

Temporally and Spatially Regulated Expression of a Candidate G-Protein-Coupled Receptor during Cerebral Cortical Development

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ABSTRACT: Genes expressed in layer-specific patterns in the mammalian cerebral cortex may play a role in specifying the identity of different cortical layers. Using PCR-differential display, we identified a cDNA that encodes rCNL3, a gene cloned previously by sequence homology to G-protein-coupled receptors. rCNL3 is expressed predominantly in layers 2–4 of the young rat cortex and in the developing and adult striatum. Cortical expression of rCNL3 begins postnatally at P3 and continues at high levels until around P15, while striatal expression begins at E20 and continues through

adulthood. rCNL3 expression is not detectable in the ventricular zone precursors that generate the neurons of layers 2–4. The expression pattern of rCNL3 in the developing cortex suggests that rCNL3 is not involved in the initial specification of laminar fate, but rather may be involved with later differentiation events within the superficial cortical layers. © 2001 John Wiley & Sons, Inc. *J Neurobiol* 46: 167–177, 2001

Keywords: cerebral cortex; layers; G-protein-coupled receptor; PCR-differential display; cell fate

INTRODUCTION

The mammalian cerebral cortex is organized into six layers, each of which is distinguished by the density and morphology of its resident neurons, as well as by the physiological properties and axonal connections of those cells (Brodmann, 1909; Gilbert and Kelly, 1975; Gilbert, 1977). Cortical neurons are produced from progenitors in the telencephalic ventricular zone; young neurons exit the cell cycle and migrate to their final positions in the cortical plate, where they establish axonal and dendritic connections. [³H]-thymidine birth-dating studies have shown that the layers form in an inside-first, outside-last gradient: early-generated

neurons populate the deepest layers of the cortex, while later-generated neurons migrate past the early-born cells to form the superficial layers (reviewed in McConnell, 1988). The birthdate of a neuron is thus highly correlated with its final laminar position.

A variety of studies have explored the correlation between cell birthday and laminar fate, in the hope of understanding the processes that specify the phenotypes of cortical neurons. Retroviral lineage experiments have revealed that precursors infected early in development produce clones that encompass many different layers, suggesting that early progenitors are multifated (Reid et al., 1997). Transplantation experiments have shown directly that precursors of deep-layer neurons are multipotent, because these cells can produce either upper- or deep-layer neurons, depending on the environment in which they complete their final mitosis (McConnell and Kaznowski, 1991). In contrast, cortical progenitors that normally give rise to the superficial layers of the cortex are restricted in their developmental potential. Late progenitors pro-

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duce upper-layer neurons exclusively, even when transplanted into a much younger environment in which deep-layer neurogenesis is ongoing (Frantz and McConnell, 1996). These results suggest that late progenitors are lineally committed to forming upper-layer neurons.

The difference in developmental potential between early and late progenitors suggests that late progenitors may be molecularly distinct from progenitors at earlier stages. Indeed, the homeobox gene *Otx1* is expressed at high levels by a subset of neurons in the deep cortical layers (5 and 6), and by their precursors in the ventricular zone (Frantz et al., 1994). The expression of *Otx1* appears to be down-regulated in the ventricular zone of older embryos, and *Otx1* is not expressed in upper-layer neurons. Although the expression pattern of *Otx1* suggested that *Otx1* might be involved in determining laminar fate, it now appears that *Otx1* plays no role in laminar specification. Instead, recent characterizations of mouse mutant for *Otx1* suggest that *Otx1* is essential for the development of normal axonal connectivity of subcortically projecting layer 5 neurons (Weimann et al., 1999).

Although *Otx1* appears not to be required for laminar specification, other genes that are expressed only in restricted layers of neurons may play an important role in the initial determination of laminar identity. Alternatively, these genes may enable the differentiation of lamina-specific phenotypes, such as the establishment of axonal outgrowth patterns or electrophysiological properties. One approach to the identification of layer-specific genes in the developing cortex has been to identify mammalian homologues of genes involved in the development of *Drosophila melanogaster*. Such studies have resulted in the cloning of the homeobox genes *Emx1*, *Emx2*, *Otx1*, and *Otx2*, which are expressed in a nested pattern in the developing mammalian forebrain (Simeone et al., 1992; Boncinelli et al., 1993). Of these, only *Otx1* also displays a layer-specific pattern of expression (Frantz et al., 1994). Another approach to the identification of genes expressed in spatially restricted patterns is to perform subtractive or comparative analyses of mRNA expression. These methods have led to the cloning of genes expressed specifically in the floor plate (Klar et al., 1992) and striatum (Porteus et al., 1991; Usui et al., 1994). The present study attempts to identify mRNAs expressed in a subset of cortical layers during development by using the technique of PCR differential display (Liang and Pardee, 1992).

MATERIALS AND METHODS

Animals

Timed-pregnant Long-Evans rats (Simonsen) were used for the developmental studies. The morning after breeding was considered embryonic day 0 (E0). RNA was isolated from 10 rats on postnatal day 6 (P6) to construct an upper-layer cDNA library. A total of 11 developing and adult rats were used for *in situ* hybridization experiments on normal animals: 1 at E20, 1 at P0, 1 at P3, 4 at P6, 1 at P15, and 3 adults. Sections for *in situ* hybridization on dark-reared rats were the gift of Dr. E. Nedivi at Cold Spring Harbor Lab (Nedivi et al., 1996). These included sections through the visual cortex from normal rats at P1, P7, P14, P21, P28, and adult; sections at the same ages from rats that had been reared in complete darkness, and sections at the same ages from dark-reared rats that were exposed to light for 6–24 h prior to sacrifice.

Preparation of RNA

Coronal 400 μm slices were prepared from P6 brains using a wire guillotine as described previously (Roberts et al., 1993). Slices were dissected in Hank's Balanced Salt Solution (HBSS) and the upper cortical layers 2–4 were separated from layers 5–6 using fine dissection knives. The tissue was frozen in liquid nitrogen. Total RNA was isolated by guanidinium isothiocyanate (Stratagene). RNA was extracted once with 25:24:1 phenol:chloroform:isoamyl alcohol, precipitated with isopropanol, and stored at -70°C until use.

Reverse Transcription and PCR Differential Display

Reverse transcription and PCR differential display were performed using the GenHunter Kit. In a total volume of 20 μL , 0.2 μg total RNA was reverse transcribed with 10 U MMLV reverse transcriptase in the presence of 1 μM T12MN as primer and 20 μM dNTP for 60 min at 37°C . After inactivation at 94°C for 5 min, 2 μL of the reaction was used for PCR amplification in the presence of 2.5 μM dNTP, 0.2 μM AP (arbitrary upstream 10mer) primer, 1 μM T12MN primer, and 12 μCi [^{35}S]-dATP (Gibco-BRL) and 1 U Taq polymerase (Amplitaq, Perkin Elmer). Forty cycles of PCR were performed with the following conditions: 94°C , 30 s; 40°C , 2 min; 72°C , 30 s; and finally, 72°C for 5 min. Three-and-a-half μL of the reaction with 2 μL loading dye was then denatured at 80°C for 2 min and electrophoresed on a 6% sequencing gel until the xylene dye was 10 cm from the bottom. The gel was dried without fixation and exposed to film. After developing the film, differentially expressed bands were cut from the gel. The gel slice was then soaked in 100 μL TE for 10 min, then boiled for 15 min. The supernatant was removed, and DNA was recovered by ethanol precipitation. Four μL of 10 was used for reamplification in a 40 μL reaction with the iden-

tical primers and conditions used in the original differential display reaction except substituting 20 μM dNTP without labeled isotopes. Four μL of this amplification was used in a second round of amplification with identical conditions. One μL of the reamplified cDNA was then cloned into the TA cloning vector (Invitrogen) and sequenced to determine its orientation. The primers used to isolate rCNL3 were AP-5 (5'-GTTGCGATCC-3') and T12MC (M = A, C, G, and T).

Cloning and Library Construction

The original 186 base pair PCR fragment (1A) was used to screen, under high stringency conditions, a cDNA library generated from upper-layer (2–4) cDNAs. The cDNA library was constructed in λ ZAPII (Stratagene) from approximately 5 μg upper-layer cortex poly (A)⁺ selected RNA. Briefly, the library was plated, transferred in duplicate to nylon filters (Amersham), and screened using [³²P]-labeled cDNA probes generated by random priming (Amersham). Positive clones were rescreened twice, and the cDNA insert of the clone (1AFL) that hybridized on the final screen was excised from the library in a pBluescript SK-vector (Stratagene). The 1A3' clone was generated by an Exonuclease III digestion of the 5' end of the full-length construct (1AFL). 1A3' corresponds to the 3' untranslated portion of the 1AFL clone (1904–2260). The rCNL3 clone was created by subcloning a 422 base pair NaeI-NsiI fragment into the PstI and EcoRV sites in the pBluescript SK⁺ vector.

DNA Sequencing of rCNL3

An ordered set of Exonuclease III deletions from both ends of the 2.1 kb 1AFL insert was made by the method of Henikoff (1987). The nucleotide sequences of both strands of the unique 3' sequence were determined using the deletions, and, where necessary, custom-synthesized oligonucleotide primers. Both double-stranded and single-stranded DNA were used as a template for T7 DNA polymerase (Sequenase, U.S. Biochemicals). Sequences were assembled on an Apple Macintosh computer using the Speakquencer program (freeware) and the Intelligenetics sequence analysis program.

In Situ Hybridization

In situ hybridization was performed as described by Frantz et al. (1994), with minor modifications. Whole embryos or brains were dissected out and frozen in OCT embedding compound on dry ice and stored at -70°C until use. Frozen sections were cut at 15 μm , mounted onto Superfrost Plus slides (Fisher), postfixed with 4% paraformaldehyde, dehydrated, and stored at -70°C . [³⁵S]-labeled 1A, 1A3', and rCNL3 probes were transcribed from plasmid clones. The plasmid containing clone 1A was linearized with XhoI and transcribed with SP6 RNA polymerase to produce antisense probe; the plasmid was linearized with BamHI and tran-

scribed with T7 polymerase for sense controls. 1A3' was linearized with SacI and antisense probe was produced by transcribing with T7. The rCNL3 plasmid was linearized with XhoI and transcribed with T3 polymerase. Conditions for *in situ* hybridization were identical to those described in Frantz et al., 1994. Autoradiography was performed with Kodak NTB2 autoradiographic emulsion, and sections were exposed for approximately 4 weeks before developing. Sense controls revealed no hybridization above background (not shown). Sections were examined and photographed using a Nikon UFX camera system with both dark- and bright-field illumination. Prints were scanned with a Nikon scanner and processed using Adobe Photoshop.

PCR Amplification of Genomic DNA

Genomic DNA was prepared from neonatal rat tails by incubating in 700 μL lysis buffer (100 mM Tris, pH 8.5; 200 mM NaCl; 5 mM EDTA; 0.2% SDS; and 50 $\mu\text{g}/\text{mL}$ proteinase K) at 55°C overnight. The sample was treated with RNaseA (100 $\mu\text{g}/\text{mL}$, 37°C , 1 h), extracted with phenol: chloroform: isoamyl alcohol twice, then chloroform once, then precipitated with 700 μL isopropanol. Genomic DNA was then spooled out, washed with 70% ethanol, and resuspended in TE. Approximately 0.2 μg genomic DNA was used for each reaction. The reaction conditions were as follows: in the presence of 250 μM dNTPs and 2 μM of each primer, 0.25 U Taq DNA polymerase (Qiagen) was added to the reaction following incubation at 94°C for 2 min. Amplification was performed for 35 cycles with the following parameters: 94°C , 4 min; 94°C , 20 s; 65°C , 20 s; 72°C , 20 s; decreasing the annealing temperature 1°C for nine cycles; carrying out 25 additional cycles with annealing temperature of 55°C ; final extension at 72°C for 10 min. The products were run on a 3% agarose gel. The following primers were used: set 1: primers corresponding to sense bases 1631–1651 (GTTGCCATCCCCAAGAGCAGG), and antisense bases 1905–1928 (CTCCATGACTCATGAATGTTACTG), yielding an expected product of 298 base pairs; set 2: sense strand 1427–1450 (ATATACAGTGATACACGTGTACA) and antisense strand 1631–1651 (CCTGCTCTTGGGGATGGCAAC), yielding an expected product of 224 base pairs. If the 3' end of 1A arose through the spurious fusion of two cDNAs during library construction, PCR amplification should fail for primer set 1 but still yield the expected product with primer set 2.

RESULTS

Isolation of a Superficial-Layer Restricted Clone

To isolate genes that exhibit a restricted laminar pattern of expression, PCR differential display (Liang and Pardee, 1992) was used to compare mRNAs derived from the superficial layers and deep layers of the postnatal day 6 (P6) rat cortex. PCR differential dis-

play was chosen because this method requires only small amounts of tissue to isolate candidate molecules that can be used to generate *in situ* probes to screen tissues. Four hundred μm slices were prepared from approximately the middle third of the P6 rat cortex, discarding slices from the rostral and caudal thirds of the cerebral vesicles. By P6, the neurons of all of the cortical layers have arrived at their final locations (Berry and Rogers, 1965; Hicks and D'Amato, 1968), and the layers are clearly distinguishable in these slices when viewed with dark-field illumination. Fine dissection knives were used to cut along layer 4 of the cortex and in the underlying white matter to separate the tissue into superficial cortex (layers 2–4) and deep cortex (layers 4–6). The efficacy of these dissections was confirmed in a subset of slices by sectioning the dissected tissue and staining the sections with Cresyl violet to identify the layers. Total RNA was isolated from each tissue and reverse-transcribed into cDNA that was used in the PCR differential display.

From an initial set of five pairs of primers and PCR amplifications, 15 bands appeared to be differentially expressed (e.g., Fig. 1). These bands were cut from the gel, subcloned, and used to generate [^{35}S]-labeled RNA probes for *in situ* hybridization studies. Of these 15 initial candidate clones, the expression of one (1A) was limited to layers 2–4 within the P6 rat cortex (Figs. 1 and 4–6). The gene is also expressed at high levels in the developing striatum. *In situ* analysis of the other candidates yielded either no signal in the cortex or hybridization to all cortical layers. The 186 base pair 1A PCR fragment was then used to probe a superficial cortex cDNA library to isolate a larger cDNA clone. Sequencing of the cDNA clone (Fig. 2) revealed that it is identical in coding sequence to rCNL3, a cDNA encoding a putative G-protein-coupled receptor that was isolated in a PCR-based screen for cannabinoid receptor family members (Song et al., 1994).

The 1A cDNA differs slightly from rCNL3 in that 1A contains an additional 484 bases at its 3' end (Fig. 2). In order to rule out the possibility that the cDNA identified here arose from a spurious fusion of two messages during the construction of the cDNA library, primers specific for 1A and rCNL3 were used to amplify fragments from genomic DNA. These primers amplified products of appropriate size, indicating that the 1A cDNA is not a fusion of two messages (Fig. 3). The differences in the 3' end of the 1A and rCNL3 cDNAs may arise from the alternative use of polyadenylation sites, because the 3' end of 1A contains a number of consensus polyadenylation sites (Fig. 2).

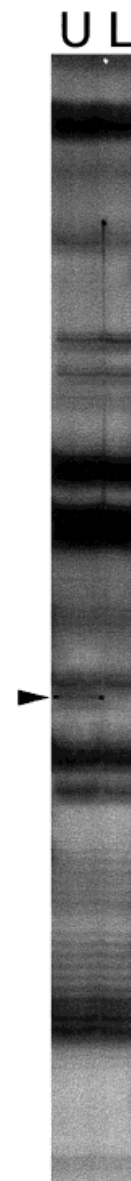


Figure 1 Differential display comparing total RNA isolated from upper layers 2–4 and deeper layers 4–6. A pair of PCR differential display amplification products from upper (U) and lower (L) cortical layers. The primers used were 5'-GTTGCGATCC-3' and T12MC. The arrowhead marks the differentially expressed 1A band, which was also marked by small holes in the gel (dots). This band was cut out, reamplified, subcloned, and used for *in situ* hybridization.

Spatial and Temporal Patterns of rCNL3 Expression

As a first step toward examining the possible role of rCNL3 in cortical development, its expression pattern was examined through a series of developmental *in situ* hybridization studies using the original 1A probe.

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1420      1430      1440      1450      1460      1470      1480      1490
CTTGTGGCA TTTTACATAT ACAGTGTATA CACGTGTACA TATATATACA AATATTTGTA TCTTCTGGAG GTGTTTCAGGG

1500      1510      1520      1530      1540      1550      1560      1570
CCTGGAGCTT CCCGTTCTGT GAAAAAACT AACAAAATGT GGTGTATAC TCAATCGTAC ATCACATTTG TCAAGTGAAG

1580      1590      1600      1610      1620      1630      1640      1650
ACATTCCAAT ACTGCTTAAT AATAGCACTT TATTTTTAGC TGCTGAAATG CCAAGACAGT GTTGCCATCC CCAAGAGCAG

1660      1670      1680      1690      1700      1710      1720      1730
GAGAAAGGGA GTCAGAGATG TATTTTTGTT GTATGTGATA GAATATTTTG CTGCACATGC ATCAGTAAAC TACAACATAT

1740      1750      1760      1770      1780      1790      1800      1810
TTTGTACACA AATAAACACA TTATAAAAGT ATGATCTTGG GTATGTGATA AATTTGTGGCA TTGTTTGGAA TCTGAATTTG

1820      1830      1840      1850      1860      1870      1880      1890
GAGTGTGTTT ATTTTAAAG TCTACTGCAC ATCTATCTAT CATCTGTCCC AGGGAGAGCC ACATGAAATA CCAGATCTAA

1900      1910      1920      1930      1940      1950      1960      1970
ACCATGGGAT GAGGCAGTAA CATTCATGAG TCATGGAGGA AGTAACATTC ACACGTGGCA TCCCCAGAAC ACGAGAAGGA

1980      1990      2000      2010      2020      2030      2040      2050
AGGCTGTTAC TGGTGCAAGT TAATGTGCTC TCTTGGTGGC CGGGTTGGGA AGACATGTTT CAGAGGTCAA AACAAITCAA

2060      2070      2080      2090      2100      2110      2120      2130
GTCACATAGG CACAGGTGTA AACTCTGAAG CCACAACTTA CCAGCATAAC TTTACAAACA TGCCAAGATT TCTCAACATG

2140      2150      2160      2170      2180      2190      2200      2210
GCTTATTCAG ACTGTTGCTA CCCAGGTCTG GTAGCCAGGT GCAGAGCCCA ATAAAACCAA TAAAATCTC CTAAAGCAGG

2220      2230      2240      2250      2260
ATTCCCAGAT CTTCATGGGG TAACTGAAAA AAAAAAAAAA AAAAAAAAAA

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Figure 2 Partial nucleic acid sequence of clone 1AFL, showing nucleotides 1411–2260. The complete sequence can be accessed in GenBank (accession number AF064706). The region corresponding to the original cDNA fragment isolated by differential display extends from nucleotides 1631 to 1800 (the downstream differential display primer contributed 16 additional bases to the amplified fragment). The rCNL3 cDNA extends to base 1752 (marked by a diamond). Sequences targeted for PCR amplification of genomic DNA are underlined (see text for details). Potential polyadenylation sites are marked with double underlines.

We reasoned that if rCNL3 regulates the laminar identity of superficial cortical neurons, it should be expressed in the committed precursors that generate the upper layers. In the rat, layer 4 neurons are generated on embryonic day 17, while layers 2 and 3 are generated from E18–E20 (Frantz et al., 1994). *In situ* hybridization of the E17 cortex failed to reveal rCNL3 expression in the ventricular zone or in other regions of the forebrain (data not shown). Further *in situ* analysis revealed that rCNL3 expression begins weakly in the striatum at E20, but is not expressed at detectable levels in cortical progenitors in the ventricular zone at any embryonic age [Fig. 4(A,B)]. These expression patterns in the embryonic cortex suggest that rCNL3 is not expressed in the progenitors that give rise to layers 2–4. Thus, it appears unlikely that rCNL3 is involved in the initial specification of laminar fate.

To examine the onset and distribution of rCNL3

expression later in development, *in situ* hybridization was performed using the 1A probe on brain sections from postnatal animals.

Birth (P0): In the newborn rat, the neurons that give rise to layers 2–4 of the cortex are migrating in the intermediate zone *en route* to their final laminar positions (Berry and Rogers, 1965; Hicks and D'Amato, 1968). *In situ* analysis revealed that rCNL3 expression is not detectable in this migratory region, nor is rCNL3 expressed in the deep-layer neurons that already populate the cortical plate [Fig. 4(C,D)]. However, rCNL3 expression remains strong in the striatum at this age [Fig. 4(C,D)]. Expression is confined to regions in which neurons are differentiating, and is excluded from progenitor cells of the ganglionic eminence.

P3: By P3, some of the first neurons of layers 2–4 have arrived within the cortical plate (Berry and Rogers, 1965). Although the layers are not clearly distin-

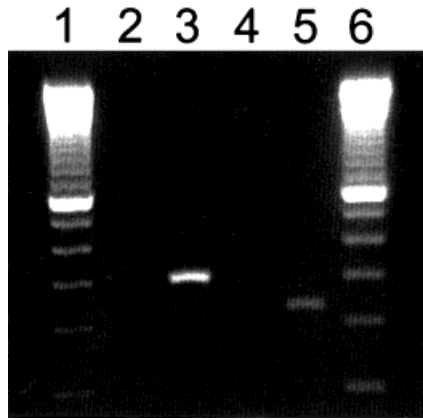


Figure 3 PCR analysis of genomic DNA using primers for full-length 1A cDNA. PCR primers overlapping a segment of full-length cDNA (1AFL) were used to amplify from genomic DNA. Primer set 1 (lanes 2 and 3) spans bases 1631–1928 on 1AFL and generates a product of 298 base pairs. Primer set 2 (lanes 4 and 5) spans 1427–1651, contained in both 1AFL and rCNL3, and generates a product of 225 base pairs. If the 1AFL cDNA arose from a spurious fusion during library construction, only primer set 2 should yield a product from genomic DNA amplification. Lanes 2 and 4 are no-DNA controls. Marker lanes (1 and 6) contain 100 base pair ladders.

guishable from each other at this age, it appears that rCNL3 hybridization is strongest in the most superficial region of the cortical plate, corresponding with the recent arrival of upper-layer neurons [Fig. 4(E,F)].

P6: By P6, all of the neurons of the cortex have reached their final laminar locations, and the layers are distinguishable from each other by cellular density and morphology (Berry and Rogers, 1965). At this time in development, the neurons of the superficial layers are elaborating axonal and dendritic processes (Miller, 1986). *In situ* hybridization with the rCNL3 probe revealed continued high expression in the striatum, and strong expression in layers 2–4 of the cortex [Fig. 4(G–J), Fig. 5]. The signal appears stronger in the more posterior cortical regions than in the anterior cortex. This regional pattern could reflect an area-specific pattern of gene expression, or it may simply reflect the maturational gradient of cortical development. Because the cortex matures in a lateral-first, medial-last and anterior-first, posterior-last gradient (Berry and Rogers, 1965), stronger rCNL3 expression in posterior regions raises the possibility that rCNL3 is highest in immature neurons and is down-regulated in the more mature anterior regions. The eventual loss of rCNL3 expression throughout the cortex (see below) is consistent with the notion that the regional pattern reflects a maturational gradient of expression.

P15: By P15, cortical neurons have adopted many of their final neurotransmitter characteristics. Both glutamate-immunoreactive cells and GABA-immunoreactive cells are distributed in an adult-like pattern by approximately 2 weeks after birth, and VIP-immunoreactive cells achieve an adult-like distribution between the second and third postnatal week (Götz and Bolz, 1994). Axonal complexity increases during the second and third postnatal weeks, and by about the end of the third postnatal week the appearance of the cell bodies and dendrites are mature (Miller, 1986). rCNL3 continues to be expressed in the superficial layers at P15, although expression still continues to be stronger in the posterior cortex [Fig. 4(K,L)]. Likewise, rCNL3 expression remains strong in the striatum [Fig. 4(K,L)].

Adult: Expression of rCNL3 is not detectable in the adult cortex [Fig. 4(M,N)]. Although we cannot distinguish directly whether rCNL3 expression has been down-regulated or whether the cells expressing rCNL3 have been eliminated via cell death, the large numbers of rCNL3-expressing cells seen at early times would suggest that the former possibility is more likely. In contrast, expression of rCNL3 remains detectable in the striatum in adulthood [Fig. 4(M,N)].

The distribution of rCNL3 expression in the cortex of postnatal animals indicates that rCNL3 is restricted to the superficial layers 2–4 during the period from P3 until P15 (Fig. 5). We were unable to ascertain directly whether rCNL3 is expressed exclusively by neurons, or by both neurons and glial cells, because cells are closely packed at early stages of cortical development and the silver grains that signify mRNA hybridization are scattered over more than one cell diameter. However, the presence of rCNL3 hybridization in the cortex at P3 and P6 suggests that expression is unlikely to be strictly glial in origin, because the cells of the early postnatal cortical plate are almost exclusively neuronal (Goldman and Vaysse, 1991). The expression of rCNL3 in the striatum begins even earlier, at E20, which is also suggestive of neuronal expression, but in contrast to the cortex the striatal expression of rCNL3 continues into adulthood.

Because our original 1A cDNA differed from that originally reported for rCNL3 in the 3'-untranslated region, we were curious to examine whether probes specific to different portions of the cDNA revealed differing cortical expression patterns. Probes to segments of the cDNAs unique to either 1A (1A3' probe) or to the coding sequence of rCNL3 were generated to examine whether the expression patterns of 1A and rCNL3 differed. *In situ* hybridization using these probes revealed that the expression patterns of each were similar but not identical in the P6 cortex (Fig. 6).

Although the signal using the rCNL3 probe was stronger than that of the 1A3' probe, both probes hybridized to cortical layers 2–4 as well as to the striatum. Surprisingly, the 1A3' probe also showed hybridization within the hippocampus. This hippocampal signal may represent either the deployment of alternative polyadenylation sites or the cross-hybridization of the 1A3' probe to related messages. However, our results do show that the hybridization patterns in neocortex and striatum are similar using the two probes.

Expression of rCNL3 in Dark-Reared Rats

The timing of rCNL3 expression in the cortex is suggestive of a role for this gene in the differentiation of neurons in layers 2–4. Because the first few weeks of life in rodents present a period in which synaptic connections are highly modifiable through experience-dependent interactions (Fagiolini et al., 1994; Gordon and Stryker, 1996), we asked whether the expression of rCNL3 is altered by changes in neuronal activity. rCNL3 expression was assessed by *in situ* hybridization on brain sections from control rats, rats that had been reared in complete darkness, and dark-reared rats exposed to light (Nedivi et al., 1996; sections were the generous gift of Dr. E. Nedivi). The patterns of rCNL3 hybridization were comparable in all conditions tested, and the time at which rCNL3 expression disappeared from the cortex was not obviously altered by any of these manipulations (data not shown).

DISCUSSION

We have isolated a cDNA from rat that encodes a gene expressed within the cortex selectively in the superficial layers (2–4) during development, as well as in the developing and adult striatum. This cDNA is identical in its coding sequence to rCNL3, a gene identified previously when using degenerate PCR in a search for members of the cannabinoid receptor family (Song et al., 1994). rCNL3 encodes a putative G-coupled receptor that shows sequence similarity to cannabinoid receptors. Here we have found that cortical expression of rCNL3 begins postnatally at P3 and continues at high levels until after P15; rCNL3 is not expressed in the adult cortex. Striatal expression of rCNL3 begins at E20 and continues into adulthood. In the cortex, rCNL3 expression is not detected in precursor cells in the ventricular zone at the time of upper-layer neurogenesis, suggesting that this gene is not involved with the initial specification of cell iden-

tity. However, rCNL3 expression is initiated in the superficial layers soon after the neurons have migrated into their final positions, and expression continues for at least 2 weeks, suggesting a role for rCNL3 in neuronal differentiation.

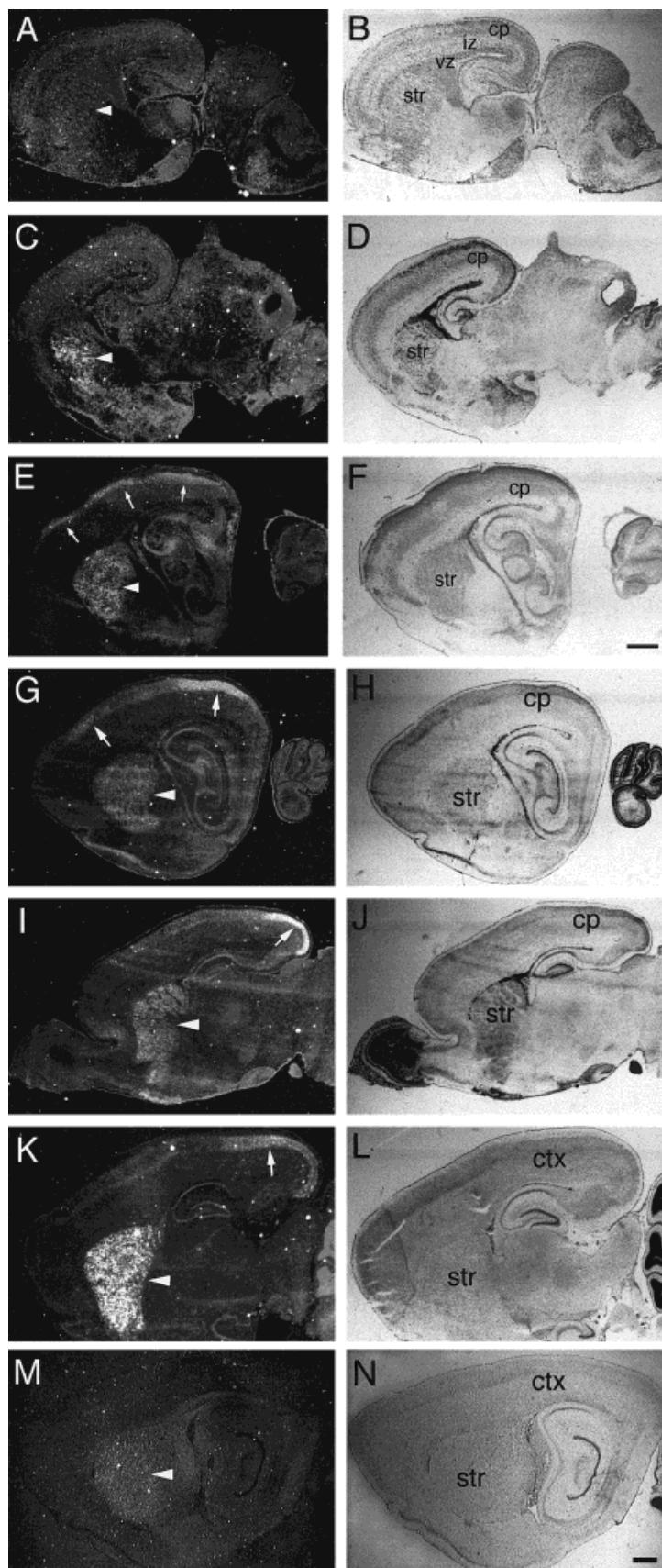
rCNL3 Expression Patterns in the Forebrain

We observed a consistent pattern of stronger hybridization in posterior regions of the cerebral cortex during development. We suspect that this gradient of expression reflects the gradient of maturation in cortical development, as the signal is weaker in anterior, more mature areas of the cortex. Although the posterior-anterior gradient continues in older P15 brains, we note that the absolute signal in P15 cortex was less than that seen at P6. Although it is difficult to compare absolute signal across different ages, the stronger relative signal at P6 was observed when identical exposure times were used, supporting the notion that more mature areas of cortex were beginning to down-regulate the message.

We also found that a probe generated to the 3' portion of the 1A cDNA showed a cortical and striatal hybridization pattern similar to the patterns revealed by coding region probes. Although the signal from the 3' probe was weaker than that seen with the rCNL3 probe, the probe hybridized to the upper layers of the cortex as well as the striatum. Interestingly, we observed that the 3' probe also showed hybridization to the hippocampus consistently. These differences in signal cannot be explained by different hybridization stringencies or dramatic differences in the probe hybridization conditions, as the probes were similar in size (356 vs. 422 bp) and GC content. The signal seen in hippocampus may represent the use of an alternative polyadenylation signal or cross-hybridization with related transcripts. These possibilities cannot be distinguished without further studies.

rCNL3 and Cannabinoid Receptors

The coding sequence of rCNL3 suggests that it is a member of the G-coupled receptor family (Song et al., 1994). It consists of seven hydrophobic transmembrane domains and conserved amino acid motifs typical of the family, and it shows several conserved features of the rhodopsin-like family of G-coupled receptors (Song et al., 1994). However, rCNL3 appears to be most similar to the cannabinoid receptors and the edg-1 family of receptors for sphingosine-1-phosphate (T.I. Bonner, personal communication). Several features suggest a greater similarity between



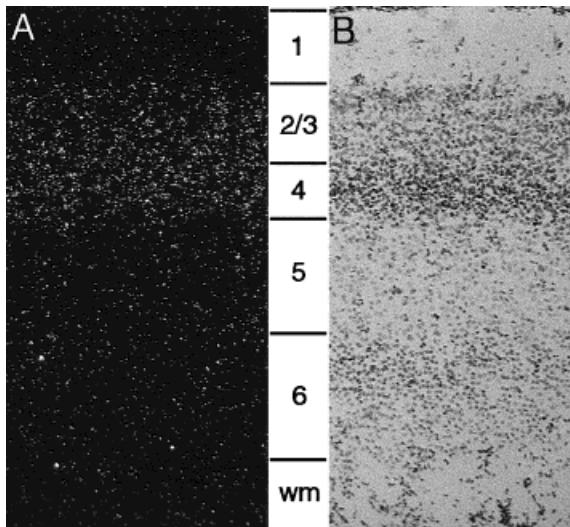


Figure 5 Higher power dark-field view of rCNL3 hybridization within layers 2–4 of P6 rat cerebral cortex. (A) Dark-field view of all cortical layers and the underlying white matter (wm), showing intense hybridization in layers 2–4 of the cortex. (B) Bright-field view of same section, counterstained with Cresyl violet.

rCNL3 and the cannabinoid and edg receptors than between rCNL3 and the rhodopsin family of receptors: the first transmembrane region contains Glu-Asn rather than the much more common Gly-Asn; the third transmembrane domain lacks a Cysteine residue highly conserved in the rhodopsin family of receptors; and a conserved GWNC motif is present at the end of transmembrane domain four.

An endogenous ligand for the cannabinoid receptors, anandamide, was isolated recently (Reisine and Brownstein, 1994). Despite the similarity between rCNL3 and cannabinoid receptors, neither any of the known ligands for cannabinoid receptors nor structur-

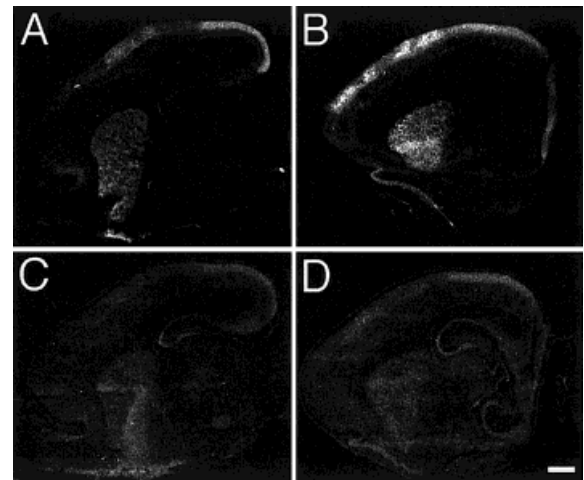


Figure 6 Hybridization using different regions of the rCNL3 cDNA in P6 rat forebrain reveals a similar pattern of expression. (A–D) Comparison of *in situ* hybridization using two different probes, 1A3' and rCNL3. These are dark-field views of parasagittal sections of P6 rat forebrain. (A,B) Probe rCNL3 hybridizes to the translated region of the rCNL3 cDNA. Hybridization is detected in the superficial layers 2–4 of the cerebral cortex and in the striatum in both lateral (A) and medial (B) sections. (C,D) Probe 1A3' hybridizes to the 3' untranslated region of the 1AFL cDNA. The hybridization of rCNL3 appears stronger than that of 1A3' throughout the forebrain and striatum in both lateral (C) and medial (D) sections. Scale bar = 1 mm.

ally related molecules were able to bind specifically or produce functional responses in cells transfected with rCNL3 (Song et al., 1994). Other putative ligands that were tested included dopamine, L-DOPA, a sigma receptor ligand 1,3, di-ortho-tolyl-guanidine (DTG), haloperidol, and the GABA-B receptor agonist baclofen; none of these agonists bound or elicited responses. By Northern analysis, rCNL3 is expressed

Figure 4 Developmental regulation of 1A expression within the developing rat forebrain. (A,C,E,G,I,K,M) Dark-field views of rCNL3 hybridization using the 1A probe; parasagittal sections, anterior is to the left. (B,D,F,H,J,L,N) Bright-field views of the same sections, counterstained with Cresyl violet. (A,B) E20: rCNL3 is expressed weakly in the developing striatum (arrowhead). No staining is visible in the cortical ventricular zone. (C,D) P0: rCNL3 expression is stronger in the striatum (arrowhead). (E,F) P3: rCNL3 hybridization is apparent in the superficial regions of the cortical plate (arrows), as well as in the striatum (arrowhead). (G–J) P6: Within the cortical plate, rCNL3 expression is restricted to the superficial layers 2–4 of the cortex (arrows). Hybridization is stronger in the posterior cortical regions. (G,H) are lateral parasagittal sections while (I,J) are medial. Expression continues in the striatum (arrowhead). (K,L) P15: Cortical expression of rCNL3 is prominent in layers 2–4 (arrow). Hybridization continues to be more abundant in the posterior cortex than in the anterior. rCNL3 is expressed strongly in the striatum (arrowhead). (M,N) Adult: rCNL3 expression is not detectable in the adult cortical plate, although hybridization continues in the striatum (arrowhead). Abbreviations: str, striatum; vz, ventricular zone; iz, intermediate zone; cp, cortical plate; ctx, cortex. Scale bar = 1 mm.

only in brain, leading Song et al. (1994) to suggest that rCNL3 may serve as a receptor for an as yet unidentified neurotransmitter or neuromodulator. They further speculated that rCNL3 may be involved in motor and learning functions, because the gene is expressed in the striatum, retrosplenial cortex, amygdala, and hippocampus (Song et al., 1994).

rCNL3 and Cortical Differentiation

Very little is known about the functions of G-coupled receptors in cortical development, and the question of whether the ligands for cannabinoid receptors play important developmental roles remains to be examined. Transient expression of the cannabinoid receptor CB1 mRNA, as well as ligand binding, has been detected in fetal rat cerebral cortex, suggesting a possible role during neuronal development (Berrendero et al., 1998; Fernandez-Ruiz et al., 2000). It is clear, however, that other G-protein-coupled receptors and their ligands can serve crucial roles in a wide variety of developmental processes. A number of G-coupled receptors are known to bind neurotransmitters or neuropeptides as ligands, and it has long been speculated that neurotransmitters may act as morphogens during development (e.g., Lauder, 1988). In addition, G-coupled receptors can serve as receptors for secreted signaling molecules. For example, the frizzled family of 7-pass transmembrane proteins serves as receptors for the signaling proteins Wingless and Wnts, which mediate a number of cell-cell interactions during development (reviewed in Bhanot et al., 1996; Orsulic and Peifer, 1996; Peifer and Polakis, 2000), including axis formation in *Xenopus* (He et al., 1997), and endoderm specification and the establishment of cell polarity in *C. elegans* (Han, 1997; Rocheleau et al., 1997; Thorpe et al., 1997). Another G-protein-coupled receptor with an essential role in development is *smoothened*, a segment polarity gene in *Drosophila* that participates in the *hedgehog* signaling pathway (Alcedo et al., 1996). Ectopic expression of a constitutively active form of *smoothened* in the dorsal mid-brain and hindbrain of mouse and chick mimics the action of Sonic Hedgehog (Shh), suggesting that *smoothened* is an important signaling component of the Shh receptor (Hynes et al., 2000).

Evidence of a developmental role for other G-protein-coupled receptors raises the possibility that rCNL3 may serve an important function during the development of neurons in the superficial layers of the cerebral cortex. Although rCNL3 is expressed at high levels in layers 2–4, the observation that it is not expressed in the progenitors in the ventricular zone argues against the possibility that rCNL3 is involved

in the initial specification of laminar identity, which occurs prior to migration (McConnell and Kaznowski, 1991). The expression of rCNL3 during the early postnatal period from P3–P15 suggests that rCNL3 may play a role in the early events of neuronal differentiation, such as the initial elaboration of axons and dendrites of neurons in layers 2–4. For example, the arrival of layer 2/3 callosal axons from the contralateral cortex coincides with the expression of rCNL3 (Hernit et al., 1996). However, it seems unlikely that rCNL3 could serve a sole role in determining the specificity of axonal outgrowth from these layers, because the targeting decisions of layer 2/3 neurons differ from those of layer 4. Layer 2/3 pyramidal neurons branch specifically in layers 2/3 and 5 and extend long-distance axons to other regions of cortex, while layer 4 spiny neurons form intrinsic projections within layers 2/3 and 4 (reviewed in Katz and Callaway, 1992). An alternative role suggested by the timing of expression of rCNL3 in cortex is that this receptor may mediate activity-dependent changes in cortical circuitry during the early postnatal period. We failed, however, to find any obvious changes in the pattern or timing of rCNL3 expression in the visual cortex of dark-reared rats, suggesting that rCNL3 itself is not regulated directly by neuronal activity. Thus, the hypothesis that rCNL3 plays a role in cortical differentiation remains to be tested with gene disruption or pharmacological studies.

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