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SEXUAL DIMORPHISM IN AREAS OF THE BRAIN OF CETARTIODACTYLA

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À Rémy, Germaine, Francis et Annick

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GENERAL ABSTRACT

ENGLISH

Comparative neuroanatomy studies have helped us see the major structures and similarities between mammals, and allowed for the confirmations of hypotheses via direct observation.

Variety in brain specialization can be seen in Cetartiodactyla, between the terrestrial artiodactyls and aquatic cetaceans. Compared to the primate brain, adapted to grasp using articulated fingers with opposable thumbs, the pure quadrupedal locomotion of ungulates and the absence of articulated movements in the limbs of cetaceans most certainly shows variations in the related brain areas. From this principle, the study of the brain of the swine, bovine, sheep or any other cetartiodactyl can help understand the basic organization coding the brain of mammals. Some species have been used more than others among cetartiodactyls. The sheep *Ovis aries* has been the subject of a fair amount of cytoarchitectural studies and functional investigations of the cortex, but also the hypothalamus. Other more exotic terrestrial species like the giraffe *Giraffa camelopardalis*, have much more rarely had their brain thoroughly studied and published. For this reason we studied the cortical folding of the giraffe brain, its brain weight and encephalization quotient (EQ), and we reviewed the literature concerning its encephalon as far as 1839. This showed that, although a relatively very small absolute number of specimen have been collected over the years, the giraffe brain was quite typical of ungulates, with a deep gyrification, and an absolute weight of 720 g. The brain of the swine *Sus scrofa* received comparatively little attention, regarding even basic data. To provide weighed data on the brain of the pig, we weighted the brain of 48 animals to reach a robust value for the average brain weight of the domestic swine a different age categories. The average adult brain weight was 135 g, which was compared to the literature, as well as published EQs. The relatively low EQ (0.38) could be related to the domestication and heavy breeding that meat production necessitated to improve body weight.

One of the findings of the cyctochemical exploration of the mammalian brain is the existence of sexually dimorphic structures. Groups of cells were found to be much larger in volume or cell number in the brain of males or females. Although their precise function is still unknown, most of them are directly involved in reproduction behaviors. Hormones have a crucial role in shaping the developing brain, and in particular androgens. The effects of male and female steroids on the development of the brain and its sexual differentiation can be put in perspective in the study of intersex freemartin bovines. During twin pregnancy of a bovine with a male and a female fetus, male sexual hormones circulating during intrauterine development in the female fetus can masculinize its genital apparatus and alter its phenotype, resulting in an intersex animal. We investigated the hypothalamus cytoarchitecture of freemartin heifers and compared them to male and female hypothalami. We found sex differences between male and female suprachiasmatic (SCN) and vasopressin-oxytocin containing (VON) nuclei. Moreover, the freemartin hypothalamus showed differences more complex than a simple masculinized female brain. While the VON was in size and cell count between male and female values, the SCN of freemartins was larger than both males and females. Using modern multivariate statistical methods, we also investigated the cytoarchitecture of male, female and freemartin cerebellar cortex. We found differences among cellular layers in size, regularity and density of the cells, across sex categories, showing that a multivariate multi-aspect approach can yield valuable results at the cellular level for large cohorts, and that a multi-disciplinary team can produce finer studies.

ITALIANO

La neuroanatomia comparata consente di caratterizzare le strutture nervose mettendo in luce le somiglianze e le differenze tra i mammiferi.

Un aspetto interessante di questo studio comparato riguarda il cervello dei Cetartiodattili sia terrestri (artiodattili) sia marini (cetacei) in cui la locomozione quadrupedale degli ungulati e l'assenza di movimenti articolati nell'arto dei cetacei mostrano variazioni morfologiche rispetto ai primati nelle aree cerebrali correlate i quali presentano un arto specializzato con dita articolate e pollici opponibili capaci di afferrare oggetti molto piccoli con precisione. Da questo punto di vista uno studio neuroanatomico dell'encefalo di artiodattili come il suino, il bovino e la pecora può aiutarci a comprendere l'organizzazione della citoarchitettura nei diversi mammiferi. La pecora *Ovis aries* come modello animale è stata oggetto di una discreta quantità di studi anatomici e indagini funzionali sul ruolo della corteccia cerebrale e dell'ipotalamo. Altre specie di artiodattili terrestri come la giraffa *Giraffa camelopardalis*, sono state meno studiate e sono rare le pubblicazioni che ne hanno studiato il cervello. Per questo motivo, uno degli obiettivi di questa tesi è stato quello di caratterizzare le circonvoluzioni corticali dell'encefalo della giraffa, valutare il suo peso ed il suo quoziente di encefalizzazione (EQ). I risultati hanno permesso di affermare che il cervello della giraffa presenta caratteristiche comuni a quelle degli altri ungulati con una notevole girificazione e un peso medio di 720 g. Un altro obiettivo è stato quello di analizzare l'encefalo del suino *Sus scrofa*. Per ottenere risultati significativi, abbiamo pesato il cervello di 48 animali appartenenti a diverse categorie di età. Il peso medio del cervello adulto è risultato di 135 g. L'EQ relativamente basso (0,38) ottenuto da questo mammifero potrebbe essere spiegato con le esigenze di produzione spinta che l'allevamento intensivo comporta, incrementando la selezione di animali sempre più pesanti. Uno degli aspetti molto studiati in questi ultimi anni da un punto di vista neuroanatomia riguarda la caratterizzazione delle aree sessualmente dimorfiche nell'encefalo dei mammiferi. Il bovino *Bos taurus* rappresenta un modello interessante per lo studio dei dimorfismi cereberali perché questo mammifero possiede un cervello grande, altamente convoluto, una gravidanza di 9 mesi. Inoltre in questa specie si manifesta la sindrome del freemartinismo. Tale sindrome si presenta perchè durante la gravidanza gemellare di un feto maschile e uno femminile, gli ormoni maschili del maschio a causa della anastomosi plaecentare circolano nel feto femminile mascolinizzando. Così il feto femmina è un individuo intersesso interessante perché il suo cervello femminile si è sviluppato naturalmente in un ambiente ormonale maschile. L'obiettivo che ci siamo posti è stato quello di studiare la citoarchitettura dell'ipotalamo di giovenche freemartin e confrontare i dati ottenuti con quelli analizzati nell'ipotalamo di bovini maschi e femmine. Questo studio ci ha permesso di caratterizzare i dimorfismi sessuali tra maschili e femminili presenti nel nucleo suprachiasmatico (SCN) e nel nucleo contenete vasopressina-ossitocina (VON). L'ipotalamo dei freemartin mostrava per quanti riguarda il nucleo VON valori intermedi in termini di dimensioni e numero di cellule tra i valori maschili e femminili. Il SCN dei freemartins è risultato più grande rispetto a quello dei bovini maschi e femmine. Un ulteriore obiettivo è stato quello di studiare la morfologia della citoarchitettura della corteccia cerebellare tra bovini maschi, femmine e freemartin. Applicando un metodo statistico multivariato e multi-aspetto, abbiamo caratterizzato le differenze di genere tra gli strati del cervelletto in termini di dimensioni, regolarità e densità delle cellule.

INTRODUCTION

Perhaps one of the greatest challenges in science has been the pursuit of the mechanisms underlying our brain. In this endeavor, immense progress has seen the progressive rise and fall of different specific paradigms: phrenology, the neuron doctrine, cytoarchitectonic maps and the localization of functions, the synapse, molecular neurobiology, and the connectome, to name a few. To this day, there is still a great amount of work to be done, as neuroscience branched out in multiple major directions including cognitive, behavioral or computational neuroscience. Countless questions are still waiting to be answered, relating to the physiology, pathology, evolution, ontology, and even the anatomy of the central nervous system. Nevertheless, today, research in neuroscience has grown exponentially with topics ranging from the single synapse to the structure and function of the brain as a whole.

The complexity of our brain stands on millions of years of evolution which translate in comparative neuroanatomy. Although the human brain could be considered its pinnacle, the mammalian brain has taken several forms in different species, in an adaptation process to their various environments. In comparative neuroanatomy, species comparisons have brought invaluable insight on the evolution and specialization of neural circuits. For instance, the existence in certain songbirds of a very large group of cells in the brain of (singing) males but not in the (silent) females sparked the search for such differences in other animals (Nottebohm and Arnold, 1976). Finer techniques such as molecular biology helped understand complex mechanisms underlying brain function. We now know that brain development and function are based on an intricate play between hormones, genes, our environment, and an even more complex intertwining of up and down regulations among them. The recent developments of epigenetics has notably unraveled the possibility of a social environment to influence gene expression via complex mechanisms of transcription, DNA methylation and histone acetylation (Forger, 2016)

For a large majority of species, sex is determinant on a wide variety of levels in life. Sex determines a vast amount of differences such as anatomy, life expectancy (Stephens *et al.*, 2018), different tasks in groups, different behaviors regarding exploration (McCarthy and Konkle, 2005), strength, stress and learning capacities (Shors, 2016) or disease prevalence (Table 1, Swaab, 2003a). Sex differences range from obvious, such as in the genital system, to encompassing a large variety of features in structure and function of some organs. Notably, the brain contains sex-specific circuitry, since it is the origin of sexual behaviors such as courting, which are usually widely different between females and males. For a certain time, differences in behavior between sexes have been attributed to hormonal differences, affecting essentially the same neural circuitry (Nelson, 1995). However, during the last 50 years, structural differences have been reported in the brain, comprising brain parts such as the corpus callosum or certain parts of the cortex (DeLacoste-Utamsing and Holloway, 1982; Kanaan *et al.*, 2012), but also brain connectivity or activity (Ingalhalikar *et al.*, 2014; Joel *et al.*, 2015; Tunç *et al.*, 2016), notwithstanding some controversy (Joel and Tarrasch, 2014). Although no structure has yet been precisely pinpointed to a detailed function (Panzica *et al.*, 1995; de Vries and Södersten, 2009), differences are unavoidable. Some of this divergence takes place in the hypothalamus. The study of the hypothalamus and pituitary gland has yielded crucial information on hormone secretion and the control of various physiological functions, comprising the biological clock, water balance, satiety, thirst, stress, lactation or the maternal bond (Swaab, 1997). It is a relatively old area of the brain present in all vertebrates, although recent consideration of neuromeric organization would place it along with the telencephalon, in a "secondary proencephalon" rather than in the *diencephalon proper*

(Nieuwenhuys and Puelles, 2016). It shares almost all of its fundamental structure and functions across a wide variety of mammals, although it seems that clades can have specific features stepping away from the *bauplan*, even closely related ones, such as in Artiodactyla (see later). The hypothalamus is considered to be the main control of the sexual behavior, together with the amygdala. Consequently, the hypothalamus is also the area of the brain concentrating some of the most sexually dimorphic structures in the encephalon. In particular, the sexually dimorphic nucleus (SDN) in rats was the first to raise substantial attention. However, other sexually related nuclei exist, such as the bed nucleus of the stria terminalis (Kruijver, Zhou, Chris W. Pool, *et al.*, 2000), which bridges the hypothalamus and the amygdala, or the anteroventral periventricular nucleus (Orikasa *et al.*, 2002). The study of these differences can help understand the functionality of sex behaviors in the brain, along with some sexually biased pathologies, as the prevalence of certain neurologic and psychiatric conditions is not equal between sexes (Table 1).

Disease	Women : Men	Reference
Rett syndrome	100 : 0	(Leonard, Cobb and Downs, 2016)
Anorexia nervosa	93 : 7	(Lucas *et al.*, 1999)
Bulimia	90 : 10	(Castle and Kreipe, 2007)
Senile dementia of the Alzheimer type	74 : 26	(Hebert *et al.*, 2013)
Multiple sclerosis	70 : 30	(Rotstein *et al.*, 2018)
Anxiety disorder	67 : 33	(Remes *et al.*, 2016)
Schizophrenia	47 : 53	(Saha *et al.*, 2005)
Substance abuse	34 : 66	(Substance Abuse and Mental Health Services Administration, 2014)
ADHD	30 : 70	(Ramtekkar *et al.*, 2010)
Autism	20 : 80	(Baron-Cohen *et al.*, 2011)
Kallmann syndrome	18 : 82	(Laitinen *et al.*, 2011)
Gilles de la Tourette syndrome	8 : 92	(Yang *et al.*, 2016)

Table 1: Ratios for women over men suffering from a selection of neurological and psychiatric conditions (modified from Swaab, 2003a)

Cetartiodactyla has been considered a "super-order", or clade, which was created to comprise both the orders *Cetacea* and Artiodactyla, that is, even-toed ungulates, such as *camelidae*, *suidae*, *giraffidae*, *bovidae* or *hippopotamidae*, after molecular phylogeny studies suggested the hippopotamus was the closest extant terrestrial mammal to cetaceans (Price, Bininda-Emonds and Gittleman, 2005). From a common ancestor about 65 million years ago, cetartiodactyls have grown to occupy a great variety of ecological niches and doing so, have evolved highly specialized features, adapting to their environment (Fordyce, 2009; Gatesy *et al.*, 2013; Cozzi, Huggenberger and Oelschläger, 2017). Cetaceans are anosmic carnivorous aquatic mammals adapted to heat conservation, with rudimentary limbs (Cozzi, Huggenberger and Oelschläger, 2017) and a peculiar sleep rhythm (Lyamin *et al.*, 2008). Artiodactyls on the other hand, are terrestrial highly osmic herbivores adapted to evacuate metabolic heat, showing REM sleep patterns (Lyamin *et al.*, 2008; Herculano-Houzel, 2015), with quadrupedal locomotion and balance. Bodily adaptations arguably comprise central nervous system modifications (Pérez-Barbería and Gordon, 2005; Isler and van Schaik, 2006; Manger, 2006), and the environmental diversity encountered by cetartiodactyls entails that their brain functions diverged enough, along with their bodies, to adapt to conditions such as aquatic life (Depasquale *et al.*, 2016). Changes in neural pathways most probably includes sensory and perception modification, depending on specific adaptations (Hof, Chanis and Marino, 2005; Marino, 2009; Ichishima, 2016; Cozzi, Huggenberger and Oelschläger, 2017).

Artiodactyls have been at the base of early humans' settlement and farming everywhere in the world (Driscoll, Macdonald and O'Brien, 2009). Humans have domesticated some ungulate species such as the goat, the sheep, the pig, the bovine, and progressively selected and bred them, for various

purposes. This has caused profound anatomical and physiological changes in the characteristics of these animals, and some of the highly specialized domestic breeds today would be unable to survive in the wild (Taberlet *et al.*, 2008). Despite these changes in conformation, behavior and physiology, we still know little of their perception of the world, we still partially understand their behavior and the processes revolving through their central nervous system (Knolle, Goncalves and Jennifer Morton, 2017). From an ethical point of view, we arguably ought to assess thoroughly the emotional states taking place in the brain of the domestic animals, during keeping, transport and especially during the slaughtering protocol implemented in Europe. In 2014, the world food production industry slaughtered over 326 million heads of cattle (F.A.O., 2014), and global demand for meat is increasing.

It is evaluated that about 100 million vertebrates are used annually in experimentation laboratories in the world, of which 10% are kept in the EU (R.S.P.C.A., 2018). A very large proportion of laboratory animals are rodents, mostly mice and rats, and a growing proportion are now purpose-bred, with specific genetic alterations. Rodents also present strategic advantages since their maintenance costs are low, and their management is easy. Yet, there have been increasing calls to broaden the spectrum of animal models in neuroscience (Manger, 2008; Bolker, 2012). Indeed, translational neuroscience would benefit from a broadening of points of view, detailing the brain structure and functions in a mammalian brain with features differing strikingly from rodents. A critical step in this direction could involve a large species with a convoluted brain (Peruffo and Cozzi, 2014).

The bovine brain can be considered for neuroscience investigation. It is relatively large in absolute value, weighing about 480 grams (Ballarin *et al.*, 2016), which is larger than in chimpanzees (Jerison, 1973; Chen *et al.*, 2013). The bovine telencephalon is layered, and has developed deep and complex sulci and gyri (Figure 1.1), and specific features (see below) formed during a long gestation period of 41 weeks. During that time, the critical period for the brain differentiation seems to be the second quarter of gestation (about the fourth month) (Peruffo, Cozzi and Ballarin, 2008), which is comparable to other large-brained species such as primates, and unlike rodents in which the aromatase activity peaks near delivery. For these reasons, the bovine represents a valuable and easily accessible animal model with large quantities of neural tissue from animals raised in relatively controlled conditions, and with a homogeneous genetic background (Peruffo and Cozzi, 2014).

Academic anatomy has tremendously changed since its premises in Padova, in the XVIth century (Castiglioni, 1936). Classic anatomy has been based on macroscopic observation, then microscopic observations and measurements, based first on chemical and later immunocytochemical stainings. Measurements done by hand and eye necessitated large amounts of time and were prone to human and mechanical error. Modern, often computational techniques attempt to limit human error by relying on automated algorithms and detection. However, modern neuroanatomy is reaching a phase in which each subject requires technical expertise and data managements hardly achievable by one researcher anymore(Leiner, Leiner and Dow, 1986; Berns *et al.*, 2015; DeFelipe *et al.*, 2016). Increasing detail and precision in each subfield such as statistics, electron microscopy, magnetic resonance imaging or programming, can be adequately combined by multi-disciplinary teams, each covering their field. In this perspective, we formed a working group of anatomists, statistician and image analyst in order to set a modern framework on the study of neuroanatomy in veterinary science.

In the light of these considerations, this thesis set out to describe the brain of some cetartiodactyls and to investigate potential sexual differences in their hypothalamus and cerebellum. Doing so, we initiated a new approach to neuromorphology which could lead to new findings in neuroanatomy.

I. THE BRAIN OF CETARTIODACTYLA

In the end of the XIXth century, a relatively broad interest in cerebral cortices resulted in the study of the brain of several species, including ungulates, first in general accounts (Owen, 1839; Cobbold, 1854; Dwight, 1892), then specifically, at the turn of the century, modern neuroanatomy focused on specific areas of the brain cortices, to map them (Snell, 1891; Weber, 1896; Warncke, 1908; Brodmann, 1909; King, 1911b, 1911a; Simpson and King, 1911; Black, 1915; Van't Hoog, 1920; Bagley, 1922; Brodmann and Garey, 2006). Followed a relative specialization in the field, corresponding to specific studies of different parts of the brain (Koikegami, 1938; Solnitzky, 1939; Lassek, 1942; Rose, 1942; Adrian, 1943; Vierling, 1956, 1957, 1958; Breazile, Swafford and Biles, 1966). At this point, new techniques, the amount of work necessary and the difficulty of handling larger animals progressively drew researchers to a few key animals, namely the cat for carnivores, the rhesus monkey for primates, and the opossum for marsupials (Voogd, 1998b), along with rodents. Indeed, in spite of existing publications on artiodactyl neuroanatomy, including atlases (Tindal, Knaggs and Turvey, 1968; Yoshikawa, 1968; Tindal, Turvey and Blake, 1987; Félix *et al.*, 1999; Okamura, 2002; Vanderwolf and Cooley, 2002) and cellular-level neuroanatomy (Gadamski and Lakomy, 1972; Haarmann, 1974; Ebinger, 1975; Junge, 1976, 1977; Grütze, 1978), little attention was drawn onto ungulates. Later, the rise of investigation on neuroendocrine mechanisms in the brain led to a wide spectrum of research, occasionally resorting to domestic ungulates, notably regarding the magnocellular neurosecretory system in the hypothalamus (De Mey, Vandesande and Dierickx, 1974; De Mey, Dierickx and Vandesande, 1975; Vandesande, Dierickx and De Mey, 1975b, 1975a; Szteyn *et al.*, 1981; Melrose and Knigge, 1989; Tillet, Caldani and Tramu, 1989; van Eerdenburg *et al.*, 1990; van Eerdenburg and Swaab, 1991; van Eerdenburg, Swaab and Van Leeuwen, 1992; Leshin *et al.*, 1995; Leshin, Kraeling and Kiser, 1995; Chaillou *et al.*, 1998). Other mentions of artiodactyl brain mostly regarded them as animal models (Polkowska, Dubois and Domański, 1980; Dees and Mcarthur, 1981; Weesner *et al.*, 1993; Tessonneaud *et al.*, 1994; Barker-Gibb and Clarke, 1996; Resko *et al.*, 1996; Urban, Hewicker-Trautwein and Trautwein, 1997; Chaillou, Tramu and Tillet, 1999).

However, besides dated veterinary treatises (Ellenberger and Baum, 1943; Bruni and Zimmerl, 1951; Getty, 1975; Nickel, Schummer and Seiferle, 1975; Brauer and Schober, 1976; Barone and Bortolami, 2004; Dyce, Sack and Wensing, 2010), relatively little data has been acquired regarding the functional neuroanatomy and general cellular architecture of Artiodactyla. In some instances, the data reported is arguably controversial (Dexler, 1927; Breazile, Jennings and Swafford, 1967).

Cetaceans separated from artiodactyls around 53 million years ago to live in water (Nikaido, Rooney and Okada, 1999; Price, Bininda-Emonds and Gittleman, 2005; Fordyce, 2009; Gatesy *et al.*, 2013; Cozzi, Huggenberger and Oelschläger, 2017). Profound changes in their body morphology over millions of years accompanied this drastic change of environment, including in organs such as the brain (Cozzi *et al.*, 2015; Montelli, Peruffo, *et al.*, 2016). Quantitatively, published studies on the morphology of the nervous system of Cetartiodactyla mostly concern the order *Cetacea*, given its peculiar organization (Kesarev, 1971; Pilleri, 1971; Würsig, Perrin and Thewissen, 2009). Indeed, no scientific publication is dedicated to ungulates or artiodactyls, but at least 3 international journals exist on marine mammals (Aquatic Mammals, Marine Mammal Science and the Journal of Cetacean Research and Management).

Since the seminal work done by Brodmann, and Ramón y Cajal over a century ago on the isocortex layers (Brodmann, 1909; Ramón y Cajal, 1911; Brodmann and Garey, 2006), part of it in

ungulates and cetaceans, the cytoarchitectural organization of specific cortical areas has profited from the advent of immunocytochemistry, staining with a high specificity types of neurons based on their expression of certain molecules. Immunocytochemistry of calcium-binding proteins has allowed to distinguish cell types and architecture (Hof *et al.*, 1999) mostly on rodent and human tissue. Comparative studies among cetaceans, humans and ungulates are even rarer (Hof *et al.*, 1999, 2000; Dell *et al.*, 2012; Butti *et al.*, 2014; Jacobs, Harland, *et al.*, 2014; Jacobs, Johnson, *et al.*, 2014; Kazu *et al.*, 2014; Cozzi *et al.*, 2017; Van Kann *et al.*, 2017). The few extant recent reports are usually focused on the isocortex, and tend to show that the relatively high cortex folding presented by Cetartiodactyla contains comparatively to other mammals, fewer neurons within (Kazu *et al.*, 2014). One common characteristic feature reaffirmed by Hof et al. (1999), is the reduction of the thalamo-receiving 4th cortical layer, with a general cytoarchitectural homogeneity, including prevailing magnocellular neurons throughout the neocortex.

This finding, using calcium-binding proteins, tends to confirm the phylogenetic lineage of cetaceans and artiodactyls found by molecular biology (Price, Bininda-Emonds and Gittleman, 2005; Gatesy *et al.*, 2013), but also hints the possibility of a phenomenon called pedomorphosis in which juvenile characteristics are passed onto the adult stage of life, such as cephalic flexures, large brain size and the predominance of calbindin- and calretinin-containing interneurons. This could be linked to the need for newborns to be physically mature at birth, termed precociality, to either escape predators and run in the case of artiodactyls, or swim and breathe in the case of cetaceans (Hof *et al.*, 1999), as opposed to altricial species, in which newborns are unable to move about by themselves and highly depend on their mother (Kruska, 2005).

I.A. The brain of Artiodactyla

A modern neuroanatomy of Artiodactyla is practically non-existent, as stated by Voogd, Nieuwenhuys, van Dongen and ten Donkelaar some twenty years ago (Voogd, 1998b). The gross anatomy of the brain structure has been published in research journals or books for the goat (Tindal, Knaggs and Turvey, 1968; Tindal, Turvey and Blake, 1987), the ovine (Vanderwolf and Cooley, 2002), the bovine (Yoshikawa, 1968; Lakshminarasimhan, 1975a, 1975b; Okamura, 2002), the pig (Félix *et al.*, 1999), and in veterinary treatises (Ellenberger and Baum, 1943; Bruni and Zimmerl, 1951; Getty, 1975; Nickel, Schummer and Seiferle, 1975; Brauer and Schober, 1976; Barone and Bortolami, 2004; Dyce, Sack and Wensing, 2010).

Some characteristic features can be outlined. All artiodactyls have a highly gyrencephalic brain with a large paleocortex (Nieuwenhuys, 1998). General observation shows that the principal fissures are oriented longitudinally (Figure 1.1). The Sylvian fissure stands vertically, much less pronounced than in primates. Typical fissures such as the diagonal sulcus can be seen on the surface of the isocortex. The rhinal sulcus is very pronounced, separating the isocortex from the paleocortex. Contrarily to cetaceans, artiodactyls show a very developed olfactory bulb with a large number of neurons, compared to other orders (Kazu *et al.*, 2014). The insular cortex can be partially seen, contrarily to *Carnivora*, *Rodentia* and *Lagomorpha*, where it is fully visible, and Primates, and *Cetacea*, where it is completely covered (Nieuwenhuys, 1998). Other principal sulci are the coronal sulcus and ansate sulcus, which together are homologous to the central sulcus of primates (Lakshminarasimhan, 1975a), and the endo-, ecto- and marginal sulci. The orientation of the ansate and coronal sulci suggests that the primate sulcus termed precentral sulcus could have in fact been pushed caudally and rotated due to the development of the motor cortex in primates (Lakshminarasimhan, 1975a).

In a study from Schmidt et al. (2012), detailed MRI imaging of sheep, goats and calves brains were however compared to a dog brain for comparison, which illustrates the need for modern ungulates brain anatomy references. The macroscopic structure of the isocortex was found to be extremely folded in sheep and goat, with particular ramification in the brain of calves (Schmidt *et al.*, 2012). Interestingly, the gyrification is similar to primates for a similar weight, but with a lower number of neurons (Kazu *et al.*, 2014).

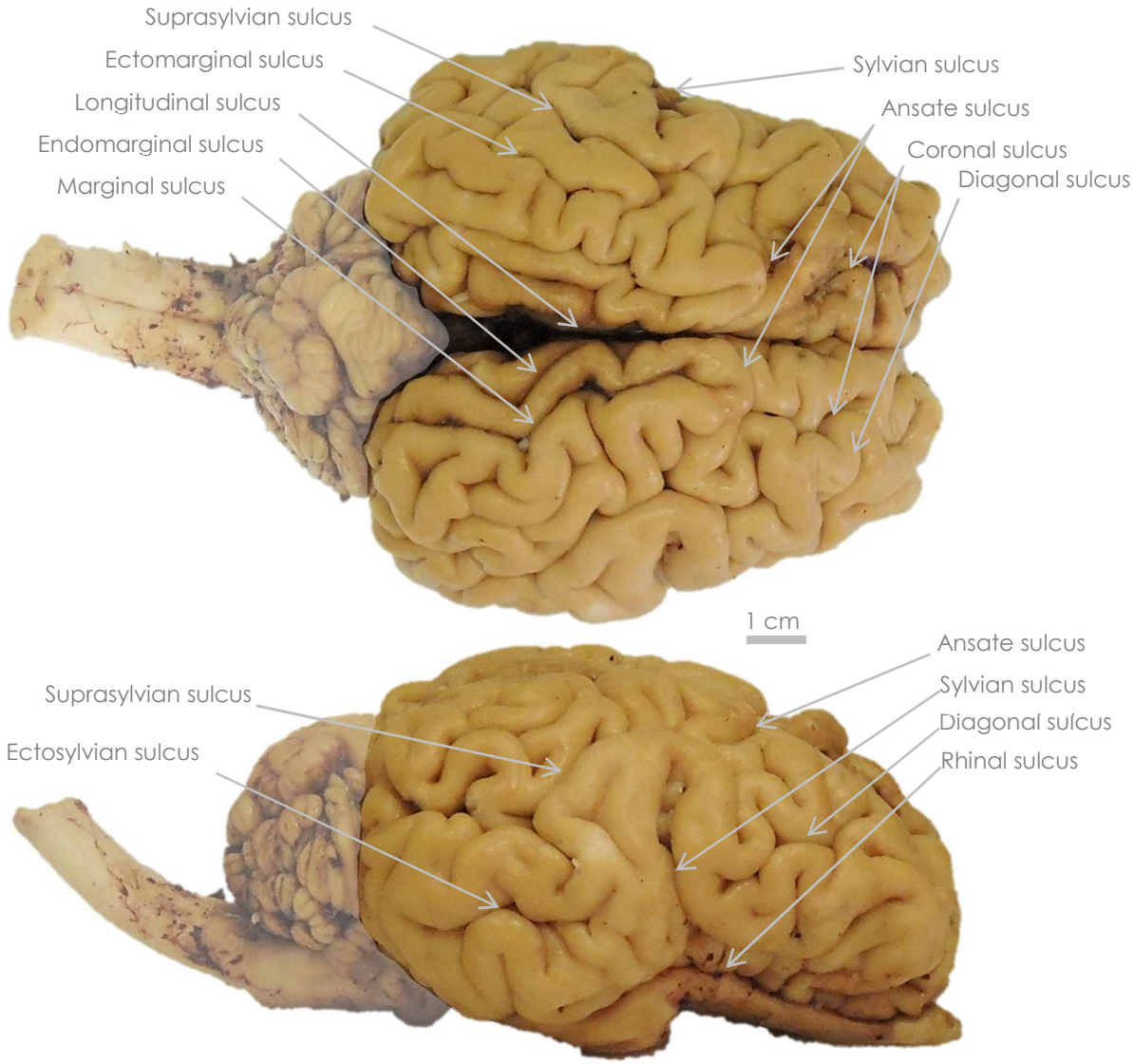

Figure 1.1: Original photograph of a bovine brain showing cortical sulci nomenclature (based on the Nomina Anatomica Veterinaria (2012), and Lakshminarasimhan, 1975a)

However these references mostly deal with macroscopic considerations, and their cytoarchitecture is still for the most part, scarcely described. Some studies on the ovine cortex do exist (Simpson and King, 1911; Van't Hoog, 1920; Lassek, 1942; Rose, 1942; Adrian, 1943; Johnson, Rubel and Hatton, 1974; Dinopoulos *et al.*, 1985), and some are reported in Johnson (1990). Taken together, they show the relatively limited scope of cytoarchitectural and electrophysiological knowledge about sheep cortex, which is only more limited in other artiodactyls. On the other hand, species with particular features for translational neuroscience such as minipigs have been more extensively described (Sauleau *et al.*, 2009; Ettrup, Sørensen and Bjarkam, 2010).

As mentioned above, over the years, few laboratories published studies on *Atriodactyla*. The scope of these investigations was either exploratory, for instance comparing orexigenic neurons organizations (Dell *et al.*, 2012), or using ungulates as model, in the case of Roselli and colleagues, observing the effects of estrogens on the brain and sexual drive of rams (Perkins and Roselli, 2007). Other models have been used, including minipigs (Ettrup, Sørensen and Bjarkam, 2010), pigs and bovines in the study of neuroendocrine processes in the hypothalamus (Leshin *et al.*, 1995; Leshin, Kraeling and Kiser, 1995), or in the sheep (Chaillou, Tramu and Tillet, 1999; Chaillou *et al.*, 2000; Chaillou and Tillet, 2005). Our laboratory has taken part in this stream of research during the last decade, mostly focusing on the bovine (Peruffo *et al.*, 2004, 2008, 2011, 2013; Peruffo, Cozzi and Ballarin, 2008; Peruffo and Cozzi, 2014; Panin *et al.*, 2015; Ballarin *et al.*, 2016; Montelli, Suman, *et al.*, 2016) but also on the horse (Cozzi *et al.*, 2014, 2017) and sheep (Cannas *et al.*, 2018).

In this context of lacking data, we first took on to describe the brain morphology and literature existing on the brain of a relatively rare artiodactyl: the giraffe *Giraffa camelopardalis*. A study of the gross morphology of the brain of the domestic pig *Sus scrofa* was also carried out, as the same type of study for *Bos taurus* and the horse *Equus caballus* had already been published by our lab (Cozzi *et al.*, 2014; Ballarin *et al.*, 2016), focusing on fundamental variables such as fresh brain weight and encephalization quotients (EQ).

I.A.1. THE BRAIN OF THE GIRAFFE *GIRAFFA CAMELOPARDALIS*

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The Brain of the Giraffe (*Giraffa Camelopardalis*): Surface Configuration, Encephalization Quotient, and Analysis of the Existing Literature

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ABSTRACT

The anatomy of the giraffe (*Giraffa camelopardalis* Linnaeus, 1758) has been poorly studied, except for the circulatory system. In particular, only a handful of studies have concerned the brain of this species since the first description in 1839. Accordingly, only a very few articles discussing encephalization mentioned the giraffe or used it in their calculations. In this article, we performed a thorough examination of the literature including old and grey, regarding the central nervous system of the giraffe. Furthermore, we examined the brain of 3 giraffes, and calculated the encephalization quotient (EQ) of the species, based on our own data and the values found in the literature. We also revised the pre-existing literature and re-mapped the main sulci based on current comparative interpretation and anatomical nomenclature. Our results were compared to those of other selected significant mammals. The mean brain weight was of 719.9 ± 12.5 g. Our data indicate that the EQ of the giraffe is 0.64 and matches that of the typical ungulate, despite having the largest brain among terrestrial Cetartiodactyla. This emphasizes that the giraffe is a highly specialized mammal, within the limitations of its clad. Anat Rec, 00:000–000, 2017. © 2017 Wiley Periodicals, Inc.

Key words: Giraffe; brain; encephalization; EQ; neuroanatomy

INTRODUCTION

The giraffe (*Giraffa camelopardalis* Linnaeus, 1758) may be the tallest and among the most iconic animals on the planet, but data concerning other subjects than its unique circulatory system is scarce, and it is not until Owen in 1839 that a scientific publication was made about the anatomical description of a rather little-described species at the time.

The giraffe is part of the order Cetartiodactyla (including Cetaceans and Artiodactyls), with a relatively broad range of phenotypes. Phylogenetically, molecular analysis based on several gene sequences set the Giraffidae as a sister group, as well as the Antilo-capridae, from a clade consisting of Bovidae and Cervidae (Hernández Fernández and Vrba, 2005). Very recently, a multi-locus and population genetic analysis concluded the existence of four separate species instead of the previously described nine subspecies (Fennessy et al., 2016).

Despite its uniquely long neck, it seems that the giraffe could be just a large ruminant adapted to a specific type of grazing (Mitchell et al. 2009). The evolution

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TABLE 1. Individual data concerning our specimen

Animal	Age	Sex	Body weight (Kg)	Nutrition status	Brain weight (g)	Cause of death	Year
Giraffe 1	7	M	630	Normal	770.4	Abscedative abomasitis and cranial trauma	2013
Giraffe 2	10	M	800	Normal	766.1	Ileo-caecal impaction and stomach ulceration	2014
Giraffe 3	10	M	680[a]	Normal	722.7	Right hypertrophic cardiomyopathy	2015
Mean ± S.E.M.			703.3 ± 50.4		753.1 ± 15.23		

Estimated with a Δ of 20 kg.
S. E. M.: standard error mean.

process distinguished the giraffe from its relatives with an extremely elongated neck, most probably adapting to its shifting environment (Mitchell and Skinner, 2003; van Schalkwyk et al., 2004; Badlangana et al., 2009; Mitchell et al., 2009).

On top of the longest neck on earth sits a rather large head. The allocation of space to the frontal sinus also contributes to separating the giraffe from other ungulates (Badlangana et al., 2011), as well as the okapi *Okapia johnstoni*, the only other extant Giraffidae. This adaptation allows an increase of the total head volume with relatively reduced cranial mass. The function associated with this adaptation is still unclear, as the thermoregulation or mastication hypothesis seem unlikely. Other explanations including sexual differentiation and a low frequency resonance case, have not been investigated. It is known that the giraffe's uniquely high-positioned perception is accompanied by unique visual features, allowing both high precision in tongue prehension as well as enhanced movement detection in the lower visual field (Coimbra et al., 2013). Although very long (up to 2.6 m and reaching as far as the sacral vertebrae), the giraffe spinal cord is a quite typical ungulate corticospinal tract (Badlangana et al., 2007a). Consequently, with an elongated but typical spinal cord, a skull with an enlarged frontal sinus and specific visual adaptations, it would be reasonable to expect some distinctive features on the giraffe brain setting it apart from the ungulate brain type. Although being a mammal of great size, it is yet not considered one of the biggest brained animals (Manger et al., 2013).

Since Owen (1839) first described the anatomy of the giraffe, over two centuries ago, there has been no thorough examination of the whole data regarding the giraffe brain. Among the few existing studies of the giraffe brain, some were performed either a rather long time ago, or on collection animals (Friant, 1968; Badlangana et al., 2009). Some were using casts of the brain case, or relying on data from other studies (Black, 1915). Other studies included one specimen as part of a comprehensive comparative study (Ariëns Kappers et al., 1967; Pérez-Barbería and Gordon, 2005; Badlangana et al., 2007b). Only a handful of studies used more than one specimen (Badlangana et al., 2007a; Bux et al., 2010; Dell et al., 2012; Coimbra et al., 2013; Jacobs et al., 2014). In some cases, studies re-quoted or used animals from previous publications. Alternatively, studies are seldom comprehensive in terms of age, sex, or conditions of life. This resulted in a meagre amount of animals used in the literature considering the time span. Voogd *et al*. (1998) stated that a modern neuroanatomy of ungulates was practically inexistent. Although a few studies have since tackled the gap (Badlangana et al., 2007a,b, 2009, 2011; Bux et al., 2010; Dell et al., 2012; Coimbra et al., 2013; Jacobs et al., 2014) the concept still stands.

The objective of this research was to compare the encephalization of the giraffe with that of other mammals. To this effect we performed a thorough search combing the available literature, including modern references and grey, discontinued journals. To calculate an encephalization quotient (EQ) of the giraffe, we pooled together values on brain and body weight found in the examined articles with our own data on three giraffe brains. Finally, we re-mapped the main sulci of the giraffe brain based on previous neuro-anatomical studies and the current anatomical nomenclature (N. A. V. 2012).

MATERIALS AND METHODS

Sampled Specimens

For this study, we used the brains of three giraffes who lived in zoological parks in Northern Italy and whose body was autopsied for diagnostic purposes (see Table 1). Two giraffes were brought to the necropsy room of the Department of Comparative Biomedicine and Food Science of the University of Padova in Legnaro, Italy, in 2013 and 2014. The other specimen was necropsied on site of death, due to technical and logistical reasons, in 2015. Two out of three specimens died for causes unrelated to neurology. The third animal died because of gastrointestinal problems and the consequence of accidental head trauma that resulted in multiple fractures of the skull. However, the brain showed no macroscopic damage or sign of edema.

All procedures performed were in accordance with the ethical standards of the University of Padova.

Data concerning the three animals can be found in Table 1. The body weight was estimated on site for one animal and measured in the necropsy room for the two others. Postmortem delay varied between 8 and 32 hr. The brains were collected after the necropsy, photographed and weighted without the dura mater, before fixation for at least 24h in buffered formalin 4% before coronal section, then kept at 4°C in formalin for weeks.

TABLE 2. Reference table for giraffe specimen brain and body weight from 1838 to 2015

Year	Body weight (g)	Brain weight (g)	Sex	Age	Fresh/Fixated	Source
1838		397				Owen (1839)
1846		710		Young		Joly and Lavocat (1845)
1854		539	-	> 2 years old		Cobbold (1854)
1864	**150,000 (3 cwt)**	**390**	**M**	**2 months old**		Crisp (1864)
	810,000 (16 cwt)	**426**	**M**	**Sub-adult.**		
1896	**529,000**	**680**	**M**	**Adult**		Weber (1896)
	300,000	**420**	**M**	**Sub-adult**		
	150,000	389	M	2 months old		
1908	529,000	680	M	Adult		Warncke (1908)
1915	529,000	680	M	Adult		Black 1915 (from Weber)
	300,000	420	M	Sub-adult		
	150,000	389	M	2 months old		
1927		665		Adult		Ariëns Kappers (1927)
		466		Neonate		
1938	**1,198,000**	**700**	**M**	**Adult**		Quiring (1938)
1940	**1,220,000**	**700**	**M**	**Adult**	Fresh	Crile and Quiring (1940)
1956	**1,200,000**	**720 (0.06 g/100 g)**	**M**	**Adult**	Fresh	Spector (1956)
1966	**600,000**	**712**		**Adult**	Fixed	Mangold-Wirz (1966)
1971	**600,000**	**655**	**M**	**Adult**	Fixed	Oboussier and Möller (1971)
1973	529,000	680	M	Adult	-	Kruska 1973 (from Warncke
	600,000	712	M	Adult	Fixed	and from Mangold-Wirz)
2005	**1,002,300**	**773**	-	-	Fixed (collection)	Pérez-Barbería and Gordon (2005) (from own database)
2006		707 (from log)	-	-	Fixed	Shultz and Dunbar (2006) (from
	575,000–439,000 (from log)					Mangold-Wirz, 1966)
2007	Approx. 400,000	509	M	Sub-adult (2 y old)	Fixed*	Badlangana et al. (2007a)
	450,000	**509**	**M**	**Sub-adult (2 y old)**	Fixed*	Badlangana et al. (2007b)
)2008		397	M	> 2 years old	Fixed	Mitchell et al. (2008) (from
		710	M	Sub-adult	Fixed*	Owen, Joly and Lavocat,
		539	M	2 months old		Cobbold, Crisp, Oboussier
		426	M	Adult		and Möller, Badlangana)
		390		Sub-adult		
		655				
		509				
2010	**480,000**	**544**	**M**	**Sub-adult**	Fixed*	Bux et al. (2010) (same as
	450,000	509	M	Sub-adult	Fixed*	Badlangana et al. 2007a,b)
	318,850	703,9	-	-		Shultz and Dunbar 2010 (estimate)
2012	480,000	544	M	Sub-adult	Fixed*	Dell et al. (2012) (same as above)
	450,000	509	M	Sub-adult	Fixed*	
2014		610	M	Sub-adult	Fixed*	Jacobs et al. (2014a) and
		527	M	Sub-adult	Fixed*	Jacobs et al. (2014b)
		480	M	Sub-adult	Fixed*	

Numbers in bold represent first hand complete data from new animals at the time. * Fixation was performed briefly by perfusion immediately after death, before weighting.

TABLE 3. Average weights and EQ estimations of different giraffe cohorts

Age group		n	Mean brain weight (g) ± S. E. M.	Mean body weight (g) ± S. E. M.	EQ
Literature	Total	12	602.4 ± 39.4	711,608.3 ± 106,348.2	0.60
	Adults	7	705.7 ± 13.9	907,042.9 ± 120,412.3	0,60
Our sample	Adults	3	753,1 ± 15,2	703,333.3 ± 50,442.5	0,76
Overall mean	Adults	10	719,9 ± 12,5	845,930 ± 88,905.4	0,64

S. E. M.: standard error mean.

Analyses of the Literature

The literature was examined using search engines (Google Scholar, Scopus, PubMed), the Padova University Library through NILDE communication software, and with the help of the library of the Civic Museum of Natural History of Milan, Italy, for references that were not searchable online. We extracted data reports of body and brain weight throughout the available accounts on the subject. This collection of data is reported and compared to our findings in Table 2.

Regarding the anatomy of the brain, we reviewed and used the specific descriptions found in Owen (1839), Black (1915), Ariëns Kappers et al. (1967), Friant (1968), Lauer (1982), and Schaller (1999).

Nomenclature

The brain main sulci, gyri and other structures were assessed using the available literature, as a complete nomenclature of the giraffe brain is not universally accepted. In the present article we referred to the international nomenclature (N. A. V., 2012). As a means of comparison, we used two bovine brains and two human brains, from the archival collection of the Department of Comparative Biomedicine and Food Science of the University of Padova.

Encephalization Quotients

To calculate EQ, we used Jerison's equation: $\text{E.Q.} = \frac{E_i}{0{,}12 \times P_i^{2/3}}$ where E_i is the mean weight of the brain and P_i the mean weight of the body (Jerison, 1973). We are aware that a 0,75 exponent has been used (Pilbeam and Gould, 1974; Martin, 1981), though we chose to maintain the original 2/3 exponent (for a review on the subject, see Kruska, 2005). The data obtained was confronted with a selected range of mammal species belonging to other orders in Table 3.

RESULTS

Sampled Specimens

The gross anatomy of the giraffe brain showed the features typical of terrestrial Cetartiodactyla, with a developed and very convoluted neocortex, well developed rhinencephalon visible from the lateral side, and the characteristic lateral enlargement of the temporal lobe. Figure 1 reports our interpretation of the sulci and gyri of the giraffe brain, together with nomenclature from older publications. The aspect of the slightly covered cerebellum shows a distinctive very convoluted vermis in an S shape on its dorsal surface (Fig. 1).

The three brains of the adult male giraffes weighted respectively 722.7, 766.1 and 770.4 g, with a mean of 753.1 ± 15.23 g (Table 1). The body weights were similar with an average weight of 703.3 ± 50.4 kg. The EQ calculated with these means resulted in a value of 0.76 (Table 3).

Analyses of the Literature

Thorough search of the literature yielded data from 24 giraffes, all males except the rare undisclosed cases (see Table 2). Of these 24 animals, 12 were identified as independent specimen (bold numbers in Table 2), and their specifics are discussed here together for the first time. Of these 12 separate animals, only seven were adults. The mean body and brain weight of this latter group (seven adults) were, respectively 907 ± 120 kg and 705.7 ± 13.9 g. The calculated EQ was correspondingly 0.60. The addition of young animals in the total group obviously reduced both body and brain weight, but the EQ (0.60) did not change (see Table 3).

After pooling adults from the literature with our specimen, we obtained a mean brain weight of 719.9 ± 12.5 g, a mean body weight of 845.9 ± 88.9 kg, and a resulting EQ of 0.64 (Table 3).

DISCUSSION

Gross Anatomy of the Giraffe Brain

Based on genetic evidence, it was recently proposed that the species now commonly identified as *Giraffa camelopardalis* should be split into four different species: *G. camelopardalis*; *G. reticulate*; *G. tippelskirchi*; and *G. giraffa* (Fennessy et al., 2016). Such a new definition of the giraffe as we know it could have deep implications regarding individual variability as well as species or subspecies differences. Based on the lack of characterization of the current subspecies in the past literature as well as in our specimens, we were forced to consider our cohort data as representative of the giraffe as one coherent group.

Our interpretation of the cerebral sulci and gyri follows the current International Nomenclature (N.A.V., 2012), largely based on the horse brain. Doubts due to the different shape of the Cetartiodactyla brain were solved by comparison with the available bovine brains (for a review of these, see Lakshminarasimhan, 1975a,b; Harper and Maser, 1976; Louw, 1989), while interpretation of uncertain sulci was based on the assessment of the human brains. Most of our choices agree with Black (1915), namely the identification of *sulcus coronalis*, *sulcus enterolateralis* (N.A.V., 2012: *endomarginalis*). Remarkably, the *sulcus suprasylvius*, presently divided in *rostralis*, *medius* and *caudalis* (N.A.V., 2012), was

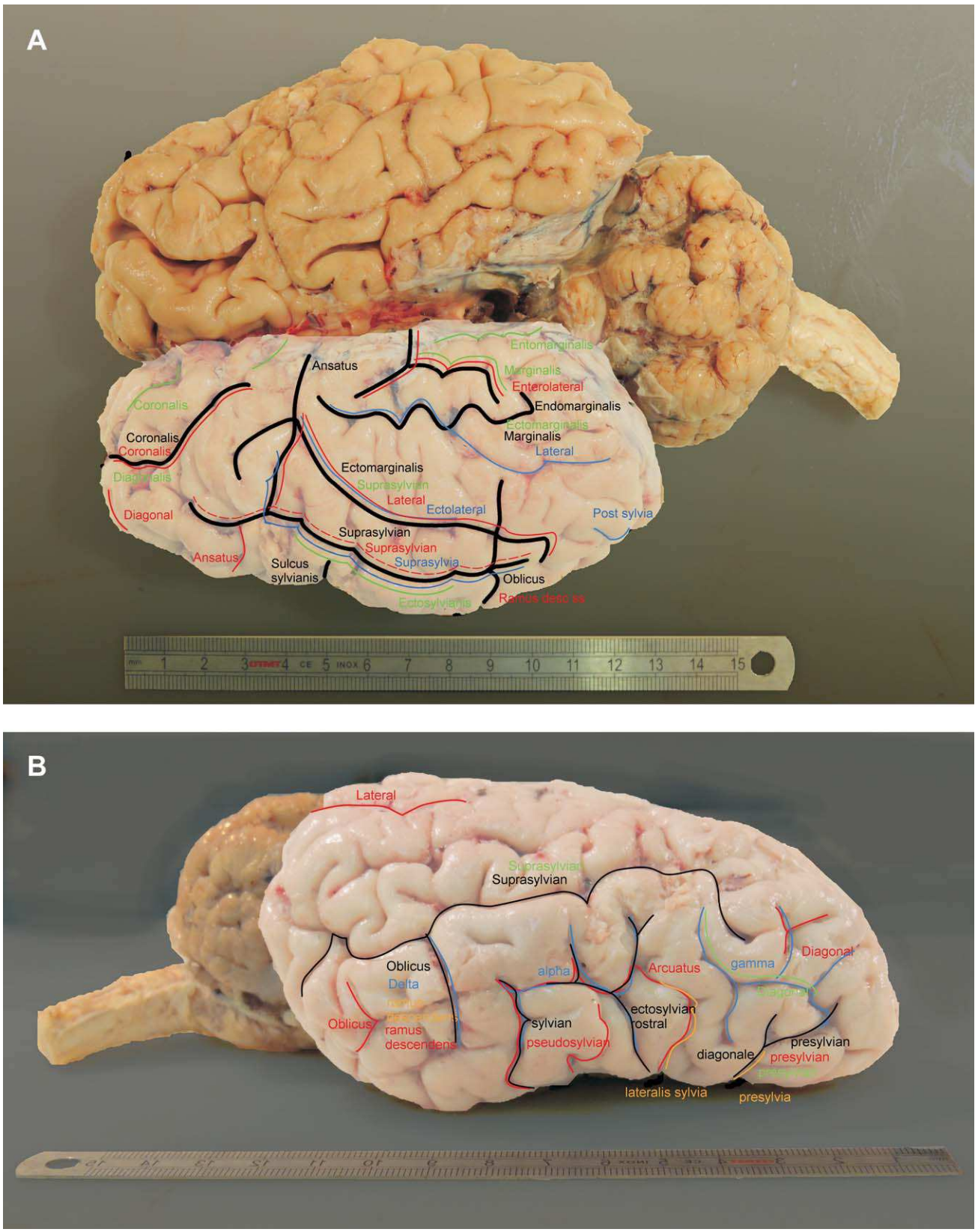

Fig. 1. Main sulci of the giraffe Giraffa Camelopardalis brain according to different articles, dorsal (A) and lateral (B) views.Our determination of sulci is in black lines and nomenclature in black lettering. Colored code for lines and names: red for Black (1915), blue for Friant (1968), orange for (Oboussier and Möller, 1971) and green for Lauer (1982). Figures were produced using Gimp 2.8.

also clearly described by Black's endocast. The limits of endocasting was maybe more evident on the lateral *sulcus*, or *sulcus marginalis* (N.A.V., 2012). On the lateral side, Black's (1915) *ramus descendens suprasylvius* seems to correspond to *sulcus obliquus*. Friant (1968) identified an arcuate gyrus furtherly divided by three minor sulci (named *delta*, *alpha* and *gamma*) in the territory called *sulcus arcuatus* originally described by Black (1915). Later again, Lauer (1982), named the same feature as the ectosylvian sulcus, actually the anterior part of the sylvian fissure. Oboussier and Möller (1971) also identified the same features on the lateral side of the brain, but their description of the dorsal part was uncertain. Lauer (1982) described a suprasylvian sulcus, but to our opinion the structure is too posterior to bear that name (see Fig. 1A). We therefore identified it as *sulcus ectomarginalis*, a terminology that better fits N.A.V. (2012) standards. Lauer (1912) described a rostro-dorsal sulcus, and named it diagonal sulcus because of its orientation. However, we do not consider it an independent structure, but just a continuation of the presylvian sulcus with the confluence of a minor additional sulcus (see Fig. 1B).

We hereby found that the coronal fissure was very deep and marked. We believe the sylvian fissure is present, although in our specimens it was substantially less marked than in the bovine or the horse.

Our observation suggests the existence of a certain variability in the smaller convolutions of the giraffe brain, and a minor asymmetry regarding the said minor sulci.

On the whole, the brain of the giraffe is comparable to that of other terrestrial Cetartiodactyla, and shows a high degree of gyrification, without the rotations and compressions found in more distant Cetartiodactyla such as the bottlenose dolphin (Cozzi et al., 2014). High folding adaptation of the brain ontology suggests surface enhancement in a relatively smaller space, compared to species with a similar brain weight (Pillay and Manger, 2007).

Analyses of the Literature

The first description of the morphology of the giraffe brain is contained in Owen (1839). The description is based on an endocast, which was fairly common in early studies of exotic animal brains. However, such methodology, although obviously still acceptable in fossils, is rather detrimental for the correct representation of the sulci and gyri and the morphology of the cerebellum (Radinsky 1981). Friant (1968) described the fetal stages of the giraffe brain, and considered that the placement of the coronal and splenial sulci were "primitive" in this species, if compared to Bovidae and Cervidae. The rather characteristic double operculization (through the ectosylvian fissure and the posterior rhinal fissure), shows similarities with some Perissodactyla, including the horse (Friant, 1968). Ariëns Kappers et al. (1967) described a cauliflower-like series of convolutions forming the vermis of the cerebellum, a feature also reported by Owen (1839).

A detailed analysis of the cytoarchitectonic of the giraffe brain is outside the scope of the present article. Several studies characterized it as resembling that of a typical ungulate with some specificities (Hof et al., 1999; Hof and Sherwood, 2005; Badlangana et al., 2007a,b; Bux et al., 2010; Dell et al., 2012; Jacobs et al., 2014), despite its early phylogenetic separation from other terrestrial Cetartiodactyla and its peculiar ecology, phenotype and brain size. The relatively large array of complex spiny neurons, organized into a vertical columnar organization (Jacobs et al., 2014), is reminiscent of that of rodents and primates. The cortical column of the giraffe is similar to that of other terrestrial Cetartiodactyla, but differences in neuron variants set it aside from cetaceans. The neuronal dendrites of the giraffe brain remain smaller than that of the elephant and the minke whale (Butti et al., 2011).

TABLE 4. Bibliographic references on giraffe weight

Reference	Weight range (mean) in kg
Dagg (1971)	M: Up to 1,220 or more 800 (independent of sex)
Cillie (1987)	M: 970–1,935 (1,452.5) F: 700–950 (825)
Nowak (1999)	500–1,930 (800)
Wilson and Reeder (2005)	500–1,100 (800)
Skinner and Chimimba (2006)	M: 800–1,930 (1,365) F: 550–1,180 (865)
Kingdon and Hoffmann (2013)	M: 1,191.8 (973–1,395), $n = 18$ F: 828.4 (703–950), $n = 18$
Miller and Fowler (2014)	M: 850–1,950 (1,400) F: 700–1,200 (950)
Kingdon (2015)	M: (1500) F: (1000)

Means in italics are arithmetic means calculated by us based on range. M: males, F: females.

Encephalization as a Reference Tool and Limits

Encephalization designates the augmentation of brain mass for a given body mass. Therefore, the EQ represents how larger or smaller the measured mass of the brain is compared to the brain mass that could be expected for the animal's body weight. Jerison's equation has been used for decades and derives from an allometry found between the brain mass and the body mass (Snell, 1891; Jerison, 1973). To date, two exponential quotients have been proposed from regression line slopes. The original quotient hypothesized by Snell and used by Jerison is 2/3. Slightly different exponents were proposed by Pilbeam and Gould (1974) and later by Martin (1981), based on a broader sample of mammal species. Although the equation remains slightly unfavorable for mammals of large body mass, here we maintained the original Jerison formula for easier comparative purposes.

Table 4 summarizes the values of body weight ranges found in reference literature. While most agree on a rough 800 kg mean, a certain disparity can be noted. Concerning age, Berry and Bercovitch (2012), came to the conclusion that, in the wild, the life expectancy is 14 to 16 years with a maximum of 20 years. The animals that we examined revealed a relatively low body weight ranging from 630 to 800 kg for adult males (Table 4), compared to average weights found in literature for animals caught in the wild (Badlangana et al., 2007b).

Our own sampled data shows a higher brain weight than what could be expected for a giraffe according to

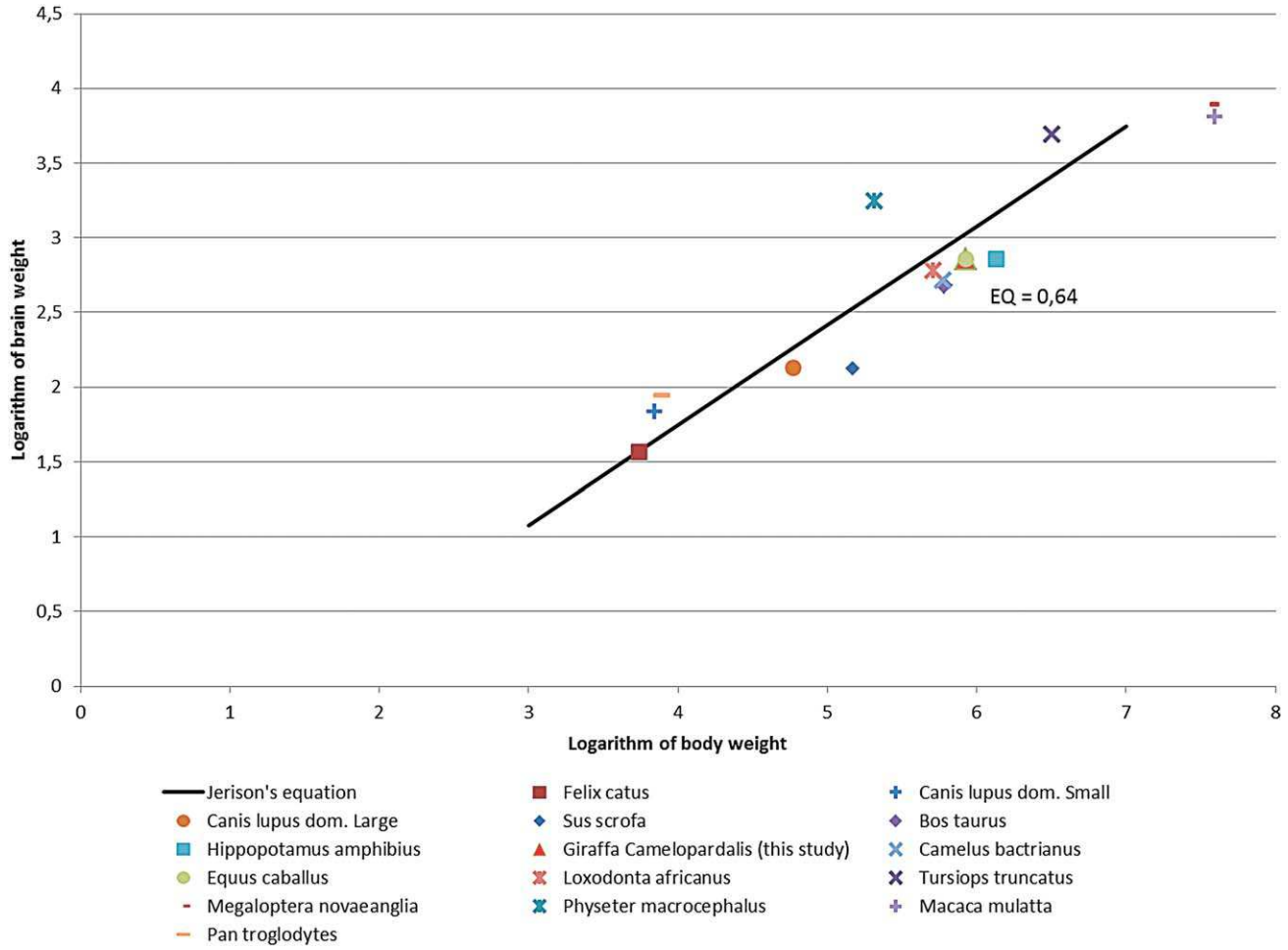

Fig. 2. Log-Log plot representation of brain to body weight for selected mammals quoted in Table 3.The black line represents expected values following Jerison's equation. It can be noted that the giraffe is closely clustered with other ungulates such as the horse, the hippopotamus, the camel and the bovine.

TABLE 5. Brain, body weight and EQ from selected mammals.

Species	Brain weight (g)	Body weight (kg)	EQ	Reference
Carnivora	37	5.5	1.00	Shultz and Dunbar (2006)
Felis catus	68–135	7–59	1.55–0.74	Seiferle 1988
Canis lupus familiaris				
Artiodactyla				
Sus scrofa	133	149	0.39	Minervini et al. (2016)
Bos Taurus	480	603,7	0.57	Ballarin et al. (2016)
Hippopotamus amphibius	720	1351	0.49	Silva and Downing (1995)
Camelus bactrianus	518	594	0.61	Xie et al. (2011)
Giraffa camelopardalis	719	845.4	0.64	This study
Perissodactyla				
Equus caballus	599	514	0.78	Cozzi et al. (2014)
Proboscidea				
Loxodonta africana	4,927	3185	1.67	Shoshani et al. (2006)
Cetacea				
Tursiops truncatus	1,759	206	4.20	Shultz and Dunbar (2006)
Physeter macrocephalus	7,818	37,094	0.6	Ridgway (1986)
Megaloptera novaeangliae	6,439	39,311	0.46	Manger (2006)
Primates				
Macaca mulata	88	7.8	1.86	Shultz and Dunbar (2006)
Pan troglodytes	382	46	2.48	

the literature (see Table 2). The brain weight of our specimens was consistently above 700 g, and thus well above the limit established by Mangeret al. (2013) to qualify "large" brains. We emphasize that although the brains that we studied were fixed in formalin, their weight was calculated immediately after removal from the brain case and should not be considered biased by either shrinkage or fluid absorption. We also add that our limited sample was constituted only by adult captive males. It is therefore possible, as recently suggested, that the EQ for females of the same species could have been even greater (Mitchell et al., 2013).

Review of the literature showed that basic data on the central nervous system of the giraffe is scarce (Owen, 1839; Joly and Lavocat, 1846; Cobbold, 1854; Crisp, 1864; Weber, 1896; Black, 1915). Moreover, the state of nutrition of the animals described is not verifiable: They were often considered exotic and housed with poor husbandry and only an approximate knowledge of their diet and requirements, frequently succumbing to the climate within a few months of their arrival in city zoos or parks (Cobbold, 1854). The consequence is that the EQ calculations based on the latter subjects could be biased. To our knowledge, only a few articles (Pérez-Barbería and Gordon, 2005; Shultz and Dunbar, 2006, 2010; Boddy et al., 2012) have used giraffes in encephalization studies so far. Published articles often rely on old literature to increase the sample size when discussing rare or wild species. The giraffe does not escape this tendency given that in almost two centuries, only seven adult animals, all of them males, were synthetically described and their data reported (Table 2). The EQ calculated based on data extracted from the literature (see Table 2) is 0.60 (Table 3). However, we stress that this number is not representative of giraffes as a population for two reasons, the first one being the total absence of female in the sample, and the second one being the large amount of captive animals, which can diverge in weight and size from the wild. More broadly, in accord with the Healy and Rowe review (2007), caution should be taken regarding the overall variability in measurement methods and scientific rigor.

In the present article, the calculated EQ based on our data is substantially higher (0.76) than in previous studies (Tables 2 and 3). What are the reasons for this important 20%–25% difference with previously reported EQ values? First of all we emphasize that our values originate from a direct calculation of brain and body weight derived from captive, but well housed, fed and groomed, mature specimens. Second, intraspecific variation may sometimes yield huge differences: In a recent study of our group (Cozzi et al., 2014), 7 out of 131 horses showed an EQ in the cat or primate range. An EQ of 0.76 for the giraffe is also more consistent with the Ruminantia clad (0.86, Boddy et al. 2012; Ballarin et al. 2016). The cluster is very obvious on the Figure 2, while the non-ruminant even-hoofed swine (*Sus scrofa*) is rather distant from this tendency (Minervini et al., 2016).

If we pool together all adult giraffes, thus including both specimens described in published articles and our own directly measured animals, we can calculate an average EQ of 0,64 (Table 3). Both our figure (0.76) and the ones obtained from either the literature (0.60) or by pooling all data together (0.64), place the giraffe along with the camel, the horse, and the bovine below the EQ = 1 line (Fig. 2), tending to confirm that, despite the rather unique morphology, the giraffe remains a ruminant comparable to others.

Table 5 reports also the EQ of other terrestrial Cetartiodactyla and other selected mammalian species. The giraffe seems to have not only the biggest brain among terrestrial Cetartiodactyla in absolute mass, together with the hippopotamus, but also the largest EQ (Table 5). It is notably lower than that of the odd-hoofed horse, with 0.78 (Cozzi et al., 2014).

An EQ below 1 implies that the giraffe shows a relatively smaller brain than would be expected for its weight. One contributing factor could be the calculation of the EQ itself, which is slightly unfavorable to larger species. This can be seen in another subgroup of the Cetartiodactyla, cetaceans, where large species show a rather low EQ, unlike smaller species (Table 5). However, a low body to brain weight ratio is to be put in perspective with the fact that ungulates have a tendency to have a more gyrencephalic brain than other clads (Pillay and Manger, 2007).

There is a vast discussion in the scientific community regarding the definition and implications of "intelligence", brain size and encephalization among other parameters like cortical thickness, neuron packing density and conduction velocity (Roth and Dicke, 2005; Roth, 2015; Dicke and Roth, 2016). The Social Brain Hypothesis brought up by Shultz and Dunbar (2006) assumes that the growth and gyrification of the neocortex, leading to allometric increase of brain weight, is caused by accentuated social structure among animals, calling for a faster computing capacity to deal with those social interactions. The social structure of giraffes does not appear directly as complex as that of other Cetartiodactyla (especially marine ones), yet it is known today that giraffes share the fission/fusion social dynamics attributed to orcas and chimpanzees (Bercovitch and Berry, 2013), with long and short term bonds, various sizes of herds, home-range sizes, mobility, habitats (van der Jeugd and Prins, 2000), and hence complex social bonds needing computational capacity allocation. The correlation between complex behaviors including complex social structures, and brain size or encephalization remains an unsolved issue (Healy and Rowe, 2007). It is expected that encephalization requires specialization of adaptive features allowed by pathway duplication (Chakraborty and Jarvis, 2015). The similarity of the organization of the giraffe (and other terrestrial Cetartiodactyla) neocortex with that of cetaceans (Hof et al., 1999), shows this possibility of specialization, as is postulated for the cetacean brain in the aquatic environment (Tsagkogeorga et al., 2015). Further studies mapping cortex functions (Badlangana et al., 2007a) could bring more insight into the specialization process, on a microscopic level, and on an evolutionary comparative scale.

In conclusion, it appears that the giraffe brain follows the typical ungulate *bauplan*, despite a larger skull case and a longer spinal cord. A recent article (Abelson, 2016) correlated the risk of endangerment of a species to their relative encephalization, at least in American mammals. Given the intrinsic value of this species, the brain of the giraffe should receive a special interest also considering its importance in social and comparative studies. Further studies on the cytoarchitectonics of the central nervous system of the giraffe could greatly contribute to

further understanding the neural substrate of behavior in this unique representative of highly specialized ungulates.

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I.A.2. THE BRAIN OF THE DOMESTIC PIG *SUS SCROFA*

The swine brain has received increasing attention in the recent years, based on the similarity of the cortex and subcortex to humans (Sauleau *et al.*, 2009; Pirone *et al.*, 2018). However, as noted by Ettrup and colleagues (2010) most studies concerning cytoarchitecture, e.g. concerning the hypothalamus, have been based on thionine staining (Solnitzky, 1939; Yoshikawa, 1968; Szteyn *et al.*, 1980; Seeger, 1987, 1990; Félix *et al.*, 1999) or limited in their scope to a type of neuron or cyctochemical marker (Cassone *et al.*, 1988; van Eerdenburg *et al.*, 1990; van Eerdenburg, Swaab and Van Leeuwen, 1992; Leshin *et al.*, 1995; Vellucci and Parrott, 1997; Rankin *et al.*, 2003; Raymond *et al.*, 2006; Su *et al.*, 2008).

From an encephalization point of view, there have been studies concerning the effect of domestication on wild animals, including on swine (Kruska and Röhrs, 1974; Kruska, 2005). The pig is a relatively common domesticated animal, and it was reported that domestication reduced the size of its brain by 34% (Kruska, 1970). This has however to been nuanced, since not all the parts of the brain are smaller but the overall size (Rehkämper, Frahm and Cnotka, 2008). To precise fundamental data concerning the brain of *Sus scrofa*, we proceeded to sample and weigh a large number of animals (n=31 adults), calculate a reliable encephalization quotient and put it in perspective among other *Cetartiodactyla*.

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RESEARCH ARTICLE

Brain Mass and Encephalization Quotients in the Domestic Industrial Pig (*Sus scrofa*)

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Abstract

In the present study we examined the brain of fetal, newborn, and adult pigs raised for meat production. The fresh and formalin-fixed weights of the brain have been recorded and used, together with body weight, to calculate the Encephalization Quotient (EQ). The weight of the cerebellum has been used to calculate the Cerebellar Quotient (CQ). The results have been discussed together with analogue data obtained in other terrestrial Cetartiodactyla (including the domestic bovine, sheep, goat, and camel), domesticated Carnivora, Proboscidata, and Primates. Our study, based on a relatively large experimental series, corrects former observations present in the literature based on smaller samples, and emphasizes that the domestic pig has a small brain relative to its body size (EQ = 0.38 for adults), possibly due to factors linked to the necessity of meat production and improved body weight. Comparison with other terrestrial Cetartiodactyla indicates a similar trend for all domesticated species.

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Introduction

The pig (*Sus scrofa domesticus*) is one of the first domesticated mammals, and represents a very diffuse, important and traditional meat resource in many countries, in which millions of individuals of the species are raised and slaughtered for production. The pig is also a useful experimental model in different areas of biomedical research world-wide.

Although several textbooks addressed the anatomy of the pig in detail [1–4], an original, exhaustive and functional description of the brain of this species is lacking. Data on the weight of the brain of the pig and relative brain to body weight ratio are reported in Table 1, with the relative relevant literature and reference texts.

There is a growing awareness of consumers and of the general public towards animal welfare, and the current European legislation includes several measures to minimize animal stress in the production farms and also during transportation and at the slaughterhouse. The behavior of the domestic swine has been actively investigated considering maternal-neonatal

Table 1. Data from literature rative to the ratio brain/body weight of the swine.

Year	Source	Brain weight (g)	Body weight (kg)	Ratio
1879	[57]	160		
1912	[58]	125–164, *domestic*	250*	1:2000–1:9000
		178, *wild*		
1913	[59]	112		
1927	[60]	112, *adult*		
		14, *neonate*		
1909	[37]	162	157,5	1:972
			74	1:705
1969	[61]	180		
1988	[18]	96–145	60–96	1:630–1:660
		105–110	126–209	1:1200–1:1900
2005	[21]	259	89,4	
2006	[4]	111–123*	80–90	1:650
			200	1:1800
2007	[53]	95,3		
2010	[27]	180	125	
2012	[15]	169.8	867,72	
		70,2	22,15	
		60,6	27,7	
		57,6	27,5	
		47,7	10	
		28,8	0.478	
2012	[16]	95,3		
2013	[55]	95,3		

*estimate

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interactions [5], disease [6], feeding [7], aggression and affiliation during social conflict [8], and during group housing [9]. However, identification of the associative areas of this species and the functional wiring of the relative circuitry is still poorly understood. Under these conditions the neural foundation of behavior, motivation and social interaction are only tentatively identified based on comparative studies performed in rodents, primates or other mammals.

The encephalization quotient (EQ), defined as the ratio between observed and expected brain mass [10,11], is a parameter widely applied in comparative mammalian neuroanatomy. The EQ value indicates whether a species possesses a brain larger (EQ > 1), equal (EQ = 1) or smaller (EQ <1) than expected for its body mass. The cerebellar quotient (CQ) is a similar parameter that assesses the relative development of the cerebellum.

In this study we considered the EQ and CQ of the adult domestic pig and compared the results to those obtained in other mammalian species, with a special attention to additional members of the order Cetartiodactyla. Furthermore, we investigated the change of EQ, CQ, and weight of the single cerebral vesicles from newborn to adult state. Since the most diffuse pig breed in the industrial world is the pure or cross-breed Landrace (LR) pig of Danish origin, we focused our investigation on this specific variety together with the similar and also very diffuse Large White (LW) pig, as they represent the "type" of animal most commonly found in commercial intensive farming.

Materials and Methods

Brain sampling

For the present study we used brains from 48 *Sus scrofa domesticus* (31 adults, 13 newborns, 4 fetuses, males and females, see Table 2). The animals were LR or LW breeds, or mixed LR x LW. Pure of cross-bred LR pigs include more than 90% of all pigs commercially raised in the Western world. The LW breed is the other popular breed used in animal production all over the world. The two breeds have the same average body weight at the age considered here.

The brain of the adults was removed at the slaughterhouse (Maselli Industrie Srl 41°7'9.956" N; 16° 28'29.975" E), where animals were treated according to the European Community Council Regulation (CE1099/2009) concerning animal welfare during the commercial slaughtering process, and were constantly monitored under mandatory official veterinary medical care. All the animals considered here were in good body condition and considered free of pathologies by the veterinary medical officer responsible for the health and hygiene of the slaughterhouse. Adult carcasses were put on the market, while newborns and fetuses were discarded in accordance with the Regulation CE 1069/2009.

Four fetuses, born from the same sow whose pregnancy was undetected prior to slaughtering, were collected at the slaughterhouse and transported to the necropsy room of the Veterinary Clinic and Animal Productions Section, Department of Emergency and Organ Transplantation, of the University of Bari "Aldo Moro", Italy. There the fetal brains were removed and weighted. Samples from newborns animals were collected in the latter location from individuals who did not survive after birth and whose death resulted unrelated to the nervous system.

The body weight of adult was determined for each animal by the staff of the abattoir, whereas the weight of fetuses and newborns was measured in the necropsy room. The age of the fetuses was determined based on references in literature using cranium-sacrum distance [12]. All brains were extracted in 3-48h *post mortem*, and if extraction was not possible during the day, samples were kept at 4°C.

Determination of brain weight

The brains were removed and treated according to an established protocol [13]. Briefly, the brains were weighed with a digital precision scale and photographed. The dura mater and the other two meningeal layers were preserved during the extraction of the brain (Fig 1).

After removal, the brains were immersed for 2 months in 4% (w/v) phosphate buffered paraformaldehyde at 4°C to allow hardening and proper fixation. The immersion in paraformaldehyde resulted in an increase in brain weight due to penetration of the fluid. Comparison between fresh and paraformaldehyde-fixed brain weights yielded the following conversion formula: $Bw_{fresh} = Bw_{fixed}/1{,}104$ where Bw is brain weight. After removal of the dura mater, this meningeal layer and fixed brains were weighed and the following conversion formula was used: $Bw_{fresh} = Bw_{fixed\text{-}dmw}/0{,}94$ where dmw is the dura mater weight. In addition, the weight of the brain and its components (telencephalon, diencephalon, mesencephalon, pons, cerebellum, and myelencephalon) was calculated from fixed brains after careful dissection.

EQ and CQ

The relationship between the weight of the brain and the body weight to obtain the EQ of each animal, was calculated with the formula $EQ = E_i/0.12P^{2/3}$, where E_i and P are the mean weight of the brain and body, respectively [10]. We kept the value of the exponent 2/3 (or 0.67) originally indicated by [10], although recent studies suggested a higher value (0.75) to better fit

Table 2. Details of the animals used in the experimental series.

Sample	Date of slaugther	Breed	Age (days)	Maturity	Body weight (kg)	Brain weight (g)	Sex
1	29/06/2013	Landrace (LR)	1	Neonate	1.100	30	
2	29/06/2013	LR	1	Neonate	1.280	33	
3	29/06/2013	LR	1	Neonate	1.100	32.91	M
4	29/06/2013	LR	1	Neonate	1.750	32.26	M
5	09/07/2013	Large White (LW)	300–360	Adult	190–200	148.53	F
6	09/07/2013	LW	300–360	Adult	190–200	115.54	F
7	09/07/2013	LW	300–360	Adult	190–200	136.08	F
8	09/07/2013	LW	300–360	Adult	190–200		F
9	14/07/2013	LR	1	Neonate	1.120	31.98	M
10	14/07/2013	LR	1	Neonate	0.900	29.8	M
11	14/07/2013	LR	1	Neonate	0.940	28.84	F
12	14/07/2013	LR	1	Neonate	1.230	34.06	F
13	14/07/2013	LR	1	Neonate	1.530	35.5	M
14	23/07/2013	LW	180–240	Young adult	74–80	116.1	F
15	23/07/2013	LW	180–240	Young adult	74–80	128.15	F
16	23/07/2013	LW	180–240	Young adult	74–80	134.39	F
17	23/07/2013	LW	180–240	Young adult	74–80		F
18	23/07/2013	LW	G 92–96	Fetus	0.12	12	M
19	23/07/2013	LW	G 92–96	Fetus	0.16	16	F
20	23/07/2013	LW	G 92–96	Fetus	0.167	16.7	M
21	23/07/2013	LW	G 92–96	Fetus	0.148	14.75	F
22	08/10/2013	LW	240–300	Adult	150–160	122.42	F
23	08/10/2013	LW	240–300	Adult	150–160	136.7	F
24	08/10/2013	LW	240–300	Adult	150–160	128.86	F
25	08/10/2013	LW	240–300	Adult	150–160	138.27	F
26	28/10/2013	LR	1	Neonate	0.850	31.17	F
27	28/10/2013	LR	1	Neonate	0.870	33.54	M
28	04/11/2013	LR	1	Neonate	1.350	34.19	F
29	04/11/2013	LR	1	Neonate	1.340	35.11	M
30	13/11/2013	LR x LW	240–300	Adult	130–150	131.32	M
31	13/11/2013	LR x LW	240–300	Adult	130–150	139.41	F
32	13/11/2013	LR x LW	240–300	Adult	130–150	120.72	M
33	13/11/2013	LR x LW	240–300	Adult	130–150	146.39	F
34	13/11/2013	LR x LW	240–300	Adult	130–150	140.15	F
35	13/11/2013	LR x LW	240–300	Adult	130–150	151.5	M
36	13/11/2013	LR x LW	240–300	Adult	130–150	125.98	F
37	13/11/2013	LR x LW	240–300	Adult	130–150	147.48	F
38	04/12/2013	LR x LW	240–300	Adult	190–200	133.78	M
39	04/12/2013	LR x LW	240–300	Adult	190–200	160.26	F
40	04/12/2013	LR x LW	240–300	Adult	190–200	139.65	F
41	04/12/2013	LR x LW	240–300	Adult	190–200	140.53	M
42	04/12/2013	LR x LW	240–300	Adult	130–150	118.93	F
43	04/12/2013	LR x LW	240–300	Adult	130–150	138.54	F
44	04/12/2013	LR x LW	240–300	Adult	130–150	126.77	M
45	04/12/2013	LR x LW	240–300	Adult	130–150	107.08	F
46	04/12/2013	LR x LW	240–300	Adult	130–150	122.35	F
47	04/12/2013	LR x LW	240–300	Adult	130–150	134.04	F

(*Continued*)

Table 2. (*Continued*)

Sample	Date of slaughter	Breed	Age (days)	Maturity	Body weight (kg)	Brain weight (g)	Sex
48	04/12/2013	LR x LW	240–300	Adult	130–150	136.58	M

G: gestational age in days (duration of the pregnancy is approx. 114 days).

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mammals with a large body mass (for reference and discussion on this topic see [14,15]. The EQ was calculated using only data from fresh brains.

To calculate the CQ, we used the following equation $CQ = Cb_{vol}/(0.145M_b^{0.978})$, proposed by [16], where Cb_{vol} is the volume of the cerebellum (Cb_{vol} x 1.04 = Cb_{mass} x 0.96) [17] and M_b as the brain mass (= brain weight). Since the weight of each brain part was measured from fixed specimens, we applied the conversion formula to determine the weight of fresh tissue (see above).

Results

Gross anatomy

The gross anatomy of the swine brain was evaluated only after removal of the dura mater (Fig 1), which was thick and resistant to dissection, as observed by [18].

The dimensions of the newborn brain (4.5 cm wide and 3.5 cm long) are proportionally comparable to those of adults (7.5 of width by 11.5 cm in length). The morphology of the brain recalls overall that of other large domestic ungulates. The olfactory bulbs and cerebellum are visible dorsally and the latter has a rather developed cerebellar vermis compared to the cerebellar hemispheres (Fig 2). The profile of the cerebral hemispheres grows in height in the cranio-caudal axis in a regular curvilinear line. The lateral expansion of the temporal lobe at the level of the inter-insular axis is typical of the Cetartiodactyla. The arrangement of the sulci (Fig 2) follows the general plan of the ungulates [19].

Fresh brain weight

The general appearance of the pig's brain after extraction is shown in Fig 3. The fresh weights of the body and brain of all animals are shown in Table 2, while Table 3 shows the average of the weights considered. Since the somatic difference between LW, LR, and LW x LR pigs of comparable age and weight class were minimal, we considered all breeds as a single

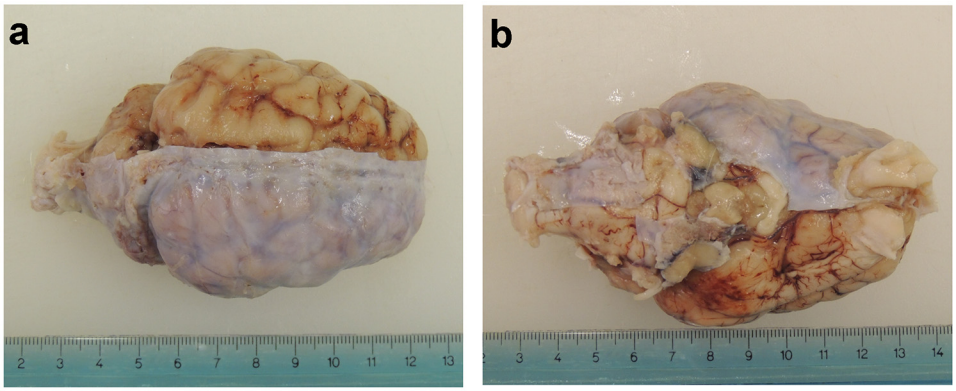

Fig 1. Fixed brain after partial removal of the dura mater. (a) Dorsal view. (b) Ventral view.

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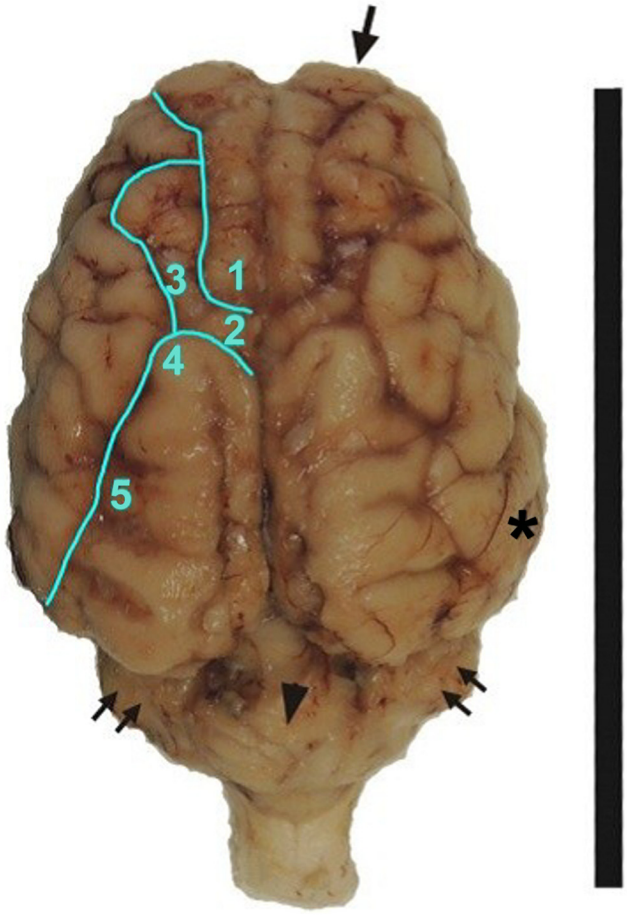

Fig 2. Fixed brain with main sulci shown. 1: cruciate sulcus; 2: ansate sulcus; 3: coronal sulcus; 4: connection sulcus with suprasylvian sulcus; 5: median suprasylvian sulcus. *: temporal lobe. Arrow: olfactory bulb. Double arrows: cerebellar hemispheres. Arrow head: cerebellar vermis. Bar: 10cm.

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experimental cohort. We found that fetuses (n = 4) had a mean brain weight of 14,9g (SEM ±1,03) for an average body weight of 150g (SEM ±1). Neonates (n = 13) had a mean brain weight of 32,5g (SEM ±0,6) for a mean body weight of 1,18kg (SEM ±0,075).

Body weight

The values related to body weight (Tables 2 and 3) were of 0.85 to 1.75kg for newborns (n = 13) with an average value of 1.18kg (SEM ± 0,075). The values for adults (n = 29) ranged from 80 to 200kg. The majority of individuals had a weight comprised between 120 and 180kg. The individuals were divided into two groups on the basis of sexual maturity: young adults (n = 3), in which are included the body weight of individuals of 74–80 kg, and proper adults (n = 26), that is, individuals of 130–200 kg. This division was also respected in the calculation of the weight of the brain and its EQ.

Weight of fixed brains

After successful fixation (Fig 4) the brains were weighed again. The values obtained for the individuals that had reached sexual maturity, representing the majority of the sample, were of 141g (SEM = ± 2.36) in average with a range of 114-168g. More specifically, for the young

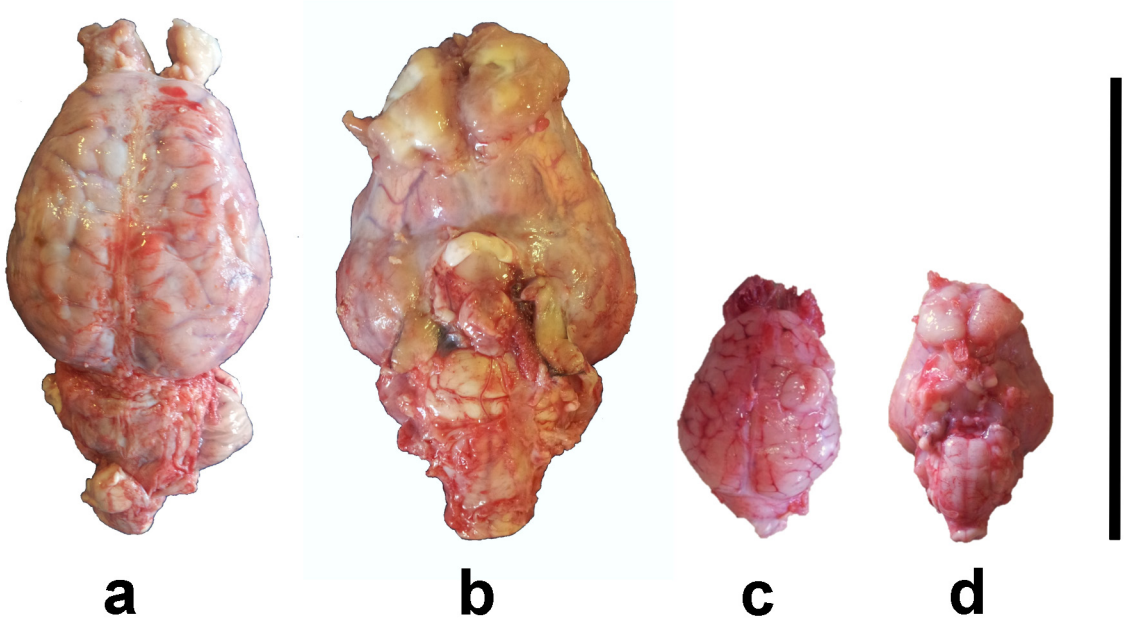

Fig 3. Aspect of the swine brain after extraction. (a) Adult, dorsal view. (b) Adult, ventral view. (c) Neonate, dorsal view. (d) Neonate, ventral view. Bar: 10cm.

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adult group the minimum and the maximum are respectively 128 and 135g, average of 132g (SEM = ± 3.18), for the adult group of 114 and 168g and 142g (SEM = ± 2.55) on average. The brains of neonates had an average value of 35.1g (SEM = ± 0.43) with a range of 33-37g.

Weight of the dura mater and brain segments

After removal of the dura mater, the main brain parts corresponding to the original neural vesicles were isolated, all structures were weighed (Table 4) and the percentages of the weight of each part compared to whole brain were calculated (Fig 5).

EQ. The EQ of the different groups was as follows: 2.42 for one day piglets (n = 13); 0,58 for young adults (n = 3); 0.38 for adults (n = 26). The EQ of the species obtained by sexually mature subjects (n = 29) is 0.39, and its position, compared to that calculated in other mammals, is shown in Table 5 and Fig 6. A comparison within the adult animals that we used for our study showed that LW adult females (n = 7) have an EQ of 0.34, and LW x LR adult females (n = 12) an EQ of 0.38 ($p < 0.05$).

CQ. Like the EQ, the CQ varied considerably in the different groups studied. The values were of 0,59 in infants; 0,62 in young adults; 0,71 in adults and 0,7 in sexually mature animals.

Table 3. Mean values for body and brain weights of each class of age. SEM: Standard Error Mean.

Age category	n	Body weight (kg) ± SEM	Brain weight (g) ± SEM
Fetuses	4	0,15 ± 0,01	14,9 ± 1,03
Neonates	13	1,18 ± 0,075	32,5 ± 0,6
Sexually mature	29	149 ± 6,69	133 ± 2,29
Young adults	3	77 ± 3	126 ± 6,57
Adults	26	158 ± 5,3	134,5 ± 2,45

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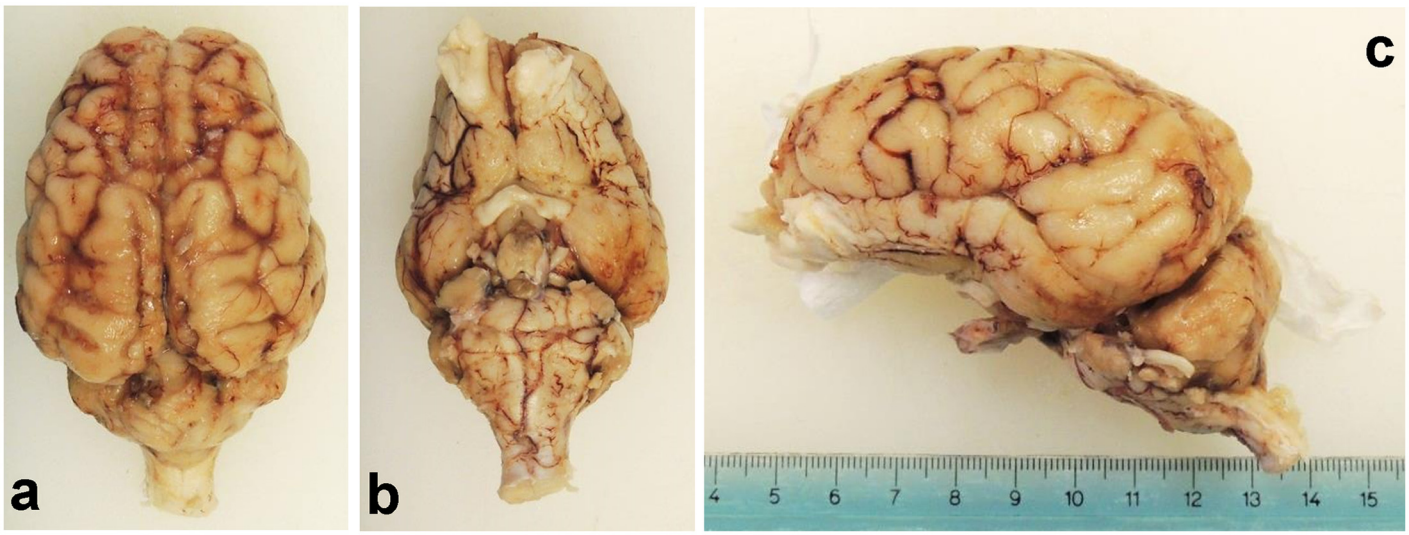

Fig 4. Views of the swine brain after fixation. (a) Dorsal. (b) Ventral. (c) Lateral left.

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Discussion

The present study, based on a statistically significant sample of 46 brains, provides a macroscopic gross anatomical description of the brain of the domestic pig *Sus scrofa domesticus*, the absolute and relative weight of its parts, the calculation of the EQ and CQ values, as well as a position relative to other mammals.

The first striking aspect of the brain of the domestic pig is its rather small size compared to the mass of the animal. Remarkably, the average adult brain weight fluctuated between 107 and 160g. This gap in absolute weight is reasonable when considering the heterogeneity of individual weights in the sample (adults weight from 70–80 kg up to 200 kg). This marked gap in addition to differences attributable to race, is due to the typical Italian pig farming system distinguishing two categories of pigs. The first is that of light-weight pigs (80–100 kg of live weight) intended for consumption as fresh meat, the second is that of heavy-weight pigs that reach up to 200 kg and are used for the production of sausages. So, the tremendous increase in body weight imposed by industrial farming condition certainly influences brain-to-body weight measures, including the EQ.

The weight of the brain at birth averaged 32,5g with a body weight of 1,18kg. As expected, the remarkable brain size for a neonate is due to the particular mode of replication of neurons which increase in number during the earlier fetal stages and, to a much lesser extent after birth,

Table 4. Mean values of the dura mater and the parts of the brain.

	Dura madre		Medulla oblongata		Pons		Cerebellum		Mesencephalon		Diencephalon		Telencephalon	
	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%
Adults														
Mean	13,12	9,22	6,55	4,66	3,11	2,23	14,06	9,97	4,31	3,10	2,53	1,81	96,52	68,70
SEM	0,58	0,30	0,18	0,11	0,13	0,13	0,29	0,19	0,13	0,12	0,11	0,09	1,58	0,46
Neonates														
Mean	2,89	8,26	1,08	3,14	0,64	1,86	3,01	8,73	0,99	2,86	0,75	2,16	25,01	72,48
SEM	0,30	0,89	0,07	0,20	0,05	0,13	0,07	0,07	0,06	0,17	0,06	0,15	0,44	0,92

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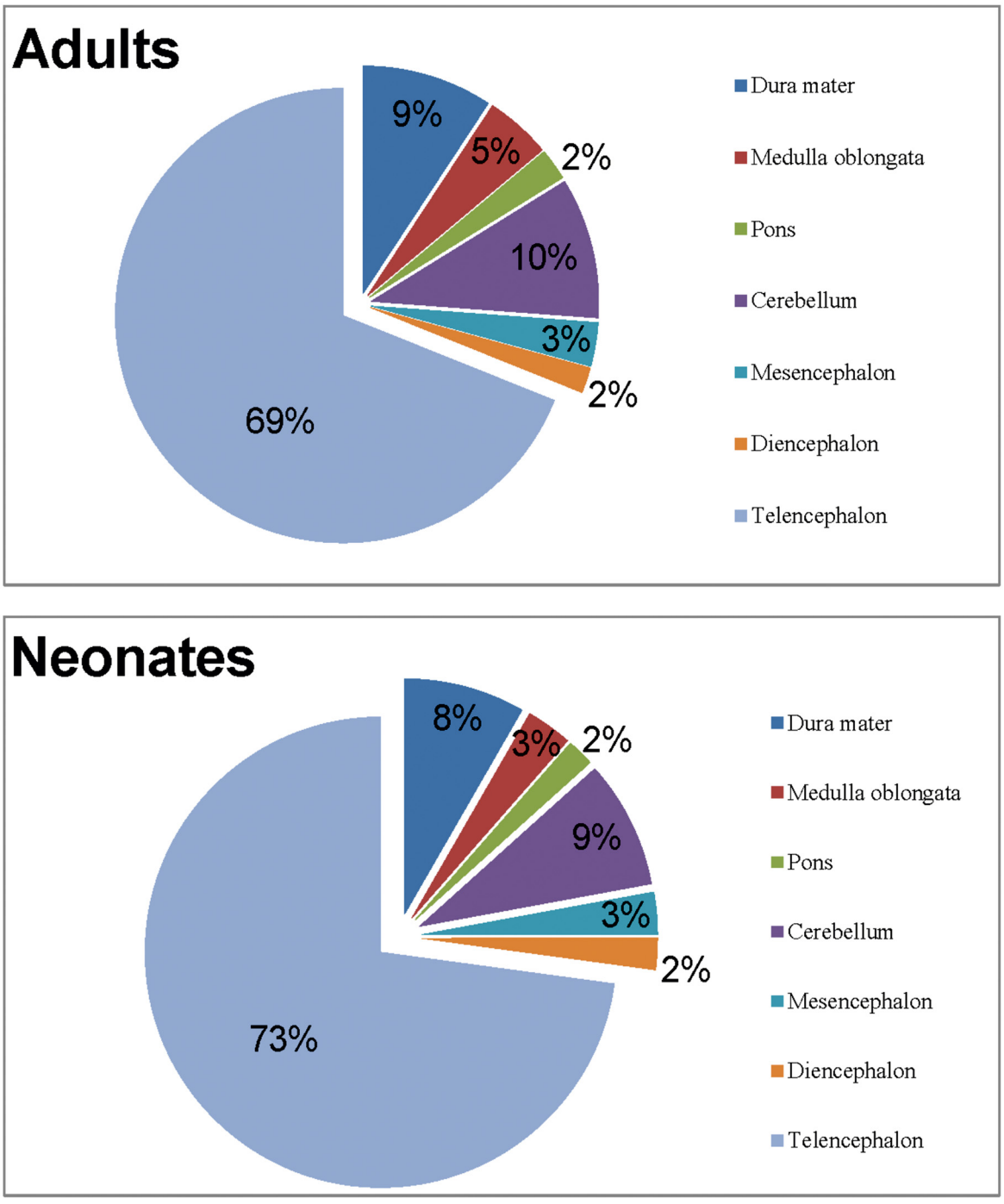

Fig 5. Percentages of the respective weights of the brain parts of adult and neonate swines.

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with the exception of the olfactory bulb. As for the body weight, the fluctuation range it is to be considered absolutely normal even if the maximum value is almost double the minimum given the relative size of the piglets depends on the number of births per sow and the number of brood [20].

Table 5. Brain mass, body weight and EQ of chosen mammals.

Species	Brain weight (g)	Body weight (kg)	EQ	Source
Carnivora—Felidae				
Felis catus	37	5,05	1	[10]
Carnivora—Canidae				
Canis lupus familiaris	68–135	7–59	1,55–0,74	[18]
Artiodactyla—Suidae				
Sus scrofa (n = 1)	180	125	0,60	[10]
Sus scrofa domesticus (n = 29)	133	149	0,39	This study
Artiodactyla—Bovidae				
Bos taurus	445	550	0,55	[18]
Ovis aries	130	50	0,80	
Capra hircus	95	37,5	0,71	[37]
Artiodactyla—Camelidae				
Camelus bactrianus	518	594	0,61	[38,39]
Proboscidea—Elephantidae				
Loxodonta africana	4927	3185	1,67	[52]
Perissodactyla—Equidae				
Equus caballus	599	514	0,78	[13]
Primates—Cercopithecidae				
Macaca mulatta	88	7,8	1,86	[10]
Primates—Hominidae				
Pan troglodytes	382	46	2,48	[10]
Homo sapiens	1300–1400	70	6,62	[62]

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Different methodologies, together with differences and variability between age classes of the specimens considered, may lead to different results: The data obtained in this study do not agree with another report including the same species in which the authors described a higher brain weight and a lower body mass [21].

In several publications consulted for the present study, the Authors do not specify how the brain weights were obtained: fresh or after fixation; after how many hours *post mortem* (if fresh); if the dura mater was included or excluded in the data disclosed [21–25]. The experimental series was often reduced in numbers, old, or derived from various studies involving different sampling methods (including the use of cranial measurements to derive the volume of brain perfusion and fixation) or unspecified methodology [23]. There is also a frequent use of database (without support of new samplings) to increase the sample size [15,21,25,26,27]. A detailed comparison between the data obtained in this study and data available is therefore made rather difficult (see [28] for discussion and criticisms).

It is widely believed that the brain has suffered a progressive increase in size over the course of evolution [10,21,24,26,27,29,30] with the consequence, more or less explicit, that at each increase of magnitude corresponds an increase in function. Learning skills, foraging strategies, habitat management capabilities have been variously linked with this aspect, and sometimes considered the primary cause. To date, the most likely hypothesis remains that the social brain (SBH, Social Brain Hypothesis) developed in primates [30], but is also applicable in varying degrees to carnivores [24] and artiodactyls [21]. It is based on the principle that in complex social groups, such as those of primates, or gregarious animals as can be ungulates, develop relational dynamics that often require the ability to manage individual conflicts and the need to remain in the group, thus the need to cope with huge computational demand, contributing

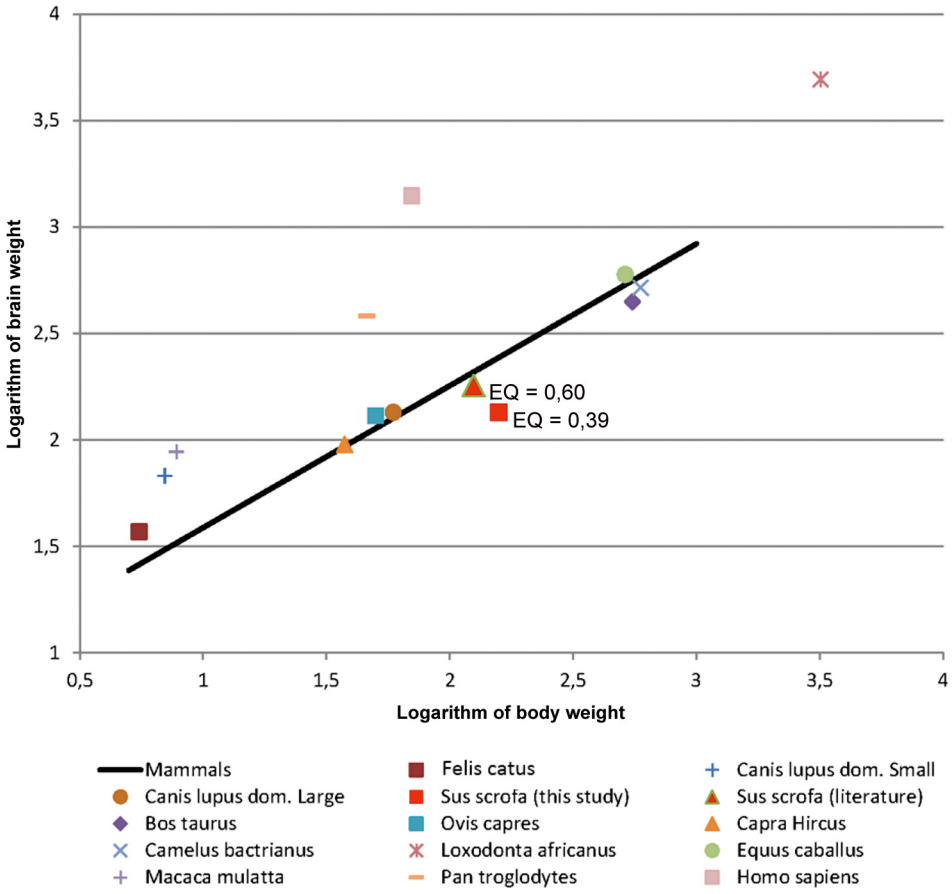

Fig 6. Logarithmic graph showing the evolution of the brain weight in function of the body weight of chosen mammal species. The regression line represents the expected value for the weight of the brain based on the body weight following the equation: $Eo = Ei/0,12Pi^{2/3}$ by [10]. The values above and under the line represent experimental findings with heavier or lighter brain weights per body weight than the theoretical value of the Jerison equation [10].

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to the progressive increase of brain size [21,26,27]. The role of a greater or lesser magnitude of the brain is still a topic widely debated. The brain is one of the organs requiring the most energy (preceded only by the heart) and the cost of its operation is about 8–10 times higher per mass unit than that of skeletal muscles. Evolution is an "economical process" and does not enlarge an organ from which the body cannot derive a real benefit [29]. In mammals the increase in size is due predominantly to the enlargement of the forebrain and neocortex, leading to an increase of the primary areas (receiving thalamic afferents: motor area, visual, auditory, somatosensory) as well as secondary ones (higher mental functions). Progressive specialization and diversification in the mammalian brain arises precisely from the differential development of these areas, and the possibility to adopt flexible behaviors. To balance this line of thought, there is the paradox of the miniaturized brain of insects. These animals are still capable of very complex (but relatively stereotyped) social behavior and have considerable spatial and visual skills. It seems therefore that "basic functions" can be allocated in very small spaces [31].

Studies on brain size may group species of different orders and apply a number of indexes to assess the evolutionary position of each group. The EQ represents how many times the brain is larger (or smaller) than what would be expected for a given species relative to its body size

[15], and encephalization has been considered to correlate with improved cognitive abilities across species and even intelligence [32]. In some species brain mass significantly differs from the expected one in a way that has been suggested to be functionally significant. A great deal of evidence implies that larger EQs or relative brain size endow species with improved cognitive abilities [33], behavioral flexibility, such as the ability to respond successfully to novel environments [34] or to alternate between feeding strategies [35]. These findings seem to agree with the fact that humans, dolphins, and chimpanzees have the largest known EQs [36]. The EQ of Ungulates differs among the species ranging from 0.91 to 0.78 in Perissodactyla (*Equus caballus*) [13,27], to 0.55–0.80 in Cetartiodactyla such as Bovidae [18,37] and Camelidae (*Camelus bactrianus*) [38,39]. In Suidae, it has been reported that the EQ of only one specimen of *Sus scrofa* (un-indicated subspecies) was 0.60 [27] (Fig 6).

The average value of 0,39 obtained in this study for the domestic pig with differences between young adults (0,58) and adults (0,38), is significantly less than 1. Such a low index nevertheless falls within the wide range indicated for Cetartiodactyla (0,14 to 4,43) compared to primates and Carnivora. At the top of the range are the odontocetes (average EQ = 3,10), alone sufficient to raise the upper limit of the range, while the terrestrial Cetartiodactyla are at much lower values [15]. Even compared to cattle, sheep and goat, the pig stands in a rather lower position.

Various reasons are responsible for a low EQ value, including the existence of very heavy animals reaching 200 kg, a strong domestication pressure [40,41], and the intrinsic nature of the Jerison model [10] which may be unfavorable for species of large size. To this effect we also note that within our samples, heavier LW sows had a lower EQ (0.34) than cross-bred LW x LR (0.38), emphasizing the direct effect of body weight (see below for further discussion).

The evolutionary pressure can alter in at least two ways the brain/body size ratio. It could directly affect brain size or alternatively be a consequence of increase or decrease of the body size [21]. In the case of domestic mammals bred for the purpose of food production, the selective pressure is directed towards the increase of body weight in order to obtain a higher yield at slaughter, affecting the EQ to a lower value. Kruska [40,41] also showed that bred animals have an absolute brain weight lower, and therefore a lower EQ compared to wild progenitors. Domestication is perhaps the longest and most important experiment in genetic selection, and involved especially Lagomorphs, Cetartiodactyla, Perissodactyla and Carnivores. This process in all cases led to a reduction in absolute brain size. The quantification of this reduction depends on the single species. In fact, species with originally low encephalization (i.e. Lagomorphs) showed a smaller decrease than Carnivores. In the case of the domestic pig we noted that the brain weight decreased by 34% compared to the wild progenitor. This fact alone is sufficient to explain the EQ found in this work.

The brain and body values reported for *Sus scrofa* by Shultz and Dunbar [27] would produce an EQ = 0,6 for the pig, which is higher than the value reported here. The measures attributed to *Sus scrofa* in their work are of 180g of brain weight (size in the original Table of [27]) for 125kg of body weight. Given the relatively large brain weight which was never matched in the sample considered in this study, and the rather reduced body size reported in [27], it is possible that the authors referred to the wild boar and not to the domestic swine. The given name of *Sus scrofa* and not of *Sus scrofa domesticus* suggests this eventuality. An alternative explanation could be the different breed considered or the origin/destination of use. In the first case if the animal had belonged to a rustic breed, rather than to one commonly used for meat production, it could have retained ancestral characteristics, including a reduced body size with a relatively heavy brain. In this regard, it is logical to ask what impact can obtain the reduction of the mass of the brain following the process of domestication [40,41]. It would be logical to expect that the decrease is associated with a reduction in functional capacity [40,41]. Studies explicitly based on wild specimens are extremely rare to come by, as the domestic and wild pig basically

belong to the same genus and—depending on the taxonomy—to the same species. A study centered on the pigmy hog (*Porcula salvanius*) indicates a similar level of encephalization for several members of the Suidae family, except *Sus scrofa* that maintains a larger brain [42]. A 16% reduction in brain size due to domestication has been reported also in domestic Perissodactyla [43]. Surprisingly, despite a 20–30% loss of brain mass relative to wild ancestors a "domestic brain" is still able to deal with wildlife if the individual is reinserted in natural context. From what emerges from experimental tests [44] in rats and dogs [45] these animals are even faster learners compared to wild animals. It appears that functional capabilities are preserved despite reduced size hence it seems appropriate to consider these changes as a form of adaptation to a particular ecological niche. While the body size alone remains the best predictor of the mass of the brain, we cannot say that the mass of the brain is a predictor for the behavioral repertoire and cognitive abilities [31].

The apparent high EQ of piglets (EQ = 2,42) and its subsequent decrease is explained by the lack of body fat at birth, by the advanced maturity of the central nervous system at birth. Pigs belong to a precocial species, as well as horses and cattle. A few minutes after birth they must be able to stand and move independently, a notable difference from altricial offspring. This is further shown by "multiplication factor", the number by which the brain size of the newborn must be multiplied to obtain the corresponding value of the adult. In precocial offspring species this number is between 1–6, while for those with altricial offspring it is 7–12 times. In the case of the pig, brain mass will increase by about 4 times while the body size (fat and muscle) will grow by 60–70 times. Somatic development occurs primarily after birth while brain growth is biphasic. The first phase sees a very rapid growth of the brain, while in the second phase growth is slower than the rest of the body and the brain reaches maturity before complete somatic development. This brain growth pattern is common to all mammal species with the due differences: in precocial species the first phase takes place during fetal development while in altricial species the first phase is carried out immediately after birth [40,41]. Therefore, mammal brain size differences are to be found mainly in embryogenesis and ontogenesis processes [21,40] and thus explain the relatively higher EQ in young individuals than adults. An important collection of data on the macroscopic anatomy of the piglet brain can be found in the online collection of University of Illinois (http://pigmri.illinois.edu/).

Consistently with evolutionary phylogeny showing that the encephalization increase is largely due to telencephalization (increased cerebrum) [40,41], our analysis of the weight of each brain part shows that the cerebrum alone occupies 70% of the entire brain. The neocortex gradually increases in size taking alongside its peculiar stratification pattern characteristic of mammals. Finlay and Darlington [46] detected an apparent regularity in the sizes of mammalian brain structures and the mass of belonging brains. These authors demonstrated this consistency in size of the cerebellum relative to total brain mass, cerebellum quotient, when examining a series of insectivore and primate brains. However, to date exceptions to this allometric regularity have been documented for cerebellar size in relation to brain mass of microchiropteran, odontocete cetacean, and African elephants' brains [12,47,48]. Our results indicate that in the pig the second heaviest part of the brain is the cerebellum with a CQ of 0,7. Macroscopically the cerebellar vermis and hemispheres appear of the same size, an aspect described also by [49] in the African elephant, for which they indicated a volume of 4,47 and 4,81 ml respectively. The development and relative expansion of the cerebellum is a parameter indicative of the general motor skills of a given species, and reflects the general capabilities in the regulation of body posture and movement coordination. In particular, the large size of the archicerebellum, including the connections to the lateral vestibular nucleus and the vestibulospinal tract, are justified by the function of rapid connection center for quadrupedal movement [40,41,49,50]. This CQ is in the same range as the bovine (0,725) [51], but falls short of

half the value for elephants (1,66–1,84) and primates (0,71–1,28) [12]. Additional considerations about the CQ value are difficult to express because they lack elements of comparison and literature on the specific topic is almost non-existent. The remaining parts seem to follow the proportions given by other authors for ungulates [13,37].

The domestic pig shows a convoluted brain characteristic of Cetartiodactyla with pronounced individual variations. One general feature of the order is the lateral extension of the temporal lobes [18]. This expansion, noteworthy in cetaceans, is not so evident in pigs compared to other terrestrial members of the order (e.g. cattle) and could show evolutionary convergence towards auditory sensitivity with different degrees according to family- and species-specific developmental trends. The design of the grooves has significant individual variations (e.g. differences between hemispheres) despite species-specific characteristics [18]. In mammals the number and width of the sulci (primary and secondary) also seem to depend on the somatic mass as well as the phylogenetic position (Proboscidata have an extremely convoluted brain) [49,52–55]. In mammals of veterinary interest while the cow and the horse have a very complex sulci arrangement with tortuosity, the pig is placed in an intermediate position with a more linear topography, unlike carnivores (dogs and cats) which show a more simple antero-posterior parallel organization.

The different sulci and gyri were definitively mapped in the man and some laboratory animals, for which stereotactic atlases are available. We tried to derive by analogy the topography in other species. The situation is further complicated by the fact that homologous convolutions should match not only for position and course but also in the cytoarchitectural structure, which is realized only in part [18]. Comparative studies have shown that the neocortex varies regarding the arrangement of the areas [13]. Based on this comparison, ten "main" sulci always recognizable and other "accessory" sulci subject to greater variability have been identified in ungulates [19,54]. In this study, the grooves nomenclature follows the NAV, but alternative names are also available in literature.

In conclusion, our data suggest that correlations between brain size and complex behaviors remain unclear, at least in the pig, and while EQ and CQ comparative measurements shed light on the position of *Sus scrofa domesticus* among other domestic and wild mammals, the relative weights and lobe developments could be related to feeding behaviors, environment and social adaptations or sensorial specialization [28]. The data proposed in this paper contributes to better characterize the poorly understood brain capabilities of a domestic species very common in the world, as public concerns regarding animal wellbeing and living conditions rise. Being separated from its wild ancestor by human heavy selection for centuries may reduce scientific interest towards the domestic pig, but the low inter-individual variability is an aspect looked for in laboratory animals, also heavily selected and relied upon for the immense majority of scientific work. The use of domestic mammals could then be a valuable alternative and preserve laboratory animal lives [56], as recommended by several neuroscience societies and the European Community regulations.

Author Contributions

Conceived and designed the experiments: SM BC SD. Performed the experiments: SM GA. Analyzed the data: SM AP BC SD. Contributed reagents/materials/analysis tools: SM BC SD. Wrote the paper: SM GA AP JMG BC SD.

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I.A.3. THE BRAIN OF THE DOMESTIC BOVINE *BOS TAURUS*

One of the most common members of *Artiodactyla* is *Bos taurus*. Since its domestication about 10 000 years ago from Aurochs (McTavish *et al.*, 2013), bovines have accompanied human farming almost everywhere in the world (Stock and Gifford-Gonzalez, 2013). However, the extent of our knowledge on the functioning of the central nervous system of the bovine is small compared to the vast amount of research that has been done concerning its reproduction or metabolism. The classical veterinary anatomy treatises (Frauchiger and Hofmann, 1941; Ellenberger and Baum, 1943; Bruni and Zimmerl, 1951; Getty, 1975; Nickel, Schummer and Seiferle, 1975; Barone and Bortolami, 2004; Dyce, Sack and Wensing, 2010) mostly cover the macroscopic aspects of the brain, along with common clinical affections of domestic ungulates, and usually use the human brain map as reference, or the dog (Schmidt *et al.*, 2012). It is not until 1975 that a precise cortical study was undertaken in the cow (Lakshminarasimhan, 1975a, 1975b). Lakshminarasimhan notes that there is absolutely no reference to cyto- or myeloarchitectural areas of the cattle cortex, or their functional attributes previous to his work (Lakshminarasimhan, 1975a). A subsequent study by Louw (1989) described the development of the sulci and gyri in fetuses from 60 days old onwards.

However, *Bos taurus* has been the subject of a certain body of studies concerning parts or the brain in its entirety. Part of this interest was directed at the extraction of hormones from the pituitary gland or hypothalamus (Dellmann, 1959; Gorski and Erb, 1959; Dean, Hope and Kazić, 1968; Pevet, Reinharz and Dogterom, 1980). Later most of the studies involving the bovine brain were focused on endocrinology, the secretion and origin of the main neuropeptides (De Mey, Vandesande and Dierickx, 1974; De Mey, Dierickx and Vandesande, 1975; Vandesande, Dierickx and De Mey, 1975a; Dierickx and Vandesande, 1977; Leshin *et al.*, 1995). Nonetheless, some studies focused on neuroanatomy (Vierling, 1956, 1957, 1958, Gadamski and Lakomy, 1972, 1973; Lakomy and Gadamski, 1973; Schneider and Beck, 1974; Schneider, 1975, 1976, 1977, Junge, 1976, 1977; Grütze, 1978). In some cases, imaging studies used bovine specimen (Tsuka *et al.*, 2002; Schmidt *et al.*, 2009, 2012). Today, few laboratories still use the bovine as a substrate for research (Russo, Paparcone and Genovese, 2008; Peruffo and Cozzi, 2014).

There is consequently a certain lack of modern data concerning the functional anatomy of domestic ruminants, even concerning the most basic aspects such as the average brain weight of these animals. For this reason, our lab introduced a study of brain mass and encephalization from a large number (n=158) of bovines (Ballarin *et al.*, 2016). Data obtained showed that the average brain weight is 480.54 ± 4g, which is slightly lower than what could be expected for an animal of its mass. Comparing it with other Cetartiodactyla, showed that *Bos taurus* had a similar encephalization quotient to other ruminants and baleen whales, but showed a relative distance from the carnivore Odontocetes. The relative lower EQ of cattle could also be explained by the heavy breeding man has subjected it to (Kruska, 2007), which could be reflected by the relative higher EQ of the horse (Cozzi *et al.*, 2014).

I.A.4. THE BRAIN OF THE DOMESTIC SHEEP *OVIS ARIES*

The most studied species among artiodactyls is most probably the sheep. The study of Rose (Rose, 1942) provided the first comprehensive cytoarchitectural mapping of the cortex in *Ovis aries*. However, previous observations contributed to the understanding of the localization of the motor cortex (King, 1911a; Simpson and King, 1911; Bagley, 1922; Ebinger, 1975) as well as the somatosensory areas(Adrian, 1943, 1946). Subsequent research has brought additional knowledge to

the thalamic connections and somatosensory afferents to the sheep brain (Johnson, Rubel and Hatton, 1974; Karamanlidis *et al.*, 1979; Gierthmuehlen *et al.*, 2014). The study of the pyramidal tract has been the subject of some attention (King, 1911b; Lassek, 1942; Towe, 1973), tending to show its full stop in the cervical vertebrae, leaving only extrapyramidal tracts to control locomotion, in particular in lower limbs (Dyce, Sack and Wensing, 2010; Cozzi, Huggenberger and Oelschläger, 2017). This statement has however been challenged by different findings, particularly in the horse (Breazile, Jennings and Swafford, 1967). From a more global point of view, a stereotaxic atlas and a magnetic resonance atlas have been published (Vanderwolf and Cooley, 2002; Nitzsche *et al.*, 2015).

The ovine hypothalamus anatomy has also been assessed, notably in the context of sex differentiation (Franceschini *et al.*, 2006; Smith, 2009; Cheng *et al.*, 2010) as well as sex behavior related to steroids exposition (Resko *et al.*, 1996; Perkins and Roselli, 2007; Roselli *et al.*, 2009).

A recent study has applied modern imaging technology to the measure of stress in sheep (Cannas *et al.*, 2018). This technique had been previously used in the measure of pain in disbudded calves (Stewart *et al.*, 2008). Nonetheless, this technique, coupled with portable cerebral imaging techniques could help in understanding the emotional and motor processes involved in reaction to the environment (Knolle, Goncalves and Jennifer Morton, 2017).

I.B. The brain of Cetacea

The brain of cetaceans has attracted the most attention out of all *Cetartiodactyla*, partially because of their extreme living conditions as mammals, but also their extreme convoluted aspect. Briefly, its most striking macroscopic features are an extreme neocorticalization, i.e. a relative domination of the neocortex over the allocortex, a rostrally rotated encephalon, and a large cerebellum (Cozzi, Huggenberger and Oelschläger, 2017). The paleocortex is in fact reduced compared to other cetartiodactyls, seemingly from the loss of the anterior olfactory system for the paleocortex(Cozzi, Huggenberger and Oelschläger, 2017), as well as the archicortex, composed mostly of a reduced hippocampus. Notably, adaptation to the marine environment prompted the development of the auditory capacities of cetaceans, notably in the form of echolocation (Montelli, Peruffo, *et al.*, 2016). Features such as the inferior colliculus, the large vestibulocochlear nerve and its associated ventral cochlear nucleus, denotes the importance of the hearing system in echolocation, while the extreme reduction of the vestibular nuclei shows adaptation to the water medium (Cozzi, Huggenberger and Oelschläger, 2017). However, much like in terrestrial Cetartiodactyla, the motor cortex and somatosensory cortex is found at the rostral extremity of the cortex (Van Kann *et al.*, 2017).

From the point of view of absolute size, the brain of cetaceans are among the largest in the world, the largest being that of the sperm whale, with up to 10 kg for the sperm whale *Physeter macrocephalus* (Ridgway and Hanson, 2014). The size and complexity of the brain of cetaceans has drained a lot of attention (Hof, Chanis and Marino, 2005; Manger, 2006; Montgomery *et al.*, 2013). The EQ originally intended to compare cognitive abilities (Jerison, 1973) and supposed to increase along the mammalian evolution (Pilbeam and Gould, 1974), was not unanimously supported (Deacon, 1990), and its relevance could be limited to clads (Dunbar and Shultz, 2007; Shultz and Dunbar, 2010). This sheds light on the potential primate-like EQ of some cetaceans, and more precisely toothed cetaceans (Odontocetes). Although large regarding their body size and weight, the cetacean brain must be placed in its context. Despite the impressive gyrification of the neocortex, the amount of grey matter is less than that of the human, since the thickness of the cortices are much smaller (Manger *et al.*, 2012; Mota and Herculano-Houzel, 2016). The cortical layers of cetaceans is similar to that of terrestrial ungulates

with traits such as a relative uniformity throughout the cortex, but showing specificities such as an even thinner aspect, a very thick layer I (up to a third of the total column), an even more pronounced agranuarity, and large pyramidal cells at the border between layers III and V (Hof, Chanis and Marino, 2005; Kern *et al.*, 2011).

II. SEXUAL DIMORPHISM AND SEX DIFFERENCES

II.A. Sex differences: implications, models and history

The visible distinctions between the phenotype of males and females, easily noticeable in several species, was first explained by Alfred Jost's experiments in the 1940s, establishing the influence of gonadal hormones on the development of the genital tract and secondary characteristics (reviewed in Josso, 2008). The anti-Müllerian hormone (AMH), secreted by Sertoli cells, was responsible for the regression of the Müllerian (female) ducts. Later, it was proposed that behavioral differences between sexes could arise from these differences in phenotype (reviewed in Nelson, 1995). However, in 1959, Phoenix and colleagues showed that male behavior could be obtained from females if treated with androgens during their ontogeny, proposing that testosterone could act on the central nervous system (Phoenix *et al.*, 1959). This finding demonstrated that there were *organizational* effects of hormones during fetal life, and *activational* effects later in life (Breedlove, 1992; Kruijver *et al.*, 2002; McCarthy, Wright and Schwarz, 2009). Although this was shown in the guinea pig, which is a precocial species with a relatively long gestation period among rodents, the rat was the animal model of choice for most laboratories (McCarthy, Wright and Schwarz, 2009), the latter showing a sensitive period well postnatally.

Nonetheless, the rodent brain allowed for the understanding of the aromatization hypothesis, which is still current today, although challenged (Wallen, 2005). The aromatization hypothesis came from observations that estrogen injections were much more potent to masculinize behaviors, together with the wide expression of estrogen receptors in the brain during critical periods and the high activity of enzyme aromatase P450 (McCarthy, 2008). The paradigm is that during male fetal life (embryonic day 18), and possibly beyond in the case of rats (postnatal day 10), gonadal testosterone enters the brain during a critical period, where it is aromatized into estradiol (MacLusky *et al.*, 1994), while maternal estrogens are sequestered by alpha-fetoprotein (Bakker *et al.*, 2006; Schwarz and McCarthy, 2008). The estrogens present in the brain will target estrogen receptors (ER-α and ER-β) which can have two effects, either as membrane receptors (Beyer, Pawlak and Karolczak, 2003; Seredynski *et al.*, 2015), or steroid receptors, in which case they will promote and repress the expression of a large array of genes, resulting in the masculinization of the brain (Ishunina *et al.*, 2013).

It has now been established that androgens act to masculinize the developing brain in males (reviewed in McCarthy, Pickett, Vanryzin, & Kight, 2016). Organizational effects of steroids reach down to differences in synaptic patterning (Matsumoto and Arai, 1980). However, there is evidence that steroids also affect the brain in adult life (Cooke, Tabibnia and Breedlove, 1999), and that the epigenetics of the sex chromosomes also heavily influence sex differences in the brain (Shen *et al.*, 2015). Globally, the human brain remains more complex and potentially influenced by experiences and the environment than any animal model (Maney, 2016). Finding sex differences in the brain does not necessarily translates into a difference in behavior (Tunç *et al.*, 2016), and moreover, the brain itself is not male or female, but a single heterogeneous population of what is seen as a mosaic brains with individual features which can differentiate towards female or male (Joel and Fausto-Sterling, 2016). New results are now expected to come from the study of epigenetics (Arnold *et al.*, 2016; Forger, 2016), as the effect of DNA methylation and histone acetylation on various gene expression have a fundamental influence on the development of the brain.

The term sex difference is usually used to describe scientific findings that differ between sexes. There is a wide variety of studies published on such differences. However, when systematic differences have been established reliably, one can start to define them as dimorphic, according to the definition of the occurrence of two forms in the same species. Historically, some structures stand out, such as the Sexually Dimorphic Nucleus (SDN) in the hypothalamus of the rat, first described by Gorski and colleagues (1978). Forty years and a large number of publications later, its function along with other sexually different structures is still not well understood. Evidence points towards an exposure and responsiveness to testosterone (aromatized to estrogens) prenatally (Roselli *et al.*, 2007) as well as an increased apoptosis in females postnatally (Tsukahara, Kakeyama and Toyofuku, 2006). In rams, the ovine SDN, the homologue of the rat SDN, was found to be twice larger in heterosexual rams than in homosexual rams (Roselli *et al.*, 2004). Another structure, the anteroventral periventricular nucleus (AVPV), has conversely a larger size in female rats than in males (Bleier, Byne and Siggelkow, 1982). A large number of structures have been given names, according to their anatomy, relation, or staining for a particular marker. The precise function of a number of these structures, especially related to sex differences, remain elusive, apart for a few nuclei (Panzica *et al.*, 1995; Simerly, 2002). Nonetheless, Gorski's experiment was as a stepping stone for a whole field to develop around sex differences in the brain.

II.A.1. MODELS

Over time, several animal models have risen following different lines of investigation of sex differentiation in the brain. Here again, most used are rodents, such as guinea pigs (Phoenix *et al.*, 1959; Hines *et al.*, 1985), rats (Gorski *et al.*, 1978), mouse (Kudwa *et al.*, 2006) and ferrets (Tobet, Zahniser and Baum, 1986). However, some birds have been studied such as songbirds like canaries (Nottebohm and Arnold, 1976) or finches (nucleus of the *archistriatum*), but also the Japanese quail (Ball and Balthazart, 2010). Only a few larger mammals were used such as the ram, where an ovine SDN was found (Perkins and Roselli, 2007).

Although the vast majority of studies were conducted on rats(Manger, 2008), these models each with their particularities, have helped researchers to reach new paradigms in the face of hormones and their influence on both the early organization of the brain circuitry, but also on their modulation the functioning brain at given times (for a review see McCarthy, 2016). Based on this circuitry, in adult life, differentiated hormonal levels determine their activation. This theory has however faced difficulties, as the same estrogen receptors have been found to have different effects depending on the area of the brain, their location in the cell, or their activation target (Mong, Nuñez and McCarthy, 2002; Amateau *et al.*, 2004; Schwarz *et al.*, 2008).

Some of the first sex differences spotted in humans have been so through syndromes and genetic abnormalities, such as XXY or congenital adrenal hyperplasia (CAH) and comparisons between 5α-reductase II deficiency and CAIS (Swaab, 2004). Homologues to mammalian model structures have been found in humans, such as Onuf's nucleus in the spinal cord, the suprachiasmatic nucleus (SCN), the Bed nucleus of the Stria Terminalis (BST), and notably the interstitial nucleus of the anterior hypothalamus 1 (INAH1) (related to the rodent SDN-POA) as well as INAH 2 and 3 (Swaab and Fliers, 1985; Allen *et al.*, 1989; LeVay, 1991). Possible other candidates, albeit under more discussion, are the hippocampus (Tan *et al.*, 2016), the amygdala (Domes *et al.*, 2009)and the corpus callosum (DeLacoste-Utamsing and Holloway, 1982; Luders, Toga and Thompson, 2014), or grey matter (Kurth, Thompson and Luders, 2018).

Most of these studies have been done on post mortem human brains, which are difficult to obtain for research despite some successful initiatives (Ravid and Swaab, 1993; Harper *et al.*, 2003), and could be affected by artifacts, or can be tedious in collecting the right sample. To a certain extent, technologies like MRIs have unraveled new possibilities, mostly for functional imaging (Gur *et al.*, 2000), DTI (Ingalhalikar *et al.*, 2014; Joel and Tarrasch, 2014) although the relationship between structural differences and behaviors remain unclear. Research on human sex differences additionally has the potential to spark controversy, in particular, research regarding sex, gender and sexual orientation (Swaab and Hofman, 1990; Zhou *et al.*, 1995; Kruijver, Zhou, Chris W Pool, *et al.*, 2000; Kruijver, Zhou, Chris W. Pool, *et al.*, 2000; Swaab *et al.*, 2001; Hines *et al.*, 2016). Therefore, research on alternative models can help confirm findings in humans.

II.A.2. THE HYPOTHALAMUS

A large part of these differences can be found in the hypothalamus. This structure has historically been placed as part of the diencephalon, although recent neuromeric observations could make the hypothalamus as part of the "secondary proencephalon", together with the telencephalon. Part of the limbic system, and a link with the endocrine system, it is concerned with basic survival functions, including growth, metabolism, fluid intake and excretion, reproduction, the sleep-wake cycle, temperature control and stress (Swaab, 1997). Because of the variety of its functions, sensory inputs and effector mechanisms, it is one of the most complex structures in the brain.

The hypothalamus (figure 1.2) is traversed by the third ventricle. Customarily, the hypothalamus is divided into the anterior part or preoptic area, the middle or tuberal part, and the posterior or mammillary part. The work presented below (Graïc *et al.*, 2018) was performed on the anterior hypothalamus.

The anterior hypothalamus hosts most of the magnocellular neuroendocrine system, grouped in principal nuclei: the paraventricular, suprachiasmatic and supraoptic nuclei. This system projects to the pituitary grand and secretes hormones and peptides, e.g. vasopressin, oxytocin, gonadotrophin releasing hormone (GnRH), or corticotrophin releasing hormone (CRH). It also has a large amount of afferent and efferent connections to the autonomic nervous system and the cortex, the hippocampal formation, and the amygdala, which is also involved in the management of fear and emotional states.

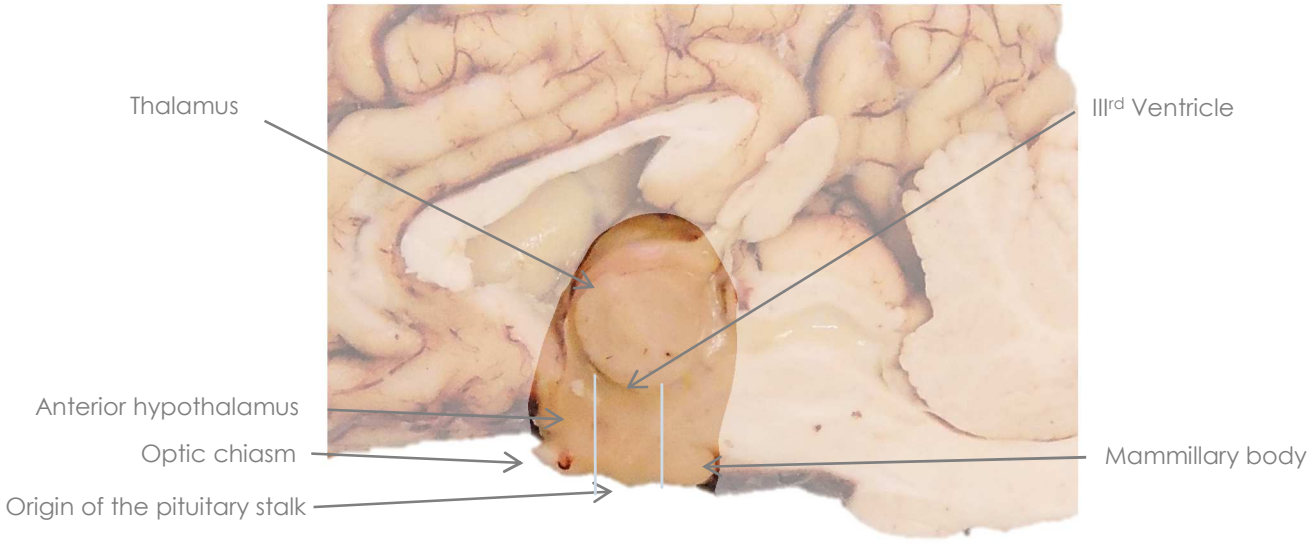

Figure 1.2: Photograph of a bovine brain in sagittal section, showing the hypothalamus gross anatomy. The vertical lines divide the preoptic, tuberal and mammillary parts.

II.A.3. FREEMARTINISM AND THE BOVINE MODEL

Freemartinism is a congenital syndrome mostly seen in cattle, although it is also reported in sheep, goats and pigs (Padula, 2005). During the gestation of a cow, heterozygotic twin pregnancy can occur. If the two concepti are of different sex, in 90% of cases in cattle (Padula, 2005), the placentas will mesh and create vascular anastomoses, which will ensure blood communication between the growing fetuses. This in turn will allow exchanges of hematopoietic precursors, besides red blood cells. Some male myeloid precursors will establish in the bone marrow of the female fetus and will produce male cells of the hematopoietic system, which is the reason why freemartins are called chimeras (Dunn, Kenney and Lein, 1968). However, this is only true for the bone marrow, as well as gonadal tissue (Cabianca *et al.*, 2007). The rest of the animal cells will remain chomosomically XX. Nonetheless, anastomoses will additionally allow the passage of testicular androgenic hormones from the male conceptus in the bloodstream of the female. Famously shown by Jost (Jost, 1947), Sertoli cells in the male fetus' developing testicular tissue secrete anti-Müllerian hormone, a glycoprotein that will induce the regression of Müllerian ducts (potential ovarian ducts), and initiate the masculinization process (Vigier *et al.*, 1984). Evidence of AMH interaction with the brain have been reported (Wittmann and McLennan, 2011). Consequently to the anastomoses, the female fetus, including its brain, will be under the influence of a male hormonal profile for the rest of the gestation, until parturition. This raises the question of the susceptibility of the female brain to masculine hormones, for which the freemartin brain proposes a good model. A lot remains to be learned as for instance, we still don't know precisely the mechanism initiating the testosterone secretion in the fetal testis (Clarkson and Herbison, 2016).

There has been a large amount of research focusing on the disturbances provoked to the development of the female genital tract (Jost, 1947; Short *et al.*, 1970; Vigier *et al.*, 1984), which yielded findings such as the AMH and its function, but none so far concerned the brain of freemartins. As stated before, several models were used to understand the development of the mammal brain, mostly by subjecting it to various doses of androgens or estrogens at precise moments of their lives(Romeo, Diedrich and Sisk, 2000). The freemartin brain has the advantage of being naturally exposed to male hormones during the whole intrauterine period and exactly in the same dose and timeframe as a male fetus. This constitutes a fair model to investigate the effects of androgens and their metabolites in the brain of a female mammal, compared to male and female specimen.

II.B. Sex differences in *Bos taurus*

Our lab has for now over a decade studied the brain of the bovine. We were able to culture fetal cortical and hypothalamic cells (neurons and astrocytes) (Peruffo *et al.*, 2004), and proposed that ER-α was not expressed in astrocytes (Peruffo *et al.*, 2008), although it was found in rodents, and sexually differentiated (Kuo *et al.*, 2010). In immortalized brain cells from endothelial origin, we also found that calcium uptake was decreased by estrogen treatment, possibly *via* mitochondrial permeability blocking (Suman *et al.*, 2012). Additional cultures of cerebellar granule neurons showed that estrogens had a trophic effect more potent in female than in male fetuses (Montelli, Suman, *et al.*, 2016). A fetal development study measured mRNA levels in the hypothalamus of bovine fetuses, but no sex difference was found (Panin *et al.*, 2015). However, the different time window in hypothalamus development in a long gestation species (285 days), showed that some CNS molecular developments such as Voltage Operated Calcium Channels (VOCC) take place in during fetal stages (Peruffo *et al.*, 2013), while they happen postnatally in rodents. While aromatase activity, a key to sex differentiation of most mammal brains, was peaking during the second quarter of gestation (Peruffo, Cozzi and

Ballarin, 2008), additional fetal studies using rtPCR revealed in male calves' frontal cortex, that aromatase activity was not correlated to estrogen receptors (ERs) expression, while ERs were highly correlated during development stages (Peruffo *et al.*, 2011). In continuity with previous efforts to describe the brain of cetartiodactyls, including *Bos taurus* (Ballarin *et al.*, 2016), the next logical step was to initiate a study at the cytoarchitectural level. Therefore, our line of study was directed at the cytochemical investigation of the still poorly described bovine hypothalamus. In the context of sex differences, we endeavored the comparison of male and female hypothalamic nuclei that were found to be sexually dimorphic in other species, and subsequently added intersex freemartins to evaluate the effects of masculinization over these areas.

In a second time, using newly developed image analysis tools and a multivariate multi-aspect statistical approach, we investigated the cytoarchitecture of the bovine cerebellum, among males, females and freemartins.

II.B.1. THE HYPOTHALAMUS OF *BOS TAURUS*

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THE BOVINE ANTERIOR HYPOTHALAMUS: CHARACTERIZATION OF THE VASOPRESSIN-OXYTOCIN CONTAINING NUCLEUS AND CHANGES IN RELATION TO SEXUAL DIFFERENTIATION

ABSTRACT

In an effort to systematically describe the neurochemical anatomy of the bovine anterior hypothalamus, we used a series of immunocytochemical markers such as Acetylcholine Esterase (AChE), Arginine-Vasopressin (AVP), Calbindin (Calb), Galanin (Gal), Neuropeptide-Y (NPY), Oxytocin (OXT), Somatostatin (SST) and Vasoactive Intestinal Peptide (VIP). We also investigated the potential sex difference present in the suprachiasmatic nucleus (SCN) and the vasopressin-oxytocin containing nucleus (VON) of 6 males and 6 female *Bos taurus*. Our study revealed that the cytochemical structure of the cattle anterior hypothalamus follows the blueprint of other mammals. The VON, which was never described before in cattle, showed a sex difference with a 33.7% smaller volume and 23.2% fewer magnocellular neurons (approximately 20-30 μm) in the male. The SCN also did show a sex difference in VIP neurons and volume with a 36.1% larger female nucleus with 28.1% more cells. Additionally, we included 5 heifers with freemartin syndrome as a new animal model relevant for sexual differentiation in the brain. This is to our knowledge the first freemartin study in relation to the brain. Surprisingly, the SCN of freemartin heifers was 32.5% larger than its control male and female counterparts with 29% more VIP cells. Conversely, the freemartin VON had an intermediary size between male and female. To analyze our data, a classical statistical analysis and a novel multivariate and multi-aspect approach were applied. These findings shed new light on sexual dimorphism in the bovine brain and present this species with freemartins as a valuable animal model in neuroscience.

ABBREVIATIONS:

AC: Anterior Commissure
Acc: Nucleus Accumbens
AChE: Acetylcholine Esterase
AVP: Arginine-Vasopressin
BST: Bed nucleus of the Stria Terminalis
Calb: Calbindin
Cau: Caudate nucleus
DBB: Diagonal Band of Broca
Gal: Galanin
INAH1: Interstitial Nucleus of the Anterior Hypothalamus 1
INF: Infundibular nucleus
LSA: Lateral Septal Area
MPOA: Medial Preoptic Area
NBM: Nucleus Basalis of Meynert
NC: Nucleus Circularis
NPY: Neuropeptide Y
OXT: Oxytocin
Pe: Periventricular nucleus
PVN: Paraventricular Nucleus
SCN: Suprachiasmatic Nucleus
SDN: Sexually Dimorphic Nucleus
SST: Somatosatin
VIP: Vasoactive Intestinal Peptide
VMH: Ventro-Medial Hypothalamic area
VON: Vasopressin-Oxytocin containing Nucleus

INTRODUCTION

The bovine species (*Bos taurus*) has been used regularly in neuroscience, since the early days of neuroendocrinology (Gorski and Erb, 1959; Legros *et al.*, 1976; Estes, Padmanabhan and Convey, 1977; Brownstein, 1983; Senders and Weber, 1987). However, the microscopic cytoarchitecture of the cattle brain is nowhere near as precisely described as that of the widely used rat and mouse brain (Okamura, 2002; Manger, 2008; Bolker, 2012). The nature of the highly gyrencephalic brain, its large size with an average weight of 476 gr (Ballarin *et al.*, 2016), and easy access through local slaughterhouses make the cattle brain an interesting animal model for neuroscience. Additionally the duration of gestation, the prevalence of aromatase P450 and its hormonal profiles make it an interesting model for the study of the interaction of hormones and brain structures in development (Kim, Yen and Benirschke, 1972; Challis *et al.*, 1974; Dominguez, Liptrap and Basrur, 1988; Peruffo *et al.*, 2008; Peruffo, Cozzi and Ballarin, 2008).

Compared to other species, little knowledge is available about the cytochemistry of the bovine hypothalamus despite the early interest in research mentioned above, apart from the chemical neuroanatomy of the vasopressin-oxytocin neurophysin systems (De Mey, Dierickx, & Vandesande, 1975; De Mey, Vandesande, & Dierickx, 1974; Dellmann, 1959; Gadamski & Lakomy, 1972, 1973; Grütze, 1978; Junge, 1976, 1977; Lakomy & Gadamski, 1973; Leshin, Rund, Crim, & Kiser, 1988; Maciag, Cerundolo, Ilsley, Kelley, & Forand, 1979; Okamura, 2002; Schmidt et al., 2012; Sofroniew & Weindl, 1980; Szteyn, Lakomy, Dynowski, & Krawczuk, 1981; Vierling, 1957, 1958). Therefore, one aim of this study was to describe systematically the bovine anterior hypothalamus using a panel of known immunocytochemical markers. This resulted in the first description of the vasopressin-oxytocin nucleus (VON) in the cow brain.

Since the pioneering studies showing that sexual behavior is shaped by the effect of hormones on the brain during early development (Phoenix *et al.*, 1959), a great body of research has been performed to explain the complex mechanisms underlying sexual behavior (for a review see McCarthy, 2016). This line of research led to the detection of sexual dimorphism in a number of brain areas during the past 40 years in the rat (Gorski *et al.*, 1978), guinea pig (Bleier, Byne and Siggelkow, 1982; Hines *et al.*, 1985), hamster (Greenough *et al.*, 1977), pig (van Eerdenburg *et al.*, 1990) and human (Swaab and Fliers, 1985; Allen *et al.*, 1989; Zhou *et al.*, 1995; Zhou, Hofman and Swaab, 1995b) as well as the effect of hormones on the brain.

The conversion of testosterone into estradiol by the enzyme aromatase P450 produces irreversible organizational effects during a critical period in the course of brain development (Lephart, 1996). These hormones may induce permanent changes in the architecture of nervous circuits, including changes in cell number, density of axonal connections, dendritic architecture and neurotransmitter phenotype (Simerly, 2002). The ontogenetic window of sensitivity to steroids is not the same across all mammalian species. In short-gestation species (i.e. rodents), there is a perinatal peak in steroids whereas in long-gestation, larger-brained species (including human and bovine), the critical period for sexual differentiation occurs earlier (Peruffo, Cozzi and Ballarin, 2008). Morphological and functional sex differences of the brain circuitry of long-gestation species therefore depend on the prenatal hormonal milieu in which the fetus develops, irrespective of perinatal or postnatal hormonal exposures. A notable difference however, is that primate brains seem to undergo masculinization without aromatization of testosterone (Wisniewski *et al.*, 2000; Wallen, 2005). This

implies that sex is an important determinant in neuroscience and its applications, in particular in the medical field (Cahill, 2006).

Freemartinism is an interesting model to bridge neuroanatomy and physiology in relation to sexual differentiation of the brain. Freemartinism occurs when a male and female (*ergo* dizygotic) fetuses create placental anastomoses, exposing the female fetus to the hormonal production of the male fetus during intra-uterine development, resulting in a masculinization of the female newborn genitalia. This condition, first correctly explained by Tandler and Keller (1911) and later more extensively by Lillie (1916), is far more frequent in cattle than in other species (Ladds, 1993; Padula, 2005). Freemartinism has been known to exist since the domestication of *Bos taurus* (Moore, Graham, & Barr, 1957) and was mostly considered for its implications in the physiology and pathology of reproduction. The potential structural effect of the freemartin syndrome on the development and organization of the brain has never been studied. It has long been known that the Anti-Müllerian hormone (AMH) is responsible for the development of the freemartin female genital tract into variably masculinized tissues, usually causing sterility, but no study focused so far on their brain (Vigier *et al.*, 1984, 1989). Chromosomically female, the freemartin brain is exposed to testosterone comparably to a male fetus during its development (Dunn, Kenney, & Lein, 1968; Rota, Ballarin, Vigier, Cozzi, & Rey, 2002), both in terms of time and intensity, from as early as 45 days post conception (Dominguez, Liptrap, & Basur, 1988) in natural conditions still unmatched today in other models of sexual differentiation of the brain. Hence, this results in genetically female brain cells receiving a testosterone surge enzymatically transformed in estrogens by aromatase P450 (Peruffo, Cozzi and Ballarin, 2008), known to mediate male development of the rodent brain (Lephart, 1996). This contrasts with primates, in which androgens seem to act directly on the brain during sexual differentiation in rhesus monkeys, without the need for aromatization to masculinize male brains, as a non-aromatizable androgen had the same masculinization effects as testosterone (Wallen, 2005). The freemartin model could help to differentiate and specify where and when the genetic, epigenetic and hormonal environment's relative influence and mechanisms take place. A second aim of this study was to compare the cell numbers and volumes of both the suprachiasmatic nucleus (SCN) and the newly described Vasopressin and Oxytocin containing nucleus (VON) between females, males and freemartins, to test whether the masculine hormonal environment may affect the organization of the female hypothalamus.

MATERIALS AND METHODS

Tissue sampling and processing

The brain of 16 adult bovines (over 12 months old) were samples for this study. They formed three groups, 6 males, 6 females and 5 freemartins. The diagnosis of freemartinism was made on living animals by expert veterinary practitioners. Diagnostic criteria included: birth with a male co-twin (mandatory), malformation of the female external genitalia (elongated penis-like clitoris, elongated vulva, upward urination), abnormally short vaginal length (5-8 cm in the newborn freemartins instead of 13-15 cm in normal calves) blindly ending without a cervix, abnormal development of the genital tract at transrectal ultrasonography performed at one year of age, and a failure to show estrus (Padula, 2005)

The animals' heads were collected from local slaughterhouses in the Veneto region. Animals were treated according to the present European Community Council directive concerning animal welfare during the commercial slaughtering process and were constantly monitored under mandatory official veterinary medical care. The brains were extracted in the necropsy room of the Department of

Comparative Biomedicine and Food Science of the University of Padova, dissected and fixated by immersion in phosphate buffered formalin 4% v/w pH 7.4 within two hours after death. After a week, the hypothalamic region was dissected using the origin of the optic chiasma and the caudal part of the mammillary body as rostral and caudal borders respectively. For the present study however, we considered the region from and including the preoptic area to the pituitary stalk. The tissue blocks were then kept in formalin for at least 3 additional weeks before further processing.

After fixation the hypothalamic blocks were dehydrated in graded ethanols, cleared in toluene, embedded in paraffin and cut in 8 µm-thick serial sections in the coronal plane. A thionine staining was performed on every 100th section for topological reference. Each section was mounted on Superfrost Plus glass (Ref. J1800AMNZ, Menzel-Gläser, Braunschweig, Germany) and dried at least overnight on a hot plate at 40 °C.

Immunocytochemistry

VON

Due to its very packed and round aspect, the VON was easily identifiable using Nissl stained (thionine) sections (Figure 2, 3a-c). Nucleolus containing neurons were counted in this material following a technique recently reaffirmed for neuron description (Jones, 1937; García-Cabezas *et al.*, 2016). Sections were deparaffinized in xylene and rehydrated in a descending alcohol series. After a short passage in distilled water, sections were submerged in a 0.4 % thionine bath for 4 minutes, then washed in tap water until the water was clear. The sections were dehydrated in an ascending alcohol series, cleared in xylene and finally coverslipped with Entellan (cat. n. 107960, Merck).

To detect the presence of Arginin-Vasopressin (AVP) and Oxytocin (OXT) expressing neurons in the VON and ascertain its boundaries, we used antibodies directed against these neuropeptides (information regarding primary antibodies used can be found in Table 2). Anti-AVP antibodies were raised in rabbits using purified Guinea pig posterior pituaitary extracts. The extracts were subjected to reverse phase HPLC and radiolabeled glycopeptide fraction isolation, pooled and automatically sequenced to confirm the glycopeptide identity, corresponding to one single N-terminal sequence (Fairhall & Robinson, 1989). The antisera obtained was tested for binding to radioiodinated glycopeptide, and cross-reactivity (AVP, k.1.7 rabbit polyclonal, Fairhall & Robinson, 1989, kindly provided by Dr. van Leeuwen, RRID: AB_2732873): Anti-OXT monoclonal antibodies were produced by mouse spleen cell hybridoma and tested for specificity via radiobinding testing, and immunoabsorption with synthetic AVP, pressinamide, OT, (2-Phe)OT, (3-Phe)OT, (8-Ile)OT, (4-Asn)OT, (7-Gly)OT, (8-Val)OT, AVT and tocinamide (A1-28, mouse monoclonal antibody, generous gift of A. J. Silverman, used in Hou-Yu, Lamme, Zimmerman, & Silverman, 1986, RRID: AB_2732874). The AVP C-terminal end is extended by a 39-peptides glycopeptide, which is not present in the OXT precursor providing a specific target for antibodies (Gordon-Weeks, Jones and Robinson, 1983; Richards, Morris and Raisman, 1985; Fairhall and Robinson, 1989). In this study, the specificity of the AVP and OXT stainings was confirmed by showing no overlap in staining of VON cells in alternating sections (Fig. 3a and b). Deparaffinized sections were rehydrated in descending alcohol series, and rinsed in distilled water 2 × 5 minutes. For antigen retrieval (Shi, Cote and Taylor, 1997), sections were kept boiling for 10 minutes in 0.05 M Tris-HCl pH 9 buffer in a microwave oven at 800 W. After cooling to room temperature, sections were washed 3 × 3 minutes in Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6), then treated with 10 % methanol, 3 % H2O2 in TBS for 10 minutes to quench

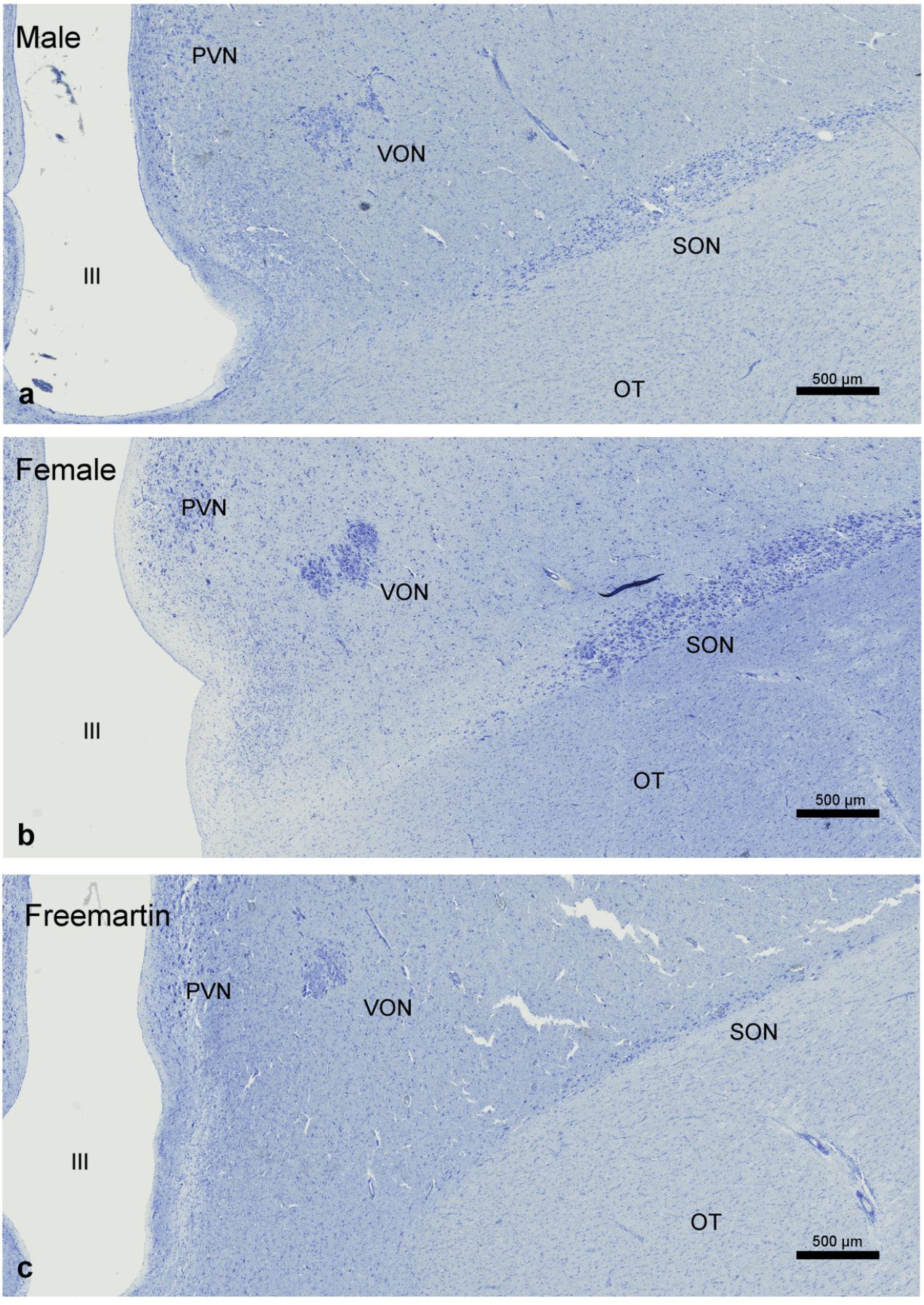


Figure 2: Microphotographs of the bovine hypothalamus of **a)** a male, **b)** a female and **c)** a freemartin.

Antibody	Immunogen	Manufacturer, RRID, Species	Concentration
AChE	ChAT	Millipore Cat# AB144P, RRID:AB_2079751, Goat polyclonal	1:500
AVP	Guinea pig C-terminal end of the glycopeptide Amino acid sequence: Ala-Gly-Asp-Arg-Ser-Asn-Val-Thr-Gln-Leu-Asp-Gly-Pro-Ala-Gly-Ala-Leu-Leu-Leu-Arg-Leu-Met-Gln-Leu-Ala-Gly-Ala-Pro-Glu-Pro-Gln-Pro-Ala-Ala-Pro-COOH	Rabbit polyclonal, thyroglobulin-coupled, kindly provided by Dr. van Leeuwen, RRID: AB_2732873	1:1000
Calbindin	Calbindin D-28k	Swant, Marly, Switzerland, (Swant Cat# CB38, RRID:AB_2721225), rabbit polyclonal	1:500
Galanin	Rabbit antiserum (Gaaltje [29-6-1993]) Amino acid sequence:Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu- Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH2	Rabbit polyclonal (Sigma galanin porcine G-1266), thyroglobulin-coupled, Gaaltje 29-06-1993, Netherlands Institute for Neuroscience. RRID: AB_2732875	1:800
Neurotensin	Neurotensin Amino acid sequence: Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH	Rabbit polyclonal (Neurotensin, Beckman Instruments) Hemocyanin-8 coupled (HC-8, 11/11/75), RRID: AB_2732876	1:1000
NPY	Porcine NPY (1-36) Amino acid sequence: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr -Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2	porcine NPY(1–36) (Sigma, St. Louis,MO) coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck, Rahwah, NJ) "Niepke", rabbit polyclonal, bleeding 26/11/1988, Netherlands Institute for Neuroscience RRID: AB_2732877	1:1000
OXT	A1-28 Oxytocin Amino acid sequence: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2	Mouse monoclonal produced by cell fusion and immunoabsorbed, kindly provided by AJ. Silverman RRID: AB_2732874	1:1000
SST	SOMAAR Amino acid sequence: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	Rabbit polyclonal,(SOMAAR, bleeding 8/2/1989), Netherlands Institute for Neuroscience in van Nes et al., 1994, RRID: AB_2732878	1:800
VIP	VIPER Amino acid sequence: His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2	VIP coupled to thyroglobulin. Rabbit polyclonal (Viper, bleeding 18/9/1986), Netherland Institute for Neuroscience, RRID:AB_2513212	1:1000

Table 2. Primary antibodies information

pseudoperoxidase activity (Streefkerk, 1972). After another wash in TBS, sections were incubated with either anti-AVP or anti-OXT, both diluted to 1:1000 in Super Mix (SuMi, TBS pH 7.6 with 0.5 % Triton X-100 and 0.25 % gelatine) for 1 hour at room temperature followed by an overnight incubation at 4 °C. The next day, after several washes in TBS, sections were incubated for 1 hour in biotinylated horse anti-rabbit IgG for AVP or horse anti-mouse IgG for OXT, both 1:400, (cat. no. BA-1100, cat. no. BA-2000, Vector Laboratories Inc., Burlingame, CA, USA) in SuMi. After a few rinses in TBS, sections were incubated in 1:800 Avidin-Biotin Complex (cat. no. PK-1600, Elite ABC kit; Vector Laboratories Inc.) in SuMi for 1 hour, followed by final washes. Staining development was achieved using immersion in a solution of 0.5 mg/ml 3,3'-diaminobenzidinetetrahydrochloride (DAB, Merck) 0.2 % nickel ammonium sulfate, and 0.01 % hydrogen peroxyde (H_2O_2; Merck, cat. no. 1.07209.0250) in TBS. Reactions were stopped in distilled water, then sections went through an ascending alcohol series, ending in xylene before coverslipping with Entellan (cat. n. 107960, Merck).

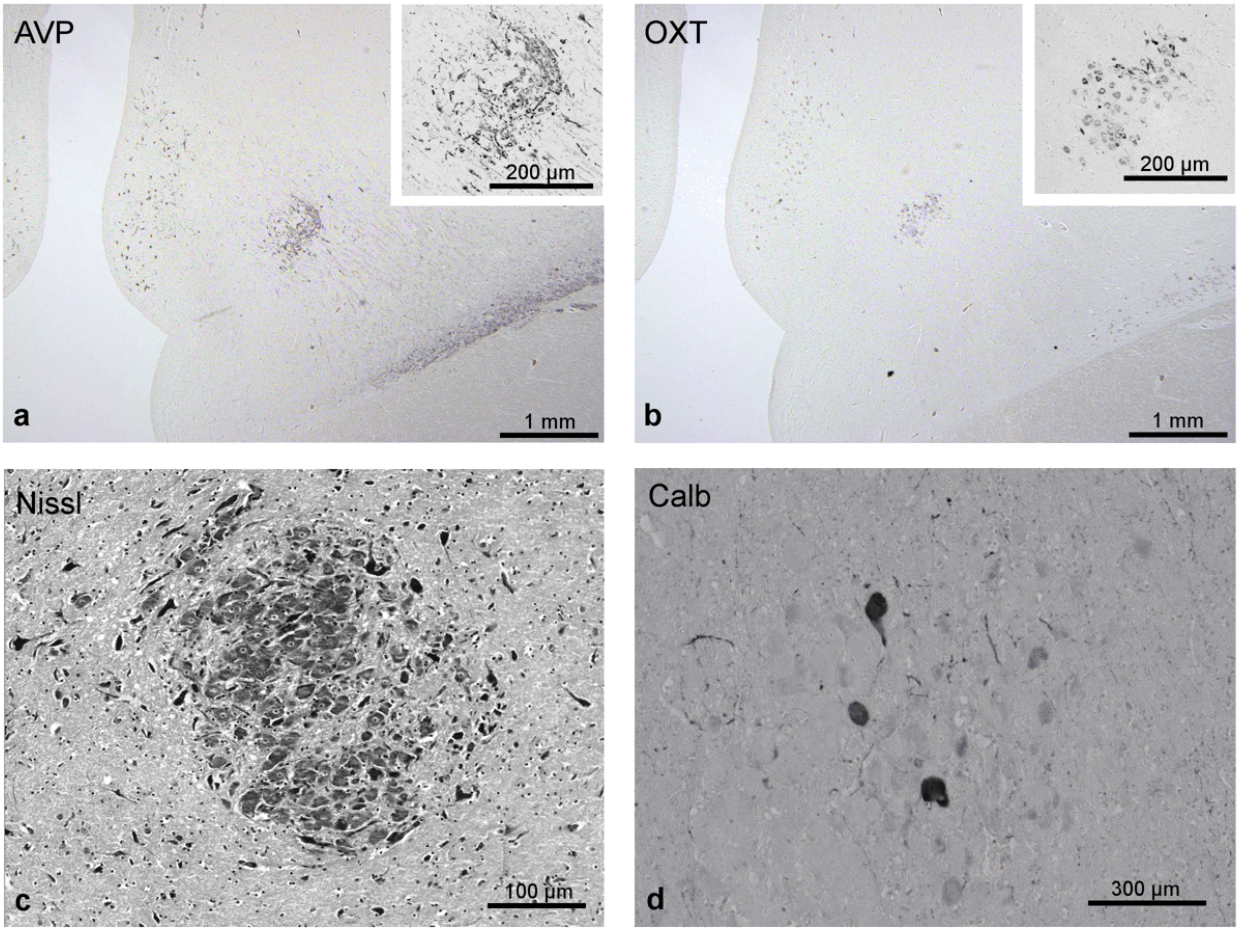


Figure 3: Pictures of the Vasopressin-Oxytocin containing Nucleus. **a)** and **b)** are adjacent sections. No double staining of cell can be seen. **a)** AVP staining, insert for details; **b)** OXT staining with insert for details; **c)** Round aspect of the VON, Nissl staining; **d)** VON neurons positive to calbindin staining. Note the unstained magnocellular neurons.

SCN

We used antibodies raised against vasopressin (AVP k.1.7), neurotensin (HC-8; 11/11/75, RRID: AB_2732876), tested in Carraway & Leeman (1976) and Kahn, Abrams, Zimmerman, Carraway, & Leeman (1980) and vasoactive intestinal peptide (VIP, Viper, 18/9/1986, Netherland Institute for Neuroscience, RRID:AB_2513212). Anti-neurotensin polyclonal antibodies were obtained from a rabbit host, via conjugation with hemocyanin and tested by radioimmunoassay and chromatography (Carraway and Leeman, 1976). Anti-VIP was raised at the Netherlands Institute for Neuroscience following conjugation to thyroglobulin by glutaraldehyde. Nonspecific antibodies were removed by adsorption with thyroglobulin Sepharose beads, treated with glutaraldehyde (Zhou, Hofman and Swaab, 1995a, 1995b). To stain the SCN (Swaab, 2003) and we chose to use VIP for the rest of the experiment since it selectively stained only the SCN (Card *et al.*, 1981). Deparaffinized sections were rehydrated in a descending alcohol series, and boiled for 10 minute in 0.05 M Tris-HCl pH 9 to retrieve antigenicity. After cooling, sections were washed in TBS and treated with 10 % methanol, 3 % H2O2 in TBS for 10 minutes. After rinses in TBS, sections were incubated with VIP antiserum at 1:1000 dilution in SuMi for 1 hour at room temperature and overnight at 4 °C. The next day, detection was done by incubating sections with biotinylated 1:400 anti-rabbit IgG (horse anti-rabbit; cat. no. BA-1100, Vector Laboratories Inc., Burlingame, CA, USA) in SuMi, and ABC, followed by nickel-enhanced DAB development.

Other neuropeptides

Immunocytochemical staining of other neuropeptides mostly followed the same procedure, except that blocking with milk was required, which included a one hour pre-incubation in TBS-milk (TBS with 5 % Elk™ powder milk, Campina bv., Eindhoven, The Netherlands) and incubation of the primary antibody in SuMi-milk (Super Mix with 5 % powder milk) to reduce background staining. The peptides below were stained in this way, including Calbindin D-28k (1:500, cat.no. CB-38a, Lot no. 9.03, Swant, Marly, Switzerland) and anti-acetylcholinesterase (AChE, goat polyclonal, cat.no. AB144P, RRID AB_2079751, 1:500), Anti-Neuropeptide Y antibodies (NPY, "Niepke", rabbit polyclonal, bleeding 26/11/1988, RRID: AB_2732877) were raised at the Netherlands Institute for Neuroscience by immunizing rabbits with porcine NPY (Sigma, St Louis, MO) coupled to thyroglobulin with gutaraldehyde, and specificity was tested by pre-incubation with protein A and G, as well as with the homologous antigen (Van der Beek *et al.*, 1992; Goldstone *et al.*, 2002). Anti-Galanin antibodies ("Gaaltje", rabbit polyclonal, bleeding 29/06/1993 RRID: AB_2732875) were raised in rabbits using galanin (Sigma, St Louis, MO, G1266).at the Netherlands Institute for Neuroscience. The peptide was coupled to thyroglobulin (Sigma T-1126) by means of glutaraldehyde (Merck, #6528). Incubations with pre-immune serum showed no staining while adsorption of the galanin antiserum, performed with galanin spotted on gelatine-coated nitrocellulose membrane and based on the press-blotting procedure of van der Sluis, Pool, & Sluiter (1988), prevented staining (Garcia-Falgueras *et al.*, 2011). Anti-somatostatin antibodies (SOMAAR, rabbit polyclonal, bleeding 8/2/1989, RRID: AB_2732878), were raised at the Netherland Institute for Neuroscience in rabbits, coupling somatostatin to thyroglobulin with glutaraldehyde (Buijs *et al.*, 1989; van de Nes *et al.*, 1994). Specificity was tested by pressblotting with an homologous antigen, abolishing staining, and pre-incubation with protein A and G (Buijs *et al.*, 1989). In all cases, omitting the primary antibody resulted in complete abolishment of staining.

The nomenclature we used was based on the available brain atlas of the species (Okamura, 2002) and historic publications (Vierling, 1958; Szteyn *et al.*, 1981).

Image analysis for the quantitative study

A quantitative study of the cell count and volume was carried out using the Cavalieri's principle, with a minimum of 10 sections (8 µm thick) evenly spaced along the rostro-caudal axis of the structure to be studied to infer a volume based on the integration of each area over its interval. The interval was every 10 sections for the VON given its small size, and every 25 sections for the SCN. All nuclei were evaluated bilaterally and mean values are presented in Figure 6. The SCN borders were drawn manually, given the absence of other VIP-immunoreactive structures in the immediate periphery of the SCN. In case of doubt, an adjacent thionine stained section was used. Since this work was done in thin sections, neuron counting was achieved by counting nucleoli within the nucleus contour on each sampled section and dividing it by the nucleus volume in the section obtain a cell density. This density was multiplied by the total volume of the nucleus to obtain a total neuron count. Only nucleolated cells were counted to prevent double counting (Jones, 1937; García-Cabezas *et al.*, 2016). No double nucleolus was ever seen in this experiment. Criteria were VIP positivity, nucleolus visibility and inclusion in the outline. The delineated nucleus was subdivided using an automated grid, into 500-pixels-wide squares, displayed full-screen on the monitor, and all of them were counted. The VON borders were delineated on thionine sections, since the densely-packed large neurons were easily distinguished from the background. All nucleolated neurons were counted within the delineated area, following the same procedure. Tiled images were recorded using the 40x magnification objective (Plan-Neofluor lense) on a Zeiss Axioscope microscope mounted with a CDD black and white video

camera (Sony-XC77) and connected to an ImageProPlus version 5.1 image analysis system (MediaCybernetics, Silver Spring, MD). All images were collected with exactly the same settings of the camera microscope.

Statistical design and data analytics

The main focus of data analytics was on the comparison among the three populations under investigation, i.e. male, freemartin and female. For this goal, as done before in other works (Swaab *et al.*, 1994; Zhou, Hofman and Swaab, 1995b; Goldstone *et al.*, 2002; Garcia-Falgueras *et al.*, 2011), we applied two traditional nonparametric rank-based tests : the Kruskal-Wallis test and the Mann-Whitney test (Bonnini *et al.*, 2014). The first and second testing procedure are devoted to compare respectively all three populations and one pair of populations. Nonparametric tests can be considered as the more recommended testing to apply to our morphometric data because of their evident non-normal distribution.

In order to try to provide some additional insights on the population comparison, we applied also some innovative nonparametric permutation-based tests that are presented in details in the appendix. This methodology consists of a powerful multivariate and multi-aspect-testing approach, able to quantify fine differences of the cell morphology in the nervous tissue (see the statistical method in appendix). For all tests, a *p*-value of less than 0.05 was considered to be significant.

Kruskal-Wallis and Mann-Whitney p-values were calculated by using Minitab 18.1 (Minitab, 2017) while permutation-based multi-aspect tests were performed by ad-hoc R codes that are available by authors on request to interested readers.

RESULTS

Anterior hypothalamus topography

The distribution of the neurochemical markers are represented in Figure 4.

Acetylcholinesterase

Large to medium sized (approx. 20-30 μm) multipolar acetylcholinesterase-positive nerve cell bodies were found in the caudate nucleus, in the *nucleus accumbens* (Acc) and most pronounced in the *nucleus basalis* of Meynert (NBM) (Fig. 5a) as well as in the diagonal band of Broca (DBB) and the septum. A posterior group of middle-sized neurons was present along the descending fornix in the lateral septal area (LSA), above the anterior commissure and along the bed nucleus of the stria terminalis (BST). AchE-ir cells were also present in the paraventricular nucleus (PVN) *pars verticalis*, although not nearly as densely as AVP or OXT neurons. Dark patches of fibers were observed in the islands of Calleja near the NBM.

Galanin

Positive neurons were small (approx.10-20 μm) and spindle-shaped (Fig. 5d and e). We found galanin positive fibers within the DBB and cells in the *nucleus accumbens*, the periventricular nucleus (Pe), the BST, the PVN *pars horizontalis* and *pars verticalis* and the medial preoptic area (MPOA). We did not reliably find any distinctive nucleus possibly homologous to the Intermediate nucleus (Braak and Braak, 1987) also named Sexually Dimorphic Nucleus (SDN) (Swaab and Fliers, 1985) or Interstitial Nucleus of the Anterior Hypothalamus 1 (INAH1) (Allen *et al.*, 1989). Immunoreactive fibers spread wide throughout the preoptic area, some in the paraventricular area, around the fornix, and the infundibular nucleus (INF). The VON did not show any reactivity to galanin, but sparse fibers

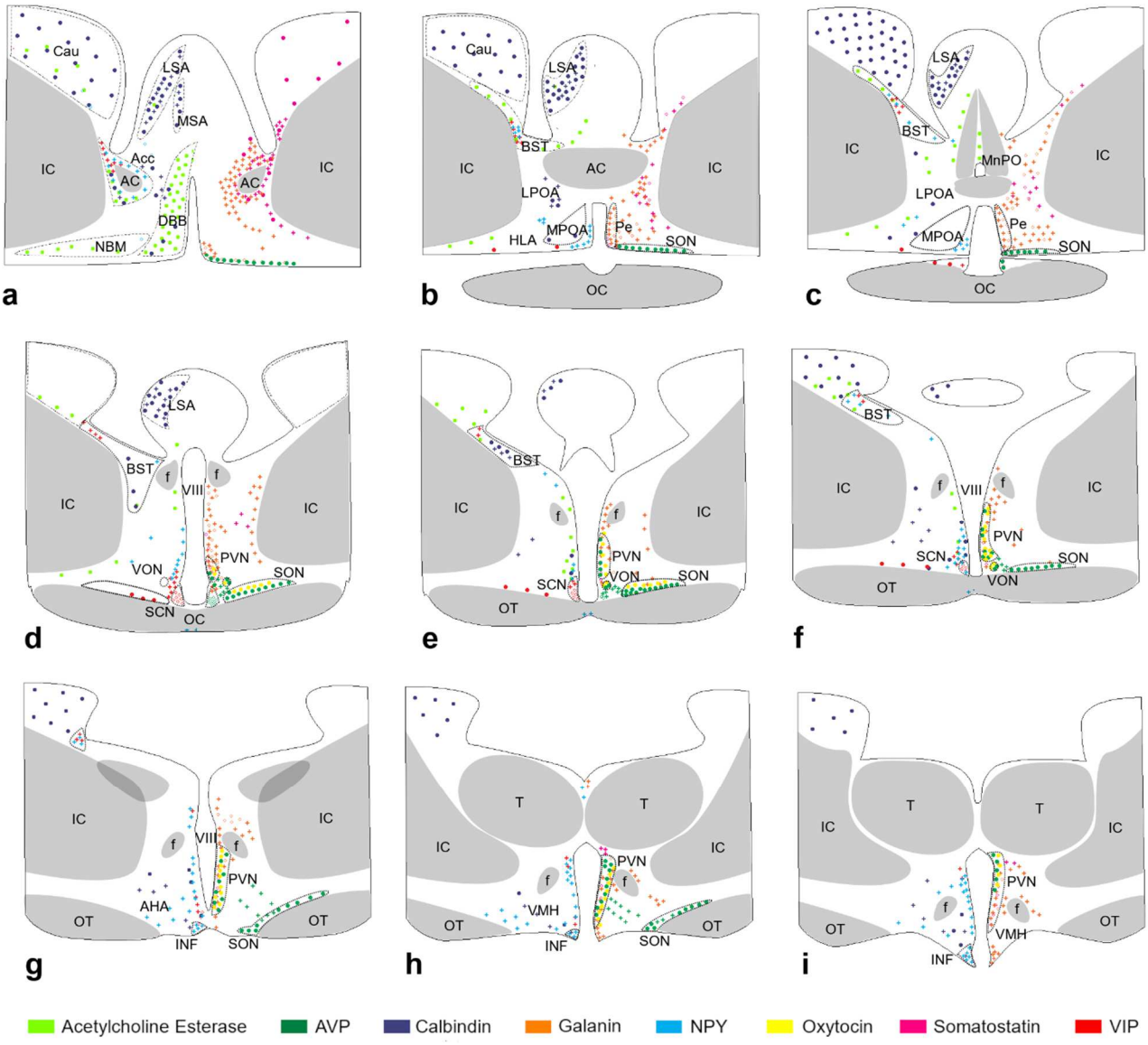


Figure 4: Schematic localization of immunoreactive perikarya in the rostral bovine hypothalamus. Dots ● represent magnocellular perikarya; O represent parvocellular perikarya; "+" represent fibers. Acc: nucleus Accumbens, AHA: Anterior Hypothalamic Area, BST: Bed nucleus of the Stria Terminalis, Cau: Caudate nucleus, DBB: Diagonal Band of Broca, LHA: Lateral Hypothalamic Area, INF: Infundibular nucleus, LPOA: Lateral Preoptic Area, LSA: Lateral Septal Area, MnPO: Median Preoptic nucleus, MPOA: Medial Preoptic Area, MSA: Median Septal Area, Pe: periventricular nucleus, PVN: paraventricular nucleus, SCN: Suprachiasmatic Nucleus, SON: Supraoptic Nucleus, VMH: Ventromedial Hypothalamic nucleus, VON: Vasopressin-Oxytocin containing Nucleus; f: fornix, OC: Optic Chiasma, T: Thalamus, IC: Internal Capsule, OT: Optic Tract, VIII: Third Ventricle.

were found in the dorsal SCN.

NPY

Extensive NPY-ir areas were found in the nucleus accumbens and in the BST, composed of fibers and parvocellular neurons (approx.10-20 µm), while fewer cells were present in the ventral DBB (Fig. 4f). Beaded neuronal fibers spread out in the preoptic area, and remained most dense around the anterior commissure (AC) and in the Pe. A thick cluster of fibers was found lateral to the Pe, but no cell bodies were present. Many fibers were present in the infundibular nucleus but cell bodies were seldom seen. Small neurons and fibers could also be seen in the PVN *pars verticalis*.

Somatostatin

For the largest part, somatostatin (SST) signal was found in the *nucleus accumbens*, the caudate nucleus (Cau) as well as the bed nucleus of the *stria terminalis*. Parvocellular neurons were found scattered around the anterior commissure in the POA but a few were also spotted in the region of the *nucleus basalis* of Meynert (NBM). The cells were small (approx. 10-20 μm) and bipolar (Fig. 5c). Further caudally at the level of the SCN, parvocellular SST-ir neurons and fibers with varicosities were detected in the PVN (Fig. 4d) all the way caudally up to the infundibular nucleus.

Calbindin

Magnocellular calbindin-immunoreactive neurons (approx. 20-30 μm) were clearly present in the LSA (Fig. 5b). They were quite densely packed and surrounded by fibers. The caudate nucleus also showed regularly spaced positive neurons. Calb-ir neurons were also seen in the BST, posterior to the anterior commissure. In the hypothalamus, some medium sized scattered cells and beaded fibers were found in the MPOA (Fig. 4e, f). In the VON we found some rare cells positive for calbindin (Fig. 3d). Conversely, positive cells were regular in the exterior part of the PVN *pars verticalis*. More caudally, Calb-ir cells were seen in the ventro-medial hypothalamic area (VMH), and fibers were present in the infundibular nucleus.

AVP

Vasopressin was widely distributed in the bovine hypothalamus. Magnocellular multipolar neurons (approx. 20-30 μm) were seen in the medial part of the paraventricular nucleus, throughout the SON and in the VON (Fig. 3a). Parvocellular spindle-like AVP-ir neurons were found in the SCN, mostly on the medial side, with a thin rim of cytoplasm (Fig 5h). Fiber tracts were seen passing between the PVN and the SON via the VON. In both cell types the cytoplasm was stained leaving the nucleus clear.

OXT

Oxytocin-containing neurons were strongly present in the cattle SON, as a thin cap of densely packed magnocellular multipolar neurons dorsal to the AVP-ir cells. OXT-ir neurons were also present in the lateral part of the PVN (Fig. 3b). The SCN showed no positive signal. The VON exhibited densely packed magnocellular OXT neurons (Fig. 3c). OXT positive staining was found in the neuronal cytoplasm, with a clear, unstained nucleus.

VIP

VIP-reactive perikarya were found in the SCN medioventral part. (Fig. 5g). Rare somas could be seen on the floor of the preoptic area. Fibers were found in the Acc and the BST mostly (Fig. 4b-d). Fibers are also present in the SCN running to the ventral PVN (Fig. 5h).

Neurochemical organization of the SCN and VON

SCN

The largest part of VIP-ir neurons was present in the medioventral part of the SCN (Fig. 4d, e). The neuron bodies were rather small (approx.10-20 μm) and spindle-shaped. We sometimes saw a thin commissural junction between the two SCNs below the ventral part of the ventricle. Dorsal to the VIP-ir cells were beaded fibers present running dorsolaterally, where AVP-ir neurons were also found in the SCN (Fig. 5g and h).

Cell counts and volumes are reported in Table 3. The female nucleus was larger than the male (p = 0.028, Table 4), while the freemartin SCN was larger than both male and female (p < 0.001 and p = 0.004 respectively).The freemartin group showed a statistically higher number of cells and area than both females and males (p FM>F = 0.006, p FM>M = 0.001; Table 5). Density across sexes was similar (Table 4 and 5).The left or right side of the nucleus did not show any difference (data not shown).

In Figure 7b, the scatterplots show more information about the morphology of the SCN. Comparison between cell count and area, and cell count and density show a positive correlation. Consequently, area and density are seemingly independent. The more cells are present, the larger the nucleus tends to be. However, the larger the nucleus cross-section does not imply a higher density in cells.

	Females		Freemartins		Males	
	Cells ± S.E.M.	Volume ± S.E.M. (mm3)	Cells ± S.E.M.	Volume ± S.E.M. (mm3)	Cells ± S.E.M.	Volume ± S.E.M. (mm3)
SCN	$20.77 \times 10^3 \pm 2.60 \times 10^3$	2.60 ± 0.37	$25.15 \times 10^3 \pm 4.28 \times 10^3$	3.17 ± 0.63	$14.92 \times 10^3 \pm 2.02 \times 10^3$	1.67 ± 0,26
VON	$5.47 \times 10^3 \pm 1.48 \times 10^3$	0.18 ± 0.05	$4.87 \times 10^3 \pm 1.01 \times 10^3$	0.16 ± 0.03	$4.20 \times 10^3 \pm 732$	0.12 ± 0.03

Table 3. Cell number and Volume of female, freemartin and male groups in the SCN and the VON. The average cell count for the SCN was 14.9 x 10^3 ± 2.0 x 10^3 neurons (mean ± S.E.M.) for males, 20.8 × 10^3 ± 2.6 × 10^3 neurons for females and 25.2 × 10^3 ± 4.3 × 10^3 neurons for freemartins. The volume of the nucleus followed the same trend with 1.7 ± 0.26 mm^3 for males, 2.6 ± 0.37 mm^3 for females and 3.2 ± 0.63 mm^3 for freemartins. The average of male and female SCN cell count (14.92 + 20.77)/2) x 10^3 = 17.85 x 10^3) is 29% smaller than the freemartin cell count (25.15 x 10^3)

VON

The bovine VON is a small (0.12-0.18 mm^3) very densely packed nucleus containing typical large (approx. 20-30 μm) Nissl-stained neurons with cytoplasm around the whole empty-looking large and lightly stained nucleus with a distinct nucleolus (Fig. 3c). The VON comprises AVP-ir and OXT-ir neurons with slightly more AVP-positive neurons. It is dorsal to the SCN and rostroventral to the PVN (Fig. 2, Fig. 3). The shape of the VON is round to ovoid anteriorly and evolves into a rather more elongated shape posteriorly.

Detailed results are reported in Table 3-5. The male group had a significantly smaller nucleus than both females and freemartins, both, in terms of area (p < 0.001 and p = 0.019 respectively, Table 4) and number of cells (p = 0.009 and p = 0.017 respectively). There was also a less significant difference in cell number and area between females and freemartins (p = 0.038, Table 5). Males showed a higher density than females and freemartins in the VON (p = 0.002 and 0.004 respectively, Table 5).

The scatterplots in Figure 7d add some valuable information on the morphology of the VON. There seems to be a positive correlation between cell number and the area of the nucleus, but a negative correlation with density, implying that where the nucleus cross-section is larger, the density tends to shrink.

	Test	Kruskal-Wallis	Mann-Whitney		
	Comparison	F vs. FM vs. M	F vs. FM	F vs. M	FM vs. M
SCN	Morph. Meas.				
	Area	**<0.001**	**0.004**	**0.028**	**<0.001**
	Cell count	0.122	0.094	0.762	0.174
	Density	0.556	0.952	0.987	0.371
VON	Morph. Meas.				
	Area	**0.001**	0.171	**<0.001**	**0.019**
	Cell count	**0.005**	0.530	**0.009**	**0.017**
	Density	0.118	0.428	0.135	0.218

Table 4. Statistical *p*-values for Area, Cell count and Density between sex groups. Two-sided p-values, for each morphometric parameter, using the Kruskal-Wallis test (comparison among all three populations) and the Mann-Whitney test (pairwise comparison between populations). Pairwise Mann-Whitney p-values were adjusted by the Bonferroni-Holm-Shaffer method. 5% and 10% significant p-values are highlighted in bold and italic respectively.

	Aspect under Testing	Location					
	Directional Comparison	F > FM	F > M	FM > M	F < FM	F < M	FM < M
SCN	Morph. Measure						
	Area & Cell count	0.999	**0.017**	**0.001**	**0.006**	0.919	0.999
	Density	0.547	0.791	0.732	0.454	0.210	0.269
VON	Morph. Measure						
	Area & Cell count	0.038	**0.001**	**<0.001**	0.999	0.999	0.984
	Density	0.999	0.999	0.996	0.054	**0.002**	**0.004**
	Aspect under Testing	Scatter					
	Comparison	F > FM	F > M	FM > M	F < FM	F < M	FM < M
SCN	Morph. Measure						
	Area & Cell count	0.447	0.031	**0.020**	0.194	0.931	0.994
	Density	0.696	0.431	0.285	0.305	0.570	0.715
VON	Morph. Measure						
	Area & Cell count	**0.014**	0.059	0.408	0.708	0.790	0.429
	Density	0.999	0.975	0.917	0.317	0.077	0.084

Table 5. One-sided location and scatter bivariate and univariate permutation p-values by nucleus. In each cell, two populations are compared following the directional comparison ">" (greater than) or "<" (smaller than). Location or "mean" will show whether a population is likely to take larger (">") or smaller ("<") morphometric values compared the other. Scatter or "variance", will show whether one population has more (">") or less ("<") scattered morphometric values around the mean, compared to the other population. 5% and 10% significant p-values are respectively highlighted in bold and italic (since we jointly considered both one-sided alternatives, the actual α-level must be $\alpha/2$). P-values were adjusted by multiplicity using the Bonferroni-Holm-Shaffer method. Further explanation can be found in the Appendix.

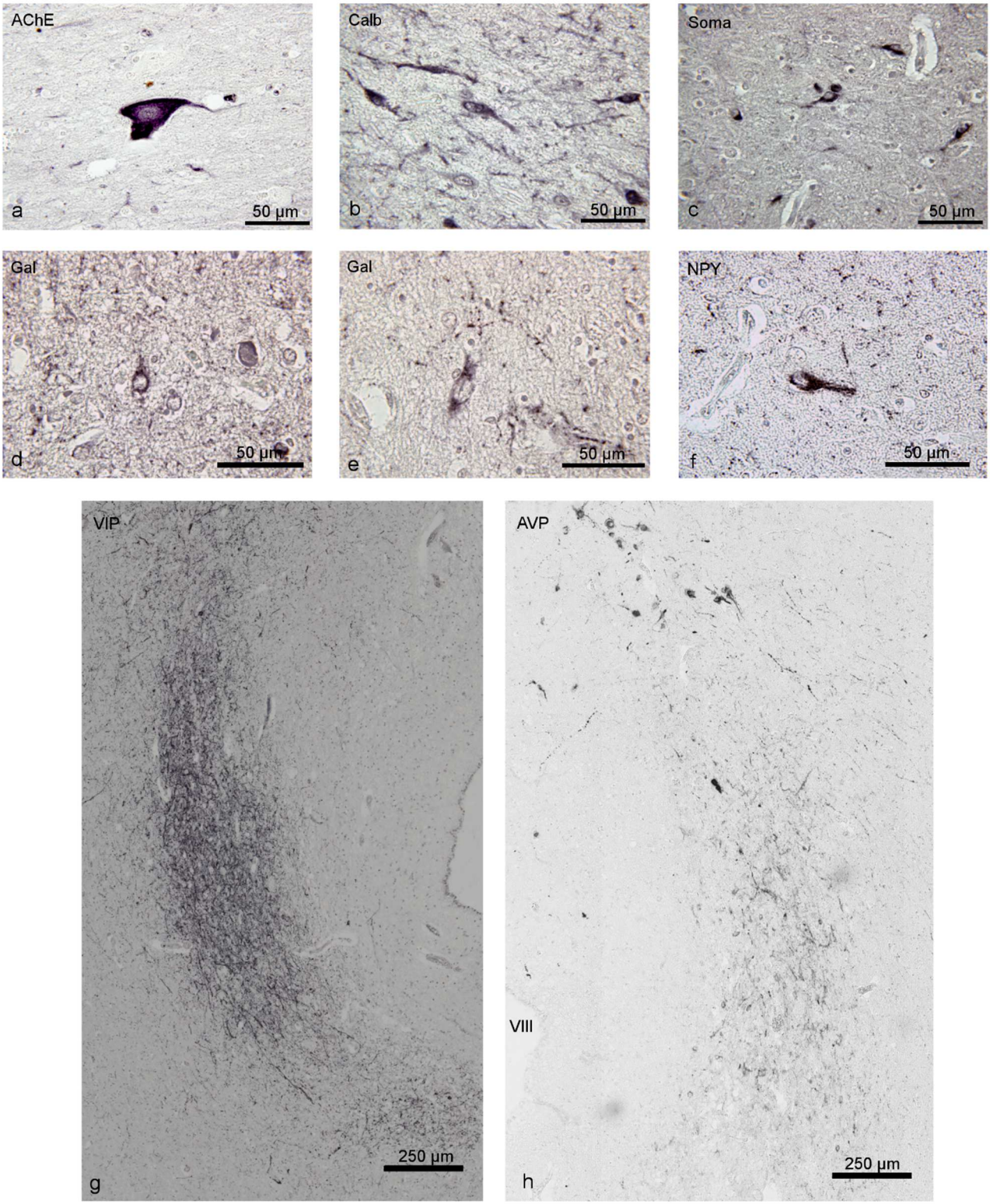


Figure 5: Immunoreactive neurons in bovine forebrain areas. **a)** AChE: Acetylcholine esterase in the NBM; **b)** Calb: Calbindin in the LSA; **c)** Soma: Somatostatin in the NBM; **d)** and **e)** Gal: Galanin in the MPOA and the DBB; **f)** NPY: Neuropeptide Y in the nucleus accumbens; **g)** VIP: Vasoactive Intestinal Peptide in the SCN. VIII:Third ventricle; **h)** AVP: Vasopressin in the SCN note the AVP magnocellular neurons marked in the upper left corner.

DISCUSSION

The main findings of this study are that in *Bos taurus* there is evidence to suggest the presence of a sexually dimorphic VON in the bovine hypothalamus that is distinct from the SON and the PVN. The VON was 33.7% larger in females than in the males. The VIP-expressing cells of the SCN had also a sex difference, since the female SCN was 36.1% larger than in the males. Moreover, in freemartins the VON was larger than in the male and smaller than in the female, and that the SCN was 32.5% larger than in both sexes with 29% more cells (Table 3).

When quantitatively studying a nucleus' morphology, it is worth noting that volume and section data are two sides of the same coin. Assuming no dimorphism does exist, one would expect to observe no difference in the mean of volumes between subjects sampled from different sex-related populations, as well as no differences in the mean of areas and cells counts measured on the set of slides obtained from the same subjects. Conversely, if one population had a larger nucleus, one would expect to observe larger area and possibly a higher number of cells as evidence of a larger nucleus. The distinction between using section data or volume data lies in the possibility of dealing with less or more aggregated information but not in the essence and interpretation of results.

Classical vs. Multi-aspect Statistical Analysis

One of the pillars of neuroanatomy is to study and understand the relation between brain function and its underlying structure. In this context, efforts in statistics can help unravel new methods able to systematically extract useful information from the neuroanatomical data.

Two-tailed non-parametric tests are widely used in biomedicine to study potential differences in one parameter between two populations (Daniel and Cross, 2013). These standard univariate statistical tests are based on the means (location) of any single morphometric descriptor. These methods have two main drawbacks; 1) they do not account for the variance (scatter) aspect of the morphometric descriptors, and 2) since they do not jointly consider the set of all morphometric descriptors, they do not support a broad scope on the problem at hand.

However, in an effort to propose more detail on the morphometric measures we acquired, we also considered a more sensitive statistical approach able to help identify finer differences between sexes. We adopted a multivariate permutation test on location and scatter, homologous to mean and variance, on the primary data collected (cell counts and areas). This allowed a deeper analysis and showed for example that males had a higher density than both females and freemartins in the VON (Table 5). The novelty of this approach lies in simultaneously testing the hypothesis on the location and scatter of a multivariate set of morphometric descriptors, taking into account the variance (scatter) of these morphometric descriptors. The results of this approach are multivariate in nature, introducing a general view on the data (Arboretti Giancristofaro, R., Bonnini, S., Corain, L. & Salmaso, L., 2014). Multivariate and univariate analyses examine different aspects of data; one is not necessarily broader or more general than the other. The advantage of the multivariate approach in this case is to examine aspects of location and dispersion of cell count and area measures that relate to the joint distribution of these variables.

Vasopressin-Oxytocin containing Nucleus

This paper is the first arguing for the distinction of the VON in cattle. Table 6 summarizes the comparative nomenclature used from 1938 in the scientific literature for the magnocellular neuron groups in different species. Frank van Eerdenburg and colleagues firstly introduced the VON in the

pig (van Eerdenburg *et al.*, 1990). Originally most authors found these cells to be accessory subnuclei to the PVN and the SON (Koikegami, 1938; Scharrer and Scharrer, 1954; Dellmann, 1959; Morton, 1969; Junge, 1977), based on various species. Other research groups chose to consider it a different entity (Vierling, 1957, 1958; Gadamski and Lakomy, 1973; Szteyn *et al.*, 1981; Braak and Braak, 1987; van Eerdenburg *et al.*, 1990) while later, the concept of islands being part of a supraoptico-paraventriculo-hypohysal system emerged (Dierickx and Vandesande, 1977; Silverman and Zimmerman, 1983; Marani, 1990; Møller *et al.*, 2018) following the human structure of these clusters. We subscribe to the argument of van Eerdenburg et al., (1990) in that the VON is obviously related to the PVN and SCN, because of its content of AVP and OXT and cell size, and is part of the hypothalamic magnocellular neurosecretory system although in the case of the pig and cattle at least, the organization into one distinct nucleus is striking (Fig. 2 and 3a,b). The nucleus itself is constituted of two clearly separated AVP-ir and OXT-ir neuron groups (adjacent sections in Fig. 3a, b). They seem to be predominantly distributed with AVP cells on the ventrolateral side sometimes tending to

Author	Year	Species	Name	Notes
Koikegami	1938	Mole rat Bat Squirrel Cat Rhesus monkey	Accessory nucleus of SON or PVN	Not present in every species.
Scharrer and Scharrer	1954	Reptiles Birds Mammals	Nucleus supraopticus accessorius	Small groups of perivascular cells
Vierling	1957 1958	Bovine	Nuclei intersupraoptico-paraventricularis	Compact clusters of cells
Dellmann	1959	Bovine	Accessory part of the PVN	
Morton	1969	Human	Accessory supraoptic nucleus	Separation in two clusters
Gadamsky and Lakomy	1973	Bovine	Nucleus intersupraoptico-paraventricularis	Oval shape, close arrangement. Cells similar to the PVN
Dierickx & Vandesande	1977	Human	Intersupraoptico-paraventricular islands	
Junge	1977	Bovine	PVN pars accessoria	
Szteyn et al.	1981	Bovine	Nucleus intersupraoptico-paraventricularis	Closely arraged large cells
Silverman and Zimmerman	1983	Human, mouse	Irregular string of cells	
Braak and Braak	1987	Human	Accessory neurosecretory nucleus	high density, large soma size, dense capillary network
Marani 1990		Reptiles Birds Mammals	Supraoptico-paraventriculo-hypophysis system	Cell nests connecting the PVN with the SON
van Eerdenburg et al. 1990		Pig	Vasopressin-Oxytocin containing Nucleus	High density, large soma, vasopressin and oxytocin reactivity
Møller et al.	2018	Human	Accessory magnocellular neurosecretory system	Scattered, different in man from other mammals

Table 6 Comparative names given to the magnocellular structures between the SON and the PVN in different species List of relevant literature concerning the magnocellular cells between the SON and the PVN. Notes have been added where comments were made about the structure of the cell groups.

surround a more dorsomedial OXT core (Fig. 3b). Its specific function has, however, still to be revealed since there did not seem to be a preferential orientation of the axonal processes, either centrally or down towards the pituitary gland. Although, tracts were especially seen in the AVP-ir neurons, with a general fiber orientation between the PVN and the SON surrounding the nucleus (Fig. 3a).

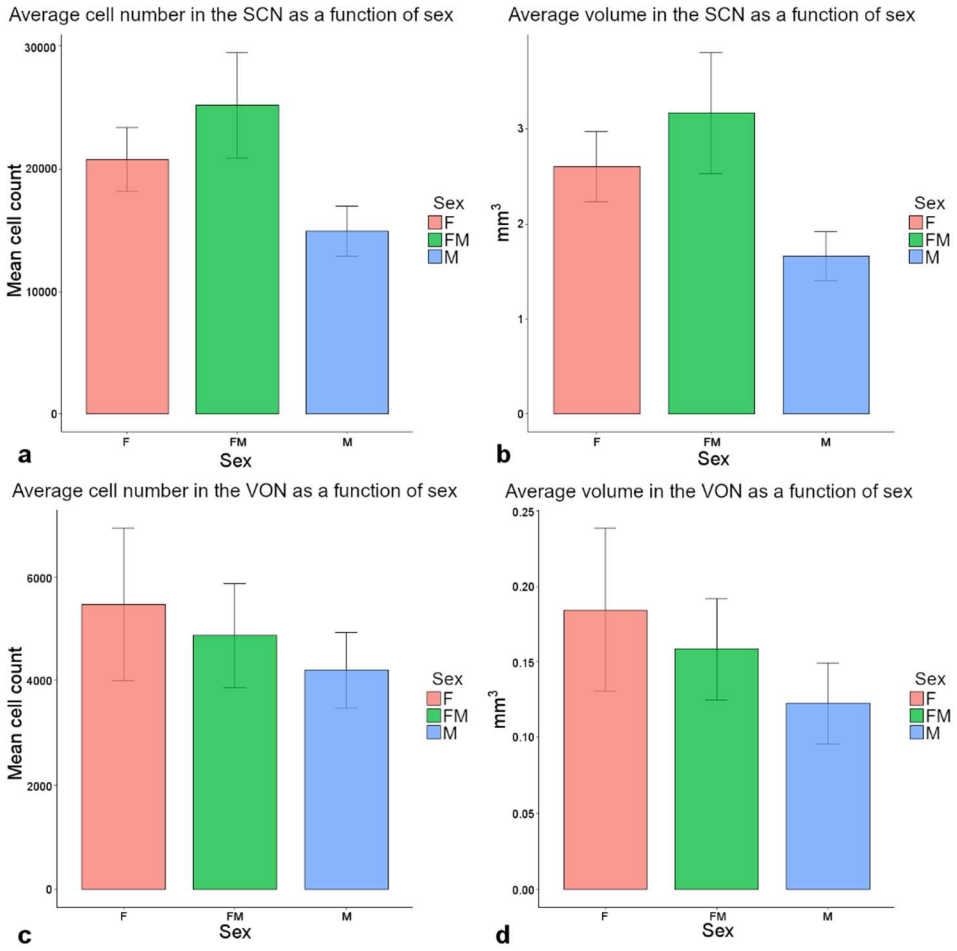


Figure 6: Histograms showing the total cell counts and volumes of the SCN and the VON. **a)** Average cell number in the SCN as a function of sex; **b)** Average volume of the SCN as a function of sex; **c)** Average cell count in the VON as a function of sex; **d)** Average volume of the VON as a function of sex . Error bars represent the standard error of the mean. FM: freemartins; F: females; M: males

Species differences

The VON was found to be sexually dimorphic but it is not homologous to the Intermediate nucleus (SDN, INAH1), since the latter does not contain AVP or OXT. (Braak and Braak, 1987) describe the intermediate nucleus in man in a clearly different position from the neurosecretory accessory nucleus. Furthermore, galanin-positive cells are abundantly present in the Intermediate nucleus of the human brain (Garcia-Falgueras *et al.*, 2011), but are absent in the VON. This does not necessarily mean a total absence of a homologous functional area, but rather a different anatomical organization, which in turn may have purposeful connectivity consequences. Cells staining for galanin in the anterior hypothalamus may be homologous to the intermediate nucleus but are arranged more diffusely in the bovine hypothalamus (Fig. 4c). In the opposite way, the cells forming the VON are probably present more diffusely in other species, such as the anterobasal PVN in the rabbit (Schimchowitsch *et al.*, 1989), or the anterior PVN in the cat (Caverson *et al.*, 1987). Another possibility is that the VON is a different entity from the

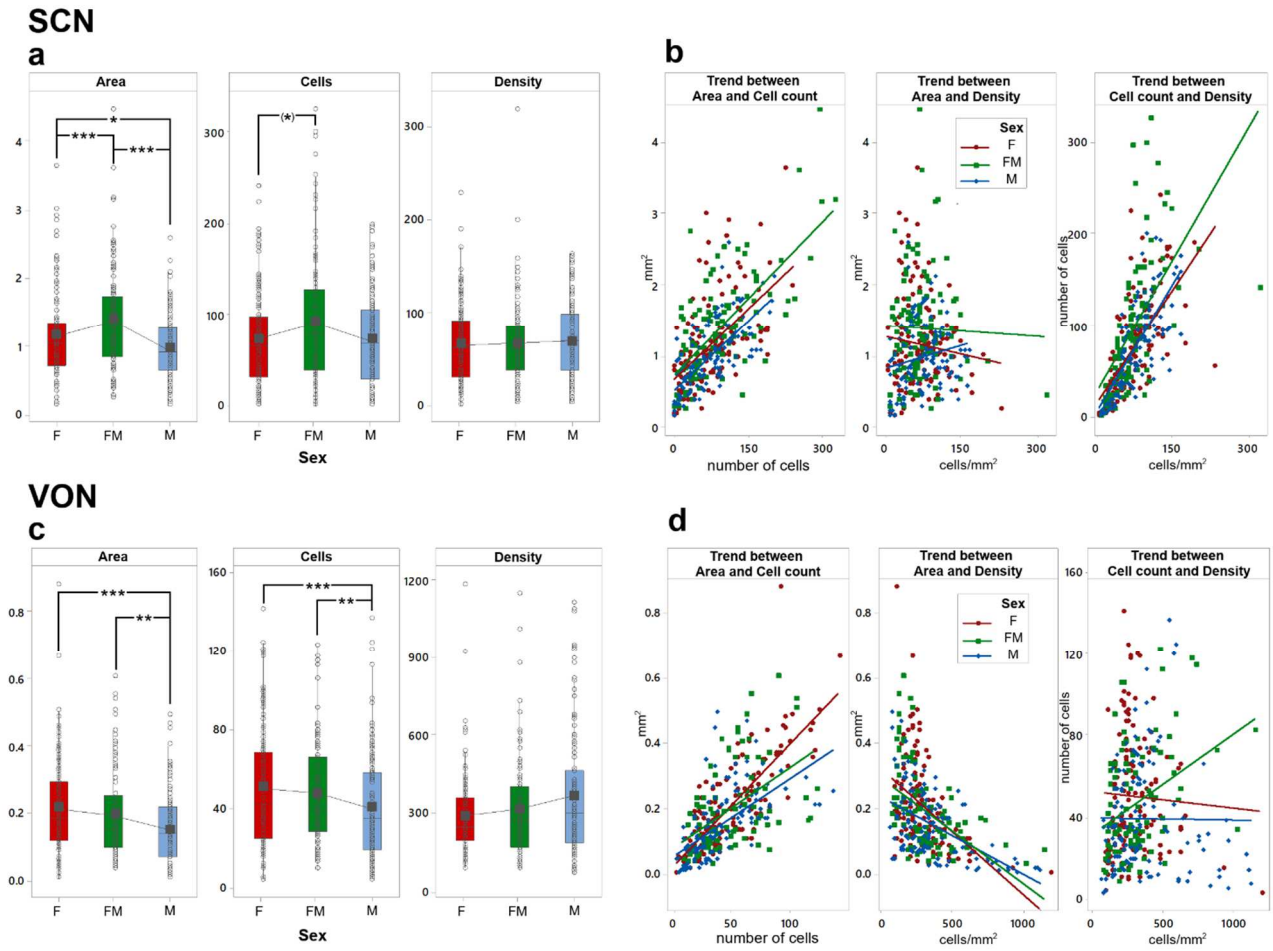


Figure 7: Graphical representation of the statistical analysis. Boxplot for SCN (**a**) and VON (**c**) nucleus by sex and morphometric feature (area, no. of cells, cell density), where solid dots connected by line do represent the sample means. As one box shows a relative upwards/downwards shift this is a descriptive indication that the related population is distributed on larger/lower morphometric values. Significance levels according to the Mann-Whitney test are indicated by asterisks: *** < 0.01, ** 0.01 to 0.025, * 0.025 to 0.05 (see Table 4 for *p*-values). Scatterplot for the SCN (**b**) and the VON (**d**) nucleus by morphometric feature pair and sex (using colored dots). In both the SCN and the VON, the area and cell number are positively correlated. Note that for the SCN area and density are not positively correlated (as one increases, the other feature does not). Conversely, the VON area and density are inversely correlated (as one feature increases the other feature tends to decrease). Finally, note the much more scattered pattern in the no. of cells vs. density plot appearing in VON than in SCN, suggesting that those features are almost independent in VON, while in SCN are strongly positively correlated.

accessory neurosecretory system, that is the more diffuse magnocellular accessory nuclei found between the SON and the PVN, the accessory neurosecretory system, in the hypothalamo-hypophysial tract (Møller *et al.*, 2018). In sheep, Vierling, (1957) described a group of cells similar to the VON although as part of the PVN, comparable to what was later described in the horse as the ventral PVN (Melrose and Knigge, 1989), which tends to show that the VON could be a common feature in large herbivores. In *Bos taurus* we found that the VON presented rare cells positive to calbindin (Fig. 3d) that were not reported in the pig (van Eerdenburg *et al.*, 1990), while calbindin has been used to stain the SDN-POA in rats (Brager, Sickel and McCarthy, 2000; Sickel and McCarthy, 2000). This finding suggests an SDN homology, although the cells were not abundant and did not cover a large part of the nucleus like in the rat.

The *nucleus circularis* (NC) (also called accessory supraoptic nucleus in the rat in Bodian & Maren, (1951)) was clearly present in the pig, disambiguating it from the VON (van Eerdenburg and Swaab, 1991). It is also present in rats (Price Peterson, 1966), hamsters (Ferris *et al.*, 1989), cats (Caverson *et al.*, 1987), dogs (Laqueur, 1954), and was recently reported in the human hypothalamus (Møller *et al.*, 2018) and has been linked to osmotic functions in rats (Wallace and Harrell, 1983) and flank marking in hamsters (Ferris *et al.*, 1989). We were not able to detect any NC in the bovine hypothalamus. Despite a comparable packed aspect, and positivity to OXT and AVP, the position of the NC in the cat and the pig is more dorsolateral than the VON in the pig and the bovine, leaving little doubt for the absence of homology. Additionally, a characteristic of the NC is the presence of a large blood vessel, which is not the case of the VON. The reduction of the number of AVP somas in the NC of hamsters infected by scrapie, a transmissible neurodegenerative disease (Ye *et al.*, 1994), may help to differentiate it from the VON.

Consequently, although this is subject to discussion, we argue, based on the existence of the VON in the pig (van Eerdenburg et al., 1990), that there is a homologous nucleus in the bovine, another terrestrial Cetartiodactyla. Such a structure could be a feature of this clad of mammals, which should be investigated.

Sex differences

Unlike in the pig, we found that cows had a larger VON than bulls. It is situated, like in the pig, between the SON and the PVN, dorsal to the SCN. The relative size of the bovine VON is similar to that of the pig, since its volume is over 3 times larger than in the pig at puberty with 0.15 mm^3 versus 0.054 mm^3, considering a brain weight 3.5 times heavier, with 476 g for the bovine (Ballarin *et al.*, 2016) and 135 g for the pig brain (Minervini *et al.*, 2016). The sex difference of the bovine VON is of a different order than that of the SCN. The VON size and cell number were larger in females while males had a significantly smaller nucleus and freemartins had an intermediate position (Fig. 6, 7).

However, the VON does not fit in the general masculinization model, given that, unlike the rat or ovine SDN (Roselli *et al.*, 2004), but more similarly to the infundibular nucleus (Taziaux, Swaab and Bakker, 2012; Taziaux *et al.*, 2016), the female nucleus is larger than the male. This could mean that the VON function might be linked to female reproduction functions such as lactation, in which oxytocin plays a crucial role, or more generally by central effects on reproductive behavior. In van Eerdenburg and Swaab (1991), 130 weeks old female pigs display a VON twice larger than males. One explanation to the increase in number of cells in the VON postnatally in females is parturition and lactation. In swine, litters are typically large (usually over 10 piglets), which is not the case in cattle (1-2 calves). Further studies with different age groups, with or without calving may yield more information on its function.

SCN

Species differences

The VIP-stained bovine SCN was much larger than what is described in Okamura (2002) using Nissl staining, and compared to the VIP-stained human SCN (Zhou, Hofman and Swaab, 1995b, 1995a). The largest part of VIP cell bodies was present in the medioventral part of the bovine SCN as in the human (Swaab et al., 1994), ovine (Tessonneaud *et al.*, 1994), the camel (El Allali *et al.*, 2017) and rodent SCN (Abrahamson and Moore, 2001). This is in contrast with the SCN of the opossum, a marsupial (Cassone *et al.*, 1988), in which VIP neurons are localized dorsally. NPY has been described in neurons of the human SCN (Mai *et al.*, 1991), while the SCN of rodents (Moore, Gustafson, & Card,

1984; Moore, Speh, & Leak, 2002), and the camel (El Allali *et al.*, 2017), contained only fibers, particularly sparse in the sheep (Tillet, Caldani and Tramu, 1989). The present study found that the SCN of cattle contained sparse NPY-ir fibers, originating from the geniculate body and transmitting non-photic information to the SCN (Saderi *et al.*, 2013), as in the sheep. Calbindin was found anteriorly to the SCN but no neuron body was found in the SCN itself, which is unlike in the camel (El allali et al., 2017) and human (Mai, Kedziora, Teckhaus & Sofroniew, 1991). In humans, the SCN does contain galanin somata (Gai, Geffen and Blessing, 1990) while in the rat only a few galanin immunoreactive neurons locate in the SCN (Melander *et al.*, 1985). Sparse galanin-ir fibers were seen in the SCN of the bovine, which is coherent with other mammals like opossums and pigs (Elmquist *et al.*, 1992; Pearson, Anderson and Jacobson, 1996). Finally, AVP was considered absent from the SCN in bovines (De Mey, Vandesande and Dierickx, 1974) but we found some AVP neurons and fibers running through the SCN with clear varicosities (Fig. 4h). The general distribution of AVP and VIP described by Abrahamson and Moore (2001) in the mouse is, despite some overlap, similar to what we found in the bovine SCN, with a ventrolateral core positive to vasoactive intestinal peptide (Fig. 4g) and a medial rather than dorsomedial shell positive to vasopressin (Fig. 4h). As expected, the suprachiasmatic nucleus showed no oxytocin positive signal, which is in accordance with most mammals, except notably in the horse (Melrose and Knigge, 1989).

Sex differences

A significant sex difference was found in the number of VIP stained neurons between males and females ($p = 0.017$, Table 5) where females had a larger nucleus than males. This is different from previous findings in humans regarding VIP-expressing neurons (Swaab et al., 1994), where the adult male SCN has been found to contain twice as many VIP neurons as the female's, although this varies with old age (Zhou, Hofman and Swaab, 1995b). Results similar to humans have been found in rats and gerbils, using Nissl staining (Gorski *et al.*, 1978; Robinson *et al.*, 1986; Holman and Hutchison, 1991) where the male SCN was larger than the female's. Although no OXT neurons can be found in the SCN, the timekeeping and synchronicity role of VIP has been demonstrated (Maywood *et al.*, 2006). The peculiarity of the situation in cattle may be related to advanced breeding selection for milk production, which is indeed influenced by the circadian rhythm (Plaut and Casey, 2012).

Other areas

The cattle hypothalamus follows the general mammalian blueprint with distinctive minute relative difference in the distribution of markers. Notably, the BST is positive for somatostatin, but does not display a well bordered nucleus in cattle like it does in the human brain (Kruijver, Zhou, Chris W. Pool, *et al.*, 2000), rather an irregular area with dense fibers and few parvocellular neurons instead (data not shown here).

As it has been described originally in details in cattle (De Mey, Vandesande and Dierickx, 1974; De Mey, Dierickx and Vandesande, 1975; Vandesande, Dierickx and De Mey, 1975b, 1975a), AVP and OXT in the hypothalamus are mostly distributed in the PVN and the SON. In accordance with De Mey et al. (1974) bovine OXT-ir cells were strongly present as a thin cap of densely packed magnocellular multipolar neurons dorsal to the AVP-ir cells of the SON, as well as in the lateral part of the PVN (Fig. 3b, Fig. 4). In their study of the neurophysin-producing neurons in the bovine hypothalamus, Vandesande et al., (1975b, 1975a) found a predominating presence of AVP in the SON and of OXT in the PVN. We did find more AVP-ir neurons in the SON, but the PVN did not exhibit qualitatively

more OXT-ir than AVP-ir somata. Moreover, it appeared that AVP cells in the PVN were localized slightly more medially than OXT-ir neurons (Fig. 3a,b, Fig. 4).

The *nucleus accumbens* is one of the largest sources of NPY after the hypothalamus in the human (Hendry, 1993), and rat brain (Brown, Coscina and Fletcher, 2000), similarly to what we found in the bovine. In the bovine hypothalamus area corresponding to the human medial subnucleus of the medial preoptic area reported by Koutcherov, Paxinos, & Mai, (2007), we found dense clusters of NPY-ir fibers as well as Calbindin-ir fibers (Fig. 4d). Compared to the rat, Koutcherov et al., (2007) also noted the more diffuse aspect of neuronal groups in the human hypothalamus.

To our knowledge, this study is the first anatomical description of galanin in cattle hypothalamus. Unlike in the sheep (Chaillou *et al.*, 1998; Chaillou, Tramu and Tillet, 1999) and humans (Gai, Geffen and Blessing, 1990), we found no galanin-immunoreactive cells but only galanin-ir fibres in the infundibular region. Galanin fibers and cells are typically found in the INF of sheep (Chaillou & Tillet, 2005) and rat (Ciofi, Leroy and Tramu, 2006). The bovine exhibits the same general organization of fibers, but we found no galanin-positive neuron somas in the INF of cattle.

AChE staining was found throughout Ch1-Ch4 described in Mesulam, Mufson, Wainer, & Levey, (1983). Namely, AChE-ir neurons were found in the medial septal area (MSA), like it was found in humans (Swaab, 2003), as well as the diagonal band of Broca and the nucleus basalis of Meynert. The topography of the islands of Calleja, characterized by AchE-if fibers (Alheid, Switzer III and Heimer, 1990) and located between the ventral pallidum, the diagonal band of Broca and the substantia innominata, was consistent with what was reported in other species and orders (Meyer *et al.*, 1989).

Sex differences and the freemartin syndrome

Freemartin heifers show an intermediary development of the VON between the male and the female regarding size and cell number. In the developing bovine hypothalamus, the critical period for sexual differentiation occurs in early gestation phases, during the second quarter of gestation (Peruffo, Cozzi and Ballarin, 2008). It has been shown that during this time, estrogens stimulate sexually dimorphic patterns of development by binding to α and β estrogen receptors. In this context, the freemartin VON may have an early intermediate development disturbed by the male hormonal fetal environment. As it is well known that steroid hormones influence neurogenesis and dendritic growth on neural cells, these effects may have influenced sexual dimorphism by increasing the overall number and size of neurons within the hypothalamic nuclei, including the VON. A recent paper showed that estrogens exposure exerts a trophic effect on developing bovine granular neurons (Montelli, Suman, *et al.*, 2016). Previous work on estrogen receptors α and β in Cetartiodactyla (including the ovine and the bovine) (Peruffo *et al.*, 2008; Schaub *et al.*, 2008; Panin *et al.*, 2015), suggest that, in freemartins, the brain of the female twin exposed to male androgens undergoes a partial masculinization either through aromatization into estrogens and activation of ER-α and ER-β (Kudwa *et al.*, 2006; Roselli, Liu and Hurn, 2009), or *via* the direct effect of androgens on androgen receptors.

Freemartins seem to develop a larger SCN that contains more VIP-expressing neurons and a larger volume than both cows and bulls. The ratio between the number of cells and the area of measure also shows that the global density remains very comparable across the 3 groups, which means that there is a certain homogeneity in the cell density of the SCN across sex categories. Thus it seems that the general cellular organization remains the same. In order to reach this larger size and number of cells, the freemartin SCN, most likely during brain development after a peak value, does not lose as many VIP-expressing neurons as male or female bovine do to reach normal levels, as it has been

proposed in humans for AVP-containing neurons in the SCN (Swaab, Hofman and Honnebier, 1990; Swaab *et al.*, 1994). There is a case of larger SCN than both male and female, which has also been described, concerning AVP-ir neurons of the SCN in relation to homosexuality. In their article on the SCN of homosexual men, Swaab & Hofman, (1990) noted an enlargement of the SCN by 2.1 times in AVP expressing neurons and 1.73 times in volume relatively to heterosexual men (Swaab & Hofman, 1990). However, this difference was not present in the VIP-containing cells (Zhou, Hofman and Swaab, 1995a). The observations showed that the SCN of homosexual men was not an intermediary of male and female SCN, which did not contain a sex difference (Swaab *et al.*, 1995). In the case of freemartin heifers, a link to the sexual behavior consequences of this difference would be hard to establish. No behavioral comparison has been made for this study, although the freemartin syndrome arises in a species in which one of the most reliable signs of estrus is the active and passive mounting between females in the herd, hence a typical male-type behavior cannot be easily characterized.

The development of the SCN seems to be influenced by sex hormones. In male rat, the prenatal administration of ATD (1,4,6-androstatriene-3,17-dione), an aromatase inhibitor that precludes the formation of estrogens from testosterone, induced a bisexual partner preference and a greater number of AVP-positive neurons in the SCN (Swaab *et al.*, 1995). In addition, a more recent study involving letrozole, an aromatase inhibitor, in prenatally treated male rats showed a reduced volume and cell number in the SDN and SCN, independently of sex preference (Olvera-Hernández *et al.*, 2017). The prenatal treatment alone of ATD did not change the partner preference, but the postnatal treatment did not change the AVP neuron number in the SCN. This fact, along with the fall of Anti-Müllerian Hormone (AMH) 9 days after birth in freemartin cows (Rota *et al.*, 2002), suggests that sexual behavior itself may be more intimately linked to the circulating hormones at the time of the behavior via binding to the expressed receptors. On the other hand, the prenatal hormonal environment could more probably influence directly or indirectly the development of the circuitry necessary for the said behavior, as described in humans by (Kruijver and Swaab, 2002), since in the case of freemartin heifers, androgens return to female levels after birth (Rota *et al.*, 2002). In both the VON and the SCN, it seems that the development of the female (XX) nuclei is disturbed by androgens, either directly through androgen receptors, or through aromatization of androgens and adhesion to estrogen receptors. The VON appears masculinized, with a reduction of the volume and number of neurons, while, the SCN shows a notable overgrowth. This difference between the VON and the SCN, exposed to the same prenatal hormonal environment, could be explained by a difference in estrogen and/or androgen receptor expression or subtype, as no estrogen receptor was found in the VON of adult pigs (van Leeuwen *et al.*, 1995) while they are present in the adult SCN (Kruijver and Swaab, 2002). Since the VIP neurons of the SCN influence daily rhythm synchronicity, their role in milk production should be further explored. Moreover, the potential effect of the sex chromosomes in the cells themselves and their reaction to one or the other hormonal environment must be taken in account, as it was shown by Moore et al., (1957) that the freemartin neuron chromatin was not altered by the prenatal male hormonal environment. This could mean that the methylation of freemartin neurons' DNA is that of females and not males (McCarthy et al., 2009), ultimately influencing gene expression. Our results show a difference in the organization of the SCN in freemartin bovine that is prenatally determined by a male hormonal environment, and might be a marker for the development of an alternative neuronal network. In details, few studies have studied the anatomy of the bovine hypothalamus from a chemoarchitectonic perspective (Leshin *et al.*, 1988, 1995). As of today, relatively few studies use bovine brain tissues for comparative purposes. However, public awareness and ethics influence the use of animals in translational studies, and consequently increasingly weigh on regulations and study

designs. In this regard, brain tissues from slaughterhouses can be a valuable ethical alternative. This brings forward the importance of multiple animal models in the understanding of physiological processes and anatomical structures. Additionally, as stated by (Short *et al.*, 1970), the freemartin syndrome has attracted in the past the attention of leading specialists and, although a large part of the puzzle has now been explained, its potential contribution to neuroscience remains unknown.

CONCLUSIONS

The neurochemical organization of the bovine hypothalamus is comparable to that of other mammal. In our investigation, we found that the SCN was sexually dimorphic, and that there was a compact group of cells between the PVN and the SCN that we called the VON, which was also sexually dimorphic. This sex difference, unlike in other species, consisted of a larger female nucleus than males'. Freemartin bovines were found to have a much larger SCN than males and females, and an intermediate VON between males and females. This finding confirms the prenatal organizational effects of androgens on the development of the brain. More broadly, the present study emphasizes the effects of prenatal steroids on the brain of precocious mammals such as cattle, and the disturbances that may arise from a misbalance of such steroids in the pregnant cow. In particular, the similarity with the increased SCN size, although in AVP cells, in homosexual men found by Swaab & Hofman, (1990) is intriguing. Further studies on this specific animal model could increase our understanding of the complex interactions between structure and function in neurology (de Vries and Södersten, 2009; Yang and Shah, 2014; McCarthy, 2016).

II.B.2. THE CEREBELLUM OF *BOS TAURUS*

Multi-aspect testing and ranking inference to quantify sexual dimorphism in the cerebellum of *Bos taurus*.

SUMMARY

We designed a novel approach to quantify the morphology of large cohorts of cells in digitalized images, and rank the populations along defined factors such as sex, age or pathology. To test this method we applied it to the study of cerebellar tissue, namely to compare molecular, Purkinje and granular layer of lobules VIII and IX among bovine males, females and freemartins, a well-known sex differentiation syndrome.

This methodology comprises a statistical multivariate and multi-aspect testing, able to co-involve several morphometric parameters and compare them jointly, to quantify cell populations more globally than a univariate method would. Traditional univariate statistical analyses are based on the mean (location) of one morphometric descriptor. This approach can only describe a group of cells over one dimension, and does not reflect the variability (scatter) and multifactorial aspect of nervous tissues. Our non-parametric method allows to test a hypothesis over both location and scatter in a set of morphometric descriptors simultaneously.

We chose a set of parameters to describe the cells individually (morphometric descriptors), regrouped in domains (size, regularity and density). We finally defined the notion of cytoarchitectural complexity to be the cells descriptors' variability in a given region. Complex populations will have a larger scatter distribution, while uniform populations will show a reduced scatter around a given form.

Females had smaller, irregular and denser cells than males in the molecular layer. Female Purkinje neurons were conversely larger than males, but more irregular and more densely positioned. In the granular layer, females had larger, more irregular and denser cells than males. Freemartins had the size and regularity of females but an intermediary density in the molecular layer, while in the granular layer, they presented the largest and most regular cells, with the most neighboring cells.

This example proves that our methodology could provide a robust base for tissue screening, notably involved in neurodegenerative pathologies.

INTRODUCTION

Cell shape can be a powerful indicator of function. Pyramidal cells in the cortex are famously recognizable for their shape. In the cerebellar cortex, Purkinje cells are also easily recognizable, and highly conserved among mammals (Jacobs, Johnson, *et al.*, 2014). However, the analysis of cellular morphology and cytoarchitecture in the brain remains a challenge, translating observed differences into quantitative measures (Lobo *et al.*, 2016). To quantify the distribution of specific neurons is also crucial to detect pathological alterations, and morphometric parameters such as reduced neuronal size, decrease in volume or density could be aspects of brain pathogenesis (Silvestri *et al.*, 2015). In this context, the capacity to gather such data reliably and to statistically extract useful information requires new methodologies (Bowman, Guo and Derado, 2007; Pincus and Theriot, 2007; Ozaki, 2014).

The concept of morphometry, i.e. the study of cell shape and volume (Haug, 1986) started at the dawn of the XXth century, however it was broadly used mostly from the 1980s. Quantitative geometrical analyses of cell structure and sub-cellular components were done using specific descriptors and tools, used to translate qualitative differences into quantitative measurements

(Pasqualato *et al.*, 2012). Recent neuroanatomy however requires the development of statistical methods focusing on several parameters simultaneously, and keep a general view on the structure studied.

The mammalian cerebellum represents about 13.5% of the total brain volume across mammals (Clark et al., 2001; Jacobs et al., 2014), and can be divided in four parts: the anterior lobe, the lobule simplex, the posterior vermis and the paired hemisphere (Voogd, 1998a). The paramedian sulcus separates the vermis from the hemispheres. The vermis is further divided into four transverse zones: the anterior zone, comprising the lobule I to V, the central zone (lobules VI-VII), the posterior zone (lobule VIII-IX) and the nodal zone (lobule X) (Figure 1). Particular to Artiodactyla is the fact that the width of the hemispheres of the anterior lobe is smaller or equal to that of the vermis. The lobule simplex is large in Perissodactyla and Artiodactyla, and even larger in Proboscidea (Maseko *et al.*, 2012). In *Bos taurus*, the folia of the vermis is divided into segments, which expand locally into lobules: the lingula (I), the lobulus centralis (II and III), the culmen (IV and V), the declive (VI), tuber (VII), pyramis (VIII), uvula (IX) and nodule (X). Frequent variation can be seen (Voogd, 1998a). Although most of the structure of the cerebellum is commonly agreed upon, results also diverge partially on functions associated with regions (Mottolese *et al.*, 2013; van der Zwaag *et al.*, 2013).

On a cytoarchitectural level, the cerebellum is composed of three layers, conserved in mammals. At least four types of inhibitory interneurons are present in the cerebellar cortex. The external layer (molecular layer) is composed of stellate cells, basket cells, and the dendrites of Purkinje neurons. The middle layer is a monolayer of large Purkinje cells, as well as candelabrum cells (Lainé and Axelrad, 1994) and Lugaro cells (Lainé and Axelrad, 2002). The innermost layer (granular layer) is composed of a great number of granule cells (Voogd and Glickstein, 1998) as well as Golgi cells.

Granule cells are small (about 10 µm) but very numerous, since they are the largest population in the brain (Sotelo, 2015). Functionally, they synapse with mossy fibers and Purkinje neurons and participate in the control of information (D'Angelo and De Zeeuw, 2009). Also present in the granular layer are Golgi cells, which are larger than granule cells (about 30 µm). While several subtypes exist (Sillitoe, Fu and Watson, 2012), all are interneurons. Both granule and Golgi cells seem to be larger in the vermis than the hemispheres (Lange, 1982; Geurts *et al.*, 2001). Additional local differences in cell types and expression markers have been reported (Cerminara *et al.*, 2015). Molecular layer interneurons (MLIs) are stellate and basket cells. Their physiology has been extensively studied and recently described (for a review see Sotelo, 2015). Their position in the molecular layer are different, as well as the topography of their dendrites, leading to their names given by Cajal. Basket cells are situated close to the Purkinje layer, in the inner part of the molecular layer and project their axons around the soma of Purkinje neurons (Komuro *et al.*, 2013), while stellate cells are spread in the molecular layer and synapse dendrites of the Purkinje cells (Voogd and Ruigrok, 2012). Purkinje neurons, generating the output of the cerebellum, have been reported to show different sizes between regions (Müller & Heinsen, 1984).

Sex differences in the mammalian brain have been reported in several regions and many aspects of normal and pathological brain function show sex differences (Swaab, 2003a; McCarthy *et al.*, 2012; de Vries and Forger, 2015; Marrocco and McEwen, 2016; McEwen and Milner, 2017), however differences are often subtle and local, and subject to controversy for some (Ingalhalikar *et al.*, 2014; Joel and Tarrasch, 2014; Chekroud *et al.*, 2016; Del Giudice *et al.*, 2016). In the cerebellum, there seems to be a larger total volume in males (Tiemeier *et al.*, 2010; Weier *et al.*, 2014; Kurth, Thompson and Luders, 2018), but a proportionally larger cerebellum in females comparatively to the intracranial

volume (Weier *et al.*, 2014), although some failed to confirm such results (Dean and McCarthy, 2008). MRI studies have however been increasingly used to search for sex differences (Fan *et al.*, 2010; Batson *et al.*, 2015), and a recent study found differences in lobules VII, VIII and IX (Kurth, Thompson and Luders, 2018), also noting a rightward asymmetry, more pronounced in males. An in vitro study on bovine cerebellar cell cultures showed that granule cells from females were significantly larger than males (Montelli, Suman, *et al.*, 2016). Indeed, the trophic effect of estrogens has been suggested before in the cerebellum, in coordination with X chromosome expression (Ikeda and Nagai, 2006; Abel, Witt and Rissman, 2011)

One pathology affecting overwhelmingly boys is autism, which has been heavily correlated with the cerebellum (Courchesne *et al.*, 1988; Bauman and Kemper, 2005; Hampson and Blatt, 2015). In particular, lobules VIII to X seem to be significantly smaller than control (Levitt *et al.*, 1999), although Courchesne and colleagues (1988) found vermal lobules VI and VII to be smaller. On MRI imagining, it was found that the vermis was larger (Sparks *et al.*, 2002). The same rationale can be found in the reduction of the vermis in schizophrenic patients, also males in majority (Womer *et al.*, 2016). However, studies focusing on the minute structure of sex differences in the cerebellum and their potential differential effect on pathologies are lacking.

The bovine brain has recently been reaffirmed as a model for translational neuroscience (Peruffo & Cozzi, 2014), although literature on the global bovine neuroanatomy is rather limited (Yoshikawa, 1968; Lakshminarasimhan, 1975a, 1975b; Okamura, 2002), and those particularly describing the cerebellum is even more scarce (Voogd, 1998a). *Bos taurus* has a large brain (480 g) slightly smaller than expected for an animal of its mass (Ballarin *et al.*, 2016), and a rather small cerebellum. One peculiarity in this species is the existence of a hormonal congenital syndrome named freemartinism (Tandler and Keller, 1911; Lillie, 1916), by which a female fetus is exposed to the male hormones of a male fetus during ontogeny, and subsequently undergoes a masculinization of its gonads and genital tract (Short *et al.*, 1970). This syndrome provides an interesting model to study sex differences in brain areas such as the cerebellum, in comparison with males and females.

Using detailed image analysis and individual morphometrics on large cohorts of cells in each layer of the cerebellum, we were able to apply multivariate multi-aspect statistical testing to investigate and precise controverted sex differences at the cellular level.

Material and methods

Brains from adult (24 months old) male (n = 10) female (n = 10) and freemartin (n= 8) bovines were sampled from local abattoirs in the Venetian region of Italy. As made statutory by the European Directive concerning animal welfare in commercial slaughtering, animals were under constant official veterinary monitoring and medical care. Cerebellums were collected and fixed in 4% v/w formaldehyde less than 2h *post-mortem*, for at least a month. Lobules VIII and IX were sampled (Figure 8) at the level of the vermis, washed in phosphate buffered saline 0.1M, pH 7.4 (PBS), processed and embedded in paraffin.

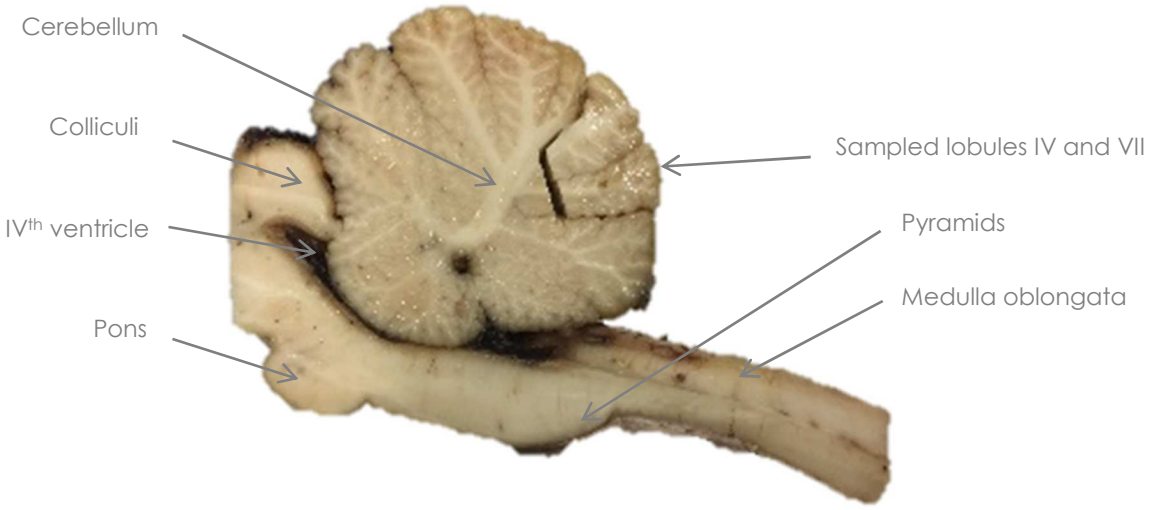


Figure 8: sampling of the cerebellum lobules in the vermis (sagittal section)

For each animal, 10 sections of 8 µm thickness were stained with Nissl technique. Briefly, deparaffinized in xylene for 3 × 5 minutes, sections were then dipped into a decreasing graded series of ethanol solutions for 3 minutes each. After a brief rinsing in distilled water, a 10 minutes bath at 50 °C in a 0.1 % cresyl violet solution was applied. Sections were then differentiated in 95 % ethanol for 20 minutes, then rinsed in distilled water, before passing through an increasing series of graded ethanol for 3 minutes each and finally in xylene 3 × 5 minutes. Sections were coverslipped using mounting medium.

Digitalization was achieved using a semi-automatic microscope (D-Sight v2, Menarini Diagnostics, Italy). Magnification was set to 40x in fast mode and automatic focus.

For the cellular detection, morphometry and statistical analysis details, see IV. B and IV.C. Briefly, ten morphometric parameters were grouped into three domains: size, regularity and density. Each parameter was tested individually, but was also weighted equally into a domain value, which was used in ranking.

Results

Generally, in the three well distinguishable layers, we could individualize two types of cells in the molecular layer, one slightly larger and closer to the Purkinje layer seemed similar to basket cells and was called “basket-like”, and one smaller and localized on the external half of the molecular layer was named “stellate-like”. The Purkinje monolayer was separating the molecular and granular layer, but no lugaro or candelabrum neuron could be reliably identified. Finally, in the granular layer, a dense population of homogeneous granule neurons was interspersed with rare larger cells that we named “Golgi-like” cells.

In the molecular layer, two distinct populations of cells seemed to stand out during the image analysis, based on their major axis size. We used this feature to segregate them in two groups: stellate-like cells were 11 µm on average, and basket like cells were 19.5 µm on average. Stellate-like cells had an average area of 73 μm^2 and a perimeter of 28 µm with a major axis length of about 11 µm. Basket-like cells had an area of 230 μm^2 for a perimeter of 55 µm, and a major axis length of about 19 µm. Stellate-like cells in females were significantly smaller than males in size (domain), except for minor axis length, while freemartins had the largest values for all parameters. Basket like cells showed the same trend, although freemartins had smaller size than males. Conversely, stellate-like cells were more

regular in females and freemartins while they were more irregular in basket-like cells. For both types, neurons from males were less dense than freemartins and females. Pearson correlation tests showed that larger stellate-like cells were more irregular, while larger basket-like cells were more regular. Multivariate testing confirms that stellate-like cells in females were smaller, regular and denser than males, although with more variation, while freemartins had higher values, also in regularity and density, with a low scatter of values. Multivariate analysis on basket-like cells indicated that they were smaller and denser in females, with a homogeneous distribution, intermediate in freemartins and larger in males. Freemartin basket-like cells showed the greatest variation around the mean in density and regularity.

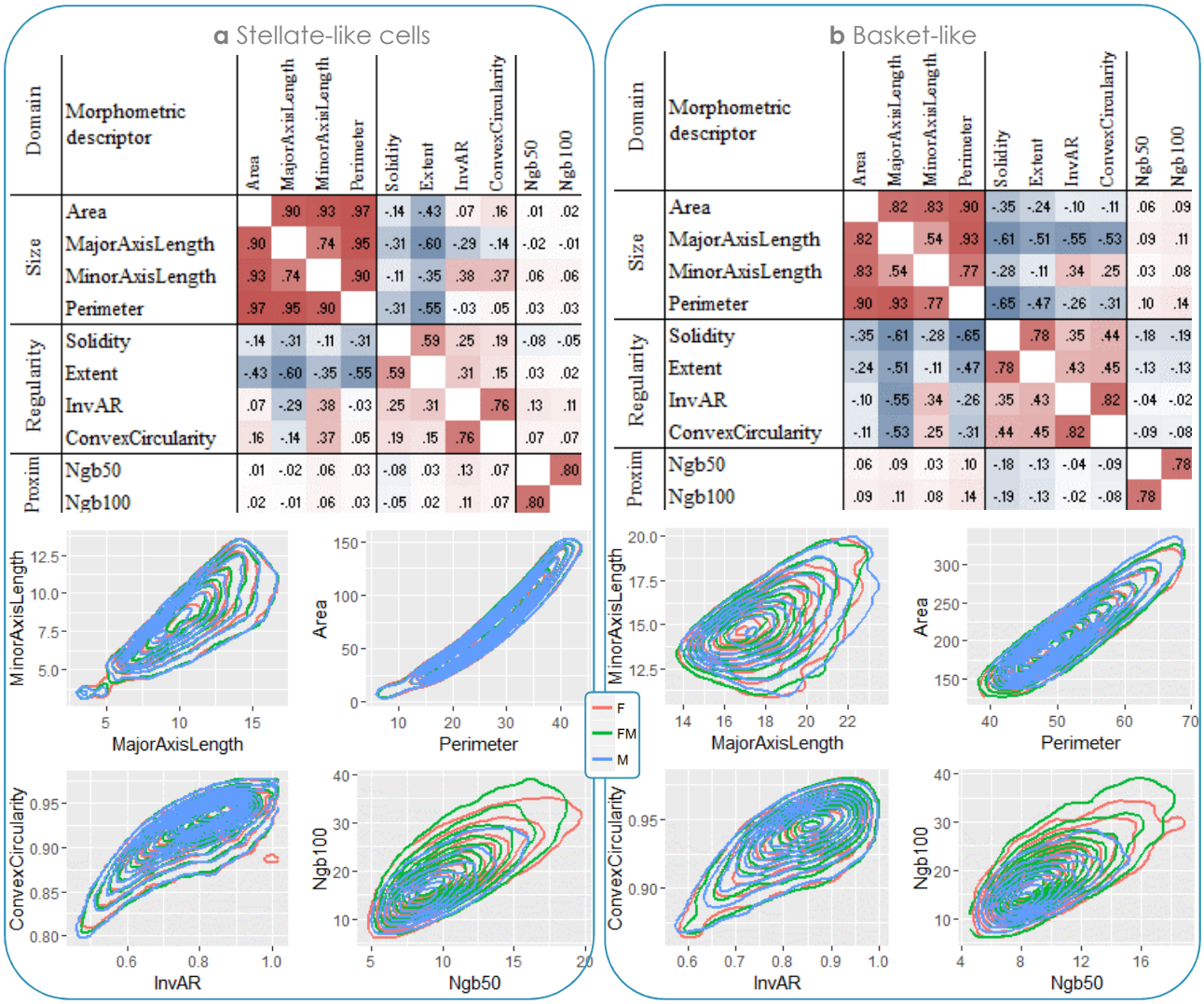


Figure 9: Pearson's correlation tests and bivariate joint distribution contour plots for stellate-like cells (a) and basket-like cells (b).

Purkinje neurons were the largest type in the cerebellum with an average area of 2500 μm^2 and perimeter of 250 μm. The average major axis length was about 70 μm. Female Purkinje neurons were significantly larger in most size parameters, and denser. However, male Purkinje cells were more regular in shape. Purkinje cells in freemartins showed an intermediate position not significantly different from males of females. We found a remarkable homogeneity in scatter, while Pearson correlations showed a tendency to irregularity with increasing size. Multivariate testing showed that at the domain level, female Purkinje cells were larger, more irregular and denser than males.

Granule neurons were the smallest in the cerebellum cortex with a soma area of 80 μm², a perimeter of about 30 μm, and a major axis length averaging 11 μm. Female granule cells were found to be larger in area, perimeter and major axis length than males. Female granule cells were also more densely organized. In fact, all parameters were smaller in males. Freemartins had a more regular soma than both males and females. Pearson correlations tended to show that larger granule cells were more irregular. All size parameters were correlated. Multivariate analysis confirmed that female granule cells were larger and denser than males, but scatter of the distribution was also larger, implying a wider variability. Freemartin granule cells were the most regular and dense, while size was intermediate between male and female.

	Size	Regularity	Density
Stellate-like cells	Location: FM > M > F Scatter: F = M > FM	Location: FM = F > M Scatter: F = F > M	Location: FM > F > M Scatter: F > FM > M
Basket-like cells	Location: M > FM > F Scatter: M = FM > F	Location: M = F > FM Scatter: FM = F > M	Location: FM > F > M Scatter: F > FM > M
Purkinje cells	Location: F > FM = M Scatter: F = FM = M	Location: M > F = FM Scatter: F = FM = M	Location: F > FM = M Scatter: F = FM = M
Granule cells	Location: F > FM > M Scatter: F > M > FM	Location: FM > M > F Scatter: F = M > FM	Location: FM > F > M Scatter: FM > F > M

Table 7: Rankings resulting from the matrix algorithm. > means larger or more, < means smaller or less, = means no difference.

DISCUSSION

We initiated this study to put in practice a new methodology allowing to study a large number of cells and their morphometric features, collected off tissue sections. We chose to implement this method on the cerebellar cortex of the bovine to compare male, female and freemartin cellular populations across layers. This was achieved by quantifying the cytoarchitecture of the vermal lobes VIII and IX.

Nissl staining was recently reaffirmed as the best technique to approach cytoarchitecture in nervous tissue, since it allows a comprehensive staining of the somata present, including glia, and a careful look can discriminate between cell types (García-Cabezas *et al.*, 2016). Its inexpensiveness along with simplicity made it the standard staining technique worldwide. It also is very effective in showing the cortical laminal organization of any type of cortex throughout the brain regions (Pilati *et al.*, 2008). This is of particular importance when considering an automated cell detection algorithm. One key feature is that most glial cells are much smaller in size than neurons, which let us to set a threshold and only consider neurons.

Our results showed there were measurable differences among groups in each layer. Namely, in the molecular layer, male basket-like and stellate-like cells were larger than females, more irregular (stellate-like) or regular (basket-like), and less densely arranged. In the granular layer however, female granule cells were larger than males, more irregular, denser. More generally, female cells tended to take values further around the mean (scatter). Freemartins had larger, more regular and dense stellate-like cells, the most irregular and dense basket-like cells and granule cells. Purkinje cells seemed to vary from male to female, but not significantly in freemartins.

Purkinje cells have been reported to be more numerous in males than in females (Wittmann and McLennan, 2011). This could have consequences functionally, since Purkinje cells are the output of the cerebellum. However, our results as well as other reports across species (Jacobs, Johnson, *et al.*, 2014), tend to show the remarkable homogeneity among Purkinje cells. Similar studies screening cells on pathological subjects could shed more light on this issue.

Studies of the mammal cerebellum suggest the existence of functional subsections controlling different systems, although the general cytoarchitectural structure is highly homogeneous (Ruigrok *et al.*, 2008). Additionally, sex dimorphisms have been suggested by other studies of both the structure (Fan et al., 2010) and physiology (Abel et al., 2011). Few studies focused on the sex differences at the cellular level, and most, albeit not all, found some in cell number (Mayhew, MacLaren and Henery, 1990; Wittmann and McLennan, 2011). Differences seem to have been found in glial cell populations (Suárez *et al.*, 1992; Nguon *et al.*, 2005) with no functional certainty. However, the differences reported are usually small, and do not reflect the male bias in cerebellar disease (Dean and McCarthy, 2008). Further studies including female samples should help understand sex differences in the cerebellar function, since a large proportion of those are exclusively male, and not necessarily for valuable reasons (Zucker and Beery, 2010; Becker, Prendergast and Liang, 2016). This expresses the need for a deeper study of the cell population making such sex differences emerge, and creating the tools to detect changes in these populations. Moreover, as a previous study identified in cultured cerebellar bovine cells (Montelli, Suman, *et al.*, 2016), the precise localization of cells in the cerebellum cortex is required for a fine characterization, and differences at the cellular level can be found.

If pathologies of the cerebellum such as autism, attention deficit hyperactivity disorder or schizophrenia (Okugawa *et al.*, 2002; Schneider *et al.*, 2006; Dean and McCarthy, 2008), there could arguably be a cellular basis to find for these pathologies, which could be identified using broad cellular screening techniques.

Moreover, a suitable analysis of cell population should be able to detect and put in relation different morphological factors, in order to detect global trends or variations. Morphometric and statistical studies tend to rely on univariate statistical analysis comparing means (location) of a single parameter. These methods are characterized by two factors: 1) they do not account for variation (scatter) of the parameter, and 2) they do not follow the distribution of several parameters simultaneously, which is biologically relevant, since it does not represent well the often multi-variable biological substrate (Benigni and Giuliani, 1994). In this sense characterizing the frequency distribution of dependent measures or score and applying suitable statistical tests are important (Maney, 2016).

The algorithm integrating staining, computational image processing and powerful morphometric data analysis represent a potential leap in the investigation of the brain tissue. The non-parametric multivariate testing and ranking approach allowed us to overcome the issues mentioned previously, and test location along with scatter in a set of morphometric parameters. We were able to discern that the cell population from the female group had a wider variation in values than males and freemartins, except in basket-like cells size. The morphometric parameters considered together showed that some cell types became increasingly regular with size, while some types became rounder with size, which could have a meaning related to function, such as more distant branching or increased metabolism.

The growth in specialization of techniques requires increasingly multi-disciplinary teams (DeFelipe, 2015), and this approach can help apply appropriate concepts on specific topics. Despite the controversy in sex difference studies, we would like to put forward the potentialities brought by specific cellular screening and large cohort detection, along with non-parametric multivariate and multi-aspect statistical testing and ranking, in reading large groups of neurons and organizations clearly, while remaining sensitive enough to detect minor yet significant differences. And in this view, the addition of the bovine freemartin model can help put male and female values in perspective.

III. A NEW COMPUTER-AIDED METHODOLOGY

III.A. Principle

When observing a complicated structure in which all parts must be accounted for, it becomes nearly impossible to measure thousands of units for several parameters while keeping a global view over different populations of units, or comparing them. Neuroanatomy is such a case. In this context, cellular morphology greatly benefits from computerized image analysis, and descriptive and inferential statistics adapted to such an array of data, since manual counting implies heavy sampling and traditional statistics imply usually mono-aspect mono-variate analysis.

Cell recognition and measurements were introduced in the early 1900s with studies on cell volume (Haug, 1986). Studies were made by hand, subject to human error on top of various biases that have been discussed over the years (Jones, 1937; Abercrombie, 1946; Glaser and Glaser, 2000). With the development of computer-aided science in the 1960's, the field of cellular morphometry rose towards more quantitative and systematic measurements. Stereology, the measure of three-dimensional objects based on two-dimensional sections followed the same general timeline from the beginning of the XXth century. And similarly, biases and good practices have been proposed over the century (Abercrombie, 1946; Gundersen, 1986). The advent of the computer helped automatize and systematize measurements also in these studies. Today, numerous tools and techniques exist with the goal to accurately measure cell composition, density, and morphology, such as isotropic fractionator, optic fractionator, etc (Schmitz and Hof, 2005).

Morphometric descriptors and software to compute them were developed to analyze quantitatively cell structure and sub-cellular components. A more objective analysis of cell shape was progressively achieved using quantitative measurements over qualitative differences or scores (Pasqualato *et al.*, 2012), which in turn allowed for reliable and transposable counting and measuring techniques of neuronal populations.

There is a vast scope of studies done on the recognition and stereological counting of cells within a tissue (Haug, 1986). One peculiarly tricky point has been to be able to count cells, not solely within a two dimensional plane but taking account for the depth of the tissue section, and the fractions created by cutting the section. Indeed, counting cells within a section implies counting parts of cells that might have been cut, and the problem of how to count reliably cell bodies arose. There is however a reliable, yet intrinsically biased way to minimize the amount of error. In sufficiently clear cells, like large neurons or small neurons, counting nucleoli assures a relative absence of double counting, given the fact that nucleoli do get cut extremely rarely (Jones, 1937; Abercrombie, 1946). Within a given image, for a sufficiently thin section such as 6 to 8 µm, all nucleoli can be seen, and counted. Additionally, this also resolves partially the issue of superposition, as in a large majority of cases, nucleoli are small enough not to overlap even if the cells are partially overlapping.

One other issue concerns cells which are too small or too dark to effectively spot a nucleolus. In this case, the overlapping of cells is more difficult to disentangle.

Together with modern image analysis techniques came the need for appropriate statistical testing for data collected on cellular morphology. Gathering particular morphometric data such as perimeter or major axis length only makes sense when considered among other parameters, to represent what is seen, i.e. groups of cells with shapes and sizes. For example a group of slender cells will have a large major axis but they will be extremely different from round cells with the same major

axis. Ideally, morphometric parameters should be considered jointly to retain a general view on the cell morphology. This is why we applied multi-aspect multi-variate non-parametric testing.

In the current state of image analysis and cell morphology studies, we took on to propose an innovative approach to systematically identify and analyze cell morphology of large cohort of cells within a designated area. In correspondence with the emergence of Big Data science came the computational power to analyze large cohorts for multiple parameters. This semi-automated method allows for manually designated regions to be objectively analyzed individually, collecting thousands of cellular shapes, sizes, areas, and other values. This we believe takes part in a broader effort to extract more objective data out of the material, and consider morphometry at the cell population level.

III.B. Image Analysis

Computational power has greatly improved in the last 10 years, opening the way to large scale analysis, including image analysis. Indeed, high-throughput imaging methods have greatly augmented in quality and quantity (Luengo-Sanchez *et al.*, 2015). Manual processing of such data has become increasingly time-consuming. Therefore, the need for high resolution automatic techniques capable of cell recognition and morphometric measure has become paramount.

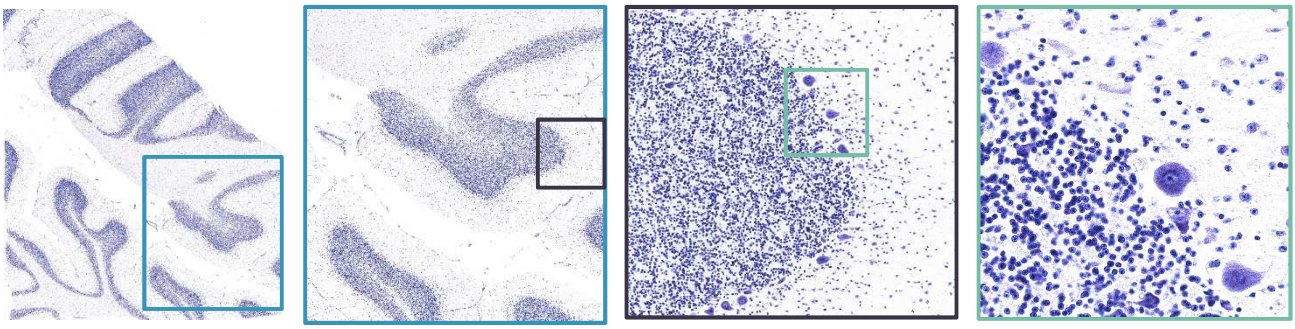
III.B.1. CELL DETECTION

The following has been published as part of the proceedings of the IEEE ISBI (2018).

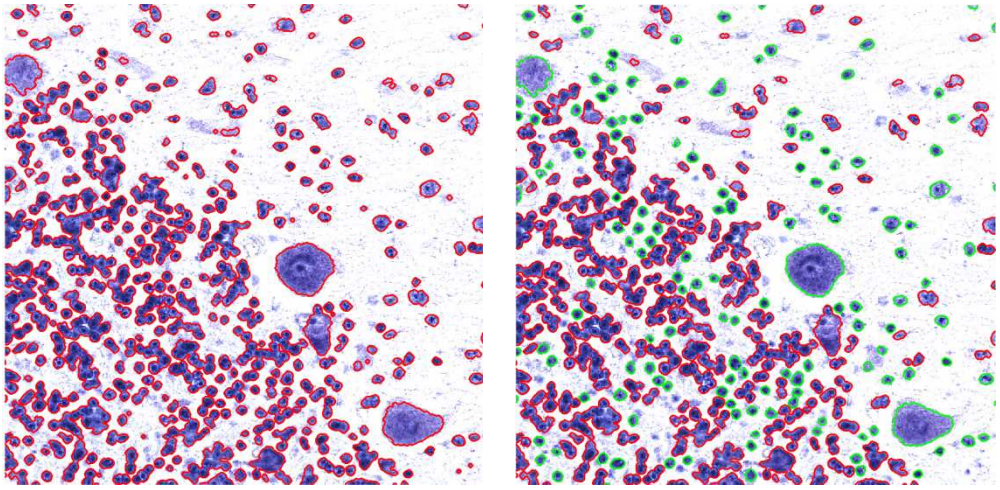
Grisan, E., Graïc, J.-M., Corain, L., & Peruffo, A. (2018). Resolving single cells in heavily clustered Nissl-stained images for the analysis of brain cytoarchitecture. In 2018 IEEE 15th International Symposium on Biomedical Imaging (ISBI 2018) (Vol. 2018–April, pp. 427–430). IEEE. https://doi.org/10.1109/ISBI.2018.8363608

If we consider the example of the cerebellar cortex (II.B.2), we first identify manually its laminar organization in an external molecular layer, a Purkinje mono-layer, and a granular layer, usually performed on Nissl stained sections, which consistently labels the whole neuronal population and exposes clearly the structure of nervous tissue. Manual processing of individual cell shapes and counts is infeasible due to the enormous amount of data and the size of histological samples. Therefore, automated image analysis presents advantages. However, automatic analysis of microscopy and histopathological images is challenging. Robust and accurate cell segmentation are difficult to achieve. Images often show background clutter with noise and artefacts, coupled with a significant heterogeneity in the size, shape and appearance of cells that can also be tightly packed together in touching and overlapping clusters.

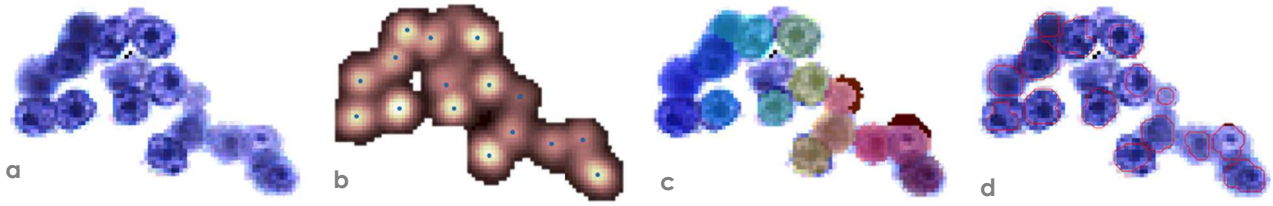
An initial separation of the stained objects from the background is obtained by applying a local space-varying threshold to the image (Poletti *et al.*, 2012). Then, by estimating the local density of the detected objects and using a constraint on the ordering of the layers, it is possible to obtain a rough separation of the most densely (possibly with clustered and cluttered cells) and most sparse regions. The application of a local threshold provides a first segmentation of the cells from the lightly stained background of the specimen (Neerad Phansalkar *et al.*, 2011), and in a second time, a small array of thresholds based on the values of eccentricity, areas and solidity of the identified objects allows the identification of single small cells from possible clusters of cells (Figure 10).



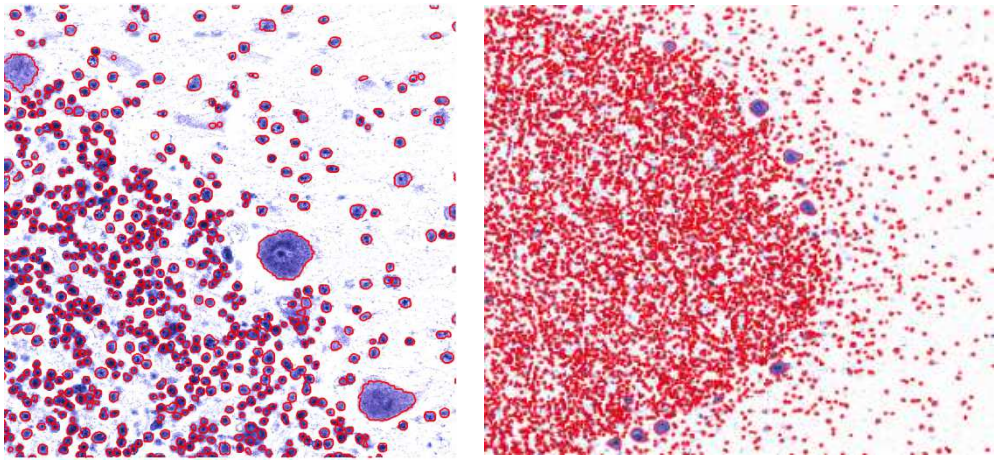
The cerebellum cortex comprises 3 layers: molecular (white), purkinje (large cells) and granular (dense blue)



Space variant thresholding Cluster (red) and single cell (green) identification



Cell clusters (a) are passed through fast radial transform, given cell centers candidates (b). These are passed through a mixed Gaussian sparse fit, and the resulting centers are identifying cells (c), which will have their borders evaluated (d)



De-clustered cells are then reconsidered in the population and this method is applied for every cluster, in order to obtain a whole cell population.

Figure 10: Cell detection algorithm in successive steps in the cerebellum.

Each object that has been estimated as a possible cluster is separately analyzed. A multi-scale Fast Radial Transform (MSFRT) (Loy and Zelinsky, 2003; Ram and Rodriguez, 2013, 2016) identifies possible positions of cell nuclei within the cluster. After a smoothing using median filtering (preserving peak information while reducing noise) All N local maxima of the filtered MSFRT are used to provide an initial estimate for the presence of separated cells. Hypothesizing that cells have a shape best approximated by an ellipsoid, each cell can be represented by a 2D Gaussian profile. Clustered cells were treated using a mixture of Gaussian profiles, with a number of mode that is the number of cells in the cluster. Hence, the image data around the cluster I_{clu}(x,y) are fit with a 2-dimensional Gaussian mixture model with a number of modes N equals to the number of the local maxima detected through MSFRT, each centered at the corresponding position $c_i = (x_i, y_i)\ i = 1, \dots N$:

$$G(x, y; c_i, \Sigma_i) = e^{-0.5\left((x,y)-c_i\right)^T \Sigma_i^{-1}\left((x,y)-c_i\right)}$$

$$GMM(x, y) = \sum_{i=1}^{N} \alpha_i G(x, y; c_i, \Sigma_i)$$

A non-negative constraint on the value of the mixing coefficients of the mixture of Gaussians enforces a sparse solution (as few non-zero modes as possible), and a cluster with as few cells as possible. An additional post-hoc check is performed, constraining all candidate cell centers to correspond to a local maximum of the mixture of Gaussians fits. By keeping the position c_i fixed, only the mixing component α_i and covariance matrix $\Sigma_i$representing the shape (dimension, eccentricity and orientation) of each possible cell need to be estimated. Additionally, by constraining the mixing component to be positive, and by checking if each candidate cell-center is still a local maximum of the MSFRT after the fit, the optimization tries to remove all candidate cells whose presence is not supported by the image data I, while constraining all others to have an elliptical shape:

$$\begin{cases} \left(\hat{\alpha}_i, \hat{\Sigma}_i\right) = \underset{\alpha_i, \Sigma_i}{argmax}\left(I(x, y) - GMM(x, y)\right)^2 \\ \alpha_i \geq 0, i = 1, \dots, N \end{cases}$$

In addition to the implicit sparse search provided by the non-negative constraint on α_i, an additional post-hoc check of the Gaussian centers c_i is performed: all the centers (candidate cells) not corresponding to a local maximum of the fit $GMM(x, y)$ are removed.

The detection performance was evaluated (Table 8) in terms of the ability of each algorithm to correctly identify a cell (true positive, TP), to reduce the number of objects that are wrongly identified as cells but belong to the background (false positives, FP), and to correctly separate different cells (objects containing more than one cell, remaining clusters); as performance scores we computed the precision as TP/(TP+FP), the recall as TP/(TP+FN) and F_1-score as 2TP/(2TP+FP+FN), where the FN is the number of false negatives (missed cells).

Method	Detected cells (TP)	Non-cell detection (FP)	Remaining	Precision	Recall	F_1-score
(Al-Kofahi *et al.*, 2010)	1837	2178	14	0.45	0.49	0.47
(Lu *et al.*, 2016)	2280	9226	94	0.20	0.60	0.30
(Ram and Rodriguez, 2016)	3561	7233	56	0.33	0.94	0.49
Proposed	3294	**488**	20	**0.87**	0.87	**0.87**

Table 8: performance comparison between other published algorithms and our own. In particular, the non-detection of cells is particularly low, and our precision and F_1 scores are improved.

III.B.2. MORPHOMETRIC PARAMETERS

After detection of the cells, morphometric measures of three order are made, based on size, regularity and density. These morphometric measures were based on Matlab® functions and gathered for tens of thousands of cells on each passage.

Morphometric domain	Morphometric measures (Matlab function)	Mathematical meaning
Size	Area	area of the cell body
	Perimeter	Total length of neural cell boundary measured in micron.
	Major axis length	Measure of the length of the major axis of the cell body.
	Minor axis length	Measure of the length of the minor axis of the cell body.
Regularity	Solidity	Proportion of pixels in the convexhull that are also in the region of the cell
	Extent	Area/area of the bounding box
	Inv. AR, 1/AR	Measure of Aspect Ratio, defined as AR=major axis/minor axis.
	Convex circularity	4 x π x convexArea/ConvexPerimeter^2
Density	Ngb_50	Quantify the cells number within a radius of 50 micron
	Ngb_100	Quantify the cells number within a radius of 100 micron

Table 8 : morphological domains

These morphometric measures are then the output of the image analysis. Each structure identified as a cell is measured.

III.C. Statistical analysis

Some early studies in which sexual dimorphism was confirmed by statistical methods were focused on identifying the gender of human bone fragments initially diagnosed via morphometric data (Gerven and Oakland, 1973). These studies provided an important area for the development of statistical procedure for morphological data.

There is however a great well-known advantage in multivariate statistics in that they can help differentiate populations with two variables that would otherwise be impossible to differentiate with only a single variable(Figure 11), which was recently published in response to the brain mosaic paper by Joel and colleagues (Joel *et al.*, 2015; Rosenblatt, 2016).

On the base of cellular morphological measures, in the case of the cerebellum, neurons, we applied a statistical method and data representation model to test the acquired data. A different of analysis was used in the study of the bovine hypothalamus. Here will be described first the general approach, then the mathematical formalization will be presented.

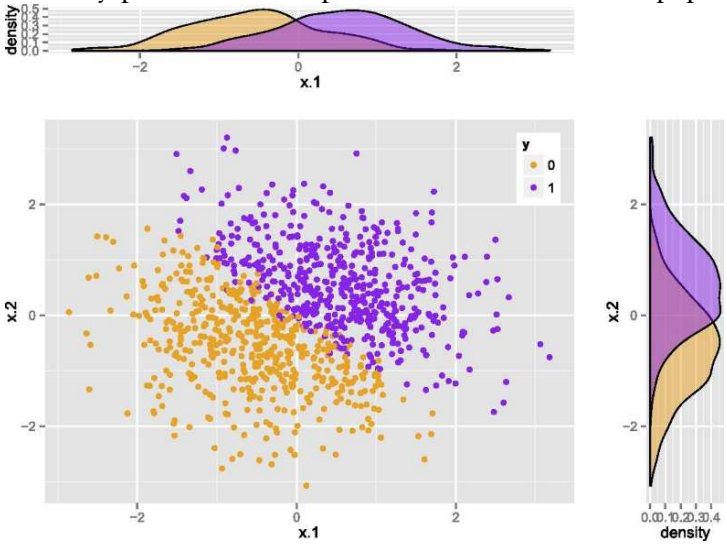


Figure 11: bivariate plotting of two populations (orange and purple). In each density distribution, the two populations are overlapping, making them undistinguishable. However, as can be seen on the dotplot, considering the joint variation of variables (x.1 and x.2), the two populations can clearly be separated (from Rosenblatt, 2016).

III.C.1. STATISTICAL DESIGN AND DATA REPRESENTATION MODEL

In inferential statistics, designed to draw valid conclusions on a population from sample data once a given method has been selected (Student-t test, Mann-Whitney test, etc…), there is a tendency to consider only univariate data, one response variable at a time. In traditional statistics, the normality of the response variable at the population level is often tested for before choosing a parametric or non-parametric test, however, there have been critics of the rationale behind such a logic (Rochon, Gondan and Kieser, 2012).

We have chosen beforehand to approach the data with a non-parametric permutation approach, referred to a multivariate mixed-effects linear model. It advantageously allows for the independent analysis of two distributional aspects of the multivariate response variable: the mean (location) and variance (scatter), hereby facing the hypotheses testing in the perspective of the "generalized Berhens-Fisher problem" (Yanagihara and Yuan, 2005). This statistical problem states that if two populations have different variances (i.e. no homoscedasticity assumption holds), investigation of the possible mean differences implies to apply a proper adjusted testing method. This is a quite general issue arising in both parametric and non-parametric approaches.

To formalize the comparison between a set of C populations (in our case three bovine populations, i.e. male, freemartin and female), first of all we assume a suitable experimental data representation model. Let us represent as $\mathbf{Y}$ a dataset of size $n=\Sigma j\ n_j$, where p morphometric features have been measured on the i-th cell belonging to the l-th subject drawn from the j-th population.

Let us assume that the p-variate response variable the can be modelled as

$$\mathbf{Y}_{ilj} = \boldsymbol{\mu} + \boldsymbol{\tau}_j + \boldsymbol{\eta}_{l(j)} + \boldsymbol{\varepsilon}_{ilj}, \quad (1)$$

where $\boldsymbol{\varepsilon}_{ilj}$ are i.i.d. possibly non-Gaussian error terms with null mean and population-dependent scale coefficients $\boldsymbol{\sigma}_j^2=\boldsymbol{\sigma}^2(\boldsymbol{\tau}_j)$ and unknown distribution $P_{\boldsymbol{\varepsilon}}$, $\boldsymbol{\mu}$ is a population-invariant constant, coefficients $\boldsymbol{\tau}_j$ represent the main population effects, $\boldsymbol{\eta}_{l(j)}$ refers to the individual/subject location effects (obviously nested within each population) that is assumed $IID(\mathbf{0};\Sigma_{l(j)})$, and $\boldsymbol{\sigma}_j^2=\boldsymbol{\sigma}^2(\boldsymbol{\tau}_j)$ are population-varying scale coefficients which may depend, through monotonic functions, on main treatment effects $\boldsymbol{\tau}_j$. Basically, according to the so-called generalized Behrens-Fisher problem (Yanagihara and Yuan, 2005), the proposed data representation model is a quite general less-demanding mixed effect nonparametric model where specific location and scale effects are both allowed to differ across populations. It is worth noting that no restriction is set up on the p-dimensional covariance/correlation matrix so that each population may differ also in its own joint-dependency distributional aspect.

Since the study's main goal was to compare the sex-related populations, we actually inferred on the main population coefficients $\boldsymbol{\tau}_j$, while $\boldsymbol{\eta}_{l(j)}$ can be considered as random nuisance parameters. In order to remove in mean the random nuisance effects, let us define the random variable $\widetilde{\mathbf{Y}}_{ilj} = \mathbf{Y}_{ilj} - \boldsymbol{\eta}_{l(j)}$ and, by using the Roy's Union-Intersection testing approach (Roy, 1953; Pesarin and Salmaso, 2010), let us formalize, separately for the location and scatter parameters, the comparison between the j-th and the h-th population with the null and alternative hypotheses as follows:

In our non-parametric testing, two aspects are to be considered: location, which is a general term for mean or median, and scatter, which is closely related to variance. We applied a permutation-

based testing approach as inferential method to test separately and rank the possible equality or dominance of two or more populations.

Formally, among the C populations, let us consider a pairwise comparison between the i-th and t-th population; hence, we set up the two separated testing problems, the first for location and the second for scatter testing, as follows.

$$\begin{cases} H_{0(jh)}^{loc}: \cap_k \tilde{Y}_{jk} \overset{loc}{=} \tilde{Y}_{hk} \equiv \cap_k [\eta_{jk} = \eta_{hk}] \\ H_{1(jh)}^{loc}: \cup_k [(\tilde{Y}_{jk} \overset{loc}{<} \tilde{Y}_{hk}) \cup (\tilde{Y}_{jk} \overset{loc}{>} \tilde{Y}_{hk})] \\ \quad \equiv \cup_k [(\eta_{jk} < \eta_{sk}) \cup (\eta_{jk} > \eta_{hk})] \end{cases} \quad \begin{cases} H_{0(jh)}^{sca}: \cap_k \tilde{Y}_{jk} \overset{sca}{=} \tilde{Y}_{hk} \equiv \cap_k [\sigma_{jk}^2 = \sigma_{hk}^2] \\ H_{1(jh)}^{sca}: \cup_k [(\tilde{Y}_{jk} \overset{sca}{<} \tilde{Y}_{shk}) \cup (\tilde{Y}_{sjk} \overset{sca}{>} \tilde{Y}_{shk})] \\ \quad \equiv \cup_k [(\sigma_{jk}^2 < \sigma_{hk}^2) \cup (\sigma_{jk}^2 > \sigma_{hk}^2)] \end{cases} \quad (2)$$

These expressions postulate that the alternative hypothesis (H1, there is a difference between populations), for either the location (left) or the scatter (right), seeks in which directions ("larger" or "smaller") H1 happens.

In order to better understand this idea, let use assume the p=2, i.e. we are considering a bivariate response variable and a number of C=3 populations three bivariate populations Π_1, Π_2 and Π_3 represented by the random variables $\mathbf{Y}_j$ for j=1;2,3, where $\mathbf{Y}_1$ is dominated by $\mathbf{Y}_2$ and $\mathbf{Y}_3$ with respect to both univariate components, i.e. Y_1 and Y_2, while $\mathbf{Y}_2$ dominates $\mathbf{Y}_3$ for the second component and the *vice versa* holds for the first component (Figure 12).

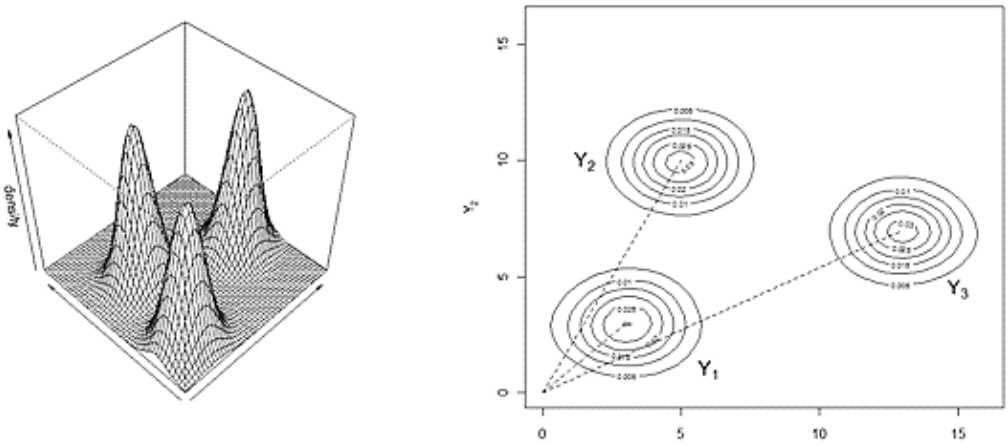


Figure 12. Density and contour plot of three hypothetical bivariate populations.

Therefore, for the comparison of two populations Π_j and Π_h, j,h=1,...,3, j≠h, there are 4 possibilities of alternative hypotheses (H_1) for two univariate responses Y_1 and Y_2 (Table 9).

H_1 alternatives	$\Pi_1 > \Pi_2$ for Y_1	$\Pi_1 < \Pi_2$ for Y_1
$\Pi_1 > \Pi_2$ for Y_2	$\Pi_1 > \Pi_2$for Y_1 $\Pi_1 > \Pi_2$ for Y_2	$\Pi_1 < \Pi_2$ for Y_1 $\Pi_1 > \Pi_2$ for Y_2
$\Pi_1 < \Pi_2$ for Y_2	$\Pi_1 > \Pi_2$for Y_1 $\Pi_1 < \Pi_2$ for Y_2	$\Pi_1 < \Pi_2$ for Y_1 $\Pi_1 < \Pi_2$ for Y_2

Table 9. H1 alternatives for two bivariate populations on two response variables.

For each possible H_1 alternative, there will be a p-value associated. In order to reject the null hypothesis, this p-value has to be smaller than $\alpha/2$, since we are jointly testing on both alternative directions.

In the present case, the response variables were grouped in morphometric domains (table 8). Therefore, each morphometric domain was assumed to be a vector with a number of dimensions p equal to number of morphometric descriptors within that domain, e. g. Size is a vector of 4 dimensions,

area, perimeter, minor and major axis length. These dimensions implicitly are weighted equally in the calculation of each p-values on Size among populations.

We used this rationale to infer dominances in morphometric domains among populations, which were then passed into a matrix to sum the p-values up in ones and zeroes (Table 10), and reach a ranking (Arboretti *et al.*, 2014).

p-values	Population 1	Population 2	Population 3
Pop 1	-	0.002	0.001
Pop 2	0.82	-	0.0018
Pop 3	0.4	0.02	-

➔

	Population 1	Population 2	Population 3	Sum of dominance
Population 1	-	1	1	2
Population 2	0	-	1	1
Population 3	0	0	-	0
Sum of dominees	0	1	2	Π1>Π2>Π3

Table 10 : sum algorithm based on relative dominance

The ranking then obtained in Table 10 gives a global and weighted comparison of the cell populations, within domains such as Size that encompass several coherent individual computer-measured morphometric descriptor. The same procedure is applied for the 3 domains (Size, Regularity and Density).

III.C.2. STATISTICAL OUTPUT GUIDELINES

For each cerebellar cortex layer, statistical outputs were tested possible bivariate neural cell morphometric differences between the three sex-related groups. For each domain, we separately carried out descriptive statistics and inferential statistics: two p-values 3 × 3 squared matrices of pairwise comparisons between groups, one matrix referred to the location and the other referred to the scatter analysis. By using all the cells above and below the diagonal, we represented in each squared matrix both the bivariate one-sided p-values to be associated to each one directional alternative ("greater than" and "lower than"). Finally, by exploiting the whole set of all relative p-value-based estimated dominances, we obtained also a ranking suitable for ordering the three populations from larger to smaller (location) and from most to least complex (scatter ranking) (Table 7).

From the inferential point of view, a larger in location or in scatter population over one other, is expected to take a higher rank in the corresponding ranking and to show a significant p-value in the associated row vs. column cell from the 3x3 squared matrix of pairwise comparisons.

III.D. Perspectives

The capabilities of image analysis are rapidly growing. These capacities could ideally highly raise our capacity to study the brain and its process. However, this requires a proper use of this computational power, with adapted high throughput algorithms and statistical analysis.

One area of improvement resides in the better recognition of cells and de-clustering. Naturally, along with a better cell recognition comes the possibility to differentiate cell types based on their shape or visual features. A robust classification of cells based on either direct computerized assessment or subsequent statistical analysis could help science objectively judge of cellular populations specifically in the brain, but also in other tissues, such as cancerous biopsies, in which it has been shown that

computers detect more accurately proliferative cell types than trained pathologists (Camp, Chung and Rimm, 2002; Turbin *et al.*, 2008).

One possible straightforward application would be on the study neurotrophic factors, such as estrogens (Montelli, Suman, *et al.*, 2016). A neurotrophic effect can be detected via the growth of the cells. This larger size, if it extends to a large enough portion of the cell population, can be detected, and confronted with another group, from a different sex, species or environment, and this on multiple variables. Our algorithm is capable of detecting subtle cell changes and discriminate between large populations. This would focus on the location aspect of the analysis in the size domain. Additional features could include dendritic trees and axons.

Another possible application of our algorithm could use scatter aspects of neuronal populations as a proxy for the complexity of their organization. Scatter can represent the heterogeneity of a population, since different types of cells will distribute in a wider pattern. While trophic effects are affecting the location aspect of density and size, complexity affects scatter from the perspective that the more different objects there are, the more complex the cell group is. However the definition of complexity in neuroscience varies (Sporns, Tononi and Edelman, 2000; DeFelipe, 2015; Gao and Ganguli, 2015).

One other key feature is the capacity to differentiate cell types. Undistinguished populations comprising different cell types can, beyond raise scatter, influence location and have an effect on the comparison with other populations. In the molecular layer of the cerebellum we identified two groups of neurons, which shows the importance of clean and organized data.

However, we are still in the process of adapting our statistical algorithm to the high throughput image analyses that have been made possible by recent gains in computing capacities. Big Data science is rising, from social sciences to internet data, including in science in general, and the need for readability and reliability increases with it. Therefore, an optimal statistical testing method must adapt to profit from this data proficiency rather than to simply treat it normally. There is increasing interest in non-parametric models and in large data handling models in neuroimaging in general, and this trend will most probably develop in the coming years (Gao and Ganguli, 2015; Bzdok and Yeo, 2017).

GENERAL CONCLUSION

Cetartiodactyla present features that can still yield view-changing results, and extreme adaptations in species such as giraffes and dolphins contribute to make it an exceptional clade (Gatesy *et al.*, 2013). Cetartiodactyla present a double interest from the veterinary anatomist's point of view. First, their neuroanatomy remains mostly to be mapped and explored at the cytoarchitectural and functional level. Secondly, the premise according to which all mammals basically function on the same nervous circuitry doesn't stand a closer look, for it can be seen that specific functional pathways differ among mammals living in different milieus (Butti *et al.*, 2014; Cozzi, Huggenberger and Oelschläger, 2017). Ideally, the brain of the giraffe, as much as that of the orca, conceal specialized and differentially developed regions, evolved from the same common ancestor, and the comparative study of such a clade surely will yield invaluable insight on the functioning of the brain in general. In this context, we used a large and systematic dataset to reach a robust average weight and encephalization quotient in the domestic pig *Sus scrofa* (Minervini *et al.*, 2016). Data showed that the pig, despite being used in translational neuroscience (Ettrup, Sørensen and Bjarkam, 2010), had a relatively small brain (135 g) for a mammal of its size. Conversely, despite appearing to be the largest of the terrestrial Cetartiodactyla with 719 ± 12.5g in absolute weight (Graïc *et al.*, 2017), extensive review of the literature proved that since the first recorded autopsy of a giraffe by Owen in 1839, only 12 specimen were used thoroughly for study and published, which tends to show that the neuroanatomy of ungulates remains under-represented.

Among Cetartiodactyla, bovines represent one of the closest domestic animals humans endeavored to keep, with sheep, goats and pigs (Larson, 2005), around 8000-10000 years ago (McTavish *et al.*, 2013). To this day, it is still a widely spread, highly selected and uniform animal, of which the brain material can be easily available. Unlike giraffes, bovines have been used as a model, as discoveries in general physiology have been made from bovine tissue (Gorski and Erb, 1959). Yet, issues related to housing and feeding costs to raise cattle only for research, and most notably the rise of rodents breeding and genetically modified models led to a dramatic reduction of their use. The direction taken by our lab over a decade ago, along with ethical considerations, led to a series of publications on the physiology of the brain in cattle (Peruffo *et al.*, 2004, 2008, 2011, 2013; Peruffo, Cozzi and Ballarin, 2008; Montelli *et al.*, 2012; Mura *et al.*, 2013; Peruffo and Cozzi, 2014; Panin *et al.*, 2015; Ballarin *et al.*, 2016; Montelli, Suman, *et al.*, 2016). In continuation with this theme, we set out to precise the chemical anatomy of the bovine hypothalamus, and concomitantly found sex differences in certain nuclei, one of which had never been described in this species (VON). These sex differences could be expected, as several instances have been found in other species, yet our results point out that females possess a larger suprachiasmatic nucleus and a larger vasopressin-oxytocin containing nucleus (Graïc *et al.*, 2018).

One additional advantage to the choice of *Bos taurus* for animal model is the existence of an intersex relatively common phenomenon that is freemartinism. Freemartin heifers present the advantageous feature of being XX females with a female brain, submitted to a male normal hormonal environment during their ontology. This condition allows to investigate the relative effects of genes, androgens and their conversion to estrogens, steroid receptors, their effects on gene expression and the intricate complexity of the masculinization of the brain. What we found however, is more complex than the masculinized version of a female brain. From sex differences we found between males and females in hypothalamic nuclei, freemartins had an intermediate vasopressin-oxytocin containing nucleus size, but a much larger suprachiasmatic nucleus than both. This in turn shows that there is not

a masculinized brain, but that the brain can be seen as a mosaic (Joel, 2011) of diversely receptive areas to hormonal cues. This view though remains challenged (Del Giudice *et al.*, 2016), and no universally accepted paradigm has emerged. Behavioral studies are possible to lead in bovines, like there have been in sheep or rams (Perkins and Roselli, 2007; Cannas *et al.*, 2018). In this context, sex preference in freemartin cows could help put in perspective the bovine model with other mammal sex preference models, including humans (Hines *et al.*, 2016).

Surely, the matter of sex differences will not be resolved unilaterally, and goes beyond the scope of the present work. In the recent past, new approaches based on functional imaging, genetics and epigenetics studies have shed new light on an old problem (McCarthy, 2016). Indeed, activation and connectivity maps, expression profiles and timing and DNA methylation bring additional insight to areas that were previously shown to be anatomically dimorphic, but also to areas or systems that where shown not to be (Clarkson and Herbison, 2016). As the gap between structure and function slowly gets bridged, additional models and particular cases to investigate provide the ground for further research, and translational approaches.

Translational neuroscience is based on similitudes between the animal model and the human brain. However, homologies depend on the area of study. For instance, the cortices of primates associated with grasping may be virtual in animals such as pigs or horses (Orban, 2016), but the morphology of these differences shed light on adaptive processes and divergence in neural pathways.

Certainly bovine and Cetartiodactyla deserve more attention for they are highly adapted superior mammals with a high diversity of ecological niche. Necessarily, their central nervous system adapted accordingly, to fit these niches, and the elusive relationship between structure and function in neuroanatomy can be found in the diversity of brain patterns within Cetartiodactyla. Regarding the modern context of increasing attention brought onto sex, gender, identity and the pressure to legislate, the pursuit to answer fundamental questions pertaining to sex and the brain will continue. In this process, valuable frameworks and models are needed to let science shed light on such basic enquiries. And freemartins can be part of them.

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APPENDIX

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RESEARCH ARTICLE

The bovine anterior hypothalamus: Characterization of the vasopressin–oxytocin containing nucleus and changes in relation to sexual differentiation

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Abstract

In an effort to systematically describe the neurochemical anatomy of the bovine anterior hypothalamus, we used a series of immunocytochemical markers such as acetylcholine esterase (AChE), arginine–vasopressin (AVP), calbindin (Calb), galanin (Gal), neuropeptide-Y (NPY), oxytocin (OXT), somatostatin (SST), and vasoactive intestinal peptide (VIP). We also investigated the potential sex difference present in the suprachiasmatic nucleus (SCN) and the vasopressin–oxytocin containing nucleus (VON) of six male and six female *Bos taurus*. Our study revealed that the cytochemical structure of the cattle anterior hypothalamus follows the blueprint of other mammals. The VON, which was never described before in cattle, showed a sex difference with a 33.7% smaller volume and 23.2% fewer magnocellular neurons (approximately 20–30 μm) in the male. The SCN also did show a sex difference in VIP neurons and volume with a 36.1% larger female nucleus with 28.1% more cells. Additionally, we included five heifers with freemartin syndrome as a new animal model relevant to sexual differentiation in the brain. This is, to the best of our knowledge, the first freemartin study in relation to the brain. Surprisingly, the SCN of freemartin heifers was 32.5% larger than its control male and female counterparts with 29% more VIP cells. Conversely, the freemartin VON had an intermediary size between male and female. To analyze our data, a classical statistical analysis and a novel multivariate and multi-aspect approach were applied. These findings shed new light on sexual dimorphism in the bovine brain and present this species with freemartins as a valuable animal model in neuroscience.

KEYWORDS

Bos taurus, freemartin; RRID: AB_2079751, hypothalamus, oxytocin, RRID: AB_2721225, RRID: AB_2732873, RRID: AB_2732874, RRID: AB_2732875, RRID: AB_2732876, RRID: AB_2732877, RRID: AB_2732878, RRID:AB_2513212, sexual differentiation of the brain, sexual dimorphism, suprachiasmatic nucleus, vasopressin, vasopressin–oxytocin containing nucleus

Abbreviations: AC, anterior commissure; Acc, nucleus accumbens; AChE, acetylcholine esterase; AVP, arginine–vasopressin; BST, bed nucleus of the stria terminalis; Calb, calbindin; Cau, caudate nucleus; DBB, diagonal band of broca; Gal, galanin; INAH1, interstitial nucleus of the anterior hypothalamus 1; INF, infundibular nucleus; LSA, lateral septal area; MPOA, medial preoptic area; NBM, nucleus basalis of meynert; NC, nucleus circularis; NPY, neuropeptide Y; OXT, oxytocin; Pe, eriventricular nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus; SST, somatosatin; VIP, vasoactive intestinal peptide; VMH, ventro-medial hypothalamic area; VON, vasopressin–oxytocin containing nucleus

1 | INTRODUCTION

The bovine species (*Bos taurus*) has been used regularly in neuroscience, since the early days of neuroendocrinology (Brownstein, 1983; Estes, Padmanabhan, & Convey, 1977; Gorski & Erb, 1959; Legros, Peeters, Marcus, De Groot, & Reynaert, 1976; Senders & Weber, 1987). However, the microscopic cytoarchitecture of the cattle brain

is nowhere near as precisely described as that of the widely used rat and mouse brain (Bolker, 2012; Manger, 2008; Okamura, 2002). The nature of the highly gyrencephalic brain, its large size with an average weight of 476 g (Ballarin et al., 2016), and easy access through local slaughterhouses make the cattle brain an interesting animal model for neuroscience. Additionally the duration of gestation, the prevalence of aromatase P450 and its hormonal profiles make it an interesting model for the study of the interaction of hormones and brain structures in development (Challis et al., 1974; Dominguez, Liptrap, & Basrur, 1988; Kim, Yen, & Benirschke, 1972; Peruffo, Buson, Cozzi, & Ballarin, 2008; Peruffo, Cozzi, & Ballarin, 2008).

Compared to other species, little knowledge is available about the cytochemistry of the bovine hypothalamus despite the early interest in research mentioned above, apart from the chemical neuroanatomy of the vasopressin–oxytocin neurophysin systems (De Mey, Dierickx, & Vandesande, 1975; De Mey, Vandesande, & Dierickx, 1974; Dellmann, 1959; Gadamski & Lakomy, 1972, 1973; Grütze, 1978; Junge, 1976, 1977; Lakomy & Gadamski, 1973; Leshin, Rund, Crim, & Kiser, 1988; Maciag, Cerundolo, Ilsley, Kelley, & Forand, 1979; Okamura, 2002; Schmidt et al., 2012; Sofroniew & Weindl, 1980; Szteyn, Lakomy, Dynowski, & Krawczuk, 1981; Vierling, 1957, 1958). Therefore, one aim of this study was to describe systematically the bovine anterior hypothalamus using a panel of known immunocytochemical markers. This resulted in the first description of the vasopressin–oxytocin nucleus (VON) in the cow brain.

Since the pioneering studies showing that sexual behavior is shaped by the effect of hormones on the brain during early development (Phoenix, Goy, Gerall, & Young, 1959), a great body of research has been performed to explain the complex mechanisms underlying sexual behavior (for a review see McCarthy, 2016). This line of research led to the detection of sexual dimorphism in a number of brain areas during the past 40 years in the rat (Gorski, Gordon, Shryne, & Southam, 1978), guinea pig (Bleier, Byne, & Siggelkow, 1982; Hines, Davis, Coquelin, Goy, & Gorski, 1985), hamster (Greenough, Carter, Steerman, & DeVoogd, 1977), pig (van Eerdenburg, Poot, Molenaar, van Leeuwen, & Swaab, 1990), and human (Allen, Hines, Shryne, & Gorski, 1989; Swaab & Fliers, 1985; Zhou, Hofman, Gooren, & Swaab, 1995; Zhou, Hofman, & Swaab, 1995b) as well as the effect of hormones on the brain.

The conversion of testosterone into estradiol by the enzyme aromatase P450 produces irreversible organizational effects during a critical period in the course of brain development (Lephart, 1996). These hormones may induce permanent changes in the architecture of nervous circuits, including changes in cell number, density of axonal connections, dendritic architecture, and neurotransmitter phenotype (Simerly, 2002). The ontogenetic window of sensitivity to steroids is not the same across all mammalian species. In short-gestation species (i.e., rodents), there is a perinatal peak in steroids whereas, in long-gestation, larger-brained species (including human and bovine), the critical period for sexual differentiation occurs earlier (Peruffo, Cozzi, & Ballarin, 2008). Morphological and functional sex differences of the brain circuitry of long-gestation species therefore depend on the prenatal hormonal milieu in which the fetus develops, irrespective of perinatal or postnatal hormonal exposures. A notable difference however is that primate brains seem to undergo masculinization without aromatization of testosterone (Wallen, 2005; Wisniewski et al., 2000). This implies that sex is an important determinant in neuroscience and its applications, in particular in the medical field (Cahill, 2006).

Freemartinism is an interesting model to bridge neuroanatomy and physiology in relation to sexual differentiation of the brain. Freemartinism occurs when a male and female (*ergo* dizygotic) fetuses create placental anastomoses, exposing the female fetus to the hormonal production of the male fetus during intra-uterine development, resulting in a masculinization of the female newborn genitalia. This condition, first correctly explained by Tandler and Keller (1911) and later more extensively by Lillie (1916), is far more frequent in cattle than in other species (Ladds, 1993; Padula, 2005). Freemartinism has been known to exist since the domestication of *Bos taurus* (Moore, Graham, & Barr, 1957) and was mostly considered for its implications in the physiology and pathology of reproduction. The potential structural effect of the freemartin syndrome on the development and organization of the brain has never been studied. It has long been known that the anti-Müllerian hormone (AMH) is responsible for the development of the freemartin female genital tract into variably masculinized tissues, usually causing sterility, but no study focused so far on their brain (Vigier et al., 1989; Vigier, Tran, Legeai, Bézard, & Josso, 1984). Chromosomically female, the freemartin brain is exposed to testosterone comparably to a male fetus during its development (Dunn, Kenney, & Lein, 1968; Rota, Ballarin, Vigier, Cozzi, & Rey, 2003), both in terms of time and intensity, from as early as 45 days post conception (Dominguez et al., 1988) in natural conditions still unmatched today in other models of sexual differentiation of the brain. Hence, this results in genetically female brain cells receiving a testosterone surge enzymatically transformed in estrogens by aromatase P450 (Peruffo, Cozzi, & Ballarin, 2008), known to mediate the male development of the rodent brain (Lephart, 1996). This contrasts with primates, in which androgens seem to act directly on the brain during sexual differentiation in rhesus monkeys, without the need for aromatization to masculinize male brains, as a nonaromatizable androgen had the same masculinization effects as testosterone (Wallen, 2005). The freemartin model could help to differentiate and specify where and when the genetic, epigenetic, and hormonal environment's relative influence and mechanisms take place. The second aim of this study was to compare the cell numbers and volumes of both the suprachiasmatic nucleus (SCN) and the newly described vasopressin and oxytocin containing nucleus (VON) between females, males, and freemartins, to test whether the masculine hormonal environment may affect the organization of the female hypothalamus.

2 | MATERIALS AND METHODS

2.1 | Tissue sampling and processing

The brain of 16 adult bovines (over 12 months old) was sampled for this study. They formed three groups, 6 males, 6 females, and 5 freemartins. The diagnosis of freemartinism was made on living animals by expert veterinary practitioners. Diagnostic criteria included: birth with a male co-twin (mandatory), malformation of the female external genitalia (elongated penis-like clitoris, elongated vulva, and upward urination), abnormally short vaginal length (5–8 cm in the newborn

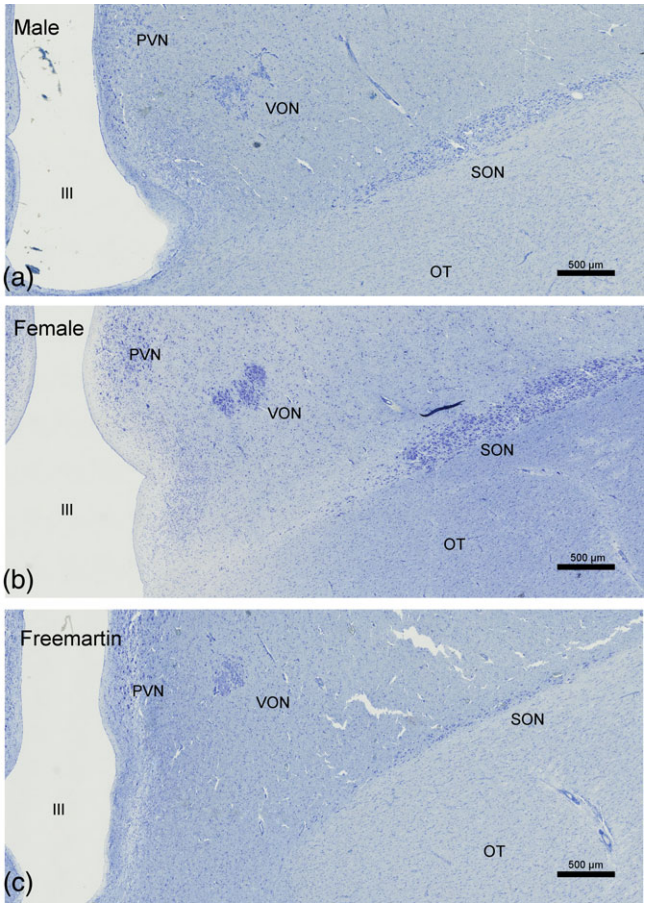

FIGURE 1 Microphotographs of the bovine hypothalamus of (a) a male, (b) a female, and (c) a freemartin [Color figure can be viewed at wileyonlinelibrary.com]

freemartins instead of 13–15 cm in normal calves) blindly ending without a cervix, abnormal development of the genital tract at transrectal ultrasonography performed at 1 year of age, and a failure to show estrus (Padula, 2005)

The animals' heads were collected from local slaughterhouses in the Veneto region. Animals were treated according to the present European Community Council directive concerning animal welfare during the commercial slaughtering process and were constantly monitored under mandatory official veterinary medical care. The brains were extracted in the necropsy room of the Department of Comparative Biomedicine and Food Science of the University of Padova, dissected and fixated by immersion in phosphate buffered formalin 4% v/w pH 7.4 within 2 hr after death. After a week, the hypothalamic region was dissected using the origin of the optic chiasma and the caudal part of the mammillary body as rostral and caudal borders respectively. For the present study however, we considered the region from and including the preoptic area to the pituitary stalk. The tissue blocks were then kept in formalin for at least three additional weeks before further processing.

After fixation the hypothalamic blocks were dehydrated in graded ethanols, cleared in toluene, embedded in paraffin and cut in 8 µm thick serial sections in the coronal plane. A thionine staining was performed on every 100th section for topological reference. Each section was mounted on Superfrost Plus glass (Ref. J1800AMNZ, Menzel-Gläser, Braunschweig, Germany) and dried at least overnight on a hot plate at 40 °C.

2.2 | Immunocytochemistry

2.2.1 | Vasopressin–oxytocin nucleus

Due to its very packed and round aspect, the VON was easily identifiable using Nissl stained (thionine) sections (Figures 1 and 2a–c). Nucleolus containing neurons were counted in this material following a technique recently reaffirmed for neuron description (García-Cabezas, John, Barbas, & Zikopoulos, 2016; Jones, 1937). Sections were deparaffinized in xylene and rehydrated in a descending alcohol series. After a short passage in distilled water, sections were submerged in a 0.4% thionine bath for 4 min, then washed in tap water until the water was clear. The sections were dehydrated in an ascending alcohol series, cleared in xylene, and finally coverslipped with Entellan (Cat. No. 107960, Merck, Kenilworth, NJ).

To detect the presence of arginin–vasopressin (AVP) and oxytocin (OXT) expressing neurons in the VON and ascertain its boundaries, we used antibodies directed against these neuropeptides (information regarding primary antibodies used can be found in Table 1). Anti-AVP antibodies were raised in rabbits using purified Guinea pig posterior pituitary extracts. The extracts were subjected to reverse phase HPLC and radiolabeled glycopeptide fraction isolation, pooled and automatically sequenced to confirm the glycopeptide identity, corresponding to one single N-terminal sequence (Fairhall & Robinson, 1989). The antisera obtained was tested for binding to radioiodinated glycopeptide, and cross-reactivity (AVP, k.1.7 rabbit polyclonal, Fairhall & Robinson, 1989, kindly provided by Dr. van Leeuwen, RRID: AB_2732873): anti-OXT monoclonal antibodies were produced by mouse spleen cell hybridoma and tested for specificity via radiobinding testing, and immunoabsorption with synthetic AVP, pressinamide, OT, (2-Phe)OT, (3-Phe)OT, (8-Ile)OT, (4-Asn)OT, (7-Gly)OT, (8-Val)OT, AVT, and tocinamide (A1–28, mouse monoclonal antibody, generous gift of A. J. Silverman, used in Hou-Yu, Lamme, Zimmerman, & Silverman, 1986, RRID: AB_2732874). The AVP C-terminal end is extended by a 39-peptides glycopeptide, which is not present in the OXT precursor providing a specific target for antibodies (Fairhall & Robinson, 1989; Gordon-Weeks, Jones, & Robinson, 1983; Richards, Morris, & Raisman, 1985). In this study, the specificity of the AVP and OXT stainings was confirmed by showing no overlap in staining of VON cells in alternating sections (Figure 2a,b). Deparaffinized sections were rehydrated in descending alcohol series and rinsed in distilled water 2 × 5 min. For antigen retrieval (Shi, Cote, & Taylor, 1997), sections were kept boiling for 10 min in 0.05 M Tris–HCl pH 9 buffer in a microwave oven at 800 W. After cooling to room temperature, sections were washed 3 × 3 min in Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.6), then treated with 10% methanol, 3% H_2O_2 in TBS for 10 min to quench pseudoperoxidase activity (Streefkerk, 1972). After another wash in TBS, sections were incubated with either anti-AVP or anti-OXT, both diluted to 1:1000 in Super Mix (SuMi, TBS pH 7.6 with 0.5% Triton X-100 and 0.25% gelatine) for 1 h at room temperature followed by an overnight incubation at 4 °C. The next day, after several washes in TBS, sections were incubated for 1 h in biotinylated horse anti-rabbit IgG for AVP or horse

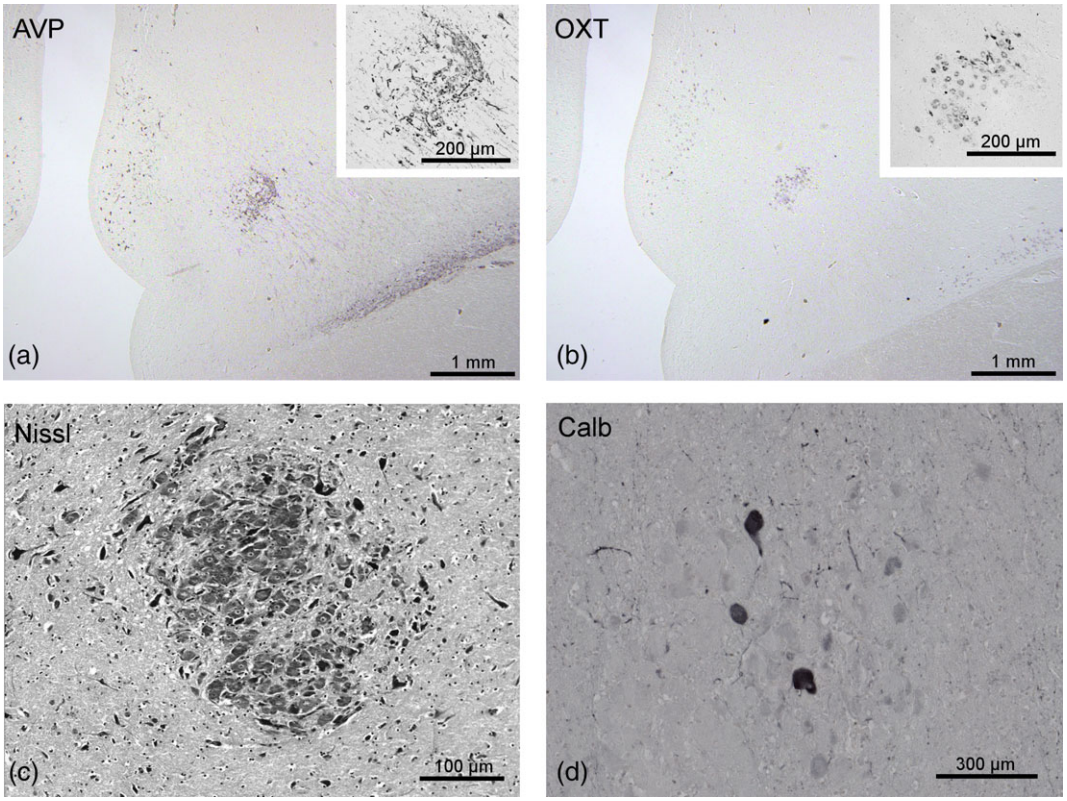

FIGURE 2 Pictures of the vasopressin-oxytocin containing nucleus. (a) and (b) are adjacent sections. No double staining of cell can be seen. (a) AVP staining, insert for details; (b) OXT staining with insert for details; (c) Round aspect of the VON, Nissl staining; (d) VON neurons positive to calbindin staining. Note the unstained magnocellular neurons [Color figure can be viewed at wileyonlinelibrary.com]

anti-mouse IgG for OXT, both 1:400 (Cat. No. BA-1100, Cat. No. BA-2000, Vector Laboratories Inc., Burlingame, CA) in SuMi. After a few rinses in TBS, sections were incubated in 1:800 avidin–biotin complex (Cat. No. PK-1600, Elite ABC kit; Vector Laboratories, Burlingame, CA) in SuMi for 1 hr, followed by final washes. Staining development was achieved using immersion in a solution of 0.5 mg/ml 3,3′-diaminobenzidinetetrahydrochloride (DAB, Merck) 0.2% nickel ammonium sulfate, and 0.01% hydrogen peroxide (H_2O_2; Merck, Cat. No. 1.07209.0250) in TBS. Reactions were stopped in the distilled water, then sections went through an ascending alcohol series, ending in xylene before coverslipping with Entellan (Cat. No. 107960, Merck).

2.2.2 | Suprachiasmatic nucleus

We used antibodies raised against vasopressin (AVP k.1.7), neurotensin (HC-8; 11/11/75, RRID: AB_2732876), tested in Carraway and Leeman (1976) and Kahn, Abrams, Zimmerman, Carraway, and Leeman (1980) and vasoactive intestinal peptide (VIP, Viper, 18/9/1986, Netherland Institute for Neuroscience, RRID:AB_2513212). Anti-neurotensin polyclonal antibodies were obtained from a rabbit host, via conjugation with hemocyanin and tested by radioimmunoassay and chromatography (Carraway & Leeman, 1976). Anti-VIP was raised at the Netherlands Institute for Neuroscience following conjugation to thyroglobulin by glutaraldehyde. Nonspecific antibodies were removed by adsorption with thyroglobulin Sepharose beads, treated with glutaraldehyde (Zhou, Hofman, & Swaab, 1995a, 1995b). To stain the SCN (Swaab, 2003) and we chose to use VIP for the rest of the experiment since it selectively stained only the SCN (Card, Brecha, Karten, & Moore, 1981). Deparaffinized sections were rehydrated in a descending alcohol series and boiled for 10 min in 0.05 M Tris–HCl pH 9 to retrieve antigenicity. After cooling, sections were washed in TBS and treated with 10% methanol, 3% H_2O_2 in TBS for 10 min. After rinses in TBS, sections were incubated with VIP antiserum at 1:1000 dilution in SuMi for 1 hr at room temperature and overnight at 4 °C. The next day, detection was done by incubating sections with biotinylated 1:400 anti-rabbit IgG (horse anti-rabbit; Cat. No. BA-1100, Vector Laboratories, Burlingame, CA) in SuMi, and ABC, followed by nickel-enhanced DAB development.

2.2.3 | Other neuropeptides

Immunocytochemical staining of other neuropeptides mostly followed the same procedure, except that blocking with milk was required, which included a 1 h preincubation in TBS-milk (TBS with 5% Elk powder milk, Campina bv., Eindhoven, The Netherlands) and incubation of the primary antibody in SuMi-milk (Super Mix with 5% powder milk) to reduce background staining. The peptides below were stained in this way, including Calbindin D-28k (1:500, Cat. No. CB-38a, Lot No. 9.03, Swant, Marly, Switzerland) and anti-acetylcholinesterase (AChE, goat polyclonal, Cat. No. AB144P, RRID AB_2079751, 1:500), anti-neuropeptide Y antibodies (NPY, "Niepke", rabbit polyclonal, bleeding; November 26, 1988, RRID: AB_2732877) were raised at the Netherlands Institute for Neuroscience by immunizing rabbits with porcine NPY (Sigma, St. Louis, MO) coupled to thyroglobulin with

TABLE 1 Primary antibodies information

Antibody	Immunogen	Manufacturer, RRID, species	Concentration
AChE	ChAT	Millipore cat# AB144P, RRID:AB_2079751, goat polyclonal	1:500
AVP	Guinea pig C-terminal end of the glycopeptide Amino acid sequence: Ala-Gly-Asp-Arg-Ser-Asn-Val-Thr-Gln-Leu-Asp-Gly-Pro-Ala-Gly-Ala-Leu-Leu-Leu-Arg-Leu-Met-Gln-Leu-Ala-Gly-Ala-Pro-Glu-Pro-Gln-Pro-Ala-Ala-Pro-COOH	Rabbit polyclonal, thyroglobulin-coupled, kindly provided by Dr. van Leeuwen, RRID: AB_2732873	1:1,000
Calbindin	Calbindin D-28k	Swant, Marly, Switzerland, (Swant cat# CB38, RRID: AB_2721225), rabbit polyclonal	1:500
Galanin	Rabbit antiserum (Gaaltje [June 29, 1993]) Amino acid sequence: Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH_2	Rabbit polyclonal (sigma galanin porcine G-1266), thyroglobulin-coupled, Gaaltje June 29, 1993, Netherlands Institute for Neuroscience. RRID: AB_2732875	1:800
Neurotensin	Neurotensin Amino acid sequence: Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH	Rabbit polyclonal (Neurotensin, Beckman instruments) Hemocyanin-8 coupled (HC-8, 11/11/75), RRID: AB_2732876	1:1,000
NPY	Porcine NPY (1–36) Amino acid sequence: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2	Porcine NPY(1–36) (sigma, St. Louis, MO) coupled to thyroglobulin (sigma) with glutaraldehyde (Merck, Rahwah, NJ) "Niepke", rabbit polyclonal, bleeding November 26, 1988, Netherlands Institute for Neuroscience RRID: AB_2732877	1:1,000
OXT	A1-28 oxytocin Amino acid sequence: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2	Mouse monoclonal produced by cell fusion and immunoabsorbed, kindly provided by AJ. Silverman RRID: AB_2732874	1:1,000
SST	SOMAAR Amino acid sequence: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	Rabbit polyclonal (SOMAAR, bleeding august 2, 1989), Netherlands Institute for Neuroscience In van de Nes et al., 1994, RRID: AB_2732878	1:800
VIP	VIPER Amino acid sequence: His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2	VIP coupled to thyroglobulin. Rabbit polyclonal (viper, bleeding 18/9/1986), Netherland Institute for Neuroscience, RRID:AB_2513212	1:1,000

glutaraldehyde, and specificity was tested by preincubation with protein A and G, as well as with the homologous antigen (Goldstone, Unmehopa, Bloom, & Swaab, 2002; Van der Beek et al., 1992). anti-galanin antibodies ("Gaaltje", rabbit polyclonal, bleeding; June 29, 1993 RRID: AB_2732875) were raised in rabbits using galanin (Sigma, St. Louis, MO, G1266) at the Netherlands Institute for Neuroscience. The peptide was coupled to thyroglobulin (Sigma T-1126) by means of glutaraldehyde (Merck, #6528). Incubations with preimmune serum showed no staining while adsorption of the galanin antiserum, performed with galanin spotted on gelatine-coated nitrocellulose membrane and based on the press-blotting procedure of van der Sluis, Pool, and Sluiter (1988), prevented staining (Garcia-Falgueras, Ligtenberg, Kruijver, & Swaab, 2011). Anti-somatostatin antibodies (SOMAAR, rabbit polyclonal, bleeding August 2, 1989, RRID: AB_2732878), were raised at the Netherland Institute for Neuroscience in rabbits, coupling somatostatin to thyroglobulin with glutaraldehyde (Buijs et al., 1989; van de Nes et al., 1994). Specificity was tested by press-blotting with a homologous antigen, abolishing staining, and preincubation with protein A and G (Buijs et al., 1989). In all cases, omitting the primary antibody resulted in complete abolishment of staining.

The nomenclature we used was based on the available brain atlas of the species (Okamura, 2002) and historic publications (Szteyn et al., 1981; Vierling, 1958).

2.3 | Image analysis for the quantitative study

A quantitative study of the cell count and volume was carried out using the Cavalieri's principle, with a minimum of 10 sections (8 μm thick) evenly spaced along the rostrocaudal axis of the structure to be studied to infer a volume based on the integration of each area over its interval. The interval was every 10 sections for the VON given its small size, and every 25 sections for the SCN. All nuclei were evaluated bilaterally and mean values are presented in Figure 5. The SCN borders were drawn manually, given the absence of other VIP-immunoreactive structures in the immediate periphery of the SCN. In case of doubt, an adjacent thionine stained section was used. Since this work was done in thin sections, neuron counting was achieved by counting nucleoli within the nucleus contour on each sampled section and dividing it by the nucleus volume in the section obtain a cell density. This density was multiplied by the total volume of the nucleus to obtain a total neuron count. Only nucleolated cells were

counted to prevent double counting (García-Cabezas et al., 2016; Jones, 1937). No double nucleolus was ever seen in this experiment. Criteria were VIP positivity, nucleolus visibility and inclusion in the outline. The delineated nucleus was subdivided using an automated grid, into 500-pixels-wide squares, displayed full-screen on the monitor, and all of them were counted. The VON borders were delineated on thionine sections since the densely packed large neurons were easily distinguished from the background. All nucleolated neurons were counted within the delineated area, following the same procedure. Tiled images were recorded using the 40× magnification objective (Plan-Neofluor lense) on a Zeiss Axioscope microscope mounted with a CDD black and white video camera (Sony-XC77) and connected to an ImageProPlus version 5.1 image analysis system (MediaCybernetics, Silver Spring, MD). All images were collected with exactly the same settings of the camera microscope.

2.4 | Statistical design and data analytics

The main focus of data analytics was on the comparison among the three populations under investigation, that is, male, freemartin, and female. For this goal, as done before in other works (Garcia-Falgueras et al., 2011; Goldstone et al., 2002; Swaab, Zhou, Ehlhart, & Hofman, 1994; Zhou, Hofman, & Swaab, 1995b), we applied two traditional nonparametric rank-based tests: the Kruskal-Wallis test and the Mann–Whitney test (Bonnini, Corain, Marozzi, & Salmaso, 2014). The first and second testing procedures are devoted to compare respectively all three populations and one pair of populations. Nonparametric tests can be considered as the more recommended testing to apply to our morphometric data because of their evident non-normal distribution.

In order to try to provide some additional insights into the population comparison, we applied also some innovative nonparametric permutation-based tests that are presented in details in the Supporting Information. This methodology consists of a powerful multivariate and multi-aspect-testing approach, able to quantify fine differences of the cell morphology in the nervous tissue (see the "Statistical Method" in Supporting Information). For all tests, a p value of less than 0.05 was considered to be significant.

Kruskal–Wallis and Mann–Whitney p values were calculated by using Minitab 18.1 (Minitab Inc., 2017) while permutation-based multi-aspect tests were performed by ad hoc R codes that are available by authors on request to interested readers.

3 | RESULTS

3.1 | Anterior hypothalamus topography

The distribution of the neurochemical markers is represented in Figure 3.

3.1.1 | Acetylcholinesterase

Large to medium-sized (approximately 20–30 μm) multipolar acetylcholinesterase-positive nerve cell bodies were found in the caudate nucleus, in the *nucleus accumbens* (Acc) and most pronounced in the *nucleus basalis* of Meynert (NBM; Figure 4a) as well as in the diagonal band of Broca (DBB) and the septum. A posterior group of middle-sized neurons was present along the descending fornix in the lateral septal area (LSA), above the anterior commissure and along the bed nucleus of the stria terminalis (BST). AchE-ir cells were also present in the paraventricular nucleus (PVN) *pars verticalis*, although not nearly as densely as AVP or OXT neurons. Dark patches of fibers were observed in the islands of Calleja near the NBM.

3.1.2 | Galanin

Positive neurons were small (approximately 10–20 μm) and spindle-shaped (Figure 4d,e). We found galanin positive fibers within the DBB and cells in the *nucleus accumbens*, the periventricular nucleus (Pe), the BST, the PVN *pars horizontalis* and *pars verticalis* and the medial preoptic area (MPOA). We did not reliably find any distinctive nucleus possibly homologous to the intermediate nucleus (Braak & Braak, 1987) also named sexually dimorphic nucleus (SDN; Swaab & Fliers, 1985) or interstitial nucleus of the anterior hypothalamus 1 (INAH1; Allen et al., 1989). Immunoreactive fibers spread wide throughout the preoptic area, some in the paraventricular area, around the fornix, and the infundibular nucleus (INF). The VON did not show any reactivity to galanin, but sparse fibers were found in the dorsal SCN.

3.1.3 | Neuropeptide-Y

Extensive NPY-ir areas were found in the *nucleus accumbens* and in the BST, composed of fibers and parvocellular neurons (approximately 10–20 μm), while fewer cells were present in the ventral DBB (Figure 3f). Beaded neuronal fibers spread out in the preoptic area, and remained most dense around the anterior commissure (AC) and in the Pe. A thick cluster of fibers was found lateral to the Pe, but no cell bodies were present. Many fibers were present in the infundibular nucleus but cell bodies were seldom seen. Small neurons and fibers could also be seen in the PVN *pars verticalis*.

3.1.4 | Somatostatin

For the largest part, somatostatin (SST) signal was found in the *nucleus accumbens*, the caudate nucleus (Cau) as well as the bed nucleus of the *stria terminalis*. Parvocellular neurons were found scattered around the anterior commissure in the POA but a few were also spotted in the region of the *nucleus basalis* of Meynert (NBM). The cells were small (approximately 10–20 μm) and bipolar (Figure 4c). Further caudally at the level of the SCN, parvocellular SST-ir neurons and fibers with varicosities were detected in the PVN (Figure 3d) all the way caudally up to the infundibular nucleus.

3.1.5 | Calbindin

Magnocellular calbindin-immunoreactive neurons (approximately 20–30 μm) were clearly present in the LSA (Figure 4b). They were quite densely packed and surrounded by fibers. The caudate nucleus also showed regularly spaced positive neurons. Calb-ir neurons were also seen in the BST, posterior to the anterior commissure. In the hypothalamus, some medium sized scattered cells and beaded fibers were found in the MPOA (Figure 3e, f). In the VON, we found some rare cells positive for calbindin (Figure 2d). Conversely, positive cells

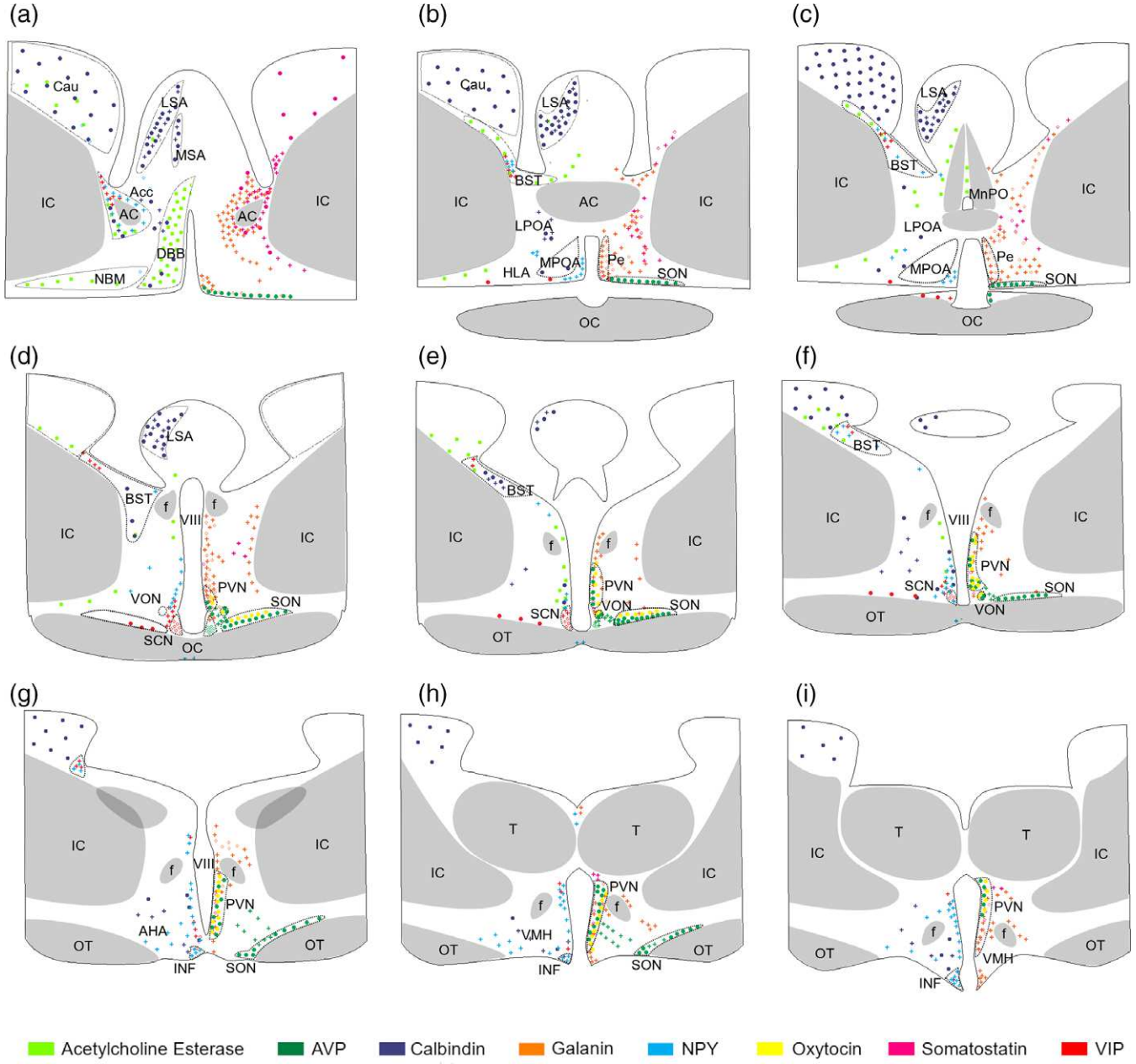

FIGURE 3 Schematic localization of immunoreactive perikarya in the rostral bovine hypothalamus. Dots (•) represent magnocellular perikarya; (○) represent parvocellular perikarya; "+" represent fibers. Acc = nucleus accumbens; AHA = anterior hypothalamic area; BST = bed nucleus of the stria terminalis; Cau = caudate nucleus; DBB = diagonal band of broca; LHA = lateral hypothalamic area; INF = infundibular nucleus; LPOA = lateral preoptic area, LSA = lateral septal area; MnPO = median preoptic nucleus; MPOA = medial preoptic area; MSA = median septal area; Pe = periventricular nucleus; PVN = paraventricular nucleus; SCN = suprachiasmatic nucleus; SON = supraoptic nucleus; VMH = ventromedial hypothalamic nucleus; VON = vasopressin-oxytocin containing nucleus; f = fornix; OC = optic chiasma; T = thalamus; IC = internal capsule; OT = optic tract; VIII = third ventricle [Color figure can be viewed at wileyonlinelibrary.com]

were regular in the exterior part of the PVN *pars verticalis*. More caudally, Calb-ir cells were seen in the ventromedial hypothalamic area (VMH), and fibers were present in the infundibular nucleus.

3.1.6 | Arginin–vasopressin

Vasopressin was widely distributed in the bovine hypothalamus. Magnocellular multipolar neurons (approximately 20–30 μm) were seen in the medial part of the paraventricular nucleus, throughout the SON and in the VON (Figure 2a). Parvocellular spindle-like AVP-ir neurons were found in the SCN, mostly on the medial side, with a thin rim of cytoplasm (Figure 4h). Fiber tracts were seen passing between the PVN and the SON via the VON. In both cell types, the cytoplasm was stained leaving the nucleus clear.

3.1.7 | Oxitocin

Oxytocin-containing neurons were strongly present in the cattle SON, as a thin cap of densely packed magnocellular multipolar neurons dorsal to the AVP-ir cells. OXT-ir neurons were also present in the lateral part of the PVN (Figure 2b). The SCN showed no positive signal. The VON exhibited densely packed magnocellular OXT neurons (Figure 2b). OXT-positive staining was found in the neuronal cytoplasm, with a clear, unstained nucleus.

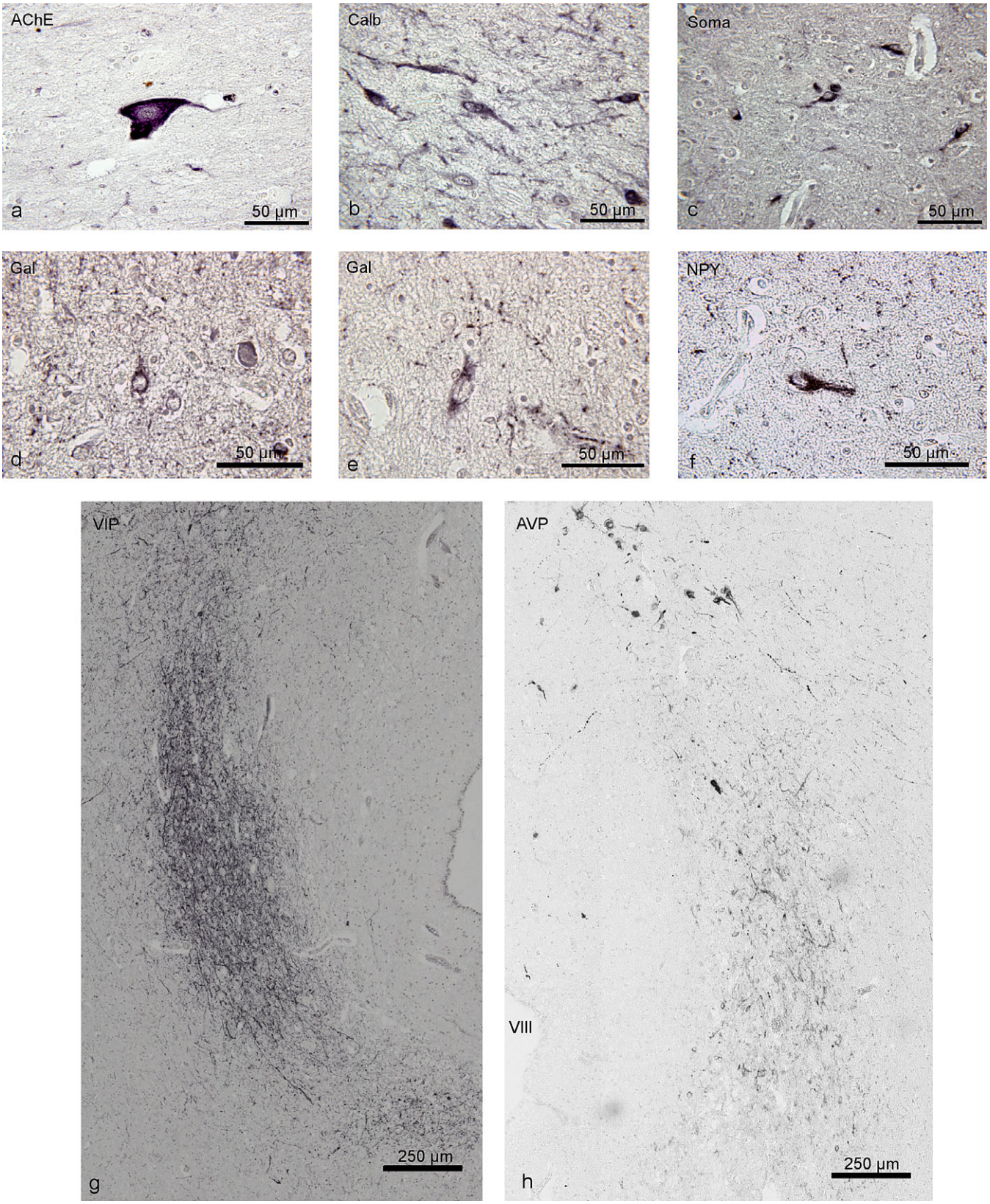

FIGURE 4 Immunoreactive neurons in bovine forebrain areas. (a) AChE = Acetylcholine esterase in the NBM; (b) Calb = Calbindin in the LSA; (c) Soma = Somatostatin in the NBM; (d, e) Gal = Galanin in the MPOA and the DBB; (f) NPY =neuropeptide Y in the nucleus accumbens; (g) VIP = vasoactive intestinal peptide in the SCN. (h) AVP = Vasopressin in the SCN note the AVP magnocellular neurons marked in the upper left corner. VIII = third ventricle [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Cell number and volume of female, freemartin and male groups in the SCN and the VON

	Females		Freemartins		Males	
	Cells ± *SEM*	Volume ± *SEM* (mm^3)	Cells ± *SEM*	Volume ± *SEM* (mm^3)	Cells ± *SEM*	Volume ± *SEM* (mm^3)
SCN	$20.77 \times 10^3 \pm 2.60 \times 10^3$	2.60 ± 0.37	$25.15 \times 10^3 \pm 4.28 \times 10^3$	3.17 ± 0.63	$14.92 \times 10^3 \pm 2.02 \times 10^3$	1.67 ± 0,26
VON	$5.47 \times 10^3 \pm 1.48 \times 10^3$	0.18 ± 0.05	$4.87 \times 10^3 \pm 1.01 \times 10^3$	0.16 ± 0.03	$4.20 \times 10^3 \pm 732$	0.12 ± 0.03

The average cell count for the SCN was $14.9 \times 10^3 \pm 2.0 \times 10^3$ neurons (mean ± *SEM*) for males, $20.8 \times 10^3 \pm 2.6 \times 10^3$ neurons for females and $25.2 \times 10^3 \pm 4.3 \times 10^3$ neurons for freemartins. The volume of the nucleus followed the same trend with 1.7 ± 0.26 mm^3 for males, 2.6 ± 0.37 mm^3 for females and 3.2 ± 0.63 mm^3 for freemartins. The average of male and female SCN cell count (14.92 + 20.77)/2) × 10^3 = 17.85 × 10^3) is 29% smaller than the freemartin cell count (25.15 × 10^3).

3.1.8 | Vasoactive intestinal peptide

VIP-reactive perikarya were found in the SCN medioventral part (Figure 4g). Rare somas could be seen on the floor of the preoptic area. Fibers were found in the Acc and the BST mostly (Figure 3b–d). Fibers are also present in the SCN running to the ventral PVN (Figure 4h).

3.2 | Neurochemical organization of the SCN and VON

3.2.1 | Suprachiasmatic nucleus

The largest part of VIP-ir neurons was present in the medioventral part of the SCN (Figure 3d,e). The neuron bodies were rather small (approximately 10–20 μm) and spindle-shaped. We sometimes saw a thin commissural junction between the two SCNs below the ventral part of the ventricle. Dorsal to the VIP-ir cells were beaded fibers present running dorsolaterally, where AVP-ir neurons were also found in the SCN (Figure 4g,h).

Cell counts and volumes are reported in Table 2. The female nucleus was larger than the male (p = .028, Table 3), while the freemartin SCN was larger than both male and female (p < .001 and p = .004, respectively).The freemartin group showed a statistically higher number of cells and area than both females and males (p FM > F = .006, p FM > M = .001; Table 4). Density across sexes was similar (Tables 3 and 4). The left or right side of the nucleus did not show any difference (data not shown).

In Figure 6b, the scatterplots show more information about the morphology of the SCN. Comparison between cell count and area, and cell count and density show a positive correlation. Consequently, area and density are seemingly independent. The more cells are present, the larger the nucleus tends to be. However, the larger the nucleus cross-section does not imply a higher density in cells.

3.2.2 | Vasopressin–oxytocin nucleus

The bovine VON is a small (0.12–0.18 mm^3) very densely packed nucleus containing typical large (approximately 20–30 μm) Nissl-stained neurons with cytoplasm around the whole empty-looking large and lightly stained nucleus with a distinct nucleolus (Figure 2c). The VON comprises AVP-ir and OXT-ir neurons with slightly more AVP-positive neurons. It is dorsal to the SCN and rostroventral to the PVN (Figures 1 and 2). The shape of the VON is round to ovoid anteriorly and evolves into a rather more elongated shape posteriorly.

Detailed results are reported in Table 2–4. The male group had a significantly smaller nucleus than both females and freemartins, both, in terms of area (p < .001 and p = .019, respectively, Table 3) and a number of cells (p = .009 and p = .017, respectively). There was also a less significant difference in cell number and area between females and freemartins (p = .038, Table 4). Males showed a higher density than females and freemartins in the VON (p = .002 and .004, respectively, Table 4).

The scatterplots in Figure 6d add some valuable information on the morphology of the VON. There seems to be a positive correlation between cell number and the area of the nucleus, but a negative correlation with density, implying that where the nucleus cross-section is larger, the density tends to shrink.

TABLE 3 Statistical p values for area, cell count, and density between sex groups

	Test Comparison	Kruskal–Wallis F vs. FM vs. M	Mann–Whitney F vs. FM	F vs. M	FM vs. M
SCN	*Morph. Meas.*				
	Area	**<0.001**	**0.004**	**0.028**	**<0.001**
	Cell count	0.122	*0.094*	0.762	0.174
	Density	0.556	0.952	0.987	0.371
VON	*Morph. Meas.*				
	Area	**0.001**	0.171	**<0.001**	**0.019**
	Cell count	**0.005**	0.530	**0.009**	**0.017**
	Density	0.118	0.428	0.135	0.218

Two-sided p values, for each morphometric parameter, using the Kruskal–Wallis test (comparison among all three populations) and the Mann–Whitney test (pairwise comparison between populations). Pairwise Mann–Whitney p values were adjusted by the Bonferroni–Holm–Shaffer method. Significant p values for 5% and 10% are highlighted in bold and italic respectively.

TABLE 4 One-sided location and scatter bivariate (nucleus area and cell count) and univariate permutation *p* values as stated in hypotheses (2) by the nucleus

	Aspect under testing Directional comparison	Location F > FM	F > M	FM > M	F < FM	F < M	FM < M
SCN	*Morph. Measure*						
	Area and cell count	0.999	**0.025**	**<0.001**	**0.004**	0.910	0.999
	Density	0.999	0.769	0.803	0.494	0.655	0.334
VON	*Morph. Measure*						
	Area and cell count	*0.044*	**<0.001**	**<0.001**	0.999	0.999	0.982
	Density	0.999	0.999	0.992	0.076	**<0.001**	**0.007**
	Aspect under testing	Scatter					
	Comparison	F > FM	F > M	FM > M	F < FM	F < M	FM < M
SCN	*Morph. Measure*						
	Area and cell count	0.443	*0.030*	**0.025**	0.190	0.933	0.993
	Density	0.639	0.545	0.999	0.999	0.647	0.671
VON	*Morph. Measure*						
	Area and cell count	**0.017**	0.071	0.415	0.716	0.798	0.368
	Density	0.777	0.985	0.999	0.238	0.045	0.108

In each cell, two populations are compared following the directional comparison ">" (greater than) or "<" (smaller than). Location or "mean" will show whether a population is likely to take larger (>) or smaller (<) morphometric values compared the other. Scatter or "variance", will show whether one population has more (>) or less (<) scattered morphometric values around the mean, compared to the other population. Significant *p* values for 5% and 10% are respectively highlighted in bold and italic (since we jointly considered both one-sided alternatives, the actual α-level must be $\alpha/2$). Significant *p* values were adjusted by multiplicity using the Bonferroni–Holm–Shaffer method. Permutation *p* values were calculated using the difference of sample means and by estimating the null permutation distribution with 10,000 random permutations (so-called CMC—Conditional Monte Carlo procedure). In order to accommodate for possible confounding effects due to the nucleus side, we set up a two-way layout where the nucleus side was considered as stratification factor (so-called stratified analysis, see Corain & Salmaso, 2004).
Further explanation can be found in the Supporting Information.

4 | DISCUSSION

The main findings of this study are that in *Bos taurus* there is evidence to suggest the presence of a sexually dimorphic VON in the bovine hypothalamus that is distinct from the SON and the PVN. The VON was 33.7% larger in females than in the males. The VIP-expressing cells of the SCN had also a sex difference since the female SCN was 36.1% larger than in the males. Moreover, in freemartins, the VON was larger than in the male and smaller than in the female, and that the SCN was 32.5% larger than in both sexes with 29% more cells (Table 2).

When quantitatively studying a nucleus' morphology, it is worth noting that volume and section data are two sides of the same coin. Assuming no dimorphism does exist, one would expect to observe no difference in the mean of volumes between subjects sampled from different sex-related populations, as well as no differences in the mean of areas and cells counts measured on the set of slides obtained from the same subjects. Conversely, if one population had a larger nucleus, one would expect to observe the larger area and possibly a higher number of cells as evidence of a larger nucleus. The distinction between using section data or volume data lies in the possibility of dealing with less or more aggregated information but not in the essence and interpretation of results.

4.1 | Classical versus multi-aspect statistical analysis

One of the pillars of neuroanatomy is to study and understand the relation between brain function and its underlying structure. In this context, efforts in statistics can help unravel new methods able to systematically extract useful information from the neuroanatomical data.

Two-tailed nonparametric tests are widely used in biomedicine to study potential differences in one parameter between two populations (Daniel & Cross, 2013). These standard univariate statistical tests are based on the means (location) of any single morphometric descriptor. These methods have two main drawbacks: (a) they do not account for the variance (scatter) aspect of the morphometric descriptors and (b) since they do not jointly consider the set of all morphometric descriptors, they do not support a broad scope on the problem at hand.

However, in an effort to propose more detail on the morphometric measures we acquired, we also considered a more sensitive statistical approachable to help identify finer differences between sexes. We adopted a multivariate permutation test on location and scatter, homologous to mean and variance, on the primary data collected (cell counts and areas). This allowed a deeper analysis and showed for example that males had a higher density than both females and freemartins in the VON (Table 4). The novelty of this approach lies in simultaneously testing the hypothesis on the location and scatter of a multivariate set of morphometric descriptors, taking into account the variance (scatter) of these morphometric descriptors. The results of this approach are multivariate in nature, introducing a general view on the data (Arboretti Giancristofaro, Bonnini, Corain, & Salmaso, 2014). Multivariate and univariate analyses examine different aspects of data; one is not necessarily broader or more general than the other. The advantage of the multivariate approach, in this case, is to examine

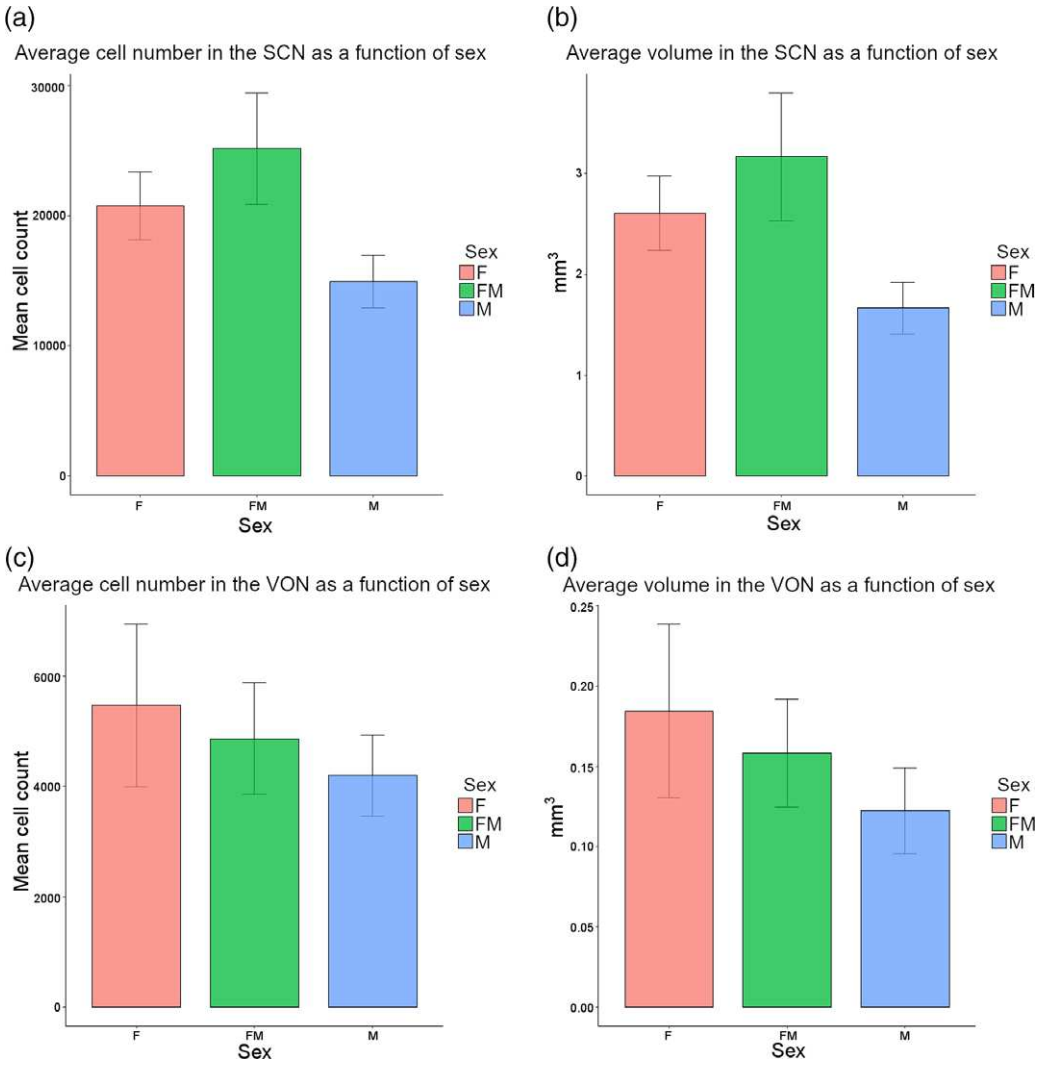

FIGURE 5 Histograms showing the total cell counts and volumes of the SCN and the VON. (a) Average cell number in the SCN as a function of sex; (b) average volume of the SCN as a function of sex; (c) average cell count in the VON as a function of sex; (d) average volume of the VON as a function of sex. Error bars represent the standard error of the mean. FM = freemartins; F = Females; M = Males [Color figure can be viewed at wileyonlinelibrary.com]

aspects of location and dispersion of cell count and area measures that relate to the joint distribution of these variables.

4.2 | Vasopressin–oxytocin containing nucleus

This article is the first arguing for the distinction of the VON in cattle. Table 5 summarizes the comparative nomenclature used from 1938 in the scientific literature for the magnocellular neuron groups in different species. van Eerdenburg et al. (1990) firstly introduced the VON in the pig. Originally most authors found these cells to be accessory subnuclei to the PVN and the SON (Dellmann, 1959; Junge, 1977; Koikegami, 1938; Morton, 1969; Scharrer & Scharrer, 1954), based on various species. Other research groups chose to consider it a different entity (Braak & Braak, 1987; Gadamski & Lakomy, 1973; Szteyn et al., 1981; van Eerdenburg et al., 1990; Vierling, 1957, 1958) while later, the concept of islands being part of a supraoptico-paraventriculo-hypohysal system emerged (Dierickx & Vandesande, 1977; Marani, 1990; Møller et al., 2018; Silverman & Zimmerman, 1983) following the human structure of these clusters. We subscribe to the argument of van Eerdenburg et al. (1990) in that the VON is obviously related to the PVN and SCN, because of its content of AVP and OXT and cell size, and is part of the hypothalamic magnocellular neurosecretory system although in the case of the pig and cattle at least, the organization into one distinct nucleus is striking (Figures 1 and 2a,b). The nucleus itself is constituted of two clearly separated AVP-ir and OXT-ir neuron groups (adjacent sections in Figure 2a,b). They seem to be predominantly distributed with AVP cells on the ventrolateral side sometimes tending to surround a more dorsomedial OXT core (Figure 2b). Its specific function has, however, still to be revealed since there did not seem to be a preferential orientation of the axonal processes, either centrally or down towards the pituitary gland. Although, tracts were especially seen in the AVP-ir neurons, with a general fiber orientation between the PVN and the SON surrounding the nucleus (Figure 2a).

4.2.1 | Species differences

The VON was found to be sexually dimorphic but it is not homologous to the intermediate nucleus (SDN, INAH1) since the latter does not

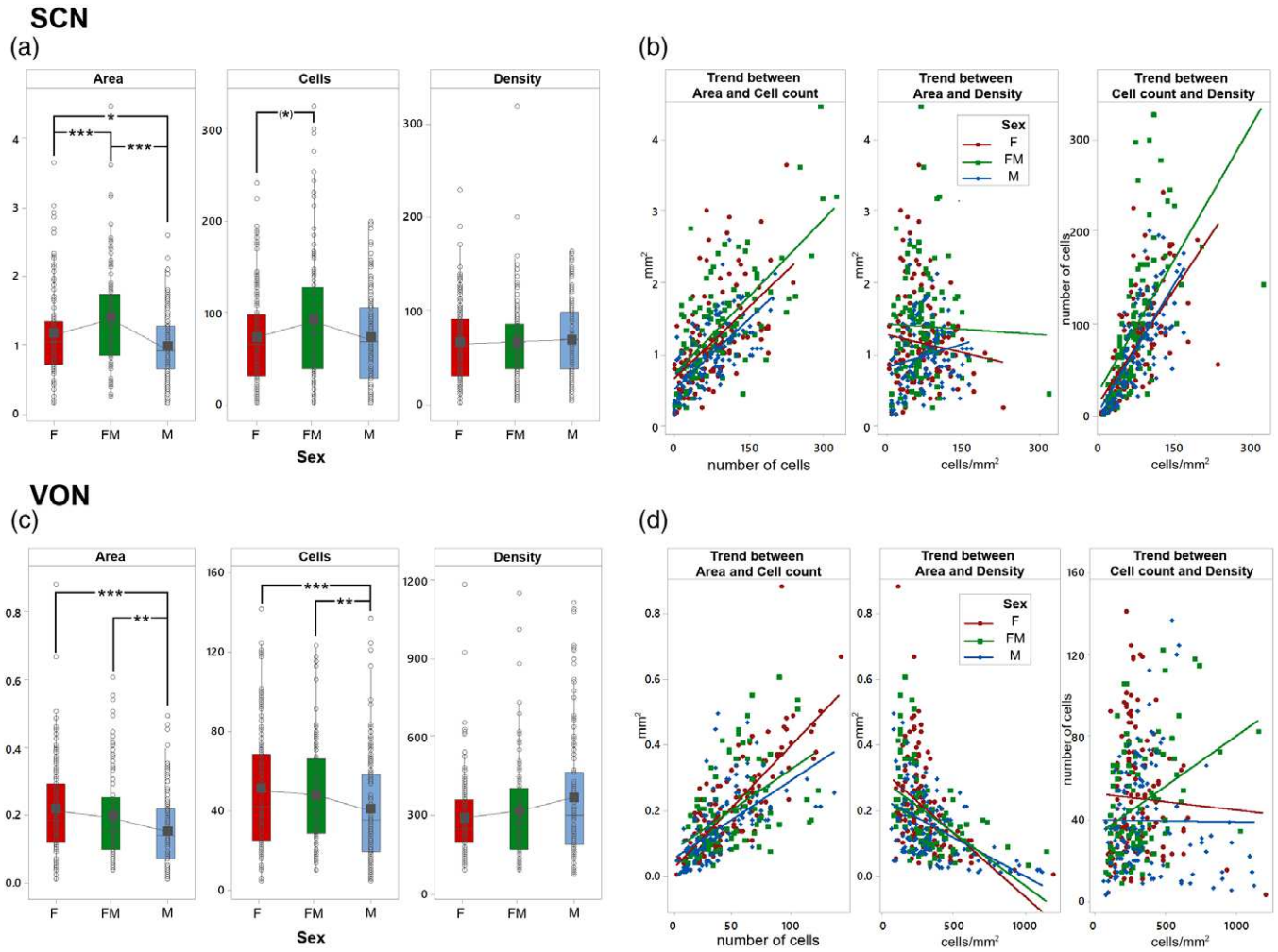

FIGURE 6 Graphical representation of the statistical analysis. Boxplot for SCN (a) and VON (c) nucleus by sex and morphometric feature (area, no. of cells, and cell density), where solid dots connected by line to represent the sample means. As one box shows a relative upwards/downwards shift this is a descriptive indication that the related population is distributed on larger/lower morphometric values. Significance levels according to the Mann–Whitney test are indicated by asterisks: *** < 0.01, ** 0.01 to 0.025, * 0.025 to 0.05 (see Table 3 for *p* values). Scatterplot for the SCN (b) and the VON (d) nucleus by morphometric feature pair and sex (using colored dots). In both the SCN and the VON, the area and cell number are positively correlated. Note that for the SCN area and density are not positively correlated (as one increases, the other feature does not). Conversely, the VON area and density are inversely correlated (as one feature increases the other feature tends to decrease). Finally, note the much more scattered pattern in the number of cells versus density plot appearing in the VON than in the SCN, suggesting that those features are almost independent in the VON, while in the SCN are strongly positively correlated [Color figure can be viewed at wileyonlinelibrary.com]

contain AVP or OXT. Braak and Braak (1987) describe the intermediate nucleus in man in a clearly different position from the neurosecretory accessory nucleus. Furthermore, galanin-positive cells are abundantly present in the Intermediate nucleus of the human brain (Garcia-Falgueras et al., 2011), but are absent in the VON. This does not necessarily mean a total absence of a homologous functional area, but rather a different anatomical organization, which in turn may have purposeful connectivity consequences. Cells staining for galanin in the anterior hypothalamus may be homologous to the intermediate nucleus but are arranged more diffusely in the bovine hypothalamus (Figure 3c). In the opposite way, the cells forming the VON are probably present more diffusely in other species, such as the anterobasal PVN in the rabbit (Schimchowitsch, Moreau, Laurent, & Stoeckel, 1989), or the anterior PVN in the cat (Caverson, Ciriello, Calaresu, & Krukoff, 1987). Another possibility is that the VON is a different entity from the accessory neurosecretory system, that is the more diffuse magnocellular accessory nuclei found between the SON and the PVN, the accessory neurosecretory system, in the hypothalamo-hypophysial tract (Møller et al., 2018). In sheep, Vierling (1957) described a group of cells similar to the VON although as part of the PVN, comparable to what was later described in the horse as the ventral PVN (Melrose & Knigge, 1989), which tends to show that the VON could be a common feature in large herbivores. In *Bos taurus*, we found that the VON presented rare cells positive to calbindin (Figure 2d) that were not reported in the pig (van Eerdenburg et al., 1990), while calbindin has been used to stain the SDN-POA in rats (Brager, Sickel, & McCarthy, 2000; Sickel & McCarthy, 2000). This finding suggests an SDN homology, although the cells were not abundant and did not cover a large part of the nucleus like in the rat.

The *nucleus circularis* (NC; also called accessory supraoptic nucleus in the rat in Bodian and Maren (1951) was clearly present in the pig, disambiguating it from the VON (van Eerdenburg & Swaab, 1991). It is also present in rats (Price Peterson, 1966), hamsters (Ferris,

TABLE 5 Comparative names given to the magnocellular structures between the SON and the PVN in different species

Author	Year	Species	Name	Notes
Koikegami	1938	Mole rat Bat Squirrel Cat Rhesus monkey	Accessory nucleus of SON or PVN	Not present in every species.
Scharrer and Scharrer	1954	Reptiles Birds Mammals	Nucleus supraopticus accessorius	Small groups of perivascular cells
Vierling	1957 1958	Bovine	Nuclei intersupraoptico-paraventricularis	Compact clusters of cells
Dellmann	1959	Bovine	Accessory part of the PVN	
Morton	1969	Human	Accessory supraoptic nucleus	Separation in two clusters
Gadamsky and Lakomy	1973	Bovine	Nucleus intersupraoptico-paraventricularis	Oval shape, close arrangement. Cells similar to the PVN
Dierickx and Vandesande	1977	Human	Intersupraoptico-paraventricular islands	
Junge	1977	Bovine	PVN pars accessoria	
Szteyn et al.	1981	Bovine	Nucleus intersupraoptico-paraventricularis	Closely arraged large cells
Silverman and Zimmerman	1983	Human, mouse	Irregular string of cells	
Braak and Braak	1987	Human	Accessory neurosecretory nucleus	High density, large soma size, dense capillary network
Marani	1990	Reptiles Birds Mammals	Supraoptico-paraventriculo-hypophysis system	Cell nests connecting the PVN with the SON
Van Eerdenburg et al.	1990	Pig	Vasopressin-oxytocin containing nucleus	High density, large soma, vasopressin and oxytocin reactivity
Møller et al.	2018	Human	Accessory magnocellular neurosecretory system	Scattered, different in man from other mammals

List of relevant literature concerning the magnocellular cells between the SON and the PVN. Notes have been added where comments were made about the structure of the cell groups.

Axelson, Martin, & Roberge, 1989), cats (Caverson et al., 1987), dogs (Laqueur, 1954), and was recently reported in the human hypothalamus (Møller et al., 2018) and has been linked to osmotic functions in rats (Wallace & Harrell, 1983) and flank marking in hamsters (Ferris et al., 1989). We were not able to detect any NC in the bovine hypothalamus. Despite a comparable packed aspect and positivity to OXT and AVP, the position of the NC in the cat and the pig is more dorsolateral than the VON in the pig and the bovine, leaving little doubt for the absence of homology. Additionally, a characteristic of the NC is the presence of a large blood vessel, which is not the case of the VON. The reduction of the number of AVP somas in the NC of hamsters infected by scrapie, a transmissible neurodegenerative disease (Ye, Carp, Yu, Kozielski, & Kozlowski, 1994), may help to differentiate it from the VON.

Consequently, although this is subject to discussion, we argue, based on the existence of the VON in the pig (van Eerdenburg et al., 1990), that there is a homologous nucleus in the bovine, another terrestrial Cetartiodactyla. Such a structure could be a feature of this clad of mammals, which should be investigated.

4.2.2 | Sex differences

Unlike in the pig, we found that cows had a larger VON than bulls. It is situated, like in the pig, between the SON and the PVN, dorsal to the SCN. The relative size of the bovine VON is similar to that of the pig, since its volume is over three times larger than in the pig at puberty with 0.15 mm^3 versus 0.054 mm^3, considering a brain weight 3.5 times heavier, with 476 g for the bovine (Ballarin et al., 2016) and 135 g for the pig brain (Minervini et al., 2016). The sex difference of the bovine VON is of a different order than that of the SCN. The VON size and cell number were larger in females while males had a significantly smaller nucleus and freemartins had an intermediate position (Figures 5 and 6).

However, the VON does not fit in the general masculinization model, given that, unlike the rat or ovine SDN (Roselli, Larkin, Resko, Stellflug, & Stormshak, 2004), but more similar to the infundibular nucleus (Taziaux et al., 2016; Taziaux, Swaab, & Bakker, 2012), the female nucleus is larger than the male. This could mean that the VON function might be linked to female reproduction functions such as lactation, in which oxytocin plays a crucial role, or more generally by central effects on reproductive behavior. In van Eerdenburg and Swaab (1991), 130 weeks old female pigs display a VON twice larger than males. One explanation for the increase in a number of cells in the VON postnatally in females is parturition and lactation. In swine, litters are typically large (usually over 10 piglets), which is not the case in cattle (1–2 calves). Further studies with different age groups, with or without calving may yield more information on its function.

4.3 | Suprachiasmatic nucleus

4.3.1 | Species differences

The VIP-stained bovine SCN was much larger than what is described in Okamura (2002) using Nissl staining and compared to the VIP-

stained human SCN (Zhou, Hofman, & Swaab, 1995a, 1995b). The largest part of VIP cell bodies was present in the medioventral part of the bovine SCN as in the human (Swaab et al., 1994), ovine (Tessonneaud, Cooper, Caldani, Locatelli, & Viguier-Martinez, 1994), the camel (El Allali et al., 2017), and rodent SCN (Abrahamson & Moore, 2001). This is in contrast with the SCN of the opossum, a marsupial (Cassone, Speh, Card, & Moore, 1988), in which VIP neurons are localized dorsally. NPY has been described in neurons of the human SCN (Mai, Kedziora, Teckhaus, & Sofroniew, 1991), while the SCN of rodents (Moore, Gustafson, & Card, 1984; Moore, Speh, & Leak, 2002), and the camel (El Allali et al., 2017), contained only fibers, particularly sparse in the sheep (Tillet, Caldani, & Tramu, 1989). The present study found that the SCN of cattle contained sparse NPY-ir fibers, originating from the geniculate body and transmitting nonphotic information to the SCN (Saderi et al., 2013), as in the sheep. Calbindin was found anteriorly to the SCN but no neuron body was found in the SCN itself, which is unlike in the camel (El Allali et al., 2017) and human (Mai et al., 1991). In humans, the SCN does contain galanin somata (Gai, Geffen, & Blessing, 1990) while in the rat only a few galanin immunoreactive neurons located in the SCN (Melander et al., 1985). Sparse galanin-ir fibers were seen in the SCN of the bovine, which is coherent with other mammals like opossums and pigs (Elmquist, Fox, Ross, & Jacobson, 1992; Pearson, Anderson, & Jacobson, 1996). Finally, AVP was considered absent from the SCN in bovines (De Mey et al., 1974) but we found some AVP neurons and fibers running through the SCN with clear varicosities (Figure 3h). The general distribution of AVP and VIP described by Abrahamson and Moore (2001) in the mouse is, despite some overlap, similar to what we found in the bovine SCN, with a ventrolateral core positive to vasoactive intestinal peptide (Figure 4g) and a medial rather than dorsomedial shell positive to vasopressin (Figure 3h). As expected, the suprachiasmatic nucleus showed no oxytocin positive signal, which is in accordance with most mammals, except notably in the horse (Melrose & Knigge, 1989).

4.3.2 | Sex differences

A significant sex difference was found in the number of VIP stained neurons between males and females (p = .017, Table 4) where females had a larger nucleus than males. This is different from previous findings in humans regarding VIP-expressing neurons (Swaab et al., 1994), where the adult male SCN has been found to contain twice as many VIP neurons as the female's, although this varies with old age (Zhou, Hofman, & Swaab, 1995b). Results similar to humans have been found in rats and gerbils, using Nissl staining (Gorski et al., 1978; Holman & Hutchison, 1991; Robinson, Fox, Dikkes, & Pearlstein, 1986) where the male SCN was larger than the females. Although no OXT neurons can be found in the SCN, the timekeeping and synchronicity role of VIP has been demonstrated (Maywood et al., 2006). The peculiarity of the situation in cattle may be related to advanced breeding selection for milk production, which is indeed influenced by the circadian rhythm (Plaut & Casey, 2012).

4.4 | Other areas

The cattle hypothalamus follows the general mammalian blueprint with the distinctive minute relative difference in the distribution of markers. Notably, the BST is positive for somatostatin but does not display a well-bordered nucleus in cattle as it does in the human brain (Kruijver et al., 2000), rather an irregular area with dense fibers and few parvocellular neurons instead (data not shown here).

As it has been described originally in details in cattle (De Mey et al., 1974, 1975; Vandesande, Dierickx, & De Mey, 1975a, 1975b), AVP and OXT in the hypothalamus are mostly distributed in the PVN and the SON. In accordance with De Mey et al. (1974), bovine OXT-ir cells were strongly present as a thin cap of densely packed magnocellular multipolar neurons dorsal to the AVP-ir cells of the SON, as well as in the lateral part of the PVN (Figures 2b and 3). In their study of the neurophysin-producing neurons in the bovine hypothalamus, Vandesande et al. (1975a, 1975b) found a predominating presence of AVP in the SON and of OXT in the PVN. We did find more AVP-ir neurons in the SON, but the PVN did not exhibit qualitatively more OXT-ir than AVP-ir somata. Moreover, it appeared that AVP cells in the PVN were localized slightly more medially than OXT-ir neurons (Figures 2a,b and 3).

The *nucleus accumbens* is one of the largest sources of NPY after the hypothalamus in the human (Hendry, 1993), and rat brain (Brown, Coscina, & Fletcher, 2000), similar to what we found in the bovine. In the bovine hypothalamus area corresponding to the human medial subnucleus of the medial preoptic area reported by Koutcherov, Paxinos, and Mai (2007), we found dense clusters of NPY-ir fibers as well as Calbindin-ir fibers (Figure 3d). Compared to the rat, Koutcherov et al. (2007) also noted the more diffuse aspect of neuronal groups in the human hypothalamus.

To the best of our knowledge, this study is the first anatomical description of galanin in cattle hypothalamus. Unlike in the sheep (Chaillou, Tramu, Thibault, & Tillet, 1998; Chaillou, Tramu, & Tillet, 1999) and humans (Gai et al., 1990), we found no galanin-immunoreactive cells but only galanin-ir fibers in the infundibular region. Galanin fibers and cells are typically found in the INF of sheep (Chaillou & Tillet, 2005) and rat (Ciofi, Leroy, & Tramu, 2006). The bovine exhibits the same general organization of fibers, but we found no galanin-positive neuron somas in the INF of cattle.

AChE staining was found throughout Ch1-Ch4 described in Mesulam, Mufson, Wainer, and Levey (1983). Namely, AChE-ir neurons were found in the medial septal area (MSA), like it was found in humans (Swaab, 2003), as well as the diagonal band of Broca and the *nucleus basalis* of Meynert. The topography of the islands of Calleja, characterized by AchE-if fibers (Alheid, Switzer III, & Heimer, 1990) and located between the ventral pallidum, the diagonal band of Broca and the *substantia innominata*, was consistent with what was reported in other species and orders (Meyer, Gonzalez-Hernandez, Carrillo-Padilla, & Ferres-Torres, 1989).

4.5 | Sex differences and the freemartin syndrome

Freemartin heifers show an intermediary development of the VON between the male and the female regarding size and cell number. In the developing bovine hypothalamus, the critical period for sexual differentiation occurs in early gestation phases, during the second quarter of gestation (Peruffo, Cozzi, & Ballarin, 2008). It has been shown that during this time, estrogens stimulate sexually dimorphic patterns

of development by binding to α and β estrogen receptors. In this context, the freemartin VON may have an early intermediate development disturbed by the male hormonal fetal environment. As it is well known that steroid hormones influence neurogenesis and dendritic growth on neural cells, these effects may have influenced sexual dimorphism by increasing the overall number and size of neurons within the hypothalamic nuclei, including the VON. A recent paper showed that estrogens exposure exerts a trophic effect on developing bovine granular neurons (Montelli, Suman, Corain, Cozzi, & Peruffo, 2016). Previous work on estrogen receptors α and β in Cetartiodactyla (including the ovine and the bovine) (Panin, Corain, Montelli, Cozzi, & Peruffo, 2015; Peruffo, Buson, et al., 2008; Schaub, Gersting, Keller-Wood, & Wood, 2008), suggest that, in freemartins, the brain of the female twin exposed to male androgens undergoes a partial masculinization either through aromatization into estrogens and activation of ER-α and ER-β (Kudwa, Michopoulos, Gatewood, & Rissman, 2006; Roselli, Liu, & Hurn, 2009), or via the direct effect of androgens on androgen receptors.

Freemartins seem to develop a larger SCN that contains more VIP-expressing neurons and a larger volume than both cows and bulls. The ratio between the number of cells and the area of measure also shows that the global density remains very comparable across the three groups, which means that there is a certain homogeneity in the cell density of the SCN across sex categories. Thus, it seems that the general cellular organization remains the same. In order to reach this larger size and number of cells, the freemartin SCN, most likely during brain development after a peak value, does not lose as many VIP-expressing neurons as male or female bovine do to reach normal levels, as it has been proposed in humans for AVP-containing neurons in the SCN (Swaab et al., 1994; Swaab, Hofman, & Honnebier, 1990). There is a case of larger SCN than both male and female, which has also been described, concerning AVP-ir neurons of the SCN in relation to homosexuality. In their article on the SCN of homosexual men, Swaab and Hofman (1990) noted an enlargement of the SCN by 2.1 times in AVP expressing neurons and 1.73 times in volume relatively to heterosexual men (Swaab & Hofman, 1990). However, this difference was not present in the VIP-containing cells (Zhou, Hofman, & Swaab, 1995a). The observations showed that the SCN of homosexual men was not an intermediary of male and female SCN, which did not contain a sex difference (Swaab, Slob, Houtsmuller, Brand, & Zhou, 1995). In the case of freemartin heifers, a link to the sexual behavior consequences of this difference would be hard to establish. No behavioral comparison has been made for this study, although the freemartin syndrome arises in a species in which one of the most reliable signs of estrus is the active and passive mounting between females in the herd, hence a typical male-type behavior cannot be easily characterized.

The development of the SCN seems to be influenced by sex hormones. In male rat, the prenatal administration of 1,4,6-androstatriene-3,17-dione (ATD), an aromatase inhibitor that precludes the formation of estrogens from testosterone, induced a bisexual partner preference and a greater number of AVP-positive neurons in the SCN (Swaab et al., 1995). In addition, a more recent study involving letrozole, an aromatase inhibitor, in prenatally treated male rats showed a reduced volume and cell number in the SDN and SCN, independently of sex preference (Olvera-Hernández, Tapia-Rodríguez, Swaab, & Fernández-Guasti, 2017). The prenatal treatment alone of ATD did not change the partner preference, but the postnatal treatment did not change the AVP neuron number in the SCN. This fact, along with the fall of anti-Müllerian hormone (AMH) 9 days after birth in freemartin cows (Rota et al., 2003), suggests that sexual behavior itself may be more intimately linked to the circulating hormones at the time of the behavior via binding to the expressed receptors. On the other hand, the prenatal hormonal environment could more probably influence directly or indirectly the development of the circuitry necessary for the said behavior, as described in humans by Kruijver and Swaab (2002), since in the case of freemartin heifers, androgens return to female levels after birth (Rota et al., 2003). In both the VON and the SCN, it seems that the development of the female (XX) nuclei is disturbed by androgens, either directly through androgen receptors, or through aromatization of androgens and adhesion to estrogen receptors. The VON appears masculinized, with a reduction of the volume and number of neurons, while, the SCN shows a notable overgrowth. This difference between the VON and the SCN, exposed to the same prenatal hormonal environment, could be explained by a difference in estrogen and/or androgen receptor expression or subtype, as no estrogen receptor was found in the VON of adult pigs (Van Leeuwen, Chouham, Axelson, Swaab, & Van Eerdenburg, 1995) while they are present in the adult SCN (Kruijver & Swaab, 2002). Since the VIP neurons of the SCN influence daily rhythm synchronicity, their role in milk production should be further explored. Moreover, the potential effect of the sex chromosomes in the cells themselves and their reaction to one or the other hormonal environment must be taken in account, as it was shown by Moore et al. (1957) that the freemartin neuron chromatin was not altered by the prenatal male hormonal environment. This could mean that the methylation of freemartin neurons' DNA is that of females and not males (McCarthy et al., 2009), ultimately influencing gene expression. Our results show a difference in the organization of the SCN in freemartin bovine that is prenatally determined by a male hormonal environment and might be a marker for the development of an alternative neuronal network. In details, few studies have studied the anatomy of the bovine hypothalamus from a chemoarchitectonic perspective (Leshin et al., 1988; Leshin, Kraeling, Kiser, Barb, & Rampacek, 1995). As of today, relatively few studies use bovine brain tissues for comparative purposes. However, public awareness and ethics influence the use of animals in translational studies, and consequently increasingly weigh on regulations and study designs. In this regard, brain tissues from slaughterhouses can be a valuable ethical alternative. This brings forward the importance of multiple animal models in the understanding of physiological processes and anatomical structures. Additionally, as stated by Short, Jost, Harris, and Ford (1970), the freemartin syndrome has attracted in the past the attention of leading specialists and, although a large part of the puzzle has now been explained, its potential contribution to neuroscience remains unknown.

5 | CONCLUSIONS

The neurochemical organization of the bovine hypothalamus is comparable to that of other mammal. In our investigation, we found that

the SCN was sexually dimorphic and that there was a compact group of cells between the PVN and the SCN that we called the VON, which was also sexually dimorphic. This sex difference, unlike in other species, consisted of a larger female nucleus than males'. Freemartin bovines were found to have a much larger SCN than males and females, and an intermediate VON between males and females. This finding confirms the prenatal organizational effects of androgens on the development of the brain. More broadly, the present study emphasizes the effects of prenatal steroids on the brain of precocious mammals such as cattle, and the disturbances that may arise from a misbalance of such steroids in the pregnant cow. In particular, the similarity with the increased SCN size, although in AVP cells, in homosexual men found by Swaab and Hofman (1990) is intriguing. Further studies on this specific animal model could increase our understanding of the complex interactions between structure and function in neurology (de Vries & Södersten, 2009; McCarthy, 2016; Yang & Shah, 2014).

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SUPPORTING INFORMATION

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APPENDIX

Advanced statistical analytics

In order to formalize the comparison among the three populations under investigation, i.e. male, freemartin and female, we assumed a suitable data representation model. More formally and without loss of generality, let us assume that the 3-dimensional nucleus-related morphometric features Y (area, no. of cells and density) measured on the *i*-th slide (our experimental unit) at *s*-th nucleus side (left or right) from the *l*-th subject belonging to the *j*-th population can be modelled as

$$\mathbf{Y}_{ilsj} = \boldsymbol{\mu} + \boldsymbol{\tau}_j + \boldsymbol{\eta}_{l(j)} + \boldsymbol{\beta}_s + \boldsymbol{\varepsilon}_{ilsj}, \quad (1)$$

where $\boldsymbol{\varepsilon}_{ilsj}$ are i.i.d. possibly non-Gaussian error terms with null mean and scale coefficients $\boldsymbol{\sigma}_j^2=\boldsymbol{\sigma}^2(\boldsymbol{\tau}_j)$ and unknown distribution P_{ε}, $\boldsymbol{\mu}$ is a population-invariant constant, coefficients $\boldsymbol{\tau}_j$ represent the *main population effects,* $\boldsymbol{\eta}_{l(j)}$ refers to the individual/subject effects (obviously nested within each population), $\boldsymbol{\beta}_s$ is the nucleus' side effect, and $\boldsymbol{\sigma}^2(\boldsymbol{\tau}_j)$ are population-varying scale coefficients which may depend, through monotonic functions, on main treatment effects $\boldsymbol{\tau}_j$. Basically, the proposed data representation model is a quite general less-demanding nonparametric model where specific location and scale effects are both allowed across populations.

Since the study's main goal was to compare the sex-related populations, we actually inferred on the *main population* coefficients $\boldsymbol{\tau}_j$. With the same inferential goal, but at an univariate level and by assuming both normality and homoscedasticity, i.e. $\sigma_j^2=\sigma^2 \ \forall j$, the biostatistics literature proposes using the so-called multilevel or mixed-effect linear model (Aarts, Verhage, Veenvliet, Dolan, & van der Sluis, 2014), where the individual effect is assumed as a random realization from a normal distribution, i.e $\eta_{l(j)}\sim N(0;\sigma^2_{\eta})$. Mixed effects models are expected to prevent faulty inference in analysis of data sampled from multiple observations per subject by accounting for intra-class correlation (Wilson, Sethi, Lein, & Keil, 2017). However, in order to keep a more flexible and reliable inferential analysis, we preferred dealing with the issue of intra-class

correlation by referring to a suitable extension to model (1) of the nonparametric combination and permutation-based testing method (Corain & Salmaso, 2015).

Consequently, we underline that the study's main goal was actually inferring on the *main population* coefficients $\boldsymbol{\tau}_j$, while $\boldsymbol{\eta}_{l(j)}$ should be considered as random nuisance parameters. In order to remove the nuisance effects, let us define the random variable $\widetilde{\mathbf{Y}}_{ilj} = \mathbf{Y}_{ilj} - \boldsymbol{\eta}_{l(j)}$ and, by using the Roy's Union-Intersection testing approach (Pesarin & Salmaso, 2010), let us formalize, separately for the location and scatter parameters, the comparison between the *j*-th and the *h*-th population with the null and alternative hypothesis as follows:

$$\begin{cases} H_{0(jh)}: \bigcap_s \bigcap_k \tilde{Y}_{sjk} \overset{loc}{=} \tilde{Y}_{shk} \equiv \bigcap_s \bigcap_k [\eta_{sjk} = \eta_{shk}] \\ H_{1(jh)}: \bigcup_s \bigcup_k [(\tilde{Y}_{sjk} \overset{loc}{<} \tilde{Y}_{shk}) \cup (\tilde{Y}_{sjk} \overset{loc}{>} \tilde{Y}_{shk})] \\ \quad \equiv \bigcup_s \bigcup_k [(\eta_{sjk} < \eta_{shk}) \cup (\eta_{sjk} > \eta_{shk})] \end{cases} \quad \begin{cases} H_{0(jh)}: \bigcap_s \bigcap_k \tilde{Y}_{sjk} \overset{scat}{=} \tilde{Y}_{shk} \equiv \bigcap_s \bigcap_k [\sigma^2_{sjk} = \sigma^2_{shk}] \\ H_{1(jh)}: \bigcup_s \bigcup_k [(\tilde{Y}_{sjk} \overset{scat}{<} \tilde{Y}_{shk}) \cup (\tilde{Y}_{sjk} \overset{scat}{>} \tilde{Y}_{shk})] \\ \quad \equiv \bigcup_s \bigcup_k [(\sigma^2_{sjk} < \sigma^2_{shk}) \cup (\sigma^2_{sjk} > \sigma^2_{shk})] \end{cases} \quad (2)$$

where k = 1,2,3, is the reference index for each individual univariate morphometric feature.

It is worth noting that hypothesis (2) refers to a nonparametric version of the so-called generalized Beherens-Fisher problem (Yanagihara & Yuan, 2005). Despite the fact that $\widetilde{\mathbf{Y}}_{isjl}$ is not an observable variable, it can be estimated by residuals from the estimate of $\boldsymbol{\eta}_{l(j)}$, so that under the null hypothesis those residuals are actually exchangeable random components that can be permuted between groups in order to derive two multivariate directional *p*-values, set to 0.025, separately for the location and scatter problems. As univariate location and scatter permutation statistic tests, we respectively used the differences of sample means and squared deviations together with Fisher's combining function (Pesarin & Salmaso, 2010), to derive the multivariate combined *p*-values.

Finally, results of pairwise testing as in (2) can be exploited to provide an extension to model (1) of the ranking methodology recently proposed by Arboretti, Bonnini, Corain & Salmaso (2014) and Corain, Arboretti & Bonnini (2016). Under different random distributions Corain, Ceccato, Salmaso & Peruffo (2018) proved the validity of the proposed testing and ranking solution (for a more in depth understanding on the testing and ranking procedure, see Arboretti et al., 2014).

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RESOLVING SINGLE CELLS IN HEAVILY CLUSTERED NISSL-STAINED IMAGES FOR THE ANALYSIS OF BRAIN CYTOARCHITECTURE

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ABSTRACT

The analysis of the cytoarchitecture of a tissue is of great importance for the understanding of development, behavior and disease. This is also true when analyzing tissue specimens of the brain, for analyzing cells morphology and their spatial organization. To this end, on the Nissl-stained each single cells present in the sample needs to be detected, classified according to its morphology and position. The dimension of typical histological images and the sheer numbers of cells present make the task impossible to be carried out manually.
Additionally, the presence of background and staining heterogeneity, clutter, heavily clustered cells, and variability in shape and appearance of cells, makes the task difficult also for automatic methods.
We present a method that building on the tentative detection obtained by local thresholding and radial symmetry transform, represent each cell cluster as a sparse mixture of gaussians. We show that the proposed method performs well both in terms of precision and recall, obtaining a F_1-score of 0.87 on Nissl-stained images of the cerebellum.

Index Terms— histology, brain, segmentation, cell detection, radial symmetry, mixture models

1. INTRODUCTION

Since the last century, numerous studies have been performed to demonstrate the correlation between structure and function: in particular, the cytoarchitecture of the cerebellum might be of particular interest in the evaluation of sexual dimorphism [1] and neurological diseases [2, 3]. The cerebellum has a uniform architecture throughout and is divided into the cerebellar vermis along the medial portion and cerebellar hemispheres laterally with the paravermis (or intermediate zone) located between the two; these regions are divided into ten lobules, arranged dorsoventrally. Further, each lobule is compartmentalized along the medial-lateral axis into sagittal zones [4]. From a cytoarchitecture perspective, the cerebellum shows a laminar structure, each lamina being characterized by the presence of specific cellular types and by a specific spatial organization of cells, dendrites and axons. Despite a uniform structural organization of the cerebellum (conserved also among different species) the density, morphology and arrangements of the different cell types seems to be linked to different underlying genotypes and functional phenotypes.

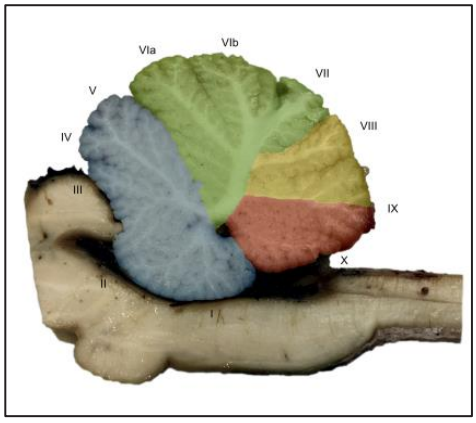

Figure 1 Sagittal section cut through the bovine cerebellar vermis with its stereotypical foliation pattern, which consists of 10 lobules (shown in false colors).

In order to investigate the differences in the morphometric and cytological organization of the cerebellum, it is required to identify and classify both the different laminar tissues and specific cellular types that are present. This is usually performed manually on samples stained with the Nissl technique that allows for consistent labeling of the whole neuronal population, showing the regional or laminar organization of the cytoarchitecture in the different brain areas. The large amount of data and the huge dimension of histological images required for this kind of investigations makes the manual processing infeasible for even small studies.

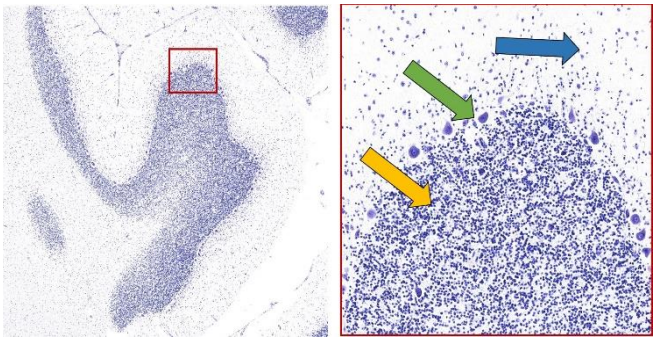

Figure 2 Representative example of a Nissl stained image of the cerebellum. In the enlarged region are pointed the granular region (orange arrow), the Purkinje cells (green arrow) and the molecular layer with sparse cells (blue arrow).

Hence, a system requiring as small human interaction as possible for analyzing the images providing a reliable identification and classification of the cells in the specimen would allow neuroanatomical investigation at the cellular level.

In general it is well recognized that automatic analysis of microscopy and histopathological images it is a challenging task, and that it is difficult to achieve robust and accurate cell segmentation. Images often exhibit background clutter with many noises, artifacts, that is coupled with a significant heterogeneity in the size, shape and appearance of cells, that can also be tightly packed together in touching and overlapping clusters. A thorough review of methods can be found in [5].

As for comparison with the proposed method we chose the classical method based on Laplacian of Gaussian filtering proposed in [6], the recently proposed [7] multi-pass evolution of the method based on structural saliency [8] [9], and the size-invariant symmetry-based based detection [10] whose refinement inspired this work: in fact, despite its appeal, the symmetry-based methods have the tendency to oversegment cells grouped into clusters, particularly in the case of Nissl-stained images.

3. METHODS

3.1. Preprocessing

Nissl-stained images of the cerebellar cortex are characterized by a layered appearance of the different cell types: from the outside inward there is the molecular layer, then a thin mono-cellular layer composed by Purkinje cells, the granular layer where the cells are most densely packed, and finally the white matter (Fig. 2). In the outermost and innermost layer, the cells are usually sparse and do not show overlaps and packing. In order to avoid further processing in these regions (while concentrating the efforts on the most dense region), it is useful to preprocess the images so to roughly identify the different layers. An estimation of a local space-varying threshold [11] to be applied to the image allows the separation of stained objects from the background. Then the local density of the detected objects is

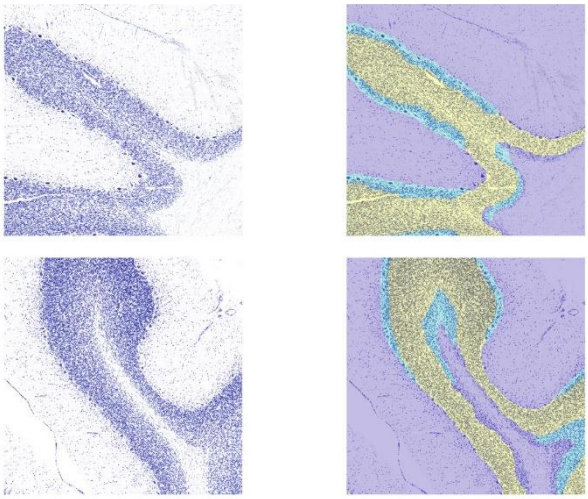

Figure 3 Two representative images of Nissl-stained samples of the VII lobe of the cerebellum, with the rough segmentation (Sec 3.1) in granular region (light yellow), region containing the Purkinje cells (cyan), and the molecular and white-matter region (purple).

estimated: based on this and on a constraint on the ordering of the layers a rough separation of the most densely (possibly with clustered and cluttered cells) and most sparse regions is obtained.

3.2. Identification of clustered cells

The application of the local threshold as in [12] provide a first segmentation of the cells from the lightly stained background of the specimen; then, a small set of threshold on the values of eccentricity, areas and solidity of the identified objects allows the identification of single small cells (limited area, high circularity and solidity), Purkinje cells (large area, high circularity, decreasing solidity with area), from possible clusters of cells.

3.2. Separate clusters through sparse Gaussian-mixture

Each object that has been estimated as a possible cluster, is separately analyzed. In order to find the all possible position of the cell nuclei within the cluster, a multi-scale Fast Radial Transform (MSFRT) [13] [14] [10] is computed. After a small smoothing through a median filtering (so to preserve the peak information reducing the noise), all N local maxima of the filtered MSFRT are used to provide an initial estimate for the presence of a separated cell.

Under the hypothesis that the cells have a regular appearance that could be approximated by an elliptical shape, we choose to represent each cell with a 2-dimensional Gaussian profile. In case of clustered cells, this leads to a representation of the cluster as a mixture of Gaussian, with a number of modes corresponding to the number of cells composing the cluster. Hence, the image data around the cluster I_{clu}(x,y) are fit with a 2-dimensional Gaussian mixture model with a number of modes N equals to the number of the local maxima detected through

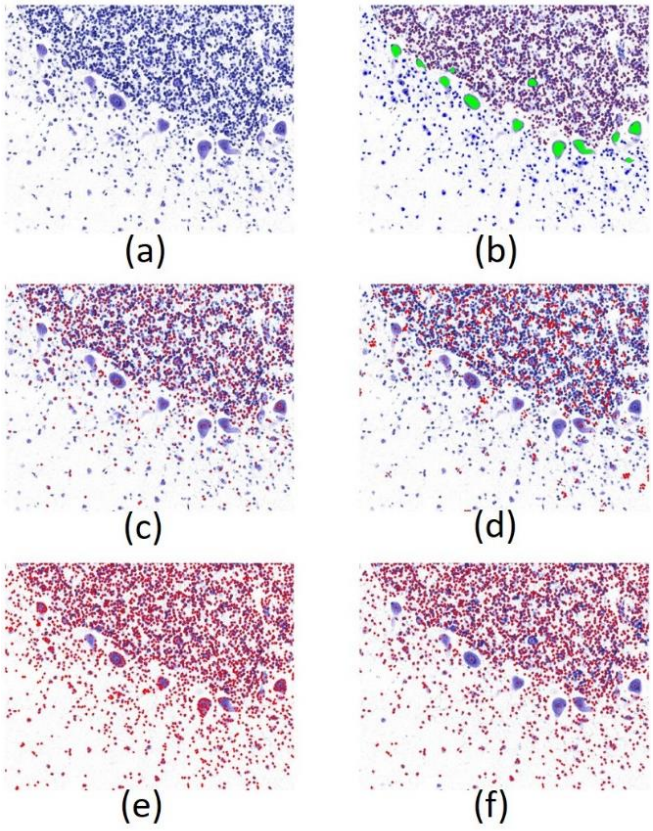

Figure 4 Results of cell detection in a representative image region (a), shown with the corresponding manual annotation (b). Al-Kifahi et al. method (c), Lu et al. method (d), Ram et al. method (e) and the proposed method (f) are shown for visual comparison. All detected cells are shown with a red cross.

MSFRT, each centered at the corresponding position $c_i = (x_i, y_i)\ i = 1, \dots N$:

$$G(x, y; c_i, \Sigma_i) = e^{-0.5((x,y)-c_i)^T \Sigma_i^{-1}((x,y)-c_i)}$$

$$GMM(x, y) = \sum_{i=1}^{N} \alpha_i G(x, y; c_i, \Sigma_i)$$

By keeping the position c_i fixed, only the mixing component α_i and covariance matrix Σ_i representing the shape (dimension, eccentricity and orientation) of each possible cell need to be estimated. Additionally, by constraining the mixing component to be positive, and by checking if each candidate cell-center is still a local maximum of the MSFRT after the fit, the optimization tries to remove all candidate cells whose presence is not supported by the image data I, while constraining all others to have an elliptical shape:

$$\begin{cases} (\hat{\alpha}_i, \hat{\Sigma}_i) = \arg\max_{\alpha_i, \Sigma_i} (I(x, y) - GMM(x, y))^2 \\ \alpha_i \geq 0\,, \quad i = 1, \dots, N \end{cases}$$

In addition to the implicit sparse search provided by the non-negative constraint on α_i, an additional post-hoc check of the
Gaussian centers c_i is performed: all the centers (candidate cells) not corresponding to a local maximum of the fit $GMM(x, y)$ are removed.

4. DATA

3 adult (24 months old) bovine brains were obtained at local abattoirs. Animals were treated according to the present European Community Council directive concerning animal welfare during the commercial slaughtering process and were constantly monitored under mandatory official veterinary medical care. The cerebellum were collected under sterile conditions, and fixed by immersion in buffered formalin. From each cerebellum, the VII lobe was serially cut in 6 µm frontal sections, and one every 50 section was mounted and stained using the Nissl technique (the purple-blue Cresyl violet stain will allow the identification of the basic neuronal structure in brain samples). The stained sections were then imaged with a semi-automated microscope equipment (D-Sight v2, Menarini Diagnostics, Italy) at a magnification of 40X in fast mode. The images will be exported as Jpeg2000, resulting in a mean dimension of 42000x42000 pixels with a resolution of 0.5 μm per pixel. Each image was downsampled to keep the computational burden low, to an equivalent resolution of 1 μm per pixel; on each of these images, a small area of approximately 1000x1000 pixels was manually annotated, identifying the center of the visible cells, for a total count of 3782 cells.

Table 1 Performance of the proposed and competing algorithm in terms absolute numbers of detected cells (first column), wrong detections (second rows), and detected areas corresponding to multiple cells that were not separated.

Method	Detected cells (TP)	Non-cell detection (FP)	Remaining clusters
Al-Kofahi et al [6]	1837	2178	14
Lu et al. [7]	2280	9226	94
Ram et al. [10]	**3561**	7233	56
Proposed	3294	**488**	**20**

5. RESULTS

The detection performance was evaluated in terms of the ability of each algorithm to correctly identify a cell (true positive, TP), to reduce the number of objects that are wrongly identified as cells but belong to the background (false positives, FP), and to correctly separate different cells (objects containing more than one cell, remaining clusters

(Tab. 1, results as absolute values). The results show that at the price of a small reduction in the number of detected cells, a dramatic reduction in the number of false positives is obtained through the proposed method. A qualitative representation of the results is given in Fig. 4.

In order to score the performance proportionally with respect to the number of annotated cells, defining the false negatives (FN) as the cells that were manually annotated but missed by the algorithm, we computed the precision as $TP/(TP+FP)$, the recall as $TP/(TP+FN)$ and F_1-score as $2TP/(2TP+FP+FN)$ (Tab. 2).

Table 2 Performance of the proposed and competing algorithms

Method	Precision	Recall	F_1-score
Al-Kofahi et al [6]	0.45	0.49	0.47
Lu et al. [7]	0.20	0.60	0.30
Ram et al. [10]	0.33	**0.94**	0.49
Proposed	**0.87**	0.87	**0.87**

6. CONCLUSIONS

The quantitative analysis of cell morphology and cytoarchitecture needs a precise detection of neuronal cell bodies in histological slides. Automatic detection and segmentation is a difficult task due to the presence of appearance and shape heterogeneity, clutter, and cell clustering. We present a method that, building on a preliminary candidate cell detection through a space variant threshold and a multi-scale radial symmetry transform, refine the results by modeling each cluster with a mixture of Gaussians model.

We expect to integrate this single-cell detection method with a multivariate nonparametric testing approach able to provide a ranking of the neuroanatomical complexity across populations defined by factors such as sex, age or species [15] [16].

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