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STUDIO NEUROANATOMICO DEI CENTRI DI REGOLAZIONE CARDIORESPIRATORIA: *BULBO ENCEFALICO E GLOMO CAROTIDEO*

DIRETTORE DELLA SCUOLA: CH.MO PROF. ANTONIO TIENGO
SUPERVISORI: CH.MO PROF. CARLO REGGIANI
CH. MO PROF. RAFFAELE DE CARO

DOTTORANDO: DOTT. ANDREA PORZIONATO

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ABBREVIATIONS

AM:	Adrenomedullin
Amb:	Nucleus Ambiguus
AP:	Area Postrema
BDNF:	Brain-Derived Neurotrophic Factor
Cu:	Cuneate Nucleus
DION:	Dorsal Inferior Olivary Nucleus
DMNV:	Dorsal Motor Nucleus of the Vagus
ERK:	Extracellular signal-Regulated Kinase
GDNF:	Glial cell line-Derived Neurotrophic Factor
Gr:	Gracile nucleus
ION:	Inferior Olivary Nucleus
LatRet:	Lateroreticular nucleus
MedVe:	Medial Vestibular Nucleus
MION:	Medial Inferior Olivary Nucleus
NCAM:	Neural Cell Adhesion Molecule
NSTT:	Nucleus of the Spinal Trigeminal Tract
NT:	Neurotensin
NTR:	Neurotensin Receptor
NTS:	Nucleus Tractus Solitarii
PDE:	Phosphodiesterase
PION:	Principal Inferior Olivary Nucleus
SD:	Standard Deviation
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labelling
XII:	Hypoglossal Nucleus

ABSTRACT

INTRODUCTION – Central and peripheral structures, as the medullary nuclei and the carotid body, strictly cohoperate for cardiovascular and respiratory regulation. In the literature, comprehensive and unbiased analyses of morphometric parameters and apoptosis phenomena in infant and adult medullary nuclei are still lacking. Data about local expression of different phosphodiesterase isoforms are also still poor. In the carotid body, many different neurotransmitters/neuromodulators, adhesion molecules and signalling transduction components have been identified but data are still lacking about the presence of some of these, such as adrenomedullin (AM), neurotensin receptors, Neural Cell Adhesion Molecule (NCAM), extracellular signal-regulated kinase (ERK), AKT. The aim of the present work was to give a comprehensive analysis of morphometric parameters and apoptosis phenomena in human medullary nuclei and to analyse the presence of the above components in the carotid body.

MATERIALS AND METHODS – Materials consisted of medullae oblongatae sampled at autopsy from 22 adults and 10 infants, and carotid bodies sampled at autopsy from 16 adult subjects and 6 foetuses. A morphometric analysis with the optical disector method was performed to calculate the neuronal densities, nuclear volumes and total neuron numbers of medullary nuclei. Apoptosis was also studied by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) and the mean percentages (± Standard Deviation) of TUNEL-positive neurons were morphometrically analysed. The regional distribution and cellular localization of four isozyme forms of the phosphodiesterase 4 (PDE4A, PDE4B, PDE4C and PDE4D) were also studied in 8 of the above adult subjects by *in situ* hybridization. In the study of the carotid body, we analysed by immunocytochemistry and double immunofluorescence the expression of AM, neurotensin receptor 1 (NTR1), NCAM, ERK and pERK, AKT and pAKT. AM, ERK/pERK and AKT/pAKT were also studied in foetal samples.

RESULTS – In both adults and infants, higher neuronal densities were found in the more ventrally located nuclei of the spinal trigeminal tract (NSTT) (mean values ± Standard Deviation: 9217±2146 n/mm^3 and 21347±5006 n/mm^3, respectively) and inferior olivary complex (PION: 9149±1341 n/mm^3 and 20910±1582 n/mm^3; MION: 10102±3024 n/mm^3 and 19267±3475 n/mm^3; DION: 11318±3704 n/mm^3

and 23124±6740 n/mm^3, respectively) than in the nuclei of the medullary tegmentum, i.e., hypoglossal nucleus (XII) (1070±343.4 n/mm^3 and 2799±1292 n/mm^3), dorsal motor nucleus of the vagus (DMNV) (1663±431.4 n/mm^3 and 2915±568.1 n/mm^3), nucleus tractus solitarii (NTS) (2982±639.9 n/mm^3 and 8150±1735 n/mm^3), medial vestibular nucleus (MedVe) (2964±380.8 n/mm^3 and 6958±1076 n/mm^3) and cuneate nucleus (Cu) (1061±238.7 n/mm^3 and 2686±843.6 n/mm^3). All the medullary nuclei showed higher volumes and lower neuronal densities in adults than in infants, without statistically significant differences in total neuron numbers. Statistically significant differences between adults and infants were found in the neuronal apoptotic indexes of the Cu (28.2±16.3% vs. 6.9±8.7%), MedVe (24.7±15.0% vs. 11.3±11.4%), NTS (11.2±11.2% vs. 2.3±2.4%), DMNV (6.8±8.5% vs. 0.1±0.2%) and XII (6.6±5.7% vs. 0.1±0.2%). Differences in neuronal apoptotic index were also statistically significant among nuclei, those with higher neuronal apoptotic indexes being the Cu, MedVe and NSTT. In the medullary nuclei PDE4B and PDE4D mRNA expression was abundant and distributed not only in neuronal cells, but also in glial cells, especially on and around blood vessels. The hybridization signals for PDE4B and PDE4D mRNAs in the AP were stronger than in any other nucleus considered. They were also found in vomiting-related nuclei such as NTS and DMNV. In the carotid body, no AM, NTR1, and NCAM immunoreactivities were visible in type II cells. Anti-NTR1 and -NCAM immunohistochemistries showed positivity in 45.6 ± 9.2% and 78.3 ± 7.2% of adult type I cells, respectively. Higher percentages of positive type I cells were found in adult than foetal subjects for anti-AM (32.3 ± 7.7% vs 11.8 ± 2.7%, $P<0.001$), -ERK (32.3 ± 7.7% vs 11.8 ± 2.7%, $P<0.001$), -pERK (32.3 ± 7.7% vs 11.8 ± 2.7%, $P<0.001$), -AKT (32.3 ± 7.7% vs 11.8 ± 2.7%, $P<0.001$) and -pAKT (32.3 ± 7.7% vs 11.8 ± 2.7%, $P<0.001$) immunohistochemistries.

DISCUSSION – The higher nuclear volumes and lower neuronal densities in adults may be ascribed to postnatal development of the neuropil and microvascularization. The higher apoptotic indexes in adults indicate higher resistance of infant neuronal populations to terminal hypoxic-ischaemic injury or post-mortem changes. Moreover, nuclei with higher apoptotic indexes are located in the lateral medullary tegmentum and share the same vascular supply from the posterior inferior cerebellar artery, suggesting

different characteristics of survival on vascular basis. High PDE4 expression in the AP suggests that cAMP signaling modification could mediate the emetic effects of PDE4 inhibitors in human brainstem. Immunohistochemical findings in the carotid body suggest that AM and NT may play a role in the regulation of chemoreceptor discharge. The high expression level of NCAM in the carotid body indicates a role in regulating adhesion between type I cells. It may be hypothetized that the ERK and AKT signalling pathways in the carotid body are activated by neuromodulator/neurotrophic factors and plays a role in producing long-term cellular modifications. The lower expression of AM, ERK/pERK, and AKT/pAKT in foetuses may be ascribed to the absence of pulmonary respiration with lack of regulatory role of the carotid body during the prenatal period.

RIASSUNTO

INTRODUZIONE – Strutture nervose centrali e periferiche, quali i nuclei bulbari ed il glomo carotideo, cooperano alla regolazione cardiovascolare e respiratoria. Le conoscenze dei parametri morfometrici e dei fenomeni apoptotici nei nuclei bulbari di adulti e bambini, così come quelle relative all'espressione locale delle isoforme delle fosfodiesterasi, sono ancora limitate. Nel glomo carotideo sono stati individuati molti neurotrasmettitori/neuromodulatori differenti, molecole di adesione e componenti di vie di trasduzione del segnale ma non ci sono ancora dati sulla presenza di alcuni di essi, quali l'adrenomedullina (AM), i recettori della neurotensina, la molecola di adesione cellulare neurale (NCAM), la chinasi regolata da segnale extracellulare (ERK) ed AKT. Scopo del presente lavoro è fornire un'analisi completa dei parametri morfometrici e dei fenomeni apoptotici dei nuclei bulbari umani e analizzare la presenza delle componenti sovrariportate nel glomo carotideo.

MATERIALI E METODI – I materiali usati sono stati bulbi encefalici prelevati in corso di autopsia da 22 adulti e 10 bambini e glomi carotidei prelevati da 16 adulti e 6 feti. E' stata effettuata un'analisi morfometrica con il metodo del dissettore ottico per calcolare le densità neuronali, i volumi nucleari e i numeri totali di neuroni dei nuclei bulbari. E' stata altresì studiata l'apoptosi mediante terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) e sono state analizzate morfometricamente le percentuali medie (± Deviazione Standard) dei neuroni TUNEL-positivi. E' stata studiata la distribuzione regionale e cellulare delle quattro isoforme della fosfodiesterasi 4 (PDE4A, PDE4B, PDE4C e PDE4D) in 8 dei soggetti adulti mediante ibridazione *in situ*. Per quanto riguarda l'analisi dei glomi carotidei si è proceduto allo studio con immunoistochimica ed immunofluorescenza doppia dell'espressione di AM, recettore della neurotensina di tipo 1 (NTR1), NCAM, ERK e pERK, AKT e pAKT. L'analisi d'espressione di AM, ERK/pERK e AKT/pAKT è stata altresì effettuata su prelievi fetali.

RISULTATI – Sia negli adulti che nei bambini densità neuronali maggiori sono state trovate nei nuclei localizzati ventralmente, ossia il nucleo del tratto spinale del trigemino (NSTT) (valori medi ± Deviazione Standard: 9217±2146 n/mm^3 e 21347±5006 n/mm^3, rispettivamente) ed il complesso olivare

inferiore (PION: 9149±1341 n/mm^3 e 20910±1582 n/mm^3; MION: 10102±3024 n/mm^3 e 19267±3475 n/mm^3; DION: 11318±3704 n/mm^3 e 23124±6740 n/mm^3, rispettivamente), che nei nuclei del tegmento bulbare, cioè il nucleo ipoglosso (XII) (1070±343,4 n/mm^3 e 2799±1292 n/mm^3), il nucleo motore dorsale del vago (DMNV) (1663±431,4 n/mm^3 e 2915±568,1 n/mm^3), il nucleo del tratto solitario (NTS) (2982±639,9 n/mm^3 e 8150±1735 n/mm^3), il nucleo vestibolare mediale (MedVe) (2964±380,8 n/mm^3 e 6958±1076 n/mm^3) ed il nucleo cuneato (Cu) (1061±238,7 n/mm^3 e 2686±843,6 n/mm^3). Tutti i nuclei bulbari presentavano volumi maggiori e densità neuronali minori negli adulti rispetto ai bambini, senza differenze statisticamente significative nel numero totale di neuroni. Differenze statisticamente significative tra adulti e bambini sono state trovate negli indici apoptotici neuronali del Cu (28,2±16,3% vs. 6,9±8,7%), MedVe (24,7±15,0% vs. 11,3±11,4%), NTS (11,2±11,2% vs. 2,3±2,4%), DMNV (6,8±8,5% vs. 0,1±0,2%) e XII (6,6±5,7% vs. 0,1±0,2%). Differenze statisticamente significative erano altresì presenti per quanto riguarda il confronto tra i diversi nuclei, con il Cu, il MedVe ed il NSTT che mostravano i maggiori indici apoptotici neuronali. Nei nuclei bulbari l'mRNA di PDE4B e PDE4D era abbondante e distribuito non solo nelle cellule neuronali ma anche nelle cellule gliali, soprattutto in vicinanza dei vasi sanguigni. I segnali di ibridazione per PDE4B e PDE4D erano più intensi nell'AP che in ogni altro nucleo considerato. Sono stati altresì trovati in nuclei implicati nei meccanismi del vomito come il NTS ed il DMNV. Nel glomo carotideo non sono state rilevate immunoreattività per AM, NTR1, e NCAM a carico delle cellule di tipo II di entrambe le casistiche. Analisi immunoistochimiche anti-NTR1 ed –NCAM hanno evidenziato positività del 45,6±9,2% e 78,3±7,2% delle cellule di I tipo dei soggetti adulti, rispettivamente. Percentuali maggiori di cellule di tipo I positive sono state trovate negli adulti rispetto ai feti all'esame immunoistochimico per AM (32,3±7,7% vs 11,8±2,7%, P<0,001), ERK (32,3±7,7% vs 11,8±2,7%, P<0,001), pErk (32,3±7,7% vs 11,8±2,7%, P<0,001), Akt (32,3±7,7% vs 11,8±2,7%, P<0,001) e pAkt (32,3±7,7% vs 11,8±2,7%, P<0,001).

DISCUSSIONE – I maggiori volumi nucleari e le minori densità neuronali rilevati negli adulti possono essere ascritti allo sviluppo postnatale del neuropilo e delle microvascolarizzazione. I maggiori indici

apoptotici neuronali rilevati negli adulti indicano una maggiore resistenza delle popolazioni neuronali infantili agli insulti ipossico-ischemici o ai cambiamenti postmortali. Inoltre i nuclei con maggiori indici apoptotici sono localizzati nel tegmento bulbare laterale e condividono la stessa irrorazione vascolare dall'arteria cerebellare posteriore inferiore, suggerendo differenti caratteristiche di sopravvivenza su base vascolare. L'elevata espressione di PDE4 nell'AP suggerisce che modificazioni delle vie del segnale che coinvolgono cAMP possano mediare gli effetti emetici degli inibitori di PDE4 nei tronchi encefalici umani. I reperti immunoistochimici nel glomo carotideo suggeriscono che l'AM e la NT possano svolgere un ruolo nella regolazione dell'attività chemorecettoriale del glomo. L'elevato livello di espressione di NCAM nel glomo carotideo indica un ruolo nella regolazione dell'adesione tra cellule di tipo I. Si può altresì ipotizzare l'attivazione delle vie di trasduzione del segnale ERK e AKT da parte di neuromodulatori/fattori neurotrofici, con possibile ruolo nella produzione di modificazioni cellulari a lungo termine. La minore espressione di AM, ERK/pERK, ed AKT/pAKT nei feti può essere ricondotta all'assenza di respirazione polmonare con mancanza del ruolo regolatorio del glomo carotideo durante il periodo prenatale.

INTRODUCTION

The centres of cardiovascular and respiratory regulation, in particular the nucleus tractus solitarii (NTS), dorsal motor nucleus of the vagus (DMNV), nucleus ambiguus (Amb), area postrema (AP), reticular formation, are mainly located in the central nervous system at the level of the medulla oblongata. The action of these nuclei is modulated by inputs arriving from peripheral structures such as the carotid sinus, a baroreceptor sensing peripheral blood pressure, and carotid body, a chemoreceptor sensing hypoxia, hypercapnia and acidosis.

The present work is focused on some neuroanatomic and neuropathologic aspects of *MEDULLARY NUCLEI*, with particular reference to those involved in cardiorespiratory regulation, and of *CAROTID BODY*.

MEDULLARY NUCLEI

The medulla oblongata contains numerous intrinsic neuron cell bodies and their processes, some of which are homolog of spinal neuronal groups. These include the sites of termination and cells of origin of axons that enter or leave the medulla oblongata through the cranial nerves. They provide the sensory and motor innervation of structures that are mostly in the head and neck. Additional groups of neurons receive inputs related to the special senses of hearing, vestibular function and taste. The reticular formation is an extensive, often ill-defined network of neurons that extends throughout the length of the medulla oblongata. Some of its nuclei are concerned with cardiac and respiratory control, others in aspects of many neural activities, and yet others provide or receive massive afferent and efferent cerebellar projections (Standring et al., 2005). In particular, the neurons involved in autonomic regulation are located approximately in the middle of each half of the medulla, extending obliquely from a region lateral and rostral to the hypoglossal nucleus (XII) across the reticular formation to the ventrolateral surface. The regions involved are the NTS, DMNV, and the intermediate reticular zone, which includes the region of the ventrolateral medulla adjacent to the surface. In a transverse section of the medulla at the caudal end of the fourth ventricle these nuclei are located in the ventricular floor

interposed between the XII and the caudal ends of the inferior and medial vestibular nuclei. Moreover at this medullary level, the total area of grey matter is increased by the presence of the large olivary nuclear complex and nuclei of the vestibulocochlear, glossopharyngeal, and accessory nerves. The arcuate nuclei are curved, interrupted bands, ventral to the pyramids. They project mainly to the contralateral cerebellum through the inferior cerebellar peduncle. The inferior olivary nucleus (ION) is a hollow, irregularly crenated grey mass. It has a longitudinal medial hilum, and is surrounded by myelinated fibres which form the olivary amiculum. Dorsolateral to the pyramid, it underlies the olive but ascends within the pons.

Gracile and cuneate nuclei

The gracile (Gr) and cuneate (Cu) nuclei are part of the pathway that is considered to be the major route for discriminative aspects of tactile and locomotor sensation. Gr and Cu are continuous with the central gray substance of the medulla spinalis, and may be regarded as dorsal projections of this, each being covered superficially by the fibers of the corresponding fasciculus. On transverse section Gr appears as a single, more or less quadrangular mass. The upper regions of both nuclei are reticular and contain small and large multipolar neurons with long dendrites. The lower regions contain clusters of large round neurons with short and profusely branching dendrites. Upper and lower zones differ in their connections but both receive terminals from the dorsal spinal roots at all levels. Cu is divided into several parts. Its middle zone contains a large pars rotunda, in which rostrocaudally elongated medium-sized neurons are clustered between bundles of densely myelinated fibres. The reticular poles of its rostral and caudal zones contain scattered, but evenly distributed, neurons of various sizes. The pars triangularis is smaller and laterally placed. There is a somatotopic pattern of termination of cutaneous inputs from the upper limb upon the cell clusters of the pars rotunda. Terminations are diffuse in the reticular poles. The nuclei also contain interneurons, many of which are inhibitory.

The accessory cuneate nucleus, dorsolateral to Cu, is part of the spinocerebellar system of precerebellar nuclei and contains large neurons like those in the spinal thoracic nucleus. These form the posterior external arcuate fibres, which enter the cerebellum by the ipsilateral inferior peduncle.

Vestibular nucleus

The medial vestibular nucleus (MedVe) continues a little from the medulla into the pontine tegmentum, and is separated from the inferior cerebellar peduncle by the lateral vestibular nucleus. It is one of the vestibular nuclei, together with the lateral (Deiters' nucleus), superior and inferior vestibular groups, laterally placed in the rhomboid fossa of the fourth ventricle, subjacent to the vestibular area. They all receive fibres from the vestibulocochlear nerve and send axons to the cerebellum, medial longitudinal fasciculus, spinal cord and lateral lemniscus. MedVe broadens, then narrows as it ascends from the upper olivary level into the lower pons, where it separates the vagal nucleus from the floor of the fourth ventricle. It is crossed by the striae medullares nearer the floor. Below, it is continuous with the nucleus intercalatus. The inferior vestibular nucleus (which is the smallest) lies between MedVe and inferior cerebellar peduncle, from the level of the upper end of Gr to the pontomedullary junction. It is crossed by descending fibres of the vestibulocochlear nerve and the vestibulospinal tract. The lateral vestibular nucleus lies just above the inferior nucleus, and ascends almost to the level of the abducens nucleus. It is composed of large multipolar neurons, which are the main source of the vestibulospinal tract. The superior vestibular nucleus is small and lies above the medial and lateral nuclei.

Nucleus of the Spinal Trigeminal Tract

The nucleus of the spinal trigeminal tract (NSTT) is embraced by the spinal tract of the trigeminal nerve formed by the descending fibres of the sensory root of the trigeminal nerve. The spinal nucleus is considered to consist of three parts: the subnucleus oralis (which is most rostral and adjoins the principal sensory nucleus); the subnucleus interpolaris; and the subnucleus caudalis (which is the most caudal part and is continuous below with the dorsal grey column of the spinal cord).

Dorsal Motor Nucleus of the Vagus

The DMNV lies dorsolateral to the XII, from which it is separated by the nucleus intercalatus. It extends caudally to the first cervical spinal segment and rostrally to the open part of the medulla under the vagal triangle. The vagal nucleus is a general visceral efferent nucleus and is the largest parasympathetic nucleus in the brainstem. Most (80%) of its neurons give rise to the preganglionic parasympathetic fibres of the vagus nerve. The remainder are interneurons or project centrally. Its fibres control the non-striated muscle of the viscera of the thorax (heart, bronchi, lungs and oesophagus) and abdomen (stomach, liver, pancreas, spleen, small intestine and proximal part of the colon). Neurons within the nucleus are heterogeneous and can be classified into nine subnuclei, which are regionally grouped into rostral, intermediate and caudal divisions. Topographic maps of visceral representation in animals suggest that the heart and lungs are represented in the caudal and lateral part of the nucleus, the stomach and pancreas in intermediate regions, and the remaining abdominal organs in the rostral and medial part of the nucleus. There may be a sparse sensory afferent supply, which arises in the nodose ganglion and projects directly to the nucleus and possibly beyond into the solitary tract nucleus.

Hypoglossal nucleus

The XII is derived from the base of the anterior column; in the lower closed part of the medulla oblongata it is situated on the ventrolateral aspect of the central canal; but in the upper part it approaches the rhomboid fossa, where it lies close to the middle line, under an eminence named the trigonum hypoglossi. Numerous fibers connect the two nuclei, both nuclei send long dendrons across the midline to the opposite nucleus; commissure fibers also connect them. The nucleus measures about 2 cm. in length, and consists of large motor neurons interspersed with myelinated fibres and whose axons constitute the roots of the hypoglossal nerve. These nerve roots leave the ventral side of the nucleus, pass forward between the white reticular formation and the gray reticular formation, some between the ION and the medial accessory olivary nucleus (MION), and emerge from the antero-lateral sulcus. It is organized into dorsal and ventral nuclear tiers, each divisible into medial and lateral subnuclei. There is

a musculotopic organization of motor neurons within the nuclei that corresponds to the structural and functional divisions of tongue musculature. Thus, motor neurons innervating tongue retrusor muscles are located in dorsal/dorsolateral nuclei, whereas motor neurons innervating the main tongue protrusor muscle are located in ventral/ventromedial regions of the nucleus. Several smaller groups of cells lie near the XII. They include the nucleus intercalatus, sublingual nucleus, nucleus prepositus hypoglossi and nucleus paramedianus dorsalis (reticularis). Gustatory and visceral connections are attributed to the nucleus intercalatus.

Inferior olivary nucleus

The olivary nuclear complex consists of the large principal inferior olivary nucleus (PION) and the much smaller MION and dorsal accessory olivary nucleus (DION). They consist of small, round, yellowish cells and numerous fine nerve fibers. They are the so-called precerebellar nuclei, a group that also includes the pontine, arcuate, vestibular, reticulocerebellar and spinocerebellar nuclei, all of which receive afferents from specific sources and project to the cerebellum. The ION is the largest, and is situated within the olive. It consists of a gray folded lamina arranged in the form of an incomplete capsule, opening medially by an aperture called the hilum emerging from the hilum are numerous fibers which collectively constitute the peduncle of the olive. The axons, olivocerebellar fibers, which leave the ION pass out through the hilum and decussate with those from the opposite olive in the raphe, then as internal arcuate fibers they pass partly through and partly around the opposite olive and enter the inferior peduncle to be distributed to the cerebellar hemisphere of the opposite side from which they arise. The fibers are smaller than the internal arcuate fibers connected with the median lemniscus. Fibers passing in the opposite direction from the cerebellum to the ION are often described but their existence is doubtful. Much uncertainty also exists in regard to the connections of the olive and the spinal cord. Many collaterals from the reticular formation and from the pyramids enter the ION. The MION lies between the PION and the pyramid, and forms a curved lamina, the concavity of which is directed

laterally. The fibers of the hypoglossal nerve, as they traverse the medulla, pass between the MION and PION. The DION is the smallest, and appears on transverse section as a curved lamina behind the PION.

Nucleus Tractus Solitarii

The NTS is a complex integration centre subserving many diverse functions. It receives numerous afferent inputs and, via oligo- and monosynaptic relations with other structures, it participates in cardiovascular, respiratory and gastrointestinal regulation. The human NTS is subdivided into the following subnuclei: commissural, paracommissural, medial, ventrolateral, ventral, dorsolateral, dorsal, intermediate, interstitial, and gelatinosus. Among the subnuclei of the NTS, the first to be described was the subnucleus gelatinosus (Olszewski and Baxter, 1954), located in the dorsal portion of the NTS and reaching at the level of the obex its largest proportions. It is characterized by pale-staining neurons, predominantly small to medium-sized and triangular to oblong in shape, with sparse large pigmented and non-pigmented cells. There are also many glial cells, with the complete or almost complete absence of myelinated axons. The most common type of axon terminal contains round clear vesicles and has asymmetrical synapses with the dendritic profiles (Leslie et al., 1982).

The dorsal portion of the NTS shows selective dendritic lesions in adults who died after an episode of acute heart failure with profound (<60 mmHg) and prolonged (>2 hours) hypotension (De Caro et al., 2000, 2003). The limited extent of these lesions accounts for selective necrosis of a group of neurons with greater vulnerability, due to their location at the watershed zone between the terminal branches of vascularization of the medullary tegmentum. These lesions are characteristic of neuronal death due to hyperexcitation (Olney, 1969, 1971), and an excitotoxic component to ischaemic cell death in the NTS has been suggested (De Caro et al., 2000, 2003; Porzionato et al., 2004a; Parenti et al., 2005).

Nucleus Ambiguus

The Amb is a group of large motor neurons, situated deep in the medullary reticular formation. It extends rostrally as far as the upper end of the vagal nucleus while caudally it is continuous with the

nucleus of the spinal accessory nerve. The Amb contains several cellular subgroups, and some topographical representation of the muscles innervated has been established. Individual laryngeal muscles are innervated by relatively discrete groups of cells in more caudal zones. Neurons that innervate the pharynx lie in the intermediate area, and neurons that innervate the oesophagus and soft palate are rostral. The Amb is connected to corticonuclear tracts bilaterally and to many brainstem centres. At its upper end, a small retrofacial nucleus intervenes between it and the facial nucleus. Although the Amb lies in line with the special visceral efferent nuclei, it is a reputed source of general visceral efferent vagal fibres.

Area Postrema

The AP is implicated as a chemoreceptor trigger zone for vomiting, through μ opioid, 5HT3 and H2 receptor binding (Borinson and Wang, 1953; Carpenter et al., 1988). It is a circumventricular organ serving as an interface between the brain parenchyma and the cerebrospinal fluid (CSF)-containing ventricles. The AP lacks a specific blood-brain diffusion barrier to large polar molecules and is thus anatomically positioned to detect emetic toxins in the blood as well as in the CSF. The AP along with the NTS and the DMNV makes up the so-called dorsal vagal complex, which is the major termination site of vagal afferent nerve fibers. Lesions of the AP prevent vomiting in response to most, but not all, emetic drugs. Moreover, many peptides, such as angiotensin, adrenomedullin, endothelin and endorphin, regulate cardiovascular function (Szilagyi and Ferrario, 1981; Sander et al., 1989; Allen et al., 1997) by binding on receptors located in the AP, the regulatory function of which is mediated through efferent projections towards other medullary centres. The AP is located in the caudal fourth ventricle floor. In rodents, it is a single midline structure at the apex of the calamus scriptorius; in humans, it is composed of bilateral areas joining in the midline of the caudal levels. The body of the AP consists of a loose network of neuroglia, with small sparse neurons. Microcirculation in the AP is characterized by high capillary density (Shaver et al., 1991; Porzionato et al., 2004b, 2005b), large pericapillary spaces and lack of a tight blood–brain barrier (Gross, 1991). Cytoarchitectonic and neurochemical studies in rat

indicate the existence of distinct anatomical and functional compartments within the AP, and even topographical diversity in its capillary organization (Gross et al., 1991; Shaver et al., 1991).

Brainstem Reticular Formation

The brainstem contains extensive fields of intermingled neurons and nerve fibres, which are collectively termed the reticular formation. The reticular regions are often regarded as phylogenetically ancient, representing a primitive nerve network upon which more anatomically organized, functionally selective, connections have developed during evolution. However, the most primitive nervous systems show both diffuse and highly organized regions, which cooperate in response to different demands.

The reticular regions tend to be ill-defined collections of neurons and fibres with diffuse connections. Their conduction paths are difficult to define, complex and often polysynaptic, and they have ascending and descending components that are partly crossed and uncrossed. Their components subserve somatic and visceral functions. They include distinct chemoarchitectonic nuclear groups, including clusters of serotoninergic neurons (group B cells), which synthesize the indolamine 5-hydroxytryptamine (serotonin); cholinergic neurons (group Ch cells), which contain acetyltransferase, the enzyme which catalyses the synthesis of acetylcholine; and three catecholaminergic groups composed of noradrenergic (group A), adrenergic (group C), and dopaminergic (group A) neurons, which synthesize noradrenaline (norepinephrine), adrenaline (epinephrine) and dopamine respectively as neurotransmitters. The reticular formation is a continuous core that traverses the whole brainstem, and is continuous below with the reticular intermediate spinal grey laminae. It is divisible, on the basis of cytoarchitectonic, chemoarchitectonic and functional criteria, into three bilateral longitudinal columns: median; medial, containing mostly large reticular neurons; and lateral, containing mostly small to intermediate neurons.

MORPHOMETRIC DATA ON MEDULLARY NUCLEI

There have been a few studies in the literature detailing morphometric parameters of the medullary nuclei, such as neuronal density, nuclear volume and total neuron number. Most studies showed intrinsic bias due to counting in a two-dimensional plane and applying of correction factors to estimate neuron numbers in three-dimensional space (Abercrombie, 1946). On the contrary, unbiased stereological methods using a three-dimensional probe and not relying on any assumptions about size and shape of the objects have been developed. Moreover, all previous studies, reviewed in Table 1, limited the morphometric analysis to one or two nuclei, without providing the possibility to compare morphometric data of different medullary nuclei, and few data are available about comparisons between infants and adults. One of the aims of the present work was to provide a morphometric analysis, through the unbiased method of the optic disector, of a wide series of medullary nuclei in both adult and infant casistics

Table 1. Review of literature on morphometric parameters (neuronal density NV_N; nuclear volume, V; total neuron number, N_N) of medullary nuclei.

INFANTS	REFERENCES	N° CASES	AGE (±SD)	NV_N (neurons/mm^3±SD)	V (mm^3±SD)	N_N (±SD)
XII	Nara et al., 1989	1	2 Months	-	-	≈6000
	O'Kusky and Norman, 1992	15	51.1±5.3 postc wks	*Motor neurons* 1200-3000 *Interneurons* 400-1200	≈2.7-≈6	*Motor neurons* 7450-7630 *Interneurons* 3050-3092
		28 (SIDS)	50.8±1.5 postc wks	*Motor neurons* 1000-2300 *Interneurons* 300-800	≈3.2-≈7	Non statistically significant differences
	Konrat et al., 1992	4	20±18 wks	1500±500	7±4	9500±2000
		6 (SIDS)	13±9 wks	2000±1000	4±2	9000±4000
	O'Kusky and Norman, 1995	6	49.9±7.1(SEM) postc wks	*Motor neurons* 2335±277 (SEM)	3.732±0.432 (SEM)	11210±637(SEM) *Motor neurons* 8256±551 (SEM)
		9 (SIDS)	49.8±2.8(SEM) postc wks	*Motor neurons* 1681±81 (SEM)	5.067±0.363 (SEM)	11354±481(SEM) *Motor neurons* 8355±430 (SEM)
	Lamont et al., 1995	11	43-112 postc wks	-	2.98±0.88	6475±1029
		11 (SIDS)	43-112 postc wks	-	3.13±0.78	6647±819
	Pamphlett et al.,1996	8	42-88 postc wks	-	1.94±0.43	-
		12 (SIDS)	43-88 postc wks	-	1.91±0.58	-
DMNV	Konrat et al., 1992	4	20±18 wks	2000±1000	6±5	11000±4000
		6 (SIDS)	13±9 wks	2500±1000	4.5±4	10400±4000
	Lamont et al., 1995	11	43-112 postc wks	-	1.99±0.50	11993±1895
		11 (SIDS)	43-112 postc wks	-	1.91±0.39	11298±1217
Amb	Lamont et al., 1995	11	43-112 postc wks	-	-	1488±355
		11 (SIDS)	43-112 postc wks	-	-	1497±381
Retram	Lamont et al., 1995	11	43-112 postc wks	-	-	244±274
		11 (SIDS)	43-112 postc wks	-	-	209±126
LatVe	Fujii et al., 1997	1	2 months		≈0.75	≈3750
LatCu	Ma et al., 2002; 2006	1	2 months	-	8.73	≈17000
Cu	Ma et al., 2005, 2006	1	2 months	-	5.5	≈8000
Gr	Ma et al., 2006	1	2 months	-	5.79	≈5000
TrPSN	Hamano et al., 1997	1	2 months	46000	1,11	51100
PION	Kinney et al., 2002	29	410±87postc days	8889±255 (S.E.M.)	-	-
		29 (SIDS)	378±62postc days	7687±255 (S.E.M.)	-	-

ADULTS	REFERENCES	N° CASES	AGE (±SD)	NV_N (neurons/mm^3±SD)	V (mm^3±SD)	N_N (neurons±SD)
XII	Nara et al., 1989	2	16 and 63 years	-	-	≈5400-≈6200
DMNV	Nara et al., 1991	1	63 years	-	≈3.8	≈11000
	Huang et al., 1993	3	59-71 years	*Motor neurons* 1601±356	8±2	16826±967 *Motor neurons* 12410±1649
SupVe	Suarez et al., 1997	8	30-55 years	2127	10.95 ± 1.45 (S.E.M.)	22309
	Alvarez et al., 2000	8	35-89 years		9.6-12.39	17323-24575
MedVe	Diaz et al., 1996	9	30-50 years	4136	30.44±0.85 (S.E.M.)	127737
	Suarez et al., 1997	8	30-55 years	4196	30.44 ± 0.85 (S.E.M.)	127737
	Alvarez et al., 1998	8	35-89 years	1617-4250	-	75915-122241
	Alvarez et al., 2000	8	35-89 years	-	24.82-38.36	75915-122241
	Tang et al., 2001/2002	13	40-93 years			151000±23400
LatVe	Fujii et al., 1997	1	63 years	-	3,03	5410
	Suarez et al., 1997	8	30-55 years	1563	16.33 ± 2.80 (S.E.M.)	25046
	Alvarez et al., 2000	8	35-89 years	-	19.28-24.77	21398-53421
DesVe	Suarez et al., 1997	8	30-55 years	3303	17.1 ± 3 (S.E.M.)	56482
	Alvarez et al., 2000	8	35-89 years		14.45-18.13	21602-48341
LatCu	Ma et al., 2002	1	63 years	-	27.65	≈16000
	Ma et al., 2006	1	63 years	-	27.65	≈16000
Cu	Ma et al., 2005, 2006	1	63 years	-	21.0	≈25000
Gr	Ma et al., 2006	1	63 years	-	28.29	≈11000
TrPSN	Hamano et al., 1997	2	16 and 63 years	17000 and 20000	2,64 and 2,32	44900 and 46400
PION	Lasn et al., 2006	6	17-57 years	-	-	760000
		4	69-79 years	-	-	600000

SIDS: Sudden Infant Death Syndrome; Postc: postconceptional; wks: weeks; SD: Standard Deviation; SEM: Standard Error of Measurement; XII: hypoglossal nucleus; DMNV: dorsal motor nucleus of the vagus; Amb: nucleus ambiguus; Retram: nucleus retroambiguus; LatVe: lateral vestibular nucleus; MedVe: medial vestibular nucleus; SupVe: superior vestibular nucleus; DesVe: descending vestibular nucleus; LatCu: lateral cuneate nucleus; Cu: medial cuneate nucleus; Gr: gracile nucleus; TrPSN: trigeminal principal sensory nucleus; PION: principal inferior olivary nucleus.

NECROSIS AND APOPTOSIS IN THE MEDULLARY NUCLEI

In the literature, quite specific anatomic patterns of necrosis have been reported in the medulla oblongata of neonates with perinatal hypoxia (Gilles, 1969; Dambska et al., 1976; Leech and Alvord, 1977; Janzer and Friede, 1980; Pindur et al., 1992; Taylor and Roessman, 1984) and of adults with hypoxic-ischaemic injury (Gilles, 1969; Revesz and Geddes, 1988; De Caro et al., 2000, 2003; Porzionato et al., 2004a; Parenti et al., 2005). Moreover, preceding studies have found specific patterns of vascularisation in the medullary nuclei, showing different microvessel densities (Porzionato et al., 2004b, 2005b). Neurons may also undergo apoptosis in response to hypoxic-ischaemic injury (Snider et al., 1999). Apoptosis is a common type of programmed cell death, characterised by chromatin condensation and margination, cell shrinkage, membrane blebbing, and release of apoptotic bodies (Kerr et al., 1972). It involves the activation of caspases, which cleave DNA molecules into small fragments and disintegrate the cell into apoptotic bodies. The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) is a method which can identify such DNA strand breaks (Wyllie, 1980). In the central nervous system, apoptosis has mainly been studied with regard to embryonic development (Chan and Yew, 1998), ageing (Anglade et al., 1997) and cerebrovascular diseases (Zhang et al., 2004), with particular reference to diencephalic and telencephalic structures; literature data regarding the topographic distribution of apoptotic phenomena in human infant and adult medulla oblongata are still lacking. The present study was also designed to evaluate the distribution of apoptosis of neurons in the medullary nuclei of infant and adult subjects who died of hypoxic-ischaemic injury, in order to identify possible anatomic and age-related patterns.

Although many studies have reported the apoptotic effect of opiates in animals and cell cultures (Atici et al., 2004; Goswami et al., 1998; Yin et al., 1997; Hu et al., 2002), apoptosis in human heroin addicts has not yet been studied. Thus, a comparison was also made between

opiate addicts who died of heroin intoxication and the other adult cases, taken as a control group.

PHOSPHODIESTERASE ISOFORMS IN THE MEDULLA OBLONGATA

Rolipram is an inhibitor of the protein family responsible for cAMP hydrolysis phosphodiesterase 4 (PDE4) (Wachtel, 1982) that has anti-depressant effects modifying PDE4 expression (Wachtel, 1983; Wachtel and Schneider, 1986; Scott et al., 1991; Takahashi et al., 1999). However, its clinical use has been hampered by the high incidence of nausea an vomiting (Hebenstreit et al., 1989). Emesis is one of the most common side effects of PDE4 inhibitors. The mechanism by which PDE4 inhibition results in increased vomiting and nausea in species such as dog and human (Bertolino et al., 1988; Hebenstreit et al., 1989; Heaslip and Evans, 1995) is not fully understood, but probably includes both central and peripheral sites of actions. Emesis induced by PDE4 inhibitors in peripheral sites was initially described with rolipram (Puurunen et al., 1978). This effect is presumably related to the ability of PDE4 inhibitors to increase parietal cell cAMP content, enhancing acid gastric secretion, an effect which correlates with the affinity for high affinity rolipram binding sites (HARBS) (Barnette et al., 1998). Other studies on the potentiation of apomorphine-induced emesis in dogs by RO20-1724 (Carpenter et al., 1988) suggest that nausea and vomiting are likely to be produced, at least in part, via the direct stimulation at the emetic centers in the brain. Consistent with these studies the administration of rolipram or 6-(4-pyridylmethyl)-8-(3-nitrophenyl)-quinoline (PMPQ) in rats, elevated the Fos-like immunoreactivity in brain regions potentially relevant to the emetic effects of PDE4 inhibition (Bureau et al., 2006). The significance of emetic central sites has been additionally validated experimentally by the use of YM976, a PDE4 inhibitor with low emetogenic potential likely due to its poor brain penetration and low affinity for HARBS (Aoki et al., 2001).

The presence of mRNAs coding for PDE4B and PDE4D has been reported in the AP in rats (Pérez-Torres et al., 2000), suggesting that cAMP signaling modification in this area could mediate the emetic effects of PDE4 inhibitors. Here, we describe experiments aimed at the analysis of both regional and cellular expression of the mRNA coding for PDE4A, PDE4B PDE4C and PDE4D in AP and in other medullary nuclei, i.e., XII, NTS, DMNV, NSTT, ION, Cu, Gr, and lateroreticular nucleus (LatRet).

CAROTID BODY

The carotid body is a small ellipsoid tissue mass, which is located posterior to the carotid bifurcation or between its branches. It is an arterial chemoreceptor which induces increases in ventilatory volume and frequency in response to hypoxia, hypercapnia, or reduction of blood pH. The carotid body is organized in lobules, separated by thin connective septa arising from the capsule (Fig. 1A). The cells of the lobules belong to two different populations: type I (or chief) cells, with much cytoplasm and a few dendritic processes extending into extracellular spaces, and type II (or sustentacular) cells, with fusiform shape and located at the edges of the clusters. Type I cells release dopamine in response to carotid body stimulation and are in turn divided into light, dark, and pyknotic cells, which can be clearly recognized on the basis of their nuclear and cytoplasmic configuration (Hurst et al., 1985; Pallot, 1987; Smith et al., 1986). Light cells have large nuclei, with pale haematoxylin staining and open vesicular pattern of chromatin. Dark cells show smaller and darkly-staining nuclei, due to dense packing of heterochromatin. As to the cytoplasm, it is faintly eosinophilic, with indistinct margin, in the light cells and more intensely stained in the dark cells. Pyknotic cells have a small condensed nucleus and a small rim of darkly-staining cytoplasm (Hurst et al., 1985; Smith et al., 1986) (Fig. 1B-C).

Molecular mechanisms of chemoreception have been proposed in low-O2 affinity mitochondrial cytochromes, NADPH oxidase and heme oxygenase 2 (reviewed in Prabhakar and Jacono, 2005). Type II cells show astrocytic markers and play a supportive role (Pallot et al., 1986; Pallot, 1987), although it has also been reported that these cells exposed to prolonged hypoxia may behave as stem cells precursors for type I cells (Pardal et al., 2007).

The carotid body is mainly innervated by sensitive glossopharyngeal fibres, which centrally project to the commissural subnucleus of the NTS. Post-ganglionic sympathetic nerve fibres from the superior cervical ganglion are present, innervating blood vessels and type I cells

(Verna, 1997). Preganglionic parasympathetic and sympathetic fibres also reach ganglionic cells, which are either isolated or found in small groups near the surface of the carotid body (McDonald and Mitchell, 1975; Standring et al., 2005). Axons from the ganglion cells innervate the blood vessels, the parasympathetic axons being vasodilator (Biscoe et al., 1969) and the sympathetic vasoconstrictor (Purves, 1970).

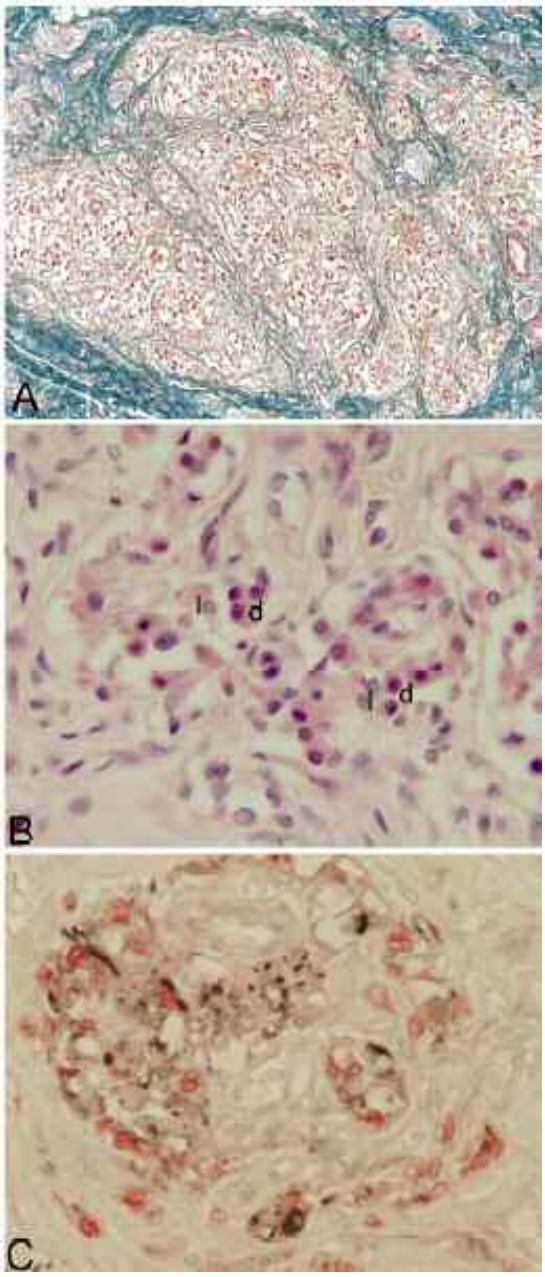

Fig. 1. A, Longitudinal section of human carotid body, showing parenchymal and connective components (azan-Mallory; original magnification 10X). B, Section of carotid body showing light (l) and dark (d) cells (original magnification 63X). C, Double-labelling immunohistochemistry with anti-neurone-specific enolase (black stain) and anti-S100 (red stain) antibodies, showing type I and II cells, respectively (original magnification 40X).

DEVELOPMENTAL CHANGES OF THE CAROTID BODY

The carotid body develops as a condensation from the mesenchyme of the third pharyngeal arch. As regards its functional development, this chemoreceptor does not contribute much to breathing during foetal life, in spite of a hypoxia level of about 25 torr (Blanco et al., 1984), and its activity is not necessary in order to establish rhythmic breathing at birth (Jansen et al., 1981). In the following postnatal period a gradual increase in hypoxic chemosensitivity develops, with a change in the hypoxic threshold to 55 torr (Blanco et al., 1984, 1988; Gauda et al., 2004) and an increase in the slope of the hypoxic stimulus response curve (Blanco et al., 1984; Bamford et al., 1999; Kholwadwala and Donnelly, 1992; Gauda et al., 2004).

Postnatal changes in the peripheral chemoreceptors are listed in Table 2. In adult cat, a three-fold increase in the volume of the carotid body has been described with respect to newborns (Clarke and Daly, 1985; Clarke et al., 1990). Some authors found very few mitoses in the carotid body of newborn kitten (Clarke and Daly, 1985), rat (von Dalnok and Menssen, 1986) and human (Heath et al., 1990), assuming improbable postnatal increase in the number of type I cells. However, it has been found that type I cells from newborn rats divide in culture (Fishman and Schaffner, 1984; Nurse and Fearon, 2002) and Wang and Bisgard (2005) identified bromodeoxyuridine-labelled type I cells in newborn rats, providing evidence of proliferation of type I cells in the postnatal period.

Some cellular and molecular changes associated with postnatal increase in hypoxic chemosensitivity have been identified in the higher density of K^+-channels (Peers and O'Donnell, 1990), a greater rise in intracellular Ca^{2+} levels in response to hypoxia (Sterni et al., 1995; Bamford et al., 1999) and changes in neurotransmitter profile (Gauda et al., 1996, 2000, 2004). Immunohistochemical and hybridisation studies have shown increased neuropeptide Y (NPY) expression (Oomori et al., 1991) and reduced tyrosine hydroxilase (Sterni et al., 1995; Holgert et al., 1995; Bamford et al., 1999), cannabinoid 1 (McLemore et

al., 2004) and dopamine 2 receptor expression (Sterni et al., 1995; Holgert et al., 1995; Bamford et al., 1999). In rats, an increase in the number of dense core vesicles of type I cells has also been found by the end of the first postnatal week (von Dalnok and Menssen, 1986). Proliferation of endothelial cells and type II cells (Wang and Bisgard, 2005) and increased number of synapses between type I and II cells (Kondo and Iwasa, 1996) have also been described in newborn rats. As regards innervation of glomic cells, some authors have found proliferation of Schwann cells lining peripheral nerve fibres (Wang and Bisgard, 2005) and glial and Schwann cells in the petrosal and superior cervical ganglia of rats (Wang and Bisgard, 2005; Lemke, 2001).

Postnatal changes in the carotid body may be ascribed partly to the role of growth factors, such as brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF), produced by type I cells (Hertzberg et al., 1994a, 1994b; Erickson et al., 2001; Wang and Bisgard, 2005). Trk B, which is the receptor of BDNF, is located in both type I cells and chemoafferent neurons, suggesting that it has autocrine and paracrine functions. Ret, which is the receptor of GDNF, is instead only found in petrosal sensory neurons and nerve fibres (Wang and Bisgard, 2005).

Table 2. Postnatal changes in carotid body

CAROTID BODY		
Changes	**Species**	**References**
Proliferation of type I cells	Rat	Wang and Bisgard, 2005
Improbable postnatal increase	Cat	Clarke and Daly, 1985
in type I cells	Rat	Von Dalnok and Menssen, 1986
	Human	Heath et al., 1990
↑ dense core vesicles in type I cells	Rat	Von Dalnok and Menssen, 1986
↑ K^+ channels	Rat	Peers and O'Donnell, 1990
↑ rise in [Ca2+]i in response to hypoxia	Rabbit	Sterni et al., 1995
	Rat	Bamford et al., 1999
↑ neuropeptide Y	Rat	Oomori et al., 1991
↓ tyrosine hydroxylase	Rabbit	Sterni et al., 1995
	Rat	Holgert et al., 1995
	Rat	Bamford et al., 1999
↓ cannabinoid 1 receptors	Rat	McLemore et al., 2004
↓ dopamine 2 receptors	Rabbit	Sterni et al., 1995
	Rat	Holgert et al., 1995
	Rat	Bamford et al., 1999
↑ endothelial cells	Rat	Wang and Bisgard, 2005
↑ type II cells	Rat	Wang and Bisgard, 2005
↑ synapses between type I and II cells	Rat	Kondo and Iwasa, 1996
↑ Schwann cells of nerve fibres	Rat	Wang and Bisgard, 2005

HYPOXIC CHANGES IN THE CAROTID BODY

In the carotid body, chronic hypoxia causes a series of morphological, cellular and biochemical changes, most of which may be explained by the action of neurotrophic factors. In chronic hypoxia due to lung disease or cyanotic congenital heart disease (Lack, 1978; Heath et al., 1982; Smith et al., 1986; Habeck, 1986), carotid bodies have been found to be enlarged, with a percentual increase in type II cells. In opiate addiction, the percentage of type II cells and intralobular and interlobular connective components have also been found to be increased (Porzionato et al., 2005a). The percentage of type I cells has been reported to decrease in chronic obstructive pulmonary disease (Heath et al., 1986; Vinhaes et al., 2002) and increase in chronic high-altitude hypoxia (Arias-Stella and Valcarcel, 1973, 1976). Chronic hypoxia has been shown to increase O2 sensitivity in the carotid body through changes in molecular chemoreceptors, ion channels and neurochemicals (reviewed in Prabhakar and Jacono, 2005; Powell, 2007). NADPH oxidase plays an important role in plasticity during chronic hypoxia, producing more superoxide which may enhance the effects of other neuromodulators. Decreased K^+ channel density and increased Na^+ and Ca^{2+} channel densities have been found in glomus cells cultured in conditions of chronic hypoxia or in carotid bodies of chronically hypoxic neonatal rats (Stea et al., 1992; Hempleman, 1995, 1996). Acetylcholine has been considered a neurotransmitter involved in increased O2 sensitivity in chronic hypoxia, due to increased nicotinic cholinergic receptors on carotid body afferent nerves. Endothelin 1 is the trophic factor which has been studied mainly with reference to hypoxic adaptations, but other trophic factors are also involved.

CAROTID BODY TRANSPLANTATION IN NEURODEGENERATIVE PATHOLOGIES

Recently, intrastriatal autotransplantation of carotid body cells has been proposed as a therapeutic approach for Parkinson's disease, as unilateral surgical resection of the carotid body has been found to produce no significant side-effects (Honda, 1992). The root cause of Parkinson's disease is degeneration of the neurons of the substantia nigra, which send dopaminergic connections to the striatum. Thus, the aim of carotid body grafts is to restore dopamine release in the striatum. Neverthless, experimental studies demonstrated that amelioration of the motor syndrome in carotid body-grafted rats mainly relies on restoration of the nigrostriatal dopaminergic pathway, due to expression of the GDNF by glomus cells, more than on simple dopamine release (Toledo-Aral et al., 2003; Villadiego et al., 2005). Thus, intracerebral transplantation of carotid body has also been proposed for reduction of stroke-induced deficits and a first experimental study demonstrated reduced stroke-induced motor deficits and cerebral infarction in rats after carotid body grafts (Yu et al., 2004). Neurotrophic effects of carotid body grafts probably rely not only on GDNF production but also on many other growth factors. In particular, Yu et al. (2004) reported increased levels of GDNF, Neural Growth Factor and BDNF in the ipsilateral ischemic striatum collected from stroke animals which received carotid body grafts. Moreover, glomus cells have been verified to be well suited for intracerebral transplantation, due to their extended viability in low oxygen conditions. Carotid body grafts show remarkable longevity, appearing to be active for almost the entire life of the animal, and maintaining a stable phenotype and typical organization in glomerula. Growth factor production by glomus cells, also sustained by hypoxia, probably plays a pivotal role in permitting long-term viability of implants.

NEUROMODULATORS IN THE CAROTID BODY

Many neurotransmitters and neuromodulators have been identified in the carotid body and chemoreception in the carotid body is known to be modulated by interaction between various factors. There is also increasing evidence in the literature that neurotransmitters and neuromodulators can have trophic effects on various cell types and also on glomic cells (reviewed in Schwartz, 1992; Porzionato et al., 2008a). In the following section we consider peptides, the role of which in glomic neuromodulation has been suggested, although some components (neuromodulators or receptors) have not yet been fully investigated. Some of these peptides have been studied in the present work, others will be evaluated in the next years.

The calcitonin peptide family comprises calcitonin, calcitonin gene-related peptide (CGRP), adrenomedullin (AM), amylin, intermedin, and calcitonin receptor-stimulating peptides. Calcitonin is a 32 amino acid peptide produced by the parafollicular cells of the thyroid gland, the main activity of which is inhibition of bone resorption by direct action on osteoclasts. CGRP is a 37 amino acid peptide derived from alternative splicing of the calcitonin gene. CGRP is widely distributed in the central and peripheral nervous systems, where it plays a role as neurotransmitter or neuromodulator. AM, a multifunctional regulatory peptide, was originally isolated in human pheochromocytoma (Kitamura et al., 1993) and has been detected in human brain, heart, kidney, lung, and adrenal cortex and medulla (reviewed in Nussdorfer, 2001). In the central nervous system, AM may play a role as a neurotransmitter, neuromodulator, neurohormone, or as a cytoprotective factor in ischemic/hypoxic conditions, through its vasodilator role (reviewed in López and Martínez, 2002). As regards the receptors of these peptides, the calcitonin receptor (CTR) belongs to the type II seven-transmembrane G protein-coupled receptors. The interaction of calcitonin

receptor-like receptor (CRLR) with subtype 1 of a family of receptor activity-modifying proteins (RAMP) gives rise to the CGRP receptor; the interactions of CRLR with RAMP2 and RAMP 3 give rise to two distinct AM receptors, AM1 and AM2 (McLatchie et al., 1998). Associations of CTR with the three RAMPs give rise to three different amylin receptors, AMY_1, AMY_2 and AMY_3 (Lerner, 2006).

Calcitonin-like immunoreactivity has been found in chief cells of the human infant carotid body by light- and electron-microscopy immunohistochemical techniques (Wang et al., 1993b), but data on the expression of the corresponding receptor are still lacking. There is evidence that CT is also involved in cell growth, differentiation and morphogenesis. It plays a role in the development of the mouse early blastocyst (Wang et al., 1998a). It shows antiproliferative activity in human breast cancer cells (Ng et al., 1983) and HEK-293 cells over-expressing the insert negative hCTR (Evdokiou et al., 1999; Findlay et al., 2002) but is mitogenic in certain prostate cancer cell lines (Shah et al., 1994). CT also has antiapoptotic effects in osteoblast/osteocyte-like cells (Plotkin et al., 1999) and prostate cancer cell lines (Salido et al., 2000), and potentiates neuronal death due to hypoxia and glucose deprivation (Asrari and Lobner, 2001). In light of the investigations herein reported, it may be hypothesized that CT expressed by carotid body type I cells may exert trophic effects also on carotid body itself.

Immunohistochemical studies have identified **CGRP** in nervous terminations proximal to blood vessels and glomic cell clusters in mammals, birds and amphibians (Kondo and Yamamoto, 1988; Kameda, 1989; Kummer and Habeck, 1991; Kusakabe, 1992; Kusakabe et al., 1995). Immunoreactivity for CGRP was not found in the glomus cells of normoxic or chronically hypoxic rat carotid bodies (Kusakabe et al., 1998). The density of CGRP fibers has been found to decrease significantly in the chronically hypoxic rat carotid body (Kusakabe et al., 1998) and to increase 4 weeks after the termination of hypoxia (Kusakabe et

al., 2004). CGRP-induced proliferation has been reported in several cell types, such as endothelial (Haegerstrand et al., 1990), Schwann (Cheng et al., 1995) and rat smooth muscle cells (Mitsuhashi and Payan, 1987), fibroblasts (Kawase et al., 1999), retinal pigment epithelial (Kishi et al., 1996) and neurogenic cancer cells (Pluder et al., 2007). However, antiproliferative effects have also been found in rat vascular smooth muscle cells (Li et al., 1997; Connat et al., 2001; Qin et al., 2004; Chattergoon et al., 2005) and human retinal pigment epithelial cells (Troger et al., 2003). Different effects have been ascribed to different species or cell types, and it has been suggested that the action of CGRP is more likely to modulate the action of other trophic factors than to have a direct effect (Connat et al., 2001). Although CGRP has been identified in nerve fibers and not in glomic cells, it cannot be excluded that release of CGRP by nerve fibers may exert some local trophic effect.

AM is a multifunctional regulatory peptide which was originally isolated in human pheochromocytoma (Kitamura et al., 1993a). AM gene expression has been detected in human heart, kidney, lung, and adrenal cortex and medulla (Kitamura et al., 1993b). Immunoreactive AM was also detected in many regions of human brain, with the highest concentrations found in thalamus and hypothalamus (Satoh et al., 1996). As a consequence it has been suggested that AM may play a role as a neurotransmitter, neuromodulator, or neurohormone, or as a cytoprotective factor in ischemic/hypoxic conditions, through its vasodilator role (Satoh et al., 1996; Kis et al., 2001). AM has been found in the chromaffin cells of the adrenal medulla (Kapas et al., 1998), a cellular type which shows a similar mechanism of response to hypoxia, with dopamine release and increased expression of tyrosine hydroxylase (Millhorn et al., 1996), and a common embryonic origin with the glomus cells from the neural crest (Crowder, 1957; Le Douarin et al., 1972). Moreover, Martinez et al. (2003) found AM immunoreactivity in type I cells of rat carotid bodies, and observed that exposure of carotid bodies to AM resulted in dopamine release from these cells.

One of the aims of the present study was to detect AM immunoreactivity also in the human carotid body, with particular reference to the different cellular populations and to the age of the subjects. It is known that AM enhances proliferation in human tumor cell lines (Miller et al., 1996), endothelial cells (Xia et al., 2006) and in cultured cells from rat and human adrenal zona glomerulosa (Nussdorfer, 2001; Trejter et al., 2002b) and in rat thymus (Belloni et al., 2003), an organ sharing a common embryonic origin with the glomus cells from the neural crest. It can also inhibit the apoptosis of several cell types, including neurons and glial cells (Xia et al., 2006). Thus, it has been suggested that AM plays a role in mediating hyperplasia of glomus cells in response to hypoxia (Martinez et al., 2003; Porzionato et al., 2006a), although further experimental studies on animals subjected to hypoxic stimuli will be necessary.

NT is a tridecapeptide, first isolated from bovine hypothalamus (Carraway and Leeman, 1973) and widely distributed in the central and peripheral nervous systems, mainly acting as a neurotransmitter and neuromodulator. Three different NT receptors, NTR1, NTR2 and NTR3, have been identified. NTR1 and NTR2 are, respectively, high- and low-affinity seven trans-membrane domain G protein-coupled receptors. NTR3 is a high-affinity single trans-membrane domain type 1 receptor (Kitabgi, 2006; Mazella et al., 1998; Martin et al., 2002; Pelaprat, 2006). Nuclear internalization of all three has been reported, and it has been suggested that they play a role in producing long-term genomic effects (Feldberg et al., 1998; Laduron, 1992; Mazella and Vincent, 2006). Neurotensin has been demonstrated to stimulate proliferation of various normal and neoplastic tissues (for a review, see Evers, 2006) (Markowska et al., 1994). It also acts as a growth factor on various normal or cancer cells and can exert immunomodulating actions (Navarro et al., 2006; Pelaprat, 2006). NT binding sites

have been widely identified in brain, intestine (Carraway and Mitra, 1994), blood mononuclear cells (Goldman et al., 1982) and endothelial cells (Schaeffer et al., 1995).

NT occurrence in glomic type I cells has been found by radioimmunoassay, immunohistochemistry and immunogold techniques in humans and experimental animals (Heath et al., 1988; Heym and Kummer, 1989; Smith et al., 1990). Thus, other aim of the present study was to investigate, through immunohistochemistry, the presence and location of NTR1 in the carotid body of both human and rat, with particular reference to the different cell types.

Galanin is a 29 amino acid neuropeptide, widely distributed in the central and peripheral nervous system. It is thought to act directly or by modifying the release of other neurotransmitters. Three G protein-coupled receptors (GalR1, GalR2, GalR3) have now been identified (Branchek et al., 2000). Galanin has been identified by immunohistochemistry in the nerve fibers of rat and chicken carotid body (Kameda, 1989; Ichikawa and Helke, 1993). In rat carotid body, Ichikawa and Helke (1993) reported the disappearance of galanin-immunoreactive nerve fibers after transection of the carotid sinus nerve, and acquisition of galanin immunoreactivity in originally immunonegative glomic cells and nerve fibers, probably originating from the superior cervical ganglion. In chicken carotid body, galanin immunoreactivity has been reported to originate from the 14^{th} cervical ganglion of the sympathetic trunk (Kameda, 1999). Galanin has antiproliferative effects on thymocytes, cultured neural stem cells, olfactory-ensheathing cells, PC12 cell lines and proproliferative action on the B104 neuroblastoma cell line, due to binding to GalR1 and GalR2/3, respectively (Trejter et al., 2002a; Shen et al., 2005; Xia et al., 2005; Cheng and Yuan, 2007). The presence of galanin in nerve fibers also suggests the possibility of local action on the carotid body. Identification of galanin receptor types in carotid body components and

experimental studies on carotid body cell cultures may also identify the presence and type (proliferative or antiproliferative) of trophic actions on glomic cells.

NPY immunoreactivity has been found in nerve fibers and type I cells of dog, monkey and rat carotid body (Oomori et al., 1991, 2002). In the rat carotid body, NPY-immunoreactive type I cells were more numerous from birth to postnatal week 1, but quickly decreased from postnatal week 2 onward. Instead, NPY-immunoreactive fibers were scarce at birth and increased mainly after postnatal week 2 (Oomori et al., 2002).

Carotid body type I cells have been shown to exhibit Met/Leu-**enkephalin**-like immunoreactivity (Lundberg et al., 1979; Wharton et al., 1980; Hansen et al., 1982; Varndell et al., 1982; Heym and Kummer, 1989; Smith et al., 1990; Scraggs et al., 1992) in cat, dog, rabbit, shrew, piglet and human. In the rat carotid body, nerve fibers but not type I cells show Met-enkephalin-like immunoreactivity (Heym and Kummer, 1989). Type I cells of pig and guinea pig show prevalent immunoreactivity for dynorphins (Heym and Kummer, 1989). Chemoinhibitory action on the cat carotid body has been revealed for morphine, enkephalins and beta-endorphin (McQueen and Ribeiro, 1980, 1981a, 1981b), and physiological chemoreceptor stimulation decreases enkephalin content in the rabbit carotid body (Hanson et al., 1986). Experimental studies in cats involving selective opioid receptor agonists and antagonists provided evidence that depression of the chemosensory discharge caused by opioids involves delta-opioid receptors (Kirby and McQueen, 1986) and such receptors have been identified by immunohistochemistry in both rat carotid body type I cells and nerve fibers (Ichikawa et al., 2005).

Tachykinins are neuropeptides encoded in mammals by three different genes, preprotachykinin A, B, and C (ppt-a, ppt-b, ppt-c). Ppt-a may encode substance P, neurokinin A, neuropeptide γ and neuropeptide K. Ppt-b is the gene of neurokinin B. Ppt-c encodes a protein which contains the sequence of hemokinin 1 and endokinins C and D (Conlon, 2004). The biological actions of tachykinins are mediated by binding with the NK1, NK2 and NK3 receptors, which show preferential affinity for substance P, neurokinin A and neurokinin B, respectively (Henry, 1987). Some novel tachykinin receptor types/subtypes have been claimed to exist, although sufficient scientific evidence has not yet been endowed (Patacchini and Maggi, 2004).

Substance P (Wharton et al., 1980; Cuello and McQueen, 1980; Chen et al., 1986; Hanson et al., 1986; Prabhakar et al., 1989; Heym and Kummer, 1989; Smith et al., 1990) and **neurokinin A** (Prabhakar et al., 1989) immunoreactivities have been found in type I cells and nerve fibers of the mammalian carotid body. In the cat carotid body, they increased the chemosensory response to hypoxia, and substance P has been found to bind to NK-1 receptors (Prabhakar et al., 1989). Some authors have considered the changes in tachykinin content following acute or chronic hypoxia. Hanson et al. (1986) reported a reduction of substance P in rabbit carotid body after 1 hour exposure to hypoxia; for the same type of stimulus, Prabhakar et al. (1989) reported increased levels of substance P in cats. Lastly, chronic hypoxia was found to dramatically reduce the number of substance P-positive type I cells in cats (Wang et al., 1998b) but did not affect substance P immunoreactivity in rats (Poncet et al., 1996). In the cat carotid body, neurokinin A content was not modified by acute hypoxia but was reduced by acute hyperoxia (Prabhakar et al., 1989).

Somatostatin-immunoreactive nerve fibers have been identified in the chicken (Kameda, 1989), guinea pig (Kummer et al., 1989) and amphibian (Kusakabe et al., 1991) carotid body, but not confirmed in humans (Kummer and Habeck, 1992). Infusion of somatostatin in humans selectively decreases the hypoxic but not the hypercapnic response, suggesting action on the carotid body, although not excluding a central effect (Maxwell et al., 1986). Somatostatin has also been shown to inhibit whole-cell Ca^{2+} current in rat type I cells (e Silva and Lewis, 1995).

Bombesin is a 14 amino acid peptide which was first isolated from amphibian skin (Anastasi et al., 1971) and then from porcine gastric and intestinal tissue (McDonald et al., 1979), with the name of gastrin-releasing peptide. An homolog protein to gastrin-releasing peptide is neuromedin B (NMB), which was first identified from porcine spinal cord (Minamino et al., 1983). In mammals, three receptors for bombesin-like peptides have been identified: gastrin-releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R), with higher affinity for the corresponding peptides, and bombesin-like peptide receptor subtype 3 (BRS-3), which shows lower affinity for bombesin than GRP-R and NMB-R (reviewed in Ohki-Hamazaki et al., 2005). In the literature, the presence of NMB in the carotid body has not been investigated. Although an early immunohistochemical study (Helen et al., 1984) in rats did not reveal bombesin in glomic type I cells, it was then found in the human carotid body through radioimmunoassay (Heath et al., 1988) and immunohistochemistry in type I cells (Smith et al., 1990). Instead, bombesin immunostaining was not found in the human carotid bodies of foetuses at gestational ages of 13-19 weeks (Scraggs et al., 1992).

Cholecystokinin (CCK) is a multifunctional regulatory peptide which acts through two main subtypes of receptors, CCK-A and CCK-B receptors. CCK has been identified by

immunohistochemistry in type I cells of dog, Callithrix (Heym and Kummer, 1989) and human infant carotid body (Wang et al., 1993b). In cats anesthetized with pentobarbitone, spontaneous chemoreceptor discharge has been reported to be increased by intracarotid injection of CCK (McQueen and Ribeiro, 1981a).

Vasoactive Intestinal Peptide (VIP) - Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) – Secretin FamilyThe VIP/PACAP/secretin family comprises VIP, PACAP, secretin, glucagon, glucagon-like peptide-1 (GLP(1)), GLP(2), gastric inhibitory peptide (GIP), growth hormone releasing hormone, and peptide histidine methionine. Except for VIP and PACAP, there are no data in the literature regarding the presence of the above peptides in the carotid body. VIP is a 28 amino acid peptide which is widely distributed in the central nervous system. PACAP is a neuropeptide, originally purified from ovine hypothalamus (Miyata et al., 1989), which has been found in both central and peripheral nervous systems and in endocrine organs, such as adrenal gland, islets of Langerhans and testis. For VIP and PACAP, three G protein-coupled receptors have been reported: PAC1-R, showing much higher affinity for PACAP than for VIP, VPAC1-R and VPAC2-R, showing equal affinity for PACAP and VIP (Harmar et al., 1998).

VIP has been found by radioimmunoassay in human carotid body (Heath et al., 1988) and by immunohistochemistry in nervous terminations in the carotid body of mammals, birds and amphibians (Wharton et al., 1980; Kameda, 1989; Kummer, 1990; Kusakabe et al., 1991; Kusakabe, 1992; Poncet et al., 1994; Kusakabe et al., 1995, 2003). Weak VIP immunoreactivity was also found by Smith et al. (1990) in type I cells of human carotid body, although their findings were not confirmed in the glomus cells of normoxic or chronically hypoxic rat carotid bodies by another team of researchers (Kusakabe et al., 1998). In the rat, the density of VIP fibers in the chronically hypoxic carotid body significantly increases

(Poncet et al., 1994; Kusakabe et al., 1998) and return to density of a normoxic state in few weeks after termination of hypoxia (Kusakabe et al., 2004). The effects of VIP on carotid chemoreceptor activity have been investigated in cats, resulting in decrease or increase of spontaneous chemoreceptor discharge by intracarotid injection of low or high doses of VIP, respectively (McQueen and Ribeiro, 1981a). VIP causes vasodilation of blood vessels and it may be involved in chemosensory mechanisms by controlling local circulation (Kusakabe et al., 1998).

It has been found that intravenous injection of **PACAP** in dogs causes an increase in ventilation, which is abolished by cutting of the carotid sinus nerve (Runcie et al., 1995), and other studies have shown that PACAP-deficient mice show a reduced respiratory response to hypoxia and hypercapnia (Cummings et al., 2004) and are more prone to sudden neonatal death (Gray et al., 2001, 2002; Cummings et al., 2004). These findings suggest that PACAP may contribute to chemosensory transduction in the carotid body. Thus, a recent experimental study on rats demonstrated that PACAP, acting via the PAC1-R coupled PKA pathway, inhibits a TWIK-related acid-sensitive K^+-like K^+ channel current and causes depolarization and voltage-gated Ca^{2+} channel activation. The additive effect of PACAP and moderate hypoxia has also been found, suggesting that PACAP may augment the hypoxia-mediated $[Ca^{2+}]_i$ elevation in type I cells (Xu et al., 2007, 2008).

Angiotensin II (ANG II), an octapeptide derived from the circulating renin-angiotensin system, plays a crucial role in the regulation of cardiovascular function and electrolyte/water homeostasis. Intrinsic angiotensin-generating systems have also been found in organs such as the pancreas, epididymis and carotid body. AT1 and AT2 receptors have been identified, which are members of the seven-transmembrane G protein-coupled receptor family (Murphy et al., 1991; Sasaki et al., 1991).

Angiotensinogen and ANG-converting enzyme, but not renin, are expressed in the rat carotid body, suggesting an intrinsic angiotensin-generating system linked to a renin-independent biosynthetic pathway (Lam and Leung, 2002). RT-PCR has identified gene expression of both AT1 and AT2 receptors in the rat carotid body and AT1 receptors have also been identified in type I cells by immunohistochemistry (Leung et al., 2000; Fung et al., 2001). In the rat carotid body, ANG II has been found to increase the afferent activity of the carotid body (Allen, 1998). In the rabbit carotid body, it increases expression of NADPH oxidase components (Li et al., 2007) and the sensitivity of voltage-gated K^+ channels to hypoxia in type I cells by binding to AT1 receptors (Li and Schultz, 2006). In rat chronically hypoxic carotid bodies, local up-regulation of angiotensinogen expression, ANG- converting enzyme expression and enzymatic activity has been found (Lam and Leung, 2003; Lam et al., 2004). In chronic heart failure in rabbits, elevation of endogenous ANG II and up-regulation of AT1 receptors in the carotid body contribute to enhanced carotid body chemosensitivity (Li et al., 2006). Chronic hypoxia increases ANG II sensitivity in both rat pups and adults (Leung et al., 2000; Fung et al., 2002). In the rat carotid body during early maturation, chronic hypoxia up-regulates AT_{1a} receptor subtype in type I cells and down-regulates AT_{1b} receptor subtype (Fung et al., 2002); in adult rats, it enhances both AT_{1a} and AT_{1b} receptor subtype expression (Leung et al., 2000).

Three mammalian **natriuretic peptides** have now been identified, i.e., atrial (ANP), B-type (BNP) and C-type (CNP). Three different natriuretic peptide receptors have also been identified, i.e., NPR-A, NPR-B and NPR-C, the first two being transmembrane guanylyl cyclases (reviewed in Takei, 2000; Potter et al., 2006). ANP-like immunoreactivity has been located in cat and human carotid body type I cells (Wang et al., 1991, 1992; Benvenuti et al., 1996). It has been showed that incubation of carotid body with synthetic ANP analog atriopeptine III elevates cGMP content, indicating the presence of receptors coupled to membrane-bound guanylyl cyclase (Wang et al., 1992). ANP superfusion in *in vitro* rabbit carotid body preparations showed inhibition of chemosensory discharge (Wang et al., 1993a; He et al., 2000), whereas intra-arterial ANP stimulated cat carotid body chemoreceptor activity in vivo (Di Giulio et al., 2003), these different effects probably being due to differing experimental conditions. There are no data available on the presence of BNP and CNP in the carotid body.

ADHESION MOLECULES IN THE CAROTID BODY

Carotid body cells are known to express many different growth factors and corresponding receptors (reviewed in Porzionato et al., 2008a). Conversely, little is known about expression of cell adhesion molecules (CAMs) by different carotid body cell types, although such proteins have been suggested to play a pivotal role in modulation of the actions of neurotransmitters/neuromodulators and growth factors. CAMs are cell surface molecules grouped into four subclasses based on their structural characteristics, i.e., cadherins, selectins, the integrin family, and the immunoglobulin (Ig) superfamily. Neural Cell Adhesion Molecule (NCAM) is a member of the Ig superfamily and shows five membrane-distal Ig modules and two membrane-proximal fibronectin type III modules. NCAM is encoded by a single gene (*NCAM1*) but alternative splicing gives rise to several different isoforms, NCAM-

120, -140, and -180 being the three major ones (Hansen et al., 2008). NCAM also undergoes posttranslational modifications, the most important of which is addition of polysialic acid (PSA). PSA is widely accepted to modify the adhesive properties of NCAM, probably decreasing NCAM omophilic binding and favoring eterophilic binding to transmembrane receptors, such as fibroblast growth factor receptor. It has been reported that NCAM triggers signaling events which can result in many different cellular responses, such as differentiation, survival, or modulation of synaptic plasticity, also through interactions with other cell surface receptors. In the nervous system, PSA content is known to decrease during maturation and its mainly roles have been identified in precursor migration, axon outgrowth/maturation, circuit maturation and plasticity (Rutishauser, 2008). Thus, expression of PSA-NCAM is considered a marker of neural progenitor cells (Izal-Azcarate et al., 2008). Izal-Azcarate et al. (2008) reported PSA-NCAM immunoreactivity in type I cells of rat carotid bodies but to the best of our knowledge there are not yet data regarding *in toto* expression of NCAM in the human carotid body. One of the aims of the present study was to analize NCAM expression in the human carotid body, with particular reference to the different cell populations, and to discuss its possible involvement in the development/differentiation of the carotid body and its changes in response to hypoxia.

SIGNAL TRANSDUCTION SYSTEMS: ERK AND AKT.

Research on the carotid body has mainly focused on neurotransmitters and receptors, while little is known about the signalling pathways involved in neurotransmission/neuromodulation and cellular proliferation, also with reference to the different developmental stages. Two important signalling pathways involved in the integration of extracellular signals and which are particularly important for cell survival and proliferation are the extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways. ERK

belongs to the family of the mitogen-activated protein kinases (MAPK), a group of serine/threonine protein kinases linking various extracellular signals to nuclear events (Waskiewicz and Cooper, 1995; Su and Karin, 1996; Nishio et al., 2001). ERK is activated by dual phosphorylation of tyrosine and threonine residues by the MAPK kinases, MEK1 and MEK2 (Derijard et al., 1995). There are two closely related ERKs, i.e., ERK1 (p44) and ERK2 (p42). Activation of the ERK signalling pathway is known to be implicated in cell proliferation (reviewed in Chambard et al., 2007) and occurs in two phases: a rapid initial phase, in response to many different stimuli, and a later sustained phase, leading to cell proliferation (Chambard et al., 2007).

The PI3K/AKT signalling pathway is upstream activated by growth factors, cytokines and other cellular stimuli. In this pathway, activated receptor tyrosine kinases (RTK) activate class I PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). The serine/threonine kinase AKT binds to PIP3 and is phosphorylated by phosphoinositide-dependent kinase-1 at Threonine 308. RTK signalling also activates mTOR complex 2, which phosphorylates AKT at Serine 473. AKT may then activate many different substrates involved in various cellular functions, such as cell survival, growth, proliferation, metabolism and migration (reviewed in Engelman et al., 2006; Manning and Cantley, 2007). In the nervous system, the PI3K/AKT pathway has also been found to be involved in synaptic plasticity, such as long-term potentiation and long-term depression (Kelly and Lynch, 2000; Sanna et al., 2002; Man et al., 2003; Opazo et al., 2003). There are no data in the literature regarding the involvement of the ERK and PI3K/AKT signalling pathways in the carotid body and in its foetal stages of development. Thus, one of the aims of the present study was to evaluate these pathways in the various cell populations of carotid body in adults and foetuses, by immunolocalization of ERK/pERK and AKT/pAKT.

AIM OF THE STUDY

The main fields of study of our research group in the Department of Human Anatomy and Physiology have been the medulla oblongata (Macchi et al., 2004, 2005, 2006, 2008; Parenti et al., 2005; Porzionato et al., 2004a, 2004b, 2005b, 2006b, 2008b, 2008c, Stecco et al., 2005; Mori et al., 2009) and the carotid body (Porzionato et al., 2005a, 2006a, 2008a, 2008d, 2008e 2009a, 2009b). The aim of the present study was to give a comprehensive and reliable analysis of some neuroanatomic and neuropathologic aspects of the medullary nuclei and carotid body which have not yet been considered in detail in the literature.

Our previous studies about MEDULLARY NUCLEI have mainly considered the local effects of hypoxic-ischaemic injuries in autoptic materials, through neuropathologic examination and analysis of the expression of immediate early genes (Fos). We also considered how neuropathologic findings could be at least partially explained on the basis of neuroanatomic parameters. In the present work, this kind of study was continued with the following aims.

- Analysis of neuroanatomic parameters, as neuronal densities, nuclear volumes, and total neuron numbers, in medullary nuclei of both adults and infants through an unbiased morphometric method called ‘optical disector’;
- Morphometrical evaluation of apoptosis in the same medullary nuclei and casistics, through TUNEL and calculation of apoptotic indexes through optical dissector method;
- Analysis of expression of different PDE4 isoforms in the medullary nuclei of a subgroup of adult medullae oblongatae.

As regards the CAROTID BODY, the review of the literature reported in the Introduction has shown how the local expression of many neuromodulators/neuromodulator receptors, adhesion molecules, trophic factors and signalling pathways components has not yet been

investigated in detail. The present study was aimed at analyse through immunohistochemistry and double immunofluorescence the expression of various proteins not yet considered in detail in the available literature, i.e.,:

- AM,
- NTR1,
- NCAM,
- ERK and pERK,
- AKT and pAKT.

Expression of AM, ERK/pERK, and AKT/pAKT was also evaluated with reference to development of the carotid body, considering two series of carotid bodies sampled from adult and foetal subjects.

MATERIALS AND METHODS

MATERIALS

Analysis of morphometric parameters and distribution of apoptosis in the medullary nuclei was performed on 32 medullae oblongatae sampled during autopsy from the following subjects: 15 adults (9 male, 6 female; age range: 25-58 years; mean age: 40 years; mean death-autopsy interval (DAI) ± Standard Deviation (SD): 29 ± 2.1 hours) who died of myocardial infarct (10 cases), haemorrhagic shock (3 cases) or bronchopneumonia (2 cases); 7 adults (7 male; age range: 20-38 years; mean age: 30 years; mean DAI ± SD: 31 ± 1.9 hours) who died of opiate intoxication; 10 infants (5 male, 5 female; age range: 1 month-1 year; mean age: 5 months; mean DAI ± SD: 30 ± 2.0 hours) who died of Sudden Infant Death Syndrome (SIDS) (5 cases), bronchopneumonia (3 cases) or cardiopathologic causes (2 cases).

Analysis of the expression of the PDE4 isoforms through *in situ* hybridization was performed on brainstems sampled during autopsy from 8 of the above adult subjects (6 males, 2 females; age range: 31-71 years; mean age: 53 years) who died of myocardial infarct (4 cases), opiate intoxication (2 cases), bronchopneumonia (1 case) or haemorrhagic shock (1 case).

As regards opiate deaths, confirmation of heroin intoxication and a search for other drugs of abuse (cocaine, methadone, amphetamine, benzodiazepines, cannabis, alcohol) were performed by toxicological immunochemical screening (Enzyme Multiplied Immunoassay Technique) and confirmatory chromatographic techniques (High-Performance Liquid-Chromatography and Gas-Chromatography, coupled with Mass Spectrometry in Selective Ion Monitoring mode), on urine and venous blood samples. In all opiate deaths, morphine was detected in blood (range: 0.3-2.7 μg/ml) and in 5 cases also in urine (range: 0.2-11.6 μg/ml). In 5 cases, alcohol was detected in blood (range: 30-175 mg/100ml) and in 4 cases also in

urine (range: 10-264 mg/100ml). In 1 case, tetrahydrocannabinol was detected in urine (0.1 μg/ml).

Autopsies were performed within 48 h of death. Brainstems were cut after fixation in 10% formalin for 7 days and paraffin-embedded. Preliminary histological examination was performed on transverse sections of the medulla oblongata, stained with haematoxylin-eosin, Nissl, Klüver-Barrera and Luxol fast blue. In all cases, examination revealed the absence of acute, chronic, localized or diffuse brain pathology.

Carotid bodies were obtained at autopsy from 16 adult subjects (10 males, 6 females; mean age 44.3 years, SD ± 3.4), clinically negative for chronic pulmonary or cardiovascular diseases, and 6 foetuses obtained from spontaneous abortion (3 males, 3 females; mean gestational age 167 days, SD ± 11). Causes of abortion were eclampsia (4 cases) and traumatic shock (2 cases). In adults, cardiac hypertrophy or preceding myocardial infarction were excluded at autopsy. Autopsies were performed between 24 and 36 hours after death. Right carotid bifurcations were sampled, fixed in neutral 10% formalin and embedded in paraffin wax.

HISTOLOGICAL AND TUNEL TECHNIQUES

In each case, 5 sections, collected 100 μm from each other at the level of the caudal portion of the IV ventricle, were examined by the TUNEL method. Sections were incubated with 20 μg/ml proteinase K for 15 min at room temperature and washed in 0.01 M PBS. They were incubated in 0.3% hydrogen peroxide in deionised H_2O to arrest endogenous peroxidase activity, and then incubated with equilibration buffer (ApopTag, Oncor, Gaithersburg, MD, USA) for 5 min at room temperature. Sections were treated with terminal-deoxynucleotidyl transferase reaction mix for 1 hour at room temperature, soaked in stop-wash buffer, and

incubated for 15 min at room temperature. Sections were then incubated with horseradish peroxidase streptoavidin, diluted 1:500 in PBS, for 30 min at room temperature. After the reaction, sections were washed with PBS, placed in 0.03% 3,3'-diaminobenzidine (DAB, Sigma, Milan, Italy) containing 0.01% H_2O_2, and counterstained with haematoxylin. For positive controls, incubation with DNase was performed. For negative controls, the terminal-deoxynucleotidyl transferase reaction mix was omitted.

MORPHOMETRIC ANALYSIS

Sections were examined under a Leica DM4500B microscope (Leica Microsystems, Wetzlar, Germany) with a Leica DFC320 high-resolution digital camera (Leica Microsystems), and image data were transmitted to a personal computer equipped with appropriate software for image acquisition and analysis (QWin, Leica Microsystems). The nuclei examined included the XII, DMNV, NTS, MedVe, Cu, NSTT, PION, MION and DION. The boundaries of these nuclei were defined according to McRitchie and Tork (1993) and Paxinos and Huang (1995). In each section, they were traced as regions of interest. Haematoxylin counterstaining in sections stained with the TUNEL method usually permitted precise delineation of the boundaries of the medullary nuclei. Moreover, tracing of nuclear boundaries was also confirmed by comparison with the adjacent serial sections, stained with haematoxylin-eosin, Nissl, Klüver-Barrera and azan-Mallory. For DMNV, dorsal and medial fringes were not considered. Sections were analysed at primary magnifications of x10-100. Apoptosis was evaluated as neuronal apoptotic index. According also to preceding studies on neuronal apoptosis in medullary nuclei (Machaalani and Waters, 2003; Kiryu-Seo et al., 2005), neurons were distinguished from glial cells on the basis of their larger size, clearly defined neuronal cytoplasm, with Nissl substance, and membrane-bound nucleus with a clear nucleolus (Toft at al. 2005). Although it must be considered that the TUNEL method may

obliterate some nuclear features which can be used to distinguish these cells, a quite acceptable estimate may be provided by referring to size and cytoplasm characteristics. The optical disector method also allows better evaluation of cytoplasm characteristics, due to the greater thickness of the section and movement of the focal plane. Apoptotic phenomena were analysed in term of percentages and not densities, because the cell densities of the nuclei are very different from each other and would not have permitted comparisons. In each section examined by the TUNEL method, all the above-mentioned nuclei were identifiable.

The optical disector method was used to count cell nuclei (Gundersen et al., 1988; Pakkenberg and Gundersen, 1988; West, 1993). The focal plane (or optical section) was moved through the thickness of the section, producing a continuous series of overlapping sections within which counting could be carried out with the following disector counting rules. Counting was performed with high numerical aperture (100X) oil immersion lenses, in order to obtain the smallest possible focal depth. An unbiased counting frame of known area was superimposed on an optical section. Profiles of nuclei in contact with the upper right edges of the frame were considered to be inside the frame, and those in contact with the lower left edges to be outside it. All cell nuclei seen in focus in the first, most superficial, look-up plane were disregarded. Then all cell nuclei which came into focus through the thickness of the section were counted, including those in the last optical section. The thickness of the upper guard area was 1 μm, so that the disector in which the nuclei were actually counted was 9 μm high. Counting was performed on 5 sections per case and 20 fields per section for each nucleus. The total numbers of profiles corresponding to neuronal (Q_N^-) nuclei in each field were counted, and the numbers of the corresponding TUNEL-positive profiles (Q_{NA}^-) were also recorded. The data obtained in each field and section were then summed over the 5

analysed sections to provide the total number of profiles observed ($\sum Q_N^-$) and the number of TUNEL-positive ones ($\sum Q_{NA}^-$).

An estimate of the total number of neuronal nuclei (N_N) and of TUNEL-positive ones (N_{NA}) was performed in a two-step process which involved estimating both the numerical densities of the objects, *NV*, and the volumes of the medullary nuclei, *V*.

For each medullary nucleus, the densities of the neuronal nuclei were calculated as follows:

Neuronal density: $NV_N = \frac{\sum Q_N^-}{\sum Vol(dis) \times \sum P}$

Apoptotic neuronal density: $NV_{NA} = \frac{\sum Q_{NA}^-}{\sum Vol(dis) \times \sum P}$

Vol(dis) was the volume of each disector, i.e., the product of the area of the counting frame and the height of the dissector; $\sum P$ was the total number of the disectors counted in the specimen.

For each nucleus, the total numbers of neuronal (N_N) nuclei were estimated as follows:

Total neuron number: $N_N = NV_N \times V$

Total apoptotic neuron number: $N_{NA} = NV_{NA} \times V$

V was the volume of each medullary nucleus, estimated using Cavalieri's point-counting method (Gundersen and Jensen, 1987; Gundersen et al. 1999):

$$V = a(p) \times \bar{d} \times \sum_{i=1}^{n} P_i(X)$$

where $a(p)$ is the area associated with each sampling point; $\bar{d}$ is the mean distance between two consecutive studied sections (0.2 or 0.8 mm, depending on medullary nucleus); *n* is the

number of sections studied for each nucleus; and $\sum_{i=1}^{n} P_i(X)$ is the sum of points hitting a given target.

For each nucleus, the neuronal apoptotic index was finally estimated as the following ratio:

Neuronal apoptotic index: $I_{NA} = \frac{N_{NA}}{N_N} \times 100$

For each nucleus, mean values (± standard deviations) were calculated for the various sample populations.

PHOSPHODIESTERASE 4 *IN SITU* HYBRIDIZATION

Analysis of PDE4 isoforms expression through *in situ* hybridization was performed in collaboration with the Departament de Neuroquimica i Neurofarmacologia, Institut d'Investigacions Biomèdiques de Barcelona (Prof. Mengod). In each of the 8 cases studied for the expression of PDE4 isoforms, 10 tissue sections, 14 µm thick, were cut on a microtome at the level of the AP and then mounted onto Histogrip-coated slides (Zymed, Carlsbad,CA, USA). Oligonucleotide probes for PDE4A, PDE4B, PDE4C and PDE4D complementary to regions of each mRNA that share little similarity among the PDE family have been used accordingly to Pérez-Torres et al. (2000). The oligonucleotides used were complementary to the following bases of the human cDNAs: 2323–2374 of PDE4A (GenBank acc. no. L20965); 2410-2455 of PDE4B (GenBank acc. no. L20971), 2333-2378 PDE4C (GenBank acc. no. U66347) and 1916-1960 of PDE4D (GenBank acc. no. U50159). They were custom-synthesized by Amersham Pharmacia Biotech (Little Chalfont, UK).

The oligonucleotides were 3'-end-labeled with terminal deoxynucleotidyltransferase and [^{33}P]α-dATP (3000 Ci/mmol, New England Nuclear, Boston, USA). Labeled probes were purified by QIAquick Nucleotide Removal Kit (QIAGEN, Hilden, Germany).

The protocol for *in situ* hybridization histochemistry was based on previously described procedures with minor modifications (Tomiyama et al., 1997). Tissue sections were deparaffinized and fixed for 20 min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (1x PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3x PBS at room temperature, twice for 5 min each in 1x PBS, and incubated for 2 min at 21ºC in a solution of proteinase K (Calbiochem, San Diego, CA, USA) at a final concentration of 100 µg/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, washed twice in 1 X PBS, 5 min each and dehydrated through a graded series of ethanol and air dried.

For hybridization, labeled oligonucleotides were diluted to a final concentration of approximately $2x10^7$ cpm/mL in a solution containing 50% formamide, 4x SSC, 1x Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 20 mM phosphate buffer pH 7.0, 250 µg/mL yeast tRNA, 500 µg/mL salmon sperm DNA. Tissues were covered with 100 µL of hybridization solution, overlaid with Nescofilm (Bando Chemical Ind, Kobe, Japan) coverslips, and incubated overnight in humid boxes at 42ºC. Sections were then washed 4 times (45 min each) in 600 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA at 60ºC. The sections were then briefly dipped in 70% and 100% ethanol, air-dried and dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Chesire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4°C for 6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

Several routine controls were carried out to determine the specificity of the hybridization signals. For a given oligonucleotide probe, the hybridization signals were completely blocked by competition of the labeled probe when hybridized in the presence of 50-fold excess of the same unlabeled oligonucleotide (data not shown) (Pérez-Torres et al., 2000).

Tissue sections were examined in bright- and dark-field in a Wild 420 macroscope (Leica, Heerbrugg, Germany) and in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan)

equipped with bright- and dark-field condensers for transmitted light and with epi-illumination. Microphotography was performed digitally using a DXM 1200 Digital still camera (Nikon, Tokyo, Japan) and analySIS® 3.1 software (Soft Imaging System GmbH, Münster, Germany). Images were captured separately as TIFF files. All images were slightly enhanced in Photoshop 7.0 (Adobe Software, Mountain View, CA, USA) with the levels and bright/contrast tools. All images were treated equally to make backgrounds appear similar. The AP localization was verified by the examination of cresyl violet-stained sections.

IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENCE PROCEDURES

For analysis of the carotid bodies, immunohistochemical examinations were carried out on longitudinal serial sections, 5 μm thick, of the whole carotid bifurcation. Sections were incubated in 0.3% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum (0.04% bovine serum albumin (A2153, Sigma-Aldrich, Milan, Italy) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA) in PBS) for 30 min. Anti-NTR1 and anti-NCAM immunohistochemical analyses were performed only in adult cases. For anti-AM and –pERK immunohistochemistry, unmasking was performed with 10mM sodium citrate buffer, pH 6.0, at 90 °C for 10 min, then sections were incubated with 0.01 M PBS containing 0.1% Triton® X-100 for 10 min. For anti-NCAM, -ERK, -AKT, and -pAKT immunohistochemistry, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90 °C for 20 min. Antigen unmasking was not necessary for anti-NTR1 immunohistochemistry. Primary antibody incubations were performed as follows: anti-human AM 1:1000 [Rabbit anti-AM 1-52 (human) serum, Peninsula Laboratories Inc, San Carlo, CA, USA] in blocking serum for 18 hours at 4° (Satoh et al., 1996); rabbit polyclonal anti-NTR1 antibody at 1:600 dilution in

blocking serum for 1 hour at room temperature; anti-NCAM primary antibody (Monoclonal Mouse Anti-CD56 (NCAM), NCL-CD56-1B6, Novocastra) diluted 1:100 in PBS for 1 hour at room temperature; anti-ERK (rabbit anti-ERK 1 (K-23) sc-94, Santa Cruz Biotechnology Inc., CA, USA), diluted 1:1500 in blocking serum for 1 hour at room temperature; anti-pERK (mouse anti-pERK (E-4) sc-7383, Santa Cruz Biotechnology), diluted 1:250 in blocking serum for 1 hour at room temperature; anti-AKT (Akt (C67E7) Rabbit mAb #4691, Cell Signaling Technology Inc., Boston, MA, USA), diluted 1:100 in blocking serum for 1 hour at room temperature; anti-pAKT (Phospho-Akt (Ser473) Rabbit mAb #4060, Cell Signaling Technology), diluted 1:100 in blocking serum for 1 hour at room temperature. Anti-NTR1 antibody was raised against amino acids 1-28 and 50-69 of rat NTR1 and also detected human NTR1 (Feldberg et al., 1998; Castagliuolo et al., 1999; Brun et al., 2005). Sections were then washed three times for 5 min in PBS. Primary antibody binding was revealed by incubation with anti-rabbit/mouse serum diluted 1:100 in blocking serum for 30 min at room temperature (DAKO® EnVision + TM Peroxidase, Rabbit/Mouse, Dako Corporation, Glostrup, Denmark) and developed in 3,3'-diaminobenzidine for 3 min at room temperature. Finally sections were counterstained with hematoxylin. Negative controls were performed by omission of primary antibody. AM immunoreaction detected in hypothalamus, NTR1 immunoreaction detected in rat and human substantia nigra, NCAM positivity in Natural Killer cells were used as positive controls. Endothelial cells served as positive internal controls for anti-ERK and -pERK immunoreactivities. Human breast carcinoma was used as positive immunohistochemical controls for AKT and pAKT. In order to verify the immunohistochemical specificity of the anti-AM reaction, absorption tests with human AM, CGRP, corticotropin releasing hormone and arginine vasopressin were performed. In order to verify the immunohistochemical specificity of the reaction for NTR1, absorption tests with

NTR1 peptide antigen (NTS1 Blocking Peptide, 2 mg/ml, P14020 Neuromics, Edina, MN) for 1 hour at room temperature were also performed.

The percentage of type I and II positive cells was evaluated at a magnification of 40X, and 5 sections and 3 fields per section were examined. The mean percentages of positive type I and II cells were calculated for each case and for the entire series.

Counterstaining with haematoxylin permitted differentiation between light and dark cells, showing both positive and negative immunoreaction, on the basis of the morphological characteristics and staining properties of the nuclei. The percentages of dark and light cells showing immunoreactivity were evaluated at a magnification of 40X, and 5 sections and 3 fields per section were examined. The mean percentages of positive dark and light cells were calculated for each case and for the entire series.

In order to confirm the cellular location of immunoreactivities in the carotid body, a double immunofluorescent staining was performed in 4 frozen carotid bodies taken at autopsy from 3 adult subjects (2 males, 1 female; mean age 45.3 years, SD ± 1.2) and one male foetus (gestational age 180 days). The other antibody used to mark type I cells was monoclonal anti-Neurone-Specific Enolase [Mouse anti-NSE; Dako, Milan, Italy] diluted 1:300 in PBS for 1 hour at room temperature, as NSE is a marker of type I cells (Abramovici et al., 1991; Porzionato et al., 2005a). Then sections were incubated with Alexa fluor 488 goat anti-rabbit (Molecular Probes, Leiden, the Netherlands) and then with Alexa fluor 594 goat anti-mouse (Molecular Probes). Both incubations were performed for 30 min at a concentration of 1:1000 in PBS. After thorough washes, the slides were mounted and observed with a Fluorescence Microscope Olympus BH-2.

STATISTICS

The Mann-Whitney U-test was performed to verify any differences in morphometric parameters and apoptotic indexes in the medullary nuclei between opiate deaths and controls, SIDS and the other infant cases, and between adults and infants. In order to reveal differences between the morphometric parameters and apoptotic indexes of the nuclei, statistical analysis was performed in both adult and infant cases by the Kruskal-Wallis test and Dunn's multiple comparison test. In all sample populations, statistical analysis of the linear correlation of apoptotic indexes with age and DAI were performed.

In the study of the carotid body, comparisons of immunopositivity between different cell types and between adult and foetuses were performed by the Mann-Whitney test. A P value of 0.05 was considered significant. Statistical calculations were carried out by Prism 3.0.3 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

MEDULLARY NUCLEI

NEUROANATOMIC MORPHOMETRIC PARAMETERS

Table 3 shows the estimates of neuronal densities, nuclear volumes and total neuron numbers with reference to adult and infant series. The Mann-Whitney U-test did not reveal any statistically significant differences in total neuron numbers between adults and infants for the medullary nuclei considered. All the medullary nuclei showed lower nuclear volume and higher neuronal density in infants than in adults ($P<0.05$).

In both adult and infant cases, the Kruskal-Wallis test revealed that the differences in morphometric parameters between the nuclei reached statistical significance ($P<0.0001$). In infants, the inferior olivary complex and NSTT showed higher neuronal densities than DMNV, XII and Cu. In adults, the neuronal densities of the inferior olivary complex and NSTT were higher than neuronal densities of NTS, MedVe, DMNV, XII and Cu. NTS and MedVe showed higher neuronal densities than XII and Cu (Fig. 2).

In infants, PION showed higher volume than Cu, DMNV, XII, MION and DION; NTS presented higher volume than XII, MION and DION; volumes of MedVe and NSTT were higher than volumes of MION and DION, and Cu had higher volume than DION. In adults, PION showed higher volume than NSTT, Cu, XII, DMNV, MION and DION; NTS showed higher volume than Cu, XII, DMNV, MION and DION; MedVe presented higher volume than XII, DMNV, MION and DION; volumes of NSTT and Cu were higher than volumes of MION and DION, and XII had higher volume than DION.

In infants, PION showed higher total neuron number than MION, DION, Cu, DMNV and XII; NSTT showed higher total neuron number than DION, Cu, DMNV and XII; NTS had higher total neuron number than Cu, DMNV and XII; MedVe presented higher total neuron

number than DMNV and XII; total neuron number of MION was higher than that of XII. In adults, PION showed higher total neuron number than MedVe, MION, DION, Cu, DMNV and XII; NSTT showed higher total neuron number than MION, DION, Cu, DMNV and XII; NTS had higher total neuron number than DION, Cu, DMNV and XII; MedVe presented higher total neuron number than Cu, DMNV and XII; total neuron number of MION was higher than those of DMNV and XII, and total neuron number of DION was higher than that of XII.

Table 3. Morphometric parameters (neuronal density NV_N; nuclear volume, V; total neuron number, N_N; ± Standard Deviation) of adult and infant medullary nuclei.

	ADULTS			**INFANTS**		
	$\boldsymbol{NV_N}$ (n/mm^3)	$\boldsymbol{V}$ (mm^3)	$\boldsymbol{N_N}$ (n)	$\boldsymbol{NV_N}$ (n/mm^3)	$\boldsymbol{V}$ (mm^3)	$\boldsymbol{N_N}$ (n)
XII	1070±343.4	12.3±2.9	12368±1972	2799±1292	5.4±1.6	13437±1722
DMNV	1663±431.4	11.5±2.9	18051±2623	2915±568.1	6.1±1.4	17063±1391
NTS	2982±639.9	38.2±5.2	111582±16833	8150±1735	15.0±2.5	119505±16881
Cu	1061±238.7	21.7±3.1	22451±2743	2686±843.6	8.1±2.1	20168±2511
MedVe	2964±380.8	30.6±3.7	89533±6819	6958±1076	13.7±2.2	93213±8441
NSTT	9217±2146	25.0±3.6	224416±29686	21347±5006	11.8±2.5	241139±27242
PION	9149±1341	82.9±8.5	748945±60529	20910±1582	36.9±4.1	768002±71935
MION	10102±3024	6.0±1.4	56823±4316	19267±3475	3.0±0.4	55685±5870
DION	11318±3704	3.3±0.8	35534±4526	23124±6740	1.8±0.4	38114±3824

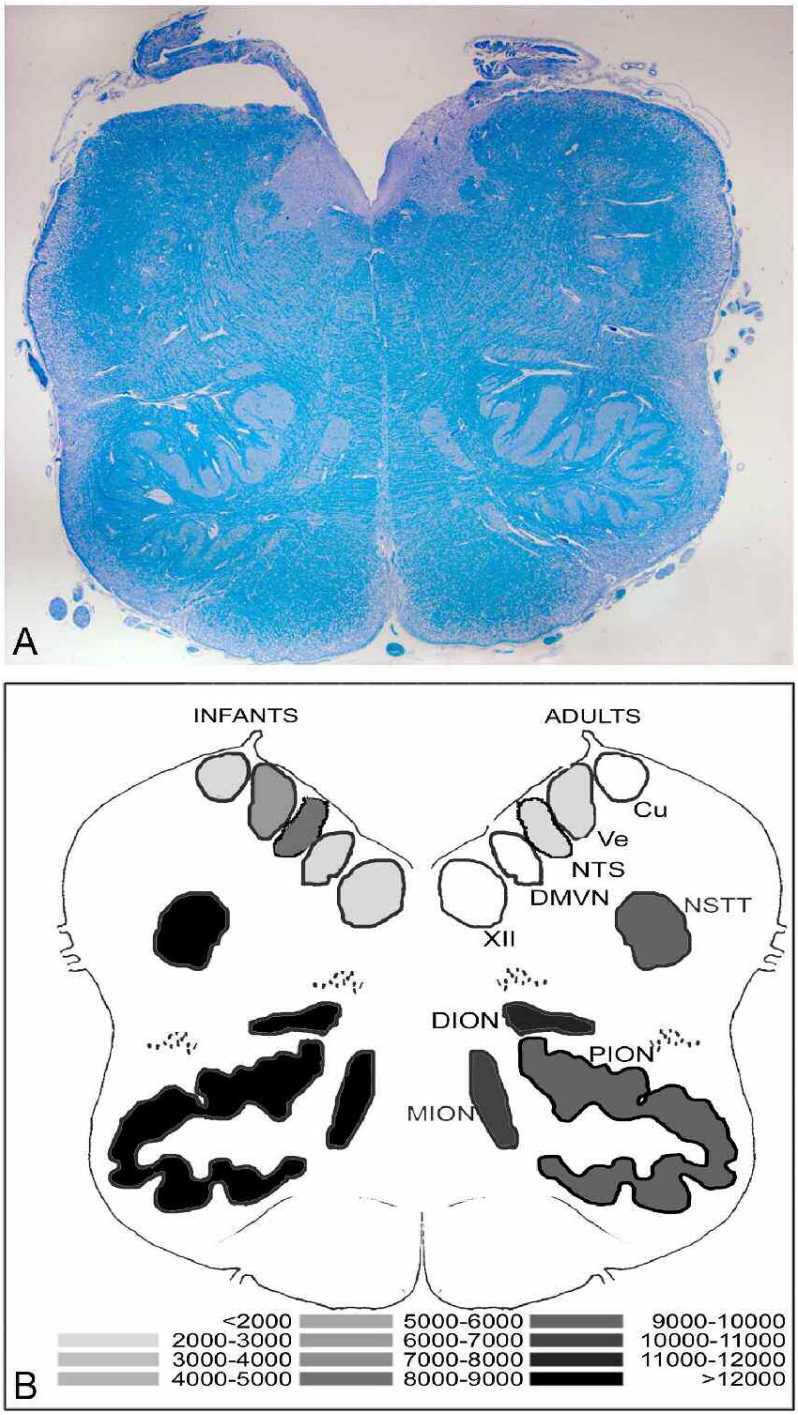

Fig. 2 A: Histological section of medulla of an adult case at level of caudal portion of IV ventricle (Klüver-Barrera, 1X). B: Sketch showing varying neuronal densities in medullary nuclei of infants (left) and adults (right) through grey scales (data expressed in neurons/mm^3). (Cu, Cuneate nucleus; MedVe, medial vestibular nucleus; NTS, nucleus tractus solitarii; DMNV, dorsal motor nucleus of vagus; XII, hypoglossal nucleus; NSTT, nucleus of spinal trigeminal tract; PION, principal inferior olivary nucleus; MION, medial accessory inferior olivary nucleus; DION, dorsal accessory inferior olivary nucleus).

DISTRIBUTION OF APOPTOSIS

Apoptotic neuronal cells were found in all medullae examined (Fig. 3-5). The majority of TUNEL-positive neurons showed morphologic characteristics of apoptosis, such as chromatin condensation and margination and nuclear fragmentation.

The Mann-Whitney U-test did not reveal any statistically significant differences in neuronal apoptotic indexes between opiate deaths and controls, or between SIDS and the other infant cases. A statistically significant difference in neuronal apoptotic index was found between adults and infants in the following nuclei: Cu (28.2±16.3% vs 6.9±8.7%; $P<0.005$), Ve (24.7±15.0% vs 11.3±11.4%; $P<0.05$), NTS (11.2±11.2% vs 2.3±2.4%; $P<0.05$), DMNV (6.8±8.5% vs 0.1±0.2%; $P<0.05$) and XII (6.6±5.7% vs 0.1±0.2%; $P<0.005$) (Table 4).

In both adult and infant cases, the Kruskal-Wallis test revealed that the differences in neuronal apoptotic indexes between the nuclei did reach statistical significance ($P<0.001$ and $P<0.005$) (Figs. 3C, 5). In adults, Dunn's multiple comparison test revealed significant differences in comparing the neuronal apoptotic index of Cu with those of PION ($P<0.05$), NTS ($P<0.05$), MION ($P<0.001$), XII ($P<0.001$), and DMNV ($P<0.001$). The neuronal apoptotic indexes of NSTT and Ve were higher than in MION, XII and DMNV ($P<0.05$ and $P<0.01$, respectively). In infants, the differences between NSTT (20.2±19.0%) and XII (0.1±0.2%) and between NSTT and DMNV (0.1±0.2%) were statistically significant ($P<0.05$). Differences in the neuronal apoptotic indexes between the other nuclei were not statistically significant ($P>0.05$).

No significant statistical correlation of apoptotic indexes with age was found in any group. There was no statistically significant difference in the mean DAI between the three groups, and no statistically significant correlation of apoptotic indexes with the DAI interval in any group.

Table 4. Total neuron numbers (N_N) and neuronal apoptotic indexes (I_{NA}) in medullary nuclei of adults and infants. (Values expressed as mean ± s.d.).

	ADULTS	**INFANTS**
	I_{NA}	I_{NA}
XII	6.6 ± 5.7	0.1 ± 0.2 *(P<0.005)*
DMNV	6.8 ± 8.5	0.1 ± 0.2 *(P<0.05)*
NTS	11.2 ± 11.2	2.3 ± 2.4 *(P<0.05)*
NSTT	24.4 ± 19.3	20.2 ± 19.0 *(P>0.05)*
Cu	28.2 ± 16.3	6.9 ± 8.7 *(P<0.005)*
Ve	24.7 ± 15.0	11.3 ± 11.4 *(P<0.05)*
PION	12.9 ± 15.0	12.1 ± 21.2 *(P>0.05)*
MION	10.0 ± 14.4	3.0 ± 6.5 *(P>0.05)*
DION	13.8 ± 14.3	4.9 ± 7.8 *(P>0.05)*

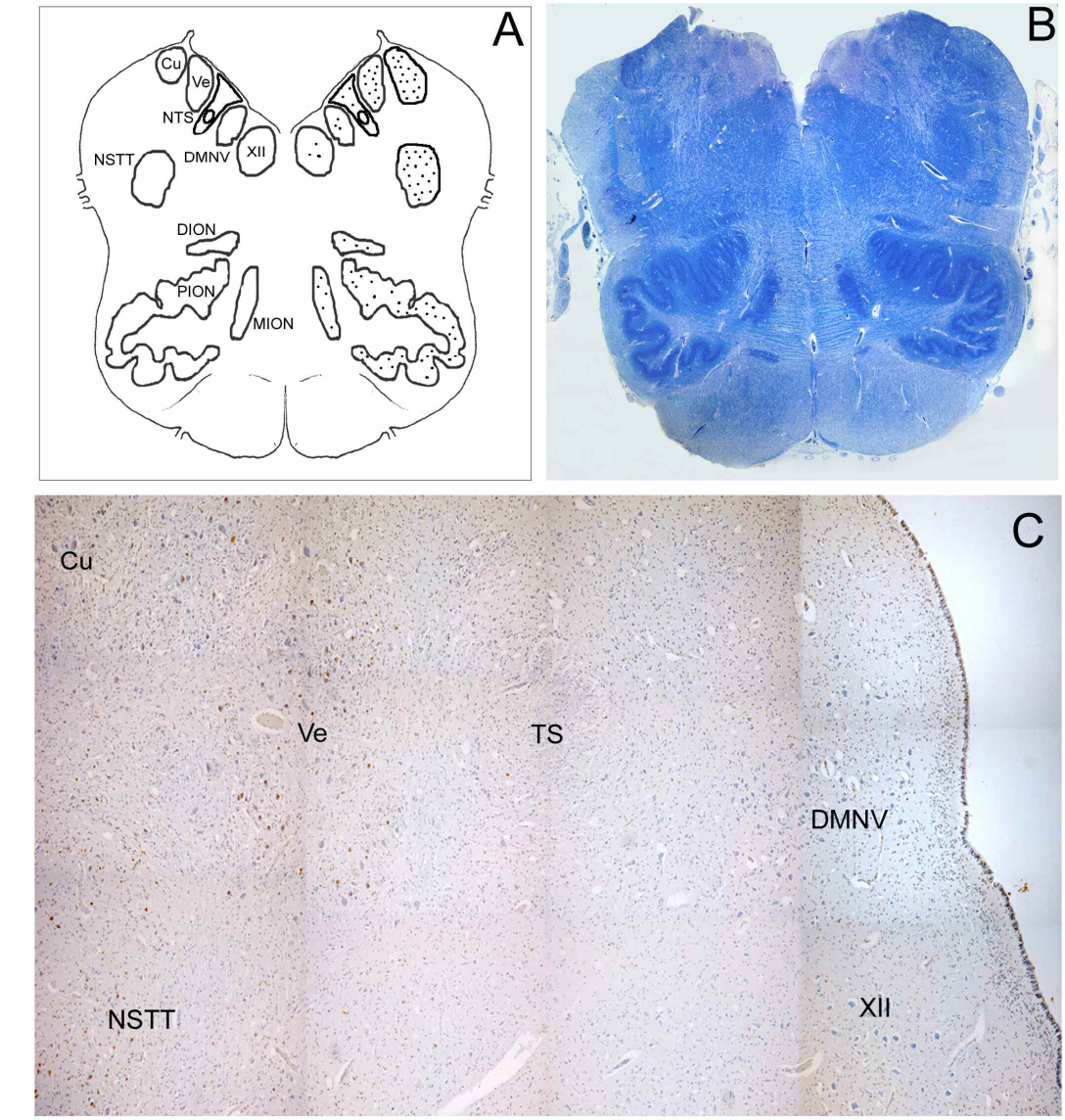

Fig. 3 A: Sketch showing varying incidence of neuronal apoptosis in medullary nuclei. (Cu, Cuneate nucleus; Ve, medial vestibular nucleus; NTS, nucleus tractus solitarii; DMNV, dorsal motor nucleus of vagus; XII, hypoglossal nucleus; NSTT, nucleus of spinal trigeminal tract; PION, principal inferior olivary nucleus; MION, medial accessory inferior olivary nucleus; DION, dorsal accessory inferior olivary nucleus). B: Histological section of medulla of an adult case at level of caudal portion of IV ventricle (Klüver-Barrera, 1X). C: Medullary tegmentum stained by TUNEL method, showing higher neuronal apoptosis incidence in Cu, Ve and NSTT, corresponding to lateral area, than in XII, DMNV and NTS. Photomicrographs (10X) mounted together by Photo Stitch 3.1, Canon Inc.

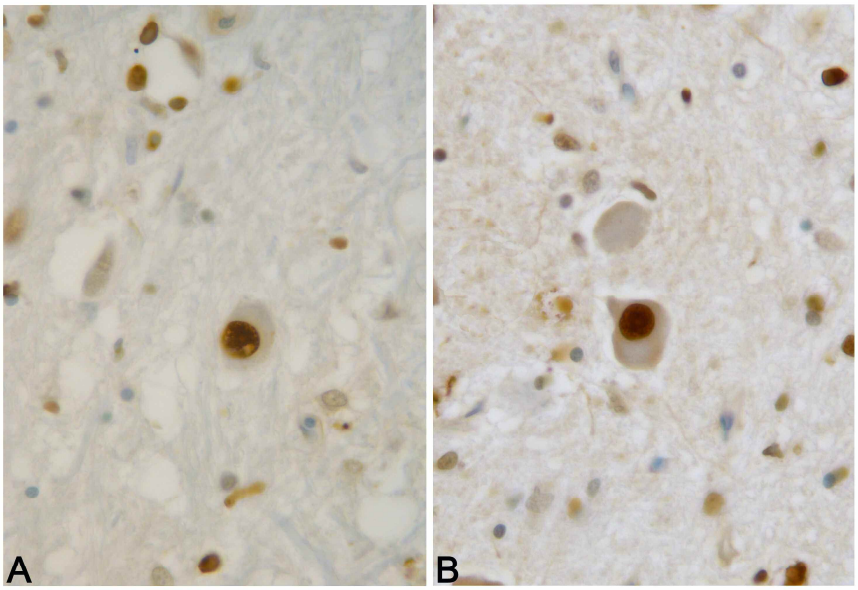

Fig. 4 TUNEL-positive neurons of inferior olivary complex, showing chromatin condensation (63X).

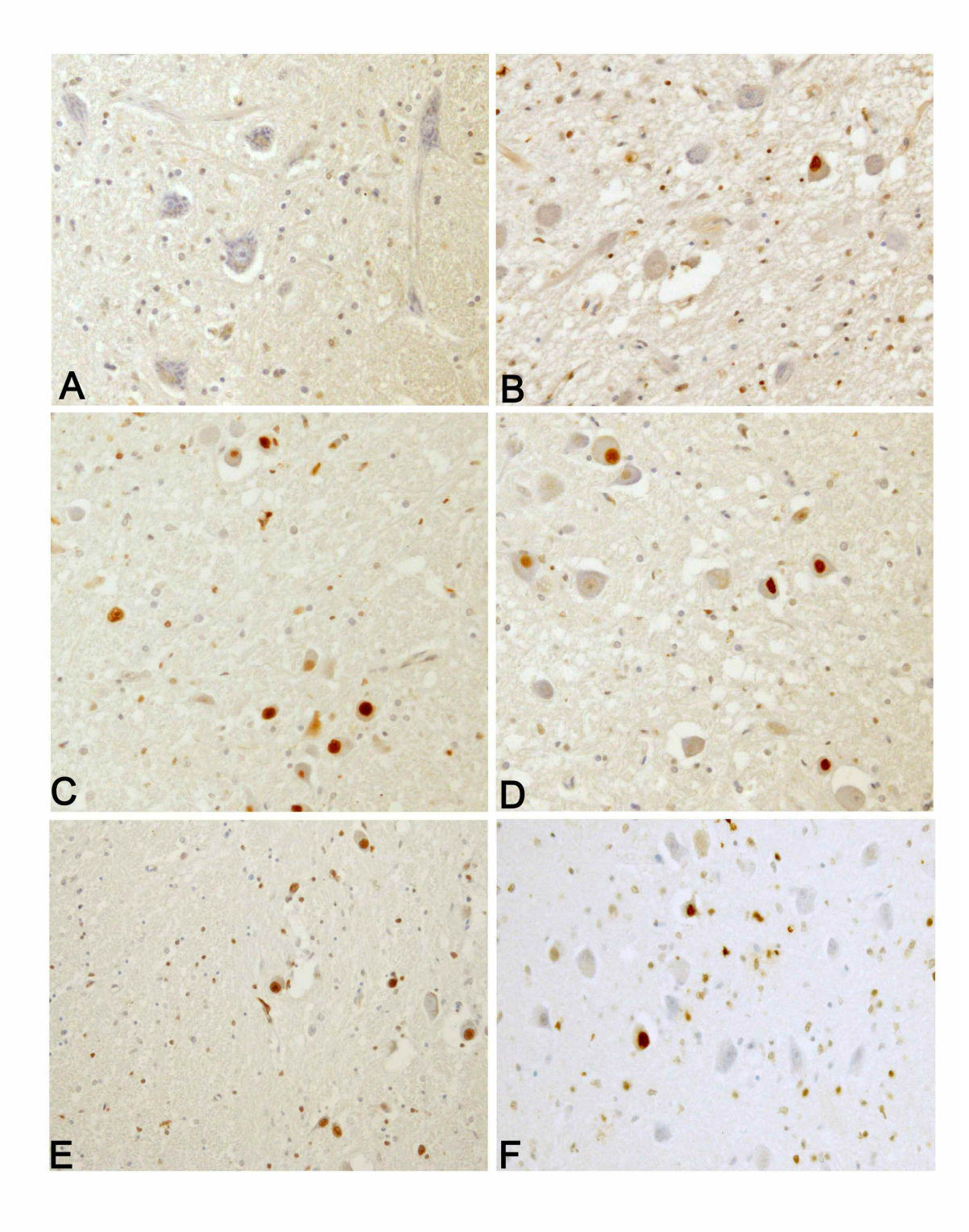

Fig. 5 Differences in incidence of neuronal apoptosis in hypoglossal nucleus (A, 20X), dorsal motor nucleus of vagus (B, 20X), cuneate nucleus (C, 20X), medial vestibular nucleus (D, 20X), nucleus of spinal trigeminal tract (E, 20X) and principal inferior olivary nucleus (F, 20X).

PHOSPHODIESTERASE ISOFORMS

Table 5 summarizes the results obtained. In the AP, very low hybridization signal for PDE4A, low for PDE4B, and strong for PDE4D were seen (Fig. 6A-I). In the NTS and the DMNV, no hybridization signal for PDE4A could be observed, whereas it was low for PDE4B and moderate for PDE4D (Fig. 6A-C). Hybridization levels for PDE4B and PDE4D were stronger in the AP than in the NTS or DMNV. PDE4C mRNA expression was totally absent in the tissue sections analyzed as in the rat tissue sections examined by Pérez-Torres et al. (2000). PDE4B and PDE4D presented a diffuse hybridization signal in the reticular (ventral, medial and dorsal) nuclei. Somatosensory nuclei, such as Cu, Gr and NSTT showed low to moderate hybridization signals for PDE4B and PDE4D. The XII weakly expressed PDE4B and PDE4D mRNA. In addition, low levels of PDE4B mRNA were observed in the inferior olivary complex, including PION and MION where also moderate hybridization signal for PDE4D was seen, especially in the PION. In the NTS, both PDE4B and PDE4D mRNAs were detected in the different subnuclei. Low to moderate levels were visualized in both paracommissural and commissural solitary nuclei and lower levels in the intermediate solitary nucleus. PDE4B and PDE4D were found expressed at low levels in the cells of the locus coeruleus. Very low hybridization signals for PDE4A mRNA were seen in the LatRet, whereas no hybridization signal for PDE4A mRNA could be observed in the other nuclei.

Very low hybridization signal for PDE4A was detected on and around blood vessels (Fig. 6G) and on neurons (Fig. 7A, D, G). Moderate number of silver grains for PDE4B were observed on and around blood vessels (Fig. 6H) and on neuronal and glial cells (Fig. 7B, E, H). Strong hybridization signal for PDE4D was also seen on and around blood vessels (Fig. 6I) and on neuronal and glial cells (Fig. 7C, F, I).

Table 5. Distribution of PDE4 mRNAs in the medullary nuclei

	PDE4A	PDE4B	PDE4D
AP	-/+	++/+	+++
XII	-	+	++
DMNV	-	+	++
NTS	-	+	++
NSTT	-	+	++
Cu	-	+	++
Gr	-	+	+
LatRet	-/+	+	++
ION	-	+	++

The levels of intensity are indicated from '+' for low intensity up to '+++' for the highest intensity. '-', not detected.

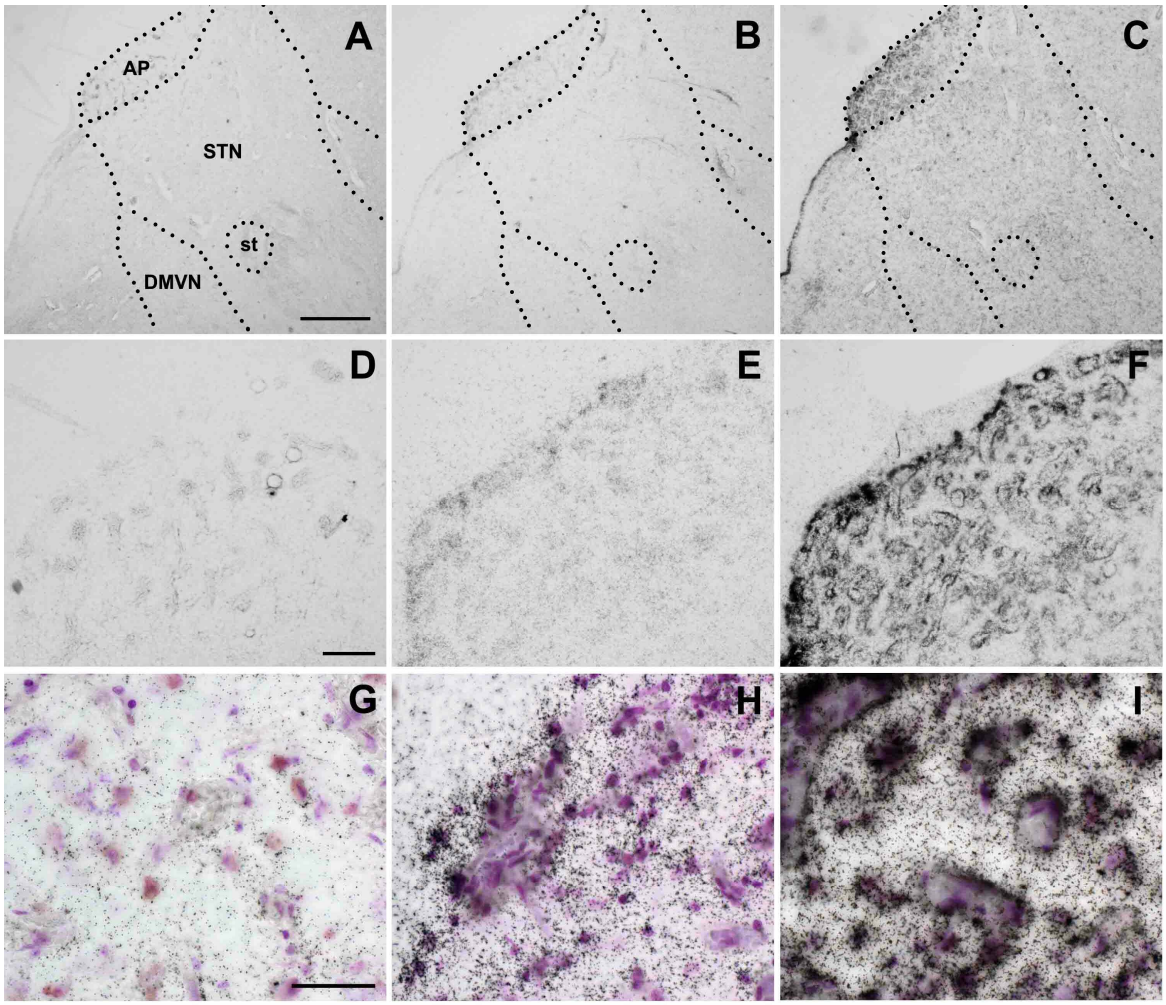

Fig. 6. Distribution of PDE4 isozyme mRNAs in the human medulla oblongata and cellular localization of PDE4 isozyme mRNAs in the area postrema (AP). A-I are bright-field photomicrographs from emulsion-dipped sections showing the presence of PDE4A (A, D, G), PDE4B (B, E, H) and PDE4D (C, F, I) mRNAs. D-I are higher magnification of A-C from AP. Note absence of the labeling of PDE4A (D, G). Low densities of the labeling of PDE4B can be observed in AP (E) and located on and around blood vessels (H). Strong levels of hybridization for PDE4D are seen on and around blood vessels of AP (F, I). Sections (G-I) are stained with cresyl violet. DMVN, dorsal motor vagal nucleus; STN, solitary tract nucleus; st, solitary tract. Bar in A = 500 µm (applies to A - C). Bar in D = 100 µm (applies to D - F). Bar in G = 40 µm (applies to G - I).

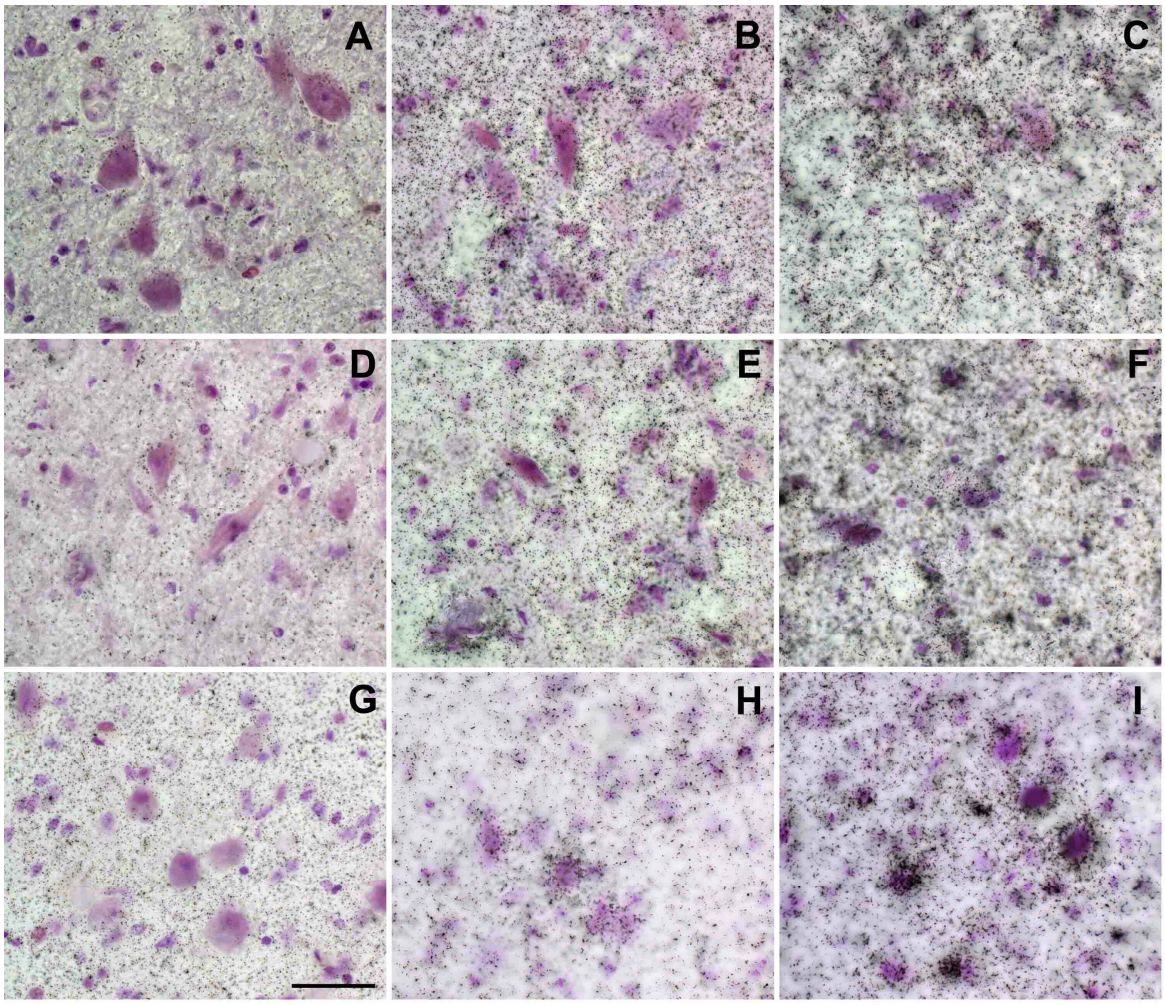

Fig. 7. Cellular localization of PDE4 isozyme mRNAs in the dorsal motor nucleus of the vagus (DMNV) (A-C), nucleus tractus solitarii (NTS) (D-F) and inferior olivary nucleus (ION) (G-I). Autoradiographic images are presented as bright-field photomicrographs from emulsion-dipped tissue sections in which autoradiographic grains are seen as black dots. All sections are stained with cresyl violet. Most neurons contain few grains for PDE4A mRNA in DVMN (A), NTS (D) and ION (G). Moderate labeling for PDE4B mRNA can be seen on neuronal and glial cells in the three nuclei (B, E, H). High levels of hybridization for PDE4D mRNA are observed on neuronal and glial cells in the three nuclei (C, F, I). Bar in G = 40 µm (applies to A-I).

CAROTID BODY

NEUROMODULATORS: ADRENOMEDULLIN AND NEUROTENSIN SYSTEMS

Immunohistochemistry revealed the coexistence of both AM+ and AM- type I cells in all specimens examined. When present, AM immunoreactivity was intense and widely distributed in the cytoplasm. The percentage of positive type I cells was higher in adult subjects (32.3 ± 7.7%) with respect to the foetuses (11.8 ± 2.7%, $P<0.01$) (Fig. 8). Immunostained cells were distributed both in the centre and in the periphery of the lobules. No AM immunostaining was observed in type II cells. Double immunofluorescent staining with anti-NSE confirmed the selective location of AM immunoreactivity in type I cells (Fig. 9).

Concerning subpopulations of type I cells, dark cells showed a higher percentage of positive immunoreaction with respect to light cells, both in adult subjects (61.7 ± 13.4% vs 19.2 ± 5.2%, $P<0.01$) and in foetuses (25.3 ± 4.4% vs 6.2 ± 2.0%, $P<0.01$). Pyknotic cells were rarely immunostained.

Sinusoids located in the context of the lobules of the carotid body showed a faint AM immunoreactivity, less intense than immunostained type I cells. Blood vessels in the connective tissue in proximity of the carotid body showed positive immunostaining in the endothelium and/or in the smooth muscle cells (Fig. 8E).

Preabsorption with human AM eliminated the immunostaining, while preabsorption with CGRP, corticotropin releasing hormone and arginine vasopressin did not affect the immunostaining.

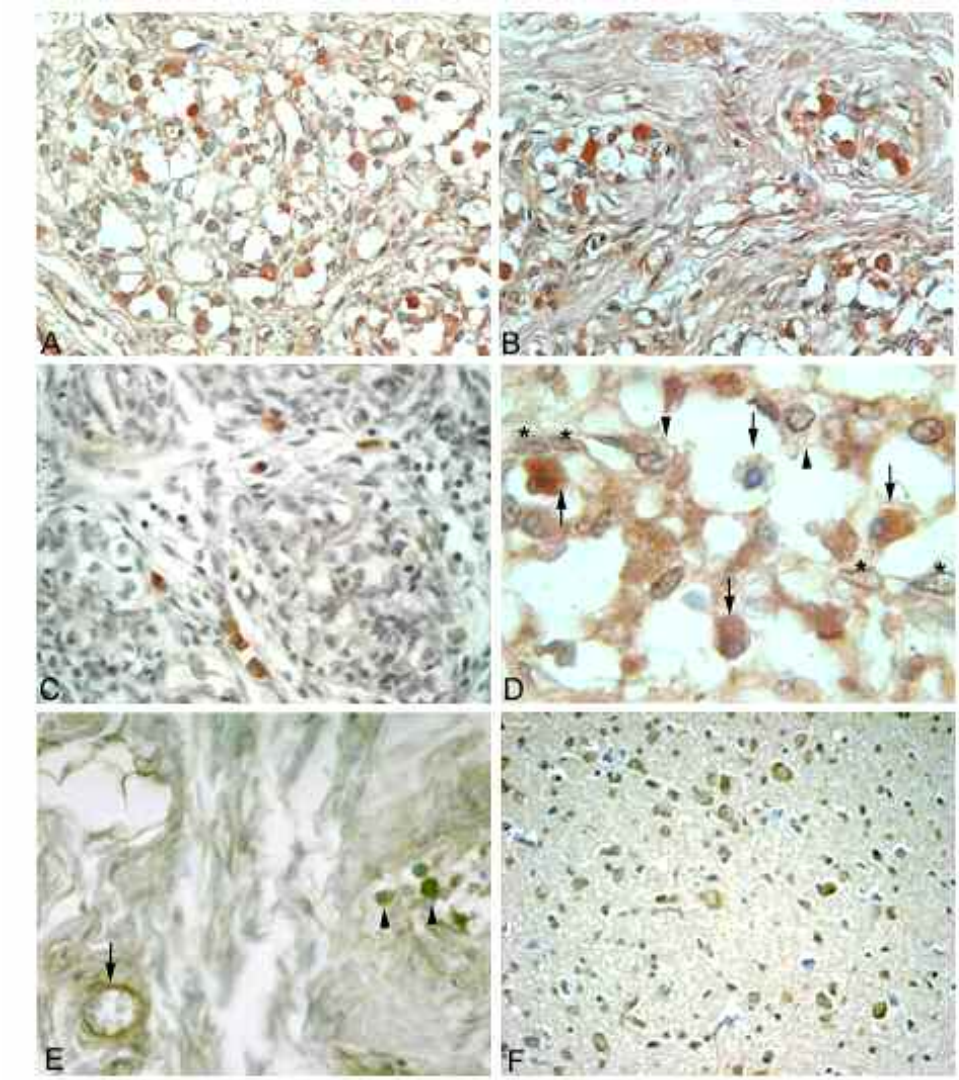

Fig. 8. Sections of the carotid body immunostained for adrenomedullin (AM) displaying higher percentages of immunostained type I cells in adult subjects (A,B) with respect to foetuses (C). At higher magnification (D) preferential immunostaining of dark cells (arrows) with respect to light cells (arrowheads) and type II cells (asterisks) is recognizable. In Fig. E positive AM immunostaining of a blood vessel (arrow) is visible, near to a glomic lobule with immunostained type I cells (arrowheads). Fig. F shows positive AM immunostaining of hypothalamus, taken as positive control. Negative controls are not shown. Magnifications: (A,B,C,E) 40x, (D) 100x, (F) 20x.

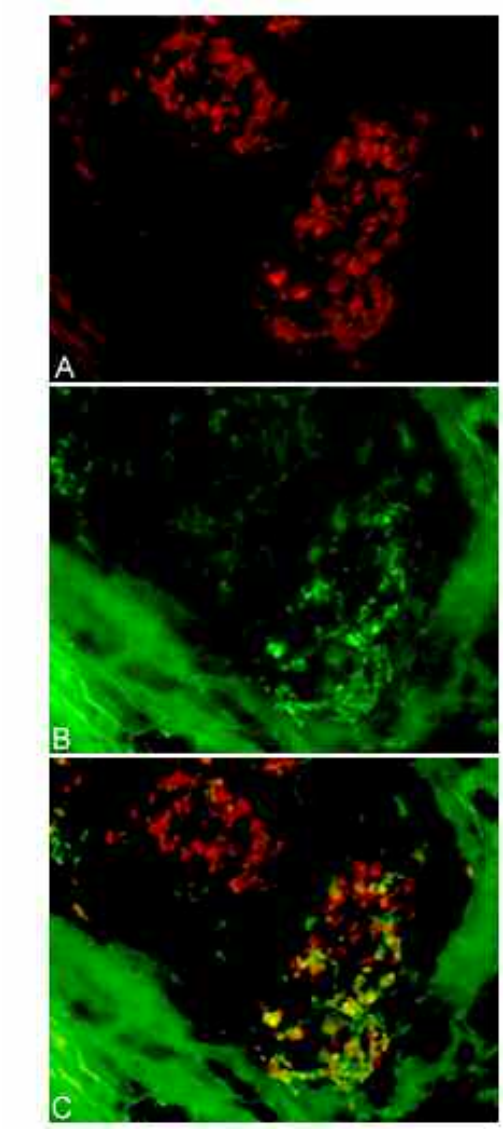

Fig. 9. Double immunofluorescence of a frozen section of an adult carotid body. Neurone-specific enolase was demonstrated in red (A), adrenomedullin (AM) in green (B). The combination of the previous images (C) shows colocalization of AM and NSE in some type I cells (yellow) and absence of AM immunostaining in other type I cells (red). Magnifications: (A,B,C) 20x.

In adult human carotid bodies, a preliminary analysis of subpopulations of type I cells did not reveal any statistically significant differences between dark, light or pyknotic cells ($P>0.05$). Immunohistochemistry revealed the coexistence of both NTR1-positive and -negative type I cells in all specimens examined. Anti-NTR1 immunohistochemistry showed positivity in 45.6 ± 9.2% type I cells (Fig. 10). When present, NTR1 immunoreactivity was intense, and distributed in the cytoplasm or nucleus. The percentage of nuclear NTR1 immunostaining on the total was 13.9 ± 11.1%. Immunostained cells were distributed in both the center and periphery of the lobules. Type II cells did not show immunostaining (Fig. 10-11). Glomic cell immunostaining was eliminated when antiserum, preabsorbed with its peptide antigen, was used.

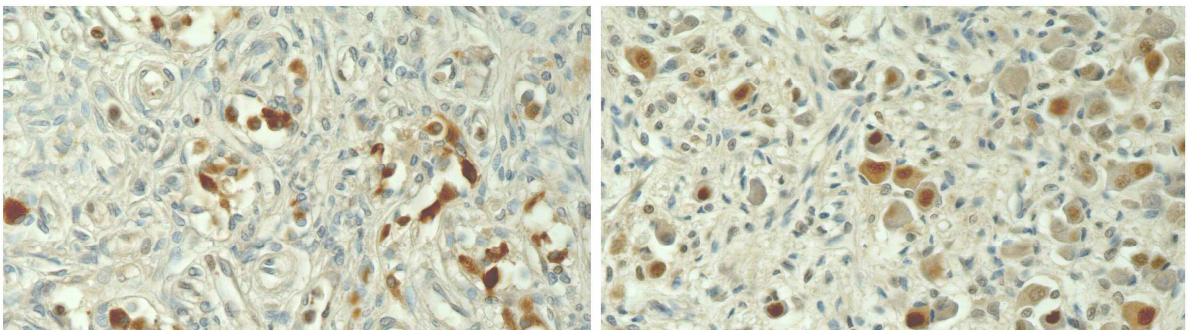

Fig. 10. Anti-NTR1 immunohistochemistry - Sections of adult carotid bodies (E,F) showing selective positivity of glomic type I cells. Scale bars = 20 μm.

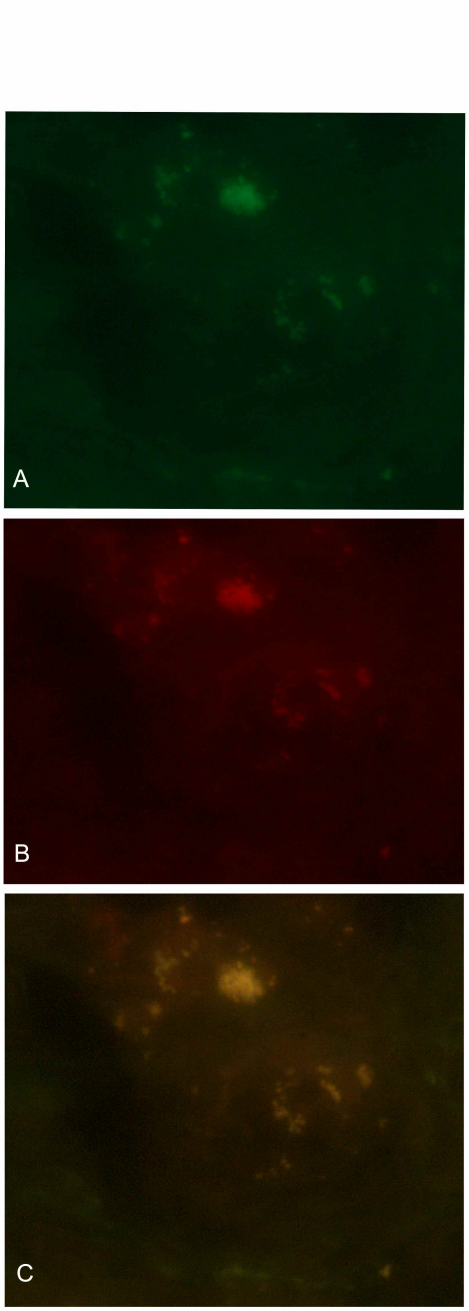

Fig. 11. Double immunofluorescence of a frozen section of human carotid body. NTR1 shown in green (A), neurone-specific enolase in red (B). Combination of the previous images (C) shows colocalization of NTR1 and NSE in type I cell (yellow). Original magnifications: 63X.

ADHESION MOLECULES: NEURAL CELL ADHESION MOLECULE

Anti-NCAM immunohistochemistry performed in adult carotid bodies revealed the coexistence of both NCAM+ and NCAM- type I cells in all specimens examined. When present, NCAM immunoreactivity involved cell membranes. The percentage of positive type I cells was very high, i.e., 78.3 ± 7.2%. Immunostained cells were distributed both in the centre and in the periphery of the lobules (Fig. 12A-B). Concerning subpopulations of type I cells, statistically significant differences were not found in anti-NCAM immunostaining of light and dark cells (80.2 ± 6.2% vs 74.7 ± 13.4%, $P>0.05$) (Fig. 12C). Pyknotic cells also frequently showed positive immunoreaction. No NCAM immunostaining was observed in type II cells. Connective tissue components were negative, immunoreactivity being present only in the glomic lobules.

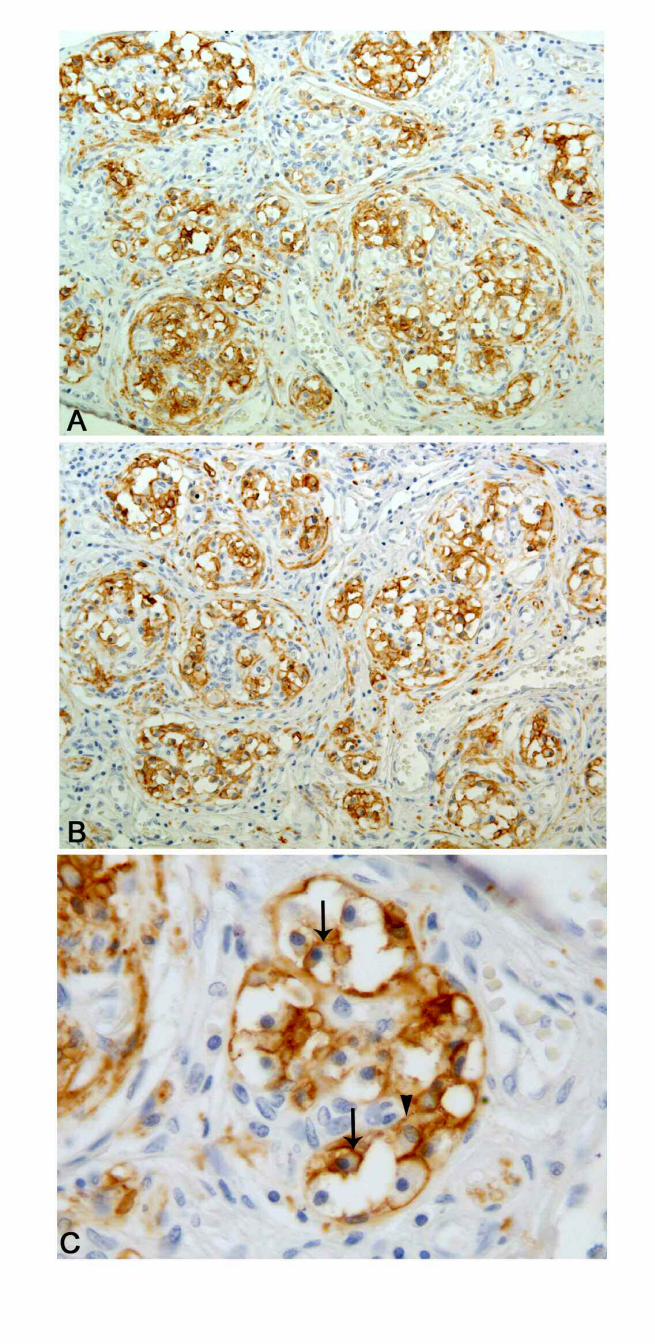

Fig. 12. A-B: Sections of the carotid body immunostained for Neural Cell Adhesion Molecule (NCAM) displaying high percentages of immunostained type I cells and negativity of type II cells. C: At higher magnification immunostaining of both dark (arrows) and light (arrowhead) cells is recognizable. Negative controls are not shown. Original magnifications: A-B, 20X; C, 63X.

SIGNAL TRANSDUCTION SYSTEMS: ERK AND AKT

Table 6 lists the mean values (± standard deviation) of immunopositive cells in foetuses and adult subjects. In adults, percentages of ERK-, pERK, AKT- and pAKT-immunopositive type II cells were lower than corresponding percentages of ERK-immunopositive type I cells ($P<0.05$). A preliminary analysis of subpopulations of type I cells did not reveal statistically significant differences between dark, light or pyknotic cells for each of the immunohistochemical analysis ($P>0.05$). ERK immunopositivity was mostly cytoplasmic, with only rare cells also showing nuclear immunoreactivity. pERK immunolabelling was also predominantly cytoplasmic in both type I and II cells. AKT immunolabelling in type I and II cells was mainly cytoplasmic, although nuclear and nucleo-cytoplasmic immunolocalisation was also present. pAKT immunolabelling in type I cells was mainly nuclear. Type II cell immunolabelling was not detectable. Immunolabelled cells were distributed both in the centre and in the periphery of the lobules. Mann-Whitney tests revealed a significantly lower percentage of ERK-, pERK-, and AKT-immunopositive type I cells in foetuses in comparison to adults ($P<0.05$). Significantly lower percentages of ERK-, and AKT-immunopositive type II cells were also found in foetuses in comparison to adults ($P<0.05$). (Fig. 13-14).

Table 6. Mean percentages (± standard deviation) of ERK, pERK, AKT and pAKT immunopositive carotid body cells in foetuses and adults

		Carotid Body	
		Type I cells	Type II cells
ERK			
	Foetuses	19.5 ± 8.5	10.3 ± 4.3
	Adults	59.4 ± 8.5	29.4 ± 5.1
pERK			
	Foetuses	5.3 ± 2.8	2.0 ± 1.3
	Adults	15.9 ± 5.1	4.9 ± 2.0
AKT			
	Foetuses	4.0 ± 2.8	0.0 ± 0.0
	Adults	38.4 ± 7.8	4.7 ± 3.1
pAKT			
	Foetuses	2.3 ± 0.5	0.0 ± 0.0
	Adults	4.6 ± 3.0	0.0 ± 0.0

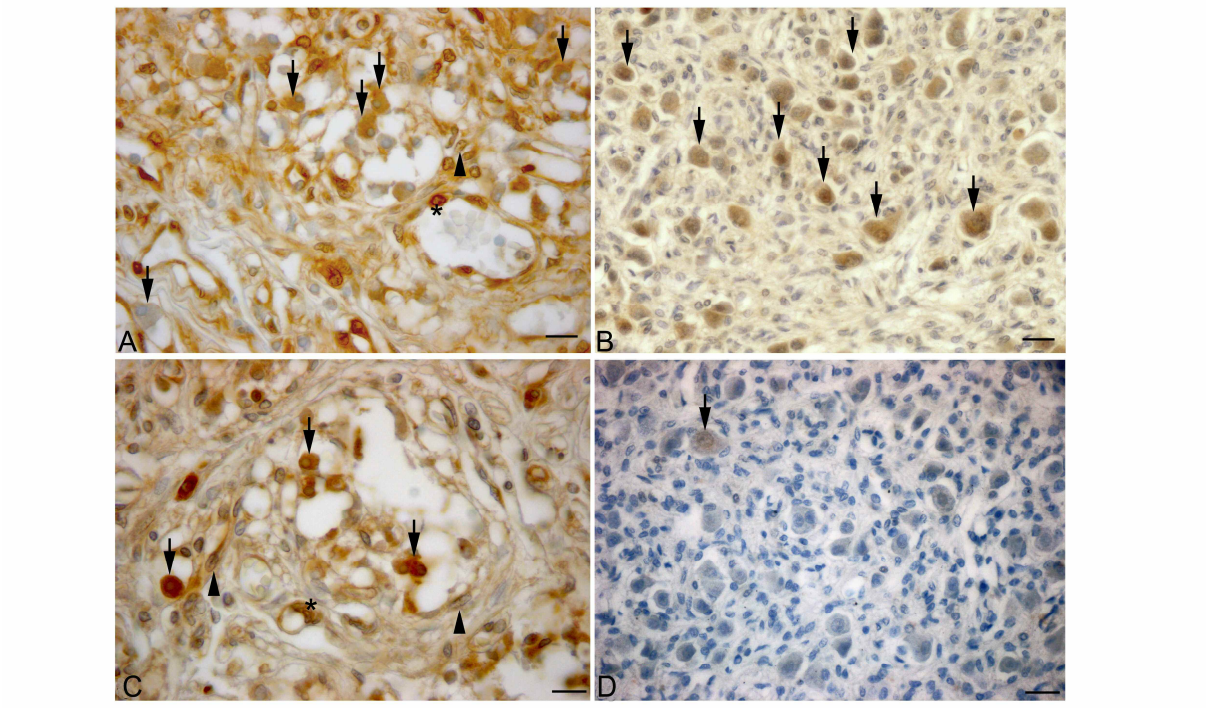

Fig. 13. ERK (A, B) and pERK (C, D) immunohistochemistry in the carotid body, displaying higher percentages of immunopositive cells in adults (A, C) than in foetuses (B, D). (arrows: type I cells; arrowheads: type II cells; asterisk: endothelial cells). Scale bars = 20µm.

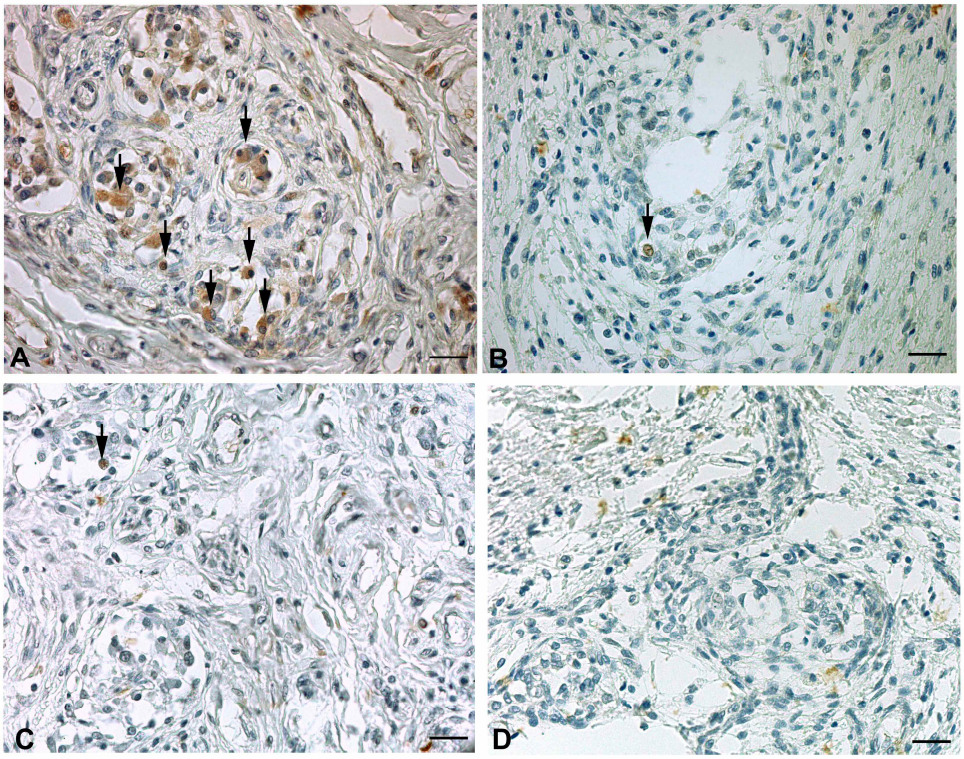

Fig. 14. AKT (A, B) and pAKT (C, D) immunohistochemistry in the carotid body, displaying higher percentages of immunopositive cells in adults (A, C) than in foetuses (B, D). (arrows: type I cells). Scale bars = 20µm.

DISCUSSION

NEUROANATOMIC MORPHOMETRIC PARAMETERS

Estimates of cell total numbers in various tissues have for much time been carried out applying indirect and biased methods based on counts in a two-dimensional plane. In fact, the probability for objects of being hit by a single section is proportional not only to their number but also to their size and form. Thus, direct and unbiased methods for cell counting have been developed, the so-called disector (Sterio, 1984) and fractionator (Gundersen, 1986). The basic principle of these methods is that the probability that an object is hit by a section but is not hit by a parallel section is independent from object size and form. The physical disector method relies on parallel physical sections separated by a distance smaller than the minimum particle height; objects counted in a section but not in the other one are considered. In the optical disector method (Gundersen, 1986) relatively thick sections are used, through which two or more parallel optical sections are performed by moving the plane of focus (reviewed in Gundersen et al., 1988; Pakkenberg and Gundersen, 1988; West, 1993). The fractionator method (Gundersen, 1986) relies on the same basic counting principles as the disector; the estimate of the total object number derives from multiplication of counted particles with fixed fractions of sections and fields counted. In the present work, the optical disector method was applied, due to the advantage of eliminating the difficult and time consuming task of identifying corresponding portions in different physical sections. In Table 1 a review of the morphometric data of the medullary nuclei from previous studies is presented. Most morphometric studies on medullary nuclei, with few exceptions (Tang et al., 2001/2002; Lasn et al., 2006), did not apply the disector or fractionator methods, indicating a not yet complete acquaintance of the importance of the unbiased principles at the basis of such methods. A research group compared analyses of total neuron numbers in MedVe with two-dimensional

and three-dimensional probes (optical fractionator), finding 18% higher total neuron number with the unbiased method (Lopez et al., 1997; Tang et al., 2001/2002). Moreover, it must be considered that preceding studies concern one or few nuclei, lacking a comprehensive and contemporary study of many medullary structures. The use of different methods in the above studies limits the reliability of the comparison of morphometric data from different nuclei. Moreover, morphometric data regarding NTS, NSTT and accessory inferior olivary nuclei are completely lacking. To the best of our knowledge this is the first study applying the unbiased optical disector method to detail reliable morphometric data of a relatively wide series of medullary nuclei.

Previous works reported noteworthy differences between the various medullary nuclei in terms of vascularization (Porzionato et al., 2004b, 2005b) and responses to hypoxic-ischaemic injuries (Gilles, 1969; Revesz and Geddes, 1988; De Caro et al., 2000, 2003; Parenti et al., 2005; Porzionato et al., 2004b, 2008a). Our present study also revealed statistically significant differences between morphometric parameters of the different medullary nuclei. In particular, the nuclei of the medullary tegmentum (XII, DMNV, NTS, MedVe, Cu) showed lower neuronal densities than the NSTT and the nuclei of the inferior olivary complex, which are located in a more ventral position (Fig. 2). Further analysis could consider the relationships between morphometric data of neuronal populations and microvascularization.

As regards comparison between adults and infants, we have not found statistically significant differences in the total neuron numbers in all the nuclei examined. In the literature, higher total neuron number was only found in the medial Cu of an adult than in a 2-month infant, and was ascribed to continuation of the development of this nucleus also after birth (Ma et al., 2005). However the small number of specimens examined in that study strongly limited the reliability of the finding. The absence of statistically significant differences in total neuron

number between adults and infants indicates the completment of the development of the medullary nuclei considered, at least for total neuron number, in the prenatal period. On the contrary, both literature data and our findings showed an increased volume and corresponding decreased neuronal density in adult medullary nuclei with respect to infant ones. Such changes may be ascribed to development of the neuropil and microvascularization (Bourrat and Sotelo, 1984; Porzionato et al., 2004b, 2005b). Some authors focused on aging effects on nervous structures. Significant age-related losses of neurons were found in the cerebral cortex (Anderson et al., 1993), cerebellar Purkinje cell population (Hall et al., 1975), putamen (Pesce and Reale, 1987), substantia nigra (McGeer et al., 1977), locus coeruleus (Mann et al., 1983), vestibular nuclei (Alvarez et al., 1998, 2000), while no age-related neuronal loss was found in the ventral cochlear (Konigsmark and Murphy, 1972), abducens (Vijayashankar and Brody, 1977), facial (Van Buskirk, 1945) and inferior olivary nuclei (Moatamed, 1966). In our study, we did not find significant age-related neuronal losses in medullary nuclei. Most of the above literature studies on aging effects on neuronal populations have been performed with biased methods. Further investigations performed with unbiased methods will be necessary to better clarify differential aging effects on the various nervous structures. At the moment, data arising from literature and our investigations, seem to indicate a lower susceptibility to aging of the rombencephalic nuclei, which are phylogenetically and ontogenetically more ancient. Moreover, further studies considering morphological and morphometric parameters could be of interest in order to fully evaluate the development of the medullary nuclei regarding synaptogenesis and maturation of the neuropil, also with reference to the different patterns of dendritic arborisation (isodendritic, allodendritic and idiodendritic).

DISTRIBUTION OF APOPTOSIS

It has been reported that the TUNEL method can potentially detect random DNA fragmentation due to necrosis (Gold et al., 1994) or post-mortem damage (Scott and Hegyi, 1997). However, in our study, TUNEL-positive cell nuclei did not show any of the characteristics of necrosis, i.e., swelling, dispersion or flocculation of chromosomal material (Clarke, 1990). Moreover, tissue fixation time was quite constant for all specimens, and the apoptotic indexes did not correlate with post-mortem delay. However, although some authors report that post-mortem periods of up to 48 hours do not influence *in situ* end-labelling in rat brain (Petito and Roberts, 1995a) and that post-mortem intervals of up to 70 hours do not have significant effects on the detection of apoptosis by the TUNEL method in human brain (Adle-Biassette et al., 1995; Gelbard et al., 1995; Petito and Roberts, 1995b; Vincent et al., 1999; Cosenza et al., 2004), we must consider that some of the apoptotic phenomena evidenced in our study are to be ascribed to post-mortem changes.

In the present study, differences in apoptotic indexes between opiate deaths and controls were analysed, as opiates have been shown to induce apoptosis of neuronal cells in experimental studies in rats (Atici et al., 2004), chick embryo cell cultures (Goswami et al., 1998), and murine (Goswami et al., 1998) and human (Yin et al., 1997; Hu et al., 2002) neuroblastoma cell lines. It has also been suggested that long-term neurobehavioural damage (Steingart et al., 2000) and developmental abnormalities (Basheer et al., 1992; Seatriz and Hammer, 1993; Harlan and Song, 1994; Malanga and Kosofsky, 1999) in the brains of neonatal animals born to pregnant animals exposed to opiates may be ascribed to morphine-induced apoptosis of neurons (Hu et al., 2002). In our study, comparison between apoptotic indexes of opiate deaths and controls did not show statistically significant differences. Thus, the *in vivo* apoptotic effect of morphine was not evidenced in our study. This finding may be ascribed to post-mortem changes or to a common effect due to terminal hypoxic-ischaemic injury. Opiate

deaths and controls shared systemic hypoxic-ischaemic injury as terminal cause of death, and the literature has clarified that this kind of injury may produce neuronal apoptosis (Snider et al., 1999).

As regards the infant cases, a higher incidence of neuronal apoptosis within the brainstem of SIDS victims has been described, with particular reference to the gracile nucleus, Cu, NSTT, NTS, lateral reticular formation and lateral Cu, and ascribed to hypoxia (Waters et al., 1999). In our study, however, we did not find higher apoptotic indexes in SIDS cases with respect to other infant cases, although this may be due to the small number of cases.

A comparison was also made between adults and infants, due to the absence of significant internal differences in the two populations. The neuronal apoptotic indexes of infants were lower than those of adults, with statistical significance for XII, DMNV, Cu, Ve and NTS. These findings indicate that neuronal populations show different characteristics of survival between adults and infants. The neurons of these infant nuclei may be more resistant to hypoxic-ischaemic injury or post-mortem changes. Instead, no statistically significant correlation between age and apoptosis was found in the adult series.

Heterogeneity was found among the neuronal apoptotic indexes of the various nuclei of the medulla oblongata. The topographical pattern of apoptosis in the medulla oblongata of adults did not differ significantly from that of infants. These findings may be explained by reference to differing hypotheses. It may be that neurons of different nuclei simply take different times to become apoptotic, as a result of post-mortem changes. Otherwise, these findings may indicate the different vulnerability of the medullary nuclei in response to hypoxic-ischaemic injury. Further studies on experimental animals will be necessary to verify this hypothesis.

Three areas of vascularisation have been described in the medulla, i.e., median, fed by the paramedian arteries; middle, fed by the short circumferential arteries; and lateral, fed by the posterior inferior cerebellar artery (PICA) (Foix and Hillemand, 1925; Lazorthes, 1961).

Although at the moment we cannot evaluate the apoptotic component due to post-mortem changes, it is intriguing that the nuclei with higher neuronal apoptotic indexes, i.e., Cu, Ve and NSTT, are all located in the lateral area of the medullary tegmentum and share the same vascular supply from the PICA, thus suggesting that local vascularisation plays a role in determining the spatial distribution of apoptosis.

PHOSPHODIESTERASE ISOFORMS

By using oligonucleotide probes that selectively recognize the transcripts of the four isozyme forms of the PDE4 family (PDE4A, PDE4B, PDE4C and PDE4D), we have examined their regional distribution and cellular localization in human brainstem. Our results show that PDE4D mRNA expression was in general stronger than PDE4B. Additionally, PDE4B and PDE4D mRNA expression was abundant and distributed not only in neuronal cells but also in glial cells, especially on and around blood vessels. An important finding is that the signals for PDE4B and PDE4D mRNAs in the AP were stronger than those in any other nuclei in the brainstem. To our knowledge, this is the first detailed report of the regional distribution and cellular localization of the PDE4 isozymes in human brainstem.

We observed positive hybridization signals for PDE4B and PDE4D in several nuclei in human brainstem, especially in the AP. The AP modulates cardiovascular responses through local action of a series of circulating peptides, such as ANG II, vasopressin and endothelins, and has been implicated as a chemoreceptor trigger zone for vomiting (emesis) (Miller and Leslie, 1994). Emesis is one of the most common side effects of PDE4 inhibitors administration. The mechanism by which PDE4 inhibition results in increased vomiting and nausea in species such as dog and human (Bertolino et al., 1988; Hebenstreit et al., 1989; Heaslip and Evans, 1995) is not fully understood, but probably includes both central and

peripheral sites of actions. Treatment of rats with rolipram elevated Fos-like immunoreactivity (an activity marker for a variety of neuronal populations in the brain) in brain regions associated with emesis such as the AP and NTS (Bureau et al., 2006). Our finding suggests that cAMP signaling modification in this area could mediate the emetic effects of PDE4 inhibitors in human brainstem.

In the present study, hybridization signal for PDE4B and PDE4D mRNAs was stronger in the AP than in any other nuclei in human brainstem and those signals were expressed in association with blood vessels. Morphologically, the AP shares many features with the subfornical organ and the vascular organ of the lamina terminalis, including rich vascularity (numerous blood vessels), modified ependyma, and a network of neuroglia (Weisinger et al., 1990). In a previous work (Porzionato et al., 2005b) we evaluated the regional differences in microvessel density of the human medullary tegmentum and reported that DMNV and AP showed higher microvessel density with respect to NTS, ST and XII. These findings indicate that the expression of PDE4B and PDE4D mRNAs in the AP might be regulated by chemical substances originated from blood vessels.

The anatomical distribution of PDE4B and PDE4D mRNAs presented in this study partially agrees with that reported by Lamontagne and coworkers (Lamontagne et al., 2001) who detected expression of PDE4D mRNA and protein in the medulla and nodose ganglion of squirrel monkey. However, and in contrast with our results, they were unable to find PDE4B expression mainly due, according to them, to the lack of selective antibodies for this isoenzyme and to inconclusive results obtained with their *in situ* hybridization experiments. Further evidence that supports the role of PDE4D in the emetic response is the increased reversion of alfa-2-mediated-anaesthesia, a behavioural surrogate of emesis in non vomiting species found with pro-emetic PDE4 inhibitors (Robichaud et al., 2002a), that has also been demonstrated using PDE4D deficient mice (Robichaud et al., 2002b). In contrast, in the same

study, PDE4B deficient mice exhibited the same sleeping time than their wild-type littermates under xylacine/ketamine induced anaesthesia (Robichaud et al., 2002b). In our study, the AP contained higher levels of PDE4D mRNA than those of PDE4B mRNA in human brainstem (Table 5) when compared to their own expression in other brainstem nuclei, similar to what it has been previously described in the AP of rat brainstem (Pérez-Torres et al., 2000). The AP is a region which is known to mediate vomiting and nausea as emesis (Borinson and Wang 1953; Carpenter et al., 1988). Based on their results, Miller and Ruggiero (1994) have proposed that there is not an unique group of neurons that might function as "vomiting center", but rather that those neurons involved in coordinating emesis could branch out from the AP and NTS to an arc in the lateral tegmental field implicated in somato-autonomic integration. The majoritary presence of PDE4D mRNA in some of these brainstem nuclei in this study points to its possible participation in emesis and other functions.

PDE4 inhibitors such as roflumilast and cilomilast have been proposed for the treatment of chronic respiratory diseases, such as asthma and COPD, where they have shown modest but significant improvement in several clinically relevant readouts (Rabe et al., 2005; Lipworth, 2005). Full exploitation of their therapeutic potential has been hampered though by the dose limitation imposed by the appearance of unwanted secondary effects, of which nausea has been consistently considered as one of the most prominent (Compton et al., 2001; Chung, 2006).

In conclusion, our studies have demonstrated that PDE4B and PDE4D mRNAs are present in human brainstem, especially in the AP in association with blood vessels. This evidence, together with the expression of PDE4D in the nodose ganglia and the functional results on KO mice suggests that PDE4D, could be associated to distinct functions of the AP such as

emesis in men. In contrast the functional role of PDE4B in human brainstem, and especially in the AP, remains to be elucidated.

NEUROMODULATORS: ADRENOMEDULLIN AND NEUROTENSIN SYSTEMS

Many neurotransmitters and neuromodulators have been identified in glomic cells, such as dopamine, serotonin, noradrenaline, adrenaline, acetylcholine, adenosine, met- and leu-enkephalins, NPY, CGRP, galanin, endothelins, bombesin, NO and AM (Bairam and Carroll, 2005; Porzionato et al., 2008a). The data of our study demonstrated AM immunoreactivity in the type I cells of human carotid body, in accordance with previous data on rats (Martinez et al., 2003). The association of amines and peptides has been considered of importance for the chemoreceptor properties of the carotid body (Wharton et al., 1980). In the carotid body there are afferent glossopharingeal nerve endings which are considered the true chemoreceptors and their sensivity is regulated through releasing of dopamine by type I cells (McDonald and Mitchell, 1975). Martinez et al (2003) evidenced that AM induced dopamine release *in vitro* from glomus cells and that AM expression and secretion were elevated after hypoxic stimuli. Thus, the detection of AM in type I cells of the human carotid body supports a role of AM in the regulation of chemoreceptor discharge.

Carotid body is characterized by an elevated blood flow (2000ml/100g/min) (Ganong, 2003). Local changes in blood flow has been considered to be involved in carotid body chemoreceptor discharge (Joels and Neil, 1963). For instance, it has been pointed out that serotonin may be involved, through its vasoactive effect, in a vascular-mediated control of carotid body function (Kirby and McQueen, 1984). AM has a known vasodilator effect which probably plays a role in regulation of local blood flow and, indirectly, in chemoreceptor discharge.

An intriguing result of our study is the lower expression of AM in foetuses with respect to adult subjects. Different patterns of expression of von Willebrand factor, in relation with age, have been found at the level of the microvasculature of the human medulla oblongata (Porzionato et al., 2005a). Also the expression of NPY in the type I cells of the rat carotid body appeared to be developmentally regulated (Oomori et al., 1991). Our results indicate that the AM is fully expressed in the carotid body only in the postnatal period. This may be ascribed to the absence of pulmonary respiration with lack of regulatory role of the carotid body during the prenatal period.

In our study, positive AM immunoreaction was mainly detected in the dark cells, both in adult subjects and foetuses. Some authors (Pallot et al., 1992) suggested that previous descriptions of light, dark and pyknotic cells in the human carotid body could represent a description of post-mortem changes in that they found an almost linear decrease in the number of light cells with the post-mortem interval in an histological study performed on rat carotid body. These data have not been confirmed in humans and light and dark cells continue to be considered as variants of the same cell type (Verna, 1997; Vinhaes et al., 2002; Porzionato et al., 2005a). In particular, it has been suggested that dark cells could represent a higher functional status of type I cells (Vinhaes et al., 2002) in that dark cell hyperplasia was observed in rabbits exposed to altitude hypoxia (Smith et al., 1993), in patients with asthma (Bencini and Pulera, 1991) and acute respiratory distress syndrome (Vinhaes et al., 2002). Moreover, Biscoe and Stehbens (1966) detected a highly complex secretory structure in the dark cells with larger number of electron-dense granules with respect to light cells.

The almost selective synthesis and storing of AM in the dark cells is consistent with a specific functional (and not artifactual) significance of this cell type. In particular, it supports the hypothesis that dark cells may be a higher and more complex functional state of type I cells and may represent the cell types mainly involved in chemoreceptor discharge. The fact

that not all dark cells are AM immunoreactive may be also explained with reference to the secretory characteristics of the peptide and to the different functional states of the cells. The different AM immunostaining between the two casistics and between cell types may not be ascribed to an artifact due to sampling and immunohistochemical technique because of the uniformity of sampling, postmortem interval and fixation time among the cases.

It has been suggested a role of AM in mediating hyperplasia of glomus cells in response to hypoxia (Martinez et al., 2003) as AM is also known as an antiapoptotic and growth promoting factor (Miller et al., 1996; Belloni et al., 2003). Hence, it could be of interest to quantify the AM in hypoxic diseases accompanied with changes in cellular populations of the carotid body and to evaluate whether a correlation with apoptosis is identifiable.

NT occurrence and distribution has been studied by radioimmunoassay (Heath et al., 1988), immunohistochemistry (Heym and Kummer, 1989) and immunogold techniques (Smith et al., 1990). In human carotid bodies, radioimmunoassay showed higher levels of NT expression than VIP and substance P, and lower levels of bombesin, met- and leu-enkephalin (Heath et al., 1988). The immunogold technique revealed the weakest immunoreactivity for neurotensin with respect to the other neuropeptides (Smith et al., 1990). A species-specific pattern of NT expression was also reported, with NT immunolabeling only in beagle glomus cells among various animal species (Heym and Kummer, 1989). The location of NTR1 in the carotid body has never been investigated before, so that our study demonstrates for the first time the selective expression of NTR1 in human and rat type I cells. In some cases, immunoreaction was cytoplasmic and in others nuclear, thus also confirming NT internalization in the carotid body. The higher cytoplasmic NTR1 positivity in humans than in rats may be explained on the basis of the different kinetics of internalization, or longer post-mortem intervals. Thus, NT produced by type I cells may act in an autocrine or

paracrine way on the same cell type. It is possible that NT acting on type I cells also derives from NT-positive sympathetic or parasympathetic fibers innervating type I cells, although there is no direct evidence of this in the literature. NT probably plays a modulatory role on chemoception by type I cells, as the neuromodulatory effects of NT have already been proposed in ganglion transmission (Bachoo and Polosa, 1988; Stapelfeldt and Szurszewski, 1989a, 1989b). Further functional studies will be necessary to understand the effect of NT in glomic cells. Binding of NT to NTR1 stimulates phosphorylation of ERK and AKT (Hassan et al., 2004) and probably contributes to the activation of ERK and AKT signalling pathways in the carotid body. Cell line PC12, which shows oxygen-sensing properties and has been considered as a model for carotid body function (Millhorn et al., 1996), cannot be used for investigation of NT action, as these cells express and release neurotensin (Tischler et al., 1991) but seem not to show functional NTR (Caillaud et al., 1995). However, it is intriguing to note that NT attenuates dopamine-induced inhibition of midbrain dopamine neurons (Seutin et al., 1989; Shi and Bunney, 1992), as dopamine is the main glomic neurotransmitter. It may be hypothesized that, also in the carotid body, NT reduces the inhibitory effect of dopamine on chemoception.

ADHESION MOLECULES: NEURAL CELL ADHESION MOLECULE

In the literature, NCAM has been identified in Natural Killer cells and in a subset of activated T lymphocytes. Moreover, it has also been identified in many different cells of neuroectodermal origin. NCAM is widely distributed in neurons and glial cells of both central and peripheral nervous systems whereas, in the adult central nervous system, PSA-NCAM is restricted to brain regions showing ongoing neurogenesis and morphofunctional plasticity, such as the subventricular zone of the forebrain or the hippocampus (Seki and Arai, 1993;

Gubkina et al., 2001). NCAM has also been detected in cells of thyroid follicular epithelium, adrenal cortex and medulla, pancreatic islets and pituitary gland (Langley et al., 1989; Grant et al., 1992; Berardi et al., 1995; Zeromski et al., 1998). Apart from chromaffin cells of the adrenal medulla, NCAM has also been found in extraadrenal chromaffin cells of paraortic bodies of adult rabbits (Moftaquir et al., 1996). PSA-NCAM immunoreactivity has been found in type I cells of rat carotid bodies and its possible role as marker of precursor cells has been stressed (Izal-Azcarate et al., 2008). The present report is the first to confirm the expression of NCAM, apart from the isoform, in the human carotid body and to detail its expression in the different subpopulations of type I cells. Confirmation in humans of expression data arisen from experimental animals is particularly important in the carotid body which is known to show morphological and molecular differences between species. For instance, type I cells of many non human carotid bodies do not show subdivision in dark, light, and pyknotic subtypes. Moreover, some neuromodulators, such as Met-enkephalin (Lundberg et al., 1979; Wharton et al., 1980; Heym and Kummer, 1989; Smith et al., 1990) and somatostatin (Kameda, 1989; Kummer et al., 1989; Kusakabe et al., 1991; Kummer and Habeck, 1992), are expressed in the carotid body of some species but not of others.

Type I cells are known to express many different neurotransmitter/neuromodulator systems and to modulate chemoreception through autocrine/paracrine mechanisms (Nurse, 2005; Porzionato et al., 2006a). NCAM specifically regulates adhesion between type I cells in glomic lobules and as a consequence probably plays a role in permitting such paracrine modulation of chemoreception through neuromodulation.

The carotid body is characterized by a peculiar development/differentiation process which can extend in the postnatal period and by the capability to undergo cellular/molecular changes in response to chronic hypoxia. It develops from a specific condensation of mesenchymal cells in the wall of the third branchial artery (Kondo, 1975); type I cells then

originate from neural crest elements and glomus cells showing serotonin immunoreactivity begin to appear in the E16.5 mouse carotid body (Kameda et al., 2002; Kameda, 2005). The carotid body does not contribute greatly to breathing during foetal life but, in the following postnatal period, gradual increases in hypoxic chemosensitivity and in the slope of the hypoxic stimulus response curve develop (Blanco et al., 1984; Bamford et al., 1999; Gauda et al., 2004). Postnatal changes (reviewed in Porzionato et al., 2008d) include carotid body volume increase, proliferation of type I, type II, endothelial and Schwann cells lining peripheral nerve fibers and increased number of synapses between type I and II cells. In the carotid body, chronic hypoxia causes a series of morphological, cellular and biochemical changes such as volume enlargement, percentual increase in type II cells, increased O_2 sensitivity through changes in molecular chemoreceptors, ion channels and neurochemicals (reviewed in Prabhakar and Jacono, 2005). In opiate addiction, which is characterized by repeated hypoxic episodes, the percentage of type II cells and intralobular and interlobular connective components have also been found to be increased (Porzionato et al., 2005a). Our study reported expression of NCAM in the great majority of human type I cells. The high expression level of NCAM and its ascertained role in neural mechanisms of differentiation, survival and cell plasticity suggest a possible involvement in the development/differentiation process of the carotid body and in determining cellular/molecular changes due to hypoxia.

Further studies will be necessary to better evaluate the expression of other CAMs in the different glomic cell types. At the moment, there are no consistent data in the literature about the presence of selectins, integrins, cadherins or other members of the Ig superfamily in the carotid body. However, the high expression level of NCAM suggests a possible role played also by other CAMs in the regulation of steric interactions between cells and cell types in the carotid body, modulating chemosensitive function and cell plasticity through modulation of the local effects of neurotransmitters/neuromodulators and growth factors.

SIGNAL TRANSDUCTION SYSTEMS: ERK AND AKT

Our study demonstrated both ERK and AKT immunolocalization and activation in the carotid body. ERK and AKT immunolabelling were mainly found in cytoplasm; pERK immunolabelling was located mainly in the cytoplasm, but also in the nucleus of some cells; pAKT immunolabelling showed different patterns of positivity. pERK and pAKT nuclear immunoreactions were consistent with nuclear translocation of the phosphorylated forms. However, nucleo-cytoplasmic pERK immunopositivity is also consistent with previous studies in the central nervous system, which revealed immunolabelling in multiple subcellular compartments, including nucleus, cytosol, proximal dendrites and terminals (Flood et al., 1998; Koh et al., 2002; Springell et al., 2005) and the cytoplasmic location of pAKT immunolabelling has also been reported by some authors (e.g. Dubé et al., 2008). ERK- and AKT-immunopositive cells were more numerous than pERK- and pAKT- immunopositive ones, in accordance with ERK and AKT activation in a subpopulation of cells expressing ERK and AKT. To our knowledge, this study is the first to reveal ERK and AKT immunolocalization and activation in cell populations of the human carotid body. Both I and II cell types showed ERK and AKT immunopositivity, although more intense immunopositivity was found in type I cells, which are considered to be the chemosensitive element of the carotid body (Verna, 1997; Wang and Bisgard, 2005).

In the literature, ERK and AKT activation has mainly been considered to be related to cell proliferation and survival, respectively. Some neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF), have been reported to activate ERK and AKT in various cell types, including neurons (Gottschalk et al., 1998; Zheng et al., 2000; Sweatt, 2001; Yamada et al., 2001; Qiao et al., 2004; Shelton et al., 2004; Cowen, 2007). Such growth factors are known to exert a local action also on carotid body type I cells (Wang and Bisgard, 2005; Nurse and Fearon, 2002). Both ERK and AKT

phosphorylation have also been reported for activation of D_2 dopaminergic (Roberson et al., 1999; Beom et al., 2004; Beaulieu et al., 2007), muscarinic (Jones et al., 1999; Roberson et al., 1999; Rosenblum et al., 2000; Krieg et al., 2002), α_2 adrenergic (Taraviras et al., 2002; Roberts, 2004), $5HT_{2A}$ serotoninergic (Martini et al., 2004; Cowen, 2007), $GABA_B$ (Balasubramanian et al., 2004; Rane et al., 2005), and A_{2A} adenosine (Canals et al., 2005; Wiese et al., 2007) receptors, which are known to be present in type I cells. Thus, activation of ERK and PI3K/AKT signalling pathways in carotid body type I cells may be ascribed to neurotransmitters/neuromodulators or neurotrophic factors.

Both ERK and AKT signalling pathways have also been reported to be involved in neuronal synaptic plasticity (Kelly and Lynch, 2000; Sweatt, 2001; Sanna et al., 2002; Man et al., 2003; Opazo et al., 2003) and the carotid body may undergo long-term sensory facilitation or depression as a consequence of chronic hypoxia (Prabhakar and Jacono, 2005; Powell, 2007) or hyperoxia (Bavis, 2005), respectively. Thus, it may be hypothesised that ERK and AKT signalling pathways are involved in such long-term cellular modifications in the carotid body, although further studies in animals and carotid body cultures need to be undertaken.

In the literature, ERK and AKT activation has been reported to be regulated on a developmental basis. For instance, basal levels of pERK are lower in CA1 cells from immature mice than from adult ones Vanhoose et al. (2002) and Kato et al. (2005) found that pERK appeared in different neuron populations of the dorsal root ganglia of the chick embryo at specific embryonic stages, i.e., on E5 and E9 in the ventrolateral and dorsomedial ganglionic regions, respectively. Other authors have found that, during the embryonic development of the retina, the insulin survival effect is selectively mediated by ERK activation at E5 and by AKT activation at E9 (Chavarria et al., 2007). To the best of our knowledge, this is the first study to evaluate the involvement of the ERK and AKT signalling pathways in the human carotid body at foetal and postnatal periods.

We found both lower ERK and AKT immunopositivity and activation in foetal cell populations of carotid body with respect to post-natal periods. In accordance with literature and our data about developmental expression of NPY (Oomori et al., 1991) and AM, respectively, and with full development of the chemosensitive function of the carotid body only in the first post-natal weeks (Blanco et al., 1984, 1988; Gauda et al., 2004), our findings indicate that ERK and AKT signalling pathways are not yet fully operative in humans during foetal life.

CONCLUSIONS AND FUTURE PERSPECTIVES

The present study is the first in the literature to give reliable data, obtained with the unbiased method of optical dissector, about morphometric parameters (neuronal densities, nuclear volume, total neuron numbers) and apoptotic distribution about a wide series of medullary nuclei. Original are also our contributions about regional expression of the different PDE4 isoforms in the AP and other medullary nuclei. About carotid body, to the best of our knowledge our study is the first to identify the presence in the carotid body of some neuromodulators or correspondent receptors (AM and NTR1), adhesion molecules (NCAM) and signaling pathways components (ERK/pERK, AKT/pAKT). Our findings and methodological approaches developed during this study will permit us in the future a widening and deepening of our research project.

During this study very numerous samples of human medullae oblongatae and carotid bodies have been collected by our Research Group. This autoptic material will also permit in the future years the analysis of other neuroanatomic and neuropathologic data. In the Introduction Paragraph entitled “Neuromodulators in the Carotid Body”, literature data about the glomic presence of a series of neuromodulators have been considered. Reliable data about expression of many neuromodulators or neuromodulator receptors are still lacking. Thus, these neuromodulators will also be analysed by immunohistochemistry and immunofluorescence in the following years. Moreover, literature data about expression of various PDE in the carotid body are also still lacking so that *in situ* hybridization and immunohistochemical studies, analogous to those performed in the medullary nuclei, will also be performed in our carotid body samples.

Apart from analysis of human materials, we are also developing new *in vivo* and *in vitro* studies. An experimental project on rats have also been begun aimed at evaluating changes in the medullary nuclei and carotid body in response to hypoxic or hyperoxic conditions, the

modulation of O_2 and CO_2 partial pressures being performed with the help of OxyCycler technology (BioSpherix, OxyCycler model A84XOV, Redfield, NY). A series of samples have been collected and first results will be available in the following months.

In the recent years, new technologies have been developed which could be applied to selective analysis of patterns of expression of different neuronal populations and cell types in the medullary nuclei and carotid body. Laser-capture microdissection has recently been applied to obtain homogeneous cell populations from endocrine and nervous structures, such as the pituitary gland (Lloyd et al., 2005), hippocampus (Kamme et al., 2003) and amygdala (Zirlinger and Anderson, 2003). Microarray analyses have also been performed on mRNA extracted from these cell populations. Laser-capture microdissection in conjunction with microarray analysis may allow genome-wide screening of transcripts from selective neuronal populations and glomus cells. Studies of proteomics could also provide complete and accurate profiles of protein expression in response to exposure to various noxious or trophic stimuli.

Techniques for producing and maintaining carotid body cell cultures have also been developed. In the Department of Human Anatomy and Physiology a protocol has been prepared for developing such cultures from carotid bodies of newborn rats. These cultures will permit the evaluation of the effects of neuromodulators, such as AM and NT, on carotid body cells. Changes of expression of various neuromodulators, trophic factors, adhesion molecules and signalling pathway components will also be analysed in response to hypoxic or hyperoxic conditions.

REFERENCES

1. Abercrombie M. (1946). Estimation of nuclear population from microtome sections. Anat Rec 94, 239-247.
2. Abramovici A, Pallot DJ, Polak JM. (1991). Immunohistochemical approach to the study of the cat carotid body. Acta Anat 140, 70-74.
3. Adle-Biassette H, Levy Y, Colombel M, Poron F, Natchev S, Keohane C, Gray F. (1995). Neuronal apoptosis in HIV infection in adults. Neuropathol Appl Neurobiol 21, 218-227.
4. Allen AM. (1998). Angiotensin AT1 receptor-mediated excitation of rat carotid body chemoreceptor afferent activity. J Physiol 510, 773-781.
5. Allen MA, Smith PM, Ferguson AV. (1997). Adrenomedullin microinjection into the area postrema increases blood pressure. Am J Physiol 272, R1698-1703.
6. Alvarez JC, Diaz C, Suarez C, Fernandez JA, Gonzalez del Rey C, Navarro A, Tolivia J. (1998). Neuronal loss in human medial vestibular nucleus. Anat Rec 251, 431-438.
7. Alvarez JC, Diaz C, Suarez C, Fernandez JA, Gonzalez del Rey C, Navarro A, Tolivia J. (2000). Aging and the human vestibular nuclei: morphometric analysis. Mech Ageing Dev 114, 149-172.
8. Anastasi A, Erspamer V, Bucci M. (1971). Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. Experientia 27, 166-167.
9. Anderson JM, Hubbard BM, Coghill GR, Slidders W. (1983). The effect of advanced old age on the neurone content of the cerebral cortex. Observations with an automatic image analyser point counting method. J Neurol Sci 58, 235-246.
10. Anderson DJ. (1993). Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. Annu Rev Neurosci 16, 129-158.
11. Anglade P, Vyas S, Hirsch EC, Agid Y. (1997). Apoptosis in dopaminergic neurons of the human substantia nigra during normal aging. Histol Histopathol 12, 603-610.
12. Aoki M, Fukunaga M, Sugimoto T, Hirano Y, Kobayashi M, Honda K, Yamada T. (2001). Studies on mechanisms of low emetogenicity of YM976, a novel phosphodiesterase type 4 inhibitor. J Pharmacol Exp Ther 298, 1142-1149.
13. Arias-Stella J, Valcarcel J. (1973). The human carotid body at high altitudes. Pathol Microbiol 39, 292-297.
14. Arias-Stella J, Valcarcel J. (1976). Chief cell hyperplasia in the human carotid body at high altitudes; physiologic and pathologic significance. Hum Pathol 7, 361-373.
15. Asrari M, Lobner D. (2001). Calcitonin potentiates oxygen-glucose deprivation-induced neuronal death. Exp Neurol 167, 183-188.

16. Atici S, Cinel L, Cinel I, Doruk N, Aktekin M, Akca A, Camdeviren H, Oral U. (2004). Opioid neurotoxicity: comparison of morphine and tramadol in an experimental rat model. Int J Neurosci 114, 1001-1011.
17. Bachoo M, Polosa C. (1988). Cardioacceleration produced by close intra-arterial injection of neurotensin into the stellate ganglion of the cat. Can J Physiol Pharmacol 66, 408-412.
18. Bairam A, Carroll JL. (2005). Neurotransmitters in carotid body development. Respir Physiol Neurobiol 149, 217-232.
19. Balasubramanian S, Teissere JA, Raju DV, Hall RA. (2004). Hetero-oligomerization between GABAA and GABAB receptors regulates GABAB receptor trafficking. J Biol Chem 279, 18840-18850.
20. Bamford OS, Sterni LM, Wasicko MJ, Montrose MH, Carroll JL. (1999). Postnatal maturation of carotid body and type I cell chemoreception in the rat. Am J Physiol 276, L875-884.
21. Barnette MS, Christensen SB, Essayan DM, Grous M, Prabhakar U, Rush JA, Kagey-Sobotka A, Torphy TJ. (1998). SB 207499 (Ariflo), a potent and selective second-generation phosphodiesterase 4 inhibitor: in vitro anti-inflammatory actions. J Pharmacol Exp Ther 284, 420-426
22. Basheer R, Yang J, Tempel A. (1992). Chronic prenatal morphine treatment decreases G alpha s mRNA levels in neonatal frontal cortex. Brain Res Dev Brain Res 70, 145-148.
23. Bavis RW. (2005). Developmental plasticity of the hypoxic ventilatory response after perinatal hyperoxia and hypoxia. Respir Physiol Neurobiol 149, 287-299.
24. Beaulieu JM, Tirotta E, Sotnikova TD, Masri B, Salahpour A, Gainetdinov RR, Borrelli E, Caron MG. (2007). Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. J Neurosci 27, 881-885.
25. Belloni AS, Trejter M, Malendowicz LK, Nussdorfer GG. (2003). Adrenomedullin stimulates proliferation and inhibits apoptosis of immature rat thymocytes cultured in vitro. Peptides 24, 295-300.
26. Bencini C, Pulera N. (1991). The carotid bodies in bronchial asthma. Histopathology 18, 195-200.
27. Benvenuti LA, Reis MM, de Lourdes Higuchi M. (1996). Immunohistochemical detection of atrial natriuretic peptide (ANP) in the chief cells of human carotid bodies. Acta Histochem 98, 89-92.
28. Beom S, Cheong D, Torres G, Caron MG, Kim KM. (2004). Comparative studies of molecular mechanisms of dopamine D2 and D3 receptors for the activation of extracellular signal-regulated kinase. J Biol Chem 279, 28304-28314.

29. Berardi M, Hindelang C, Laurent-Huck FM, Langley K, Rougon G, Félix JM, Stoeckel ME. (1995). Expression of neural cell adhesion molecules, NCAMs, and their polysialylated forms, PSA-NCAMs, in the developing rat pituitary gland. Cell Tissue Res 280, 463-472.
30. Bertolino A, Crippa D, di Dio S, Fichte K, Musmeci G, Porro V, Rapisarda V, Sastre M, Schratzer M. (1988). Rolipram versus imipramine in inpatients with major, "minor" or atypical depressive disorder: a double-blind double-dummy study aimed at testing a novel therapeutic approach. Int Clin Psychopharmacol 3, 245-253
31. Biscoe TJ, Bradley GW, Purves MJ. (1969). The relation between carotid body chemoreceptor activity and carotid sinus pressure in the cat. J Physiol 203, 40P.
32. Biscoe TJ, Stehbens WE. (1966). Ultrastructure of the carotid body. J Cell Biol 30, 563-578.
33. Blanco CE, Dawes GS, Hanson MA, McCooke H.B. (1984). The response to hypoxia of arterial chemoreceptors in fetal sheep and new-born lambs. J. Physiol. 351, 25-37.
34. Blanco CE, Hanson MA, McCooke HB. (1988). Effects on carotid chemoreceptor resetting of pulmonary ventilation in the fetal lamb in utero. J Dev Physiol 10, 167-174.
35. Borinson HL, Wang SC. (1953). Physiology and pharmacology of vomiting. Pharmacol Rev 5, 193-230.
36. Bourrat F, Sotelo C. (1984). Postnatal development of the inferior olivary complex in the rat. III. A morphometric analysis of volumetric growth and neuronal cell number. Brain Res 318, 241-251.
37. Branchek TA, Smith KE, Gerald C, Walker MW. (2000). Galanin receptor subtypes. Trends Pharmacol Sci 21, 109-117.
38. Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I. (2005). Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288, G621-629.
39. Bureau Y, Handa M, Zhu Y, Laliberte F, Moore CS, Liu S, Huang Z, Macdonald D, Xu DG, Robertson GS. (2006). Neuroanatomical and pharmacological assessment of Fos expression induced in the rat brain by the phosphodiesterase-4 inhibitor 6-(4-pyridylmethyl)-8-(3-nitrophenyl) quinoline. Neuropharmacology 51, 974-985
40. Caillaud T, Opstal WY, Scarceriaux V, Billardon C, Rostene W. (1995). Treatment of PC12 cells by nerve growth factor, dexamethasone, and forskolin. Effects on cell morphology and expression of neurotensin and tyrosine hydroxylase. Mol Neurobiol 10, 105-114.
41. Canals M, Angulo E, Casado V, Canela EI, Mallol J, Vinals F, Staines W, Tinner B, Hillion J, Agnati L, Fuxe K, Ferre S, Lluis C, Franco R. (2005). Molecular mechanisms involved in the adenosine A_1 and A_{2A} receptor-induced neuronal differentiation in neuroblastoma cells and striatal primary cultures. J Neurochem 92, 337-348.

42. Carpenter DO, Briggs DB, Knox AP, Strominger N. (1988) Excitation of area postrema neurons by transmitters, peptides, and cyclic nucleotides. J Neurophysiol 59, 358-369
43. Carraway R, Leeman SE. (1973). The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J Biol Chem 248, 6854-6861.
44. Carraway RE, Mitra SP. (1994). Binding and biologic activity of neurotensin in guinea pig ileum. Peptides 15, 1451-1459.
45. Castagliuolo I, Wang CC, Valenick L, Pasha A, Nikulasson S, Carraway RE, Pothoulakis C. (1999). Neurotensin is a proinflammatory neuropeptide in colonic inflammation. J Clin Invest 103, 843-849.
46. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. (2007). ERK implication in cell cycle regulation. Biochim Biophys Acta 1773, 1299-1310.
47. Chan WY, Yew DT. (1998). Apoptosis and bcl-2 oncoprotein expression in the human fetal central nervous system. Anat Rec 252, 165-175.
48. Chattergoon NN, D'Souza FM, Deng W, Chen H, Hyman AL, Kadowitz PJ, Jeter JR Jr. (2005). Antiproliferative effects of calcitonin gene-related peptide in aortic and pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 288, L202-211.
49. Chavarría T, Valenciano AI, Mayordomo R, Egea J, Comella JX, Hallböök F, de Pablo F, de la Rosa EJ. (2007). Differential, age-dependent MEK-ERK and PI3K-Akt activation by insulin acting as a survival factor during embryonic retinal development. Dev Neurobiol 67, 1777-1788.
50. Chen II, Yates RD, Hansen JT. (1986). Substance P-like immunoreactivity in rat and cat carotid bodies: light and electron microscopic studies. Histol Histopathol 1, 203-212.
51. Cheng L, Khan M, Mudge AW. (1995). Calcitonin gene-related peptide promotes Schwann cell proliferation. J Cell Biol 129, 789-796.
52. Cheng S, Yuan CG. (2007). Differential effect of galanin on proliferation of PC12 and B104 cells. Neuroreport 18, 1379-1383.
53. Chung KF. (2006). Phosphodiesterase inhibitors in airways disease. Eur J Pharmacol 533, 110-117.
54. Clarke JA, Daly MD. (1985). The volume of the carotid body and periadventitial type I and type II cells in the carotid bifurcation region of the fetal cat and kitten. Anat Embryol 173, 117-127.
55. Clarke JA, de Burgh Daly M, Ead HW. (1990). Comparison of the size of the vascular compartment of the carotid body of the fetal, neonatal and adult cat. Acta Anat 138, 166-174.
56. Clarke PG. (1990). Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 181, 195-213.

57. Compton CH, Gubb J, Nieman R, Edelson J, Amit O, Bakst A, Ayres JG, Creemers JP, Schultze-Werninghaus G, Brambilla C, Barnes NC. (2001). Cilomilast, a selective phosphodiesterase-4 inhibitor for treatment of patients with chronic obstructive pulmonary disease: a randomised, dose-ranging study. Lancet 358, 265-270.
58. Conlon JM. (2004). The tachykinin peptide family, with particular emphasis on mammalian tachykinins and tachykinin receptor agonists. In “Handbook of Experimental Pharmacology - Tachykinins” (P. Holzer, Ed.), pp. 25-61. Springer-Verlag, Berlin Heidelberg.
59. Connat JL, Schnüriger V, Zanone R, Schaeffer C, Gaillard M, Faivre B, Rochette L. (2001). The neuropeptide calcitonin gene-related peptide differently modulates proliferation and differentiation of smooth muscle cells in culture depending on the cell type. Regul Pept 101, 169-178.
60. Cosenza MA, Zhao ML, Lee SC. (2004). HIV-1 expression protects macrophages and microglia from apoptotic death. Neuropath Appl Neurobiol 30, 478-490.
61. Cowen DS. (2007). Serotonin and neuronal growth factors - a convergence of signaling pathways. J Neurochem 101, 1161-1171.
62. Crowder RE. (1957). The development of the adrenal gland in man, with special reference to origin and ultimate location of cell types and evidence in favour of the ‘cell migration’ theory. Contrib Embryol Carnegie Inst Washington 36, 193-210.
63. Cuello AC, McQueen DS. (1980). Substance P: a carotid body peptide. Neurosci Lett 17, 215-219.
64. Cummings KJ, Pendlebury JD, Sherwood NM, Wilson RJ. (2004). Sudden neonatal death in PACAP-deficient mice is associated with reduced respiratory chemoresponse and susceptibility to apnoea. J Physiol 555, 15-26.
65. Dambska M, Dydyk L, Szretter T, Wozniewicz J, Myers RE. (1976). Topography of lesions in newborn and infant brains following cardiac arrest and resuscitation: damage to brainstem and hemispheres. Biol Neonate 29, 194-206.
66. De Caro R, Parenti A, Montisci M, Guidolin D, Macchi V. (2000). Solitary tract nuclei in acute heart failure. Stroke 31, 1187-1193.
67. De Caro R, Parenti A, Montisci M, Guidolin D, Macchi V. (2003). Symmetrical selective neuronal necrosis in solitary tract nuclei. Int J Legal Med 117, 253-254.
68. Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267, 682-685.
69. Diaz C, Suarez C, Navarro A, Gonzalez Del Rey C, Alvarez JC, Mendez E, Tolivia J. (1996). Rostrocaudal and ventrodorsal change in neuronal cell size in human medial vestibular nucleus. Anat Rec 246, 403-409.

70. Di Giulio C, Huang W, Waters V, Mokashi A, Bianchi G, Cacchio M, Macri MA, Lahiri S. (2003). Atrial natriuretic peptide stimulates cat carotid body chemoreceptors in vivo. Comp Biochem Physiol A Mol Integr Physiol 134, 27-31.
71. Dubé PE, Rowland KJ, Brubaker PL. (2008). Glucagon-like peptide-2 activates {beta}-catenin signaling in the mouse intestinal crypt: Role of insulin-like growth factor-1. Endocrinology 149, 291-301.
72. e Silva MJ, Lewis DL. (1995). L- and N-type Ca2+ channels in adult rat carotid body chemoreceptor type I cells. J Physiol 489, 689-699.
73. Engelman JA, Luo J, Cantley LC. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet 7, 606-619.
74. Erickson JT, Brosenitsch TA, Katz DM. (2001). Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor are required simultaneously for survival of dopaminergic primary sensory neurons in vivo. J Neurosci 21, 581-589.
75. Evdokiou A, Raggatt LJ, Atkins GJ, Findlay DM. (1999). Calcitonin receptor-mediated growth suppression of HEK-293 cells is accompanied by induction of p21WAF1/CIP1 and G2/M arrest. Mol Endocrinol 13, 1738-1750.
76. Evers BM. (2006). Neurotensin and growth of normal and neoplastic tissues. Peptides 27, 2424-2433.
77. Feldberg RS, Cochrane DE, Carraway RE, Brown E, Sawyer R, Hartunian M, Wentworth D. (1998). Evidence for a neurotensin receptor in rat serosal mast cells. Inflamm Res 47, 245-250.
78. Findlay DM, Raggatt LJ, Bouralexis S, Hay S, Atkins GJ, Evdokiou A. (2002). Calcitonin decreases the adherence and survival of HEK-293 cells by a caspase-independent mechanism. J Endocrinol 75, 715-725.
79. Fishman MC, Schaffner AE. (1984). Carotid body cell culture and selective growth of glomus cells. Am J Physiol 246, C106-113.
80. Flood DG, Finn JP, Walton KM, Dionne CA, Contreras PC, Miller MS, Bhat RV. (1998). Immunolocalization of the mitogen-activated protein kinases p42MAPK and JNK1, and their regulatory kinases MEK1 and MEK4, in adult rat central nervous system. J Comp Neurol 398, 373-392.
81. Foix C, Hillemand P. (1925). Les artères de l'axe encéphalique jusqu'au diencéphale inclusivement. Rev Neurol 11, 705-739.
82. Fujii M, Goto N, Onagi S, Okada A, Kida A. (1997). Development of the human lateral vestibular nucleus: a morphometric evaluation. Early Hum Dev 48, 23-33.
83. Fung ML, Lam SY, Chen Y, Dong X, Leung PS. (2001). Functional expression of angiotensin II receptors in type-I cells of the rat carotid body. Pflugers Arch 441, 474-480.

84. Fung ML, Lam SY, Dong X, Chen Y, Leung PS. (2002). Postnatal hypoxemia increases angiotensin II sensitivity and up-regulates AT_{1a} receptors in rat carotid body chemoreceptors. J Endocrinol 173, 305–313.
85. Ganong WF. Review of medical physiology. 21th ed, New York-London: McGraw-Hill, 2003.
86. Gauda EB, Bamford O, Gerfen CR. (1996). Developmental expression of tyrosine hydroxylase, D2-dopamine receptor and substance P genes in the carotid body of the rat. Neuroscience 75, 969-977.
87. Gauda EB, McLemore GL, Tolosa J, Marston-Nelson J, Kwak D. (2004). Maturation of peripheral arterial chemoreceptors in relation to neonatal apnoea. Semin Neonatol 9, 181-194.
88. Gauda EB, Northington FJ, Linden J, Rosin DL. (2000). Differential expression of a(2a), A(1)-adenosine and D(2)-dopamine receptor genes in rat peripheral arterial chemoreceptors during postnatal development. Brain Res 872, 1-10.
89. Gelbard HA, James HJ, Sharer LR, Perry SW, Saito Y, Kazee AM, Blumberg BM, Epstein LG. (1995). Apoptotic neurons in brains from paediatric patients with HIV-1 encephalitis and progressive encephalopathy. Neuropathol Appl Neurobiol 21, 208-217.
90. Gilles FH. (1969). Hypotensive brain stem necrosis: selective symmetrical necrosis of tegmental neuronal aggregates following cardiac arrest. Arch Pathol 88, 32-41.
91. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H. (1994). Differentation between cellular apoptosis and necrosis by combined use of *in situ* tailing and nick translation techniques. Lab Invest 71, 219-225.
92. Goldman R, Bar-Shavit Z, Shezen E, Terry S, Blumberg S. (1982). Enhancement of phagocytosis by neurotensin, a newly found biological activity of the neuropeptide. Adv Exp Med Biol 155, 133-141.
93. Goswami R, Dawson SA, Dawson G. (1998). Cyclic AMP protects against staurosporine and wortmannin-induced apoptosis and opioid-enhanced apoptosis in both embryonic and immortalized (F-11kappa7) neurons. J Neurochem 70, 1376-1382.
94. Gottschalk W, Pozzo-Miller LD, Figurov A, Lu B. (1998). Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. J Neurosci 18, 6830-6839.
95. Grant NJ, Leon C, Aunis D, Langley K. (1992). Cellular localization of the neural cell adhesion molecule L1 in adult rat neuroendocrine and endocrine tissues: comparisons with NCAM. J Comp Neurol 325, 548-558.
96. Gray SL, Cummings KJ, Jirik FR, Sherwood NM. (2001). Targeted disruption of the pituitary adenylate cyclase-activating polypeptide gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism. Mol Endocrinol 15, 1739-1747.

97. Gray SL, Yamaguchi N, Vencova P, Sherwood NM. (2002). Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 143, 3946-3954.
98. Gross PM, Wall KM, Wainman DS, Shaver SW. (1991). Subregional topography of capillaries in the dorsal vagal complex of rats: II. Physiological properties. J Comp Neurol 306, 83-94.
99. Gross PM. (1991). Morphology and physiology of capillary systems in subregions of the subfornical organ and area postrema. Can J Physiol Pharmacol 69, 1010-1025.
100. Gubkina O, Cremer H, Rougon G. (2001). Mutation in the neural cell adhesion molecule interferes with the differentiation of anterior pituitary secretory cells. Neuroendocrinology 74, 335-346.
101. Gundersen HJG, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, West MJ. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857-881.
102. Gundersen HJG, Jensen EB. (1987). The efficiency of systematic sampling in stereology and its prediction. J Microsc 147, 229-263.
103. Gundersen HJG, Jensen EBV, Kieu K, Nielsen J. (1999). The efficiency of systematic sampling in stereology - reconsidered. J Microsc 193, 199-211.
104. Gundersen HJG. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompsen. J Microsc 143, 3-45.
105. Habeck JO. (1986). Morphological findings at the carotid bodies of humans suffering from different types of systemic hypertension or severe lung diseases. Anat Anz 162, 17-27.
106. Haegerstrand A, Dalsgaard CJ, Jonzon B, Larsson O, Nilsson J. (1990). Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. Proc Natl Acad Sci U S A 87, 3299-3303.
107. Hall TC, Miller KH, Corsallis JAN. (1975). Variation in the human Purkinje cell population according to age and sex. Neuropathol Appl Neurobiol 1, 267-292.
108. Hamano S, Goto N, Nara T, Okada A, Maekawa K. (1997). Development of the human principal sensory trigeminal nucleus: a morphometric analysis. Early Hum Dev 48, 225-235.
109. Hansen SM, Berezin V, Bock E. (2008). Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-Cadherin. Cell Mol Life Sci 65, 3809-3821.

110. Hansen JT, Brokaw J, Christie D, Karasek M. (1982). Localization of enkephalin-like immunoreactivity in the cat carotid and aortic body chemoreceptors. Anat Rec 203, 405-410.

111. Hanson G, Jones L, Fidone S. (1986). Physiological chemoreceptor stimulation decreases enkephalin and substance P in the carotid body. Peptides 7, 767-769.

112. Harlan RE, Song DD. (1994). Prenatal morphine treatment and the development of the striatum. Regul Pept 54, 117-118.

113. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. (1998). International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 50, 265-270.

114. Hassan S, Dobner PR, Carraway RE. (2004). Involvement of MAP-kinase, PI3-kinase and EGF-receptor in the stimulatory effect of Neurotensin on DNA synthesis in PC3 cells. Regul Pept 120, 155-166.

115. He L, Dinger B, Fidone S. (2000). Cellular mechanisms involved in carotid body inhibition produced by atrial natriuretic peptide. Am J Physiol Cell Physiol 278, C845-C852.

116. Heaslip RJ, Evans DY. (1995). Emetic, central nervous system, and pulmonary activities of rolipram in the dog. Eur J Pharmacol 286, 281-290

117. Heath D, Khan Q, Smith P. (1990). Histopathology of the carotid bodies in neonates and infants. Histopathology 17, 511-519.

118. Heath D, Quinzanini M, Rodella A, Albertini A, Ferrari R, Harris P. (1988). Immunoreactivity to various peptides in the human carotid body. Res Commun Chem Pathol Pharmacol 62, 289-293.

119. Heath D, Smith P, Hurst G. (1986). The carotid bodies in coarctation of the aorta. Br J Dis Chest 80, 122-130.

120. Heath D, Smith P, Jago R. (1982). Hyperplasia of the carotid body. J Pathol 138, 115-127.

121. Hebenstreit GF, Fellerer K, Fichte K, Fischer G, Geyer N, Meya U, Sastre Schony W, Schratzer M, Soukop W. (1989). Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine. Pharmacopsychiatry 22, 156-160.

122. Helen P, Panula P, Yang HYT, Rapoport S. (1984). Bombesin/gastrin-releasing peptide (GRP)- and Met^5-enkephalin-Arg^6-Gly^7-Leu^8-like immunoreactivities in small intensely fluorescent (SIF) cells and nerve fibers of rat sympathetic ganglia. J Histochem Cytochem 32, 1131-1138.

123. Hempleman SC. (1995). Sodium and potassium current in neonatal rat carotid body cells following chronic in vivo hypoxia. Brain Res 699, 42-50.

124. Hempleman SC. (1996). Increased calcium current in carotid body glomus cells following in vivo acclimatization to chronic hypoxia. J Neurophysiol 76, 1880-1886.

125. Henry JL. (1987). Discussions of nomenclature for tachykinins and tachykinin receptors. In “Substance P and neurokinins” (J. L. Henry, R. Couture, A. C. Cuello, R. Pelletier, R. Quiron and D. Regoli, Eds.), pp. 17-18. Springer-Verlag, New York.

126. Hertzberg T, Fan G, Finley JC, Erickson JT, Katz DM. (1994a). BDNF supports mammalian chemoafferent neurons in vitro and following peripheral target removal in vivo. Dev Biol 166, 801-811.

127. Hertzberg T, Finley JC, Katz DM. (1994b). Trophic regulation of carotid body afferent development. Adv Exp Med Biol 360, 305-307.

128. Heym C, Kummer W. (1989). Immunohistochemical distribution and colocalization of regulatory peptides in the carotid body. J Electron Microsc Tech 12, 331-342.

129. Holgert H, Hokfelt T, Hertzberg T, Lagercrantz H. (1995). Functional and developmental studies of the peripheral arterial chemoreceptors in rat: effects of nicotine and possible relation to sudden infant death syndrome. Proc Natl Acad Sci USA 92, 7575-7579.

130. Honda Y. (1992). Respiratory and circulatory activities in carotid body-resected humans. J Appl Physiol 73, 1-8.

131. Hu S, Sheng WS, Lokensgard JR, Peterson PK. (2002). Morphine induces apoptosis of human microglia and neurons. Neuropharmacology 42, 829-836.

132. Huang XF, Törk I, Paxinos G. (1993). Dorsal motor nucleus of the vagus nerve: a cyto- and chemoarchitectonic study in the human. J Comp Neurol 330, 158-182.

133. Hurst G, Hearth D, Smith P. (1985). Histological changes associated with ageing of the human carotid body. J Pathol 147, 181-187.

134. Ichikawa H, Helke CJ. (1993). Distribution, origin and plasticity of galanin-immunoreactivity in the rat carotid body. Neuroscience 52, 757-767.

135. Ichikawa H, Schulz S, Höllt V, Sugimoto T. (2005). Delta-opioid receptor-immunoreactive neurons in the rat cranial sensory ganglia. Brain Res 1043, 225-230.

136. Izal-Azcárate A, Belzunegui S, San Sebastián W, Garrido-Gil P, Vázquez-Claverie M, López B, Marcilla I, Luquin MA. (2008). Immunohistochemical characterization of the rat carotid body. Respir Physiol Neurobiol 161, 95-99.

137. Jansen AH, Ioffe S, Russell BJ, Chernick V. (1981). Effect of carotid chemoreceptor denervation on breathing in utero and after birth. J Appl Physiol 51, 630-633.

138. Janzer RC, Friede RL. (1980). Hypotensive brain stem necrosis or cardiac arrest encephalopathy? Acta Neuropathol 50, 53-56.

139. Joels N, Neil E. (1963). The excitation mechanisms of the carotid body. Br Med Bull 19, 21-24.

140. Jones MW, French PJ, Bliss TV, Rosenblum K. (1999). Molecular mechanisms of long-term potentiation in the insular cortex in vivo. J Neurosci 19, RC36.

141. Kameda Y, Nishimaki T, Takeichi M, Chisaka O. (2002). Homeobox gene hoxa3 is essential for the formation of the carotid body in the mouse embryos. Dev Biol 247, 197-209.

142. Kameda Y. (1989). Distribution of CGRP-, somatostatin-, galanin-, VIP-, and substance P-immunoreactive nerve fibers in the chicken carotid body. Cell Tissue Res 257, 623-629.

143. Kameda Y. (2005). Mash1 is required for glomus cell formation in the mouse carotid body. Dev Biol 283, 128-139.

144. Kameda Y. (1999). VIP-, galanin-, and neuropeptide-Y-immunoreactive fibers in the chicken carotid bodies after various types of denervation. Cell Tissue Res 298, 437-447.

145. Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M. (2003). Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. J Neurosci 23, 3607-3615.

146. Kapas S, Martinez A, Cuttitta F, Hinson JP. (1998). Local production and action of adrenomedullin in the rat adrenal zona glomerulosa. J Endocrinol 156, 477-484.

147. Kato T, Ohtani-Kaneko R, Ono K, Okado N, Shiga T. (2005). Developmental regulation of activated ERK expression in the spinal cord and dorsal root ganglion of the chick embryo. Neurosci Res 52, 11-19.

148. Kawase T, Okuda K, Wu CH, Yoshie H, Hara K, Burns DM. (1999). Calcitonin gene-related peptide acts as a mitogen for human Gin-1 gingival fibroblasts by activating the MAP kinase signalling pathway. J Periodontal Res 34, 160-168.

149. Kelly A, Lynch MA. (2000). Long-term potentiation in dentate gyrus of the rat is inhibited by the phosphoinositide 3-kinase inhibitor, wortmannin. Neuropharmacology 39, 643-651.

150. Kerr JFT, Wyllie AH, Currie AR. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26, 239-257.

151. Kholwadwala D, Donnelly DF. (1992). Maturation of carotid chemoreceptor sensitivity to hypoxia: in vitro studies in the newborn rat. J Physiol 453, 461-473.

152. Kinney HC, McHugh T, Miller K, Belliveau RA, Assmann SF. (2002). Subtle developmental abnormalities in the inferior olive: an indicator of prenatal brainstem injury in the sudden infant death syndrome. J Neuropathol Exp Neurol 61, 427-441.

153. Kirby GC, McQueen DS. (1984). Effects of the antagonists MDL 7222 and ketanserin on responses of cat carotid body chemoreceptors to 5-hydroxytryptamine. Br J Pharmacol 83, 259-269.

154. Kirby GC, McQueen DS. (1986). Characterization of opioid receptors in the cat carotid body involved in chemosensory depression in vivo. Br J Pharmacol 88, 889-898.

155. Kiryu-Seo S, Hirayama T, Kato R, Kiyama H. (2005). Noxa is a critical mediator of p53-dependent motor neuron death after nerve injury in adult mouse. J Neurosci 25, 1442-1447.

156. Kis B, Abraham CS, Deli MA, Kobayashi H, Wada A, Niwa M, Yamashita H, Ueta Y. (2001). Adrenomedullin in the cerebral circulation. Peptides 22, 1825-1834.

157. Kishi H, Mishima HK, Sakamoto I, Yamashita U. (1996). Stimulation of retinal pigment epithelial cell growth by neuropeptides in vitro. Curr Eye Res 15, 708-713.

158. Kitabgi P. (2006). Functional domains of the subtype 1 neurotensin receptor (NTS1). Peptides 27, 2461-2468.

159. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 192, 553-560.

160. Kitamura K, Sakata J, Kangawa K, Kojima M, Matsuo H, Eto T. (1993). Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. Biochem Biophys Res Commun 194, 720-725.

161. Koh YH, Ruiz-Canada C, Gorczyca M, Budnik V. (2002). The Ras1-mitogen-activated protein kinase signal transduction pathway regulates synaptic plasticity through fasciclin II-mediated cell adhesion. J Neurosci 22, 2496-2504.

162. Kondo H, Iwasa H. (1996). Re-examination of the carotid body ultrastructure with special attention to intercellular membrane appositions. Adv Exp Med Biol 410, 45-50.

163. Kondo H. (1975). A light and electron microscopic study on the embryonic development of the rat carotid body. Am J Anat 144, 275-293.

164. Kondo H, Yamamoto M. (1988). Occurrence, ontogeny, ultrastructure and some plasticity of CGRP (calcitonin gene-related peptide)-immunoreactive nerves in the carotid body of rats. Brain Res 473, 283-293.

165. Konigsmark BW, Murphy EA. (1972). Volume of the ventral cochlear nucleus in man: its relationship to neuronal population and age. J Neuropathol Exp Neurol 31, 304-316.

166. Konrat G, Halliday G, Sullivan C, Harper C. (1992). Preliminary evidence suggesting delayed development in the hypoglossal and vagal nuclei of SIDS infants: a necropsy study. J Child Neurol 7, 44-49.

167. Krieg T, Qin Q, McIntosh EC, Cohen MV, Downey JM. (2002). ACh and adenosine activate PI3-kinase in rabbit hearts through transactivation of receptor tyrosine kinases. Am J Physiol Heart Circ Physiol 283, H2322-H2330.

168. Kummer W, Gibbins IL, Heym C. (1989). Peptidergic innervation of arterial chemoreceptors. Arch Histol Cytol 52S, 361-364.

169. Kummer W. (1990). Three types of neurochemically defined autonomic fibres innervate the carotid baroreceptor and chemoreceptor regions in the guinea-pig. Anat Embryol 181, 477-489.

170. Kummer W, Habeck JO. (1991). Substance P- and calcitonin gene-related peptide-like immunoreactivities in the human carotid body studied at light and electron microscopical level. Brain Res 554, 286-292.

171. Kummer W, Habeck JO. (1992). Chemoreceptor A-fibres in the human carotid body contain tyrosine hydroxylase and neurofilament immunoreactivity. Neuroscience 47, 713-725.

172. Kusakabe T, Anglade P, Tsuji S. (1991). Localization of substance P, CGRP, VIP, neuropeptide Y, and somatostatin immunoreactive nerve fibers in the carotid labyrinths of some amphibian species. Histochemistry 96, 255-260.

173. Kusakabe T, Hirakawa H, Matsuda H, Kawakami T, Takenaka T, Hayashida Y. (2003). Peptidergic innervation in the rat carotid body after 2, 4, and 8 weeks of hypocapnic hypoxic exposure. Histol Histopathol 18, 409-418.

174. Kusakabe T. (1992). Ontogeny of substance P-, CGRP-, and VIP-containing nerve fibers in the amphibian carotid labyrinth of the bullfrog, Rana catesbeiana. An immunohistochemical study. Cell Tissue Res 269, 79-85.

175. Kusakabe T, Hayashida Y, Matsuda H, Gono Y, Powell FL, Ellisman MH, Kawakami T, Takenaka T. (1998). Hypoxic adaptation of the peptidergic innervation in the rat carotid body. Brain Res 806, 165-174.

176. Kusakabe T, Hirakawa H, Oikawa S, Matsuda H, Kawakami T, Takenaka T, Hayashida Y. (2004). Morphological changes in the rat carotid body 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia. Histol Histopathol 19, 1133-1140.

177. Kusakabe T, Kawakami T, Takenaka T. (1995). Peptidergic innervation in the amphibian carotid labyrinth. Histol Histopathol 10, 185-202.

178. Lack EE. (1978). Hyperplasia of vagal and carotid body paraganglia in patients with chronic hypoxemia. Am J Pathol 91, 497-516.

179. Laduron PM. (1992). Toward genomic pharmacology: from membranal to nuclear receptors. Adv Drug Res 22, 107–148.

180. Lam SY, Leung PS. (2002). A locally generated angiotensin system in rat carotid body. Regul Pept 107, 97-103.

181. Lam SY, Leung PS. (2003). Chronic hypoxia activates a local angiotensin-generating system in rat carotid body. Mol Cell Endocrinol 203, 147-153.

182. Lam SY, Fung ML, Leung PS. (2004). Regulation of the angiotensin-converting enzyme activity by a time-course hypoxia in the carotid body. J Appl Physiol 96, 809–813.

183. Lamont P, Murray N, Halliday G, Hilton J, Pamphlett R. (1995). Brain stem nuclei in sudden infant death syndrome (SIDS): volumes, neuronal numbers and positions. Neuropathol Appl Neurobiol 21, 262-268.

184. Lamontagne S, Meadows E, Luk P, Normandin D, Muise E, Boulet L, Pon DJ, Robichaud A, Robertson GS, Metters KM, Nantel F. (2001). Localization of phosphodiesterase-4 isoforms in the medulla and nodose ganglion of the squirrel monkey. Brain Res 920, 84-96.

185. Langley OK, Aletsee-Ufrecht MC, Grant NJ, Gratzl M. (1989). Expression of the neural cell adhesion molecule NCAM in endocrine cells. J Histochem Cytochem 37, 781-791.

186. Lasn H, Winblad B, Bogdanovic N. (2006). Neuroglia in the inferior olivary nucleus during normal aging and Alzheimer's disease. J Cell Mol Med 10, 145-156.

187. Lazorthes G. (1961). Vascularisation et circulation cérébrales. Paris: Masson & C^{ie} Editeurs.

188. Le Douarin N, Le Lièvre C, Fontaine J. (1972). Recherches experimentales sur l'origine embryologique du corps carotidien chez les oiseaux. CR Acad Sci Ser D 275, 583-586.

189. Leech RW, Alvord ECJr. (1977). Anoxic-ischaemic encephalopathy in the human neonatal period: the significance of brain stem involvement. Arch Neurol 34, 109-113.

190. Lemke G. (2001). Glial control of neuronal development. Ann Rev Neurosci 24, 87-105.

191. Lerner UH. (2006). Deletions of genes encoding calcitonin/alpha-CGRP, amylin and calcitonin receptor have given new and unexpected insights into the function of calcitonin receptors and calcitonin receptor-like receptors in bone. J Musculoskelet Neuronal Interact 6, 87-95.

192. Leslie RA, Gwyn DG, Hopkins DA. (1982). The ultrastructure of the subnucleus gelatinosus of the nucleus of the tractus solitarius in the cat. J Comp Neurol 206, 109-118.

193. Leung PS, Lam SY, Fung ML. (2000). Chronic hypoxia upregulates the expression and function of AT_1 receptor in rat carotid body. J Endocrinol 167, 517–524.

194. Li YL, Schultz HD. (2006). Enhanced sensitivity of Kv channels to hypoxia in the rabbit carotid body in heart failure: role of angiotensin II. J Physiol 575, 215-227.

195. Li YL, Gao L, Zucker ICH, Schultz HD. (2007). NADPH oxidase-derived superoxide anion mediates angiotensin II-enhanced carotid body chemoreceptor sensitivity in heart failure rabbits. Cardiovasc Res 75, 546-554.

196. Li YL, Xia XH, Zheng H, Gao L, Li YF, Liu D, Patel KP, Wang W, Schultz HD. (2006). Angiotensin II enhances carotid body chemoreflex control of sympathetic outflow in chronic heart failure rabbits. Cardiovasc Res 71, 129-138.

197. Li Y, Fiscus RR, Wu J, Yang L, Wang X. (1997). The antiproliferative effects of calcitonin gene-related peptide in different passages of cultured vascular smooth muscle cells. Neuropeptides 31, 503-509.

198. Lipworth BJ. (2005). Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. Lancet 365, 167-175

199. Lloyd RV, Qian X, Jin L, Ruebel K, Bayliss J, Zhang S, Kobayashi I. (2005). Analysis of pituitary cells by laser capture microdissection. Methods Mol Biol 293, 233-241.

200. Lopez I, Honrubia V, Baloh RW. (1997). Aging and the human vestibular nucleus. J Vestib Res 7, 77-85.

201. López J, Martínez A. (2002). Cell and molecular biology of the multifunctional peptide, adrenomedullin. Int Rev Cytol 221, 1-92.

202. Lundberg JM, Hokfelt T, Fahrenkrug J, Nilsson G, Terenius L. (1979). Peptides in the cat carotid body (glomus caroticum): VIP-, enkephalin-, and substance P-like immunoreactivity. Acta Physiol Scand 107, 279-281.

203. Ma X, Goto N, Goto J, Ezure H, Nonaka N. (2002). Development of the human lateral cuneate nucleus: a morphometric evaluation. Brain Res Dev Brain Res 131, 69-75.

204. Ma X, Goto N, Goto J, Ezure H, Nonaka N. (2005). Development of the human cuneatus medialis nucleus: a morphometric evaluation. Early Hum Dev 81, 369-377.

205. Ma X, Goto N, Goto J, Nonaka N, Shibata M. (2006). Morphometric development of the posterior funicular nucleus in the human medulla oblongata. Okajimas Folia Anat Jpn 83, 35-42.

206. Macchi V, Porzionato A, Parenti A, De Caro R. (2004). The course of the posterior inferior cerebellar artery may be related to its level of origin. Surg Radiol Anat 26, 60-65.

207. Macchi V, Porzionato A, Guidolin D, Parenti A, De Caro R. (2005). Morphogenesis of the posterior inferior cerebellar artery with three-dimensional reconstruction of the late embryonic vertebrobasilar system. Surg Radiol Anat 27, 56-60.

208. Macchi V, Porzionato A, Belloni AS, Stecco C, Parenti A, De Caro R. (2006). Immunohistochemical mapping of adrenomedullin in the human medulla oblongata. Peptides 27, 1397-1404.

209. Macchi V, Porzionato A, De Caro R, Clemente A, Parenti A. (2008). Comment on "the relationship of the posterior inferior cerebellar artery to the cranial nerves VII-XII". Clin Anat 21, 218-220;

210. Machaalani R, Waters KA. (2003). Increased neuronal cell death after intermittent hypercapnic hypoxia in the developing piglet brainstem. Brain Res 985, 127-134.

211. Malanga CJ 3rd, Kosofsky BE. (1999). Mechanisms of action of drugs of abuse on the developing fetal brain. Clin Perinatol 26, 17-37.

212. Man HY, Wang Q, Lu WY, Ju W, Ahmadian G, Liu L, D'Souza S, Wong TP, Taghibiglou C, Lu J, Becker LE, Pei L, Liu F, Wymann MP, MacDonald JF, Wang YT. (2003). Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. Neuron 38, 611-624.

213. Mann DM, Yates PO, Hawkes J. (1983). The pathology of the human locus ceruleus. Clin Neuropathol 2, 1-7.

214. Manning BD, Cantley LC. (2007). AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274.

215. Markowska A, Nussdorfer GG, Malendowicz LK. (1994). Different effects of neurotensin and neuromedin-N on the proliferative activity of rat adrenal cortex. Histol Histopathol 9, 449-452.

216. Martin S, Navarro V, Vincent JP, Mazella J. (2002). Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line. Gastroenterology 123, 1135-1143.

217. Martinez A, Saldise L, Ramirez MJ, Belzunegui S, Zudaire E, Luquin MR, Cuttitta F. (2003). Adrenomedullin expression and function in the rat carotid body. J Endocrinol 176, 95-102.

218. Martini C, Trincavelli ML, Tuscano D, Carmassi C, Ciapparelli A, Lucacchini A, Cassano GB, Dell'Osso L. (2004). Serotonin-mediated phosphorylation of extracellular regulated kinases in platelets of patients with panic disorder versus controls. Neurochem Int 44, 627-639.

219. Maxwell DL, Chahal P, Nolop KB, Hughes JM. (1986). Somatostatin inhibits the ventilatory response to hypoxia in humans. J Appl Physiol 60, 997-1002.

220. Mazella J, Vincent JP. (2006). Internalization and recycling properties of neurotensin receptors. Peptides 27, 2488-2492.

221. Mazella J, Zsurger N, Navarro V, Chabry J, Kaghad M, Caput D, Ferrara P, Vita N, Gully D, Maffrand JP, Vincent JP. (1998). The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. J Biol Chem 273, 26273-26276.

222. McDonald DM, Mitchell RA. (1975). The innervation of glomus cells, ganglion cells and blood vessels in the rat carotid body: a quantitative structural analysis. J Neurocytol 4, 17-23.

223. McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, Mutt V. (1979). Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochem Biophys Res Commun 90, 227-233.

224. McGeer PL, McGeer EG, Suzuki JS. (1977). Aging and extrapyramidal function. Arch Neurol 34, 33-35.

225. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393, 333-339.

226. McLemore GL, Cooper RZ, Richardson KA, Mason AV, Marshall C, Northington FJ, Gauda EB. (2004). Cannabinoid receptor expression in peripheral arterial chemoreceptors during postnatal development. J Appl Physiol 97, 1486-1495.

227. McQueen DS, Ribeiro JA. (1981a). Effects of beta-endorphin, vasoactive intestinal polypeptide and cholecystokinin octapeptide on cat carotid chemoreceptor activity. Q J Exp Physiol 66, 273-284.

228. McQueen DS, Ribeiro JA. (1980). Inhibitory actions of methionine-enkephalin and morphine on the cat carotid chemoreceptors. Br J Pharmacol 71, 297-305.

229. McQueen DS, Ribeiro JA. (1981b). Effect of adenosine on carotid chemoreceptor activity in the cat. Br J Pharmacol 74, 129-136.

230. McRitchie DA, Tork I. (1993). The internal organization of the human solitary nucleus. Brain Res Bull 31, 171-193.

231. Miller AD, Leslie RA. (1994). The area postrema and vomiting. Front Neuroendocrinol 15, 301-320

232. Miller MJ, Martinez A, Unsworth EJ, Thiele CJ, Moody TW, Cuttitta F. (1996). Adrenomedullin expression in human tumor cell lines: its potential role as an autocrine growth factor. J Biol Chem 271, 23345-23351.

233. Miller AD, Ruggiero DA. (1994). Emetic reflex arc revealed by expression of the immediate-early gene c-fos in the cat. J Neurosci 14, 871-888.

234. Millhorn DE, Conforti L, Beitner-Johnson D, Zhu W, Raymond R, Filisko T, Kobayashi S, Peng M, Genter MB. (1996). Regulation of ionic conductances and gene expression by hypoxia in an oxygen sensitive cell line. Adv Exp Med Biol 410, 135-142.

235. Minamino N, Kangawa K, Matsuo H. (1983). Neuromedin B: a novel bombesin-like peptide identified in porcine spinal cord. Biochem Biophys Res Commun 114, 541-548.

236. Mitsuhashi M, Payan DG. (1987). The mitogenic effects of vasoactive neuropeptides on cultured smooth muscle cell lines. Life Sci 40, 853-861.

237. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164, 567-574.

238. Moatamed F. (1966). Cell frequencies in the human inferior olivary nuclear complex. J Comp Neurol 128, 109-116.

239. Moftaquir A, Langley K, Boutroy MJ. (1996). Immunocytochemical localization of NCAM and catecholamine-synthesizing enzymes in rabbit intra- and extra-adrenal chromaffin tissue. Histochem J 28, 661-669.

240. Mori F, Perez-Torres S, De Caro R, Porzionato A, Macchi V, Beleta J, Gavalda A, Palacios JM, Mengod G. (2009). cAMP phosphodiesterases 4 (PDE4) isoform expression in the area postrema and the nuclei related with the emetic reflex in human brainstem. Eur J Neurosci *In press.*

241. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE. (1991). Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. Nature 16, 233-236.

242. Nara T, Goto N, Hamano S. (1991). Development of the human dorsal nucleus of vagus nerve: a morphometric study. J Auton Nerv Syst 33, 267-275.

243. Nara T, Goto N, Yamaguchi K. (1989). Development of the human hypoglossal nucleus: a morphometric study. Dev Neurosci 11, 212-220.

244. Navarro V, Martin S, Mazella J. (2006). Internalization-dependent regulation of HT29 cell proliferation by neurotensin. Peptides 27, 2502-2507.

245. Ng KW, Livesey SA, Larkins RG, Martin TJ. (1983). Calcitonin effects on growth and on selective activation of type II isoenzyme of cyclic adenosine 3':5'-monophosphate-dependent protein kinase in T 47D human breast cancer cells. Cancer Res 43, 794-800.

246. Nishio H, Matsui K, Tsuji H, Tamura A, Suzuki K. (2001). Immunolocalization of the mitogen-activated protein kinase signaling pathway in Hassall's corpuscles of the human thymus. Acta Histochem 103, 89-98.

247. Nurse CA, Fearon IM. (2002). Carotid body chemoreceptors in dissociated cell culture. Microsc Res Tech 59, 249-255.

248. Nurse CA. (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. Auton Neurosci 120, 1-9.

249. Nussdorfer GG. (2001). Proadrenomedullin-derived peptides in the paracrine control of the hypothalamo-pituitary-adrenal axis. Int Rev Cytol 206, 249-284.

250. Ohki-Hamazaki H, Iwabuchi M, Maekawa F. (2005). Development and function of bombesin-like peptides and their receptors. Int J Dev Biol 49, 293-300.

251. O'Kusky JR, Norman MG. (1992). Sudden infant death syndrome: postnatal changes in the numerical density and total number of neurons in the hypoglossal nucleus. J Neuropathol Exp Neurol 51, 577-584.

252. O'Kusky JR, Norman MG. (1995). Sudden infant death syndrome: increased number of synapses in the hypoglossal nucleus. J Neuropathol Exp Neurol 54, 627-634.

253. Olney JW. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164, 719-721.

254. Olney JW. (1971). Glutamate-induced neuronal necrosis in the infant mouse hypothalamus: an electron microscopic study. J Neuropathol Exp Neurol 30, 75-90.

255. Olszewski J, Baxter DW. (1954). Cytoarchitecture of the human brainstem. Basel, Switzerland, JB Lippincott.

256. Oomori Y, Ishikawa K, Satoh Y, Matsuda M, Ono K. (1991). Neuropeptide-Y-immunoreactive chief cells in the carotid body of young rats. Acta Anat 140, 120-123.

257. Oomori Y, Murabayashi H, Ishikawa K, Miyakawa K, Nakaya K, Tanaka H. (2002). Neuropeptide Y- and catecholamine-synthesizing enzymes: immunoreactivities in the rat carotid body during postnatal development. Anat Embryol 206, 37-47.

258. Opazo P, Watabe AM, Grant SG, O'Dell TJ. (2003). Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23, 3679-3688.

259. Pakkenberg B, Gundersen HJG. (1988). Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. J Microsc 150, 1-20.

260. Pallot DJ. (1987). The Mammalian Carotid Body. Springer-Verlag. Berlin.

261. Pallot DJ, Al Neamy K, Blakeman N. (1986). Quantitative studies of the rat carotid body Type I cells. Acta Anat 126, 187-191.

262. Pallot DJ, Seker M, Abramovici A. (1992). Post-mortem changes in the normal rat carotid body: possible implications for human histopathology. Virchows Arch A Pathol Anat Histopathol 420, 31-35.

263. Pamphlett R, Treloar L. (1996). Astrocytes in the hypoglossal nuclei of sudden infant death syndrome (SIDS) infants: a quantitative study. Neuropathol Appl Neurobiol 22, 136-143.

264. Pardal R, Ortega-Sáenz P, Durán R, López-Barneo J. (2007). Glia-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. Cell 131, 364-377.

265. Parenti A, Macchi V, Snenghi R, Porzionato A, Scaravilli T, Ferrara SD, De Caro R. (2005). Selective stroke of the solitary tract nuclei in two cases of central sleep apnoea. Clin Neuropathol 24, 239-246.

266. Patacchini R, Maggi CA. (2004). The nomenclature of tachykinin receptors. In "Handbook of Experimental Pharmacology - Tachykinins" (P. Holzer, Ed.), pp. 121-139. Springer-Verlag, Berlin Heidelberg.

267. Paxinos G, Huang XF. (1995). Atlas of the Human Brainstem. San Diego: Academic Press.

268. Peers C, O'Donnell J. (1990). Potassium currents recorded in type I carotid body cells from the neonatal rat and their modulation by chemoexcitatory agents. Brain Res 522, 259-266.

269. Pelaprat D. (2006). Interactions between neurotensin receptors and G proteins. Peptides 27, 2476-2487.

270. Pérez-Torres S, Miró X, Palacios JM, Cortés R, Puigdomènech P, Mengod G. (2000). Phosphodiesterase type 4 isozymes expression in human brain examined by *in situ* hybridization histochemistry and[^{3}H]rolipram binding autoradiography. Comparison with monkey and rat brain. J Chem Neuroanat 20, 349-374.

271. Pesce C, Reale A. (1987). Aging and the nerve cell population of the putamen: a morphometric study. Clin Neuropathol 6, 16-18.

272. Petito CK, Roberts B. (1995a). Effect of post-mortem interval on *in situ* end-labeling of DNA oligonucleosomes. J Neuropathol Exp Neurol 54, 761-765.

273. Petito CK, Roberts B. (1995b). Evidence of apoptotic cell death in HIV encephalitis. Am J Pathol 146, 1121-1130.

274. Pindur J, Capin DM, Johnson MI, Rance NE. (1992). Cystic brainstem necrosis in a premature infant after prolonged bradycardia. Acta Neuropathol 83, 667-669.

275. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. (1999). Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest 104, 1363-1374.

276. Pluder F, Sinz A, Beck-Sickinger AG. (2007). Proliferative effect of calcitonin gene-related peptide is induced by at least five proteins as identified by proteome profiling. Chem Biol Drug Des 69, 14-22.

277. Poncet L, Denoroy L, Dalmaz Y, Pequignot JM, Jouvet M. (1994). Chronic hypoxia affects peripheral and central vasoactive intestinal peptide-like immunoreactivity in the rat. Neurosci Lett 176, 1-4.

278. Poncet L, Denoroy L, Dalmaz Y, Pequignot JM, Jouvet M. (1996). Alteration in central and peripheral substance P- and neuropeptide Y-like immunoreactivity after chronic hypoxia in the rat. Brain Res 733, 64-72.

279. Porzionato A, Macchi V, Ferrara SD, Parenti A, De Caro R. (2004a). C-FOS expression in the subnucleus gelatinosus of the human nucleus tractus solitarii. Ital J Anat Embryol 109, 125-134.

280. Porzionato A, Macchi V, Parenti A, De Caro R. (2004b). The distribution of mast cells in the human area postrema. J Anat 204, 141-147.

281. Porzionato A, Macchi V, Guidolin D, Parenti A, Ferrara SD, De Caro R. (2005a). Histopathology of carotid body in heroin addiction. Possible chemosensitive impairment. Histopathology 46, 296-306.

282. Porzionato A, Macchi V, Morsut L, Parenti A, De Caro R. (2005b). Microvascular patterns in human medullary tegmentum at the level of the area postrema. J Anat 206, 405-410.

283. Porzionato A, Macchi V, Belloni AS, Parenti A, De Caro R. (2006a). Adrenomedullin immunoreactivity in the human carotid body. Peptides 27, 69-73.

284. Porzionato A, Macchi V, Barzon L, Masi G, Iacobone M, Parenti A, Palù G, De Caro R. (2006b). Immunohistochemical assessment of parafibromin in mouse and human tissues. J Anat 209, 817-827.

285. Porzionato A, Macchi V, Parenti A, De Caro R. (2008a). Trophic factors in the carotid body. Int Rev Cell Mol Biol 269, 1-58.

286. Porzionato A, Macchi V, Guidolin D, Sarasin G, Parenti A, De Caro R. (2008b). Anatomic distribution of apoptosis in medulla oblongata of infants and adults. J Anat 212, 106-113.

287. Porzionato A, Macchi V, Stecco C, Vigato E, Tiengo C, Parenti A, De Caro R. (2008c). Morphometric analysis of the inferior olivary complex in infants and adults. Ital J Anat Embryol 113, 65-73.

288. Porzionato A, Macchi V, Parenti A, Matturri L, De Caro R. (2008d). Peripheral chemoreceptors: postnatal development and cytochemical findings in Sudden Infant Death Syndrome. Histol Histopathol 23, 351-365.

289. Porzionato A, Macchi V, Parenti A, De Caro R. (2008e). Neural Cell Adhesion Molecule in the human carotid body. Ital J Anat Embryol 113, *In press.*

290. Porzionato A, Macchi V, Parenti A, De Caro R. (2009a). Extracellular signal-regulated kinase and phosphatidylinositol-3-kinase/AKT signalling pathways in the human carotid body and peripheral ganglia. Acta Histochem *In press.*

291. Porzionato A, Macchi V, Parenti A, De Caro R. (2009b). Chronic carotid glomitis in heroin addiction. Histol Histopathol *In press.*

292. Potter LR, Abbey-Hosch S, Dickey DM. (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. Endocr Rev 27, 47-72.

293. Powell FL. (2007). The influence of chronic hypoxia upon chemoreception. Respir Physiol Neurobiol 157, 154-161.

294. Prabhakar NR, Jacono FJ. (2005). Cellular and molecular mechanisms associated with carotid body adaptations to chronic hypoxia. High Alt Med Biol 6, 112-120.

295. Prabhakar NR, Landis SC, Kumar GK, Mullikin-Kilpatrick D, Cherniack NS, Leeman S. (1989). Substance P and neurokinin A in the cat carotid body: localization, exogenous effects and changes in content in response to arterial pO2. Brain Res 481, 205-214.

296. Purves MJ. (1970). The role of the cervical sympathetic nerve in the regulation of oxygen consumption of the carotid body of the cat. J Physiol 209, 417-431.

297. Puurunen J, Lucke C, Schwabe U. (1978). Effect of the phosphodiesterase inhibitor 4-(3-cyclopentyloxy-4- methoxyphenyl)-2-pyrrolidone (ZK 62711) on gastric secretion and gastric mucosal cyclic AMP. Naunyn Schmiedebergs Arch Pharmacol 304, 69-75.

298. Qiao M, Shapiro P, Kumar R, Passaniti A. (2004). Insulin-like growth factor-1 regulates endogenous RUNX2 activity in endothelial cells through a phosphatidylinositol 3-kinase/ERK-dependent and Akt-independent signaling pathway. J Biol Chem 279, 42709-42718.

299. Qin XP, Ye F, Hu CP, Liao DF, Deng HW, Li YJ. (2004). Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. Eur J Pharmacol 488, 45-49.

300. Rabe KF, Bateman ED, O'Donnell D, Witte S, Bredenbroker D, Bethke TD. (2005). Roflumilast--an oral anti-inflammatory treatment for chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 366, 563-571.

301. Rane MJ, Gozal D, Butt W, Gozal E, Pierce WM Jr, Guo SZ, Wu R, Goldbart AD, Thongboonkerd V, McLeish KR, Klein JB. (2005). Gamma-amino butyric acid type B receptors stimulate neutrophil chemotaxis during ischemia-reperfusion. J Immunol 174, 7242-7249.

302. Revesz T, Geddes JF. (1988). Symmetrical columnar necrosis of the basal ganglia and brain stem in an adult following cardiac arrest. Clin Neuropathol 6, 294-298.

303. Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD. (1999). The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19, 4337-4348.

304. Roberts RE. (2004). The role of Rho kinase and extracellular regulated kinase-mitogen-activated protein kinase in alpha2-adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein. J Pharmacol Exp Ther 311, 742-747.

305. Robichaud A, Savoie C, Stamatiou PB, Lachance N, Jolicoeur P, Rasori R, Chan CC. (2002a). Assessing the emetic potential of PDE4 inhibitors in rats. Br J Pharmacol 135, 113-118.

306. Robichaud A, Stamatiou PB, Jin SL, Lachance N, Macdonald D, Laliberte F, Liu S, Huang Z, Conti M, Chan CC. (2002b). Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. J Clin Invest 110, 1045-1052.

307. Rosenblum K, Futter M, Jones M, Hulme EC, Bliss TV. (2000). ERKI/II regulation by the muscarinic acetylcholine receptors in neurons. J Neurosci 20, 977-985.

308. Runcie MJ, Ulman LG, Potter EK. (1995). Effects of pituitary adenylate cyclase-activating polypeptide on cardiovascular and respiratory responses in anaesthetised dogs. Regul Pept 60, 193-200.

309. Rutishauser U. (2008). Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat Rev Neurosci 9, 26-35.

310. Salido M, Vilches J, López A. (2000). Neuropeptides bombesin and calcitonin induce resistance to etoposide induced apoptosis in prostate cancer cell lines. Histol Histopathol 15, 729-738.

311. Sander GE, Lowe RF, Given MB, Giles TD. (1989). Interaction between circulating peptides and the central nervous system in hemodynamic regulation. Am J Cardiol 64, 44–50.

312. Sanna PP, Cammalleri M, Berton F, Simpson C, Lutjens R, Bloom FE, Francesconi W. (2002). Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. J Neurosci 22, 3359-3365.

313. Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T. (1991). Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. Nature 351, 230-233.

314. Satoh F, Takahashi K, Murakami O, Totsune K, Sone M, Ohneda M, Sasano H, Mouri T. (1996). Immunocytochemical localization of adrenomedullin-like immunoreactivity in the human hypothalamus and the adrenal gland. Neurosci Lett 203, 207-210.

315. Schaeffer P, Laplace MC, Savi P, Pflieger AM, Gully D, Herbert JM. (1995). Human umbilical vein endothelial cells express high affinity neurotensin receptors coupled to intracellular calcium release. J Biol Chem 270, 3409-3413.

316. Schwartz JP. (1992). Neurotransmitters as neurotrophic factors: a new set of functions. Int Rev Neurobiol 34, 1-23.

317. Scott RJ, Hegyi L. (1997). Cell death in perinatal hypoxic-ischaemic brain injury. Neuropathol App Neurob 23, 307-314.

318. Scott AI, Perini AF, Shering PA, Whalley LJ. (1991). In-patient major depression: is rolipram as effective as amitriptyline? Eur J Clin Pharmacol 40, 127-129.

319. Scraggs M, Smith P, Heath D. (1992). Glomic cells and their peptides in the carotid body of the human fetus. Pediatr Pathol 12, 823-834.

320. Seatriz JV, Hammer RPJr. (1993). Effects of opiates on neuronal development in the rat cerebral cortex. Brain Res Bull 30, 523-527.

321. Seki T, Arai Y. (1993). Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci Res 17, 265-290.

322. Seutin V, Massotte L, Dresse A. (1989). Electrophysiological effects of neurotensin on dopaminergic neurones of the ventral tegmental area of the rat in vitro. Neuropharmacology 28, 949-954.

323. Shah GV, Rayford W, Noble MJ, Austenfeld M, Weigel J, Vamos S, Mebust WK. (1994). Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3',5'-monophosphates and cytoplasmic Ca2+ transients. Endocrinology 134, 596-602.

324. Shaver SW, Pang JJ, Wall KM, Sposito NM, Gross PM. (1991). Subregional topography of capillaries in the dorsal vagal complex of rats. I. Morphometric properties. J Comp Neurol 306, 73–82.

325. Shelton JG, Steelman LS, White ER, McCubrey JA. (2004). Synergy between PI3K/Akt and Raf/MEK/ERK pathways in IGF-1R mediated cell cycle progression and prevention of apoptosis in hematopoietic cells. Cell Cycle 3, 372-379.

326. Shen PJ, Yuan CG, Ma J, Cheng S, Yao M, Turnley AM, Gundlach AL. (2005). Galanin in neuro(glio)genesis: expression of galanin and receptors by progenitor cells in vivo and in vitro and effects of galanin on neurosphere proliferation. Neuropeptides 39, 201-205.

327. Shi WX, Bunney BS. (1992). Roles of intracellular cAMP and protein kinase A in the actions of dopamine and neurotensin on midbrain dopamine neurons. J Neurosci 12, 2433-2438.

328. Smith P, Heath D, Fitch R, Hurst G, Moore D, Weitzenblum E. (1986). Effects on the rabbit carotid body of stimulation by almitrine, natural high altitude, and experimental normobaric hypoxia. J Pathol 149, 143-153.

329. Smith P, Heath D, Williams D, Bencini C, Pulera N, Giuntini C. (1993). The earliest histopathological response to hypobaric hypoxia in rabbits in the Rifugio Torino (3370 M) on Monte Bianco. J Pathol 170, 485-491.

330. Smith P, Gosney J, Heath D, Burnett H. (1990). The occurrence and distribution of certain polypeptides within the human carotid body. Cell Tissue Res 261, 565-571.

331. Smith P, Hurst G, Heath D, Drewe R. (1986). The carotid bodies in a case of ventricular septal defect. Histopathology 10, 831-840.

332. Snider BJ, Gottron FJ, Choi DW. (1999). Apoptosis and necrosis in cerebrovascular disease. Ann N Y Acad Sci 893, 243-253.

333. Springell DA, Costin NS, Pilowsky PM, Goodchild AK. (2005). Hypotension and short-term anaesthesia induce ERK1/2 phosphorylation in autonomic nuclei of the brainstem. Eur J Neurosci 22, 2257-2270.

334. Standring S, Ellis H, Healy H, Johnson D, Williams A. (2005). Gray's Anatomy. 39th ed. Churchill Livingstone. London. p 547.

335. Stapelfeldt WH, Szurszewski JH. (1989a). The electrophysiological effects of neurotensin on neurones of guinea-pig prevertebral sympathetic ganglia. J Physiol 411, 301-323.

336. Stapelfeldt WH, Szurszewski JH. (1989b). Neurotensin facilitates release of substance P in the guinea-pig inferior mesenteric ganglion. J Physiol 411, 325-345.

337. Stea A, Jackson A, Nurse CA. (1992). Hypoxia and N6,O2'-dibutyryladenosine 3',5'-cyclic monophosphate, but not nerve growth factor, induce Na+ channels and hypertrophy in chromaffin-like arterial chemoreceptors. Proc Natl Acad Sci USA 89, 9469-9473.

338. Stecco C, Porzionato A, Macchi V, Sarasin G, Calcagno A, Parenti A, De Caro R. (2005). Detection of apoptosis in human brainstem by TUNEL assay. Ital J Anat Embryol 110, 255-260.

339. Steingart RA, Abu-Roumi M, Newman ME, Silverman WF, Slotkin TA, Yanai J. (2000). Neurobehavioral damage to cholinergic systems caused by prenatal exposure to heroin or phenobarbital: cellular mechanisms and the reversal of deficits by neural grafts. Brain Res Dev Brain Res 122, 125-133.

340. Sterio DC. (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134, 127-136.

341. Sterni LM, Bamford OS, Tomares SM, Montrose MH, Carroll JL. (1995). Developmental changes in intracellular Ca2+ response of carotid chemoreceptor cells to hypoxia. Am J Physiol 268, L801-808.

342. Su B, Karin M. (1996). Mitogen-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 8, 402-411.

343. Suarez C, Diaz C, Tolivia J, Alvarez JC, Gonzalez del Rey C, Navarro A. (1997). Morphometric analysis of the human vestibular nuclei. Anat Rec 247:271-288.

344. Sweatt JD. (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem 76, 1-10.

345. Szilagyi JE, Ferrario CM. (1981). Central opiate system modulation of the area postrema pressor pathway. Hypertension 3, 313–317.

346. Takahashi M, Terwilliger R, Lane C, Mezes PS, Conti M, Duman RS. (1999). Chronic antidepressant administration increases the expression of cAMP- specific phosphodiesterase 4A and 4B isoforms. J Neurosci 19, 610-618.

347. Takei Y. (2000). Structural and functional evolution of the natriuretic peptide system in vertebrates. Int Rev Cytol 194, 1-66.

348. Tang Y, Lopez I, Baloh RW. (2001-2002). Age-related change of the neuronal number in the human medial vestibular nucleus: a stereological investigation. J Vestib Res 11, 357-363.

349. Taraviras S, Olli-Lähdesmäki T, Lymperopoulos A, Charitonidou D, Mavroidis M, Kallio J, Scheinin M, Flordellis C. (2002). Subtype-specific neuronal differentiation of PC12 cells transfected with alpha2-adrenergic receptors. Eur J Cell Biol 81, 363-374.

350. Taylor SR, Roessman U. (1984). Hypotensive brainstem necrosis in a stillborn. Acta Neuropathol 65, 166-167.

351. Tischler AS, Ruzicka LA, DeLellis RA. (1991). Regulation of neurotensin content in adrenal medullary cells: comparison of PC12 cells to normal rat chromaffin cells in vitro. Neuroscience 43, 671-678.

352. Toft MH, Gredal O, Pakkenberg B. (2005). The size distribution of neurons in the motor cortex in amyotrophic lateral sclerosis. J Anat 207, 399-407.

353. Toledo-Aral JJ, Mendez-Ferrer S, Pardal R, Echevarria M, Lopez-Barneo J. (2003). Trophic restoration of the nigrostriatal dopaminergic pathway in long-term carotid body-grafted parkinsonian rats. J Neurosci 23, 141–148.

354. Tomiyama M, Palacios JM, Cortés R, Vilaró MT, Mengod G. (1997). Distribution of AMPA receptor subunit mRNAs in the human basal ganglia: an *in situ* hybridization study. Mol Brain Res 46, 281-289.

355. Trejter M, Brelinska R, Warchol JB, Butowska W, Neri G, Rebuffat P, Gottardo L, Malendowicz LK. (2002a). Effects of galanin on proliferation and apoptosis of immature rat thymocytes. Int J Mol Med 10, 183-186.

356. Trejter M, Markowska A, Belloni AS, Nussdorfer GG, Malendowicz LK. (2002b). Investigation of the effect of different regulatory peptides on adrenocortical cell proliferation in immature rats: evidence that endogenous adrenomedullin exerts a stimulating action. Int J Mol Med 10, 81-84.

357. Troger J, Sellemond S, Kieselbach G, Kralinger M, Schmid E, Teuchner B, Nguyen QA, Schretter-Irschick E, Göttinger W. (2003). Inhibitory effect of certain neuropeptides on the proliferation of human retinal pigment epithelial cells. Br J Ophthalmol 87, 1403-1408.

358. Van Buskirk C. (1945). The seventh nerve complex. J Comp Neurol 82, 303-333.

359. Vanhoose AM, Emery M, Jimenez L, Winder DG. (2002). ERK activation by G-protein-coupled receptors in mouse brain is receptor identity-specific. J Biol Chem 277, 9049-9053.

360. Varndell IM, Tapia FJ, De Mey J, Rush RA, Bloom SR, Polak JM. (1982). Electron immunocytochemical localization of enkephalin-like material in catecholamine-containing cells of the carotid body, the adrenal medulla, and in pheochromocytomas of man and other mammals. J Histochem Cytochem 30, 682-690.

361. Verna A. (1997). The mammalian carotid body: morphological data. In: The Carotid Body Chemoreceptors. Gonzalez C. (ed). MIU Landes Bioscience. Austin, TX. pp 1-29.

362. Vijayashankar N, Brody H. (1977). A study of aging in the human abducens nucleus. J Comp Neurol 173, 433-438.

363. Villadiego J, Méndez-Ferrer S, Valdés-Sánchez T, Silos-Santiago I, Fariñas I, López-Barneo J, Toledo-Aral JJ. (2005). Selective glial cell line-derived neurotrophic factor production in adult dopaminergic carotid body cells *in situ* and after intrastriatal transplantation. J Neurosci 25, 4091-4098.

364. Vincent VAM, De Groot CJ, Lucassen PJ, Portegies P, Troost D, Tilders FJ, Van Dam AM. (1999). Nitric oxide synthase expression and apoptotic cell death in brains of AIDS and AIDS dementia patients. AIDS 13, 317-326.

365. Vinhaes ENG, Dolhnikoff M, Saldiva PHN. (2002). Morphological changes of carotid bodies in acute respiratory distress syndrome: a morphometric study in humans. Braz J Med Biol Res 35, 1119-1125.

366. von Dalnok GK, Menssen HD. (1986). A quantitative electron microscopic study of the effect of glucocorticoids in vivo on the early postnatal differentiation of paraneuronal cells in the carotid body and the adrenal medulla of the rat. Anat Embryol 174, 307-319.

367. Wachtel H. (1982). Characteristic behavioural alterations in rats induced by rolipram and other selective adenosine cyclic 3', 5'-monophosphate phosphodiesterase inhibitors. Psychopharmacology (Berlin) 77, 309-316.

368. Wachtel H. (1983). Potential antidepressant activity of rolipram and other selective cyclic adenosine 3',5'-monophosphate phosphodiesterase inhibitors. Neuropharmacology 22, 267-272.

369. Wachtel H, Schneider HH. (1986). Rolipram, a novel antidepressant drug, reverses the hypothermia and hypokinesia of monoamine-depleted mice by an action beyond postsynaptic monoamine receptors. Neuropharmacology 25, 1119-1126.

370. Wang J, Rout UK, Bagchi IC, Armant DR. (1998a). Expression of calcitonin receptors in mouse preimplantation embryos and their function in the regulation of blastocyst differentiation by calcitonin. Development 125, 4293-4302.

371. Wang WJ, He L, Chen J, Dinger B, Fidone S. (1993a). Mechanisms underlying chemoreceptor inhibition induced by atrial natriuretic peptide in rabbit carotid body. J Physiol 460, 427-441.

372. Wang YY, Perrin DG, Cutz E. (1993b). Localization of cholecystokinin-like and calcitonin-like peptides in infant carotid bodies: a light- and electron-microscopic immunohistochemical study. Cell Tissue Res 272, 169-174.

373. Wang ZY, Bisgard GE. (2005). Postnatal growth of the carotid body. Respir Physiol Neurobiol 149, 181-190.

374. Wang ZZ, Dinger B, Fidone SJ, Stensaas LJ. (1998b). Changes in tyrosine hydroxylase and substance P immunoreactivity in the cat carotid body following chronic hypoxia and denervation. Neuroscience 83, 1273-1281.

375. Wang ZZ, He L, Stensaas LJ, Dinger BG, Fidone SJ. (1991). Localization and in vitro actions of atrial natriuretic peptide in the cat carotid body. J Appl Physiol 70, 942-946.

376. Wang ZZ, Stensaas LJ, Wang WJ, Dinger B, de Vente J, Fidone SJ. (1992). Atrial natriuretic peptide increases cyclic guanosine monophosphate immunoreactivity in the carotid body. Neuroscience 49, 479-486.

377. Waskiewicz AJ, Cooper JA. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Curr Opin Cell Biol 7, 798-805.

378. Waters KA, Meehan B, Huang JQ, Gravel RA, Michaud J, Cote A. (1999). Neuronal apoptosis in sudden infant death syndrome. Pediatr Res 45, 166-172.

379. Weisinger RS, Denton DA, Di Nicolantonio R, Hards DK, McKinley MJ, Oldfield B, Osborne PG. (1990). Subfornical organ lesion decreases sodium appetite in the sodium-depleted rat. Brain Res 526, 23-30.

380. West MJ. (1993). New stereological methods for counting neurons. Neurobiol Aging 14, 275-285.

381. Wharton J, Polak JM, Pearse AGE, McGregor GP, Bryan MG, Bloom SR, Emson PC, Bisgard GE, Will JA. (1980). Enkephalin, VIP and substance P-like immunoreactivity in the carotid body. Nature 284, 269-271.

382. Wiese S, Jablonka S, Holtmann B, Orel N, Rajagopal R, Chao MV, Sendtner M. (2007). Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. Proc Natl Acad Sci USA 104, 17210-17215.

383. Wyllie A. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555-556.

384. Xia CF, Yin H, Borlongan CV, Chao J, Chao L. (2006). Postischemic infusion of adrenomedullin protects against ischemic stroke by inhibiting apoptosis and promoting angiogenesis. Exp Neurol 197, 521-530.

385. Xia CY, Yuan CX, Yuan CG. (2005). Galanin inhibits the proliferation of glial olfactory ensheathing cells. Neuropeptides 39, 453-459.

386. Xu F, Tse FW, Tse A. (2007). Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates the oxygen sensing type I (glomus) cells of rat carotid bodies via reduction of a background TASK-like K+ current. J Neurochem 101, 1284-1293.

387. Xu F, Tse FW, Tse A. (2008). Stimulatory actions of pituitary adenylate cyclase-activating polypeptide (PACAP) in rat carotid glomus cells. Adv Exp Med Biol 605, 69-74.

388. Yamada M, Tanabe K, Wada K, Shimoke K, Ishikawa Y, Ikeuchi T, Koizumi S, Hatanaka H. (2001). Differences in survival-promoting effects and intracellular signaling properties of BDNF and IGF-1 in cultured cerebral cortical neurons. J Neurochem 78, 940-951.

389. Yin DL, Ren XH, Zheng ZL, Pu L, Jiang LZ, Ma L, Pei G. (1997). Etorphine inhibits cell growth and induces apoptosis in SK-N-SH cells: involvement of pertussis toxin-sensitive G proteins. Neurosci Res 29, 121-127.

390. Yu G, Xu L, Hadman M, Hess DC, Borlongan CV. (2004). Intracerebral transplantation of carotid body in rats with transient middle cerebral artery occlusion. Brain Res 1015, 50-56.

391. Zeromski J, Lawniczak M, Galbas K, Jenek R, Golusiński P. (1998). Expression of CD56/N-CAM antigen and some other adhesion molecules in various human endocrine glands. Folia Histochem Cytobiol 36, 119-125.

392. Zhang F, Yin W, Chen J. (2004). Apoptosis in cerebral ischemia: executional and regulatory signalling mechanisms. Neurol Res 26, 835-845.

393. Zheng WH, Kar S, Quirion R. (2000). Stimulation of protein kinase C modulates insulin-like growth factor-1-induced akt activation in PC12 cells. J Biol Chem 275, 13377-13385.

394. Zirlinger M, Anderson D. (2003). Molecular dissection of the amygdala and its relevance to autism. Genes Brain Behav 2, 282-294.