

Review

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# A one-health approach to using sheep in research, with a focus on neuroscience studies

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**How to cite this article:** Chatzimanou T, Tsingotjidou A. A one-health approach to using sheep in research, with a focus on neuroscience studies. *One Health Implement Res* 2023;3:107-24. <https://dx.doi.org/10.20517/ohir.2023.13>

**Received:** 23 Mar 2023 **First Decision:** 19 Jul 2023 **Revised:** 31 Jul 2023 **Accepted:** 20 Oct 2023 **Published:** 30 Oct 2023

**Academic Editors:** Despoina Iatridou, Jose M. Martin-Moreno, Zengyun Hu, Xiaolong Wang **Copy Editor:** Pei-Yun Wang  
**Production Editor:** Pei-Yun Wang

## Abstract

Sheep have been an important animal for both academic and basic sciences education, with a positive impact on the public health sector and, subsequently, One Health. This review presents the impact of sheep on research with a specific focus on neuroscience studies. Disorders, as well as neuroendocrine and environmental factors affecting the brain, the spinal cord, and the peripheral nervous system, are selected, and relevant research and sheep models mimicking human diseases are described. The review discusses various sheep models, encompassing prion, Parkinson's, Alzheimer's, Huntington's disease, and neuronal ceroid lipofuscinoses (Batten Disease), along with ischemic stroke. Sheep play a pivotal role in elucidating the pathogenesis and/or treatment for the aforementioned diseases. Furthermore, this research is underpinned by solid neuroanatomy knowledge. Consequently, we outline the main reasons why sheep are such robust research models. In conclusion, we demonstrate the important role that sheep models fulfill in advancing the mission of the One Health Initiative.

**Keywords:** Sheep, large animal models, neuroscience, neurodegenerative disorders, basic sciences research, One Health, postgraduate education, translational research



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## INTRODUCTION

The One Health initiative centers around the tenet that humans, animals, and the environment are all integral parts of the same system impacting one another<sup>[1,2]</sup>. This tenet arose to address complicated interdisciplinary problems affecting these parts of the system<sup>[3]</sup>. Additionally, in the “One Health, One Medicine” concept, animals and humans develop diseases sharing a common etiology, pathology, and/or even related treatment, therefore establishing a link between them<sup>[4]</sup>.

For millennia, sheep have been used as farm animals, providing both excellent nutrition and clothing to a growing human population, and strongly influencing the economy of countries<sup>[5]</sup>. Sheep research, with a One Health impact, has been conducted in various scientific fields, including infectious agents, like Ngari virus, that affects sheep and humans<sup>[6]</sup>, antimicrobial resistance research<sup>[3]</sup>, and comparative research between species, like the study of the mastitis microenvironment, leading to progress in human research<sup>[7]</sup>. Additionally, the cognitive capacity of the sheep makes them well-suited for the implementation of advanced technologies. For example, experiments using the ME20.4 immunoglobulin G (IgG)-saporin, a toxin that induces specific basal forebrain cholinergic (BFC) lesions, were conducted to investigate the role of the BFC system in social recognition training. Olfactory recognition of lambs by their mother serves as an appropriate model for employing this advanced “molecular surgery” technique<sup>[8,9]</sup>.

Neuroscience research is undeniably dominated by rodents<sup>[10,11]</sup>. These small animals have numerous reasons for being the most commonly used model, which include: their low costs as laboratory animals, well-defined biology, fast reproductive rates, a plethora of available research tools, and above all, their genetic manipulation to better recapitulate the human condition<sup>[12,13]</sup>. Rodents provide detailed insight into the physiological aspects of human disease and are integral to human drug development. However, there is still a need for animal models with brain structures comparable to those of humans<sup>[10,11]</sup>. While non-human primates (NHPs) appear as the most relevant phylogenetic candidates for preclinical studies, research on them is fraught with ethical concerns<sup>[4,14,15]</sup>. Public opinion and disapproval of NHP research are based on the phenotypic/genetic proximity of NHPs to humans, their highly developed social skills, and many more characteristics<sup>[16]</sup>.

It is noteworthy that sheep can fulfill a didactical role. In veterinary medicine, sheep are an established reference animal for anatomic and histologic education. Moreover, ovine models are being used in postgraduate fields outside the umbrella of veterinary medical education. Training human surgical techniques described by Ianacone *et al.* (2016) and Isaacson *et al.* (2022) are simulated in ovine tissue to model human surgical approaches<sup>[17,18]</sup>. Consequently, sheep provide an advantageous tool for translational research and educational purposes.

Many large animals have been used in neuroscience research: horses, cattle, pigs, sheep, dogs, cats, *etc.*<sup>[4,14,19]</sup>. Their use in research is a prerequisite since the U.S. Food and Drug Administration (FDA) requires toxicity tests carried out on both a rodent and a non-rodent species for novel human drug approval<sup>[20]</sup>. In this review, the authors emphasize sheep’s role in neuroscience research with reference to neurological disorders, spinal cord injuries, ischemic stroke, and neuroendocrine studies. Thus, sheep provide an emerging, convenient, and applicable disease model reflecting human disease due to neuroanatomical and physiological similarities.

## NEUROLOGICAL DISORDERS

In line with the One Health notion, neuroscience studies on sheep are presented to deduce knowledge applicable mainly to humans, to either fill gaps regarding the nervous system’s function or collect

neurologic disorder data and apply potential therapeutic strategies. The first disorder discussed here, though, ticks an extra One Health box<sup>[2]</sup>, and helps to elucidate the prion agent's transmission from sheep to humans<sup>[21]</sup> [Figure 1].

### Prion diseases

Prion disorders can affect many different mammals, from Scrapie in sheep to Creutzfeldt-Jakob disease in humans<sup>[22]</sup>. This fatal neurodegenerative disorder, known as Scrapie, is the result of the misfolding of the normal prion protein (PrP<sup>C</sup>) into the disease-associated form (PrP<sup>Sc</sup>), and accumulation of this PrP<sup>Sc</sup> form. The misfolding can be caused by a mutation in the *PRNP* gene, an infection with the misfolded protein, or a combination of both factors<sup>[21]</sup>. It entails a long asymptomatic subclinical phase/incubation period, followed by a comparatively shorter clinical phase leading to death<sup>[21-23]</sup>. Under certain conditions, Scrapie may pose a zoonotic threat by the consumption of prion-infected (PrP<sup>Sc</sup>) material<sup>[21,23]</sup>. This explains the extra surveillance measures worldwide to detect the prevalence and geographical disease distribution in small ruminants and cattle in order to avoid further human exposure to prion agents<sup>[21]</sup>.

Prions were first observed in sheep. Isolation of the scrapie strains from sheep has been characterized through experimental transmission studies to rodents<sup>[22,24]</sup>. Prion diseases are a well-known category of proteinopathies, which also includes other human neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Creutzfeldt-Jakob disease, and more. These proteinopathies may be influenced by different multifactorial aetiologies, but they commonly share the accumulation of abnormal proteins, which plays a key role in their pathogenesis<sup>[22,24-26]</sup>. Subsequently, a deeper understanding of Scrapie pathogenesis and treatment approaches may additionally prove beneficial for other proteinopathies<sup>[25]</sup>.

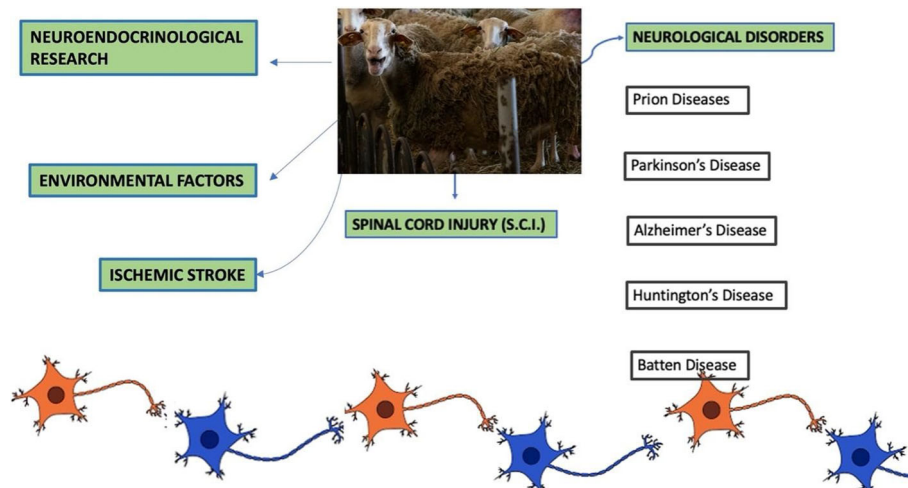
The ability to cross the species barrier when BSE infection was orally transmitted to genetically susceptible sheep was demonstrated by Jeffrey *et al.* (2001). One hundred percent infection rate was observed, with abnormal prion accumulation detected 22 months post inoculation<sup>[27]</sup>. Previous studies showed that the initial accumulation of the infectious agent occurs in Peyer's patch follicles. Therefore, Åkesson *et al.* (2011) conducted an experimental study in lambs, which involved surgically manipulating intestinal loops and then inoculating these isolated loops with scrapie brain pool inoculum, normal brain homogenate, or sucrose solution. They concluded that the intestinal epithelium remained undisturbed, and thus formed different hypotheses on the infection of these structures<sup>[28]</sup>.

Few diagnostic tools exist for the detection of prion proteins. In 2015, an effort was made to create a Scrapie blood plasma test based on previous studies, indicating infectivity was present in the blood before CNS and viscera deposition, even three months post inoculation<sup>[29,30]</sup>. Ultimately, Lim *et al.* (2015) showed positive results on the detection of Scrapie-infected sheep using a multimer detection system<sup>[31]</sup>.

It is important to highlight that non-prion nervous system infections (i.e., bacteria, viruses, *etc.*) may ignite or intensify human prion disorders<sup>[32]</sup>. This is particularly important in the post-COVID era, where a strong correlation between COVID-19 patients and prion-related diseases is emerging<sup>[33]</sup>. Therefore, understanding prion disease may pave the way for the development of novel therapeutic or prophylactic approaches. The use of sheep in this field of study is of paramount importance.

### Parkinson's disease

Parkinson's disease causes a different kind of abnormal aggregated-protein deposition, where the misfolded  $\alpha$ -synuclein causes the degeneration of dopaminergic neurons in the substantia nigra, leading mainly to



**Figure 1.** Sketch showing the utility of sheep as experimental models in a broad range of disciplines. All this research is grounded on a neuroanatomical basis.

motor function decline<sup>[24,26,34]</sup>. One study examines the ovine brain bioavailability of intranasally administered ropinirole. Rao *et al.* (2017) proved that their ropinirole nasal gel resulted in higher drug concentrations in sheep brains, suggesting a promising non-invasive alternative to intravenous injection<sup>[35]</sup>. Moreover, additional experiments using sheep as an intranasal drug bioavailability model continue to emerge.

Bourke (2018) proposes a molybdenum-deficient sheep PD model, causing symptoms of motoric and motor neuron dysfunction and dementia through astrocyte dysfunction<sup>[36]</sup>. The most commonly used PD model involves exposure to the neurotoxic agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively targets PD-involved neurons<sup>[37,38]</sup>. This substance promotes the manifestation of PD symptoms in aged PD sheep model<sup>[39-41]</sup>. It is noteworthy that sheep, like humans, naturally develop age-related diseases, e.g., PD and AD<sup>[41]</sup>.

### Alzheimer's disease

Furthermore, in aged sheep brains, neurofibrillary structures similar to those observed in human AD were observed<sup>[42]</sup>. Davies *et al.* (2022) have shown that humans and sheep have common AD pathological components, while Reid *et al.* (2017) demonstrated age-dependent plaque aggregation in sheep<sup>[43,44]</sup>. All the above strengthen sheep's importance as an AD model.

### Huntington's disease

Mutant Huntingtin protein accumulation in brain neurons causes HD, a fatal genetic neurodegenerative disorder characterized by motor dysfunction and cognitive decline<sup>[45,46]</sup>. After many years of studying this disorder in rodents, the absence of an effective therapeutic agent against HD is leading to a shift in the use of large animal models. There are a number of scientific papers indicating the adoption of sheep as a more suitable HD model, based on sheep's neuroanatomical similarities to the human brain, experimental convenience, and mainly for its longevity, allowing for long-term effect measurements of potential therapeutic agents<sup>[12,46-48]</sup>. Therefore, in 2010, a transgenic ovine (OVT73) HD model was developed<sup>[49]</sup>. This transgenic model, the OVT73 sheep, carries the human genetic variation, something that has not been achieved by the other transgenic HD models<sup>[47]</sup>. Studies on this sheep HD model demonstrate that, by five years of age, these animals exhibit brain pathology similar to that observed in human HD patients, and

metabolic abnormalities, although without the neurodegenerative symptomatology, hinting at the diagnostic potential for presymptomatic human disease and indicating the potential for identifying novel biomarkers that can enhance the assessment of disease progression<sup>[45,47,50,51]</sup>. Another symptom of human HD is also exhibited in the sheep model: sleep disturbances. In general, HD causes presymptomatic circadian rhythm abnormalities, worsened with age in OVT73<sup>[52]</sup>. A sleep study using electroencephalography on HD and normal sheep revealed significant differences, indicating them as an important prognostic factor for HD patients<sup>[53]</sup>. A different study comparing normal and HD sheep showed elevated plasma melatonin levels in the latter, suggesting melatonin neuroprotective properties for huntingtin toxicity<sup>[54]</sup>.

The development of this new transgenic ovine model revealed the lack of preclinical cognitive tests in sheep, which are necessary for assessing disease-related cognitive decline and, more importantly, the effect of potential treatments<sup>[55]</sup>. Morton *et al.* (2011) demonstrated sheep can perform executive cognitive tasks, making the cognitive decline of sheep in neurodegenerative diseases quantifiable<sup>[47,55]</sup>. Additionally, McBride *et al.* (2016) created a mobile semi-automated system to test the cognitive abilities of sheep and other animals<sup>[56]</sup>.

Sheep magnetic resonance imaging (MRI) imaging revealed discrepancies between aged HD and normal sheep brains. These discrepancies allow for therapeutic potency testing in HD sheep models at the early disease stages, prior to the onset of evident clinical symptoms<sup>[46]</sup>. Moreover, the establishment of an OVT73 multi-omic dataset provides additional practical information that can be beneficial for future HD research<sup>[57]</sup>. Finally, researchers evaluated viral therapies in HD sheep. Mondo *et al.* (2018) have studied the neuronal uptake and distribution of three adeno-associated virus (AAV) therapies, while Pfister *et al.* (2018) showed that AAV9 can safely silence human mutant HD protein<sup>[58,59]</sup>.

### Neuronal ceroid lipofuscinosis (Batten disease)

The final neurological disorder in this review is Batten Disease (BD), also known as neuronal ceroid lipofuscinoses, which has been extensively studied on sheep. Batten Disease refers to a group of rare inherited neurodegenerative diseases with limited treatment options, stemming from dysfunctional storage disorders in lysosomes. Common symptomatology of this group of diseases includes dementia, visual impairment, seizures, and progressive motor decline, resulting in premature death<sup>[60-62]</sup>. In humans, there is also a rare juvenile manifestation of the disease linked with the *CLN3* gene, occurring in children between the ages of 5 and 10, unfortunately, with no available treatment options. Patients present the common symptoms of the group and, in rare cases, heart malfunction, with the majority of individuals not exceeding a life expectancy beyond their early 20s<sup>[63,64]</sup>. Large animal models for this disease primarily include dogs and sheep<sup>[65,66]</sup>. Both genetically modified and naturally occurring sheep models are available<sup>[61,65-68]</sup>.

The translational relevance of the CLN sheep, [referring to the family of mutated *CLN* (ceroid lipofuscinosis, neuronal) genes, which causes Batten disease] is amplified through several studies. Table 1 provides a concise summary of them:

Therapeutic BD approaches include enzyme replacement therapy and gene therapy, both of which have been extensively studied in murine models, with limited translation potential in humans<sup>[66,80]</sup>. A cross-species approach by Nelvagal *et al.* (2022) treated mice and sheep with enzyme replacement therapy and obtained positive results when assessed with MRI and neuropathology<sup>[70]</sup>. Gene therapy was also applied to sheep through lentiviral or AAV injections<sup>[72]</sup>. These techniques slowed disease progression<sup>[73,81]</sup>. Finally, a neural cell culture from BD sheep showed a reversal of pathological storage body accumulation following the application of lentiviral gene therapy<sup>[82]</sup>.

**Table 1. Ovine animal models of BD**

Mutation in gene	Breed	Occurrence	Experimental therapies	Onset of clinical symptoms	Examples of studies/model characteristics
CLN1 <sup>[68]</sup>	Texel-cross <sup>[69]</sup>	Gene editing	Enzyme Replacement Therapy <sup>[70]</sup>	N/A	It produces common phenotypical human disease characteristics
CLN5 <sup>[71]</sup>	New Zealand Borderdale	Natural	Gene Therapies <sup>[72,73]</sup>	11-12 months <sup>[74]</sup>	In a comparative EEG study, CLN5 homozygous sheep present sleep abnormalities comparable to the brain activity of epileptic children <sup>[75]</sup> . They manifest similarity to the molecular pathways of synapse pathophysiology with less developed animals (mouse and <i>Drosophila</i> models), allowing for target therapeutics on a larger animal <sup>[60]</sup>
CLN6 <sup>[71,76]</sup>	1. New Zealand Borderdale 2. Australian Merino 3. New Zealand South Hampshire	Natural	1. Gene Therapies 2. Gene Therapies 3. Gene Therapies, Anti-inflammatory drug <sup>[67]</sup>	1. 6 months 2. 7-10 months 3. 10-14 months	A longitudinal MRI study in two different time points in South Hampshire sheep revealed atrophy in the cortex and other brain anatomical areas, which is a key point to observe the progression of the disease and alterations or deceleration caused by treatment <sup>[77]</sup>
CLN10 <sup>[78]</sup>	White Swedish Landrace	Natural	N/A	At birth <sup>[79]</sup>	Brain atrophy and early death are the result of a mutation in the cathepsin D gene, in this model of disease

BD: Batten disease; EEG: electroencephalogram; MRI: magnetic resonance imaging.

## SPINAL CORD INJURY

Spinal cord injury (SCI) [Figure 1] refers to damage occurring directly by mechanical impact or indirectly by the body's attempt to repair the damage over time. It can be inflicted by trauma, disease, degeneration, or ischemia<sup>[83-85]</sup>. This uncommon medical problem can lead to overwhelming financial costs arising from high rates of mortality, hospitalization, and patient care and the productivity loss of the affected individuals. Added costs arise from post-SCI complications, including neurological dysfunction like spasticity, paralysis, paraparesis and fatigue, and potential future contractures, pressure ulcers, and infections<sup>[83,86,87]</sup>. A deeper understanding of the biology, pathology, and post-SCI repair mechanisms is required due to the lack of a well-defined treatment. This knowledge deficit is driving researchers to a shift towards large animal models for better human translational outcomes<sup>[83]</sup>.

Animal SCI models include rats, mice, rabbits, dogs, cats, pigs, and NHPs, while far fewer studies have been conducted in sheep. Although the most commonly used SCI animal model is the rat, its suitability for preliminary studies is uncertain<sup>[83,84,86]</sup>. The preference for rats in acute SCI studies has been questioned by Petteys *et al.* (2017). They hinted that the vast majority of rat SCI research has limited benefits for the human patient<sup>[86]</sup>. Advantages of using rodents include their cost-effectiveness, well-studied SCI biology, low ethical restrictions for research, and standardized methods to effectively replicate SCI conditions<sup>[83,84,86]</sup>. The disadvantages include significant anatomical differences between humans and rodents. In terms of size, rodent spinal cord is far shorter than that of humans. This size differential is vital for regenerative spinal cord studies because rodents require shorter nerve fiber regeneration distances; therefore, restoration of function is more probable in smaller species, impairing translational results<sup>[86,88]</sup>. Sheep have a large-diameter spinal cord, spine, and spinal cord anatomical similarities to humans; they have well-studied CNS electrophysiology, and follow similar injury and repair patterns as humans<sup>[85-87]</sup>. The ovine body



size permits chronic human-scale device implantation for electrophysiological studies, playing a crucial role in implant testing<sup>[87]</sup>. Conclusively, large animal models, like sheep, more effectively approximate human SCIs.

There is a pivotal hurdle to overcome when studying sheep SCI. Many studies lack consistent approaches because, in contrast to rodents, sheep have not been widely studied and standardized sheep SCI methods do not exist<sup>[86]</sup>. In previous studies, a weighted drop object falling from a certain height was used onto the exposed spinal cord to cause its injury<sup>[89,90]</sup>. To overcome this problem, Petteys *et al.* (2017) created a controlled electromagnetic spinal cord impactor for large animals. This device will allow future therapeutical studies for acute traumatic SCI in sheep<sup>[86]</sup>.

An important treatment for this injury is intradural or epidural spinal cord stimulation using electrodes to alleviate post-SCI chronic pain and spasticity. Sheep have been used to test the efficacy and safety of these implantable devices<sup>[91,92]</sup>. Several factors require consideration: proximity to the stimulation area, magnitude of the stimuli, durability and longevity of the device, and prevention of ischemic effects due to pressure from the device<sup>[91,92]</sup>. Safayi *et al.* (2015) have examined the gait of sheep walking on a treadmill, comparing sheep's walking patterns before and after a SCI through video analysis, the method of which is applicable to future novel therapeutic assessment<sup>[90]</sup>. This has an observable One Health approach because it has already been applied to SCI intervention of other quadrupedal species<sup>[90]</sup>.

Human spinal cord ischemia is a common sequelae to an accident or trauma requiring prompt action to restore oxygenation and blood supply to avoid secondary SCI<sup>[85]</sup>. An adult Dorset sheep model was used to develop a fiber optic device for monitoring spinal cord blood supply; such devices provide an early diagnostic tool for this damage<sup>[85]</sup>. In another study, Merino sheep were used to understand SCI pathophysiology following endovascular descending thoracic aortic surgery<sup>[93]</sup>.

## ISCHEMIC STROKE RESEARCH

The majority of human brain ischemia clinical cases are caused by vasculature blockage in the brain from ischemic stroke (IS) [Figure 1]<sup>[94]</sup>. As of May 2023, there is only one therapeutic agent for IS, called tissue plasminogen activator, according to the National Institute of Health. This sole medicinal contribution is extrapolated from rodent IS models<sup>[95,96]</sup>. To increase novel therapy research, large animal models are required. This is suggested by the Stroke Therapy Academic Industry before proceeding with clinical trials for Acute Ischemic Stroke<sup>[94,95]</sup>. Large animal IS models with high predictive value include NHPs, dogs, swine, sheep, and cats. Blood sampling is easier due to larger blood volumes, compared to rodents, which is very important for prognostic or recovery markers testing<sup>[95]</sup>. Additionally, there are plenty of anatomical and physiological similarities between sheep and humans, many of which are later reported in this review, like size, morphology, and gyrification.

Experimental IS models are either endovascular or surgical. A limiting factor for sheep endovascular IS studies is the rostral epidural rete mirabile (RM) formation, an anatomical structure that assists in selective brain cooling and body water balance maintenance, especially during high temperatures<sup>[97]</sup>. The RM is the result of many small-diameter arteries anastomoses between the maxillary and the internal carotid arteries, which prohibits the incidence of thromboembolic stroke and the required passage of microcatheters, allowing only for surgical approaches<sup>[94,95,97]</sup>. Boltze *et al.* (2008) described their results after permanent middle cerebral artery occlusion (MCAO) in sheep. They demonstrated that this novel focal cerebral ischemia model allows for controlled lesion size, with the analogous neural function effect, according to behavioral assessment. Positron emission tomography, MRI, and pathological, histological, and

immunohistological testing revealed stroke-induced changes similar to human findings<sup>[94,98]</sup>. Wells *et al.* (2012) also verified the development of an ovine IS model while additionally comparing the effects between permanent and transient MCAO, using physiological and histopathological changes as well as infarct volumes in the brain. Both groups showed ischemic injury characteristics; the permanent occlusion group showed larger infarct volumes, whereas the second group, of aneurysm clip application, additionally allowed for future reperfusion studies<sup>[94,99]</sup>. Neither Boltze nor Wells reported mortality or complications during the experiments, and they both established sheep as a validated model in the stroke research field<sup>[98,99]</sup>.

## NEUROENDOCRINOLOGICAL RESEARCH

Sheep play a crucial role in research associated with the discipline of neuroendocrinology [Figure 1], which orchestrates and regulates vital functions and physiological behaviors through the constant interactions between the nervous and endocrine systems. Neuroendocrinological research topics include the suppression of reproductive activity due to stress, undernutrition, and inflammation<sup>[100-103]</sup>, and seasonal sheep neuroendocrine regulation<sup>[104,105]</sup>. Their outcomes assist in understanding mechanisms governing neuroendocrine human and ovine biology. More specifically, the regulation and control of ovine reproductive characteristics contribute to increased food and wool production<sup>[104,105]</sup>. In contrast to rodents, sheep show similarities in the mediation of progesterone negative feedback with primates during the luteal phase<sup>[106]</sup>. As previously mentioned, the large blood volumes, which allow for extensive serial sampling, provide repeated measurements of blood plasma to characterize the animal hormonal secretory profiles<sup>[104]</sup>. The direct hypophyseal portal blood sampling, coupled with jugular blood sampling, is another significant benefit. This can be achieved on unanesthetized and unrestrained/freely moving sheep for extended periods of time, omitting stress and anesthetic substances<sup>[104,107-109]</sup>. Even more importantly, the large size of the sheep brain is suitable for central agent administration, for example, intracerebroventricular norepinephrine injection, concomitant with frequent hypophyseal portal and peripheral blood sampling for extended periods<sup>[110]</sup>. Lastly, the ovine skull is suitable for chronic intubation through neurosurgical approaches, for repeated sampling of larger volumes of cerebrospinal fluid (CSF) compared to rodents, to counterbalance the physiologically low concentrations of substances found in CSF<sup>[111,112]</sup>.

The works of Clarke, Goodman, Lehman, and other researchers utilize the above advantages to thoroughly study sheep hypothalamic, hypophyseal, and gonadal interactions<sup>[113-118]</sup>. These studies founded the hypothesis regarding the synchronized episodic gonadotropin-releasing hormone (GnRH) secretion<sup>[109]</sup>. The data acquired suggest that GnRH neuron activity is modulated by the KNDy neurons, containing kisspeptin, neurokinin B (NKB), and dynorphin. These neurons are located in the sheep arcuate nucleus of the medial basal hypothalamus, which is believed to play an essential role as the “GnRH pulse generator” similar to that in humans<sup>[119]</sup>. More recently, sheep research in this field has integrated the implementation of newer techniques in neuroanatomical analysis, such as RNAscope, a fluorescent *in situ* hybridization technique to assess mRNA at the cellular level<sup>[120]</sup>. For example, Merkley *et al.* (2020) and Harlow *et al.* (2022) have utilized this technique along with immunohistochemical analysis to examine KNDy cells in young male and female sheep during undernutrition, which align with a global impact of nutritional challenges in adolescents<sup>[121-123]</sup>. Another sheep study by Aerts *et al.* (2021) using RNAscope also utilized frequent blood sampling and immunohistochemistry to conclude that even though these neurons play an important role during pubertal development, they are not responsible for the initiation of puberty<sup>[124]</sup>. Moreover, kisspeptin, among other neuropeptides and neurotransmitters, is regulated by photoperiod, i.e., alterations in melatonin secretion, therefore linked to seasonal reproductive changes<sup>[125]</sup>.



In the context of circadian rhythms and their importance in research, Esposito *et al.* (2020) experimented on rodents using three distinct cerebral ischemia therapeutic agents, yielding neuroprotective results. However, this study raised an issue regarding the translational potential due to the ineffectiveness of the same therapeutic scheme in humans<sup>[126]</sup>. Ultimately, they suggested that the opposite circadian rhythms between humans (diurnal) and rodents (nocturnal) need to be taken into consideration for translational studies on IS and CNS disease<sup>[126]</sup>. Research on sheep circadian rhythm is conclusive that they are mainly diurnal animals, like humans<sup>[127-129]</sup>. The latest technological approaches can be implemented in this field. For example, the use of GPS collars to extensively monitor sheep during free grazing can provide information on circadian rhythms in natural environments, which is nonapplicable in rodents<sup>[129]</sup>. Furthermore, Helm *et al.* (2013) proposed the potential seasonality of humans as an approach to better interpret human medical and psychological problems<sup>[130]</sup>. Therefore, it could be assumed that sheep circadian research might be more beneficial for human health.

Furthermore, oxytocin secretion is one of the major neuroendocrine mechanisms in mammals. Sheep as an animal model contributes vastly to the relevant research and the neuroanatomical basis of the milk ejection reflex has been elucidated<sup>[131,132]</sup>. Electrophysiological studies also exist to describe the milk-ejection reflex in sheep<sup>[133]</sup>, where oxytocin is released only once or twice during each suckling session. Based on these data, modeling of oxytocin release in sheep has also been published<sup>[134]</sup>, and the Artificial Neural Network produced by this work could be used for training on physiological data, leading to the improvement of milk production.

From a different perspective, the gut microbiome is shown to affect many physiological functions, including the neuroendocrine system of different animal species, establishing a microbiome-gut-brain axis (MGBA). In addition, MGBA affects the hypothalamic-pituitary-adrenal axis, as elucidated in microbiota-deficient germ-free animals<sup>[135,136]</sup>. The ovine gut microbiome composition has been studied through DNA/RNA sequencing techniques and compared to other polygastric and monogastric animals<sup>[137]</sup>. Gut microbiome metabolome studies were performed on Dorper and Tan sheep. Results on the performance of these animals were affected by microbiome composition and distinctive differences between the fermentation patterns of these two breeds were shown<sup>[138]</sup>. Conclusively, gut microbiome studies on sheep are extensive but restricted to animal production performance, mostly overrunning the MGBA<sup>[135]</sup>.

## ENVIRONMENTAL FACTORS WITH IMPACT ON THE NERVOUS SYSTEM

Finally, it is known that there are environmental factors [Figure 1]; (both to sheep and humans) such as bisphenols, affecting the function of many animal body systems, even from the embryonic stage<sup>[138]</sup>. For this reason, nutritional experiments were performed from earlier years in order to study the effects of macroelements, e.g., ammonium metavanadate, on lambs' diet (mineral concentrations in animal tissues)<sup>[139]</sup>. In another study, sheep were given organophosphorus compounds and their subsequent neurological effects were monitored<sup>[140]</sup>.

In later years, a sheep study has shown the results from nutritional exposure in biosolids containing heavy metals, which were associated with altered expression of GnRH, GnRH receptors, galanin receptors, and kisspeptin mRNA within the hypothalamus and pituitary gland. The effects of biosolid exposure at the hypothalamic level have concentrated on the GnRH neurosecretory system, as this is the primary system through which the reproductive axis is controlled and it is sensitive to the effects of endogenous and exogenous steroids both during development and in adult life. In most cases, these anatomical and functional differences do not result in altered peripheral hormone concentrations or reproductive function (like the lambing rate), indicating physiological compensation under the conditions tested<sup>[141]</sup>.

In another study, gestational sheep exposure to a low dose of bisphenol A (BPA) is shown to affect maternofetal thyroid function and fetal brain development in a region-specific manner. The authors state that the use of a larger animal model enabled the examination of specific brain areas, leading to the generation of evidence indicating distinct region-specific effects of fetal BPA exposure on the brain metabolome<sup>[142]</sup>.

Lastly, a publication focused on the results of heavy metal consumption (Cd, Pb, Cu, and Zn) both on humans and animals due to their vicinity in a smelting facility, implementing the One Health Initiative. Although it does not present effects on animals' and humans' nervous systems, it evaluates the contamination of pastures and farmlands with heavy metals and, in this regard, identifies the necessity of equivalent research to benefit both humans and animals<sup>[143]</sup>.

## FINAL REMARKS

In brief, we summarize the points that make sheep an essential animal model.

### Why are sheep an important animal research model?<sup>[4,41,144-146]</sup>

1. Their docile nature permits easier and stress-free handling, saving time and money (e.g., purchase of restraints).
2. They have a size similar to humans, making sheep good candidates for:
  - (1) use of imaging techniques (e.g., MRI, CT)
  - (2) testing of medical devices (e.g., stents, pacemakers, heart valves)
  - (3) testing for surgical trials
  - (4) human-scale implantation electrophysiological tests
3. They are anatomically, physiologically, and genetically closer to humans than rodents.
4. They are relatively cost-efficient and easy to maintain compared to other large animals.
5. Their use as animal models is ethically more acceptable compared to NHPs and dogs.
6. Their long lifespan:
  - (1) allows for age-related studies (Scrapie)
  - (2) is more similar to macaque monkeys (10-30 years)
  - (3) requires longer studies for data accumulation compared to rodents
  - (4) may result in additional risk of population mortality in studies
7. They have naturally occurring diseases, similar to human diseases (BD).
8. They play an important role in preclinical trials.
9. They have a fully sequenced genome providing additional information.
10. Genome engineering is feasible in sheep, even supporting CRISPR/Cas9 to generate disease models.
11. They are relatively outbred, providing diverse populations for research, showing a wider spectrum of responses, and reflecting human heterogeneity.
12. There are available behavioral and cognitive tests.
13. They have a low litter size like humans.

Although there are reviews on the importance of sheep as an experimental animal, such as the one by Murray and Mitchell (2022)<sup>[147]</sup>, our manuscript introduces sheep into the One Health system. We also believe the neuroanatomical similarities between humans and sheep are decisive for translational research. The similarities below are particularly relevant in establishing animal models.

### Neuroanatomical and physiological significance of the sheep

The sheep brain anatomy, specifically its structure and organization, has significant similarities to NHPs and humans including<sup>[55,146,148-150]</sup>:

1. large brains
2. thick and well-developed meninges
3. human-like basal ganglia
  - (1) a dorsal striatum divided to caudate nucleus and putamen
  - (2) anatomical and neurochemical similarities of the substantia nigra
4. gyrencephalic cerebral cortexes
5. a brain with the capacity to remember the faces of other sheep and even humans.
6. white matter distribution like humans
7. well-defined sulci and gyri
8. relatively round skull, like humans, despite their elongated brain
9. lobulated cortexes with human-like landmarks
10. a brain region responsible for decision-making and emotional control similar to the corresponding human orbitofrontal cortex

### What are some limitations when using sheep in neuroscience research?<sup>[4,20,147]</sup>

An extensive list of all the limitations and disadvantages of sheep in neuroscience research would result in a lengthy and time-consuming read. Nonetheless, the reader who is inexperienced with their use should get a general idea of the hurdles of using this large animal model. This includes:

1. higher expenses resulting from:
  - (1) housing facilities that are specialized for their larger body size, including proper surgical/operating facilities, imaging rooms, and specialized husbandry. Regarding the infrastructure needed for the housing of sheep in scientific institutions, more detailed and thorough guidelines can be found, for example, by the Animal Ethics Infolink online<sup>[151]</sup>.
  - (2) a high skilled team of specialists for operations and surgeries, e.g., veterinarian assistance
  - (3) increasing maintenance expenses for long-term studies
  - (4) biosecurity measures and practices (examples listed below)<sup>[152,153]</sup>:
    - quarantine/ acclimatization zones for animals before entering the main facility, including separate utensils and tools used only in this area
    - vaccination, prophylactic treatment (anthelmintics), infectious disease testing
    - isolation area for animals that are sick or suspected of having a disease, including separate utensils and tools used only in this area
    - manure should be composted for at least a year before spreading on the environment
    - the average costs of obtaining and keeping sheep as laboratory animals varies greatly between countries and different regions. In the references listed here, including the supplemental material by Taha *et al.* (2022), some indicative costs may provide the reader with a general idea of the expenses<sup>[95,154]</sup>.
2. difficulty in finding and selecting proper antibodies and/or reagents for routine techniques, which are often more costly than rodents.
3. more time required for the accumulation of data, due to seasonality and longer gestation periods and lifespans.
4. anatomical differences to humans:
  - (1) due to their quadrupedal walk, they have a different neuraxis than humans, which may affect the outcomes of SCI models.

- (2) regarding their gastrointestinal anatomy, which may play an important role in research about the MGBA.
- (3) the presence of the rostral epidural rete mirabile, hindering endovascular IS research.
- 5. less available neurological and cognitive tests for sheep, compared to rodents.

## CONCLUSION

This review serves to challenge the world's general idea that sheep are low-intelligence farm animals, providing a significant contribution to neuroscience. The increased utilization of experimental techniques in several research disciplines [e.g., electroencephalogram (EEG)] demonstrates that sheep are more cognitively robust than previously thought. Moreover, several naturally occurring diseases and novel gene-manipulating technologies for disease induction, in combination with innate neuroanatomical and physiological properties of the sheep that approximate those of humans, underscore the importance of sheep, not only for neuroscience research contributions but biomedical research in general. The conclusions drawn from these sheep experiments will allow for a significant understanding of disease mechanisms, and hopefully result in the advancement of therapeutic tools for human patients. Definitively, sheep research translational outcomes strengthen the One Health notion.

## DECLARATIONS

### Acknowledgment

We thank Dr. Patrick Sharp, Attending Staff Veterinarian, University of California, Merced, USA for the critical review of the main part of the manuscript, and Giannis Graidis for capturing and editing the sheep photos used in the graphical abstract and [Figure 1](#). The sheep photos are from the Farm Animal Clinic - Kolchiko, School of Veterinary Medicine, Aristotle University of Thessaloniki.

### Authors' contributions

Design, writing, and editing of the manuscript: Chatzimanou T, Tsingotjidou A

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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