying progenitor cells (type C cells), and neuroblasts (type A cells) (Doetsch et al., 1997; Mirzadeh et al., 2008). B1 cells are reminiscent embryonic neuroepithelial cells with a subependymal localization and astrocytic appearance which is in contact with the ventricle, intercalating into the differentiated ependymal layer cells. These cells proliferate at a slow rate and are considered to be adult resident neural stem cells. Type B2 cells, with a low proliferative activity, form a subependymal band and support the neuroblasts that migrate to the olfactory bulb. Type C cells are rapidly proliferating transient amplifying cells that arise from B1 cells and produce neuroblasts.

Few reports have described the cellular components of the SVZ in domestic animals apart from bovine (Rodriguez-Perez et al., 2003), ovine (Low et al., 2013), and porcine (Costine et al., 2015) species, in which immunological techniques have been used to describe the SVZ. In rabbits, this has been described at the immunocytochemical and ultrastructural levels (Luzzati et al., 2003; Bonfanti et al., 2006; Bonfanti and Ponti, 2008). The cellular composition and cytoarchitecture of the SVZ have also been studied in human (Quiñones-Hinojosa et al., 2006; Nogueira et al., 2014) and non-human primates (Gil-Perotin et al., 2009; Sawamoto et al., 2011).

The main differences observed in the SVZ of mammals lie in the extent and distribution of their components, apparently in relation to their anatomical and physiological variations. While rodents display a niche

mainly organized in large chains of neuroblasts supported by a dense meshwork of glial tubes (Jankovski and Sotelo, 1996; Lois et al., 1996), bovines, ovines, rabbits, and primates display a scarcely cellular subependymal area with the formation of a subjacent 'ribbon' of astrocytic cells (Barbaro et al., 2004; Gil-Perotin et al., 2009; Martínez-Cerdeño et al., 2012), highly similar to the hypocellular gap layer in humans. These differences have been related with differences in brain size (Paredes et al., 2016) and with their longer life span. Thus, long-living species that generally have a larger brain possess a specialized architecture of the SVZ allowing the formation of new neural cells during the entire life of the individual and not restricted only to the young life of the animal (Bonfanti and Peretto, 2011).

The dog has been used as an animal model for aging and human diseases (Franco et al., 2015) and seems also to be an accurate animal model in the field of adult neurogenesis. Previous studies have examined the neurogenic areas of dogs in early postnatal period, providing new information about their cytoarchitecture and their neurogenic potential. The results of some of these studies allude to the expression of stem markers by neuroepithelial cells of the SVZ (Walton et al., 2013). Also, the RMS has been characterized in the brain of canine and feline species as a longer track than in humans and primates (Malik et al., 2012). To date there is only a single ultrastructural description of the SVZ in dogs in which the presence of a subependymal cell plate and its relationships are described (Blakemore and Jolly, 1972).

Furthermore, the capacity to form neurospheres *in vitro* with a neuroepithelial cell subpopulation has been described in canines through

the isolation of grafts from the SVZ (Lim et al., 2012), olfactory bulb (Walton and Wolfe, 2008), and hippocampus (Lowe et al., 2015).

In the present study, we have identified and characterized, using histology, immunohistochemistry (IHC), electron microscopy and primary cell culture, the cell types that constitute the postnatal canine rostral SVZ (cSVZ) with three main objectives: (i) to describe the cellular composition of the striatal wall of canine lateral ventricles and to compare it with that described in other species including humans, (ii) to describe their age-related morphological changes in order to study the variabilities of the neurogenic potential with age, and (iii) to demonstrate the presence of multipotent NSCs with the capacity to form neurospheres in adult canine individuals.

Material and methods

Animals

The entire brains from 12 dogs aged between 3 months and 17 years were studied. Breed, sex and ages of the animals included in this study are summarized in Table 1. In order to evaluate the age-related changes of the SVZ with aging, we divided the animals into 4 groups: young (animals number 1 and 2), young-adult (animals 3-8), middle-aged (animals number 9-11), and aged (animal number 12). These animals were not known to have shown any previous neurological signs during their lives and were humanely euthanatized for other reasons and with their owners' consent. Dogs were deeply anesthetized by administration of a IV overdose of

sodium pentobarbital (dolethal). A complete necropsy was performed immediately after euthanasia, and the brain was removed and dissected grossly (less than 30 minutes *post-mortem*). Samples of all organs were collected during the necropsy for histological examination. All experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the *Universitat Autònoma de Barcelona*

Sampling

Serial transverse sections of the brain were made at the level of the frontal area (Fig 1A). The most rostral section (Figure 1A, section a) was used to take fresh samples from the SVZ. Each sample was approximately 8 mm^3. For cell cultures, tissue samples were immersed in control medium (CM) consisting of neurobasal medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.3 mg/ml glutamine (Life Technologies, Madrid, Spain), and preserved in ice until processed. Another, more caudal section (Fig. 1A, section b) was fixed in a solution of 2% glutaradehyde, 1% paraformaldehyde sodium phosphate buffer at pH 7.3 for 15 days at 4ºC for the ultrastructural study. The rest of the brain samples were fixed by immersion in 10% buffered formalin.

Histology

After fixation, one additional transversal section was performed in the frontal lobe (Fig 1A, section c). The microscopic morphology of the canine rostral SVZ was evaluated on 5 µm paraffin-embedded sections, stained

with haematoxylin and eosin (HE). In every section including the SVZ we analyzed the dorsolateral and ventrolateral areas of the striatal ventricular wall (Figs. 1C, 1D and 1E).

Immunohistochemistry

For the immunohistochemical study the cell markers used were nestin, as stem cell marker, doublecortin (DCX) and βIII-tubulin (βIII) as neuronal progenitor cell markers, glial fibrillary acidic protein (GFAP) as atrocytic marker, Olig2 protein as a glial precursor marker, and Laminin as a vascular associated ECM marker. The nuclear antigen Ki-67 was used as a marker of cellular proliferative activity.

Sections 5-µm thick were mounted on capillary glass slides, deparaffinised, and rinsed with water. When antigen retrieval was necessary, sections were heated for 20 min in a water-bath or 4 min in a pressure cooker with 10 mM citrate buffer pH 6.0, cooled for 30 min at room temperature, and rinsed in phosphate-buffer saline (PBS). The technical details of the primary antibodies used are summarised in Table 2. Previously, sections were treated for 35 min with 3% peroxide to block endogenous peroxidase activity. Non-specific binding was blocked by normal goat or rabbit serum 30% diluted with PBS for 1 h. Samples were incubated overnight with primary antibodies at 4ºC. Sections were rinsed with PBS and incubated for 40 min with a labelled polymer according to the manufacturer's instructions (mouse kit K4007 or rabbit kit K011, Dakocytomation). Incubation was performed using a secondary goat anti-rabbit antibody and then a standard ABC Peroxidase staining kit (Thermo Scientific, kit number 32020) diluted with PBS, for 1 h at room

temperature. Staining was completed by a 10 min incubation with 3,3'-diaminobenzidine (DAB) and counterstaining in haematoxylin for 3 s. The positive control used for DCX, βIII-tubulin, GFAP, and Olig2 was normal canine brain tissue including grey and white matter, for nestin it was the lateral ventricular walls of an adult dog, and for laminin normal canine skin tissue. In all experiments, negative controls were obtained by omitting the primary antibody.

Ultrastructure

Fresh samples from the SVZ of four animals included in this study were taken (animals number 5, 8, 10, 11 from Table 1). After fixation, transversal slides 200 μm thick of the lateral ventricle walls were made using a vibratome. The sections were postfixed in 2% osmium for 1.5 hours, rinsed, dehydrated, and embedded in araldite. Serial 1.5-μm semi-thin sections were stained with 1% toluidine blue and examined under a light microscope to study the overall organization of the SVZ. Ultrathin (70-nm) sections were cut with a diamond knife, stained with lead citrate, and examined under a Jeol 1010 electron microscope (Jeol, Tokyo, Japan). Digital pictures were taken with a TEM digital camera (Morada, Soft Image System-Olympus, Tokyo, Japan) and processed with ITEM software (Soft Image System). Adjustment of brightness and contrast of the pictures, if needed, was performed with Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Cell culture

Samples from the SVZ of dogs 3 and 4 were processed for cell culture expansion. Cortical and caudate nucleus samples from the same dogs were processed as negative controls. Conditions for primary tissue culture were adapted from Stoica *et al.* (2009) and were carried out following the same protocols as published for samples of canine gliomas (Herranz et al., 2016). Samples were cut into small pieces and subsequently dissociated mechanically in control medium. The resulting cell suspension was washed twice with phosphate buffered saline (PBS), filtered through a 40 μm filter, and resuspended in complete media (CTM) consisting of CM with 2% B27 supplement, 20 ng/ml epidermal growth factor (EGF; Life Technologies), and 20 ng/ml fibroblast growth factor (FGF; Sigma, Madrid, Spain). Floating sphere cultures were obtained by seeding 10^4 cells/cm^2 in CTM. Cells were expanded in T75 flasks (TPP Techno, Trasadingen, Switzerland) at 37ºC in 5% CO_2. These cultures were considered as pass 0 (P0). Every 3–4 days, one half of the medium was changed. The spheres tended to adhere to the plastic wall of the flasks, so the expansion methodology was improved by using ultralow attachment surface culture flasks (Corning, New York, USA). After 10–15 days, spheres were collected, centrifuged, incubated with Accutase 1X (PAA Laboratories, Dartmouth, Rhode Island, USA) for 5–10 min at 37ºC, and dissociated into a single cell suspension by vigorous pipetting. Cell viability was evaluated by trypan blue exclusion, and 10^4 cells/cm^2 were seeded into fresh CTM. The same procedure was repeated for several passes up to a maximum of 5 (P5).

Cell culture differentiation

A protocol described by Martín-Ibáñez *et al.* (2010) was used for differentiation studies. Briefly, dissociated spheres were disaggregated using Accutase and seeded at a cell density of 12.5×10^3 cells/cm^2 onto coverslips that were precoated with Matrigel (BD Biosciences, Madrid, Spain). Cells were allowed to differentiate for 3 days in differentiation media (DM) 1, which consisted of CM supplemented with 2% B27 and 20 ng/ml fibroblast growth factor (FGF). The culture medium was then changed to DM2, which consisted of CM supplemented with 2% B27 and 2% fetal bovine serum (FBS; Life Technologies), for the last 3 days. Under these conditions adult NSC were able to differentiate spontaneously towards the three neural lineages: neurons, astrocytes, and oligodendrocytes, as described previously (Herranz C *et al.*, 2016).

Immunocytochemistry

Differentiated cell cultures were characterized by immunocytochemistry according to the protocol described by Martín-Ibáñez *et al.* (2010). The panel of primary antisera employed is shown in Table 2. Cy3-conjugated donkey anti-rabbit IgG (1 in 500 dilution) and Cy3-conjugated donkey anti-mouse IgG (1 in 500 dilution), both from Jackson Immunoresearch Laboratories (Suffolk; Newmarket, UK) were used as secondary reagents. Cell cultures were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (Sigma). Fluorescent photomicrographs were taken on a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems, Barcelona, Spain).

Results

Histological description of the cSVZ

None of the brains evaluated showed neuropathological lesions and all of them displayed lateral ventricles of normal size and morphology. The rostral ventricular area was dorsally limited by the corpus callosum, medially by the interventricular septum, and laterally by the caudate nucleus (Fig. 1B). At low power fields, we observed that the two sides of the rostral SVZ showed different amounts of cellularity. While the lateral side showed large amounts of cells, the medial side appeared as a pale continuous band (Fig. 1E). In each animal we differentiated the dorsal SVZ (dSVZ) and the ventral SVZ (vSVZ) (Fig 1C). In each region of the SVZ we considered three layers: ependymal, subependymal, and subjacent glial layers (Fig 1F). The ependymal layer (E) was composed of a continuous monolayer of ciliated cuboidal to prismatic ependymal cells. The subependymal layer (SE) generally appeared as a continuous band showing a dense fibrillar network of ramified cellular elongations with a variable orientation below the E. Along this layer an undifferentiated cellular population formed by small rounded cells with a dense nuclei and scarce cytoplasm was identified. These rounded cells were observed in all animals but differences in the amount, distribution and localization along the SVZ were observed in different SVZ regions and age groups. Underneath this layer and touching the caudate nucleus, a thick deep glial layer (GL) was present in all animals.

Age-related morphological features in cSVZ

Young animal dSVZ showed undifferentiated cells frequently organized in highly cellular prominent clusters located in SE and extending to the GL (Fig 2A). Local ependymocytes overlying these groups protruded towards the ventricular lumen. These cells were surrounded by a dense network of astrocytic-like elongations. Considering these morphological features we identified these structures as SVZ neurogenic niches. In vSVZ, neurogenic niches were also present but with a lower cellularity (Fig 3A).

In young-adult and middle-aged animals, vSVZ also showed neurogenic niches (Fig 3B and 3C) but they were hypocellular compared with the previous group. In vSVZ only some hypocellular groups of undifferentiated cells were observed (Fig 3B).

A particular feature was observed in both 9-year-old animals from the middle-aged group (animals 10 and 11). In those animals, groups of undifferentiated cells were located outside the SVZ showing a multifocal to diffuse distribution in the dorsomedial area of the caudate nucleus. These cells were frequently localized at the perivascular area (Fig 6D). In accordance with their histological similarity to undifferentiated cells located in the SVZ, we identified these groups as putative striatal neurogenic niches.

A single 17-year-old individual represented the aged group. This animal showed the lowest SE cellularity cells of all animals included in the study, with the presence of occasional clusters in the ventral SVZ area (Fig 4B). However in this animal the number of neurogenic niches in the caudate nucleus was greatly increased (Fig 6J).

Immunophenotype of cellular components in cSVZ

In general, some of the ependymocytes located in the E presented discontinuous and scarce cytoplasmic $GFAP^+$ (Table 3). Multifocal strong $GFAP^+$ intercellular areas were observed on the lateral sides of some ependymal cells. In this layer ependymal $nestin^+$ nuclei were identified more frequently in dSVZ (Figs 2G-2I) than in vSVZ (Fig. 3G-3I). In SE most of the undifferentiated cells showed expression of DCX confirming that these cells were neural precursors. This layer showed a high presence of $GFAP^+$ astrocytic elongations, some of them arising from the GL layer and displaying a perpendicular orientation with respect to the ventricle. Most of these structures showed continuity with those $GFAP^+$ areas observed in the E, indicating that these elongations were in contact with the lumen of the ventricle (Figs 2G-2I and 3G-3I). A few cells showed $Olig2^+$ nuclear expression in areas near the GL layer along the entire lateral ventricular walls and in the subjacent areas corresponding to the caudate nucleus (data not shown).

Age-related immunohistochemical features in cell populations in the cSVZ

The most evident differences among the various age groups corresponded to the cellularity within the SE and caudate nucleus. DCX^+ cells were identified in the SVZ of all animals, indicating that neural precursors remain in the canine brain during postnatal life.

In agreement with the histology, young animals showed the highest presence of SVZ DCX^+ cells (Figs. 2D and 3D, Table 3). IHC also evidenced the presence of a few DCX^+ neural precursors intermixed with the

components of the GL. In one of the 1-year-old dogs of this group, a few DCX^+ cells were dispersed in adjacent areas and outside dSVZ neurogenic niches. This feature was not evident in the histological evaluation. These cells represented a minority compared with those located in the SVZ and were organized in small groups or chains (Fig $2E_1$ and 6A). In this group occasional βIII^+ cells were present in the SE layer with a low intensity. Some ependymal nuclei were strongly $nestin^+$ (Fig $2M_1$) while a lower proportion of $nestin^+$ cells were observed in the SE and GL layers (Figs 2M and 3M). A large number of proliferating $Ki67^+$ cells were identified in the dSVZ neurogenic niches (Fig. 2P) and a lower number of them in the GL of vSVZ (Fig 3P).

In young-adult dogs a decrease of neural progenitors was confirmed by the decrease of DCX^+ cells in SE layer, especially in vSVZ (Fig 3F). In contrast, the cells located in the neurogenic niches of this group showed the highest expression of βIII^+ (Figs 2K and 3K). A low proportion of $nestin^+$ cells showed nuclear positivity both in the E in dSVZ (Fig. 2M) and in the SE of vSVZ (Fig 3M). A lesser presence of $Ki67^+$ cells was identified and some of them were located in the GL of vSVZ.

Compared with the previous younger groups, middle-aged dogs showed a marked decrease of SVZ DCX^+ neural progenitors. However a high proportion of cells were located in the perivascular niches of the caudate nucleus, as they were identified surrounding the $Laminin^+$ basal lamina. Most of them showed a high presence of cells with $DCX^+/GFAP^-$ immunophenotype and only some nuclear $nestin^+$ cells. Occasional $Olig2^+$ nuclei were intermixed with these cells.

Aged dog vSVZ showed a low number of DCX^+ cells. In the same area, a prominent group of βIII^+ cells was identified (Figs. 4E and 4F). As described

above, neurogenic niches in the caudate nucleus were hypercellular and more evident and showed similar immunophenotypical features to middle-aged animals (Figs 6J-6M).

Ultrastructural study

General ultrastructural architecture of the cSVZ

Under electronic microscopy, the SVZ composition of 3 differentiated layers previously identified in the histological study was confirmed (Fig 5A): The EL was made up of ovoid to cuboidal ependymocytes (type E cells) with unramified microvilli and several cilia located in the apical cell pole. SE layer contained a dense network of astrocytes (type B cells) intermixed with ependymal ramifications. In this layer widely extended hypocellular areas were observed, mainly composed of cellular glial expansions forming a dense network. Along this layer, a variable number of migratory cells (type A cells) was identified. The GL layer contained the nuclei of the astrocytic cells. These layers were differentiated from the nervous parenchyma of the underneath caudate nucleus. Other normal brain components such as microglial cells, oligodendroglial cells, and myelinated axons were also present throughout this layer. Moreover, we identified an underlying GL layer composed of a ribbon of astrocytic cells.

Cellular components in the cSVZ

Ependymal cells lining the ventricular wall showed similar ultrastructural features to those previously described in other mammalian species (Fig

5B- 5E). Although found in young and young-adult dogs, some of these cells showed expression of nestin in some of the SVZ samples evaluated, with ultrastructural features of undifferentiated neural [noun missing]. Some ectopic ependymal cells with a multifocal distribution were identified in the SE and GL layers in dorsal and ventral SVZ areas of 1-year-old and 9-year-old animals (Fig 5C-5D). These cells were not recognized as ependymal cells in the histological and immuhistochemical studies.

The astrocytic component in the SE layer was mainly composed of a dense network of glial ramifications with a larger amount of intermediate filaments (Fig. 5). Some of these elongations were interlaced with ependymal elongations arising from the EL (Fig. 5E), and GAP junctions were frequently observed between them (Fig 5H). These features were similar of those described in human gap layer (Quiñones-Hinojosa et al., 2006). Most of the SVZ astrocytic nuclei were located in the GL layer, forming a ribbon of cells (B2 cells). Moreover, in the dSVZ and vSVZ of the 1-year-old dog we observed some astrocytic elongations arising from underlying areas and located between ependymal cells, in contact with the lumen of the lateral ventricle (Fig 5J, Table 4). Considering the similar GFAP structures described in the immunohistochemical study, we identified these cells as B1 astrocytes.

Migratory cells showed similar ultrastructural characteristics to those previously described in the brains of non-human primates (Gil-Perotin et al., 2009) (Fig 5). They were identified in all of the animals included in our study, indicating their presence in young-adult and middle-aged dogs. These results complement those obtained in the histological and IHC

studies in which DCX$^+$ neural progenitors were present in all age groups. Moreover, these cells were always associated with astrocytic cellular components (Fig 8A), a feature that correlates with the IHC description of GFAP$^+$ elongations around neural precursors. Groups of migratory cells were also observed in subjacent areas of the SVZ, distributed in the neuropil of the caudate nucleus. These groups where present mostly in ventral areas of 9-year-old dogs but also in the dorsal area of one of them. A single group of these cells was identified in the dorsal area of the 1-year-old dog (Table 4). These groups of cells showed similar ultrastructural features to the migrating and astrocytic cells described in the SVZ. They appeared most frequently surrounding blood vessels (Figs.6 D, 6H and 6J) and occasionally surrounding neuronal bodies (Figs. 6F-6G). These results, previously observed in the histological and IHC studies, confirm that adult neural progenitors have the capability to perform their migration outside the SVZ.

Finally, along the examined portions of SVZ other structures were observed in animals of different ages. Transversal and longitudinal fragments of supraependymal axons were identified in dorsal areas of the SVZ in both 1-year-old and 9-year-old animals (Figs. 9A and 9B). These axons were located in the lateral ventricular lumen and were linked and surrounded by the ramifications of ependymal microvilli.

Cell culture

Neural stem cells are characterized by their potential to expand and differentiate towards the three neural lineages: astrocytes, neurons, and

oligodendrocytes. Therefore, we next studied the ability of adult cSVZ samples to proliferate, forming neurospheres and their differentiation potential *in vitro*. In addition, samples dissected from the cortex and caudate nucleus of the same animals were examined as negative controls. Primary neurospheres were obtained from all the SVZ samples studied and no differences were found between them (Figs. 8A-8C). Expansion of SVZ cultures was achieved for up to 5 passes. However, the ratios of proliferation were very low and we could not expand them beyond pass 6 (data not shown).

Negative control samples derived from the cortex and caudate nuclei of the same animals were used. We did not observe any neurospheres growing in these cultures (Fig. 8D).

The differentiation potential of adult canine SVZ derived-expanded spheres was characterized in adherent cultures after 6 days. All cultures made were able to differentiate into neurons, astrocytes, and oligodendrocytes after *in vitro* expansion for several passes (Fig. 9). Our results showed that NSC can be isolated from adult canine SVZ, and expanded and differentiated towards the three neural phenotypes.

Discussion

The present study describes for the first time the cytoarchitecture of the cSVZ together with description of age-related changes, using routine histological techniques, a combination of immunohistochemical neural markers, and observation under electronic microscopy. We also describe the neurogenic potential in adult animals, evidenced by the *in vitro*

formation of neurospheres and their differentiation into the neuronal, astrocytic, and oligodendroglial lineages.

Our results demonstrate that cSVZ is stratified in layers similar to those described in humans (Quiñones-Hinojosa et al., 2006; Gil-Perotin et al., 2009; Sawamoto et al., 2011) and differing from rodents (Doetsch et al., 1997). Canine SE layer showed a dense GFAP$^+$ network corresponding with multiple astrocytes admixed with basal ependymal elongations, as previously described (Blakemore and Jolly, 1972). In this layer we observed a moderate amount of desmosomes and gap junctions between cell elongations. This demonstrates that the SE layer in dogs is similar to the gap layer described in humans (Quiñones-Hinojosa et al., 2006) and probably represents an important area of exchange of signals that modulate different aspects of the neurogenic process. In all dogs examined the crSVZ showed a ribbon of cells underneath the SE layer, a feature that has been described in the human SVZ (Barbaro et al., 2004) but which was not described in previous studies (Blakemore and Jolly, 1972). Considering these morphological aspects, the dog could be an accurate model of adult neurogenesis.

Neurogenic niches were characterized by multifocal groups of DCX$^+$ cells surrounded by GFAP$^+$ processes. DCX protein is selectively expressed in neural cells that migrate out from the SVZ, into the RMS, and olfactory bulb (Koizumi et al., 2006). Moreover, DCX has been demonstrated to show expression in suspected neurogenic areas in the human brain (Nogueira et al., 2014). The ultrastructural examination of these areas showed a variable presence of migratory cells in SE and GL layer

ensheathed by astrocytic elongations. This cellular arrangement indicates that in dogs, the niche cytoarchitecture is similar to that described in other mammalian species (Doetsch et al., 1997; Mirzadeh et al., 2008). In dogs neurogenic niches are made up of a DCX$^+$ cell population. These cells have been previously described as dark cells of the subependymal plate (Blakemore and Jolly, 1972). In our study we identified them as migratory neuroblasts (type A cell) distributed along the SVZ and supported by GFAP$^+$ glial elongations from B2 cells. B1 cells were identified in the 1-year-old animal (animal number 5) indicating the persistence of radial glia in adult ages, as described in other mammalian species (Ponti et al., 2006a; Quiñones-Hinojosa et al., 2006; Tavazoie et al., 2008; Gil-Perotín et al., 2013). As noted, astrocytic cells can maintain the properties of radial glia acting as stem cells in neurogenic areas, so the differences in the astrocytic organization have to be considered as important features in sustaining neurogenesis (Go et al., 2002; Garcı et al., 2004; Malatesta et al., 2008). Although B1 cells were only identified by ultrastructure in one animal, dogs in the young and middle-aged groups showed Ki67$^+$ in SE and GL layers, suggesting the proliferative progenitors in all ages. Nevertheless, the immunohistochemical exam did not differentiate wether proliferating cells were B1 or B2. In humans, B2 cells have astrocytes described as multipotent cells *in vitro* (Barbaro et al., 2004) so it remains unclear which cell population is proliferating. We evaluated the presence of nestin expression in order to identify them as neural stem cells, as previously described (Couillard-Despres et al., 2005). We frequently observed a strong nuclear nestin$^+$ in ependymal cells of dSVZ of young animals and a weak positivity in the SE layer of all animals, except in the aged dog. These results could be related with a decrease in the

neurogenic pool of NSCs in the canine brain. Nevertheless it is known that nestin represents an unspecific neural marker that could be also expressed by reactive endothelial astrocytes, as it was overexpressed in the areas which were strongly GFAP positive, with ependymal cells in response to reactiveness (Gilyarov, 2008). Regarding the variability in the cellular location and the intensity of labeling of this marker, we concluded that it is not an exclusive NSC marker in paraffin-embedded samples

These niches were more frequently observed in dSVZ than in vSVZ both in young and young-adult animals. Age-related differences were observed in these SVZ neurogenic cell populations. While DCX^+ cells content decreased in older animals, βIII^+ cells showed a significant increase in the vSVZ. Thus, the cell population in dSVZ progressed from $DCX^+/\beta III^-/Ki67^+$ to $DCX^-/\beta III^+/Ki67^-$, while older animals showed a predominantly $DCX^-/\beta III^+/Ki67^-$ cell population in vSVZ. These results suggests that a dorsoventral migration of DCX^+ neuroblasts occurs in dogs, as described in rodents (Sang et al., 2007). Increased βIII tubulin is expressed in early formed post-mitotic neurons (Menezes and Luskin, 1994) so their greater expression indicates a greater neuronal differentiation rate in aged vSVZ .

We found some groups of migrating cells outside the SVZ, immersed in the neuropil of the caudate nucleus, and often located around blood vessels. This feature has been previously described with light microscopy in prior studies (Blakemore and Jolly, 1972), but its cellular components and have not been detailed. Our ultrastructural study revealed that they were formed by migratory cells and B2 cells. This localization has been described in other animal species such as rodents (Yang et al., 2004).

Similar findings have been scantily described in a few animal species but widely described in adult rabbits (Bonfanti et al., 2006; Ponti et al., 2006a; b) and in one study of ferrets (Takamori et al., 2014). Moreover, the presence of these ectopic chains of cells was age-related, appearing in adult animals from 1 year of age, and increasing in middle-aged and aged animals. In humans only one study has noted the presence of migratory chains outside the SVZ, and only in children (Sanai et al., 2011). Interestingly, these chains migrate to the prefrontal cortex, the same encephalic cortical area that shows more neuronal decline in aged dogs (Head, 2011). Although further studies are needed to determine where these neural precursors are migrating, this indicates a conserved migrating pattern in humans and animals such as dogs. Moreover, migratory cells have been observed in the striatum of adult humans (Ernst et al., 2014) and primates (Cossette et al., 2003), indicating that similar events related with adult neurogenesis could be maintained across these species.

In general terms, these chains of migratory cells were composed of DCX^+/$GFAP^-$ cells and a lower proportion of $nestin^+$ and $Olig2^+$ cells. As we did not observe the expression of βIII or markers of mostly differentiated neuronal stages, we concluded that they corresponded to a mixed population of progenitors composed of glial and neural precursors. These results are in agreement with studies performed in rodents and ferrets that only revealed the presence of DCX^+ neural precursors (Yang et al., 2004; Takamori et al., 2014). In humans, while a higher formation of oligodendroglial precursors from the SVZ has been described in the aged brain (Bergmann et al., 2012) in these chains a greater presence of DCX^+

progenitors was observed (Ernst et al., 2014), suggesting that both in humans and dogs different subpopulations of neural progenitors follow the same migratory paths along the caudate nucleus. In dogs those cell chains were usually associated to blood vessels. This indicates that in perivascular areas there probably exists a specialized micro-environment that allows these cells to survive and migrate. In relation to the maintenance of progenitor cell biology, the existence of elements of the extracellular matrix (ECM) related to the presence of laminin^{+} basal laminae components was confirmed (data not shown). The niches are associated with blood vessels, whose basal lamina proteins have been proposed as maintaining and promoting the proliferation of these migratory cells (Faissner and Reinhard, 2015).

As in humans and primate counterparts, in adult canine SVZ we found displaced ependymal cells located in the SE layer. Similar results have been described in humans and interpreted as cells retained in this area during development (Spassky, 2005). Regarding their position, they may display interactions with the other cell types located in this zone and participate in the signaling network (Quiñones-Hinojosa et al., 2006). Another feature observed in our ultrastructural study was the presence of axons at the supraependymal level. In mice and humans, these axons have been described as serotoninergic terminals that interact with the ependymal and B1 cells, suggesting their participation in the regulation of neurogenic activity (Tong et al., 2014). Additional studies are needed to test whether they participate in similar functions in canines.

Canine NSCs have been obtained using different techniques from normal embryonic (Wilcox et al., 2011) and perinatal (Milward et al., 1997; Zhang et al., 1998) brains, and from adult olfactory bulb (Walton and Wolfe, 2008). Our results demonstrate that the canine SVZ contains a cellular component with the capability to form neurospheres, as previously described in rodents (Milward et al., 1997), pigs (Liard et al., 2009; Yin et al., 2011), teleost fish (Hinsch and Zupanc, 2006), and humans (Vik-Mo et al., 2011). This finding provides additional evidence to previous studies on early post-natal periods in dogs (Lim et al., 2012; Walton et al., 2013). Their capability of growth and differentiation in the three neural lineages cells confirms the presence of multipotent NSCs in the SVZ of normal adult canine individuals.

In conclusion, the canine species shares anatomical and cytoarchitectural features with humans and contains a neurogenic constitutive potential that seems to be maintained during different post-natal phases. Moreover, our results indicate that this neurogenic potential is highest till 1 year of life but then starts to decline in subsequent adult phases. During this time, dorsal areas of the anterior SVZ apparently contain greater neurogenic activity which could be relevant in future studies. All our findings confirm the dog as a better animal model than rodents for studies of neurogenesis and their translational application to human beings.

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Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Tables

Table 1. Data of animals included in the study and techniques used for the evaluation of the SVZ; m: months, y: years, H: histology, IHC: Immunohistochemistry; EM: Electronic microscopy; NA: Neurosphere assay

Group	Animal	Breed	Sex	Age	Technique
Young	1	Maltese	M	3m	H, IHC
	2	German Shepherd	F	4m	H,IHC
Young-adult	3	Beagle	M	9m	H, IHC, NA
	4	Beagle	M	9m	H, IHC, NA
	5	Beagle	M	1y	H, IHC, EM
	6	Beagle	M	1y	H, IHC
	7	Beagle	M	2y	H, IHC,
	8	Beagle	M	3y	H,IHC, EM
Middle-aged	9	Beagle	M	6a	H, IHC
	10	Boxer	M	9y	H, IHC, EM
	11	French Bulldog	M	9y	H, IHC, EM
Aged	12	Crossbreed	M	17y	H, IHC

Table 2. Primary antibodies used for immunohistochemistry and immunocytochemistry.

Marker	*Antibody name*	*Manufacturer*	*Dilution*	*Pretreatment*
Nestin	Rabbit polyclonal to nestin (neural stem cell marker)	Abcam (Cambridge, UK) ab5968	1 in 500	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
DCx	Rabbit polyclonal to CD133 stem cell marker	Abcam ab18723	1 in 1000	Citrate buffer 0.01 M pH 6.0, 20 min water bath 96–98°C + 30 min room temperature
ßIII (Tuj1)	Mouse monoclonal anti-tubulin ßIII isoform	Merck Millipore MAB1637 (Barcelona, Spain)	1 in 200	Citrate buffer 0.01 M pH 6.0, 20 min water bath 96–98°C + 30 min room temperature
ßIII (Tuj1)	Mouse monoclonal anti-tubulin ßIII isoform	Sigma T8660	1 in 200	Used for immunocytochemistry (without pretreatment)
Olig2	Rabbit Olig 2 polyclonal antibody	Merck Millipore AB 9610	1 in 100	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature and used for immunocytochemistry with no pretreatment.
GFAP	Rabbit anti-bovine glial fibrillary acidic protein	Dako Z0334 (Barcelona, Spain)	1 in 500	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature and used for immunocytochemistry with no pretreatment
Laminin	Rabbit polyclonal anti-laminin	Dako Z0097 (Barcelona, Spain)	1 in 800	Citrate buffer 0.01 M pH 6.0, 20 min water bath + 30 min room temperature
Ki67	Mouse anti-human Ki67	Dako M7240	1 in 100	Citrate buffer 0.01 M pH 6.0, pressure cooker 4 min + 30 min room temperature

Table 3. Immunohistochemical features of dorsal and ventral areas of the canine SVZ.

Group	Area		DCx	GFAP	βIII tubulin	Nestin	Ki67
Young	Dorsal						
		E	-	*	-	*	-
		SE	***	*	*	*	**
		GL	*	*	-	*	*
	Ventral						
		E	-	*	-	*	-
		SE	***	*	*	*	*
		GL	*	*	-	-	*
Adult	Dorsal						
		E	-	*	-	*	-
		SE	**	**	**	*	*
		GL	-	*	-	-	-
	Ventral						
		E	-	*	-	-	-
		SE	-	**	**	*	*
		GL	*	*	-	-	*
Middle-aged	Dorsal						
		E	-	*	-	-	-
		SE	*	*	*	*	*
		GL	-	*	-	-	*
	Ventral						
		E	-	*	-	*	-
		SE	*	*	**	-	-
		GL	-	*	-	-	-
Aged	Dorsal						
		E	-	*	-	-	-
		SE		*	-	-	-
		GL	-	*	-	-	-
	Ventral						
		E	-	*	-	-	-
		SE	*	*	*	-	-
		GL	-	*	-	-	-

-: absent; *: scarce;**: intermediate presence; ***: abundant; (CN): caudate nucleus

Table 4. Ultrastructural specialized features of cSVZ. y: years

Case	Age	Supraependymal axons	B1 cells	SE cellularity	Migratory cells	Ectopic ependymocytes	Mitosis
5	1y						
Dorsal		*	*	*	**(CN)	*	*
Ventral		-	*	*	*	*	-
8	3y						
Dorsal		-	-	*	*	-	-
Ventral		-	-	-	*	-	-
10	9y						
Dorsal		*	-	*	**	*	-
ventral		-	-	*	***(CN)	*	-
11	9y						
Dorsal		-	-	*	*(CN)	*	-
Ventral		-	-	*	**	*	-

-: absent; *: scarce;**: intermediate presence; ***: abundant;
(CN): migratory chain in the caudate nucleus

Figures

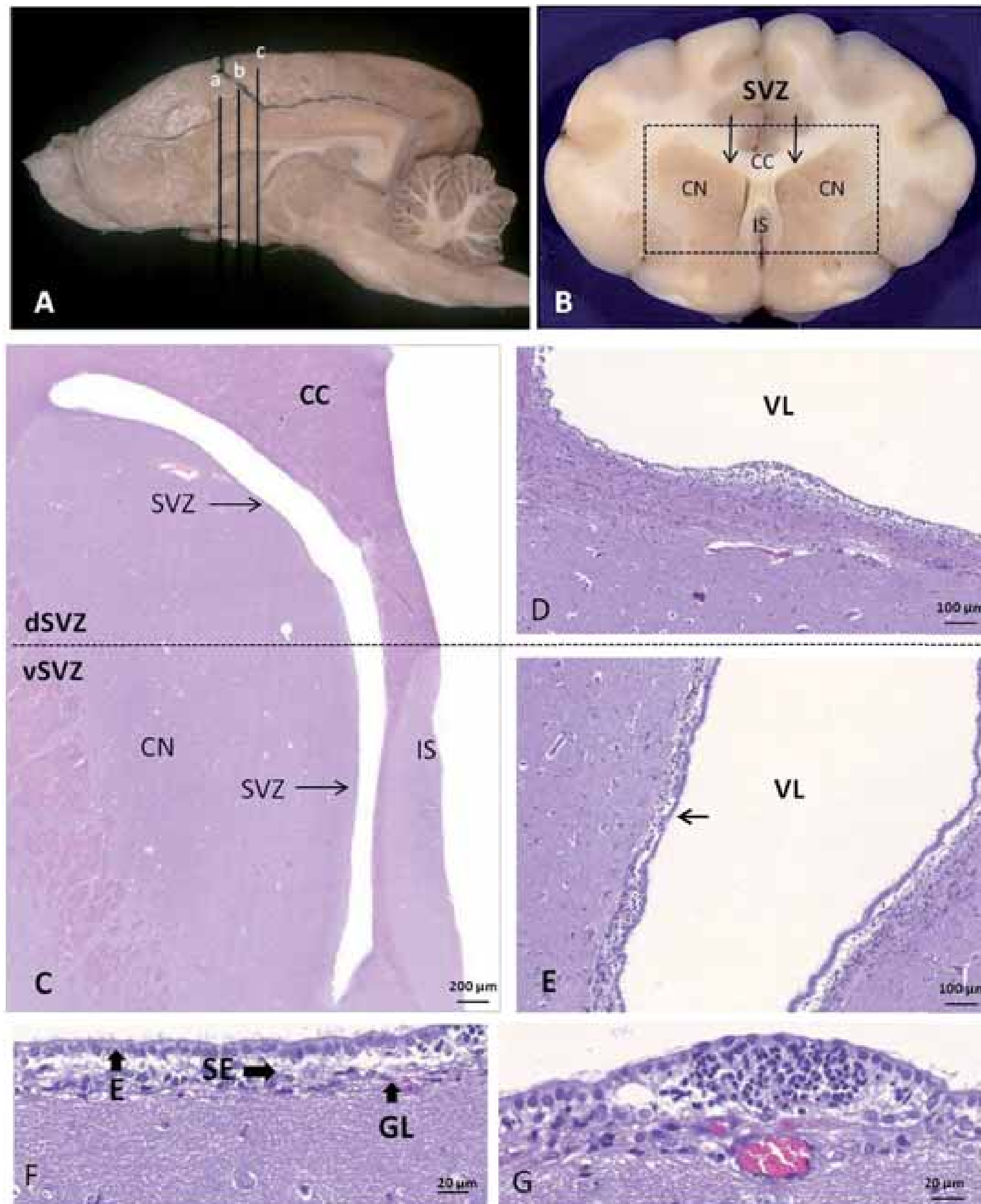


Fig 1. (A) Sections performed in canine brains for the neurosphere assay (a), ultrastructural (b), and histological and IHC (c) studies. (B) Representative transversal section of canine brains at the level of frontal area. The anterior SVZ is limited laterally by the caudate nucleus (CN), medially by the interventricular septum (IS), and dorsally by the corpus callosum (CC). (C) Panoramic view of the histological sections stained with HE. The SVZ lines the ventricular wall of the lateral ventricles. (D) View of the dSVZ with presence of cellular clusters of cells protruding towards the ventricular lumen (VL). (E) View of the vSVZ showing a continuous area of subependymal hypercellularity in lateral ventricular wall (black arrow) in contrast to the medial wall. (F) Histological appearance of the ventricular wall layers in which an ependymal (E), subependymal (SE), and deep glial layer (GL) can be identified. (G) Histological appearance of hypercellular clusters identified as neurogenic niche in dSVZ.

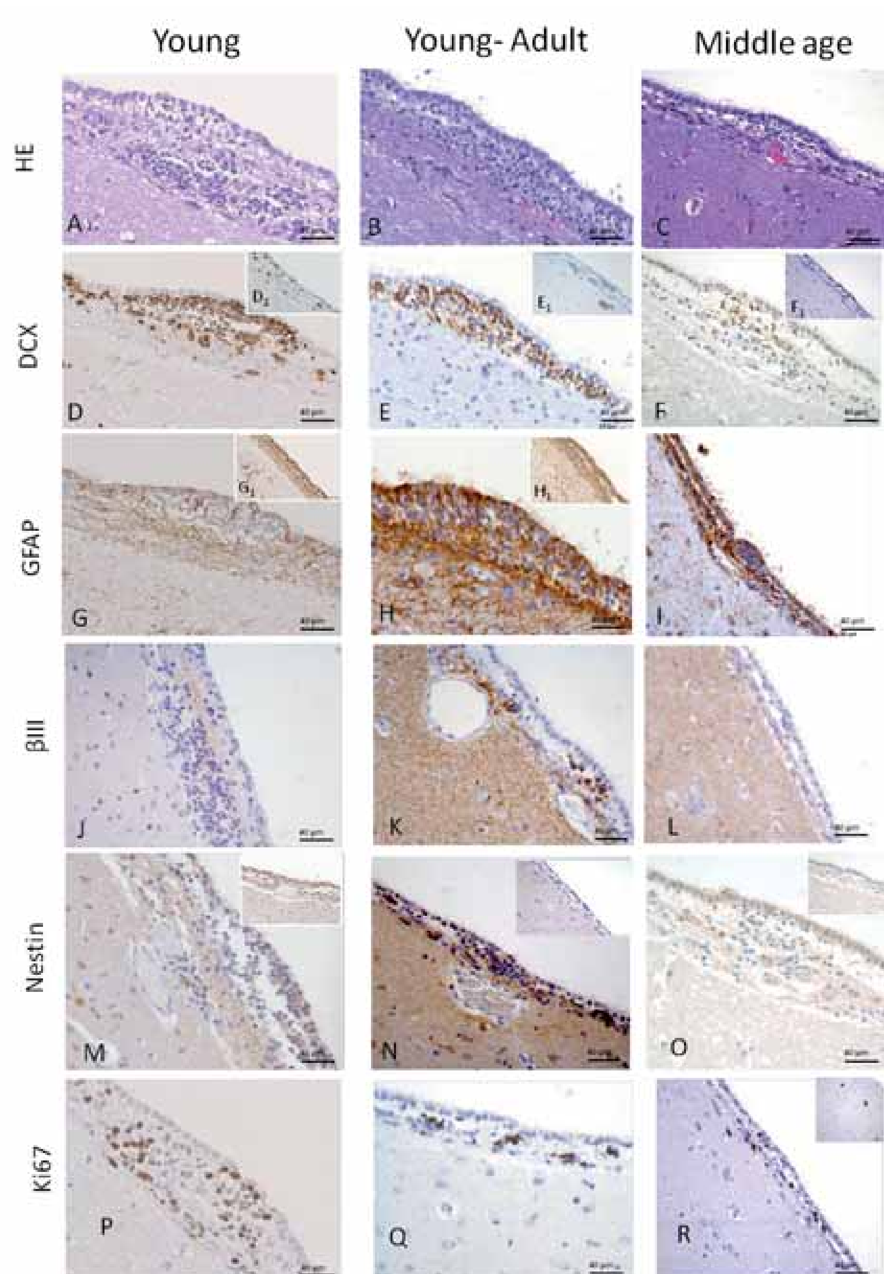


Fig 2. Histological and inmunohistochemical features of dSVZ in young, adult, and middle-aged dogs. (A, B, C; HE) dSVZ showing large amount of undifferentiated cells occupying the SE and GL layers. Middle-aged dogs show a hypocellularity compared with young and adult dogs. (D, E, F; DCX) DCX^+ cells predominate in this area and decrease in amount with aging. (D1, E1, F1) Adjacent SVZ areas from the cellular clusters also show insolated DCX^+ cells, extending to the GL layer in young and adult dogs. (G, H; GFAP) Astrocytic component is highly abundant in the SE and GL layers displaying fibrillary morphology. In young and adult animals radial $GFAP^+$ elongations originating in the GL layer and reaching the E layer and in contact with the ventricular lumen can be observed. (G1, H1) Adjacent areas show a continuous thick GFAP+ band. (I; GFAP). The astrocytic fibrillary band in middle-aged animals shows a dense, compact network of astrocytic processes. (J, K, L; βIII tubulin) Adult animals shows an evident cellular subpopulation of βIII^+ cells, in contrast with the low expression in young and middle-aged animals. (M, N, O; Nestin) The expression of nestin is highly variable with expression in ependymal nuclei. (P, Q, R; Ki67) Proliferating $Ki67^+$ cells are present in young, adult, and middle-aged animals. The number of positive cells is lower in the middle-aged group. (R1) Adjacent areas in this group show few Ki67 cells in the nearby areas of the caudate nucleus.

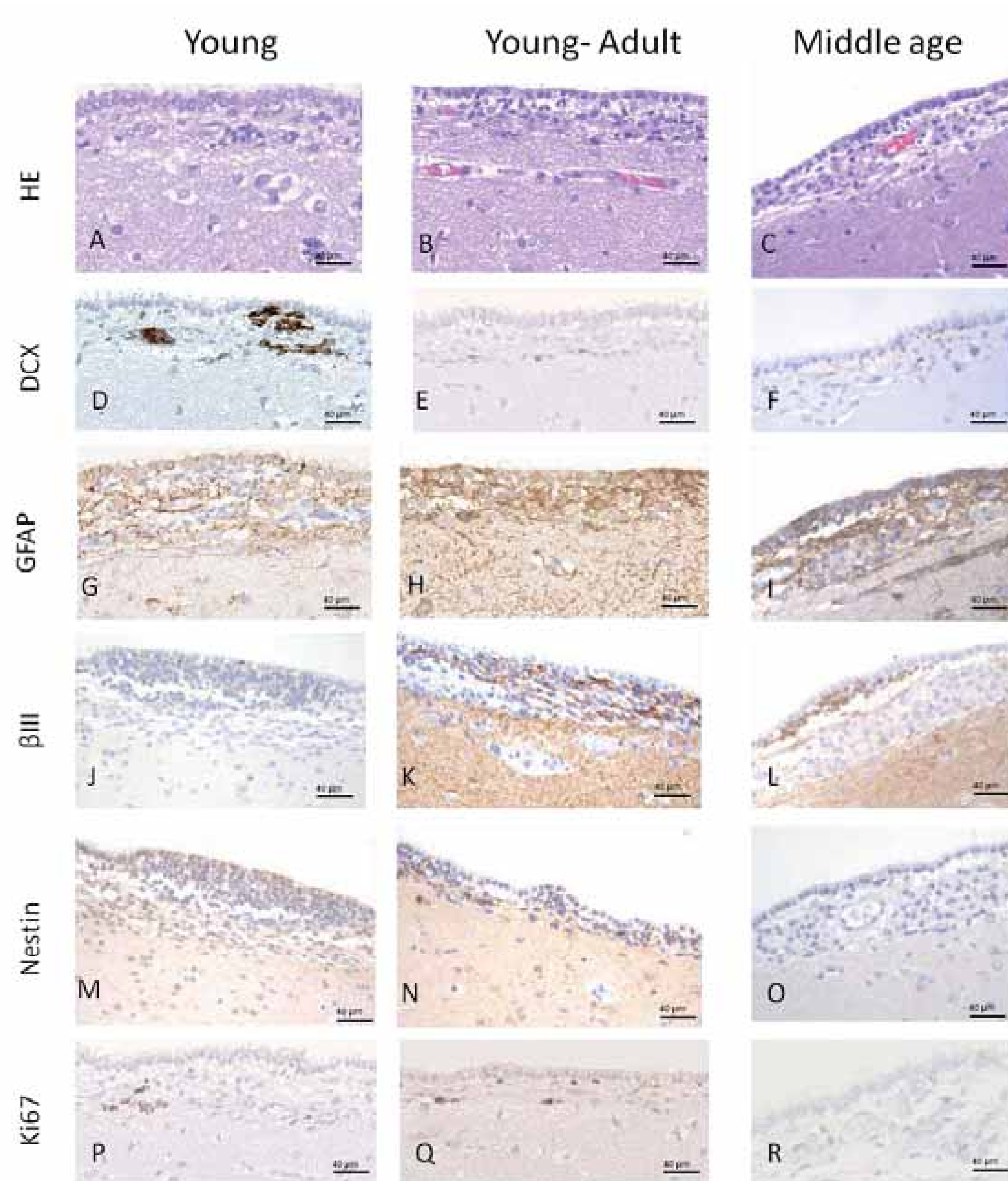


Fig 3. Histological and immunohistochemical features of vSVZ in young, young-adult and middle-aged dogs. (A, B, C; HE) In young and adult dog vSVZ there is less cellularity compared with dSVZ. Conversely, middle-aged dogs show a greater amount of cells than in dorsal areas. (D, E, F; DCX) DCX^+ cells are more abundant in young dogs and are organized in groups in the SE and GL layers. (G, H; GFAP) Astrocytic component is highly abundant in the SE and GL layers, displaying a fibrillary morphology, similar to that observed in dorsal areas. (I; GFAP) The astrocytic processes ensheath the cellular population in middle-aged animals. (J, K, L; βIII tubulin) Adult animals show an evident cellular subpopulation of βIII^+ cells while low expression is detected in young animals. (M, N, O; Nestin) In contrast with the dorsal area, ventral zones in middle-aged animals show a subpopulation of nestin cells in the SE layer. (P, Q, R; Ki67) Proliferating $Ki67^+$ cells are present in ventral areas in lesser numbers than in dorsal areas, and middle-aged animals show lowest positivity.

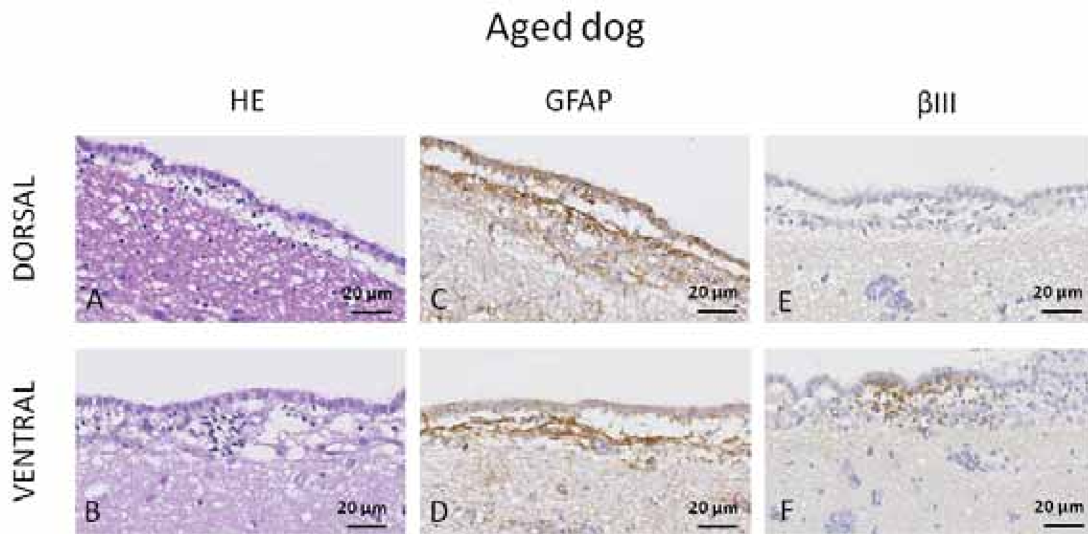


Fig 4. Histological and immunohistochemical features of dSVZ and vSVZ in aged dog. (A; HE) In aged dog the SE cellularity is scarce. (B; HE) Ventral areas contain some clusters of undifferentiated cells. (C, D; GFAP) GFAP in SE and GL of aged dog is the most evident immunohistochemical marker. (E, F; βIII) While dSVZ was completely acellular, in vSVZ a prominent group of βIII^{+} cells was identified.

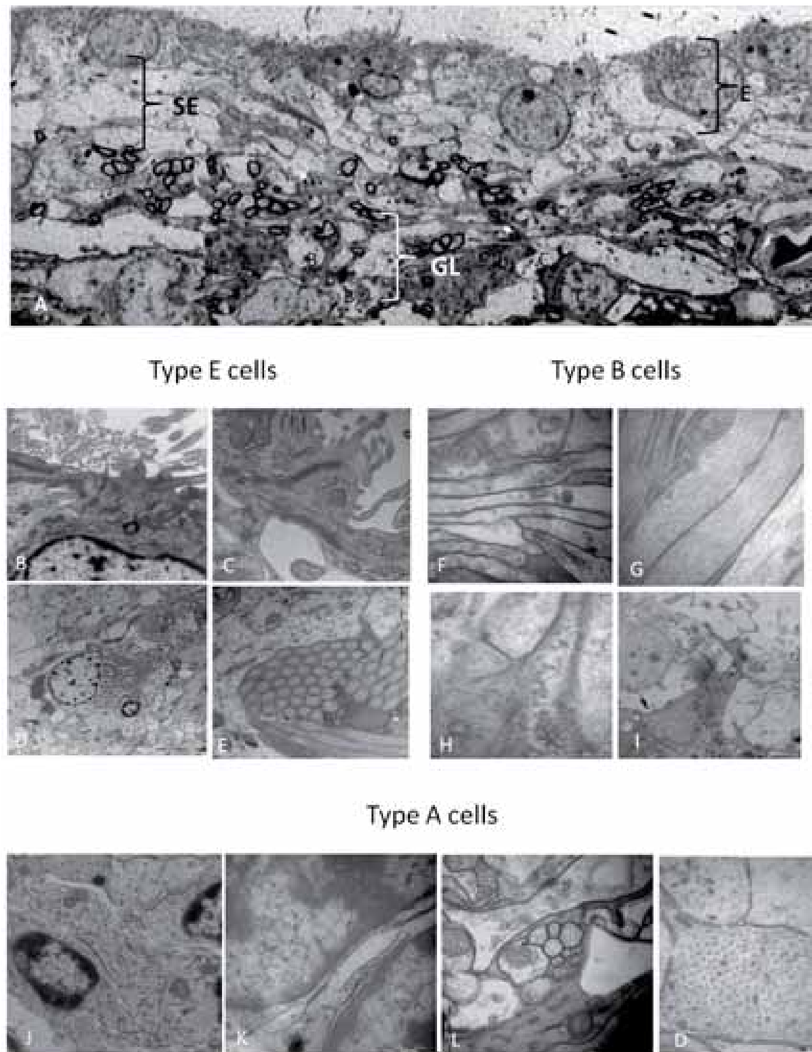
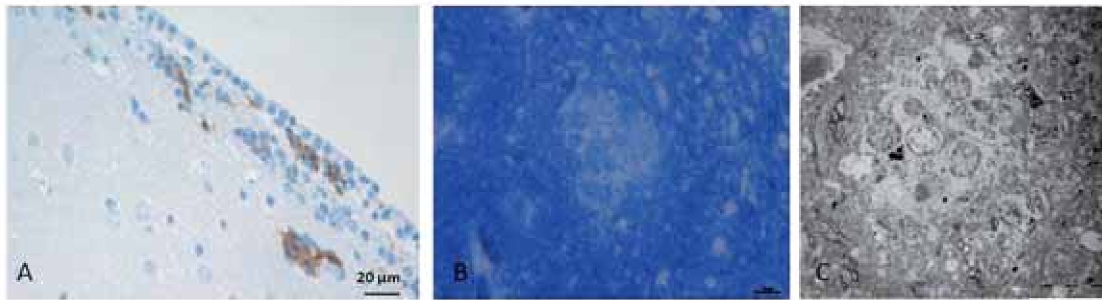
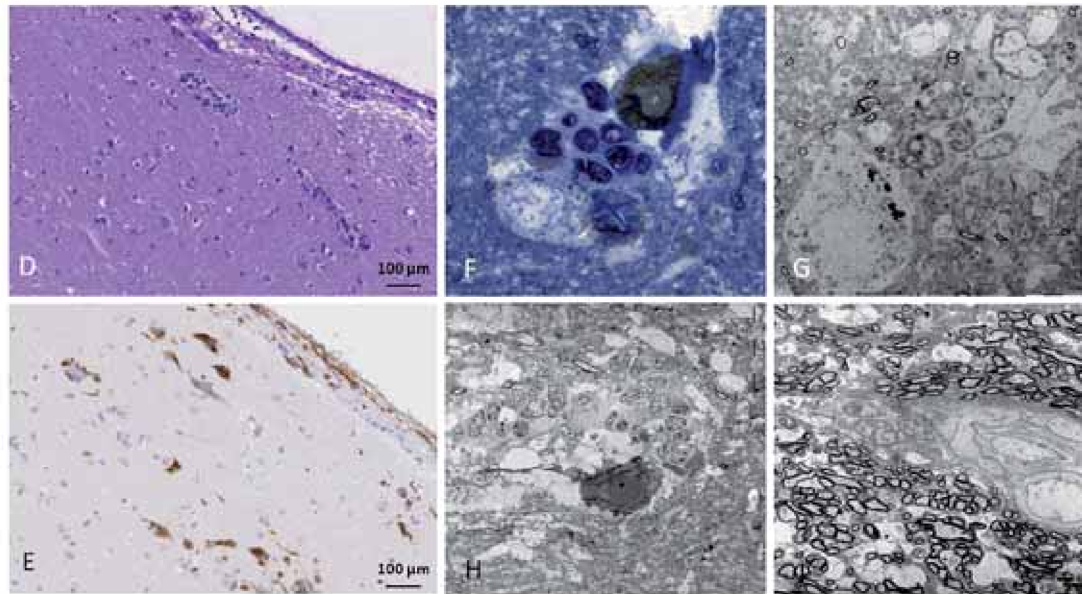


Fig 5. Ultrastructure and cell components in cSVZ. (A) Three differentiated layers were identified: ependymal (E), subependymal (SE), and a deep glial cell layers (GL). (b) Ependymal cells characterized by the presence of multiple cilia and ramified microvilli in the apical pole. The cilia show a typical 9+2 structure and are in contact with the ventricular lumen. (C) Several discontinuous tight junctions (desmosomes) are present between the ependymal cells (arrows). (D) Displaced ependymal cells located in the SE and GL layers, with an abnormal organization of the cellular components. (E) Detail of the ectopic ependymal cell shows the presence of abnormal cilia in the cytoplasm. (F) Dense network composed of parallel processes of astrocytic cells in the SE layer. (G) Interdigitated astrocytic and ependymal ramifications located in the subependymal layer. This layer is mostly composed of pale longitudinal astrocytic expansions made up of abundant intermediate filaments (black asterisk) mixed with dark ramifications of ependymal cells (white asterisk). (I) B1 cell characterized the presence of elongations that reach the ventricular lumen. (J) Migratory cells showed a dark nucleus with varying amounts of chromatin irregularly distributed. Groups of polyribosomes were also present, generally located next to the nucleus and taking up different areas of the cytoplasm (K). Around these cells, several intercellular spaces were identified between their external surface and peripheral supporting astrocytic cell expansions. (L) The evidence of structures corresponding to migrating cells was also observed in areas where no cell bodies were found. In different areas of the SE multiple migrating cell expansions were identified containing several microtubules orientated in the axial plane. The cytoplasmic membrane showed discontinuity and empty spaces demonstrating the connection with other migrating cells and also with surrounding astrocytic cells containing intermediate filaments. (M) Detail of the cytoplasm content of migrating cells. It was mainly composed of several microtubules orientated in the axial plane. The cytoplasmic membrane showed empty spaces, demonstrating the connection with other migrating cells and also with other astrocytic cells.

Adult young: 1 year-old



Middle age: 9 years-old



Aged: 17 years-old

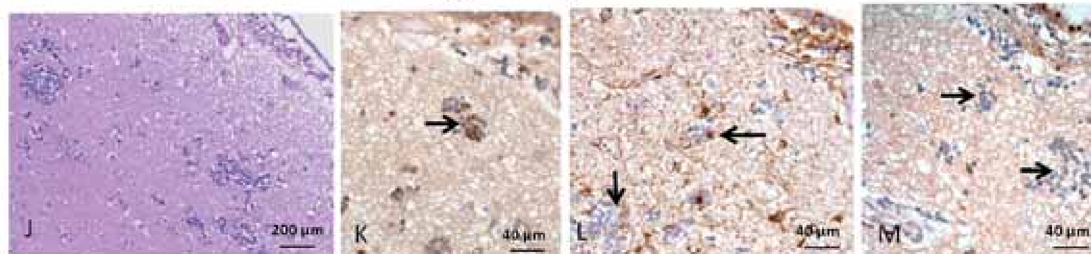


Fig. 6. Migratory chains located in the caudate nucleus. (A; HE) DCX+ cells in the dSVZ are not only restricted to SVZ areas and occupy the subjacent caudate nucleus. (B) The semithin section shows the grouping of cells with different cellular features in some point of the neuropil. (C) Ultrathin section where the migrating chain was observed and astrocytic cells in the neuropil of the caudate nucleus. (D; HE) Group of migrating cells identified in a 9-year-old dog, close to a neuronal body and a blood vessel. (E; DCX) DCX+ neuroblasts in the caudate nucleus. (F) Ultrathin section of migrating chain near blood vessel and neuronal body. (G) Semithin section showing migratory chains associated with neuronal body (H) The same animal showed multifocal chains located in deeper areas of the neuropil. (I) Also, a migratory chain was located in the periventricular white matter. (J; HE) Aged animal shows higher presence and highly cellular chain in the caudate nucleous. (K; DCX) Groups of cells are predominantly formed by DCX+ cells (arrow) (L; GFAP) The same cells are GFAP- and are ensheathed by a dense astrocytic network (arrows) (M; Nestin) Occasional nestin+ cells in migratory chains.

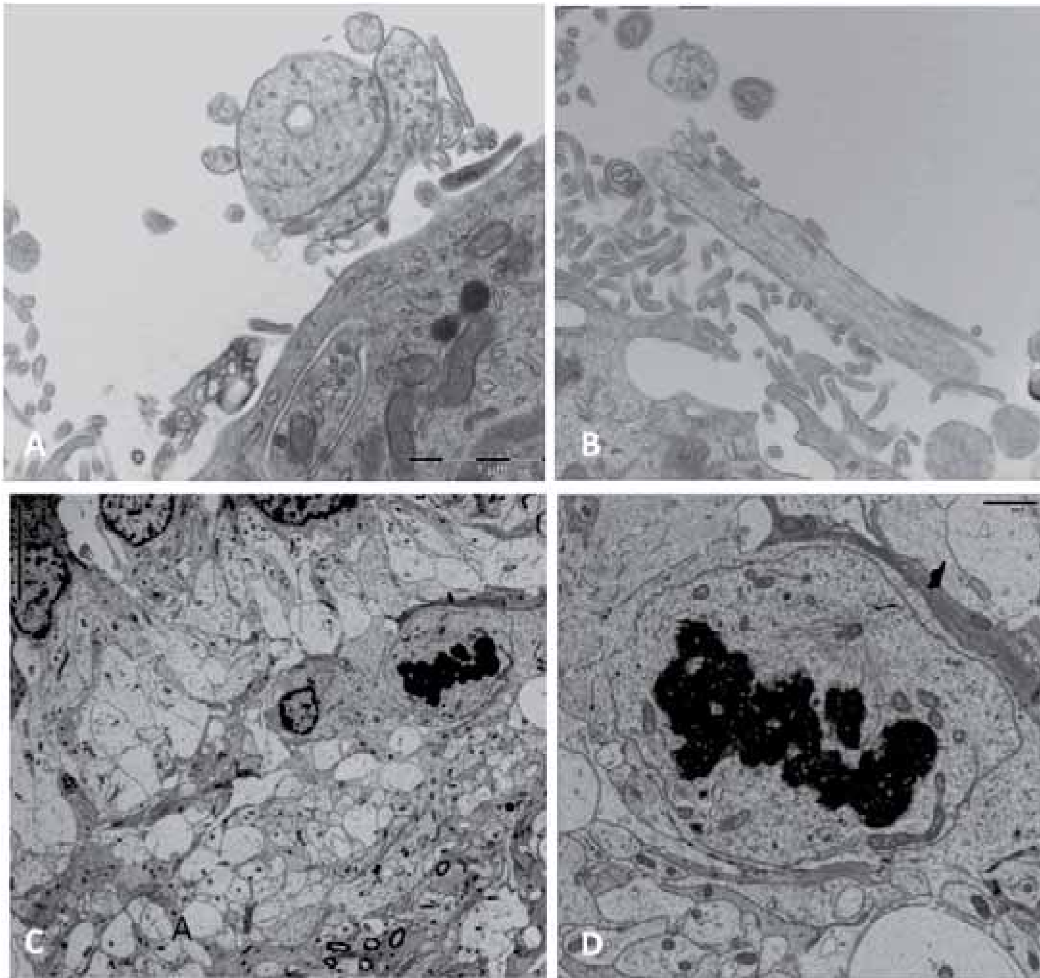


Fig. 7. Ultrastructural features of findings in canine SVZ previously described in mammals. (A, B) Supraependymal axons. Axial and transversal fragments of axons were found in the lateral ventricular lumen, surrounded by the ependymal microvilli. (C, D) Mitosis. Canine adult SVZ displays occasional mitotic cells in different phases of the cell cycle. The image shows an anaphasic dividing cell with separated nuclear material and invagination of the cell membrane.

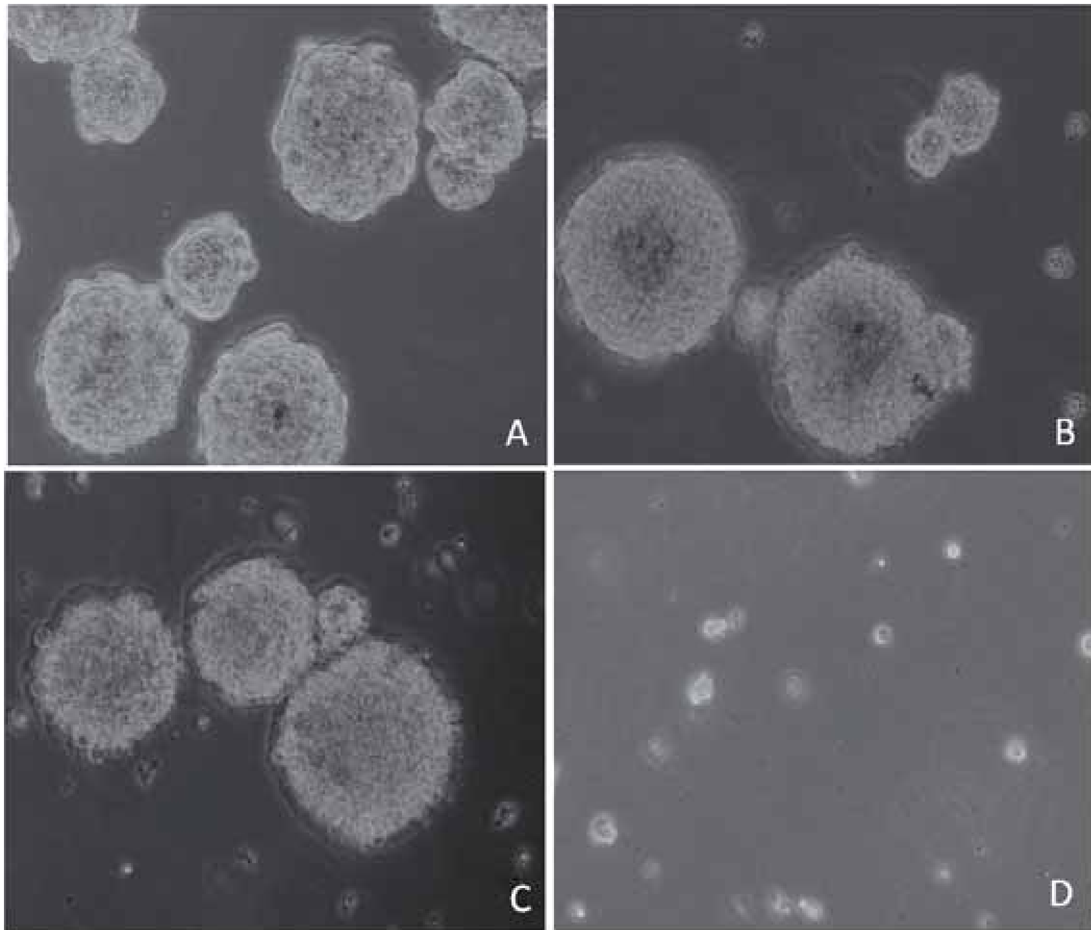


Fig. 8. Sphere formation and expansion potential of cultures obtained from cases 3 and 4. Representative pictures show the ability of NSCs to divide and grow in suspension, forming cell aggregates called spheres. (A-C) Neurospheres derived from dog SVZ. (B–D) Spheres from contralateral cerebral tissue were obtained. Representative image of spheres obtained from contralateral cerebral tissue.

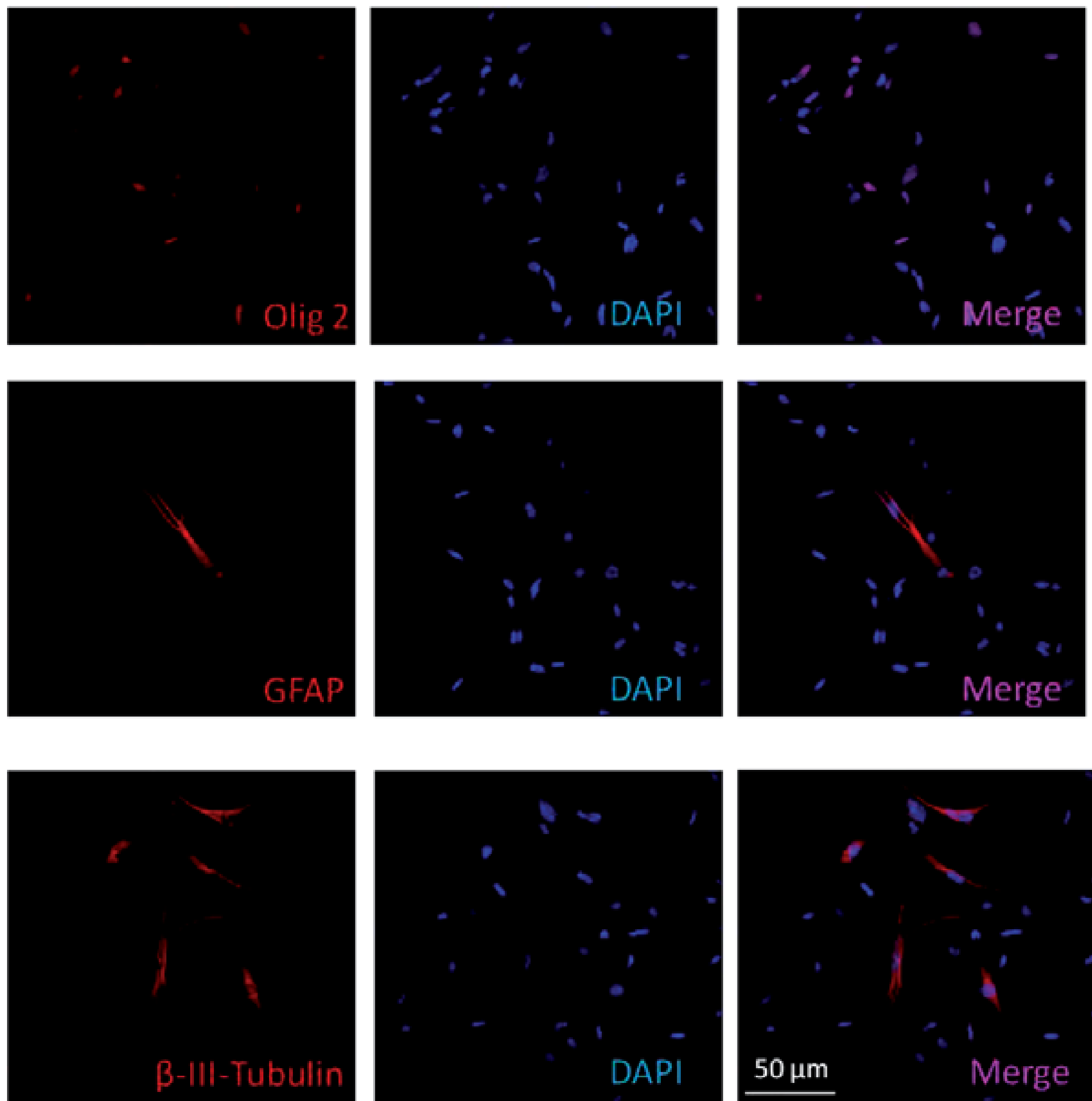


Fig. 9. Expanded spheres obtained from the SVZ are multipotent under differentiation conditions. Images show the ability to generate Olig2 positive oligodendrocytes, glial fibrillary acidic protein (GFAP) positive astrocytes, and β-III-tubulin positive neurons (Tuj-1). All images are representative and counterstained with nuclear marker 4’, 6-diamidino-2-phenylindole (DAPI).

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DISCUSSION

6. Discussion

In this doctoral thesis, the study of spontaneous canine glioma has provided valuable data that permit us to establish comparisons with results previously obtained in other species, including humans. One of the main aims of this study was to establish a comparative approach from the canine to human through the study of pathological aspects of canine glioma and also with the physiological features of the adult neurogenesis in the canine species.

Firs at all, although the incidence of gliomas in the canine species is similar to that described in humans, canine samples were not always available for use in research. Some procedural problems, such as the agreement of owners to allow taking samples from their pets and the *post-mortem* delay of necropsies, result in their underutilization and produce losses of valuble samples. These were the main reasons for the low number of available canine gliomas for the present study. Further, their random appearance made it difficult to control and balance the subgroups, number of subtypes, and grades of canine gliomas investigated. These aspects have been taken into account in the interpretation of our results.

The cancer stem cell hypothesis represents a compelling paradigm for the development and progression of neoplastic disease (Rahman et al., 2011; Singh et al., 2014). One of the main difficulties in the evaluation of the CSC hypothesis arises from the lack of complete specific markers that

unequivocally define the hierarchy of cancer stem cells, and of reliable techniques to assess clonal expansion.

This is a first approach to the study of the CSC hypothesis. One of the aims of this study was to evaluate the phenotypic features of canine gliomas in order to compare our observations with those described in human gliomas. The second motivation was the evaluation of the expression by neoplastic cells of different immunohistochemical markers that allow identification of the presence of neural progenitors in canine gliomas.

We evaluated the histopathological features of 20 spontaneous canine gliomas. This histopathological evaluation allowed us to identify similar morphological aspects of canine gliomas that had been described in human gliomas (Louis et al., 2007), indicating that spontaneous canine tumors demonstrate a comparable biological behavior as that described in humans.

On this point, different aspects of the histological exam allowed differentiation between different groups of tumors. On the one hand, astrocytic tumors versus oligodendroglial tumors were discriminated. On the other, differences between low-grade gliomas and high-grade gliomas could be established. This permitted comparisons between oligodendrogliomas (WHO grade II), anaplastic oligodendrogliomas (WHO grade III), mixed gliomas (WHO grade III), and glioblastomas (WHO grade IV).

The semiquantitative evaluation, according to the number of positive cells and the intensity of expression of the markers used, allowed the calculation of median proportion scores, making the presentation of IHC results more accessible. Moreover, the use of semiquantitative scores reflects the reliability of the IHC studies.

Similarities of histological and immunohistochemical features between human and canine gliomas confirmed that the dog can be a relevant animal model for preclinical evaluation of new treatment strategies (Dickinson et al., 2010; Stoica et al., 2011; Hicks et al., 2015; Schiffman and Breen, 2015).

The interpretation of the results provided new insights into the tumorigenesis of canine CNS neoplasm in accordance with the evaluation of lineage commitment of tumor cells in spontaneous canine gliomas. Our results suggested that, as in human infiltrating gliomas (Mao et al., 2007; Hu et al., 2009; Lu et al., 2011; He et al., 2012; Jin et al., 2013a), canine glioma cells contains glial-progenitor cells with enhanced nestin and CD133 expression in high-grade as opposed to low-grade gliomas. Thus, we concluded that these two markers are relevant in the study of the CSCs in canine glioma, in agreement with published works on humans (Jin et al., 2013b; Tamura et al., 2013; Matsuda et al., 2015; Miconi et al., 2015; SIBIN et al., 2015). Moreover, the variable expression of the IHC markers used in this study such as DCX, Vimentin, and S100 demonstrated a heterogeneous degree of differentiation of the neoplastic cells in canine gliomas. This scenario is compatible with a hierarchical organization of

neoplastic cells, compatible with the tumor growth model attributed to the involvement of CSCs in solid tumors (Venere et al., 2011).

Secondly, and corresponding with study 2, apart from the examination of canine gliomas using histological and immunohistochemical techniques, we included the neurosphere assay in our protocols. Using these techniques we expanded the glioma-derived spheres. Moreover, we demonstrated their potential in differentiating derived neurosphere cells into different neuronal and glial cell phenotypes. In conclusion, we fulfilled some of the required criteria for the demonstration of the CSC hypothesis in canine gliomas. Nevertheless, for a fuel demonstration, tumor growth in xerographs mice should possibly have been accomplished.

As we had previously observed that canine gliomas could be histologically simolar to human gliomas, several of these canine tumors were studied: two oligodendrogliomas, one anaplastic oligodendroglioma, one mixed glioma and one glioblastoma.

We obtained neurospheres from all the tumors included in our study. These results were in concordance with the expression of nestin and CD133 in tumors of all grades described in our previous study (Fernández et al., 2015). However, high grade gliomas showed a greater ability to expand, suggesting a correlation between the grade of the tumor and the capacity for self-renewal of the neural progenitors evolving in gliomas, as has been proposed in human studies (Azari et al., 2011; Pavon et al., 2014)

As there exist no standardized protocols for neurospheres generation from canine species material, we adapted previously described protocols used in murine models (Martin-Ibanez et al., 2010). Although we obtained high rates of cell attachment, viability of neurospheres was achieved and derived neurosphere cells could be driven to differentiate into the three neural lineages. This raises two points to consider: (i) the similar underlying biological properties in canine gliomas and other animal models (Milward et al., 1997; Gil-Perotín et al., 2013) and humans (Azari et al., 2011; Collet et al., 2014; Pavon et al., 2014) , and (ii) the capacity for extrapolation of technical procedures for neurosphere generation from mice to the canine model. In future studies in this field we propose the comparison of our protocols with those used in human studies.

Based on this second work, and taking into account that only 3 of our cultures were able to reach 6 passes, our results constitute a prior step in this field that requires further examination for valuable data. For example, it remains unclear whether the presence of progenitors in gliomas really constitutes stem cells. Nonetheless, despite the percentages of each cell type, with our differentiation experiment we demonstrated that all glioma-derived expanded spheres are multipotent, as had previously been asserted (Stoica et al., 2011; Hicks et al., 2015; Schiffman and Breen, 2015). The samples obtained from canine gliomas should be included in a multicentre study that includes evaluation of the capacity for neurosphere formation, multi-lineage differentiation, and tumor initiation in immunocompromised xenografted rodents to definitively demonstrate

the CSC hypothesis in canine gliomas, as has been done with canine glioblastoma (Stoica et al., 2009).

Moreover, two interesting additional observations were carried out in this study:

First one was the different growth potential between the center and periphery of canine gliomas. This feature had been previously described in human gliomas (Glass et al, Ann Neurol 2010). As neural progenitors need to be included in special microenvironments to mantain the properties that define them as stem cells, we hypothesize that the center of the tumor provides structural and diffusible factors that allow the mantained surveillance of neural progenitors in these areas. Based on previous studies (Inukai et al., 2015), we hypothesize that it is probably due to a hypoxic status. These differences could be relevant for the evaluation of new therapeutic approaches. In this respect to the study of microvessel formation, organization, and density in the tumor together with morphological properties of the vascular component should be necessary. In fact, the role of angiogenesis in gliomas is being studied in human (Hardee and Zagzag, 2012; Wang et al., 2013), so a complete study of this phenomenon in canine gliomas remains to be developed in parallel. Furthermore, the capability for expansion on the periphery of the tumor opens the possibility that differing growth behavior is due to phenotypic

tumor cell diversity, distinct malignant subentities present in gliomas (Verhaak et al., 2010), or distinct topographically diverse environmental cues *in situ* that could lead to a diversification of some of the cellular and molecular properties in the gliomas (Phillips et al., 2006; Morokoff et al., 2015b). These results address an important field of research in tumor biology in terms of progression, therapy escape, and phenotypic shifting.

The second results of interest were those obtained in the samples originating from the contralateral SVZ of the brain of dogs with glioma. This apparently normal SVZ proved on morphological examination to contain progenitor proliferating cells in cellular culture studies, since all neurospheres derived from them were able to expand to between 3 and 5 passes.

In order to investigate these data in more detail, we included a third study in this thesis in which we examined the biological properties of the cellular components and cytoarchitecture of the rostral SVZ in normal adult dogs.

The histological evaluation of the anterior lateral ventricular walls in these dogs allowed us to identify groups of undifferentiated cells in this localization. The observations under electron microscopy allowed us to see in detail the cytological and cytoarchitectonic features of canine SVZ. Moreover, this allowed to confirm the composition of the collected samples from canine SVZ in order to perform *in vitro* studies using the neurosphere assay and associated differentiation studies.

The results obtained from this third study produced additional new interesting observations.

At the histological and ultrastructural level, our results confirm that canine SVZ is organized in layers similar to the human one (Quiñones-Hinojosa et al., 2006) and that of primates (Gil-Perotin et al., 2009), and differs from the murine species (Doetsch et al., 1997b; Mirzadeh et al., 2008). This organization is also similar to those described in other mammal species (Rodriguez-Perez et al., 2003; Ponti et al., 2006a; Sawamoto et al., 2011; Low et al., 2013; Takamori et al., 2014; Costine et al., 2015).

The cellular components of the neurogenic niches investigated in the canine species are similar to those described in other mammals (Doetsch et al., 1997b).

The amount of neural progenitors declines with age as has been described in rodents (Maslov et al., 2004; Capilla-Gonzalez et al., 2014; Daynac et al., 2016) and in the SGZ of aged dogs (Siwak-Tapp et al., 2007). The progenitors can be located outside the SVZ in adult individuals, as has been widely described in rodents (Inta et al., 2015), ferrets (Takamori et al., 2014), rabbits (Bonfanti et al., 2006), primates, and adult humans (Cossette et al., 2003; Ernst et al., 2014).

Canine SVZ contains cellular components with the ability to form neurospheres, as previously described in rodents (Milward et al., 1997), pigs (Liard et al., 2009; Yin et al., 2011), teleost fish (Hinsch and Zupanc, 2006), and humans (Vik-Mo et al., 2011). This finding provides new data to add to previous studies of the early post-natal period in dogs (Lim et al., 2012; Walton et al., 2013). The capability of growth and differentiation into the three neural lineages cells confirms the presence of multipotent NSCs in the SVZ of adult normal canine individuals.

An interesting observation is that although the major components of the niche are located in the subependymal area of the SVZ , in adult dogs we also observed chains of migratory cells localized in the adjacent caudate nucleus with a perivascular pattern of distribution. Migratory chains outside the SVZ have been described in other animal species such as rodents (Yang et al., 2004), rabbits (Bonfanti et al., 2006; Ponti et al., 2006a; b), ferrets (Takamori et al., 2014), and primates (Cossette et al., 2003). While in these species there was observed to be a predominance of DCX^+ neuronal precursors, in our adult dogs these chains of migratory cells were composed of DCX^+/$GFAP^-$ cells, and a lower proportion of $nestin^+$ and $Olig2^+$ cells. As we did not observe expression of βIII or markers of mostly differentiated neuronal stages, we concluded that they corresponded to a mixed population of progenitors composed of glial and neural precursors. In humans, migratory chains of DCX^+ cells are not commonly found in the SVZ (Quiñones-Hinojosa et al., 2006), but migratory chains with similar cell components have been observed outside the SVZ in adults (Ernst et al., 2014). Furthermore, in children, the prefrontal cortex seems to receive new neural progenitors in early post-natal stages (Sanai et al., 2011). So we hypothesize that these chains are formed by subpopulations of neural progenitors different from those present in the SVZ, in both humans and dogs. This could indicate neurogenic events related to post-natal development of the brain in humans, and may be compensatory to the loss of grey matter in the canine prefrontal. Nevertheless, additional studies are needed to characterize these cells, the perivascular environment of these niches, and their pathways of migration.

Additionally, our results suggest that neural precursor striatal chains in dogs have a special ability to establish relations with the ECM components even in assumed to be non-neurogenic areas, especially surrounding the vessels, where the components that characterize the neurogenic niches are not present.

This adaptability could be related to the presence of perivascular niches in gliomas, indicating that neural precursors isolated from tumors are able to maintain their stem properties due to the enrichment of the perivascular microenvironment, facilitating their invasion of adjacent normal nervous tissue. Thus, the ECM components, together with other vessel characteristics, and their relation with the neurogenic potential, could be an important field for future research in order to investigate how gliomas invade nervous tissue and to develop new therapeutic approaches.

Finally, these results definitively confirm that the canine species is an appropriate, relevant animal model to include in the field of comparative oncology, as it is capable of reproducing several pathological and biological phenomena comparable to tumorigenesis and neurogenesis in humans.

CONCLUSIONS

7. Conclusions

The results obtained during the three studies that make up this doctoral thesis allow us to conclude:

1. Spontaneous canine gliomas share histological and immunohistochemical features with human gliomas. This allows us to use the same neuropathological and grading criteria as with human tomors and facilitates the interpretation of the results and their comparison with human gliomas.

2. Dogs are companion animals that are clinically controlled for the owner and veterinarian. This provides a potentially large supply of epidemiological data such as incidence, age, breed, localization, and prognosis that can be compared with human glioma.

3. *In vitro* human glioma studies are based mainly on the evaluation of tumoral biopsies. Canine glioma study allows working with the whole brain generating wide samples that can be used simultaneously in different studies. Speed in collecting the samples is very important to guarantee results, especially when cell culture related studies.

4. Immunohistochemistry is a good approach to the study of the lineage of cellular composition of canine SVZ, to identify cells with a stem

phenotype both in canine gliomas and in normal dog brains. Despite the absence of a specific marker for neural stem cells, we consider nestin, CD133, Doublecortin, and Olig2 to be indicators of neural precursors in canine tissues.

5. The neurosphere assay demonstrates that canine gliomas contain a subpopulation of neural progenitors that have the capability to grow in cell culture. These cells show different growth potential depending of their malignancy grade.

6. The different growth capability of neural precursors located in the center compared with those of the periphery of gliomas could be related with different microenviromental conditions, related changes in the tumor vasculature, or intratumoral heterogeneous cell populations.

7. Immunohistochemical and neurosphere assay studies confirm the presence of multipotent neural progenitors in canine gliomas. These results are in accordance with the cancer stem cell hypothesis. Nonetheless, succesful stereotactical xerograph transplantation of these neurospheres in immunodepressed mice is required to confirm definitively the CSC hypothesis for canine derived samples.

8. The canine SVZ of the anterior part of lateral ventricles has similar cell types detected in other mammalian species. Nonetheless, its cytoarchitecture is much closer to that of human than to murine models both histologically and ultrastructurally. It has three differentiated layers: ependymal, subependymal, and transitional.

9. The canine subependymal layer contains chains of neural progenitors organized in niches surrounded by astrocytes and adjacent to capillaries. In this layer, astrocytic and ependymal cells are interlaced and show desmosomes and tight junctions, simulating the gap layer observed in humans.

10. Neurogenesis exists in adult canine brain. Neural progenitors from SVZ reveal neurogenic properties when investigated with cell culture techniques: neurosphere assay and neural differentiation.

11. Canine SVZ neural progenitors decline with age, as occurs in other mammals, including humans.

12. In adult, middle-aged, and aged dogs, neural progenitors are arranged in perivascular chains forming niches outside the SVZ. This feature indicates their survival and adaptability outside classical neurogenic areas, as in our canine gliomas.

13. The dog represents a relevant animal model not only in the field of comparative oncology but also to study the adult neurogenesis.

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LIST OF ABBREVIATIONS

9. List of abbreviations

ANISO: anisocariosis

ASCs: adult stem cells

C: compresive

CNS: central nervous system

CP: capillary proliferation

CSCs: cancer stem cells

DCX: doublecortin

DG: dentate gyrus

E: ependymal layer

ECM: extracellular matrix

EGF: epidermal growth factor

FGF: fibroblast growth factor

G: glomeruloid capillaries

GFAP: glial fibrillar acidic protein

GL: deep glial layer

GR: growth

H: haemorrages

HC: honeycomb

HGGs: high-grade gliomas

I: infiltrative

IHC: immunohistochemistry

LGGs: low-grade gliomas

MIT: mitosis

MSEC: mucinous secretion

NEC: necrosis

NSCs: neural stem cells

OPCs: oligodendrocyte precursor cells

PLEO: pleocitosis

S: solid growth

SCs: stem cells

SE: subependymal layer

SGZ: subgranular zone

cSVZ: canine subventricular zone

dSVZ: dorsal subventricular zone

vSVZ: ventral subventricular zone

SVZ: subentricular zone

VASC: vascular features

VIM: vimentin