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CADHERIN EXPRESSION IN THE SOMATOSENSORY CORTEX: EVIDENCE FOR A COMBINATORIAL MOLECULAR CODE AT THE SINGLE-CELL LEVEL

K. KRISHNA-K, N. HERTEL AND C. REDIES*

Institute of Anatomy I, University of Jena School of Medicine and Jena University Hospital, Teichgraben 7, D-07743 Jena, Germany

Abstract—Cadherin superfamily genes play a role in a wide variety of developmental processes and mature functions of the vertebrate brain. In the present study, we mapped *in situ* the expression pattern of five classic cadherins (Cdh4, Cdh6, Cdh7, Cdh8, Cdh11) and eight δ-protocadherins (Pcdh1, Pcdh7, Pcdh8, Pcdh9, Pcdh10, Pcdh11, Pcdh17 and Pcdh19) in the primary somatosensory cortex of the adult mouse. All of these cadherins show layer-specific expression profiles in primary somatosensory cortex. Some cadherins (for example, Cdh4, Cdh7, Pcdh8) mark subsets of cells within a given lamina, while other cadherins (Cdh11 and Pcdh10) are expressed more widely in multiple layers. Results from tyramide-based double-fluorescence *in situ* hybridization (FISH) provide evidence that most single neurons express more than one cadherin in a combinatorial fashion in all layers of cerebral cortex. This combinatorial code is rather comprehensive because pairwise expression of cadherins can assume any type of combination (complementarity, partial or complete overlap, subset-specific expression, cell-size specific expression, etc.). We propose that the combinatorial expression of multiple cadherin genes contributes to the molecular specification of the vast complexity of neurons in cerebral cortex.

Key words: cerebral cortex, cortical layers, neural specification, cell adhesion, δ-protocadherins.

Like other areas of the cerebral cortex, the primary somatosensory cortex (S1) of mammals displays a layered cytoarchitecture. Sensory information processing in S1 depends on the exact positioning and connectivity of different classes of neurons within the six neocortical layers, which develop in an inside-out fashion (Angevine and Sidman, 1961; Rakic, 1974; Caviness et al., 1995; Rakic and Caviness, 1995; Rubenstein and Rakic, 1999). The cortical layers can be distinguished by their histology, cellular composition, connectivity, gene expression, and time period of development. They typically segregate neurons that share characteristic dendritic morphologies, physiological properties, and axonal connections (McConnell, 1988, 1991). Within a neocortical area, the axonal and dendritic processes of individual neurons or subpopulations of neurons arborize in precise lamina-specific patterns (Freund and Gulyás, 1991; Callaway, 1998a,b; Borrell and Callaway, 2002; Schubert et al., 2007). Layer-specific axonal arbors are believed to be an important factor contributing to the specificity of cortical connections (Gilbert, 1983; Burkhalter, 1989; Callaway, 1998a,b; Price et al., 2006). In primary somatosensory cortex, laminar specificity is regulated by a combination of neuronal activity-dependent cues, which are provided by innervating axons of the thalamus, and various intrinsic molecular cues, including transcription factors, secreted gene regulatory proteins, and cell adhesion molecules (Rakic, 1988; O'Leary et al., 1994; Monuki and Walsh, 2001; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002; Funatsu et al., 2004; Garel and Rubenstein, 2004).

Although relatively little is known about the molecular determinants responsible for the laminar and cell-type specificity in the cortex, the differences in neuronal composition, gene expression, developmental timing and connectivity across various cortical layers of S1 strongly suggest the existence of a vast number of genes with layer-specific and neuronal subtype-specific patterns of expression. A number of studies have identified numerous potential candidate genes for regulating layer-specific circuit formation in the cortex of mouse, such as ER81, 5HT2C, Nurr1, CTGF, OTX1, CUTL2, CALB1, LAMB1, NR2E1, NR2F2, VIP, CNR1, LIX1, Cux-1, Cux-2, Emx1, Npy, Wnt7b, Bcl-6, MT4-MMP, Nrf3, Reelin, Sidekicks, EphrinA5 and cadherins (Rakic, 1988; Bulfone et al., 1993; Chan et al., 2001; Bishop et al., 2002; Tissir and Goffinet, 2003; Arlotta et al., 2005; Molnár and Cheung, 2006; Rash and Grove, 2006; Arion et al., 2007; Molyneaux et al., 2007; Takeuchi et al., 2007; Watakabe et al., 2007; Hevner et al., 2003; Hevner, 2007; Krishna-K et al., 2009). Some of these molecules, in particular cadherins, are expressed predominantly in a region- and layer-specific fashion in the developing and adult cerebral cortex (e.g. N-cadherin, Redies and Takeichi, 1993; R-cadherin, Stoykova et al., 1997; Cdh6, Cdh8, Cdh11, Suzuki et al., 1997; Pcdh1 and Pcdh8, Krishna-K et al., 2009) and were used as markers for cortical regions in genetically manipulated mice (Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Bishop et al., 2000).

Cadherins are a large superfamily of Ca^{2+}-dependent cell adhesion glycoproteins, with more than 100 members in vertebrates. They mediate homophilic cell-cell adhesion

*Corresponding author. Tel: +49-3641-93-8511; fax: +49-3641-93-8512.
E-mail address: redies@mti.uni-jena.de (C. Redies).

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Cdh, cadherin; cDNA, complementary deoxyribonucleic acid; cRNA, complementary ribonucleic acid; DIG, digoxigenin; FISH, fluorescent *in situ* hybridization; HRP, horse radish peroxidise; NBT, nitroblue tetrazolium; NeuN, neuronal nuclei; Pcdh, protocadherin; PCR, polymerase chain reaction; S1, primary somatosensory cortex.

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by interacting with each other or with other molecules at cell-cell interfaces through their hallmark cadherin repeats. Cadherins play a role also in signal transduction by generating different intracellular signals through distinct cytoplasmic domains (for reviews, see Frank and Kemler, 2002; Hirano et al., 2003; Takeichi, 2007). The specific nature of homotypic cadherin-based adhesion and the strikingly restricted expression patterns of cadherins in the CNS are indicative of an adhesive code that mediates the selective association of neuronal structures, providing an adhesive framework for neuronal connectivity and synaptic specificity (Redies et al., 1993; Redies, 2000; Takeichi, 2007). Recent expression analyses and functional studies have led to the idea that the function of cadherins extends well beyond their developmental roles to encompass the dynamic regulation of synaptic strength and plasticity in the adult cortex (Takeichi, 2007). Consequently, the large diversity and complex molecular structure of cadherins may possibly explain the emergence and maintenance of the intricate and highly specific network of connections between billions of cortical neurons.

There are two basic possibilities of how complexity can be accomplished by gene expression. First, complexity may be subserved by a huge number of molecules, with each cell expressing one of them. Second, a restricted number of molecular players may set up a "combinatorial code" that involves co-expression of genes at the single-cell level. On the regional level, many members of the cadherin gene family are expressed in a combinatorial fashion in different areas of the brain (Arndt et al., 1998; Redies, 2000), including the cerebral cortex (Suzuki et al., 1997; Kim et al., 2007; Krishna-K et al., 2009). However, at the cellular level, co-expression of cadherins has not been studied in cerebral cortex. In the present study, we therefore undertook an extensive screening approach of 13 classic cadherins and δ-protocadherins to analyze their expression profiles in single cells of the mouse somatosensory cortex by tyramide-based fluorescent double-label *in situ* hybridization, using highly sensitive tyramide signal amplification (Krishna-K et al., 2009; Krishna-K and Redies, 2009).

EXPERIMENTAL PROCEDURES

Animals and preparation of tissues

All experiments were carried out in accordance with German guidelines for the care of animals in research. The number of animals used and the amount of suffering of the animals were minimized. Brains of 6 adult wild-type mice were cut into parasagittal and transverse sections of 20 μm thickness in a cryostat (HM 560 Cryo-Star, Microm International, Walldorf, Germany) and thawed directly onto SuperFrost Plus slide glasses (Menzel, Braunschweig, Germany). The sections were dried at 50–56 °C.

PCR, cloning and probes

To generate complementary DNA (cDNA) plasmids for cadherin probes, total RNA from adult mouse brain was isolated using the RNeasy protect mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, as described earlier (Krishna-K et al., 2009; Krishna-K and Redies, 2009). First-strand cDNA was synthesized from the total RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, CA, USA) according to the manufacturer's protocol. The degenerate primers and polymerase chain reaction (PCR) conditions for amplifying cDNA fragments of cadherin-7 (Cdh7), protocadherin-8 (Pcdh8), Pcdh17 and Pcdh19 were described elsewhere (Krishna-K et al., 2009). The obtained 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. The following cDNA fragments were obtained: Cdh7 (1.8 kb fragment containing nucleotide positions 182–1,998 of GenBank sequence Acc. No. AK137369), Pcdh8 (1.7 kb), Pcdh17 (2.0 kb) and Pcdh19 (2.8 kb) (sequences to be submitted).

In addition to the above cDNA fragments, the following plasmids obtained from other sources were used to generate cadherin cRNA probes for *in situ* hybridization: Full-length mouse Cdh4 (R-cadherin) cDNA (positions 55–2,794 of sequence Gen-Bank Acc. No. D14888) in plasmid pBSMR4 (gift of Dr. M. Takeichi, RIKEN Institute, Kobe, Japan); a 1.6 kb fragment of mouse Cdh8 cDNA (positions 504–1,583 of sequence GenBank Acc. No. X95600) in plasmid cad8-12 (Korematsu and Redies, 1997); full-length mouse Cdh11 cDNA (positions 452–2,840 of sequence GenBank Acc. No. D31963) in plasmid BSSK11 (gift of Dr. M. Takeichi, RIKEN, Kobe, Japan); a 1.6 kb fragment of Pcdh1 cDNA (positions 1,195–2,781 of sequence GenBank Acc. No. NM029357) in plasmid pGEMte-mPcdh1-ISH (Redies et al., 2008); a 1.6 kb fragment of Pcdh7 cDNA (positions 1,947–3,575 of sequence GenBank Acc. No. NM018764) in plasmid pGEMte-mPcdh7 (Vanhalst et al., 2005); a 1.2 kb fragment of Pcdh9 cDNA (positions 961–2,258 of sequence GenBank acc. No. NM001081377) in plasmid pGEMte-mPcdh9 (Vanhalst et al., 2005); a 1.2 kb fragment of Pcdh11 cDNA (positions 1,581–2,867 of sequence GenBank Acc. No. NM001081385) in plasmid pGEMte-mPcdh11 (Vanhalst et al., 2005); and full-length mouse Pcdh10 (OL-protocadherin) cDNA (GenBank Acc. No. NM001081377) in plasmid mOLe11 (kind gift of S. Hirano, RIKEN Institute, Kobe, Japan; Hirano et al., 1999).

In vitro transcription

To generate cRNA probes, we used the digoxigenin (DIG) RNA Labeling Kit or the Fluorescein-RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions (Krishna-K et al., 2009). Plasmids were transcribed with T7 or SP6 RNA polymerase (New England Biolabs, Ipswich, MA, USA) followed by labeling with digoxigenin or fluorescein to generate sense and antisense probes, as described earlier (Krishna-K et al., 2009; Krishna-K and Redies, 2009). Probes were purified by LiCl/EtOH precipitation or by using Quick Spin Columns (Roche Diagnostics).

In situ hybridization

In situ hybridization was performed as described earlier (Krishna-K et al., 2009; Krishna-K and Redies, 2009). Briefly, 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 EDTA, 3× 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 (NBT) and 0.02% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) 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). Contrast and brightness were