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Migration of Branchiomotor Neurons to the Alar Plate in the Chicken Embryo

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1-6	rhombomeres 1-6
A	alar plate
α	α-catenin
B	basal plate
β	β-catenin
b/a	basal/alar plate boundary
BCIP	5-bromo-4-chloro-3-indolyl phosphatetoluidine salt
Bl	lateral part of basal plate
Bm	medial part of basal plate
bm	branchiomotor
cad6B	cadherin-6B
cad7	cadherin-7
CNS	central nervous system
d	dorsal band of Phox2b expression
D/V	dorsoventral
E	embryonic day (day of incubation)
F	floor plate
flm	medial longitudinal fascicle
fp/FP	floor plate
gV	trigeminal ganglion
gVII	facial ganglion
gIX	glossopharyngeal ganglion

gX	vagus ganglion
Hb	hindbrain
HBSS	HEPES-buffered salt solution
HH	stage according to Hamburger and Hamilton (1951)
III	oculomotor complex
IV	trochlear complex
Isl1	Islet-1
mbm	migrating branchiomotor neurons
MN	motor neuron column
mz	mantle zone
nc/NC	notochord
NBT	nitroblue tetrazolium salt
p0-p3	ventral progenitor cell domain
PBS	phophate-buffered saline
pMN	motorneuron progenitor domain
Rcad	R-cadherin
RP	roof plate
SG	spinal ganglion
Shh	Sonic hedgehog
s.m.	somatomotor
s.s.	somatosensory
TBS	Tris-buffered saline
Tect	tectum
Thio	thionine

v4	fourth ventricle
Vm	laterally/dorsally migrated branchiomotor nucleus of the trigeminal nerve
Vmn	ventral motor nucleus of the trigeminal nerve
v.m.	visceromotor
VIn	abducens nerve
VIIn	facial nerve
VIIm	laterally/dorsally migrated branchiomotor nucleus of the facial nerve
vr	ventral root
v.s.	viscerosensory
vz	ventricular zone
XII	hypoglossal nucleus
XIIn	hypoglossal nerve
Xm	laterally/dorsally migrated branchiomotor nucleus of the vagus nerve

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1 Introduction

1.1 Development of the spinal cord and hindbrain from the neural tube

The vertebrate central nervous system develops from a sheet of cells called the neural plate. It invaginates soon to form the neural tube, a bilaterally symmetrical structure that extends in the center of the body along the ongitudinal axis. The following parts of the central nervous system (CNS) emerge from the neural tube from rostral to caudal: forebrain, midbrain, hindbrain and spinal cord. The hindbrain and spinal cord enclose the fourth ventricle and the central canal, respectively. Underlying both structures is the notocord that stops rostrally just before the transition from the hindbrain to the midbrain. In the developing spinal cord and hindbrain, the lateral wall of theneural tube can be divided, along the dorsoventral dimension, into a roof plate, an alar plate, a basal plate, and a floor plate.

In the hindbrain, a thin sheet above the fourth ventricle forms the roofplate and the paired lateral walls are flapped open like a book so that the alar plate comes to lie lateral to the basal plate. It is generally believed that basal plate contains the somatic and visceral motor neurons, whereas the alar plate consists of somatic and visceral sensory neurons (Fig. 1) (His, 1888; Kuhlenbeck, 1975). Consequently, in the hindbrain, four columns of nuclei with specific functions are discerned from medial to lateral: a somatomotor column, a visceromotor column, a viscerosensory column and a somatosensory column (Fig. 1) (Gaskell, 1886, 1889; Johnston, 1905; Herrick,

1922). The striated muscles associated with the branchial arches are innervated by specialized group of visceromotor neurons that are called "special visceral motor" neurons or "brachiomotor" neurons. In higher vertebrates, these motorneurons are born at a medial position close to the floor plate and then migrate laterally (Ramon y Cajal, 1911; Heaton and Moody, 1980; Covell and Noden, 1989; Fritzsch, 1998). Their final position is thought to be in the ventrolateral basal plate.

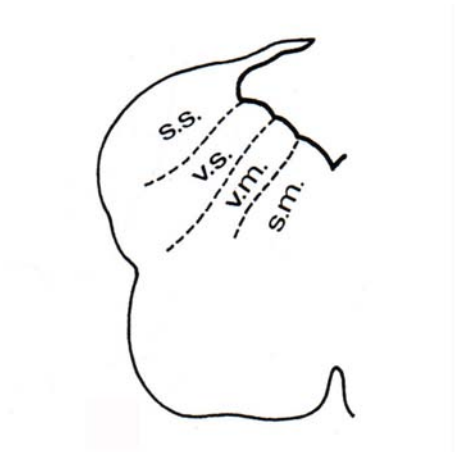

Figure 1. Schematic diagram of His's (1888) concept of the arrangement of sensory and motor neurons in the hindbrain. A similar diagram is depicted also in most modern neuroanatomical text books. Abbreviations: s.m., somatomotor; s.s., somatosensory; v.m., visceromotor; v.s., viscerosensory. Reproduced from Nieuwenhuys et al. (1998).

In the present work, I investigated the relation of cadherins, a family of cell adhesion molecules that regulate morphogenesis in a variety of organs, to nervous system development in the embryonic chicken CNS. As an introduction, I will give a brief overview of dorsoventral (D/V) patterning in the neural tube (see Sections 1.2 and 1.3) and of cadherins and their role as morphoregulatory molecules (see Section 1.4). Several studies have shown

that cells in the spinal cord express differentially several cadherins (Fig. 2) (Redies, 2000).

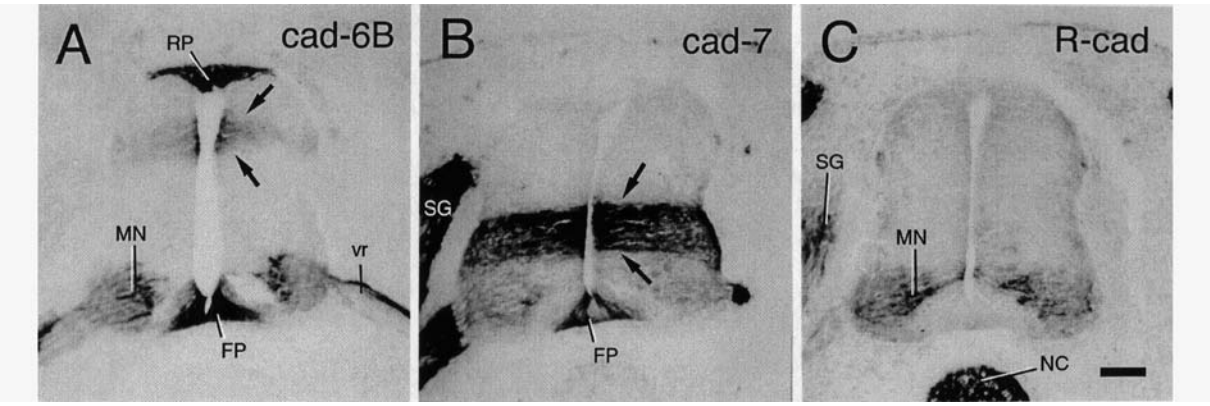

Figure 2. Differential expression of cadherin-6B (cad6B, A), cadherin-7 (cad7, B) and R-cadherin (Rcad, C). A transverse section through the spinal cord of a stage 21 chicken embryo (at 3.5 days of incubation) is shown. Immunostains are shown for a series of consecutive sections. Abbreviations: FP, floor plate; MN, motor neuron column; NC, notochord; RP, roof plate; SG, spinal ganglion; vr, ventral root. Scale bar (in C), 50 µm for A-C. Reproduced from the publication by Redies (2000).

In the current work, I focus on the expression of two cadherins, cadherin-7 and cadherin-6B, during the early development of the spinal cord and hindbrain. At early stages of development, cadherins were shown to play multiple roles in neural tube regionalization and in the migration and aggregation of neurons (for review, see Redies, 2000; Hirano et al., 2003). My results show that the two cadherins are expressed in different dorsoventral domains of the developing neural tube wall and that they might possibly play a role in branchiomotor neuron migration and differentiation. Surprisingly, results of my research contradict the century-old belief that all primary motor neurons come to lie in the basal plate of the hindbrain (His, 1888; Gaskell, 1886, 1889; Johnston, 1905; Herrick, 1922; Kuhlenbeck, 1975).

1.2 Dorsoventral patterning in the spinal cord and hindbrain

D/V patterning results in multiple gene expression domains in the neural tube wall. With the advent of immunohistochemical and molecular genetic techniques, a series of molecular markers for D/V patterning in the hindbrain and spinal cord has become available and some of the genetic mechanisms inducing this patterning have been elucidated. The spinal cord is patterned along the D/V axis by the secretion of Sonic hedgehog (Shh) from the notochord and the floor plate (Fig.3) (Marti et al., 1995; Ericson et al., 1996, 1997a; Briscoe et al., 2000; Poh et al., 2002) and by the secretion of BMPs and dorsalin from the roof plate (Basler et al., 1993; Lee et al., 2000; Liem et al., 2000; Litingtung and Chiang, 2000; Timmer et al., 2002). In the basal plate of the spinal cord, various molecular markers allow the distinction of at least five classes of motoneurons and interneurons that are arranged in the D/V zones (Fig. 3C) (Simeone et al., 1994; Pattyn et al., 1997; Briscoe et al., 2000; Garel et al., 2000; Marthiens et al., 2002). The position of the motorneuron zones was shown to depend on the concentration of Shh (Ericson et al., 1997a; Briscoe and Ericson, 1999; Briscoe et al., 2000). Similar patterning mechanisms are thought to take place in the hindbrain. As previous studies have shown (Redies, 2000), different classic cadherins are expressed in different D/V domains of the spinal cord in the chicken embryo (Fig. 2). Specifically, cadherin-7 was shown to be expressed in a complete and sharply demarcated radial domain of the spinal cord (Fig. 2B).

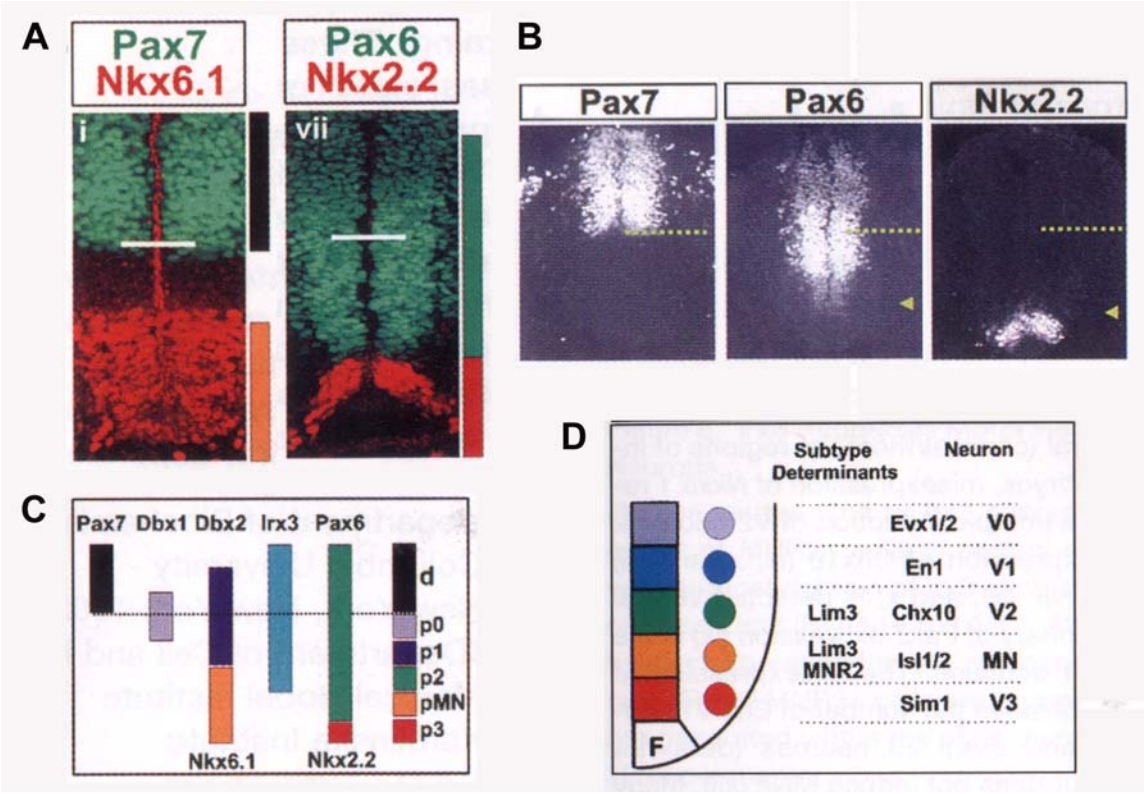

Figure 3. A: Combinatorial expression of transcription factors (i.e., Pax7, Pax6 and Nkx2.2, A) in the spinal cord of a stage 22 (HH22) chick embryo after retroviral transduction and in ovo electroporation. The white line (A) indicates the alar/basal boundary. B: Localization of Pax7, Pax6 and Nkx2.2 in the spinal cord of a stage 20 embryo (HH20). The dotted line indicates the basal/alar boundary. Yellow arrowheads indicate the ventral boundary of Pax6 expression and the dorsal boundary of Nkx2.2 expression. C: Schematic diagram of various transcription factors. Abbreviations: d, dorsal; p, progenitor domain. D: Differential neuronal fate. Distinct neuronal subtypes are generated from each progenitor domain. In the present work, I focused on the expression of Lim3 and Isl1. Reproduced from the study by Briscoe et al. (2000).

In my research, I first tried to define this domain in comparison with various transcription factors mapped previously in the spinal cord. I then studied the same domain also in the hindbrain of the chicken embryo at different stages of development. Thirdly, I followed the migration of branchiomotor neurons, which also express the two cadherins, with respect to the basal/alar plate

boundary, which is marked medially by the expression border of cadherin-7 (see below).

1.3 Markers for dorsoventral patterning and development of branchiomotor neurons

To visualize dorsoventral D/V patterning in the neural tube, various transcription factors have been used. For example, Pax7 was used as a marker for sensory neurons in the alar plate (Fig. 3A,B,C). The basal plate in spinal cord has been divided into five progenitor cell domains which were termed the p0, p1, p2, pMN and p3 domains (Fig. 3C) (Ericson et al., 1996; Briscoe et al., 2000). Pax6 was shown to be expressed in the alar plate, some parts of basal plate in the hindbrain and in the p0, p1, p2 and pMN of basal plate in the spinal cord (Fig. 3A,B,C) (Briscoe et al., 2000). Nkx2.2 is expressed in the p3 domain of the spinal cord and its dorsal limit coincides with the ventral limit of Pax6 expression in the basal plate (Fig. 3A,B,C) (Briscoe et al., 2000). Lim3 has been used as subtype determinant, which was positioned within the p2 and pMN domains in basal plate and includes motor neurons (Briscoe et al., 2000). The antibody against Lim3, which I used in my research, also recognizes the closely related transcription factor Lhx4 (Johan Ericson, Karolinska Institute, Sweden; personal communication). The homeobox genes Nkx2.2 and Lim3 start to be expressed in mitotic cells. It is well established that Lim3 is expressed in the hypoglossal and accessory abducens nuclei, and Lim1 and Lim2 are not expressed by cranial motor neurons (Varela-Echavarria

et al., 1996). Isl1 was used also as a molecular marker of motor neurons (Fig. 3D). Shh expression marks the floor plate and the notocord (Ericson et al., 1997a; Briscoe and Ericson, 1999; Briscoe et al., 2000). Otp expression was used as an indicator for the basal/alar plate boundary (Simeone et al., 1994) and Phox2b served as a marker for branchiomotor neurons (Pattyn, 1997; Garel, 2000). Cadherin-6B expression has been described in the roof plate, in the floor plate and in the motor columns of the spinal cord (Redies, 2000; Marthiens, 2002). In the present study, I defined the exact position of the basal/alar plate boundary in the hindbrain and spinal cord at several stages of development by using a panel of markers whose expression borders coincide with the basal/alar plate boundary (Pax7, cadherin-7 and Otp).

Moreover, I investigated the relationship between the migration of "branchiomotor" (bm) neurons and the basal/alar plate boundary in the chicken embryo. Going back to an idea of His (1888), it is generally believed that all primary motor neurons are located in the basal plate (Fig.1) (for review, see Kuhlenbeck, 1975; Nieuwenhuys et al., 1998). The bm neurons are a specialized group of visceromotor neurons, which innervate the striated muscles associated with the branchial arches. Branchiomotor neuron are born on either side of floor plate from 48 hours of incubation (stage 12 according to Hamburger and Hamilton, 1951) until E3 (Heaton and Moody, 1980) or E5 in the chicken (Arens and Straznicky, 1986; Covell, 1989). Branchiomotor neurons migrate along stereotyped pathways, first following a leading axon that exists the hindbrain laterally (Ramon y Cajal, 1911; Heaton and Moody, 1980; Covell and Noden, 1989; Fritzsch, 1998). For the trigeminal

bm neurons, migration was shown to depend on the presence of sensory fibers entering the hindbrain from the trigeminal ganglion (Moody and Heaton, 1983b). Upon reaching a more lateral terrority, most of the bm neurons migrate ventrally to their final position in the lateral bm nuclei (Heaton and Moody, 1980; Arens and Straznicky, 1986; Covell and Noden, 1989; Lumsden and Keynes, 1989; Guthrie et al., 1991), which are generally thought to be in the lateral basal plate (reviewed in Nieuwenhuys, 1998; Kuhlenbeck, 1975; see, however, Varela-Echavarria et al., 1996). A few laterally migrating bm neurons end up dorsally where they form the dorsal bm nuclei (Heaton and Moody, 1980; Covell and Noden, 1989).

In the present study, I investigated the relationship between the migration of branchiomotor neurons and the position of the basal/alar plate boundary. For this purpose, cadherin-7 was used as a molecular marker to define the basal/alar plate boundary (see Section 3.2).

1.4 Cadherins

1.4.1 Structure of cadherins

Cadherins represent a large family of cell adhesion molecules that are involved in the regional and functional specification of the vertebrate CNS (for review, see Redies, 1995, 2000; Hirano et al., 2003). Cadherins are Ca^{2+}-dependent cell surface glycoproteins that regulate cell binding in many morphogenetic processes (for review, see Suzuki, 1996; Humphries and

Newham, 1998; Nollet, 2000). Recent work has shown that more than about 80 members of the cadherin family are expressed in the vertebrate CNS. The cadherin family consists of several subfamilies, e.g. classic type I and type II cadherins types, protocadherins, desmosomal cadherins and variety of cadherin-related molecules. Most subfamilies of cadherins have specific cytoplasmic domains, which distinguish them from each other and possibly participate in distinct signaling pathways and protein interactions (Fig. 4) (Hirano et al., 2003).

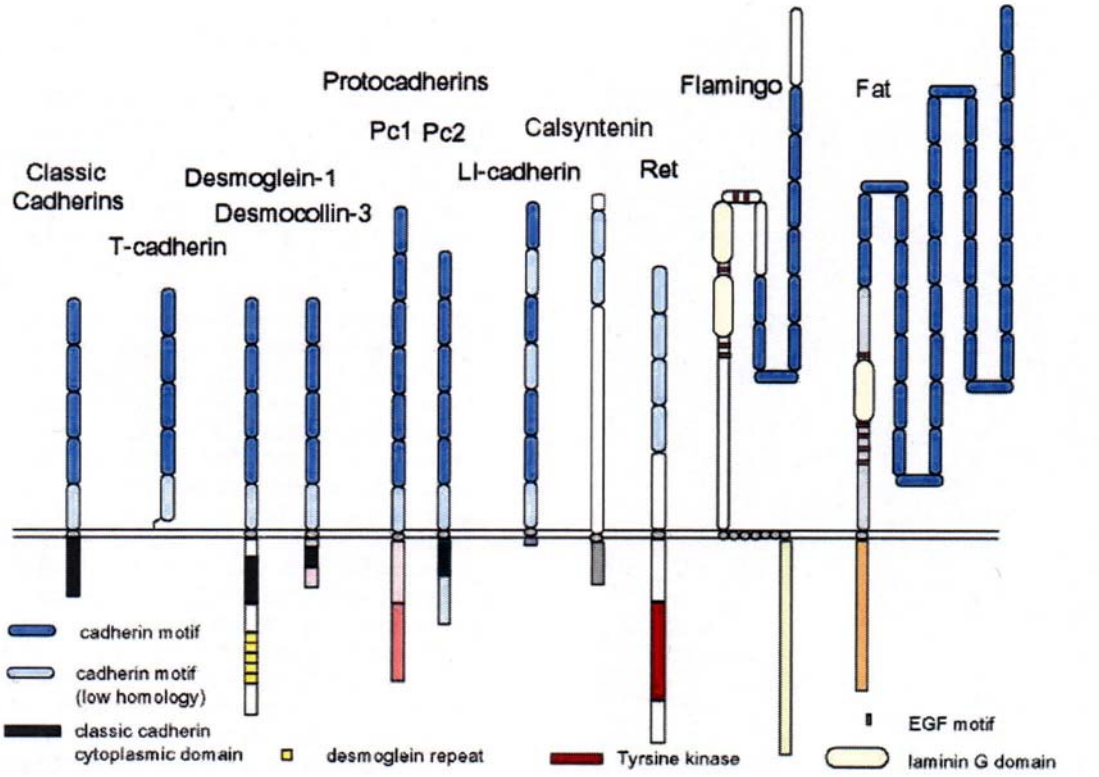

Figure 4. Overview of the cadherin superfamily in the vertebrates. Note that each cadherin subfamily has a characteristic cytoplasmic domain, which distinguishes it from other cadherin subfamily. The different cytoplasmic domains are possibly involved in distinct signaling pathways. Reproduced from the study by Hirano et al. (2003).

Cadherins are composed of three domains, a calcium-binding extracellular domain, a cytoplasmic domain and a transmembrane domain

(for review, see Suzuki, 1996; Humphries and Newham, 1998; Redies, 2000). The extracellular domain contains about 110 amino acid-long repeats, called "cadherin repeats". The amino acid sequences at the N terminus determine the adhesion specificity of each cadherin type. Two cadherin monomers can dimerize in *cis* configuration to form dimers, which mediate the interaction of adjacent cells expressing the same cadherin type.

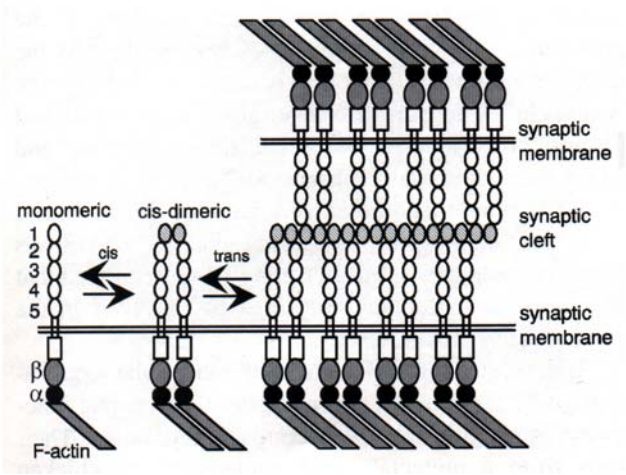

Figure 5. Schematic model for the structural basis of cadherin-cadherin interaction at the synaptic junction. The extracellular cadherin domains form *cis*-dimers. The intracellular cadherin domains bind to β-catenin, thus forming a cadherin-catenin complex, and to α-catenin, which interacts with the cytoskeletal protein F-actin. *Cis*-dimers are built from monomeric cadherin molecules and interact with dimers located in the opposing membrane in *trans* configuration. Abbreviations; α, α-catenin; β, β-catenin. Reproduced from the review by Obst-Pernberg and Redies (1999).

On the other hand, the intracellular cadherin domain binds to cytoplasmic components, for example, to β-catenin, which is involved in intracellular signal transduction, or to α-catenin, which interacts with the F-actin-based cytoskeletal network. These and other catenins are the major cadherin-associated molecules (for review, see Obst-Pernberg and Redies, 1999; Redies, 2000; Hirano, 2003). *Cis*-dimers can interact with dimers located in the

opposing membrane in *trans* configuration to mediate the binding between cells and their processes (Fig. 5) (for review, see Obst-Pernberg and Redies, 1999; Zigmond et al., 1999; Redies, 2000; Hirano et al., 2003).

1.4.2 Roles of cadherins in development

The general role of cadherins in development is to control cell contacts in tissue undergoing cell delamination, boundary formation, cell arrangements, and motility over cell surfaces. This role is closely linked to the formation of adherens junctions and to the interaction of cadherins with the force-generating, actin-based cytoskeletal network (for review, see Gumbiner, 1996). During CNS development, cadherins are involved in neural tube regionalization, in the migration and aggregation of neurons, in the outgrowth and fasciculation of neurites, in axonal target recognition and in synaptogenesis. Generally, cadherins are markers of functional circuits in the CNS (for review, see Redies, 1997).

Cadherins are calcium-dependent molecules, i. e., there is no aggregation of cadherin-expressing cells in the absence of Ca^{2+} from the culture medium (Fig. 6C). Moreover, cadherins bind in a type-specific fashion. Cadherin-mediated cell binding can be monitored in an aggregation assay. No aggregation takes place with non-adherent cells (Fig. 6A). In contrast, the same cells form aggregates after a few hours in the cell adhesion assay if they are induced to express a cadherin by transfecting with cadherin cDNA (Fig. 6B).

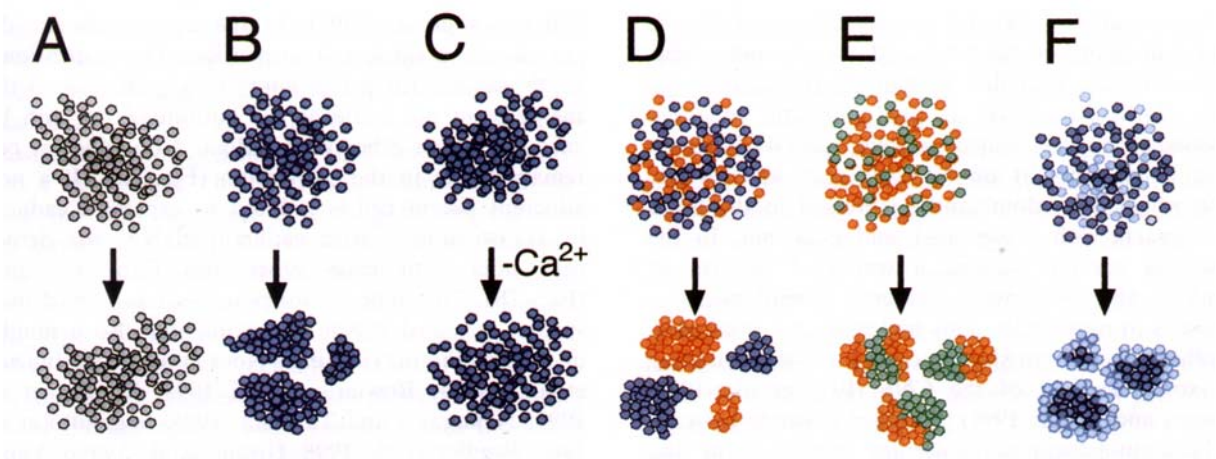

Figure 6. Binding between cadherins in a cell adhesion assay. Non-adherent cells remain in single cell suspension (A) whereas the same type of cells adhere to each other and form aggregates when they are induced to express a cadherin by cDNA transfection with (B). Cadherins are calcium-dependent molecules. There is no aggregation of cells in the absence of Ca^{2+} (C). Homotypic binding (D). Heterotypic binding (E). Homotypic binding is stronger than heterotypic binding with quantitative differences in the level of cadherin expression (F), the cadherins with the higher expression levels aggregate into the inner part surrounded by a shell of cells showing weaker expression. Reproduced from the study by Redies (2000).

Additionally, the binding between cadherins is specific. Cells, which express the same type of cadherin, form cell clusters while cells, which express different cadherin subtypes, segregate from each other (i. e., cadherins mediate preferentially "homotypic" binding, Fig. 6D). Particular combinations of cadherins form mixed aggregates, suggesting that "heterotypic" binding can also take place (Fig. 6E), but the different cell types will later segregate from each other according to which cadherin they express. This result indicates that, with few exceptions, "homotypic" binding is stronger than "heterotypic" binding for cadherins.

Quantitative differences in cadherin expression also cause cells to segregate within common aggregates. The cells, which express a given

cadherin at a higher expression level, aggregate into the inner part of aggregates, while the cells, which express the cadherin type more weakly, gather in an outer covering of the aggregate (Fig. 6F) (Steinberg and Takeichi, 1994).

Each cadherin has its stereotypic expression pattern (for review, see Takeichi, 1988; Redies, 2000). A classic example is the differential expression of N-cadherin and E-cadherin during neurulation. N-cadherin is expressed in neural tissue but not in epidermal tissue, whereas E-cadherin shows the opposite pattern of expression (Miyatani et al., 1989). Therefore, cadherins are thought to provide the molecular basis for the differential adhesion of embryonic cells, as originally observed in cell aggregation assays. Differential cadherin expression can be observed during neurulation at the earliest stages of neural tube morphogenesis (for review, see Takeichi, 1988). At first, E-cadherin is expressed by the surface ectoderm at the midline of the embryo and this E-cadherin-positive ectoderm forms the neural fold. When the two lateral ridges of the neural fold fuse to form the neural tube, the prospective neural tissue switches expression from E-cadherin to N-cadherin. N-cadherin expression in the neural tissue continues, but only in the proliferative neuroepithelium (for review, see Redies, 2000). Cadherin-6B expression appears at the neural crest-generating area when the neural tube is formed from the ectoderm. The cadherin expression switches again. Cadherin-7 expression rises and persists when neural crest cells appear and migrate to reach their final positions. It has thus been proposed that the emigration of

neural crest cells from the neural tube may rely on a modulation of cell adhesion mediated by cadherin expression (Zigmond et al., 1999).

Neural tube formation is followed by the compartmentation of the neuroepithelium into transverse segments, which are called “neuromeres”. The hindbrain (“rhombencephalon”) is composed of seven neuromeres which are called “rhombomeres” (for review, see Redies and Puelles, 2001). In addition, longitudinal domains are formed, such as the alar and basal plates. In many cases, the borders of restricted cadherin expression domains coincide with the boundaries of the neuromeres or longitudinal subdivisions of the early embryonic CNS (Gänzler et al., 1995; Matsunami et al., 1995). The expression of other types of cadherins therefore can be linked to the early neuromeric organization. Neuroepithelial cells, radial glia and early neurons also express cadherins in a regionally restricted fashion. The radial glial cells and their processes from dense palisades of fibers, when the mantle layer has formed and is growing in size at later stages (Gänzler and Redies, 1995; Yoon et al., 2000). These palisades show changes of cadherin expression at the divisional borders. For example, the rostral boundary of cadherin-6 expression in rhombomere 6 coincides with the caudal boundary of rhombomere 5 (Matsunami et al., 1995).

A relation of cadherin expression to gene regulatory protein expression has been demonstrated. Mouse embryonic brain morphology is altered when the gene regulatory protein Wnt-1 is deficient. This gene regulatory protein tends to be expressed in the same regions as E-cadherin and E-cadherin expression is altered in the Wnt-1-deficient mice. These results suggest that

gene regulatory proteins or transcription factors regulate, either directly or indirectly, cadherin expression (Shimamura et al., 1994; for review, see Redies, 2000; Redies and Puelles, 2001).

Cadherins are involved in several developmental processes that regulate the formation of gray matter structures and functional systems in the developing CNS. The newly born neuroblasts in the ventricular zone migrate into the mantle zone where they accumulate. Cadherin-mediated adhesive specificity may be involved when early neurons that express the same cadherin subtype aggregate into gray matter structures like brain nuclei and cortical layers (Gänzler and Redies, 1995; Yoon et al., 2000). Besides, patchy gray matter architecture relates also to cadherin expression (Redies et al., 2002; Heyers et al., 2003).

As already mentioned above, cadherins play a role in the formation of functional systems in the CNS, including the regulation of processes such as the induction and guidance of neurite outgrowth, axon fasciculation, target recognition and synaptogenesis. Functional systems and neural circuits are formed by fiber tracts in gray matter structures. Gray matter structures and their connecting fiber tracts that belong to the same functional circuit or system often express the same cadherin subtype (Redies et al., 1993; Arndt and Redies, 1996). Cadherins may have two functions during the neural circuit formation. First, neurites are induced to grow along pathways that express the same cadherin (Treubert-Zimmermann et al., 2002) and may recognize their targets based on a recognition mechanisms mediated by cadherins (Inoue and Sanes, 1997). Second, the homophilic binding

specificity of cadherins may induce the specific binding of pre- and postsynaptic membranes during synaptogenesis (for review, see Colman, 1997). Synaptogenesis is the last step during the formation of functional connections between neurons. N-cadherin was identified as an integral component of the synaptic complex at the neuromuscular junction (Beesley et al., 1995). Moreover, N-cadherin expression was demonstrated to sharply border and partially surround the transmitter release zone associated with synaptic vesicles (Uchida et al., 1996; Fannon et al., 1996).

1.4.3 Cadherins and disease

Cadherins play a role not only during the development of CNS, but also in the patterning and organogenesis of other organs and in disease. A role of cadherins in organogenesis, tumor formation and progression, in particular in the invasiveness of tumor cells, has been established experimentally in various studies. One example for the role of cadherins in organogenesis is the pancreas. In N-cadherin-deficient mice, dorsal pancreas agenesis or lack of a dorsal pancreas was observed. During pancreatic ontogeny, N-cadherin acts as a survival factor in the dorsal pancreatic mesenchyme (Esni et al., 2001). Epithelialmesenchymal interactions are needed for the formation of many organs which include differentiation, proliferation, apoptosis, cell migration, cell sorting, cell shape changes and cell aggregation. To study the role of R-cadherin in kidney ontogeny, R-cadherin-deficient mice were generated. Morphological analysis of nephrogenesis in these mice suggests that

R-cadherin is involved in the differentiation of mesenchymal and epithelial components of the kidney. The deficient mice show dilated proximal tubules with an accumulation of large intracellular vacuoles and the proximal parts of the ureteric bud epithelium were also altered. Moreover, significantly fewer epithelial structures developed (Dahl et al., 2002).

Several studies describe a role for cadherins and catenins in carcinogenesis. They demonstrate that the cadherin/catenin complex plays a role in signal transduction and tumor progression. Dysregulation or mutation of the E-cadherin and the alpha-, beta-, gamma-catenin complex interrupts cell adhesion in carcinomas (Behrens, 1999) and deficiency of immunoreactivity at least in one of these four proteins has a significant association to the presence of metastases. If one of these proteins is down-regulated, the function of the others is altered in suppressing metastasis. The best studied example today is E-cadherin. Downregulation of E-cadherin plays a role in tumor cell invasion and metastasis in bladder carcinomas and prostatic carcinomas. Besides, reduction of E-cadherin expression is associated with a poor prognosis, i.e., a higher risk for patients with bladder carcinoma (Otto et al., 1993, 1994). Similarly, strong E-cadherin expression was observed in the normal prostate and benign prostatic hyperplasia, while a decrease in or loss of E-cadherin was demonstrated in prostatic carcinoma (Otto et al., 1993). Analyses of E-cadherin tranfectants showed that E-cadherin transfection suppressed invasive potential of prostata cancer and matrix metalloproteinase 2 activity, an important marker associated with invasive and metastatic potential (Luo et al., 1999). Hence, E-cadherin was

suggested to be used as a marker for differentiation and invasiveness of carcinoma cells.

In addition, some dermatological diseases, e.g. Pemphigus vulgaris and Dyskeratosis bullosa hereditaria, were shown to relate to an abnormal expression of desmosomal cadherins (Hossein et al., 1999).

2 MATERIALS AND METHODS

2.1 Chemicals and instruments

2.1.1 Chemicals

- ABC reagent kit — Vector Laboratories, Burlingame, CA, USA
- Benzyl alcohol — Merck, Darmstadt
- Benzyl benzoate — Merck, Darmstadt
- Calcium chloride — Merck, Darmstadt
- 3-3`Diaminobenzidin tetra hydrochloride — Sigma, Darmstadt
- Dimethyl sulfoxide (DMSO) — Merck, Darmstadt
- Entellan embedding medium — Merck, Darmstadt
- Ethanol — Roth, Karlsruhe
- Gelatin — Merck, Darmstadt
- Glucose — Merck, Darmstadt
- HEPES — Sigma, Deissenhofen
- Histomount — Shandon, Pittsburgh, PA, USA

• Horse serum	Vector Laboratories, Burlingame, CA, USA
• Hoechst 33258	Sigma, Deissenhofen
• Hydrogen peroxide 30%	Merck, Darmstadt
• Isopropanol	Merck, Darmstadt
• Methanol	Merck, Darmstadt
• Magnesium chloride	Sigma, Deissenhofen
• Na_2HPO_4	Merck, Darmstadt
• NaH_2PO_4	Merck, Darmstadt
• NaOH	Merck, Darmstadt
• Nickel chloride	Merck, Darmstadt
• Paraformaldehyde	Merck, Darmstadt
• Phenol red	Merck, Darmstadt
• Potassium chloride	Merck, Darmstadt
• Potassium chromium(III) sulfate	Merck, Darmstad
• Sodium acide	Merck, Darmstadt
• Sodium chloride	Merck, Darmstadt
• Thionine acetate	Sigma, Deissenhofen
• Tissue-Tec O.C.T. medium	Miles, Elkhart, IN, USA
• Triton X-100	Merck, Darmstadt
• Tween-20	Roth, Karlsruhe
• Vectastatin ABC-Elitekit	Vector Laboratories, Burlingame,CA, USA

- Vectashield medium — Vector Laboratories, Burlingame, CA, USA
- Xylenes — Merck, Darmstadt; Roth, Karlsruhe

2.1.2 Instruments

- Binocular stereomicroscope — Stemi SV 6, Zeiss, Oberkochen
- Centrifuge — Biofuge Fresco, Heraeus, Hanau
- Confocal laser scanning microscope — Zeiss, LSM 510, Oberkochen
- Egg incubator — Ehret, Emmendigen
- Filter for sterilization — Schleicher & Schuell, Dassel
- Fluorescence microscope — Axiophot, Zeiss, Oberkochen
- McIlwain tissue chopper — Gomshall, Surrey, England
- Refrigerated microtome — Leica, Nussloch
- Seesaw — GFL 3010, Burgwedel
- Thermal unit — Strech A, Alessandrini, I-S, Prospero, Italy
- Ultraphot microscope — Zeiss, Oberkochen

2.1.3. Computer software and hardware

• Adobe Photoshop 5.5	Adobe, Mountainview, CA, USA
• Freehand 8.0	Macromedia, San Francisco, CA, USA
• Microsoft Word	Microsoft, Redmond, WA, USA
• Power Macintosh 8000/120	Apple, Silicon Valley, CA, USA
• Scanner Sharp JX	Sharp, Japan

2.2 Solutions for immunohistochemistry

2.2.1 HEPES-buffered stock solution (HBSS stock solution (10X))

- 50 mM glucose
- 0.1 M HEPES
- 1.4 M NaCl
- 4 mM $Na_2HPO_4 \times 2H_2O$
- 50 mM KCl
- 0.4 mM phenol red
- pH 7.4

2.2.2 HEPES-buffered solution with Ca^{2+} and Mg^{2+} (HBSS solution (1X))

- 100 mL HBSS stock solution (10X)
- 10 ml 1 M $CaCl_2$ (stock solution)
- 10 ml 1 M $MgCl_2$ (stock solution)
- add distilled water to 1l

2.2.3 TBS stock solution (10X)

- 1.5 M NaCl
- 0.5 M Tris
- 35 ml HCl (concentrated solution)
- add distilled water to 1l
- pH 7.4

2.2.4 TBS solution (1X)

- 1 mM $CaCl_2$
- 1l TBS stock solution (10X)
- add distilled water to 10l

2.2.5 Sucrose solution

- 12 % sucrose solution: 12 g sucrose in 100 ml HBSS solution (1X)

- 15 % sucrose solution: 15 g sucrose in 100 ml HBSS solution (1X)
- 18 % sucrose solution: 18 g sucrose in 100 ml HBSS solution (1X)

2.2.6 PBS stock solution (10X)

- 1.3 M NaCl
- 70 mM Na_2HPO_4
- 30 mM NaH_2PO_4
- pH 7.4

2.2.7 PBT solution (1000 ml)

- 10 ml 100 mM $CaCl_2$ (stock solution)
- 10 ml 100 mM $MgCl_2$ (stock solution)
- 100 ml PBS stock solution (10X)
- 10 ml dimethyl sulfoxide
- 5 ml 10 % Tween 20 stock solution
- add distilled water to 1l

2.2.8 4% PFA in HEPES-buffered solution

- 10 ml 100 mM $CaCl_2$ (stock solution)
- 880 ml distilled water (60°C)
- 100 ml HBSS stock solution (10X)

- 10 ml 100 mM $MgCl_2$ (stock solution)
- 0.5 ml 1N NaOH solution
- 40 g paraformaldehyde
- pH 7.4

2.2.9 ABC reagent

- 100 µl solution A (avidin)
- 100 µl solution B (biotin with horseradish peroxidase)
- 4.8 ml blocking solution (see 2.2.10)

2.2.10 Blocking solution for immunohistochemistry

- 150 µl horse serum
- 9.85 ml TBS solution

2.2.11 Solution for diaminobenzidine reaction

- 75 µl DAB 40%
- 1 µl 30% H_2O_2 solution in H_2O
- 50 µl $NiCl_2$ 0.04 %
- add 10 ml TBS solution

2.2.12 Thionine solution for Nissl staining

- 4.8 ml acetic acide
- 100 ml 99 % ethanol
- 2.7 g sodium acetate
- 1 g thionine
- add distilled water to 1l

2.2.13 Hoechst solution

- 50 µg Hoechst 33258
- 100 ml TBS

2.2.14 Blocking solution for double immunofluorescence labeling

- 2.5 g dried skim milk
- 200 µl sodium acide (10% solution)
- add TBS solution (1X) to 1l
- 1.6 ml Triton X-100 (10 % solution)

2.3 Animals.

Fertilized eggs from White Leghorn chicken (Gallus domestics) were obtained from a local breeder (Soerries-Trockels, Moehnesee-Hewingsen, Germany)

and incubated in a forced-draft incubator at 37°C and 65% humidity until the desired stage (according to Hamburger and Hamilton, 1951). Embryos were transferred into ice-cold HEPES-buffered salt solution supplemented with 1 mM $CaCl_2$ (HBSS, pH 7.6; see 2.2.2). Late stage embryos were killed by decapitation according to institutional and national guidelines for the use of animals in research. Specimens were fixed in 4% formaldehyde dissolved in HBSS for 2 hours (see 2.2.8). For cryoprotection, heads were immersed in an ascending series of HBSS-buffered sucrose solutions [12% (w/v), 15%, 18%] (see 2.2.5) and embedded in Tissue Tec O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands). Specimens were frozen in liquid nitrogen and stored at –80 °C. Sections of 18-20 µm thickness were cut on a refrigerated microtome and directly thawed onto gelatine-coated slide glasses for immunohistochemistry and onto silane-coated slide glasses for *in situ* hybridization. Embryos at the following stages according to Hamburger and Hamilton (1951) were used: stage 15 (ca. 50-55 hours of incubation); stage 19 (ca. 68-72 hours of incubation); stage 20 (ca. 70-72 hours of incubation); stage 21 (3.5 days of incubation; E3.5); stage 22 (3.5 days of incubation; E3.5); stage 23 (E3.5-E4); stage 24 (E4); stage 26 (E4.5-E5); stage 27 (E5), stage 28 (E5.5) and stage 30 (E6.5)

2.4 Antibodies

The following antibodies were used for immunostaining: Mouse monoclonal antibodies CC6B-1 (1:3000 dilution) and CC7-1 (1:1000 dilution) raised against

chicken cad6B and cad7, respectively (Nakagawa and Takeichi, 1998; kind gift of Drs. S. Nakagawa and M. Takeichi, Kyoto University, Kyoto, Japan); mouse monoclonal antibody PAX6 (1:30 dilution) raised against chicken Pax6 (Ericson et al., 1997b); mouse monoclonal antibody PAX7 (1:10 dilution) raised against chicken Pax7 (Ericson et al., 1996); mouse monoclonal antibody 5E1 (1:1000 dilution) raised against chicken Sonic Hedgehog (Ericson et al., 1996); mouse monoclonal antibody 67.4E12 (1:300 dilution) raised against Lim3 (Ericson et al., 1997b) which also recognizes Lhx4 (Johan Ericson, personal communication); mouse monoclonal antibody 74.5A5 (1:10 dilution) raised against chicken Nkx2.2 (Ericson et al., 1997b); and mouse monoclonal antibody 39.4D5 (1:300 dilution) raised against Islet-1 (Ericson et al., 1992). Antibodies PAX6, PAX7, 5E1, 67.4E12, 74.5A5 and 39.4D5 were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD. As secondary antibodies polyclonal biotinylated horse antibody against mouse IgG (Vector Laboratories, Burlingame, CA, USA) for cadherin-7 (1:1000 dilution), Pax6 (1:1000 dilution), Pax7 (1:1000 dilution), Nkx2.2 (1:1000 dilution), Shh (1:1000 dilution), Lim3/Lhx4 (1:1000 dilution), and Islet-1 (1:1000 dilution) and secondary antibody polyclonal biotinylated goat antibody against rat IgG (Dianova, Hamburg, Germany) for cadherin-6B (1:200 dilution). For double immunofluorescence labeling against cadherin-6B and Islet-1, the following antibodies were used: Rabbit polyclonal antiserum raised against chicken cadherin-6B (1:3000 dilution) and mouse monoclonal antibody against Islet-1 (1:300 dilution). As

secondary antibodies, Cy3-conjugated polyclonal goat antibody against mouse IgG (Dianova, Hamburg, Germany) for cadherin-6B (1:300 dilution) and FITC-conjugated polyclonal goat antibody against Islet-1 (1:100 dilution) were used.

2.5 Immunohistochemistry

Procedures for immunohistochemistry have been published previously (Redies et al., 1993; Gänzler and Redies, 1995). Briefly, dried sections were rehydrated in HBSS (see 2.2.2). Sections were then postfixed in 4% formaldehyde in HBSS (see 2.2.8) for 30 min and washed in Tris-buffered saline (TBS; see 2.2.4) supplemented with 1 mM $CaCl_2$ (pH 7.5; TBS). Endogenous peroxidase activity was suppressed by immersing the sections in 0.3% H_2O_2 in methanol for 30 min at –20°C. To block unspecific binding and to dilute antibodies, 1.5% horse serum in TBS was used (see 2.2.10). Primary antibodies were applied for 1.5 hours at room temperature or overnight at 4 °C. After sequential incubation with appropriate biotinylated secondary antibodies (see 2.4) and avidin-coupled peroxidase complex, which was prepared in TBS containing 1.5% horse serum, 2% solution A and 2% solution B (see 2.2.9) (ABC Elite kit; Vector Laboratories, Burlingame, CA), sections were treated with a substrate solution containing 0.7% 3-3‘ diaminobenzidine tetrachloride, 0.5% nickel chloride, and 0.1% peroxide in TBS (see 2.2.11). After enough reaction product had formed, sections were washed, dehydrated in an ascending series of ethanol (70%, 96%, 100%) for 10 minutes each, in isopropanol for 10 minutes, and in xylenes

for 20 minutes. The slices were embedded in Histomount (Shandon, Frankfurt, Germany).

2.6 Double-labeling immunostaining

Double-labeling immunostaining procedures were described previously (Redies et al., 1992, 1993). Slides were stained using rabbit polyclonal antiserum against cad6B and mouse monoclonal antibody against Isl1. Before the slides were incubated with primary antibody, a solution containing TBS, 5% dried skim milk, 0.3% Triton and 0.04% sodium acide (see 2.2.14) was used to block nonspecific binding and to dilute antibodies. Primary antibodies were applied for 1 hour at room temperature, and then the slices were washed for 20 minutes in TBS and TBS containing Triton X-100 (see 2.2.4). Appropriate Cy3-conjugated (Dianova, Hamburg, Germany) and FITC-conjugated secondary antibodies were applied for 2 hours at room temperature to doubly label antigens. After sequential incubation with appropriate secondary antibodies, sections were counterstained with nuclear dye Hoechst 33258 (Sigma, Deisenhofen, Germany, see 2.2.13). The sections were washed and mounted in Vectashield medium (Vector Laboratories). Fluorescence was visualized with a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Germany).

2.7 Nissl staining

For neuroanatomic orientation, sections adjacent to those used for immunohistochemistry were stained with thionine acetate for Nissl substance, as described previously (Redies et al., 1993). In brief, dried sections were washed and dehydrated in ethanol 70% for 10 minutes. After washing in distilled water, the sections were incubated in thionine solution (see 2.2.12) for 5 minutes. They were then washed for 2 minutes each in an ascending ethanol series (70% and 96% ethanol in distilled water and 100% ethanol). The sections were incubated in isopropanol and washed twice in xylenes. The slices were embedded in Histomount and photographed (Axiophot and Ultraphot, Zeiss, Oberkochen, Germany).

2.8 Whole mount immunostaining

Whole mount immunostaining specimens were prepared previously in the Redies laboratory by a laboratory technician. Whole mount immunostaining was performed as described (Gänzler and Redies, 1995). Briefly, embryonic brain stems from stages 9 to 27 (Hamburger and Hamilton, 1951) were prepared in HEPES-buffered salt solution (HBSS, see 2.2.2). They were dissected and fixed in 4% formaldehyde in HEPES-buffered salt solution on ice (HBSS, see 2.2.8). Wholemounts were incubated with 0.3% H_2O_2 in methanol for 20 minutes at room temperature and for 1 hour at –20°C to inactivate endogenous peroxidases. The specimens were rehydrated in a graded series

of methanol in phosphate-buffered saline (PBS; 75%[v/v], 50%, 25%, see 2.2.6) supplemented with 0.05% Tween-20, 1mM $CaCl_2$, 1mM $MgCl_2$, and 1% dimethyl-sulfoxide (PBT, see 2.2.7) for 15 minutes each at room temperature. Thereafter, an incubation in a solution of 5% horse serum in PBT was carried out for 4 hours to reduce nonspecific binding of antibodies. After blocking endogenous peroxidases, specimens were incubated at 4°C overnight with monoclonal antibody CC7-1 against chicken cad7. Following incubation at 4°C overnight with appropriate biotinylated secondary antibody and ABC reagent, specimens were washed in PBT with 1.5% horse serum for 5 hours and incubated for 4 hours with ABC reagent (see 2.2.9). The specimens were washed overnight at 4°C one more time. On the fourth day, the specimens were washed again and were reacted with substrate solution (0.025% diamino-benzidine and 0.04% $NiCl_2$ in PBS) until enough reaction product had formed. The specimens were dehydrated in an ascending series of methanol solutions and then stored in 100% methanol at –20°C. They were viewed and photographed under a stereomicroscope (Stemi SV, Zeiss).

2.9 In situ hybridization

One series of sections from stage 22 brain was hybridized *in situ* with probe for the transcription factor Otp by a laboratory technician (Ulrike Laub) in the Redies laboratory at the University of Duisburg-Essen. To detect Otp mRNA, a plasmid containing a 180 bp insert of chicken Otp cDNA was used (kind gift of Dr. A. Simeone, Instituto Internazionale di Genetica and Biofisica, Naples,

Italy). Digoxigenin-labeled and fluorescein-labeled, sense and anti-sense RNAs were prepared by using commercially available kits (Stratagene, La Jolla, CA). The *in situ* hybridization procedure for sections was described previously (Redies et al., 1993). Briefly, the digoxigenin-labeled probe for Otp, which was hybridized *in situ*, was detected with anti-digoxigenin alkaline-phosphatase-conjugated Fab fragments (Boehringer, Mannheim, Germany). The sections were reacted with a solution containing nitroblue tetrazolium salt (75 mg/ml; NBT) and 5-bromo-4-chloro-3-indolyl phosphate-ptoluidin salt (50 mg/ml; BCIP) in alkaline buffer until enough colored precipitate had formed. Sections were embedded in Entellan (Merck). Sections adjacent to those used for *in situ* hybridization were immunostained for cad7 and Pax7, and stained for Nissl substance, as described above.

2.10 Flatmount double RNA in situ hybridization

Flatmount double RNA *in situ* hybridization for Otp and Phox2b was prepared in the Puelles laboratory at the University of Murcia, Spain, by Pilar Aroca. Procedures were performed basically following the protocols of Shimamura et al. (1994) and Jowett and Lettice (1994), adding minor modifications. Chicken embryos of stages 20, 24 and 28 were hybridized with 2 µg/ml of each probe for 12 hours. Embryos were then washed in the hybridization buffer to remove the remaining probe. Fluorescein-labeled probe was detected with alkaline-phosphatase anti-fluorescein antibodies, followed by a reaction with NBT/BCIP (blue precipitate). To decrease the background in the tissue, NBT

was used at a lower concentration (33 µg/ml). Fluorescein-labeled probe was detected with alkaline-phosphatase-conjugated anti-fluorescein antibody, followed by a reaction with a solution containing 248 µg/ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride and 33 mg/ml BCIP (redishbrown precipitate).

2.11 Photomicrograph production

All stains shown in the figures were digitized and adjusted in contrast and brightness with the Photoshop software (Adobe Systems, Mountain, CA). Labeling of the figures was done with the Freehand software (Macromedia, San Francisco, CA) and the Photoshop software (Adobe Systems, Mountain, CA).

3 RESULTS

3.1 Cad7 expression in the spinal cord

In both the spinal cord and the hindbrain, cad7 is expressed in a well-demarcated domain at early stages of development, confirming previous preliminary data from the spinal cord (Fig. 2B)(Nakagawa and Takeichi, 1998; Redies, 2000). I studied the boundaries of this expression domain first in the spinal cord because numerous well-established molecular markers for the ventrodorsal domains are available in this part of the CNS (Ericson et al., 1997b; Briscoe and Ericson, 1999; Briscoe et al., 2000; Liem et al., 2000; Takahashi and Osumi, 2002).

At stage 19, the cad7 expression domain of the spinal cord marks a distinct radial domain of the neural tube wall (Nakagawa and Takeichi, 1998; Redies, 2000). I used transcription factors to define the boundaries of this domain. Results show that the dorsal boundary of the cad7 expression domain (arrow in Fig. 7A,I) coincides with the ventral boundary of Pax7 expression (arrow in Fig. 7B,I), which, in turn, defines the border between the ventral and dorsal spinal cord (basal/alar plate boundary; see Discussion)(Ericson et al., 1996; Briscoe and Ericson, 1999; Liem et al., 2000). I assume that the alar and basal plates are the major D/V domains of the neural tube, each with characteristic fate and function (i.e., sensory versus motor). In the alar and basal plates, dorsalizing and ventralizing patterning

effects predominate, respectively. The basal/alar boundary would thus

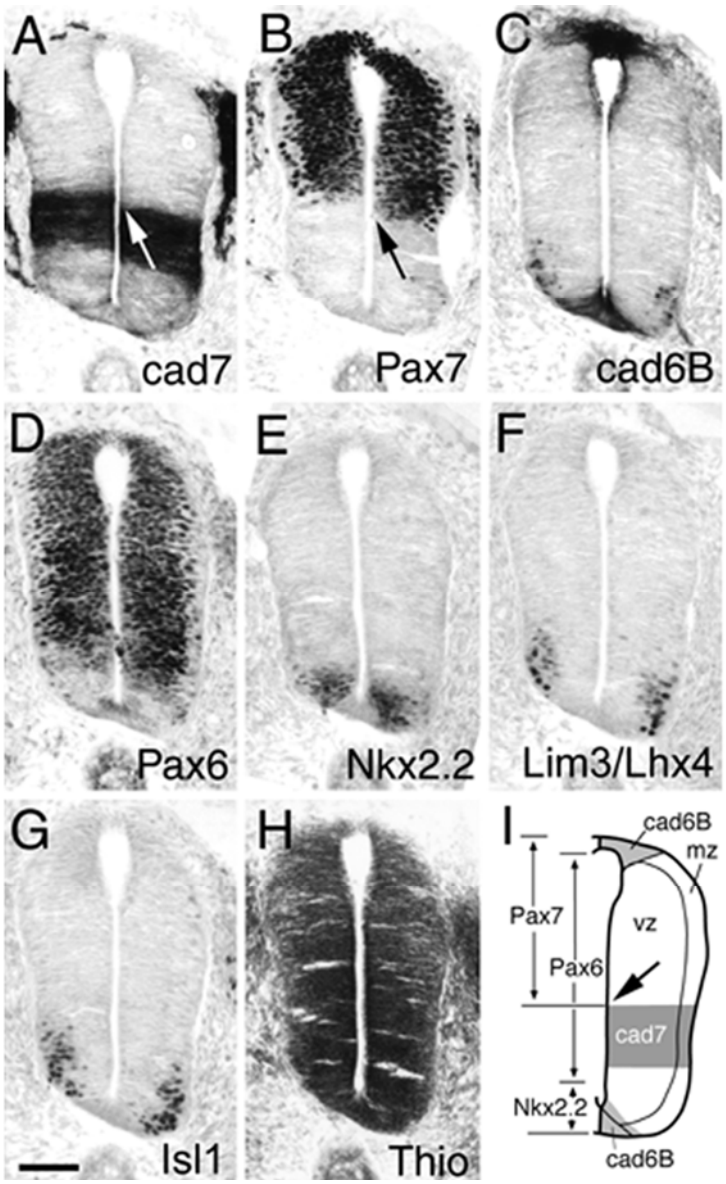

Figure 7. Expression of cadherin-7 (cad7, A) in the cervical spinal cord of a stage 19 (HH19) embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; Nkx2.2, E; Lim3/Lhx4, F; and Islet-1, Is11, G). Immunostains are shown for a series of consecutive sections. H, Nissl stain (thionine, Thio) of an adjacent section. The arrows in A, B and I point to the basal/alar plate boundary. I, Schematic diagram of the expression domains (see also Figure 20A). Abbreviations: mz, mantle zone; vz, ventricular zone. Scale bar (in G), 100 µm for A-H.

represent the molecularly-determined turning point of the alternative fates. This result indicates that the cad7 expression domain represents the most

dorsal part of the ventral spinal cord (i.e., the basal plate). The basal plate has been divided into five progenitor cell (p) domains termed p0, p1, p2, pMN and p3 (Fig. 3C).

These domains each give rise to a corresponding group of neurons termed V0, V1, V2, VMN and V3 (Ericson et al., 1997a; Briscoe et al., 2000). The ventral boundary of the cad7 expression domain is located dorsal to the border between Nkx2.2 expression and Pax6 expression (Fig. 7D,E,I). In the mantle zone, this border coincides with the dorsal expression border of the Lim3/Lhx4 domain (Fig. 7F). Lim3/Lhx4 is a marker for the VMN and V2 neurons (Sharma et al., 1998). The motor (VMN) neurons also express Isl1 (Fig. 7G) and cad6B (Fig. 7C) signals, which do not overlap with cad7 at this stage. Thus the cad7 signal corresponds to the dorsal part of the basal plate and includes the p0 and p1 domains that give rise to the V0 and V1 interneurons (Takahashi and Osumi, 2002).

At stage 23, the radial cad7 expression domain is still present and shares the same dorsal boundary with the Pax7 expression domain (arrows in Fig. 8A, B,H). In addition, the V2 and VMN neurons now express cad7, and a subset expresses cad6B (Fig. 8C) (Marthiens et al., 2002), while the Nkx2.2-positive p3 domain is cad7 negative. The lateral part of the floor plate, which can be defined by the expression of Shh (Fig. 8E), is cad7 immunoreactive also. Results are schematically represented in Figures 8H and 20A. The domain of cad7 expression persists in the ventricular zone at least until stage 28 (Fig. 9).

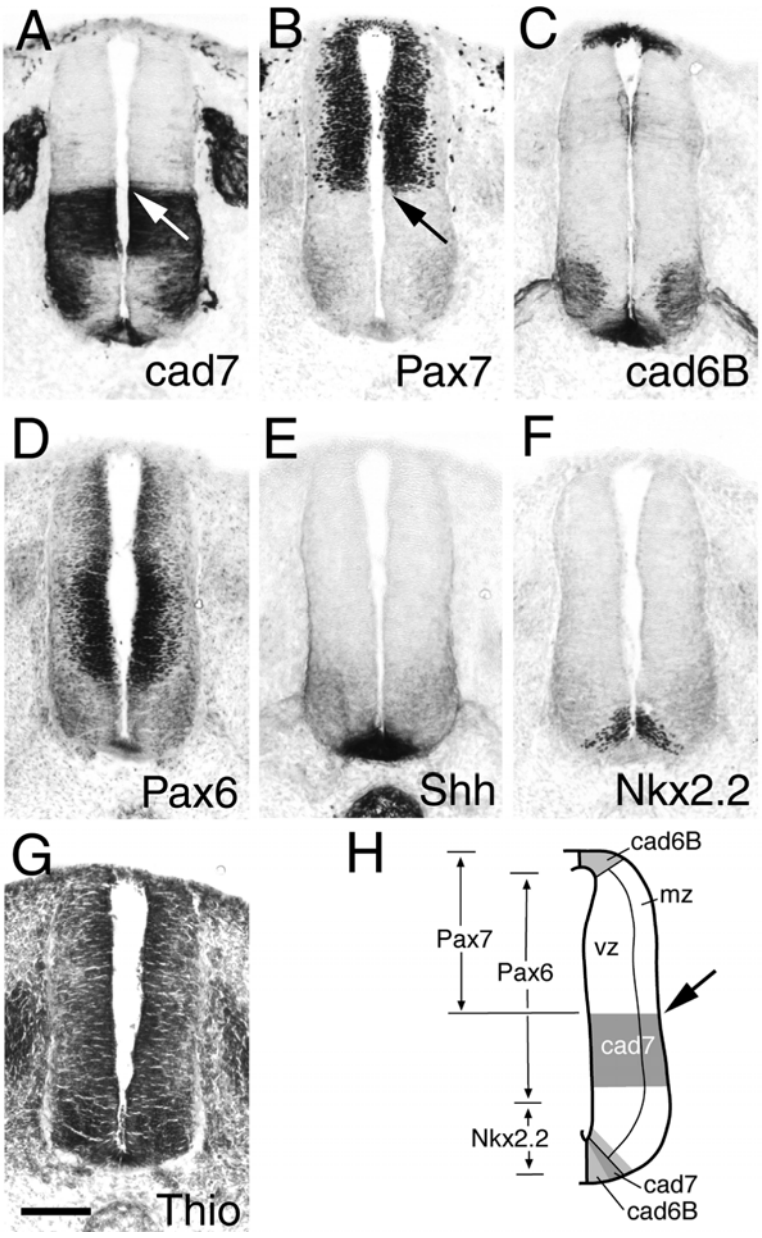

Figure 8. Expression of cadherin-7 (cad7, A) in the lumbal spinal cord of a stage 23 (HH23) embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; Sonic hedgehog, Shh, E; and Nkx2.2, F). Immunostains are shown for a series of consecutive sections. G, Nissl stain (thionine, Thio) of an adjacent section. H, Schematic diagram of the expression domains (see also Figure 20A). The arrows in A, B and H point to the basal/alar plate boundary. Abbreviations: mz, mantle zone; nc, notochord; vz, ventricular zone. Scale bar (in G), 100 µm for A-G.

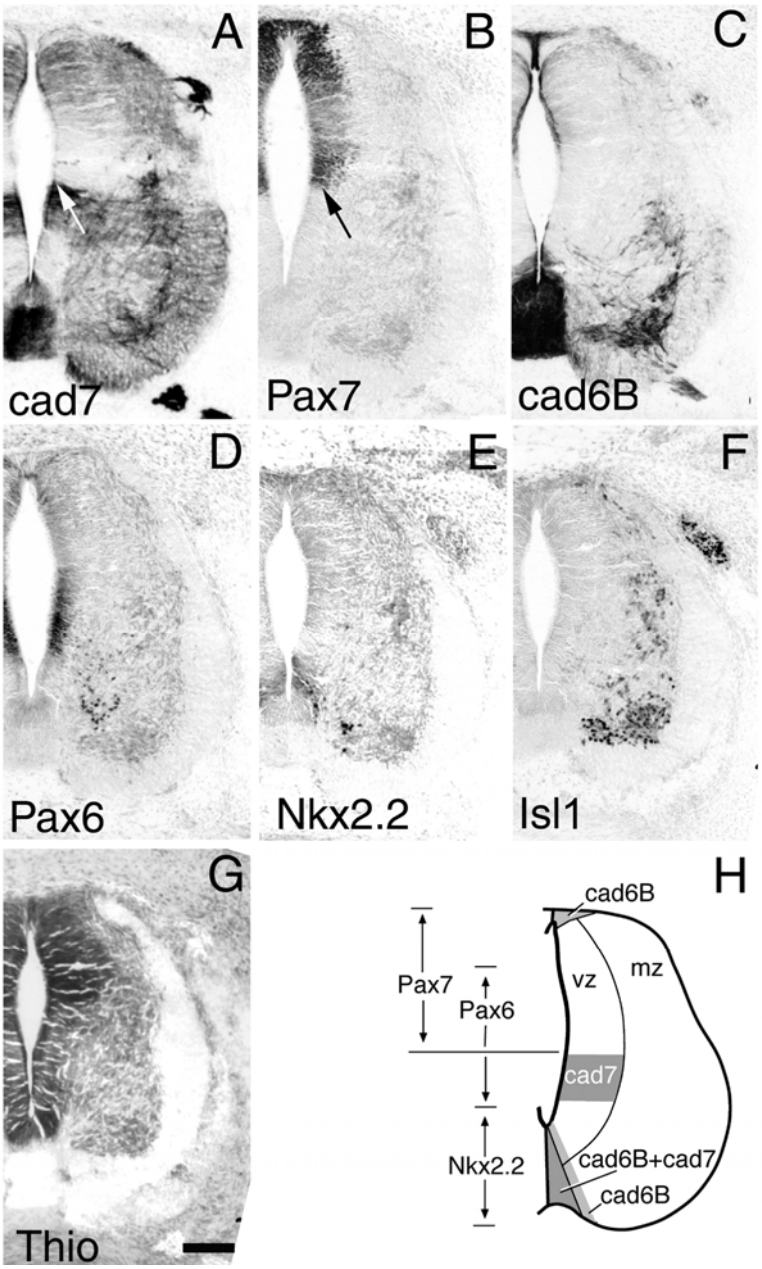

Figure 9. Expression of cadherin-7 (cad7, A) in the cervical spinal cord (a part of cervical mark) of a stage 28 (HH28) embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; Nkx2.2, E; and Islet-1, Isl-1,F). Immunostains are shown for a series of consecutive sections. G, Nissl stain (thionine, Thio) of an adjacent section. H, Schematic diagram of the expression domains (see also Figure 20A). The arrows in A and B point to the basal/alar plate boundary. Abbreviations: mz, mantle zone; vz, ventricular zone. Scale bar (in G), 100 µm for A-G.

At this stage the dorsal boundary of the cad7 expression domain (arrow in Fig. 9A) coincides also with the ventral boundary of Pax7 expression, like at the earlier stages (arrow in Fig. 9B). In addition, the development of mantle layer has advanced and motorneuron pools appear clearly at this stage (Fig. 9) in the ventral spinal cord. Using Isl1 as marker for motor neurons, I found that a subset of motorneurons expresses cad6B at the stage 26 (Fig. 10), confirming previsions results in the frog (Marthiens et al., 2002).

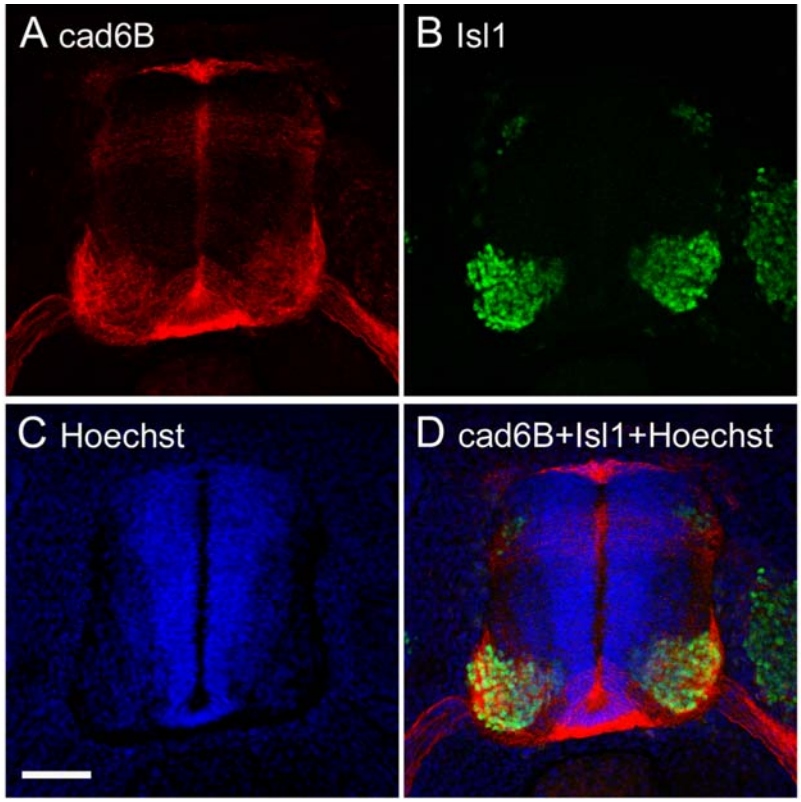

Figure 10. Subpopulations of motorneurons in the spinal cord co-express cadherin-6B (cad6B, red in A, D) and Islet-1(Isl1, green in B, D) at stage 26. Nuclei were stained with Hoechst 33258 (blue in C). An overlay of the double-label immunostaining for this frontal section is shown in D. Scale bar (in C), 100 µm for A-D.

3.2 Expression of cad7 in hindbrain

Like the spinal cord, the hindbrain contains a bilateral, complete radial domain that expresses cad7 (Figs. 11-16,18). This domain appears in the hindbrain at around stage 11-12 (around 45 hours of incubation) and it has become prominent at stage 15 (Fig. 11A). In the ventricular layer, the domain persists at least until stage 30 (E6.5-7; Fig. 15G).

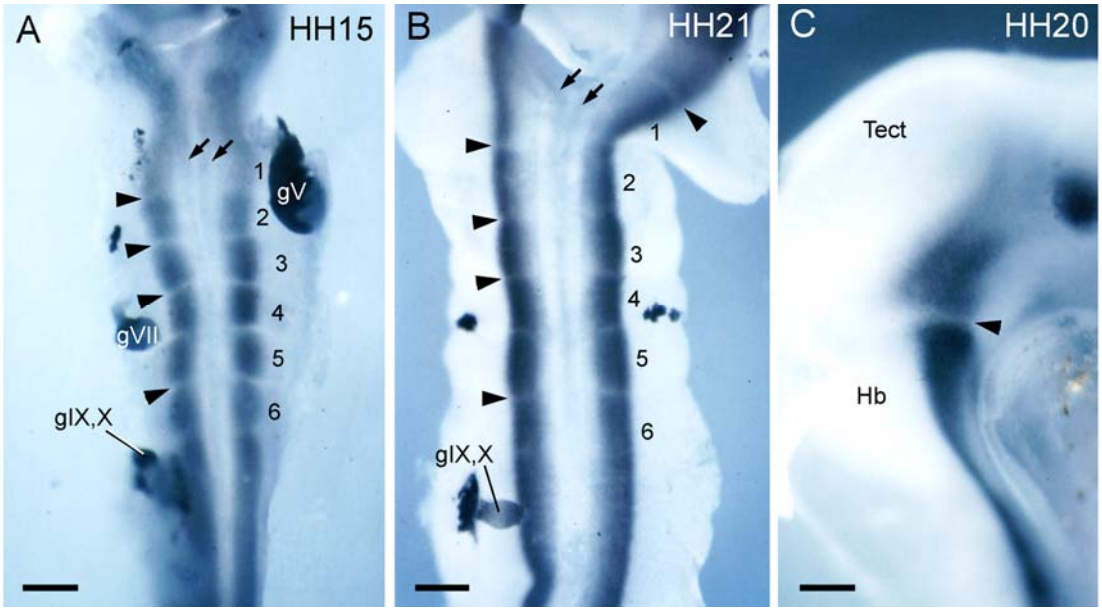

Figure 11. Whole mount immunostaining for cadherin-7 of the hindbrain at stage 15 (HH15; A) and stage 21 (HH21; B) and of the hindbrain/midbrain transition area at stage 20 (HH20; C). A, B show dorsal views and C shows a lateral view. The arrowheads point to cad7-negative boundary regions between individual rhombomeres and between midbrain and hindbrain. The arrows point to bilateral stripes of cadherin-7 expression in the lateral floor plate. Abbreviations: 1-6, rhombomeres 1-6; gV, trigeminal ganglion; gVII, facial ganglion; gIX/X, ganglion of the glossopharyngeal/vagus nerves; Hb, hindbrain; and Tect, tectum. Scale bars 200µm (in A-C).

Whole mount immunostaining of hindbrain (Fig. 11) demonstrates that the cad7-positive domain extends from the spinal cord to the rostral limit of the

hindbrain. It is interrupted at the rhombomere boundaries (arrowheads in Fig. 11A,B). The midbrain/hindbrain boundary is also cad7 negative (arrowhead in Fig. 11C). In addition, the floor plate of the hindbrain expresses cad7 weakly. Rostrally, the immunostaining becomes stronger in the lateral regions of the floor plate. From about rhombomere 3 (r3) to the midbrain/hindbrain boundary, the median epichordal strip (Puelles, 1978) in the caudal hindbrain and the paramedian floor domains are cad7 positiv (arrows in Fig. 11A,B).

To define the limits of the cad7-positive lateral stripe in the hindbrain, I used the same panel of genetic markers as in the spinal cord, but added Otp. Like in the spinal cord, I also examined expression of cadherin-7 of several stages in the hindbrain. The lateral (dorsal) border of the cad7 expression domain (Fig. 12A; 13A; 14A) coincides with the medial (ventral) border of Pax7 expression in the ventricular layer (Fig. 12B; 13B; 14B). Similarly, in the mantle layer, the lateral border of cadherin7 exactly coincides with the medial border of Pax7 of the stage 19, although the location of the common border is slightly more medial in the mantle layer than in the ventricular layer (Fig. 14A, B). In the mantle layer, the lateral border of the cad7 domain coincides with the lateral border of Otp expression (Fig. 18). The medial border of the cad7 domain coincides with the boundary between Pax6 and Nkx2.2 expression (Figs. 13D, E; 14D, E; 15D, E; 20B). At the medial border of the radial cad7 domain in the mantle layer, the exit points of the somatomotor nerves are found (abducens nerve and hypoglossal nerve, arrows in Fig. 16A and Fig. 16D, respectively). The paramedian stripes of cad7 expression in the floor plate coincide approximately with the domain of intense Shh expression

(Fig. 12F), while the cad6B-positive floor plate domain (Fig. 12C; 13C; 14C) extends further laterally in the ventricular zone. These results are summarized schematically in Figures 12H, 13I, 14F and 20B.

The mantle layer, in which postmitotic neurons differentiate has strongly increased in thickness at the stage 28 (Fig. 13). The postmitotic neurons accumulate more and more in the mantle layer and begin to aggregate in morphologically distinct groups of early neurons, e.g. in branchiomotor nuclei, which express cadherin-6B (Fig. 13C), Lim3/Lhx4 (Fig. 13F) and Isl1 (Fig. 13G).

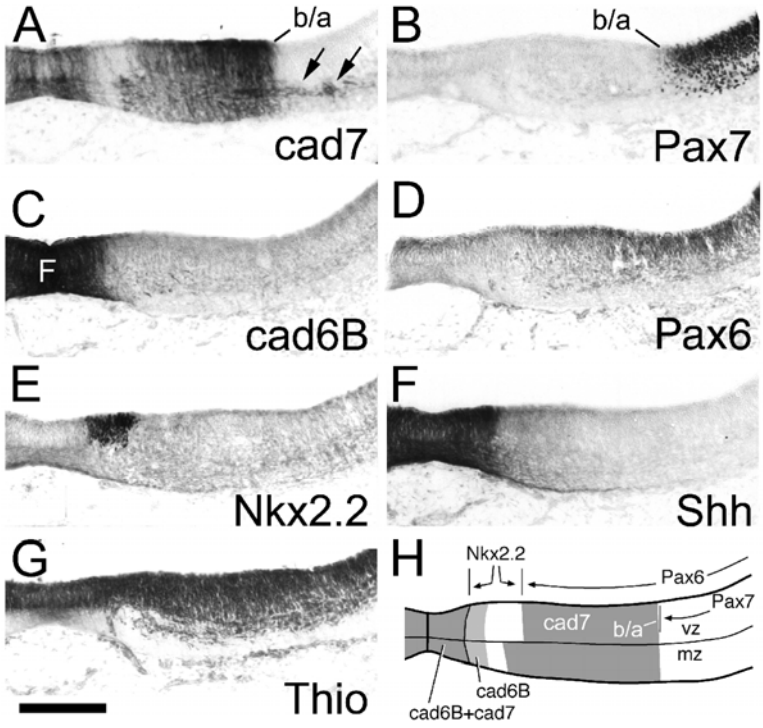

Figure 12. Expression of cadherin-7 (cad7, A) in the hindbrain of a stage 23 embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; Nkx2.2, E ; and Sonic hedgehog, Shh, F). Immunostains are shown for a series of consecutive sections. G, Nissl stain(thionine, Thio) of an adjacent section. H, Schematic diagram of the expression domains. The arrows in A point to migrating branchiomotor neurons of the trigeminal nerve. Abbreviations: b/a, basal/alar plate boundary; mz, mantle zone; vz, ventricular zone. Scale bar (in G), 100 µm for A-G.

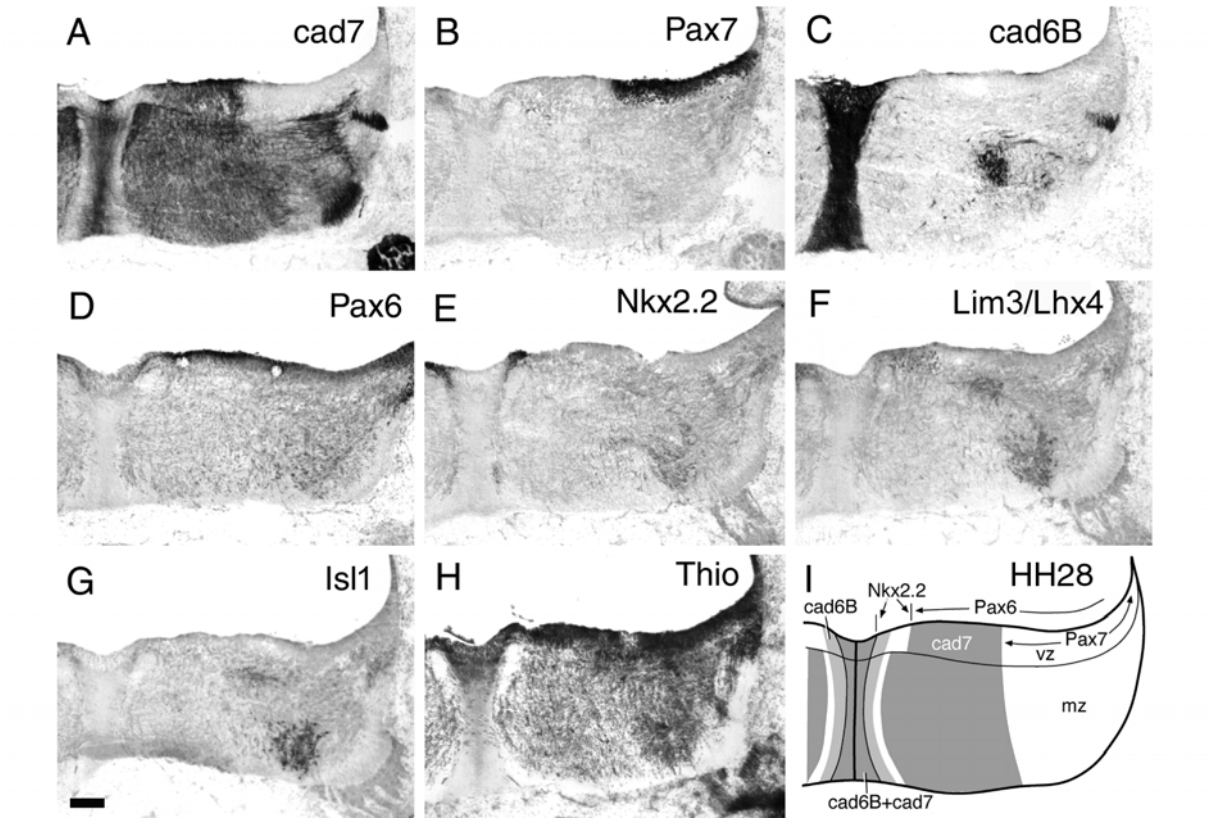

Figure 13. Expression of cadherin-7 (cad7, A) in the hindbrain of a stage 28 embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; Nkx2.2, E ; Lim3/Lhx4, F; and Islet-1, Isl1,G). Immunostains are shown for a series of consecutive sections. H, Nissl stain (thionine, Thio) of an adjacent section. I, Schematic diagram of the expression domains. Abbreviations: mz, mantle zone; vz, ventricular zone. Scale bar (in G), 100 µm for A-H.

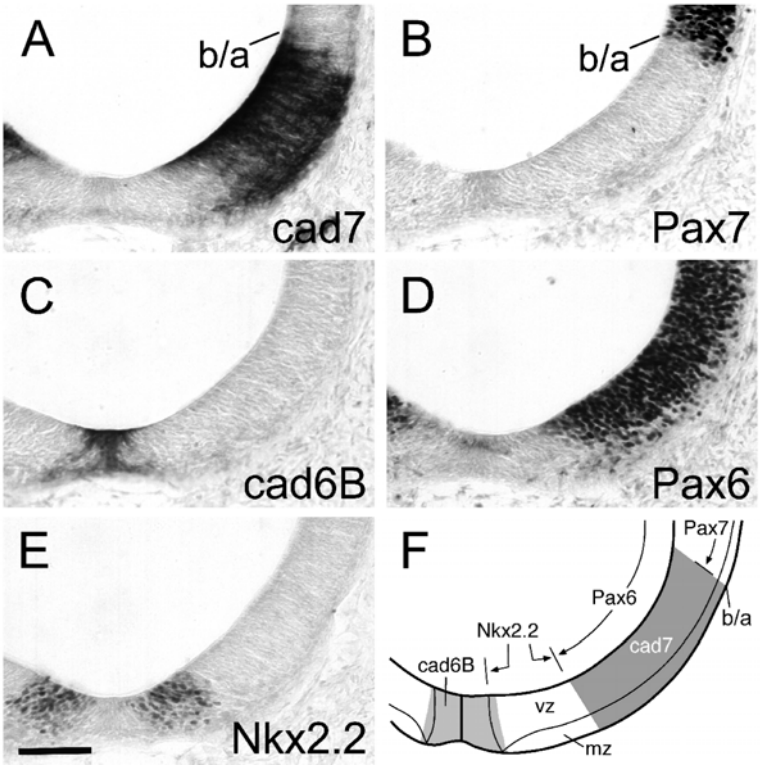

Figure 14. Expression of cadherin-7 (cad7, A) in the hindbrain of a stage 19 embryo, compared to the expression of other markers (Pax7, B; cadherin-6B, cad6B, C; Pax6, D; and Nkx2.2, E). F, Schematic diagram of the expression domains. Abbreviations: b/a, basal/alar plate boundary; mz, mantle zone; vz, ventricular zone. Scale bar (in E), 100 µm for A-E.

3.3 Cadherin expression by branchiomotor neurons

I next studied the migration of the branchiomotor neurons with respect to the cad7 domain of the hindbrain. At their place of birth laterally adjacent to the floor plate, the branchiomotor neurons of the trigeminal nerve do not express cad7 or cad6B (Fig. 15). However, as they extend axons and migrate laterally through the cad7 domain, both the cell bodies and their axons begin to express cad7 (Fig. 15A).

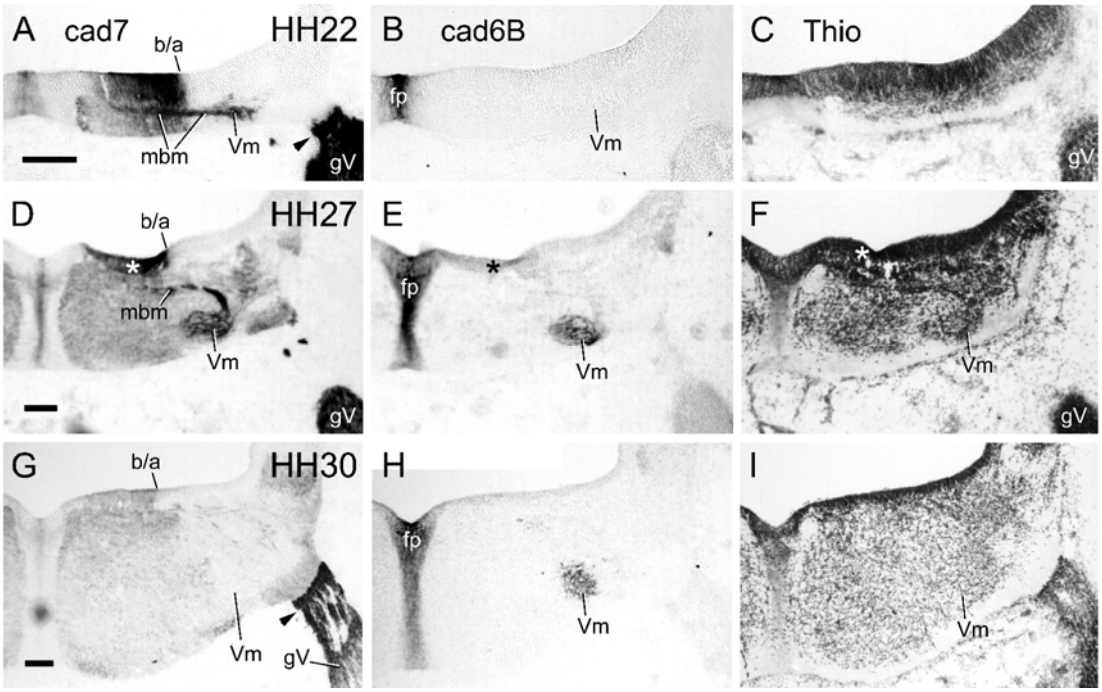

Figure 15. Cadherin expression during and after the migration of trigeminal branchiomotor neurons at different stages of development (stage 22, HH22, A-C; stage 27, HH27, D-F; and stage 30, HH30, G-I). Immunostains for cadherin-7 (cad7; A, D, G), cadherin-6B (cad6B; B, E, H) and a Nissl stain (thionine, Thio; C, F, I) are shown for series of consecutive sections. The arrowheads in A and G point at cad7-positive cells in the trigeminal nerve. The asterisks in D-F indicate an artefact (fold in the section). Abbreviations: b/a, basal/alar plate boundary; fp, floor plate; gV, trigeminal ganglion; mbm, migrating branchiomotor neurons; Vm, ventral motor nucleus of the trigeminal nerve. Scale bars 100 µm (in A for A-C, in D for D-F, and in G for G-I.

Similar observations were made for the migrating branchiomotor neurons (mbm) of the facial nerve (Fig. 16A) and of the glossopharyngeal/vagus nerves (Fig. 16D). This expression persists until after the branchiomotor neurons have reached their final laterodorsal location. Here, they begin to express cad6B, as they slowly downregulate cad7. Note that the migrating neurons and their axons (Figs. 15B,E; 16B,E) do not express cad6B. Thus, cad6B expression is upregulated by the postmigratory neurons. After migration of the trigeminal motor neurons has been completed at around stage 28 (E 5,5-6

days) (Heaton and Moody, 1980; Covell and Noden, 1989), cad7 expression is no longer observed in the trigeminal motor nucleus at stage 30, while cad6B expression persists until at least stage 37 (E11; C. Redies and L. Puelles, unpublished data). To confirm that the cad6B-positive neurons are motor neurons, double immunostaining for cad6B and Isl1 or Lim3/Lhx4 was performed (Varela-Echavarria et al., 1996; Marthiens et al., 2002). Results demonstrate that all cad6B-positive neurons express Isl1 (Fig. 17) and Lim3/Lhx4 (Fig. 13C, F, G). Similar observations were made for the other branchiomotor cell groups (data not shown).

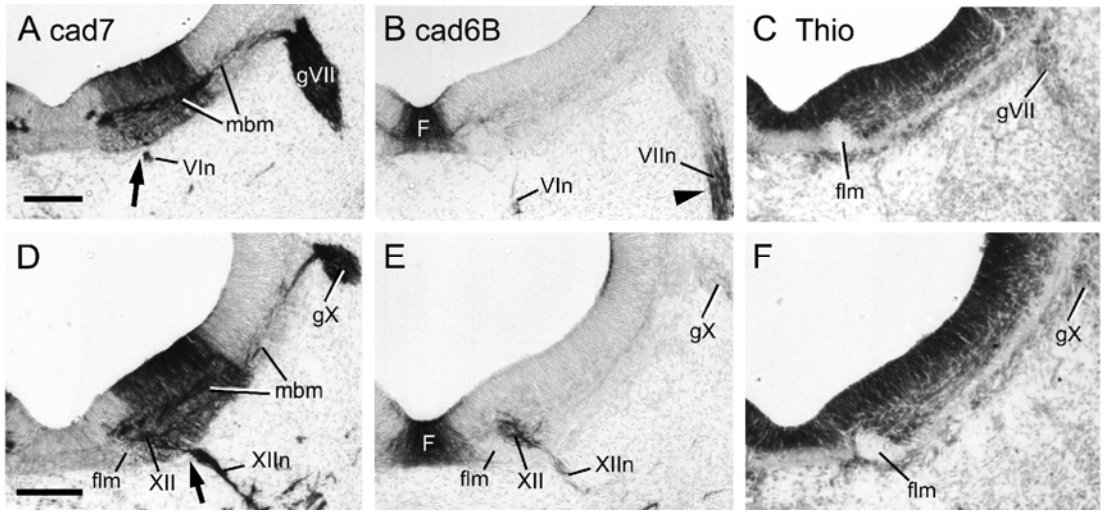

Figure 16. Immunostains for cadherin-7 (cad7; A,D), cadherin-6B (cad6B; B, E) and Nissl stains (thionine, Thio; C, F) for a series of consecutive frontal sections at stage 24 (HH24). Note that the exit of the somatomotor nerves (abducens nerve, VIn; A-C; and hypoglossal nerve, XIIn; D-F) are located medial to the cadherin-7 expression domain (arrows). The arrowhead in A points to cadherin-7-positive cells at the exit point of the facial nerve (VIIn). The arrowhead in B points to cadherin-6B-positive cells in the more distal facial nerve. Other abbreviations: F, floor plate; flm, medial longitudinal fascicle; gVII, facial ganglion; gIX/X, ganglion of the glossopharyngeal/vagus nerves; mbm, migrating branchiomotor neurons; XII, hypoglossal nucleus. Scale bars 100 µm (in A for A-C, and in D for D-F).

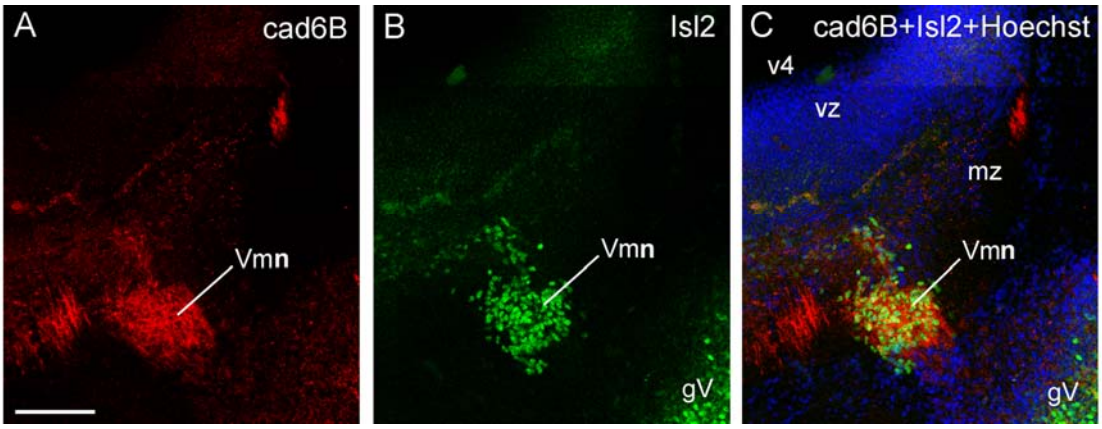

Figure 17. Branchiomotor neurons of the ventral trigeminal motor nucleus co-express cadherin-6B (cad6B, red in A, C) and Islet-1 (green in B, C) at stage 26. An overlay of the double-label immunostaining for this frontal section is shown in C (nuclear stain [Hoechst 33258] in blue). Abbreviations: gV, trigeminal ganglion; mz, mantle zone; v4, fourth ventricle; Vm, ventral motor nucleus of the trigeminal nerve; vz, ventricular zone. Scale bar (in A), 100 µm for A-C.

3.4 Postmigratory localization of branchiomotor neurons

The results described above demonstrate that the branchiomotor neurons of the chicken hindbrain, which migrate first laterally and then radially pialward, assume a final position lateral to the cad7 domain. As described above, the lateral border of the cad7 domain coincides with the lateral border of the Otp domain in the mantle layer (Fig. 18). In collaboration with Pilar Aroca and Luis Puelles, University of Murcia, we next asked whether all branchiomotor neurons come to reside lateral to this border. To answer this question, flatmount double RNA *in situ* hybridization for Otp and for Phox2b at stages 22, 24 and 28 (Fig.19) were prepared by Pilar Aroca. The Phox2b gene encodes a homeodomain transcription factor, which is expressed in progenitors and mature branchiomotor neurons, apart of in other cell types (Fig. 19) (Pattyn et al., 1997; Garel et al., 2000; Pattyn et al., 2000).

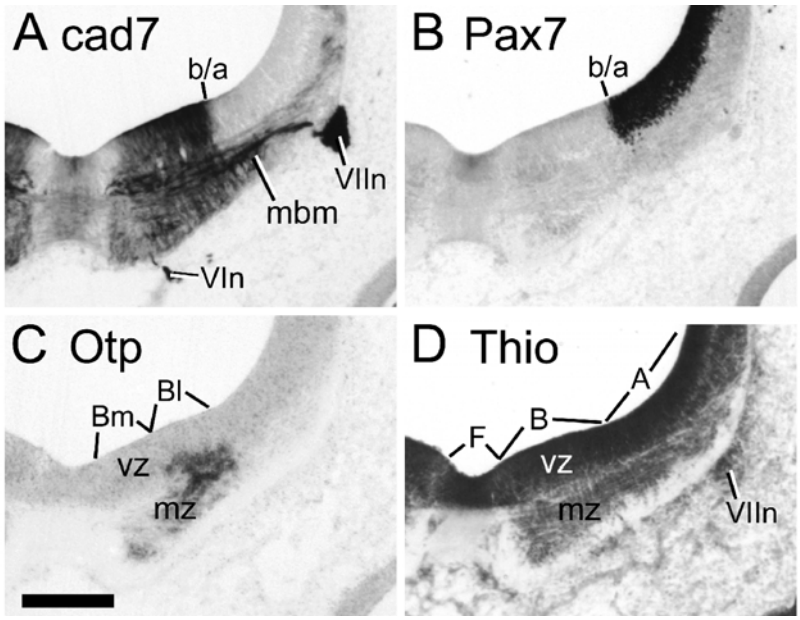

Figure 18. Expression of cadherin-7 (cad7, A), Pax7 (B), and Otp (C) in the hindbrain of a stage 24 embryo. Immunostains are shown for a series of consecutive sections. D, Nissl stain (thionine, Thio) of an adjacent section. Note that the lateral border of the Otp expression domain coincides approximately with the lateral border of the cad7 expression domain in mantle layer. Abbreviations: A, alar plate; B, basal plate; b/a, basal/alar plate boundary; Bm, medial part of basal plate; Bl, lateral part of basal plate; F, floor plate; mbm, migrating branchiomotor neurons; mz, mantle zone; VIn, abducens nerve; VIIn, facial nerve; and vz, ventricular zone. Scale bar (in C), 200 µm for A-D.

Results suggest that the majority of Phox2b-positive motorneurons eventually assume positions intercalated between the Otp expression domain, medially, and the lateral longitudinal band of the Phox2b expression, laterally (Fig. 19C). This also applies to the dorsally migrating brachiomotor neuron population, which stop their migration and stabilize periventricularly (data not shown).

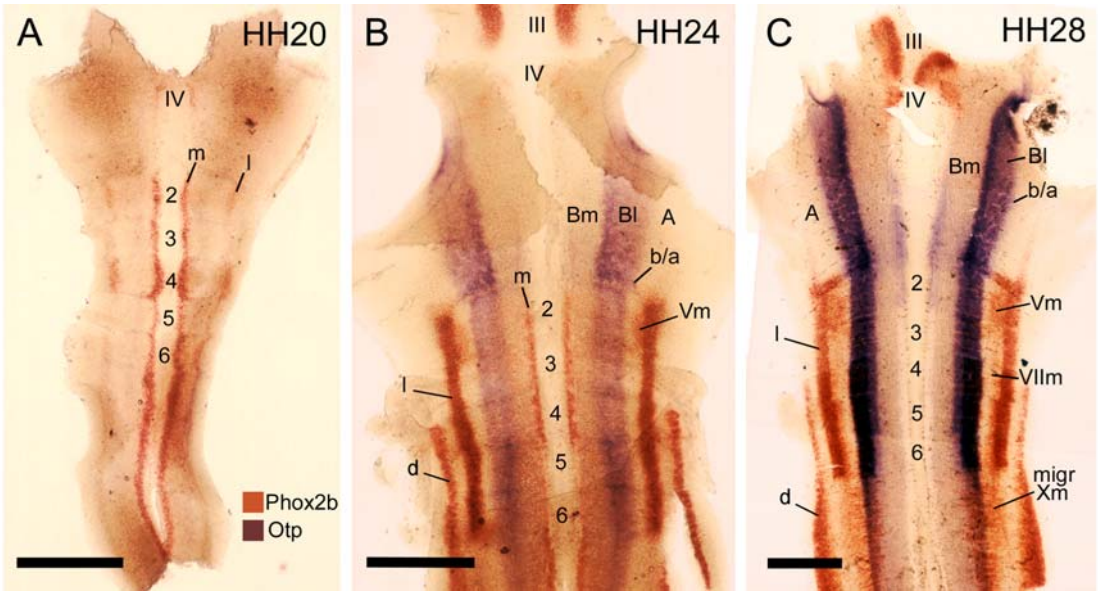

Figure 19. Expression of Phox2b and Otp in flatmount specimens of hindbrain from embryonic stage 20 (HH20; A), stage 24 (HH24; B), and stage 28 (HH28; C). Specimens were hybridized with cRNA probes for Phox2b (brown) and Otp (purple). Abbreviations: 2-6, rhombomeres 2-6; alar, alar plate; b/a, basal/alar plate boundary; Bm, medial part of basal plate; Bl, lateral part of basal plate; d, dorsal band of Phox2b expression; III, oculomotor complex; IV, trochear complex; Xm, laterally/dorsally migrated branchiomotor nucleus of the vagus nerve; m, medial band of Phox2b expression; migr, migrating branchiomotor neurons; l, lateral band of Phox2b expression; Vm, laterally/dorsally migrated branchiomotor nucleus of the trigeminal nerve; and VIIm, laterally/dorsally migrated branchiomotor nucleus of the facial nerve. Scale bars, 600 µm for A-C.

4 DISCUSSION

In the present study, I investigated the relation of branchiomotor (bm) neuron migration to mediolateral patterning of the chicken embryonic hindbrain. Although a number of expression markers have been available for the different mediolateral domains of the proliferative (ventricular) zone of the hindbrain (Simeone et al., 1994; Osumi et al., 1997; Takahashi and Osumi, 2002) and for the different motoneuron populations (Varela-Echavarria et al., 1996; Osumi et al., 1997; Pattyn et al., 1997, 2000; Garel et al., 2000; Takahashi and Osumi, 2002), the present study is the first to use such markers in order to examine in which domain of the mantle layer the bm neurons assume their final position with respect to the basal/alar plate boundary.

This issue has been obscured by both the "sulcus limitans of His" concept (His, 1888) and the four functional columns of cranial nerve nuclei defined in the adult hindbrain (reviewed in Nieuwenhuys, 1998). The sulcus limitans is a variable morphological feature (Hugosson, 1955) that does not correlate clearly with any relevant molecular boundary. The concept of adult functional columns, which are long known not to correlate with primary histogenic phenomena (Hugosson, 1955, 1957), disregards the existence of tangential cell migrations and D/V genetic patterning mechanisms (Puelles and Rubenstein, 1993; Shimamura et al., 1995; Briscoe and Ericson, 2001). There is sound evidence on complexly regulated alternative "ventral" or "dorsal" molecular fates of the neural tube lateral wall, thus validating a molecular redefinition of the old alar-basal concept (Briscoe et al., 2000;

Litingtung and Chiang, 2000; Muhr et al., 2001; Vallstedt et al., 2001; Persson et al., 2002).

An analysis of recent literature suggests that the ventral limit of the Pax7 expression domain across the spinal cord and hindbrain is a credible candidate for the postulated basal/alar plate boundary (see below). The terms "alar"/"basal" seem semantically advantageous, mainly because "dorsal"/"ventral" are relative terms (i.e., there is a ventral part of the "dorsal" neural tube) and, in the hindbrain, dorsal/ventral changes to lateral/medial as the two halves of the neural tube open like a book. The mapping of three specific markers (Pax7, cad7 and Otp) allowed me to visualize the basal/alar plate boundary along its entire radial extent from the ventricular to the pial surface in the developing hindbrain.

4.1 Cad7 expression marks the most dorsal (lateral) portion of the basal plate in the spinal cord and hindbrain

In the spinal cord, ventrodorsal patterning has been well investigated with several markers (Fig.3C)(Ericson et al., 1997b; Briscoe et al., 2000; Marthiens et al., 2002). Specifically, the ventral border of the Pax7 expression domain has been used to define the boundary between dorsal and ventral spinal cord (Jostes et al., 1990; Ericson et al., 1997b; Kawakami et al., 1997; Briscoe and Ericson, 1999; Briscoe et al., 2000; Mizuguchi et al., 2001), which is thought to correspond to the basal/alar plate boundary. Other markers that approximate this boundary at both spinal and hindbrain levels are *Dbx1* (Fig. 3C),

the determinant of the p0/p1 progenitor domain, and *Evx1* (Fig. 3D), the determinants of the dorsal/p0 progenitor domain and V0 interneurons (Bastian and Gruss, 1990; Puelles and Rubenstein, 1993; Briscoe et al., 2000; Moran-Rivard et al., 2001; Muhr et al., 2001; Pierani et al., 2001; Vallstedt et al., 2001). It is presently a matter of controversy whether the basal/alar boundary is defined at the dorsal/p0 limit, across the p0 domain itself or at the p0/p1 limit. My conclusions with regard to the migration of the bm neurons into the alar plate (see below) would not change by selecting one of the alternatives.

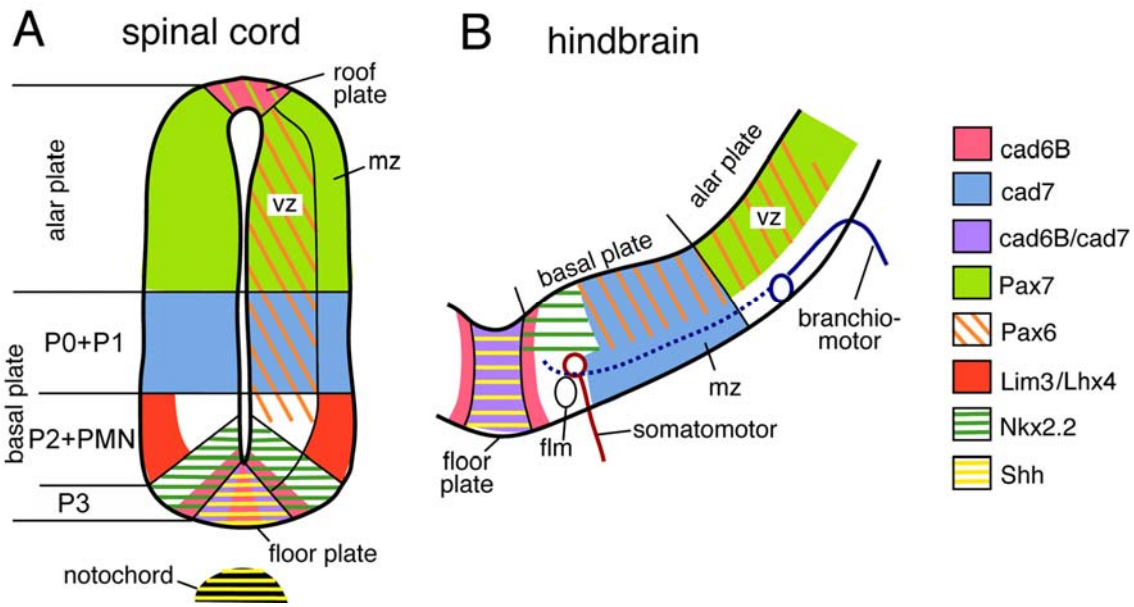

Figure 20. Schematic representation of the expression domains of cadherin-7 (cad7) and other genes (cadherin-6B [cad6B], Pax7, Pax6, Lim3/Lhx4, Nkx2.2 and Sonic hedgehog [Shh]; for color coding, see right side of the figure) in the spinal cord (A) and in the hindbrain (B) at stage 23. The blue dotted line in B represents the migratory path of branchiomotor neurons. Note that the branchiomotor neurons (dark blue in B) come to lie in the alar plate. The exit point of the somatomotor neurons (red in B) is just medial to the cad7 expression domain. Abbreviations: flm, medial longitudinal fascicle; mz, mantle zone; p0-p3, ventral progenitor cell domains; pMN, motoneuron progenitor domain; vz, ventricular zone.

Results from the present study show that the radial cad7 domain in the spinal cord represents the p0 and p1 regions of the proliferative basal plate. The cad7 domain includes the V0 and V1 neurons in the mantle layer. It thus marks the most dorsal region of the basal plate. The complete radial labeling in this region is likely due to the expression of cad7 by radial glia that extends processes from the ventricular to the pial surface. A regionally restricted expression of classic cadherins by radial glia has been observed also in other brain regions (Shimamura and Takeichi, 1992; Gänzler and Redies, 1995; Yoon et al., 2000). I conclude that the dorsal border of the cad7 domain coincides with the basal/alar plate boundary throughout the entire depth of the neural tube wall.

The cad7 domain continues into the hindbrain, where it is only interrupted by the rhombomere boundaries (Fig. 11A,B) that constitute a specialized population of boundary cells (Guthrie et al., 1991; Heyman et al., 1995). Like in the spinal cord, the lateral (dorsal) border of cad7 expression coincides closely with the medial border of Pax7 expression in the ventricular zone (Figs. 12,13,14,18). The medial border of the cad7 domain coincides with the lateral border of Nkx2.2 expression and with the medial border of Pax6 (Figs. 12A, D, E; 13A,D,E; 14A,D,E) and it colocalizes with the exit point of the somatomotor nerves of the hindbrain (Fig. 16).

In conclusion, the cad7 domain represents the most lateral (dorsal) domain of the basal plate also in the hindbrain. In the mantle layer of the hindbrain, the lateral border the cad7 domain coincides with the lateral border of the Otp-positive domain (Fig. 18). In the embryonic mouse

hindbrain, this lateral Otp expression border has been previously localized to the position of the sulcus limitans (Simeone et al., 1994) that marks the basal/alar plate boundary on the ventricular surface of the hindbrain (His, 1888). Together, these results demonstrate that the overlapping domains of cad7 and Otp expression abut laterally the basal/alar plate boundary in the mantle layer.

4.2 Branchiomotor neurons assume their final positions in the alar plate

The bm neurons are born adjacent to the floor plate and later migrate lateralward (Windle and Austin, 1936; Hugosson, 1955, 1957; Windle, 1970; Millet and Alvarado-Mallant, 1995; Varela-Echavarria et al., 1996; Pattyn et al., 1997, 2000; Qui et al., 1998; Puelles et al., 2001; Marthiens et al., 2002). The migration is initially guided by their axons exiting the hindbrain alar plate close to the respective afferent root (Moody and Heaton, 1983b; Moody and Heaton, 1983a).

It is generally assumed that the bm axons exit the hindbrain in the alar plate. Results from the present study demonstrate that the final location of the bm cell bodies is lateral to the overlapping cad7 and Otp expression domains in the mantle layer (Fig. 18). Because this limit marks the basal/alar plate boundary (see above), I conclude that bm neurons in the chicken take up their final positions in the alar plate. This conclusion applies to the trigeminal nuclei (Figs. 15; 17) as well as to all bm neurons of the facial nerve and glossopharyngeal/vagus nerves (Data not shown), including their dorsally

migrating bm neuron populations (data not shown). In the present study, these neurons and their progenitors were visualized by immunostaining with antibodies against Phox2b, a specific marker for bm neurons and their progenitors (Pattyn et al., 1997; Pattyn et al., 2000). In addition, I used monoclonal antibodies against Isl1 (Figs. 13G; 17) and Lim3/Lhx4 (Fig. 13F) to confirm the branchiomotor identity of the neurons in the lateral nuclei (Varela-Echavarria et al., 1996; Marthiens et al., 2002).

In the mouse hindbrain, trigeminal bm neurons are born in rhombomere 2 (r2) and migrate laterally within this rhombomere (Lumsden and Keynes, 1989; Marín and Puelles, 1995). Facial bm neurons, however, take a morecomplex course of migration in the mouse. They first migrate caudally from r4 through r5 to r6 where they then migrate radially towards the surface of the hindbrain (Auclair et al., 1996; Goddard et al., 1996; Pattyn et al., 1997; Garel et al., 2000). It is unknown at present on which side of the basal/alar plate boundary these cells come to lie after they complete migration. Unlike their mouse counterparts, facial bm neurons remain in r4 in the chicken embryo (Lumsden and Keynes, 1989; Guthrie and Lumsden, 1992; Fritzsch, 1998; Jacob and Guthrie, 2000; Studer, 2001).

My results make it necessary to revise the century-old concept that all primary motor neurons, including the visceromotor column, are part of the basal plate of the vertebrate brain (Gaskell, 1886, 1889; His, 1888; Johnston, 1905; Herrick, 1933; Kuhlenbeck, 1975; Nieuwenhuys et al., 1998). The visceromotor column of neurons is generally thought to contain the branchiomotor ("special visceromotor") neurons and the parasympathetic

preganglionic ("general visceromotor") neurons. Note that sympathetic preganglionic neurons migrate also dorsalward in the thoracic spinal cord, settling in an intermediate position between the dorsal and ventral horns (Levi-Montalcini, 1950).

At least for the chicken brain, I show that, during their migration from medial to lateral, the bm neurons migrate through the basal/alar plate boundary (Fig. 15). To settle in a medial subdomain of the alar plate (Fig. 18), either periventricularly or at intermediate/superficial radial levels, after an additional radial migration step with trailing axons (Windle and Austin, 1936). This particular radial domain is characterized by the presence of catecholaminergic neurons near the bm neurons (Puelles, 1978; Puelles and Verney, 1998) and lies just medial relative to the "lateral" longitudinal band expressing Phox2b in the ventricular zone (l in Fig. 20), a possible source of such neurons (Pattyn et al., 1997, 2000). In mammals, a chemoarchitectonically distinct "intermediate" sector (or "lateral paracore"; Nieuwenhuys, 1988) of the reticular formation develops in this alar location (Foster, 1998; Jacobowitz and Abbott, 1998; Paxinos and Watson, 1998; Paxonis and Franklin, 2001). The bm neurons migrating into this alar domain thus encounter a distinct cellular and molecular environment. This result implies that the radial domain apparently is able to either attract or at least stabilize specific migrating elements. Site-specific guidance by radial glia may be a potentially basis for the pialward migration of subgroups of bm neurons within the alar plate. As a result of the partial reshuffling of the diverse cell populations via tangential and radial migrations, a neuronal complex is

formed that might possibly carry out novel functions, which may depend on the interaction of the neurons of hybrid developmental origin. Since the migration pattern of bm neurons in the chicken seems to be rather different from that in the mouse (see above), it remains to be examined whether bm neurons of other species also settle in the alar plate. With the recent availability of molecular markers that show phylogenetically conserved expression patterns (see, for example, Puelles et al., 2000), future studies will be able to define the basal/alar plate boundary and other limits between longitudinal hindbrain domains more clearly in different species.

4.3 Change of cadherin expression during branchiomotor neuron migration and differentiation

It has been reported that facial bm neurons in the mouse successively change their expression of cell surface molecules (TAG-1, Ret and cadherin-8) as they migrate from r4 to r6 (Garel et al., 2000). An examination of transgenic mice suggests that this change of gene expression is an adaptation to the different rhombomeric environments and may play a role in the selection of the local migratory pathway. In the present study, I report a similar switch of expression from cad7 during the migratory phase to cad6B in the post-migratory phase of bm neuron differentiation. Cad7 is downregulated after the bm neurons have traversed the cad7-positive basal plate region. The axons of the migrating bm neurons also express cad7 and exit the hindbrain at a point where cad7-positive Schwann cells are found (arrowheads in Fig.

15)(Nakagawa and Takeichi, 1995). The presence of a cad7-positive substrate along the axonal trajectory may induce or facilitate axon outgrowth. An axonal growth promoting effect has been shown previously for N-cadherin *in vitro* and *in vivo* (Matsunaga et al., 1988; Bixby and Zhang, 1990; Redies et al., 1992; Riehl et al., 1996; Iwai et al., 1997; Broadbent and Pettitt, 2002). A role of classic cadherins in promoting the selective outgrowth of axons expressing the same cadherin subtype has been demonstrated *in vivo* (Treubert-Zimmermann et al., 2002). In this context, it is interesting to note that more peripheral Schwann cells express strongly cad6B (arrowhead in Fig. 16B; Nakagawa and Takeichi, 1995) and may provide a homotypic substrate for outgrowth of more distal bm axons after they switch expression from cad7 to cad6B.

It remains unclear whether the absence of cad7 expression in the medial alar plate modifies the gene expression or migratory behavior of the bm cells. It is conceivable that the bm neurons slow down or stop their translocation of cell bodies after they have reached the cad7-negative medial alar plate. Likewise, it is possible that the bm neurons change expression of cell surface molecules in response to the different environment, as demonstrated in the mouse for the facial bm neurons (Garel et al., 2000; Studer, 2001). Whether such modifications in gene expression contribute to the functional difference between branchiomotor and general visceromotor neurons remains to be examined.

The somatomotor (sm) axons, which exit the hindbrain ventrally in the basal plate, exhibit also a switch from cad7 to cad6B and also encounter

cad7-positive Schwann cells at their exit points (arrows in Fig. 16A, D). It has been proposed that other factors, such as the ephrins and their receptors, differentially attract and repel sm and bm neurons, respectively, from the midline (Kury et al., 2000). Cadherins may thus carry generally instuctive cues for axon and neurite migration in this area, rather than determine the gross direction of axon outgrowth. In the spinal cord, cadherins were proposed to regulate motor neurons cell sorting. Here, only a subpopulation of sm neurons expresses cad6B (Figs. 7-10) (Marthiens et al., 2002; Price et al., 2002). In contrast, all Lim3/Lhx4-positive bm neurons were cad6B-positive at E5. Whether there is differential expression of cadherins in the different subnuclei of bm neurons at later stages (Heaton and Moody, 1980) was not examined in the present study.

5 Summary

It is generally believed that the spinal cord and hindbrain consist of a motor basal plate and a sensory alar plate. This assumption was tested in the chicken embryo by mapping the expression of cadherin-7 and cadherin-6B, in comparison to genetic markers of ventrodorsal patterning (Otp, Pax6, Pax7, Nkx2.2, Shh) and markers for specific motoneuron subpopulations (Phox2b, Islet-1, and Lim3/Lhx4). Results show that cadherin-7 is expressed in a complete radial domain, which occupies the dorsal region of the basal plate. The dorsal limit of this cadherin-7 domain coincides with the ventral border of Pax7 expression, i.e. with the basal/alar plate boundary. The branchiomotor neurons of the chicken hindbrain are born at a medial position close to the floor plate. They extend a cadherin-7-positive axon that grows laterally and exits the hindbrain from the alar plate. Following this axonal trajectory, the cadherin-7-positive neuronal cell bodies translocate laterally to pass through the cadherin-7-positive basal plate domain. Subsequently, the cell bodies traverse the basal/alar plate boundary and assume their final position in the alar plate. After migration is completed, branchiomotor neurons switch expression from cadherin-7 to cadherin-6B.

These findings demonstrate that a specific subset of primary motor neurons, the branchiomotor neurons, migrate into the alar plate of the chicken embryo. Consequently, the century-old concept that all primary motor neurons come to reside in the basal plate should be revised.

6 References

1. Arens M, Straznicky C (1986)

The development of the trigeminal (V) motor nucleus in normal and tubocurare treated chick embryos.

Anat & Embryol 174:67-72.

2. Arndt K, Redies C (1996)

Restricted expression of R-cadherin by brain nuclei and neural circuits of the developing chicken brain.

J Comp Neurol 373:373-399

3. Auclair F, Valdes N, Marchand R (1996)

Rhombomere-specific origin of branchial and visceral motoneurons of the facial nerve in the rat embryo.

J Comp Neurol 369:451-461.

4. Basler K, Edlund T, Jessell TM, Yamada T (1993)

Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member.

Cell 73:687-702.

5. Bastian H, Gruss P (1990)

A murine even-skipped homologue, Evx 1, is expressed during early embryogenesis and neurogenesis in a biphasic manner.

EMBO J 9:1839-1852.

6. Beesley PW, Mumery R, Tibaldi J, Chapman AP, Smith, SJ (1995)

The postsynaptic density-putative involvement in synapse stabilization via cadherins and covalent modification by ubiquitination.

Biochem Soc Trans 23:59-64

7. Behrens J (1999)

Cadherins and catenins: role in signal transduction and tumor progression.

Cancer Metastasis Rev 18:15-30

8. Bixby JL, Zhang R (1990)

Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth.

J Cell Biol 110:1253-1260.

9. Briscoe J, Ericson J (2001)

Specification of neuronal fates in the ventral neural tube.

Curr Opin Neurobiol 11:43-49.

10. Briscoe J, Pierani A, Jessell TM, Ericson J (2000)

A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube.

Cell 101:435-445.

11. Briscoe J, Ericson J (1999)

The specification of neuronal identity by graded Sonic Hedgehog signalling. Semin Cell Dev Biol 10:353-362.

12. Broadbent ID, Pettitt J (2002)

The C. elegans hmr-1 gene can encode a neuronal classic cadherin involved in the regulation of axon fasciculation.

Current Biol 12:59-63.

13. Colman DR (1997)

Neurites, synapses, and cadherins reconciled.

Mol Cell Neurosci 10:1-6

14. Covell D, Jr., Noden DM (1989)

Embryonic development of the chick primary trigeminal sensory-motor complex.

J Comp Neurol 286:488-503.

15. Dahl U, Sjödin A, Laure L, Radice GL, Cajander S, Takeichi M, Kemler R, Semb H (2002)

Genetic dissection of cadherin function during nephrogenesis.

Mol Cell Biol 22:1474-1487

16. Ericson J, Briscoe J, Rashbass P, van Heyningen V, Jessell TM (1997a)

Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube.

Cold Spring Harb Symp Quant Biol 62:451-466

17. Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J (1997b)
Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling.
Cell 90:169-180.

18. Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM (1996)
Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity.
Cell 87:661-673.

19. Ericson J, Thor S, Edlund T, Jessell TM, Yamada T (1992)
Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1.
Science 256:1555-1560.

20. Esni F, Johansson BR, Radice GL, Semb H (2001)
Dorsal pancreas agenesis in N-cadherin-deficient mice
Dev Biol 238:202-212

21. Fannon AM, Colman DR, (1996)
A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins.
Neuron 17:423-434

22. Foster GA (1998)

Chemical neuroanatomy of the prenatal rat brain.

Oxford: Oxford University Press.

23. Fritzsch B (1998)

Of mice and genes: evolution of vertebrate brain development.

Brain Behav Evol 52:207-217.

24. Gänzler SII, Redies C (1995)

R-cadherin expression during nucleus formation in chicken forebrain neuromeres. J Neurosci 15:4157-4172.

25. Garel S, Garcia-Dominguez M, Charnay P (2000)

Control of the migratory pathway of facial branchiomotor neurones.

Development 127:5297-5307.

26. Gaskell W (1886)

On the structure, distribution and function of the nerves which innervate the visceral and vascular systems.

J Physiol 7:1-81.

27. Gaskell W (1889)

On the relation between the structure, function, distribution and origin of the cranial nerves; together with an theory of the origin ot the nervous system of vertebrata.

J Physiol 10:153-211.

28. Goddard JM, Rossel M, Manley NR, Capecchi MR (1996)

Mice with targeted disruption of Hoxb-1 fail to form the motor nucleus of the VIIth nerve.

Development 122:3217-3228.

29. Gumbiner B. M. (1996)

Cell adhesion: The molecular basis of tissue architecture and morphogenesis.

Cell 84:345-357

30. Guthrie S, Butcher M, Lumsden A (1991)

Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain.

J Neurobiol 22:742-754.

31. Guthrie S, Lumsden A (1992)

Motor neuron pathfinding following rhombomere reversals in the chick embryo hindbrain.

Development 114:663-673.

32. Hamburger V, Hamilton H (1951)

A series of normal stages in the development of the chick embryo.

J Morphol 88:49-92.

33. Heaton MB, Moody SA (1980)

Early development and migration of the trigeminal motor nucleus in the chick embryo.

J Comp Neurol 189:61-99.

34. Herrick CJ (1922)

Functional factors in the morphology of the forebrain of fishes.

In: Libro en honor de D. Santiago Ramón y Cajal ed), Vol. pp 142-204. Madrid: Jiméney Molina.

35. Herrick CJ (1933)

Morphogenesis of the brain.

J Morphol 54:233-258.

36. Heyers D, Kovjanic D, Redies C (2003)

Cadherin expression coincides with birth dating patterns in patchy compartments of the developing chicken telencephalon.

J Comp Neurol 462:155-166

37. Heyman I, Faissner A, Lumsden A (1995)

Cell and matrix specialisations of rhombomere boundaries.

Dev Dynam 204:301-315.

38. Hirano S, Suzuki ST, Redies C (2003)

The cadherin superfamily in neural development: diversity, function and interaction with other molecules

Front Biosci 8:306-356.

39. His W (1888)

Zur Geschichte des Gehirns sowie der centralen und periferischen Nervenbahnen beim menschlichen Embryo.

Abh Math Phys Kl Kgl Sächs Ges Wiss 14:339-393.

40. Hossein CN, Grant JA (1999)

Pemphigus and bullous pemphigoid.

Lancet 354:667-672

41. Hugosson R (1955)

Studien über die Entwicklung der longitudinalen Zellsäulen und der Anlagen der Gehirnnervenkerne in der Medulla oblongata bei verschiedenen Vertebraten.

Z Anat Entwickl-Gesch 118:543-566.

42. Hugosson R (1957)

Morphologic and experimental studies on the development and significance of the rhombencephalic longitudinal columns.

Dissertation, University of Lund.

43. Humphries M. J., Newham P (1998)

The structure of cell-adhesion molecules.

Trends Cell Biol 8:685-691

44. Inoue A, Sanes JR (1997)

Lamina-specific connectivity in the brain: regulation by N-cadherin, neurotrophins, and glycoconjugates.

Science 27:1428-1431

45. Iwai Y, Usui T, Hirano S, Steward R, Takeichi M, Uemura T (1997)

Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the Drosophila embryonic CNS.

Neuron 19:77-89.

46. Jacob J, Guthrie S (2000)

Facial visceral motor neurons display specific rhombomere origin and axon pathfinding behavior in the chick.

J Neurosci 20:7664-7671.

47. Jacobowitz DM, Abbott LC (1998)

Chemoarchitectonic Atlas of the Developing Mouse Brain.

Boca Raton: CRC Press.

48. Johnston JB (1905)

The cranial nerve components of Petromyzon.

Morphologisches Jahrbuch 34:149-203.

49. Jostes B, Walther C, Gruss P (1990)

The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system.

Mech Dev 33:27-37.

50. Jowett T, Lettice L (1994)

Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes.

Trends Genetics 10:73-74.

51. Kawakami A, Kimura-Kawakami M, Nomura T, Fujisawa H (1997)

Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development.

Mech Dev 66:119-130.

52. Kuhlenbeck H (1975)

The central nervous system of vertebrates, Vol. 4: Spinal cord and deuterencephalon.

Basel: Karger.

53. Kury P, Gale N, Connor R, Pasquale E, Guthrie S (2000)

Eph receptors and ephrin expression in cranial motor neurons and the branchial arches of the chick embryo.

Mol Cell Neurosci 15:123-140.

54. Lee KJ, Dietrich P, Jessell TM (2000)

Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification.

Nature 403:734-740.

55. Levi-Montalcini R (1950)

The origin and development of the visceral system in the spinal cord of the chick embryo.

J Morphol 86:253-283.

56. Liem KF, Jr., Jessell TM, Briscoe J (2000)

Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites.

Development 127:4855-4866.

57. Litingtung Y, Chiang C (2000)

Control of Shh activity and signaling in the neural tube.

Dev Dyn 219:143-154.

58. Lumsden A, Keynes R (1989)

Segmental pattern of neuronal development in the chick hindbrain.

Nature 337:424-428.

59. Luo J, Lubaroff DM, Hendrix MJ (1999)
Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection.
Cancer Res 59:3552-3556

60. Marín F, Puelles L (1995)
Morphological fate of rhombomeres in quail/chick chimeras: a segmental analysis of hindbrain nuclei.
Eur J Neurosci 7:1714-1738.

61. Marthiens V, Padilla F, Lambert M, Mege RM (2002)
Complementary expression and regulation of cadherins 6 and 11 during specific steps of motoneuron differentiation.
Mol Cell Neurosci 20:458-475.

62. Marti E, Takada R, Bumcrot DA, Sasaki H, McMahon AP (1995)
Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo.
Development 121:2537-2547.

63. Matsunaga M, Hatta K, Takeichi M (1988)
Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina.
Neuron 1:289-295.

64. Matsunami H, Takeichi M (1995)
Fetal subdivisions defined by R-and E-cadherin expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion.
Dev Biol 172:466-478

65. Millet S, Alvarado-Mallart RM (1995)
Expression of the homeobox-containing gene En-2 during the development of the chick central nervous system.
Eur J Neurosci 7:777-791.

66. Miyatani S, Shimamura K, Hatta M, Nagafuchi A, Nose A, Matsunaga M, Hatta K, Takeichi M (1989)
Neural cadherins role in selective cell-cell adhesion.
Science 245:631-635

67. Mizuguchi R, Sugimori M, Takebayashi H, Kosako H, Nagao M, Yoshida S, Nabeshima Y, Shimamura K, Nakafuku M (2001)
Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons.
Neuron 31:757-771.

68. Moody SA, Heaton MB (1983a)
Developmental relationships between trigeminal ganglia and trigeminal motoneurons in chick embryos. I. Ganglion development is necessary for motoneuron migration.
J Comp Neurol 213:327-343.

69. Moody SA, Heaton MB (1983b)
Developmental relationships between trigeminal ganglia and trigeminal motoneurons in chick embryos. II. Ganglion axon ingrowth guides motoneuron migration.
J Comp Neurol 213:344-349.

70. Moran-Rivard L, Kagawa T, Saueressig H, Gross MK, Burrill J, Goulding M (2001)
Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord.
Neuron 29:385-399.

71. Muhr J, Andersson E, Persson M, Jessell TM, Ericson J (2001)
Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube.
Cell 104:861-873.

72. Nakagawa S, Takeichi M (1995)
Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins.
Development 121:1321-1332.

73. Nakagawa S, Takeichi M (1998)
Neural crest emigration from the neural tube depends on regulated cadherin expression.
Development 125:2963-2971.

74. Nieuwenhuys R, ten Donkelaar HJ, Nicholson C (1998)

The Central Nervous System of Vertebrates.

Heidelberg: Springer.

75. Nieuwenhuys R, Veening JG, van Domburg P (1988-1989)

Core and paracores; some new chemoarchitectural entities in the mammalian neuraxis.

Acta Morphol Neerl Scand 26:131-163

76. Nollet F, Kools P, van Roy F (2000)

Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members.

J Mol Biol 299 : 551-572

77. Obst-Pernberg K, Redies C (1999)

Cadherins and synaptic specificity.

J Neurosci Res 58:130-138

78 Osumi N, Hirota A, Ohuchi H, Nakafuku M, Iimura T, Kuratani S, Fujiwara M, Noji S, Eto K (1997)

Pax-6 is involved in the specification of hindbrain motor neuron subtype.

Development 124:2961-2972.

79. Otto T, Birchmeier W, Schmidt U, Hinke A, Schipper J, Rübben H, Raz A (1994)
Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas.
Cancer Res 54:3120-3123

80. Otto T, Rembrink K, Goepel M, Meyer-Schwickerath M, Rübben H (1993)
E-cadherin: a marker for differentiation and invasiveness in prostatic carcinoma.
Urol Res 21:359-362

81. Pattyn A, Hirsch M, Goridis C, Brunet JF (2000)
Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b.
Development 127:1349-1358.

82. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1997)
Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis.
Development 124:4065-4075.

83. Paxinos G, Watson C (1998)
The Rat Brain in Stereotaxic Coordinates.
San Diego: Academic Press.

84. Paxonis G, Franklin KBJ (2001)
The Mouse Brain in Stereotaxic Coordinates.
San Diego: Academic Press.

85. Persson M, Stamataki D, te Welscher P, Andersson E, Bose J, Ruther U, Ericson J, Briscoe J (2002)
Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity.
Genes & Devel 16:2865-2878.

86. Pierani A, Moran-Rivard L, Sunshine MJ, Littman DR, Goulding M, Jessell TM (2001)
Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1.
Neuron 29:367-384.

87. Poh A, Karunaratne A, Kolle G, Huang N, Smith E, Starkey J, Wen D, Wilson I, Yamada T, Hargrave M (2002)
Patterning of the vertebrate ventral spinal cord.
Int J Devl Neurosci 46:597-608.

88. Price SR, De Marco Garcia NV, Ranscht B, Jessell TM (2002)
Regulation of motor neuron pool sorting by differential expression of type II cadherins.
Cell 109:205-216.

89. Puelles E, Rubenstein JL, Puelles L (2001)
Chicken Nkx6.1 expression at advanced stages of development identifies distinct brain nuclei derived from the basal plate.
Mech Dev 102:279-282.

90. Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000)
Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes D2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1.
J Comp Neurol 424:409-438

91. Puelles L, Verney C (1998)
Early neuromeric distribution of tyrosine-hydroxylase-immunoreactive neurons in human embryos.
J Comp Neurol 394:283-308.

92. Puelles L, Rubenstein JLR (1993)
Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization.
Trends Neurosci 16:472-479.

93. Puelles L (1978)
Velate glioblasts in the developing chick optic tectum: probable immature forms of oligodendroglia.
Neurosci 3:41-47.

94. Qiu M, Shimamura K, Sussel L, Chen S, Rubenstein JL (1998)
Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development.
Mech Dev 72:77-88.

95. Ramon y Cajal S (1911)

Histologie du système nerveux de l´homme et des vertébrés.

Vol. I. Paris. Reprint. Madrid, 1972.: Maloine.

96. Redies C, Kovjanic D, Heyers D, Medina L, Hirano S, Suzuki ST, Puelles L (2002)

Patch/matrix patterns of gray matter differentiation in the telencephalon of chicken and mouse.

Brain Res Bull 57:489-493

97. Redies C, Puelles L (2001)

Modularity in vertebrate brain development and evolution.

BioEssays 23:1100-1111

98. Redies C (2000)

Cadherins in the central nervous system.

Prog Neurobiol 61:611-648.

99. Redies C (1997)

Cadherins and the formation of neural circuitry in the vertebrate CNS.

Cell Tissue Res 290:405-413

100. Redies C (1995)

Cadherin expression in the developing vertebrate CNS: from neuromeres to brain nuclei and neural circuits.

Exp Cell Res 220:243-256

101. Redies C, Engelhart K, Takeichi M (1993)
Differential expression of N- and R-cadherin in functional neuronal systems and other structures of the developing chicken brain.
J Comp Neurol 333:398-416.

102. Redies C, Inuzuka H, Takeichi M (1992)
Restricted expression of N- and R-cadherin on neurites of the developing chicken CNS.
J Neurosci 12:3525-3534.

103. Riehl R, Johnson K, Bradley R, Grunwald GB, Cornel E, Lilienbaum A, Holt CE (1996)
Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo.
Neuron 17:837-848.

104. Sharma K, Sheng HZ, Lettieri K, Li H, Karavanov A, Potter S, Westphal H, Pfaff SL (1998)
LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons.
Cell 95:817-828.

105. Shimamura K, Hartigan DJ, Martínez S, Puelles L, Rubenstein JLR (1995)
Longitudinal organization of the anterior neural plate and neural tube.
Development 121:3923-3933.

106. Shimamura K, Hirano S, McMahon AP, Takeichi M (1994)
Wnt-1-dependent regulation of local E-cadherin and aN-catenin expression in the embryonic mouse brain.
Development 120:2225-2234.

107. Shimamura K, Takeichi M (1992)
Local and transient expression of E-cadherin involved in mouse embryonic brain morphogenesis.
Development 116:1011-1019.

108. Simeone A, D'Apice MR, Nigro V, Casanova J, Graziani F, Acampora D, Avantaggiato V (1994)
Orthopedia, a novel homeobox-containing gene expressed in the developing CNS of both mouse and Drosophila.
Neuron 13:83-101.

109. Steinberg MS, Takeichi M (1994)
Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression.
Proc Natl Acad Sci USA 91: 206-209

110. Studer M (2001)
Initiation of facial motoneurone migration is dependent on rhombomeres 5 and 6.
Development 128:3707-3716.

111. Suzuki S (1996)

Structural and functional diversity of cadherin superfamily: are new members of cadherin superfamily involved in signal transduction pathway?

J Cell Biochem 61:531-542

112. Takahashi M, Osumi N (2002)

Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains.

Development 129:1327-1338.

113. Takeichi M (1988)

The cadherins: cell-cell adhesion molecules controlling animal morphogenesis.

Development 102:639-655

114. Timmer JR, Wang C, Niswander L (2002)

BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors.

Development 129:2459-2472.

115. Treubert-Zimmermann U, Heyers D, Redies C (2002)

Targeting axons to specific fibre tracts in vivo by altering cadherin expression.

J Neurosci 22:7617-7626.

116. Uchida N, Honjo Y, Johnson KR, Wheelock MJ, Takeichi M (1996)
The catenin/cadherin adhesion system is located in synaptic junctions bordering transmitter release zones.
J Cell Biol 135:767-779

117. Vallstedt A, Muhr J, Pattyn A, Pierani A, Mendelsohn M, Sander M, Jessell TM, Ericson J (2001)
Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification.
Neuron 31:743-755.

118. Varela-Echavarria A, Pfaff SL, Guthrie S (1996)
Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem.
Mol Cell Neurosci 8:242-257.

119. Windle WF (1970)
Development of neural elements in human embryos of four to seven weeks gestation.
Exp Neurol 28:44-83.

120. Windle WF, Austin MF (1936)
Neurofibrillar development in the central nervous system of chick embryos up to 5 days´ incubation.
J Comp Neurol 63:431-463.

121. Yoon MS, Puelles L, Redies C (2000)

Formation of cadherin-expressing brain nuclei in diencephalic alar plate subdivisions.

J Comp Neurol 421:461-480.

122. Zigmond M. J., Bloom F.E., Landis S. C., Roberts J. L., Squire L. R. (1999)

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Academic Press, pg. 426-427

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