Experience-dependent plasticity of the adult visual cortex underlies perceptual learning and recovery of function following central nervous system lesions. To reveal the signal transduction cascades involved in adult cortical plasticity, we utilized a model of remapping of cortical topography following binocular retinal lesions. In this model, the lesion projection zone (LPZ) of primary visual cortex (V1) recovers visually driven activity by the sprouting of horizontal axonal connections originating from the cells in the surrounding region. To explore the molecular mechanism underlying this process, we used gene microarrays from an expression library prepared from Macaque V1. By microarray analysis of gene expression levels in the LPZ and the surrounding region, and subsequent confirmation with Quantitative Real-Time polymerase chain reaction and in situ hybridization, the participation of a number of genes was observed, including the Rho GTPase family. Its role in regulation of cytoskeleton assembly provides a possible link between the alteration of neural activity and cortical functional reorganization.

**Keywords:** adult visual plasticity, microarray, Rho GTPases

Experience-dependent plasticity in the adult brain is a phenomenon that is general to the entire cerebral cortex, including primary sensory cortex. This plasticity is associated with perceptual learning occurring during normal sensory experience, as well as with recovery of function following lesions of the central nervous system (CNS).

In the visual system, one model of adult cortical plasticity is the remapping of topography of primary visual cortex (V1) following retinal lesions. Retinal lesions deprive input to the portions of V1 representing the lesioned part of the retina, which is referred to as the lesion projection zone (LPZ). Electrophysiological mapping has shown that the size of LPZ shrinks over time and recovers its ability to respond to visual stimuli, where the new input arises from parts of the retina surrounding the lesion (Gilbert et al. 1990; Kaas et al. 1990; Heinen and Skavenski 1991; Chino et al. 1992; Gilbert and Wiesel 1992; Calford et al. 2000). The circuitry underlying this recovery includes the plexus of long-range horizontal connections, which mediate a lateral spread of visual information within each cortical area (Darian-Smith and Gilbert 1995). The preexisting framework of horizontal connections linking the LPZ with the surrounding area (the peri-LPZ) has been shown to sprout within the LPZ (Darian-Smith and Gilbert 1994; Yamahachi et al. 2008), thereby strengthening the input originating from the area of retina surrounding the lesion.

To determine the signal transduction mechanisms underlying the sprouting of horizontal connections and the consequent remapping of cortical topography following retinal lesions, we utilized a gene expression library obtained from the V1 of the Macaque. Here, we were able to take advantage of the distinct topography of the reorganization process—a well-delineated region of cortex undergoing reorganization, within which synaptogenesis is taking place (the LPZ), and the surrounding region containing neurons responsible for the sprouting and providing visual input into the LPZ. To limit the diversity of cell types used in our analysis, we labeled neurons with fluorescent tags, which then allowed us to compare the levels of gene expression of superficial layer pyramidal cells within and outside the LPZ. The cells were selected by laser capture microdissection (LCM). In the results that follow, this revealed the participation of a number of gene families in adult cortical plasticity, which was confirmed by subsequent Quantitative Real-Time Polymerase Chain reaction (qRT-PCR) and in situ hybridization.

**Materials and Methods**

**Retinal Lesion and Neuron Labeling**

Four *Macaca fascicularis* were used for this work. All retinal lesions and viral injections were carried out in anesthetized animals in accordance with institutional and federal guidelines for the treatment of animals.

Animals were anesthetized with Nembutal, incubated, and placed on a heating pad. A patient monitor provided continuous monitoring of heart rate, breathing rate, and expired CO₂ concentration. The animals were placed in a stereotaxic apparatus and a craniotomy was made over the area of visual cortex representing the parafocal area of the visual field. Retinal lesions were performed as previously described (Gilbert and Wiesel 1992). Briefly, the topographic organization of the exposed cortical area was determined by making a series of electrode penetrations and mapping the receptive field (RF) positions at each recording site on a tangent screen. The retinal lesions were made so that the boundary of the LPZ would traverse the center of the exposed cortical region. After mapping the cortical topography, focal binocular lesions were made at homologous positions in the 2 retinas in 4 Macaque monkeys, positioned to include a portion of the mapped area. After making the lesions, the boundary of the cortical representation of the retinal lesion (the LPZ) was established in the mapped area, using the cortical vasculature as a fiducial reference (Fig. 1). The visuotopic location of the lesion was determined with a fundus camera (Zeiss), by back projecting its boundary on the tangent screen. This enabled us to determine the relative positions of the lesion boundary and the mapped RFs, and consequently the position of the LPZ boundary relative to the cortical recording sites.

Immediately after placing the lesions, we injected genetically engineered adenoviruses in the cortex to label neurons lying within and outside of the LPZ. The virus was nonreplicating and was engineered to have either the gene encoding green fluorescent eGFP or the gene encoding red fluorescent tdTomato integrated in its genome. After an incubation period of a few days, the fluorescent proteins would start to be expressed in the infected neurons, and the expression could last for several weeks. To distinguish neurons in the...
LPZ from those in the peri-LPZ, we injected adeno-eGFP or adeno-
tdTomato into either side of the LPZ boundary so that cells in LPZ and
peri-LPZ were labeled with different colors. Virus was pressure
injected with a glass micropipette at 10--12 locations along the axis
of the LPZ boundary. The injections were made 1--2 mm inside or
outside the boundary and spaced approximately 1 mm apart. Each
injection contained 100--150 nL of virus with $1.6 \times 10^9$ viral particles.

Neurons transduced with the virus begin to express the genes
encoding the fluorescent probes within a few days, and expression
peaks at around 2 weeks.

Two weeks after making the retinal lesions and viral injections, the
animals were perfused with phosphate buffered saline (PBS) followed by
2% paraformaldehyde in PBS.

**Tissue Preparation**

After perfusion, the portion of V1 containing the lesion and the
injections, as well as the surrounding area, was blocked. It was then
postfixed in 2% paraformaldehyde in PBS for 2 h and immersed in 30%
sucrose in PBS for another 2 h for cryoprotection. The tissue was then
frozen in the OCT compound (Tissue Tek) on dry ice with ethanol and
stored at $-80\,^\circ C$ until further use. We processed the tissue with this
short protocol to preserve the mRNA in the cells. A typical example of
the RNA we used for microarray hybridizations is shown in Figure 2.

**LCM**

LCM was used to select superficial layer pyramidal neurons for analysis
(the Molecular Machine Industries, MMI). On the day of or the night
before LCM, frozen tissues were cut at 8--10 $\mu$m with cryostat and
sections were mounted on the RNase-free membrane slides (MMI).
Slides were kept at $-80\,^\circ C$ throughout the LCM procedure. Before
capturing cells, every section was immersed in 100% ethanol for 30 s
and left to dry at room temperature over a desiccant for a few minutes
before being mounted on the LCM microscope. A few sections in the
series were stained beforehand with cresyl violate to ascertain the
cortical layers, and measurements were taken to locate the superficial
layers. Pyramidal cells were selected in the superficial layers from LPZ
and peri-LPZ, respectively, according to their fluorescent color. About
20--50 cells from each region were captured and collected in the same
tube. The RNA extraction buffer (Arcturus, Mount View, CA) was then
added into the tube, and the tube was kept at $-80\,^\circ C$. LCM continued
this way until 200--300 cells were collected.

**RNA Extraction and Amplification**

Cells from the same region (LPZ or peri-LPZ) were pooled and total
RNA was extracted (Arcturus, # KIT0204). mRNA from LPZ and peri-
LPZ was simultaneously amplified twice (Arcturus, # KIT0205), and the
quality of amplified RNA was checked on a Bioanalyzer (Fig. 2).

**Microarrays**

We made a cDNA library out of total RNA extracted from a Macaque V1.
The library was cloned into the vector pCMV-Sport 6.1 (Invitrogen,
Carlsbad, CA) and was normalized to increase the representation of low
copy number genes and decrease that of high copy number ones, using
subtraction hybridization at 2 different Cot values (15 and 7.5,
Invitrogen technology). DNA from each clone was then extracted

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**Figure 1.** Retinal lesion and LCM. A surface view of the mapped part of V1 is shown
in panel a. Scale bar = 1 mm. The white dots represent electrode penetrations, with
the map of corresponding RFs shown in panel b, the left eye on the left and the right
eye on the right. Because the animal is anesthetized and paralyzed for the purpose of
RF mapping, the eyes are not converged on the tangent screen, and the RFs from
either eye fall on different locations. The degree of overlap between the lesions in the
2 eyes was evaluated by the position of the lesion relative to the RFs. The retinal
lesions were placed such that the boundary between the LPZ and peri-LPZ fell along
the line marked by penetrations 4, 11, 7, and 10. The boundary is indicated by the
solid black line in a. The injection sites of adenoviruses carrying eGFP or tdTomato
are shown in green and red, respectively, with the eGFP injections within the LPZ and
the tdTomato injections on the other side of the LPZ boundary. Sections showing labeled
neurons in the superficial layers of V1 before and after LCM in LPZ and peri-LPZ are
shown in panel c.

**Figure 2.** RNA quality. Before microarray hybridization, amplified RNA was checked
for quality on a Bioanalyzer. Shown is a typical example of the RNA quality, from 300
laser-captured cells and after 2 rounds of amplification.
using miniprep columns (NuGen, San Carlos, CA) and the cDNA insert was PCR amplified using primers that flanked the multiple cloning site (M13 forward and reverse). A total of 19,200 cDNA clones produced fragments in PCR. They were purified and dissolved in saline sodium citrate at a concentration of 100–150 ng/μL. We print all these clones on a microarray slide (Corning, cat# 40015) using a Microgrid II machine (Genomic Solutions, Ann Arbor, MI). Spots were arranged in 48 blocks with each block containing a 20 by 20 matrix. After printing, DNA was fixed to the slides by UV cross-linking. The quality of the prints was checked by hybridization with random oligos immediately after printing. For microarray hybridizations, we labeled equal amount of amplified RNA from LPZ and peri-LPZ with cy3 (green fluorescent dye) and cy5 (red fluorescent dye), respectively, and hybridized them to one microarray. The slide was then scanned (Perkin Elmer, Waltham, MA) to obtain a 2-color image. Each image was carefully checked for quality of the spots and flagged manually if a spot was not well rounded or fully saturated. The intensity of every unflagged spot was read by Genepix (Axon Instruments, Sunnyvale, CA) and we thus obtained data for subsequent analyses.

**Statistical Analyses**

Data normalization was performed with Matlab (Math Works). Each set of microarray data was normalized with a locally weighted algorithm (LOWESS) (Yang et al. 2002) to remove the artificial effects posed by factors such as the dye bias. Normalized data were then calculated to obtain the ratio of each gene's expression in LPZ and peri-LPZ. We established a threshold at 2-fold in either direction (LPZ/peri-LPZ or peri-LPZ/LPZ). We have a total of 6 sets of microarray data obtained from 6 hemispheres of 4 animals. To reduce the probability of getting false positives, we selected genes that passed the 2-fold threshold in at least 2 hemispheres. For the normal control, we used an animal where no lesion was made. Total RNA was extracted from the same region from which the experimental tissue was taken—the opercular surface of macaque V1. This tissue was used for determining the appropriatness of primer sets used in qRT-PCR for each selected gene. We were able to normalise the expression of each gene to the level of a housekeeping gene, glyceral phosphate dehydrogenase (GAPDH). This enabled us to compare the baseline expression levels in the LPZ/peri-LPZ samples and therefore to obtain an estimate of whether a given gene was upregulated or downregulated in the experimental tissue.

**qRT-PCR**

mRNA from the same animals above, but amplified independently from those used for microarrays, was used for qRT-PCR. cDNA was obtained using SuperScript III First-Strand Synthesis System (Invitrogen). PCR was carried out on an ABI 7900 model according to manufacturer’s instruction (ABI, Carlsbad, CA), with SYBR Green (ABI, #4309155) as the fluorescence dye to indicate the cDNA amount. For each sample, PCR was run for the selected genes and for GAPDH as a control.

**In Situ Hybridization**

Tissues were processed as described above. In situ hybridization was performed as previously described (Ishii et al. 2004). Sections were cut at 12 μm with cryostat and mounted on precoated glass slides (Fisher, Pittsburgh, PA, #12-550-15). Slides were fixed in 2% paraformaldehyde for 15 min, washed in 1× PBS 3 times, acetylated in 50 mL triethanolamine with 125 μL acetic anhydride for 10 min, washed in 1× PBS for 5 min, dehydrated in a series of ethanol with graded concentrations, and left to dry in air. Digoxigenin (DIG) labeled probes (100 ng/μL) were added to the hybridization buffer (Amresco, Solon, OH) and hybridized on the sections in a humid chamber at 60 °C overnight. Slides were then washed and incubated with anti-DIG-AP (Roche) antibody at 4 °C overnight. A color reaction was performed by incubation of the alkaline phosphate chromogen reaction substrate NBT/BCIP until signal could be detected.

To subclone HD-PTP, the cDNA clone from the cDNA library was streaked and the plasmid extracted with miniprep (Qiagen, Valencia, CA). A 150-bp fragment was excised with AccI and religated into the vector pCMV-SPORT6.1. For all the other genes, double-stranded DNA was amplified with primers listed below. They were then cloned into the pGEM-T vector (Promega, Madison, WI). To make the RNA probes, all the subclone plasmids were linearized and RNA was transcribed with either T7 or Sp6 RNA polymerase to obtain the sense and antisense probes, whereas DIG labeling mix (Roche, #11277073910) was provided. Primers used to make the probes are as follows:

**MRCKα:** forward 5’-gcatgaaaataagagccaaacagg-3’, reverse 5’-tgagttgagaaaggaaggaaggg-3’
**ARHGAP10:** forward 5’-agaqcagacacgctcggaat-3’, reverse 5’-gaggaaagggggtctct-3’
**MIR:** forward 5’-agcagcttggagatagaga-3’, reverse 5’-aggctccaaggaact-3’
**GGAl:** forward 5’-ttccccccaccccgttagc-3’, reverse 5’-atgaccccaagttcctccaa-3’
**Cdc42:** forward 5’-taaggttttcaggtcctcagttc-3’, reverse 5’-cctctcctctctctctctct-3’
**Rac1:** forward 5’-tqaggctcttaaatgctgtctga-3’, reverse 5’-cctctcctctctctctctctct-3’
**RhoA:** forward 5’-ttacccttctgccgcccagctgatc-3’, reverse 5’-gttggagcaggttagccacactc-3’

**Results**

**Microarrays Identified Differentially Expressed Genes after Retinal Lesions**

To explore the patterns of gene expression associated with cortical reorganization, we employed the model of remapping of visual topography following retinal lesions. We made focal binocular lesions at homologous positions in the 2 retinas in 4 Macaque monkeys (see Materials and Methods), and chose a time point of 2 weeks following retinal lesion to analyze changes in gene expression in the LPZ and surrounding areas. Although the functional reorganization has been shown to progress over a period up to a year, we have seen changes over a shorter term. The functional remapping of cortical topography spreads from the original LPZ boundary inwards toward the center of the LPZ, and recovery of visually driven activity has been shown to invade the LPZ over a distance of several millimeters within 3 weeks (Gilbert et al. 1990). Concomitant changes have been seen in the sprouting of axons in the LPZ: Although we originally documented sprouting of axons 3–9 months following the lesion (Darian-Smith and Gilbert 1994), we have seen substantial changes beginning from the day of the lesion (Yamahachi et al. 2008). A massive process of axonal sprouting and pruning occurs continuously over the first few weeks following the lesion, and rapid changes are seen at the 2-week time point. We therefore believe that this is an optimal time to observe the changes in gene expression associated with the sprouting.

To reduce dilution effects associated with analyzing gene expression in the highly heterogeneous neuronal population present in the cerebral cortex, and to focus on the population of neurons most closely associated with the sprouting of axons into the LPZ, we selected superficial layer pyramidal neurons for microarray analysis. In order to distinguish different neuronal groups according to their position relative to the LPZ boundary, we labeled cells in the LPZ and peri-LPZ with green or red fluorophores by injecting an adenovirus engineered to carry genes encoding either eGFP or tdTomato (Fig. 1). The labeling enabled us to select pyramidal cells in the superficial layers with LCM. From laser-captured cells, we extracted total RNA and amplified mRNA twice to obtain enough material for microarray hybridization.
We optimized conditions to process the tissue and to obtain the RNA (see the Materials and Methods section). Typically, we modified the standard protocol of tissue treatment in order to keep intact the mRNA inside cells while retaining the fluorescent labeling. mRNA was extracted and amplified in a carefully managed RNase-free condition. The quality of the RNA was monitored after amplifications. Figure 2 shows an example of a well-preserved RNA from 300 laser-captured cells, after being amplified twice. Only after we confirmed the RNA quality did we proceed to microarray hybridization.

For the microarray analysis of gene expression, we made a cDNA library out of the total RNA extracted from a Macaque V1. Although the library cannot be characterized for each clone’s identity, we normalized it to increase the representation of low copy number genes and decrease that of high copy number genes. A total of 19,200 clones were printed on a microarray slide. Equal amounts of amplified RNA, obtained from LPZ and peri-LPZ, were labeled with cyan (green fluorescent dye) and rhodamine (red fluorescent dye), respectively, and hybridized to the array (Fig. 3).

We performed microarray hybridization with independent RNA samples from 6 hemispheres of 4 animals. After statistical analyses (see Methods and Materials), we chose those genes that had at least a 2-fold difference in expression between LPZ and peri-LPZ in 2 or more hemispheres for further analysis (Fig. 3). Of the 192 gene candidates, we sequenced 67. About half of the sequenced genes encoded known proteins with diverse functional roles (Tables 1 and 2). Many of the candidates bore a potential relationship to axonal sprouting. For example, a total of 7 genes appeared to be involved in cytoskeleton assembly. These genes are relevant because axons are composed of microtubules and neurofilament bundles, and actins are enriched in the growth cone that guides axon branching and outgrowth. They are therefore likely involved in regulating the axonal sprouting post lesion.

We also identified genes that are involved in cell-surface signaling in the nervous system, such as ephrin B1 receptor (EPHB1) and latent transforming growth factor beta binding protein 3 (LTBP3). Genes involved in the control of gene expression included transcription factors and histone deacetylase 5 (HDAC5), which regulates transcription through altering the chromosomal structure. These genes may be directly involved in signaling cascades after retinal lesions. We also identified genes in protein processing, such as trafficking and ubiquination. Some of the genes, such as B2-microglobulin (B2M), are known to be involved in immune responses. Interestingly, we also found genes that have been implicated in neural degenerative diseases, such as reticulon3 (RTN3). All the above signaling pathways may influence axon sprouting at different levels (see Discussion).

Validation with qRT-PCR
We tested the expression levels of the microarray candidates with qRT-PCR for validation, with RNA samples from LPZ and peri-LPZ of 3 hemispheres, but independently amplified from the preparation used for microarrays. To normalize the level of
expression to a constitutively expressed gene, GAPDH as a control. We tried to test all the candidates with qRT-PCR; however, because we were working with primate samples that were obtained by LCM, which had very low level of transcripts, we could only obtain results with a subset of genes.

We considered a gene validated when the sign of the difference was the same in all samples, that is, higher expression in LPZ or in peri-LPZ, both in qRT-PCR samples and in microarrays. The exception occurred in some instances where the gene’s expression was below the level of detection with qRT-PCR in one or 2 hemispheres. However, we only considered a gene fully validated when we saw the difference in at least 2 hemispheres. Figure 4 shows all the genes that were validated with qRT-PCR in either all 3 hemispheres (GGA1 and Alzheimer gene) or in 2 of them (the rest).

Genes that were validated by qRT-PCR, and with functions that can be linked to processes involved in neurite outgrowth and functional recovery, were chosen for further investigation.

There are 2 genes on our validated list, GGA1 and HD-PTP, that are both related to the Rho GTPase pathway. This gene group has been found to play a role in neurite outgrowth in vitro and consequently could represent the molecular mechanism for the sprouting of the horizontal axon collateral network that

### Table 1

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Higher expression in LPZ</th>
<th>Higher expression in peri-LPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeleton</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Cell morphology/polarity</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Receptor/signaling</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Transcription</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Trafficking/folding</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ubiquination</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nucleotide synthesis/structure</td>
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<td>3</td>
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<tr>
<td>Immune response</td>
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<td>2</td>
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<tr>
<td>Metabolism</td>
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<tr>
<td>Disease related</td>
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<td>1</td>
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<tr>
<td>Novel</td>
<td>6</td>
<td>17</td>
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</table>

* Twenty-three candidate genes with no known functional association are not listed in this table.

### Table 2

<table>
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<th>Groups</th>
<th>Genes</th>
<th>Functions</th>
<th>Fold change (LPZ/peri)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeleton</td>
<td>MACF1</td>
<td>Cytoskeleton</td>
<td>2.3 ± 0.09</td>
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<td>Cdc42 binding protein kinase (MRCKα1)</td>
<td>Cytoskeleton</td>
<td>2.2 ± 0.02</td>
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<td></td>
<td>Rho GTPase activating protein (ARHGAP10)</td>
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<td>MRCL interacting protein (MIR)</td>
<td>Cytoskeleton</td>
<td>0.45 ± 0.14</td>
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<td>GSA1</td>
<td>Cytoskeleton</td>
<td>0.29 ± 0.04</td>
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<td>Tyrosine phosphatase, nonreceptor type (HD-PTP)</td>
<td>Cytoskeleton</td>
<td>0.37 ± 0.02</td>
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<td></td>
<td>Quattro</td>
<td>Cytoskeleton</td>
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<td>Cell morphology, polarity</td>
<td>DOK1 (downstream of Stk11)</td>
<td>Cell polarity, tumor suppressor</td>
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<td>EPH B1</td>
<td>Growth factor associated</td>
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<td>LTBP3</td>
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<td>Splicing factor, arg/ser rich 46 kDa (SpRP46)</td>
<td>Splicing factor, arg/ser rich 46 kDa (SpRP46)</td>
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<tr>
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<td>NIF3</td>
<td>Transcriptional factor</td>
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<td>E1 enzyme</td>
<td>Protein folding, neural differentiation</td>
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<td>Tyrosol-DNA phosphodiesterase 1 (TDP1)</td>
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<td>Isocitrate dehydrogenase</td>
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<td>Pyruvate-CoA dioxygenase domain containing 1</td>
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<td>Zinc-binding alcohol dehydrogenase</td>
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<td>24-Dehydrocholesterol reductase precursor (DHCR24)</td>
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</tbody>
</table>
underlies remapping of V1 topography. We therefore enlarged our search for genes related to the same pathway in our candidate pools. We found 3 more genes related to the Rho GTPases, ARHGAP10, MRCKa, and MIR. ARHGAP10 and MRCKa were partially validated with qRT-PCR. MRCKa was detected in 1 of the 3 hemispheres tested and had higher expression in the peri-LPZ, consistent with its microarray behavior; ARHGAP10 showed good expression level in peri-LPZ from all 3 hemispheres used in qRT-PCR, but its expression could not be detected in any LPZ sample, suggesting its elevated expression level in peri-LPZ compared with LPZ, an observation in agreement with its microarray behavior. MIR was not detectable with qRT-PCR, presumably because only trace amounts of its mRNA were obtained from the limited number of laser-captured cells. Because of the known association of the Rho GTPases with neurite outgrowth, we focused our attention on the above 5 genes.

Rho GTPase-Related Genes

Five of the genes involved in the Rho GTPase pathway were identified in the microarray analysis. They were Rho GTPase activating protein 10 (ARHGAP10), Cdc42 binding protein kinase (MRCKa), myosin regulatory light chain interacting protein (MIR), Golgi-localizing, gamma-adaptin ear homology domain, Arf-binding protein 1 (GGA1), and protein tyrosine phosphatase, nonreceptor type 23 (HD-PTP).

The Rho GTPases are small guanosine triphosphate (GTP)-bound molecules that control a variety of cellular activities through regulating the actin cytoskeleton (Hall 1998). In neuronal cells, the cytoskeleton is important for a cell’s morphology, targeted movement toward guidance cues, and for neuron branching. Three Rho GTPases, Cdc42, Rac, and RhoA, are thought to be required for proper neurite outgrowth (Koh 2007). Because of their potential role in the axonal sprouting and synaptogenesis underlying cortical reorganization, the 5 candidates identified by microarray analysis, as well as the 3 additional Rho-associated proteins Cdc42, Rac, and RhoA, were of particular interest.

Rho GTPase-Related Genes Are Upregulated in the Peri-LPZ and Downregulated in the LPZ

When validating the Rho GTPase-related genes with qRT-PCR, we also tried to obtain a measure of the levels of expression of GGA1, HD-PTP, MRCKa, and ARHGAP10 relative to normal cortex. We always performed qRT-PCR with cDNA samples obtained from V1 of a normal, nonlesioned animal to ensure that the primer sets were working properly. At the same time, these normal samples enabled us to estimate the level of expression of genes of interest within V1 as a whole. As shown in Figure 5, the expression levels were expressed in terms of the number of PCR amplification cycles required to reach threshold relative to GAPDH (ΔCT). If ΔCT was 1 in the normal sample, but 2 in the peri-LPZ and –1 in the LPZ sample, it means that in normal cortex, this gene is expressed at a level twice as much as that of GAPDH, but 4 times as much as that of GAPDH in the peri-LPZ and only half of GAPDH in the LPZ. These measurements gave us an estimation of the expression levels of the genes relative to GAPDH within each sample (normal or LPZ/peri-LPZ), but it could not be used as a direct indication of the direction of change within the laser-captured population from the experimental tissue. As such, a change in the LPZ/peri-LPZ ratio could be due to either an upregulation on one side or a downregulation on the other. One may be able to evaluate this from some of the in situ hybridization results, however, because when there is an increase in the peri-LPZ/LPZ expression ratio, one frequently sees the highest levels of staining in the peri-LPZ, dropping to lower levels as one moves

Figure 4. Validation with qRT-PCR. Genes that showed differential expression in qRT-PCR in 2 or 3 hemispheres, in agreement with microarray results. (a) Genes that had higher expression in LPZ than peri-LPZ; (b) genes that had higher expression in peri-LPZ than LPZ.

Figure 5. Differential expression of the Rho GTPases-related genes. The expression levels were measured relative to a housekeeping gene, GAPDH, with qRT-PCR. ΔCT is the difference in the cycle numbers relative to GAPDH, more negative levels indicate lower expression than GAPDH. The Rho pathway genes, ARHGAP10, GGA1, MRCKa, and HD-PTP, were expressed at higher levels in the peri-LPZ than in either normal tissue or LPZ. (ND: nondetectable).
farther from the LPZ, as well as decreased expression in the LPZ. This would indicate that the ratio change was due to both an increase in the peri-LPZ and a decrease in the LPZ.

We also performed in situ hybridization on these genes to test if they were expressed in a pattern coincident with the LPZ topography, as well as to validate MIR's differential expression. Because of the distinct topography of cortical plasticity induced by retinal lesions, with a central LPZ undergoing reorganization and a surrounding peri-LPZ with neurons contributing to the reorganization by axonal sprouting, we were able to use in situ hybridization to confirm the involvement of various genes in the reorganization. If the in situ of the gene showed a distribution of expression coincident with the topography of the LPZ, this was taken as evidence of the involvement of the gene in the reorganization process. We tested and confirmed the LPZ-related distribution of the putative plasticity related genes with in situ hybridization on at least 2 experimental hemispheres. Because the tissues for in situ hybridization came from the same hemispheres where eGFP and mRFP were used to differentially label neurons in LPZ and peri-LPZ, the boundary between the 2 regions on sections for in situ was easily established by taking measurements of eGFP or mRFP labeled region in previous LCM experiments where labeled neurons were collected. The same measurements could be applied to differentiate LPZ from peri-LPZ in the sections used for in situ hybridization although no fluorescent labeling is present.

All of the 5 genes had lower mRNA levels in the LPZ than the neighboring region, a pattern that is consistent with microarrays and qRT-PCR results (Fig. 6). As one scans across the section of V1, there is a reduction in the levels of gene expression within the LPZ, an elevation in the peri-LPZ (see, e.g., insets in Fig. 6A), and a drop to intermediate levels as one moves farther from the LPZ boundary into normal cortex.

A Link of the Rho GTPase Pathway to the Postlesion Gene Regulation

Because all the 5 Rho-related proteins were confirmed with in situ hybridization to have a differential expression pattern coincident with LPZ/peri-LPZ topography, we wondered whether these genes reflected a regulated pattern of the Rho GTPase pathway. In order to test that, we reasoned that if the Rho GTPase pathway was targeted to be differentially regulated in the LPZ versus peri-LPZ, the 3 Rho GTPases, Cdc42, Rac1, and RhO, would be likely to have an expression pattern coincident with the LPZ topography. We therefore did in situ hybridizations for these 3 additional genes. Like the above Rho-related genes, they all had a lower mRNA level in the LPZ than in the neighboring region (Fig. 6). This finding suggests that the differential expression of ARHGAP10, MBCKa, MIR, GGA1, and HD-PTP was likely due to changes in the signaling pathway of the Rho GTPases. These results thus link the role of the Rho GTPase pathway to the cortical reorganization post lesion.

Discussion

Functional recovery of visual cortex following retinal lesions provides an ideal model for revealing signal transduction pathways involved in adult cortical plasticity. We can take advantage of the topography of the functional reorganization, with a sharply delineated region of cortex representing the lesioned part of the retina (the LPZ) and a surrounding area (the peri-LPZ) containing neurons that mediate the reorganization via axonal sprouting and synaptogenesis. Utilizing microarrays to measure gene expression, we identified an array of genes that showed differential expression in the LPZ and peri-LPZ. These genes have a wide range of functional roles. A number have functions that bear an intriguing relationship to cortical plasticity, including those associated with the Rho GTPases. This finding establishes a potential link between the alteration in neuronal activity induced by retinal lesions and the axonal sprouting associated with functional recovery of the LPZ.

Our work also provided means for extending the understanding of signal-transduction cascades involved in adult cortical plasticity beyond testing candidate genes. Previous efforts (Zhang et al. 1995; Obata et al. 1999; Massie et al. 2003; Cnop et al. 2007) focused on neurotransmitters, growth factors, synaptic vesicles, and genes related with neurite outgrowth. Among growth factors, several associated with promoting and regulating neurite outgrowth have been shown to be upregulated in the LPZ, including brain-derived neurotrophic factor, neurotrophin 3, nerve growth factor, and insulin-like growth factor 1, collapsin response mediator protein 2 (CRMP2), and CRMP4. Among neurotransmitters, inhibitory neurotransmitter, γ-aminobutyric acid had a decreased concentration in the LPZ, thus effectively magnifying the effect of excitatory inputs to the LPZ from the surrounding area and providing a pathway for visual input from nonlesioned parts of the retina. But it is likely that many more genes are involved in translating alterations in visual experience to modifications of cortical circuits and to obtain a fuller characterization of the molecular mechanisms involved requires an unbiased approach. This is the advantage afforded by gene expression measurements with large-scale microarrays. To optimize the utility of these arrays for the current experiment, we made a cDNA library of genes expressed in the V1 of the Macaque; the library was normalized to expand the representation of genes expressed at lower levels, and we printed a large number of clones to include as many genes involved in visual cortical function as possible. Even so, the microarray approach, when applied to brain tissue, is problematic because of the diversity of cell types found in any brain region. This leads to dilution effects when differentially expressed genes involve only a subpopulation of neurons and the entire tissue is used for analysis. We therefore chose to restrict our analysis to the neurons responsible for the long-range horizontal connections that are likely to be involved in cortical remapping following retinal lesions, cortical pyramidal neurons. The most pronounced axonal sprouting was found among superficial layer pyramidal neurons lying in the peri-LPZ (Darian-Smith and Gilbert 1994), so these neurons were selected for LCM, which has been successfully used to isolate specific cell types in brain tissues (Luo et al. 1999; Sugino et al. 2006) and subsequent microarray analysis.

A large range of functions was represented by the 67 sequenced candidates. Among them, we found 5 genes in the Rho GTPase pathway to be differentially expressed in the LPZ versus peri-LPZ post lesion. Their differential expressions were validated and further confirmed with qRT-PCR and in situ hybridization. An illustration of the mechanism of the Rho GTPase pathway is shown in Figure 7. All the 5 genes seem to be effectors of the Rho proteins. ARHGAP10 was first identified as
a member of the Rho GTPase--activating proteins (Rho-GAPs) (Basseres et al. 2002). The Rho-GAPs (Rho-GTPase-activating proteins) facilitate the hydrolysis of Rho-bound GTP to GDP and therefore inhibit the activity of Rho GTPases (Lamarche and Hall 1994). ARHGAP10 is a Cdc42 GAP localized on the Golgi membrane (Dubois et al. 2005). MRCKα acts as an effector of Cdc42 to promote cytoskeleton reorganization (Leung et al. 1998). MIR is an interacting protein of MRLC protein, whose phosphorylation is mediated by Rho-associated p160 kinase (ROCK) (Uehata et al. 1997). MIR has also been shown to regulate neurite outgrowth (Olsson et al. 1999). GGA1 is one of the GGA proteins that are associated with ARFs on the Golgi structure to regulate formation of filopodia (Nakayama and Takatsu 2005), a process further regulated by Cdc42 (Miura et al. 2002). HD-PTP is a nonreceptor type of the tyrosine phosphatase protein family (Toyooka et al. 2000). It shares with other proteins in this family an N-terminal domain that is similar to a Rho-binding protein, Rhophilin (Nakamura et al. 1999). Other proteins of the family have already been shown to directly link to the Rho GTPases (Nimnual et al. 2003).

Figure 6. In situ hybridizations. The 5 Rho-related genes and 3 Rho GTPases were tested with in situ hybridization for their expression levels on the brain sections from animals with retinal lesions. Hybridizations with both the sense probe and the antisense probe are shown. The extent of the LPZ within the section is shown by the horizontal black line. For each gene, a magnified view of the labeling pattern in LPZ and peri-LPZ is shown in the insets on the left and right, with the position of the magnified area indicated by the black rectangles. The blue bar = 1 mm.
We found with in situ hybridization that the 3 Rho proteins had a similar expression pattern, supporting the involvement of the Rho-related genes in adult cortical plasticity. Studies in neuronal cell lines have shown that Cdc42 and Rac1 promote neurite outgrowth (Sarner et al. 2000; Aoki et al. 2004), whereas blocking RhoA activity or using a dominant negative form of RhoA induces neurite outgrowth (Jalink et al. 1994). Although Cdc42/Rac1 and RhoA were once thought to have antagonistic effects on neurite outgrowth, recent data have shown that both inhibition and overexpression of Rac1, like perturbation of RhoA, reduce lamellar protrusions and disrupt neurite outgrowth (Woo and Gomez 2006), suggesting that coordinated activities of Rho GTPases are needed to maintain proper neural branches. Our findings that all the Rho-related candidates and the 3 Rho proteins showed higher expression levels in peri-LPZ may reflect such a coordination. Negative feedback mechanism between Cdc42/Rac and RhoA may be involved in their function. High-level activities of Cdc42/Rac and their associated activators may result in an elevated activity of RhoA and other opposing factors. The elevation of these proteins can inhibit further enrichment of Cdc42/Rac proteins. Eventually, a balance between the three Rho GTPases is reached to maintain proper neurite outgrowth. This may account for why we saw elevation of expression of all the Rho GTPase genes in the peri-LPZ.

These structural genes, or genes involved in control of neurite outgrowth, were elevated in the peri-LPZ, rather than the LPZ, presumably because the neurons in the peri-LPZ are the ones responsible for axonal sprouting into the LPZ. One gene among our microarray candidates, microtubule-actin cross-linking factor (MACF1), was elevated in the LPZ. The neurons in the LPZ have not yet been characterized in terms of their dendritic and axonal changes.

The Rho GTPases and their effectors are signaling cascades downstream of receptor activation. Different signaling ligands might be involved. One of our microarray candidates that had higher expression in LPZ than peri-LPZ, EPHB1, is an ephrin B1 receptor. Ephrin receptors and their ligands mediate numerous developmental processes, especially important in the nervous system during the critical period (Klein 2004). Although we are studying adult plasticity, mechanisms similar to those involved in developmental plasticity might be recapitulated in adulthood. Another candidate involved in cell-surface signaling is LTBP3, a protein associated with TGF-beta (Yin et al. 1995). The TGF-beta signaling pathway is important in injury responses and is thought to play an important role in neural degeneration (Tesseur and Wyss-Coray 2006). Retinal lesions may represent a model for brain injuries in general, and the TGF-betas are likely to be targeted for postlesion regulation.

Following the signals for axonal sprouting, many genes are targeted for transcriptional changes. In our assays, we found several transcription factors. Transcription factors can play both repressive and activating roles. We saw upregulation of different factors in both LPZ and peri-LPZ. Among proteins associated with transcription regulation, we identified HDAC5, which had higher expression in peri-LPZ than in LPZ. Histones play a pivotal role in regulating gene transcription. Their acetylation and deacetylation alter chromosome structure, thereby opening up or blocking access of transcription factors to DNA, resulting in transcription activation or repression (Kuo and Allis 1998). Posttranslational modification of histones has been implicated in regulating visual cortical plasticity in the critical period (Putignano et al. 2007) although the target molecular pathway has not been identified. Because every transcriptional activation or repression involves changes of the chromatin structure and...
hence modifications of histones, the fact that HDAC5 showed up in our assay may reflect just such a mechanism of gene regulation involved in adult cortical plasticity.

Another gene group potentially involved in synaptic plasticity, and showing differential regulation in our assay, are the histocompatibility genes. We found, for example, B2M differentially regulated in our assay. B2M is the light chain component of the major histocompatibility complex class I (MHC I) protein. MHC I molecules have been largely known to play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen (Simpson 1988). B2M was once used as an internal control for qRT-PCR as it was considered to be constitutively expressed. However, a recent study has shown that the expression level of B2M in the frontal cortex is not stable (Johansson et al. 2007). We confirmed with qRT-PCR that B2M had much higher expression in peri-LPZ than in LPZ. Recent studies have shown that MHC I molecules are important for synaptic plasticity (Huh et al. 2000; Oliveira et al. 2004; Thams et al. 2008). Our finding extends these studies to adult plasticity in early sensory areas.

In addition to the above categories that have direct connections to cortical plasticity, we have identified genes that have been shown to be involved in neural degenerative diseases. RTN3, for example, which showed higher expression in LPZ, is a member of the reticulon family and plays roles in proteomic processing of amyloid precursor protein (APP) (Watari and Yutsudo 2003; Tang and Liu 2007), whose irregular processing is a key point in the development of Alzheimer’s disease. As previously discussed, mechanisms mediating cortical reorganization after retinal lesions may be similar to those engaged in recovery from neural degenerative diseases.

Using microarrays, we have identified genes that may guide the cortical reorganization and axon sprouting following retinal lesions. Although retinal lesions represent an abnormal disruption of sensory input, similar mechanisms may underlie the normal processes of experience-dependent changes, such as those involved with perceptual learning. Moreover, the retinal lesion model may represent an exemplar of functional adaptation to CNS lesions, including those associated with stroke or neurodegenerative disease. As such, it is a useful model for characterizing the signal transduction pathways involved in both normal and disease-related functional changes.

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