Molecular correlates of cortical network modulation by long-term sensory experience in the adult rat barrel cortex

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Modulation of cortical network connectivity is crucial for an adaptive response to experience. In the rat barrel cortex, long-term sensory stimulation induces cortical network modifications and neuronal response changes of which the molecular basis is unknown. Here, we show that long-term somatosensory stimulation by enriched environment up-regulates cortical expression of neuropeptide mRNAs and down-regulates immediate-early gene (IEG) mRNAs specifically in the barrel cortex, and not in other brain regions. The present data suggest a central role of neuropeptides in the fine-tuning of sensory cortical circuits by long-term experience.

[Supplemental material is available for this article.]

The storage and retrieval of information in and from the cerebral cortex is crucial for cognition. To permit effective adaptive responses, cortical networks must remain plastic throughout life, and their functional organization needs to be accurately adjusted depending on experience (Barnes and Finnerty 2010). The functional and morphological changes in cortical connectivity following sensory experience have been well established, especially in the rodent visual and somatosensory (barrel) cortices (Fox and Wong 2005). At the level of the barrel cortex, these alterations have been observed following various sensory manipulations (Feldman and Brecht 2005). One of the most striking changes takes place when animals are reared in an enriched environment (EE), after which reduced cortical whisker representations, suppressed evoked neuronal responses, as well as smaller and better-defined receptive fields are observed (Polley et al. 2004). Similar effects occur upon single-whisker experience. When all but one whisker is clipped on one side of the snout, that whisker's functional representation contracts if the animal is allowed to explore the environment (Polley et al. 1999). Similarly, contracted whisker functional representations are also observed after single-whisker stimulation (Welker et al. 1992), which is accompanied by an increase in inhibitory synapses (Knott et al. 2002). Therefore, increased whisker use leads to cortical whisker receptive fields that are spatially focused and more metabolically efficient (Frostig 2006), which at a population level can translate into weaker overall excitability (Polley et al. 2004). The increased specificity of whisker representations is critically dependent on experience, as the spared whisker representation shows expansion rather than contraction in animals housed under standard conditions (i.e., with limited whisker use). Although the exact behavioral consequences of experience-induced refinement of whisker representations caused by EE are not known, this refinement may reduce the

signal-to-noise ratio, improve cortical information processing, and thereby enhance discriminative performance.

At the molecular level, the mechanisms steering this functional refinement are poorly understood. We have previously described the global changes in gene expression in the barrel cortex after a short period of EE. Compared to controls, EE leads to the 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 blood vessel morphogenesis (Vallès et al. 2011). However, genome-wide information on the molecular changes observed in the primary somatosensory cortex after long-term EE is lacking. This knowledge is crucial to understand the mechanisms underlying the functional and structural changes induced by long-term experience. Given the reported increase in inhibitory synapse number (Knott et al. 2002) after continuous whisker stimulation, we hypothesized that the contracted cortical receptive fields observed upon increased whisker use may be, at least partially, due to an increased signaling from interneurons.

To test this hypothesis, and to understand the molecular basis of the functional and morphological changes of EE rearing, in experiment 1 we performed mRNA expression profiling (microarray analysis) of the barrel cortex of adult Long-Evans rats placed in an EE environment for 28 d (large cage with enrichment, $n = 8$ animals/cage). As a control, we used animals kept in their home cage (home-caged controls, HCC) for 28 d (standard cage, $n = 2$ animals/cage) (see Supplemental Methods for details).

The 28-d period of sensory enrichment induced, in the rat barrel cortex, significant changes (≥ 1.3 fold, $P < 0.05$, SAM analysis) in the expression of only a limited number of genes, namely 29

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of the ~22,000 genes present on the array (Fig. 1). Gene ontology (GO) enrichment analysis included significant enrichment of the GO categories neuropeptide signaling, synaptic transmission, and regulation of synaptic plasticity and transmission (Supplemental Table 1). The 13 genes (out of the 29 regulated genes) selected for qPCR analysis were all validated (*t*-test, $P < 0.05$) (Fig. 2A,B), confirming the results of the microarray analysis. Remarkably, a large portion (44%) of the transcripts up-regulated in the EE group consisted of neuropeptide mRNAs. These included transcripts encoding cortistatin (*Cort*), cocaine- and amphetamine-regulated transcript (*Cart*), corticotrophin-releasing hormone (*Crh*) and its binding protein *Crhbp*, adenylate cyclase activating polypeptide 1 (*Adcyap1*, also known as *Pacap*), neuropeptide Y (*Npy*), and prepronociceptin (*Pnoc*). The remaining up-regulated genes code for transporters (such as the vesicular glutamate transporter 2, *VGlut2*), enzymes (such as phosphodiesterase 7A, *Pde7a*, and lysyl oxidase, *Lox*), or are involved in oxygen binding, among others (Fig. 1).

The up-regulation of neuropeptide mRNAs was accompanied by a striking down-regulation of a number of immediate-early genes (IEGs) (54% of the down-regulated genes) (Fig. 1; Supplemental Table 1). Several of these are known to be involved in regulating synaptic plasticity and transcription, including the IEGs encoding activity-regulated cytoskeleton-associated protein (*Arc*), early growth response 1 (*Egr1*, also known as *Zif268*), FBJ osteosarcoma oncogene (*Fos*), immediate early response 5 (*Ier5*), and ring finger protein 39 (*Rfp39*, also named *Lirf*). The clock genes period homolog 1 and 2 (*Per1*, *Per2*) and basic helix–loop–helix domain containing, class B2 and B3 (*Bhlhb2*, *Bhlhb3*), were also down-regulated, as was the gene coding for the type 4 muscarinic receptor (*Chrm4*), among others (Fig. 1).

To assess the specificity of these changes in terms of (1) somatosensory enrichment and (2) brain region, experiment 2 was performed, in which the enrichment procedure was similar to experiment 1 (EE group, large cage with enrichment, $n = 8$ animals/cage), but in which, as a control group, animals placed in a large cage without enrichment (large cage control group, LCC, $n = 8$ animals/cage) were used (instead of $n = 2$ animals/cage in standard cages as in experiment 1). Expression of the top three up- and top three down-regulated genes from the microarray results from the previous experiment was measured by qPCR in experiment 2. This was done not only in the barrel cortex but also in primary motor (M1) and visual (V1) cortex, striatum, and ventral posteromedial thalamic nucleus (VPM). In the barrel cortex, the top three up-regulated genes from the first experiment (neuropeptides *Cart*, *Cort*, and *Crh*) were, although to a lesser extent, also significantly up-regulated in EE animals in comparison to LCC rats (*t*-test, $P < 0.05$) (Fig. 2C). However, none of the top three down-regulated genes from the first experiment (IEGs *Arc*, *Egr1*, and *Per2*) were significantly regulated in EE vs. LCC animals (Fig. 2C). In the two other cortical areas studied (M1 and V1), only the mRNA of the neuropeptide *Cort* was differentially expressed (up-regulated) in M1 from EE vs. LCC groups (*t*-test, $P < 0.05$), while the rest of the measured genes showed no significant differences (Fig. 2C). In the two additional noncortical regions analyzed (striatum and VPM) none of the measured genes showed significant differences between the EE and LCC groups (data not shown).

The two experiments showed important parallels and some differences. Importantly, our finding in experiment 1 that neuropeptides are up-regulated in primary somatosensory (barrel) cortex due to prolonged somatosensory experience was validated in experiment 2. However, although up-regulation of neuropeptides in both experiments was significant, the effects appeared smaller in experiment 2 (Fig. 2, A vs. C). To test the difference between experiments, we computed for the two experiments the
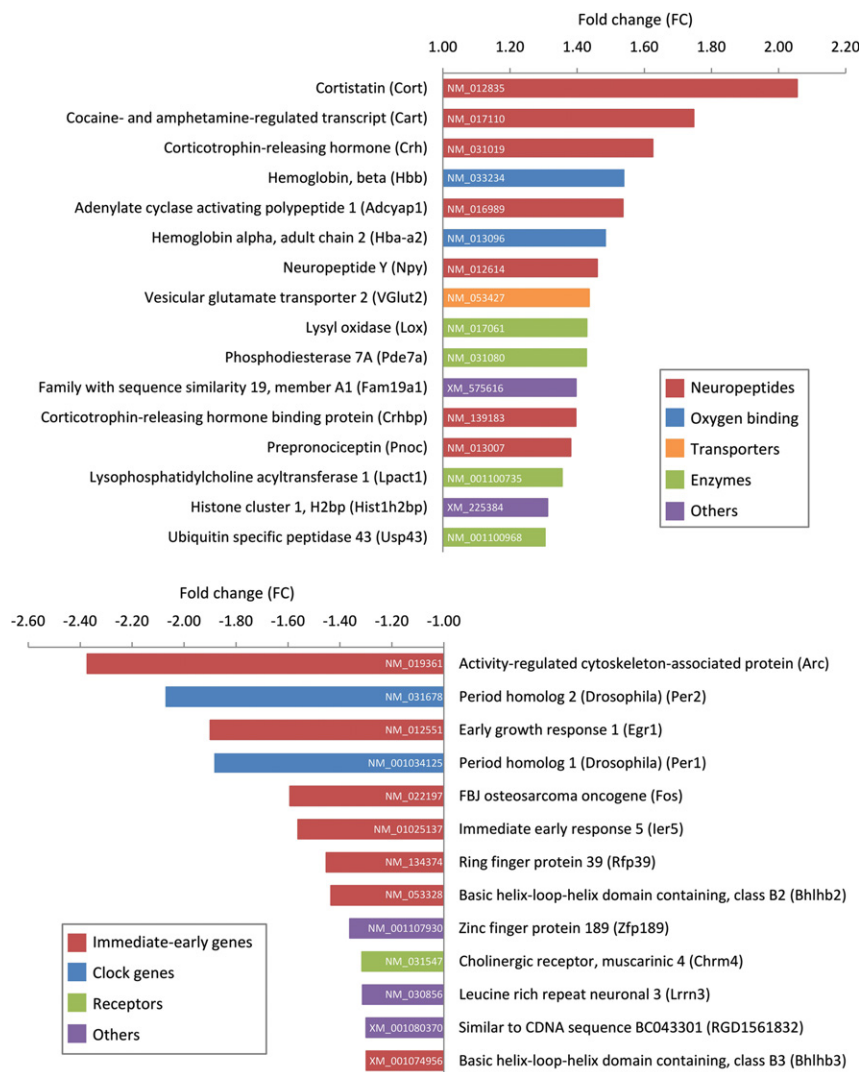

Figure 1. Long-term sensory enrichment induces significant changes in gene expression. Microarray analysis revealed differentially expressed genes in the rat barrel cortex following 28 d of enriched environment (EE) rearing (large cage with enrichment, $n = 8$ animals/cage), in comparison to home-caged controls (HCC) (standard cage, $n = 2$ animals/cage). Gene names, abbreviations, fold changes, and color-coded gene ontologies for up-regulated (*top* panel) and down-regulated (*bottom* panel) genes are indicated, together with the accession number of each gene.
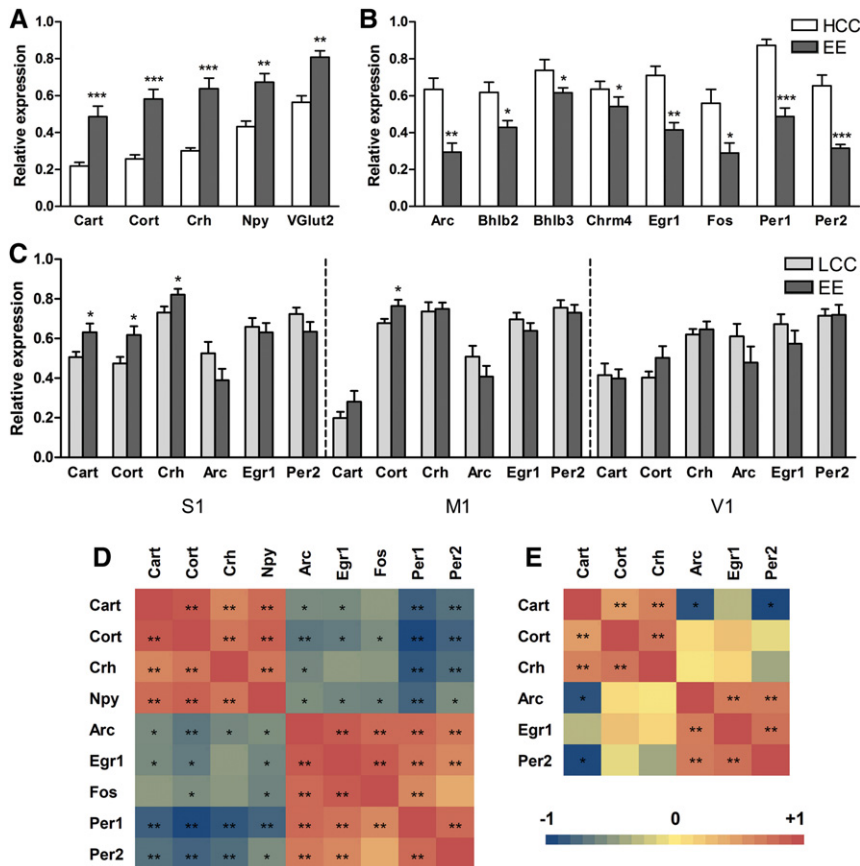
Figure 2. qPCR analysis of gene expression in different cortical regions after 28 d of enriched environment (EE). Normalized expression values of selected up-regulated (*A*) and down-regulated (*B*) genes in the barrel cortex after 28 d of EE (enrichment in large cage, $n = 8$ animals/cage), in comparison to home-caged controls (HCC) (housed in standard cages, $n = 2$ animals/cage) (experiment 1). (*C*) In experiment 2, expression of the top three up-regulated and down-regulated genes was measured in the barrel cortex (S1) and primary motor (M1) and visual (V1) cortices after 28 d of EE in comparison to control animals housed ($n = 8$/cage) in large cages (LCC group). For normalization, β-*actin* or *Ywhaz* was selected as the housekeeping gene (most stable gene chosen). Bars represent average normalized expression values ± SEM ($n = 8$ animals/group). Asterisks represent significant differences between home-caged control (HCC) and EE groups or large cage control (LCC) and EE groups (Student's *t*-test; [*] $P < 0.05$, [**] $P < 0.01$, [***] $P < 0.001$). A graphical view of Pearson correlations after multiple testing correction between all measured mRNA levels in the barrel cortex in the HCC vs. EE comparison (*D*) and LCC vs. EE comparison (*E*) is shown. The correlations are color-coded as indicated in the bar at the *bottom left* (from −1 to +1), and statistically significant correlations are tagged with asterisks (Pearson correlation, [*] corrected $P < 0.05$, [**] corrected $P < 0.01$).

by the finding that the neuropeptide effects were overall quite specific for the barrel cortex (Fig. 2C). With respect to the IEGs, the results of the two experiments differ. We found no significantly reduced mRNA levels for the IEGs in experiment 2, and a repeated measures ANOVA, as described above, showed a significantly larger down-regulation of IEGs in experiment 1 than in experiment 2 ($F_{(1,24)} = 9.804$, $P = 0.005$) and a significant effect of gene ($F_{(2,48)} = 19.071$, $P < 0.001$), but no significant interaction ($F_{(2,48)} = 2.131$, $P = 0.130$). These findings may again reflect the higher similarity between control and EE groups in experiment 2 than in experiment 1.

To further explore the relationship between neuropeptide and IEG levels, we performed correlation analyses among mRNA levels of all the measured genes in the barrel cortex, in both experiment 1 (HCC vs. EE comparison) and experiment 2 (LCC vs. EE comparison). As expected, mRNA levels of most of the analyzed neuropeptides positively correlated among themselves, and similarly also the levels of the majority of IEG mRNAs showed positive correlations among themselves (Pearson correlation, corrected $P < 0.05$) (Fig. 2D,E). Interestingly, mRNA levels of neuropeptide and IEGs displayed negative correlations for many of the analyzed genes (Pearson correlation, corrected $P < 0.05$), thus identifying two clusters of genes with opposite responses to EE. This clustering was pronounced in experiment 1 (HCC vs. EE comparison) (Fig. 2D), but less clear in experiment 2 (LCC vs. EE comparison) (Fig. 2E).

The genome-wide approach used thus revealed new insights into the mRNA changes underlying the neurophysiological effects of long-term somatosensory enrichment in the barrel cortex. The genomic changes induced by long-term EE were limited to a small

expression level for each gene in each individual animal as a percentage of the control expression level averaged over all animals. These percentages were log-transformed (to homogenize variance) and entered in a repeated measures ANOVA with the within-subject factor gene (3) and the between-subjects factor experiment (2). This analysis showed a main effect of experiment ($F_{(1,26)} = 43.995$, $P < 0.001$) in line with a greater neuropeptide up-regulation in experiment 1 than in 2, no main effect of gene ($F_{(2,52)} = 1.713$, $P = 0.190$), and no significant interaction ($F_{(2,52)} = 0.171$, $P = 0.843$). This result suggests that the smaller neuropeptide up-regulation in experiment 2 is more specifically linked to sensory than to social enrichment, because the social factors in experimental and control groups were better matched in experiment 2 (8 animals in EE and LCC groups) than in experiment 1 (8 animals in EE group, pairs of animals in HCC group). Conversely, the larger effect in experiment 1 may be seen as reflecting the addition of a social aspect to the enrichment as compared to experiment 2. The sensory interpretation of experiment 2 is supported

number of genes, and specific to the barrel cortex. Strikingly, nearly half of the genes up-regulated following 28 d of EE were related to neuropeptide signaling, whereas the majority of the down-regulated genes represented IEGs. At the cortical level, neuropeptides are primarily expressed in interneurons, which play crucial roles in neuronal sensory transmission (Markram et al. 2004; Fries 2005) and in the specificity of sensory neurons (Sillito 1975). Therefore, an increased tone of neuropeptide signaling may well play an important role in the functional refinement of the cortical network and enhanced specificity of neural responses in a variety of stimulation and learning conditions. The overall reduction of excitability reported by Polley et al. (2004) after long-term EE fits with these ideas.

How specific interneuron types act on cortical network depends on their molecular signature (expression of neuropeptides and calcium-binding proteins), their morphology and (electro)-physiological features, as well as their cortical location/distribution (connectivity). Our hypothesis that increased neuropeptide

signaling in interneurons may contribute to altered cortical network activity after long-term sensory experience is strongly supported by the cortical distributions of the neuropeptides upregulated in our study (Fig. 3), and their known functions in other brain systems (de Lecea et al. 1997; Yan et al. 1998a,b; Hannibal 2002; Markram et al. 2004; Karagiannis et al. 2009). For example, *Crh*, up-regulated here together with its binding protein *Crhbp*, depresses evoked excitatory responses in the rat sensorimotor cortex (Froc and Christie 2005). This is consistent with the reduced evoked responses found in the somatosensory cortex following EE rearing (Polley et al. 2004), although excitatory effects of *Crh* in the cortex have also been reported (Gallopin et al. 2006). Similarly, *Cort* and *Npy*, which were up-regulated in our study, decrease excitability in cortical circuits (Spier and De Lecea 2000; Bacci et al. 2002). Among the other up-regulated neuropeptides, *Pnoc* modulates glutamatergic functions (Kuzmin et al. 2009), and *Adcyap1* modulates evoked responses in the hippocampus in a dose-dependent manner (Roberto et al. 2001), although excitatory effects of *Adcyap1* have also been described (Hu et al. 2011). The observed up-regulation of *Cort*, *Npy*, *Pnoc*, and *Adcyap1* mRNAs is therefore generally in line with the previously reported decrease in cortical excitability following long-term enrichment (Polley et al. 2004). Furthermore, mRNAs of the neuropeptide *Cart* and the regulatory enzyme *Pde7a*, both highly expressed in cortical layer 4 of the barrel cortex (Couceyro et al. 1997; Miro et al. 2001), were also up-regulated, but their role in modulating cortical signaling remains to be established. Interestingly, the up-regulated gene *Lox* may be related to (neuro)peptide signaling (Vora et al. 2010) and has been implicated in Purkinje cell dendritic development

(Li et al. 2010), while the present study suggests its involvement in cortical network reorganization. Finally, besides neuropeptide-related genes, *VGlut2* (also named *Slc17a6*) was up-regulated as well, in line with previous observations showing that the expression of vesicular glutamate transporters is regulated by experience (Erickson et al. 2006), and therefore may affect glutamate signaling in cortical networks. In this regard, *VGlut2* up-regulation has been linked to feedforward inhibition in cultured cortical neurons, where increased *VGlut2* mRNA and protein levels have been described at excitatory synapses onto inhibitory interneurons after prolonged increases in glutamatergic synaptic activity induced by $GABA_A$ receptor activity blockade (Doyle et al. 2010). This is very much in line with our findings that *VGlut2* mRNA is up-regulated after a long period of EE (increased sensory activity) and suggests, together with the involvement of neuropeptide signaling, its contribution to the reduced neuronal excitability found after a long period of sensory stimulation.

Interestingly, modulation of inhibition is a critical factor in regulating plasticity in other forms of long-term adult plasticity (Rosier et al. 1995; Arckens et al. 2000; Mitchell et al. 2007) as well as during development (Yuste and Sur 1999). Along the same lines, a large number of studies have demonstrated the role of neuropeptide signaling in learning and memory. For instance, Crh has been implicated in cognitive functional impairment after stress (Chen et al. 2010), while Cort impairs long-term memory, likely through inhibition of glutamatergic neurotransmission (de Lecea 2008). In contrast, both positive and negative effects of Npy on learning and memory have been reported (Flood et al. 1987; Thorsell et al. 2000). The effects of nociceptin, also known
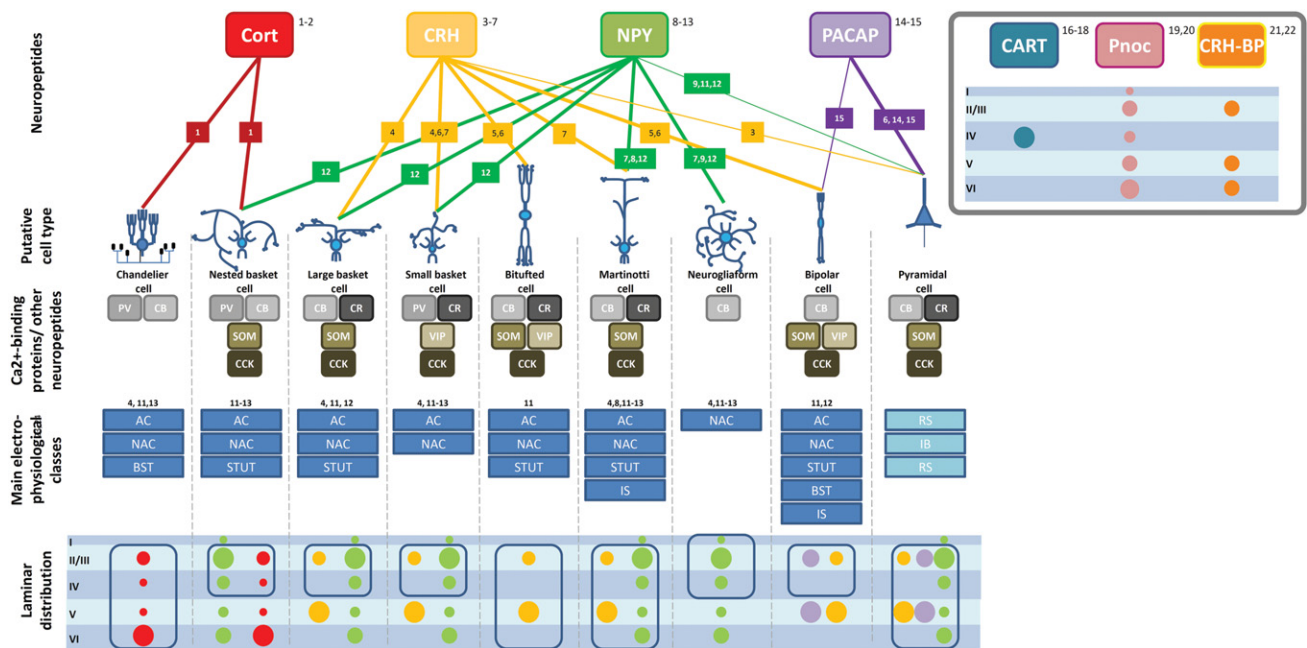
Figure 3. Scheme summarizing the laminar distribution and the main molecular, anatomical, and electrophysiological properties of the various neuronal types in which the EE-up-regulated neuropeptides are expressed. On *top* are the differentially regulated neuropeptides. The lines indicate in which cell type the peptides can be found: (thick line) common, (thin line) rare. Next, calcium-binding proteins ([PV] parvalbumin, [CB] calbindin, [CR] calretinin) and other neuropeptides which can be co-expressed with the EE-regulated neuropeptides are indicated, followed by the main electrophysiological classes corresponding to the putative cell type ([AC] accommodating, [NAC] nonaccommodating, [BST] bursting [STUT] stuttering, [IS] irregular spiking, [RS] regular spiking, [IB] intrinsic burst spiking). Finally, the colored circles indicate in which cortical layer the corresponding neuropeptide is expressed (laminar distribution), the size of the circles being proportional to the amount of expression; a blue rectangle defines the layers where the putative cell type can be found. There is insufficient information on putative cell type, electrophysiological classes, and additional molecular markers for the neuropeptides/neuropeptide-binding protein Cart, Pnoc and Crhbp. The information has been summarized on the basis of several studies; each study where the specific information has been derived from is indicated by a number, and the list of numbers with corresponding references, details of the study, and hyperlinks can be found in Supplemental Table 2.

as orphanin FQ (N/OFQ), have been extensively investigated, and reported effects include memory impairments (Goeldner et al. 2008) and suppression of LTP (Bongsebandhu-phubhakdi and Manabe 2007). Adcyap1, or Pacap, has been shown to facilitate learning (Telegdy and Kokavszky 2000), and *Pacap* mutants display impaired hippocampal LTP (Matsuyama et al. 2003). Finally, Cart promotes spatial learning and memory in rats (Upadhya et al. 2011). Although the ensemble of these observations is in line with our findings, and further supports that neuropeptides have a crucial role in modulating neuronal function, direct demonstrations of enhanced neuronal specificity (in tuning or RF profile) during up-regulation of specific neuropeptides are currently lacking.

Bain-derived neurotrophic factor (*Bdnf*) mRNA is up-regulated in the somatosensory cortex after 2, 7, and 28 d of EE rearing in a "naturalistic habitat" (Gomez-Pinilla et al. 2011). Although we also found that *Bdnf* mRNA is up-regulated in the barrel cortex already after 30 min of EE (Vallès et al. 2011), in the present study we did not observe up-regulation of *Bdnf* mRNA after a long period of EE (28 d). This could be due to differences in experimental design, such as the use of a "naturalistic habitat" instead of traditional EE rearing, timing of the sampling, or differences between rat strains studied (i.e., Sprague-Dawley vs. Long-Evans). In view of the long EE-period, we may have observed the downstream effects of earlier *Bdnf* up-regulation, because *Bdnf* induces the expression of two neuropeptide mRNAs (*Npy* and *Pnoc*) (Barnea et al. 2004; Ring et al. 2006) that were up-regulated in our study.

The up-regulation of neuropeptide signaling was accompanied by a striking down-regulation of a number of IEGs, classically seen as markers of neuronal activation and neuronal plasticity (Loebrich and Nedivi 2009). The down-regulated IEGs included *Arc*, *Egr1* (*Zif-268*), *Fos*, *Ier5*, and *Rnf39* (also named *Lirf*). This observation is another feature in our data that agrees well with the reduced cortical excitability following prolonged periods of sensory stimulation (Polley et al. 2004). These changes in neuronal excitability may increase signal-to-noise ratio and improve information processing in cortical networks. Besides IEGs, other genes were also down-regulated; the interpretation of these findings can be found in the Supplemental Discussion.

Strikingly, we observed a small number of regulated genes in the present study, whereas we previously found many differentially expressed genes following a short period of EE (Vallès et al. 2011). This suggests that during the initial phases of plasticity (i.e., after short-term EE) a complex set of molecular cascades is activated, while the long-term EE changes observed in the present study reflect the stable steady-state molecular signature resulting from the plastic reorganization of the circuitry. The molecular effects observed after short-term and long-term EE exposure could be analogous to those underlying the "acquisition" phase (early learning) and the "asymptotic phase" in skill learning, respectively, in which the latter phase results in long-term memory traces that can include sensory and/or motor cortex (Karni and Bertini 1997; Schoups et al. 2001). In addition, the molecular changes that we describe here are in line with the previously reported morphological and functional changes after long-term sensory experience (Polley et al. 2004). Without excluding the contributions of higher-level areas to implicit memory formation (Law and Gold 2008; Xiao et al. 2008; Reed et al. 2011), our data support the idea that low-level sensory cortical areas can contribute to the long-term maintenance of memory traces acquired during stimulus exposure and skill learning (Karni and Bertini 1997; Schoups et al. 2001). Along these lines, we suggest that after learning or experience, increased inhibitory function may be an integral part of the manner in which memory traces are maintained in cortical networks (Tremere et al. 2003).

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