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Development/Plasticity/Repair

Genomewide Analysis of Rat Barrel Cortex Reveals Time- and Layer-Specific mRNA Expression Changes Related to Experience-Dependent Plasticity

Astrid Vallés,1,2 Arjen J. Boender,1 Steef Gijsbers,1 Roy A. M. Haast,1 Gerard J. M. Martens,2,* and Peter de Weerd1,*

1Department of Neurocognition, Faculty of Psychology and Neurosciences, Maastricht University, 6200 MD Maastricht, The Netherlands, and 2Department of Molecular Animal Physiology, Radboud University Nijmegen, Donders Institute for Brain, Cognition, and Behaviour (Centre for Neuroscience), Nijmegen Centre for Molecular Life Sciences, 6525 GA Nijmegen, The Netherlands

Because of its anatomical organization, the rodent whisker-to-barrel system is an ideal model to study experience-dependent plasticity. Manipulation of sensory input causes changes in the properties of the barrels at the physiological, structural, and functional levels. However, much less is known about the molecular events underlying these changes. To explore such molecular events, we have used a genomewide approach to identify key genes and molecular pathways involved in experience-induced plasticity in the barrel cortex of adult rats. Given the natural tendency of rats to explore novel objects, exposure to an enriched environment (EE) was used to stimulate the activity of the whisker-to-barrel cortex in vivo. Microarray analysis at two different time points after EE revealed differential expression of genes encoding transcription factors, including nuclear receptors, as well as of genes involved in the regulation of synaptic plasticity, cell differentiation, metabolism, and, surprisingly, blood vessel morphogenesis. These expression differences reflect changes in somatosensory information processing because unilateral whisker clipping showed EE-induced differential expression patterns in the spared versus deprived barrel cortex. Finally, in situ hybridization revealed cortical layer patterns specific for each selected gene. Together, the present study offers the first genomewide exploration of the key genes regulated by somatosensory stimulation in the barrel cortex and thus provides a solid experimental framework for future in-depth analysis of the mechanisms underlying experience-dependent plasticity.

Introduction

Experience-dependent plasticity enables organisms to adapt their behavior to the environment. However, how experience shapes the functional organization of different brain systems is still poorly understood. At the level of sensory systems, the rodent primary somatosensory cortex (S1) is a particularly suited model to investigate the mechanisms underlying experience-dependent plasticity (Feldman and Brecht, 2005). The whisker-to-barrel system plays an important role in various types of rodent behavior, from foraging to object recognition (Brecht, 2007; Diamond et al., 2008). Its organization allows for the precise characterization of experience-dependent plasticity, thanks to the one-to-one correspondence of each whisker to its cortical receptive field in layer 4 of S1, the “barrel” (Woolsey and Van der Loos, 1970). In the barrel cortex, plasticity can be induced by modifying sensory input through simple manipulations, such as exposure to enriched environment (EE) or whisker clipping (Fox, 2002; Polley et al., 2004).

Because of its easy accessibility, the whisker-to-barrel system is an ideal model to integrate a wide range of experimental approaches to dissect plasticity mechanisms at different levels, such as genetics, molecular biology, electrophysiology, and imaging (Brecht et al., 2004; Schubert et al., 2007; Petersen, 2009). Most studies investigating experience-dependent plasticity in barrel cortex make use of electrophysiological methods (Petersen, 2007). More recently, gene targeting and viral vector strategies have also been applied, permitting layer- and column-specific manipulation of the expression of selected genes to study their function (Aronoff and Petersen, 2008), visualization of structural plasticity (Knott and Holtmaat, 2008), and control of neuronal activity to examine network connectivity and behavior through optogenetic approaches (Huber et al., 2008; Petreanu et al., 2009). For such studies, knowledge of the genes expressed in the barrel cortex both under resting conditions and after neuronal stimulation is of crucial importance, in particular of those induced by sensory experience and their cortical layer and cellular expression patterns.

A limited number of studies have determined the expression of selected genes in barrel cortex after activation of the whisker system. Single-whisker experience induces CAMP responsive element (CRE)-dependent gene expression in the spared barrel of transgene reporter mice (Barth et al., 2000), whereas neurtin, a plasticity-related gene, shows differential regulation in spared and deprived barrels (Harwell et al., 2005). Passive whisker stimulation increases brain-derived neurotrophic factor (Bdnf)
Materials and Methods

Animals. Male Long–Evans rats (Harlan) were used, ranging in age from 65 to 75 d. A total of \( n = 36 \) animals were used for microarray and qPCR analysis (group size, \( n = 6–8 \)) and \( n = 24 \) for \textit{in situ} hybridization experiments (group size, \( n = 4 \)). The animals were housed two per cage (37.8 \( \times \) 21.7 \( \times \) 18.0 cm) in a controlled environment under a 12 h light/dark cycle with lights on at 6:30 A.M. Food and water were provided \textit{ad libitum}. Experimental procedures were performed between 7:30 A.M. and 2:30 P.M. All the experimental groups were constructed using matched weight criteria. The experiments were approved by the Animal Ethics Committee of the Radboud University Nijmegen (Nijmegen, The Netherlands), according to Dutch legislation.

Experimental procedure. A schematic overview of the experimental procedure is depicted in Figure 1A. To reduce stress levels, all rats underwent two habituation periods. During the first period, lasting 7 d, the rats were handled and weighed daily. In the second period, lasting 5 d, the rats were placed per two in the empty test cage (round, plastic cage; 0.88 m\(^2\)) for 30 min on a daily basis. Note that two rats from the same home cage were placed together in the test cage and not individually or in a larger group, to avoid stress caused by isolation or by exposure to “stranger” rats (rats from other home cages). To prevent any visual stimulation and promote mainly the use of the somatosensory system, the test cage was located in a room without illumination. The procedures to which the rats were subjected during the five habituation sessions in the empty test cage during the second habituation period were identical to the procedures applied during the actual EE session. Because of this, stress during the EE session can be assumed to be very low. For clarity, the habituation sessions are not part of the sensory enrichment manipulation. After the habituation period, all the rats underwent a short (2–3 min) 2% isoflurane mixed gas \( O_2 \) anesthesia (Pharmachemie BV). During anesthesia, the right whiskers of half of the animals were clipped as close to the skin as possible (CLIP), whereas the whiskers of the other rats were left intact (UNCLIP). The following day, both groups were transferred to a room without illumination, where they either stayed in their home cage (CTR) or were allowed to explore the enriched test cages, per two (EE), in a single session of 30 min. Exploration of the EE took place in the dark to increase somatosensory (vs visual) stimulation. The behaviors of all EE animals (both UNCLIP and CLIP groups) were quantified using The Observer 5.0 (Noldus Information Technology). A period of 10 min was analyzed, starting 10 min after the beginning of the EE exposure. The behavior was divided into six different categories, namely “fighting,” “gnawing,” “grooming,” “rear-
**Table 1. Primers for quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
<th>Amplicon size (bp)</th>
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<td>Abcg2</td>
<td>TCTTCACTGACGACCTTCTTTGTGT</td>
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<td>ApoD1</td>
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<td>Arc</td>
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<tr>
<td>BDNF</td>
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<td>TGGCGATGAGGAGGTTCCAGA</td>
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<td>GGGTGACGAGGGAGCTTG</td>
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</tr>
<tr>
<td>Fos</td>
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</tr>
<tr>
<td>Chz2h</td>
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<td>CAGGGCGAGATGAGAGGAAA</td>
<td>135</td>
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<tr>
<td>Chrm4</td>
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<tr>
<td>CycA</td>
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</table>

*Shown are the names of the genes selected for qPCR analysis, the sequences of the forward and reverse primers from 5’ to 3’, and the size of the final product. The tested housekeeping genes are in bold.*

**Table 2. Primers for probe template generation (in situ hybridization)**

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<th>Genes</th>
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<th>Internal primers (5’→3’)</th>
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<td>702</td>
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</tbody>
</table>

*Shown are the names of the genes selected for in situ hybridization, the sequences of the forward and external and internal primers from 5’ to 3’, and the size of the final product after the second (internal) PCR reaction. The T3 (5’ ATTTTTTTTTTTTTTTT T) and T7 (5’ TTTTTTTTTTTTTTTTTT) RNA polymerase sequences at the 5’ of the forward internal and reverse primers are highlighted in bold. The Arc plasmid containing the full-length rat Arc cDNA subcloned into the EcoRI–XhoI site of pBluescriptII SK(+) (Stratagene) was provided by Dr. P. F. Worley (John Hopkins University). The plasmid was linearized with EcoRI or XhoI and transcribed with T7 or SP6 RNApolymerases to generate antisense and sense probes, respectively.*

**Tissue processing.** All procedures were performed under RNase-free conditions. For microarray and quantitative PCR analysis, the brains were sliced into 300 µm coronal sections using a cryotome (Leica) at −15°C and mounted on glass slides. Cytochrome oxidase-stained reference sections were used as a template to locate the barrel cortex, following stereotactic coordinates (Paxinos and Watson, 1998). Punches of barrel cortex (Fig. 1C) were taken bilaterally using a 2 mm micropunch (Harris Inc.), and samples from each hemisphere were collected separately and stored at −80°C before RNA isolation took place. For *in situ* hybridization, the brains were sliced into 40 µm coronal sections using a sliding microtome (Microm HM440E; Thermo Fisher Scientific). Sections were stored at −20°C in cryoprotectant.
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<th>Gene title</th>
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Table continued
solution (30% ethyleneglycol, 20% glycerol in sodium phosphate buffer, pH 7.3) until additional analysis.

**RNA isolation.** Tissue samples were homogenized with a TissueLyser (Retsch GmbH) in TRIzol Reagent (Invitrogen), according to the protocol of the manufacturer. The procedure was modified for small amounts of tissue by using 800 μl of TRIzol Reagent and adding 1 μl of glycogen (Fermentas). RNA concentration and quality was determined with a NanoDropTM ND-1000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis, respectively. The samples were kept at −80°C until additional analysis.

**Microarray analysis.** Samples from UNCLIP groups (with and without EE) were processed for microarray analysis. To reduce variability, RNA samples were pooled (three samples per pool), resulting in 12 pools (two to four pools per experimental group). The pooled samples were purified with the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co.) and sent to ServiceXS BV for additional processing. Sample concentration and integrity was checked with a Bioanalyzer 2100 (Agilent Technologies), followed by labeling using the Affymetrix Transcript (WT) Single-Stranded Target Labeling kit, hybridization to the GeneChip Rat Gene 1.0 ST Array (both from Affymetrix), and scanning. The Affymetrix Command Console and Expression Console software were used for the performance of the washing, staining, and scanning of the chips. Data quality controls were within Affymetrix specifications.

Normalization of the microarray data was performed with GeneSpring GX version 11 (Agilent Technologies). The raw data were summarized using ExonRMA as a summarization algorithm, followed by log and baseline transformation to the median of all the samples. After transformation, data were filtered on expression (20–100th percentile). Normalized data were analyzed by Significance Analysis of Microarrays (SAM) as described previously (Tusher et al., 2001), using the “samr” package (http://www-stat.stanford.edu/~tibs/SAM) in R version 2.12.0 (http://www.r-project.org). Criteria for differential expression were a p value ≤ 0.05 (delta 2.05) and a fold change (FC) ≥ 1.2. Gene ontology (GO) enrichment analysis of the differentially expressed genes was performed using the Web-based gene ontology tool from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov) (Dennis et al., 2003; Huang et al., 2009). This analysis was performed by using the Functional Annotation Chart (in which GO enrichment in the list of differentially expressed genes is tested) and the Functional Annotation Clustering tool (which groups redundant GO terms in clusters to facilitate the interpretation of the results). For the enrichment analysis (Functional Annotation Chart tool), default software settings were used, and GO terms with a p value [or EASE score (for Expression Analysis Systematic Explorer)] ≤ 0.05 were considered to be overrepresented (enriched). For the Functional Annotation Clustering tool, the classification stringency was set as high, and only clusters with an enrichment score ≥ 1.3 (equivalent to non-log scale p of 0.05) were considered.

**Quantitative PCR.** Before cdNA synthesis, 2 μg of each RNA sample was treated with 2 U of DNase (Fermentas) in the presence of Ribolock RNase Inhibitor (20 U/μl) (Fermentas). For cdDNA synthesis, through random priming, the RevertAid H Minus First Strand cdDNA Synthesis kit (Fermentas) was used, following the guidelines of the manufacturer. Before analysis, 15 μl of each cdDNA sample was diluted with 185 μl of MilliQ water. qPCR reactions were performed with the Rotor–Gene 6000 Series (Corbett Life Science Pty. Ltd.). For each reaction, 2.5 μl of each diluted sample of cdDNA was added to a mix containing 6.25 μl of 2× Maxima SYBR Green qPCR Master Mix (Fermentas), 1 μl of each primer (5 μm), and 1.75 μl of MilliQ water. Primers were designed using NCRI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthe-

### Table 3. Continued

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<td>1.21E-03</td>
<td>1.42 Up</td>
<td>1.02 Down</td>
</tr>
<tr>
<td>10795289</td>
<td>Hist1h2ail</td>
<td>Histone cluster 1, H2ai-like</td>
<td>1.22E-03</td>
<td>1.34 Down</td>
<td>1.00 Down</td>
</tr>
<tr>
<td>10826672</td>
<td>Alp1k</td>
<td>β-Kinase 1</td>
<td>1.23E-03</td>
<td>1.03 Up</td>
<td>1.25 Down</td>
</tr>
<tr>
<td>10849988</td>
<td>Lrp1b</td>
<td>Low-density lipoprotein-related protein 1B (deleted in tumors)</td>
<td>1.24E-03</td>
<td>1.31 Up</td>
<td>1.38 Down</td>
</tr>
<tr>
<td>10882511</td>
<td>Mom2</td>
<td>MORN repeat containing 2</td>
<td>1.25E-03</td>
<td>1.11 Down</td>
<td>1.27 Down</td>
</tr>
<tr>
<td>10875420</td>
<td>LOC502940</td>
<td>Pro-histogranin 1</td>
<td>1.26E-03</td>
<td>1.21 Down</td>
<td>1.13 Up</td>
</tr>
<tr>
<td>10878565</td>
<td>Abcc4</td>
<td>ATP-binding cassette, subfamily C (CFTR/MRP), member 4</td>
<td>1.28E-03</td>
<td>1.23 Down</td>
<td>1.11 Up</td>
</tr>
<tr>
<td>10854303</td>
<td>Cpa4</td>
<td>Carboxypeptidase A4</td>
<td>1.28E-03</td>
<td>1.19 Down</td>
<td>1.21 Down</td>
</tr>
<tr>
<td>10936346</td>
<td>Lonf1</td>
<td>LON peptidase N-terminal domain and ring finger 3</td>
<td>1.31E-03</td>
<td>1.09 Up</td>
<td>1.27 Down</td>
</tr>
<tr>
<td>10852580</td>
<td>Hiph1</td>
<td>Homeodomain interacting protein kinase 1</td>
<td>1.34E-03</td>
<td>1.15 Up</td>
<td>1.30 Down</td>
</tr>
<tr>
<td>10752654</td>
<td>Znf654</td>
<td>Zinc finger protein 654</td>
<td>1.36E-03</td>
<td>1.25 Down</td>
<td>1.06 Down</td>
</tr>
<tr>
<td>10877896</td>
<td>Fam29a</td>
<td>Family with sequence similarity 29, member A</td>
<td>1.40E-03</td>
<td>1.13 Up</td>
<td>1.49 Down</td>
</tr>
<tr>
<td>10879966</td>
<td>Hnprl</td>
<td>Heterogeneous nuclear ribonucleoprotein L-like</td>
<td>1.42E-03</td>
<td>1.22 Up</td>
<td>1.02 Up</td>
</tr>
<tr>
<td>10764404</td>
<td>Zbb9</td>
<td>Zinc finger and BTB domain containing 41</td>
<td>1.42E-03</td>
<td>1.26 Up</td>
<td>1.11 Down</td>
</tr>
<tr>
<td>10763127</td>
<td>RGD156237</td>
<td>Similar to chromosome 6 open reading frame 70</td>
<td>1.43E-03</td>
<td>1.13 Up</td>
<td>1.25 Down</td>
</tr>
<tr>
<td>10843125</td>
<td>Pcmtd2</td>
<td>Protein-c-isoaspartate (o-aspartate) O-methyltransferase domain containing 2</td>
<td>1.43E-03</td>
<td>1.10 Up</td>
<td>1.24 Down</td>
</tr>
<tr>
<td>10868669</td>
<td>Zchc7</td>
<td>Zinc finger, CCHC domain containing 7</td>
<td>1.44E-03</td>
<td>1.04 Up</td>
<td>1.25 Down</td>
</tr>
<tr>
<td>10902614</td>
<td>Rap1b</td>
<td>RAP1B, member of RAS oncogene family</td>
<td>1.45E-03</td>
<td>1.22 Up</td>
<td>1.06 Down</td>
</tr>
<tr>
<td>10702126</td>
<td>LOC687333</td>
<td>Similar to zinc finger protein 59</td>
<td>1.45E-03</td>
<td>1.26 Up</td>
<td>1.07 Up</td>
</tr>
<tr>
<td>10868007</td>
<td>Casp8ap2</td>
<td>Caspase 8 associated protein 2</td>
<td>1.46E-03</td>
<td>1.15 Up</td>
<td>1.25 Down</td>
</tr>
<tr>
<td>10746626</td>
<td>Ivnslabp</td>
<td>Influenza virus NS1A binding protein</td>
<td>1.47E-03</td>
<td>1.22 Up</td>
<td>1.01 Down</td>
</tr>
<tr>
<td>10901039</td>
<td>LOC100364912</td>
<td>rCG29233-like</td>
<td>1.50E-03</td>
<td>1.21 Up</td>
<td>1.05 Down</td>
</tr>
<tr>
<td>10815442</td>
<td>Spg20</td>
<td>Spastic paraplegia 20 (Trayer syndrome) homolog (human)</td>
<td>1.50E-03</td>
<td>1.11 Up</td>
<td>1.25 Down</td>
</tr>
<tr>
<td>10850492</td>
<td>RGD1308023</td>
<td>Similar to G5S21-PA</td>
<td>1.51E-03</td>
<td>1.15 Up</td>
<td>1.24 Down</td>
</tr>
<tr>
<td>10850585</td>
<td>Pnx4</td>
<td>Peroxoxosomal membrane protein 4</td>
<td>1.56E-03</td>
<td>1.21 Down</td>
<td>1.06 Down</td>
</tr>
</tbody>
</table>

*Shown are the Affymetrix transcripts cluster ID, gene symbol, gene title, SAM q value, and fold change at t = 0 h and t = 4 h, together with the direction of regulation (up or down), of the differentially expressed genes obtained by microarray analysis in the barrel cortex after enriched environment. Genes are ordered by significance (most significant genes on top). Only well-annotated genes are included.*
Table 4. Functional clusters of enriched GO categories (most significant category of each cluster shown)

<table>
<thead>
<tr>
<th>Annotation cluster #</th>
<th>Term</th>
<th>Category</th>
<th>Count</th>
<th>Genes</th>
<th>p value</th>
<th>Fold enrichment</th>
<th>Enrichment score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transcription</td>
<td>GOTERM_BP_FAT</td>
<td>29</td>
<td>Per1, Ddit3, Eg2, Jun, Arid4b, Klf2, Nr4a3, Eid3, Ccnl1, Rax, Med14, Eg4, Tmxip, Nr4a2, Nf63, Fos, JunB, Npax4, Nr4a1, Btg2, Eg3, Zbt10, Cdy2, Gtf2a2, Crem, Stat1/Stat4, Afl3, FosB, Eg1</td>
<td>9.08E-09</td>
<td>3.49</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>Positive regulation of macro molecule metabolic process</td>
<td>GOTERM_BP_FAT</td>
<td>28</td>
<td>Ddit3, Comp1, Klf4, Eg2, Zfp36, Rnf6, Jun, Jr2a, F2, Klf2, Nr4a3, Hnpp1, Med14, Eg4, Barx2, Cdx1, Nr4a2, Rel, Gmpra2/Tnf, Klf4, Fos, JunB, Npax4, Nr4a1, Inhba, Gtf2a2, Pdgfb, Sertad1, Egr1</td>
<td>2.23E-06</td>
<td>2.74</td>
<td>5.9</td>
</tr>
<tr>
<td>3</td>
<td>Basic-leucine zipper (bZIP) transcription factor</td>
<td>Regulation of apoptosis</td>
<td>INTERPRO</td>
<td>Sgk1, Tnp1, Ddit3, Hif1k, Nr4a2, Rel, Jun, Rock1, Casp8ap2, Nr4a1, Btg2, Bdnf, Skil, Inhba, Dusp1, Hspb1, G2e3, Hspa1a/Hspa1l/Hspa1b, Ptg2, Bir2, Stat1/Stat4</td>
<td>1.34E-06</td>
<td>13.89</td>
<td>5.1</td>
</tr>
<tr>
<td>4</td>
<td>Membrane-enclosed lumen</td>
<td>GOTERM_CC_FAT</td>
<td>27</td>
<td>Kir4, Rnf6, Jun, Dld, Ptg2, Ccn1, Med14, Barx2, Tmxip, Dusp6, Zbtb33, Hif1k, Dusp4, Rps6ka5, Rel, Mdn, Gmpra2/Tnf, Klf4, Fos, Npax4, Nr4a1, Cdx2/2a, Gtf2a2, Pdgfb, Fxl1-1/Fx1, Crem, Stat1/Stat4, Afl3</td>
<td>1.90E-04</td>
<td>2.13</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>Negative regulation of cellular biosynthetic process</td>
<td>GOTERM_BP_FAT</td>
<td>18</td>
<td>Per1, Bax2, Tnp1, Klf4, Dnajb5, Gmpra2/Tnf, Sikt, Klf4, Kdm6b, Inv1q/L0688922, Skil, Foxn3, Bcl6b, Zbtb10, Pdgfb, Pdcd4, Edf1, Egr1, Hbegf</td>
<td>9.11E-03</td>
<td>3.00</td>
<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>Response to oxidative stress</td>
<td>GOTERM_BP_FAT</td>
<td>8</td>
<td>N6d, Tnp1, Ddit3, Dusp1, Ptg2, Jun, Stat1/Stat4, Fos</td>
<td>6.38E-03</td>
<td>3.63</td>
<td>2.7</td>
</tr>
<tr>
<td>7</td>
<td>Regulation of synaptic transmission</td>
<td>GOTERM_BP_FAT</td>
<td>8</td>
<td>Pkia, Bdnf, Plat, Arc, Egr2, Ptg2, Edf1, Egr1</td>
<td>3.68E-03</td>
<td>4.02</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>Transcription from RNA polymerase II promoter</td>
<td>GOTERM_BP_FAT</td>
<td>7</td>
<td>Med14, Ddit3, Gtf2a2, Jun, FosB, JunB, Fos</td>
<td>1.10E-05</td>
<td>5.95</td>
<td>2.4</td>
</tr>
<tr>
<td>9</td>
<td>Phosphate metabolic process</td>
<td>GOTERM_BP_FAT</td>
<td>20</td>
<td>Sgk1, Dusp6, Dusp4, Pim3, Gpd1, Rps6ka5, Apk1, Sikt, Rock1, Fam13b, Dld, Bdnf, Pkia, Kdm6b, Gadd45b, Gadd45g, Dusp1, Pkis, Lats1, Stat1/Stat4, Trib1</td>
<td>4.08E-03</td>
<td>2.02</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>Blood vessel morphogenesis</td>
<td>GOTERM_BP_FAT</td>
<td>8</td>
<td>Gx2a, Plat, Cyn61, Tiparp, Jun, Edn1, Jun1, Apd1</td>
<td>6.98E-03</td>
<td>3.57</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>Negative regulation of protein kinase activity</td>
<td>GOTERM_BP_FAT</td>
<td>6</td>
<td>Dusp6, Gadd45g, Gadd45b, Hmgcr, Pdcd4, Trib1</td>
<td>1.64E-03</td>
<td>6.94</td>
<td>1.7</td>
</tr>
<tr>
<td>12</td>
<td>Positive regulation of apoptosis</td>
<td>GOTERM_BP_FAT</td>
<td>10</td>
<td>Nr4a1, Tnp1, Inhba, Ddit3, Dusp1, Hif1k, Ptg2, Jun, Stat1/Stat4, Stat1/Stat4, Afl3</td>
<td>1.91E-02</td>
<td>2.48</td>
<td>1.7</td>
</tr>
<tr>
<td>13</td>
<td>Zinc finger, C2H2-type/integrase, DNA-binding</td>
<td>INTERPRO</td>
<td>7</td>
<td>Egr4, Egr3, Zbtb10, Eg2, Klf2, Eg1</td>
<td>4.49E-02</td>
<td>3.07</td>
<td>1.7</td>
</tr>
<tr>
<td>14</td>
<td>Response to bacterium</td>
<td>GOTERM_BP_FAT</td>
<td>7</td>
<td>Defb10, Ptg2, Jun, Stat1/Stat4, Fos, Pck1, Trib1</td>
<td>4.62E-02</td>
<td>2.67</td>
<td>1.6</td>
</tr>
<tr>
<td>15</td>
<td>Response to progesterone stimulus</td>
<td>GOTERM_BP_FAT</td>
<td>4</td>
<td>Tmxip, FosB, JunB, Fos</td>
<td>6.56E-03</td>
<td>10.30</td>
<td>1.6</td>
</tr>
<tr>
<td>16</td>
<td>ATPase activity, coupled to transmembrane movement of substances</td>
<td>GOTERM_BP_FAT</td>
<td>5</td>
<td>Abcc4, Atp11b, Atp13a3/L068704, Abcg2, Abcb1</td>
<td>3.19E-02</td>
<td>4.15</td>
<td>1.6</td>
</tr>
<tr>
<td>17</td>
<td>Dual specificity protein phosphatase (MAP kinase phosphatase)</td>
<td>PIR_SUPERFAMILY</td>
<td>8</td>
<td>Dusp6, Dusp1, Dusp4</td>
<td>1.83E-03</td>
<td>42.71</td>
<td>1.4</td>
</tr>
<tr>
<td>18</td>
<td>ABC transporters</td>
<td>KEGG_PATHWAY</td>
<td>4</td>
<td>Abcc4, Abcd2, Abcg2, Abcb1a</td>
<td>8.77E-03</td>
<td>9.14</td>
<td>1.4</td>
</tr>
<tr>
<td>19</td>
<td>Nerve growth factor IB-like nuclear receptor</td>
<td>KEGG_PATHWAY</td>
<td>3</td>
<td>Nr4a1, Nr4a2, Nr4a3</td>
<td>5.58E-04</td>
<td>71.18</td>
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<tr>
<td>20</td>
<td>Sterol biosynthetic process</td>
<td>KEGG_PATHWAY</td>
<td>4</td>
<td>Inv1q/L0688922, Id1, Hmgcr, Ceh25</td>
<td>5.98E-03</td>
<td>10.64</td>
<td>1.3</td>
</tr>
<tr>
<td>21</td>
<td>Negative regulation of apoptosis</td>
<td>GOTERM_BP_FAT</td>
<td>9</td>
<td>Btg2, Sgk1, Bdnf, Hspb1, Nr4a2, G2e3, Hspa1a/Hspa1l/Hspa1b, Hspa1a/Hspa1b, Atp13a3/L068704, Lats1, Arl1b, Hnpp1, Abca1a, Trib1, Pim3, Gpd1, Rps6ka5, Kif5b, Arl4d, Arl13b, Alpk1, Sikt1, Mat2a, LOC499330, Prkx, Mat2a, Abcd2, Cyd2, Rabb18, Dynclh1</td>
<td>1.81E-02</td>
<td>1.47</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Shown are the different functional clusters of significantly enriched GO categories of the differentially expressed genes. For each cluster, the term, category, count, and genes (Gene symbol) are shown, together with the p value and the fold enrichment.
for variance homogeneity using Levene’s test, and, in case of non-homogeneous data, a log transformation was applied. To directly compare the values (FC) obtained by microarray and qPCR analysis, a two-tailed Student’s t test, with a significance level of 0.05, was used to test with a one-sample t test (test value 1, significance level p ≤ 0.05) whether expression levels in the spared (R) side were higher than in the deprived (L) side.

In situ hybridization. The plasmid containing the full-length rat activity-regulated cytoskeleton-associated protein (Arc) cdNA subcloned into the EcoRI–Xhol site of pBluescript SK+ (Stratagene) was kindly provided by Dr P. F. Worley (John Hopkins University, Baltimore, MD). The plasmid was linearized with EcoRI or Xhol and transcribed with T7 or SP6 RNA polymerases to generate antisense and sense probes, respectively. For the other genes, nested PCR was used to obtain DNA templates for sense and antisense probe generation of the gene of interest.

Primers were designed using NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized at Biologico BV, adding T3 and reverse internal primers, respectively. For the other genes, nested PCR was used to obtain DNA templates for sense and antisense probe generation of the gene of interest. Primers were designed using NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized at Biologico BV, adding T3 and reverse internal primers, respectively. For the other genes, nested PCR was used to obtain DNA templates for sense and antisense probe generation of the gene of interest.

For variance homogeneity using Levene’s test, and, in case of non-homogeneous data, a log transformation was applied. To directly compare the values (FC) obtained by microarray and qPCR analysis, a two-tailed Student’s t test, with a significance level of 0.05, was used to test with a one-sample t test (test value 1, significance level p ≤ 0.05) whether expression levels in the spared (R) side were higher than in the deprived (L) side.

Table 5. Significantly overrepresented pathways

<table>
<thead>
<tr>
<th>Pathway #</th>
<th>Term</th>
<th>Category</th>
<th>Count</th>
<th>Genes</th>
<th>p value</th>
<th>Fold enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MAPK signaling pathway</td>
<td>KEGG_PATHWAY</td>
<td>16</td>
<td>Rap1b, Dusp6, Ddit3, Dusp4, Rps6ka5, Jun, Fos, Nra1, Bdnf, Hspb1, Gadd45g, Dusp1, Gadd45b, Prkx, Hspa1l, Pdgfb</td>
<td>2.20E-08</td>
<td>5.98</td>
</tr>
<tr>
<td>2</td>
<td>Platelet-derived growth factor receptor signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>5</td>
<td>Txnip, Plat, Csrnp1, Tiparp, Pdgfb</td>
<td>1.41E-04</td>
<td>18.14</td>
</tr>
<tr>
<td>3</td>
<td>Enzyme linked receptor protein signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>12</td>
<td>Rap1b, Dusp6, Ddit3, Dusp4, Rps6ka5, Jun, Fos, Nra1, Bdnf, Hspb1, Gadd45g, Dusp1, Gadd45b, Prkx, Hspa1l, Pdgfb</td>
<td>5.43E-04</td>
<td>3.55</td>
</tr>
<tr>
<td>4</td>
<td>Transmembrane receptor protein tyrosine kinase signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>8</td>
<td>Tnixip, Plat, Csrnp1, Tiparp, Pdgfb, Jun, Tob1, Fos, Dok1, Ins2, Hbegf</td>
<td>8.31E-03</td>
<td>3.45</td>
</tr>
<tr>
<td>5</td>
<td>Transmembrane receptor protein serine/threonine kinase signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>5</td>
<td>Skil, Pdgfb, Jun, Tob1, Fos</td>
<td>1.82E-02</td>
<td>4.93</td>
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<tr>
<td>6</td>
<td>Transforming growth factor beta receptor signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>4</td>
<td>Skil, Pdgfb, Jun, Tob1, Fos</td>
<td>1.83E-02</td>
<td>7.10</td>
</tr>
<tr>
<td>7</td>
<td>Neurotrophin signaling pathway</td>
<td>KEGG_PATHWAY</td>
<td>5</td>
<td>Rap1b, Bdnf, Rps6ka5, Jun, Ins2</td>
<td>3.63E-02</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Shown are the pathways that were overrepresented from the GO analysis of the differentially expressed genes. For each pathway, the term, category, count, and genes (Gene symbol) are shown, together with the p value and the Fold enrichment.
kit (Qiagen Benelux BV), measuring their concentration with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Sense and antisense digoxigenin (DIG)-labeled probes were generated by *in vitro* transcription using SP6, T3, or T7 RNA polymerases, depending on the probe. The 20 μl of mixture included 0.3–1 μg of probe template, 1 mM each of ATP, CTP, and GTP, 0.7 mM UTP, 0.3 mM DIG–UTP (Roche Applied Science), 40 U of RiboLock RNase inhibitor, 1/1000 transcription buffer, and 40 U of SP6, T7, or T3 RNA polymerase (all from Fermentas). After 2 h incubation at 37°C, the probe template was digested with 2 U of RNase-free DNase I (Fermentas) for 15 min at 37°C. The labeled cRNA probe was then precipitated overnight by adding 1 μl of glycogen (Fermentas), 2.5 μl of 4 M LiCl, and 75 μl 100% ethanol and recovered by centrifuging at 4°C for 15 min. The pellet was washed twice and resuspended in 25 μl of DEPC H2O. The amount of labeled probe was quantified by spot blot using DIG-labeled control RNA of known concentration (Roche Applied Science) for comparison. The resulting DIG-labeled probes were stored in nuclease free water at −20°C.

*In situ* hybridization was performed in free-floating tissue sections using DIG-labeled riboprobes as described previously (Schaeren-Wildi et al., 1993). Shown is the amount of time that the animals displayed a certain behavior (namely, fighting, gnawing, grooming, rearing, whisking, and other behaviors). The measurements were performed during a period of 10 min, starting 10 min after the beginning of the EE exposure, in both UNCLIP and CLIP animals (white and gray bars, respectively). Bars represent average ± SEM (n = 6–8). Homogeneous subsets are indicated with the same characters (a–c) above the bars (two-way ANOVA, p ≤ 0.001; post hoc SNK test).
Wiemers and Gerfin-Moser, 1993; Korosi et al., 2006), with minor modifications. All the steps were performed under RNase-free conditions and at room temperature unless stated otherwise. Throughout the procedure, the sections were kept under gentle agitation in six-well plates using Netwells (both from Corning Inc.). Briefly, sections were washed in PBS followed by a 30 min postfixation with 4% PFA in 0.1 M Borax. Next, sections were permeabilized with 0.1 M TEA containing 0.25% acetic anhydride (Sigma-Aldrich), followed by overnight incubation at 58°C in hybridization buffer [50% deionized formamide (Ambion), 1× Denhardt’s solution and 10% dextran sulfate (both from Sigma-Aldrich), 0.5 mg/ml tRNA (Roche Applied Science), 0.3 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 8.0] containing 1 ng/ml of the DIG-labeled probe. The next day, sections were washed in 4× SSC and treated with 0.01 mg/ml RNase A (Sigma-Aldrich) in 0.5 M NaCl, 0.01 M Tris, and 1 mM EDTA buffer, pH 8.0, for 15 min at 37°C, followed by washing steps in decreasing concentrations of SSC, including a 30 min wash in 0.1× SSC at 58°C, several rinses in TBS, and 1 h blocking [0.05% blocking reagent (Roche Applied Science) in TBS]. Sections were then incubated for 3 h with sheep anti-DIG–AP (1:5000; Roche Applied Science), followed by several rinses in alkaline phosphatase (AP) buffer (50 mM MgCl₂ in TBS, pH 9.5) and overnight incubation with nitroblue-tetrazolium-chloride/5-bromo-4-chlor-indolyl-phosphate (NBT/BCIP) medium [175 μl NBT/BCIP stock solution (Roche Applied Science) in 10 ml of AP buffer, containing 0.24 mg/ml levamisole] in the dark. Staining was stopped by several washes in 0.1 M Tris, 0.01 M EDTA buffer, pH 8.0, and sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific), air dried, dehydrated in increasing ethanol concentrations, fixed briefly with isopropanol and acetone, cleared in xylene, and coverslipped with Entellan (Merck Chemicals). The sections were examined under a Leica DM 6000B microscope and representative pictures captured with a Leica DFC480 CCD camera using Leica IM500 imaging software (Leica Microsystems). In addition, a qualitative analysis was performed by a blind observer to determine expression levels, ranked as undetectable (0), low (1), medium (2), high (3), and very high (4), in various brain regions (striatum, cortex, hippocampus, and cerebellum) and in the different cortical layers.

Results

Microarray analysis reveals differentially expressed genes in rat barrel cortex after in vivo somatosensory experience

To study experience-dependent changes in mRNA expression at the level of the
barrel cortex, adult rats were placed in the dark, per two, in an enriched cage during a short (30 min) period. The animals had been habituated previously to the empty test cages to minimize stress-induced artifacts. In contrast to caged-control (CTR) animals, EE exposure induced strong exploratory behavior, especially through active whisking. Importantly, stress-induced behaviors such as self-grooming (Spruijt et al., 1992) were hardly observed, indicating that the habituation procedure successfully reduced novelty-associated stress.

To determine which genes were differentially expressed in the barrel cortex after EE, mRNA expression profiling was performed using microarrays. Expression levels were determined at two time points after the EE session, namely 0 and 4 h, and compared with those observed in CTR rats. Significant differences in gene expression were observed at both time points after EE compared with the respective CTR groups, with 170 genes upregulated and 31 downregulated at t = 0 h and 29 upregulated and 98 downregulated genes at t = 4 h (Table 3). Of all the differentially expressed genes, 40 were identified at both time points after EE.

Validation of microarray results by qPCR analysis
To validate our results, we verified the differential expression of a subset of genes by qPCR analysis. The genes were selected on the basis of their functional annotation (a wide range of biological functions was chosen, including transcription factor activity, nuclear receptors, cholesterol metabolism, and regulation of synaptic plasticity). A total of 18 genes, ranging from low to high FC and p values, were selected for validation, together with three housekeeping gene candidates for normalization (for a list of selected genes and primer pairs, see Table 1). To estimate the validation rate, the normalized expression levels obtained by qPCR (normalized against CycA and Ryhwhaz as the two most stable housekeeping genes) were used to calculate an FC for each gene at each time point, similar to the microarray analysis (i.e., EE vs CTR at t = 0 h and t = 4 h). The FCs obtained by the microarray and qPCR analyses were highly correlated (Pearson’s correlation, two-tailed, r² = 0.992, p < 0.001 at t = 0 h and r² = 0.927, p < 0.001 at t = 4 h), strongly supporting the validity of the criteria used for the microarray data analysis. In summary, these results show that the expression of a number of genes is modified by increased sensory exploration in the rat barrel cortex, the majority of genes being upregulated at t = 0 h and downregulated at t = 4 h.

Overrepresented GO categories of the differentially expressed genes
The observed massive EE-induced regulation of gene expression in the barrel cortex suggests that a short but intense period of sensory experience is able to trigger major cellular changes, which could potentially lead to changes in neuronal circuits. To classify these changes, we used a GO enrichment and functional clustering analysis [DAVID version 6.7 (Dennis et al., 2003; Huang et al., 2009)] as a tool to test whether particular functional categories were overrepresented (enriched) in our dataset. From the list of differentially expressed genes (both at t = 0 h and t = 4 h), as much as 249 significantly overrepresented GO terms were identified, using as a background list all genes present on the array (modified Fisher’s exact test, EASE score ≤ 0.05). These GO terms were grouped into 23 functional clusters (DAVID Fuzzy clustering, enrichment score ≥ 1.3), including regulation of cel-
lular metabolic processes (nucleic acid, phosphate, and cholesterol metabolic processes), gene expression (transcription factors and nuclear receptors), apoptosis, synaptic plasticity, and blood vessel morphogenesis, among others (Table 4). Some signaling pathways were also overrepresented, such as the mitogen-activated protein kinase (MAPK) and the platelet-derived growth factor (PDGF) receptor signaling pathways (Table 5). With a similar analysis of 288 randomly selected genes (25 iterations), we obtained 29.9 ± 10.7 significantly enriched GO terms and 3.0 ± 1.7 functional clusters, indicating a false-positive rate of ~10%. The GO enrichment and functional clustering analysis indicates that many of the EE-induced changes in gene expression are involved in general cellular processes, including cell growth and differentiation, regulation of transcription, and several metabolic processes. In addition, overrepresented clusters include functional categories related to synaptic plasticity, strongly linking our findings at the molecular level to EE-induced anatomical and physiological changes in rat barrel cortex (Fox, 2002; Polley et al., 2004).

In situ hybridization of selected genes reveals cortical layer-specific patterning of mRNA expression

To study the expression patterns of EE-induced genes at the cellular level, in situ hybridization was performed for selected genes. This information will reveal in which cortical layers these genes are expressed and, more specifically, link the time-specific induction of these genes with specific locations in the cortical network of the barrel cortex. From the list of differentially expressed genes, we selected nine differentially expressed genes based on (1) qPCR validation, (2) strong regulation on EE (high FC), (3) different peaks of activation (0 h, 4 h, or both time points), and (4) different functional annotations. Included were genes previously known to be involved in neuronal plasticity and neuronal activation, such as the transcription factors Fos and Egr3, the cytoskeletal protein Arc, the nuclear factor Nr4a2, and the protein neuronal pentraxin 2 (Nptx2, also known as Narp, for neuronal activity-regulated protein). In addition, the vascular-related protein apolipoprotein L domain containing 1 (Apoldl, also known as Verge, for vascular early response gene protein), the anti-proliferative protein Btg2, the enzyme Ch25h (cholesterol metabolism), and the heparin-binding protein cysteine-rich angiogenic inducer 61 (Cyr61) were selected for in situ hybridization analysis.

The immediate-early gene and transcription factor Fos has been used extensively as a marker of neuronal activation (Sheng and Greenberg, 1990; Curran and Morgan, 1995). We used this marker to examine the main brain areas that were activated on EE exposure at $t = 0$ h. In addition to cerebral cortex, we found evidence of activity in striatum, hippocampus, and cerebellar cortex, all areas strongly associated with locomotor activity and spatial learning (Fig. 2). Interestingly, in stress-related areas such as the hypothalamic paraventricular nucleus and the amygdala, the Fos transcript was undetectable (data not shown), indicating low stress levels in these animals (most likely because of the habituation of the animals preceding EE). In addition, we investigated the expression of the other eight selected genes in cerebral cortex, all areas strongly associated with locomotor activity and spatial learning (Fig. 2). Unfortunately, Apoldl expression was below detection levels (data not shown); note that detection of Apoldl also failed in the Allen Mouse Brain Atlas (Lein et al., 2007). For the remaining seven

Figure 7. qPCR analysis of gene expression in the rat barrel cortex after EE. Normalized expression values of Bdnf, Egr3, Nr4a2, and Pcsk1 mRNA in rat somatosensory cortex at $t = 0$ h and $t = 4$ h after exposure to EE for 30 min, in UNCLIP (right group of bars) and CLIP (left group of bars) animals. For normalization, CypA and Ywhaz were selected as housekeeping genes. Measurements were made separately for left (L, white bars) and right (R, gray bars) barrel cortices. Note that, in CLIP groups, L barrel cortex is deprived of sensory input attributable to unilateral clipping of the right whiskers. Bars represent average normalized expression values ± SEM. * represents significant differences between CTR and EE groups (two-way ANOVA, $p = 0.05$; post hoc SNK test). # represents significant differences between left and right barrel cortices within the same group and time point ($p = 0.05$, Student’s t test). In the deprived (L) barrel cortex of CLIP animals, for all analyzed genes, expression levels were significantly higher in EE than in CTR groups at $t = 0$ h and $t = 4$ h, except for Bdnf at $t = 0$ h and Pcsk1 at $t = 4$ h, indicative of some activation in the deprived cortex ($p = 0.05$, Student’s t test); for the sake of clarity, these significant differences have not been marked by a symbol in the figure.
genes, specific expression was detected in at least one of the time points studied, and all negative controls (sense probes) failed to detect any specific signal. Interestingly, whereas all seven genes were responsive to EE in the cerebral (barrel) cortex, each gene had a specific expression pattern in the other brain areas. Some genes (Arc, Btg2, Cyr61, and Nptx2) were upregulated in all areas except cerbellum, Nr4a2 in all areas except striatum, Egr3 in striatum and cortex, and Ch2sh in cortex only.

We next focused on the patterns of mRNA expression of the selected genes in the different cortical layers of the barrel cortex (Fig. 3). Fos and Ch2sh were undetectable in CTR groups; Fos was rapidly and strongly upregulated after EE at t = 0 h, whereas Ch2sh levels increased only slightly at this time point. Interestingly, Fos levels were primarily increased in layers 2/3 and 4 and less in layers 5 and 6, whereas Ch2sh levels increased similarly in all cortical layers. The two genes returned to basal (undetectable) levels at t = 4 h. Expression of Btg2, Cyr61, Egr3, and Nr4a2 was barely detectable in CTR animals; only residual to low levels in scattered cells were detected primarily in layer 2/3 (Btg2, Egr3), layer 6 (Cyr61), or both layers 2/3 and 6 (Nr4a2). EE-induced upregulation of Egr3 was observed at both t = 0 h and t = 4 h and took place primarily in layers 2/3 and 6 and to a lesser extent in layers 4 and 5. In contrast, Btg2 and Cyr61 and Nr4a2 were only upregulated at t = 0 h. At this time point, Btg2-positive cells increased in layers 2/3, 6, and, to a lesser extent, 4 and 5, whereas Cyr61 increased primarily in layer 6. Nr4a2 was strongly increased in layers 2/3 and 4 and less in layers 5 and 6. The plasticity-related genes Arc and Nptx2 displayed a clear expression already in the CTR groups. Whereas Arc was primarily expressed in layers 4 and 6 and less in layers 2/3 and 5, Nptx2 levels were detected to an equal extent in layers 2/3, 5, and 6. During EE exposure, Arc levels were upregulated in all cortical layers, except layer 1 at t = 0 h, and returned to basal levels at t = 4 h. In contrast, Nptx2 levels did not increase during EE at t = 0 h but did increase dramatically at t = 4 h, especially in layer 2/3 and also layers 5 and 6.

Expression levels were comparable in both brain hemispheres for all the expression patterns described above. In summary, the in situ hybridization results show time- and layer-specific molecular changes related to experience-induced plasticity in the barrel cortex.

EE-induced mRNA expression changes in the barrel cortex are attributable to differential somatosensory processing

Because EE is known to induce plasticity in the entire brain, we investigated whether the changes in mRNA expression at the level of the barrel cortex reflected specific modifications in somatosensory processing or simply reflected general cortical activation. For this purpose, we performed experiments not only in rats without whisker manipulation (UNCLIP group) but also with rats in which the whiskers had been removed unilaterally 18 h before the EE session (CLIP group). Importantly, we performed a behavioral analysis showing that such a short time of deprivation did not affect exploratory behavior in CLIP animals because, similar to UNCLIP rats, CLIP animals spent most of the time actively whisking rather than displaying the other behaviors (two-way ANOVA, p ≤ 0.0001; SNK) (Fig. 4), and they had no preference for a particular location in the test cage (data not shown). We then performed detailed qPCR analysis of the 18 differentially expressed and validated genes selected on the basis of the microarray results (see above) and compared the expression levels in the spared versus the deprived sides of the barrel cortex. The direction (up or down) and time point (t = 0 h or t = 4 h) of the changes in expression determined by qPCR were the same as those found by microarray analysis and were similar between genes in UNCLIP and CLIP groups. Indeed, we observed for a number of genes that mRNA expression was upregulated after EE at t = 0 h (Fig. 5, Arc, Apol1, Btg2, Ch2sh, Chrm4, Cyr61, Dusp1, Fos, FoxB, and Plat), at t = 4 h (Fig. 6, Nptx2 and phosphodiesterase 7B, Pde7b), or at both time points (Fig. 7, Bdnf, Egr3, Nr4a2, and Psk1) and significantly downregulated at t = 4 h (Fig. 6, ABC transporter G2, Abcg2) (two-way ANOVA, followed by SNK post hoc tests, p ≤ 0.05). Only promin1 (Fig. 6, Prom1), which was significantly downregulated at t = 4 h in UNCLIP animals, did not show significant differences in CLIP animals. Importantly, when compared with UNCLIP animals, EE-induced changes in gene expression in CLIP groups were more prominent in the spared (R side) than in the deprived (L side) barrel cortex (Student’s t test, p ≤ 0.05). However, for most genes, there was residual activation in the deprived side when comparing L side of EE animals with respect to CTR animals, Student’s t test, p ≤ 0.05). To quantify and statistically test the specificity of the effects in the spared side, we computed a specificity index for each gene. The index was defined as the ratio of the difference between EE and CTR expression levels in spared versus deprived side (Fig. 8). As expected, in

![Figure 8](image-url)
were similar as in UNCLIP animals and that L barrel cortex only displayed residual activation. We performed separately for left (L, deprived) and right (R, spared) barrel cortices. Note that the expression levels in the R barrel cortex studied, selecting genes at a cellular (cortical layer) level and validating the results in deprived versus spared barrel cortices of whisker-clipped animals. Differentially expressed genes are involved in metabolic processes, regulation of transcription, and intracellular cascades such as PDGF and MAPK signaling pathways, but also in the regulation of synaptic plasticity and blood vessel morphogenesis. This is the first time that a genomewide study is applied at the level of the barrel cortex. Remarkably, the genes found to be differentially expressed in our study hardly overlap with those found by microarray analysis of whole cortex of adult EE-exposed mice (Rampone et al., 2000), possibly as a result of differences in experimental design (e.g., stress levels, length of EE exposure, timing of when the animals were killed, tissue and species used). Interestingly, some genes from our study do overlap with genes known to be regulated by sensory experience in rodent barrel cortex, such as Bdnf, Crem, Egr1, Fos, FosB, and JunB (Rocamora et al., 1996; Pinaud et al., 2006). Our results also overlap, at least partly, with other genomewide studies in related models of experience-induced plasticity. For instance, in the visual cortex, a 4 d monoocular deprivation at the peak of the critical period induced differential expression of genes also found in our dataset, namely Bdnf, Btg2, Cyr61, Egr2, Fos, FosB, Ier2, JunB, and Nr4a1 (Tropea et al., 2006). Strikingly, in the same model, a set of genes (Bdnf, Dusp1, Dusp6, Egr1, Egr2, Fos, FosB, Gadd45b, Ier2, JunB, and Nr4a1) is regulated independently of the developmental stage (Majdan and Shatz, 2006), and in the barrel cortex, we found that these MAPK signaling pathway components were also regulated by experience. Moreover, during singing behavior, some of these genes were also differentially expressed in the songbird brain (Wada et al., 2006; Dong et al., 2009), supporting their involvement in experience-dependent plasticity in the non-mammalian brain as well.

Discussion
Using microarray mRNA expression profiling, we have provided a comprehensive view on the genes induced in the adult rat barrel cortex by sensory experience. The involvement of these genes in the processing of somatosensory information was supported by studying selected genes at a cellular (cortical layer) level and val-

Figure 9. EE-induced layer-specific differential gene expression in the rat barrel cortex of CLIP groups. In situ hybridization analysis showing expression levels of selected genes in the barrel cortex, namely Arc, Btg2, Ch25h, Cyr61, Egr3, Fos, Nptx2, and Nr4a2. Expression levels were evaluated in CLIP animals, under CTR, EE t = 0 h, and EE t = 4 h conditions. The analysis was performed separately for left (L, deprived) and right (R, spared) barrel cortices. Note that the expression levels in the R barrel cortex were similar as in UNCLIP animals and that L barrel cortex only displayed residual activation.

By studying gene expression at the level of the barrel cortex and validating the results by combining EE with whisker clipping, showing strong EE-induced mRNA expression in spared barrel cortex (contralateral to clipping), we have obtained information on genes involved in the processing of somatosensory input. However, although to a much smaller extent, differential expression was still observed in deprived barrel cortex (ipsilateral to clipping), suggesting residual processing of somatosensory input in the deprived hemisphere. This could be attributable to several reasons. First, because whisker clipping leaves the whisker follicles intact, whiskers may still have transmitted sensory information during attempted whisking attributable to follicle stimulation. Second, passive activation attributable to skin contact may have effects similar to passive whisker deflections, which are known to cause postsynaptic potentials in the ipsilateral barrel, although with smaller amplitudes and longer latencies (Manns et al., 2004). Third, it is possible that activation from the spared cortex can...
spread to the contralateral side via callosal projections. The paralemniscal pathway has strong interhemispheric connections (Li and Ebner, 2006), conveys information about the temporal frequency of whisker movements (Ahissar et al., 2000), and is involved in the sensorimotor control of whisker movement by providing reference signals during object recognition (Yu et al., 2006). Such information could be relevant in an EE in which several novel stimuli are present, possibly leading to a highly active paralemniscal pathway and the observed residual processing.

Sensory experiences and the resulting synaptic activity are critical for the shaping of neuronal networks in the barrel cortex. Several processes, including dendritic branching, synaptogenesis, maturation, and elimination of synapses, lie at the base of this shaping (Flavell and Greenberg, 2008). Transcription precedes experience-dependent plasticity by regulating the expression of several downstream genes that subsequently modulate synaptic properties. In fact, many of the transcription factor genes detected in our microarray analysis have been linked previously to synaptic plasticity, in either the barrel cortex or other brain regions. For instance, Btg2 is involved in neuronal differentiation (Bradbury et al., 1991) and plays a crucial role in contextual memory (Farioli-Veccioili et al., 2008, 2009). The upregulation of Crem during EE confirms previous observations, also in barrel cortex (Bisler et al., 2002), supporting its key role in neuronal plasticity (Mioduszewska et al., 2003). Fos and Jun induce expression of Bdnf (Zhang et al., 2002), one of the pivotal molecules in neuronal plasticity (Greenberg et al., 2009). In the barrel cortex, Bdnf regulates the balance between excitatory and inhibitory neurotransmitter systems (Genoud et al., 2004) and was also upregulated in our study, together with two of its processing enzymes, Pcsk1 (Seidah and Chretien, 1999) and Plat (Pang et al., 2004). Interestingly, Bdnf activation increases expression of Egr3 (Roberts et al., 2006), which is involved in short-term memory (Poirier et al., 2008). Egr3 has been shown to induce expression of Arc, which is essential for long-term potentiation (LTP) persistence (Li et al., 2005). EE upregulates Arc expression in rat barrel cortex and hippocampus (Ramirez-Amaya et al., 2005). Finally, all members of the inducible orphan nuclear receptor family of transcription factors (Nr4a1, Nr4a2, and Nr4a3) were also upregulated during EE. This, together with their implication in neuronal development (Perlmann and Wallen-Mackenzie, 2004) and responsiveness to depolarization (Lam et al., 2010), suggests a key role for these transcription factors in experience-dependent plasticity.

Apart from transcription factors, the MAPK and PDGF receptor signaling pathways were also significantly regulated during enrichment, as evidenced by the GO analysis. Activation of the MAPK pathway by glutamate receptors has been associated with synaptic plasticity (Wang et al., 2007). The phosphatases Dusp1 and Dusp6 were both upregulated by EE; Dusp1 targets the MAPK pathway (Farooq and Zhou, 2004) and is in turn inhibited by Dusp6 (Vogt et al., 2005). Given that a short activation of the MAPK pathway is enough to ensure LTP (Wang et al., 2007), expression of these phosphatases probably serves to fine tune the activation of this signaling cascade and overcome detrimental effects of sustained cellular activation. As to the PDGF-B receptor signaling pathway, recent studies suggest that PDGF-B plays a role in regulating NMDA receptor excitability (Egawa-Tsuzuki et al., 2004) and that PDGF-B is also able to induce Arc expression (Peng et al., 2010), linking this pathway to neuronal plasticity.

An intriguing finding in our study was the differential expression of genes related to blood vessel morphogenesis, namely Cyr61 and Apold1. Cyr61 is important for extracellular matrix production (Chen and Du, 2007) and is under the control of muscarinic acetylcholine receptor (Chrm) signaling in cortical neurons (Albrecht et al., 2000); interestingly, Chrm4 was also upregulated in our study. The possible reorganization of neural networks in barrel cortex may increase the need for energy supply and thus for the formation of novel blood vessels in which Cyr61 could be of central importance. This could hold for Apold1 as well, which regulates the differentiation of brain endothelial cells and has been shown to respond to seizures and hypoxia in brain vasculature (Regard et al., 2004). Genes involved in cholesterol biosynthesis, shown to be expressed in neuronal cells (Valdez et al., 2010), such as Ch25h or Hmgcr, were also differentially expressed. The link between cholesterol turnover, LTP, and learning has been established previously (Kotti et al., 2006), and more recently the activity of presynaptic protein kinases has been found to be sensitive to changes in membrane cholesterol content (Smith et al., 2010).

Finally, although not clearly overrepresented in a particular functional category, Ptgs2 and Nptx2 have also been linked previously to neuronal plasticity. For instance, Ptgs2 plays a crucial role in the modulation of hippocampal synaptic transmission and plasticity by regulating prostaglandin signaling (Sang and Chen, 2006). Nptx2 overexpression induces the formation of excitatory synapses (O’Brien et al., 1999) and clustering of AMPA receptors in vitro (Fox and Umemori, 2006).

In addition to knowledge about their functional background, knowing the timing and cellular expression patterns of the differentially expressed genes is crucial to understand their role in S1 plasticity. Our detailed analysis of selected genes indicates that both input (layer 4) and output layers are activated early after EE. For instance, at t = 0 h, Fos and Nrd42a are activated in input and output layers, whereas Btg2 is primarily activated in output layers. In contrast, at later time points, mainly output layers are activated, as evidenced from the Egr3 and Nptx2 expression patterns at t = 4 h. These findings suggest that initial gene activation would reflect changes in presynaptic and postsynaptic neurons in S1, whereas later in time it would reflect postsynaptic changes. Interestingly, changes in layer 2/3, the most plastic layer in adult barrel cortex (Fox, 2002), seem to be more long lasting, linking gene expression to neuronal plasticity.

In summary, we show that, in vivo, somatosensory processing in the rat barrel cortex activates a wide variety of genes in a time- and layer-specific manner. Thus, the present data provide a solid experimental framework for future genetic, electrophysiological, and imaging studies that will give insight into the mechanisms underlying experience-dependent reorganization of sensory systems.

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