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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

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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).

Due to 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 neuritin, 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)
ad libitum

light/dark cycle with lights on at 6:30 A.M. Food and water were provided

experiment (group size, 65 to 75 d). A total of 36 animals were used for microarray and qPCR analysis (group size, n = 6–8) and n = 24 for in situ hybridization experiments (group size, n = 4). The animals were housed two per cage (37.8 × 21.7 × 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 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 m²) 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 (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 O₂ 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 objects used for enrichment are depicted in Figure 1B; the position of the objects was kept as similar as possible between the tests. To quantify behavior (see below, Behavioral analysis), all sessions were videotaped using infrared cameras (Velleman). The animals from the experimental (EE) group consistently displayed high levels of activity. In contrast, we observed that, most of the time, CTR animals were inactive in their home cage, and therefore the behavior of CTR animals was not quantified. For microarray and qPCR analysis, the rats were decapitated either immediately after the end of the EE session (t = 0 h) or 4 h later (t = 4 h). After decapitation, the brains were carefully dissected, frozen on dry ice, and stored at ~80°C until additional analysis. For in situ hybridization analysis, CTR animals were killed immediately (t = 0 h), whereas EE animals were killed either immediately (t = 0 h) or 4 h (t = 4 h) after enrichment. The animals were killed by terminal anesthesia with sodium pentobarbital (90 mg/kg, i.p.), followed by intracardiac perfusion with ice-cold 0.9% saline for 1–2 min and perfusion fixation with 4% ice-cold paraformaldehyde (PFA), pH 7.4, for 10 min (perfusion rate, 25–30 ml/min). The brains were dissected, postfixed overnight in 4% PFA at 4°C, and transferred to 30% sucrose in PBS at 4°C until sectioning.

Behavioral analysis. 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--
ing,” “whisking,” and a final category with all other behaviors, called “other.” The time spans of these different categories were measured. Next to this, the cage was divided into different quadrants (Fig. 1 B), and the total time spent in these quadrants was also quantified. Levene’s test was performed to test the homogeneity of the data. In case of non-homogeneous data, a log transformation was executed. Subsequently, two-way ANOVAs (with “behavior” and “clipping” or “behavior” and “quadrant” as factors), followed by Student–Newman–Keuls (SNK) post hoc analyses when appropriate, were performed, with a significance level of $p \leq 0.05$. All tests were executed using SPSS 17.0 (SPSS Inc.).

**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. Cryochrome 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. 1 C) 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.

**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>Arc</td>
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<td>BDNF</td>
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<tr>
<td>Btg2</td>
<td>TCGAGATGGGCTGCTGCTGC</td>
<td>GCGATAGGGCCAGAATGGTTC</td>
<td>131</td>
</tr>
<tr>
<td>Cav2</td>
<td>TCAATGTTACTGGAATGGG</td>
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<tr>
<td>Cav3</td>
<td>CTGCCAGGTCCTCAGTATGACT</td>
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<td>135</td>
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<td>Chn4</td>
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<td>Cyca</td>
<td>AGACCTGAGGGAAGAAGGATT</td>
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<td>Cyrf6</td>
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<td>AGGAGCATACGGGAACATGCA</td>
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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)**

<table>
<thead>
<tr>
<th>Genes</th>
<th>External primers (5′→3′)</th>
<th>Internal primers (5′→3′)</th>
<th>Amplicon size (bp)</th>
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<tr>
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<tr>
<td>Fos</td>
<td>AATTAAACCTCAGGAGGAGG</td>
<td>TAATACGCCCTACTATAAGGTT</td>
<td>605</td>
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</table>

Shown are the names of the genes selected for *in situ* hybridization, the sequences of the forward and reverse external and internal primers from 5′ to 3′, and the size of the final product after the second (internal) PCR reaction. The T3 (5′-TAATACGACTCTATAGGTT-3′) and T7 (5′-TAATACGACTCTATAGGTT-3′) RNA polymerase sequences at the 5′ of the internal forward and reverse primers are highlighted in bold. The Arc plasmid containing the full-length rat Arc cDNA was 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.
<table>
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<tr>
<th>Affymetrix transcripts cluster ID</th>
<th>Gene symbol</th>
<th>Gene title</th>
<th>SAM q value</th>
<th>FC $t=0$ h</th>
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<td>10767767</td>
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<td>10904511</td>
<td>Arc</td>
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<td>10732652</td>
<td>Dusp1</td>
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Table 3. Continued

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<td>1.10 Up</td>
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Table continued
solution (30% ethenylene glycol, 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 manufacturers. 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), to ServiceXS BV for additional processing. Sample concentration and integrity was checked with a Bioanalyzer 2100 (Agilent Technologies), and only clusters with an enrichment score and only clusters with an enrichment score ≥ 1.3 (equivalent to non-log scale p of 0.05) were considered to be overrepresented (enriched). For the Functional Annotation Clustering tool, the classification stringency was set as high, 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 cDNA synthesis, through random priming, the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas) was used, following the guidelines of the manufacturer. Before analysis, 15 μl of each cDNA 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 cDNA was added to a mix containing 6.25 μl of 2X 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 NCBI Primer-Blast (www.ncbi.nml.nih.gov/tools/primer-blast/) and synthesis.
Table 4. Functional clusters of enriched GO categories (most significant category of each cluster shown)

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<td>28</td>
<td>Ddit3, Cmp1, Klf4, Eg2, Zfp36, Rnf6, Jun, Jrs2, F2, Klf2, Nr4a3, Hnppl, Med14, Egr4, Barx2, Cdx1, Nr4a2, Rel, Gmp2r/Tnf, Klf4, Fos, JunB, Np4a, Nr4a1, Inhha, Gtf2a2, Pdgfb, Sertad1, Egr1</td>
<td>2.23E-06</td>
<td>2.74</td>
<td>5.9</td>
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<td>3</td>
<td>Basic-leucine zipper (bZIP) transcription factor</td>
<td>Regulation of apoptosis</td>
<td>INTERPRO</td>
<td>Ddit3, Crem, Jun, FosB, Atf3, Jun, Fos, Nr3f3</td>
<td>1.34E-06</td>
<td>13.89</td>
<td>5.1</td>
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<td>4</td>
<td>Regulation of apoptosis</td>
<td>GOTERM_BP_FAT</td>
<td>21</td>
<td>Sgk1, Tnip3, Ddit3, Hic1, Nr4a2, Rel, Jun, Rock1, Casp8ap2, Nr4a1, Btg2, Bdnf, Skil, Inhba, Dusp1, Hspb1, G2e3, Hsp1a/Hsp1b/Hsp1d, Ptg2, Bir2, Stat1/Stat4</td>
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<td>5</td>
<td>Membrane-enclosed lumen</td>
<td>GOTERM_CC_FAT</td>
<td>27</td>
<td>Klf4, Rnf6, Jun, Dld, Ptg2, Ccn1, Med14, Barx2, Tmip, Dusps6, Zbtb33, Hic1, Dusp1, Rpsk5a5, Rel, Midn, Gmp2r/Tnf, Klf4, Fos, Np4a, Nr4a1, Cdx2, Gtf2a2, Pdgfb, Fxl-1/Fxl1, Crem, Stat1/Stat4, Aft3</td>
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<td>6</td>
<td>Negative regulation of cellular biosynthetic process</td>
<td>GOTERM_BP_FAT</td>
<td>18</td>
<td>Per1, Barx2, Tmip, Klf4, Dnajb5, Gmp2r/Tnf, S1k1, Klf4, Kdm6b, Insig1/LOC688922, Skil, Foxn3, Bcl6b, Zbtb10, Pdgfb, Pdc4d, Edn1, Eg1, Hbegf</td>
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<td>3.00</td>
<td>2.9</td>
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<tr>
<td>7</td>
<td>Response to oxidative stress</td>
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<td>8</td>
<td>N6d, Dusps6, Ddit3, Dusp1, Ptg2, Jun, Stat1/Stat4, Fos</td>
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<td>Regulation of synaptic transmission</td>
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<td>Transcription from RNA polymerase II promoter</td>
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<td>7</td>
<td>Med14, Ddit3, Gtf2a2, Jun, FosB, JunB, Fos</td>
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<td>10</td>
<td>Phosphate metabolic process</td>
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<td>20</td>
<td>Sgk1, Dusps6, Dusp4, Pim3, Gpd1, Rpsk5a5, Alpk1, S1k1, Rock1, Fami3b, Dld, Bdnf, Plk3, Gadd45b, Gadd45g, Dusp1, Prgx, Lats1, Stat1/Stat4, Trib1</td>
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<td>11</td>
<td>Blood vessel morphogenesis</td>
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<td>Gx2A, Plat, Cyrf61, Tiparp, Jun, Edn1, JunB, Apol1d1</td>
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<td>12</td>
<td>Negative regulation of protein kinase activity</td>
<td>GOTERM_BP_FAT</td>
<td>6</td>
<td>Dusps6, Gadd45g, Gadd45b, Hmgcr, Pdc4d, Trib1</td>
<td>1.64E-03</td>
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<td>13</td>
<td>Positive regulation of apoptosis</td>
<td>GOTERM_BP_FAT</td>
<td>10</td>
<td>Nr4a1, Tnip3, Inhba, Ddit3, Dusp1, Hic1, Ptg2, Jun, Stat1/Stat4, Fos, Crem</td>
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<td>14</td>
<td>Zinc finger, C2H2-type/integrase, DNA-binding</td>
<td>INTERPRO</td>
<td>6</td>
<td>Egr4, Gtf2a2, Zbtb10, Eg2, Klf2, Eg1</td>
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<td>3.07</td>
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<td>15</td>
<td>Response to bacterium</td>
<td>GOTERM_BP_FAT</td>
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<td>Defb10, Ptg2, Jun, Stat1/Stat4, Fos, Pck1, Trib1</td>
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<td>16</td>
<td>Response to progesterone stimulus</td>
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<td>4</td>
<td>Tmip, FosB, JunB, Fos</td>
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<td>17</td>
<td>ATPase activity, coupled to transmembrane movement of substances</td>
<td>GOTERM_BP_FAT</td>
<td>5</td>
<td>Abcc4, Atp11b, Atp13a3/LOC68704, Abcg2, Abcb1a</td>
<td>3.19E-02</td>
<td>4.15</td>
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<tr>
<td>18</td>
<td>Dual specificity protein phosphatase (MAP kinase phosphatase)</td>
<td>PIR_SUPERFAMILY</td>
<td>8</td>
<td>Dusp6, Dusp1, Dusp4</td>
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<td>42.71</td>
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<td>19</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>
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<tr>
<td>20</td>
<td>Nerve growth factor IB-like nuclear receptor</td>
<td>PIR_SUPERFAMILY</td>
<td>3</td>
<td>Nr4a1, Nr4a2, Nr4a3</td>
<td>5.58E-04</td>
<td>71.18</td>
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<tr>
<td>21</td>
<td>Sterol biosynthetic process</td>
<td>GOTERM_BP_FAT</td>
<td>4</td>
<td>Insig1/LOC688922, Idi1, Hmgcr, Ch25h</td>
<td>5.98E-03</td>
<td>10.64</td>
<td>1.3</td>
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<tr>
<td>22</td>
<td>Negative regulation of apoptosis</td>
<td>GOTERM_BP_FAT</td>
<td>9</td>
<td>Btg2, Sgk1, Bdnf, Hsp1b, Nr4a2, G2e3, Hsp1a/Hsp1b, Hsp1a/Hsp1b, Hsp1a/Hsp1b, Atp13a3/LOC68704, Lats1, Aft3, Lats1, Hsp1b, Hsp1a, Ptg1, Ptg2, Trib1, FosB, Skil, Inhba, Dusp1, Pgd1, Rpsk5a5, Klf5f, Arf4d, Arlb3, Alpk1, S1k1, Mat2a, LOC499330, Pkkx, Mat2a, Abcd2, Cry2, Rab18, Dynch2h</td>
<td>4.99E-02</td>
<td>2.21</td>
<td>1.3</td>
</tr>
<tr>
<td>23</td>
<td>Nucleotide binding</td>
<td>GOTERM_BP_FAT</td>
<td>34</td>
<td>Htatsf1, Abcc4, Sgk1, Rap1b, Atp11b, Hmgcr, Abcg2, Rock1, Ddx50, Dld, Ptk3, LOC306096, Hsp1a/Hsp1b/Hsp1a/Hsp1b, Abcc1a, Abcb1a, Trib1, Pim3, Gpd1, Rpsk5a5, Klf5f, Arf4d, Arlb3, Alpk1, S1k1, Mat2a, LOC499330, Pkkx, Mat2a, Abcd2, Cry2, Rab18, Dynch2h</td>
<td>1.81E-02</td>
<td>1.47</td>
<td>1.3</td>
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</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.

Relative expression of the genes of interest was calculated after obtaining the corresponding Ct values and correcting for unequal sample input using geNorm (Vandesompele et al., 2002), which identifies the two most stably expressed housekeeping genes (Ywhaz and CycA) from a set of three tested candidate genes reported previously to be stably expressed in the brain (Bonefeld et al., 2008) to calculate a normalization factor for each sample. This normalization factor was then used to obtain the relative differences between the samples for each primer pair. Statistics were performed using SPSS 17.0 (SPSS Inc.). Normalized data were tested...
Table 5. Significantly overrepresented pathways

<table>
<thead>
<tr>
<th>Pathway #</th>
<th>Term</th>
<th>Category</th>
<th>Count</th>
<th>Genes (Gene symbol)</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, Dusp9, Ddit3, Dusp4, Rps6ka5, Jun, Fos, Nra1, Bdnf, Hspa1, Gad645g, Dusp1, Gad445b, Prxk, Hspa31, Pdgfb</td>
<td>2.20E-08</td>
<td>5.98</td>
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<tr>
<td>2</td>
<td>Platelet-derived growth factor receptor signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>5</td>
<td>Txnip, Plat, Cmp1, Tiparp, Pdgfb</td>
<td>1.41E-04</td>
<td>18.14</td>
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<tr>
<td>3</td>
<td>Enzyme linked receptor protein signaling pathway</td>
<td>GOTERM_BP_FAT</td>
<td>12</td>
<td>Txnip, Skil, Plat, Cmp1, Tiparp, Pdgfb, Jun, Tob1, Fos, Dok1, Ins2, Hbegf</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>GUPERM_BP_FAT</td>
<td>8</td>
<td>Skil, Pdgfb, Jun, Tob1, Fos</td>
<td>8.31E-03</td>
<td>3.45</td>
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<tr>
<td>5</td>
<td>Transmembrane receptor protein serine/threonine kinase signaling pathway</td>
<td>GUPERM_BP_FAT</td>
<td>5</td>
<td>Skil, Pdgfb, Jun, Tob1, Fos</td>
<td>1.82E-02</td>
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<td>6</td>
<td>Transforming growth factor receptor signaling pathway</td>
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<td>4</td>
<td>Hbegf</td>
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<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>
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</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.

Neurotrophin signaling pathway

Figure 2. Representative brain areas activated by enrichment. A. Shown are representative images of Fos mRNA obtained by in situ hybridization in CTR and EE animals at t = 0 h. In CTR animals, Fos levels were barely detectable, whereas in EE animals, increased levels were observed in striatum, barrel cortex, hippocampus, and cerebellum. Magnifications are shown at the bottom left corner on each image. B. Expression levels of the eight genes (Arc, Btg2, Ch25h, Cyr61, Egr1, Fos, Nptx2, and Nr4a2) selected for in situ hybridization were quantified in CLIP animals in the four above-mentioned brain areas, showing different regional and temporal patterns of expression under CTR and EE conditions.

In situ hybridization. The plasmid containing the full-length rat activity-regulated cytoskeleton-associated protein (Arc) cDNA subcloned into the EcoRI–XhoI 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 XhoI 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 Biologe BV, adding T3 (5’-AATTAACCCTCACTAAAGGG3’) and T7 (5’-TAATACGACTCACTATAGGG3’) RNA polymerase sequences at the 5’ end of the forward and reverse internal primers, respectively (primer sequences are listed in Table 2). For the first (external) run, 3 μl of cDNA was mixed with 1.6 μl of each external primer (5 μM), 2 μl of 10× PCR buffer, 0.2 μl of dNTP mix, 0.1 μl of Taq polymerase, and 11.5 μl of DEPC water to a final volume of 20 μl (all from Fermentas). The same components were used for the internal reaction, using a 1:100 dilution of the external run end product as a template and the internal primers. A touchdown protocol was used for both runs. To activate the enzyme, the mix was heated to 94°C for 2 min (hot start), followed by 10 cycles of 60 s at 94°C, 30 s at 68°C (−1°C per cycle), and 90 s at 72°C. Subsequently, 30 cycles were added of 60 s at 94°C, 30 s at 58°C, and 90 s at 72°C, with a final step of 10 min at 72°C. After the PCR reaction, the samples were separated on a 1% agarose gel, and the correct products were gel extracted using the QIAEX II kit.
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, 1/100 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-Wildheer et al., 2000).

Figure 3. Layer-specific differential gene expression in rat barrel cortex after EE. A, Representative images of in situ hybridization analysis of Arc, Btg2, Nr4a2, and Nptx2 mRNA in UNCLIP groups: CTR and EE animals at t = 0 h (Arc, Btg2, Nr4a2) or t = 4 h (Nptx2); cortical layers are indicated on each image (magnification, 5×). B, Quantification of Arc, Btg2, Nr4a2, Nptx2, Ch25h, Cyr61, Egr3, and Fos expression levels (in situ hybridization) in different cortical layers of UNCLIP animals, under CTR, EE t = 0 h, and EE t = 4 h conditions.

Figure 4. Behavioral analysis of UNCLIP and CLIP animals during exposure to EE. 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), 0.5 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 2× 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 Rywhaz as the two most stable housekeeping genes) were used to calculate an FC for 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 ≤ 0.0001 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-
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 (*Apol1*, 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, *Apol1* expression was below detection levels (data not shown); note that detection of *Apol1* also failed in the Allen Mouse Brain Atlas (Lein et al., 2007). For the remaining seven
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 cerebellum, Nr4a2 in all areas except striatum, Egr3 in striatum and cortex, and Ch25h 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 Ch25h were undetectable in CTR groups; Fos was rapidly and strongly upregulated after EE at t = 0 h, whereas Ch25h 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 Ch25h 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 and CLIP and CTR groups. Indeed, we observed for a number of genes significant upregulation after EE at t = 0 h (Fig. 5, Arc, Apold1, Btg2, Ch25h, Chrm4, Cyr61, Dusp1, Fos, FosB, and Ptdhad1) at t = 4 h (Fig. 6, Nptx2 and phosphodiesterase 7B, Pde7b), or at both time points (Fig. 7, Bdnf, Egr3, Nr4a2, and Psk1 and significant downregulation 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. Specificity index showing the right/left ratio of activation in the two hemispheres of UNCLIP and CLIP groups. A specificity index was calculated, based on qPCR data, to explore the degree of activation of the genes selected for qPCR validation in right (R) versus left (L) barrel cortices (for details, see Materials and Methods). In CLIP animals, if the index is higher than 1, activation is higher in spared (R) than in deprived (L) cortex. In UNCLIP animals, the specificity index quantifies hemispheric differences in the absence of clipping. Shown are the results of this index for genes significantly regulated after EE at t = 0 h (A), t = 4 h (B), or both time points (C) (bottom). Bars represent average specificity index values ± SEM. * represents values significantly higher than 1 (one-sample t test, reference value 1, p ≤ 0.05). # represents significant differences between UNCLIP and CLIP groups (p ≤ 0.05, Student’s t test). Marginally significant differences are indicated by & symbol (0.05 < p ≤ 0.1).

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CLIP groups, the specificity index was significantly higher than 1 for most genes tested (Student’s one-sample t test, reference value 1, \( p \approx 0.05 \)) (Fig. 8), indicating that in these groups the degree of activation in the spared side was substantially higher than in the deprived side. Similar results were found by in situ hybridization, showing only residual activation in the deprived side and prominent activation in the spared side (Fig. 9). These observations support the view that EE-induced changes in gene expression observed by microarray analysis at the level of the barrel cortex are specific for changes in sensory processing and do not merely reflect general changes in cortical activity.

**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 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 (Rampon 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 mono-nuclear 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, Insig1, Nptx2, and Pdgfb (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.

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-Vecchioli 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). Fox 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, Pkci (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, Ptg2 and Nptx2 have also been linked previously to neuronal plasticity. For instance, Ptg2 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 SI 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, Fox and Nrr4a2 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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