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# Morphological Relationships between the Cholinergic and Somatostatin-28(1-12) Systems in the Alpaca (*Lama pacos*) Brainstem

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**Abstract:** In the alpaca brainstem, the distribution of the cholinergic system by the immunohistochemical detection of the enzyme choline acetyltransferase (ChAT) has been described, and its relationship with the distribution of somatostatin-28(1-12) is analyzed by double-immunostaining techniques. Overlapping distribution patterns for both substances were observed in many brainstem regions, suggesting that interactions between them may occur in the reticular formation, nucleus ambiguus or laterodorsal tegmental nucleus. Colocalization of the two substances in the same cell bodies was only observed in restricted areas, such as the nucleus of the solitary tract, reticular formation and nucleus ambiguus. In addition, in several regions, an apparent high innervation of the peptidergic fibers on cholinergic neurons has been observed. The results suggest that chemospecific interactions could be crucial for the control of specific cardiorespiratory and/or digestive functions in alpacas. These interactions may represent brain-adaptive mechanisms to particular environments and have a potential therapeutic use in respiratory disorders.

**Keywords:** alpaca; brainstem; somatostatin; acetylcholine; cardiovascular regulation; respiratory control



**Citation:** Marcos, P.; Coveñas, R. Morphological Relationships between the Cholinergic and Somatostatin-28(1-12) Systems in the Alpaca (*Lama pacos*) Brainstem. *Anatomia* **2022**, *1*, 54–67. https://doi.org/10.3390/anatomia1010006

Academic Editor: Francesco Cappello

Received: 5 May 2022
Accepted: 23 May 2022
Published: 25 May 2022



## 1. Introduction

Cetaceans (dolphins, whales) and artiodactyls (even-toed ungulates, e.g., sheep, giraffe) belong to the order Cetartiodactyla. The family Camelidae is part of artiodactyls, and the alpaca (*Lama pacos*) is included in this family [1–5]. Alpacas are important animals for the economy of numerous South American countries due to the excellent quality of their wool, and in this sense, numerous studies focused on their maintenance and reproductive cycles have been performed [6,7]. The members of the Camelidae family have specific morphological characteristics, such as long necks and seven cervical vertebrae, and moreover alpacas can live at sea level and at 5000 m above sea level [6–12]. These characteristics suggest the existence of important and unique adaptation mechanisms, mainly related to cardiovascular and respiratory mechanisms, which are controlled by the central nervous system, specifically brainstem centers. Other members of the Certiodactyla order also exhibit brain specializations that help them to survive within their respective environments [1–5].

Since all these physiological functions are regulated by neuroactive substances, several studies have reported the distributions of different classical neurotransmitters and neuropeptides by means of immunohistochemical methods in the alpaca brain [6–12]. These studies complement previous works regarding the mapping of neuro-modulatory systems in the brain of Artiodactyla and confirm that the nuclear complement of neurotransmitters, such as acetylcholine or catecholamines, detected in the alpaca brainstem and diencephalon

was similar to that found in other members of the same order, and this finding supports Manger's hypothesis [13]. In addition, the distribution of some neuropeptides has been studied in the alpaca brainstem [6–9,12] and diencephalon [10,11]. Moreover, the morphological relationship between neuropeptides and neurotransmitters has been reported, and double-immunolabeling for tyrosine hydroxylase, which is the rate-limiting enzyme of the catecholaminergic synthesis, and somatostatin-28(1-12) (Som-28(1-12)) has been carried out in the diencephalon of the alpaca [11]. In the brainstem of this species, a double-labeling for choline acetyl transferase (ChAT, a marker for the cholinergic system) and calcitonin gene-related peptide (CGRP), as well as for CGRP and tyrosine hydroxylase [9,12], has been described. According to the distribution of double-labeled perikarya, the results observed in the brainstem in these studies suggest that CGRP might interact more with the catecholaminergic system than with the cholinergic system. However, although the physiological interactions between somatostatin and catecholamines have been reported in the literature [13], no double-labeled neurons for these two substances were detected in the alpaca diencephalon [11]. A possible explanation may be that the interaction between the two substances may occur with another somatostatin fragment different from the one studied in that work.

Somatostatin, in addition to inhibiting the growth hormone, acts as a neuromodulator in numerous physiological functions, blocking the release of noradrenaline and stimulating the release of serotonin and acetylcholine [14]. The precursor named pro-somatostatin is cleaved into somatostatin-28, somatostatin-12 (corresponding to the first 112 amino acids of somatostatin-28) and somatostatin-14 [15]. These fragments can elicit different responses in relation to the same mechanism, such as the cardiovascular regulation [15], probably due to the activation of different somatostatin receptor subtypes [16]. In addition, the interaction of somatostatin with other neurotransmitters such as acetylcholine is well-known [16–21], but the studies that described such interactions were more focused on memory processing.

Studies previously performed in the alpaca brainstem suggest a similar distribution pattern for CGRP and Som-28(1-12) [7,8]. The presence of CGRP and ChAT in the same perikarya was reported in some of the alpaca brainstem nuclei; thus, the goal of the present work was to know whether there is a neuroanatomical basis for possible interactions between Som-28(1-12) and ChAT in the alpaca brainstem. Moreover, due to the participation of these two substances in cardiovascular and respiratory functions, another aim of this study is to know whether these interactions can constitute a morphological basis for the control of cardiovascular and/or respiratory mechanisms in this species. The results obtained will help to understand the distinctive control mechanisms that exist in the alpaca as physiological adaptations to living in such different habitats in terms of altitude, which leads to changes in air quality and composition. Knowledge of these adaptive mechanisms and their morphological basis will contribute to a better understanding of the neuroanatomy and physiology of the alpaca. Comparison with the results obtained in other mammalian species will allow to assess whether these mechanisms may constitute therapeutic targets for the possible treatment of cardiovascular, respiratory and/or digestive disorders.

## 2. Materials and Methods

### *2.1. Animals*

As reported previously in similar studies [8,12], six adult male alpacas (*Lama pacos*) (70–80 kg; 5–8 years) were used here. From birth to the perfusion day, animals were maintained at 0 m on the sea level and kept under standard conditions of temperature and light and with free access to water and food. The study was performed following the guidelines of the ethical and legal recommendations of the Spanish legislation [8,12].

### *2.2. Tissue Processing*

The protocol has previously been published in the reports showing the distribution of immunoreactive structures containing Som-28(1-2) [8] and ChAT [12] in the alpaca brainstem. Animals were intravenously anaesthetized with ketamine (10 mg/kg) and xylazine

(4 mg/kg), heparinized, and perfused (NaCl (0.9%): 3 L, paraformaldehyde (4%): 5 L in phosphate-buffered saline (PBS, pH 7.2, 0.15 M)) through the carotid artery [8]. Brainstems were dissected out, post-fixed in the latter fixative solution (overnight) and cryoprotected (using increasing sucrose baths). Using a cryostat, 50 µm frontal brainstem sections were obtained, kept at 4 °C in PBS and processed for the immunohistochemical detection of Som-28 (1-12) [8]. Then, these sections were used for double-immunohistochemistry with ChAT labeling.

### *2.3. Immunohistochemistry*

In the present work, the same frontal sections in which Som-28(1-12)-immunoreactivity was previously observed, after applying the diaminobenzidine (DAB) developing methodology [8], were used to detect ChAT according to a previously published protocol [22]. Som-28(1-12)-immunoreactive slides were immersed in PBS until the coverslip became detached, then sections were carefully removed from the surface of the slides. The traces of the mounting medium were removed with several rinses in PBS (6 × 10 min). To inactivate endogenous peroxidases, Som-28(1-12)-immunoreactive sections were treated with $NH_3$ (20%), NaOH (1%) and $H_2O_2$ (30%). Sections were extensively rinsed in PBS (6 × 10 min) and pre-incubated in PBS containing normal horse serum (1%) and Triton X-100 (0.3%). This solution was also used for further dilution of streptavidin and antibodies. Sections were incubated with primary polyclonal antibody against choline acetyl transferase (ChAT, Millipore, ref. AB144 P, raised in goat; dilution 1/75; overnight, 4 °C), washed in PBS and incubated in biotinylated donkey anti-goat antibody (Jackson Laboratories; dilution 1/2000; 90 min, room temperature). After washing in PBS, sections were incubated in peroxidase-coupled streptavidin (Jackson Laboratories, dilution 1/2000; 90 min, room temperature). Finally, sections were washed in PBS and Tris-HCl buffer (pH 7.6, 0.05 M), and revealed with 4-chloro-1-napthol. This chromogen shows a blue precipitate, easily distinguishable from the DAB brown product used for the detection of Som-28(1-12). Sections were mounted on gelatin-coated slides and cover-slipped with glycerol/PBS. The specificity of the ChAT immunoreactivity was confirmed by the following histological controls [12]: (1) first incubation bath: omission of the antibody, (2) PBS instead of the secondary biotinylated antibody and (3) a non-appropriated secondary antibody instead of the secondary biotinylated antibody. No immunoreactivity was found. The specificity of the Som-28(1-12) antiserum was previously checked [8]: (1) the first antiserum was preabsorbed with the synthetic peptide, (2) omission of the antibody in the first incubation bath, (3) PBS instead of the secondary biotinylated antibody and (4) the Som-28(1-12) antiserum was preabsorbed with an excess of related peptides (somatostatin-28, somatostatin-14, methionine-enkephalin, substance P, angiotensin II, cholecystokinin and neuropeptide Y). These controls confirmed the specificity of the immunoreactivity for Som-28(1-12) [8]. In addition, the distributions of the cholinergic [12] and somatostatinergic [8] systems in the alpaca brainstem were verified by comparison to the previously published distribution of each substance.

### *2.4. Mapping*

Frontal planes of the alpaca brainstem that were previously published were followed [6–9]. Atlases of non-camelid mammals and the brain atlas of *Lama glama* were also used [23,24]. For the nomenclature of the nuclei studied, previous published articles on the alpaca brainstem were used [6–9,12]. As the main references, the mapping of de Souza et al. [6–8] and Marcos and Coveñas [12] were used for both the location of the immunoreactivity and nomenclature. The distribution of cholinergic cell groups in other mammals was consulted [1–5,25–40]. To study the distribution of the immunoreactive neurons, the computerized digital mapping system Accustage MDPlot v5.2 (MD3-Digitizer, Accustage, Minnesota Datametrics, Saint Paul, MN, USA) was applied. A digital camera attached to a Nikon Eclipse 80i microscope was used to take photographs without any

further manipulation of them. Image files have been prepared using Canvas 11 Build 1173 software (Deneba ADC Systems of America, Seattle, WA, USA).

## 3. Results

### *3.1. Single Immunolabeling for Som-28(1-12) and ChAT*

The distribution of Som-28(1-12)- and ChAT-immunoreactive structures was studied according to the anatomical description performed by de Souza et al. [8] and Marcos and Coveñas [12]. Since the detailed mapping of the distribution of the immunoreactive structures containing Som-28(1-12) and ChAT in the alpaca brainstem has been performed previously in these two studies, only a brief description will be reported in the present work, more focused on the coexistence of ChAT and Som-28(1-12) in nerve cells. As in previous studies performed using a similar methodology, the brown precipitate (for Som-28(1-12)) and the blue staining (for ChAT) are easily distinguishable. Som-28(1-12)-immunoreactive profiles showed a typically peptidergic morphology, with Som-28(1-12)-positive cell bodies containing visible secretion granules and immunoreactive fibers of varicose appearance. In contrast, profiles containing ChAT showed no varicose labeling and a more homogeneous precipitate.

### *3.2. Colocalization of Som-28(1-12) and ChAT in Cell Bodies*

Comparing the distribution patterns of ChAT- and Som-28(1-12)-immunoreactive structures in the alpaca brainstem (Table 1), perikarya containing ChAT or Som-28(1-12) were detected in the nucleus of the solitary tract (Figure 1A–C), nucleus ambiguus (Figure 1D–F), dorsal motor nucleus of the vagus, the entire reticular formation (Figure 2A,B), laterodorsal tegmental nucleus (Ch6 cholinergic cell group) (Figure 2C,D), pedunculopontine tegmental nucleus (Ch5 cholinergic cell group) (Figure 2C,D) and parabigeminal nucleus (Ch8 cholinergic cell group) (Figure 2E). However, coexistence of the two neuroactive substances into the same cell bodies was only observed in isolated neurons located in the nucleus of the solitary tract (Figure 1A–C), nucleus ambiguus (Figure 1D–F) and reticular formation (Figure 2A,B). In the remaining regions, neuronal populations containing ChAT or Som-28(1-12) displayed separate distributions.

### *3.3. Fibrilar Immunolabeling*

Almost all the cholinergic regions of the alpaca brainstem contained numerous immunoreactive fibers for Som-28(1-12) (Table 1). This was especially noted in the nuclei of motor cranial nerves (Figures 2F and 3A) and associated regions (e.g., Edinger–Westphal nucleus) (Figure 3B). In some of these nuclei, a rich innervation of ChAT-immunoreactive perikarya and dendrites by fibers containing Som-28(1-12) was observed, such is the case of the nucleus ambiguus and medial division of the facial nucleus (Figure 3C,D). The opposite pattern (peptidergic neurons innervated by ChAT-immunoreactive fibers) was more difficult to observe.

**Table 1.** Distributions of immunoreactive profiles for Som-28(1-12), ChAT and double-labeled neurons detected in the alpaca brainstem. CB: cell body; F: fibers. For nomenclature of the brainstem nuclei, see the list of abbreviations.

| | Som-28(1-12) | | ChAT | | Double-Labeling |
|---|---|---|---|---|---|
| **NUCLEI** | **CB** | **F** | **CB** | **F** | |
| III | − | + | + | + | |
| IV | − | + | + | + | |
| 5M | − | + | + | + | |
| 5SL | − | + | − | − | |
| 5SP | − | + | − | − | |
| VI | − | − | + | + | |
| 7L | − | + | + | + | |
| 7M | − | + | + | + | |
| XII | − | + | + | + | |
| Amb | + | + | + | + | + |
| BC | − | + | − | − | |
| BCL | + | + | − | − | |
| BCM | + | + | − | − | |
| CAE | − | + | − | − | |
| Cu | − | + | − | − | |
| CX | − | + | − | − | |
| DMV | + | + | + | + | |
| DRM | − | + | − | − | |
| EW | − | + | + | + | |
| FRet Mesencephalon | + | + | + | + | + |
| FRet Medulla | + | + | + | + | + |
| FRet Pons | + | + | + | + | + |
| Gr | − | + | − | − | |
| IC | + | + | − | − | |
| IO | − | + | − | − | |
| IP | + | + | − | + | |
| LDT | + | + | + | + | |
| LRet | − | + | − | + | |
| MLF | − | + | − | − | |
| NR | − | + | − | − | |
| NTS | + | + | + | + | + |
| P | − | + | − | − | |
| PAG | + | + | − | + | |
| PBG | + | + | + | + | |
| Ped | − | + | − | − | |
| PG | − | + | − | − | |
| PGL | − | + | − | − | |
| PGM | − | + | − | − | |
| PH | + | + | − | − | |
| PPT | + | + | + | + | |
| s | − | + | − | − | |
| SC | + | + | − | + | |
| SNC | + | + | − | + | |
| SNR | − | + | − | + | |
| SO | − | + | − | − | |
| T | + | + | − | − | |
| TB | − | + | − | − | |
| TDC | − | + | − | − | |
| TDP | + | + | − | − | |
| TRC | − | + | − | − | |
| Ves | + | + | − | − | |

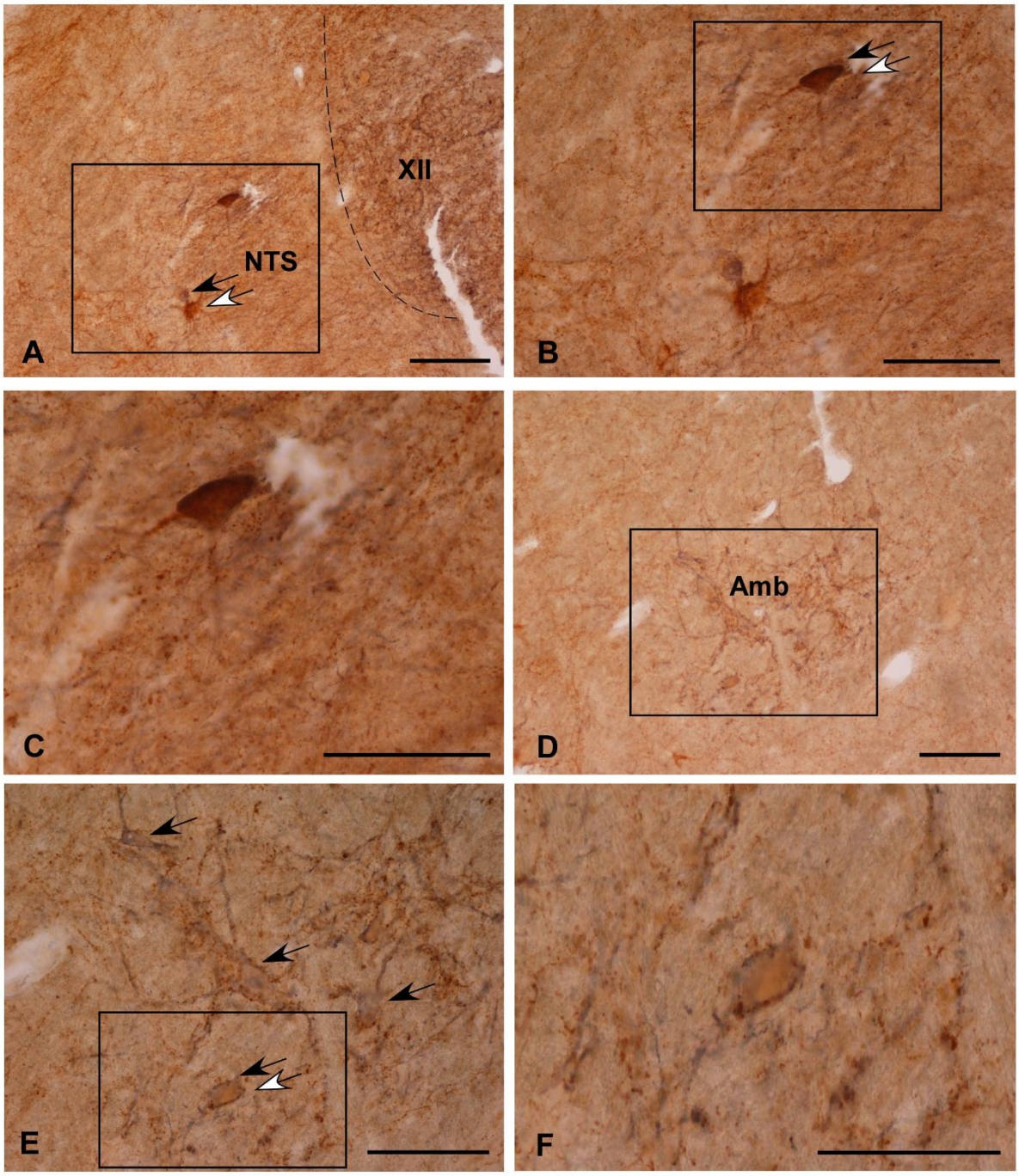


**Figure 1.** Pictures showing immunoreactivity for Som-28(1-12) and ChAT. (**A**) Image of the nucleus of the hypoglossal nerve (XII) and nucleus of the solitary tract (NTS). Abundant ChAT immunolabeling is present in XII, and in NTS, cell bodies containing ChAT (black arrow) and Som-28(1-12) (white arrow) were observed. (**B**) High-power image of the area squared in (**A**). A double-labeled neuron (white and black arrows) containing Som-28(1-12) and ChAT was detected. (**C**) Amplification of the double-immuno-stained neuron pointed out in (**B**). (**D**) Immunolabeling observed in the nucleus ambiguus. (**E**) High-power magnification of the region in the square in (**D**). Abundant immunoreactivity for ChAT was detected in this nucleus, in fibers and cell bodies (black arrows). Som-28(1-12) was detected in some neurons (white arrow), and in a high density of fibers. In some cases, these peptidergic fibers seemed to strongly innervate cell bodies containing ChAT. (**F**) Magnification of the double-labeled neuron in the square in (**E**), containing ChAT and Som-28(1-12). Scale bar = 100 μm in (**A**,**D**). Scale bar = 50 μm in (**B**,**C**,**E**,**F**).

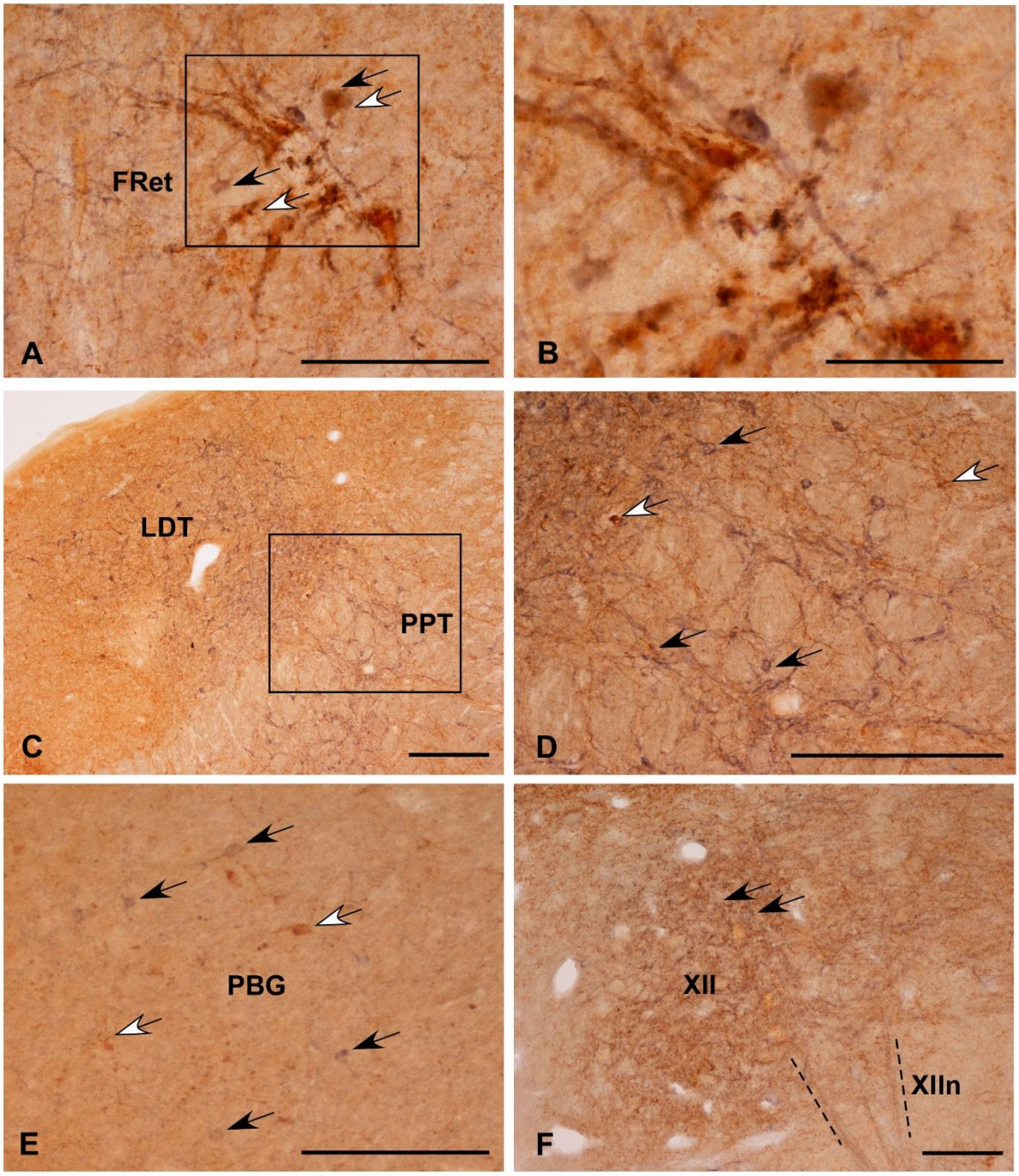


**Figure 2.** Images of Som-28(1-12)- and ChAT-immunoreactive structures in the alpaca brainstem. (**A**) Neurons positive for Som-28(1-12) (white arrows) and for ChAT (black arrows) as well as a double-labeled cell body were found in the reticular formation (FRet). (**B**) High-power magnification of the region in the square in (**A**). (**C**) Strong ChAT immunoreactivity in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT). (**D**) Amplification of the region in the square in (**C**). Numerous cholinergic cell bodies (black arrows) and scarce neurons containing Som-28(1-12) (white arrows) were found in these two regions. No double-labeled structures were detected. (**E**) Immunoreactivity in the parabigeminal nucleus (PBG). Peptidergic neurons (white arrows) and cell bodies containing ChAT (black arrows) were observed in separated populations in this nucleus. (**F**) Strong cholinergic fibers and cell bodies (black arrows) detected in the hypoglossal nucleus (XII). Hypoglossal nerve (XIIn), also containing ChAT, can be observed coming out from the nucleus (dashed lines). Scale bar in (**B**): 50 µm. Scale bar in (**A**,**C**–**F**): 100 µm.

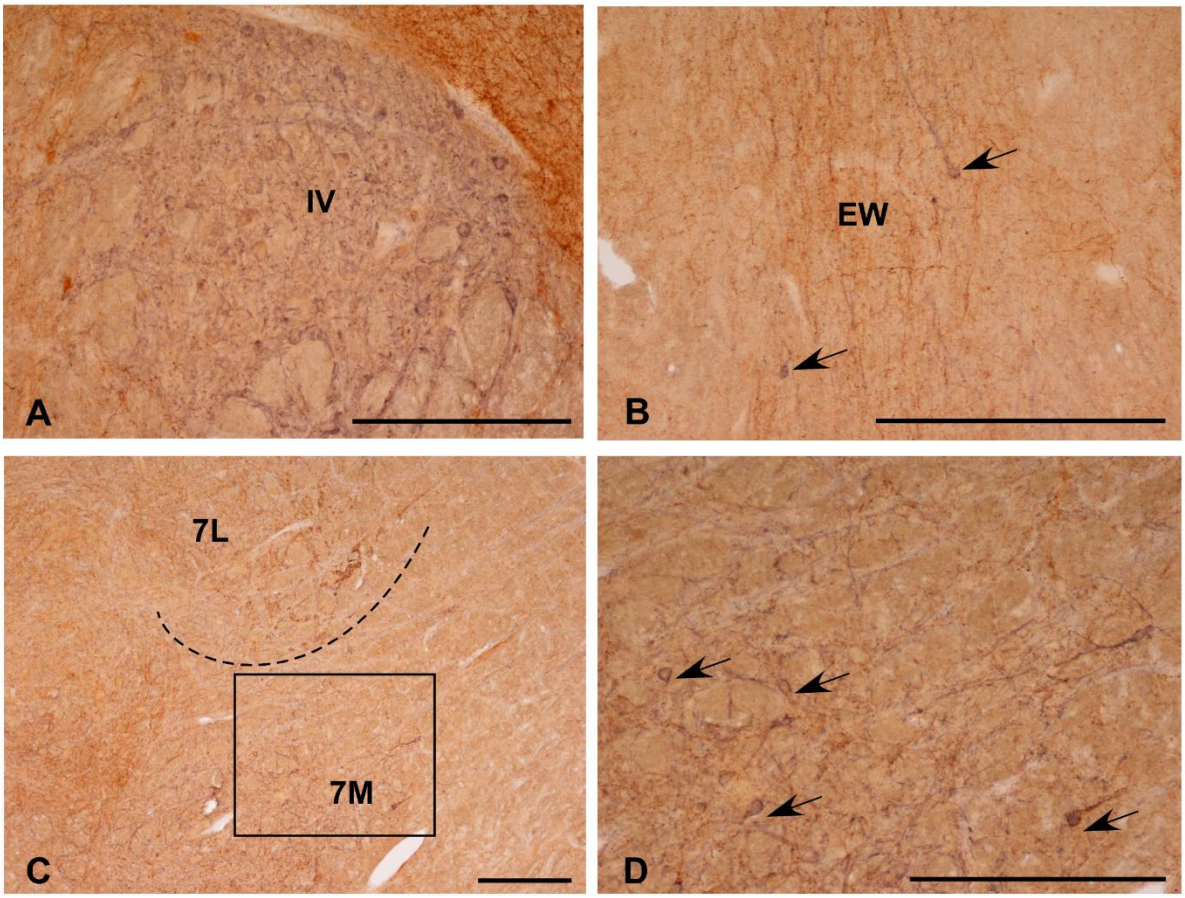


**Figure 3.** Pictures displaying immunoreactive profiles for Som-28(1-12) and ChAT in the alpaca brainstem. (**A**) Strong cholinergic immunostaining in the trochlear nucleus (IV). (**B**) Cell bodies containing ChAT (black arrows) detected in the Edinger–Westphal nucleus. (**C**) Immunoreactive profiles observed in the lateral (7 L) and medial (7 M) parts of the facial nucleus. (**D**) High-power magnification of the region in the square in (**C**). Abundant cholinergic immunoreactivity was detected in fibers and cell bodies (black arrows) of 7 M, and in some cases (lower right part of the picture), a strong peptidergic innervation on ChAT-immunoreactive neurons was observed. Scale bar: 100 µm.

## 4. Discussion

The distribution of immunoreactive profiles for ChAT and Som-28(1-12) is widespread in the alpaca brainstem. According to their respective distributions, these neuroactive substances could participate in the regulation of nociceptive, motor, autonomic, visual, auditory, cardiovascular or respiratory mechanisms [8,12]. The distribution of cholinergic cell bodies suggests that acetylcholine might be implicated, among others, in the regulation of rapid eye movement (REM) sleep and wakefulness as well as in motor functions, since ChAT was observed in the nuclei of several motor cranial nerves and in the pedunculopontine and laterodorsal tegmental nuclei [12]. Moreover, neurons containing Som-28(1-12) detected in the brainstem could be the main source for the somatostatinergic innervation of the alpaca diencephalic and other brainstem nuclei [8,10,11].

Although immunoreactive cell bodies for ChAT or Som-28(1-12) were observed in several regions of the alpaca brainstem, the presence of the two substances in the same cell bodies was only detected in the nucleus ambiguus, reticular formation and nucleus of the solitary tract, suggesting that the interaction between the cholinergic and the somatostatinergic systems in the same neurons is restricted in the alpaca brainstem. The relation between the two systems in the mammalian brain is well-known, mainly in memory processes due to the important role played by acetylcholine in this function: somatostatin stimulates the release of acetylcholine from cholinergic terminals [18,19], and somatostatin

administered intracerebrally improved memory failures in rodents showing brain cholinergic deficits [21]. In addition, a monosynaptic relationship between cholinergic forebrain neurons and somatostatin-containing axons has been described [41]. The results reported in the present study suggest that the intracellular interaction between acetylcholine and somatostatin could be mainly related with the regulation of cardiovascular, respiratory, gastrointestinal and gustatory systems [20,42]. This has been reported in other mammals [43] such as cats [20,42] and rodents [18,19,44,45]. A study performed in rats described the relationship between acetylcholine and several somatostatin molecules in the solitary tract complex (including the nucleus of the solitary tract and dorsal motor nucleus of the vagus) and reported that Som-28 and Som-14 could be considered as inhibitory neurotransmitters in the solitary tract complex, but neither Som-28(1-12) nor Som-28(1-10) hyperpolarized the same cells that showed Som-28- or Som-14-evoked hyperpolarization in this region [45]. This suggests that the different somatostatin fragments might exert different actions and that several somatostatin receptors could participate in these responses. It has been reported that cholinergic parasympathetic neurons in the dorsal motor nucleus of the vagus are immunoreactive for somatostatin receptor subtypes 2A, 2B, 4 and 5 [16]. The results obtained in the alpaca brainstem displayed neurons containing Som-28(1-12) and ChAT in the nucleus of the solitary tract but not in the dorsal motor nucleus of the vagus, although the two substances were detected in this later region in separated cellular populations. In cats, it has been suggested that the source of cholinergic elements for the nucleus of the solitary tract could arise from the dorsal motor nucleus of the vagus [42]. The methodology used in the present work does not allow to know whether the immunoreactive neurons detected in the alpaca brainstem are projection neurons or not. However, the results presented here suggest that the interactions between somatostatin and acetylcholine could be different in both regions, and that acetylcholine might be related to Som-28(1-12) in the nucleus of the solitary tract and/or to other somatostatin fragment(s) in the dorsal motor nucleus of the vagus. In other brain regions, different distribution patterns have been reported for distinct somatostatin fragments, at least in humans [43], and this could also be the case in alpaca.

The coexistence of somatostatin and acetylcholine in centers involved in autonomic regulation suggests that they could also be implicated in the regulation of the gastrointestinal tract. Esophageal afferents terminate in the nucleus of the solitary tract, which participates in deglution, eliciting the entire sequence of muscle activity. This nucleus projects to esophageal motoneurons located in the rostral portion of the nucleus ambiguus. It has been reported that this projection contains somatostatin, which can inhibit the neuronal firing in this pathway [19,46]. On the other hand, acetylcholine is present in motoneurons of the nucleus ambiguus that project to the esophagus and stimulates the contraction of the striated regions of the esophagus [46]. Somatostatin also participates in the generation of ambigual excitatory postsynaptic potential [19]. According to the results obtained in the brainstem of the alpaca, it can be suggested that acetylcholine and somatostatin might interact on neurons of the nucleus of the solitary tract and nucleus ambiguus, since both regions displayed double-labeled cells, and then could play a role in the central control of deglution and esophageal motility. In this sense, it has been reported that the somatostatinergic neurons of this circuit modulate viscerosensory signaling and provide a strong postsynaptic inhibition of this signal [44]. However, this regulatory function has been assigned to the somatostatinergic neurons containing GABA located in the nucleus of the solitary tract connected to neurons of the dorsal motor nucleus of the vagus that project to the antrum [44]. The direct stimulation of the latter nucleus increases phasic contractions and gastric tone, effects that are independent from changes in heart rate and blood pressure, which are also regulated by these regions of the brainstem [47,48]. The results obtained in the alpaca agree with these findings, since Som-28(1-12) has been detected in very few cholinergic neurons of the nucleus of the solitary tract and no colocalization has been observed in the dorsal motor nucleus of the vagus. Thus, although the interaction between both substances cannot be discarded, it seems more likely that the regulation of the gastrointestinal tract was mainly related to the somatostatin-GABAergic neurons and/or

other somatostatin fragments different from Som-28(1-12). According to the literature, the results presented here emphasize the complex regulatory mechanisms carried out in the alpaca by brainstem structures, since the same neuroactive substances detected in the same regions might exert different regulatory roles on distinct physiological functions. Further studies are needed to confirm whether these complex regulatory mechanisms are common to several species or whether they are specific adaptations of the alpacas to their habitat, since these animals can live at sea level and at altitudes of more than 5000 m. Life in these environments, together with the special morphological characteristics of the alpaca, make it a good candidate to study the brain morphological characteristics underlying these adaptations.

The results obtained in the present paper describe an abundant somatostatinergic innervation in the alpaca brainstem. In two regions, the nucleus ambiguus and the medial division of the facial nucleus, these peptidergic fibers are especially numerous surrounding the perikarya of the cholinergic neurons, suggesting that these cell bodies may be strongly innervated by somatostatinergic terminals. Supporting this interaction, the colocalization of somatostatin receptors 2A in cholinergic neurons in the nucleus ambiguus of rats has been reported [16], as well as the innervation of the vagal motoneurons of the nucleus ambiguus by somatostatin in cats [46]. Below the facial nucleus, a region displaying numerous peptidergic and cholinergic fibers in the alpaca brainstem, a small group of neurons called the retrotrapezoid nucleus, are activated by increases in $CO_2$ levels and regulate the breathing cycle [49]. These neurons are mainly glutamatergic and receive cholinergic inputs as well as somatostatinergic innervation from the pre-Bötzinger complex, and it has been reported that this could be a neuroanatomical substrate to interact with the chemosensory control of breathing [50,51]. This region is unknown in the alpaca brainstem, but the abundance of fibers containing Som-28(1-12) and ChAT as well as the possible somatostatinergic innervation on cholinergic cells detected in this area support the suggestion of a neuroanatomical basis for the chemosensory control of breathing, where somatostatin and acetylcholine might interact. Somatostatin has been involved in the chemosensory drive to breathe [49], and it should be clarified whether these results are again something common in several mammalian species or whether they are adaptive mechanisms of alpacas to their particular living conditions.

The opposite pattern (peptidergic neurons innervated by ChAT-immunoreactive fibers) was more difficult to observe in the alpaca brainstem. This was probably due to the appearance of the fibers containing the enzyme, that are usually harder to visualize near the Som-28(1-12)-immunoreactive cell bodies. The distinct appearance of cholinergic and peptidergic terminals has been reported in optical and electron microscopy [42], but the possibility of methodological aspects to this difficult visualization cannot be ruled out. It may be possible that the DAB precipitate prevents the correct visualization of cholinergic terminals when they are very thin, since the fibers containing ChAT of a larger diameter are easily visible.

The cholinergic projection from the laterodorsal tegmental nucleus links the forebrain limbic circuit with the limbic midbrain [17], and the presence of ChAT in the laterodorsal tegmental and pedunculopontine nuclei has been involved in the control of sleep, especially important in Artiodactyls to modulate the initiation of REM sleep, but not REM sleep maintenance [1]. It has been reported that putative sleep factors have hypnotic properties that could be related to changes in blood pressure, such as urotensin II [52], but this does not seem to be the case for Som-28(1-12) in the alpaca brainstem considering the results reported in the present study, since apparently the peptide was detected in a different population than acetylcholine. However, in the alpaca brainstem, the regulation of the initiation of REM sleep carried out by the cholinergic neurons of the laterodorsal tegmental nucleus seemed to be more related to CGRP, since double-labeled cholinergic neurons containing this peptide have been detected in this region [12]. Other regions such as the reticular formation and the nucleus ambiguus also displayed neurons double-labeled with CGRP and ChAT. It may be possible that some cholinergic neurons of the reticular formation

and/or the nucleus ambiguus containing Som-28(1-12) are also immunoreactive for CGRP, and thus a possible influence of the two peptidergic systems (somatostatin and CGRP) on cholinergic cell bodies can be suggested associated with the regulation of cardiovascular, digestive and respiratory functions, and this could be related to other regions of the brainstem. In this regard, the pedunculopontine tegmental nucleus modulates breathing by releasing acetylcholine into the retrotrapezoid nucleus in rats [49]. As discussed previously, in the alpaca, these latter regions showed immunoreactive profiles for ChAT, CGRP and Som-28(1-12), suggesting the involvement of these neuroactive substances in the regulation of the breathing homeostasis. The deep knowledge of the morphological basis underlying these mechanisms may potentially have a therapeutic use for the treatment of respiratory control problems, especially those associated with disorders of breathing during sleep [49]. In this regard, the morphological study of the alpaca brainstem can be very useful for the comprehension of cardiorespiratory control mechanisms, given the anatomophysiological peculiarities of these animals.

## 5. Conclusions

Although the distributions of Som-28(1-12) and ChAT in the alpaca brainstem are similar, the colocalization of both substances in the same cell bodies is very scarce, suggesting a very limited interaction at the intracellular level. However, the abundant somatostatinergic innervation detected in some regions containing cholinergic cells points to a possible regulation of these neurons by the peptide, which may be related to respiratory control. Nevertheless, results obtained in previous studies suggest that the interaction between ChAT and CGRP would be more important than the interaction between ChAT and somatostatin in the alpaca brainstem, although ChAT, CGRP and somatostatin could be involved in the regulation of the sleep cycle. The detailed knowledge of these mechanisms may contribute to the development of therapies related to respiratory problems, especially those related to breathing disorders affecting some patients during sleep.

**Author Contributions:** Conceptualization, P.M. and R.C.; methodology, P.M. and R.C.; software, P.M. and R.C.; validation, P.M. and R.C.; formal analysis, P.M. and R.C.; investigation, P.M. and R.C.; resources, P.M. and R.C.; data curation, P.M. and R.C.; writing—original draft preparation, P.M and R.C.; writing—review and editing, P.M. and R.C.; visualization, P.M. and R.C.; supervision, P.M and R.C.; project administration, P.M. and R.C.; funding acquisition, P.M. and R.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Experimental design, procedures and protocols were carried out under the guidelines of the legal and ethics recommendations of the Spanish legislation. Ethical review and approval were waived for this study due to the use of histological sections from a previous study.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| III | nucleus of oculomotor nerve (III cranial nerve) |
| IV | nucleus of the trochlear nerve (IV cranial nerve) |
| 5M | motor trigeminal nucleus |
| 5SL | laminar spinal trigeminal nucleus |
| 5SP | spinal trigeminal nucleus |
| VI | abducens nerve (VI cranial nerve) |

| | |
|---|---|
| 7L | facial nucleus, lateral division |
| 7M | facial nucleus, medial division |
| XII | nucleus of the hypoglossal nerve (XII cranial nerve) |
| Amb | nucleus ambiguus |
| BC | brachium conjunctivum |
| BCL | marginal nucleus of the brachium conjunctivum, lateral division |
| BCM | marginal nucleus of the brachium conjunctivum, medial division |
| CAE | locus coeruleus |
| ChAT | choline acetyl transferase |
| Cu | cuneate nucleus |
| CX | external cuneate nucleus |
| DMV | dorsal motor nucleus of the vagus nerve |
| DRN | dorsal raphe nucleus |
| EW | nucleus of Edinger–Westphal |
| FRet | reticular formation |
| Gr | gracile nucleus |
| IC | inferior colliculus |
| IO | inferior olive |
| IP | interpeduncular nucleus |
| LDT | laterodorsal tegmental nucleus (Ch6 cholinergic cell group) |
| LRet | lateral reticular nucleus |
| MLF | medial longitudinal fascicle |
| NR | red nucleus |
| NTS | nucleus of the solitary tract |
| P | pyramidal tract |
| PAG | periaqueductal gray |
| PBG | parabigeminal nucleus (Ch8 cholinergic cell group) |
| Ped | cerebral peduncle |
| PG | pontine gray |
| PGL | pontine gray, lateral division |
| PGM | pontine gray, medial division |
| PH | nucleus praepositus hypoglossi |
| PPT | pedunculopontine tegmental nucleus (Ch5 cholinergic cell group) |
| S | solitary tract |
| SC | superior colliculus |
| SNC | substantia nigra, pars compacta |
| SNR | substantia nigra, pars reticulata |
| SO | superior colliculus |
| Som-28(1-12) | somatostatin-28 (1-12) |
| T | nucleus of the trapezoid body |
| TB | trapezoid body |
| TDC | dorsal tegmental nucleus, central division |
| TDP | dorsal tegmental nucleus, pericentral division |
| TRC | tegmental reticular nucleus, central division |
| Ves | vestibular nucleus |

## References

1. Davimes, J.G.; Alagaili, A.N.; Bennett, N.C.; Mohammed, O.B.; Bhagwandin, A.; Manger, P.R.; Gravett, N. Neurochemical organization and morphology of the sleep related nuclei in the brain of the Arabian oryx, *Oryx leucoryx*. *J. Chem. Neuroanat.* **2017**, *81*, 53–70. [CrossRef] [PubMed]
2. Dell, L.A.; Karlsson, K.A.; Patzke, N.; Spocter, M.A.; Siegel, J.M.; Manger, P.R. Organization of the sleep-related neural systems in the brain of the minke whale (*Balaenoptera acutorostrata*). *J. Comp. Neurol.* **2015**, *524*, 2018–2035. [CrossRef] [PubMed]

3. Dell, L.A.; Patzke, N.; Spocter, M.A.; Bertelsen, M.F.; Siegel, J.M.; Manger, P.R. Organization of the sleep-related neural systems in the brain of the river hippopotamus (*Hippopotamus amphibius*): A most inusual Certiodactyl species. *J. Comp. Neurol.* **2015**, *524*, 2036–2058. [CrossRef] [PubMed]
4. Dell, L.A.; Patzke, N.; Spocter, M.A.; Siegel, J.M.; Manger, P.R. Organization of the sleep-related neural systems in the brain of the harbour porpoise (*Phocoena phocoena*). *J. Comp. Neurol.* **2016**, *524*, 1999–2017. [CrossRef] [PubMed]
5. Malungo, I.B.; Gravett, N.; Bhagwandin, A.; Davimes, J.G.; Manger, P.R. A preliminary description of the sleep-related neural systems in the brain of the blue wildebeest, *Connochaetes taurinus*. *Anat. Rec.* **2020**, *303*, 1977–1997. [CrossRef]
6. De Souza, E.; Yi, P.; Aguilar, L.A.; Coveñas, R.; Lerma, L.; Andrade, R.; Mangas, A.; Narváez, J.A. Mapping of leucine-encephalin in the alpaca (*Lama pacos*) brainstem. In *Focus on Neuropeptide Research*; Coveñas, R., Mangas, A., Narváez, J.A., Eds.; Transworld Research Network: Trivandrum, India, 2007; pp. 103–113.
7. De Souza, E.; Coveñas, R.; Yi, P.; Aguilar, L.A.; Lerma, L.; Andrade, R.; Mangas, A.; Díaz-Cabiale, Z.; Narváez, J.A. Mapping of CGRP in the alpaca (*Lama pacos*) brainstem. *J. Chem. Neuroanat.* **2008**, *35*, 346–355. [CrossRef]
8. De Souza, E.; Sánchez, M.L.; Aguilar, L.A.; Díaz-Cabiale, Z.; Narváez, J.A.; Coveñas, R. Mapping of Somatostatin-28(1-12) in the alpaca (*Lama pacos*) brainstem. *Microsc. Res. Tech.* **2015**, *78*, 363–374. [CrossRef]
9. Marcos, P.; Arroyo-Jiménez, M.M.; Lozano, G.; Aguilar, L.A.; Coveñas, R. Mapping of tyrosine hydroxylase in the alpaca (*Lama pacos*) brainstem and colocalization with CGRP. *J. Chem. Neuroanat.* **2011**, *41*, 63–72. [CrossRef]
10. Coveñas, R.; Mangas, A.; Medina, L.E.; Sánchez, M.L.; Aguilar, L.A.; Díaz-Cabiale, Z.; Narváez, J.A. Mapping of somatostatin-28 (1-12) in the alpaca diencephalon. *J. Chem. Neuroanat.* **2011**, *42*, 89–98. [CrossRef]
11. Marcos, P.; Arroyo-Jiménez, M.M.; Lozano, G.; González-Fuentes, J.; Lagartos-Donate, M.J.; Aguilar, L.A.; Coveñas, R. Mapping of tyrosine hydroxylase in the diencephalon of alpaca (*Lama pacos*) and co-distribution with somatostatin-28 (1-12). *J. Chem. Neuroanat.* **2013**, *50–51*, 66–74. [CrossRef]
12. Marcos, P.; Coveñas, R. Immunohistochemical study of the brainstem cholinergic system in the alpaca (*Lama pacos*) and colocalization with CGRP. *Eur. J. Histochem.* **2021**, *65*, 3266. [CrossRef] [PubMed]
13. Manger, P.R. Establishing order at the systems level in mammalian brain evolution. *Brain Res. Bull.* **2005**, *66*, 282–289. [CrossRef] [PubMed]
14. Reichlin, S. Somatostatin. In *Brain Peptides*; Krieger, D.T., Browstein, M.J., Martin, J.B., Eds.; John Wiley and Sons: New York, NY, USA, 1983; pp. 711–752.
15. Benoit, R.; Ling, N.; Bakhit, C.; Morrison, J.H.; Alford, B.; Guillemin, R. Somatostatin-28(1-12)-like immunoreactivity in the rat. *Endocrinology* **1982**, *111*, 2149–2151. [CrossRef] [PubMed]
16. Spary, E.J.; Maqbool, A.; Batten, T.F.C. Expression and localization of somatostatin receptor subtypes sst1-sst5 in areas of the rat medulla oblongata involved in autonomic regulation. *J. Chem. Neuroanat.* **2008**, *35*, 49–66. [CrossRef] [PubMed]
17. Sutin, E.L.; Jacobowitz, D.M. Immunocytochemical localization of peptides and other neurochemical in the rat laterodorsal tegmental nucleus and adjacent area. *J. Comp. Neurol.* **1988**, *270*, 243–270. [CrossRef]
18. Wang, Y.T.; Neuman, R.S.; Bieger, D. Somatostatin inhibits nicotinic cholinoceptor mediated-excitation in rat ambigual motoneurons in vitro. *Neurosci. Lett.* **1991**, *123*, 236–239. [CrossRef]
19. Wang, Y.T.; Zhang, M.; Neuman, R.S.; Bieger, D. Somatostatin regulates excitatory amino acid receptor-mediated fast excitatory postsynaptic potential components in vagal motoneurons. *Neuroscience* **1993**, *53*, 7–9. [CrossRef]
20. Batten, T.F.C. Immunolocalization of putative neurotransmitters innervating autonomic regulating neurons of cat ventral medulla. *Brain Res. Bull.* **1995**, *37*, 487–506. [CrossRef]
21. Matsuoka, N.; Yamazaki, M.; Yamaguchi, I. Changes in brain somatostatin in memory-deficient rats: Comparison with cholinergic markers. *Neuroscience* **1995**, *66*, 617–626. [CrossRef]
22. Marcos, P.; Corio, M.; Dubourg, P.; Tramu, G. Reciprocal synaptic connections between neurotensin- and tyrosine hydroxylase-immunoreactive neurons in the mediobasal hypothalamus of the guinea pig. *Brain. Res.* **1996**, *715*, 63–70. [CrossRef]
23. Jasper, A.L.; Ajmone-Marsan, C. *A Stereotaxic Atlas of the Diencephalon of the Cat*; National Research Council of Canada: Ottawa, ON, Canada, 1966.
24. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*; Academic Press: San Diego, CA, USA, 1998.
25. Badlangana, N.L.; Bhagwandin, A.; Fuxe, K.; Manger, P.R. Observations on the giraffe central nervous system related to the corticospinal tract, motor cortex and spinal cord: What difference does a long neck make? *Neuroscience* **2007**, *148*, 522–534. [CrossRef] [PubMed]
26. Bux, F.; Bhagwandin, A.; Fuxe, K.; Manger, P.R. Organization of cholinergic, putative catecholaminergic and serotonergic nuclei in the diencephalon, midbrain and pons of sub-adult male giraffes. *J. Chem. Neuroanat.* **2010**, *39*, 189–203. [CrossRef] [PubMed]
27. Dell, L.A.; Gruger, J.L.; Bhagwandin, A.; Jillani, N.E.; Pettigrew, J.D.; Manger, P.R. Nuclear organization of cholinergic, putative catecholaminergic, and serotonergic systems in the brains of two megachiropteran species. *J. Chem. Neuroanat.* **2010**, *40*, 177–195. [CrossRef] [PubMed]
28. Houser, C.R.; Crawford, G.D.; Barber, R.P.; Salvaterra, P.M.; Vaughn, J.E. Organization and morphological characteristics of cholinergic neurons: An immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* **1983**, *266*, 97–119. [CrossRef]
29. Kimura, H.; Maeda, T. Aminergic and cholinergic systems in the dorsolateral pontine tegmentum. *Brain Res. Bull.* **1982**, *9*, 493–499. [CrossRef]

30. Levey, A.I.; Wainer, B.H.; Mufson, E.J.; Mesulam, M.M. Co-localization of acetylcholinesterase and choline acetyltransferase in the rat cerebrum. *Neuroscience* **1983**, *9*, 9–22. [CrossRef]
31. Manaye, K.F.; Zweig, R.; Wu, D.; Hersh, L.B.; de Lacalle, S.; Saper, C.B.; German, D.C. Quantification of cholinergic and select non-cholinergic mesopontine neuronal populations in the human brain. *Neuroscience* **1999**, *89*, 759–770. [CrossRef]
32. Marcos, P.; Coveñas, R. Neuroanatomical relationship between the cholinergic and tachykininergic systems in the adult human brainstem: An immunohistochemical study. *J. Chem. Neuroanat.* **2019**, *102*, 101701. [CrossRef]
33. Mesulam, M.M. Cholinergic pathways and the ascending reticular activating system of the human brain. *Ann. N. Y. Acad. Sci.* **1995**, *757*, 169–179. [CrossRef]
34. Motts, S.D.; Slusarczyk, A.S.; Sowick, C.S.; Schofield, B.R. Distribution of cholinergic cells in guinea pig brainstem. *Neuroscience* **2008**, *154*, 186–196. [CrossRef]
35. Oda, Y. Choline acetyltransferase: The structure, distribution and pathologic changes in the central nervous system. *Pathol. Int.* **1999**, *49*, 921–937. [CrossRef]
36. Sakai, K.; Luppi, P.H.; Salvert, D.; Kimura, H.; Maeda, T.; Jouvet, M. Localization of cholinergic neurons in the cat lower brainstem. *CR Acad. Sci. III* **1986**, *303*, 317–324.
37. Tago, H.; McGeer, P.L.; McGeer, E.G.; Akiyama, H.; Hersh, L.B. Distribution of choline acetyltransferase immunopositive structures in the rat brainstem. *Brain Res.* **1989**, *495*, 271–297. [CrossRef]
38. Yasuhara, O.; Aimi, Y.; Matsuo, A.; Kimura, H. Distribution of a splice variant of choline acetyltransferase in the trigeminal ganglion and brainstem of the rat: Comparison with calcitonin gene-related peptide and substance P. *J. Comp. Neurol.* **2008**, *509*, 436–448. [CrossRef] [PubMed]
39. Mesulam, M.M.; Geula, C.; Bothwell, M.A.; Hersh, L.B. Human reticular formation: Cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei and some cytochemical comparisons to forebrain cholinergic neurons. *J. Comp. Neurol.* **1989**, *283*, 611–633. [CrossRef] [PubMed]
40. Ichikawa, T.; Ajiki, K.; Matsuura, J.; Misawa, H. Localization of two cholinergic markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: In situ hybridization histochemistry and immunohistochemistry. *J. Chem. Neuroanat.* **1997**, *13*, 23–39. [CrossRef]
41. Zaborsky, L. Afferent connections of the forebrain cholinergic projection neurons, with special reference to monoaminergic and peptidergic fibers. *Experientia* **1989**, *57*, 12–32. [CrossRef]
42. Maley, B.E. Immunohistochemical localization of neuropeptides and neurotransmitters in the nucleus solitarius. *Chem. Senses* **1996**, *21*, 367–376. [CrossRef]
43. Cebada-Sánchez, S.; Insausti, R.; González-Fuentes, J.; Arroyo-Jiménez, M.M.; Rivas-Infante, E.; Lagartos, M.J.; Martínez-Ruiz, J.; Lozano, G.; Marcos, P. Distribution of peptidergic populations in the human dentate gyrus (Somatostatin [SOM-28, SOM-12] and neuropeptide Y [NPY]) during postnatal development. *Cell Tissue Res.* **2014**, *358*, 25–41. [CrossRef]
44. Gillis, R.A.; Dezfuli, G.; Bellusci, L.; Vicini, S.; Sahibzada, N. Brainstem neuronal circuitries controlling gastric tonic and phasic contractions: A review. *Cell. Mol. Neurobiol.* **2022**, *42*, 333–360. [CrossRef]
45. Jacquin, T.; Champagnat, J.; Madamba, S.; Denavit-Saubié, M.; Siggins, G.R. Somatostatin depresses excitability in neurons of the solitary tract complex through hyperpolarization and augmentation of $I_M$, a non-inactivating voltage-dependent outward current blocked by muscarinic agonists. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 948–952. [CrossRef] [PubMed]
46. Cunningham, E.T.; Sawchenko, P.E. Central neural control of esophageal motility: A review. *Disphagia* **1990**, *5*, 35–51. [CrossRef] [PubMed]
47. Pagani, F.D.; Norman, W.P.; Gillis, R.A. Medullary parasympathetic projections innervate specific sites in the feline stomach. *Gastroenterology* **1988**, *95*, 277–288. [CrossRef]
48. Norman, W.P.; Pagani, F.D.; Ormsbee, H.S., III; Kasbekar, D.K.; Gillis, R.A. Use of horseradish peroxidase to identify hindbrain sites that influence gastric motility in the cat. *Gastroenterology* **1985**, *88*, 701–705. [CrossRef]
49. Moreira, T.S.; Sobrinho, C.R.; Falquetto, B.; Oliveira, L.M.; Lima, J.D.; Mulkey, D.K.; Takakura, A.C. The retrotrapezoid nucleus and the neuromodulation of breathing. *J. Neurophysiol.* **2021**, *125*, 699–719. [CrossRef]
50. Tan, W.; Pagliardini, S.; Yang, P.; Janczewski, W.A.; Feldman, J.A. Projections of preBötzinger complex neurons in adult rats. *J. Comp. Neurol.* **2010**, *518*, 1862–1878. [CrossRef]
51. Yang, C.F.; Feldman, J.L. Efferent projections of excitatory and inhibitory preBötzinger Complex neurons. *J. Comp. Neurol.* **2018**, *526*, 1389–1402. [CrossRef]
52. de Lecea, L.; Bourgin, P. Neuropeptide interactions and REM sleep: A role for urotensin II? *Peptides* **2008**, *29*, 845–851. [CrossRef]