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Central nervous system development in rabbits (*Oryctolagus cuniculus* L. 1758)

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Abstract

The present study describes the embryonic and fetal development of the central nervous system in rabbits from the seventh day after conception until the end of the full-term fetal period. A total of 19 embryonic and fetal samples were carefully dissected and microscopically analyzed. Neural tube closure was observed between 7.5 and 8 days of gestation. Primordial encephalic vesicle differentiation and spinal canal delimitation were observed on the 12th day of gestation. Histologically, on the 15th day of gestation, the brain, cerebellum, and brain stem were delimited. On the 18th day of gestation, the cervical and lumbar intumescences of the spinal cord were visible. On the 28th day of gestation, four-cell layers could be distinguished in the cerebral cortex, while the cerebellar cortex was still differentiating. Overall, the morphological aspects of the embryonic and fetal developmental phases in rabbits were highly similar to those in humans. Thus, the present study provides relevant information highlighting rabbits as an excellent candidate animal model for preclinical research on human neurological diseases given the high adaptability of rabbits to bioterium conditions and the similarity of morphological events between rabbits and humans.

KEYWORDS

animal models, brain, neurological studies, rabbit, spinal cord

1 | INTRODUCTION

Recently, the need to elucidate the detailed morphological aspects of embryonic development in laboratory animals has intensified for gaining a better understanding of emerging neurological diseases (Edwards, 1968). The growing interest in the neurotoxic effects of state-of-the-art drugs is now a fundamental part of experimental trials of novel drugs (Hanley, Carney, & Johnson, 2000). However, it is impossible to evaluate these effects without the knowledge of the highly complex stages of central nervous system development (Konefal, Elliot, & Crespi, 2013; Ming & Song, 2011).

The embryological phases of central nervous system development in humans have already been described (Menu, 2009; O'Rahilly & Müller, 2010; Shiraishi et al., 2015; Ten Donkelaar, Yamada, Shiota, & Van Der Vliet, 2014). These descriptions are also available for laboratory animals, such as mice (Chen et al., 2017; Chen et al., 2017; Ybot-Gonzalez et al., 2007), rats (Alicelebić, Mornjaković, & Kundurović, 2004; Altman & Bayer, 1985; Bâ & Seri, 1993; Dziegielewska, Ek, Habgood, & Saunders, 2001; Favaron, Rodrigues, Oliveira, Biasi, & Miglino, 2012), and guinea pigs (Silva et al., 2016), as well as for livestock (Ferreira et al., 2018; Franciolli et al., 2011; Greenstein & Foley, 1958; Rigoglio et al., 2017) and other domestic animals (Knospe, 2002; Marin-Padilla, 1971).

Rabbits are small and common mammals with few needs for experimental accommodation. They have a short and easily programmable reproductive cycle, produce large litters, and show placental development similar to humans (Fischer, Chavatte-Palmer, Viebahn, Navarrete Santos, & Duranthon, 2012; Julik et al., 2012; Seiler, Fischer, Lindenau, & Beier, 1994). Therefore, rabbits have been used as models in biomedical research, primarily in studies on in vitro fertilization, organogenesis, and regenerative medicine (Badran, Waki, Hamamoto, Manz, & Wong, 2014; Fischer et al., 2012; Manning, Ringler, & Newcomer, 1994; Puschel et al., 2010; Seiler et al., 1994).

However, there are fewer studies on the central nervous system development in rabbits than in other species. Some previous studies focused on the early stages of development (Beaudoin, Barbet, & Bargy, 2003; Bryden, Evans, & Binns, 1972; Noden & Lahunta, 1990) or the external morphological aspects of embryos in rabbits (Edwards, 1968), and the stages of neurulation alone have been described (Peeters, Viebahn, Hekking, van Straaten, & Christoph, 1998). Specifically, broad descriptions of the intrinsic factors, such as the apical contraction of neuroepithelial cells, formation of the points of articulation of the neural plate, and differentiation and proliferation of cells, as well as extrinsic factors, such as the expansion of the mesoderm and ectoderm (Van Straaten, Hekking, Consten, & Copp, 1993), are available.

Another aspect of neurulation is its association with the craniocaudal curvatures of the body axis. In rabbit and human embryos, the rate of neural tube closure decreased during development with increased axial curvature (Peeters, Hekking, Van-Straaten, Shum, & Copp, 1996). The similarity between these events could be exploited to study diseases originating from the failure of embryonic neural tube closure, as observed in cases of anencephaly and spina bifida (Juriloff & Harris, 2018).

In the present study, the primary aim was to propose an animal model showing phenocopies under controlled conditions and exhibiting developmental characteristics close to humans for experimental use. By describing the macroscopic and microscopic events occurring during the development of the central nervous system in rabbit embryos and fetuses at all stages of gestation, we hope to elucidate the pathological processes related to this system and compare these events with those in other mammals.

2 | MATERIAL AND METHODS

2.1 | Animals

Twelve New Zealand rabbit females at different phases of gestation, submitted to natural mating for experimental cuniculture at the University of São Paulo, Pirassununga, were used. The experimental protocol was approved by the Honorary Ethics Committee (no. 13.11910.74.9).

The day of mating and the age of embryos and fetuses were considered in the data analysis. At the beginning of gestation, it was impossible to obtain the measurements of embryos and fetuses (7.5, 8, 9, and 10 days of gestation). Thus, to confirm the gestational age, specific samples (larger embryos and fetuses) were measured using the methodology recommended by Evans and Sack (1973); the distance between the highest point on the head and the most caudal extent of the buttocks at the base of the tail (crown-rump), associated with the external features, was measured as the reference. After confirming the gestational age, samples at 12, 15, 16, 18, 20, 22, 25, and 28 days of gestation were selected.

To describe the central nervous system development, 19 samples were divided into three distinct groups according to external morphological features: the beginning of gestation (7.5–12 days), mid-gestation (15–-22 days), and the end of gestation (25–28 days) (Table 1).

TABLE 1 Biometric data and estimated age according to the crown-rump length (Evans & Sack, 1973) of rabbit (*Oryctolagus cuniculus*) embryos and fetuses

Gestational stage	Number of animals	Weight (g)	Crown-rump (mm)	Gestational age (days)
Beginning of gestation	1	—	—	7.5
	1	—	—	8
	1	—	—	9
	1	—	—	10
	3	0.012–0.025	3.5–4.5	12
Mid-gestation	2	0.580–0.705	16–17	15
	3	0.632–1.216	18	16
	1	3.34	32	18
	1	5.33	39	20
End of gestation	2	13.147	47	22
	2	22.63–22.66	70	25
	1	38.58	88	28 (term)

2.2 | Macroscopic analyses of the central nervous system

Embryos at the early stages of development (7.5, 8, 9, and 10 days) were processed along with the uterus due to their small size. The analyses were performed directly on 5-μm-thick histological sections of the fetal–maternal set obtained by fixation and paraffin embedding.

Larger embryos and fetuses were photographed with a digital camera (Canon DSLR EOS Rebel T5i) to analyze the shape and external features. A caliper was used to measure the crown-rump length (mm) and an analytical balance (Mettler® Toledo New Classic ME) to measure the weight (g). The brain and spinal cord of samples at 15, 16, 18, 20, 22, 25, and 28 days of gestation were dissected to characterize the structures (brain stem, pons, piriform lobe, brain peduncle, cerebellum, and cervical and lumbar intumescences).

The nomenclature established by the International Committee on Veterinary Gross Anatomical Nomenclature (2017a), International Committee on Veterinary Histological Nomenclature (2017b), and International Committee on Veterinary Embryological Nomenclature (2017c) was followed.

2.3 | Microscopic analyses of the central nervous system

Embryos at the early stages of development (7.5, 8, 9, and 10 days) were processed along with the uterus due to their small size. Other embryos and fetuses were fixed separately from the uterus in 10% formaldehyde solution for 48 hr, dehydrated in a series of increasing concentrations of ethanol (70–100%), and diaphonized in xylol for subsequent paraffin embedding (Histotec) and sectioning in the longitudinal plane (Tolossa, Rodrigues, Behemer, & Freitas-Neto, 2003).

Sections (thickness, 5 μm) of the paraffin blocks were obtained with an automatic microtome (Leica RM 2165, Germany) and stained with hematoxylin–eosin (HE) and toluidine blue. Microscopic photo documentation was performed using a photomicroscope (Olympus® BX40).

2.4 | Staging

The macroscopic and microscopic features of the central nervous system development in rabbits were compared with those in humans according to the Carnegie stages as described by O'Rahilly and Müller (2010).

3 | RESULTS

3.1 | Biometric data and external morphological features

The key morphological features and biometric data (weight and crown-rump length) of each group are described in Table 1.

3.1.1 | Beginning of gestation

This group included embryos between 7.5 and 12 days of gestation. However, since the embryos could be

macroscopically characterized only after 12th day of gestation, the optic vesicles could not be observed in this group. The thoracic and pelvic limbs appeared paddle-like (Figure 1-1).

3.1.2 | Mid-gestation

This group included embryos between 15 and 20 days of gestation. The crown-rump length was 16–47 mm, and the weight was 0.58–13.14 g. In these specimens, the phenotypic characteristics of embryonic to fetal transition were observed. On the 15th day of gestation, hepatic prominence was observed in the embryos, and the cervical curvature was more evident. On the 18th day of gestation, the digits could be distinguished in embryos (Figure 1-3). At 22 days of gestation, the nasal and mandibular prominences were more developed, and the optic vesicle was clearly visible due to retinal pigmentation. The external acoustic meatus was observed, but the auricle had not yet formed (Figure 1-4).

On 22nd day of gestation, the cervical curvature was less pronounced but the cephalic region was well-developed. Due to the development of the thoracic and abdominal cavities with the consequent growth of the intracavitary organs, the cardiac and hepatic prominences could be observed at this stage (Figure 1-4).

3.1.3 | End of gestation

This group included specimens, classified as fetuses, presenting the key features of the order Lagomorpha. In these fetuses, the outer ear was formed and the nostrils and tactile hair were delimited. The oral cavity was well-delimited, which enabled the distinction between the nasal and mandibular regions. In fetuses at term, the skin was no longer reddish and turned yellowish. The distal segments of the thoracic and pelvic limbs were well-formed, with fully separated digits and claws. At this stage, the crown-rump length was 70–88 mm and the weight was 22.63–38.58 g (Figure 1-5).

3.2 | Macroscopic features of the central nervous system

3.2.1 | Beginning of gestation

The primitive brain vesicle was developed and could be distinguished into the prosencephalon, mesencephalon, and rhombencephalon (Figure 2a).

3.2.2 | Mid-gestation

At 15 days of gestation, the brain, cerebellum, and brain stem structures were delimited. The cerebellum showed

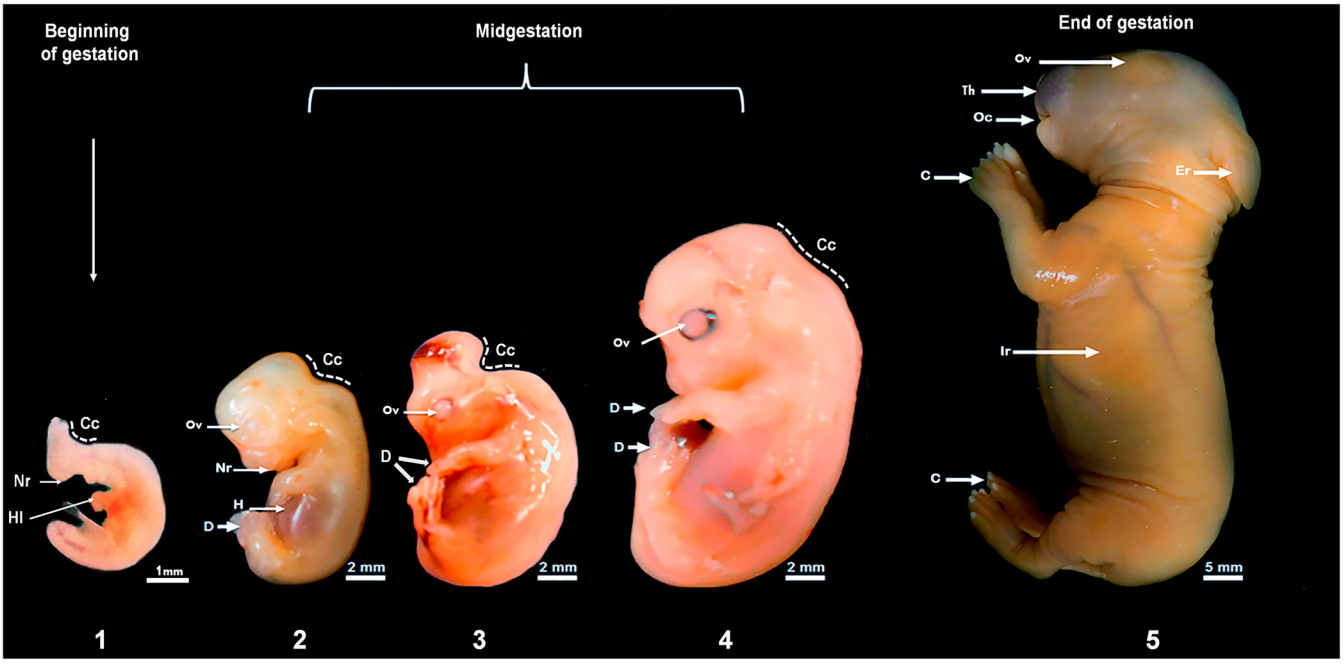

FIGURE 1 Developmental stages in New Zealand rabbits during gestation. 1—12 days of gestation, 2—15 days of gestation, 3—18 days of gestation, 4—22 days of gestation, and 5—28 days of gestation. C, claws; Cc, Cervical curvature; D, Digit buds; Er, Ear; H, Hepatic prominence; Hl, Hind limb bud; Ir, Impression of ribs; Nr, Nasal region together with the mandible; Ov, Optical vesicle; Oc, Oral cavity; Th, Tactile hair

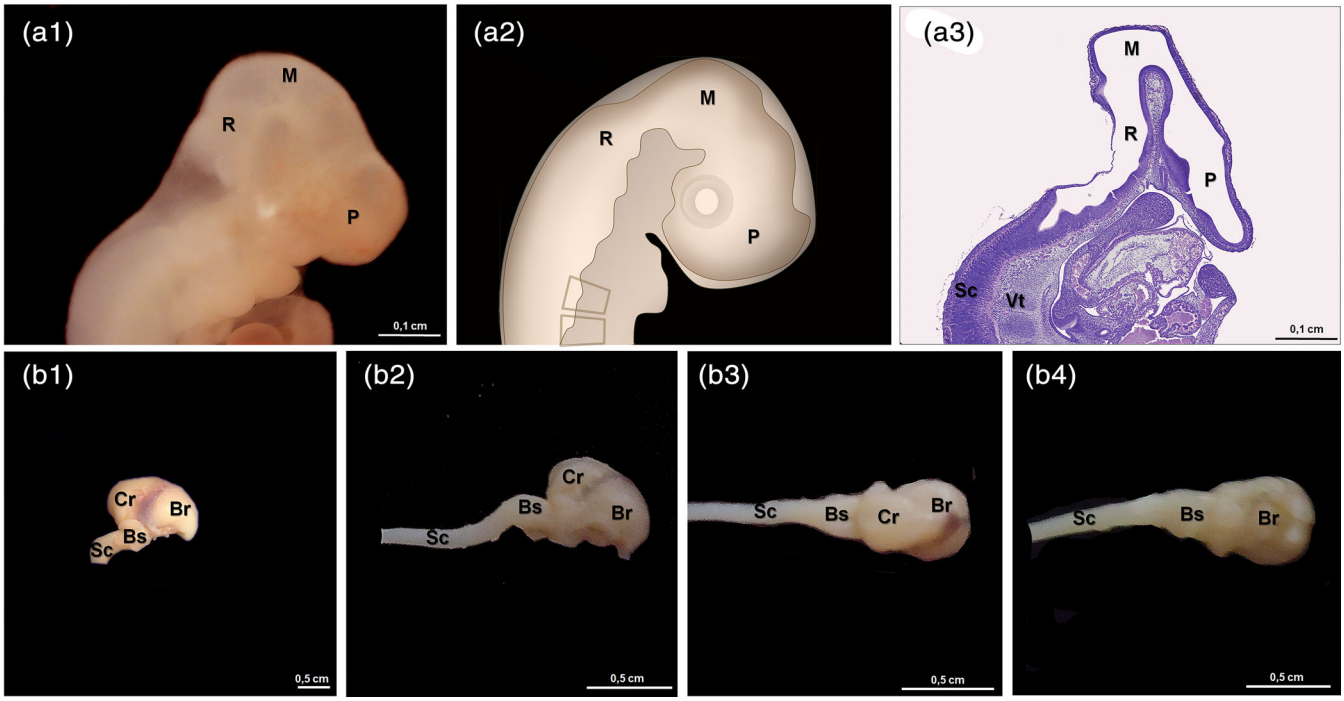

FIGURE 2 Development of the central nervous system in New Zealand rabbits. a1–a3: Macroscopic and histological (hematoxylin–eosin) views of the brain vesicles in an embryo on the 12th day of gestation. a2: Schematic representation of the brain vesicles in an embryo on 12th day of gestation. b1: Lateral view of the fetal encephalon in 15th days of gestation. b2: Lateral view of the fetal encephalon on the 16th day of gestation. b3: Dorsal view of the fetal encephalon on the 16th day of gestation. b4: Ventral view of the fetal encephalon on the 16th day of gestation. Bs, Brain stem; Cr, Cerebellum; M, Mesencephalon; P, Prosencephalon; R, Rhombencephalon; Sc, Spinal cord; Vt, Vertebra

no folds and fissures, and the brain stem was ventral to the cerebellum. The brain was divided into two cerebral hemispheres.

At 16 days of gestation, the brain stem was more caudal to the cerebellum than at the previous stage (Figure 2b1). At the same stage, in the dorsal view, the cerebellum appeared greater than the other regions of the brain, and in the ventral view, the olfactory bulb was forming (Figure 2b3).

At 18 days of gestation, fissures were observed in the caudal portion of the cerebellum. More prominent fissures separated the regions of the cerebral hemispheres from the cerebellum (Figure 3a). The spinal cord was dorsoventrally elongated and flattened, and the intumescences of the spinal cord were visible (Figure 6).

At 20 days of gestation, in the lateral view, the cerebellar paraflocculus had formed and the olfactory bulb was more prominent (Figure 3b2, b3). In the dorsal view, the cerebellum appeared more developed than in previous gestational stages (Figure 3b2). In the ventral view, the piriform lobe and olfactory bulb were observed. In addition, in the brain stem region, the basilar artery was present (Figure 3b3).

3.2.3 | End of gestation

At 25 days of gestation, the longitudinal brain fissure became more prominent (Figure 4a2). In the ventral view, the pons located caudal to the brain peduncle was observed (Figure 4a3).

At 28 days of gestation, the cerebellar vermis and cerebellar paraflocculus were delimited (Figure 4b1, b2). The longitudinal brain fissure was more evident in the dorsal view, and the two cerebral hemispheres could be distinguished from the olfactory bulb, which was located rostrally and more delimited (Figure 4b2). Moreover, the cerebellum showed more fissures, and the cerebellar paraflocculus became more evident (Figure 4b2). The pons formed successive portions of the brain stem located rostrally to the spinal cord and ventrally to the cerebellum (in the ventral view). The olfactory bulb and piriform lobe were more developed (Figure 4b3).

In the sagittal section, the corpus callosum in the frontal lobe of the brain could be identified, with four main parts, namely, the splenium, trunk, genu, and fornix. In addition, the olfactory bulb was more prominent. The cerebellar vermis was located dorsal to the cerebellar paraflocculus and showed both folds and fissures.

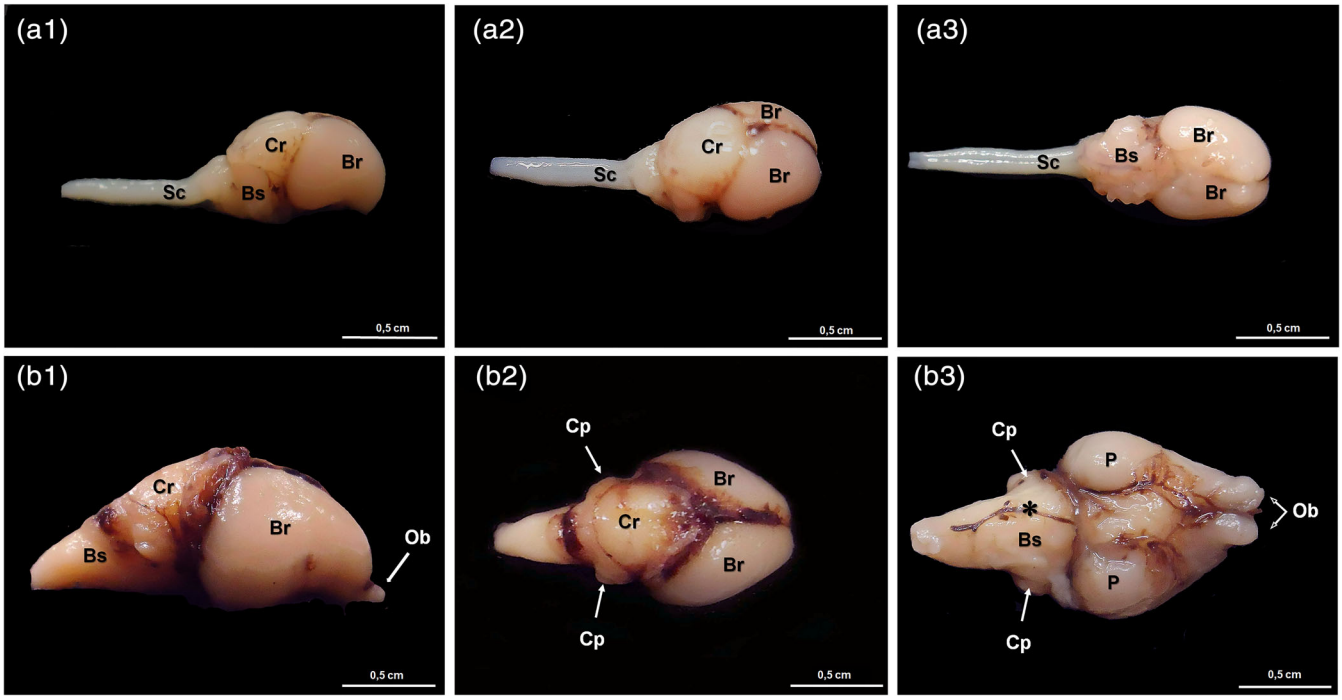

FIGURE 3 Development of the encephalon in New Zealand rabbits. a: 18th day of gestation; b: 20th day of gestation. a1, b1: Lateral view. a2, b2: Dorsal view. a3, b3: Ventral view. Br, Brain; Bs, Brain stem; Cr, Cerebellum; Cp, Cerebellar paraflocculus; Ob, Olfactory bulb; P, Piriform lobe; Sc, Spinal cord; *Basilar artery

Moreover, the ventricular foramina for the flow of cerebrospinal fluid were visible (dashed line, Figure 5).

3.3 | Microscopic features of the central nervous system

3.3.1 | Beginning of gestation

At 7.5 days of gestation, the embryos were apparently trilaminar, showing the three primitive germ layers, namely, the ectoderm, endoderm, and mesoderm. The developing neural crest with the thickening and invagination of the ectodermal cells was observed. The lateral edges were joined, forming the neural tube (Figure 7a).

At 8 days of gestation, the neural tube was completely closed. At this stage of neurulation, the embryo was still flat. Superficial ectoderm, mesoderm, and somites, which originated through paraxial mesoderm differentiation, were observed (Figure 7b).

At 9 days of gestation, dorsoventral folding, mesoderm expansion, and gastrulation onset occurred (Figure 8a). At 10 days of gestation, the neural tube wall was composed of neuroepithelial cells (asterisk, Figure 8) and had the internal limiting membrane (dark arrow, Figure 8).

At 12 days of gestation, the primitive encephalic vesicles (prosencephalon, mesencephalon, and rhombencephalon) were observed. The spinal cord primordium had a well-defined central canal, and the vertebral bodies were forming (Figure 2a3).

3.3.2 | Mid-gestation

At 15 days of gestation, the caudal portion of the prosencephalon formed the telencephalon structures (paired cerebral hemispheres) and diencephalon (epithalamus, thalamus, and hypothalamus). The pineal gland, which was derived from the epithalamus, was present (Figure 9a). The rhombencephalon was subdivided into the metencephalon (cerebellum and pons) and myelencephalon (medulla oblongata). A greater ossification of the vertebral column was observed (Figure 9c).

At 16 days of gestation, the spinal cord showed a higher density than at previous stages. Moreover, spinal ganglia and nerves were observed (Figure 9d). The choroid plexus located between the third and fourth ventricles comprised columnar epithelial cells with tufts of capillaries (Figure 9e).

At 22 days of gestation, the telencephalon was more delimited and the neopallial cortex was differentiating.

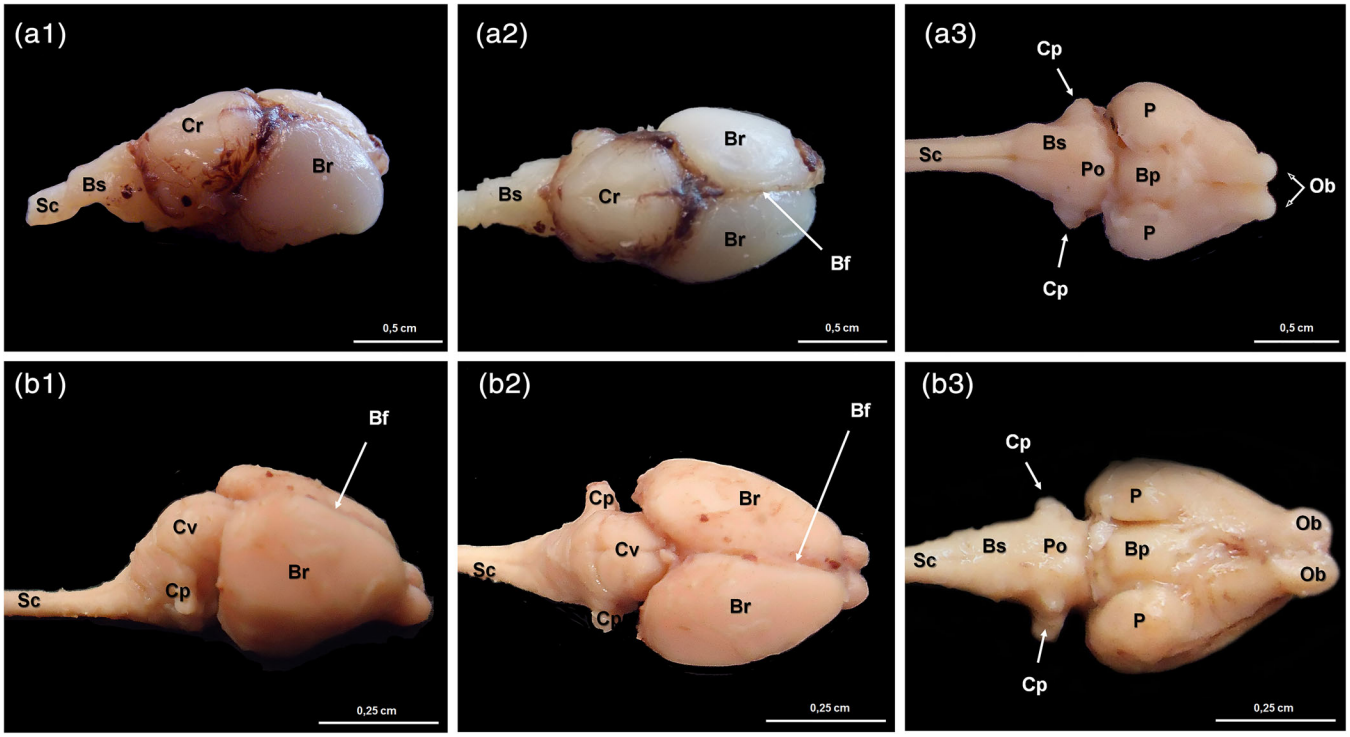

FIGURE 4 Development of the encephalon in New Zealand rabbits. a: 25th day of gestation; b: 28th day of gestation. a1, b1: Lateral view. a2, b2: Dorsal view. a3, b3: Ventral view. Bf, Brain fissure; Br, Brain; Bp, Brain peduncle; Bs, Brain stem; Cp, Cerebellar paraflocculus; Cr, Cerebellum; Cv, Cerebellar vermis; Ob, Olfactory bulb; P, Piriform lobe; Po, Pons; Sc, Spinal cord

The choroid plexus was present on the roof plate, and the volume of the third ventricle increased dramatically with roof plate thickening (Figure 9b).

3.3.3 | End of gestation

At 28 days of gestation, the cerebral cortex was differentiated (Figure 9f) and comprised four distinct layers, classified according to the cell types. The molecular layer contained few neuronal cell bodies; these cells were pyramidal with an ill-defined contour, were distributed in a scattered manner, and contained numerous ovoid nuclei with less condensed chromatin. The outer granular layer comprised small pyramidal neuroglial cells, the outer pyramidal layer comprised medium-sized pyramidal cells, and the fine internal granular layer comprised small granular cells.

At the same stage, the cerebellum was not completely differentiated. The cortex was formed by the external granular, molecular, and internal granular layers. The external granular layer was subjacent to the pia mater and contained many neuronal cell bodies. The molecular layer contained few small granular cells with dark-staining nuclei and scanty cytoplasm. Finally, the internal granular layer contained densely populated small granular cells with many dark-staining nuclei and scanty cytoplasm (Figure 9h).

At this age, the spinal cord was completely differentiated, and the external white matter could be distinguished from the central gray matter. The right and left antimeres of the spinal cord gray matter were perforated by the central canal, which was lined with a simple layer of cubic ependymal cells (Figure 9i).

3.4 | Staging

Based on the above-mentioned data, the key features of each central nervous system developmental stage in rabbits corresponds to that in humans according to the Carnegie stages (Table 2).

4 | DISCUSSION

Neurological conditions such as traumatic brain injury, stroke, Parkinson's disease, epilepsy, multiple sclerosis, Alzheimer's disease, and Zika virus-induced microcephaly are severe clinical problems that affect people worldwide (Sun et al., 2020; Taylor, Bell, Breiding, & Xu, 2017). Majority of the clinical trials on these injuries have failed

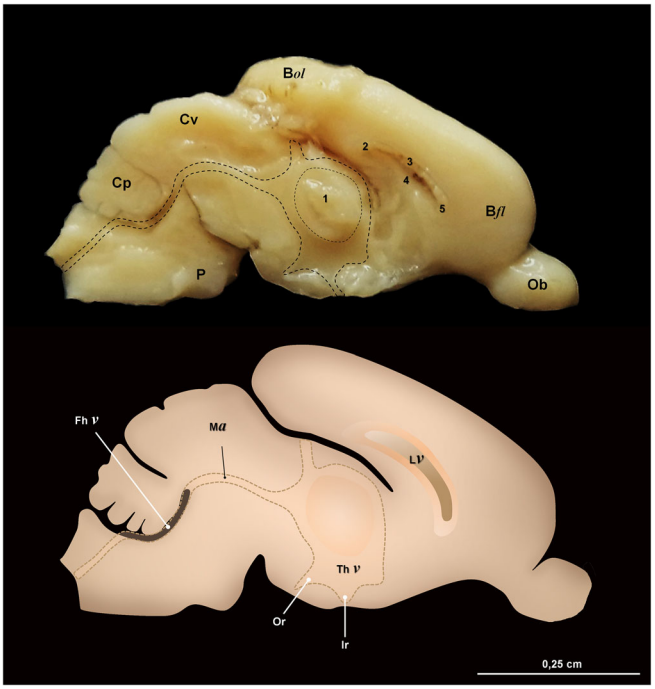

FIGURE 5 Development of the encephalon in New Zealand rabbits. Sagittal view of the encephalon on the 28th day of gestation. 1: Interthalamic adhesion; 2: Splenium of the corpus callosum; 3: Trunk of the corpus callosum; 4: Fornix of the corpus callosum 5: Genu of the corpus callosum. B*fl*, Brain frontal lobe; B*ol*, Brain occipital lobe; Cp, Cerebellar paraflocculus; Cv, Cerebellar vermis; Fh*v*, Fourth ventricle; Ir, Infundibular recess; L*v*, Lateral ventricle; M*a*, Mesencephalic aqueduct; Ob, Olfactory bulb; Op, Optical recess; P, Pons; Th*v*, Third ventricle

FIGURE 6 Macroscopic view of the central nervous system in New Zealand rabbits. 1: 15th day of gestation; 2: 18th day of gestation; 3: 28th day of gestational. (a) Cervical intumescence; (b) Lumbar intumescence

(Charvin, Medori, Hauser, & Rascol, 2018; Clossen & Reddy, 2017; Pfeuffer, Ruck, Kleinschnitz, Wiendl, & Meuth, 2016; Xiong, Zhang, Mahmood, & Chopp, 2015), and an animal model with highly similar physiological conditions to humans is urgently required (Sun et al., 2020).

Several species, from primates to small animals such as rats, mice, and rabbits, have been used in neuroscientific research (Muñoz-Moreno et al., 2013). An ideal experimental model must be selected to better understand specific pathological processes (Konefal et al., 2013; Ming & Song, 2011; Silva et al., 2016). Thus, the similarity of morphological aspects and embryological events is one of the criteria used to select the best-suited animal model for biomedical research, specifically preliminary studies of the effects of drugs or substances on organogenesis (Edwards, 1968).

In this study, we analyzed the development of neurological structures (primitive streak, neural tube closure, three primary brain vesicles, cerebellar primordium, and five secondary brain vesicles) and observed that these structures developed earlier in rabbits than in other short-gestation animals such as rats, mice, and pigmy mice (Table 3). The time of occurrence of these events is crucial and can provide novel information on the phylogeny of the concerned species (Butler & Hodos, 2005).

Furthermore, studies on the development of the central nervous system are imperative for understanding the processes of organogenesis and the probable causes of congenital abnormalities (Cagnoto et al., 2009). HE staining can be used to study the normal development of the central nervous system (Costa et al., 2019; Ekici et al., 2014; Lakshmi, Sumitra, & Victor, 2013). Hematoxylin stains the cell nuclei, and eosin stains the extracellular matrix, indicating the nuclear and cellular integrity, respectively. For instance, this method can be used to detect injuries of the brain, to monitor the integrity of the meninges and neurons (number of nuclei and cytoplasm), and to determine the presence or absence of inflammatory cells. However, for qualitative diagnoses of the cerebral nervous system, other stains can be used; for example, toluidine blue staining detects acid mucopolysaccharides based on metachromatic properties (Ghnenis, Czaikowski, Zhang, & Bushman, 2018). In this study, we used both HE and toluidine blue staining to examine the normal development of the central nervous system in rabbits.

By comparing the time of morphological configuration of the major structures of the central nervous system in short-gestation animals, the species exhibiting the most similar developmental pattern to humans could be identified. For instance, the appearance of the primitive

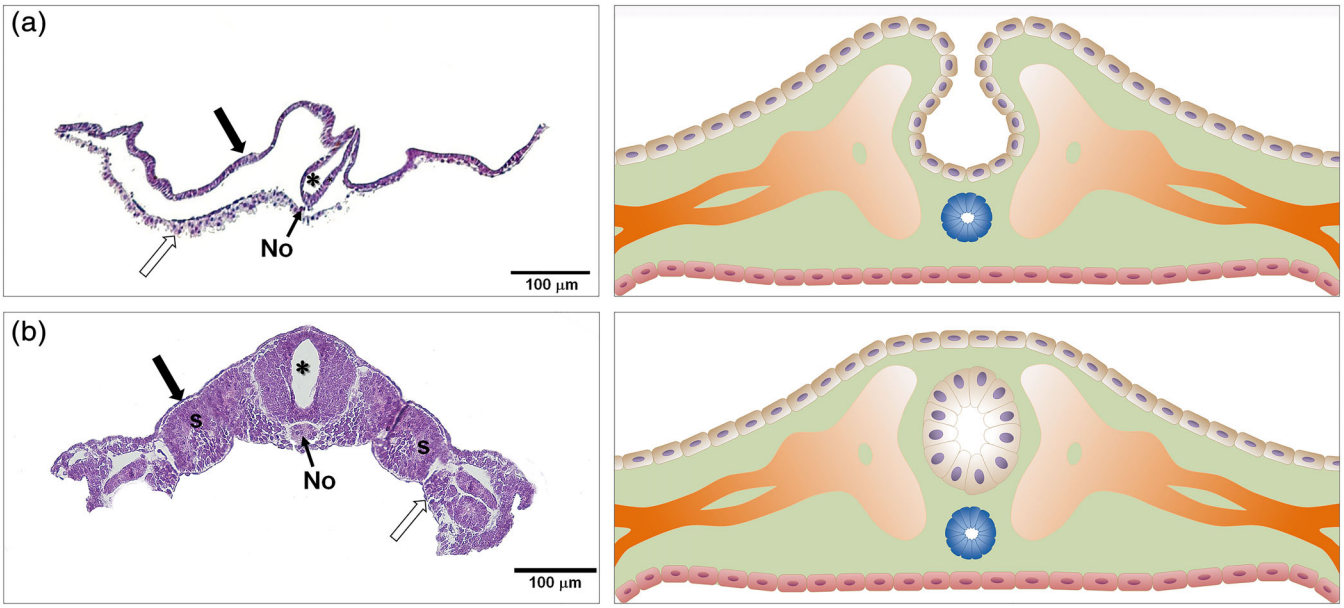

FIGURE 7 Transverse and schematic views of the embryos in New Zealand rabbits. (a) 7.5th day of gestation; (b) 8th day of gestation. Dark arrow: Ectoderm; White arrow: Mesoderm; No: Notochord; S: Somites; *Neural tube

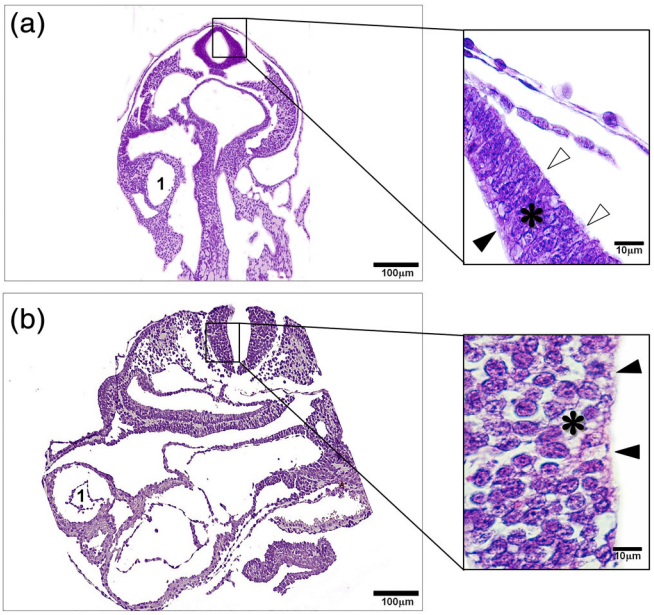

FIGURE 8 Transverse sections of the embryos in New Zealand rabbits. (a) Ninth day of gestation; (b) 10th day of gestation. *Neuro-epithelial cells of the neural tube. Dark arrow: internal limiting membrane; white arrow: external limiting membrane. 1: Cardiac area

streak, neural tube closure, development of the three primary brain vesicles, development of the cerebellar primordium, and differentiation of the five secondary brain vesicles in domestic cats, guinea pigs (Knospe, 2002; Silva et al., 2016), and rabbits are chronologically similar to the corresponding events in humans. However, olfactory bulb development during the gestational period is distinct in rabbits, rats, and mice (Alicelebić et al., 2004; Altman & Bayer, 1985; Dziegielewska et al., 2001), while choroid plexus development is distinct in domestic cats, rabbits, and mice.

In addition, we compared the chronological development of the central nervous system between humans and other long-gestation animals. The time of primitive streak appearance was highly similar between alpacas, bovids, and humans (Ferreira et al., 2018; Greenstein & Foley, 1958; Montelli et al., 2019). Moreover, the development of the three primary brain vesicles was very similar between humans and horses. However, the development of the choroid plexus and cerebellum was distinct in horses and bovids (Franciolli et al., 2011; Rigoglio et al., 2017). Such morphological characterization of the major structures of the central nervous system can help better understand the normal development of this system in various species, and the knowledge of events occurring during central nervous system organogenesis may help find alternatives to animal models in translational studies.

We next compared the features of the normal development of the central nervous system between rabbits and humans and determined the stages of development in rabbits corresponding to those in humans (Table 2). We believe that this knowledge can aid research on diseases originating from the failure of embryonic neural tube closure including neural tube defects, such as anencephaly and spina bifida (Juriloff & Harris, 2018), and may also be applied in molecular studies, such as whole-

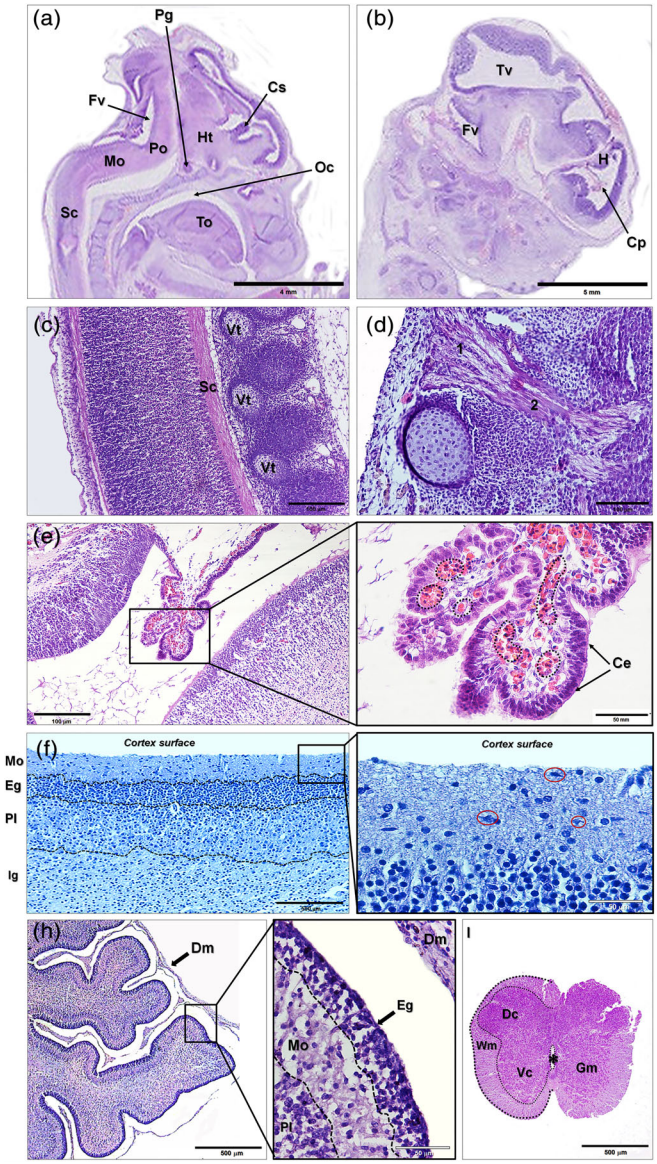

FIGURE 9 Light microscopy of the central nervous system in New Zealand rabbits. (a) Sagittal view of the embryo at 15 days of gestation (hematoxylin–eosin). (b) Sagittal view of the embryo at 22 days of gestation (hematoxylin–eosin). (c–e) Embryos at 16 days of gestation (hematoxylin–eosin) showed ossification of the vertebrae (Vt); spinal ganglion (1); and spinal nerve (2). Cp, Choroid plexus; Pointed circle: Capillary tufts; Ce, Columnar epithelium. (f) Embryos at 28 days of gestation (toluidine blue) showed the layers of the cerebral hemisphere: Mo, Molecular layer; Eg, External granular layer; Pl: Pyramidal layer; Ig: Internal granular layer; Red circle: Pyramidal neurons. (h) Sagittal view of the cerebellar cortex showed the layers of the cerebellar hemisphere; Dc, Dorsal column; Dm, Dura mater; Eg, External granular layer; Gm, Gray matter; I, Transverse view of the spinal cord; Mo, Molecular layer; Pl, Pyramidal layer; Vc, Ventral column; Wm, White matter; *Central canal

mount in situ hybridization (Ferreira et al., 2018; Kawamura & Matsumoto, 2018). Furthermore, such techniques based on the knowledge of the central nervous system organogenesis may help discover the genes or proteins involved in neurological pathologies affecting humans as well as explore the underlying signaling pathways and physiological mechanisms of hypoxic–ischemic encephalopathy, prenatal brain infections, and cerebral palsy (Saadani-Makki et al., 2008).

TABLE 2 Key features of each central nervous system developmental stage in rabbits corresponding to that in humans according to the Carnegie stages

Main features of the development of nervous system		
	Stage in rabbit/day	**Corresponding stage in humans**
Neural tube closed	7.5	Stages 9 and 10
Three brains vesicles	12	Stage 9
Primordium of cerebellum	15	Stage 13
Olfactory bulb	16	Stage 19

Specifically, based on our findings, preclinical studies in rabbits can be performed in the future to detect active fetal viral infections after exposure during gestation and to characterize fetal pathologies in different regions of the brain following maternal exposure to different viruses. The proposed model can be applied to the testing of various chemical substances for the treatment of multiple sclerosis (Gold et al., 2015), testing of vaccines and antiviral drugs in pregnant rabbits to identify possible neurological effects on the embryos or fetuses, and assessment of the effects of maternal anesthesia or surgery at different stages of gestation on the embryos or fetuses (Audouze, Taboureau, & Grandjean, 2018; Barenys et al., 2021; McArthur, 2017). Of note, in addition to the embryological similarities in the central nervous system development between rabbits and humans demonstrated here, rabbits are considered an ideal model for maternal vaccine development, as they constitute one of the only few models of maternal–fetal immunology for humans (Peng, Knouse, & Hernon, 2015).

The presence of the corpus callosum in the fetuses was observed macroscopically. Located at the center of the cerebral hemispheres, this structure is pivotal for communication between the cerebral hemispheres during the transfer of information (Bénézit et al., 2015). As a result of its anatomical position, this structure is not visible at the early stages of development, because the brain contains high amounts of liquids (Chen, Luo, et al., 2017; Chen, Morrison, et al., 2017), rendering a sagittal incision of the samples difficult. This is consistent with reports in other studies that the development of the corpus

TABLE 3 Comparative analysis of the central nervous system development between long- and short-gestation species

	Short gestation species						Long gestation species			
	Mouse	Rat	Pygmy mice	Cat	Guinea pig	Rabbit	Bovine	Human	Equine	Alpaca
Gestation period	19	22	25	64	66	32	263	267	283	335
Primitive streak	7	9	—	10–12	13–14	7–8	18	16	—	20
Three brain vesicles	9	10–11	12–13	14–15	20	12	—	20	19	30
Neural tube closed	9–10	11–12	14–15	15–17	15–16	8	22	28	22	—
Olfactory bulbs formation	10–11	12–13	—	—	45	16	—	46	40	—
Five brain vesicles	10–11	12–13	—	16–18	22–25	15	58	35	—	45
Choroid plexus formation	12–13	12–16	14–15	28–32	—	15–16	24	24–25	26	90
Cerebellar primordium	15–16	15	14–16	22–25	—	15	60	32	38	90
References	Chen, Luo, et al. (2017); Chen, Morrison, et al. (2017)	Alicelebić et al. (2004); Altman and Bayer (1985); Dziegielewska et al. (2001)	Favaron et al. (2012)	Knospe (2002)	Silva et al. (2016)	Our results	Ferreira et al. (2018); Greenstein and Foley (1958)	O'Rahilly and Müller (2010); Shiraishi et al. (2015); Ten Donkelaar et al. (2014)	Franciolli et al. (2011); Rigoglio et al. (2017)	Montelli et al. (2019)

callosum could only be described at the end of the fetal stage (Rigoglio et al., 2017).

The morphometric differences of the olfactory bulb across species reflect the differential olfactory functional requirements necessary for survival in a particular habitat. Herbivores capture olfactory stimuli that help reproductive processes and escape from predation. Meanwhile, in carnivores, the olfactory functions are more relevant for tracing and capturing prey (Kavol & Jameela, 2011). In the present study, olfactory bulb development in rabbits was demonstrated at 16 days of gestation, which is earlier than that in rodents (Table 3).

One of the advantages of using rabbits instead of rodents as an experimental model to study neurological diseases is that the development of the white matter in rabbits is closer to that in humans (Derrick, Drobyshevsky, Ji, & Tan, 2007). Moreover, in rodents, brain development occurs in the postnatal period, with most myelination occurring after the first week of life (Demir & Demir, 1998; Rasband et al., 1999). In rabbits, meanwhile, the brain development occurs in the perinatal period, as in humans (Harel, Shapira, Tomer, Donahue, & Quilligan, 1985; Van Marthens, Harel, & Zamenhofan, 1975). In samples near birth, the cerebral cortex was differentiated into four layers. Studies on the normal populations of cerebral cortical cells in rabbits are particularly important, as they can aid preclinical testing of drug efficacy against Alzheimer's disease (Woodruff-pak, Agelan, & DelValle, 2007).

Regarding the development of the cerebellar cortex, the external granular cell layer contained many granular cells, which can be explained by the fact that the development of the cerebellar cortex occurs in the postnatal period, with the migration of the granular cells into the cerebellar cortex (Ponti, Crociara, Armentano, & Bonfanti, 2010). This event also occurs in humans (Haldipur et al., 2019; Marzban et al., 2015; Marzban, Chung, Watanabe, & Hawkes, 2007).

Analysis of the spinal cord development indicated that the spinal canal was filled by cells at 12 days of gestation, while the cervical and lumbar intumescences were present at 18 days of gestation. This information can be useful in translational studies addressing issues related to preclinical pharmacological therapies (e.g., riluzole, glyburide, and magnesium) for spinal cord injuries (e.g., blunt force trauma, penetrating trauma, or ischemia) (Huang et al., 2017; Kwon et al., 2011; Tator et al., 2012; Üstün, Gürbilek, Ak, Vatansev, & Duman, 2001). In addition, these data can be useful in future preclinical studies on the risk of congenital disorders (e.g., spina bifida occulta, meningocele, and myelomeningocele) associated with the maternal use of antiepileptic drugs (e.g., valproic acid or carbamazepine) during gestation (Gilboa et al., 2011).

5 | CONCLUSION

In the present study, the key morphological aspects of the embryonic and fetal development of the central nervous system in rabbits were described. These data provide novel information on the normal development of this system, which may contribute to future studies on neurological diseases in humans and other long-gestation animals. The proposed rabbit model shows greater neurological maturity than other short-gestation animal models, such as rodents.

Further studies implementing immunohistochemical analyses and in situ hybridization techniques are warranted to identify proteins involved in neurulation and the related cell signaling pathways, which would contribute to improving the experimental design of preclinical trials of neurological diseases in humans.

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AUTHOR CONTRIBUTIONS

Adriana Anunciação: Conceptualization; formal analysis; investigation; methodology; validation; writing-original draft; writing-review and editing. **Phelipe Favaron:** Methodology; supervision; visualization; writing-original draft. **Luciano de Morais-Pinto:** Software; visualization; writing-review and editing. **Carla Maria Miranda:** Investigation; writing-original draft; writing-review and editing. **Daniele Martins:** Formal analysis; methodology. **Daniel Conei:** Validation; visualization; writing-original draft. **Mariano del Sol:** Validation; visualization; writing-original draft; writing-review and editing. **Bélgica Pastene:** Methodology; visualization; writing-original draft; writing-review and editing. **Maria Angelica Miglino:** Supervision; visualization; writing-original draft; writing-review and editing.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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