Topological Analysis of the Brainstem of the Australian Lungfish *Neoceratodus forsteri*

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Keywords

Anamniote vertebrates · Comparative neuroanatomy · Herrick-Johnston concept · Longitudinal zones · Neuromeres

Abstract

This paper presents a survey of the cell masses in the brainstem of the Australian lungfish *Neoceratodus forsteri*, based on transversely cut Bodian-stained serial sections, supplemented by immunohistochemical data from the recent literature. This study is intended to serve a double purpose. First it concludes and completes a series of publications on the structure of the brainstem in representative species of all groups of anamniote vertebrates. Within the framework of this comparative program the cell masses in the brainstem and their positional relations are analyzed in the light of the Herrick-Johnston concept, according to which the brainstem nuclei are arranged in four longitudinal, functional zones or columns, the boundaries of which are marked by ventricular sulci. The procedure employed in this analysis essentially involves two steps: first, the cell masses and large individual cells are projected upon the ventricular surface, and next, the ventricular surface is flattened out, that is, subjected to a one-to-one continuous topological transformation [J Comp Neurol. 1974;156:255–267]. The second purpose of the present paper is to complement our mapping of the longitudinal zonal arrangement of the cell masses in the

brainstem of *Neoceratodus* with a subdivision in transversely oriented neural segments. Five longitudinal rhombencephalic sulci – the sulcus medianus inferior, the sulcus intermedius ventralis, the sulcus limitans, the sulcus intermedius dorsalis and the sulcus medianus superior – and four longitudinal mesencephalic sulci – the sulcus tegmentalis medialis, the sulcus tegmentalis lateralis, the sulcus subtectalis and the sulcus lateralis mesencephali – could be distinguished. Two obliquely oriented grooves, present in the isthmic region – the sulcus isthmi dorsalis and ventralis – deviate from the overall longitudinal pattern of the other sulci. Although in *Neoceratodus* most neuronal perikarya are situated within a diffuse periventricular gray, 45 cell masses could be delineated. Ten of these are primary efferent or motor nuclei, eight are primary afferent or sensory centers, six are considered to be components of the reticular formation and the remaining 21 may be interpreted as “relay” nuclei. The topological analysis showed that in most of the rhombencephalon the gray matter is arranged in four longitudinal zones or areas, termed area ventralis, area intermedioventralis, area intermediodorsalis and area dorsalis. The sulcus intermedius ventralis, the sulcus limitans, and the sulcus intermedius dorsalis mark the boundaries between these morphological entities. These longitudinal zones coincide largely, but not entirely, with the functional columns of Herrick and Johnston. The most obvious incongruity is that the area intermediodorsalis contains, in addition to the viscerosensory nucleus of the solitary tract, several general somatosensory and spe-

cial somatosensory centers. The isthmus region does not exhibit a clear morphological zonal pattern. The mesencephalon is divisible into a ventral, primarily motor zone and a dorsal somatosensory zone. The boundary between these zones is marked by the sulcus tegmentalis lateralis, which may be

considered as an isolated rostral extremity of the sulcus limitans. The results of this study are summarized in a “classical” topological map, as well as in a “modernized” version of this map, in which neuromere borders are indicated.

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Abbreviations used in this paper

ap	area postrema	nIX	nervus glossopharyngeus
aurcb	auricula cerebelli	nX	nervus vagus
bol	bulbus olfactorius	oli	oliva inferior
cb	cerebellum	p	possible ‘pre-Mauthner cells’
ccb	corpus cerebelli	rai	nucleus raphes inferior
cmisp	columna motoria spinalis	raint	nucleus raphes intermedius
crCb	crista cerebellaris	ras	nucleus raphes superior
drNAG	dorsal rhombencephalic noradrenergic cell group	rdV	radix descendens nervi trigemini
ep	epiphysis	rhomb	rhombencephalon
EW	nucleus of Edinger-Westphal	ri	nucleus reticularis inferior
flt	fasciculus lateralis telencephali	rm	nucleus reticularis medius
flm	fasciculus longitudinalis medialis	rs	nucleus reticularis superior
fMth	fiber of Mauthner	rub	nucleus ruber
fs	fasciculus solitarius	r0, r1 etc.	rhombomeres
gcr	griseum centrale rhombencephali	sfgs	stratum fibrosum et griseum superficiale
gsim	griseum superficiale isthmi et mesencephali	sgc	stratum griseum centrale
hypoth	hypothalamus	sgp	stratum griseum periventriculare
int	nucleus intermedius areae octavolateralis	sid	sulcus intermedius dorsalis
isth	isthmus rhombencephali	sisd	sulcus isthmi dorsalis
ip1	nucleus interpeduncularis 1	sisv	sulcus isthmi ventralis
ip2	nucleus interpeduncularis 2	siv	sulcus intermedius ventralis
lc	locus coeruleus	slH	sulcus limitans of His
ldt	nucleus laterodorsalis tegmenti	slHacc	sulcus limitans of His, pars accessoria
ll	lemniscus lateralis	slm	sulcus lateralis mesencephali
loblinlat	lobus lineae lateralis	slt	sulcus lateralis tecti
lobX	lobus vagi	smi	sulcus medianus inferior
lrz	lateral reticular zone	sms	sulcus medianus superior
m	mesomere	sst	sulcus subtectalis
mcbr	median cerebellar ridge	stegl	sulcus tegmentalis lateralis
mes	mesencephalon	stegm	sulcus tegmentalis medialis
mesV	nucleus mesencephalicus nervi trigemini	t	taenia
msp	medulla spinalis	tect	tectum mesencephali
Mth	cell of Mauthner	tel	telencephalon
nllad	nervus lineae lateralis anterior, pars dorsalis	tsc	torus semicircularis
nllav	nervus lineae lateralis, pars ventralis	tviscs	tractus visceralis secundarius
nllp	nervus lineae lateralis posterior	vem	nucleus vestibularis magnocellularis
nnsp	nervi spinales	viscs	nucleus visceralis secundarius
nnspoc	nervi spino-occipitales	vrNAG	ventral rhombencephalic noradrenergic cell group
nufd	nucleus funiculi dorsalis		
nuflm	nucleus fasciculi longitudinalis medialis	vta/sn	ventral tegmental area/substantia nigra complex
nuisth	nucleus isthmi	zl	zona limitans
nurdV	nucleus of the radix descendens nervi trigemini	III	nucleus nervi oculomotorii
nutsc	nucleus tori semicircularis	IV	nucleus nervi trochlearis
nufs	nucleus fasciculi solitarii	Vm	nucleus motorius nervi trigemini
nII	nervus opticus	Vpr	nucleus sensorius principalis nervi trigemini
nIII	nervus oculomotorius	VI	nucleus nervi abducentis
nIV	nervus trochlearis	VII _{mc}	nucleus motorius nervi facialis caudalis
nVm	nervus trigeminus, pars motoria	VII _{mr}	nucleus motorius nervi facialis rostralis
nVs	nervus trigeminus, pars sensoria	IX	nucleus motorius nervi glossopharyngei
nVII	nervus facialis	Xm	nucleus motorius nervi vagi
nVIII	nervus octavus		

Introduction

The lungfishes or Dipnoi form an extremely ancient group of fish, which appeared in the Lower Devonian and reached the zenith of its evolution in Late Devonian and Carboniferous times [Moy-Thomas and Miles, 1971; Lee et al., 2006]. In the recent fauna it is represented by only six species, belonging to two different orders: the Ceratodontiformes and the Lepidosireniformes. The first order has only one species, the Australian lungfish *Neoceratodus forsteri*, whereas the second order is represented by five species belonging to two different genera, the South American genus *Lepidosiren*, with a single species: *L. paradoxo*, and four African lungfish species of the genus *Protopterus*. (*P. annectens*, *P. aethiopicus*, *P. dolloi* and *P. amphibius*). The study of lungfishes is of extraordinary interest, because they are considered to represent the closest living relatives of the amphibians and other tetrapods [Brinkmann et al., 2004; Chen et al., 2012; Biscotti et al., 2016].

In the present study an analysis will be presented of the rhombencephalic and mesencephalic cell masses of the Australian lungfish *N. forsteri*. This study concludes and completes a long series of publications on the structure of the brain stem in representative species of all groups of anamniotes: Nieuwenhuys [1972]: the lamprey *Lampetra fluviatilis*; Smeets and Nieuwenhuys [1976]: the sharks *Squalus acanthias* and *Scyliorhinus canicula*; Smeets et al. [1983]: the cartilaginous fishes *S. acanthias*, *S. canicula*, *Raja clavata* and *Hydrolagus collei*; Nieuwenhuys and Oei [1983]: the reedfish *Erpetoichthys calabaricus*; Nieuwenhuys [1998a]: the shovelnose sturgeon *Scaphirhynchus platyrhynchus*; Heijdra and Nieuwenhuys [1994]: the bowfin *Amia calva*; Nieuwenhuys and Pouwels [1983]: the gar *Lepisosteus osseus*; Kremers and Nieuwenhuys [1979]: the coelacanth *Latimeria chalumnae*; Thors and Nieuwenhuys [1979]: the lungfish *Lepidosiren paradoxa*; Opdam and Nieuwenhuys [1976]: the axolotl *Ambystoma mexicanum*; Opdam et al. [1976]: the frogs *Rana esculenta* and *Rana catesbeiana*; Nikundiwe and Nieuwenhuys [1983]: the clawed frog *Xenopus laevis*.

In all of the publications just mentioned, the cell masses in the brainstem and their positional relations have been analyzed. The method employed in these analyses, which is based on the principles of the branch of mathematics known as topology, has been extensively described in a previous paper [Nieuwenhuys, 1974]. Suffice it to mention here that within the frame of this method the cell masses and large individual neurons are first projected upon the ventricular surface, and that next the ventricular

surface, including its sulci and nuclear outlines, is flattened out (see Fig. 7).

Most of the studies on the structure of the central nervous system of dipnoans are devoted to representatives of the genera *Protopterus* and *Lepidosiren*. These studies are reviewed in Nieuwenhuys [1998b] and Northcutt [2011]. Until recently (see below), the literature on the structure of the Australian lungfish *N. forsteri* – the species with which we will concern ourselves in the present publication – was essentially confined to the classical paper of Holmgren and van der Horst [1925]. In that paper a detailed description is presented of the cell masses and fiber connections in the brain of *Neoceratodus*. It is based on two transversely sectioned brains, the one stained for myelin according to Weigert-Pal, the other impregnated with silver, according to Bielschowsky.

During the last decade, a number of immunohistochemical studies have been published on the brains of dipnoans, aimed at the localization of neurotransmitters and related molecules, as well as other neuroregulators. All of these studies are based on material derived from the spotted African lungfish *Protopterus dolloi*, as well as the Australian lungfish *N. forsteri*.

The neuromarkers studied include: The acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) [López et al., 2012], tyrosine hydroxylase, the first and rate-limiting enzyme for catecholamine synthesis [López and González, 2017], serotonin or 5-hydroxytryptamine (5-HT) [López and González, 2015], nitric oxide synthase, the nitric oxide synthesis enzyme [López et al., 2019], the calcium-binding proteins, calbindin D28K and calretinin [Morona et al., 2018] and DARPP-32, a phosphoprotein related to the dopamine D1 receptor [López et al., 2017a]. Most of the literature just cited is reviewed in López et al. [2017b].

Material and Technique

This study is based on a young adult specimen of the Australian lungfish *N. forsteri*. This specimen had a total length of 70 cm (Fig. 1a). The animal was anaesthetized in a 0.025% solution of M.S. 222 (Sandoz) and perfused through the heart with Bouin's fluid. The brain was removed, embedded in paraffin, cut transversely at a thickness of 15 µm, and stained with silver proteinate according to Bodian.

Procedure

Detailed drawings of 40 equidistant sections of our series were made with the aid of a projection apparatus at a magnification of 100 diameters. The position of these sections is indicated in Figure 7 by bilateral sets of short horizontal lines. The cell masses and large individual cells were identified and delineated in these draw-

Fig. 1. **a** The Australian lungfish, *Neoceratodus forsteri*. Dorsal (**b**) and lateral (**c**) view of the brain of the Australian lungfish. The levels of the transverse sections shown in Figures 2 and 3 have been indicated, where 2a–2f correspond with 2 to 7 and 3a–3d with 8 to 11.

ings. In the drawings of all individual sections the ependymal and meningeal surfaces were connected by a number of radially oriented curves, termed vectors. With the aid of these curves, the outlines of the cell masses and of the large individual cells were projected upon the ventricular surface. In each drawing, the deepest point of the ventricular midline groove of the brain stem, i.e. the sulcus medianus inferior, was defined as the zero point. At levels where this groove was lacking or indistinct, the zero point was defined as the ventricular end of the raphe, which was always clearly discernable. With the aid of a curvimeter, the distances from the zero point to the deepest point of other sulci, and to the projections of the outlines of the nuclei and individual neurons upon the ventricular surface, were determined on both sides of the sections. All the distances were measured along the ventricular surface and plotted graphically on a line. In the final reconstruction (Fig. 7) these transverse lines derived from the individual sections were placed in their correct rostrocaudal sequence and spaced appropriately, with their zero points connected by a vertical line, which forms the axis of the reconstruction. Finally, best fitting curves were drawn through the sets of points belonging to one and the same structure. In cases of doubt, additional sections were analyzed. For a more detailed description and a critical evaluation of the topological reconstruction procedure just outlined, the reader is referred to Nieuwenhuys [1974].

Due to its rather simple configuration, almost the entire ventricular surface of the brain stem of *Neoceratodus* could be included in our topological reconstruction (Fig. 7); only the corpus cerebelli and part of the auriculae had to be omitted.

Ten sections were selected to provide an atlas of the brain stem of *Neoceratodus*. They are represented in Figures 2, 3; their levels are indicated in Figures 1b, c and 7 by lines numbered 2 to 11.

In order to gain insight into the size of the cells in the various grisea, ten cells of each cell group were drawn at a magnification of 500 diameters, using a Zeiss drawing prism. In these drawings, the size of the somata was determined by averaging their diameters measured in two diameters perpendicular to each other. When a particular cell group appeared to contain more than one cell type, ten elements of each type were sampled and measured. Even though this procedure cannot be considered a rigorous quantitative analysis, it appeared to us preferable to the common practice of employing terms like small or fairly large without any quantification.

The cell sizes ranged from 8 to 165 μm , excluding the Mauthner cells. For convenience, we have subdivided the cells into four categories, viz. small (8–19 μm), medium-sized (20–59 μm), large (60–99 μm), and very large (100–165 μm). The medium-sized, large, and very large cells detected in the medial reticular formation are individually plotted with dots of three different sizes in our topological reconstruction (Fig. 7).

Results and Comments

Gross Features

The brain of *Neoceratodus* is, except for the telencephalon, slender and elongated (Fig. 1b, c). The well-devel-

Fig. 2. Transverse sections through the rhombencephalon of the Australian lungfish. The levels of these sections have been indicated in Figures 1 and 7.

oped rhombencephalon includes bilaterally a horizontal basal plate and an alar plate that is vertically oriented throughout most of its extent. Dorsally, the fourth ventricle is closed by a folded tela chorioidea. The ventricular

surface of the alar plate shows two elongated bulges, the rostr dorsally situated lobus lineae lateralis (Fig. 2c–e) and the caudoventral lobus vagi (Fig. 2b, c). Rostrally, the alar plate region containing the cerebellar plate curves

Fig. 3. Transverse sections through the rostral part of the rhombencephalon (**a**), the isthmus region (**b**) and the mesencephalon (**c, d**) of the Australian lungfish. The levels of these sections have been indicated in Figures 1 and 7.

around a lateral recess of the fourth ventricle and continues into a thick, horizontally oriented lamella of tissue, which fuses in the median plane with its counterpart of the opposite side. The rostralateral wall and the rostral part of the bottom of the lateral recess represent the auricula cerebelli. The corpus cerebelli is formed by the fused horizontally oriented lamellae of tissue mentioned above and caps the rostral part of the fourth ventricle (Fig. 2f). The ventricular (luminal) part of the corpus cerebelli forms an unpaired, median ridge, which protrudes into the fourth ventricle (Fig. 3a). Immediately rostral to the cerebellum, the width of the brain narrows, forming

a transitional region between the hindbrain and the midbrain, known as the isthmus rhombencephali (Fig. 1b).

The mesencephalon is small and includes bilaterally a dorsal tectal and a ventral tegmental region. The tectal region of *Neoceratodus* is, contrary to that of lepidosirenid lungfishes, differentiated into bilateral lobes (Fig. 1b, 3c, 3d).

The mesencephalon grades over the cephalic flexure into the diencephalon (Fig. 1c, 9), which limits rostrally with the secondary prosencephalon, composed of the hypothalamus and the dorsally evaginated telencephalic hemispheres.

Ventricular Sulci

Our analysis revealed the presence of the following ventricular sulci in the brain stem of *Neoceratodus* (Fig. 2, 3, 7).

The *sulcus medianus inferior* varies considerably in depth and distinctness. It is shallow in the caudal rhombencephalon and entirely absent in the intermediate rhombencephalon but is well-marked in the rostral rhombencephalon and throughout the midbrain.

The *sulcus intermedius ventralis* and the *sulcus limitans of His* are both distinct grooves, which extend throughout most of the rhombencephalon. The most rostral part of the sulcus limitans is on its medial side accompanied by a short groove, which marks the lateral boundary of the motor trigeminal and rostral facial nuclei. We designate this short groove as *sulcus limitans accessorius* (Fig. 7).

The *sulcus intermedius dorsalis* is only present in the intermediate part of the rhombencephalon where it marks the ventral boundary of the lobus lineae lateralis (Fig. 2c–e).

The *sulcus medianus superior* marks the dorsal line of fusion of the lateral walls of the neural tube. It is distinct in the region behind the obex (Fig. 2a) and throughout the midbrain (Fig. 3c, d). It is, however, lacking in the isthmus and cerebellar regions (Fig. 3a, b).

Two sulci, the short *sulcus isthmi ventralis* and the much longer *sulcus isthmi dorsalis*, pass obliquely over the surface of the isthmus region. The ventral sulcus and the rostral part of the dorsal sulcus mark the boundaries of the large nucleus visceralis secundarius (Fig. 3b, 7).

The ventricular surface of the midbrain is sculptured by four more or less longitudinally oriented sulci. These are, from dorsal to ventral, the *sulcus lateralis mesencephali*, the *sulcus subtectalis*, the *sulcus tegmentalis lateralis* and the *sulcus tegmentalis medialis* (Fig. 3c, d, 7). The sulcus lateralis mesencephali is an intratectal sulcus. The sulcus subtectalis and the sulcus tegmentalis lateralis mark the dorsal and ventral boundaries of the torus semicircularis, respectively. The sulcus tegmentalis ventralis extends rostrally into the diencephalon where it marks the dorsal boundary of the nucleus of the fasciculus longitudinalis medialis (Fig. 7).

Holmgren and van der Horst [1925] indicated to have traced the sulcus limitans of His “with some difficulty” (l.c. p 71) rostrally into the midbrain. They designated the sulcus tegmentalis lateralis of the present description as the mesencephalic continuation of the sulcus limitans (cf. their Fig. 22 and 43). According to our observations, the sulcus limitans is in *Neoceratodus*, and in all of the nu-

merous other anamniotes we investigated (see the introduction), strictly confined to the hindbrain.

Cell Masses, General Notes

In the brain stem of *Neoceratodus*, most of the neuronal perikarya are located in a continuous periventricular zone. Although the majority of the cells are small, the zone is not homogenous, and a considerable number of cell masses can be delimited within its confines. Some appear as local condensations of small cells, but most of them are composed of larger elements. These large-celled centers characteristically occupy a superficial position in the stratum griseum. A number of neuron groups have migrated away from the central gray and are embedded in the stratum album. One of these, the griseum superficiale isthmi et mesencephali, occupies a submeningeal position throughout (Fig. 3b–d).

Somatic Motor Nuclei

The somatic efferent cell masses include the most rostral part of the columna motoria spinalis and those motoneuronal groups which supply the external eye muscles.

The *columna motoria spinalis* continues for a considerable distance rostral to the obex. Its encephalic part (Fig. 2a, b) consists of large (62 μm) and very large (110 μm) fusiform cells (Fig. 4a). It gives rise to the three motor roots of the nervi spino-occipitales.

The *nucleus nervi abducentis* consists of a small group of loosely arranged, medium-sized (36 μm) ellipsoid and polygonal cells (Fig. 4i), situated immediately caudal to the Mauthner cell.

The *nucleus nervi trochlearis* occupies a paramedian position in the isthmus region. Its fusiform, medium-sized cells (36 μm) are situated between the ependymal surface and the dorsomedial aspect of the fasciculus longitudinalis medialis (Fig. 5h).

The *nucleus nervi oculomotorii* is situated in the rostromedial part of the tegmentum of the midbrain (Fig. 3d). Its medium-sized (38 μm) triangular and fusiform neurons cover, just like those of the trochlear nucleus, the dorsomedial aspect of the fasciculus longitudinalis medialis (Fig. 6d).

Branchiomotor Nuclei

The branchiomotor nuclei, i.e. the efferent centers of the Xth, IXth, VIIth and Vth cranial nerves, constitute an elongated column of cells situated in the lateral part of the rhombencephalic basal plate (Fig. 2). This column shows an interruption at the level of the Mauthner cell and the abducens nucleus. The rostral part of the column is

Fig. 4. Details of some cell masses in the brainstem of the Australian lungfish. **a** Spinal motor column. **b** Motor vagal nucleus. **c** Inferior raphe nucleus. **d** Inferior olive. **e** Lobus vagi. **f** Intermedius nucleus of the lobus octavolateralis. **g** Motor nucleus of the glossopharyngeal nerve. **h** Superior raphe nucleus. **i** Abducens nucleus.

Fig. 5. Details of some cell masses in the brainstem of the Australian lungfish. **a** A very large cell from the caudal part of the nucleus reticularis medius. **b** Nucleus vestibularis magnocellularis. **c** A large element from the rostral part of the nucleus reticularis medius. **d** Motor trigeminal nucleus. **e** A large cell from the caudal part of the nucleus reticularis superior. **f** Nucleus princeps nervi trigemini. **g** A medium-sized cell from the rostral part of the nucleus reticularis superior. **h** Trochlear nucleus. **i** Griseum superficiale isthmi et mesencephali.

Fig. 6. Details of some cell masses in the brainstem of the Australian lungfish. **a** Nucleus visceralis secundarius. **b** Nucleus mesencephalicus nervi trigemini. **c** Nucleus interpeduncularis. **d** Nucleus nervi oculomotorii. **e** Nucleus ruber. **f** Nucleus of the fasciculus longitudinalis medialis.

formed by the motor trigeminal and the rostral motor facial nuclei, whereas the caudal part harbors the caudal motor facial nucleus and the motor glossopharyngeal and vagal nuclei (Fig. 4b).

The *nucleus motorius nervi vagi* is very elongated and consists of medium-sized (45 µm) fusiform and polygonal cells (Fig. 4b).

The *nucleus motorius nervi glossopharyngei* is constituted by fusiform to multipolar cells, which are somewhat

larger (54 µm) than those in the motor vagal nucleus (Fig. 4g).

The *nucleus motorius nervi facialis caudalis* and *rostralis* are both composed of large (72 µm) triangular and polygonal cells.

The *nucleus motorius nervi trigemini* forms the somewhat enlarged most rostral part of the branchiomotor column. It produces a small elevation of the ventricular surface known as the eminentia trigemini (Fig. 2f). It is pri-

marily composed of a layer of superficially situated large (71 μm) cells, which are oriented perpendicular to the ventricular surface, but it also comprises some more deeply situated medium-sized (48 μm) neurons (Fig. 5d) and an occasional very large (104 μm) ellipsoid element (Fig. 2f).

On the basis of comparative inference, it might be expected that within or close to the branchiomotor nuclei just discussed, visceromotor or parasympathetic preganglionic elements are present. However, I failed to identify and to locate the cells of this category.

Immunohistochemical studies, using antibodies against the enzyme choline acetyltransferase indicate that the somatic motor and branchiomotor nuclei in the brain stem of *Neoceratodus* are cholinergic [López et al., 2012]. These authors also found assemblages of small cholinergic cells located at the lateral aspect of the motor VII, IX and X nuclei, which in their opinion might represent autonomic visceromotor elements, as well as bilateral groups of small cholinergic cells situated directly dorsolaterally to the oculomotor nuclei, which were interpreted as putative parasympathetic *Edinger-Westphal nuclei* (Fig. 8).

Reticular Formation

For convenience of description, the reticular formation will be divided into three longitudinal zones, median, medial and lateral.

The *median reticular zone* consists mainly of scattered small cells located in or close to the raphe, but comprises in addition two delimitable nuclei, the nucleus raphe inferior and the nucleus raphe superior.

The *nucleus raphe inferior* is situated in the caudal part of the rhombencephalon and is composed of loosely arranged, medium-sized (26 μm) ellipsoid cells (Fig. 4c).

The *nucleus raphe superior* is located in the intermediate part of the rhombencephalon, just caudal to the abducens nuclei. Its texture is similar to that of the inferior raphe nucleus although its constituent cells are somewhat larger (37 μm) (Fig. 4h).

López and González [2015] published an immunohistochemical study on the localization of 5-HT in the CNS of *Neoceratodus*. They found that in this species, a population of serotonergic elements situated in and near the raphe forms a conspicuous column extending from the caudal midbrain to the spinal cord (Fig. 8). They distinguished superior, median and inferior subdivisions within this column (the designation *median* is infelicitous and has been replaced here by *intermediate*). The cells forming the superior subdivision, which extends from the caudal midbrain to the level of the Mauthner cell, are embedded in the median and paramedian periventricular gray,

whereas the elements constituting the intermediate and caudal subdivisions form a narrow band in the raphe. The most caudal cells of the inferior subdivision, which is situated at the level of the obex, occupy a paramedian submeningeal position. It is noteworthy that the subdivisions of the serotonergic raphe column, distinguished by López and González [2015], show little correspondence with the superior and inferior raphe nuclei delimited by us (cf. Fig. 7, 8).

The *medial reticular formation* is represented by a continuous cell column, which extends over nearly the entire length of the rhombencephalon. Medially, this column begins at some distance from the raphe; its lateral boundary is marked by the sulcus intermedioventralis. Its constituent cells characteristically occupy the outermost zone of the central gray and the adjacent stratum album. These cells vary considerably in size and include very large (144 μm ; Fig. 5a, e), large (87 μm ; Fig. 5c) as well as medium-sized elements (55 μm ; Fig. 5g). The reticular cells generally show a spherical or ellipsoid shape. From the opposite poles of the very large and large cells typically two transversely and horizontally oriented dendritic trunks arise which again, just as with the soma, emit a number of ramifying dendrites that invade the white matter. Plotting the individual very large, large and medium-sized cells in our topological map revealed that these elements are not equally dispersed over the medial reticular formation. On this account, a division into three moieties, which are designated here as *nucleus reticularis superior*, *nucleus reticularis medius* and *nucleus reticularis inferior*, appeared to be warranted (Fig. 7). The

Fig. 7. Topological chart of the brainstem of the Australian lungfish, *Neoceratodus forsteri*. The heavy lines, which constitute the axis of the figure, represent the sulcus medianus inferior. The curves, which form the lateral limits of the figure, represent the taenia rhombencephali (continuous parts) and the sulcus medianus superior (dashed parts). The interruptions in these curves, marked by short horizontal lines, indicate the areas where the cerebellum has been omitted from the reconstruction. The remaining heavy curves indicate the position of ventricular sulci. The cell masses have been projected on the flattened out ventricular surface. The thin, continuous curves indicate the boundaries of periventricular cell masses; the outlines of migrated cell masses are indicated by interrupted curves; the position of the griseum superficiale isthmi et mesencephali is indicated by dotted curves. The delimitable parts of the reticular formation are indicated by curves of alternate dots and dashes. The small, intermediate and large black dots represent the medium-sized (20–59 μm), large (60–99 μm) and very large (100–165 μm) reticular cells, respectively. The vertical lines to the right of the figure indicate the rostrocaudal extent of the entrance/exit sides of cranial nerves.

(For figure see next page.)

basal part of the caudal diencephalon contains a cell group which may be considered as a rostral continuation of the medial reticular formation. This cell group, which is known as the *nucleus fasciculi longitudinalis medialis*, is composed of medium-sized (24 μm) and large (67 μm) ellipsoid cells (Fig. 6f). The medial reticular formation, which is also known as the nucleus motorius tegmenti, subserves a motor coordinating function. Accordingly, all of its parts project strongly to the spinal cord [Ronan and Northcutt, 1985].

The *lateral reticular zone* consists of a narrow strip of periventricular, small (19 μm), granular cells, which is located between the branchiomotor nuclei and the sulcus limitans (Fig. 2c). Holmgren and van der Horst [1925] believed that the cells in this zone are embedded in visceral sensorimotor reflex arcs.

The bilateral giant *cells of Mauthner* are situated directly behind the rostral motor facial nucleus (Fig. 7). Their transversely oriented, fusiform somata (368 μm) grade into coarse medially and laterally directed dendritic trunks, which together span the entire width of the basal plate. Distal ramifications of the lateral dendritic trunk contact entering fibers of the octavus nerve. The lateral as well as the medial dendritic trunks give rise to numerous ventrally directed, ramifying branches. The axon originates from the dorsomedial aspect of the soma, passes medially, decussates and passes within the contralateral fasciculus longitudinalis medialis toward the spinal cord. It is noteworthy that the Mauthner fibers in *Neoceratodus*, and in dipnoans in general, are of an exceptionally large diameter (Fig. 2a, b), and that their thin, loosely arranged myelin sheaths harbor not only the axon of the Mauthner cell, but also numerous other fibers, the nature of which is not certain (see Nieuwenhuys [1998] for details and references).

The area situated directly medial to the mediocaudal tip of the motor V nucleus contains bilaterally a large reticular cell, which occupies a position corresponding to that of the Mauthner neurons. We designated these elements provisionally as *pre-Mauthner neurons* (Fig. 7: p). It is known that the rhombencephalon of early embryonic anamniotes contains small bilateral groups of segmentally arranged neurons [Metcalf et al., 1986; Hanneman et al., 1988]. The “pre-Mauthner cells” just described, may well represent persistent elements of one of these pairs of segmental groups.

Visceral Sensory Nuclei

Under this heading, two centers, the nucleus fasciculi solitarii and the nucleus visceralis secundarius, will be considered.

The fasciculus solitarius, which consists of the afferent fibers of the VIIth, IXth and Xth nerves, forms in *Neoceratodus* a conspicuous descending bundle in the caudal rhombencephalon (Fig. 2d). The loosely arranged medium-sized (21 μm), ellipsoid cells surrounding this bundle form the *nucleus fasciculi solitarii* (Fig. 4e). This nucleus forms throughout most of its rostrocaudal extent a protrusion of the lateral wall of the fourth ventricle, known as the lobus vagi (Fig. 2b, c).

Holmgren and van der Horst [1925] observed that numerous secondary fibers leave the nucleus fasciculi solitarii in a ventrolateral direction. These fibers assemble in the submeningeal area, situated ventromedially to the descending root of V, where they form a large, thin-fibered bundle. Holmgren and van der Horst remained unable to trace this bundle to its destination; yet they felt sure that it represents the ascending tractus visceralis secundarius (Fig. 2d, f, 3a), a fiber system first described by Herrick [1905] in teleosts and known to terminate in an isthmus nucleus. Holmgren and van der Horst identified this *nucleus visceralis secundarius* in *Neoceratodus* on positional grounds as a sheet of densely arranged, small (13 μm) granular cells situated in the dorsomedial isthmus region (Fig. 3b, 6a). Morona et al. [2018] have demonstrated that this nucleus contains dense populations of calbindin- and calretinin-immunoreactive cells (Fig. 8). Double labeling experiments revealed extensive colocalization of these two proteins in the same cells. It should be mentioned that the nucleus cerebelli, as delineated by Thors and Nieuwenhuys [1979] in *Lepidosiren*, actually represents the nucleus visceralis secundarius.

General Somatic Sensory Nuclei

This category includes the nucleus funiculi dorsalis and the nucleus tractus descendens, the nucleus sensorius principalis, and the nucleus mesencephalicus of the trigeminal nerve.

The *nucleus funiculi dorsalis* represents the terminal center of primary afferent somatosensory fibers, ascending in the dorsal funiculus of the spinal cord. It is situated in the dorsolateral obex region (Fig. 2a) and consists of periventricularly situated small (17 μm), spherical, cholinergic cells [López et al., 2012].

Fig. 8. Modified version of the topological map shown in Figure 7. Neuromere borders are added, and the position of several cell masses, as determined by recent immunohistochemical studies quoted in the text, are indicated.

(For figure see next page.)

A considerable portion of the entering fibers of the Vth nerve constitute the large, superficially situated tractus descendens nervi trigemini, which can be traced as far caudal as the obex region (Fig. 2a–d).

The *nucleus tractus descendens nervi trigemini* is formed by small (19 µm), spherical cells situated among and directly medially to the fibers of the descending V bundle. These cells are generally rather loosely arranged but tend to be more concentrated in the caudal part of the rhombencephalon (Fig. 7).

The *nucleus sensorius principalis nervi trigemini* occupies a periventricular position in the most rostral and lateral part of the rhombencephalic basal plate (Fig. 2f, 3a). It is composed of medium-sized (21 µm), spherical and ellipsoid cells (Fig. 5f). This nucleus has been shown to contain a large population of calbindin- and calretinin-immunoreactive cells, many of them showing coexpression of both proteins [Morona et al., 2018].

The *nucleus mesencephalicus nervi trigemini*, finally, forms an elongated median strip in the caudal part of the tectum mesencephali (Fig. 3c). Its medium-sized (37 µm), spherical and polygonal cells are embedded in the most dorsomedial parts of the small-celled periventricular tectal zone (Fig. 6b).

Special Somatic Sensory Nuclei

The special somatic sensory area, or area octavolateralis, occupies a large part of the lateral rhombencephalic wall. It begins a little in front of the obex and extends to the cerebellar region. Three delimitable cell masses are present in this area, termed here the nucleus dorsalis areae octavolateralis, the nucleus intermedius areae octavolateralis and the nucleus vestibularis magnocellularis. The first two of these nuclei represent end stations of the lateral line nerves, whereas the last one receives vestibular fibers. The lateral line nerve nuclei are externally covered by a highly characteristic, cell-poor layer, known as the crista cerebellaris (Fig. 2c–e). This layer strongly resembles the molecular layer of the cerebellum with which it is rostrally directly continuous. In the areas between the intermediate and dorsal lateral line nerve nuclei as well as caudal to the latter nucleus, the crista cerebellaris lies immediately against the ependymal lining of the fourth ventricle. These subependymal parts of the cerebellar crest have been included in our topological map (Fig. 7).

The large *nucleus dorsalis areae octavolateralis* extends along the ventricular surface of the lateral line lobe (Fig. 2c–e). It is composed of small (16 µm), granular and medium-sized (32 µm) ellipsoid cells.

The elongated *nucleus intermedius areae octavolateralis* accompanies the sulcus intermedius dorsalis throughout its extent (Fig. 2c–e). It is composed of the same types of cells as the dorsal octavolateral nucleus (Fig. 4f).

The *nucleus vestibularis magnocellularis* (Fig. 2e) is represented by a small group of large (58 µm), bipolar cells (Fig. 5b), which occupy a periventricular position at the level of entrance of the eighth cranial nerve.

All three of the special somatosensory or octavolateral nuclei just discussed have been shown to contain populations of calbindin- and calretinin-immunoreactive cells (Fig. 8) [Morona et al., 2018].

Remaining Rhombencephalic Nuclei

Under this heading, the following cell masses will be discussed: the oliva inferior, the griseum centrale rhombencephali, the griseum superficiale isthmi et mesencephali, the nucleus isthmi, the laterodorsal tegmental nucleus, and the locus coeruleus, as well as a few catecholaminergic cell groups in the caudal rhombencephalon.

The *oliva inferior* is an elongated nucleus, which occupies a submeningeal paramedian position in the caudal part of the rhombencephalon (Fig. 2b). It consists of small (13 µm), granular, and medium-sized (20 µm), ellipsoid cells (Fig. 4d).

The *griseum centrale rhombencephali* is a condensation of small (14 µm), granular cells, situated in the periventricular zone of the rostral part of the rhombencephalic basal plate (Fig. 3a–c). Its caudal boundary is fairly distinct, but rostrally it grades into the mesencephalic central gray.

The *griseum superficiale isthmi et mesencephali* is represented by a conspicuous, superficially situated cell mass, which covers the dorsolateral aspect of the isthmus region (Fig. 3b). Rostrally, it extends as a tongue-shaped layer along the lateral surface of the midbrain, attaining the level of the oculomotor nucleus (Fig. 3c, d). Its very small (8 µm), compactly arranged cells surround irregularly shaped small patches of neuropil (Fig. 5i). The *griseum superficiale isthmi et mesencephali* is caudally continuous with the granular layer of the corpus and auricula cerebelli. It is noteworthy that granular cells in these cerebellar formations as well as those in the *griseum superficiale isthmi et mesencephali* express the Pax6 protein [López et al., 2020]. Holmgren and Van der Horst [1925] considered this superficial griseum to be homologous with the ganglion or nucleus isthmi of anurans and reptiles and designated it accordingly as ganglion isthmi. The cell mass in question has a clear homologue in lepidosirenid lungfishes

[Nieuwenhuys, 1998b; Northcutt, 2011] and in the coelacanth *Latimeria* [Kremers and Nieuwenhuys, 1979], but is entirely lacking in the many other amniote species investigated by us (see Introduction). We consider it likely that another group of cells may well represent the nucleus isthmi in *Neoceratodus*. It has been demonstrated that the rostral tip of the griseum superficiale isthmi et mesencephali is in receipt of retinal afferents [Northcutt, 1980], but the remaining connections of this large cell mass are entirely unknown.

The putative *nucleus isthmi* indicated above occupies a position halfway between the secondary visceral nucleus and the griseum superficiale isthmi et mesencephali (Fig. 3b). It is composed of rather loosely arranged small (13 μm) spherical cells. López et al. [2012] found a scarce population of weakly ChAT-immunoreactive cells in the same location, which they likewise considered to represent a putative nucleus isthmi (Fig. 8). Slightly more caudal and ventral to the putative isthmic nucleus, López et al. [2012] detected a conspicuous group of intensively labeled ChAT-immunoreactive cells (Fig. 8). This group appeared to be similar in position and cholinergic nature to the *nucleus laterodorsalis tegmenti* of amphibians, perhaps including the likewise cholinergic and nitroergic pedunculopontine tegmental nucleus of tetrapods. This correspondence is further supported by immunohistochemical evidence indicating that these cells produce nitric oxide in dipnoans, amphibians as well as amniotes [López et al., 2019]. It is also noteworthy that the lateral basal plate area, situated directly rostral to the motor trigeminal nucleus contains a small group of tyrosine hydroxylase-immunoreactive (likely noradrenergic) cells, which presumably represents the lungfish homologue of the *locus coeruleus* ([López and González, 2017]; Fig. 8). Neither the laterodorsal tegmental nucleus nor the locus coeruleus could be delimited in our silver-impregnated material of *Neoceratodus*. López and González [2017] also demonstrated the presence of three tyrosine hydroxylase-immunoreactive (putatively noradrenergic) cell populations in the caudal rhombencephalon, a small group of weakly labeled cells in the dorsomedial part of the vagal lobe, a larger group of intensely labeled cells in the ventral part of the same lobe and a caudal group occupying a superficial, dorsomedian position in the obex region. We designate the two groups first mentioned here provisionally as the *dorsal and ventral rhombencephalic noradrenergic cell groups*; the third group represents the *area postrema* (Fig. 8).

Mesencephalic Cell Masses

The bilobed *tectum mesencephali* of *Neoceratodus* is a clearly laminated structure. It contains, from the ependymal surface outward, the following three layers: (1) a compact stratum griseum periventriculare, consisting of small (13 μm) granular cells; (2) a stratum griseum centrale, which is mainly composed of small (17 μm), loosely arranged piriform elements, and (3) a wide stratum fibrosum et griseum superficiale, in which zones of small (15 μm) scattered cells alternate with layers of optic fibers and terminal fields (Fig. 3c, d). The periventricular tectal layer contains a prominent population of nitroergic cells [López et al., 2019]. These cells are intermingled with 5-HT-immunoreactive cells of similar morphology but forming a separate subpopulation [López and González, 2015]. López et al. [2017a] reported that the periventricular tectal zone harbors in addition a conspicuous group of DARPP-32-immunoreactive cells (Fig. 8). It has been experimentally established that the tectum receives strong, predominantly contralateral retinal afferents [Northcutt, 1980], and projects to the contralateral side of the spinal cord [Ronan and Northcutt, 1985].

The *nucleus tori semicircularis* is located directly ventral to the tectum. It is mainly composed of a layer of periventricular, small (13 μm) cells, which at caudal levels are situated at some distance from the ependymal surface (Fig. 3c, d). Experimental data on the connections of the dipnoan torus semicircularis are lacking. It may be expected, however, that this structure receives, just as in other fishes [Wullimann, 1998], bilateral afferents from the rhombencephalic octavus and lateral line nuclei, which ascend via the lateral lemniscus to the midbrain (Fig. 3b, c).

The *nucleus ruber* consists of a small group of medium-sized (21 μm), ellipsoid cells, situated in the rostro-medial part of the tegmentum mesencephali, at some distance from the ventricular surface (Fig. 3d, 6e). The identity of this nucleus as representing the nucleus ruber, was substantiated by Ronan and Northcutt [1985], who demonstrated experimentally that its cells project to the contralateral side of the spinal cord.

As a possible *nucleus interpeduncularis*, we interpreted a group of medium-sized (21 μm), ellipsoid cells, situated in and near the mesencephalic raphe, between the levels of the oculomotor and trochlear nuclei (Fig. 3c, 6c). We are well aware of the fact that this is an atypical position for the interpeduncular nucleus, which in almost all other vertebrates, including lepidosirenid lungfishes [Northcutt, 2011] is located *caudally* to the trochlear nucleus in the most rostral part of the rhombencephalon. It is re-

markable, however, that Holmgren and van der Horst [1925], who also studied silver-impregnated material, located the interpeduncular nucleus in *Neoceratodus* at precisely the same location as we did. These authors established, moreover, that fibers of the fasciculus retroflexus, i.e. the principal afferent system of the interpeduncular nucleus, terminate in *Neoceratodus* at the level of entrance of the oculomotor nerve (cf. their Fig. 20). Morona et al. [2018] established on the other hand, that the habenular ganglia contain numerous calbindin-immunoreactive cells and that the axons of these cells descend with the fasciculus retroflexus to form a conspicuous terminal neuropil in the mediobasal part of the rostral rhombencephalon. They designated the neurons surrounding this neuropil as the interpeduncular nucleus. We label the possible mesencephalic and rhombencephalic positions of the interpeduncular nucleus here provisionally as ip1 (Fig. 3c, 7) and ip2 (Fig. 8), respectively.

The immunohistochemical studies of López and González [2017] have shown that the most ventral parts of the mesencephalic periventricular zone are occupied by a group of densely packed dopaminergic cells. Rostrally, this group extends for some distance into the dienkephalon; caudally, it ends abruptly just rostral to the trochlear nucleus (Fig. 8). González and Northcutt [2009] have demonstrated that this cell group has reciprocal connections with the telencephalon. Taken together, these findings indicate that the dopaminergic elements in question correspond to the *area tegmentalis ventralis* and the *substantia nigra pars compacta* (vta/sn) of amniotes.

General Comment on the Cell Masses

From the preceding paragraphs, it appears that in the brain stem of the lungfish *Neoceratodus*, 45 cell masses could be distinguished. Eighteen out of these 45 cell masses represent cranial or spinal nerve nuclei; ten of these are primary efferent and eight are primary afferent centers; six nuclei can be regarded as components of the reticular formation. The remaining 21 cell masses may be interpreted as “relay nuclei.” For some of these, as for instance the nucleus ruber and the sn/vta complex, experimental data are available to warrant this interpretation, but for most it should be regarded as tentative.

General Discussion

Longitudinal Zones

As has been pointed out in the introduction, the present paper forms part of a program of research within the

frame of which the morphological pattern of the brainstem is studied in a number of representative anamniote vertebrates. This program and its specific aims have been discussed in a general, introductory paper [Nieuwenhuys, 1974] to which the reader is referred for details. Briefly, it may be stated that for each species studied the following questions are to be tested: (1) Is the brainstem divisible into a motor basal plate and a sensory alar plate? (2) To what extent are the nuclei contained within the basal and alar plates arranged in a longitudinal zonal pattern? (3) To what extent are the boundaries of such zones marked by ventricular sulci? (4) To what extent do the nuclei, falling under common functional denominators, fit into a longitudinal zonal pattern? With the aid of the topological analysis, represented in Figure 7, an attempt will now be made to answer these questions for *Neoceratodus*.

Basal Plate – Alar Plate. In *Neoceratodus* a distinct sulcus limitans extends uninterruptedly from the obex level to the rostral part of the rhombencephalon. Considering with His [1893] this sulcus as a landmark indicating the boundary between the basal plate and the alar plate, most of the rhombencephalon of *Neoceratodus* is divisible into these two basic entities. The designation of the basal plate as “motor” and alar plate as “sensory” is correct, in so far that all primary efferent centers are situated within the former and most primary afferent centers within the latter. Exceptions to this rule include the nucleus vestibularis magnocellularis and the nucleus sensorius principalis nervi trigemini, the former lying partly and the latter lying entirely in the lateral part of the basal plate.

Subdivision of Basal Plate. Our topological chart (Fig. 7) shows that the cell masses in the basal plate fit into three longitudinal zones, median, medial and lateral. The median zone contains the raphe nuclei. The medial zone is formed by the spinal motor column, the abducens nucleus, the medial reticular formation and the inferior olive. The lateral zone encompasses the motor nuclei of V, VII, IX and X, the Mauthner cells, and, most laterally, the lateral reticular zone. According to the purely morphological brainstem concept of Kühlenbeck [1973], the median and medial zones constitute together the area ventralis, whereas the lateral zone forms the area intermedioventralis. Kühlenbeck pointed out that a ventricular groove, the sulcus intermedius ventralis, marks the boundary between the ventral and intermedioventral zones. Our map shows clearly that such is the case indeed in *Neoceratodus*.

The functional designation of the area ventralis as the somatomotor column is appropriate in that it contains the most rostral part of the spinal motor column, the ab-

ducens nucleus and the medial reticular formation, and the latter subserves a somatomotor coordinating function. However, this area contains three centers, the inferior olive and the inferior and superior raphe nuclei, which cannot be characterized as somatomotor. The inferior olive is a “precerebellar” nucleus, which sends climbing fibers to the contralateral half of the cerebellum. It is an extrinsic component of the area ventralis because its constituent cells have migrated ventrally from a progenitor zone situated in the most dorsal part of the rhombencephalon. The two raphe nuclei mentioned are embedded in, and form part of a continuous column of serotonergic cells, which extends throughout the rhombencephalon [López and González, 2015]. The function of this serotonergic column is unknown. In mammals, the derivatives of this column are involved in multifarious neuromodulatory functions.

The area intermedioventralis contains, first and foremost, the motor nuclei of V, VII, IX, and X. Together, these nuclei constitute a branchiomotor or special visceromotor column. López et al. [2012] have presented evidence that the most lateral parts of the motor VII, IX and X nuclei also contain assemblages of small, autonomic, general visceromotor elements. The lateral reticular zone, which occupies the most lateral part of the area intermedioventralis, consists of small cells, which according to Holmgren and van der Horst [1925], are probably intercalated in visceral reflex arcs. Taken together, the facts just discussed justify the designation of the area intermedioventralis as a visceromotor zone.

Subdivision of Alar Plate. Along most of the rhombencephalon, the alar plate contains two longitudinally arranged cell zones which, according to Kühlenbeck [1973], may be designated as the area intermediodorsalis and the area dorsalis. The boundary between these areas is marked by the sulcus intermediodorsalis. The constituents of the intermediodorsal area do not form a functional entity. Its caudal part is occupied by the nucleus fasciculi solitarii, which receives viscerosensory afferents via VII, IX and X, but also harbors the general somatosensory nucleus of the tractus descendens of V and most of the special somatosensory nucleus vestibularis magnocellularis. The two cell masses situated in the area dorsalis, i.e. the nucleus dorsalis and the nucleus intermediodorsalis areae octavolateralis, receive their inputs exclusively from the lateral line nerves. On that account, this area may be designated as a special somatosensory zone.

The *isthmus region* does not exhibit a clear morphological zonal pattern. Functionally, the nucleus trochlearis belongs to the somatomotor zone, whereas the nucle-

us visceralis secundarius, possibly representing the homolog of the parabrachial nuclear complex of tetrapods, may be considered as the most rostral part of the discontinuous viscerosensory column. The nucleus isthmi is reciprocally connected with the tectum in all nonmammalian groups studied [López et al., 2012]. If it is confirmed that the tentative nucleus isthmi delineated in *Neoceratodus*, has similar connections, this center may be considered to belong to the special somatosensory category.

The *mesencephalon* is divisible into a ventral, primarily motor, and a dorsal sensory zone. The ventral zone contains the somatomotor oculomotor nucleus, the somatomotor coordinating nucleus ruber, and the visceromotor nucleus of Edinger-Westphal. The dorsal zone is composed of the general somatosensory nucleus mesencephalicus of V, and two special somatosensory centers, viz. the tectum and the nucleus of the torus semicircularis. The sulcus tegmentalis lateralis, which marks the boundary between the ventral and dorsal zones just discussed, may be considered to represent a separate mesencephalic portion of the sulcus limitans.

Neural Segments

At the time that we started the research program, of which the present analysis of the brainstem of *Neoceratodus* forms part, it was generally held that the brainstem (and in fact the entire brain) is essentially composed of longitudinally oriented structural and functional zones, which are separated by ventricular sulci. The central aim of our program was to test the validity of this “zonal concept” for a number of anamniote vertebrates. In the meantime, a notable paradigm shift with regard to the fundamental pattern of the vertebrate brain has occurred. Studies on the expression patterns of a large number of developmental regulatory genes and their products (morphogens, transcription factors) have shown that the CNS of vertebrates is essentially organized into transverse neural segments or neuromeres and longitudinal zones [Puelles, 2013; Nieuwenhuys and Puelles, 2016]. The intersection of the transverse and longitudinal patterning processes in the embryonic brain leads to the formation of a checkerboard pattern of distinct progenitor domains called “fundamental morphological units.” The topologically invariant pattern formed by the FMUs represents the Bauplan or morphotype of the vertebrate brain. The most important longitudinal landmark in this Bauplan is the molecularly defined alar-basal boundary [Puelles, 2013]. It is noteworthy that at rhombencephalic levels, this zone does not coincide with the classical sulcus limitans of His, but is rather situated more ventrally, roughly

Fig. 9. Sagittal scheme of the brainstem of *Neoceratodus* showing the boundaries of the neuromeres, the course of the molecularly defined zona limitans, and the distribution of main cholinergic cell groups. Note that the branchiomotor nuclei Vm, VIIIm, IXm and Xm are erroneously positioned under, i.e. medial to the zona lim-

itans. Redrawn from López et al. [2012]. DCN, dorsal column nucleus; Is, nucleus isthmi; LDT, laterodorsal tegmental nucleus; m, mesomere; mo, medial octavolateral nucleus; OT, optic tectum; Nsol, nucleus of the solitary tract; r0, r1 etc., rhombomeres; smn, somatomotor nucleus of spinal cord; zI, zona limitans.

just ventral to the branchiomotor nuclei. Due to this shift, the branchiomotor nuclei, which according to the Herriek-Johnston concept are situated in the basal plate, have now come to lie in the alar plate.

On the basis of detailed ontogenetic studies, several authors [Gilland and Baker, 1993; Aroca and Puelles, 2005; Straka et al., 2006; Puelles and Rubinstein, 2015] have succeeded in tracing the neuromeric borders from their origin in the neural plate and early neural tube, to their ultimate position in the adult brain. These data have been used for the construction of models of the segmental organization of the brain of several vertebrates, including the Australian lungfish *Neoceratodus* [López et al., 2012]. The caudal part of this neuromeric model, which is based on a series of sagittal sections, is shown in Figure 9. It will be seen that in this model the mesencephalon consists of a single mesomere (m), that the rhombencephalon is composed of eight rhombomeres (r1-r8), and that an isthmial neuromere, designated as r0, is intercalated between m and r1. López et al. [2012] used this model to visualize the results of their immunohistochemical investigations on the localization of cholinergic cell masses and fiber systems in the brain of *Neoceratodus*. The same sagittal scheme has been used in several later immunohistochemical studies on the brain of *Neoceratodus* [López and González, 2015, 2017; López et al. 2017a, b, 2019; Morona et al., 2018].

We have used the same sources as López et al. [2012] for introducing neuromere borders in our topological map of *Neoceratodus* (Fig. 8), and we have plotted the re-

sults of some of the immunohistochemical studies just quoted in this new map. Contrary to López et al. [2012], and in harmony with Cambroner and Puelles [2000], we subdivided the rhombencephalon into 11 rather than eight rhombomeres.

A special feature, in which our topological maps differ from sagittal schemes, such as the one depicted in Figure 9, is that in our maps the cell masses are projected *in a natural way* upon a *natural plane*. The natural plane is the ventricular surface, which represents the “starting” or “zero” plane of the histogenesis of the CNS. The mode of projection is natural because, given the fact that most cell groups migrate radially outward from their origin in the ventricular matrix to their ultimate location, we project these cell masses “back to their origin,” using a system of radially oriented vectors.

Concluding Remark

Topological maps, such as the one depicted in Figure 7, were originally designed to study the zonal arrangement of grisea in the brainstem. It should be emphasized that modified versions of such maps, in which the neuromere borders are indicated, and in which the ventricular sulcal pattern is replaced by molecularly defined longitudinal borders, provide a structural framework which reflects a richer checkerboard pattern of the fundamental morphological units alluded to above, and therewith the set of invariants, which constitutes the Bauplan or mor-

photype of the vertebrate brainstem. Given these properties, modern maps contemplating molecular boundaries and patterns of tangential cell migration are particularly useful for the determination of the topological position of grisea, and hence for the establishment of their homology [Nieuwenhuys, 2011, 2017].

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Statement of Ethics

This study is entirely based on non-experimental animal material; the microscopical preparations were processed before 2000 and belong to the collection of the Department of Medical Imaging, Anatomy in Nijmegen.

Conflict of Interest Statement

The author has no conflict of interest to declare.

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