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Review Article

On the Colorimetry of Neuropeptide Y Hypothalamus and Ligamentous Parts in Male and Female Deer: Immunohistochemical Analysis Report- May 2021

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Abstract

It is since 1992 that this research began after reading numerous articles which placed Neuropeptide Y (NPY) antibody in the regulation of calcium and stomach function. Research in posturology and osteopathy carried out since 1988 highlights the oral afferents in the postural balance of verticalization and hominization.

In parallel, paleontological research, on the ventro-caudal concavity flexion of the sphenoid, by Pr Anne Dambricourt, CNRS and the study on the evolution of the neurocranium by Pr Yves Lignereux of the Toulouse natural history museum, have allowed this research to position itself on the anatomical and embryological level.

Researchers such as Gulgun Sengul and Charles Watson took up the work of Dr.Yew Chan, et al., in 1999 showed the relevance of NPY, at the high dorsals level, up to the 12th week of gestation in humans.

In their work on the spine, Lumbar Spine Discover, the authors Aspden and Porter showed that this ligament relevance would be verifiable on the whole of the supraspinous ligament up to the lumbar vertebrae, which was further studied in the 2016.

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Keywords

Neuropeptides; Neuropetide Sequence; Deer; White Fats

Introduction

The NPY is also described in the human CSF at the level of lumbar disc pain and then corresponds to an informational, inflammatory process in the sense of the healing process, which is summarized by H Von Holst, et al., [1-3].

This is included in the chapter:

It is within the dental joint and especially the periodontal ligament that Dr P Bouchard in his book, "Parondontologie et implant Dentaire", Ed: Lavoisier Médecine, shows the presence of NPY on this ligament level. It would have as such the stomach functions on the muscle of the stomach by the recapture of potassium.

So, the NPY participates in the stimulation of the appetite by regulating satiety and stimulating digestive, salivary and stomach functions. It is a powerful requirement, which is summarized by H Von Holst, et al., [2,4].

This is included in the chapter:

It is within the dental joint and especially the periodontal ligament that Dr. P Bouchard in his book, "Parondontologie et implant Dentaire", Lavoisier Médecine, shows the presence of NPY on this ligament level, it would have as such the stomach functions on the muscle of the stomach by recapture of potassium.

So the NPY participates, thus in the stimulation of the appetite, regulates satiety and participates in the stimulation of the digestive, salivary and stomach functions. It is a powerful requirement, because it increases the rate of insulin and consequently the storage, lipido-glucidic, in the adipose cells, especially in the so-called white fats [5].

This increase becomes the hypothalamic-pituitary feedback, which decreases the influence of NPY on digestion. Its production leads to the increase of leptin in the blood, by said fatty tissue, which leads to the feeling of satiety.

NPY with its chain of 36 amino acids, would act on digestive stimulation by slowing down the sympathetic information of stomach functions and therefore satiety, and/or by stimulating the noradrenergic information of digestibility [6].

It is especially its hypotensive natriuretic activity on the muscularis of the stomach, which positions the NPY in the neuropeptides with digestive prevalence.

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Researchers from the “Laboratory of Neuroscience and Behavioral Studies at Rockefeller University” in New York, under the signature of the team of Laura B Duval, have shown that NPY could strongly modify the behaviour of the female mosquito, which bites before laying eggs, by similarly increasing its satiety as well as its aggressive behaviour and the transfer of various infections.

NPY is also a vasoconstrictor in the skin, and becomes a thermo regulator which acts on the contraction of striated type muscles and a stimulant on the vascularization of smooth muscle capillaries, thereby becoming a vector for stimulating lymph node secretions, especially in the digestive system.

The action of NPY on the Ca++ chain is also highlighted by Dr Isabelle Castan, PhD, temporary teaching and research associate, by Dr Philippe Valet, PhD, Lecturer at Paul-Sabatier University and by Prof. Max Lafontan, Research Director at INSERM on Adrenergic Regulation and Metabolic Adaptations, INSERM U317, Louis-Bugnard Institute, CHU Rangueil, Toulouse [7-11].

The work of Pr Remy Quirion, Chair of Psychiatry and Researcher Emeritus at the Douglas Institute and since 2011, Chief Scientist of the Government of Quebec, has been 1986 on calcitonin and NPY which have, among other things, made it possible to understand cholinergic interactions of the brain, with the NPY, in Alzheimer's disease and its influence on depression and memory stability, this on more than 750 articles.

The links that Dr Yvan Dumont, and Pr Rémi Quirion discover between NPY and calcitonin, by the same CGRP gene are yet another complementary axis in this coherence of research.

The research does not forget the studies of Professor Gerard Karsenty, of the Medical Center of Columbia University, New York, in: September 26, 2013, edition of “the journal cell”, resumed in August 2018 “on the development of the adolescent brain”, showed the influences of calcitonin and calcium on the brain, in depression, learning and memory.

The sequence of the NPY of the alligator is identical to that of man, while that of sheep is distinguished by two variants of amino acid.

Beyond these details, the relations between these peptides noted above, and their actions are preserved, and the same physiological conclusions remain valid, according to their finding.

It is on this basis of reflection that the sequencing of the NPY was orchestrated with the spectrography laboratory of the Monnot Institute, with Dr Thibault Leger's PhD research.

After trypsin digestion, for spectrographic analysis, the masses of the sequence differences appear constant at 3 distinct positions [12].

The 3 sequences of the same weight are also in the same place, except for the rat

- SSPETLISDLLMR in position 68-80

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- HYINLITR in position 54-61
- YYSALR in position 48-53

The SSPETLISDLLMR and HYINLITR sequences are 100% blasted in all recorded mammalian cases and 92% in some bats, hamsters, and white mice.

Embryologically the development of cardiac, stomach and mandibular buds originate from the same place at the level of the chord during neurulation.

The Meckel cartilage of the 1st branchial arch, which is the constituent base of the mandible, will also make the hammer and the incus of the inner ear, the stirrup being issued from the 2nd branchial arch, with the hyoid bone.

Associated with research work on the presence of NPY, at the level of the spinal ligament and periodontal system, research began to create links during dissections carried out at the René Descartes University of Paris, under the direction of Pr Delmas, responsible for the anatomy pole, for the preparation of my DU in clinical anatomy.

The large intraspinal yellow ligament widens at the level of the metamer of the 6th thoracic that of the stomach, and this is for anatomists to dorsal kyphosis. The link could also be chemical to NPY.

Research in osteopathy and on tensegrity in biology led us to meet Pr Anick Abourachide, from the CNRS of the Natural History Museum of Paris [13,14].

The latter uses these proposals on the consistency of the curvatures of the withers in the balance of the spine, and this according to the calculation of Euler's law, on the Resistance of materials (Rm), equal to the square of the Curvatures (Cb^2)+ 1, that is Rm= Cb^2+1.

After having studied 214 skeletons in the reserves of the Museum, to study the angle of the spines of the withers of deer in relation to the weight of the antlers, it is with Pr A. Abourachid that we practiced the dissection of the daguet.

This after the having scanned, at Dr. Stéphanie Walczak of the EIFFELVET veterinary clinic in Paris 15° and our dissection allows us to consider a spinal model, to highlight the importance of the nuqual ligament and its extension on the supraspinous ligament.

This is how the choice of this species is imposed and is mainly linked to the fact that their woods are deciduous and seasonal. As previously presented, their compositions are mainly calcium even if their density is the lightest.

Dr Wang's work identified the DNA sequence of timber thumb. The study of this species has led us around the world to study the different behaviors, in Canada, Europe and Asia.

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Through the deer species Hydropote of a monotypic genus belonging to the deer family, only member is the hydropote (Hydropotes inermis), and native to Asia which has saber canines instead of antlers. This would not be a paleontological vestige, but rather a species regression.

It is rather the sexual behaviour of this species, which allowed us to put forward a hypothesis, that the anthers would be a diffusion of pheromones.

The hydropot deer, Chinese deer that can also be seen, in an almost wild state, in the zoological reserve of Haute Touche 36260 Obterre, in France using its frontal area to spread this information on tree trunks (Fig. 1). This informations are in captivity By F. Feer Laboratory of General Ecology of the Museum, Brunoy, France Reception of Ms. 5. 10. 1981.

This behaviour allowed us to consider that the fight between two red deer is a way to spread these pheromones and that this dance is a hierarchy of competence and not of power.

This analysis is currently being studied with Dr Loïc Costeur of the Basel Natural History Museum, a specialist in deer.

Figure 1: The hydropot deer.

This discernment between a battle of hierarchy of competence and the anthropomorphic analysis of a hierarchy of power and domination is specific to a reading of human prevalence in biology.

Moreover, it is highlighted in zoopharmacognosy by the studies of Pr DV Sabrina Krief primatologist, a researcher at the CNRS of the MNHN of Paris that the non-intellectual intelligence of animals defines a horizontal consciousness of sharing, where we reason in pyramidal and vertical needs [15].

Our study on red deer antlers included the June 2020 article takes up the relationship between calcitonin and NPY.

Current research is mainly around the stomach and intestinal receptors for lipid disorders in obese people and insulin disorders. However, the role of the healing of the NPY and on the aroused calcitonin directed us towards a search for its presence in the ligament tissue.

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In the "Quantitative Proteomic Analysis of Human Tendon and Ligament"; Division of Disease Proteomics, Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan; Poster No. 2336 • ORS 2012 Annual Meeting.

In a 1st step:

Samples were taken by the FICIF of Rambouillet on male and female red deer and put in paraffin at the Biopôle of the Maison Alfort veterinary school in the capacity of Hélène Huet PhD in immunofluorescence research.

These samples from 1 male, around 5 years old, and 1 female, around 3 years old, allow us to compare the presence of neuropeptides Y and compare anatomical parts of the two sexes [16].

Since the age can only be done to the border of the dentin, an estimate specific to the hunting habits and the counting directives that the FICIF makes each year is defined.

The anatomical samples were taken by Mr Olivier Marcand responsible for the FICIF on:

1. Desmodondal ligament, take a molar from the half mandible
2. Nuchal ligament, at the level of the 4° cervical
3. Supraspinatus ligament at top of withers.
4. The suspensory ligament of the fetlock of the forehand is the ligament close to the bone and not under the skin
5. The rope of the hock, on the hindquarters

This set is transported to the Biopôle on the day of collection in tubes provided by the laboratory and in a 10% ether preparation (Fig. 2).

Figure 2: Red deer.

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In a 2nd step:

To validate the rabbit NPY polyclonal antibody, (LS-C184009-50), from the "CliniScience laboratory in Nanterre", research had to confirm its validity by dissecting two heads of male deer, from the breeding of Mr Becquet at the "Villaine farm", under the supervision of Dr Veterinary Guillaume Brachet.

This dissection is done at the "slaughterhouses of Craons, in France" under the veterinary supervision of Dr Veterinaty Fabienne Wery, to extract the hypothalamus and check the colourimetry of the said antibody.

This verification is being validated by our research to date, we are studying the other ligaments. This allows us to consider the study of NPY according to other scientific paradigms.

The ligament axis allows us to understand spinal reactions and decalcification processes in inflammatory disorders, learning and memorization processes in adolescents and gerontology.

The importance of Ca++ in muscle contraction and its recovery process in athletes and space research are all areas suggesting that we continue to research.

All the anatomical samples must be cut with the scalpels provided, having previously removed as much blood as possible, using physiological serum, then placed in the blue tubes, with surgical gloves provided and covered with physiological serum [17].

The tubes were labelled with a number and the following Table 1 shows the designation of the said sample.

- The animals come from the "Villaine farm, 49150 Baugé in Anjou", the owner Mr François Becquet, raises more than 100 animals
- The slaughter and the removal of the heads are done under the control and the signature of the Veterinary Doctor, Guillaume Brachet, veterinarian of the Villaine farm
- The dissection is made under acceptance, by Fabienne WERY, Official Veterinarian, DDCSPP of Mayenne, Food Quality and Safety Department and the director of "the municipal slaughterhouse of Craon"
- Species: Red deer,
- Gender of animals: MALE
- The estimated age of the 2 specimens: is between 3 to 5 years
- Date of collection: 23/11/20

The Anatomical Area Studied

The postmortem hypothalamus was performed under this control at the Craon slaughterhouse.

Situation caudal and medial to the lateral ventricles, behind and slightly cranial to the sella turcica and to the pituitary, itself medio-sphenoidal.

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The anatomical approach is based on a comparative anatomy protocol specific to mammalian vertebrates.

The bibliographic bases are then human, bovine and equine.

The objective constants are:

Neuropeptide Y is found and analyzed in the hypothalamus in all of the studies described above the brain of vertebrates has a constant on the ventricles, with lateral ventricles that are discovered by the anterior route with the rhinencephalon area, therefore olfactory.

The latter extends posteriorly caudally and laterally towards the rostral anterior horn of the lateral ventricles.

These join ventrally and, in the centre, to form the 3rd ventricle, which itself extends dorsally, in its caudal part to join the cerebellar ventral part, realizing the 4th ventricle and this by the aqueduct of Sylvius, caudal to the 3rd ventricle.

We also describe the temporal horns of the lateral ventricles which make it possible to locate and differentiate the underlying and ventral 3° ventricle in a Charcot-type frontal slice.

Note that the neurobulbar orientation is vertical in humans and horizontal in quadripeds.

The hypothalamus is frontocranial to the medulla oblongata above the sella turcica and pituitary gland.

The vasculo-nervous axes between the hypothalamus and pituitary become the anatomical landmarks of the said sample.

Note that the hypothalamus is caudal to the optic chiasm.

Also, to be described, the easily identifiable meninges, by their double layer structures, containing the cerebrospinal fluid, are peripheral and surround the 3° and 4° ventricle complex in their ventral part.

Description of Dissection

The separation of the head of the spine will take place between C2 and C3, to preserve the integrity of the medulla oblongata and its caudal continuity, which is the medulla oblongata.

Thus, all the ventricles will be preserved. Note that the cleaning of the anatomical parts will always be done using physiological saline, to avoid any risk of osmotic shock by using water rinsing.

The opening of the skull will be done medially, after the removal of cutaneous elements, the platysma and all other external muscles, to release the bone sutures.

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Separation, using the circular saw under saline cleaning, of the incisor bone, the nasal bones, the medial axis of the frontal, the parietals and the medial axis of the occiput, up to dorsally to the spinal plane.

A triangular-type incision is made on the large wing of the sphenoid in its frontal-parietal and temporal part, bilaterally, to help release the median opening without risking altering the Turkic zone medial to the sphenoid.

The nasal speculum and the 13 cm surgical retractor or the 26 cm "Milkulitcz retractor" will allow you to finish the opening and release the brain from the cranial box.

It is through the frontal route that the brain will be lifted to discern the olfactory bulbs.

The cerebellar and frontal grips allow the cortical-bulbo-cerebellar whole to be turned over and weighed. This action is done delicately so as not to damage the Turkish axis and the medulla oblongata.

The identification and removal of the hypothalamus are done, using magnifying glasses, by the ventral axis and frontal to the pituitary gland. The sample is then stored in a vial of 10% ether for transport.

Histological Analysis Report

About the research work and the publications, cited above, of Mr PIANEL Dominique PhD, 75006 PARIS, and the Biopôle laboratory in the person of Dr Hélène HUET, PhD study engineer and Dr. Veterinarian. Edouard REYES-GOMEZ Veterinary anatomo-cytopathologist Dipl. ECVP, PhD carried out, on March 15, 2021, in Maisons-Alfort, "the ligament analysis N°: H21-0087-78, of the NPY in immunohistochemistry" [18].

Description of the material received 12 deer samples were received in formalin pots, supplied by the Biopôle laboratory, in two stages. Each pot contained one or more samples.

To technique all the samples at the same time and to avoid over-fixation, the samples were transferred to 70° alcohol after appropriate fixation.

The direct debits received come from

1. From a female (series B)
2. A male (series C), for samples taken within the FICIF on deer killed the same day
3. And, for the brain samples, secondarily taken from two male stags, from the farm and under the direction of the slaughterhouses, (series A)

The latter had to concern the hypothalamic part of the brain, but only one of the 2 samples allowed the analysis. The samples were cross-sectioned and then embedded in paraffin.

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All 12 samples made it possible to produce 17 blocks.

Analysis number: H21-0087-78			
	Formalin tubes	Sampling	Blocks
1	Brain 1	Hypothalamus	A1 and A2
2	Brain 2	Hypothalamus	Not eligible
3	Female 1	nuchal ligament	B1 and B2
4	Female 2	Supraspinatus ligament	B3 and B4
5	Female 3	periodontal ligament	B5
6	Female 4	fetlock suspender	B6 and B7
7	Female 5	hock rope	B8
8	Male 1	nuchal ligament	C1 and C2
9	Male 2	Supraspinatus ligament	C3 and C4
10	Male 3	periodontal ligament	C5
11	Male 4	fetlock suspender	C6 and C7
12	Male 5	hock rope	Not eligible

Table 1: Sample analysis.

The purpose of the study was to study the expression of neuropeptide Y (NPY) by immunohistochemistry in the various samples using the anti-neuropeptide Y antibody orb11154 (supplied).

Immunohistochemical Analysis

Development of the "Rabbit polyclonal antibody" required development on the Roche Discovery automaton using deer hypothalamus as a positive control where the neuropeptide is supposed to be expressed, knowing that the antibody used shows labelling in cattle and equines.

Different pre-treatments were tested

- Tris/Borate/EDTA buffer, pH 8

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- Citrate Buffer, pH 6
- Without pre-treatment

The pre-treatment lasted 36 min at a high temperature (95-100°C).

The antibody was used at a concentration of 1/100th on tissue sections with a thickness of 5 µm. The chosen chromogen is DAB (3,3'-diaminobenzidine) which makes it possible to obtain a brown colour where the primary antibody has bound. Clear cytoplasmic labelling of hypothalamic neurons (brown staining) was obtained using the buffer pre-treatment. Tris/Borate/EDTA at pH 8.

Deer brain treated with primary antibody.

Deer brain treated without primary antibody.

Immunohistochemical Analysis of Samples for NPY

The ligaments are dense connective tissues with a lot of collagen and elastin fibres, and taking into account the treatments carried out which could be responsible for tissue detachments, it was not possible to make satisfactory slides of the B8 samples (cord of the hock) and C5 (periodontal ligament.

For all the samples studied, none of the slides showed any marking.

The nuchal ligament is used, below, as a representative example (Fig. 3-5).

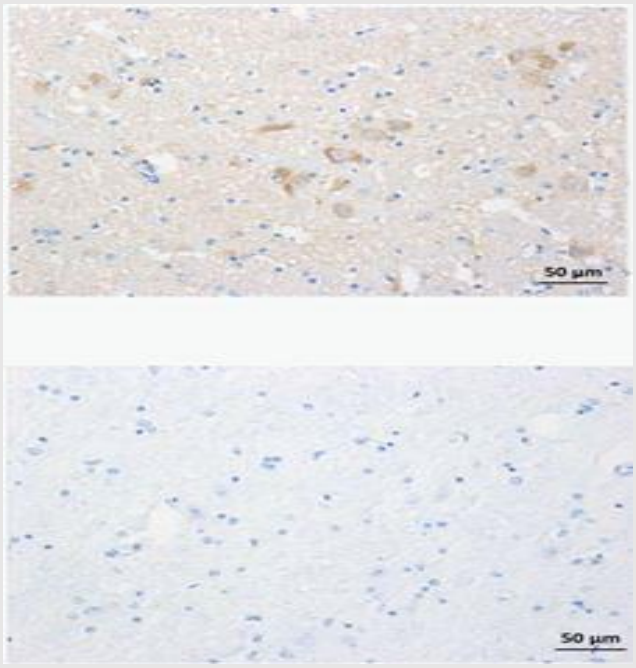

Figure 3: Deer brain treated with primary antibody (up) and treated with primary antibody (down).

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Figure 4: Use of nuchal ligament.

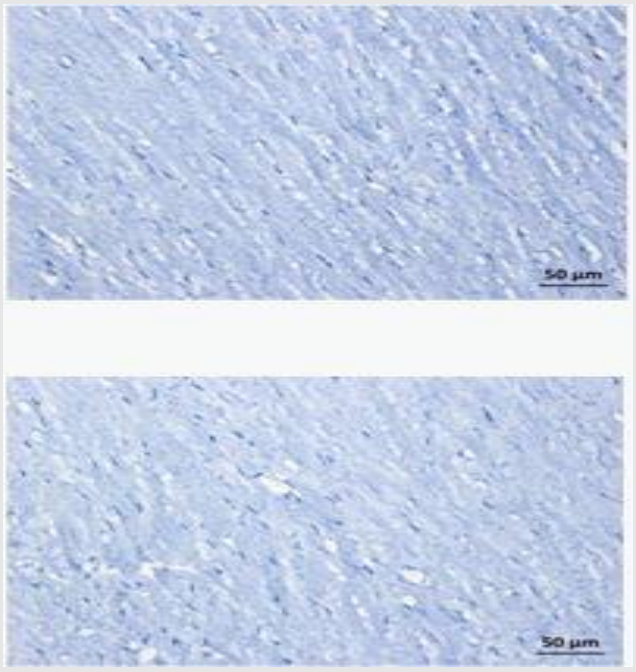

Figure 5: Nuchal ligament treated without primary antibody (up) and treated with primary antibody.

Conclusion of the Analysis

The anti-NPY orb11154 antibody has been validated in the species of deer studied, which confirms and demonstrates that cytoplasmic neuronal immunohistochemical staining is clear in the hypothalamus.

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On the cervid ligaments and tendons studied, no expression of neuropeptide Y was shown by immunohistochemistry.

These results do not support NPY expression in these tissues.

Discussion

Why did we not have any validating results on the ligaments?

• Is it a sampling error on the ligament system, specific to the slaughter of the species or the methodology for processing samples for this style of analysis? How long would the NPY last post-mortal?

• The periodontal sample not have been positive, despite the assertions of Dr P Bouchard, in his article and the biological and clinical consistency with the action of NPY on the stomach functions,

We ask about the relevance of the sample, by scraping the roots dental, this analysis at the ligament level could be modified only because of the amount of ligament during periodontal tearing.

In addition, the samples that were taken by hunters who usually know the exact age of the animal will take samples from the massacre at the level of the upper jaw.

Work on the evolution of the neurocranium by Pr Yves Lignereux shows that the exoskeleton and the exocranium are a source of organization of the dentin when the skeleton becomes endo in vertebrates.

The dental is already well developed in the Lower Permian, in the Pelycosaurians (Dimetrodon). The coronoid process for them. Temporal appears in the Upper Permian, in Cynarioids (primitive Therapsid) [19-21].

The dental complete almost the entire mandible and approaches the squamosal in Cynognathus (Therapsid of the Triassic infr), but the articular and the other bones, reduced, are always present.

The craniomandibular joint is always of the reptilian type.

In Thrinaxodon and Probainognathus, Cynodonts of the Middle Triassic, in Diarthrognathus, Ictidosaurian of the Upper Triassic and Morganucodon or Eozostrodon, Jurassic Mammal,

We observe, side by side, two joints the reptilian, quadrato-articular, and the mammalian, squamoso-dental, more lateral.

In mammals, the mandible is simplified, the dentary remaining alone.

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The coronoid fuses with the dental (coronoid process), and the articular migrate into the middle ear to form the malleus (malleus), always squarely articulated which has become the anvil (incus) and the angular, the ectotympanic, starting from the Meckel's cartilage, mandibular.

According to N. Démeusy, (Laboratory of animal biology UFR of Science University of Caen 1988 29:77-92), the specificity of the moult in the exoskeletons of crustacean decapod types, such as the crab, Xanthidae Pilumnus Hirtellus, have a gland which triggers the moult and helps it to finalize, the “Y organ” is in this type of exoskeleton in place at the level of the mandible.

Thus, the sample should have been taken from the mandible and this was not the case. This specificity was not characterized either at the beginning of this research or in the article by Dr P Bouchard.

The choice of the rabbit antibody is perhaps a mistake because the latter covers all species on the hypothalamic level, certainly, due to the concentration.

The rabbit has constant dental growth is shown in the work of Esther van Praag published in MediRabbit.com of September 2014, that the modification of vitamin D leads to a significant modification of the maxillary tooth growth.

Thus, the relevance of the immune reaction of this antibody can be called into question, in our posterior periodontal and maxillary samples. The work could be updated with other antibodies to define the possible interactions.

• Current work is mainly on NPY receptors and not on the presence of the latter in ligament tissues.

• The study of Mr Gulgun Sengul and Mr Charles Watson, resulting from the presence of NPY on the ligaments up to the 12° dorsal and of H Von Holst, A Rudehill, J M Lundberg, on the presence of NPY in the CSF, shows us that the receptors receive their hypothalamic information, thus the NPY must circulate, the blood pathway being at this level on a portal system, it seems to us justified to seek the NPY at the level of this system, by a protein profile and we push analyzes in this area.

• We validated a sample at the top of the withers. Comparative anatomy research, on several different species, could identify a different location which should be on the caudal and cranial parts of the nuchal ligament.

• The work on the TAU protein, ABETA 1-42, on CSF having to be done on a sample at -20°C, it seems likely to us that the extra hypothalamic sampling mode must be carried out according to other technical approaches, allowing NPY insulation.

• However, the interaction of the NPY with the markers, P1NP (total procollagen type 1 N-terminal propeptide), marker of bone formation, which reflects modifications in the synthesis of new type 1 collagen. B and CrossLaps (β- CTX), a marker of bone resorption, which

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produces type 1 collagen degradation, and which are the markers of bone metabolism are analyzed by blood samples. This is in Swiss Medical Review; Osteoporosis; #207; June 2009

• The hypo-vascularization of the ligament system, associated with the adrenergic shock of the slaughter, and/or the hunt, the vasoconstriction by stimulation of alpha-adrenaline which leads to peripheral hypotension and would be a lead in the discussion.

• The relationship between NPY with stomach motor function and mainly in the reuptake of K+. The adrenergic shock aroused is para sympathicolytic, in which way this neurovegetative action could modify the interpretation of non-results.

• The protein profile traditionally used in immune research can be used by changing the detectable molecular weight and research at the Monot Institute is yet another entry point into this area of intervention done at the Anatomo-Cytopathology Laboratory BioPôle 7, avenue du General de Gaulle, Camille GUERIN building, 94704 MAISONS-ALFORT Cedex.

Conflict of Interest

Author declares no conflicts of interest.

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