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DISTRIBUTION AND EXPRESSION PATTERN OF NEUROGLOBIN IN THE BACTRAIN CAMEL BRAIN

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ABSTRACT

Neuroglobin (Ngb) is a member of the vertebrate globin family involved in cellular oxygen homeostasis and reactive oxygen/nitrogen scavenging. It is an intracellular haemoprotein expressed in the central and peripheral nervous system, cerebrospinal fluid, retina and endocrine tissues. The study examines the distribution of Ngb in the brain of Bactrian Camel and compared the results with published results of yak, and cattle. The immunohistochemical staining method was used to observe the distribution of Ngb in the brain of healthy adult Bactrian camels. Ngb is significantly expressed in all tissues of the telencephalon except the hypothalamus. The cerebellar cortex, hippocampus, amygdala, cerebellum, and corpus callosum recorded the highest expression and each plays an important role in the Bactrian camel. In the Yak and cattle brain, Ngb were scattered in the cerebral cortex and were significantly higher than that in the cerebellar cortex, hippocampus, medulla oblongata, striatum, and olfactory bulb.

Key words: Bactrian camel, cattle, immunohistochemistry, neuroglobin, retina, yak

Neuroglobin (Ngb) is a kind of specific protein involved in the transport of oxygen (Burmester *et al*, 2000) and was found in mammalian brain. Further studies also observed Ngb expression in the retina, nerve system, testicles, and uterus (Geuens *et al*, 2003; Schmidt *et al*, 2003; Gao, 2015). Zhao *et al* (2012) reported that Ngb is highly expressed in the brains of mice with traumatic brain injury (Zhao *et al*, 2012) while in the hypothalamus, amygdala, and pontine tegmental nuclei of human, Ngb was significantly expressed (Hundahl *et al*, 2013). In the pig brain, Ngb levels in the hypothalamus were higher than the frontal cortex. The lowest difference was found in sheep, which showed Ngb expression in the hypothalamus and cerebrum (Fabrizius *et al*, 2016). The expression level of NGB in the brain retina was much higher than in the brain tissue (Wei *et al*, 2010; Anderson, 1968), suggesting that there exists a close relationship between NGB and retinal functions. Studies have shown that Ngb is involved in the elimination of reactive oxygen species (ROS) regulation which may play an important role in the oxygen homeostasis (Greenberg *et al*, 2008). Further research have demonstrated the important functions of Ngb in oxygen supply, anti-oxidative stress, apoptosis and signal transduction. At present, several researches have been conducted on the human, adult sheep, rabbits and rats about Ngb expression (Ran *et al*, 2005; Li *et al*, 2006; Ostoji *et al*, 2008; Ostoji *et al*, 2006; Yang *et al*, 2015a; Yang *et al*, 2015b). Ngb mRNA was detected in all brain especially the peripheral nervous system of rat (Ostoji *et al*, 2006), suggesting that Ngb could serve a neuroprotective role as scavengers of reactive oxygen species and developing therapeutic strategies for treatment of hypoxia-related ocular diseases. Despite all these results, there is no report of Ngb expression in the brain of the Bactrian camel. The current research examined Ngb expression in the brain of healthy adult Bactrian camels by employing Immunohistochemical staining procedures and IPP analysis. The researchers compared the existing results with those of the yak and cattle.

Materials and Methods

Animals and setting

The Animal Ethics and Welfare Committee of Gansu Agricultural University in October of 2019 (AEWC-GAU-2019-057) reviewed and approved all experimental procedures performed in this study. All animals were housed in a full facility at the Zhangye areas of Gansu Province, China. Three healthy adult Bactrian camel at the age of 3 years were purchased from the centre. The animals were housed and monitored by trained personnel and fed on grasses and sedges, such as Carex, Stipa, and Kobresia. In the Zhangye environment, the altitude

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was 3000m. Experiments were carried out using adult Bactrian camel weighing 550-720 kg. The weight ranges from 10-15µm. The animals were maintained at a temperature between -7° C and -8° C and had free access to food and water.

Treatment and Specimen Techniques

Animals were retrieved one at a time from their living areas and minimally immobilised to facilitate sacrificing and then extraction of the brain by craniotomy. Subsequently, the cerebral cortex, frontal lobe, temporal lobe among others were extracted. Tissue samples prepared for immunohistochemistry were fixed in 4% paraformaldehyde (PH 7.4, w/v) and samples for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western-blotting were stored at -80 °C.

Reagents and instruments

The description of instruments and reagent is given below with manufacture in parenthesis.

(1) KD-BM bio-tissue embedding machine, kd-h bio-tissue baking machine and KD-P bio-tissue spreading machine (Zhejiang Jinhua Cody Equipment Co. LTD). (2) DHP- 9082 thermostatic incubator (Shanghai Duofu Industrial Co., LTD). (3) Rm-2235 precision rotary semi-automatic helical slicer and 13395H2X optical microscope (LEICA, Germany). (4) PBS phosphate buffer (zli-9062), immunohistochemical staining kit (sp-9001) and DAB colour developing kit (zli-9018) (Beijing Zhongshan Jinqiao Biotechnology Co., LTD). (5) Trypsin (t8150-25) (Beijing Solabao Biotechnology Co., LTD). (6) Rabbit anti-mouse polyclonal antibody (bs-1859r) (Beijing Boorson Biotechnology Co., LTD). (7) Formaldehyde solution, glacial acetic acid, anhydrous ethanol, n-butanol and xylene, and other conventional chemical reagents (Tianjin Damao Chemical Industry co., LTD).

Immunohistochemical staining

Tissue samples from the brain of Bactrian camel were fixed (4% paraformaldehyde) and trimmed (2 cm×2 cm). Then, the tissue samples used conventional gradient alcohol dehydration, made tissue wax blocks with paraffin embedding, cutting tissues with serial sections (thickness 4 µm), exhibiting, patching, baking sheet processing, hematoxylin-eosin (HE) routine staining, microscopy. The paraffin-embedded tissue sections were deparaffinised in xylene and then rehydrated in graded alcohol. The PBS (0.01mol/L, pH =7.2) was rinsed 3 times, each time 5minutes. 0.125% trypsin antigen was repaired 30 minutes and rinsed in PBS for 2 times. The endogenous peroxidase activity was blocked by incubating the sections for 10 minutes in 30mL/L hydrogen peroxide blocking solution, followed by rinsing 3 times with PBS for 5 minutes each time to reduce non-specific binding of the first antibody. Normal sheep serum was added for blocking and incubated at room temperature for 15 minutes. The corresponding primary antibody was added in the sections, incubated at 37 °C for 2 hours, and rinsed in PBS 3 times. The appropriate secondary antibody was added after been removed from PBS and incubated at 37 °C for 15 minutes. The streptomyces avidin-peroxidase solution was added in the sections, incubated at 37 °C for 15 minutes, PBS was rinsed 3 times, 5 minutes each time. The immuno-peroxidase colour reaction was developed with the HRP-DAB substrate chromogen solution after removed PBS. Distilled water stopped the reaction and the sections were lightly counterstained with hematoxylin, dehydrated, in increasing concentrations of ethanol, cleared and covered with mounting medium and coverslips (at 4 °C). Then the sections were stored at 20 °C until used for taking photographs and microscopic analysis. To assess the specificity of the immunolabelling, the negative controls were performed by substituting the primary antibody with PBS. Other procedures remained constant.

Statistical analysis

SPSS 19.0 was utilised to analyse and compare the differences between MD values. The level of significant was cheked (P >.05).

Results

The trend of Ngb expression were widely distributed in the brain tissues of the adult Bactrian camel. Ngb expressions were significantly expressed in the cerebellar cortex, hippocampus, amygdala, cerebellum, and corpus callosum while other regions demonstrated less expression. However, it was recorded that the hypothalamus showed higher but without significant (Table 1).

The Ngb expression levels in different areas of the adult yak brain were significantly different (Liang *et al,* 2013). The relative expression of the Ngb gene in the cerebral cortex was significantly higher than that in the cerebellar cortex, medulla oblongata, striatum, and olfactory bulb. The expression level in the hippocampus was different from that in the other regions, with a high level of significance.

Ngb was mainly expressed in the following areas of the brain as compared to the study done

by the researchers (Tong *et al,* 2015). The quantities of expression and distribution pattern differed but localisation of distribution was almost similar.

Table 1. The comparison of the Ngb expression in the brain of Bactrian camel.

Regions	Mean ± SD	Significant
Cerebellar Cortex	12.179 ± 0.150	000***
Hippocampus	11.538 ± 0.118	002**
Amygdala	11.125 ± 0.470	0.003**
Olfactory lobe	10.690 ± 0.321	0.000***
Basal ganglia	11.022 ± 0.152	0.015**
Thalamus	10.884 ± 0.108	0.008**
Hypothalamus	11.134 ± 0.043	0.210
Cerebellum	11.805 ± 0.212	0.000***
Frontal lobe	10.707 ± 0.065	0.014**
Corpus Callosum	11.961 ± 0.008	0.000***

Discussion

According to Reuss *et al* (2002) Ngb was solely expressed in the cerebellar cortex of the rodent brain. Purkinje cells of the cerebellar cortex also showed a level of Ngb mRNA expression (Reuss *et al*, 2002). A study performed by Christian *et al* (2013) also confirmed the significant Ngb expression in the cerebellar cortex of the adult mouse brain but Fabrizius *et al* (2016) interestingly revealed a lower Ngb expression in the cerebellar cortex during foetal development of the mouse brain and had a tendency to increase as the mouse approaches adulthood. The current study displayed a significant level of Ngb expression in the cerebellar cortex of Bactrian camel. Ngb played a protective role in the control movement and influences many other functions in the cerebellar cortex (Hundahl *et al*, 2013). Its expression might play a role in protecting the cerebellar cortex and other nerve cells from permanent demage as suggested by the current findings. Ngb expression had neuroprotective and antiapoptotic functions also (Alekseeva *et al*, 2017). The expression also involved the metabolism of reactive oxygen and nitrogen species. Burmester *et al* (2000) reported that Ngb is expressed at 11% in the hippocampus of the human brain while finding reported by Reuss *et al* (2002) recorded positive expression of Ngb in the formation of the rodent's hippocampus. The present findings revealed a significant level of Ngb levels in the hippocampus of the Bactrian camel. In the Bactrian camel hippocampus, growth hormones such as age, sex, and stress require a significant level of oxygen for these changes to occur. Ngb played a role in oxygen supply and may detoxify reactive oxygen or nitric oxide (Burmester and Hankeln, 2009). Significant expression Ngb has a protective function as these changes take place in the hippocampus and influence Bactrian camel behaviour. In the hippocampus, it was observed that Ngb can decrease for long days after physiological changes but increase after a few days (Brayn *et al*, 2012). In the mouse brain, Ngb revealed its highest concentration in the amygdala and other regions (Hundahl *et al*, 2013) while a considerable level of Ngb mRNA expression was seen in the amygdala of rodent brain (Reuss *et al*, 2002). Similarly, the current result found a significant level of Ngb in the amygdala of the Bactrian camel. The behaviour changes and other functions in the Bactrian camel, such as protecting young adults from predators, long-distance movement at high-altitude, mitochondrial dysfunctions, and neurodegenerative disorders require strong protection of the neuron tissues from damage. Ngb mRNA was found distributed in the olfactory lobe and it was suggested that Ngb is a conserved gene in evolution and is very important in the nervous system (Chenggang *et al*, 2002). The present results showed a significant level of Ngb in the olfactory bulb of Bactrian camel and the expression contribute to essential neuronal senses. A significant level of oxygen was needed when the Bactrian camel was covering a long distance and the breath rate increase as the Bactrian camel moves. The Ngb expression in the olfactory bulb regulates the oxygen rate during breathing. The response to oxygen stimuli depended on the ability to successfully adapt to hypoxia. The pattern of Ngb expression facilitated oxygen movement between neural tissues and provided a level of neuronal protection during hypoxia.

In a 26 years old male, Ngb was found highly expressed in the basal nuclei while a female of 42 years showed low expression (Hu *et al*, 2017). Age factors may have an influence on the expression of Ngb in neuronal tissues especially the basal ganglia. The current findings reported a significant Ngb expression in the basal nuclei of the Bactrian camel. The expression pattern may participate in protecting neuron tissues during transportation or movement in the high altitude environment. During transportation, the breath rate of Bactrian camel often increased and oxygen was paramount in this process. The significant expression of Ngb was involved in protecting the movement and coordination of neuronal tissues in the basal ganglia.

The present findings recorded that Ngb expression in the Bactrian camel thalamus had shown

a significant rate of expression. During sensory signals in the Bactrian camel, Ngb regulated oxygen expression and played a neuroprotective function. Activities such as responding to predators can be stressful and require a significant level of oxygen. Ngb not only responded to the oxygen demand but protected neurons tissues from damage. It was recorded that Ngb expression in the yak, mouse, and murine thalamus read similarly (Della *et al*, 2010; Reuss *et al*, 2002). Although researches had a focus on Ngb expression in the thalamus, there exists limited references.

The current results showed a higher Ngb expression in the hypothalamus of Bactrian camel but were non significant. Ngb expression in the hypothalamus promoted neuronal survival (Eliana *et al*, 2016). Christian *et al (*2013) reported that Ngb was highly expressed in the hypothalamus of human while in the adult mouse brain the Ngb expression was non significant (Fabrizius *et al*, 2016). The high expression of Ngb in the Bactrian camel hypothalamus could help in supply of oxygen to the blood flow in the body and may act as endogenous protectants in the nerve cells while Hif-1α in the hypothalamus can have an oxygen-independent regulation such as oxidative stress and because the hypothalamus was located at the base of the forebrain and around the walls of the third ventricle which received signals from the periphery through the bloodstream (Cramer *et al*, 2003; Catrina, 2014). Ngb expression in the Bactrian camel hypothalamus may also be involved in preventing an imbalance in the blood flow and nutrients such as glucose and lactate, leading to biochemical and molecular changes that caused neuronal damage.

The distribution and expression of Ngb in various regions of the adult yak brain, were demonstrated by the immunohistochemical staining ISPs method and real-time fluorescence quantitative PCR (Tong-fang *et al*, 2015). The results indicated that Ngb was widely distributed in different regions of the adult yak brain (Tong-fang *et al*, 2015), while in the human brain (Hundahl *et al*, 2013), Ngb had a more limited levels of distribution, weaker expression, and fewer effects on neuronal morphology (Tong-fang *et al*, 2015). These differences could be the result of the varied methods and laboratory procedures used in each study. Ngb participation in the uptake and storage of oxygen by nerve cells can improve the rate of oxygen usage by nerve cells (Sun *et al*, 2001). Ngb upregulation can protect nerve cells, improving the tolerance of brain tissue to ischemia and hypoxia and reducing damage to the brain under these conditions (Greenberg *et al*, 2008). As yaks live in a high-altitude hypoxic environment for a long period, the levels of Ngb in different regions of the brain perform key functions in enhancing the oxygen utilisation rate. The nervous system maintained the normal physiological function of the brain (Zhang *et al*, 2008). Due to the high expression of Ngb in functional nuclei, the function of oxygen storage may be closely related (Dewilde *et al*, 2001); however, this expression may also reflect the difference in activity and oxygen consumption in different areas of the brain. In addition, the distribution of Ngb in other regions of the brain and in the cells of the yak may also be related to the oxygen-consuming activities of these regions and cells (Zivin *et al*, 2009). First, the expression of the Ngb gene in different regions of the yak brain was found by fluorescence quantitative PCR, and the results showed significant differences in the expression of Ngb in various areas of the yak brain. The level of Ngb quantity of expression in the cerebral cortex was the most significant (Tong-fang *et al*, 2015). Compared to its expression in humans, a large amount of Ngb was observed in the hypothalamus, but the difference was non significant (Hundahl *et al*, 2013). Both yaks and mice showed Ngb in the cerebral cortex, but the levels of expression differed (Tong-fang *et al*, 2015; Wei-De *et al*, 2013). The rat brain also showed higher expression in the cerebral cortex, but the difference was non significantly compared to the other brain regions (Guo *et al*, 2011). The positive expression of Ngb in the cerebral cortex of yaks was significantly higher than that in the cerebellar cortex, hippocampus, medulla oblongata, striatum and olfactory bulb (Wang *et al*, 2008).

The study of Liang *et al* (2013) used the immunohistochemistry to show the distribution of Ngb in the brain of the cattle and yak. All six layers of the cerebral cortex contained Ngb-positive cells that were distributed throughout the layers, and the level of expression was significantly higher than that in the cerebellar cortex, hippocampus, and striatum. Ngb-positive cells were also found in the medulla. The Ngb distribution and localisation were similar in the cerebral cortexes of the cattle and yaks (Tong-fang *et al*, 2015). The overall levels of Ngb expression in the brains of cattle were lower than those in the brain of yaks. In the cerebellar cortex of the yak, Ngb-positive cells showed high levels of expression in purkinje cell layers and lower levels in the granular layers. The distribution and localisation

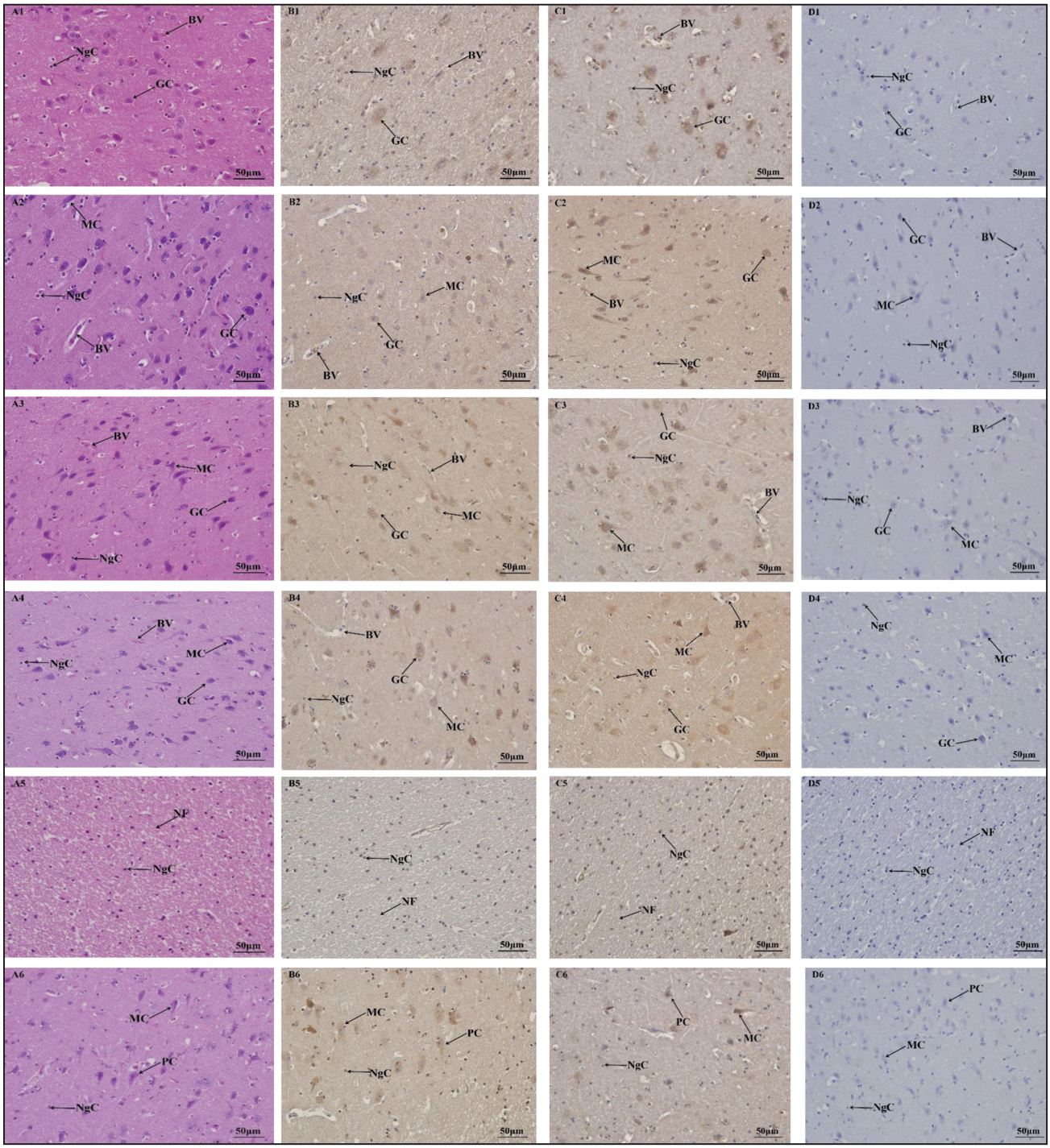

Fig 1. A1-10. Ngb expression in the brain tissues of Bactrian camel.

Plate A1-D1. The expression of Ngb in the cerebellar cortex of bactrain. Positive and negative controls are indicated by arrows.
Plate A2-D2. Ngb is found in the upper regions of hippocampus.
Plate A3-D3. Ngb is observed in the middle region of the amaydala.
Plate A4-D4. Ngb are revelaed in the middle region of cerebral.
Plate A5-D5. Ngb as seem are located in lower region of the White matter.
Plate A6-D6. Ngb are heavily found in the entire region of the Basal ganglia

of Ngb-positive cells in the cerebellar cortex of cattle was similar to that in the yak, but the intensity of the reaction was weaker overall. In various regions of the hippocampus in the yak, Ngb-positive cells were mostly found in pyramidal cells, with positive reaction sites but weak Ngb expression found in nerve

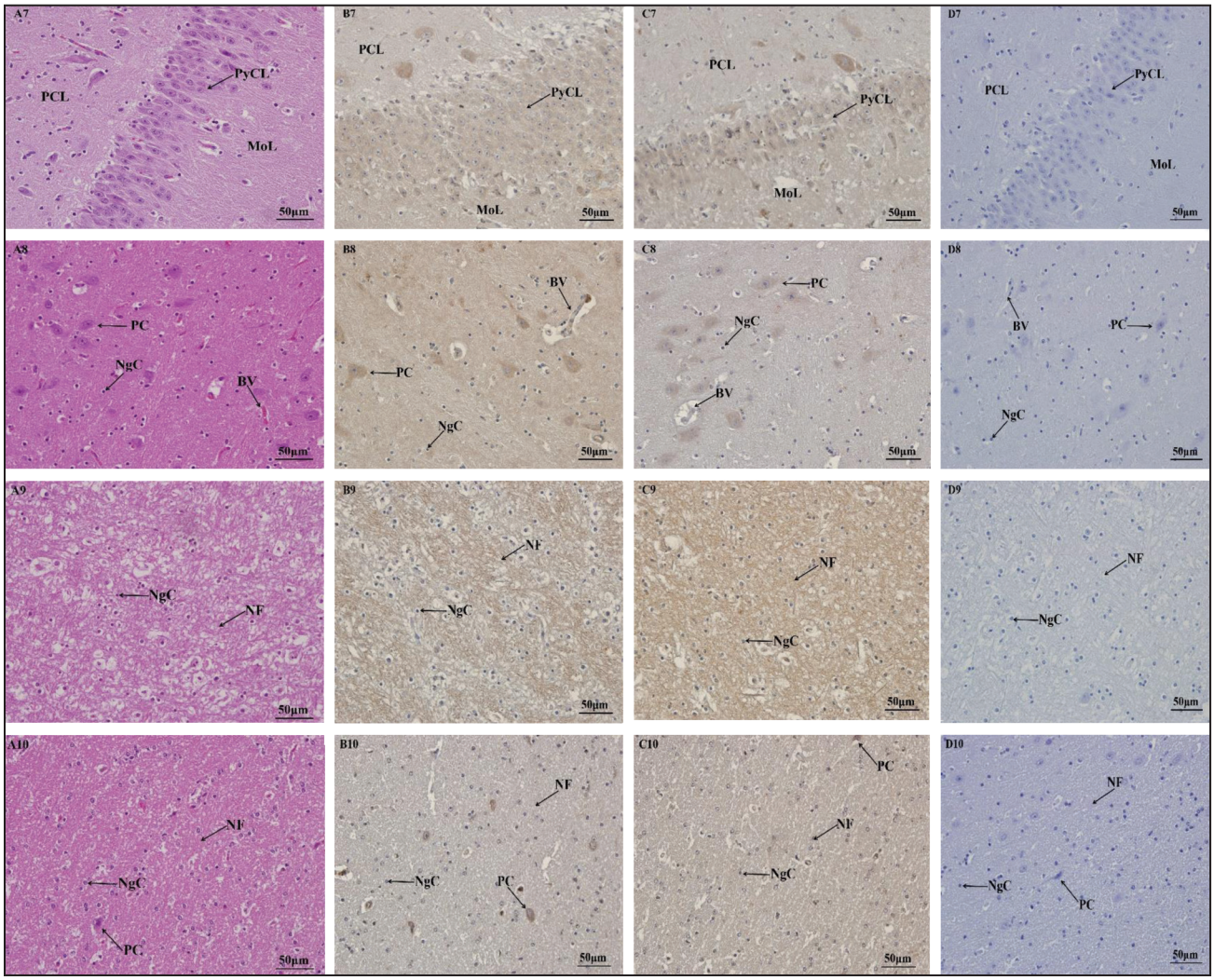

Plate A7-D7. Ngb is found in the middle region of the cerebrum.
Plate A8-D8. Ngb is observed in the middle region of the cerebellum.
Plate A9-D9. Ngb is distributed in the lower region of the frontal lobe.
Plate A10-D10. Ngb is found in the upper region of the corpus callosum.

processes (Liang *et al*, 2013). The similarities of these results might be due to the identical methods and laboratory procedures used. In separate areas of the cattle hippocampus, the distribution and localisation of Ngb-positive cells were similar to those of the yak, but the intensity of the reaction was weaker in the yak brain. Additionally, in the medulla oblongata of cattle and yaks, the distribution and localisation of Ngb-positive cells were weakly expressed, and the overall intensity was weaker in the cattle than in the yak. Ngb-positive cells in the striatum of the yak were widely distributed in the caudate nucleus and the Ngb-positive reactions were stronger than those in the hippocampus, medulla oblongata, and olfactory bulb, but Ngb was more weakly expressed than in the cerebral cortex and cerebellar cortex. In the medulla oblongata of the yak, Ngb-positive cells were mainly distributed in the gray matter and Ngb-positive cells were also scattered in the white matter. Ngb was also expressed in the mitral cell layer of the yak olfactory bulb, with notable staining and large cells; however, the staining intensity of the Ngb-positive cells was weaker than that of medulla oblongata and stronger than that of the hippocampus. The distribution and localisation of Ngb-positive cells in the mitral cell layer of the olfactory bulb of the cattle was similar to that of the yak; the staining intensity was higher than that of the hippocampus, weaker than that of the medulla oblongata and significantly weaker than that of yak (Tong-fang *et al*, 2015). Ngb-positive cells were distributed primarily in the peripheral nerve plexus and ganglia and mostly scattered in some of the nerve cells in the peripheral nervous system but at low quantities (Liang *et al*, 2013). In the peripheral nervous

system of the cattle, the distribution and localisation of the Ngb-positive cells were similar to those of the yak, but the intensity of the reactions was on average weaker than that of the yak (Tong-fang *et al*, 2015).

Conclusion

Camel

The study documented that Ngb may have a significant function in the maintenance of oxygen homeostasis and participation in the brain tissues. The study further provided explanations for Ngb physiological function and its relationship for the Bactrain camel to adapt to the extreme environmental conditions.

Yak and Cattle

The high expression of Ngb in different brain tissues of adult yak and cattle is suggested to play an important role in the utilisation of oxygen and physiological functions. Additionally, It enables the mammals to adapt to the extreme hypoxia conditions.

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions

JBM, DX, LX WH and HAM contributed to the literature search. JBM, DX and HAM organised, investigated and interpreted the data. JBM, LX and WH wrote the first draft of the manuscript. DX and LX performed the study methodology and formal analysis. JBM designed the study concept. All authors contributed to this manuscript revision and read and approved the submitted version.

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