Expression von Genen der Cadherin-Superfamilie während der Gehirnentwicklung des Frettchens

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)
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von

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Contents

ABBREVIATIONS

BBB	Blood-brain barrier
bp	Base pair
C. elegans	*Caenorhabditis elegans*
CADS	Calcium-dependent cell adhesion system
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CDH	Cadherin
cDNA	Complementary deoxyribonucleic acid
CIDS	Calcium-independent adhesion system
CM	Conserved motif
CNS	Central nervous system
CP	Cortical plate
CR	Cajal-Retzius cells
cRNA	Complementary ribonucleic acid
D. melanogaster	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
DP	Desmoplakin
E	Embryonic day
EC	Extra cellular domain
E-Cdh	Epithelial cadherin
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
Eph	Ephrin
FGF	Fibroblast growth factor
Fig.	Figure
FISH	Fluorescent in-situ hybridization
Fn	Fibronectin
Ig	Immunoglobulin
ISH	In situ hybridization
IZ	Intermediate zone
kDa	KiloDalton

LTP	Long-term potentiation
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
N-Cdh	Neural cadherin
P	Postnatal day
PBS	Phosphate-buffered saline
PCDH	Protocadherin
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PG	Plakoglobin
PK	Proteinase K
PP1α	Protein phosphatase-1α
R-Cdh	Retinal cadherin
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SMA	Alpha smooth muscle actin
SSC	Standard sodium citrate
SVZ	Subventricular zone
TBS	Tris-buffered saline
Tcr	T-cell receptor
V1	Primary visual cortex
V2	Secondary visual cortex
VE-Cdh	Vascular endothelial cadherin
VEGFR	Vascular endothelial growth factor receptor
VZ	Ventricular zone

1. PREFACE

1.1. General introduction

1.1.1. Historical perspectives

All forms of life can be roughly classified as unicellular and multicellular organisms. Single-celled organisms are independent and autonomous individual cells that do not need to assemble in groups to survive and function. Multicellular organisms have bodies formed by aggregation of many cells to form tissues and organs. Only after all the cells, tissues and organs are established by aggregating in place, is the multicellular organism capable of surviving. One of the most fundamental mechanisms underlying developmental processes in multicellular organisms is the adhesion between cells. The regulation of cell-cell adhesion controls many morphogenetic processes during the development and growth of tissues (Gumbiner, 1996; 2005). As cell-cell adhesion is a crucial process required for development, it was natural to postulate that specific adhesion molecules mediate this interaction. Various types of cell adhesion molecules and cell junctions control the physical interactions between cells. However, a family of cell adhesion molecules called “cadherins” was identified as particularly important for adhesiveness of cells and found to mediate the dynamic regulation of adhesive contacts that are associated with diverse morphogenetic processes (Takeichi, 1994; Hirano et al., 2003; Gumbiner, 2005; Redies et al., 2005; Suzuki and Takeichi, 2008). Cadherins are adhesive molecules that can glue cells together. They link cells together in their proper orientation, guiding the shape and form of the growing organism during embryonic development. The function of cadherins is required already in the first few hours of the life of higher organisms. In adult organisms, cadherins connect cells to give form and structure to the different tissues throughout the body. When cadherins fail to perform these functions, cells might lose their ability to hold onto one another. In the case of cancer, loss of adhesiveness allows individual cells to separate from a solid tumor. The cells are then free to wander through the body to form metastases.

In order to reveal the importance of cell-cell adhesion and possible principles of adhesive mechanisms, a number of pioneering studies using various experimental and theoretical approaches were performed. This led to the identification of cell adhesion molecules such as selectins, molecules of the immunoglobulin (Ig) family and cadherins. Several independent groups identified cadherins in the early 80’s using different approaches. Edelman and coworkers discovered L-CAM (chicken E-cadherin) using an antibody that inhibited cell adhesion (Gallin et al., 1983). Jacob and Kemler’s group

identified uvomorulin (now called E-cadherin) as a cell-adhesion molecule that mediates compaction in early embryos (Peyrieras et al., 1983). Lilien's group identified a 130-kDa molecule (N-cadherin) in the chick neural retina that was protected by Ca^{2+} from proteolysis (Grunwald et al., 1982). Damsky's group identified the same molecule as Cell-CAM 120/80 from a peptide released into the culture medium (Damsky et al., 1983). Geiger and coworkers then identified A-CAM (N-cadherin) as a molecule that was localized at adherens junctions (Volk and Geiger, 1984). Takeichi found differences in trypsin resistance between different cell types in cell culture using calcium ions in the medium. Consequently, he proposed that there are 2 adhesion systems, a calcium-dependent cell adhesion system (CADS) and a calcium-independent adhesion system (CIDS). Finally, in 1984, Chikako Yoshida from Takeichi's group at Kyoto University, Japan, isolated and studied a protein involved in Ca^{2+}-dependent cell adhesion using a monoclonal antibody against it. They later proposed the name 'cadherin' for the molecule responsible for calcium-dependent adhesion by combining letters from calcium, to adhere and protein (Yoshida-Noro et al., 1984). Subsequently, they showed that a molecule termed E-cadherin was responsible for the calcium-dependent aggregation of the F9 teratocarcinoma cell line. In the course of their studies, they also identified other cadherin subtypes in different tissues, e.g., N-cadherin, P-cadherin and R-cadherin. The sequence analysis of these molecules revealed that these cadherins constitute a large molecular family, which was named "cadherin superfamily". The development of modern molecular biological techniques, molecular cloning and the polymerase chain reaction (PCR) methods in particular, led to the discovery of more and more cadherins in the followig years. The so-called "classic" cadherins (E-, N-, P- and R-cadherin) were the first to be discovered, but it was later found that they constitute only a fraction of the cadherin superfamily. Later, Suzuki's group isolated a large family of ''non-classic'' cadherins called protocadherins that are present only in vertebrates (Sano et al., 1993). Protocadherins are predominantly expressed in the nervous system and constitute the largest subfamily within the cadherin superfamily. Currently, more than 100 different cadherin genes have been identified and classified into several subfamilies.

1.1.2. Evolution of cadherins

Cadherins must have evolved with or before the appearence of the animal kingdom, as they are found throughout all of the animal kingdom (Hirano et al., 2003; Hulpiau and van Roy, 2008). But the precise origin of cadherins has yet to be determined. Moreover, cadherin-

like molecules have been found also in many other species like the slime mold *Dictyostelium*. However, it has been difficult to follow the evolution of the cadherins of *Dictyostelium* and animals from a common ancestor. In the animal kingdom cadherin superfamily molecules have expanded very successfully. At least 15 and 13 cadherins have been found in the genomes of *C. elegans* and *D. melanogaster,* respectively, and most of them can be classified into several major subgroups in these species, whereas the rest represents much diverged members of the cadherin superfamily, suggesting species- or lineage-specific evolution. Notably, *C. elegans* and *D. melanogaster* have only 2 and 3 classic cadherins, respectively. There are also other members of the cadherins superfamily that are common to vertebrate and invertebrate species, such as Fat-type cadherins, flamingo cadherins, calsyntenin and Ret. These genes seem to have evolved at an early phase of evolution. In contrast, protocadherins are thought to have evolved rapidly within the vertebrate lineage because of their genomic structure and arrangement. Hypotheses on how cadherins evolved can be deduced from their molecular structure and genomic organization. The genes encoding the extracellular (EC) domains of classic cadherins have many introns at various positions (see below). In contrast, the genomic structure of many other (nonclassic) cadherins shows that their extracellular domain has few introns. In particular, most members of the protocadherin subfamily are encoded by a single exon (see below). Therefore, it has been hypothesized that the cadherin domains or motifs were multiplied initially to generate the various cadherin subfamily molecules, and that introns were later introduced after the emergence of the classic cadherins (Hirano et al., 2003; Hulpiau and van Roy, 2008). Desmosomal cadherins are found only in the vertebrate lineage, suggesting their specific function in vertebrate development. The substantial sequence similarity between desmosomal cadherins and vertebrate classic cadherins indicates that they have evolved from a common ancestor. In conclusion, the different lineages of the animal kingdom comprise common as well as species/lineage-specific members of the cadherin superfamily (Hirano et al., 2003; Hulpiau and van Roy, 2008).

1.2. Overview of the cadherin superfamily

1.2.1. Cadherin superfamily

Cadherins constitute a superfamily of integral membrane proteins with a molecular weight of about 130 kDa. The cadherin superfamily comprises the classic cadherins, protocadherins, desmogleins, desmocollins, CNRs, Fats, seven-pass transmembrane cadherins, and Ret tyrosine kinase (Redies, 1995; 1997; 2000; Hirano et al., 2003; Redies et

al., 2005). In total, the cadherin superfamily comprises more than 100 members and at least 80 of them are known to be expressed within a single mammalian species. Most cadherins are composed of a large extracellular domain (EC) at the N-terminal side, a relatively small intracellular domain at the C-terminal side, and a short transmembrane domain connecting these two domains (Hirano et al., 2003; Redies et al., 2005; see Fig. 1). Cadherins are characterized by the so-called "cadherin domains" or "cadherin repeats" in the extracellular region (Redies, 1995; 1997; 2000). The number of these repeats varies considerably between the cadherin subfamilies. The EC domain contains the cell-cell interaction site that determines the binding specificity of cadherins and is involved in Ca^{2+} binding (Nollet et al., 2000; Redies, 2000).

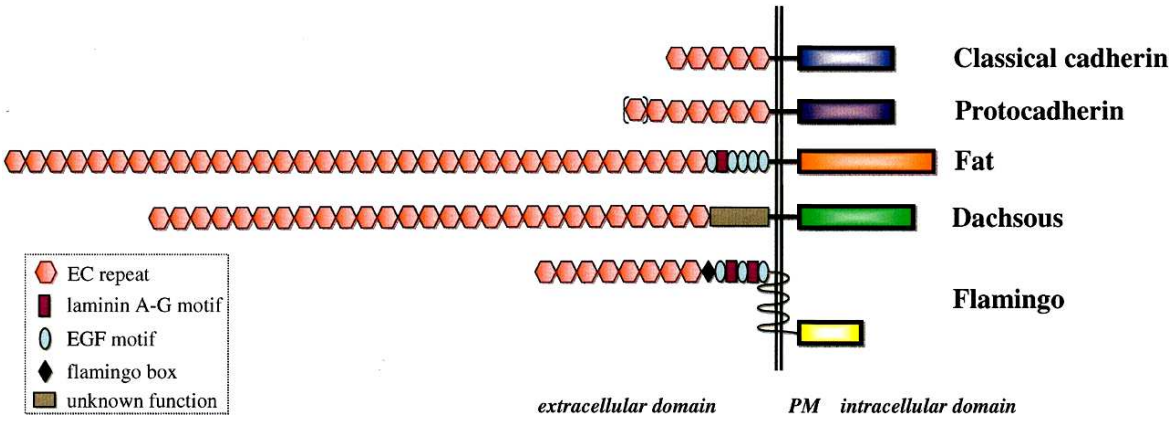

Figure 1. Cadherin structure. All cadherins possess calcium-binding EC repeats of varying number. Nonclassical cadherins have additional EC motifs including laminin and EGF domains and flamingo boxes. Brackets indicate that protocadherins have either six or seven ECs. Reproduced from (Halbleib and Nelson, 2006).

Cadherins are classified according to the organization of their extracellular cadherin motifs as well as according to sequence similarities in their extracellular and cytoplasmic domains (see Fig. 2). Cadherins have been broadly divided into two classes, namely, classic cadherins and nonclassic cadherins, for historical reasons (Takeichi et al., 1997; Hirano et al., 2005; Redies et al., 2005). Classic cadherins are type I transmembrane proteins involved in homotypic cell adhesions (see below). Non-classic cadherins comprise a variety of molecules, including protocadherins, desmosomal cadherins, fat-type cadherins, ret, flamingo and calsyntenin (Nollet et al., 2000; Hirano et al., 2005; Redies et al., 2005). Protocadherins appear to be developmentally important cadherins and are expressed in a widespread fashion throughout the nervous system and elsewhere (see below; Hirano et al., 2003; Redies et al., 2005). Fat-type cadherins are large cadherins with many cadherin

repeats. Flamingos are cadherins having a seven-pass transmembrane domain and a huge extracellular domain. Desmosomal cadherins are cadherins found to be localized only at desmosomes. Ret is a unique cadherin with a tyrosine kinase domain. Calsyntenin is another unique cadherin with just two cadherin motifs. Non-classic cadherins have considerably different cytoplasmic regions, suggesting that these cadherins might also have functions different from those of the classic cadherins.

1.3. Cadherin classification

1.3.1. Classic cadherins

The founding members of the superfamily, classic cadherins, are the most extensively studied and are named according to the tissue, in which they were identified first. Twenty different classic cadherin subtypes have been found in a single vertebrate species so far (see Fig. 2). For example, E-cadherin in epithelial cells, N-cadherin in neural epithelium, and P-cadherin in placenta; other members are retinal (R-)cadherin, brain (B-)cadherin and vascular endothelial (VE-)cadherin (Hatta and Takeichi, 1986; Geiger and Ayalon, 1992; Grunwald, 1996). However, their expression is not limited exclusively to these tissues. The extracellular domain (EC) domains of classic cadherins play a crucial role in their homotypic interactions (see below). The EC domain has five cadherin repeats and the last repeat has diverged most. The unique characteristic features lie with the third and fifth repeats (EC3 and EC5) of classic cadherins. One amino acid is deleted from EC3 at the C terminus end and the DRE sequence in the middle of the repeat is replaced by a DFE or DYE sequence. EC5 contains four cysteine residues that are characteristic for this repeat. Also, classic cadherins have a characteristic cytoplasmic sequence that binds to its signaling partner catenin (see below).

Classic cadherins can be further subdivided into 2 subfamilies on the basis of characteristic amino acid sequences in the cytoplasmic domain such as type I and type II classic cadherins. Type I cadherins are E-, P-, N- and R-cadherin (also called cadherin-1 to cadherin-4). Type II cadherins are numbered cadherin-5 to cadherin-12 based on their timeline of discovery (Suzuki et al., 1997; Yagi and Takeichi, 2000; Hirano et al., 2003; Redies et al., 2005); they differ from type I cadherins in their lack of a HAV cell adhesion recognition sequence. Both types of classic cadherins are highly conserved between vertebrate species (human, mouse, rat, chicken, and *Xenopus*) and may have important functional roles. The available experimental studies are not sufficient to determine a functional difference between type I and type II classic cadherins. Some type II cadherins,

for example cadherin-8 and cadherin-11, have splice variants and isoforms, which are hypothesized to modify and regulate cadherin-mediated adhesion (Hirano et al., 2003; Redies et al., 2005; Lin et al., 2008).

1.3.2. Nonclassic cadherins

1.3.2.1. Protocadherins

The term “protocadherin” was first coined by Shintaro Suzuki and co-workers in their search for additional cadherin-like molecules, using PCR with degenerate primers for the extracellular repeats of cadherins (Sano et al., 1993). Unexpectedly, they found PCR products coding for cadherin ectodomains (EC) with features grossly different from those of classic cadherins. Strikingly, several of these genes display an unusual genomic structure reminiscent of that of already existing superfamily molecules such as immunoglobulin and T-cell receptor genes. Similar PCR fragments were later found in a wide range of species including vertebrate and invertebrates, suggesting that these sequences potentially representing an ancient, primordial cadherin motif, from which other families might have evolved. This led to the name ‘protocadherins’ (‘protos’ [Greek] - the first). Full-length cloning of protocadherin cDNAs revealed that their molecular structures differed from each other and from classic cadherins. With the discovery of three large protocadherin clusters (α, β and γ) in mammalian genomes and the non-clustered δ-protocadherins, the protocadherin subfamily can now be divided into three subgroups: the 'clustered' protocadherins (α-, β- and γ-protocadherins), δ-protocadherins, and other protocadherins (Frank and Kemler, 2002; Hirano et al., 2003; Redies et al., 2005; see Fig. 2). To date, more than 80 protocadherins are known in mouse and man, making them the largest subfamily of the cadherin superfamily. In mammals, multiple protocadherins are highly expressed in the nervous system, and are found to be far more numerous in the brain than in any other tissues.

Protocadherins have unique features that are distinct from classic cadherins. These molecules typically contain six or seven repeats in the EC domain (see Fig. 1). The cadherin repeat sequences of protocadherins are very similar to each other, and none of them contains the unique features of the EC3 or EC5 domains of classic cadherins (Sano et al., 1993). The cytoplasmic domains of protocadherins are highly variable and contain various cytoplasmic sequences. Studies indicate that the EC domains of protocadherins can also undergo homotypic interactions, as found for classic cadherins, but the function of the cytoplasmic domains is not identical in the subfamilies. Genome analysis of protocadherins

reveal single large exons coding for multiple ectodomains as a characteristic feature of this subfamily, as well as a transmembrane domain and a short cytoplasmic tail (see below). Interestingly, protocadherins contain a conserved N-terminal RGD motif that may play a role as a membrane-associated ligand for integrins (Shapiro and Colman, 1999).

Compared to classic cadherins, protocadherins generally contribute only moderate adhesive activity that is independent of calcium ions (Sano et al., 1993). Protocadherins may need to make clusters on the cell surface to generate strong cell adhesion activity. However, protocadherins may have a role in more general cell-cell interactions, considering other features such as their capability of homophilic interaction and the expression of many protocadherins with different cytoplasmic sequences in various organisms (Frank and Kemler, 2002; Hirano et al., 2003; Redies et al., 2005). But it remains unclear if the protocadherins function as just adhesion molecules, like classic cadherins, or act only as signaling receptors through their characteristic cytoplasmic signaling partners. Recent studies reveal that the cytoplasmic domains of protocadherins interact with several cytoplasmic proteins that are different from the previously known catenins (see below; Sago et al., 1995; Hirano et al., 2003; Redies et al., 2005). The biological role of protocadherins is not well studied yet, although a large number of protocadherins are expressed in a variety of organisms.

1.3.2.1.1. Clustered protocadherins

The clustered protocadherin family is the largest subgroup of the cadherin superfamily. Clustered protocadherins are predominantly expressed in the brain and their gene structures in vertebrates are diversified (Frank and Kemler, 2002; Hirano et al., 2003; Redies et al., 2005). As discussed earlier, the clustered protocadherin family consists of three gene clusters: pcdh-α, pcdh-β, and pcdh-γ. Gene clustering was observed for human protocadherins at three chromosomal loci: 5q31, 13q21 and Xq21. At 5q31 (mouse chromosome 18c) the α-, β- and γ-protocadherins are organized in three large, sequential clusters with a total of 52 protocadherin genes (Wu and Maniatis, 1999). In the α- and γ-clusters, full-length transcripts are generated by unknown mechanisms from one large ‘variable’ exon, encoding the individual protocadherin, and from three constant exons that code for a family-specific cytoplasmic domain (Frank and Kemler, 2002). Multiple variable first exons of the clustered pcdh genes are alternatively spliced (Wu et al., 2001). Similar to that of the immunoglobulin (*Ig*) and T-cell receptor (*Tcr*) gene clusters, the α and γ clusters of pcdh have also variable and “constant” genomic organization (Wu and Maniatis, 1999).

The phylogenetic analysis of the EC domains reveals that the three subfamilies are closely related and indicates that they are likely to have evolved from multiple gene duplications (Frank and Kemler, 2002). Among these subfamilies, the protocadherins differ in their EC domains but share an identical cytoplasmic domain, which offers each family a multitude of possible extracellular interactions (Wu and Maniatis, 1999). These unique features of clustered protocadherins may provide the molecular basis for generating individual cellular diversity and the complex neural circuitry of the CNS (see below).

1.3.2.1.2. Non-clustered protocadherins or δ-protocadherins

The δ-protocadherin family is comprised of nine protocadherins (Pcdh-1, -7, -8, -9, -10, -11, -17, -18, and -19) with six or seven EC-domains (Hirano et al., 2003; Redies et al., 2005). The amino acid sequences of their cytoplasmic tails show similarities in that they contain conserved (CM1 and CM2) motifs (Hirano et al., 2003; Redies et al., 2005). This family is again subdivided into two subfamilies, based on their structure and phylogenetic analysis (see Fig. 2). The first subfamily (δ1-protocadherins) is characterized by seven EC-domains. In addition to the CM1 and CM2 motifs, the cytoplasmic domain of δ1-protocadherins contains a small but highly conserved motif (RRVTF), which is also termed CM3 motif. This RRVTF motif is necessary for the binding of δ1-protocadherins to protein phosphatase-1α (PP1α). The remaining members of the δ-protocadherins (Pcdh-8, -10, -17, -18 and -19) are now called δ2-protocadherins (Hirano et al., 2003; Redies et al., 2005). Unlike δ1-protocadherins, all δ2-protocadherins have six extracellular domains. At the genomic level, the δ-protocadherins share several features that discriminate them both from genes encoding classic cadherins and from genes encoding clustered protocadherins. They are expressed as multiple splice forms, differing mostly in their cytoplasmic domains. These genes encode no variable extracellular domains, resulting in only small variations in the extracellular gene products. Moreover, due to the presence of conserved motifs (CM1-CM3) in the cytoplasmic domains, individual δ-protocadherins might play important roles in signaling pathways, as they bind to proteins such as TAF1/Set, PP1α, and the Frizzled 7 receptor (see below).

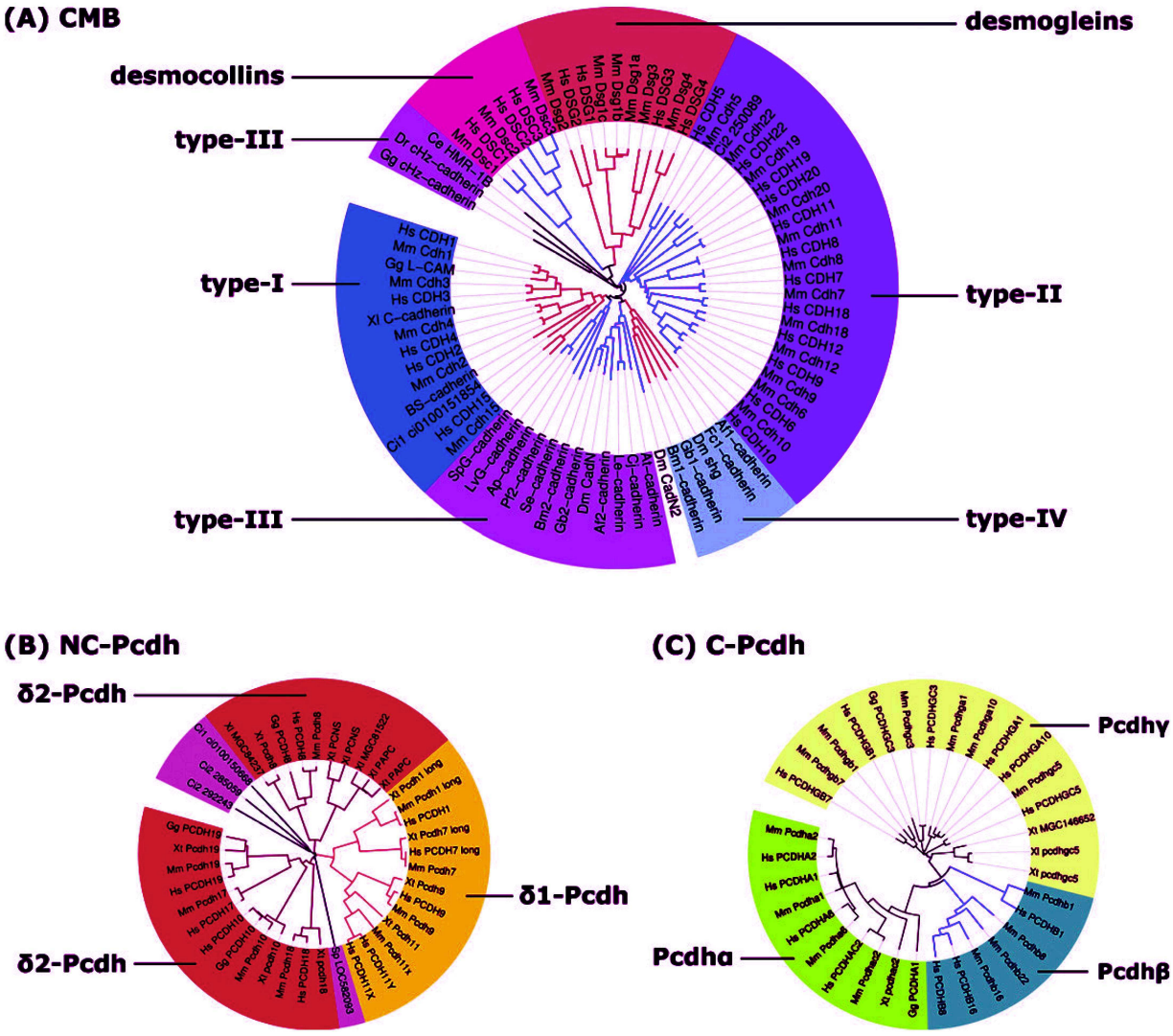

Figure 2. Circular phylogenetic trees of selected members of the cadherin superfamily on the basis of homologies in cytoplasmic domains of comparable length and sequence composition. (A) Members of the major cadherin molecules with comparable cytoplasmic domains; (B) members of the non-clustered protocadherins; (C) members of the clustered protocadherins. Reproduced from (Hulpiau and van Roy, 2008).

1.4. Interactive partners and roles

Cadherins can pass information and are involved in intracellular signaling by interacting with a complex network of cytoskeletal and signaling molecules. There are a large number of partner molecules that participate in cadherin-based adhesion (Hirano et al., 2003; Redies et al., 2005; see Fig. 3). Cadherin regulation is achieved not only through transcriptional control, but also through processes such as tyrosine phosphorylation of intracellular partner molecules. In the regulation of cadherin-mediated adhesion, signaling runs in the inside-out direction from the signaling molecule to the cadherin. The cytoplasmic domain of many cadherins interacts with a group of proteins called catenins;

for example, the binding of classic cadherins to catenins is a critical feature of cadherin-mediated adhesion and other functions (Huber et al., 1996; Hirano et al., 2003; Redies et al., 2005). The catenins interact, both directly and indirectly, with other signaling molecules that influence the state of the actin-based cytoskeleton. These pathways play roles in basic cellular processes, such as migration, neural induction, determination of the neural crest, brain patterning, apoptosis and cell proliferation (see below; Barth et al., 1997).

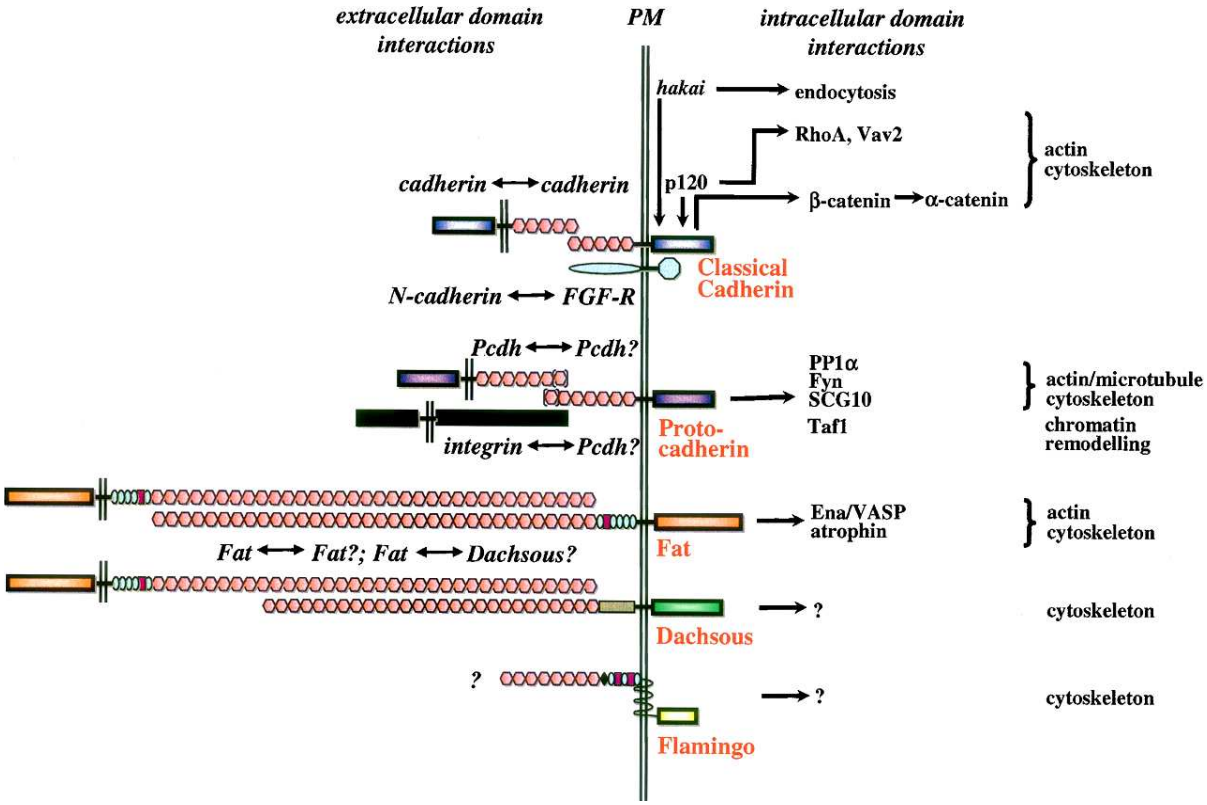

Figure 3. Cadherin-binding partners. Cadherins interact intracellularly with the catenins p120 and Hakai, which regulate the actin cytoskeleton and cadherin endocytosis. Pcdh-αs also interact with the Fyn tyrosine kinase, Reelin receptor, and integrins in vitro. The cytoplasmic domain of Fat interacts directly with Atrophin transcriptional corepressors and members of the Ena/Vasp family of actin regulators. Ds and Fmi may be linked to the cytoskeleton. Interactions that have not been shown definitively in vivo are indicated with a question mark. Brackets indicate that protocadherins have either six or seven ECs. Reproduced from (Halbleib and Nelson, 2006).

Cadherin-mediated cell adhesion activates the Rho family GTPases, is involved in Wnt signaling, and functions in receptor tyrosine kinase signaling. Rho small GTPases are key regulators of the actin and microtubule cytoskeleton. They play a role as a mediator of cadherin-signaling to coordinate the dynamics of cell-cell adhesion, and are involved in various dynamic cellular processes such as epithelio-mesenchymal transition, cell motility, membrane ruffling and neurite retraction through many effectors (Kuroda et al., 1998;

Fukata et al., 1999; Kaibuchi et al., 1999; Vasioukhin et al., 2000). Wnt signaling is important in cell fate decisions and patterning during development. Phosphorylation/dephosphorylation of cadherins and cadherin-associated molecules is a key regulatory event for proper cadherin function. Major targets of kinases/phosphatases are the tyrosine residues of the armadillo family proteins, including β-catenin, δ-catenin and p120ctn. For example, VE-cadherin has multiple possible cytoplasmic binding partners; it binds to β-catenin and thus allows a link to α-catenin and the actin filament network and a connection to the Wnt signaling pathway. In addition, it interacts with p120ctn, another member of the armadillo family, which includes β-catenin (Yap et al., 1998). Interestingly, VE-cadherin can also bind to PG (plakoglobin), which in turn binds and recruits the desmosomal plaque protein desmoplakin (DP) to the cell surface. VE-cadherin plays an important role in vasculogenesis and vascular remodeling (see below).

Several recent reports have made it clear that unlike classic cadherins, protocadherins are potentially more involved in intracellular signaling than in strong homotypic cell adhesion. Most of the cytoplasmic binding partners are members of well-known signaling pathways. For example, as mentioned above, the CM3 consensus motif of δ1-protocadherins has been reported to interact with protein phosphatase-1α (PP1α; Vanhalst et al., 2003). Protocadherin-7 was found to interact intracellularly with TAF1/Set [TATA-binding protein (TBP)-associated factor-1], a histone-binding protein promoting transcriptional access to chromatin. This molecule is also involved in ectoderm formation in the early *Xenopus laevis* embryo. Earlier, TAF1/Set has been shown to modulate Cdk5 activity during neuronal differentiation, neurocytoskeleton dynamics, and neuronal degeneration and apoptosis (Qu et al., 2002). Recently, protocadherin-8 was shown to interact with the Frizzled 7 receptor. Intracellularly, Pcdh8 and the Frizzled receptor synergistically activate the small GTPase RhoA, Jun N-terminal kinase (JNK) and protein kinase C (PKC), leading to tissue separation during the convergence-extension process of *Xenopus* embryos (Medina et al, 2004). Another family of clustered protocadherins, pcdh-α interacts intracellularly with the tyrosine kinase Fyn, a key molecule in regulation of synaptic plasticity and long-term potentiation (LTP; Kohmura et al., 1998). The number of cadherin-associated intracellular signaling molecules is continuing to grow as the signal transduction pathways involving cadherin superfamily molecules are studied in greater detail.

1.5. Structural uniqueness and domains

As discussed earlier, cadherins consist of an ectodomain, a transmembrane domain, and a cytoplasmic domain that interacts with signaling factors and the cytoskeleton, but the number of tandemly repeated extracellular (EC) domains varies considerably between the members. Each EC domain is composed of about 110 amino acids and consists of multiple, highly conserved amino acids. The EC domain contains negatively charged DXD, DRE, and DXNDNAPXF sequence motifs, which are involved in Ca^{2+} binding (Takeichi, 1990). The tryptophan residues present in the EC1 domain are responsible for homophilic adhesion and for *trans*-cadherin binding (Nose et al., 1988; Chen et al., 2005; Patel et al., 2006), although heterophilic interactions between classic cadherins have also been recently reported (Shimoyama et al., 2000; Niessen and Gumbiner, 2002; Foty and Steinberg 2005). Other extracellular domains of different cadherin subfamily members specify interactions with other binding partners, which translate into unique functionality. Although the presence of the EC domains is the hallmark of cadherins, the amino acid sequences of other parts, particularly the cytoplasmic domain, diverge significantly among the subfamilies, suggesting that their functional diversification might have occurred during evolution.

The multiple functions of cadherins, which include adhesion, selectivity, clustering, and signaling, are attributed to the extracellular and cytoplasmic domains (see below). Signaling of cadherin is localized to the cytoplasmic domain, whereas adhesion and selectivity map is attributed to the extracellular region. The EC1 domain at the N terminus determines binding specificity. The sequence homology of the different domains of cadherin is 50%-60%. The current notion is that cadherin molecules mediate cell adhesion by first joining with cadherins on the same cell surface to form lateral or *cis* dimers. Later, they in turn adhere to dimers on an adjacent cell to form *trans* adhesive bonds (Nose et al., 1988; Chen et al., 2005; Patel et al., 2006).

However, even this seemingly simple cell adhesion process of cadherin involves a hierarchy of binding events through its various extracellular domains that each contributes to its binding selectivity and adhesion strength. The complexity of cadherin adhesion is not determined by a simple mechanism, in which a single binding interaction decides the adhesion strength, selectivity, and the ability to rapidly remodel tissue interfaces. Cadherin functioning requires the binding Ca^{2+} ions to rigidify their protein structure, activate the adhesive activity, and to confer protease resistance. Each extracellular domain has three calcium ion-binding sites. A total of 12 calcium ion-binding sites have an impact both on global cadherin architecture and adhesive function. Cadherin proteins undergo staged

changes in its global architecture and structure while calcium ions binding to the different sites (Pokutta et al., 1994).

1.6. Role of cadherins

1.6.1. General functions

In the three decades since their discovery, the functions of cadherins have been extended from cell-cell adhesion to multiple aspects of tissue morphogenesis, including cell recognition, cell sorting, cell survival, formation of intercellular junctions, maintenance of tissue integrity and tumorigenesis, tissue boundary formation and maintenance, coordinated cell movements, and the induction and maintenance of structural and functional cell and tissue polarity (Redies, 1995; 1997; 2000; Gumbiner, 2002; Hirano et al., 2003; Redies et al., 2005; see Figs. 4 and 5). Cadherins are also involved in the formation and maintenance of various types of tissues and organs ranging from polarization of simple epithelia, to connecting hair cells in the cochlea mechanically, to providing an adhesive code for the formation of neural circuitry during the wiring of the CNS (Yagi and Takeichi, 2000; Gumbiner, 2005). In addition, as a result of interaction of cadherin molecules with various intracellular signaling molecules and pathways, cadherins are also involved in many basic cellular processes such as cell locomotion, proliferation and differentiation (Gumbiner, 2002; Hirano et al., 2003; Redies et al., 2005). In principle, cadherins preferentially bind to another molecule of the same subtype. When cells expressing one subtype of cadherin are mixed with cells expressing other subtypes of cadherin, they tend to aggregate separately. This result shows that cells tend to adhere preferentially to cells expressing the identical cadherin type ("homotypic" adhesion), and suggests that cadherins possess binding specificity (Nose et al., 1988; Miyatani et al., 1989; Hirano et al., 2003; Redies et al., 2005).

The expression patterns of different cadherin subtypes greatly overlap with each other, suggesting that cadherin expression is functionally redundant. In general, it should be noted that a single cell type can expresses multiple cadherin subtypes. It is possible that each cell might express more complex combinations of cadherins molecules than known to date. So, a possible combinatory role of multiple cadherins is likely to be important in the determination of adhesive specificity of cells in vivo (Redies et al., 2005).

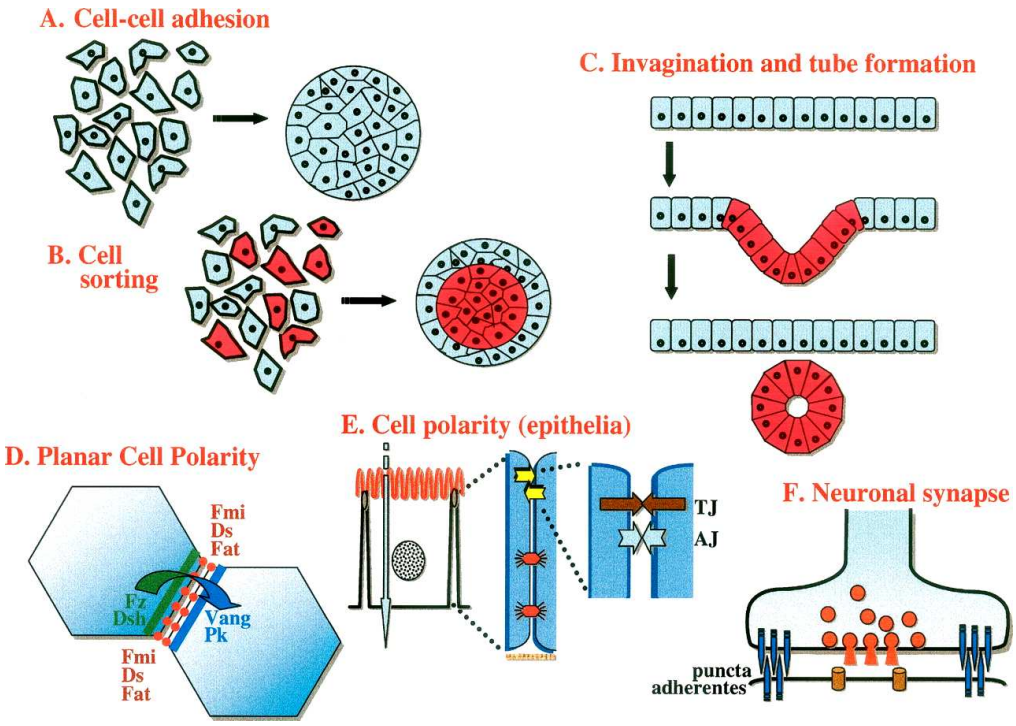

Figure 4. General functions of cadherins. (A) Cadherins mediate cell adhesion. (B) Cell sorting by differential expression of cadherins. (C) Cadherin subtype switching from one cadherin to another such as during neurulation leading to the invagination of the neural tube. (D) Cadherins function in planar cell polarity. (E) Classical cadherins have specialized distributions at the adherens junction (light-blue arrows) of polarized epithelia. (F) At puncta adherentes (blue arrows), cadherins surround the active zone at the neuronal synapse. Reproduced from (Halbleib and Nelson, 2006).

During embryonic development, cadherins mediate the separation of distinct tissue layers or the fusion of related tissues and the formation of tissue boundaries, conformational changes in the tissue leading to cell rearrangements, the conversions between histological cell states, and the long-range migration of cells. In adult tissues, cadherins are known to have a role in controling the orderly turnover of rapidly growing tissues, for example, the lining of the gut and the epidermis, the plasticity and regulation of neuronal synapses, the physiological regulation of cell junctions in epithelial and endothelial cells to allow controlled passage of nutrients to the cells, and the maintenance of stable tissue organization to prevent the dissociation and metastasis of tumor cells. In support of these roles for cadherins, it has been often experimentally demonstrated that each cadherin subset is expressed by specific cell types and tissues at a specific stage of development (Takeichi, 1991).

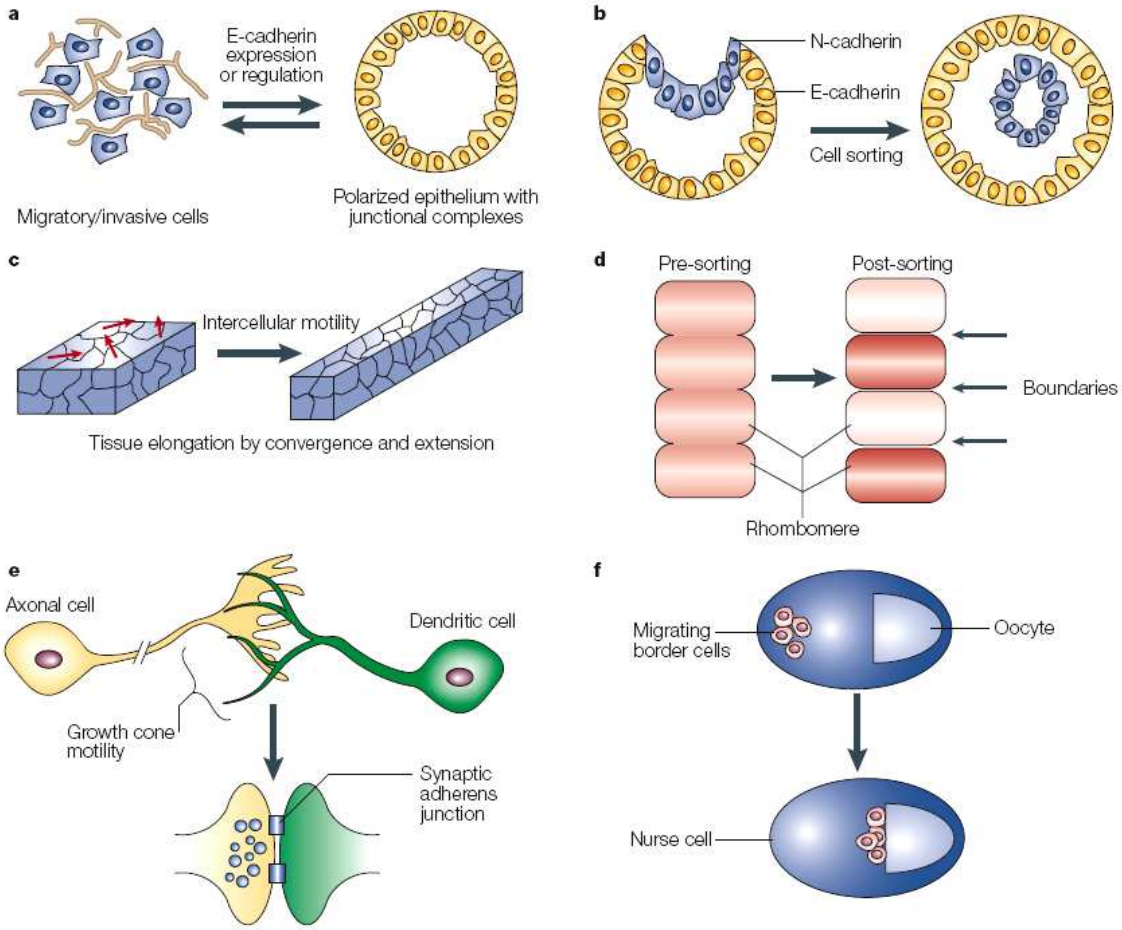

Figure 5. General functions of cadherins. **a**. Reversible epithelial–mesenchymal transition (EMT) by E-cadherin during tumour cell invasion and metastasis. The yellow cells express E-cadherin, in contrast to the blue cells that do not. **b.** Cell sorting owing to differential expression of N-cadherin (blue) in the presumptive neural epithelium allows it to separate from the E-cadherin-expressing ectoderm (yellow). **c**. Cell rearrangements cause the tissue to narrow and elongate. **d.** Formation of compartment boundaries in rhombomeres in the developing vertebrate hindbrain. **e.** Growth cone motility and synapse formation. **f.** Cell migration; the border cells (pink) migrate from one end of the egg chamber between the nurse cells until they reach the oocyte. Reproduced from (Gumbiner, 2005).

1.6.2. Function in cell sorting

The cadherin superfamily expression mediates selective cell recognition responsible for the sorting of different groups of cells in the developing tissues. Cell sorting can be attributed to the homophilic binding specificity of the extracellular domain, as the identity of the EC1 domain determines cell-sorting specificity when experimentally transferred from one cadherin to another. Homophilic cell adhesion favours cells to assort according to predetermined patterns, establishing areas, patches and boundaries eventually leading to organogenesis (Hirano et al., 2003; Redies et al., 2005). The level of cadherin expression

and the overall adhesion strength have been found to strongly influence cell-sorting behaviour, irrespective of the type of cadherin expressed. For each subtype of classic cadherin, the expression level is at a maximum in distinct tissue types during development. For example, E-, N-, R- and VE-cadherin are expressed in the epithelial cells, neural tissue and muscle, forebrain and bone, and endothelial cells, respectively (Takeichi, 1988; Hirano et al., 2003). However, as multiple cadherins are expressed by each cell types, the combinatorial property of cadherin expression that controls cell-sorting behaviour has not yet been identified. For example, during the development of the spinal cord, spinal motor-neuron cell bodies express several classic cadherins and sort into different functional groups leading to various functional areas.

1.6.3. Function in cell migration

Cadherins have also been shown to be involved in the regulation of cell migration, though cadherins are often thought to mediate only stable cell interactions, such as in the adherens junctions and the desmosomes of epithelial cells. For example, in gastrulating *X. laevis* embryos the convergence and extension of tissue movements that underlie the elongation of the body axis is attributed to the regulation of C-cadherin-mediated adhesion in response to growth factors and fibronectin. Another example is the migration of neural crest cells from the neural tube in early embryonic development. During neural crest cell migration, cadherin-7 replaces N-cadherin and cadherin-6B to favour the emigration process, which is inhibited when this expression pattern is altered (Nakagawa and Takeichi, 1998). Arterial wound healing requires the upregulation of N-cadherin expression, a process specific to cell migration, which can be impaired by the N-cadherin inhibiting antibodies (Jones et al., 2002). Similarly, N-cadherin has also been reported to play a role in growth cone motility, and interestingly E-cadherin, which is usually thought to involve mainly in epithelial junctions, mediates the long-range migration of border cells in the developing Drosophila ovary. The continuous breaking and reforming of C-cadherin adhesive bonds drives the convergence and extension movements involving local rearrangements of cells with respect to adjoining cells. Less is known about the dynamics of adhesion that undergo ordered movements and turnover in adult tissues, but E-cadherin certainly has a major role in the homeostasis and turnover of tissues such as the intestinal lining and the epidermis (Hermiston et al., 1996; Tinkle et al., 2004). Therefore, the migrating cells require the dynamic regulation of cadherin adhesions to break and remake adhesive bonds continually to change cell neighbours. This process of cadherin-mediated cell migration is analogous to the integrin-mediated cell migration in the extracellular matrix called 'intercellular motility'.

For example, during Zebrafish epiboly, though epiblasts could intercalate but are not stabilized in the external layer, thus often deintercalate, if the E-cadherin expression is disrupted (Kane et al., 2005; Shimizu et al., 2005).

1.6.4. Function in cell signaling

Recent studies focusing on cellular signaling have shown that several pathways are activated by cadherin-mediated cell-cell adhesion inferring additional signaling roles for cadherins in morphogenesis (Gumbiner, 2002; 2005; Hirano et al., 2003). Cadherins themselves participate in transducing extracellular signals to the cell interior apart from cadherins being acted upon by signaling events to regulate cadherin adhesive function. The inter-relationships between cadherins and cell signaling fall in two categories. (1) Signaling events regulating cadherin adhesive function. (2) Cadherins themselves participating in transducing extracellular signals to the cell. The capacity for cadherin function to be regulated by cell signaling was first raised by the observation that many proteins of the cadherin-catenin complex are subject to post-translational modification, especially to protein tyrosine phosphorylation (Daniel and Reynolds, 1997).

The different hypotheses that suggest how cadherins could be involved in cell signaling events are as follows: First, cadherins will bring the opposing membranes of neighboring cells in close proximity through their homophilic binding, and enable transmembrane or membrane-associated ligands to interact with membrane-bound receptors on opposing cell and stimulate juxtacrine signaling (Hirano et al., 2003). This is achieved by the association of several signaling molecules with the cadherin-catenin complex and by the high concentration of substrates for tyrosine kinases at the adherens junction. Second, as cadherins can control the polarization of cells, they might also affect signaling through their influence on the cellular distribution of membrane proteins such as transmembrane receptors. Finally, cadherins can exert direct signaling activity by directly behaving as ligands or receptors themselves in cells.

Cytoplasmic tail of the cadherins binding to intracellular signaling partners, serve to link the cadherin to the actin cytoskeleton leading to cellular signaling. Generally, cadherin-mediated cell adhesion activate the Rho family GTPases, regulate the β-catenin participation in Wnt signaling, and also has a role in receptor tyrosine kinase signaling, as discussed earlier. Cadherin function can potentially be regulated by a diverse range of other cell signals (Gumbiner, 2000). These include ubiquitination of the E-cadherin cytoplasmic tail (Fujita et al., 2002), which may regulate cadherin endocytosis, as well as signaling by

small GTPases of the Rho family (Kaibuchi et al., 1999), which influence cadherin-actin cooperativity. N-Cadherin can regulate axon outgrowth by direct interaction with the EGF and FGFR-1 receptors, thereby activating the cascade of mitogen-activated protein kinases (MAPK). In addition, E-cadherin can stimulate MAPK by ligand-independent activation of EGF receptors. It also activates Cdc42, a low-molecular-weight GTPase belonging to the Rho family, which regulates the cytoskeleton structure. Although the molecular organization of the cadherin/partner complexes appear to be similar in different cell contexts, it is becoming increasingly clear that cells receive different information from different cadherin subtypes and also processes the information specifically depending upon the cell type.

1.7. Cadherins in the central nervous system

1.7.1. General role in neural development

The human brain possesses approximately 10 billion neuronal cells that are selectively and specifically interconnected by neural circuits to control cognitive and other vital functions. One of the intriguing challenges faced by modern neurobiology is to understand the intricate network of neuronal connections established during embryonic development and modulated in the adult. These complex neuronal interactions with their intended targets and recognition are predicted to be mediated primarily by cell surface proteins, defined as cell-cell adhesion molecules, the primary functions of which is to bring the apposed cell membranes into contact via their homophilic or heterophilic interactions. Recent studies show that a variety of cell-cell adhesion molecules of the cadherin superfamily are abundantly expressed in the developing CNS (Redies, 1995; 1997; 2000; Hirano et al., 2003). More than 100 cadherins and cadherin-like molecules that have now been identified in the nervous system potentially allow for a myriad of interactions to regulate neuronal specificity and functions (see below). The molecular properties of cadherins and their function in cadherin-based adhesion systems also suggest that cadherins are potentially involved in multiple aspects of cellular behavior during development. The preferential homotypic adhesion specificity can provide an “adhesive code” that can account for various aspects of neural morphogenesis (Hirano et al., 2003; Redies et al., 2005).

During early vertebrate development, cadherins are implicated in multiple events of brain morphogenesis such as the formation of the neuroepithelium and its maintenance, neurite extension and migration of neuronal cells. Cadherins play a role in the invertebrate

nervous system also, by mediating important roles in wiring among neurons. Moreover, recent studies have offered valuable insights into the functions of cadherins in the formation of complex, diversified and organized networks in the vertebrate central nervous system (Redies and Takeichi, 1996; Redies, 1997; 2000; Takeichi, 2007; Suzuki and Takeichi, 2008). In addition, recent studies have added many novel members of the cadherin superfamily, like protocadherin subtypes, which are also found to be involved in various aspects of neural developmental mechanisms. The protocadherin diversity is suggested as a possible explanation for complex neuronal connectivity. The diversity of these cadherins and its complex molecular structures also suggest that they regulate the contacts or signaling between neurons in a variety of complicated ways. Developing neurons extend dendrites leading to formation of the complex arborizations which in turn form the dendritic fields (specific for each neuronal type), attributed to the homotypic binding specificity of cadherin adhesion. Cadherins-mediated attractive and repulsive cues may drive axonal growth cones to migrate towards their target neurons and eventually to make connections with them to form synapses, in a process which might require elaborate specific recognition mechanisms to ensure their correct pairing (see below; Suzuki et al., 1997; Tang et al., 1998; Honjo et al., 2000; Manabe et al., 2000; Tanaka et al., 2000). In the following sections, I will discuss the role of cadherins in the development of nervous system and its maintenance in the following processes: (1) Regionalization and boundary formation, (2) neural circuit formation, and (3) synaptogenesis.

1.7.2. Regionalization and boundary formation

Most of the cadherin superfamily molecules analyzed so far in the CNS are expressed in a regionally restricted fashion (spatio-temporally) and are expressed at different levels (qualitatively and quantitatively) during nervous system development. An important aspect of neurogenesis, in which cadherins show a unique expression pattern and play a fundamental role, is the regionalization of the neural tube. The embryonic neural tube is subdivided into a defined set of longitudinal and transverse subdivisions as a result of early embryonic pattern formation. At the gross anatomical level, bulges and constrictions appear and eventually produce different brain regions including cell populations, which rearrange themselves to form different regions, and neuroepithelial cells differentiate into numerous types of neurons and glial cells at the tissue and cellular level. Expression of specific combination of various gene regulatory proteins and morphogenetic molecules (Redies and Takeichi, 1996) characterizes the different regions of the brain. Various cadherins, for

example E-cadherin, R-cadherin, cadherin-6, cadherin-8, are expressed in the different structural regions of the developing brain like the neuromers. From these studies, it is now quite conceivable that intercellular adhesion dictates the regionalization of brain. The different sets of cadherins might actually lead to the boundaries by establishing the gross structural and functional borders between the regions in the developing brain. Moreover, localization of specific classic cadherins to different regions of the embryonic brain and peripheral nervous system have been reported in various studies (Hirano et al., 2003), and its disruption leading to cell mixing at domain boundaries (Stoykova et al., 1997; Inoue et al., 2001). There are many recent studies showing that multiple cadherins are expressed in one brain region that receives information from (Arndt et al., 1998; Redies et al., 2000) or sends information to regions expressing the same set of cadherins (Redies et al., 1993; Wohrn et al., 1999).

1.7.3. Neural circuit formation (Growth cone development, axon guidance and target recognition)

During the embryonic development, neuronal circuits are formed when neurons extend processes that navigate along stereotyped pathways to their synaptic targets. The ability of receptors on neuronal growth cones to recognize and selectively respond to cues in their environment is one of the bases of axon navigation and target selection (Goodman and Shatz, 1993; Goodman, 1996). Neurons respond with directional movements to both soluble factors and membrane-bound molecules such as cell adhesion molecules. In particular, growth cones form transient adhesive contacts with molecules in their environment during axon extension. The positive (permissive or attractive), negative (inhibitory or repulsive), or guiding (affecting the advance of the growth cone) signals from the extracellular space through adhesive molecules such as cadherins lead to the initiation and guidance of a neurite. The signals might also arise from the surface of other cells, or be diffusible secreted factors. Cadherins are known to have prominent expression and localization in the concerned areas. Particularly, the combinatorial cadherin expression pattern correlates with the organization of neurons into functional circuits. Based on this, I briefly describe growth cone development, axon guidance and target recognition signaling cues focusing on the role of cadherin molecules.

Growth cones are located at the tip of the growing axons and sense its target. Cadherin expression in the growth cones are reported to positively influence the process of axonal guidance (Letourneu et al., 1990; Benson and Tanaka, 1998). Distinct cadherins,

often multiple subtypes, are known to be expressed by axons of different origin during axon growth and target selection. Axons expressing multiple cadherin molecules make contact with cells positive for the same cadherin set. Thus, specific cadherin combinations in an individual neuron may provide neuronal specificity by restricting interactions to neurons/cells expressing an identical set of cadherins. Many cadherins studied so far are expressed during active neurite outgrowth, for example, when fiber tracts are formed in the CNS. Typically, when neurons differentiate and send out their processes, there is an upregulation of cadherin expression by nerve cells (Matsunaga et al., 1988; Shimamura et al., 1992; Gänzler and Redies, 1995). N- and R-cadherin are known to provide positive signals that are also experimentally demonstrated to be the permissive substrates for growth cone navigation (Letourneau et al., 1990; Arndt and Redies, 1996), while cadherin-11 promotes axon elongation (Marthiens et al., 2005) and cadherin-13 acts as a repellant cue for growth cones (Fredette et al., 1996). In Drosophila, flamingo cadherins are expressed by growth cones of R1-R6 axons; these molecules are required to select appropriate targets in the visual system. Cadherin expression by glia at boundaries may also provide cues for axonal navigation, since some early fiber systems elongate preferentially within or along these boundaries (Wilson et al., 1993). It is conceivable that neurites expressing a particular cadherin combination approach or cross boundaries that express the same set of cadherin, whereas they avoid or repel borders expressing other cadherins. Thus, the expression of cadherins by cells such as glial cells, by neurite fascicles, by specific neuronal layers and by specific target brain nuclei along the path of the axon are believed to guide the axons by stimulating growth in a specific direction.

Cadherins are also known to mediate the formation of neural circuits by regulating axon fasciculation and targeting during growth cone navigation and pathfinding. The homotypic binding specificities of cadherins lead to the precise establishment of neural circuitry where it is necessary that growing axons recognize their partner neurons in the appropriate target areas. It is reasonable to assume that the preferentially homotypic binding mechanisms mediated by cadherin subtypes might play a major role in axonal target recognition, as cadherins are not only expressed by growing neurites but also by their target areas. Interestingly, it has been shown in previous studies that a given cadherin is often not just expressed by a subset of nerve fascicles, but also by their target area (Redies et al., 1993; Arndt and Redies, 1996). For example, R-cadherin is expressed by specific neural circuits within the visual system, the motor system, and several other functional systems of the embryonic chicken brain (Arndt and Redies, 1996). There is also some evidence that

cadherins function as axonal targeting molecules in both the chick and Drosophila, as the disruption of N-cadherin adhesion leading to mistargeting of axons in the visual systems of both organisms (Inoue and Sanes, 1997; Lee et al., 2001).

1.7.4. Synaptogenesis

Synaptogenesis occurs at the specialized adhesion site between neurons, where signals are transmitted from the presynaptic neuron to the postsynaptic neuron. It is the final step in the establishment of functional connections between neurons. There is increasing evidence for the role of cadherin members in the formation of individual synapses. By virtue of a number of properties attributed to cadherins, which are enriched at synapses, including homotypic specificity and recognition and molecular diversity, cadherins have been implicated in the generation of synaptic specificity (Redies, 1997; Obst-Pernberg and Redies, 1999). Synaptogenesis occurs during the transformation of growth cones or axonal surface membranes when they come into contact with their target. The role of cadherins in synaptogenesis might extend beyond synapse formation as it also involves remodeling the synaptic architecture and modifying the strength of the synaptic signal, leading to an active role in synaptic structure, function, and plasticity. In addition, the expression of each cadherin is restricted to a subset of synapses, even during their formation, suggesting that cadherin expression contributes to the determination of synaptic specificity. Cadherin expression and localization are actively regulated during the formation of the synapse. Synaptic contacts are very similar to adherens junctions and several cadherins such as N-, E-, R-cadherin, cadherin-7, -11 and protocadherins, CNR, γ-protocadherins, protocadherin-8 and -10 have been found to be associated with the synapse. Among these, at least N-cadherin, R-cadherin and cadherin-7 are localized at the region adjacent to the active, transmitter-releasing zone of the synapse. The cadherin/catenin system is an integral component of the synaptic complex and two of the intracellular molecules connected to cadherins in the CNS, αN- and β-catenin, are also widely expressed and found in the synaptic complex of mouse and chicken (Uchida et al., 1996). N-cadherin is the first protein to localize the most nascent synapses, and is an important component of the NMDA receptor multiprotein complex (Fannon and Colman, 1996; Uchida et al., 1996; Benson and Tanaka 1998). NMDA receptor activation is critically required for synaptic plasticity as it provides the strength for adhesion across the synaptic cleft through N-cadherin. N-cadherin is upregulated during synaptogenesis when the barrel structures of the thalamic projections to the somatosensory cortex are formed and is downregulated after the establishment of this

wiring. N-cadherin is also known for its localization at synapses in the early phase of hippocampal development and may mediate the induction of long-term potentiation (LTP) of hippocampal synaptic strength, a cellular model for learning and memory. During LTP, N-cadherin is synthesized and recruited to newly forming synaptic junctions; changes in N-cadherin expression may lead to augmented adhesive force, which can be prevented when the expression is blocked. Since cell adhesion molecules such as Igs, cadherins and integrins, are localized at mature synapses, it has been speculated that adhesive mechanisms are involved not only in the formation of synapses but also in their plasticity. If typical homotypic cadherin interactions play a pivotal role in synapse formation, we might expect to find the same cadherin pre- and postsynaptically in each synapse (Fannon and Colman, 1996; Uchida et al., 1996).

1.8. Aims of the present study

1.8.1. Corticoneurogenesis

The formation and organization of mammalian neocortex into six layers is a highly orchestrated process and one of its most salient anatomical features. Each layer possesses a distinct function in cortical information processing. The function of the cortical neural networks in higher cognition depends on the exact positioning of different classes of neurons in a radial array of six cortical layers extending from the pial surface to the white matter. The different layers receive and send projections to specific targets in and out of the cerebral cortex (Marin-Padilla, 1998). The infragranular layers (V and VI) develop first and project to sub-cortical targets. The supragranular layers (II and III) develop later and project primarily to other regions in cortex (Angevine and Sidman, 1961; Jones 1981; Caviness et al., 1995; Anderson et al., 1997). The layers of neocortex are generated by the sequential migration of developing neurons. Neuroepithelial cells that are born in the ventricular and subventricular zones after mitosis leave their birthplace and are guided pialward along radial glial fibers, passing the subplate. The earliest-born neurons form the preplate. Later-born neurons migrate into the preplate to form the cortical plate that splits the preplate into the marginal zone (prospective layer I) and the subplate. The six different layers are formed in an inside-out fashion in the neocortex when more neurons migrate and arrive in the cortical plate (Angevine and Sidman, 1961; Rakic, 1988; Caviness and Takahashi, 1995; Caviness et al., 1995; Rakic and Caviness, 1995; Bolz and Castellani, 1997; Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Job and Tan, 2003; Kriegstein and Noctor, 2004).

Several cell surface molecules that are expressed in specific layers of the cortex were proposed as candidate determinants of layer-specific circuit formation and targeting (Bolz et al, 1996; Castellani and Bolz, 1996; 1997; Donoghue and Rakic, 1999; Molyneux et al. 2007). However, the precise roles of most of these molecules *in vivo* remain to be determined experimentally. Also, the diversity of neuron types in each layer is vast and the available markers do not cover all neuron types with restricted laminar distribution (Funatsu at al., 2004; Guillemot et al., 2006; Molnar et al, 2006; Mühlfriedel et al., 2007). There is some evidence suggesting that at least the initial partition of the cortex into discrete layers is regulated by cadherin-mediated adhesion. In an attempt to identify additional markers for cortical regions, layers and neuron subtypes, we focussed on cadherins in the present study. Many of the cadherin molecules are expressed predominantly in particular layers of the developing cortex. Typically, the laminar-specific distribution of cadherins changes from region to region within cortex. Some cadherins have been widely used as regional markers for developing cerebral cortex in genetically altered mice (cad6, cad8, cad11; Rubenstein et al., 1999; Gilmore and Herrup, 1997; 2000).

All these previous studies on caderin expression in cerebral cortex have focussed on single or a few cadherins, often only at specific stages of cortical development and in particular cortical areas. Here, I map systematically the expression of 16 novel members of two subgroups of cadherins, classic cadherins and δ-protocadherins, by in situ hybridization in the developing primary visual cortex (V1) of the ferret, which serves as a model for cortical development.

1.8.2. Cerebrovascular development

During evolution, organisms are required to perform more specialized tasks, demanding an increased level of information processing by neurons and supply of nutrients by blood vessels. Thus, one of the crucial requirements of embryogenesis is the development of a functional vascular system. Vascular development supports the concomitant brain development by providing the metabolic needs of growing neuronal populations. During embryogenesis, the two consecutive and distinct mechanisms that implement the formation of the vascular network are vasculogenesis and angiogenesis. Vasculogenesis is the migration and in situ differentiation of angioblasts (endothelial precursor cells; EPCs) and formation of primitive vascular plexus in response to local cues. Angiogenesis is achieved by the pruning, remodeling and extension of existing primary vascular trees. The brain is one of the unique organs that are exclusively vascularised by angiogenesis which starts at

the beginning of corticogenesis and actively proceeds up to the last waves of neuronal migration (Bär, 1980; Risau, 1993). Angiogenesis and vasculogenesis are affected by various factors in an autocrine or paracrine manner, such as cell surface receptors and cell adhesion molecules. During vascular development, endothelial cells must adhere firmly to one another by adhesive molecules to form intact and fully functional blood vessels. The molecular regulators of these distinct mechanisms have been discussed in various studies, for example the role of specific cell adhesion molecules, such as CD34, PECAM, VE-cadherin, N-cadherin, cadherin-10, T-cadherin, R-cadherin and protocadherin-1 (Breier et al., 1996; Williams et al., 2005; Redies et al., 2008, Cavallaro et al., 2006). Also, Tie-1, Tie-2, Eph, VEGR-1, and SCL/tal-1 are involved in the subsequent events of angiogenesis.

In general, cadherins play versatile roles in vascular development (see above). Gene silencing studies revealed that functional knock-down of VE-cadherin and N-cadherin in mice leads to early embryonic death with associated severe vascular anomalies (Dejana et al., 1999). Possibly, cadherin function varies in different cell types depending on the cadherin repertoire of the cell. Their prominent role in the maintenance and control of endothelial cell contacts and their essential role during morphogenesis of the vasular system has made cadherins a prime research target among adhesion molecules in the vasculature. Thus, in an attempt to identify additional cadherin markers involved in the development of the vascular system, I focused on the expression of other cadherins during cerebral development in the present study.

However, in the last few years, it has become increasingly evident that cadherins role as the mechanical pro-adhesive activity in the angiogenic cascade extends beyond that, and involves in various aspects of vascular cell biology (Gumbiner, 2005). Besides their adhesive properties, cadherins may act by transferring intracellular signals through interaction with a complex network of cytoskeletal and signaling molecules, as discussed above for neuronal development. Anchoring of the cadherins to the cytoskeleton is generally essential for optimal cadherin-mediated adhesive functions in the endothelial adherens junctions. Phosphorylation of the cadherin-catenin complex has been proposed as a crucial mechanism that regulates the stability of adherens junctions (Daniel and Reynolds, 1997). Endothelial cells express different cadherins during angiogenesis which may transfer specific signals and exert distinct functional roles. For example, the ability of both VE and N-cadherin to interact with signaling molecules such as catenins, VE-PTP, VEGFR, FGF, receptor tyrosine kinases, phosphatases and others, has been implicated in endothelial cell growth, survival, migration and morphogenesis. With the already known set of intracellular

binding partners of cadherins, the newly studied cytoplasmic partner of δ-protocadherins, PP1α, could also have a similar regulatory role in the angiogenesis and neurogenesis.

Furthermore, with regard to its precise wiring in highly orchestrated and stereotyped networks, vascular development closely parallels neuronal development. Generally, the nerve fibres and blood vessels course throughout the body often alongside one another in an orderly pattern. Although superficially distinct, the involvement of common mechanisms in wiring neural and vascular networks can not be ruled out as they seem to share some deep similarities (Carmeliet and Tessier-Lavigne, 2005). Moreover, many of the molecules regulating vascular system development are also implicated in neuronal development, such as VEGFR, FGF, EGF, Eph, N-cadherin, Dlx1/2, Nkx2.1 and Pax6 (Carmeliet, 2003; Cavallaro et al., 2006; Vasudeven et al., 2008). Together, these findings support the notion that there are more shared mechanisms regulating vascular and neuronal morphogenesis than originally expected. Therefore, studying the expression pattern of new cadherins could help us to understand the other molecular cues regulating neurovascular development. My study focused on cadherin expression with the aim of identifying which cadherins are expressed during brain blood vessel formation in relation to the main events of brain development and cerebral corticogenesis.

1.8.3. Study on cerebellum and basal ganglia

In addition to the above-mentioned work, I have also contributed to studies on the expression patterns of cadherins in different areas of CNS, in order to understand the unique expression profile of cadherins and their possible roles. In one such study with our co-workers, we compared the connectivity patterns in the mouse and ferret cerebellar system by mapping the expression of three cadherins (cdh8, pcdh7, and pcdh10) at comparable stages of development, to understand the cerebellar development and morphology. The expression patterns were determined at similar postnatal stages by in situ hybridization and immunostaining in both whole-mount specimens and cryostat sections. We chose intermediate stages of cerebellar development for analysis because any differences in the bauplan or in embryonic pattern formation can be more easily recognized. In another study with our co-workers, we mapped systematically the expression profile of twelve different classic cadherins and protocadherins (Cdh4, Cdh7, Cdh8, Cdh11, Pcdh1, Pcdh7, Pcdh8, Pcdh9, Pcdh10, Pcdh11, Pcdh17 and Pcdh19) in the basal ganglia of the postnatal and adult mouse by in situ hybridization, in order to identify novel candidate molecules regulating the morphogenesis and functional connectivity within the striatum and

other parts of the basal ganglia (globus pallidus and ventral pallidum). We focused on the postnatal day 5 (P5) basal ganglia because, at this stage of development, the striatum has already acquired its mature functional architecture, but neurons are more densely packed than at later stages, facilitating the analysis of cadherin expression patterns by in situ hybridization.

1.9. Ferret as an animal model

The ferret (*Mustela putorius furo*) is a small carnivore, which is a relatively inexpensive, easily maintained laboratory animal with a relatively large brain and a long gestation period. Animals with very short gestational periods, like rodents, are inexpensive and readily available, but cortical neurogenesis occurs at such a rapid pace that gradients of cortical neuron production are much less distinct. In contrast, the ferret's relatively short gestational period (41-42 days), coupled with a protracted (35 days) period of cortical neurogenesis, affords distinct spatiotemporal gradients of neuronal production and an extended period of postnatal cortical neurogenesis. This makes the study easier while following the cadherin expression pattern during development and permits a high temporal resolution of developmental events and stages of growth (McSherry, 1984; Jackson and Hickey, 1985; Atkinson et al., 1989; Jackson et al., 1989).

2. PUBLICATION OVERVIEW

2.1. **Krishna-K**, Nuernberger M, Weth F, Redies C (2008) Layer-specific expression of multiple cadherins in the developing visual cortex (V1) of the ferret. Cerebral Cortex, published online, doi:10.1093/cercor/bhn090.

In this study, we focused on the ferret as the experimental animal model to investigate the expression patterns of multiple classic cadherins and all known δ-protocadherins (CDH4, CDH6, CDH7, CDH8, CDH11, CDH14, CDH20, PCDH1, PCDH7, PCDH8, PCDH9, PCDH10, PCDH11, PCDH17, PCDH18 and PCDH19) in the primary visual cortex (V1) by in situ hybridization and tyramide-coupled FISH. We cloned and identified the partial sequences of all of the above-mentioned cadherin molecules for the first time from ferret. Fifteen out of the sixteen cadherins are expressed in a spatiotemporally restricted fashion throughout development and in the adult ferret. Each layer of V1 can be characterized by the combinatorial expression of a subset of cadherins at any of the 10 developmental stages studied. A few cadherins are expressed by subsets of neurons in specific layers or by neurons dispersed throughout all cortical layers. At the V1/V2 boundary, changes in layer-specific cadherin expression are observed. In conclusion, our results suggest that cadherins provide a code of potentially adhesive cues for layer formation in ferret visual cortex. The persistence of expression in the adult suggests a functional role also in the mature cortex.

Own contribution to the manuscript:

1. RT-PCR from ferret RNA and cDNA isolation
2. Designing of degenerate primer for the ferret cadherins
3. Cloning of different cadherin molecules
4. In vitro transcription and production of digoxigenin/fluorescein/biotin-labeled cRNA probes
5. In situ hybridization and double tyramide-FISH in ferret brain sections
6. Expression profile analysis in the visual cortex
7. Preparation of the manuscript

2.2. **Krishna-K** and Redies C (2008) Expression of cadherin superfamily genes in brain vascular development. Journal of Cerebral Blood Flow and Metabolism, published online, doi: 10.1038/jcbfm.2008.123.

The objective of this study was to investigate the expression profile of various cadherin markers during neurovascular development. I cloned VE-cadherin (CDH5), CDH19 and an intracellular binding partner of δ-protocadherins, protein phosphatase 1α (PP1α), from ferret brain for the first time, in addition to the set of cadherins used in the earlier study on visual cortical development. By in situ hybridization and tyramide-FISH combined immunofluorescence staining, I studied the expression profiles of these molecules in the developing ferret brain at 10 different developmental stages. Seven members of the cadherin superfamily (CDH4, CDH5, CDH6, CDH7, CDH11, PCDH1 and PCDH17) and PP1α were found to be expressed by developing blood vessels in ferret brain. The expression of some of the cadherin molecules is restricted to specific brain regions or a subset of blood vessels. The expression levels show a peak during perinatal vascular development. My results suggest that multiple cadherins, which are also involved in neurogenesis, are regulators of angiogenesis in developing vertebrate brain, supporting the idea of a common mechanism behind neurogenesis and angiogenesis ("neuroangiogenesis").

Own contribution to the manuscript:

1. RT-PCR from ferret RNA and cDNA isolation
2. Designing of degenerate primer for the ferret cadherin and PP1α molecules
3. Cloning of different cadherin and PP1α molecules
4. In vitro transcription and production of digoxigenin/fluorescein/biotin-labeled cRNA probes
5. In situ hybridization and tyramide-FISH coupled immunohistochemistry in ferret brain sections
6. Expression profile analysis in brain blood vessels
7. Preparation of the manuscript

2.3. Hertel N, **Krishna-K**, Nuernberger M, Redies C (2008) A cadherin-based code for the divisions of the mouse basal ganglia. Journal of Comparative Neurology 508:511-528.

In this study, we studied the expression of multiple cadherins, particularly classic cadherins and δ-protocadherins (Cdh4, Cdh7, Cdh8, Cdh11, Pcdh1, Pcdh7, Pcdh8, Pcdh9, Pcdh10, Pcdh11, Pcdh17 and Pcdh19), in the basal ganglia of the postnatal and adult mouse by in situ hybridization. For this purpose, we cloned and identified the partial sequences of several of the above-mentioned cadherin molecules from mouse using degenerate primers. Our analysis of the expression patterns focused on the caudoputamen that consists of patches (striosomes) and a histologically uniform matrix. Results show that the patch and matrix compartments of the caudoputamen express the 12 cadherins differentially, although partial overlap is observed. Moreover, the cadherins are expressed in multiple and diverse gradients within the caudoputamen and other parts of the basal ganglia. The persistence of the expression patterns in the adult basal ganglia suggests the possibility that cadherins play a role also at adult stages. In conclusion, our study demonstrates that cadherins provide a code of potentially adhesive cues that specify not only patch and matrix compartments but also multiple molecular gradients within the basal ganglia. This code may relate to patterns of connectivity of the basal ganglia in the mouse brain.

Own contribution to the manuscript:

1. RT-PCR from mouse RNA and cDNA isolation
2. Designing of degenerate primer for the mouse cadherin and PP1α molecules
3. Cloning of different cadherin and PP1α molecules
4. In vitro transcription and synthesis of digoxigenin-labeled cRNA probes

2.4. Neudert F, **Krishna-K**, Nuernberger M, Redies C (2008) Comparative analysis of cadherin expression and connectivity patterns in the cerebellar system of ferret and mouse. Journal of Comparative Neurology 511:736-752.

In this study, our objective was to compare the cerebella of two mammals (ferret and mouse) by mapping the expression of three cadherins (cadherin-8, protocadherin-7, and protocadherin-10) at similar postnatal stages. We cloned both ferret and mouse cadherin molecules by RT-PCR using degenerate primers. The three cadherins are expressed differentially in parasagittal stripes in the cerebellar cortex, in the portions of the deep cerebellar nuclei, in the divisions of the inferior olivary nucleus, and in the lateral vestibular nucleus. The expression profiles suggest that the cadherin-positive structures are interconnected. The expression patterns resemble each other in ferret and mouse, although some differences can be observed. Based on the cadherin expression patterns, a model of corticonuclear projection territories in ferret and mouse is proposed. In summary, our results indicate that the cerebellar systems of rodents and carnivores display a relatively large degree of similarity in their molecular and functional organization.

Own contribution to the manuscript:
1. RT-PCR from ferret RNA and cDNA isolation
2. Designing of degenerate primer for the ferret cadherin molecules
3. Cloning of different cadherin molecules
4. In vitro transcription and synthesis of digoxigenin-labeled cRNA probes

3.1.

Layer-specific expression of multiple cadherins in the developing visual cortex (V1) of the ferret

Krishna-K, Nuernberger M, Weth F, Redies C

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Layer-Specific Expression of Multiple Cadherins in the Developing Visual Cortex (V1) of the Ferret

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Cadherins are superfamily of Ca^{2+}-dependent transmembrane glycoproteins with more than 100 members. They play a role in a wide variety of developmental mechanisms, including cell proliferation, cell differentiation, cell–cell recognition, neurite outgrowth and synaptogenesis. We cloned 16 novel members of the classic cadherin and δ-protocadherin subgroups from ferret brain. Their expression patterns were investigated by in situ hybridization in the developing primary visual cortex (V1) of the ferret. Fifteen out of the 16 cadherins are expressed in a spatiotemporally restricted fashion throughout development. Each layer of V1 can be characterized by the combinatorial expression of a subset of cadherins at any given developmental stage. A few cadherins are expressed by subsets of neurons in specific layers or by neurons dispersed throughout all cortical layers. Generally, the expression of protocadherins is more widespread, whereas that of classic cadherins is more restricted to specific layers. At the V1/V2 boundary, changes in layer-specific cadherin expression are observed. In conclusion, our results suggest that cadherins provide a code of potentially adhesive cues for layer formation in ferret V1. The persistence of expression in the adult suggests a functional role also in the mature cortex.

Keywords: cell adhesion, cortical plate, corticogenesis, germinal zones, δ-protocadherins

Introduction

One of the most salient anatomical features of the mammalian neocortex is its organization into 6 layers. Most cortical cells are born in the ventricular and subventricular zones of the proliferative neuroepithelial layer. A fraction of the neuroepithelial cells leaves the mitotic cycle and differentiates into early neurons, which are guided to migrate pialward by radial glial fibers. The earliest born neurons form the preplate. Later-born neurons migrate into the preplate to form the cortical plate that splits the preplate into the marginal zone (MZ) (prospective layer I) and the subplate. As more neurons arrive in the cortical plate, the 6 neocortical layers are formed in an inside-out fashion (Angevine and Sidman 1961; Rakic 1974; Caviness et al. 1995; Rakic and Caviness 1995). In addition, the cortical plate becomes populated by interneurons that are born in the ganglionic eminences and migrate tangentially into neocortex (Anderson et al. 1997; Nadarajah and Parnavelas 2002).

The molecular events that regulate the interaction of the migrating cortical cells with their environment as well as their final positioning within the cortical plate are beginning to be understood. The differences in neuronal composition, developmental timing and connectivity across cortical layers strongly suggest the existence of a vast number of genes with layer- and region-specific patterns of expression. Consistent with this idea, several gene regulatory proteins and morphogenetic molecules, which are expressed in specific layers, were identified. These molecules include Sidekicks, EphrinA5, OTX1, CUTL2, CALB1, N-cadherin, R-cadherin, PCDH8, Reelin, LAMB1, NR2E1, NR2F2, VIP, CNR1, and LIX1 (Rakic 1988; Obst-Pernberg et al. 2001; Hevner et al. 2003; Rash and Grove 2006; Hevner 2007; Molyneaux et al. 2007; Watakabe et al. 2007; Zhou et al. 2007).

In an attempt to identify additional markers for cortical layers and regions, we focused on cadherins in the present study. Cadherins are a large family of Ca^{2+}-dependent cell adhesion glycoproteins, with more than 100 members in vertebrates. Cadherins mediate cell–cell adhesion and signal transduction and are grouped into subfamilies that are designated as classic cadherins, desmosomal cadherins, protocadherins, Flamingo cadherins and FAT molecules (for reviews, see Nollet et al. 2000; Frank and Kemler 2002; Hirano et al. 2003). Among these subfamilies, protocadherins are the largest one and contain several subgroups, such as α-, β-, and γ-protocadherins that form one large cluster of genes in both the mouse and human genome. More recently, a subgroup of nonclustered protocadherins was recognized and classified as δ-protocadherins (Redies et al. 2005; Vanhalst et al. 2005).

The vast majority of cadherins is expressed in distinct patterns in the developing and mature central nervous system of vertebrates, where they play multiple roles in the segregation of neuronal precursor populations, neurite outgrowth, axon guidance and synapse formation (for reviews, see Redies 1997, 2000; Hirano et al. 2003; Takeichi 2007). Expression analysis and functional studies have led to the idea that the homotypic adhesions mediated by cadherins may provide an adhesive code for the selective association of neuronal structures during the functional differentiation of the nervous system (Redies et al. 1993; for reviews, see Redies and Takeichi 1996; Redies 2000). Therefore, studying the expression of cadherins can help us to understand the molecular cues regulating corticogenesis.

Several cadherins have been mapped in the rodent forebrain, for example, Rcad (*Cdh4*) and Ncad (*Cdh2*; Redies and Takeichi 1993; Obst-Pernberg et al. 2001), *Cdh6, Cdh8,* and *Cdh11* (Korematsu and Redies 1997; Suzuki et al. 1997), α-protocadherins (Kohmura et al. 1998; Zou et al. 2007), γ-protocadherins (Zou et al. 2007), and δ-protocadherins (Hirano et al. 1999; Redies et al. 2005; Vanhalst et al. 2005; Gaitan and Bouchard 2006; Kim et al. 2007; Hertel et al. 2008). Each of these cadherins shows a spatially restricted expression in a specific subset of gray matter structures. Most cadherins

are expressed also in a layer-specific fashion in the developing cortex. Typically, the layer-specific distribution of cadherins changes from region to region within cortex. Some cadherins have been used as markers for cortical regions in genetically altered mice (*Cdh6*, *Cdh8*, and *Cdh11*; Miyashita-Lin et al. 1999; Nakagawa et al. 1999; Rubenstein et al. 1999; Bishop et al. 2000).

All these previous studies on cadherin expression in cerebral cortex have focused on single or a few cadherins, often only at a specific stage of cortical development and in a particular cortical area. Here, we map systematically the expression of fifteen novel classic cadherins and δ-protocadherins by in situ hybridization in the developing primary visual cortex (V1) of the ferret, which serves as a model system for cortical development (Rockland 1985). Ferrets have a relatively short gestational period (41–42 days), coupled with a protracted period (35 days) of postnatal cortical neurogenesis. Unlike the mouse, the ferret has a large cerebral cortex and the duration of neuron production permits a high temporal resolution of developmental events and stages of growth (McSherry 1984; Jackson et al. 1989).

Materials and Methods

Animals and Preparation of Tissues

Ferrets bred in captivity were obtained from the Federal Institute of Risk Research in Berlin-Marienfelde, Germany. All animals used in this study were deeply anesthetized by an overdose of intraperitoneal pentobarbital followed by decapitation, according to institutional and national guidelines on the welfare of animals. Embryos were removed from timed pregnant ferrets at 23 days after conception (E23), at E30 and at E38. Postnatally, brains from the following stages were obtained: postnatal day 2 (P2), P13, P25, P33, P46, P60 and adult. The day of birth was designated as P0. The number of animals used in this study was kept at a minimum and efforts were made to minimize animal suffering.

For embryonic stages, the skull above the brain was opened for better diffusion of the fixative. For postnatal stages, brains were removed from the skull. Specimens from brains up to P33 were fixed by immersion in ice-cold 4% formaldehyde solution in phosphate-buffered salt solution (PBS; 13 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.4) and frozen in TissueTek compound (Science Services, Munich, Germany). Brains from P46 animals and older were flash frozen in 2-methyl-butane chilled to about –40 °C by adding dry ice. All specimens were stored at –80 °C until sectioning.

Parasagittal and transverse sections of 20 μm thickness were cut in a cryostat (HM 560 Cryo-Star Cryostat, Microm International, Walldorf, Germany) and thawed directly onto SuperFrost plus slide glasses (Menzel, Braunschweig, Germany). The sections were dried at 50–56 °C. Totally, 36 entire brains or isolated occipital lobes were cut with each specimen yielding about 160–200 sections.

RNA Isolation and cDNA Synthesis

Brains of E38 pups or adult ferrets were flash frozen in liquid nitrogen. Total RNA was isolated using the RNeasy protect mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purity of the RNA samples was assessed spectrophotometrically and total RNA was quantified from the absorbance at 260 nm. First-strand complementary DNA was synthesized from the ferret total RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, CA) according to the manufacturer's protocol in a thermocycler (Mastercycler personal, Eppendorf, Hamburg, Germany).

PCR Amplification

Several classic cadherins and all known δ-*PCDH* molecules were amplified by PCR using both specific and degenerate primers designed for regions, which are highly conserved between mouse, rat, dog, and human for different cadherin molecules. Degeneracy levels up to 16 were used. Degenerate primers were designed manually by using the ClustalX multiple alignment tool and verified by the CODEHOP online primer designing software tool. The degeneracy levels of type 2 classic cadherin primers (Price et al. 2002) were 192-fold. The primers are listed in Table 1.

For amplification, the REDTaq ReadyMix PCR system (Sigma-Aldrich, St Louis, MO) was used in a gradient thermocycler (Mastercycler, Eppendorf). Each amplification commenced with an initial denaturation step at 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 3 min, primer annealing for 50 s at temperatures ranging from 54–60 °C, depending upon the primers, and extension at 72 °C for 2 min. Finally, extension was performed at 72 °C for 10 min to complete the synthesis of all strands. The PCR products were analyzed by electrophoresis in 1.2% agarose gels stained with ethidium bromide, and bands were visualized and photographed under ultraviolet light excitation (BioDoc Analyzer, Biometra, Germany). The size of the different cadherin fragments varied from 1.1 to 3 kb (Table 1).

Cloning of Cadherins

The resulting PCR products were purified (QIAquick gel extraction kit, Qiagen) and ligated into the pCR II-TOPO vector (TOPO TA Cloning Kit, Invitrogen) in accordance with the manufacturer's instructions. Several clones were picked after transformation of chemically competent *Escherichia coli* TOP 10F cells (Invitrogen) based on blue-white colony selection. The isolated plasmids (Qiaprep miniprep kit, Qiagen) were checked by restriction digestion to select a single plasmid harboring the desired sequence.

DNA Sequencing

All the inserts were sequenced using the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Biotechnologies, Madison, WI) according to

Table 1
Primers used to obtain cadherin fragments by RT-PCR

Cadherin	Primer sequence	Size (bp)	Accession number
CDH4	5′ AAG CGT GAC TGG GTC ATC C 3′ TTG AGG ATC TTT TCG C	1501	EU665238
CDH6	5′ TGG (AG)T(AGCT) TGG AA(CT) CA(AG) (AT)T(GCT) 3′ CC(AGCT) CC(AGCT) CC(CT) TC(AG) TC(AG) T(CT)(AG) TA	1824	EU665239
CDH7	5′ TGG (AG)T(AGCT) TGG AA(CT) CA(AG) (AT)T(GCT) 3′ CC(AGCT) CC(AGCT) CC(CT) TC(AG) TC(AG) T(CT)(AG) TA	1824	EU665240
CDH8	5′ TGG (AG)T(AGCT) TGG AA(CT) CA(AG) (AT)T(GCT) 3′ CC(AGCT) CC(AGCT) CC(CT) TC(AG) TC(AG) T(CT)(AG) TA	1825	EU665241
CDH11	5′ CCT GAC CCT GTG CTC GTG 3′ ACC GTC CTC TGG ATT GAT AGT G	1110	EU665242
CDH14	5′ TGG (AG)T(AGCT) TGG AA(CT) CA(AG) (AT)T(GCT) 3′ CC(AGCT) CC(AGCT) CC(CT) TC(AG) TC(AG) T(CT)(AG) TA	1474	EU665243
CDH20	5′ TGG (AG)T(AGCT) TGG AA(CT) CA(AG) (AT)T(GCT) 3′ CC(AGCT) CC(AGCT) CC(CT) TC(AG) TC(AG) T(CT)(AG) TA	2000	EU665244
PCDH1	5′ GAC CTC ACC ATC AAG GT 3′ TGG GGG CAT ACA GGT CC	1961	EU665245
PCDH7	5′ TGA TCG TGA AGG GGG CGC TGG ACC G 3′ CCT GCT CCC ACA AAT GTG TTG GCT GG	2326	EU665246
PCDH8	5′ TTY AGY CTY TGC TGG GTG CTC TC 3′ GY TGG CGC AGM GTC TCA TAG TC	1702	EU665247
PCDH9	5′ CTG GTG CTA CCA GAT GCA TGG C 3′ CCT CTT GTC CGG AGA GGC CTG G	1749	EU665248
PCDH10	5′ GGA GAT CGA IGT GCT GGA 3′ CCG CCC TGG GGC TCC ACG	1860	EU665249
PCDH11	5′ CAT GCC ACA GAT GCT GAC ATA GGT G 3′ GCA ACC AKG ATC TTG ACA TAG TCA C	2044	EU665250
PCDH17	5′ CGA CGG CAC CAA GTT CCC 3′ CCC ATG TAA TTG GGC TCT G	2038	EU665251
PCDH18	5′ GCA GCA GTT GGG ACT CG 3′ GGC ATC CAG CAC TGG TCA GAG	2351	EU665252
PCDH19	5′ AGC GCG CCG GGA CGG TGA TCG C 3′ GGG CTG CAG ATG GTC ACA TCG ACA G	2896	EU665253

the manufacturer's protocol in a DNA sequencer (LI-COR Biotechnology, Lincoln, NE). All confirmed cadherin molecules were sequenced again by a commercial company (MWG-Biotech, Ebersberg, Germany) using M13 forward, reverse and specific internal primers. The sequences have been submitted to the NCBI GenBank database. The accession numbers are listed in Table 1.

cRNA Probe Synthesis

Nonradioactive cRNA probes were produced for all the cadherin molecules with the digoxigenin (DIG) RNA Labeling Kit or the Fluorescein-RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The linearized plasmids were transcribed with T7 or SP6 RNA polymerase (New England Biolabs, Ipswich, MA) followed by labeling with digoxigenin or fluorescein to generate sense and antisense probes. Probes were purified by LiCl/EtOH precipitation or by using Quick Spin Columns (Roche Diagnostics). Correct probe size was verified by formaldehyde-agarose gel electrophoresis.

In Situ Hybridization

In situ hybridization was performed as described previously (Redies et al. 1993). Cryostat sections of 18- or 20-μm thickness were (post-) fixed with 4% formaldehyde in PBS and were pretreated with proteinase K and acetic anhydride. Sections were hybridized with cRNA probes at a concentration of about 1 ng/μL overnight at 70 °C in hybridization solution (50% formamide, 10 mM ethylene diaminetetraacetic acid [EDTA], 3× saline-sodium citrate (SSC), 1× Denhardt's solution, 10× dextran sulfate, 42 μg/mL yeast RNA, and 42 μg/mL salmon sperm DNA). After the sections were washed, alkaline phosphatase-coupled anti-digoxigenin Fab fragments were applied. For visualization of the labeled cRNAs, the sections were incubated with a substrate mixture of 0.03% nitroblue tetrazolium salt and 0.02% 5-bromo-4-chloro-3-indolyl phosphate for 1–3 days at room temperature or at 4 °C, until enough reaction product had formed. The sections were viewed and photographed under a microscope (BX40, Olympus, Hamburg, Germany) equipped with a digital camera (DP70, Olympus). Digitized images from in situ hybridization were adjusted in contrast and brightness with the Photoshop software (Adobe Systems, Mountain View, CA).

Tyramide Signal-Amplified Double-Fluorescent in Situ Hybridization

The in situ hybridization protocol was modified in the pre- and posthybridization steps by introducing a fluorescent reaction product by CARD in order to visualize the expression of more than one cadherin in a single section. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide. Then, sections were hybridized with a mixture of both DIG- and fluorescein-labeled cRNA probes at a concentration of about 1 ng/μl each, overnight at 70 °C in hybridization solution (50% formamide, 10 mM EDTA, 3× SSC, 1× Denhardt's solution, 10× dextran sulfate, 42 μg/mL yeast RNA, and 42 μg/mL salmon sperm DNA). After the hybridized sections were washed, horseradish peroxidase (HRP)–coupled anti-digoxigenin Fab fragments were applied to bind to the digoxigenin-labeled cRNA probes. Enzymatic activity was visualized by incubating sections with a substrate solution containing the tyramide-coupled Alexa fluorophore A488 (Invitrogen) and H_2O_2, for 1 h at room temperature. Subsequently, for the detection of the fluorescein-labeled probe, HRP-coupled anti-fluorescein Fab fragments and tyramide-coupled Alexa fluorophore A568 were used. The sections were viewed and photographed under a confocal laser scanning microscope (SP5, Leica Microsystems, Wetzlar, Germany).

Results

Out of the 16 cadherins studied in the present work, all except *PCDH18* are expressed in the primary visual cortex of the adult ferret. Each cadherin shows a distinct and layer-specific expression profile. In general, classic cadherins show a more restricted expression pattern than δ-protocadherins. Each cortical layer is thus marked by the combinatorial expression of multiple cadherins. However, not all cells in a given layer express the same combination of cadherins. Rather, in some layers, a given cadherin may be expressed only by a subset of cells.

Expression of the different cadherins begins at different times during development. Some cadherins are already expressed by the cells in the preplate (for example, *CDH4*, *CDH11*, *CDH14*, *PCDH7*, *PCDH9*, and *PCDH10*) or by the ventricular zone (e.g., *CDH4*, *CDH6*, *CDH20*, and *PCDH1*) at the earliest stage examined (E23). Most cadherins (for example, *CDH8, CDH11, CDH20, PCDH1, PCDH7, PCDH9*, *PCDH10*, *PCDH11*, *PCDH17*, and *PCDH19*) are expressed in the subventricular zone, the intermediate zone or the subplate before birth. All fifteen cadherins are expressed in the cortical plate (cortical layers II–VI) at birth. Layer I contains cells positive for a subset of cadherins (e.g., *CDH4*, *CDH6*, *CDH7*, *CDH11*, *CDH14*, *CDH20*, *PCDH1*, *PCDH7*, *PCDH8*, *PCDH9*, and *PCDH10*) in the mature visual cortex. During postnatal development, expression profiles are relatively stable overall, although gradual changes are observed for some cadherins. In the first part of the Results section, we will describe the ontogenetic expression of each cadherin in the primary visual cortex in detail. Figures 1–3 give an overview of the expression of each cadherin at representative stages of development. Figures 4 and 5 summarize the expression patterns for selected stages of development in schematic diagrams.

The expression pattern described is specific for the primary visual cortex of the ferret. Other cortical areas express the same cadherins, but in different layer-specific patterns (Krishna-K. and Christoph Redies, unpublished data). To demonstrate the specific expression profile in primary visual cortex, we examined the boundary between the primary and secondary visual cortex in the second part of the Results section (Figs 6, 7).

Cells in white matter and embryonic blood vessels were also found to express several of the cadherins studied. A detailed analysis of these cells is beyond the scope of the present work and will be published elsewhere (Krishna-K. and Christoph Redies, unpublished data).

Expression in Primary Visual Cortex (V1)

Cadherin-4 (CDH4)

Expression of *CDH4* (R-cadherin) is already prominent and ubiquitous at the earliest stage examined (E23; Fig. 1*A*). Notably, the cells in the preplate express *CDH4* strongly. From E30 to P2, strong expression persists in the ventricular zone. The subventricular zone, intermediate and MZs are moderately positive. The cortical plate is positive in superficial and deep layers. At P13, cells in the MZ (prospective layer I) and in layer II show prominent signal for *CDH4* and layers IV–VI show moderate signal. From P33 to the adult stage, layer I cells, a subset of cells in layer II, and layers IV and VI remain positive.

Cadherin-6 (CDH6)

At E23, only the ventricular zone (neuroepithelium) expresses *CDH6*. At E30 and E38, expression in the entire cortical mantle is weak. At P2, moderate expression is observed in the cortical plate, subplate, and MZ (Fig. 1*B*). The cortical plate is more strongly positive in the superficial and deep laminae. At P13, *CDH6*-positive cells are scattered in all cortical layers, except

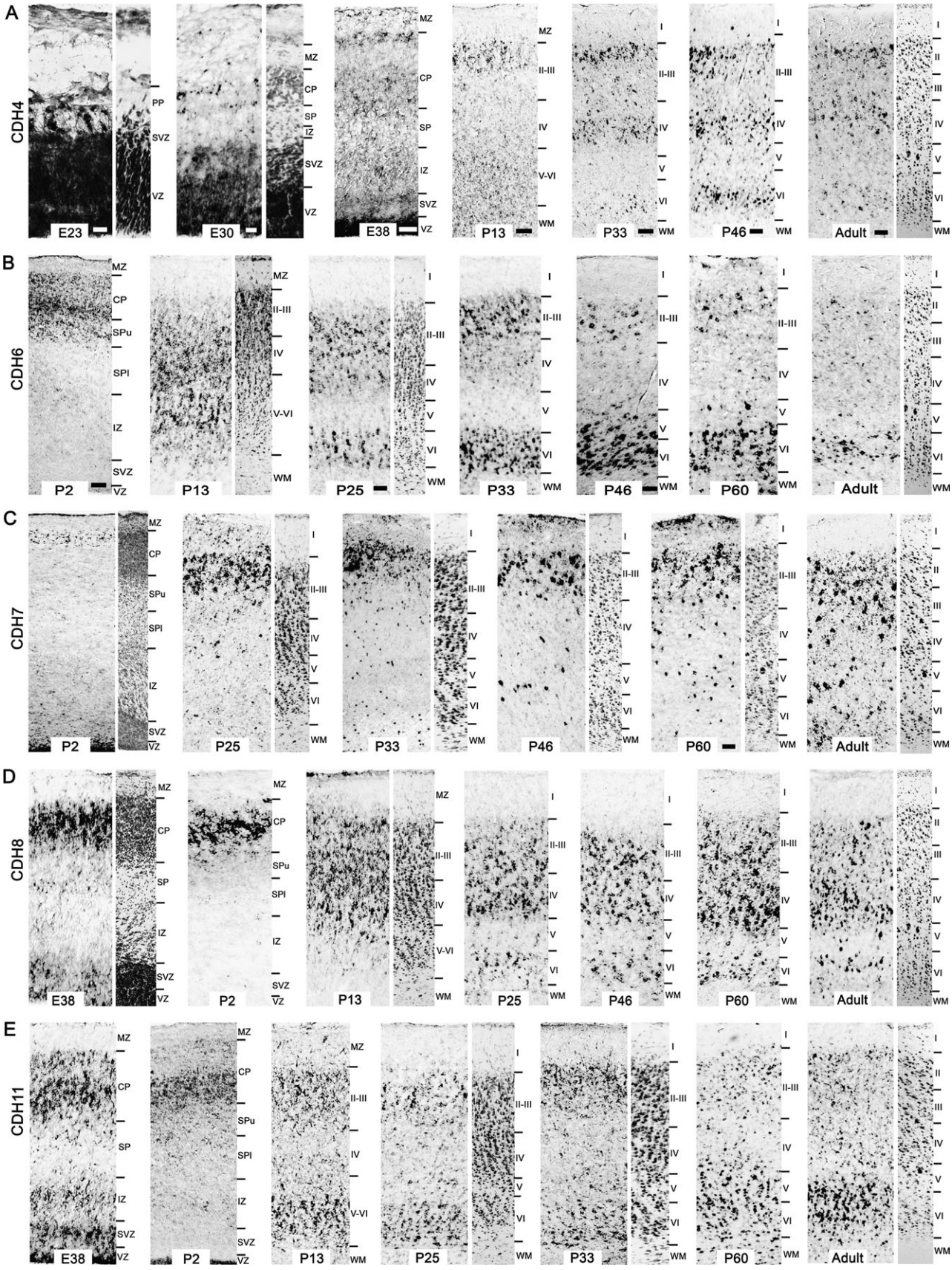

Figure 1. Expression mapping in the layers of the primary visual cortex with cRNA probes for cadherin-4/R-cadherin (*CDH4*; *A*), cadherin-6 (*CDH6*; *B*), cadherin-7 (*CDH7*; *C*), cadherin-8 (*CDH8*; *D*), and cadherin-11 (*CDH11*; *E*). In situ hybridization was carried out at different embryonic stages (E) and postnatal stages (P). Developmental stages are indicated at the bottom of each panel. The layers are indicated at the right of each panel. To facilitate the identification of cortical layers, a thionin (Nissl) stain of an adjacent section is shown to the right of the in situ hybridization results for selected stages. I–VI, cortical layers I–VI; CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; SPl, lower subplate; SPu, upper subplate; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bars are 25 μm for stage E23, 50 μm for stage E30, 100 μm for stage E38, 200 μm for stage P2, and 100 μm for stages from P13 to the adult stage.

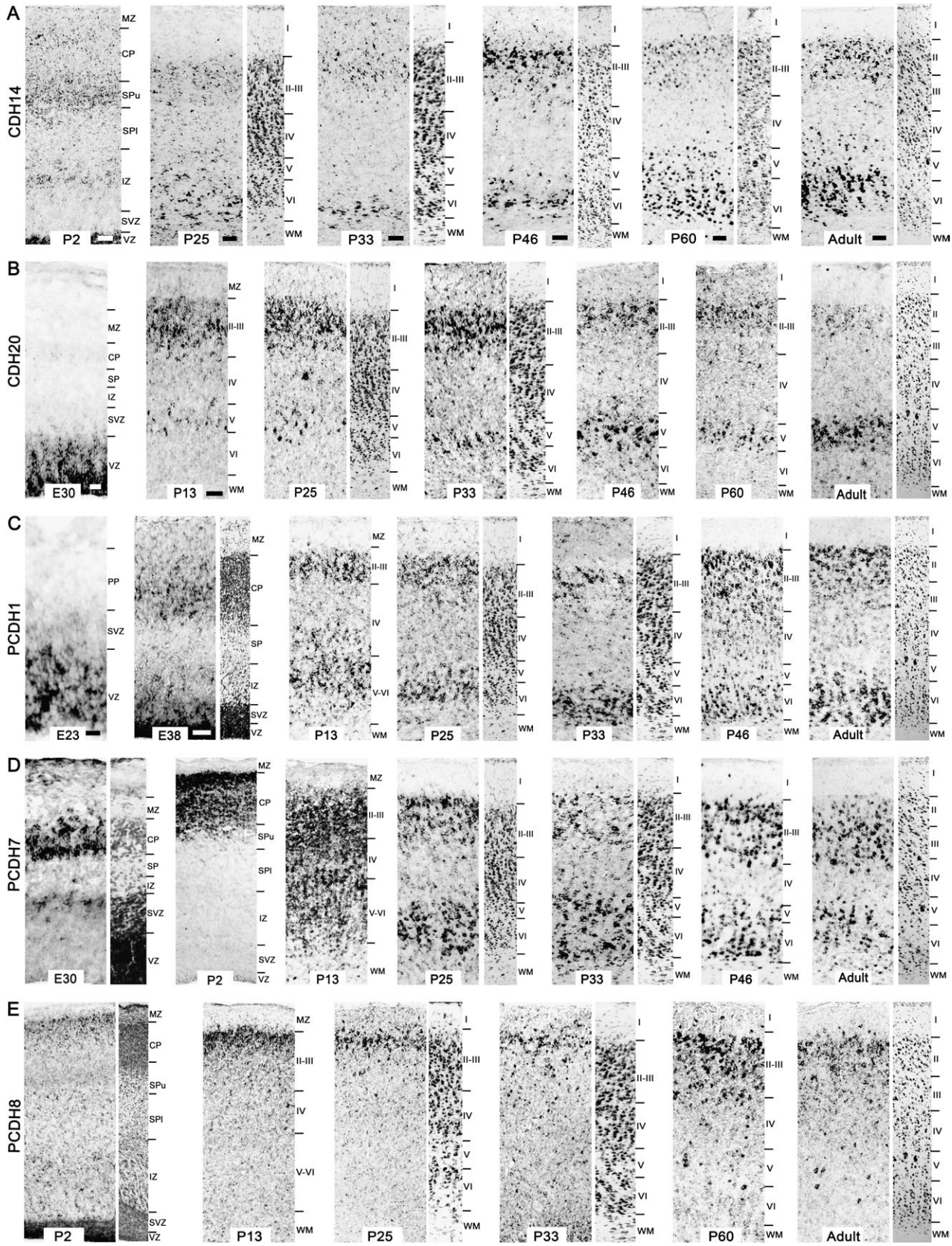

Figure 2. Expression mapping in the layers of the primary visual cortex with cRNA probes for cadherin-14 (*CDH14*; *A*), cadherin-20 (*CDH20*; *B*), protocadherin-1 (*PCDH1*; *C*), protocadherin-7 (*PCDH7*; *D*), and protocadherin-8 (*PCDH8*; *E*). In situ hybridization was carried out at different embryonic stages (E) and postnatal stages (P). Developmental stages are indicated at the bottom of each panel. The layers are indicated at the right of each panel. To facilitate the identification of cortical layers, a thionin (Nissl) stain of an adjacent section is shown to the right of the in situ hybridization results for selected stages. I–VI, cortical layers I–VI; CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; SPl, lower subplate; SPu, upper subplate; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bars are 25 μm for stage E23, 50 μm for stage E30, 100 μm for E38, 200 μm for stage P2, and 100 μm for stages from P13 to the adult stage.

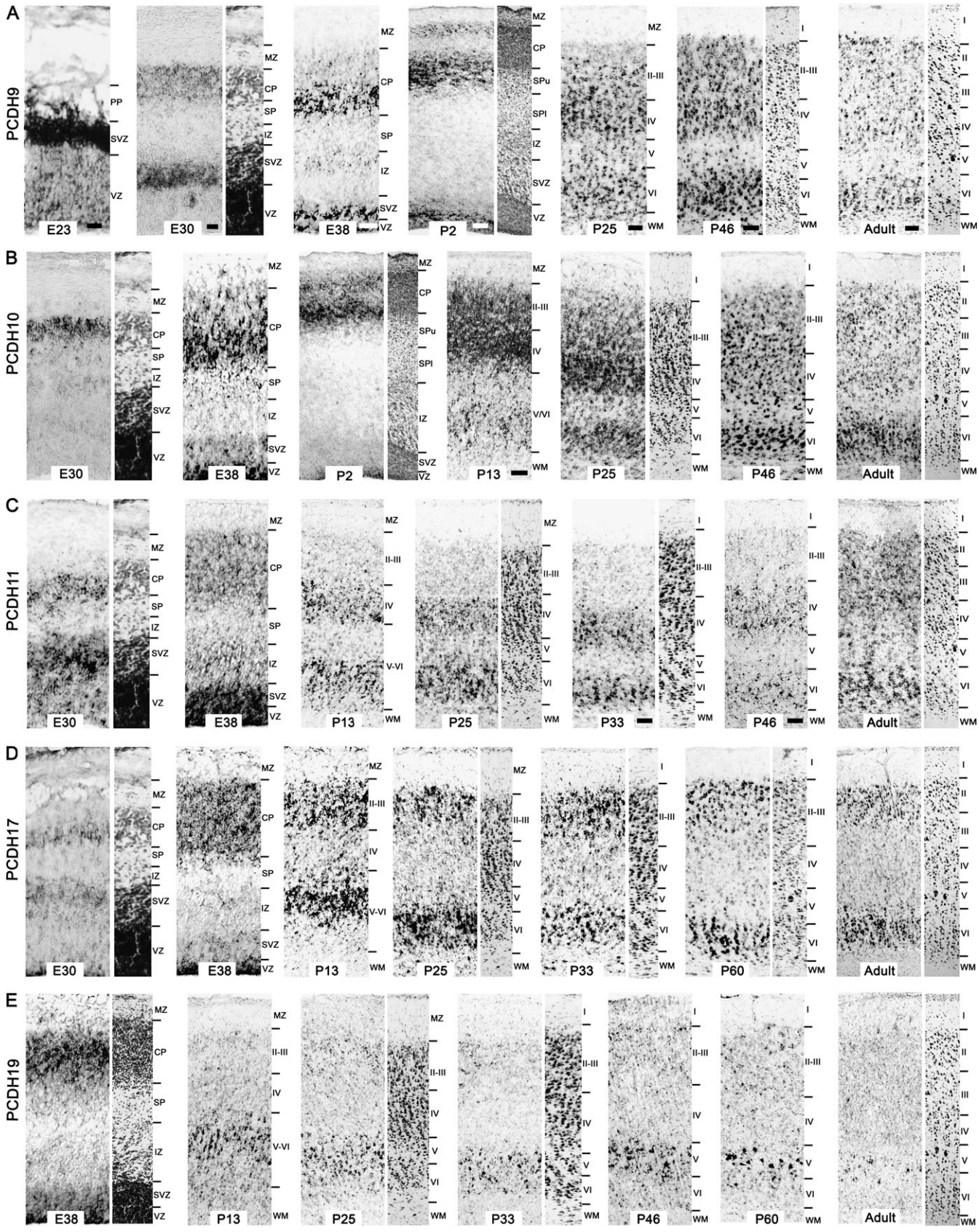

Figure 3. Expression mapping in the layers of the primary visual cortex with cRNA probes for protocadherin-9 (*PCDH9*; *A*), protocadherin-10 (*PCDH10*; *B*), protocadherin-11 (*PCDH11*; *C*), protocadherin-17 (*PCDH17*; *D*), and protocadherin-19 (*PCDH19*; *E*). In situ hybridization was carried out at different embryonic stages (*E*) and postnatal stages (P). Developmental stages are indicated at the bottom of each panel. The layers are indicated at the right of each panel. To facilitate the identification of cortical layers, a thionin (Nissl) stain of an adjacent section is shown to the right of the in situ hybridization results for selected stages. I–VI, cortical layers I–VI; CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; SPl, lower subplate; SPu, upper subplate; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bars are 25 μm for stage E23, 50 μm for stage E30, 100 μm for E38, 200 μm for stage P2, and 100 μm for stages from P13 to the adult stage.

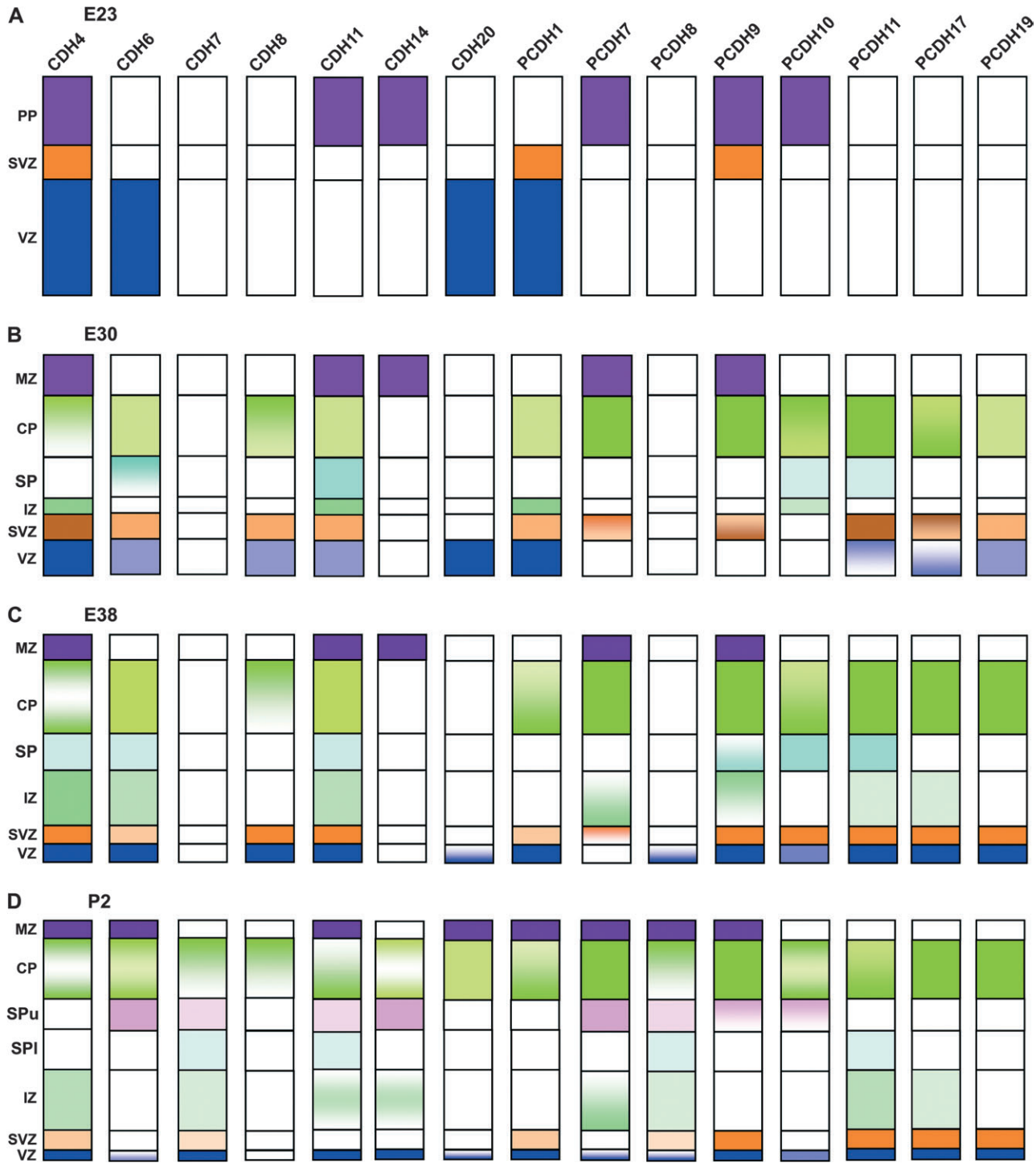

Figure 4. Schematic diagram of the cadherin expression patterns in the layers of the primary visual cortex at embryonic day 23 (E23; *A*), E30 (*B*), E38 (*C*), and postnatal day 2 (P2; *D*). The layers are indicated at the left side of the panels. Layer-specific staining is represented by different colors. The intensity of the colors indicates the approximate general level of expression. CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; SPl, lower subplate; SPu, upper subplate; SVZ, subventricular zone; VZ, ventricular zone.

in layer I, which remains negative until stage P46. During development of the cortical plate, expression becomes gradually restricted to particular layers. At P25, layers II–IV and VI contain numerous *CDH6*-positive cells, whereas positive cells are scarce in layer V. At P33, only a few cells remain positive in layer IV. Thereafter, the number of *CDH6*-positive cells in layers II and III decreases. At P60 and in the adult, expression is restricted to numerous cells in layer VI, to layer I cells and to very few, large cells in layer V.

Cadherin-7 (CDH7)

At the occipital pole of the cerebral cortex, weak staining is first observed at P2 in the upper layers of the cortical plate (Fig. 1*C*). A few scattered cells are also found in the other

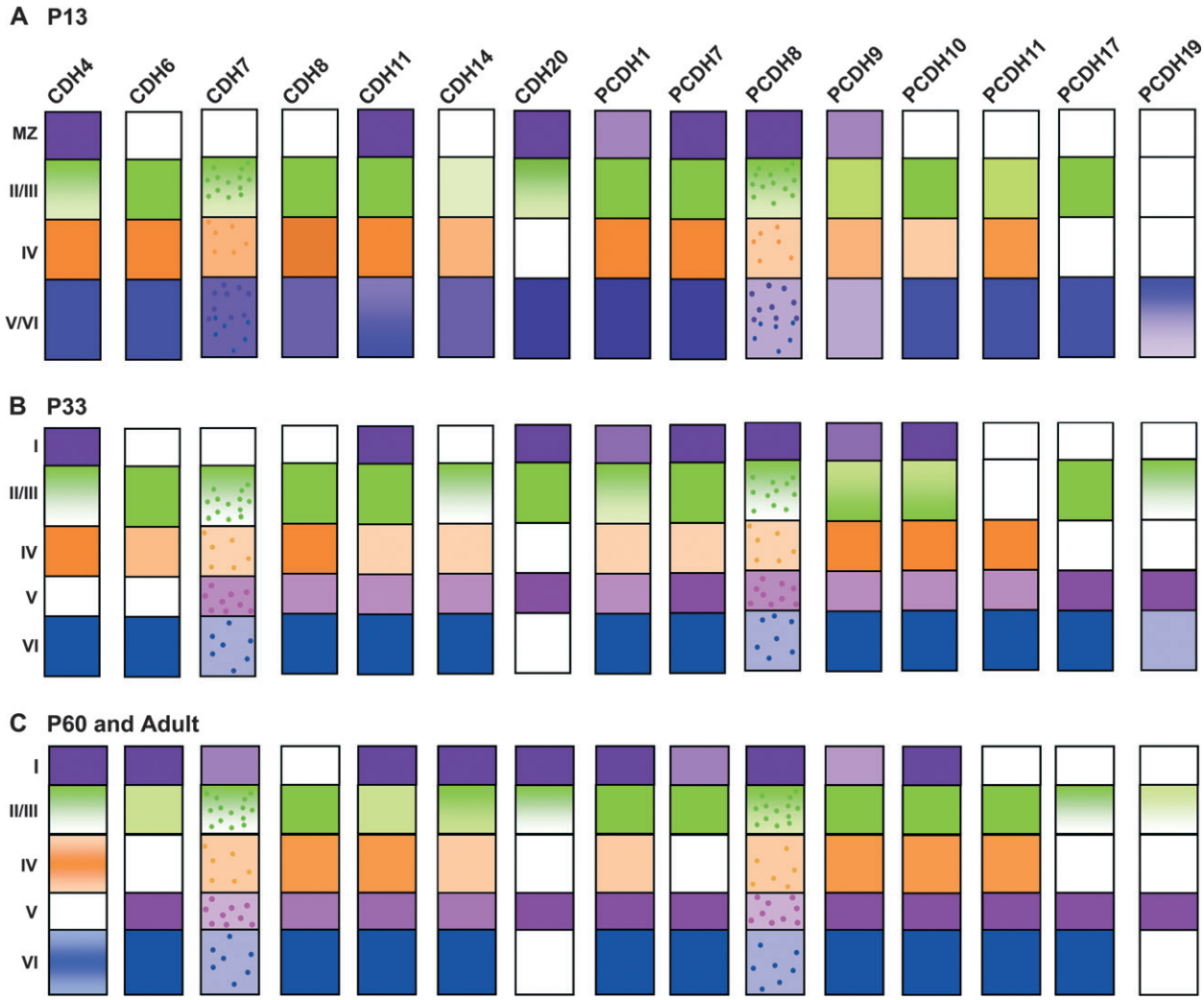

Figure 5. Schematic diagram of the cadherin expression patterns in the layers of the primary visual cortex at postnatal day 13 (P13; *A*), P33 (*B*), and P60/adult (*C*). The layers are indicated at the left side of the panels. Layer-specific staining is represented by different colors. The intensity of the colors indicates the approximate general level of expression. The dotted patterns for *CDH7* and *PCDH8* indicates that a small subset of large cells is labeled. I–VI, cortical layers I–VI.

germinal layers. Staining then increases until it is moderately strong in layers II/III at P25. From P25 to the adult stage, signal in layers II/III decreases to low levels. In addition, from P13 to P60, a few scattered *CDH7*-positive cells with large somata are found in all cortical layers. In the adult, scattered *CDH7*-positive cells remain visible in all cortical layers. Layer I shows a weak staining only for stage P60 and in the adult.

Cadherin-8 (CDH8)

Moderate staining is observed first at E30 in the outer half of the cortical plate, ventricular zone and subventricular zone. At this and later stages, all other layers of V1 do not show signal (Fig. 1*D*). At E38 and P2, the signal in the cortical plate has become stronger. At P13, most cells in layers II–VI express *CDH8*; the most strongly labeled cells are located in layer IV. At P25, layer VI cells are still positive, but in layer V, only a few cells of large size retain signal. The expression profile observed at P25 persists until the adult stage.

Cadherin-11 (CDH11)

CDH11 is expressed by the cells in the preplate at E23. Apart from this, cells in the cortical plate, subplate, intermediate zone, subventricular zone and ventricular zone show weak to moderate expression of *CDH11* from E30 onwards. At P2, the deeper layers of the cortical plate exhibit stronger staining than the upper layers (Fig. 1*E*). From P13 to P33, layers II/III and VI contain many *CDH11*-positive cells, whereas only few cells express *CDH11* in layers IV and V. Expression becomes stronger as development proceeds. In particular, the number of positive cells in layer V increases from P46 to the adult stage. Overall, expression in infragranular layers is stronger than in supragranular layers. Also there is a moderate expression of *CDH11* in the MZ and layer I from stage E30 to the adult stage.

Cadherin-14 (CDH14)

Initially, the preplate shows signal for *CDH14* at E23. This signal persists in the MZ until E38. At P2, weak signal is seen in the upper subplate, intermediate zone and ventricular zone (Fig. 2*A*). Expression becomes more prominent at P25 when signal appears in layers II and VI; layers III and V contain a few positive cells. This expression profile persists till the adult stage. At stage P60 and in the adult, layer I also shows signal.

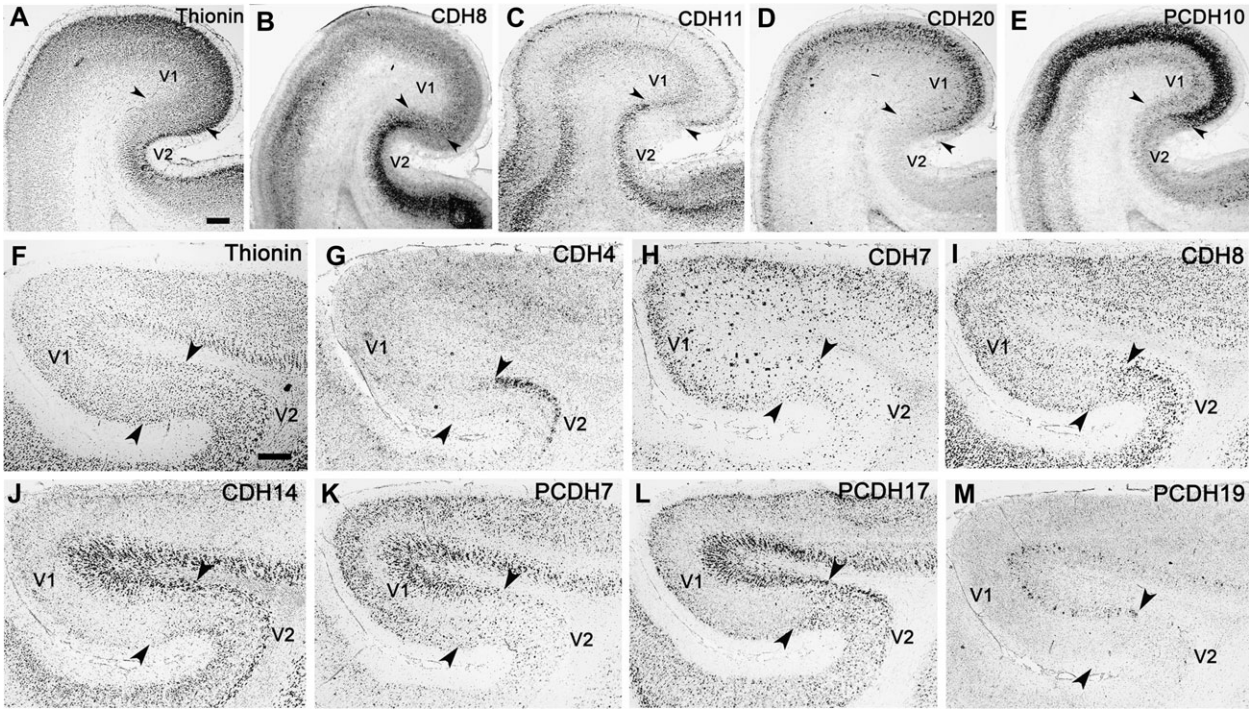

Figure 6. Expression mapping of cadherins at the boundary between primary (V1) and secondary visual cortex (V2) at postnatal day 13 (P13; *A–E*) and at P60 (*F–M*). Adjacent sections were hybridized with cRNA probes for cadherin-8 (*CDH8*; *B*, *I*), cadherin-11 (*CDH11*; *C*), cadherin-20 (*CDH20*; *D*), protocadherin-10 (*PCDH10*; *E*), cadherin-4 (*CDH4*; *G*), cadherin-7 (*CDH7*; *H*), cadherin-14 (*CDH14*; *J*), protocadherin-7 (*PCDH7*; *K*), protocadherin-17 (*PCDH17*; *L*), and protocadherin-19 (*PCDH19*; *M*). The boundary between V1 and V2 is indicated by arrowheads. To facilitate the identification of the boundary, a Nissl stain (Thionin) of adjacent sections is shown in *A* and *F*, respectively. Scale bars are 300 μm (in *A* for *A–E*) and 400 μm (in *F* for *F–M*).

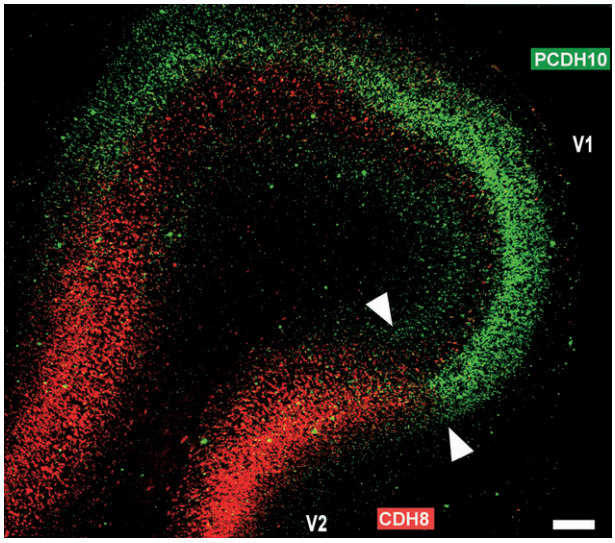

Figure 7. Tyramide signal-amplified fluorescent in situ hybridization at the boundary between primary (V1) and secondary visual cortex (V2) at postnatal day 13 (P13). The section was doubly hybridized in situ with cRNA probes for cadherin-8 (*CDH8*; red) and protocadherin-10 (*PCDH10*; green). The boundary between V1 and V2 is indicated by arrowheads. The scale bar is 1 mm.

Cadherin-20 (CDH20)

Already at E23, the entire ventricular layer of the occipital pole shows strong signal. At E30, the other layers of the developing cortical mantle, including the subventricular zone, are negative (Fig. 2*B*). The signal in the ventricular zone has receded to the subependymal layer at E38 and P2. From P13 to P33, layers II/III and a few large pyramidal cells in layer V show *CDH20* signal. Expression in layers II/III becomes weaker until the adult stage, especially in layer III, where only a subpopulation of cells is positive. Layer I cells are also positive from P2 to the adult stage. Strongly labeled cells persist in layer V until the adult stage.

Protocadherin-1 (PCDH1)

At E23, the ventricular zone is positive (Fig. 2*C*). At E38 and P2, the innermost (subependymal) lamina of the ventricular zone shows the strongest signal. Beginning at E38, the deeper layers of the cortical plate are *PCDH1* positive. At P13, when the layering of the cortical plate becomes more distinct, cells in the supragranular layers (II/III) and the infragranular layers (V/VI) express *PCDH1*, although a few scattered cells are positive in layer IV as well. This pattern of expression remains basically the same from P13 onwards, but layers II and VI are more strongly stained in the adult. Layer I cells are moderately positive from P2 until the adult stage.

Protocadherin-7 (PCDH7)

Expression is first seen at E23 in the preplate. For other zones, expression starts at E30, when moderate signal is seen in the cortical plate, upper layers of the subventricular zone and intermediate zone (Fig. 2*D*). The signal in the cortical plate becomes considerably stronger during further development. Some cells are also positive in the upper subplate at P2. At P13, *PCDH7* is expressed by cells in all layers of the cortical plate. From P25 to the adult stage, expression in layer IV becomes weak, whereas prominent signal persists in layers II, III, V, and

VI. Cells in the MZ and in layer I are also positive from E30 to the adult stage.

Protocadherin-8 (PCDH8)
The cortical mantle is negative at E30. At P2, the ventricular, subventricular and intermediate zones, the subplate and the upper layers of the cortical plate are positive (Fig. 2*E*). From P13 to the adult, cells in layer II show prominent signal. In addition, from P2 to the adult stage, a few scattered positive cells are also found in all other layers. Layer I cells are also positive from P2 to the adult stage.

Protocadherin-9 (PCDH9)
Strong signal for *PCDH9* is seen in the subventricular zone and in the preplate at E23 (Figs 3*A* and 4*E*). In addition, the cortical plate is also positive from its inception (E30). At E38, the ventricular and subventricular zones show prominent signal. The intermediate zone and lower subplate express *PCDH9* weakly. Also, the cortical plate is strongly positive at E38 and at P2; prominent signal is seen in the deeper layers, including the upper subplate at P2. Signal in the ventricular layer has become weaker. From P25 to the adult stage, all cortical layers show expression; cells in layers III/IV and VI are the most strongly labeled. The MZ and layer I cells are also moderately positive from E30 to the adult stage.

Protocadherin-10 (PCDH10)
At E23, expression is seen in the preplate. For the other zones, staining is observed at E30 in the upper cortical plate, subplate and intermediate zone (Fig. 3*B*). Signal can be detected in the deep layers of the cortical plate, including the upper subplate, and the ventricular layer at E38 and P2. At P13, the newly formed layers II/III and IV are strongly *PCDH10* positive. Staining in layer VI persists, whereas expression in layer V is weaker. From P13 to P60, this expression profile remains basically the same, but signal in layers II and III becomes less strong. At the adult stage, almost all cells in layers II–VI express *PCDH10*, except for some cells in layer V. Layer I cells are also positive from P25 to the adult stage (data not shown).

Protocadherin-11 (PCDH11)
At E30, a moderate staining is observed in the ventricular zone, the subventricular zone, subplate and the cortical plate (Fig. 3*C*). In addition, at E38, signal appears in the intermediate zone and the signal has intensified in the ventricular zone. From P13 to P46, expression in the developing cortical plate is most prominent in layers IV and VI. At P60 and in the adult, almost all cortical cells express *PCDH11* homogeneously, except in layer I.

Protocadherin-17 (PCDH17)
At E23, the meninges show a strong signal for *PCDH17*. Expression in the cortical mantle begins at E30, when the subependymal layer of the ventricular zone shows strong signal; the subventricular zone and the cortical plate are only moderately positive (Fig. 3*D*). At E38 and P2, expression in the cortical plate is rather homogeneous and moderately strong and a few positive cells are seen in the intermediate zone. From P13 onwards, expression becomes restricted to cortical layers II/III and V/VI; later in development, expression is strongest in layers II and VI. This is the expression profile also seen in the adult.

Protocadherin-19 (PCDH19)
Expression sets in at E30, when weak signal is observed first in the cortical plate, subventricular zone and ventricular zone. Later in development, signal becomes stronger, especially in the deeper layers (Fig. 3*E*). Layers V/VI remain positive until stage P33. In addition, layer II is weakly labeled from P25 onwards. From P46 to the adult stage, there are strongly positive, large cells in layer V; cells in the other layers are only weakly or moderately stained.

Expression at the V1/V2 Boundary

A comparison of the cadherin expression patterns between V1 and the secondary visual cortex (V2) in a series of consecutive sections reveals that the layer-specific expression of most cadherins differs between the 2 areas, thereby allowing a demarcation of the V1/V2 boundary. Figures 6 and 7 show the V1/V2 boundary at P13 and at P60.

At P13, *CDH8* expression is particularly strong in layers IV–VI of V2, but more moderate and ubiquitous in V1 (Fig. 6*B*). *CDH11* is strongly expressed only in layers V/VI of V2; in V1, the same layer is weakly positive but, in addition, layers II/III express *CDH11* (Fig. 6*C*). Unlike V1, V2 does not express *CDH20* (Fig. 6*D*). Similarly, expression of *PCDH10* falls off at the V1/V2 area (Fig. 6*E*). Double-labeling of a single section for both *CDH8* and *PCDH10* mRNA confirms the abrupt transition of the staining patterns at the boundary between V1 and V2 (Fig. 7).

A similar regional difference in cadherin expression can be observed at P60 (Fig. 6*F–M*). For example, *CDH4* expression is prominent in layer V of V2 but absent from the same layer of V1 (Fig. 6*G*). *CDH7* shows no significant expression for V2 in any of its layers, whereas strongly labeled cells are dispersed in all layers of V1 (Fig. 6*H*). *CDH8* is generally expressed more strongly in V2 than in V1 (Fig. 6*I*). Layer IV is positive for *CDH14* signal in V2 but not in V1 (Fig. 6*J*). *PCDH7* shows weak staining in all layers of V2; in V1, expression is generally stronger but absent from layer IV (Fig. 6*K*). *PCDH17* shows overall strong signal in all layers of V2; in contrast, there is weak expression in layers IV and V of V1, but strong expression only in layer VI (Fig. 6*L*). Strongly *PCDH19*-positive cells are absent in layer V of V2 but present in V1 (Fig. 6*M*).

Discussion

For the first time, 15 members of 2 cadherin subfamilies, classic cadherins and δ-protocadherins, were cloned and sequenced from the ferret brain, in order to study their expression in the developing primary visual cortex. Results from in situ hybridization revealed that the expression of the cadherins is subject to a tight temporal, layer-specific and region-specific regulation during corticogenesis. Not only the layers of the differentiating and mature cortical plate, but also the germinal zones of the early embryonic cortical mantle express the cadherins differentially. In addition, we provide evidence that some of the cadherins are expressed in subtypes of cells dispersed in specific cortical layers or throughout all cortical layers. The present identification of cadherins as a set of markers for cortical layers and neuronal subpopulations adds to the goal of obtaining a panel of markers that allows a comprehensive analysis of all neuron types in the mammalian neocortex (Hevner 2007).

Cadherins Provide a Code of Potentially Adhesive Cues for the Developing and Adult Ferret Primary Visual Cortex

Each of the 15 cadherins studied exhibits a unique, spatially restricted expression pattern that is dissimilar from that of other cadherins, although partial overlap between the cadherins is observed. The expression patterns are relatively stable throughout development. Changes in layer-specific expression are usually minor, if they occur at all, and take place slowly during development (Figs 1–3).

The cadherin-based code for specifying cortical laminae is probably a combinatorial one because each lamina is characterized by the expression of a subset of multiple cadherins (Figs 4, 5). Similar results have been obtained for other layered central nervous system structures, for example for the retina (Matsunaga et al. 1988; Wöhrn et al. 1998; Faulkner-Jones et al. 1999; Ruan et al. 2006) and the chicken tectum (Wöhrn et al. 1999).

Most of the cadherins studied here are expressed also in various other regions throughout the ferret brain and in neural circuits outside the visual system (Krishna-K. and Christoph Redies, unpublished data). A similarly widespread, but region-specific expression of cadherins was described before in the embryonic and postnatal brain of other vertebrates (Redies et al. 1993; Korematsu and Redies 1997; Suzuki et al. 1997; Hirano et al. 1999; Redies et al. 2000, 2005; Obst-Pernberg et al. 2001; Bekirov et al. 2002; Vanhalst et al. 2005; Kim et al. 2007). Based on these results, it has been proposed that cadherins provide an adhesive code for developing brain structures, neural circuits and synapses (for reviews, see Redies 1997, 2000; Hirano et al. 2003; Takeichi 2007).

Layer-Specific Expression of Cadherins in the Visual Cortex

As summarized in Figure 4, cadherins are markers for the different embryonic germinal zones of the developing cortical mantle. For example, the cells of the E23 preplate express *CDH4*, *CDH11*, *CDH14*, *PCDH7*, *PCDH9*, and *PCDH10*. Possibly, at least some of the cadherin-expressing cells in the preplate (prospective layer I) are Cajal-Retzius. The subependymal layer that is marked by the combinatorial expression of *CDH7*, *CDH14*, *PCDH17*, and *PCDH19*, and the ventricular zone expresses *CDH4*, *CDH11*, *CDH20*, *PCDH1*, and *PCDH11*. Previously, 2 classic cadherins, N-cadherin and CDH6, have been shown to play roles in the maintenance of the neuroepithelial layer, the proper lamination of neural tissue and the migration of neural cells (Barami et al. 1994; Radice et al. 1997; Gänzler-Odenthal and Redies 1998; Coles et al. 2007; Ruan et al. 2006; Kadowaki et al. 2007). Moreover, the cadherin-mediated adhesive system regulates cell proliferation and cell death in the neuroepithelium (Babb et al. 2005; Lien et al. 2006; Noles and Chenn 2007). It remains unclear at present whether the cadherins investigated in this study have similar functions.

A large number of other genes like Emx1, Emx2, Pax6, Dlx-2, IgCAM, and MDGA1 are expressed in a layer-specific fashion in the germinal zones (Bulfone et al. 1993; Panganiban and Rubenstein 2002; Bishop et al. 2003; Hevner et al. 2003; Takeuchi et al. 2007) and in the differentiating and mature cortical plate (for a review, see Funatsu et al. 2004). The cadherins studied in the present work are the first molecules from a single gene family that differentially distinguish the various germinal zones during development. In addition, most of the previously described genes code for gene regulatory proteins and are involved in embryonic pattern formation. In contrast, cadherins are a family of morphoregulatory molecules, possibly acting downstream of genetic patterning mechanisms (Shimamura et al. 1994; Stoykova et al. 1997; Miyashita-Lin et al. 1999; Nakagawa et al. 1999; Rubenstein et al. 1999; Bishop et al. 2000; Bishop et al. 2002; Garel et al. 2003; Luo et al. 2006; Rasin et al. 2007).

Cadherins are likely to be involved also in target recognition and intracortical circuit formation during corticogenesis. It has been shown previously that pre- and postsynaptic neurons often express the same cadherin in a matching fashion (Redies et al. 1993; Wöhrn et al. 1998; for a reviews, see Redies 1997, 2000; Hirano et al. 2003). For example, thalamic afferents and their cortical targets were shown to express matching cadherins in the cerebral cortex of rodents (Suzuki et al. 1997; Gil et al. 2002; Kim et al. 2007). In the chicken optic tectum, N-cadherin mediates layer-specific target recognition (Yamagata et al. 1995). It is possible, but remains to be demonstrated experimentally, that the expression of cadherins observed in the present study also regulates the formation of layer-specific cortical connectivity. The persistence of cadherin expression in the adult visual cortex supports the notion that cadherins play a role also in mature cortical function. Several cadherins have been found at the synapse, also during synaptogenesis (Fannon and Colman 1996; Uchida et al. 1996; Bozdagi et al. 2000; Togashi et al. 2002). A role for cadherins in dendritic sprouting and synapse plasticity has been proposed (for a review, see Takeichi 2007). We are currently generating antibodies against some of the cadherins in order to localize the cadherin proteins at the synapse also in the ferret visual cortex.

The function of classic cadherins and δ-protocadherins in the above processes may be different, as suggested by differences in the intracellular binding partners. Classic cadherins are linked intracellularly to a variety of molecules, including some catenins, which play a role in signal transduction and gene regulation; for example, one of the binding partners, β-catenin, is an integral part of the canonical Wnt pathway (for reviews, see Hirano et al. 2003; Nelson and Nusse 2004). Known intracellular binding partners of δ-protocadherins include the synaptic molecule protein phosphatase 1α, TAF1/set, β-catenin, Xfz7 and mDab1 (for a review, see Redies et al. 2005).

Region-Specific Expression of Cadherins in Primary Visual Cortex

Previous studies demonstrated that cadherins are regional markers for cortical areas during early embryonic development of the mouse. Examples are N-cadherin, *Cdh4*, *Cdh6*, *Cdh8*, and *Cdh11* and several δ-protocadherins (Korematsu and Redies 1997; Suzuki et al. 1997; Simonneau and Thiery 1998; Rubenstein et al. 1999; Obst-Pernberg et al. 2001; Bekirov et al. 2002; Kim et al. 2007). In the present study, we show that differences in cadherin expression demarcate the boundary of the V1 and V2 subregions within the visual cortex. Cadherin expression thus reflects the functional compartmentation of the cerebral cortex into functional subregions. The V1/V2 boundary is also marked by the expression of other molecules like Cat-301, alkaline phosphatase and cytochrome oxidase (Hockfield et al. 1990; Fonta and Imbert 2002).

One particularly striking feature of ferret visual cortex is its columnar functional architecture (Redies et al. 1990; Chapman et al. 1996; Weliky et al. 1996). Surprisingly, at this highest level of cortical regionalization, we did not obtain any evidence for a differential expression of cadherins at the mRNA level.

Cadherin Expression by Subsets of Cortical Neurons

A closer look at the expression of cadherins reveals that not all cadherins are expressed by all neurons in a given cortical layer. A particularly striking example is the expression of *CDH7* by scattered cells in all cortical layers (Figs 1*C*, 5, and 6). It is conceivable that these cells represent a particular type of neurons, for example interneurons. This suggestion, which requires confirmation by a double-labeling study with interneurons markers, is supported by the finding that similarly distributed cells are found in other cortical areas.

In some cortical layers, subsets of neurons express a particular cadherin. For example, *CDH4*, *CDH6*, *CDH7*, *CDH8*, *CDH11*, *CDH14*, *CDH20*, *PCDH1*, *PCDH8*, *PCDH9*, and *PCDH19* mark subsets of neurons in layer V. This layer contains a mixture of pyramidal neurons, which project to various subcortical targets. It has been shown previously in the chicken tectum that subpopulations of projection neurons and their axons express cadherins differentially (Wöhrn et al. 1999) and that the cadherins target the tectofugal axons to specific axonal pathways (Treubert-Zimmermann et al. 2004). Future studies employing antibodies will show whether a similar differential cadherin expression is also implemented in the subcortical fiber projection systems that originate in the ferret visual cortex. Another possibility is that cadherins, which are expressed by subsets of neurons in a given cortical layer, mediate the formation of intracortical microcircuitry, as has been proposed for α-protocadherins (Kohmura et al. 1998).

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3.2.

Expression of cadherin superfamily genes in brain vascular development

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Brief Communication

Expression of cadherin superfamily genes in brain vascular development

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Cadherins are Ca^{2+}-dependent cell adhesion molecules that are important in vertebrate nervous system development. We identified seven members of the cadherin superfamily (cadherin-4, cadherin-5, cadherin-6, cadherin-6, cadherin-11, protocadherin-1, and protocadherin-17) and an intracellular binding partner of δ-protocadherins, protein phosphatase 1α, as novel markers for developing blood vessels in the ferret brain. Some of the cadherin molecules are restricted to specific brain regions or a subset of blood vessels. The expression levels show a peak during perinatal vascular development. Our results suggest that multiple cadherins, which are also involved in neurogenesis, are regulators of angiogenesis in developing vertebrate brain.
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Keywords: cell adhesion; ferret brain; *in situ* hybridization; neuroangiogenesis; protein phosphatase 1α; δ-protocadherins

Introduction

The development of a functional vascular system is a complex process and a primary requirement for embryogenesis. In the brain, concomitant vascular development meets the metabolic needs of growing neuronal populations. The vascularization of the brain takes place exclusively by means of angiogenesis (Plate, 1999). Angiogenesis is achieved by the pruning, remodelling, and extension of an existing primary vascular plexus in response to local cues, such as growth factors, adhesion molecules, and guidance factors. It is regulated by the capacity of endothelial cells to adhere to each other and to surrounding cells. Examples of adhesion molecules involved in angiogenesis are CD34, PECAM, VE-cadherin (cadherin-5, CDH5; Breier *et al*, 1996), N-cadherin, cadherin-10 (Williams *et al*, 2005), T-cadherin, and R-cadherin (Cavallaro *et al*, 2006) and protocadherin-1 (PCDH1; Redies *et al*, 2008). The presence of some cadherins in developing brain vasculature prompted us to ask whether other recently identified cadherins are also expressed during brain angiogenesis.

Cadherins are a large family of Ca^{2+}-dependent cell adhesion glycoproteins, with more than 100 members in vertebrates. Cadherins are grouped into subfamilies that are designated as classic cadherins, desmosomal cadherins, flamingo cadherins, FAT cadherins, and protocadherins (Hirano *et al*, 2003). They play a role in a wide variety of developmental mechanisms, including cell proliferation, differentiation, recognition, migration and sorting, morphogenesis, signal transduction, and axon outgrowth (Gumbiner, 2005). The interaction of cadherins with the cytoskeleton through intracellular partners is crucial for their functioning. During vascular development, cadherins have been reported to regulate the formation of the primitive capillary plexus and its pruning, endothelial integrity, vascular permeability, formation of blood–brain barrier, pericyte stabilization, and tumor angiogenesis (Cavallaro *et al*, 2006). For example, gene silencing of VE-cadherin and N-cadherin in mice leads to early embryonic death with associated severe vascular anomalies (Cavallaro *et al*, 2006).

With regard to its precise wiring in highly ordered and stereotyped networks, the formation of vasculature closely parallels neuronal development. Indeed, many of the molecules regulating vascular system development, like VEGFR, Eph, N-cadherin, Dlx1/2, Nkx2.1, Shh, and Pax6, are implicated also in neural development. It has therefore been proposed that common mechanisms are shared by vascular and neuronal development (Carmeliet, 2003). A large number of classic cadherins and δ-protocadherins (Vanhalst *et al*, 2005) are differentially expressed in brain structures and subtypes of neurons in the developing mammalian brain

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(Kim *et al*, 2007; Hertel *et al*, 2008; Krishna-K *et al*, 2008). In this study, we asked whether some of these cadherins and an intracellular binding partner of δ1-protocadherins, protein phosphatase 1α (PP1α; Vanhalst *et al*, 2005), are also expressed by developing blood vessels in the ferret, an animal model suitable for cerebrovascular research (Atkinson *et al*, 1989).

Materials and methods

Animals

The study was performed in 36 ferrets at 10 developmental stages (at 23 days after conception (E23), E30, E38, at postnatal day 2 (P2), P13, P25, P33, P46, P60, and at the adult stage). The series of sections analyzed was used previously to study visual cortical development (Krishna-K *et al*, 2008).

Reverse Transcriptase-PCR and Cloning

Probe synthesis and plasmids used were described previously (Krishna-K *et al*, 2008). In addition to the existing probes, we obtained cDNA fragments for CDH5, cadherin-19 (CDH19), and PP1α by reverse transcriptase-PCR and cloned them into pCR II-TOPO vector. Degenerate primers used and sequences obtained were submitted to GenBank (Table 1).

In Situ Hybridization

cRNA probes were produced with T7 or SP6 RNA polymerase followed by labeling with digoxigenin or fluorescein to generate antisense probes. *In situ* hybridization was performed as described previously with antisense and sense probes for all cadherins (Krishna-K *et al*, 2008).

Double Tyramide-FISH and Fluorescent Immunohistochemistry

The *in situ* hybridization protocol was modified by introducing tyramide-coupled Alexa fluorophore A488 or A568 as reaction products by CARD (Krishna-K *et al*, 2008). Subsequently, fluorescent immunostaining was performed on the same slices by using anti-mouse fibronectin rabbit antiserum (kind gift of Richard Hynes, MIT, USA). The sections were viewed and photographed under a confocal laser scanning microscope (SP5; Leica Microsystems, Germany).

Results

The expression of eight classic cadherins, nine δ-protocadherins and PP1α was mapped by *in situ* hybridization from early embryonic stages to the adult stage in the ferret brain (Table 1). We found that 7 of the 18 cadherins (cadherin-4 (CDH4), CDH5, cadherin-6 (CDH6), cadherin-7 (CDH7), cadherin-11 (CDH11), protocadherin-1 (PCDH1), and protocadherin-17 (PCDH17); Figure 1A–1R) and PP1α (Figure 1S–1U) are expressed by blood vessels in the developing brain (black arrows in Figure 1; Table 1) and meninges (blue arrows in Figure 1). No specific labeling was seen with sense probes (Figure 1D′). Embryonic blood vessels showed strong and ubi-

Table 1 Expression profile of 18 cadherins and PP1α in blood vessels of the developing ferret brain[a]

Cadherin/GenBank no.	*E23*	*E30*	*E38*	*P2*	*P13*	*P25*	*P33*	*P46*	*P60*	*Adult*
CDH4 (EU665238)	−	−	+	+	+	−	−	−	−	−
CDH5 (FJ170102)	+++	+++	+++	+++	+++	++	++	+	−	−
CDH6 (EU665239)	+	++	++	+	+	+	−	−	−	−
CDH7 (EU665240)	++	+++	+++	++	++	+	−	−	−	−
CDH8 (EU665241)	−	−	−	−	−	−	−	−	−	−
CDH11 (EU665242)	++	+++	++	++	++	++	+	−	−	−
CDH14 (EU665243)	−	−	−	−	−	−	−	−	−	−
CDH19 (bankit1128002)	−	−	−	−	−	−	−	−	−	−
CDH20 (EU665244)	−	−	−	−	−	−	−	−	−	−
PCDH1 (EU665245)	++	++	++	+++	+++	+	+	−	−	−
PCDH7 (EU665246)	−	−	−	−	−	−	−	−	−	−
PCDH8 (EU665247)	−	−	−	−	−	−	−	−	−	−
PCDH9 (EU665248)	−	−	−	−	−	−	−	−	−	−
PCDH10 (EU665249)	−	−	−	−	−	−	−	−	−	−
PCDH11 (EU665250)	−	−	−	−	−	−	−	−	−	−
PCDH17 (EU665251)	+++	+++	+++	+	+	−	−	−	−	−
PCDH18 (EU665252)	−	−	−	−	−	−	−	−	−	−
PCDH19 (EU665253)	−	−	−	−	−	−	−	−	−	−
PP1α (FJ170103)	+++	+++	+++	+++	++	+	−	−	−	−

CDH4, cadherin-4; CDH5, cadherin-5; CDH6, cadherin-6; CDH7, cadherin-7; CDH8, cadherin-8; CDH11, cadherin-11; PCDH1, protocadherin-1; PCDH7, protocadherin-7; PCDH8, protocadherin-8; PCDH9, protocadherin-9; PCDH17, protocadherin-17; PCDH18, protocadherin-18; PCDH19, protocadherin-19; PP1α, protein phosphatase 1α.

[a]Expression level: −, absent ; +, weak, ++ moderate; +++ strong.

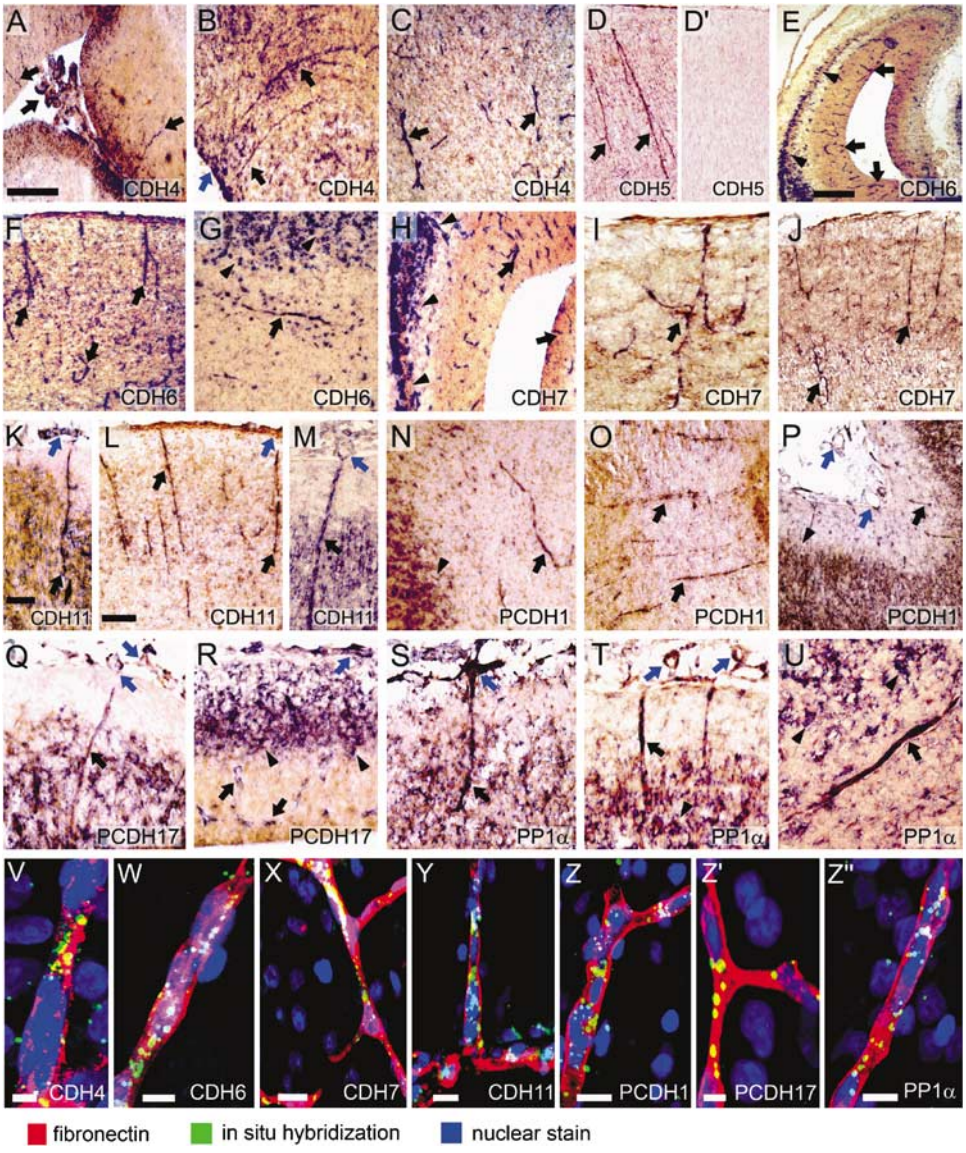

Figure 1 (**A**–**U**) Expression of cadherins and protein phosphatase 1α in blood vessels of the developing ferret brain. Expression was mapped *in situ* with cRNA antisense probes for cadherin-4 (CDH4) at embryonic day 38 (E38; **A**), postnatal day 2 (P2; **B**) and P13 (**C**); cadherin-5 (CDH5) with antisense probe (**D**) and sense probe (**D′**) at P2; cadherin-6 (CDH6) at E23 (**E**), P2 (**F**), and P25 (**G**); cadherin-7 (CDH7) at E30 (**H**), E38 (**I**), and P2 (**J**); cadherin-11 (CDH11) at E23 (**K**), P2 (**L**), and P25 (**M**); protocadherin-1 (PCDH1) at P2 (**N**), P25 (**O**), and P33 (**P**); protocadherin-17 (PCDH17) at E23 (**Q**) and E30 (**R**); and protein phosphatase 1α (PP1α) at E23 (**S**), E30 (**T**), and P13 (**U**). (**V**–**Z″**) Double fluorescent labeling of blood vessels with an antibody against fibronectin and tyramide-FISH for different cRNA probes (CDH4, **V**; CDH6, **W**; CDH7, **X**; CDH11, **Y**; PCDH1, **Z**; PCDH17, **Z′**; PP1α, **Z″**) at P13. The black arrows point at brain blood vessels. The blue arrows point at the meninges or meningeal blood vessels. The arrowheads point at neurons. Scale bars are 200 μm (**D**, **P**); 100 μm (**A**–**D**, **D′**, **E**–**H**, **J**, **N**, **O**); 50 μm (**I**, **K**, **Q**–**U**); 25 μm (**L**, **M**); 20 μm (**X**, **Y**); 10 μm (**W**, **Z**–**Z″**); and 5 μm (**V**).

quitous expression of CDH11, PCDH17, and PP1α, whereas expression of CDH4, CDH6, and PCDH1 was restricted to blood vessels in specific brain regions. Double labeling with antibodies against fibronectin, a marker for embryonic brain blood vessels, confirmed the vascular expression of the seven cadherins and PP1α (Figure 1V–1Z″). However, particularly at postnatal stages, only a subset of fibronectin-positive blood vessels expressed the cadherins. Note that many of the cadherins are also expressed by neurons in the same brain regions (arrowheads in Figure 1).

Cadherin-4 (R-Cadherin)

The expression of CDH4 (R-cadherin) starts to appear in brain blood vessels at E38 (Figure 1A). The

meninges are also positive. The expression is weak and restricted to cerebral cortex. The signal becomes stronger at P2 (Figure 1B), decreases until P13 (Figure 1C) and disappears by P25.

Cadherin-5 (VE-Cadherin)

Strong staining was observed from the earliest stage studied (E23) and persists until P13 in most brain regions (Figure 1D). Thereafter, expression decreases and is not seen after P46.

Cadherin-6

At the earliest stage examined (E23), weak staining was observed in some blood vessels of the brain stem. At E30, expression is prominent in the telencephalon, where positive blood vessels are seen in the mantle zone and ventricular zone (Figure 1E). In the hindbrain, signal becomes weak from E30 to E38. Staining in the telencephalon gradually decreases until P13 (Figure 1F). Some blood vessels in the white matter are also weakly to moderately positive, for example at P25 (Figure 1G). At later stages, most of the blood vessels do not show any expression.

Cadherin-7

Moderate staining for CDH7 was seen in hindbrain blood vessels at E23. At E30 (Figure 1H), E38 (Figure 1I), and P2 (Figure 1J), moderate to strong expression was observed in blood vessels throughout the brain, both in the ventricular zone and mantle zone. At P2 and P13, blood vessels in frontal cortical regions, including white matter, are stained more strongly than in caudal telencephalic regions. Signal decreases at P25 and disappears at P33.

Cadherin-11

Ubiquitously strong expression by blood vessels in all brain regions and in the meninges is seen from E23 to E38, both in the ventricular layer and mantle layer (Figure 1K). At P2, signal has become moderate in most areas, but remains strong in some blood vessels in the cortical mantle (Figure 1L). At P13 and P25, only blood vessels in the cortex remain weakly to moderately positive (Figure 1M). No signal is observed after P33.

Protocadherin-1

From E23 and E38, blood vessels in the telencephalon are moderately positive, and a weak to moderate signal is seen in meninges. The expression is moderately strong between P2 (Figure 1N) and P25 (Figure 1O), particularly in the cerebral cortex and diencephalon; it is no longer observed after P33 (Figure 1P).

Protocadherin-17

PCDH17 expression is very strong and ubiquitous in blood vessels of all brain regions and in the meninges at E23, both in the ventricular and mantle layer (Figure 1Q). This expression profile persists at E30 and E38 (Figure 1R). The staining becomes weak at P2 and is no longer seen after P13.

Protein Phosphatase 1α

PP1α shows strong ubiquitous staining in the blood vessels and meninges at E23 (Figure 1S). Staining persists at E30 and E38 (Figure 1T). At P2, vessels exhibit only a weak signal, except for superficial blood vessels in the neocortex that maintain a strong expression. At P13, a subset of blood vessels retains moderate staining, also in the white matter (Figure 1U). The expression becomes weak at P25 and disappears thereafter.

The expression of the above cadherins and PP1α by blood vessels was confirmed by double labeling with a marker for brain blood vessels (fibronectin; red in Figure 1V–1Z″).

Discussion

For the first time, expression of 18 members of two cadherin subfamilies, classic cadherins and δ-protocadherins, were studied in blood vessels at different stages of vertebrate brain development. Using *in situ* hybridization and immunohistochemistry, we show that 7 out of the 18 cadherins and PP1α (Table 1) are expressed under a tight spatiotemporal control by cerebral blood vessels during development.

Cadherin Expression by Blood Vessels During Development

The cadherins and PP1α are expressed from the earliest stage studied (E23) until about P25. In higher vertebrates, brain angiogenesis starts at the beginning of neurogenesis and proceeds up to the last wave of neuronal migration, when the basic scheme of vascular network is completed (Plate, 1999). As the brain grows in thickness, unbranched vessel, which migrate radially into the nervous wall, express the cadherins (Figure 1J–1M, 1P, 1Q, 1S and 1T). During this migration, cadherins may contribute to the adhesion between endothelial cells or between endothelial cells and surrounding pericytes, as shown for VE-cadherin and N-cadherin, respectively (Breier *et al*, 1996; Cavallaro *et al*, 2006). Cadherin expression reaches highest levels at perinatal stages (Table 1). At corresponding stages in the rat, maximal endothelial cell proliferation and gliogenesis takes

place. Similarly, expression of Flk-1, Flt-1, PECAM-1, and VEGF peaks in vessels of the premature brain and then declines (Ogunshola *et al*, 2000; Yang *et al*, 2003). The peak during this critical developmental period suggests that cadherins are proangiogenic factors that regulate the growth, migration, and controlled pruning of the newly forming vascular network. Because the cadherins are not expressed by astrocytes (Krishna-K and Redies C, unpublished data), they are unlikely to play a role in the interaction between endothelial cells and astrocytes (Zerlin and Goldman, 1997). The identification of PP1α in developing blood vessels leads to the question of which molecules are regulated downstream by this phosphatase.

Region- and Subtype-Specific Expression of Cadherins by Blood Vessels

Expression of CDH4, CDH6, and PCDH1 is restricted to particular areas of developing brain, in particular to cerebral cortex. Another cadherin that shows a regionally restricted expression pattern is cadherin-10; it is found at the surface of the mouse cortex but not of the cerebellum (Williams *et al*, 2005). Interestingly, some cadherins (CDH7 and CDH11) are expressed by specific blood vessels although the neighboring ones do not show expression. Several studies have shown that arteries and veins are likely to differ in their expression of molecular markers. For example, ephrinB2 expression is confined to the arterial endothelium whereas EphB4 expression is higher in veins (Wang *et al*, 1998). Whether the cadherins are expressed also by a specific blood vessel type or during a specific stage of angiogenesis remains to be investigated.

A Common Cadherin-Based Mechanism Behind Angiogenesis and Neurogenesis?

The seven cadherins expressed by blood vessels are also known for their spatiotemporally regulated expression by neurons in restricted brain regions (arrowheads in Figure 1; Kim *et al*, 2007; Hertel *et al*, 2008; Krishna-K *et al*, 2008) and for their roles in brain regionalization, cell-specific expression and guided migration. Other genes, like ephrins, netrins, slits, and semaphorins, are also concomitantly expressed and reported to play dual roles in both neural and vascular development (Carmeliet, 2003). This similarity led to the suggestion that there might be an intrinsic or operative relationship or both between neurogenesis and angiogenesis (Carmeliet, 2003; Vasudevan *et al*, 2008). The identification of a novel panel of cadherin markers for developing brain blood vessels provides a basis for the design of functional assays to study the role of these genes in neuroangiogenesis, also in brain tumors.

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Disclosure/conflict of interest

The authors declare that they have no conflict of interest.

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3.3.

A cadherin-based code for the divisions of the mouse basal ganglia

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A Cadherin-Based Code for the Divisions of the Mouse Basal Ganglia

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ABSTRACT

The expression of 12 different classic cadherins and δ-protocadherins was mapped in consecutive series of sections through the basal ganglia of the postnatal and adult mouse by in situ hybridization. A particular focus was the caudoputamen, which consists of patches (striosomes) and a surrounding matrix that is histologically uniform. The different areas within the caudoputamen are connected specifically to other parts of the basal ganglia and to other brain regions, for example, the substantia nigra. The molecules regulating the morphogenesis and functional connectivity of the basal ganglia are largely unknown. Previous studies suggested that cadherins, a large family of adhesion molecules, are involved in basal ganglia development. In the present work, we study the expression of 12 cadherins and show that the patch and matrix compartments of the caudoputamen express the cadherins differentially, although partial overlap is observed. Moreover, the cadherins are expressed in multiple and diverse gradients within the caudoputamen and other parts of the basal ganglia. The persistence of the expression patterns in the adult basal ganglia suggests the possibility that cadherins also play a role at adult stages. Our results suggest that cadherins provide a code of potentially adhesive cues that specify not only patch and matrix compartments but also multiple molecular gradients within the basal ganglia. This code may relate to patterns of connectivity. J. Comp. Neurol. 508:511–528, 2008.

Indexing terms: forebrain development; caudoputamen; accumbens nucleus; globus pallidus; ventral pallidum; substantia nigra

In the central nervous system of vertebrates, three main types of gray matter architecture can be distinguished: brain nuclei (for example, in the thalamus), laminated structures (for example, the layers of the mammalian cerebral cortex), and a more complex patch/matrix organization. An example for the latter type is found in the basal ganglia, especially in the mammalian striatum (Graybiel and Ragsdale, 1978; Gerfen, 1985, 1992; Bolam et al., 1988; Kawaguchi et al., 1989).

The basal ganglia consist of the striatum and pallidum. The striatum includes the caudoputamen (CPu; dorsal striatum), the accumbens nucleus, and a part of the olfactory tubercle in rodents. The pallidum includes the globus pallidus and the ventral pallidum that encompasses the islands of Calleja (for review, see Gerfen, 2004).

In the caudoputamen, neurons form multiple small aggregates (called patches or striosomes) that are embedded in a relatively uniform matrix (Graybiel and Ragsdale, 1978; Gerfen, 1985, 1992; Bolam et al., 1988; Kawaguchi et al., 1989). This striatal compartmentation can be visualized by several molecular markers, for example, opiate receptors, substance P and tyrosine hydroxylase (TH) (striosomes), and somatostatin, acetylcholinesterase, enkephalin, and calbindin (matrix; Graybiel et al., 1981; Graybiel, 1984, 1990; Gerfen, 1985; Gerfen et al., 1985; Liu and Graybiel, 1992). Striatal compartmentation is also reflected in connectivity. Patch neurons receive afferent projections from limbic regions and from the deeper parts of layer V and from layer VI of the neocortex. The matrix compartment receives afferents mainly from the sensorimotor cortex, in particular from superficial parts of layer V and from layers II and III (Gerfen, 1984, 1992; Donoghue and Herkenham, 1986).

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The patch/matrix organization of the caudoputamen is also reflected in functionally connected areas. A patch/matrix-directed system is the dopaminergic nigrostriatal pathway (Gerfen et al., 1987a,b; Jimenez-Castellanos and Graybiel, 1987). This system originates from the A8, A9, and A10 dopaminergic cell groups (Dahlström and Fuxe, 1964). The projecting dopaminergic neurons are organized in two subgroups, a dorsal and a ventral tier. Different portions of these A8–A10 cell groups innervate the striatal matrix and target the striosomes (Gerfen et al., 1985, 1987a,b; Jimenez-Castellanos and Graybiel, 1987; Langer and Graybiel, 1989; Gerfen, 1992; Song and Haber, 2000; Prensa and Parent, 2001).

In vivo evidence suggests that patch and matrix neurons possess differential adhesiveness (Krushel et al., 1989; Krushel and van der Kooy, 1993). One major group of adhesive factors is the cadherins, a family of cell surface glycoproteins that regulate cell adhesion and cell sorting by a preferentially homotypic binding mechanism (Nose et al., 1988). Cadherins play a role in the development of many organs and tissues, including the central nervous system (for reviews, see Takeichi, 1988; Redies, 2000; Frank and Kemler, 2002). Cadherins are involved in intracellular signaling, cell differentiation, and other developmental processes (for review, see Hirano et al., 2003).

Some cadherins are expressed differentially in the patch/matrix compartments of the rodent caudoputamen. Examples are members of the classic cadherin subfamily (cadherin-4 [R-cadherin], Obst-Pernberg et al., 2001; cadherin-8, Korematsu and Redies, 1997; and cadherin-11, Suzuki et al., 1997) and protocadherin-10 (Hirano et al., 1999). Protocadherin-10 belongs to the recently identified subgroup of δ-protocadherins (Vanhalst et al., 2005; Redies et al., 2005). Expression of other δ-protocadherins has been reported for the rat caudoputamen (Kim et al., 2007).

In the present paper, we describe a number of additional classic cadherins and δ-protocadherins, which are expressed in the basal ganglia and their connected areas. Because a systematic study comparing the expression of multiple members of the cadherin superfamily in the basal ganglia of a single species is lacking to date, we mapped the expression of 12 different cadherins. We focused on postnatal day 5 (P5) brain because, at this stage of development, the basal ganglia have already acquired their mature functional architecture, but neurons are more densely packed than at later stages, facilitating the analysis of cadherin expression patterns by in situ hybridization. Results were compared with cadherin expression in the adult basal ganglia. The expression of an intracellular binding partner of δ-protocadherins, protein phosphatase 1α (Yoshida et al., 1999; Vanhalst et al., 2005), was also studied.

MATERIALS AND METHODS

Animals

Mouse pups and adult mice of the NMRI strain were obtained from the animal facilities of the University Hospital Jena. The day of birth was defined as P0. At P5, pups were deeply anesthetized on ice and quickly decapitated. Adult mice were deeply anesthetized with chloroform and decapitated. These procedures were in accordance with current versions of institutional regulations and national laws on the use of animals in research. Efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

P5 brains were dissected and fixed overnight in 4% formaldehyde dissolved in HEPES-buffered salt solution supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS, pH 7.4). For cryoprotection, the brains were incubated in an ascending series of HBSS-buffered sucrose solutions (12% [w/v], 15%, 18%). The brains were embedded in Tissue Tec O.C.T. compound (Science Services, München, Germany), frozen in liquid nitrogen, and stored at −80°C.

Adult brains were frozen quickly by immersion in 2-methyl butane chilled with dry ice to about −35°C and stored at −80°C. For sectioning, frozen adult brains were mounted onto the cutting stage of the cryostat by Tissue Tec O.C.T. compound.

Abbreviations

aca	anterior commissure, anterior part
AcbC	accumbens nucleus, core
AcbSh	accumbens nucleus, shell
Amy	amygdala
CB	cell bridges of the ventral striatum
cc	corpus callosum
Cdh	cadherin
Cl	claustrum
CPu	caudoputamen (dorsal striatum)
Cx	cerebral cortex
d	dorsal
DEn	dorsal endopiriform nucleus
ec	external capsule
fi	fimbria of the hippocampus
fmi	minor forceps of the corpus callosum
GP	globus pallidus
HDB	nucleus of the horizontal limb of the diagonal band
HI	hippocampus
ic	internal capsule
ICj	islands of Calleja
ICjM	islands of Calleja, major island
IPAC	interstitial nucleus of the posterior limb of the anterior commissure
l	lateral
LAcbSh	lateral accumbens, shell
LH	lateral area of the hypothalamus
lo	lateral olfactory tract
LSI	lateral septal nucleus, intermediate part
LSS	lateral stripe of the striatum
LSV	lateral septal nucleus, ventral part
LV	lateral ventricle
m	medial
Pcdh	protocadherin
Pir	piriform cortex
RRF	retrorubral field
RT	room temperature
SNCD	substantia nigra, compact part, dorsal tier
SNCV	substantia nigra, compact part, ventral tier
SNR	substantia nigra, reticular part
st	stria terminalis
T	thalamus
TH	tyrosine hydroxylase
Thio	thionine
Tu	olfactory tubercle
v	ventral
VEn	ventral endopiriform nucleus
VP	ventral pallidum
VTA	ventral tegmental area

Sections of 20-μm thickness were cut in a cryostat and collected directly onto SuperFrost® Plus slide glasses (Menzel, Braunschweig, Germany). The sections were dried at 50°C and 56°C for immunohistochemistry and in situ hybridization, respectively. Complete series of adjacent frontal sections were obtained (P5, 15 series; and adult, 2 series).

Immunohistochemistry

For immunohistochemistry, the sections were fixed in ice-cold 4% formaldehyde in HBSS for 20 minutes and washed in Tris-buffered saline (TBS, pH 7.4) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Endogenous peroxidase activity was suppressed by immersing the sections in 0.3% H_2O_2 in methanol for 30 minutes. Nonspecific binding of antibodies was blocked by incubating the sections with 1.5% horse serum in TBS ("blocking solution") for 30 minutes. Rabbit polyclonal antibody against TH purified from rat pheochromocytoma (catalogue number 657012, lot number D29976, Calbiochem, La Jolla, CA) was appropriately diluted in blocking solution (1:2,000). The antibody recognizes a ~60-kDa band, which corresponds to the molecular weight of the TH protein (manufacturer's technical information). The staining pattern obtained closely matches that of TH in the caudoputamen and other parts of the brain (Jimenez-Castellanos and Graybiel, 1987; Korematsu et al., 1998).

Sections were incubated with this solution at 4°C overnight. Three washing steps in TBS were followed by incubation of the sections with biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, Cambridgeshire, UK) for 30 minutes at room termperature (RT). The sections were then reacted with avidin-biotin-horseradish peroxidase complex (ABC Kit, Vector, Burlingame, CA) by using 0.03% 3,3′-diaminobenzidine, 0.04% $NiCl_2$ and 0.01% H_2O_2 as a substrate, according to the manufacturer's instructions. The sections were dehydrated in an ascending ethanol series, cleared in xylenes, and mounted in Entellan (Merck, Darmstadt, Germany).

In situ hybridization

For in situ hybridization, a previously published protocol was followed (Redies et al., 1993). In brief, digoxigenin-labeled antisense cRNA probes were synthesized in vitro by using the following plasmids:

1. pBSMR4 containing full-length mouse cadherin-4 (R-cadherin) cDNA (positions 55–2,794 of sequence GenBank Acc. No. D14888; Matsunami et al., 1993; kind gift from Drs. H. Matsunami and M. Takeichi; RIKEN Center for Developmental Biology, Kobe, Japan).
2. mcad8-12 containing a 1.6-kb fragment of mouse cadherin-8 cDNA from the 5′ region (positions 504–1,583 of sequence GenBank Acc. No. X95600; Korematsu and Redies, 1997).
3. BSSK11 containing full-length mouse cadherin-11 cDNA (positions 452–2,840 of sequence GenBank Acc. No. D31963; Kimura et al., 1995; kind gift from Drs. H. Kimura and M. Takeichi).
4. pGEMte-mPcdh1-ISH comprising a 1.6-kb polymerase chain reaction (PCR) fragment encoding a large part of the cytoplasmic domain plus the transmembrane domain of protocadherin-1 (positions 1,195–2,781 of sequence GenBank Acc. No. NM029357; Vanhalst et al., 2005, and unpublished data).
5. pGEMte-mPcdh7 comprising a 1.6-kb PCR fragment encoding part of the extracellular domain of protocadherin-7 (positions 1,947–3,575 of sequence GenBank Acc. No. NM018764; Vanhalst et al., 2005).
6. pGEMte-mPcdh9 comprising a 1.2-kb PCR fragment encoding part of the extracellular domain of protocadherin-9 (positions 961–2,258 of sequence GenBank Acc. No. NM001081377; Vanhalst et al., 2005).
7. pGEMte-mPcdh11 comprising a 1.2-kb PCR fragment encoding part of the extracellular domain of protocadherin-11 (positions 1,581–2,867 of sequence GenBank Acc. No. NM001081385; Vanhalst et al., 2005; kind gifts of Dr. F. van Roy; Department for Molecular Biomedical Research, Ghent, Belgium).
8. mOLe11 containing full-length mouse protocadherin-10 (OL-protocadherin; Hirano et al., 1999; kind gift from Dr. S. Hirano; RIKEN Center for Developmental Biology, Kobe, Japan).

Complementary DNA fragments of different length were amplified by reverse transcriptase (RT)-PCR with specific and degenerate primers for mouse cadherin-7 (1.8-kb length; positions 182–1,998 of sequence GenBank Acc.No. AK137369), protocadherin-8 (1.7 kb; positions 201–1,901 of sequence GenBank Acc. No. NM001042726.1), protocadherin-17 (2.0 kb; positions 985–3,015 of sequence GenBank Acc. No. NM001013753.1), protocadherin-19 (2.8 kb; positions 1,827–4,722 of sequence GenBank Acc. No. NM001105246.1), and protein phosphatase 1α (972 bp; positions 42–1,014 of sequence GenBank Acc. No. NM031868.2). Appropriate bands were ligated into TOPOII-vector. The generation of these plasmids will be described elsewhere (M. Nuernberger, Krishna K. and C. Redies, unpublished data).

The sections were postfixed in phosphate-buffered formaldehyde solution (4% [w/v]) at 4°C. For better probe penetration, the sections were treated with 1 μg/ml proteinase K (Sigma, Steinheim, Germany) in 100 mM Tris (pH 8.0), 50 mM EDTA for 5 minutes. After acetylation with 0.25% acetic anhydride in triethanolamine buffer, the sections were hybridized overnight at 70°C in a humid chamber with antisense cRNA probes in hybridization buffer. The hybridization was followed by several washing steps and RNase A treatment (20 μg/ml in NTE buffer [10 mM Tris, 1 mM EDTA, 0.5 mM NaCl, pH 8.0]). Sections were incubated overnight with alkaline phosphatase-conjugated Fab fragments against digoxigenin (Roche, Mannheim, Germany) at 4°C and reacted with nitroblue tetrazolium salt (75 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine salt (50 mg/ml) in alkaline buffer. Sections were mounted in Entellan (Merck). For each series of sections, one set of adjacent sections was counterstained with thionine for neuroanatomical orientation, as described previously (Redies et al., 1993).

Digital photomicrographs of the sections were taken with a light transmission microscope (Olympus BX40) equipped with a digital camera (Olympus DP70). Images were adjusted in contrast and brightness for optimal display of the staining patterns with the Photoshop software (Adobe Systems, Mountain View, CA). For the identification of brain structures, atlases of the developing and adult mouse and rat brain were consulted (Paxinos and Franklin, 2001; Paxinos and Watson, 1998). The nomenclature and the abbreviations of the atlas by Paxinos and Watson (1998) were followed.

RESULTS

In the following sections, we will describe the expression of each cadherin that was mapped by in situ hybridization in the dorsal and ventral parts of the basal ganglia, as well as in the A8–A10 dopaminergic cell groups.

For postnatal day 5 (P5), four representative Nissl stains are shown for a rostral-to-caudal series of frontal sections in Figure 1. These sections provide an overview of the neuroanatomy of the basal ganglia. The rostralmost section (section level 1) cuts through the rostral caudoputamen (CPu; Fig. 1A–D). Section level 2 comprises the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; Fig. 1B). Level 3 cuts through the caudal caudoputamen and includes the rostral amygdala. Level 4 includes the caudalmost portion of the caudoputamen.

First we will describe the expression of cadherins in the dorsal part of the basal ganglia (caudoputamen and globus pallidus). Figure 2 summarizes the P5 expression patterns for these structures in color-coded overlays for all cadherins studied. Results for individual cadherins are displayed in Figures 3–7. For P5, an overview of all four section levels mentioned above is shown in Figures 3, 6, and 7. Each column represents results for one cadherin. These overviews are supplemented by Figure 4, which confirms the identity of the cadherin-expressing regions in the caudoputamen as patch or matrix compartment by TH immunostaining of adjacent sections. In order to find out whether the expression patterns found at P5 persist in the adult basal ganglia, we also performed in situ hybridization in the adult; results for one representative section level (corresponding to level 2; see above) are shown in Figure 5.

Second, cadherin expression in the ventral basal ganglia (accumbens nucleus and islands of Calleja) was studied (Figs. 8–10 and S1). Results for the accumbens nucleus are displayed in Figure 8 for P5 and in Supplementary Figure 1 (Fig. S1) for the adult. Results for the islands of Calleja are shown in Figures 9 and 10.

Third, we mapped cadherin expression in the A8, A9, and A10 dopaminergic cell groups, which are connected to the basal ganglia at the adult stage. Results are shown in Figure 11.

Expression in the dorsal basal ganglia (caudoputamen and globus pallidus)

Each cadherin, except cadherin-11 (Cdh11) and protocadherin-1 (Pcdh1), is expressed in a distinct subregion of the striatal matrix in the caudoputamen, often in a gradient-like fashion, with partial overlap between the cadherins. Only two cadherins (Pcdh1 and Pcdh10) are expressed in the striosomes. In the adult caudoputamen,

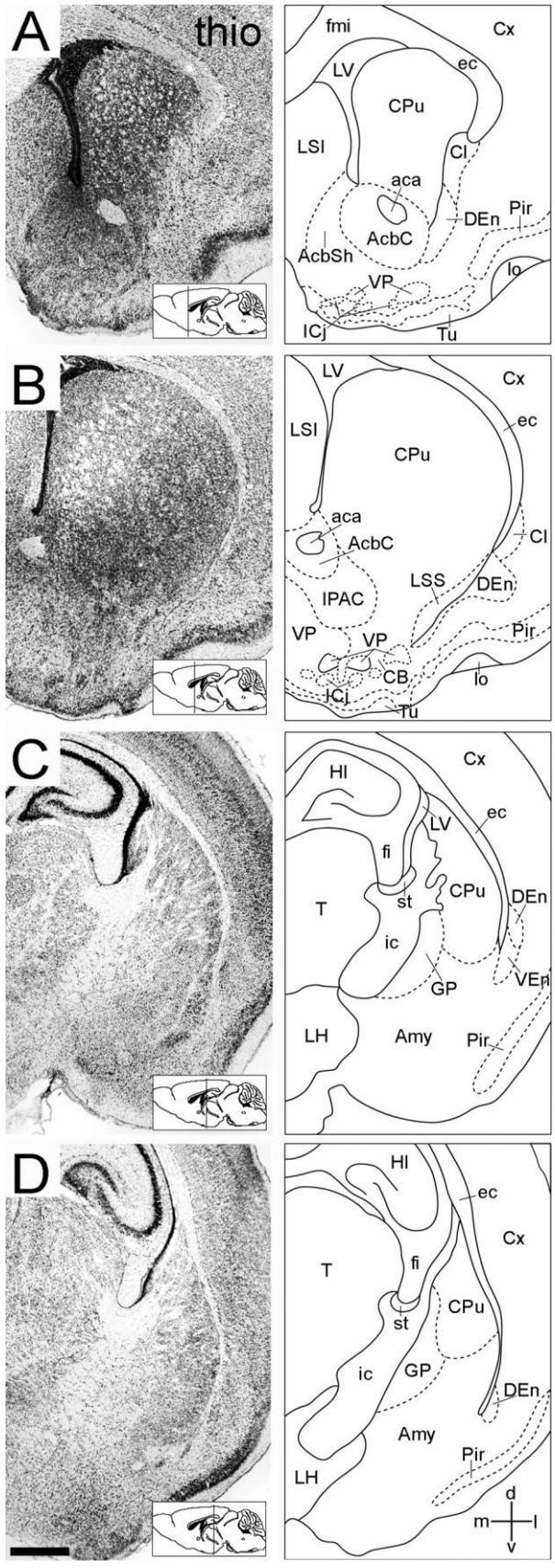

Fig. 1. Neuroanatomical description of the representative sections displayed in Figures 3, 6, and 7. Four levels of frontal sectioning through postnatal day 5 (P5) basal ganglia were stained for Nissl substance and are shown in a rostral-to-caudal sequence (**A**, section level 1; **B**, section level 2; **C**, section level 3; and **D**, section level 4). The approximate level of sectioning is indicated on the lower right corner of the Nissl stains in the schematic diagram of a midsagittal view (modified after Paxinos and Watson, 1998). The anatomical orientation of the sections is given in D. For abbreviations, see list. Scale bar = 500 μm in D (applies to A–D).

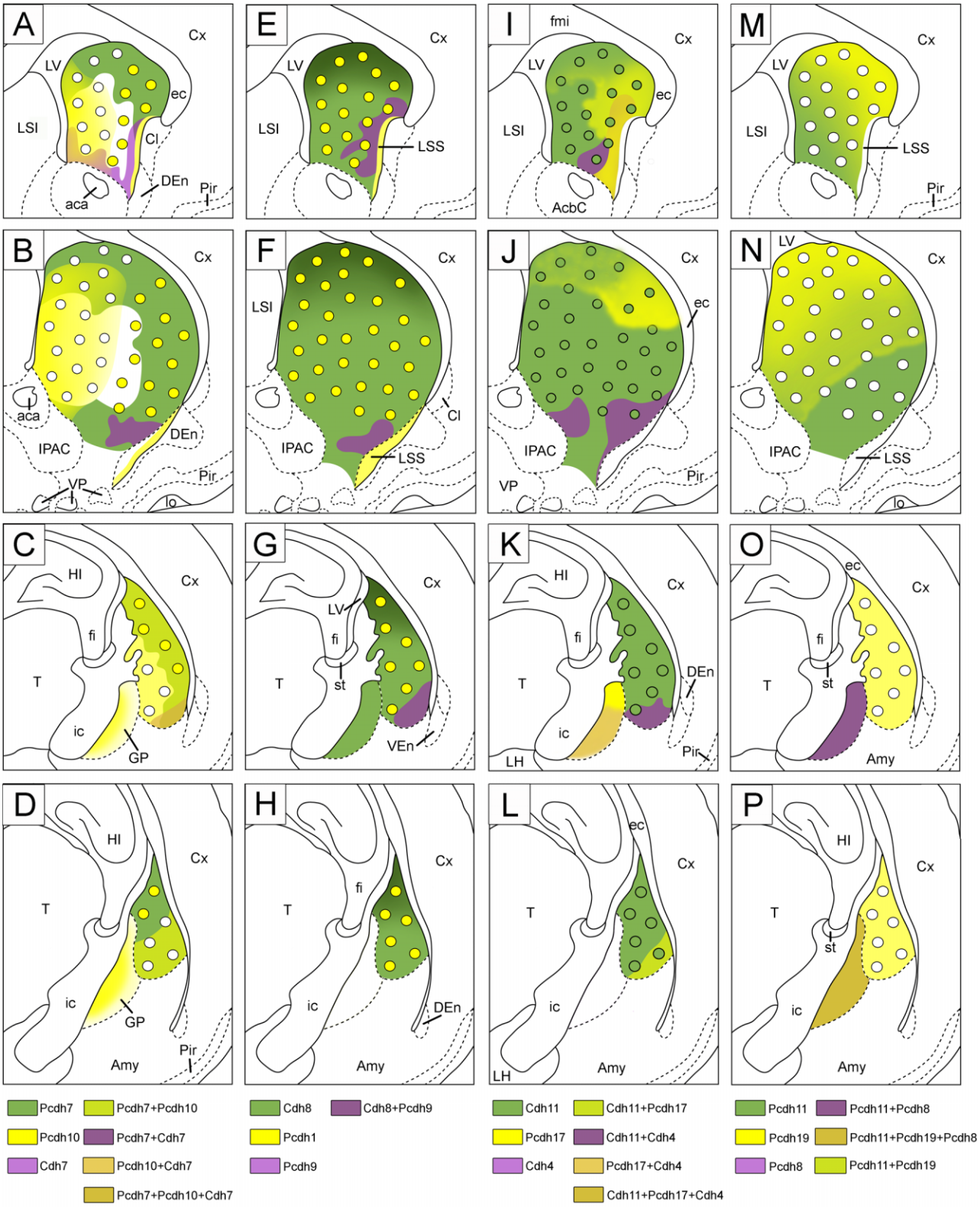

Fig. 2. Schematic overview of cadherin expression in the caudoputamen and globus pallidus of postnatal day 5 (P5) mouse brain. The diagrams summarize the data shown in Figures 3, 6, and 7. Each column shows the expression patterns for three cadherins in different colors (for color coding, see bottom of each column). Overlapping expression is represented by mixed colors, as indicated. The levels of sectioning correspond to those displayed in Figure 1. For abbreviations, see list.

expression patterns are highly similar to those at P5. Eight cadherins (Cdh4, Cdh8, Cdh11, Pcdh8, Pcdh10, Pcdh11, Pcdh17, and Pcdh19) show an expression in the globus pallidus at P5. In the following sections, these results are described in detail for each cadherin.

Cadherin-4 (Cdh4). In the rostral P5 caudoputamen (Fig. 3A,B), few Cdh4-positive perikarya can be observed in the dorsal part. The lateralmost region (Figs. 2I, 3A) and the ventrolateral corner (Figs. 2J, 3B) show strong Cdh4 expression. In these areas, positive regions border upon negative patches, which can be identified as striosomes on the basis of the TH immunostaining of an adjacent section (Fig. 4A,B). The Cdh4-negative lateroventral streak corresponds to the lateral stripe of the striatum (Fig. 3A,B; arrows in Fig. 4A,B; Paxinos et al., 1998), which is a large striosome. These results confirm the suggestion by Obst-Pernberg et al. (2001) that Cdh4 is expressed in the striatal matrix. At level 3, matrix expression is limited to the outermost ventrolateral corner (Figs. 2K, 3C). The striatal matrix is negative for Cdh4 in the caudalmost caudoputamen (Figs. 2L, 3D). A few large Cdh4-positive perikarya are dispersed throughout the caudal area.

In the adult caudoputamen, Cdh4 (Fig. 5B) is also strongly expressed in the ventral part, and only a few scattered Cdh4-positive perikarya can be observed in the dorsal part.

In the rostral part of the P5 globus pallidus, Cdh4 (Figs. 2K, 3C) is expressed moderately (Fig. 3C). Few Cdh4-positive perikarya are present in the dorsal region, whereas expression becomes more prominent in the ventral area (Fig. 2K). The entire caudal part of the globus pallidus (Fig. 3D) is Cdh4 negative.

Cadherin-7 (Cdh7). The expression of Cdh7 is limited to medial, ventral, and lateral boundary regions of the rostral striatal matrix (Figs. 2A, 3E, 4C,D, 5C) in the P5 and adult caudoputamen. These Cdh7-positive areas surround negative striosomes (Fig. 4C,D). At level 2 and 3, Cdh7-positive perikarya form a narrow stripe at the outermost lateral edge of the caudoputamen (Fig. 3F,G), adjacent to the external capsule (ec; Fig. 3F,G). The caudal part of the caudoputamen shows no signal (Figs. 2D, 3H).

Cadherin-8 (Cdh8). Cdh8 is expressed strongly in the entire caudoputamen at P5 and in the adult. This pattern can be seen at all four levels of sectioning (Fig. 3I–L), with a shallow gradient decreasing from dorsal to ventral (Figs. 2E–H, 3K). Cdh8-poor patches are embedded into these Cdh8-positive areas (Fig. 3I,J). Immunostaining against TH demonstrates that the negative patches are striosomes (Fig. 4E,F). These findings agree with previous results by Korematsu et al. (1998).

Similar to the Cdh4 expression pattern, Cdh8 shows strong and homogeneous expression only in the rostral globus pallidus (Figs. 2G, 3K). The caudal part of the globus pallidus is Cdh8 negative (Fig. 3L).

Cadherin-11 (Cdh11). Cdh11 is expressed ubiquitously and strongly in the caudoputamen (Figs. 2I–L, 3M–P) at the postnatal stage. Expression is stronger rostrally (Fig. 3M,N) than caudally (Fig. 3O,P). Both patches and matrix express Cdh11 at similar strength (Figs. 2I–L, 4G,H). In the adult caudoputamen, Cdh11 shows expression in the dorsal, medial, and ventral part (Fig. 5E). In the lateral part, the expression is only moderate, compared with the other areas.

Only few scattered positive perikarya show expression for Cdh11 in the rostral part of the globus pallidus (Fig. 3O). The caudal region is Cdh11 negative (Fig. 3P).

Protocadherin-1 (Pcdh1). The only cadherin that is expressed exclusively in the striosomes is Pcdh1 (Fig. 4I,J). All striosomes at all four levels show signal in the postnatal brain (Figs. 2E–H, 6A–D). In the rostral part of the caudoputamen, the lateral stripe of the striatum (LSS) is also Pcdh1 positive (Figs. 2E,F, 6A). At intermediate levels, Pcdh1 is expressed additionally at the outermost lateral edge beneath the external capsule (ec in Fig. 6B,C). In the representative section of the adult brain, Pcdh1 expression can be observed only in the patches (Fig. 5F). Compared with P5 brain (Fig. 6A–D), expression in the adult caudoputamen is not as strong.

Protocadherin-7 (Pcdh7). Matrix at all four levels of the caudoputamen expresses Pcdh7 at P5 and in the adult. Rostrally, the dorsal part of the caudoputamen shows moderate expression (Figs. 2A, 5G, 6E). This matrix area (Fig. 4K–N) contains a small number of perikarya, which express Pcdh7 strongly. At intermediate levels, the region of expression assumes a kidney-shaped appearance (Figs. 2B,C, 6F,G). In the caudal part of the caudoputamen, the entire matrix is Pcdh7 positive (Figs. 2D, 6H).

Protocadherin-8 (Pcdh8). The expression of Pcdh8 is limited to a few scattered perikarya at all four levels (Fig. 6I–L).

As in the striatum, Pcdh8 shows only a moderate signal in the globus pallidus (Fig. 6K). In rostral sections, individual positive perikarya are distributed over the entire core (Fig. 6K).

Protocadherin-9 (Pcdh9). Pcdh9 is expressed at the lateral edge of the rostral caudoputamen (Figs. 2E, 5H, 6M). This Pcdh9-positive area belongs to the matrix compartment (arrowhead in Fig. 4O,P), although the TH immunostaining shows a slight overlap with the Pcdh9-positive region. Caudally, the striatal expression pattern of Pcdh9 changes. At level 2, the expression is limited to the ventrolateral corner (Figs. 2F, 6N). At level 3, Pcdh9 expression becomes weaker and is restricted to the outermost ventral area (Figs. 2G, 6O). Level 4 shows no expression (Figs. 2H, 6P).

Protocadherin-10 (Pcdh10). Interestingly, Pcdh10 is expressed by both matrix and striosomes in the caudoputamen at the postanatal and adult stage. At rostral levels (levels 1 and 2), prominent matrix staining is visible medially, with a gradient decreasing from ventromedial to dorsolateral (Figs. 2A,B, 5I, 7A,B). In contrast, Pcdh10 is expressed exclusively in the striosomes in the lateral part of the rostral striatum (Figs. 4Q,R, 5I, 7A,B); in particular, the lateral stripe of the striatum shows distinct signal. At caudal levels (levels 3 and 4), Pcdh10 expression is observed ventrally in the matrix and dorsally in the patches (Fig. 7C,D). A gradient of staining in the matrix cannot be recognized (Fig. 2C,D). These results confirm and extend earlier observations by Hirano et al. (1999).

Pcdh10 is expressed only moderately in the globus pallidus; in accordance with the pattern in the striatal matrix, expression decreases from medial to lateral in the globus pallidus (Figs. 2C,D, 7C,D).

Protocadherin-11 (Pcdh11). Expression of Pcdh11 can be detected only at rostral levels in the P5 caudoputamen, but expression is relatively weak compared with that seen in other brain regions, for example, in the ventral pallidum (see below; Figs. 2M,N, 7E,F). The immuno-

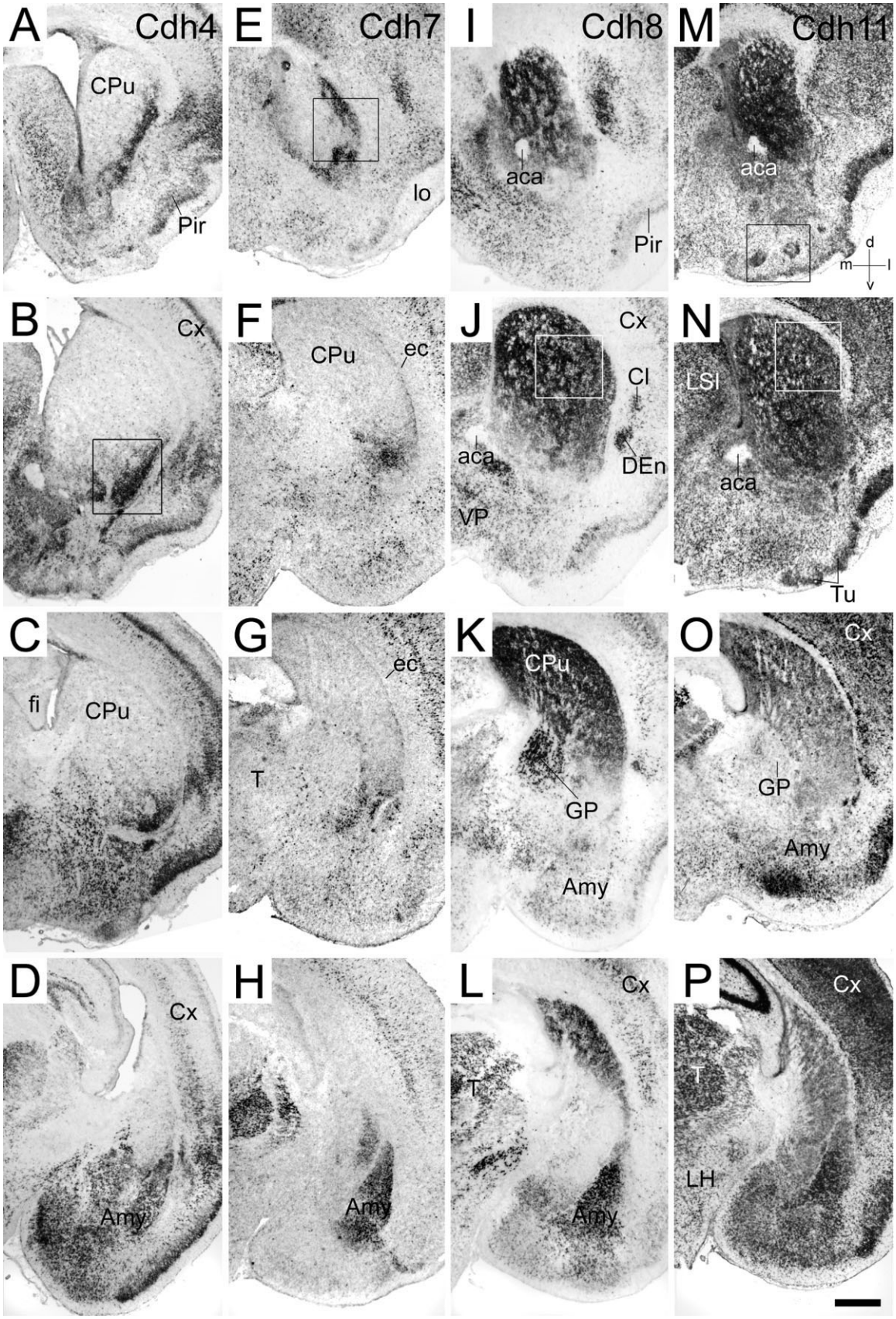

Fig. 3. Results of in situ hybridization with cRNA probes for cadherin-4 (Cdh4; **A–D**), cadherin-7 (Cdh7; **E–H**), cadherin-8 (Cdh8; **I–L**), and cadherin-11 (Cdh11; **M–P**). Frontal sections through postnatal day 5 (P5) basal ganglia are shown. The levels of sectioning correspond to those displayed in Figure 1. The boxed areas are shown at a higher magnification in Figure 4 (A,C,E,G; striatum) and in Figure 9 (A; ventral pallidum), respectively. The anatomical orientation of the sections is given in M. For abbreviations, see list. Scale bar = 500 μm in P (applies to A–P).

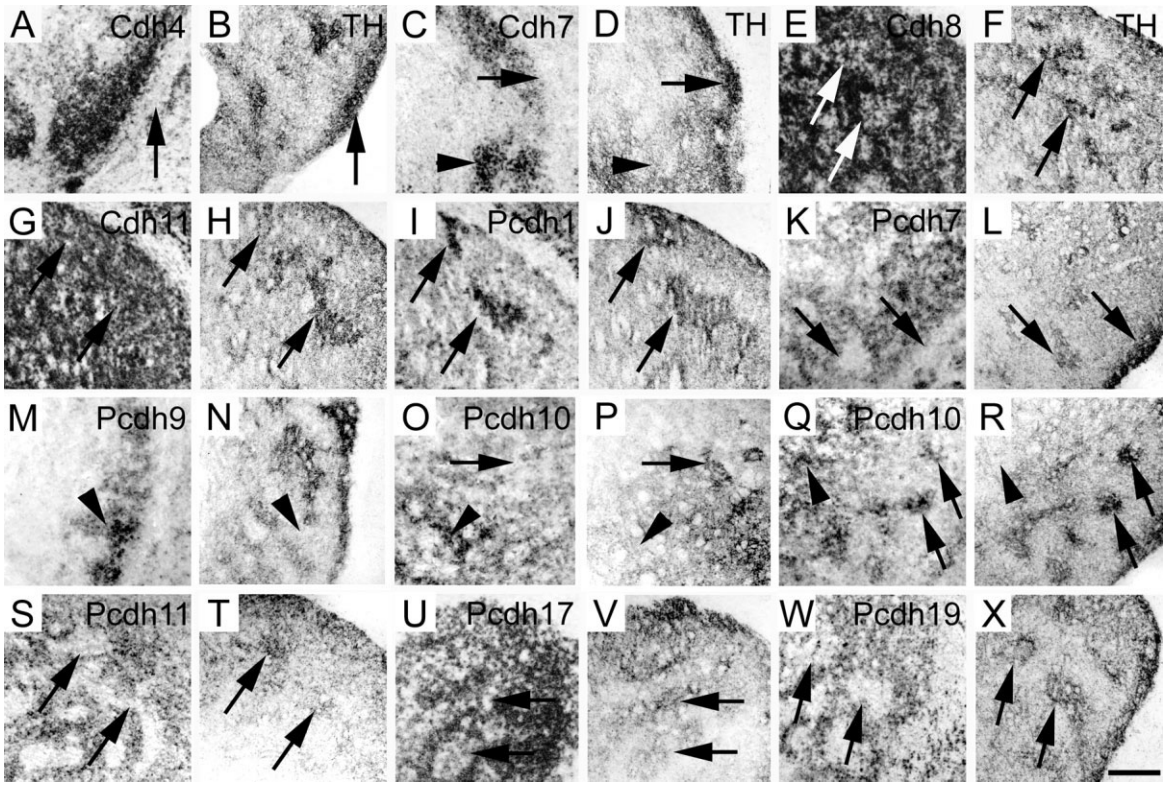

Fig. 4. Frontal sections through the caudoputamen at postnatal day 5 (P5). Cadherin expression (in situ hybridization; **A,C,E,G,I,K, M,O,Q,S,U,W**) is compared with immunostaining for TH (**B,D,F, H,J,L,N,P,R,T,V,X**). The in situ hybridization results are magnifications of the areas boxed in Figures 3, 6, and 7 (for A, C, E, and G, see Fig. 3; for I, K, M, and O, see Fig. 6; for Q, S, U, and W, see Fig. 7). For each pair of stainings, adjacent sections are displayed. The arrows point to striosomes and the arrowheads to striatal matrix. Scale bar = 200 μm in X (applies to A–X).

staining against TH indicates that Pcdh11 is expressed only in the matrix compartment (Fig. 4S,T). A gradient of expression increasing in strength from medial to lateral can be observed (Fig. 7E,F).

Pcdh11 shows a strong homogeneous expression in both rostral and caudal parts of the globus pallidus (Fig. 7G,H).

Protocadherin-17 (Pcdh17). At the rostralmost level of the postnatal and adult caudoputamen, Pcdh17 signal is restricted to the matrix (Fig. 4U,V) in an area that occupies dorsal, lateral, and ventral striatal regions (Figs. 2I, 5K, 7I). The Pcdh17-negative striosomes are clearly demarcated in the Pcdh17-positive matrix. At level 2, the matrix expression recedes to the dorsal and dorsolateral area (Figs. 2J, 7J). At both levels, expression diminishes gradually from lateral to medial. The caudal part of the striatum is Pcdh17 negative (Figs. 2K,L, 7K,L). A rostral-to-caudal gradient of Pcdh17 expression has been reported previously (Kim et al., 2007).

The expression of Pcdh17 (Fig. 7K,L) in the globus pallidus is similar to that of Cdh4 and Cdh8; only the rostral part is Pcdh17 positive.

Protocadherin-19 (Pcdh19). There is a general increase of Pcdh19 expression from rostral to caudal at P5 and in the adult (Figs. 2M–P, 5K, 7M–P). Pcdh19 marks the matrix compartment (Fig. 4W, X). In the rostral striatum, Pcdh19 exhibits a gradient of expression that decreases from dorsolateral to ventral (level 1; Figs. 2M, 7M) and from dorsomedial to ventral (level 2; Figs. 2N, 7N). In the caudal parts, the entire striatum is Pcdh19 positive (Figs. 2O,P, 5K, 7O,P).

Pcdh19 (Fig. 7O) shows only moderate signal in the rostral part of the globus pallidus. A shallow gradient from lateral to medial can be observed.

Protein phosphatase 1α (PP1α). At the adult stage, PP1α is expressed ubiquitously in the entire caudoputamen (Fig. 5L).

Expression in the accumbens nucleus

The expression in the accumbens nucleus varies for the different cadherins. Patchy and gradient-like signals can be observed in the core and shell region of this nucleus. For individual cadherins, the expression patterns are highly similar at postnatal and adult stages (Figs. 8, S1).

The ventral border of the core region of the accumbens nucleus shows a strong signal for Cdh4, with a gradient decreasing from ventral to dorsal (Figs. 8A, S1B). The shell region is Cdh4 positive. For Cdh7, only a few positive perikarya can be observed in the core region next to the anterior commissure (black asterisk in Figs. 8B, S1C) and in the ventral part of the shell region (Figs. 8B, S1C). Cdh8 is expressed strongly at the lateral edge of the core region next to the caudoputamen (Figs. 8C, S1D). Weak expression can be observed around the anterior commissure. The shell region is Cdh8 negative. The entire accumbens nucleus is Cdh11 positive (Figs. 8D, S1E). A shallow

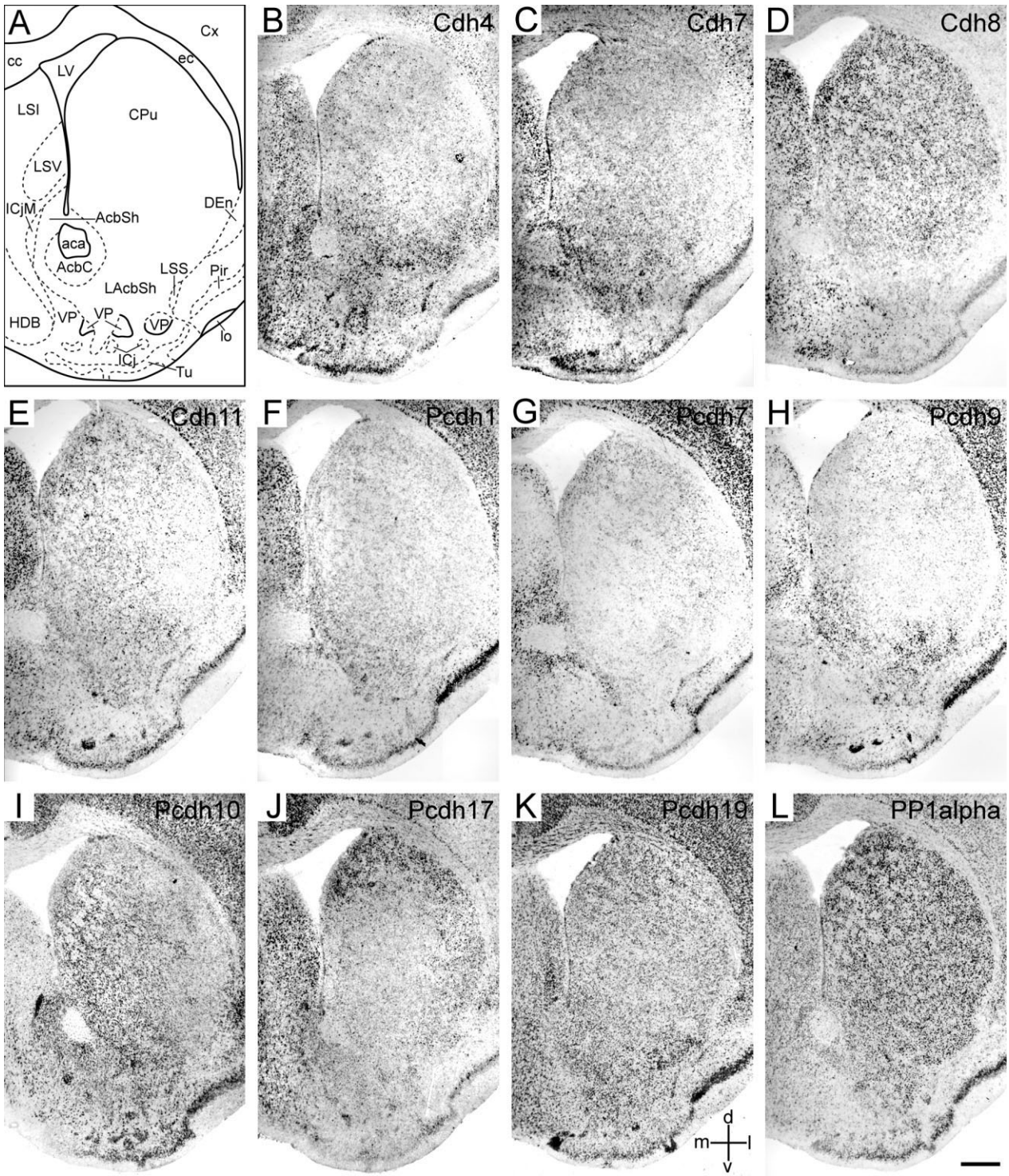

Fig. 5. Cadherin expression in a consecutive series of frontal sections through the adult caudoputamen. Results of in situ hybridization with cRNA probes for cadherin-4 (Cdh4; **B**), cadherin-7 (Cdh7; **C**), cadherin-8 (Cdh8; **D**), cadherin-11 (Cdh11; **E**), protocadherin-1 (Pcdh1; **F**), protocadherin-7 (Pcdh7; **G**), protocadherin-9 (Pcdh9; **H**), protocadherin-10 (Pcdh10; **I**), protocadherin-17 (Pcdh17; **J**), protocadherin-19 (Pcdh19; **K**), and protein phosphatase 1α (PP1α; **L**) are shown. **A:** A schematic overview of the anatomical structures present at this section level. The asterisk in B indicates a staining artefact. The anatomical orientation of the sections is given in K. For abbreviations, see list. Scale bar = 500 μm in L (applies to A–L).

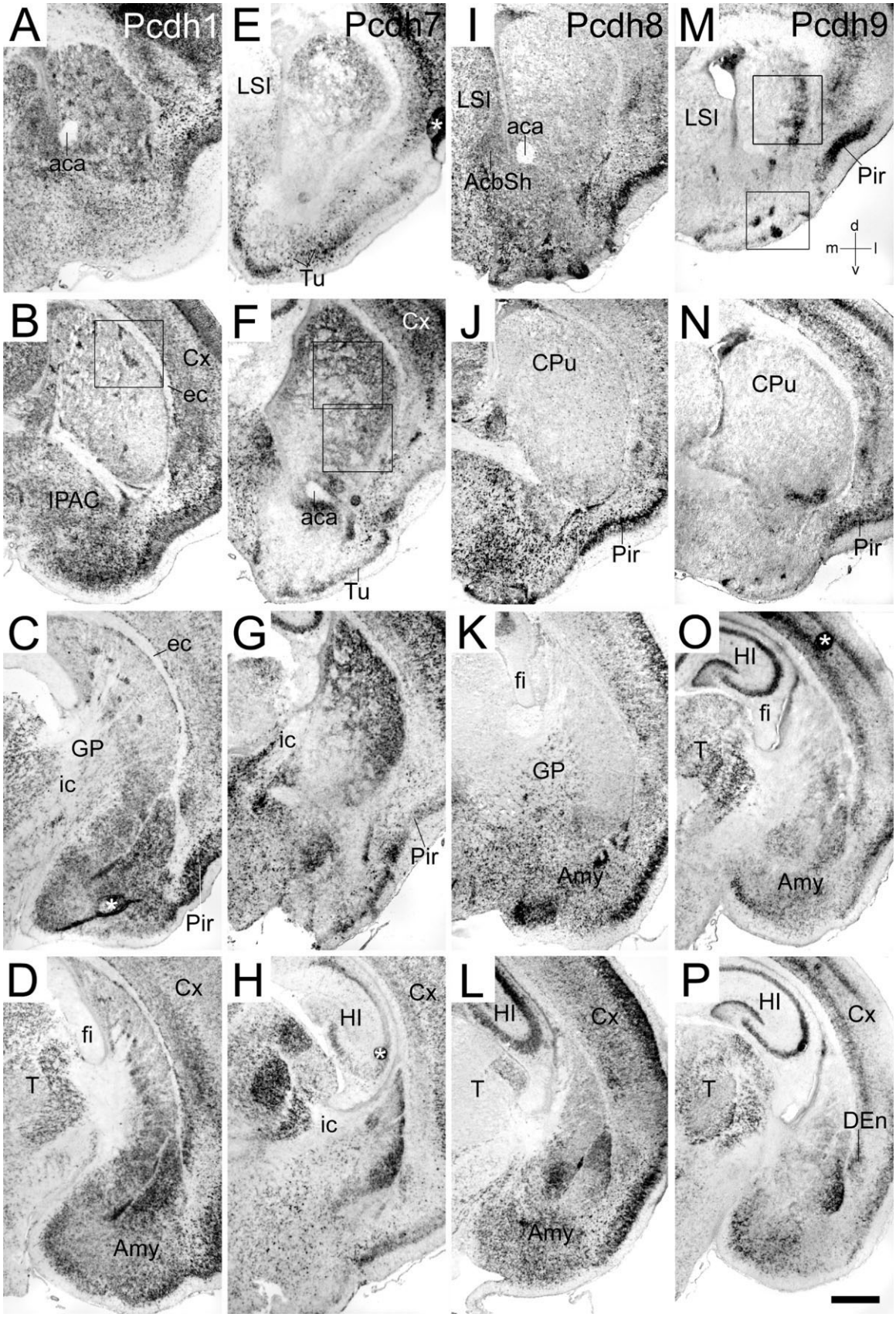

Fig. 6. Results of in situ hybridization with cRNA probes for protocadherin-1 (Pcdh1; **A–D**), protocadherin-7 (Pcdh7; **E–H**), protocadherin-8 (Pcdh8; **I–L**), and protocadherin-9 (Pcdh9; **M–P**). Frontal sections through postnatal day 5 (P5) basal ganglia are shown. The levels of sectioning correspond to those displayed in Figure 1. The boxed areas are shown at a higher magnification in Figure 4 (I,K,M,O; striatum) and in Figure 10 (C; ventral pallidum), respectively. The asterisks in C, E, H, and O indicate staining artefacts. The anatomical orientation of the sections is given in M. For abbreviations, see list. Scale bar = 500 μm in P (applies to A–P).

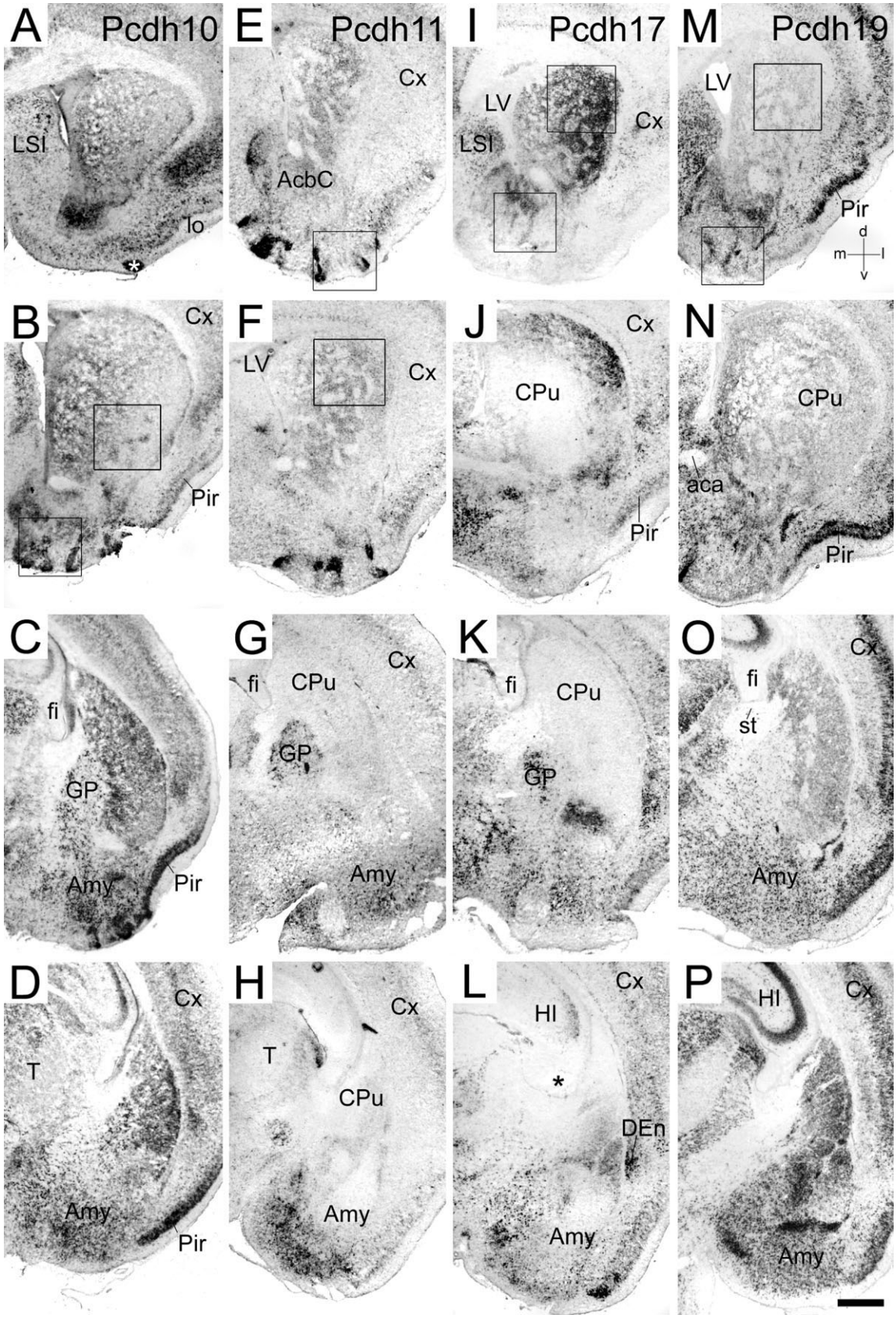

Fig. 7. Results of in situ hybridization with cRNA probes for protocadherin-10 (Pcdh10; **A–D**), protocadherin-11 (Pcdh11; **E–H**), protocadherin-17 (Pcdh17; **I–L**), and protocadherin-19 (Pcdh19; **M–P**). Frontal sections through postnatal day 5 (P5) basal ganglia are shown. The levels of sectioning correspond to those displayed in Figure 1. The boxed areas are shown at a higher magnification in Figure 4 (Q,S,U,W; striatum) and in Figure 10 (E,G,I,K; ventral pallidum), respectively. The asterisks in A and L indicate staining artefacts. The anatomical orientation of the sections is given in M. For abbreviations, see list. Scale bar = 500 μm in P (applies to A–P).

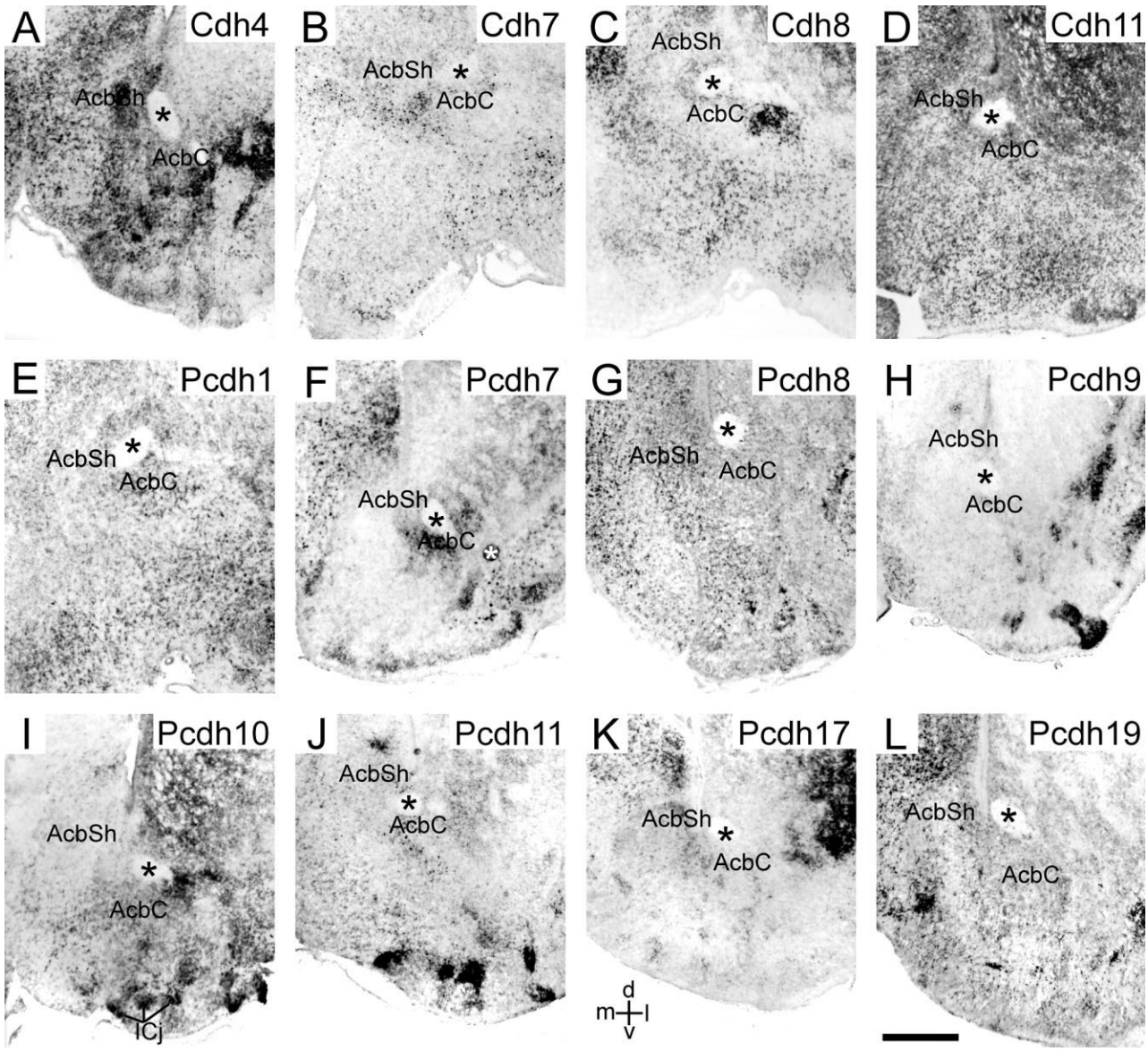

Fig. 8. Frontal sections through the accumbens nucleus at postnatal day 5 (P5). Results of in situ hybridization with cRNA probes for cadherin-4 (Cdh4; **A**), cadherin-7 (Cdh7; **B**), cadherin-8 (Cdh8; **C**), cadherin-11 (Cdh11; **D**), protocadherin-1 (Pcdh1; **E**), protocadherin-7 (Pcdh7; **F**), protocadherin-8 (Pcdh8; **G**), protocadherin-9 (Pcdh9; **H**), protocadherin-10 (Pcdh10; **I**), protocadherin-11 (Pcdh11; **J**), protocadherin-17 (Pcdh17; **K**), and protocadherin-19 (Pcdh19; **L**) are shown. The black asterisks indicate the anterior part of the anterior commissure. The white asterisk in F indicates an artefact. The anatomical orientation of the sections is given in K. For abbreviations, see list. Scale bar = 500 μm in L (applies to A–L).

gradient from dorsolateral to ventromedial can be observed postnatally (Fig. 8D). The core, but not the shell region, is Pcdh1 positive (Figs. 8E, S1F). Similar to Pcdh1, only the core region shows expression for Pcdh7 (Figs. 8F, S1G). A gradient increasing from dorsal to ventral can be observed at the postnatal stage. Pcdh8 is expressed weakly in the core region at the postnatal stage. The boundary between the core and the shell regions shows signal for Pcdh8. The accumbens nucleus is negative for Pcdh9 (Fig. 8H) at the postnatal stage. Only at the adult stage, the shell region shows weak expression (Fig. S1H).

Pcdh10 expression is prominent in the lateral part of the core region at P5, with a gradient decreasing from lateral to medial (Fig. 8I). The shell region is Pcdh10 negative. At the adult stage, the entire accumbens nucleus shows signal for Pcdh10 (Fig. S1I). Only a few Pcdh11-positive perikarya can be observed in the ventral part of the core region (Fig. 8J). The shell region shows no signal for Pcdh11. A moderate expression can be observed for Pcdh17 at both stages in the entire accumbens nucleus (Figs. 8K, S1J). In the P5 brain, the signal displays a patchy distribution (Fig. 8K). The entire nucleus shows expression for Pcdh19 (Figs. 8L, S1K). At the adult stage, PP1α is expressed in the core region (Fig. S1L). The shell region is also positive for PP1α but the signal is weaker compared with the core region (Fig. S1L).

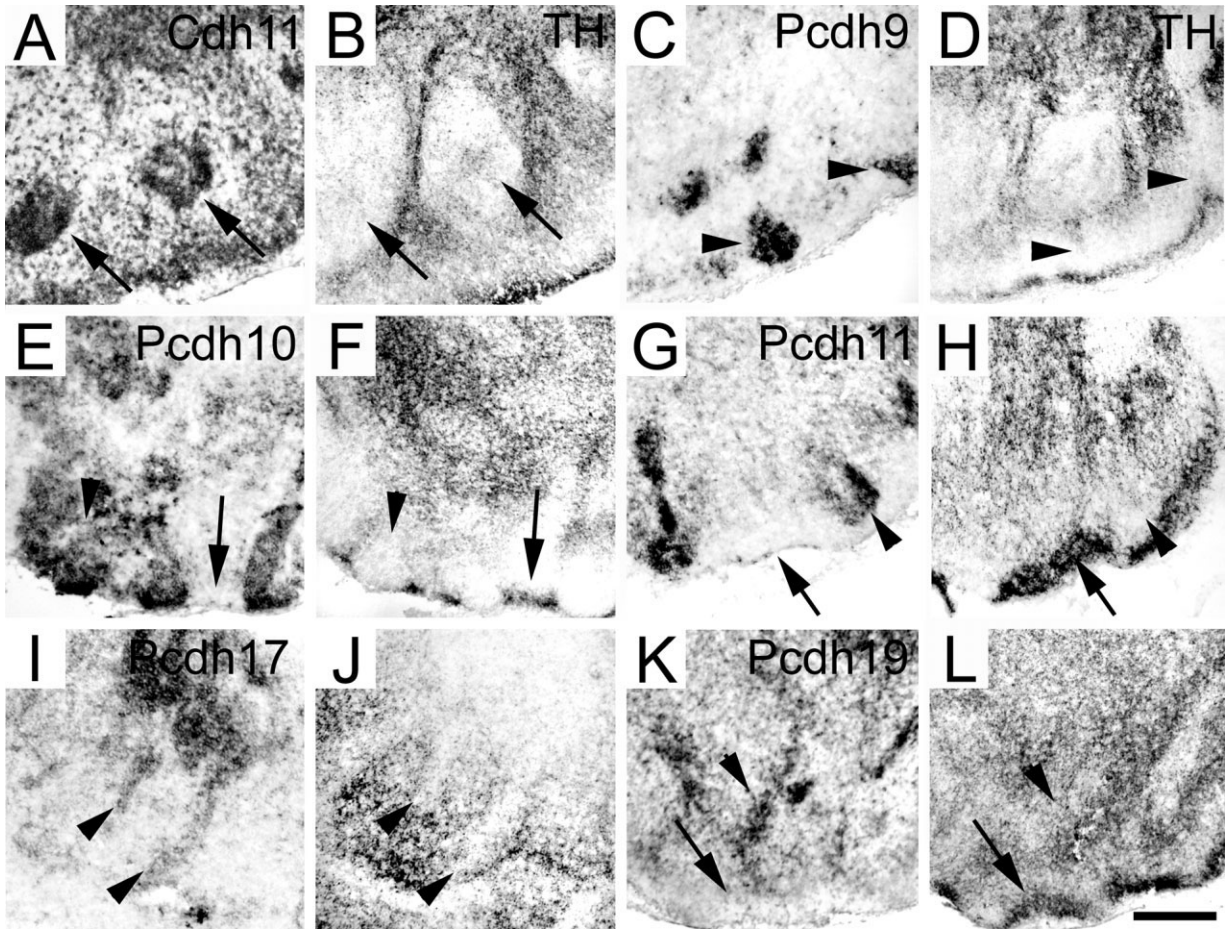

Fig. 9. Adjacent frontal sections through the ventral pallidum at postnatal day 5 (P5). Cadherin expression (in situ hybridization; **A,C,E,G,I,K**) is compared with immunostaining for TH (**B,D,F, H,J,L**). The in situ hybridization results are magnifications of the areas boxed in Figures 3, 6, and 7 (for A, see Fig. 3; for C, see Fig. 6; and for E, G, I, and K, see Fig. 7). The arrows point to TH-positive regions, and the arrowheads point to TH-negative regions. Scale bar = 200 μm in L (applies to A–L).

Expression in the ventral pallidum

The ventral pallidum exhibits a patchy cadherin-expression pattern at postnatal and adult stages. Small round, clearly delimited regions are positive for Cdh11 (Figs. 3M, S1E), Pcdh1 (Fig. S1F), Pcdh8 (Fig. 6I), Pcdh9 (Figs. 6M, S1H), Pcdh10 (Figs. 7B, S1I), and Pcdh11 (Fig. 7E,F). Pcdh17 (Figs. 7I, S1J) is expressed in stripes. Pcdh19 shows a stripe-like expression at P5 (Fig. 7M) and a patchy signal in the adult (Fig. S1K). A comparison of the expression patterns with TH immunostaining indicates that the cadherin-positive areas are TH negative (Fig. 9A–L).

To compare the expression patterns of the cadherins with each other at P5, we carried out in situ hybridization on alternating sections (Fig. 10A–F). Cdh11, Pcdh9, Pcdh10, and Pcdh11 are expressed in the islands of Calleja, and Pcdh19 is expressed by the cell bridges (CBs) in between the islands. The stripe-like pattern of Pcdh17 is located dorsal to the islands of Calleja and, therefore, cannot be assigned clearly to the islands or the intervening stripes (Fig. 7I).

Adjacent sections of the adult brain (Fig. 5) show a pattern similar to that observed at P5. The islands of Calleja show signal for Cdh11 (Fig. 5E), Pcdh1 (Fig. 5F), Pcdh9 (Fig. 5H), and Pcdh10 (Fig. 5I); the cell bridges in between the islands are positive for Pcdh19 (Fig. 5K). Note that these cadherins, except Pcdh19, are also expressed in the major island of the Calleja islands (ICjM; arrowheads in Supplementary Fig. S1A,E,I,J).

Expression in the A8, A9, and A10 dopaminergic cell groups

The dopaminergic cell groups in the midbrain were visualized by mapping TH expression (Fig. 11B,O). They show distinct signals for the different cadherins (Fig. 11C–N). Results are displayed in a rostral-to-caudal sequence of adjacent sections. At rostral levels (Fig. 11B), the dorsal tier and the ventral tier of the compact part of the substantia nigra (A9; SNCD, SNCV; Fig. 11B) are both located dorsal to the reticular part (SNR; Fig. 11B). In contrast, at caudal levels (Fig. 11O), the ventral tier is split. The first portion is subjacent to the dorsal tier of the compact part, and the second portion lies within the reticular part. Each cadherin is expressed in a subset of these anatomical divisions (Table 1).

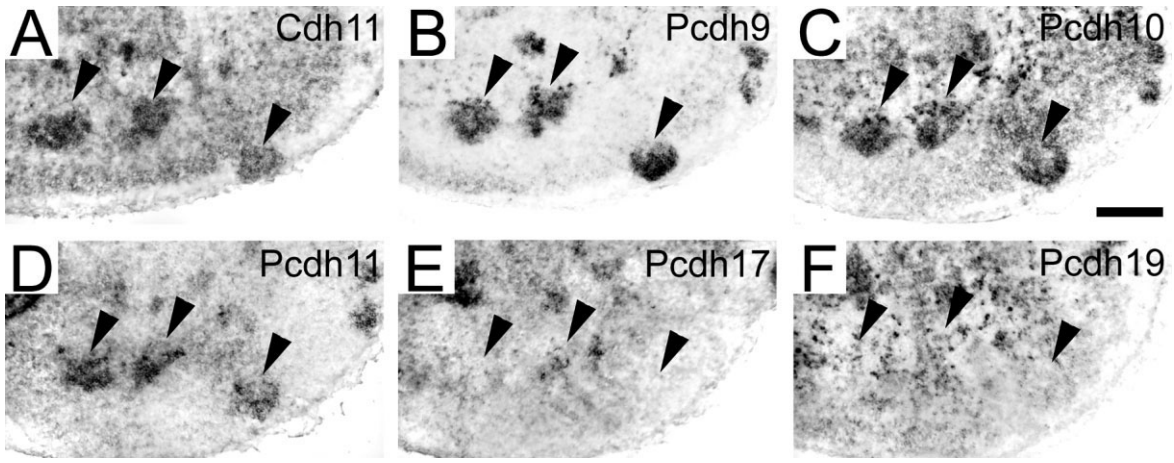

Fig. 10. Comparison of the expression of cadherin-11 (Cdh11; **A**), protocadherin-9 (Pcdh9; **B**), protocadherin-10 (Pcdh10; **C**), protocadherin-11 (Pcdh11; **D**), protocadherin-17 (Pcdh17; **E**), and protocadherin-19 (Pcdh19, **F**). A series of consecutive frontal sections through the ventral pallidum at postnatal day 5 (P5) is shown. The arrowheads point to islands of Calleja. Scale bar = 200 μm in C (applies to A–F).

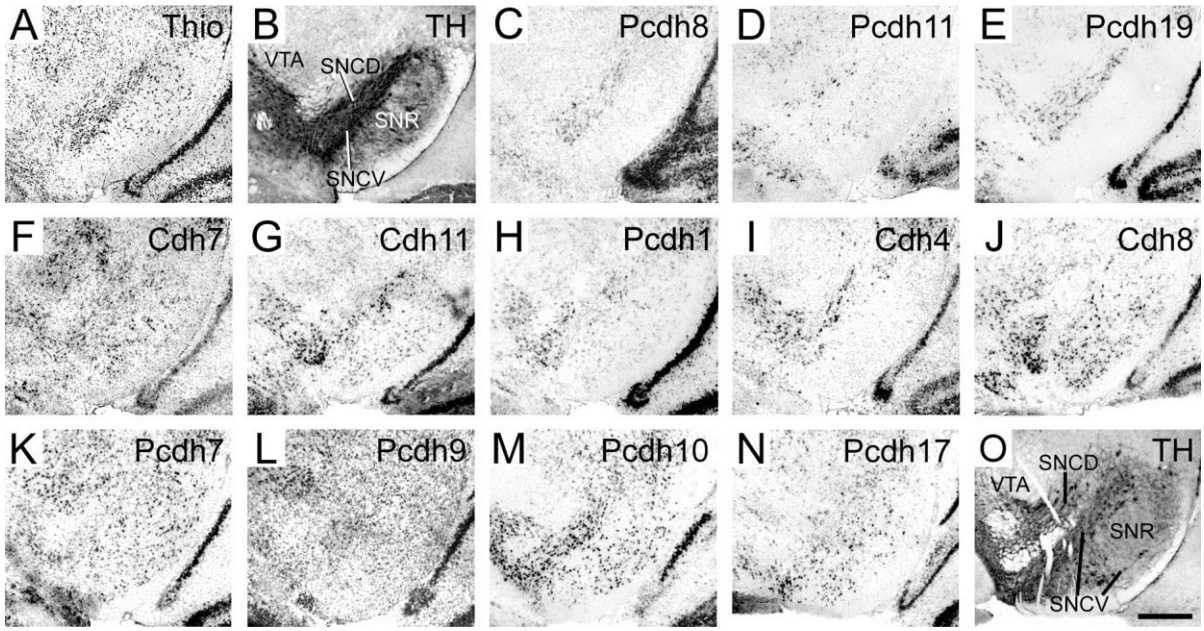

Fig. 11. A rostral-to-caudal sequence of adjacent sections of the A8 and A9 dopaminergic cell groups in the adult mouse midbrain. **A,B,O:** TH immunostaining shows the distribution of dopaminergic neurons at a rostral level (B) and a caudal level (O) in coronal sections. Medial is to the left. An adjacent Nissl (Thio) stain is shown in A. **C–N:** Results of in situ hybridization with cRNA probes for cadherin-4 (Cdh4; I), cadherin-7 (Cdh7; F), cadherin-8 (Cdh8; J), cadherin-11 (Cdh11; G), protocadherin-1 (Pcdh1; H), protocadherin-7 (Pcdh7; K), protocadherin-9 (Pcdh9; L), protocadherin-10 (Pcdh10; M), protocadherin-17 (Pcdh17; N), and protocadherin-19 (Pcdh19; E). For abbreviations, see list. Scale bar = 500 μm in O (applies to A–O).

DISCUSSION

In the present study, the expression of four classic cadherins and eight δ-protocadherins was studied in the basal ganglia of postnatal day 5 (P5) and adult mouse brain. Results show that each cadherin exhibits a distinct and spatially restricted expression pattern that differs from that of the other cadherins, although partial overlap is observed. This overall staining pattern is similar to results described previously for other brain regions.

Our results confirm and extend previous findings for Cdh4 (R-cadherin; Obst-Pernberg et al., 2001), Cdh8 (Korematsu and Redies, 1997; Korematsu et al., 1998), Cdh11 (Suzuki et al., 1997), and Pcdh10 (OL-protocadherin; Hirano et al., 1999; Redies et al., 2002) in the mouse striatum. By systematically mapping the expression of eight additional members of the cadherin superfamily in serial sections, we provide evidence that multiple cadherins represent a code of potentially adhesive cues for the divisions of the striatum, as previously proposed for other

TABLE 1. Cadherin Expression in the A8 (RRF), A9 (SNC), and A10 (VTA) Dopaminergic Cell Groups in the Adult

Cadherin	SNCD	SNCV	SNR	VTA	RRF
Cdh4	−	+	−	+	−
Cdh7	+	−	+	+	−
Cdh8	+	−	+	+	+
Cdh11	+	−	+	+	−
Pcdh1	−	+	+[1]	+	+[1]
Pcdh7	+[1]	−	+	+	+[1]
Pcdh8	+	+	−	−	+
Pcdh9		+	+	+	+
Pcdh10	−	+	+	+	+
Pcdh11	+	−	+[2]	−	−
Pcdh17	−	+[2]	+	−	+
Pcdh19	+	+	−	−	−

Abbreviations: +, positive; −, negative; Cdh, cadherin; RRF, retrorubral field; SNCD, substantia nigra, compact part, dorsal tier; SNCV, substantia nigra, compact part, ventral tier; SNR, substantia nigra, reticular part; VTA, ventral tegmental area.
[1]Only the dorsal part of the substantia nigra, compact part is positive.
[2]Few perikarya are positive.

brain regions (for reviews, see Redies, 2000; Hirano et al., 2003).

Cadherins are candidate adhesive factors for mediating the sorting of the striosome and matrix compartments

Many of the cadherins investigated in the present study can mediate cell-cell adhesion in vitro in a Ca^{2+}-dependent manner (Cdh4, Inuzuka et al., 1991; Cdh7, Cdh8, and Cdh11, Shimoyama et al., 2000; Pcdh1, Sano et al., 1993; Pcdh7, Yoshida, 2003; Pcdh8, Yamagata et al., 1999; Pcdh10, Hirano et al., 1999). Classic cadherins bind preferentially in a homotypic fashion, thereby causing different cell types to sort out from each other (Nose et al., 1988; Shimoyama et al., 2000). However, heterophilic binding between some pairs of classic cadherins has also been observed (Shimoyama et al., 2000). Moreover, several δ-protocadherins were shown to be involved in the segregation of different cell types in vivo and in vitro (Pcdh1, Kuroda et al., 2002; Pcdh7, Bradley et al., 1998; Pcdh8, Kim et al., 1998; Pcdh10, Hirano et al., 1999; for review, see Redies et al., 2005). Striatal cells have already acquired differential adhesive properties before their sorting (Krushel et al., 1995). It is therefore conceivable that the differential expression of the cadherins regulates the sorting of the initially mixed striatal cell populations (van der Kooy and Fishell, 1987; Krushel et al., 1995) into the patch and matrix compartments during development, as suggested previously (Redies et al., 2002).

Do the cadherin expression gradients represent functional organization within the basal ganglia?

Most of the cadherins exhibit prominent gradients or regionalized expression within the striatum. This regionalization is not evident in the histology and cytoarchitecture of the striatum but may rather reflect its functional differentiation and the establishment of striatal connectivity, as previously shown for other brain regions (for reviews, see Redies, 2000; Hirano et al., 2003). For example, different parts of the cerebral cortex, including primary and higher order sensory areas, motor, premotor, and prefrontal regions, and limbic cortical areas, project to different parts of the striatum. This input is organized in a topographic manner. The projections that originate in functionally related areas overlap with each other. For example, frontal areas provide input to the rostral regions of the striatum, sensorimotor cortical areas project to the dorsal lateral region, and the parietal cortex provides input to more caudal regions (Kemp and Powell, 1970; Malach and Graybiel, 1986; Ebrahimi et al., 1992; Flaherty and Graybiel, 1993; Takada et al., 1998a,b; Inase et al., 1999). This topography reflects the existence of parallel functional loops through the basal ganglia (Alexander et al., 1986; Ebrahimi et al., 1992).

Apart from a regionalized projection, there is also a compartment-specific projection from the cortex to the striatum. The patch compartment receives afferents predominantly from the deeper strata of layer V and layer VI of the neocortex, whereas neurons in superficial layer V provide input to the matrix (Gerfen, 1984, 1989, 1992; Donoghue and Herkenham, 1986). Interestingly, the cerebral cortex expresses all the cadherins examined in the present study in a region- and layer-specific fashion (Korematsu and Redies, 1997; Suzuki et al., 1997; Hirano et al., 1999; Vanhalst et al., 2005; C. Redies, M. Nuernberger, and Krishna K., unpublished data). In many brain circuits, fiber projections and their targets show a matching cadherin expression profile (for review, see Redies, 2000). A detailed study of cadherin expression in the various cortical regions is in preparation (M. Nuernberger, Krishna K., and C. Redies, unpublished data). A description of their projections to the striatum is beyond the scope of the present study.

The globus pallidus expresses cadherins in distinct subregions (Cdh4, Cdh8, Pcdh1, Pcdh8, Pcdh10, Pcdh11, Pcdh17, and Pcdh19) or in scattered populations of cells (Cdh4 and Pcdh8). A patch/matrix organization has not been observed in this brain region. The globus pallidus receives input from the caudoputamen. This input shows a dual pattern of arborization, a high degree of specificity, and high densitiy of innervation (Chang et al., 1981; Wilson and Phelan, 1982; Gerfen, 1985). Fibers that arise from striatal neurons project to a region immediately adjacent to the striatum as well as to the central part of the globus pallidus (Chang et al., 1981). It is conceivable, but remains to be demonstrated experimentally, that the adhesive code provided by cadherins plays a role in the compartmentation of the globus pallidus and/or its functional connectivity.

Dopaminergic neurons in the midbrain, which we labeled with antibodies against TH, are at the origin of the nigrostriatal dopamine system. These midbrain areas include the retrorubral field, the compact part of the substantia nigra, and the ventral tegmental area that corresponds to the A8, A9, and A10 dopaminergic cell groups, respectively (Dahlström and Fuxe, 1964). In the nigrostriatal system, the underlying patch/matrix organizational scheme is reflected in a ventral tier and a dorsal tier (Gerfen et al., 1987a,b; Jimenez-Castellanos and Graybiel, 1987). The dorsal tier includes the ventral tegmental area, the dorsal part of the substantia nigra, compact part, and the retrorubral area; it innervates the striatal matrix compartment. The ventral tier is situated in the ventral part of the substantia nigra, compact part, and includes some cell groups in the reticular part; it projects to the patch compartment (Gerfen et al., 1985, 1987a,b; Jimenez-Castellanos and Graybiel, 1987; Langer and Graybiel, 1989; Gerfen, 1992; Song and Haber, 2000; Prensa and Parent, 2001). Cadherin expression can be related to this topographical organization. This is most

clearly seen for Cdh8 that is expressed exclusively in the dorsal tier (Fig. 11J). Accordingly, only the matrix compartment of the caudoputamen is Cdh8 positive (Fig. 3I–L, 5D). Pcdh1 shows signal only in the patch compartment (Fig. 6A–D, 5F); its expression is restricted to the ventral tier (Fig. 11H). Similar correspondences can be observed for the other cadherins.

Cadherin expression in the adult basal ganglia

The expression pattern of most cadherins studied in the present work persists in the adult striatum, with little or no change from the postnatal stage. Which role do the cadherins play in the mature striatum? Many classic cadherins, including Cdh4, Cdh7, Cdh8, and Cdh11, and some δ-protocadherins (for example, Pcdh8 and Pcdh10) are expressed at the synapse in the developing and mature nervous system (Yamagata and Sanes, 1995; Fannon and Colman, 1996; Uchida et al., 1996; Arndt et al., 1998; Kohmura et al., 1998; Tang et al., 1998; Hirano et al., 1999; Yamagata et al., 1999; Manabe et al., 2000; Wang et al., 2002). Interestingly, a subgroup of δ-protocadherins, termed δ1-protocadherins (Pcdh1, Pcdh7, Pcdh9, and Pcdh11), possesses an intracellular motif that binds to PP1α (Yoshida et al., 1999; Vanhalst et al., 2005). This molecule, which is expressed in the entire adult caudoputamen (Fig. 5L), is enriched in the dendritic spines on medium spiny neurons in the striatum (da Cruz e Silva et al., 1995). PP1α regulates synaptic plasticity (Terry-Lorenzo et al., 2002), and isoform C of Pcdh7 was shown to inhibit the activity of PP1α (Yoshida et al., 1999). Together, these results suggest the possibility that the cadherins studied by us play a role at the synapse in the mature striatum.

General conclusion and outlook

In conclusion, the spatially restricted expression patterns in the basal ganglia indicate that cadherins provide an adhesive code for the patch/matrix architecture of the caudoputamen and also possibly for the differentiation and connectivity of the basal ganglia in general. The latter suggestion requires confirmation by neuroanatomical tracing studies. Also, the precise functional role of cadherins in contributing to the development of the basal ganglia remains to be studied by genetic or other experimental approaches. Interestingly, both dorsal and ventral parts of the basal ganglia show a complex and patchy expression of cadherins. This similarity suggests the presence of similar adhesive mechanisms in the dorsal and ventral parts of the subpallium, underlining the common ontogenetic origin of these structures from the ganglionic eminences (Smith-Fernandez et al., 1998; Puelles et al., 2000).

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3.4.

Comparative analysis of cadherin expression and connectivity patterns in the cerebellar system of ferret and mouse

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Comparative Analysis of Cadherin Expression and Connectivity Patterns in the Cerebellar System of Ferret and Mouse

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ABSTRACT

The cerebellum shows remarkable variations in the relative size of its divisions among vertebrate species. In the present study, we compare the cerebella of two mammals (ferret and mouse) by mapping the expression of three cadherins (cadherin-8, protocadherin-7, and protocadherin-10) at similar postnatal stages. The three cadherins are expressed differentially in parasagittal stripes in the cerebellar cortex, in the portions of the deep cerebellar nuclei, in the divisions of the inferior olivary nucleus, and in the lateral vestibular nucleus. The expression profiles suggest that the cadherin-positive structures are interconnected. The expression patterns resemble each other in ferret and mouse, although some differences can be observed. The general resemblance indicates that cerebellar organization is based on a common set of embryonic divisions in the two species. Consequently, the large differences in cerebellar morphology between the two species are more likely caused by differential growth of these embryonic divisions than by differences in early embryonic patterning. Based on the cadherin expression patterns, a model of corticonuclear projection territories in ferret and mouse is proposed. In summary, our results indicate that the cerebellar systems of rodents and carnivores display a relatively large degree of similarity in their molecular and functional organization. J. Comp. Neurol. 511:736–752, 2008.

Indexing terms: cadherin expression; cerebellum; Purkinje cell domains; transverse zones; deep cerebellar nuclei; inferior olivary nucleus

The cerebellum is an evolutionarily ancient brain structure. Its different parts show remarkable variations in size among vertebrate species. For example, large cerebellar hemispheres relative to the vermis have been described in higher mammals such as monkeys and in aquatic mammals but not in lower mammals (Larsell, 1967, 1970; for review see Jansen, 1969). The cerebellum of the cat exhibits a broad and well-developed vermis, with convolutions on the posterior surface and relatively small but clearly delimited hemispheres (Larsell, 1970; Altman and Bayer, 1997), whereas the rodent cerebellum consists of a vermis and hemispheres that are less elaborate. It has been suggested that the cerebellar hemispheres (neocerebellum) have emerged and increased in size in parallel to the enlargement of the neocortex of higher mammalian species (Edinger, 1910; Comolli, 1910; Altman and Bayer, 1997). Despite the morphological differences, cerebellar histology is remarkably similar across higher vertebrate species.

During the last decade, several candidate genes that regulate cerebellar development have been identified, mostly in the mouse (Millen et al., 1995; for reviews see Wang and Zoghbi, 2001; Larouche and Hawkes, 2006; Sillitoe and Joyner, 2007). It is possible that the above-mentioned size differences in cerebellar morphology are based on differences in embryonic pattern formation. More likely, morphological differences might be caused by differential growth in a similar set of embryonic divisions,

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which are derived from a common bauplan (for review see Redies and Puelles, 2001). The latter model has been established in the vertebrate forebrain, based on the spatial expression profiles of multiple developmentally relevant genes (for reviews see Puelles and Rubenstein, 1993, 2003; Rubenstein et al., 1994). To distinguish between these two possibilities, we used the gene mapping approach and compared the cerebellum of two mammalian species, the mouse and the ferret, that exhibit large differences in cerebellar morphology and the relative size of the parts of the cerebellar cortex.

The adult cerebellar cortex is divided into transverse zones and multiple parasagittal domains (for reviews see Jansen, 1954; Voogd and Ruigrok, 1997). These divisions can be characterized by their expression of specific subsets of molecular markers (for reviews see Voogd and Ruigrok, 1997; Voogd and Glickstein, 1998). For example, zebrin II (aldolase C; Brochu et al., 1990; Ahn et al., 1994) shows a parasagittal expression pattern in different zones of the adult mammalian cerebellum (for review see Sillitoe et al., 2005). Similar types of striping patterns have been reported for a number of other molecules, including pcp-2/L7 (Smeyne et al., 1991); *Drosophila* segmentation gene homologs lsuch as En-1, En-2, and Shh (Millen et al., 1995; Lin and Cepko, 1998); members of the ephrin family and their receptors (Lin and Cepko, 1998; Karam et al., 2000); and several cell adhesion molecules (Arndt and Redies, 1996, 1998; Chedotal et al., 1996; Suzuki et al., 1997; Luckner et al., 2001; Plagge et al., 2001) in the developing and mature mouse and chicken cerebellum. Whether these parcellations are based on a similar morphogenetic pattern in the different species is not known.

The parcellation of the cerebellar cortex is also reflected in its functional connectivity. The cerebellar cortex receives input via climbing and mossy fibers that originate in the divisions of the inferior olivary nucleus and in other brain areas, respectively. These inputs show a striking parasagittal organization. Moreover, parasagittal domains of Purkinje cells project differentially to the deep cerebellar nuclei that exhibit the main source of cerebellar efferents. Many of the above-mentioned molecules, for example, cadherins, are expressed differentially also in portions of the deep cerebellar nuclei as well as in divisions of the vestibular complex and the inferior olivary nucleus (Millen et al., 1995; Chedotal et al., 1996; Arndt et al., 1998; Lin and Cepko, 1998; Luckner et al., 2001; Plagge et al., 2001).

In a preceding study, we compared the cerebellar expression patterns of three cadherins [cadherin-8 (cdh8), protocadherin-7 (pcdh7), and protocadherin-10 (pcdh10)] with connectivity patterns that were visualized by tracing the corticonuclear axonal projections in the chicken embryo (Neudert and Redies, 2008). Our results confirm directly that cadherin expression reflects functional cerebellar connectivity. In the present study, we compare the connectivity patterns in the mouse and ferret cerebellar system by mapping the expression of the same three cadherins previously studied in chicken, at comparable stages of development [postnatal day 3 (P3) in mouse, and P2/P3 in ferret]. We show that, in both species, the cadherins are expressed differentially in parasagittal Purkinje cell domains of the cerebellar cortex, in portions of the deep cerebellar nuclei, in the divisions of the inferior olivary nucleus, and in the lateral vestibular nucleus. The observed expression patterns reflect functional connectivities within the cerebellar system. We determined, based on the cadherin expression patterns, the relative size of the cortical territories that project to the deep nuclei (medial, interposed, and lateral nucleus) in the two species. The comparison of these projection territories provides novel insight into the developmental mechanisms that possibly underlie cerebellar evolution.

Abbreviations

4v	fourth ventricle
β	cell group β of the inferior olivary nucleus
ba	basilar artery
c	caudal
d	dorsal
DC	dorsal cap
dIOPr	dorsal part of the principal olive
DMCC	dorsomedial cell column
Fl	flocculus
icp	inferior cerebellar peduncle
Int	interposed cerebellar nucleus
IntA	interposed cerebellar nucleus, anterior part
IntP	interposed cerebellar nucleus, posterior part
IOD	dorsal accessory olive
IOM	medial accessory olive
IOPr	principal olive
l	lateral
Lat	lateral cerebellar nucleus
LVe	lateral vestibular nucleus
m	medial
Med	medial cerebellar nucleus
MVe	medial vestibular nucleus
PFl	paraflocculus
r	rostral
thio	thionin
v	ventral
va	vertebral artery
vIOPr	ventral part of the principal olive

MATERIALS AND METHODS

Postnatal mice (*Mus musculus*) of the NMRI strain (up to P7) were obtained from the animal facilities of the University of Jena School of Medicine. Postnatal albino and sable ferrets (*Mustela putorius furo*) were obtained from the breeding colony of the Federal Institute of Risk Assessment, Berlin-Marienfelde, Germany (head: Dr. Dieter Wolff). The day of birth was designated as P0.

Mice were deeply anesthetized on ice and decapitated. Ferrets were deeply anesthetized by intraperitoneal injection of pentobarbital and killed by decapitation. The killing of animals was in accordance with national and institutional guidelines on the use of animals in research.

Antibodies and cDNAs

For immunostaining of mouse and ferret tissues, we used the rat monoclonal antibody 5G10 (Aoki et al., 2003) against pcdh10 at a dilution of 1:500. The antibody was a kind gift of Dr. Shinji Hirano, RIKEN Institute for Developmental Biology, Kobe, Japan. It was raised against a GST fusion protein with the cytoplasmic domain of the longer isoform of mouse pcdh10 (Nakao et al., 2005; amino acids 877–1,040 of the NCBI protein accession number AAK57195; Shinji Hirano, RIKEN, unpublished). In both species, the immunostaining pattern matched the expression pattern of the pcdh10 mRNA as visualized by in situ hybridization. This result indicated that the antibody is specific, as shown previously for the mouse by Aoki et al.

(2003). The mouse polyclonal antiserum against pcdh7 was raised against a partial recombinant protein with a GST tag (amino acids 31–125 of the NCBI protein accession number NP_002580; Abnova Corporation, Taipei, Taiwan; catalog No. H00005099-A01; lot No. BNOVA060630QCS1; dilution 1:300). The immunostaining pattern in ferret matched the expression pattern of the pcdh7 mRNA visualized by in situ hybridization.

The primary antibodies were detected by using biotinylated secondary antibodies against rat IgG (Dianova, Hamburg, Germany) or mouse IgG (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and avidin-coupled peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories). The following plasmids were used to produce cRNA probes for in situ hybridization in mouse tissue: mcad8-12 containing a 1.6-kb fragment of mouse cdh8 cDNA from the 5′ region (positions 504–1,583 of NCBI nucleotide sequence accession number X95600; Korematsu and Redies, 1997); pGEMte-mPcdh7 comprising a 1.6-kb PCR fragment encoding part of the extracellular domain of pcdh7 (positions 1,947–3,575 of NCBI nucleotide sequence accession number NM018764; Vanhalst et al., 2005); and mOLe11 containing full-length mouse pcdh10 (OL-protocadherin; NCBI nucleotide sequence accession number U88549; Hirano et al., 1999; kind gift from Dr. S. Hirano; RIKEN Center for Developmental Biology). For in situ hybridization in ferret tissue, plasmids comprising PCR fragments that encode parts of the extracellular, transmembrane, and cytoplasmic domains of ferret cdh8 (1.8 kb), pcdh7 (2.3 kb), and pcdh10 (1.9 kb) were used (full sequence of NCBI nucleotide accession numbers EU665241, EU665246, and EU665249, respectively; Krishna et al., 2008).

Staining of sections

To obtain series of sections, dissected brainstems with cerebella were fixed for 4–6 hours in 4% formaldehyde in HEPES-buffered salt solution (pH 7.4) supplemented by 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS). Fixed brains were incubated for several hours each in a graded series of sucrose solutions (12%, 15%, and 18% w/v sucrose in HBSS). Specimens were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), frozen in liquid nitrogen, and stored at −80°C.

Twenty-micrometer-thick sections were obtained in a refrigerated microtome and dried on coated slide glasses. For ferrets, series of consecutive sections were immunostained for pcdh10 and pcdh7 and hybridized in situ with probes for cdh8, pcdh7, and pcdh10. For mouse, sections were immunostained for pcdh10 and hybridized for cdh8 and pcdh7. The pcdh7 antibody did not bind specifically in mouse brain with our staining protocol. Adjacent sections were treated with thionine acetate, as described previously (Redies et al., 1993), for neuroanatomical orientation. Horizontal sections through hindbrains from P3 mouse (three series), P2 ferret (two series), and P3 ferret (one series) as well as transverse sections from P3 mouse (three series) and P5 mouse (two series) were stained.

Sections were immunostained according to a previously published procedure (Redies et al., 1997), with minor modifications (Neudert and Redies, 2008). To visualize the antibodies, sections were treated with a solution of 0.03% 3-3′-diaminobenzidine tetrahydrochloride (DAB), 0.04% nickel chloride, and 0.01% peroxide in Tris-buffered salt solution (pH 7.4; TBS; DAB solution). For in situ hybridization of digoxigenin-labeled antisense cRNA probes, a previously published protocol was followed (Redies et al., 1993; Neudert and Redies, 2008). To visualize mRNA, sections were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche, Mannheim, Germany) and reacted with a solution containing 0.03% nitroblue tetrazolium (NBT; Fermentas, St. Leon-Rot, Germany) and 0.02% 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP-T; Fermentas) as substrates.

Whole-mount staining

For immunostaining, whole-mount specimens were fixed for 4–6 hours in 4% formaldehyde in HBSS supplemented by 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Specimens were incubated in 0.3% hydrogen peroxide in methanol overnight at –20°C, transferred to methanol, and stored at –20°C. After rehydration in a descending methanol series, specimens were processed as described previously (Neudert and Redies, 2008). Briefly, specimens were permeabilized by treatment with proteinase K [10 μg/ml in phosphate-buffered salt solution supplemented with 0.05% Tween 20 and 1% dimethylsulfoxide (PBS-TD), pH 7.4] and incubated with primary antibody, followed by incubation with biotinylated secondary antibody. Secondary antibody was visualized with the Vectastain Elite ABC Kit (Vector Laboratories), according to the manufacturer's instruction. The following cerebella were stained with antibody against pcdh10: mouse at P3, P5, and P7 (six, eleven, and three specimens, respectively) and ferret at P2 (one specimen).

For whole-mount in situ hybridization, specimens were fixed overnight in 4% formaldehyde in PBS, pH 7.4, followed by dehydration in an ascending methanol series. Cerebella were stored in methanol at –20°C. Dehydrated specimens were processed as described previously (Neudert and Redies, 2008). In brief, cerebella were bleached with 6% hydrogen peroxide in PBS-T (PBS supplemented with 0.1% Tween 20) and permeabilized with proteinase K (10 μg/ml in PBS-T). After postfixation in 4% formaldehyde/0.2% glutaraldehyde, cRNA (0.5–1 μg/ml) probe was applied overnight, followed by extensive washing. mRNA was visualized basically as described above for in situ hybridized sections. Mouse cerebella were stained for cdh8 (four specimens at P3 and three specimens at P5) and for pcdh7 (four specimens at P3) and ferret cerebella for cdh8 (one specimen at E38, and two specimens at P2) and pcdh7 (one specimen at P2).

Photomicrograph production and terminology

Staining of sections was imaged using a light transmission microscope (BX40; Olympus, Hamburg, Germany) equipped with a digital camera (DP70; Olympus) and appropriate software (DP-Soft; Olympus). Whole-mount specimens were photographed with a high-performance digital 12-bit CCD camera (Pixelfly; PCO, Kelheim, Germany) mounted on a stereomicroscope (MZ FLII; Leica, Wetzlar, Germany). If required, the bright-field photomicrographs were enhanced for brightness and contrast in Photoshop (Adobe Systems, Mountain View, CA). In addition, unevenly illuminated photomicrographs were improved with the Photoshop dodging tool. Schematic drawings were prepared using selected photomicrographs as templates. Labeling of the images and schematic drawings was done with Photoshop and

Illustrator (Adobe Systems). With the nomenclature by Larsell (1952, 1953), the cerebellar lobules of ferret and mouse were numbered by roman numerals I–X (Altman and Bayer, 1997; Christensson et al., 2007). Generally, the neuroanatomical terminology of Paxinos and Watson (1998) was used.

RESULTS

In the present study, we compared the expression patterns of cdh8, pcdh7, and pcdh10 in the cerebellar system of ferret and mouse. The mouse data extend previous findings by Luckner et al. (2001) and Vanhalst et al. (2005) and provide a complete reconstruction of the expression patterns. Expression was determined at similar postnatal stages of ferret and mouse by in situ hybridization (ferret: cdh8, pcdh7, pcdh10; mouse: cdh8, pcdh7) and immunostaining (ferret: pcdh7, pcdh10; mouse: pcdh10) in both whole-mount specimens and cryostat sections. We chose intermediate stages of cerebellar development for analysis because any differences in the bauplan or in embryonic pattern formation can be more easily recognized. In the adult, morphological differences resulting from differential growth are larger. Moreover, a previous study in the mouse (Luckner et al., 2001) showed that the cadherin expression patterns are relatively distinct postnatally but already resemble those found in the mature cerebellum.

Comparable stages of cerebellar development

The length of time during which neuronal differentiation and maturation occur varies considerably among mammalian species. For example, in ferrets, postnatal cerebellar development takes notably longer than in rodents such as the rat (Clancy et al., 2001; Christensson et al., 2007), although pups are born at similar stages of cerebellar neurogenesis (Clancy et al., 2001). In carnivores and rodents, cerebellar development occurs mainly postnatally. For ferrets, we used pups at P2 or P3. These stages were readily obtained from an external breeder. To select a comparable stage of cerebellar development in the mouse, cerebella at different postnatal stages (P1, P3, P5, P7) were analyzed with respect to cortical layer formation and gross morphology. Figure 1 shows that the histological architecture and relative width of the different cortical layers resemble each other in P2 ferret cerebellum and P3 mouse cerebellum. Furthermore, the gross morphology of the cerebella is also similar (Fig. 2).

Cdh8, pcdh7, and pcdh10 show parasagittal expression patterns in the cerebellar cortex of ferret and mouse

The cortical expression patterns of cdh8, pcdh7, and pcdh10 in the postnatal cerebellum of ferret and mouse were determined by analyzing both whole-mount specimens (Fig. 2) and series of consecutive sections (Figs. 3–7). In both species, all three cadherins are expressed in subsets of Purkinje cells that are visible on the cerebellar surface as arrays of parasagittal stripes. The stripe patterns are highly reproducible among animals of the same species. The stripes continue through several lobules and are symmetrical about the midline. Medially, the cadherin-expressing stripes run roughly in parallel to the midline, whereas the laterally located stripes are slightly curved. For pcdh7 and pcdh10, the stripes were numbered by Arabic numerals from medial to lateral (Fig. 8). An exception is the stripe that runs along the midline (called "M" in the present study). Cadherin-positive stripes are denoted with "+" (Fig. 8).

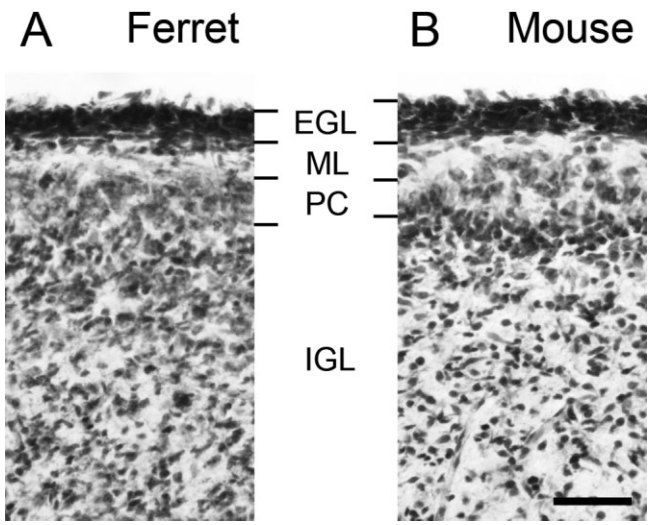

Fig. 1. Nissl staining of horizontal sections through the cerebellar cortex of ferret (P2; **A**) and mouse (P3; **B**). Note that the relative thickness and histoarchitecture of the different cortical layers are comparable. EGL, external granule cell layer; IGL, internal granule cell layer; ML, molecular layer; PC, Purkinje cell layer. Scale bar = 50 μm.

Based on the expression patterns of markers for parasagittal Purkinje cell domains such as zebrin II/aldolase C and calbindin, the adult as well as the neonatal cerebellum can be divided into four transverse zones (Ozol et al., 1999). From anterior to posterior, these are the anterior zone (AZ; ~lobules I–V), the central zone (CZ; ~lobules VI–VII), the posterior zone (PZ; lobules ~VIII–IX), and the nodular zone (NZ; ~lobule X). In the following sections, we describe the cortical expression patterns of the three cadherins in ferret and mouse for each transverse zone.

Whole-mount specimens are shown only for pcdh7 and pcdh10 but not for cdh8. The cdh8 surface staining is indistinct because the expression by the granule cells masks that of the Purkinje cell domains. For pcdh7 and pcdh10, the results are summarized in the schematic drawings displayed in Figures 2 and 8. The drawings are based on the analysis of both whole mounts and sections; in general, sections revealed a more clearcut and complete pattern than whole-mount specimens.

Anterior zone (AZ; ~lobules I–V). In ferret, there are four pcdh7-positive stripes (pc7-1$^+$-pc7-4$^+$) on each side of the midline that extend from lobule I to lobule V (Figs. 2A,C, 3B,D,G,I, 4C, 5A,E, 8A). The lateralmost stripe (pc7-4$^+$) is broad in comparison with the more medially located stripes (Figs. 2A, 3D,G). The staining intensity of stripe pc7-1$^+$ is relatively strong in lobules I–IV but decreases within lobule V (Fig. 8A).

Pcdh10 staining (Figs. 2B,C, 3E,H,K, 4D, 5B, 8B) reveals one narrow positive stripe that runs along the midline (pc10-M$^+$) from lobule I to lobule V. On both sides, it

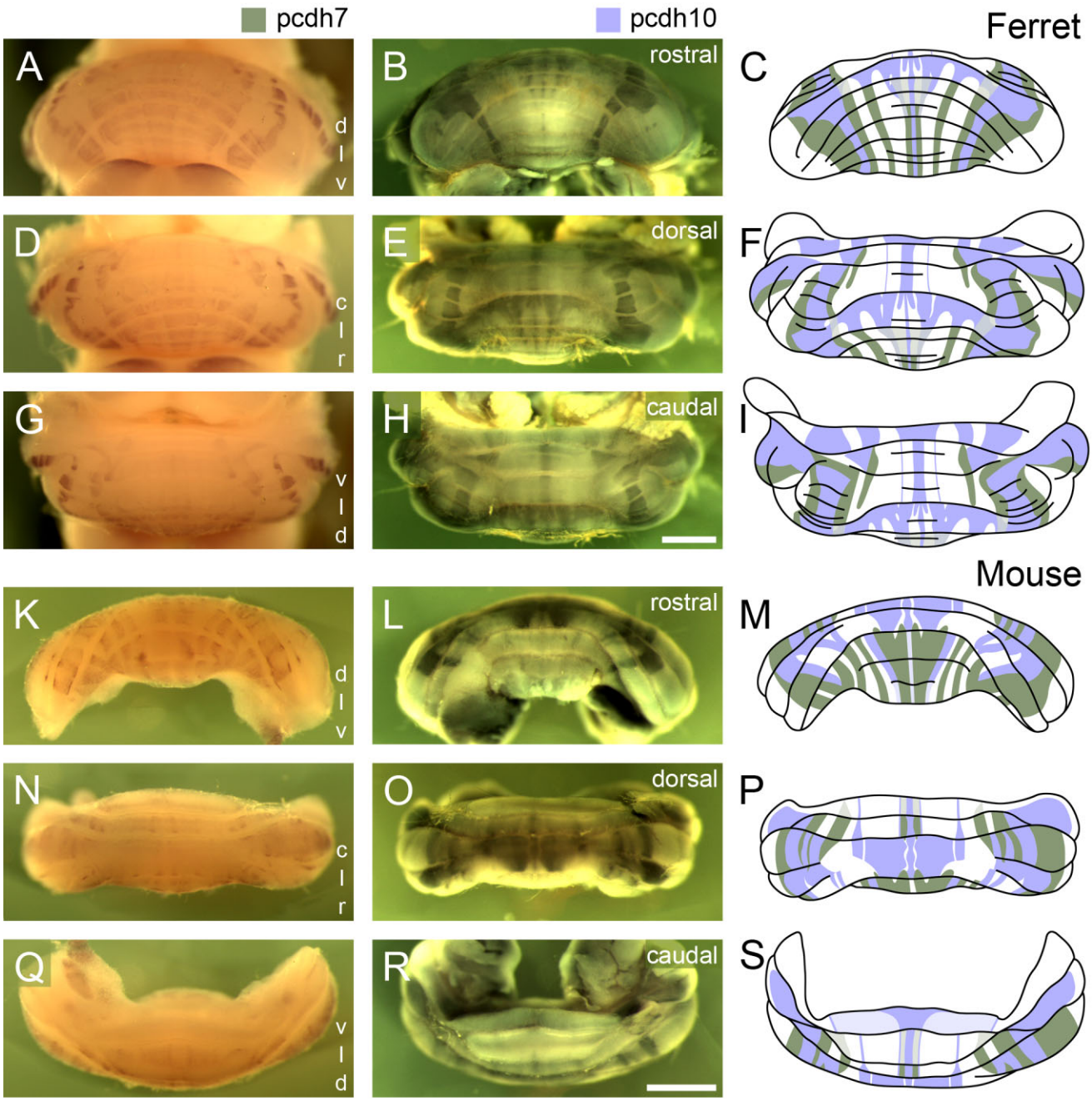

Fig. 2. Pcdh7 and pcdh10 show a complementary expression pattern at the surface of the ferret cerebellum (**A–I**) and the mouse cerebellum (**K–S**) at P2 and at P3, respectively. Whole-mount specimens were hybridized in situ with probes for pcdh7 mRNA (A,D,G,K,N,Q) or immunostained with an antibody against pcdh10 protein (B,E,H,L,O,R). Combined results of the analysis of whole-mount specimens and sections (for examples see Figs. 3–7) are summarized in the schematic diagrams for pcdh7 and pcdh10 (C,F,I,M,P,S). Colors represent cadherin staining, as indicated at the top. The same diagrams are shown separately for each cadherin in Figure 8, together with the numbering of the lobules and the cadherin-positive stripes. Each row of panels represents rostral, dorsal, or caudal views, as indicated in the panels of the second column. The orientation of the specimens is given in the panels of the first column. c, caudal; d, dorsal; r, rostral; v, ventral. Scale bars = 1 mm in H (applies to A–I); 1 mm in R (applies to K–S).

is bordered by the pc7-1$^+$ stripe (Fig. 2C). More laterally, there are two additional pcdh10-positive stripes (pc10-1$^+$, pc10-3$^+$) on each side (Figs. 2B,C, 3E,H,K, 8B). The pc10-1$^+$ stripe appears in lobule V and exhibits a very low expression signal (Figs. 2B,C, 8B). Stripe pc10-3$^+$ strongly expresses pcdh10 from lobule I to lobule V (Fig. 2B); it is enclosed by the pc7-3$^+$ and pc7-4$^+$ stripes (Fig. 2C). Cdh8 expression (arrowheads in Figs. 3A, 4B, 5D) largely overlaps with the pc7-3$^+$ and pc7-4$^+$ stripes (Figs. 3B, 4C, 5E; respectively).

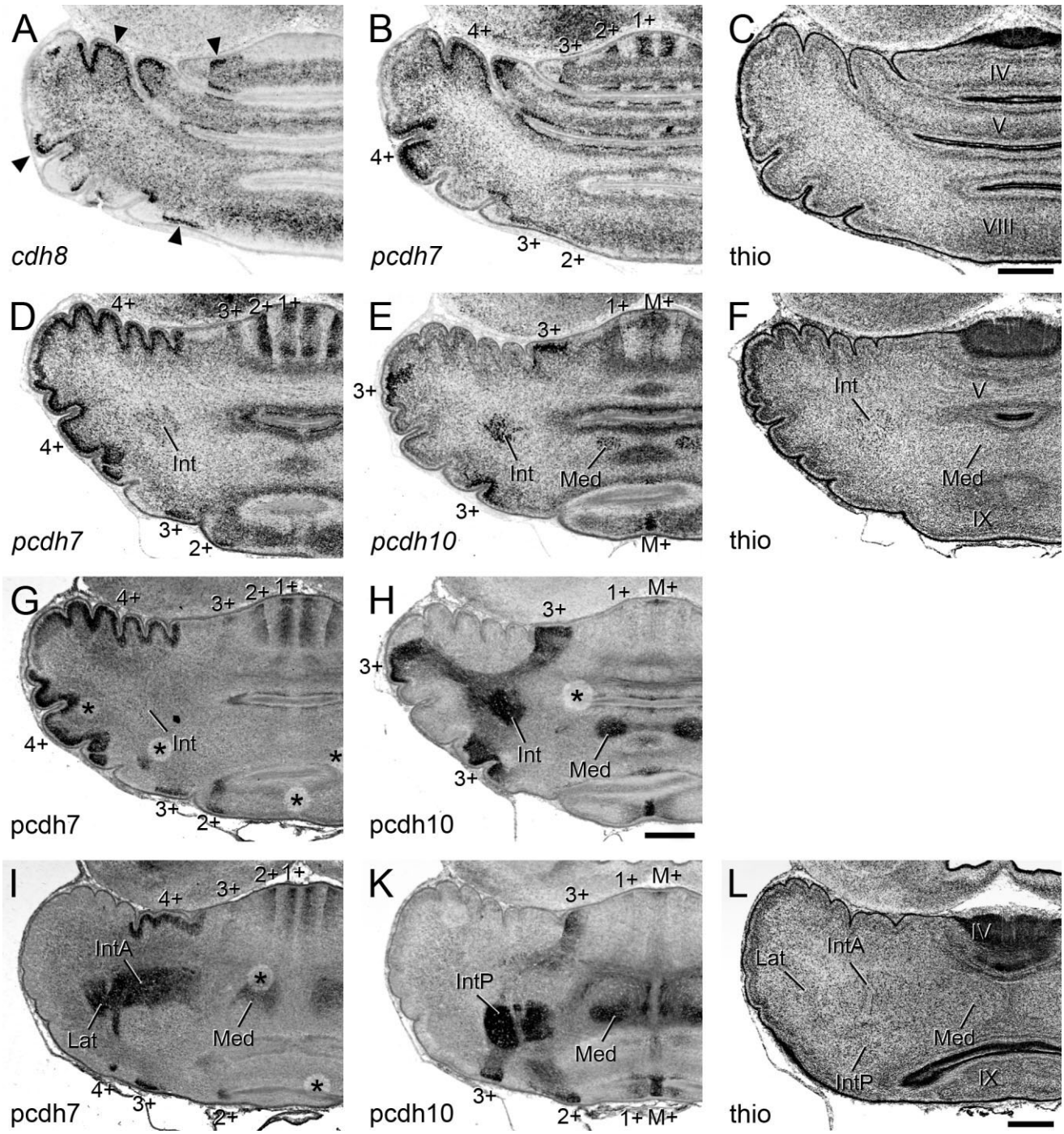

Fig. 3. Cadherin expression in the cerebellum of ferret at postnatal day 3. Consecutive horizontal sections through the dorsal cerebellum (lobules IV–IX) were hybridized with probes for cdh8 mRNA (**A**), pcdh7 mRNA (**B,D**), or pcdh10 mRNA (**E**) or immunostained with antibodies against pcdh7 protein (**G,I**) and pcdh10 protein (**H,K**). **C,F,L:** Nissl stains (thio) of adjacent sections (in C for A,B; in F for D,E,G,H, and in L for I,K). Cadherin-positive Purkinje cell domains are numbered by Arabic numerals or "M" (for the median stripe; for a summary diagram see Fig. 8A–F). Arrowheads in A point to cdh8-expressing Purkinje cell domains. The lobules are numbered by Roman numerals, as indicated on the Nissl stains (C,F,L). The asterisks indicate artefacts. Int, interposed cerebellar nucleus; IntA, anterior Int; IntP, posterior Int; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus. Scale bars = 500 μm in C (applies to A–C); 500 μm in H (applies to D–H); 500 μm in L (applies to I–L).

In mouse, the expression pattern of pcdh7 is complementary to that of pcdh10 and laterally overlaps with that of cdh8 (Figs. 2K–M, 6, 7, 8G,H), as observed in the ferret. The overall arrangment of stripes is similar in the two species (compare Fig. 2C and M). However, there are also differences. For example, in mouse, the pc10-1^{+} stripe is

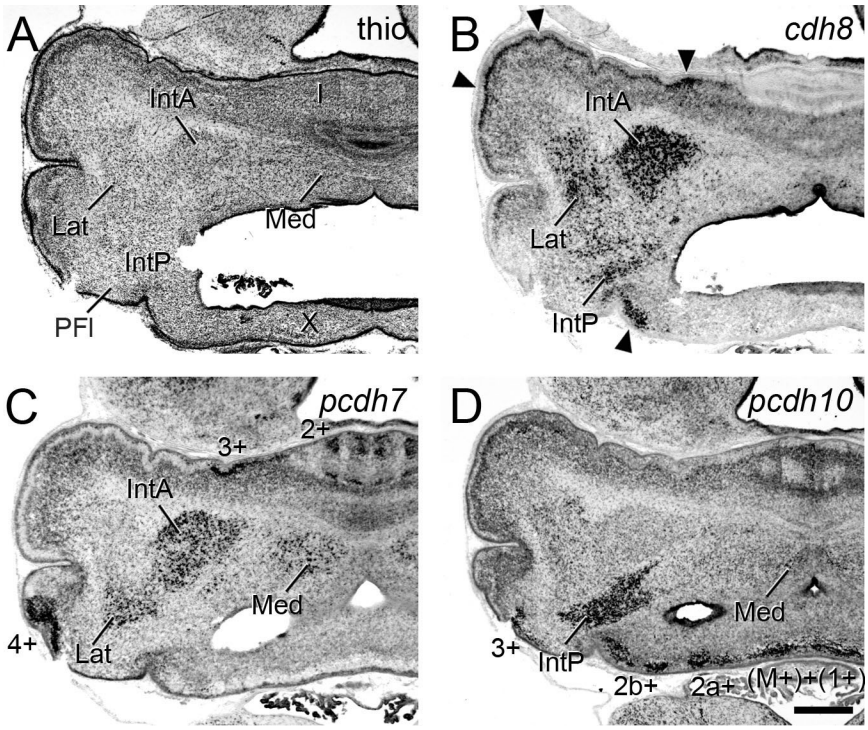

Fig. 4. Cadherin expression in the cerebellum of ferret at postnatal day 3. Consecutive horizontal sections through the ventral cerebellum (lobules I and X) were hybridized with probes for cdh8 mRNA (**B**), pcdh7 mRNA (**C**), or pcdh10 mRNA (**D**). **A:** Nissl stain (thio) of an adjacent section. Cadherin-positive Purkinje cell domains are numbered by Arabic numerals or "M" (for the median stripe; for a summary diagram see Fig. 8A–F). Arrowheads in B point to cdh8-expressing Purkinje cell domains. The lobules are numbered by Roman numerals, as indicated on the Nissl stain (A). IntA, anterior interposed cerebellar nucleus; IntP, posterior interposed cerebellar nucleus; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus; PFl, paraflocculus. Scale bar = 500 μm.

not visible in the AZ, and the pc10-2^+ stripe is seen also in lobules IV and V (Fig. 8B,H). The pc7-1^+ shows a stronger expression in lobule V in mouse than in ferret (Fig. 8A,G). Another difference is observed in the pc7-4^+ stripe that exhibits three parts (pc7-4a,b,c^+) in mouse (Fig. 8G).

Central zone (CZ; ~lobules VI, VII). Generally, the cadherin-positive stripes present in the anterior zone of the ferret cerebellum can be followed into the central zone (Fig. 2D–F). Stripes pc7-1^+ and pc7-2^+ become weaker and finally disappear in lobule VII (Figs. 2F, 8C). In lobule VII, stripe pc7-3^+ is only weakly positive (Figs. 2D, 8C). Stripe pc7-4^+ is wide in the anterior part of the lateral CZ and then narrows; it bends laterally and again becomes wider in the posterior part of the lateral CZ (Figs. 2A,C,D,F, 8C). All pcdh10-positive stripes that are detectable in the AZ widen in the CZ. Some of the stripes become partially subdivided (Figs. 2E,F, 8B,D). In addition, vermal lobule VII exhibits a new pcdh10-expressing stripe (pc10-2^+), which is located between pc10-1^+ and pc10-3^+ (Figs. 2E,F, 8B,D). As in the anterior lobules, cdh8 is coexpressed with pcdh7 in stripes pc7-3^+ and pc7-4^+ (data not shown).

In contrast to the case in ferret, stripes pc7-1^+, pc7-2^+, pc7-3^+, and pc7-4a^+ of the mouse disappear in the anterior part of the CZ; stripes pc7-1^+, pc7-2^+, and pc7-3^+ emerge again in the posterior part of CZ (Figs. 2K,M,N,P, 8G,I). Stripes pc7-4b^+ and pc7-4c^+ fuse to form one pc7-4^+ stripe in the anterior part of the lateral CZ (Figs. 2M, 8G). Stripe pc10-1^+ appears first in vermal lobule VI, whereas stripes pc10-M^+, pc10-2^+, and pc10-3^+ are already present in the AZ (Figs. 2O,P, 8H,K).

The number of pcdh7-positive and pcdh10-positive stripes in the mouse AZ and CZ is different from that of ferret in crus 2. The pc7-3^+ and pc10-3^+ stripes are divided into two or three substripes in the mouse (Figs. 2O,P, 8I,K). In the mouse CZ, cdh8 and pcdh7 show a coexpression similar to that observed in the ferret (data not shown).

Posterior zone (PZ; ~lobules VIII, IX). Three pcdh7-positive stripes are visible on each side of the midline in the posterior lobules of the ferret brain. Two of the stripes (pc7-3^+ and pc7-4^+) continue from the CZ and AZ. The pc7-4^+ stripe changes direction in the posterior part of the lateral PZ and bends laterally (Figs. 2G,I, 4C, 5E, 8C,E). Stripe pc7-3^+ becomes stronger again in the PZ (Figs. 2D,F,G,I, 8C,E). The medially located pcdh7-positive stripe possibly corresponds to stripe pc7-2^+ of the AZ (Figs. 2G,I, 3G, 8E). Lateral to the pcdh10-positive midline stripe (pc10-M^+), a weakly pcdh10-positive stripe can be observed (Figs. 2H,I, 3K, 5B, 8D,F). Although it is extremely thin, it may represent the continuation of stripe pc10-1^+ from the AZ and CZ. Laterally, the pc10-3^+ stripe of the AZ and CZ continues into the PZ. In the posterior part of the PZ, the pc10-3^+ stripe bends laterally to end in the ventral part of the paraflocculus (Figs. 2E,F,H,I, 3E,H,K, 4D, 5B, 8D,F). It is enclosed by the pc7-3^+ and pc7-4^+ stripes (Figs. 2I, 3G,H, 8E,F). Cdh8 exhibits coexpression with pcdh7 in the pc7-3^+ stripe of the anterior

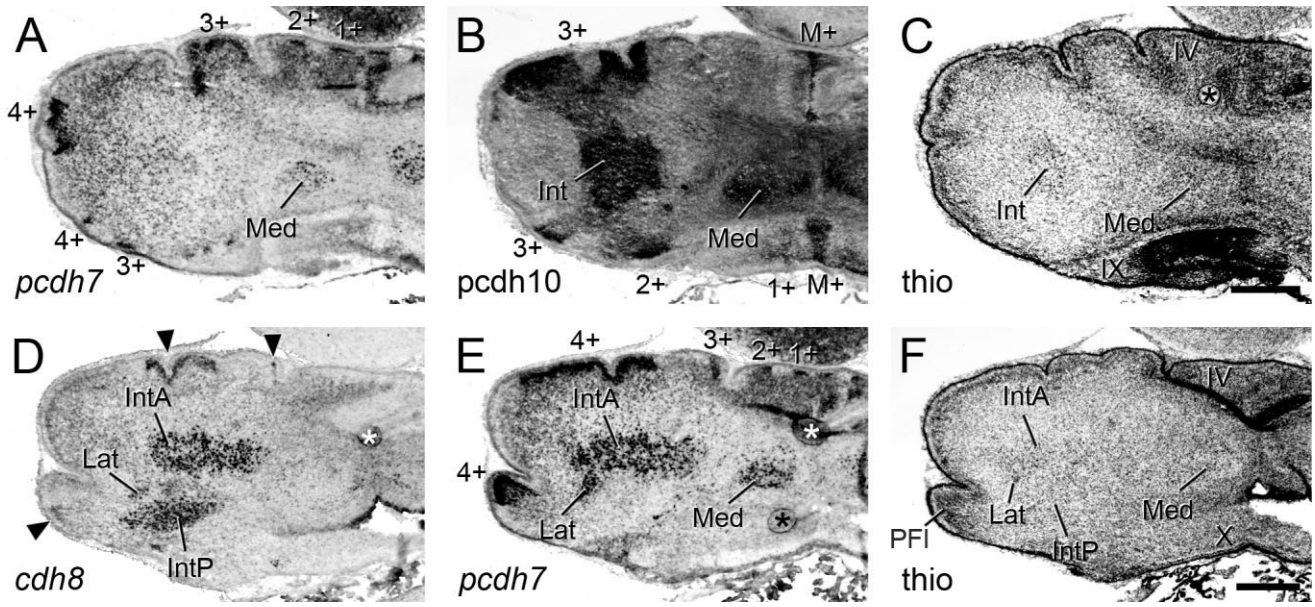

Fig. 5. Cadherin expression in the cerebellum of ferret at postnatal day 2. Consecutive horizontal sections through a level intermediate to those shown in Figures 3 and 4 were hybridized with probes for cdh8 mRNA (**D**) or pcdh7 mRNA (**A,E**) or immunostained with an antibody against pcdh10 protein (**B**). **C,F:** Nissl stains (thio) of adjacent sections. Cadherin-positive Purkinje cell domains are numbered by Arabic numerals or "M" (for the median stripe; for a summary diagram see Fig. 8A–F). Arrowheads in D point to cdh8-expressing Purkinje cell domains. The lobules are numbered by Roman numerals, as indicated on the Nissl stains (C,F). The asterisks indicate artefacts. Int, interposed cerebellar nucleus; IntA, anterior Int; IntP, posterior Int; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus; PFl, paraflocculus. Scale bars = 500 μm in C (applies to A–C); 500 μm in F (applies to D–F).

part of the PZ (Fig. 3A,B) and with pcdh10 in the pc10-3$^+$ stripe of the posterior part of the lateral PZ (data not shown).

In the mouse cerebellum, the expression patterns of cdh8, pcdh7, and pcdh10 in the PZ show many similarities to those of ferret. However, in contrast to ferret, a pc7-1$^+$ stripe is clearly detectable in the vermal lobules VIII and IX (Figs. 2Q,S, 8L), and stripe pc7-4$^+$ appears to be absent in the PZ (Figs. 2S, 8L).

Nodular zone (NZ; ~lobule X). There is no pcdh7 expression in lobule X of the cerebellum of ferret and mouse (Figs. 2I,S, 4C, 6D, 8E,L). In ferret, pcdh10 is expressed in a broad medial stripe (pc10-M1$^+$) and two broad lateral stripes (pc10-2ab$^+$; Figs. 2H,I, 4D, 8F). The medial stripe covers about the same area as stripes pc10-M$^+$ and pc10-1$^+$ of the more anterior lobules, whereas the pc10-2ab$^+$ stripes are located at a mediolateral position similar to that of stripe pc10-2$^+$ of the PZ. Cdh8 expression overlaps partially with the pc10-2b$^+$ stripe (arrowhead in Fig. 4B; cf. Fig. 4D).

As in the ferret, a broad medially located pc10-M1$^+$ stripe is present in lobule X of the mouse. It is bordered by a weakly pcdh10-positive stripe that covers the remaining vermal part of lobule X (Figs. 2S, 6E,H, 8M). Cdh8 expression was below detection levels in lobule X (Fig. 6G).

Cdh8, pcdh7, and pcdh10 are differentially expressed in portions of the deep cerebellar nuclei of ferret and mouse

Differential staining of the three cadherins was also observed for the deep cerebellar nuclei of ferret and mouse. The nuclei were identified in horizontal sections on the basis of a rat brain atlas (Paxinos and Watson, 1998). With the terminology of this atlas, the nuclear complex can be divided roughly into three nuclear areas, the medial (Med), interposed (Int), and lateral (Lat) cerebellar nucleus. The interposed nucleus can be subdivided into an anterior part (IntA) and a posterior part (IntP).

In ferret, the medial cerebellar nucleus is negative for cdh8. It expresses pcdh7 throughout, except for a pcdh10-positive cap, which covers the pcdh7-positive portion dorsally, medially, and caudally (Fig. 3E,H,I,K). The interposed cerebellar nucleus is positive for cdh8 (Fig. 4B). Pcdh7 expression is restricted to its anterior part, whereas pcdh10 is expressed only in its posterior part (Figs. 3E,F, H,I,K,L, 4C,D). The lateral cerebellar nucleus expresses cdh8 throughout (Fig. 4B). More ventrally, the signal is very strong. Only a posterior portion is also positive for pcdh7 (Figs. 3I, 4C). Pcdh10 staining is not detectable in the lateral nucleus. In conclusion, the expression of pcdh10 is complementary to that of pcdh7, whereas cdh8 partially overlaps with the other two cadherins.

Additionally, by performing immunostaining for the pcdh7 and the pcdh10 protein, positive signal in white matter between the cadherin-expressing cortical domains and nuclear portions can be visualized (Fig. 3D–L). This staining was likely associated with corticonuclear fiber connections. Because the pcdh7 immunostaining of white matter was relatively weak, we focused on the pcdh10-positive corticonuclear connections. Stripes pc10-M$^+$ and pc10-1$^+$ are connected to portions of the medial nucleus (Figs. 3H,K, 5B). The more laterally located stripe pc10-3$^+$ is connected to the posterior interposed nucleus (Fig. 3D–L). Note that the pcdh7 staining in the white matter below

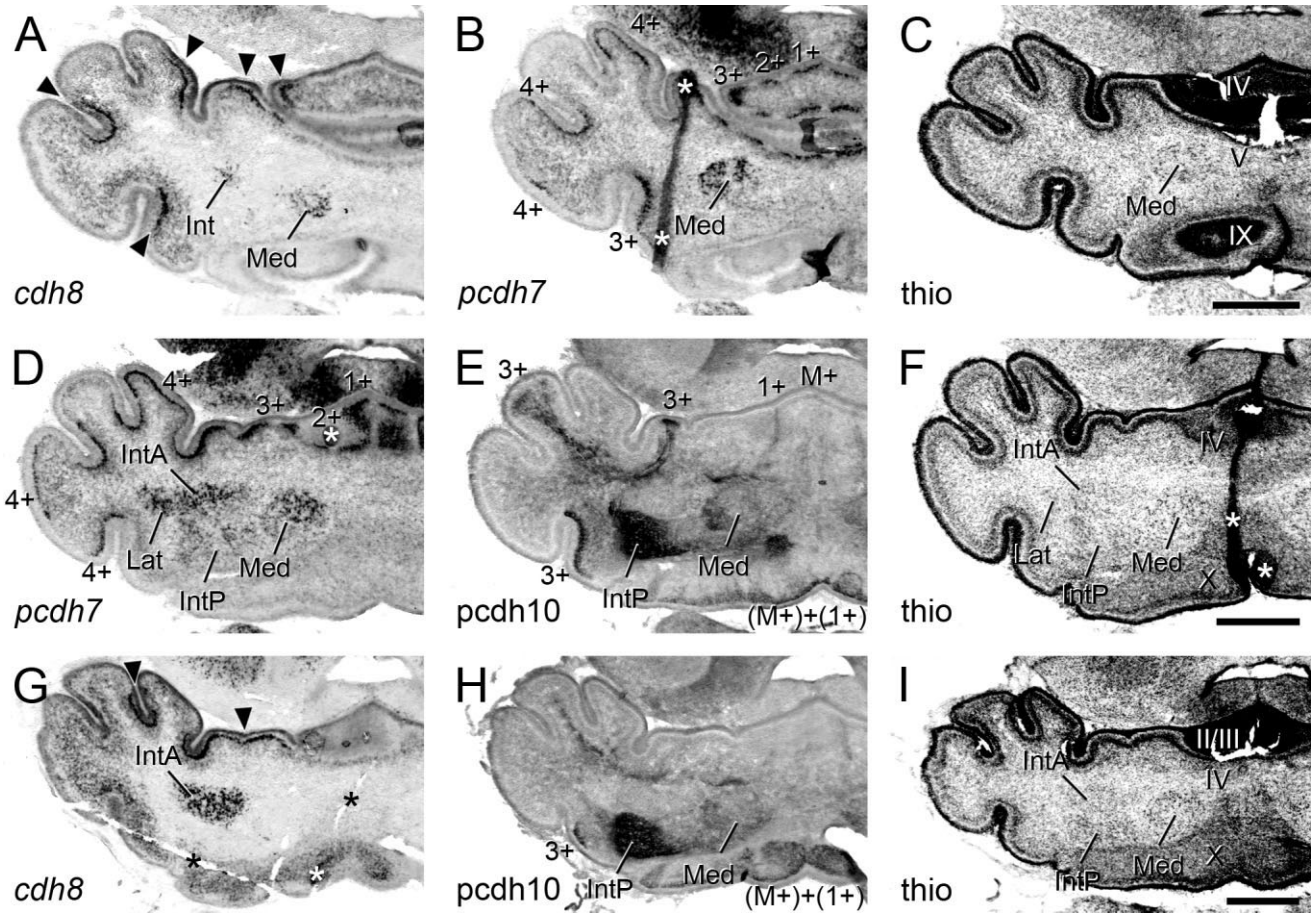

Fig. 6. Cadherin expression in the cerebellum of mouse at postnatal day 3. Consecutive horizontal sections through the midcerebellum (lobules II–V, IX, and X) were hybridized with probes for cdh8 mRNA (**A,G**) or pcdh7 mRNA (**B,D**) or immunostained with an antibody against pcdh10 protein (**E,H**). **C,F,I:** Nissl stains (thio) of adjacent sections. Cadherin-positive Purkinje cell domains are numbered by Arabic numerals or "M" (for the median stripe; for a summary diagram see Fig. 8G–M). Arrowheads in A and G point to cdh8-expressing Purkinje cell domains. The lobules are numbered by Roman numerals, as indicated on the Nissl stains (C,F,I). The asterisks indicate artefacts. Int, interposed cerebellar nucleus; IntA, anterior Int; IntP, posterior Int; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus. Scale bars = 500 μm in C (applies to A–C); 500 μm in F (applies to D–F); 500 μm in I (applies to G–I).

the stripe pc7-4^+ is complementary to that below stripe pc10-3^+, as also observed for the cortical domains (see above). With horizontal sections, we were not able to detect pcdh10-positive fibers that link the dorsally located stripe pc10-2^+ in the CZ to one of the deep nuclear regions, possibly because these fibers run perpendicular to the plane of sectioning. Only for the interposed nucleus, the fiber connections were in the plane of sectioning. Results for the other nuclei were based on the analysis of series of sections (data not shown).

As in the ferret, the medial cerebellar nucleus of mouse is positive for pcdh7 (Fig. 6B,D). Dorsally, pcdh7 expression is restricted to the anterior part, whereas pcdh10 expression is found in the posterior part (Fig. 6D,E). Unlike the case in the ferret, the dorsalmost portion of the medial cerebellar nucleus expresses cdh8 (Fig. 6A). The anterior interposed nucleus shows expression of cdh8 and pcdh7 (Figs. 6D,G, 7B,C). However, there is a small region medially adjacent to the lateral nucleus, which is positive for pcdh7 but negative for cdh8 (Fig. 7B,C). The posterior part of the interposed nucleus expresses pcdh10 strongly (Figs. 6E,H, 7D). In the lateral cerebellar nucleus, expression of all three cadherins is detectable (Fig. 7). Ventrally, the expression of cdh8 and pcdh10 overlaps to a large extent. The more dorsally located pcdh7 expression is complementary to that of pcdh10.

Pcdh10 immunostaining also reveals positive fiber connections in the mouse (Fig. 6E). The pcdh10-positive fiber connections are less well demarcated than those in ferret. However, similar connectivity patterns can be found, especially between the posterior interposed nucleus and stripe pc10-3^+ (Fig. 6E; compare with Fig. 3H for ferret).

Cdh8, pcdh7, and pcdh10 are differentially expressed in divisions of the inferior olivary nucleus and in the lateral vestibular nucleus

Cdh8, pcdh7, and pcdh10 show a regionally restricted expression in the inferior olivary nucleus of ferret and mouse (Fig. 9). In mammals, this nucleus is composed of three major nuclei and several minor nuclei. The main divisions are the dorsal accessory olive (IOD), the princi-

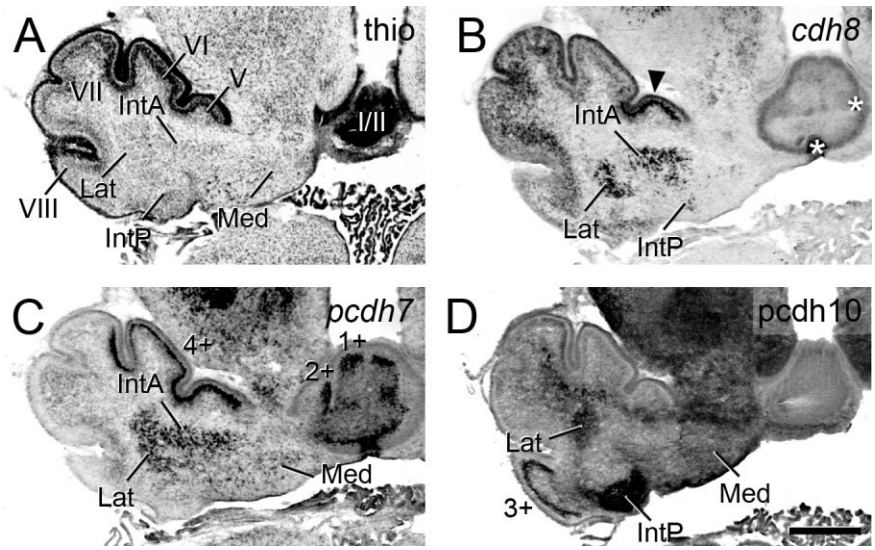

Fig. 7. Cadherin expression in the cerebellum of mouse at postnatal day 3. Consecutive horizontal sections through the ventral cerebellum were hybridized with probes for cdh8 mRNA (**B**) or pcdh7 mRNA (**C**) or immunostained with an antibody against pcdh10 protein (**D**). **A:** Nissl stain (thio) of an adjacent section. Cadherin-positive Purkinje cell domains are numbered by Arabic numerals (for a summary diagram see Fig. 8G–M). The arrowhead in B points to a cdh8-expressing Purkinje cell domain. The lobules are numbered by Roman numerals, as indicated on the Nissl stain (A). The asterisks indicate artefacts. IntA, anterior interposed cerebellar nucleus; IntP, posterior interposed cerebellar nucleus; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus. Scale bar = 500 μm.

pal olive (IOPr), and the medial accessory olive (IOM; Ruigrok, 2004). The climbing fibers that originate in the various subdivisions of the inferior olivary nucleus project to distinct parasagittal domains of the cerebellar cortex in the rat, mouse, and ferret (Buisseret-Delmas and Angaut, 1993; Garwicz, 1997; Voogd et al., 2003; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007). These domains, in turn, are connected to the different portions of the deep cerebellar nuclei and to the lateral vestibular nucleus (Buisseret-Delmas and Angaut, 1993; Voogd and Ruigrok, 2004; Sugihara and Shinoda, 2007).

Series of sections through the inferior olivary nucleus of the ferret are shown at three different rostrocaudal levels in Figure 9A–M and at a caudal level for the mouse (Fig. 9N–Q). In ferret, the IOD and the dorsal cap (DC) show strong cdh8 staining (Fig. 9A,E,I); the two olivary divisions are also completely positive for pcdh7 (Fig. 9B,F,K) but negative for pcdh10 (Fig. 9C,G,L). The IOPr is positive for cdh8 in both its dorsal part (dIOPr) and its ventral part (vIOPr) throughout their rostrocaudal extent (Fig. 9A,E,I), with stronger signal in the ventral part. The dorsal part becomes strongly cdh8 positive only at caudal levels, where it fuses with the cdh8-positive IOD (Fig. 9I). The ventral part and the dorsomedial cell column (DMCC) are positive for pcdh7 (Fig. 9B,F) but do not express pcdh10 (Fig. 9C,G).

The rostral IOM expresses cdh8, with staining more prominent ventrolaterally than dorsomedially (Fig. 9E). In the rostral IOM, pcdh10 signal is also prominent (Fig. 9C,G). Caudally, the pcdh10-positive area recedes to the β cell group dorsomedially, giving a place for an expanding pcdh10-negative ventrolateral area in the IOM (Fig. 9L). The staining disappears at approximately the same level as the cdh8 staining (Fig. 9E,I). The pcdh10-negative part of the caudal IOM expresses pcdh7 (Fig. 9K,L).

The olivary divisions are less clearly demarcated in the postnatal mouse. In contrast to the ferret, IOD is negative for cdh8 in the mouse (Fig. 9N); however, the IOD resembles its ferret counterpart by prominently expressing pcdh7 (Fig. 9O). The other olivary divisions of the mouse show an expression profile roughly similar to those of the ferret. Except for the rostralmost levels, the IOPr is positive for cdh8 in both its dorsal and its ventral parts (Fig. 9N), whereas pcdh7 (Fig. 9O) is expressed only in the ventral part. The IOM is positive for pcdh10 (Fig. 9P), whereas cdh8 staining is more prominent ventrally than dorsally (Fig. 9N). We did not identify other olivary divisions in the postnatal mouse.

Because Purkinje cells in zone B of the cerebellar cortex are known to project to the lateral vestibular nucleus in the rat (Buisseret-Delmas and Angaut, 1993; Voogd and Ruigrok, 2004), we extended our analysis to this nucleus (LVe in Fig. 10). The lateral vestibular nucleus contains giant cells (Brodal and Pompeiano, 1957) that express pcdh7 in the ferret (Fig. 10B) and in the mouse (Fig. 10F), but not cdh8 or pcdh10 (Fig. 10A,C,E,G). A complete mapping of cadherin expression in all vestibular nuclei was beyond the scope of this study.

DISCUSSION

In the present work, we show that cdh8, pcdh7, and pcdh10 are expressed in several parts of the ferret cerebellar system at an intermediate level of cerebellar development. Results from mapping cadherin mRNA expression revealed differential staining in parasagittal Purkinje cell domains of the cerebellar cortex, in portions of the deep cerebellar nuclei, in the divisions of the inferior olivary nucleus, and in the lateral vestibular nucleus. In addition, immunostaining for pcdh10 revealed positive fi-

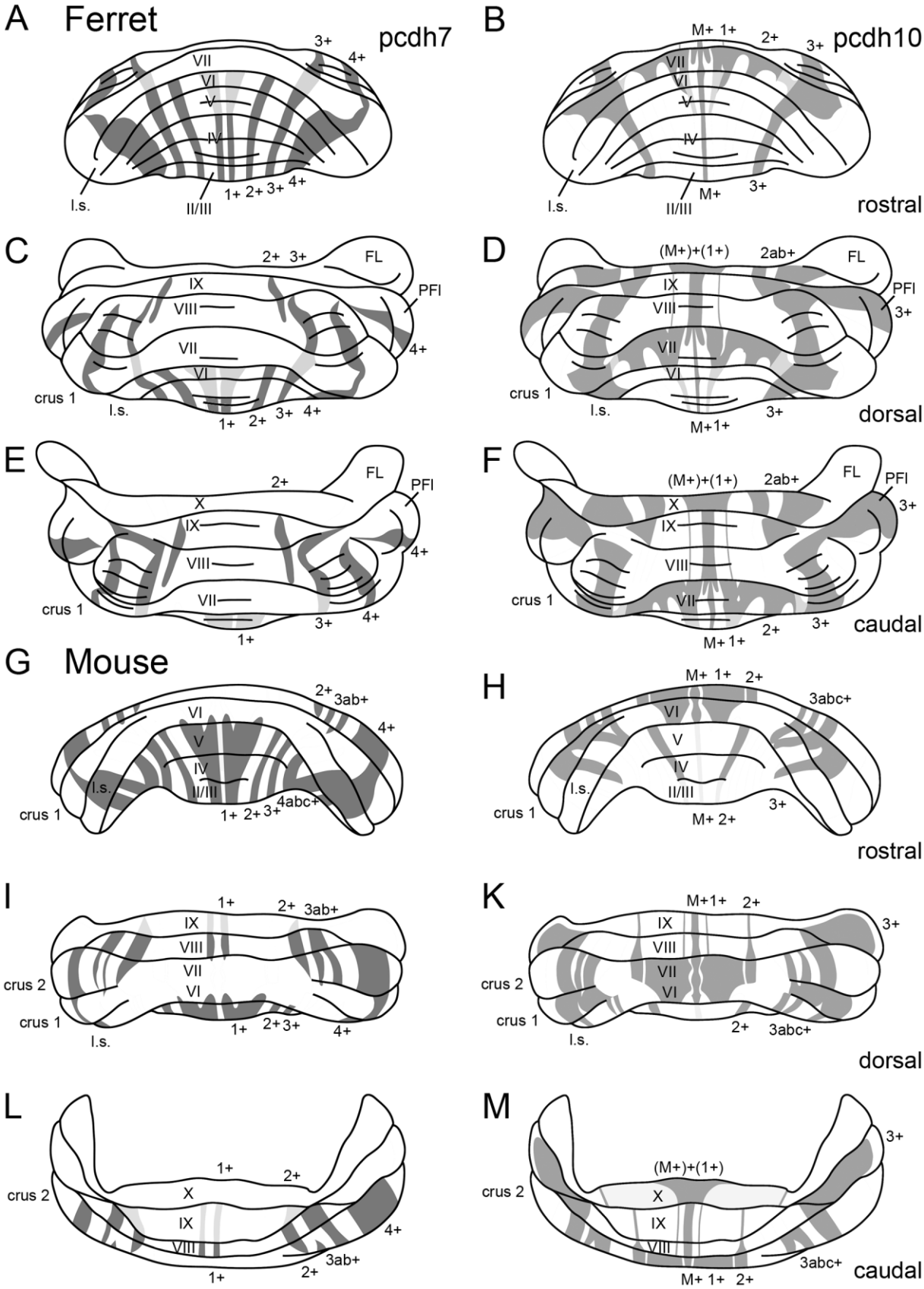

Fig. 8. **A–M:** Schematic drawings of pcdh7 and pcdh10 expression at the cerebellar surface. Data are the same as those displayed in Figure 2C,F,I (for ferret) and Figure 2M,P,S (for mouse). The lobules are numbered by Roman numerals (I–X; anterior-to-posterior sequence). Cadherin-positive Purkinje cell domains are numbered by Arabic numerals or "M" (for the median stripe). Fl, flocculus; l.s., lobulus simplex; PFl, paraflocculus. The different views (caudal, dorsal, and rostral) are indicated at right.

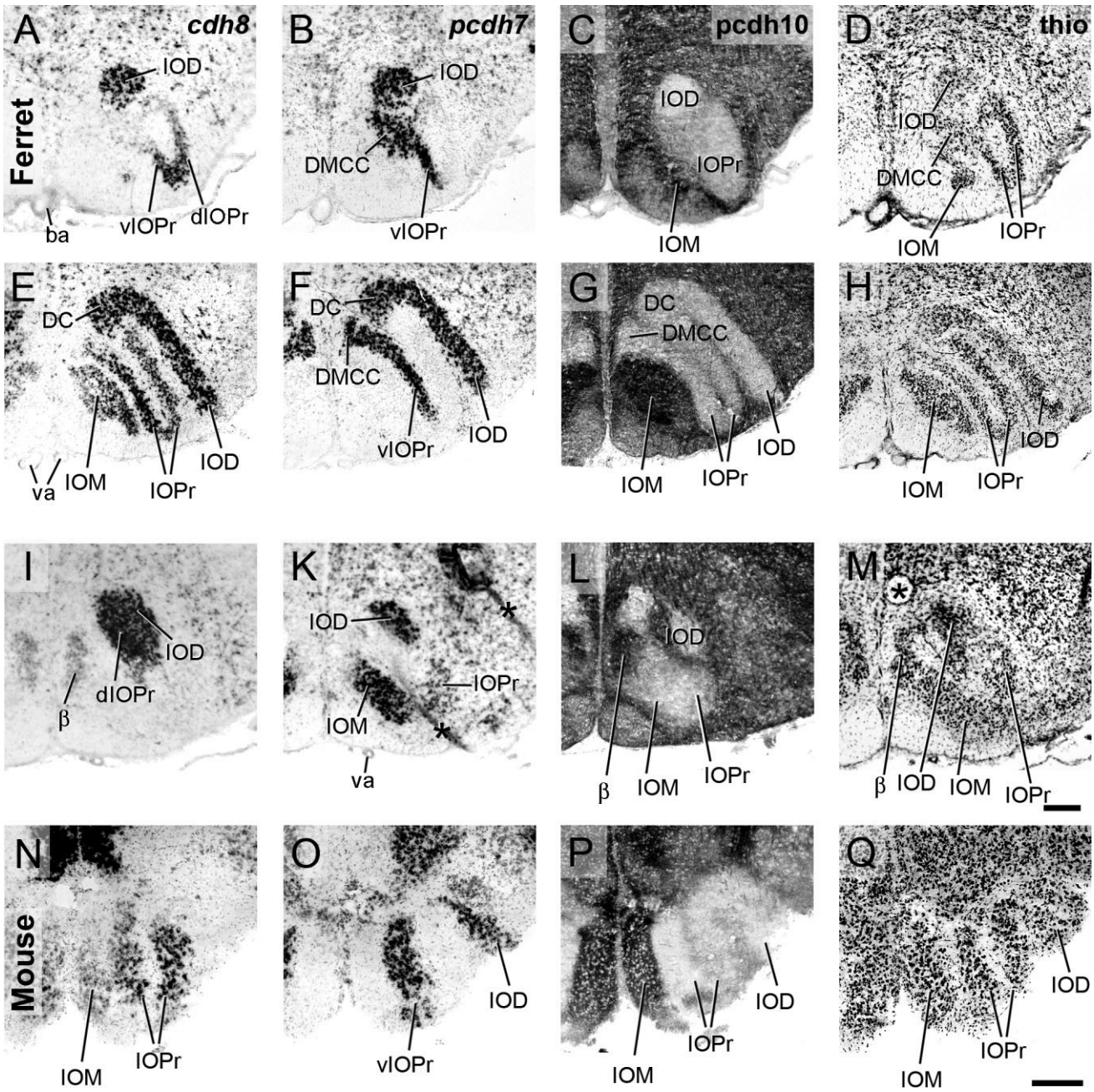

Fig. 9. Cadherin expression in the inferior olivary nucleus of ferret and mouse. Consecutive sections at a rostral level (**A–D**), an intermediate level (**E–H**), and a caudal level (**I–M**) of the ferret inferior olivary nucleus at P2 and at a caudal level of the mouse inferior olivary nucleus at P3 (**N–Q**) were hybridized with probes for cdh8 mRNA (A,E,I,N) or pcdh7 mRNA (B,F,K,O) or immunostained with an antibody against pcdh10 protein (C,G,L,P). The asterisks in K and M indicate artefacts. D,H,M,Q: Nissl stains (thio) of adjacent sections (in D for A–C, in H for E–G, in M for I–L, and in Q for N–P). β, cell group β; ba, basilar artery; DC, dorsal cap; dIOPr, dorsal part of the principal olive; DMCC, dorsomedial cell column; IOD, dorsal accessory olive; IOM, medial accessory olive; IOPr, principal olive; va, vertebral artery; vIOPr; ventral part of the principal olive. Scale bars = 200 μm in M (applies to A–M); 200 μm in Q (applies to N–Q).

ber fascicles in the cerebellar white matter that connect cortical domains and portions of the deep cerebellar nuclei, which are also positive for pcdh10.

Comparison with other molecules

Several other molecules are known to be expressed in parasagittal Purkinje cell domains of the cerebellar cortex, in cells of the deep cerebellar nuclei, and in cells of the inferior olivary nucleus (Millen et al., 1995; Arndt and Redies, 1996, 1998; Chedotal et al., 1996; Suzuki et al., 1997; Lin and Cepko, 1998; Karam et al., 2000; Luckner et al., 2001; Plagge et al., 2001). The most extensively studied example is zebrin II/aldolase C (Brochu et al., 1990; Ahn et al., 1994). Sarna et al. (2006) showed that the

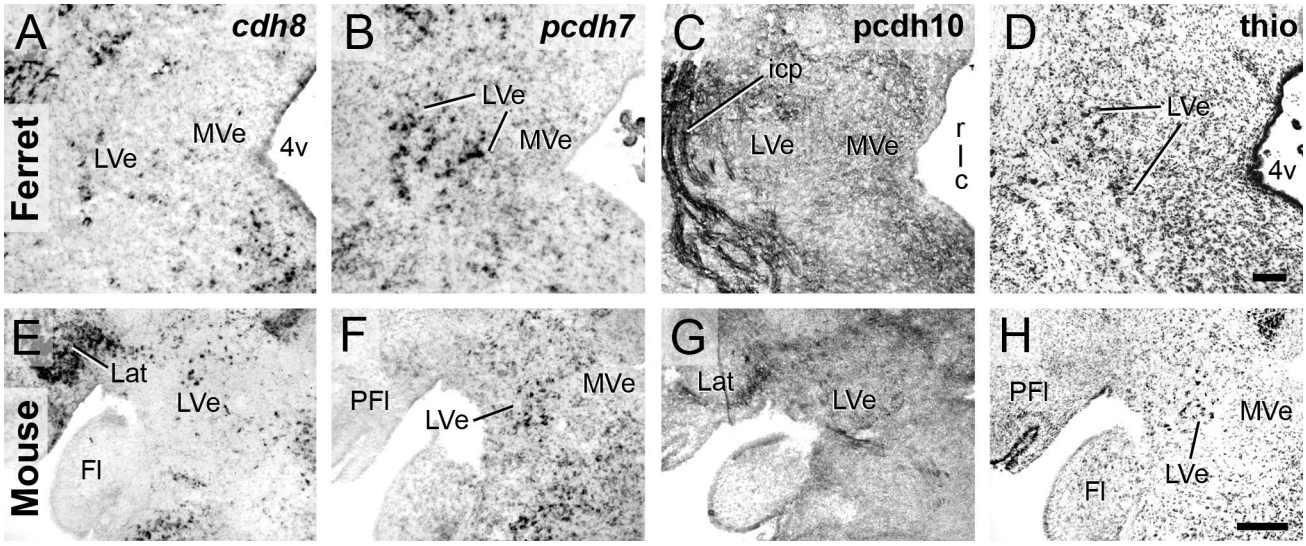

Fig. 10. Cadherin expression in the lateral vestibular nucleus of ferret and mouse. Consecutive horizontal sections of P2 ferret and P3 mouse were hybridized with probes for cdh8 mRNA (**A,E**) or pcdh7 mRNA (**B,F**) or immunostained with an antibody against pcdh10 protein (**C,G**). **D,H:** Nissl stains (thio) of adjacent sections (in D for A–C and in H for E–G). 4v, fourth ventricle; c, caudal; Fl, flocculus; icp, inferior cerebellar peduncle; Lat, lateral cerebellar nucleus; LVe, lateral vestibular nucleus; MVe, medial vestibular nucleus; PFl, paraflocculus; r, rostral. Scale bars = 100 μm in D (applies to A–D); 200 μm in H (applies to E–H).

zebrin II/aldolase C-immunopositive Purkinje cell domains are coextensive with phospholipase Cβ3 (PLCβ3) expression in the adult mouse, whereas they are complementary to those of PLCβ4. At postnatal day 3 (P3), the (PLCβ4) expression pattern (Marzban et al., 2007) shows general similarities to pcdh7 expression in the anterior part of the mouse cerebellum. This is consistent with the observation that the pcdh7 expression pattern is complementary to the expression of pcdh10 that partially overlaps with the zebrin II/aldolase C pattern in the developing and adult mouse cerebellum (Eisenman and Hawkes, 1993; Luckner et al., 2001; F. Neudert and C. Redies, unpublished data). Whether these molecules are members of different synexpression groups (Niehrs and Pollet, 1999) in the cerebellum remains to be studied by more detailed double-labeling experiments.

Cadherin expression patterns reflect functional connectivities in the cerebellar system

Cadherins are calcium-dependent cell adhesion molecules that are markers for functional brain regions, their axonal projections, and their neural circuits and play a role in their formation (for reviews see Redies, 2000; Takeichi, 2007). In our previous study of the embryonic chicken cerebellum, we directly compared the pcdh10 immunostaining data with axonal tracing (Neudert and Redies, 2008). Results revealed that the cadherin expression patterns reflect functional connectivity in the cerebellar system. In the present study, the existence of cadherin-positive corticonuclear connections was visualized, at least in part, by immunostaining of pcdh10-positive fiber projections. For example, in the ferret, stripes pc10-M^+, pc10-1^+, and pc10-2^+ are connected to the pcdh10-positive medial cerebellar nucleus and stripe pc10-3^+ is connected to the pcdh10-positive posterior interposed nucleus (Figs. 3H,K, 5B).

Based on tracing studies of olivocerebellar and corticonuclear connectivities in the rat, the cerebellar cortex has been divided into parasagittal zones (termed zones "A–D"; for reviews see Buisseret-Delmas and Angaut, 1993; Garwicz, 1997; Voogd, 2004; Sugihara and Shinoda, 2004, 2007). The following findings suggest that some of the structures, which are interconnected with each other in this scheme of cerebellar connectivities, have a matching cadherin expression profile. 1) The rostral part of our IOM (medial accessory olive in Buisseret-Delmas and Angaut, 1993) and the posterior interposed nucleus (IntP) both express pcdh10 strongly. These structures are connected to zone C2 of the cerebellar cortex (Voogd and Ruigrok, 2004; Sugihara and Shinoda, 2007; Sugihara and Quy, 2007). The position of this zone and its extention into the paraflocculus resemble those of stripe pcdh10-3^+ in the present study. 2) Stripe pcdh7-4^+ that is laterally adjacent to stripe pcdh10-3^+ possibly corresponds to zones C3, D0, and/or D1. Consistent with a correspondence to zone D1, pcdh7 is also expressed in the target nucleus of this zone, the caudal lateral cerebellar nucleus (Voogd et al., 2003; Sugihara and Shinoda, 2004, 2007), and, by the source of its climbing fibers, the ventral part of the principle olive (vIOPr). Furthermore, zone D1 and stripe pcdh7-4^+ both extend into the paraflocculus (Voogd, 1969). Zone C3 projects to the anterior interposed nucleus (IntA) and the source of its climbing fibers is the rostral dorsal accessory olive (IOD; Voogd et al., 2003; Sugihara and Shinoda, 2004, 2007); both structures also show matching pcdh7 expression. Stripe pcdh7-4^+ may thus be a composite region. Zone D0 has not been described in ferret and is not discussed here. Stripe pcdh7-3^+ that is medially adjacent to stripe pcdh10-3^+ may correspond to zone C1; this zone resembles zone C3 in its connectivity

(connectivity group IV of Sugihara and Shinoda, 2007). 3) Stripe pcdh7-2$^+$ may correspond to zone B. Consistent with this notion, its target nucleus, the lateral vestibular nucleus (Fig. 10), and the caudal IOD (Voogd et al., 2003; Sugihara and Shinoda, 2004, 2007) also express pcdh7. Similar to stripe pcdh7-2$^+$, zone B is present in lobule VIII but absent from the crus 1/lobule VII region (Fig. 8). 4) The pcdh10 and pcdh7 labeling of two narrow stripes medial to a larger pcdh10-positive stripe in crus 2 and lobule VI in mouse (stripes pcdh10-3abc$^+$ and pcdh7-3ab$^+$ in Fig. 8G–L) resembles aldolase C-positive stripes in this region (Eisenman and Hawkes, 1993; Voogd et al., 2003; Sugihara and Shinoda, 2007; Sugihara and Quy, 2007). Similar stripes are not found in ferret. This region, which seems unique to the rodent cerebellum, was termed the "lateral A zone" by Buisseret-Delmas and Angaut (1993) and receives climbing fibers from the caudal medial accessory olive (Fig. 9O,P). In ferret, this nucleus shows a complementary expression pattern for pcdh7 and pcdh10 (Fig. 9F,G,K,L). The medial accessory olive possibly projects also to stripes pc10-M$^+$, pc7-1$^+$, pc10-1$^+$, and pc7-2$^+$ in the anterior zone (AZ).

In summary, the findings described above suggest that cadherins are markers for functional connectivities also in the ferret and mouse cerebellar system. A relation of cerebellar connectivity with gene expression has also been established for zebrin II/aldolase C (Voogd et al., 2003; Sugihara et al., 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Sugihara and Quy, 2007). As pointed out above, most of the pcdh10-positive stripes possibly coincide with zebrin-positive stripes, whereas the pcdh7-positive stripes are, at least in part, complementary.

Hypothetical model of corticonuclear projection territories and possible phylogenetic implications

We propose, based on the data obtained by in situ hybridization and immunostaining, a hypothetical model of corticonuclear projection territories (Fig. 11). This model takes into account two pieces of evidence, mainly from the AZ. First, the model is based on fiber connections that were directly visualized by pcdh10 immunostaining. Second, it was assumed that the portions of the deep cerebellar nuclei and their cortical projection domains have a matching cadherin expression for all three cadherins. The presence or absence of mRNA expression was therefore taken as an indication of whether cortical and nuclear regions are connected.

The boundary between the medial and the intermediate projection territories was localized between stripes pc7-2$^+$ and pc7-3$^+$ of the AZ, because it separates pcdh7-positive stripes, which are located medially and do not express cdh8, from lateral stripes that coexpress both molecules (compare Fig. 11A with Fig. 8A,C,E). Correspondingly, the medial nucleus expresses only pcdh7, whereas the interposed nucleus expresses both molecules (Figs. 3A,B, 5D,E). Furthermore, stripes pcdh10-M$^+$ and pcdh10-1$^+$, which are located within this medial cortical territory, are connected to the medial nucleus by weakly pcdh10-positive fibers (Figs. 3H,K, 5B, 8B,D,F).

The boundary between the intermediate and the lateral projection territories was localized lateral to stripe pc10-3$^+$ of the AZ (Fig. 11A), because, on the one hand, the lateral nucleus does not express pcdh10 (Fig. 4D) and the lateralmost cortical pcdh10 stripe is stripe pc10-3$^+$; in addition, the pc10-3$^+$ stripe is connected to the posterior interposed nucleus by pcdh10-positive fibre connections (Figs. 3H,K, 5B). On the other hand, the lateral nucleus expresses pcdh7 and cdh8 (Fig. 4B,C); the overlying cortical domain contains stripe pc7-4$^+$ that coexpresses cdh8 (Figs. 3A,B, 5D,E). In the AZ, no pcdh7-positive or cdh8-positive stripes are found more laterally.

The borders identified in the AZ were extrapolated to more posterior lobules (Fig. 11E). This is in agreement with the general finding that the parasagittal Purkinje cell domains and the territories of their afferent and efferent connections can be followed approximately from one lobule into one or more neighboring lobules (Buisseret-Delmas and Angaut, 1993; Voogd et al., 2003; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Sugihara and Shinoda, 2004, 2007; Sugihara and Quy, 2007; Neudert and Redies, 2008). However, the exact number and position of the Purkinje cell domains may vary from lobule to lobule. The nodular zone was excluded from our hypothetical model because the expression patterns in this lobule are strikingly different from those of the rest of the cerebellar cortex. The boundaries of the projection territories in mouse were defined by a similar analysis (Fig. 11D,H). The hypothetical model shown in Figure 11 requires confirmation and refinement by axonal tracing and/or additional expression studies and must thus be considered a gross approximation at present. In particular, if the territories of the deep nuclei turn out to interdigitate at the borders, the model will have to be revised or abandoned.

The overall expression patterns of cdh8, pcdh7, and pcdh10 in the cerebellar system of mouse (Fig. 11B,F) are largely similar to those of ferret. Examples for differences between the two species include the lateral A zone that seems unique to rodents and contains more numerous cadherin-positive stripes (see above). Moreover, in contrast to ferret, cdh8 expression is detectable in the medial cerebellar nucleus but not in the medial area of the cerebellar cortex of mouse at P3. However, Suzuki et al. (1997) described cdh8 expression in the medial area of the postnatal mouse cerebellar cortex until P2. Another difference is that pcdh10 is expressed in the lateral cerebellar nucleus of the mouse but not in ferret.

The general resemblance of the cadherin expression patterns between ferret and mouse suggests that the differences in pattern formation between the two species are relatively small at an intermediate stage of cerebellar development. The postnatal expression patterns reflect patterns of molecular and functional differentiation that persist in the adult cerebellum (Luckner et al., 2001; Larouche and Hawkes, 2006; Marzban et al., 2007). As a consequence, differences in cerebellar morphology between the adult ferret and the adult mouse likely are due to differential growth phenomena within a common bauplan rather than to substantial variations in embryonic pattern formation. A comparison of the cadherin expression patterns between chicken (Neudert and Redies, 2008) and the present data in mammals, however, failed to reveal similarities at the intermediate stage of cerebellar development (F. Neudert and C. Redies, unpublished data). It remains to be studied whether a cerebellar bau-

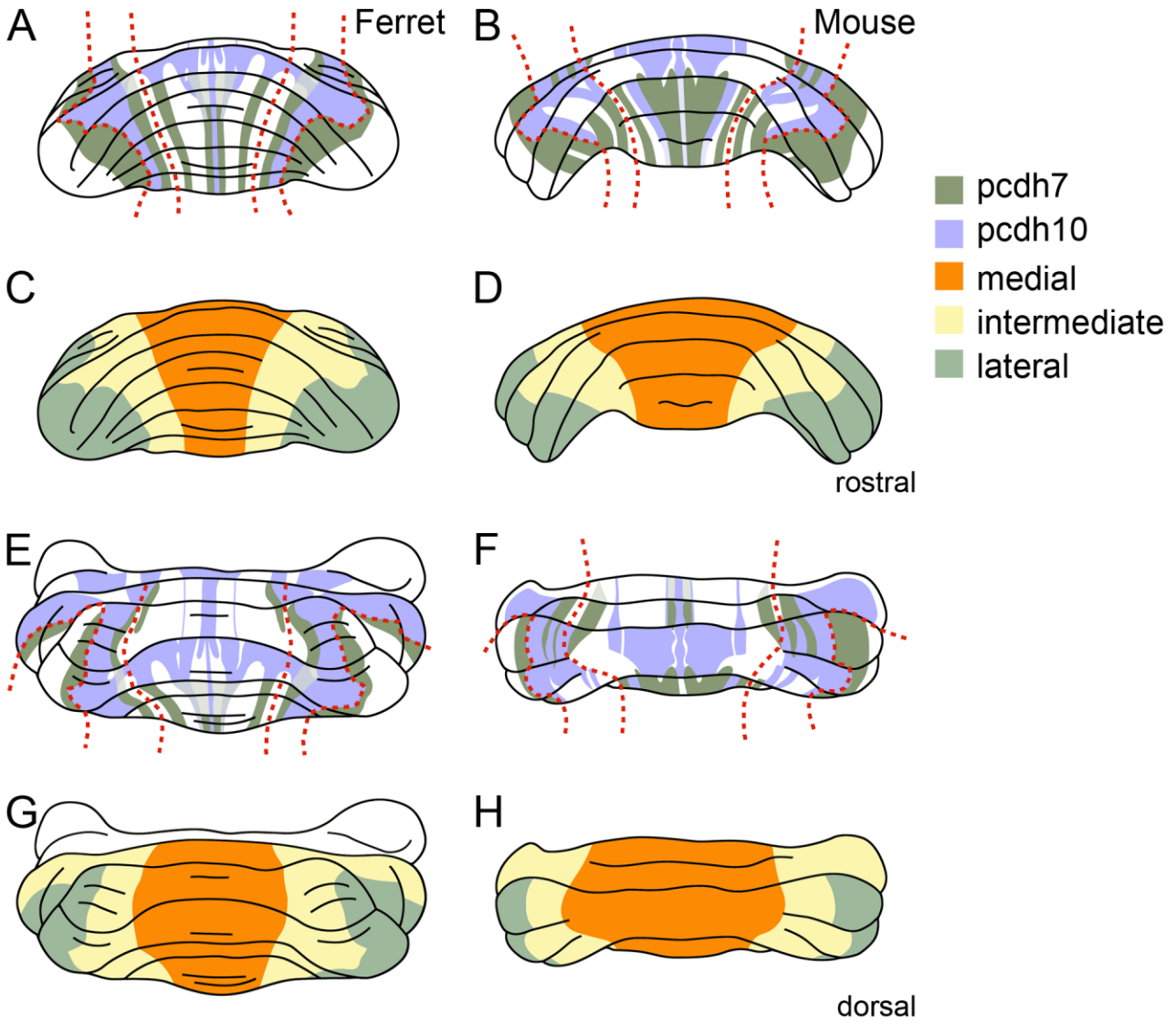

Fig. 11. Hypothetical model of the corticonuclear projection territories in the ferret and mouse cerebellum. **A,B,E,F:** Complementary expression patterns of pcdh7 and pcdh10 in the anterior (A,B) and dorsal (E,F) lobules of the postnatal ferret (A,E) and mouse (B,F) cerebellum (compare with Figure 2C,F,M,P). The dashed lines (red) indicate the approximated borders of the corticonuclear projection territories that were defined on the basis of the cadherin expression patterns and pcdh10-positive connecting fiber fascicles (see Discussion). **C,D,G,H:** Corticonuclear projection territories in the ferret (C,G) and mouse (D,H) cerebellum. Colors represent the medial, intermediate, and lateral corticonuclear projection territories, as indicated at right. A–D show rostral views and E–H show dorsal views.

plan common to all vertebrates becomes evident at earlier stages.

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4. DISCUSSION

Seventeen members of the classic cadherin and δ-protocadherins subfamilies of the cadherin superfamily genes were cloned and sequenced for the first time from the ferret brain. In addition, the intracellular binding partner of δ1-protocadherins, protein phosphatase-1α (PP1α), was cloned. In situ hybridization was carried out to study their expression during the development of the primary visual cortex and of brain blood vessels (angiogenesis). The ferret cerebral cortex is a good model system to investigate both corticogenesis (McSherry, 1984; Rockland 1985; Law et al., 1988; Jackson et al., 1989) and cerebrovascular development (Atkinson et al., 1989). Unlike the mouse, the large cerebral cortex of ferret and the duration of neuron production permit a high temporal resolution of developmental event during corticogenesis and cerebrovascular angiogenesis (McSherry, 1984). Results from in situ hybridization revealed that the expression of the cadherins is subject to a tight temporal, layer-specific regulation during corticogenesis. The germinal zones of the early embryonic cortical mantle also express the cadherins differentially, in addition to the layers of the differentiating and mature cortical plate. The cadherin expression also shows a clear region-specific pattern, as demonstrated by the change in the layer-specific expression profile at the boundary between the primary and secondary visual cortex. In addition, I demonstrate that some of the cadherins are expressed in subtypes of cells dispersed in specific cortical layers or throughout all cortical layers. My results confirm and extend previous studies on the expression of cadherins in the mammalian cerebral cortex, as discussed in detail below. Although corticogenesis can be expected to involve a large number of molecular determinants, only a limited number of candidate genes have been identified so far. The identification of new molecular determinants of corticogenesis is an important step for understanding how the neural complexity of the mature cerebral cortex is generated.

Also, I demonstrate that six out of the seventeen cadherins (VE-cadherin, CDH6, CDH7, CDH11, PCDH1 and PCDH17) and PP1α are expressed under a tight spatiotemporal control by blood vessels in specific brain regions and/or a subset of blood vessels during angiogenesis. CDH11, PCDH17 and PP1a show a strong and ubiquitous expression, whereas CDH6, CDH7 and PCDH1 show a more restricted, region-specific expression. Moreover, I provide preliminary evidence that some of the cadherins are expressed in subtypes of blood vessel. Together, my results confirm and extend the notion that neurogenesis and angiogenesis in the brain might share common molecular mechanism

(Carmeliet, 2003; Vasudevan et al., 2008; Zacchigna et al., 2008), as discussed in detail below.

4.1. Cadherins provide a code of potentially adhesive cues for visual cortex development

Each of the 15 out of 16 cadherins studied during visual cortex development is expressed in a spatio-temporally restricted pattern that is dissimilar from that of other cadherins, although partial overlap between the cadherins is observed. It has been shown that one of the basic mechanisms of brain morphogenesis is the selective and regulated adhesion of cell surface membranes of neural cells. This cadherin-based mechanism, which is preferentially homotypic (see Introduction), causes cells to adhere or segregate from each other in a highly selective manner (Hirano et al., 2003; Redies et al., 2005). For example, the selective aggregation or segregation of neural cells in the brain regulates the formation of cell clusters that segregate from other cell populations in their environment, e.g., during the formation of embryonic divisions, brain nuclei or cortical layers (Hirano et al., 2003). In addition to their role in morphogenetic processes, which are dynamic in space and time (for example, in cell migration and cell sorting), the cadherin-based adhesive code also tends to fix morphogenetic patterns into permanent anatomical structures in the mature brain.

Cell sorting takes places not only if cells express different cadherins (qualitative differences), but also if they express the same cadherin in different amounts (quantitative differences; Steinberg and Takeichi, 1994). It is this concept of qualitative and quantitative differences in cellular adhesiveness (Steinberg, 1978) that is central to our ideas about the developmental processes regulating visual cortex development (see below). Results from my study support the notion that the cadherin-based adhesive code contributes to regionalization, lamination and functional specification of developing cerebral cortex.

As observed in previous studies, several cadherins are differentially expressed in a restricted fashion by subpopulations of neural cells during the development of the vertebrate nervous system, not only in cerebral cortex but also in many other brain regions (Redies et al., 2005). Early in development, the distribution of these populations of neural cells reflects the relatively simple segmental (neuromeric) structure of the primordial neuroepithelium (Redies and Takeichi, 1996). It has been proposed that during embryonic development, the adhesive cues provided by cadherins lead to several morphoregulatory functions in the maintenance of segmentation and in the gradual emergence of functional

structures such as brain nuclei, fiber tracts and neural circuits from the primordial neuroepithelium.

Later in the development, the expression of all fifteen cadherins in different layers of visual cortex in various patterns reflects the complex functional anatomy of the vertebrate brain. This cadherin expression pattern is relatively stable throughout development. The large diversity of cadherins expressed in the brain may correlate with the vast complexity of this organ. In order to account for the staggering complexity of neuronal processes and their connections in the brain, and in view of the fact that growing nerve fibers are extremely selective in choosing their targets, it has been postulated that there exist many distinct cell surface molecules specifying neural identity. Accordingly, the adhesive coding mediated by cadherin molecules has been implicated as a candidate mechanism for the “lock-and-key” components of Sperry's chemoaffinity hypothesis (Redies et al., 1993; Tepass et al., 2000). We speculate that the adhesive specificity of the fifteen cadherins studied in the present work also regulates aggregation and sorting of cells and contribute to the functional anatomy of the cerebral cortex. Indeed, several of the classic and δ-protocadherins studied here are known to mediate the segregation of different cadherin-expressing cell types in vivo and in vitro (for classic cadherins, see Hirano et al., 2003; Pcdh1, Kuroda et al., 2002; Pcdh7, Bradley et al., 1998; Pcdh8, Strehl et al., 1998; Pcdh10, Hirano et al., 1999; Redies et al., 2005).

Most of the cadherins studied here are expressed also in various other regions throughout the ferret brain and in neural circuits outside the visual system (Krishna-K, Christoph Redies, unpublished data). This type of widespread but region-specific expression of cadherins was described before in the embryonic and postnatal brain of other species, like rodents (Korematsu and Redies 1997; Suzuki et al., 1997; Hirano et al., 1999; Obst-Pernberg et al., 2001; Bekirov et al., 2002; Vanhalst et al., 2005; Redies et al., 2005; Kim et al., 2007).

4.2. Layers of the developing visual cortex express specific cadherins

4.2.1. Germinal zones of the embryonic cerebral cortex

In the early embryonic cerebral cortex, a number of cadherins are expressed differentially in the embryonic germinal zones of the developing cortical mantle. Cadherins thus represent markers for the various lamina of the developing embryonic cortex (PP, VZ, SVZ, IZ, CP, SP, and MZ). For example, the cells of the E23 preplate (PP) express CDH4, CDH11, CDH14, PCDH7, PCDH9 and PCDH10. The subependymal layer (part of VZ) is

marked by the combinatorial expression of CDH7, CDH14, PCDH17 and PCDH19, and the rest of the ventricular zone (VZ) expresses CDH4, CDH11, CDH20, PCDH1 and PCDH11.

Another cadherin, which is expressed in the ventricular zone, is N-cadherin (Redies and Takeichi 1993). This cadherin, which is highly concentrated at the adherens junctions of the ependymal layer (Akiyata and Bronner-Fraser, 1992; Redies et al., 1993; Aaku-Saraste et al., 1996), is required for the maintenance of the neuroepithelial layer as a coherent sheet of tissue; a lack of N-cadherin results in aberrant histogenesis of the neural tube in mouse, chicken and zebrafish (Radice et al., 1997; Gänzler-Odenthal and Redies, 1998; Lele et al., 2002; Erdmann et al., 2003; Malicki et al., 2003; Masai et al., 2003; Kadowaki et al., 2007). Another classic cadherin, CDH6, is required for proper lamination of neural tissue in zebrafish (Ruan et al., 2006). In the neuroepithelium, the cadherin-mediated adhesive system also regulates cell proliferation and cell death (Babb et al., 2005; Teng et al., 2005; Lien et al., 2006; Noles and Chenn, 2007). It remains to be investigated whether the other cadherins expressed in the germinal zones of the ferret visual cortex have similar functions.

One of the essential mechanisms of development in the early embryonic cortical plate is cell migration (see Introduction). Several classic cadherins are known to play a role in cell migration. For example, a lack of N-cadherin and CDH6B leads to enhanced migration in the developing nervous system (Barami et al., 1994; Taniguchi et al., 2006; Coles et al., 2007). In contrast, overexpression of cadherins slows down migration (Taniguchi et al., 2006; Coles et al., 2007). Several cadherins were identified in the ventricular and subventricular zones in the present study (CDH11, PCDH7, PCDH9 and PCDH17). Interestingly, PCDH7 and PCDH17 are only expressed in the upper layers of the subventricular zone. Similarly restricted expression patterns are observed for CDH6 and CDH14 in the upper subplate and for CDH14 in a sublayer of the intermediate zone at P2. In conclusion, cadherins are well-positioned candidate molecules to regulate the timing and extent of migration in the developing cerebral cortex.

There are several other known markers for the different embryonic germinal zones of the developing cortex. For example, the ventricular zone expresses Otx1, Otx2, Dlx2 and Noggin (Frantz et al., 1994; Porteus et al., 1994), the subventricular zone Svet1, Bcl-2, Dlx2, Cux-1 and Cux-2 (Bulfone et al., 1993; Porteus et al., 1994; Tarabykin et al., 2001; Panganiban and Rubenstein, 2002; Nieto et al., 2004), the preplate Lhx9, Lhx2, VGLUT1, VGLUT2, Egr-1, Reelin, Novel IgCAM, MDGA1 (Bertuzzi et al., 1999; Bishop et al., 2003; Hevner et al., 2003; Ina et al., 2007; Takeuchi et al., 2007), the subplate KAT-1,

Tbr1, golli-lacZ and COUP-TFI; (Zhou et al., 1999) and the intermediate zone GDE2, AMPA receptors, Tbr1, Cux2, NeuroM (Zimmer et al., 2004; Englund et al., 2005). The cadherins studied in the present work are the first molecules from a single gene family that differentially distinguish the various germinal zones during development. In addition, most of these previously described molecules are gene regulatory proteins involved in embryonic pattern formation. But cadherins are a family of morphoregulatory molecules, and are likely acting down-stream of genetic patterning mechanisms (Shimamura et al., 1994; Stoykova et al., 1997; Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Bishop et al., 2000; Bishop et al., 2002; Garel et al., 2003; Luo et al., 2006).

4.2.2. Layers of the postnatal and adult cortical plate

Fifteen out of sixteen cadherins studied are expressed differentially in the layers of the postnatal cerebral cortex. Although layer-specific expression of cadherins in the developing cortical plate has been described for several cadherins previously, the present study is the first to map systematically the expression of multiple cadherins during the development of the cortical plate in one particular cortical region from early embryonic stages to the adult in a single species.

A large number of other genes are expressed in a layer-specific fashion in the differentiating and mature cortical plate. Layer-specific expression in the developing neocortex has been demonstrated for genes including cell-cell recognition molecules such as cadherins (Suzuki et al., 1997; Inoue et al., 1998; Nakagawa et al., 1999; Rubenstein et al., 1999), immunoglobulin superfamily members (Pimenta et al., 1996), Ephs/Ephrins (Donoghue and Rakic, 1999; O'Leary and Wilkinson, 1999; Prakash et al., 2000; Vanderhaeghen et al., 2000), Wnt receptors and their inhibitors (Kim et al., 2001), transcription factors and some other classes of genes (Nakagawa et al., 1999; Rubenstein et al., 1999). Funatsu et al. (2004) classified these and other layer-specific genes into three groups according to their potential functions: (1) Secreted molecules and their receptors; (2) transcription factors; and (3) cell adhesion molecules and their regulators.

For example, the supragranular layers (prospective layers I-III) are marked by their expression of *CUTL2* (Nieto et al., 2004; Zimmer et al., 2004), *CALB1* (Stichel et al., 1987; DeFelipe et al., 1989; Conde et al., 1994), *PCDH8* (Arcadlin; Yamagata et al., 1999), *reelin* (Impagnatiello et al., 1998; Rodriguez et al., 2000; Martinez-Cerdeno et al., 2002), *LAMB1* (Luckenbill-Edds et al., 1995), *NR2E1* (Roy et al., 2002, 2004), *NR2F2* (Lopes da Silva et al., 1995), *VIP* (De Souza et al., 1985) and *CNR1* (Eggan and Lewis,

2007). Other layer-specific genes include *ER81* (Weimann et al., 1999; Xu et al., 2000; Sugitani et al., 2002), serotonin *(5-HT) 2C* receptor, *Nurr1* (Xing et al., 1997; Arimatsu et al., 2003), and *CTGF* genes (Heuer et al., 2003) in rodent cortex. In the monkey and human neocortices, layer-specific expression of *5-HT2C* receptor mRNA had been reported (Pasqualetti et al., 1999; Lopez-Gimenez et al., 2001; Wright et al., 1995). In rodents layer V, another layer-specific gene, *fezl*, is critically involved in the fate determination of corticospinal motor neurons (Molyneaux et al., 2005; Molnar and Cheung, 2006). Mutations or any other impairments of these developmentally important layer-specific genes could cause profound deficits in cortical development, including altered cortical layer formation (Dobyns et al., 1993; Dobyns and Truwit, 1995; Gupta et al., 2002; Mukaetova-Ladinska et al., 2004). As mentioned above for the germinal zones, the multiple cadherins studied in the present work are one of the first examples of molecules from a single gene family that differentially distinguish various lamina of the cortex at different developmental stages.

Although the present expression study of cadherins is purely descriptive, a number of other studies suggest multiple roles of cadherins in cortical development. Differences in their expression profiles suggest the possibility that the two subfamilies of cadherins studied (cadherins and δ-protocadherins) may have different functions in cortical development. In general, expression patterns are more restricted for classic cadherins than for δ-protocadherins. Moreover, the expression of non-classic protocadherins starts generally at an embryonic stage earlier than that of classic cadherins. Differences in the function of classic cadherins and δ-protocadherins are also suggested by differences in the intracellular binding partners. On the one hand, classic cadherins are linked intracellularly to a variety of molecules, including some catenins, which play a role in signal transduction and gene regulation (see Introduction). For example, the binding partner β-catenin is an integral part of the canonical Wnt pathways (Hirano et al., 2003; Nelson and Nusse 2004). On the other hand, known intracellular binding partners of δ-protocadherins include the synaptic molecule protein phosphatase 1α, TAF1/set, Xfz7 and mDab1 (Yoshida et al., 1999; Homayouni et al., 2001; Heggem and Bradley 2003; Medina et al., 2004; Vanhalst et al., 2005; Redies et al., 2005) that are involved in other signaling pathways.

One of the possible roles of cadherins in cortical differentiation may be in target recognition and intracortical circuit formation. In general, pre- and postsynaptic neurons express the same cadherin, in a matching fashion (Redies, 2000; Hirano et al., 2003). For example, in the cerebral cortex of rodents, thalamic afferents and their cortical targets were

reported to express matching cadherins (Suzuki et al., 1997; Gil et al., 2002; Kim et al., 2007). In the chicken optic tectum, which also displays a laminated organization, N-cadherin mediates layer-specific target recognition (Yamagata et al., 1995). It is possible, but remains to be demonstrated experimentally, that the expression of the cadherins investigated in the present study also regulates the formation of layer-specific cortical connectivity. Interestingly, the deep layers of cerebral cortex where the long-distance projection neurons reside, express all the classic cadherins and protocadherins examined in the present study in a region- and layer-specific fashion (Redies und Takeichi, 1993; Korematsu und Redies, 1997; Suzuki et al., 1997; Hirano et al., 1999; Vanhalst et al., 2005; Redies, Nürnberger and Krishna, unpublished data). Whether a match of cadherin expression also exists for the different cortical layers and their respective targets in extracortical areas remains to be examined.

Furthermore, my present results show that the expression of the cadherins persists in the adult visual cortex. A persistence of expression has been shown previously for classic cadherins and δ-protocadherins in the adult mouse forebrain (Hirano et al., 1999; Kim et al., 2007; Hertel et al., 2008). Together, these findings support the notion that cadherins play a role not only in the formation of neural circuitry, but also in its mature function. Several cadherins have been found at the synapse, also during synaptogenesis (Fannon and Colman. 1996; Uchida et al., 1996; Bozdagi et al., 2000; Togashi et al., 2002; Prakash et al., 2005). A role for cadherins in dendritic sprouting and synaptic plasticity has been proposed (Yamagata et al., 1999; Bozdagi et al., 2000; Manabe et al., 2000; Tanaka et al., 2000; Togashi et al., 2002; Tanabe et al., 2006; Yasuda et al., 2007). We are currently producing antibodies against some of the cadherins in order to localize the cadherin proteins at the synapse.

4.3. Region-specific expression of cadherins in primary visual cortex

The mammalian neocortex is organized into distinct divisions referred to as areas and regions that are distinguished from each other by differences in their architecture, axonal connections, and functioning. The distinct areas and regions serve specialized functions, such as sensory processing and motor control. The interplay between extrinsic and intrinsic factors (molecules) is hypothesized to control the specification and differentiation of neocortical areas.

Regional specification of the developing cortex is known to be reflected in the expression of multiple genes, including cell-cell recognition molecules such as cadherins

(Suzuki et al., 1997; Inoue et al., 1998; Nakagawa et al., 1999; Rubenstein et al., 1999), immunoglobulin superfamily members (Pimenta et al., 1996), Ephs/Ephrins (Donoghue and Rakic, 1999; O'Leary and Wilkinson, 1999; Prakash et al., 2000; Vanderhaeghen et al., 2000), Wnt receptors and inhibitors (Kim et al., 2001), transcription factors and some other classes of genes (Nakagawa et al., 1999; Rubenstein et al., 1999).

For example, earlier work by other researchers has shown that the embryonic forebrain expresses, in a regionally restricted manner, several putative regulatory molecules such as Dlx-I, Dlx-2, Gbx-2, Wnt-3, Nkx-2, Nkx-2.1, Nkx-2.2, Emx-I and Emx-2 (Simeone et al., 1992a), Otx-1 and Otx-2 (Simeone et al., 1992b), Dbx (Lu et al., 1992) Hox-7 (Mac-Kenzie et al., 199 I), Pax-3 (Goulding et al., 1991), Pax-6 (Walther and Gruss, 199), Pax- 7 (Jostes et al., 1990), Ott-6 (Suzuki et al., 1990), and Bruin 4 (Mathis et al., 1992). Previous studies have also demonstrated that cadherins are regional markers for cortical areas during early embryonic development of the mouse. Examples are N-cadherin, CDH4, CDH6, CDH8, and CDH11, and several δ-protocadherins (Suzuki et al., 1997; Korematsu et al., 1997; Simonneau and Thiery 1998; Rubenstein et al., 1999; Obst-Pernberg et al., 2001; Bekirov et al., 2002; Kim et al., 2007). In the mouse, mutations in intrinsic gene regulatory factors, which regulate early cortical regionalization, result in corresponding alterations of cadherin expression (Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Bishop et al., 2000). These findings suggest that cadherins are controlled down-stream of early cortical regionalization processes.

In another study with our co-workers (Hertel et al., 2008), we showed that the patch and matrix compartments of the striatum express the cadherins differentially (Cdh4, Cdh7, Cdh8, Cdh11, Pcdh1, Pcdh7, Pcdh8, Pcdh9, Pcdh10, Pcdh11, Pcdh17 and Pcdh19), although a partial overlap is also observed. All these cadherins showed multiple and diverse gradients of expression pattern within the striatum including in the cell aggregates of the ventral basal ganglia. The persistence of the cadherin expression patterns in the adult striatum and ventral pallidum indicates that cadherins play a role in the mature basal ganglia. The ventral pallidum, the islands of Calleja and the olfactory tubercle, which forms the superficial (ventral) derivatives of the ganglionic eminences, also show a complex patchy expression of the cadherins. The similarity in cadherin expression suggests the presence of similar developmental mechanisms in the dorsal and in the ventral parts of the subpallium, underlining the common ontogenetic origin of these subpallial areas. With these facts, we have concluded that the spatially restricted expression patterns of cadherins in the striatum represent a molecular code for the divisions of the basal ganglia and also

possibly for the functional differentiation and connectivity of this part of the mammalian brain.

In the present study, I show that layer-specific differences in cadherin expression demarcate the boundary of the V1 and V2 subregions of the visual cortex. The differences in cadherin expression can be observed at the V1/V2 boundary as early as at developmental stage P2. Cadherin expression thus reflects the functional compartmentation of the cerebral cortex at a relatively high level of cortical regionalization. The V1/V2 boundary is also marked by the expression of other molecules like Cat-301, alkaline phosphatase and cytochrome oxidase (Hockfield et al., 1990; Fonta and Imbert 2002). Thus, our results provide further support for the model that, during late corticogenesis, regional expression of molecular determinants such as cadherin molecules are the result of an assignment of positional identity at earlier stages of development.

Columnar functional architecture in ferret visual cortex is one unique and particularly striking feature as studied earlier (Redies et al., 1990; Chapman et al., 1996; Weliky et al., 1996). Surprisingly, we did not get any evidence for a differential expression of cadherins at the mRNA level, at this highest level of cortical regionalization. But in another study, patchy expression patterns were observed, however, in other cortical areas of the ferret and mouse cortex (Monique Nuernberger, Krishna-K. and Christoph Redies, unpublished data), suggesting that the in situ hybridization method used by us has a resolution high enough to detect such patterns in principle. So, if the columnar expression patterns of cadherin can be detected in ferret visual cortex at the protein level remains to be studied.

It should be noted that several cadherin molecules investigated in this study exhibit abrupt transitions in their expression patterns within the cortical plate between the primary and secondary visual cortex. Histologically, similar abrupt borders are observed. However, many of the gene regulatory proteins found to be expressed in the early cortical plate exhibit gradients rather than abrupt borders, for example *Emx2* and *Pax6* (Rubenstein et al., 1999; Sestan et al., 2001). The graded expression pattern of *Emx2* and *Pax6* might be translated to generate downstream gene expression and respective roles in restricted patterns with abrupt borders (Rubenstein et al., 1999; Sestan et al., 2001). Cadherins with an expression pattern restricted to only one of the visual cortical areas (for example, to primary visual cortex or to secondary visual cortex) have not been identified. Nevertheless, it has been reported that cells derived from different regions of the embryonic forebrain showed differential adhesiveness (Whitesides and LaMantia, 1995). H-2Z1 transgene is the

only genetic marker with an expression pattern restricted to one area in neocortex, marking the granular parts of postnatal mouse S1 (Cohen-Tannoudji et al., 1994). Earlier, it has been speculated that the neocortical areas and the identity of the neurons that comprise it are defined by the expression of a unique subset of genes, each of which is also expressed in other areas (Liu et al., 2000). Since each layer of the visual cortex has a unique profile of cadherin expression, the actual scenario seems to be more complex: most cadherins studied here are expressed in multiple cortical areas but the layer-specific expression profile varies from region to region. Thus, it appears that cadherin expression pattern may provide the neurons with a distinct positional or area identity, though neurons in different layers are generated by the same progenitors (Monuki and Walsh, 2001). This result has significant implications for the genetic regulation of how area identity is encoded in the ventricular zone by the regulatory molecules such as cadherins and imparted by progenitors to their progeny. An understanding of these mechanisms will require the definition of areas and regions at the level of gene expression, and of the relationship between the specification of layer-specific and area-specific properties. A better understanding of the roles of cadherin subtypes in regulating neocortical regionalization will require the identification of the patterning mechanisms that establish their differential expression, of downstream receptors, and of mechanisms by which cadherins autonomously or combine with other (intrinsic and/or extrinsic) factors, control the process of regionalization of the visual cortex.

In conclusion, results from my study suggest that cadherins provide an adhesive code for the specification of primary and secondary visual cortical areas in the ferret. Thus, it is conceivable that region-specific cellular adhesiveness exists and plays a role in the regionalization and boundary formation between cerebral cortical areas. However, the number of regionally expressed genes is still obscure and the molecular machinery involved in neocortical regionalization remains poorly understood.

4.4. Cadherin expression by subsets of visual cortical cells

Although there is a layer-specific expression of the cadherins examined in the present study, a closer look at the expression of the cadherins reveals that not all cadherins are expressed by all neurons in a given cortical layer. In some layers, many of the cadherins studied (CDH4, CDH6, CDH7, CDH8, CDH11, CDH14, CDH20, PCDH1, PCDH8, PCDH9 and PCDH19) are markers of subsets of cells. It is therefore conceivable that some cadherins are involved in the specification of subtypes of neurons and/or glial cells (Hirano et al., 2005; Redies et al., 2005; Takeichi et al., 2007).

A particularly striking example is the expression of CDH7 by a minority of cells scattered throughout the developing and mature cortical plate. A similar expression pattern can be observed for protocadherin-8. It is conceivable that the cells expressing the cadherins represent a particular type of neuron, for example interneurons. The finding that similarly distributed cells are found in other cortical areas supports this suggestion, which requires confirmation by double-labeling studies with markers of interneuron. Although both CDH7 and PCDH8 are expressed by similarly scattered cells, the expression patterns are not identical. It is known that there are distinct interneuron subgroups that can be labeled with different molecular markers, such as parvalbumin, somatostatin, and calretinin, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), somatostatin (SS), and cholecystokinin (CCK) (Kubota et al., 1994; Gonchar and Burkhalter, 1997). These interneuron subgroups have distinct physiological characteristics (Kawaguchi and Kubota, 1996; Cauli et al., 1997). The number of cells expressing CDH7 and PCDH8 is higher in both layer II and V of the cortex. A similar distribution has been observed for Satb2-positive cortical cells. Satb2 is expressed in a subset of interneurons in layers II through V during differentiation and is a regulatory determinant of corticocortical connections in the developing cerebral cortex (Britanova et al., 2006). Other molecular markers of cortical interneurons are Dlx, Lhx6, and Arx (Anderson et al., 1997; Lavdas et al., 1999).

Neuron-type specific cadherin expression has been previously described in the chicken cerebellar cortex, where expression of cadherin-7 and cadherin-10 is restricted to subsets of Purkinje cells and granule cells (Fushimi and Arndt, 1997), and expression of R-cadherin to Purkinje cells and interneurons (Arndt et al., 1998). In the mouse cerebellar cortex, cadherin-8 expression is first restricted to Purkinje cell clusters and later also observed in most cells of the internal granular layer (Korematsu and Redies, 1997). In the retina, R-cadherin is expressed by horizontal cells, whereas N-cadherin and cadherin-11 are expressed by Müller cells (Honjo et al., 2000). Wöhrn et al. (1998) demonstrated that a particular type of ganglion cells in the chicken retina expresses CDH6. In the visual system of the chicken, the differential expression of cadherins by specific types of neurons has been extended to the synaptic level, suggesting a role for cadherins in the development of neuronal circuitry (Heyers et al., 2004).

Of particular interest is the expression of CDH4, CDH6, CDH7, CDH8, CDH11, CDH14, CDH20, PCDH1, PCDH8, PCDH9 and PCDH19 in subsets of neurons in layer V. This layer contains a mixture of pyramidal neurons, which project to various subcortical targets. In mouse, Layer V pyramidal neurons are categorized into two major

classes based on their projection site, morphology and physiological properties (Hallman et al., 1988; Klein et al., 1986; Larkman and Mason, 1990). Type I cells project to the superior colliculus, spinal cord or basal pons and are characterized by thick tufted apical dendrites, and a burst-like firing pattern (Kasper et al., 1994). Type II neurons are projecting their axons to the contralateral hemisphere or to the ipsilateral striatum, their slender with fewer oblique branches apical dendrite end without terminal tufts in the upper part of layer II/III, and never fire bursts (Kasper et al., 1994). Other known layer V pyramidal cell markers are aspartate and glutamate, Otx-1, Er81, Tbr1, SCIP, Ctip2, Fez1, Lmo4, calretinin, Clim1, Crim1 and S100a10 (Molnar and Cheung, 2006). Co-localization studies by various groups suggest that several other subclasses of layer V neurons exist in cerebral cortex. For example, co-localization studies on OTX-1 and ER81 indicate that these two markers are not expressed within the same postnatal layer V neurons (Hevner et al., 2003). Retrograde labeling and immunohistochemistry using ER81 and N200 or SMI-32 show that they are expressed in type I layer V neurons but they never co-express in the same projection neurons (Rolph et al., 2005). This suggests that there are at least two distinct neurochemical subpopulations within type I pyramidal cells in layer V. Similarly, results showed that the absence of CTIP2, a pyramidal cell marker in layer V, leading to defects in the organization and fasciculation of subcerebral fiber tracts, including axonal projections. Also, the *Ctip2*$^{-/-}$ mice exhibit striking abnormalities in their axonal projections to subcerebral targets and to the spinal cord (Arlotta et al., 2005). In the chicken tectum, subpopulations of projection neurons and their axons express cadherins differentially (Wöhrn et al., 1999). Whether similar results can be obtained for layer V pyramidal neurons remains to be investigated. Future studies employing antibodies will show whether cadherin protein expression can be associated with specific subcortical fiber projection systems that originate in the ferret visual cortex.

Another possible example for neuron type-specific cadherin expression can be found in the preplate. Early in the development, many of the cadherin molecules, for example, CDH4, CDH11, CDH14, PCDH7, PCDH9 and PCDH10, are expressed by subsets of cells in the preplate, continue to be expressed by cells in the marginal zone (prospective layer I) after the splitting of the preplate. Cajal-Retzius (CR) cells are one of the most prominent cell types found in the marginal zone. However, many other cells are present in the preplate besides CR cells. Whether cadherins are markers for some of these cell populations remains to be established.

Earlier studies have reported that cadherins can be used to map functional connections across brain regions (Redies et al., 1993; Arndt and Redies, 1996; Suzuki et al., 1997). Another possibility is that cadherin subtypes specifically expressed by subsets of neurons in different cortical layers might mediate the formation of intracortical microcircuitry, as was postulated for α-protocadherins (Kohmura et al., 1998). The differential adhesiveness conferred to cortical neurons by cadherin-expression may similarly serve to establish selective neuronal connections with targets outside the cortex or within cortical circuitry (Redies et al., 1993; Arndt and Redies, 1996; Suzuki et al., 1997).

A similar observation was made in the cerebellum in one of the earlier studies with our co-worker (Neudert et al., 2008). In this study, we showed that the cadherins are expressed differentially in parasagittal Purkinje cell domains of the cerebellar cortex, in portions of the deep cerebellar nuclei, in the divisions of the inferior olivary nucleus, and in the lateral vestibular nucleus, by comparing the connectivity patterns in the cerebellar system of two mammals, mouse and ferret through mapping the expression of three cadherins (cdh8, pcdh7, and pcdh10) at comparable postnatal stages. The observed expression profiles indicate that the cadherin-positive structures are interconnected within the cerebellar system. Our results confirm that cadherin expression reflects functional cerebellar connectivity. The expression patterns are nearly similar in both the species. The general resemblance may possibly be due to the common set of embryonic divisions in the cerebellar organization of both the species. We have concluded that the cerebellar systems of rodents and carnivores display a relatively large degree of similarity in their molecular and functional organization.

4.5. Outlook

In conclusion, the layer-, region- and cell type-specific expression patterns of cadherins in the visual cortex suggest that cadherins possibly provide an adhesive code not only for the laminar architecture but also for the functional differentiation and connectivity of this brain region. The latter suggestion requires confirmation by neuroanatomical tracing studies. Also, the precise functional role of cadherins in contributing to the above-mentioned processes remains to be studied by genetic or other experimental approaches. Signal transduction through cadherin-based adhesive cues may be equally important mechanisms, even though they have not been studied extensively for cadherins in the CNS. As noted in the previous studies, cadherins have the potential of interacting with many other molecules and systems; it is largely unclear how these interactions with other systems influence

cortical development. Therefore, in order to discuss the roles of cadherins in cortical development, we need to keep in mind both known functions of these molecules and other, still obscure, functions.

4.6. Spatio-temporal expression of cadherin by blood vessels during angiogenesis

Six cadherins (CDH4, CDH6, CDH7, CDH11, PCDH1 and PCDH17) and PP1α are expressed by blood vessels from the earliest stage studied (E23) until about P33. In higher vertebrates, brain angiogenesis starts at the beginning of neurogenesis and proceeds up to the last wave of neuronal migration, when the basic scheme of vascular network is completed (Plate, 1999). The vasculature undergoes considerable expansion in the premature brain to support the growth of the rapidly developing cortex until it reaches a constant density, which is maintained throughout adulthood. My study confirms the observation that vascularization of brain takes place by means of an angiogenic mechanism, which starts at the beginning of corticogenesis. As the brain grows in thickness, unbranched vessels, which migrate radially into the nervous wall, express several cadherins. During this migration, cadherins may possibly contribute to the adhesion between endothelial cells or between endothelial cells and surrounding pericytes, as shown previously for VE-cadherin and N-cadherin, respectively (Breier et al., 1996; Cavallaro et al., 2006). Cadherins may also contribute to endothelial cell migration by mediating cell-extracellular-matrix interactions and enabling them to migrate from the primary vascular plexus towards angiogenic stimuli and to proliferate (Klagsbrun and Moses, 1999).

Cadherin expression reaches highest levels at the perinatal stage, particularly at around P2-P13. This developmental window is similar to the time period, during which capillaries invade the rat brain (at E11) and endothelial cell proliferation is maximal (at P6-8). At about the same time of development, expression of Flk-1, Flt-1, PE-CAM-1 and VEGF peaks in vessels of the premature brain (Ogunshola et al., 2000; Yang et al., 2003). The peak of cadherin expression during this critical developmental period suggests that cadherins are proangiogenic factors that regulate the growth, migration and/or controlled pruning of the newly forming vascular network (Plate, 1999). Because cadherins are not expressed by astrocytes (Krishna-K. and Christoph Redies, unpublished data), they are unlikely to play a role in the interaction between endothelial cells and astrocytes (Zerlin and Goldman, 1997). The identification of PP1α in developing blood vessels leads to the question of which molecules are regulated down-stream by this phosphatase.

Strikingly, the expression of CDH4, CDH6 and PCDH1 are restricted to particular areas of developing brain, in particular to cerebral cortex. Another cadherin that shows a regionally restricted expression pattern is cadherin-10; it is found at the surface of the mouse cortex but not of the cerebellum, revealing the possibility of differential organization of blood vessels (Williams et al., 2005). Interestingly, some cadherins (CDH7 and CDH11) are expressed by specific blood vessels although the neighboring ones do not show expression. Several studies have shown that arteries and veins are likely to differ in their expression of molecular markers. For example, ephrinB2 expression is confined to the arterial endothelium whereas EphB4 expression is higher in veins (Wang et al., 1998; Palmer and Klein, 2003). Whether the cadherins are expressed also by a specific blood vessel type or during a specific stage of angiogenesis remains to be investigated.

4.7. A common cadherin-based mechanism behind angiogenesis and neurogenesis?

All the six cadherins expressed by blood vessels are known also for their spatiotemporally regulated expression by neurons and for their roles in brain regionalization, cell-specific expression and guided migration (Kim et al., 2007; Hertel et al., 2008). Cadherin molecules were also reported in various studies for their role in axonal guidance, axon outgrowth, neuronal migration, cell sorting, pattern and boundary formation (Redies et al., 2005). Other genes, like ephrins, netrins, slits, and semaphorins, are also concomitantly expressed and reported to play dual roles in both neural and vascular development (Carmeliet, 2003). This similarity led to the suggestion that there might be an intrinsic and/or operative relationship between neurogenesis and angiogenesis (Carmeliet, 2003; Vasudevan et al., 2008). For example, telencephalic angiogenesis has been shown to be under significant intrinsic regulation by homeobox transcription factors Dlx1/2, Nkx2.1 and Pax6, which also regulate neurogenesis and neuronal migration (Vasudevan et al., 2008). The regional-specific and subtype-specific expression of cadherins (CDH6, CDH7, CDH11 and PCDH1) in the brain blood vessels likewise suggests similarities between neurogenesis and vasculogenesis. In both processes, adhesive guidance cues may coordinate the complex spatiotemporal interaction of cells to create a precisely wired and stereotypic pattern of cellular complexity. In conclusion, the identification of a novel panel of cadherin markers for developing brain blood vessels may provide a basis for the design of functional assays to study the role of these genes in neuroangiogenesis, also in brain tumors.

5. GENERAL CONCLUSION AND OUTLOOK

I speculate that the cadherin superfamily genes identified in the present study play roles in the final determination of specific neuron types and sorting these cells into specific areas and/or layers in the cortical plate. This hypothesis must be tested by loss of function and/or gain of function analyses *in vitro* and *in vivo*, for example in transgenic mouse models, in order to identify novel genetic mechanisms involved in neocortical patterning and neuron specification. Specifically, future studies may be designed to elucidate the relationships between cadherin expression and different classes of projection neurons and interneurons and to define the time points, at which individual lineages branch from each other during development.

The present set of cadherins adds to the goal of obtaining a comprehensive panel of markers that allows analysis of the majority of neuron types in the mammalian neocortex. It is hoped that such a panel of markers will reveal important details of cortical organization and neuronal composition that influence not only interpretations of pathogenesis but also diagnostic classification. Cadherin molecular markers of neuron identity may be valuable for identifying the neuron types affected in neurodegeneration, hypoxia/ischemia, and other causes of neuron loss, and for evaluating the efficacy of neural tissue regeneration using exogenous stem cell or endogenous progenitors. Eventually, layer-, region- and cell type-specific markers may become standard immunohistochemical probes in the neuropathologic diagnosis of various brain diseases. Such a panel of markers will help to detect abnormalities in the position and number of each cortical neuron type and can be applied to mouse mutants, which show abnormalities in cortical development, as well as to related genetic diseases of the human brain. The markers will not only help in the dissection of functional cortical circuits, but will eventually lead to the means of restoring normal function. The recent recognition that angiogenesis and neurogenesis have more mechanisms and molecules in common than previously anticipated may help to unravel the molecular mechanisms, by which these mechanisms regulate both processes and lead to new insight in neurovascular medicine.

6. SUMMARY

Introduction: Cadherins are a superfamily of Ca^{2+}-dependent transmembrane glycoproteins with many subfamilies and more than 100 known members. They are multifunctional homotypic adhesion molecules playing a crucial role in the development of both the nervous system and the vascular system of vertebrates. Cadherins mediate a wide variety of developmental mechanisms, including cell division, cell proliferation, cell migration, cell differentiation, cell-cell recognition, neurite outgrowth, neural circuit formation and synaptogenesis. The formation of cortical regions and layers is a highly orchestrated and complex developmental process that involves many of the processes attributed to cadherins.

Cadherin genes are also important regulators of vascular development, mediating various angiogenic mechanisms, including the formation of the primitive capillary plexus, endothelial integrity, vascular permeability, the blood-brain barrier and pericyte stabilization. In the brain, concomitant vascular development meets the increasing metabolic needs arising during the corticogenesis. Recently, a common role for molecules such as cadherins has been predicted for the development of the nervous system and the vascular system ("neuroangiogenesis"). However, the exact role and the precise expression patterns of cadherin molecules are still unknown during these processes. Thus, in this work, I studied the expression profile of 18 cadherins during ferret brain development, focusing on the visual cortex, a model system for corticogenesis.

Moreover, in a collaborative study with other members of the laboratory, we (1) mapped the expression of three cadherins in the mouse and ferret cerebellar system to compare their connectivity patterns, and (2) compared the expression profile of 12 different cadherins with the functional architecture of the mouse basal ganglia.

Materials and methods: The main part of my study was carried out in 36 ferrets at 10 different developmental stages, from early embryonic stages to the adult stage. Total RNA was isolated from brains and first-strand complementary DNA (cDNA) was synthesized. Novel classic cadherins and all known δ-protocadherins were amplified by PCR using both specific and degenerate primers. In total, I cloned 18 novel cadherins and an intracellular binding partner of δ-protocadherins, protein phosphatase 1α (PP1α). The expression patterns of these molecules were investigated by *in situ* hybridization in the developing primary visual cortex (V1) and other brain regions of the ferret. Tyramide double-fluorescent in-situ hybridization (FISH) and FISH-coupled immunohistochemistry were

also performed to study the V1/V2 boundary and to confirm cadherin expression by blood vessels.

Results and conclusion: Fifteen cadherins (CDH4, CDH6, CDH7, CDH8, CDH11, CDH14, CDH20, PCDH1, PCDH7, PCDH8, PCDH9, PCDH10, PCDH11, PCDH17 and PCDH19*)* were found to be expressed in a spatiotemporally restricted fashion in the visual cortex throughout development. I showed that all these cadherin molecules exhibit a lamina-specific expression pattern in the developing primary visual cortex (V1). Generally, the expression of protocadherins sets in at early embryonic stages and is more widespread than the expression of classic cadherins, which begins in late gestation or following birth and is more restricted to specific layers. There is a combinatorial expression of subsets of cadherins in each layer at any given developmental stage. Some cadherins are expressed by subsets of neurons dispersed throughout all cortical layers. Changes in layer-specific cadherin expression at the V1/V2 boundary are observed, resulting in a molecular demarcation of this boundary. My results suggest that cadherins represent a large set of layer- and region-specific markers from a single gene family and provide a potentially adhesive code for the formation of layers in the primary visual cortex. Because expression persists in the adult, cadherins may also play a functional role in the mature cortex.

I also identified seven members of the cadherin superfamily (CDH4, CDH5, CDH6, CDH7, CDH11, PCDH1 and PCDH17) and PP1α as novel markers for developing blood vessels in the ferret brain. Some of the cadherin molecules are restricted to specific brain regions or a subset of blood vessels, for example in the cortical plate. The expression levels show a peak during perinatal vascular development. My results suggest that multiple cadherins, which are also involved in neurogenesis, are regulators of angiogenesis in developing vertebrate brain, supporting the idea of a common mechanism existing behind neurogenesis and angiogenesis ("neuroangiogenesis").

In collaborative work, we used three cadherins as markers for functional regions and neural circuits in the developing cerebellar system of mouse and ferret. Results showed that the embryonic divisions and derived functional organization is phylogenetically highly conserved in the cerebellar system of rodents and carnivores. Moreover, in another study of the developing and adult mouse brain, we demonstrate that cadherins provide a molecular code for the divisions of the basal ganglia and also for the functional differentiation and connectivity of this part of the mammalian brain.

7. ZUSAMMENFASSUNG

Einleitung: Cadherine bilden eine Superfamilie von Ca^{2+}-abhängigen, transmembranen Glycoproteinen, die in mehrere Unterfamilien unterteilt werden können und über 100 Mitglieder besitzen. Sie sind multifunktionelle homophile Adhäsionsmoleküle, die eine entscheidende Rolle in der Entwicklung des Nervensystems und des vaskulären Systems bei Vertebraten spielen. Cadherine vermitteln eine Vielzahl von Entwicklungsmechanismen, einschließlich Zellteilung, Zellproliferation, Zellmigration, Zelldifferenzierung, Zell-Zell-Erkennung, Neuritenwachstum, Bildung neuronaler Schaltkreise und Synaptogenese. Die Bildung von corticalen Regionen und Schichten ist ein höchst komplexer Entwicklungsprozess, der viele der Abläufe einschließt, die den Cadherinen zugeschrieben werden.

Die Cadherin-Gene sind wichtige Regulatoren auch der vaskulären Entwicklung. Sie vermitteln vielfältige angiogene Mechanismen, einschließlich der Ausbildung des primitiven kapillaren Plexus, der endothelialen Integrität, der vaskulären Permeabilität, der Blut-Hirn-Schranke und der Stabilisierung der Kapillaren durch Perizyten. Im Gehirn sichert die vaskuläre Entwicklung die erhöhten metabolischen Anforderungen, die während der Corticogenese entstehen, ab. Für die Entwicklung des Nervensystems und des vaskulären Systems wird seit kurzem angenommen, dass die gleichen Moleküle, wie z.B. die Cadherine, daran beteiligt sind („Neuroangiogenese“). Allerdings sind die genaue Rolle und die konkreten Expressionsmuster der Cadherin-Moleküle während dieses Prozesses bisher unbekannt. Deshalb untersuchte ich in dieser Arbeit die Expressionsprofile von 18 Cadherinen während der Hirnentwicklung des Frettchens. Mein Fokus lag dabei auf dem visuellen Cortex, einem Modelsystem der Corticogenese.

In Zusammenarbeit mit anderen Mitgliedern der Arbeitsgruppe, (1) kartierten wir die Expression von drei Cadherinen im Cerebellum der Maus und des Frettchens für einen Vergleich der Konnektivitätsmuster und (2) verglichen wir die Expressionsprofile 12 verschiedener Cadherine mit der funktionellen Architektur der murinen Basalganglien.

Material und Methoden: Der Hauptteil meiner Arbeit wurde an 36 Frettchen in 10 verschiedenen Entwicklungsstadien durchgeführt, beginnend bei frühen embryonalen Stadien bis hin zu adulten Stadien. Aus Gehirnen wurde die komplette RNA isoliert und einzelsträngige komplementäre DNA (cDNA) synthetisiert. Neue klassische Cadherine und alle bisher bekannten δ-Protocadherine wurden mit Hilfe der PCR unter Nutzung von

spezifischen und degenerierten Primern amplifiziert. Insgesamt klonierte ich 18 neue Cadherine und Proteinphosphatase 1α (PP1α), einen intrazellulären Bindungspartner der δ-Protocadherine. Die Expressionsmuster dieser Moleküle wurden mittels *In-situ*-Hybridisierung im sich entwickelnden primären visuellen Cortex (V1) und anderen Hirnregionen im Frettchen untersucht. Um die V1/V2- Grenze zu untersuchen, sowie die Expression von Cadherinen in Blutgefäßen zu bestätigen, wurde Tyramid-gekoppelte Fluoreszenz-*in-situ*-Hybridisierung (FISH) und FISH-gekoppelte Immunhistochemie durchgeführt.

Ergebnisse und Schlussfolgerung: Es konnten fünfzehn Cadherine (CDH4, CDH6, CDH7, CDH8, CDH11, CDH14, CDH20, PCDH1, PCDH7, PCDH8, PCDh9, PCDH10, PCDH11, PCDH17 und PCDH19) ermittelt werden, die räumlich und zeitlich begrenzt im visuellen Cortex während der Entwicklung exprimiert werden. Ich konnte zeigen, dass alle diese Cadherin-Moleküle ein Lamina-spezifisches Expressionsmuster im sich entwickelnden primären visuellen Cortex (V1) aufweisen. Generell kann man sagen, dass die Expression der Protocadherine in frühen embryonalen Phasen beginnt und sich auf mehr Schichten verteilt als die Expression der klassischen Cadherine. Diese ist erst in späten Phasen der Schwangerschaft oder nach der Geburt ausgeprägt und auf weniger Schichten begrenzt. In jedem gegebenen Entwicklungsstadium wird eine kombinatorische Expression von Cadherinen in jeder corticalen Schicht beobachtet. Einige Cadherine werden nur von bestimmten Neuronen, die über alle corticale Schichten verteilt sind, exprimiert. Veränderungen in der Schicht-spezifischen Cadherin-Expression konnten an der V1/V2-Grenze beobachtet werden und führten zu einer molekularen Abgrenzung dieser Grenze. Meine Ergebnisse deuten darauf hin, dass Cadherine eine große Gruppe von Schicht- und Region-spezifischen Markern einer einzigen Genfamilie darstellen und einen potentiellen adhäsiven Code für die Bildung von Schichten im primären visuellen Cortex liefern. Da die Expression auch im adulten Stadium bestehen bleibt, spielen Cadherine möglicherweise auch eine funktionelle Rolle im ausgereiften Cortex.

Ich identifizierte sowohl sieben Mitglieder der Cadherin-Superfamilie (CDH4, CDH5, CDH6, CDH7, CDH11, PCDH1 und PCDH17) als auch PP1α als neue Marker für sich entwickelnde Blutgefäße im Frettchenhirn. Einige der Cadherin-Moleküle sind auf spezifische Hirnregionen oder ausgewählte Blutgefäße begrenzt, zum Beispiel in der corticalen Platte. Die Expression zeigt hier einen Höhepunkt während der perinatalen Gefäßentwicklung. Meine Ergebnisse weisen darauf hin, dass Cadherine, die ebenfalls an

der Neurogenese beteiligt sind, Regulatoren der Angiogenese im sich entwickelnden Vertebratenhirn sind, und unterstützen die Hypothese, dass es gemeinsame Mechanismen für Neurogenese und Angiogenese gibt („Neuroangiogenese“).

In einer Kooperationsstudie nutzten wir drei Cadherine als Marker für funktionelle Regionen und neuronale Schaltkreise im sich entwickelnden Cerebellum der Maus und des Frettchens. Die Ergebnisse zeigen, dass die embryonale Einteilung und die erzielte funktionelle Organisation des Cerebellums in Nagern und Karnivoren phylogenetisch hoch konserviert sind. Darüber hinaus konnte in einer weiteren Untersuchung im jungen postnatalen und adulten Mausgehirn gezeigt werden, dass Cadherine einen molekularen Code für die Einteilung der Basalganglien, sowie für die funktionelle Differenzierung und Konnektivität dieses Teils des Säugerhirns bilden.

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Statement

I am familiar with the Promotionsordnung of the Faculty of Biology and Pharmacy of the University of Jena. All parts of the dissertation were produced by myself. I hereby declare that this thesis does not contain any material previously submitted for a degree or diploma at another university or any material previously written or published by any other person, except where due acknowledgment or reference is made in the text. I also declare that I did not obtain the assistance of a dissertation counselling agent and that I did not provide any direct or indirect financial remuneration to any third party in connection with the content of my dissertation.

Jena, 25 November 2008 …….

Krishna-K