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Effects of visual experience on activity-dependent gene regulation in cortex

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There are critical periods in development when sensory experience directs the maturation of synapses and circuits within neocortex. We report that the critical period in mouse visual cortex has a specific molecular logic of gene regulation. Four days of visual deprivation regulated one set of genes during the critical period, and different sets before or after. Dark rearing perturbed the regulation of these age-specific gene sets. In addition, a 'common gene set', comprised of target genes belonging to a mitogen-activated protein (MAP) kinase signaling pathway, was regulated by vision at all ages but was impervious to prior history of sensory experience. Together, our results demonstrate that vision has dual effects on gene regulation in visual cortex and that sensory experience is needed for the sequential acquisition of age-specific, but not common, gene sets. Thus, a dynamic interplay between experience and gene expression drives activity-dependent circuit maturation.

In visual system development, neural activity is crucial for the reorganization of synapses and connections into adult circuits[1]. Manipulation of visual input during a restricted time, the critical period, alters the patterning of synaptic connectivity within visual cortex (reviewed in ref. 2). For example, an imbalance of input between the two eyes by monocular deprivation (MD) during the critical period results in a functional shift in the ocular dominance (OD) of cortical neurons in favor of the open eye, accompanied anatomically by wider OD columns representing that eye and narrower ones for the closed eye[3,4].

There has been intense focus on candidate genes and gene expression programs that may be required for these processes[5–8]. To learn about molecular mechanisms underlying activity-dependent synapse remodeling, recent studies have used the mouse as a genetically accessible experimental model. As in other mammals, a critical period for OD plasticity has been defined in the mouse, assessed electrophysiologically (henceforth called the 'physiologically defined' critical period). For instance, within the binocular zone of cortex, 2–4 d of MD any time between postnatal day (P) 19 and 32 results in a functional shift in cortical neurons' visually driven responses toward the open eye[9,10]. This OD shift is thought to be triggered by a weakening of the synaptic inputs driven by the closed eye, followed by a strengthening of the inputs driven by the open eye[11,12]. Another form of plasticity, homeostatic scaling of synaptic strength, also occurs following MD or dark rearing[13].

Here we used microarrays to identify, in an unbiased way, sets of genes whose expression is regulated by visual deprivation produced by monocular enucleation (ME) in visual cortex. The rationale for this search for activity-regulated genes was based on the knowledge that visual experience is required for the normal functional and structural maturation of cortical circuits. Therefore, we reasoned that experience might also regulate the expression of genes required for this process. A pioneer molecule exemplifying this point is brain-derived neurotrophic factor (BDNF), which is not only regulated by visually driven activity, but is also required for the formation of OD columns[14], for OD plasticity in the critical period following MD (ref. 15), and for homeostatic scaling *in vitro*[13]. Using this approach, we found that visual deprivation regulates target genes belonging to a MAP kinase signaling pathway at all ages, whereas other genes are regulated only at specific ages. Moreover, dark rearing had different effects on the regulation of MAP kinase target genes and age-specific transcripts.

RESULTS

Regulation of *Bdnf* and *Fos* is similar after MD or ME

Four-day MD during the critical period caused a shift in the visual responsiveness of binocular neurons toward the open eye[9,16]. Because mouse retinal projections are largely crossed, cortex opposite the open eye remained visually driven whereas that opposite the closed eye was deprived of vision, with the exception of the narrow binocular zone (**Fig. 1a**). As a preamble to microarray experiments, we investigated the possibility of comparing gene expression in the two hemispheres following deprivation by taking advantage of the well-known down-regulation of *Bdnf* mRNA within visual cortex following blockades of vision[17]. Monocular eyelid suture from P20–P24, sufficient to cause a shift in the visual responsiveness of binocular neurons toward the open eye[9,16], resulted in a large decrease in *Bdnf* (and *Fos*) mRNA in visual cortex contralateral to the deprived eye, as monitored by *in situ* hybridization (**Fig. 1b–e**). mRNA expression in cortex contralateral to the nondeprived eye remained nearly indistinguishable from that seen in unmanipulated brains (**Fig. 1b–e**) because of the largely crossed axonal projections of the retinal ganglion cells (**Fig. 1a**). Contralateral

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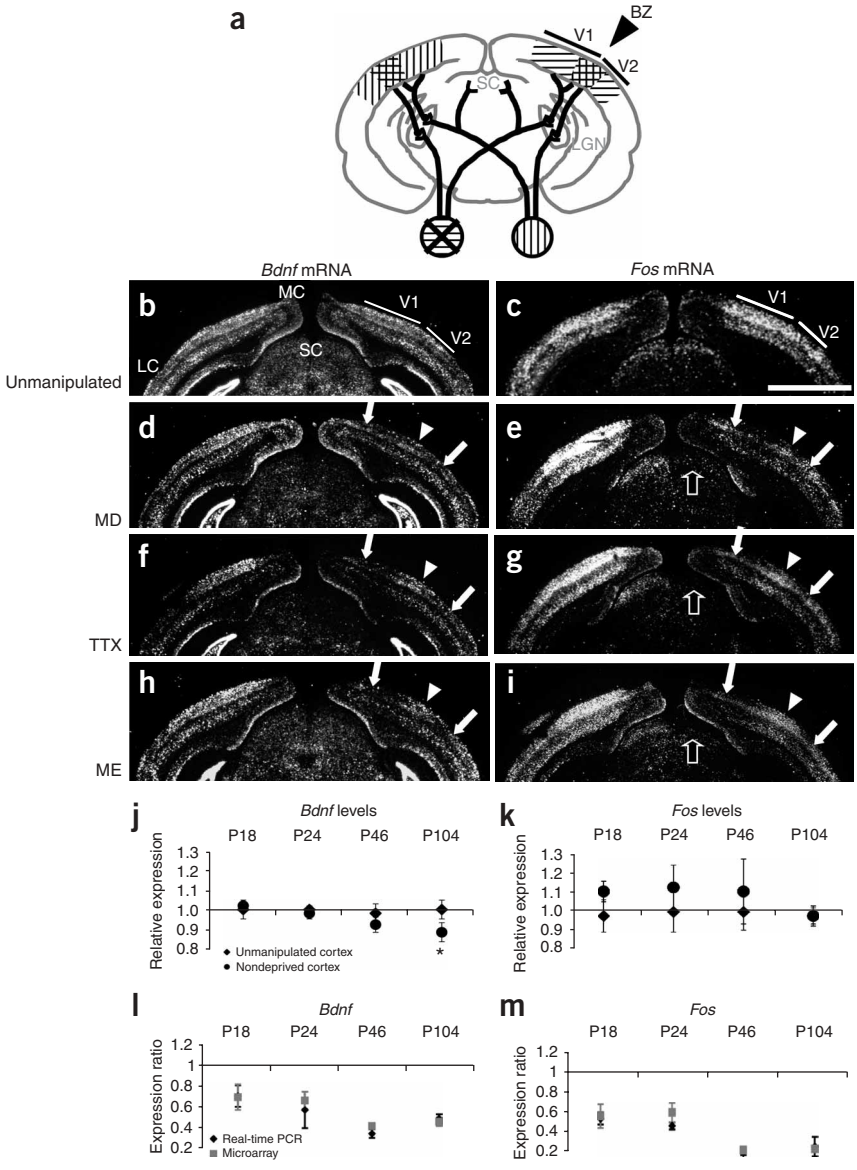

Figure 1 Mouse visual system connections and gene regulation by 4 d of visual deprivation. (**a**) LGN neurons receive bilateral retinal ganglion cell inputs and project to both primary (V1) and secondary (V2) visual cortex. Each visual cortex (hatched) receives inputs almost entirely from the contralateral eye; a small uncrossed projection forms a binocular zone (BZ) at the V1/V2 border (cross-hatched). (**b**,**c**) Equal and high expression of *Bdnf* (**b**) and *Fos* (**c**) mRNA in V1/V2 and superior colliculus (SC) in both hemispheres of unmanipulated cortex; lower expression in nonvisual cortex medial (MC) or lateral (LC) to V1/V2. (**d**–**g**) Four days (P20–P24) of MD or unilateral retinal TTX blockade decreased *Bdnf* (**d**,**f**) and *Fos* (**e**,**g**) mRNA in V1/V2 contralateral to the manipulated eye (arrows); *Fos* was also decreased in contralateral SC (open arrow in **e**,**g**,**i**). Note remaining expression in binocular zone within deprived cortex (arrowheads). Four days of ME decreased *Bdnf* and *Fos* in deprived visual cortex and superior colliculus (**h**,**i**). Autoradiographs of representative sections ($n = 3$–6 mice per treatment). (**j**,**k**) Similar *Bdnf* and *Fos* levels in nondeprived visual cortex (contralateral to intact eye; circles) and unmanipulated cortex (diamonds). At P104, *Bdnf* mRNA was slightly lower in nondeprived cortex than in unmanipulated cortex (asterisk: $P = 0.04$); *y*-axis: expression ratio in nondeprived cortex (contralateral to intact eye) versus unmanipulated visual cortex. (**l**,**m**) Quantification of ME-induced *Bdnf* (**l**) and *Fos* (**m**) regulation by microarray (gray) or real-time PCR (black); *y*-axis: expression ratio in deprived versus visually driven cortex ($n = 4$–5 mice each). Error bars represent s.d. Scale bar: 2 mm.

to the deprived eye, *Bdnf* and *Fos* mRNA expression was maintained only in the binocular zone due to remaining inputs driven by the open, ipsilateral eye (**Fig. 1d**,**e**).

Manipulations that entirely block visually driven inputs from one eye to cortex, including tetrodotoxin (TTX) retinal activity blockade or ME, resulted in *in situ* hybridization patterns and levels of *Bdnf* and *Fos* mRNA expression indistinguishable from those caused by MD (**Fig. 1d–i**), in which the deprived eye retained low levels of spontaneous activity. ME was chosen to induce changes in gene expression for several reasons. First, large regions of visual cortex contralateral to the enucleated eye are deprived of all visual input, while visual cortex contralateral to the intact eye remains functionally active even within the binocular zone due to the strong bias in favor of the contralateral eye. Thus, information on how vision regulates gene expression could be obtained by comparing microarray analysis of the two hemispheres in the same mouse following ME. mRNA for both *Bdnf* and *Fos* showed the predicted downregulation following 4 d of ME in visual cortex contralateral to the enucleated eye (**Fig. 1h**,**i**); little or no change in expression was detected contralateral to the intact eye (**Fig. 1j**,**k**). Second, gene regulation was more consistent in mice after a 4-d period of ME than after MD or TTX, in which retinas can retain low levels of spontaneous activity or vary in the extent or duration of blocked activity. In the experiments described below, ME was performed for 4 d beginning at the following ages: P14 (this entire deprivation period occurs before the onset of the physiologically defined critical period), P20 (during the critical period), P42 (after the close of the critical period) and P100 (adult). All these ages are beyond the time when retinal projections to the LGN can undergo rearrangements caused by ME (ref. 18). Therefore, LGN neurons normally receiving inputs from the removed eye are no longer visually driven[16].

We used real-time polymerase chain reaction (PCR), which permits quantitative comparison of mRNA levels in deprived versus visually driven cortex, to corroborate predicted changes in gene expression following ME in independent samples of visual cortex: at all ages studied, *Bdnf* and *Fos* mRNAs were downregulated in visual cortex contralateral to ME, with the greatest downregulation seen at P46 and older (**Fig. 1l**,**m**) In contrast, there was no significant difference in *Bdnf* or *Fos* mRNA expression when visual cortex contralateral to the intact eye in an enucleated mouse was compared to visual cortex from an unmanipulated mouse (**Fig. 1j**,**k**; $P > 0.05$; with an exception at P104: $P = 0.04$). These observations validated the approach of comparing gene expression between two hemispheres in the same mouse, using the hemisphere contralateral to the intact eye as a baseline 'control' that

receives close to normal amounts of visually driven activity. Together, these results also indicated that real-time PCR is an accurate method for assessing activity-dependent gene regulation within the samples to be used for microarray analyses.

Vision regulates different sets of genes at specific ages

Next, we searched for sets of genes regulated by visual deprivation. By 'regulated' we mean genes whose level of expression, determined by Affymetrix criteria (Methods), differs in the hemisphere deprived of vision for 4 d versus the hemisphere still receiving visual inputs. Microarray results indicated that each age tested was characterized by an age-specific group of genes maximally regulated (up or down) by ME (**Fig. 2**, top). Small subsets of transcripts, ranging from kinases and other signaling molecules to cytoskeleton and synaptic proteins, fell into this category. One example is the transcript for Ena-VASP-like protein (*Evl*), downregulated at P18 after ME at P14 ($P = 0.014$, $n = 4$ by real-time PCR) but not regulated significantly at other ages (**Figs. 2** and **3**; $P > 0.05$ at P24, P46 and P104, $n = 4$). This gene encodes a protein linking signaling pathways to the remodeling of the actin cytoskeleton[19].

Another subset of genes was maximally regulated at P24 (**Figs. 2** and **3**), during the critical period for the effects of MD. This set included genes with known functions, such as synuclein-alpha (*Snca*), implicated in the pathology of Parkinson's disease and involved in synaptic function and plasticity[20]; insulin-like growth factor binding protein 2 (*Igfbp2*); the gene encoding Mig12, a new protein thought to stabilize tubulin during development[21]; and *Dynll1*, a component of dynein light chain and a protein inhibitor of nitric oxide synthase, highly expressed at these ages and involved in synaptic plasticity (reviewed in ref. 22). In addition, olfactomedin-1 (*Olfm1*), enriched in the developing neocortex[23], was also downregulated following ME at P24.

The largest number of age-specific visually regulated genes was found at P46, after the end of the physiologically defined critical period: 36 transcripts were maximally downregulated, and 3 were upregulated (**Figs. 2** and **3**). The former set included genes encoding for synaptic and signaling molecules, such as gephyrin (*Gphn*), a postsynaptic scaffold protein required for clustering of GABA and glycine receptors[24]; the Rho-associated GTP-binding protein kinase Rock-2 (ref. 25); Mapk6/extracellular signal-related kinase (erk3), an atypical serine/threonine kinase[23]; secretogranin III, a member of the granin family, mediators of the regulated secretory pathway[26]; pleiotrophin (*Ptn*), a ligand for the chondroitin sulfate proteoglycan protein tyrosine phosphatase-ζ (ref. 27); *Rgs4*, a member of the family of G-protein signaling regulators[28]; and connexin-43, the main constituent of brain astrocytic gap junctions. In addition, three transcripts were upregulated following ME at P46: *Dbp*, a putative clock-controlled gene showing circadian regulation[29]; *Ghpr*, associated with synaptic vesicles in cortex[30]; and *Hba* . Finally, three genes were regulated at both P24 and P46: *Cdkn1a* (cyclin-dependent kinase inhibitor P21); *Hmgcl* (3-hydroxy-3-methylglutaryl coenzyme-A synthase), implicated in neurodegenerative disease[31]; and *Matn2* (matrilin-2) precursor, implicated in extracellular matrix assembly[32]. One transcript, *Tsnax* (translin-associated factor X), involved in

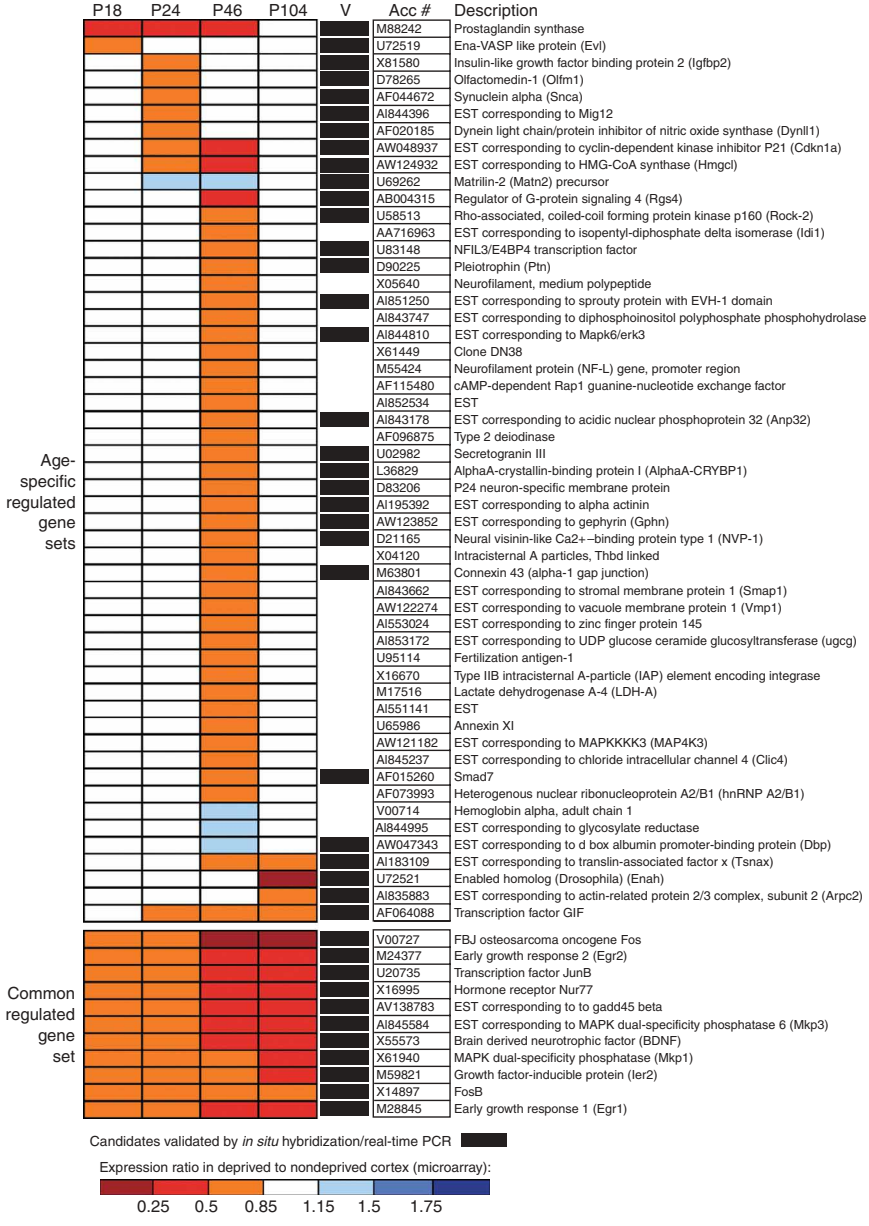

Figure 2 Visual input regulates a common gene set and age-specific gene sets. We performed monocular enucleation (ME) 4 d before the assessment of gene expression and used Affymetrix microarrays to compare regulation between visual cortex contralateral and ipsilateral to ME (at P18, P24, P46 and P104). We validated the regulation of 41 of 42 candidates ('V'; black boxes) by real-time PCR or *in situ* hybridization at every age studied. Color-coding in left columns corresponds to expression ratios as indicated in legend (white: no significant regulation). Note that a common gene set of 11 transcripts was regulated by vision at all ages.

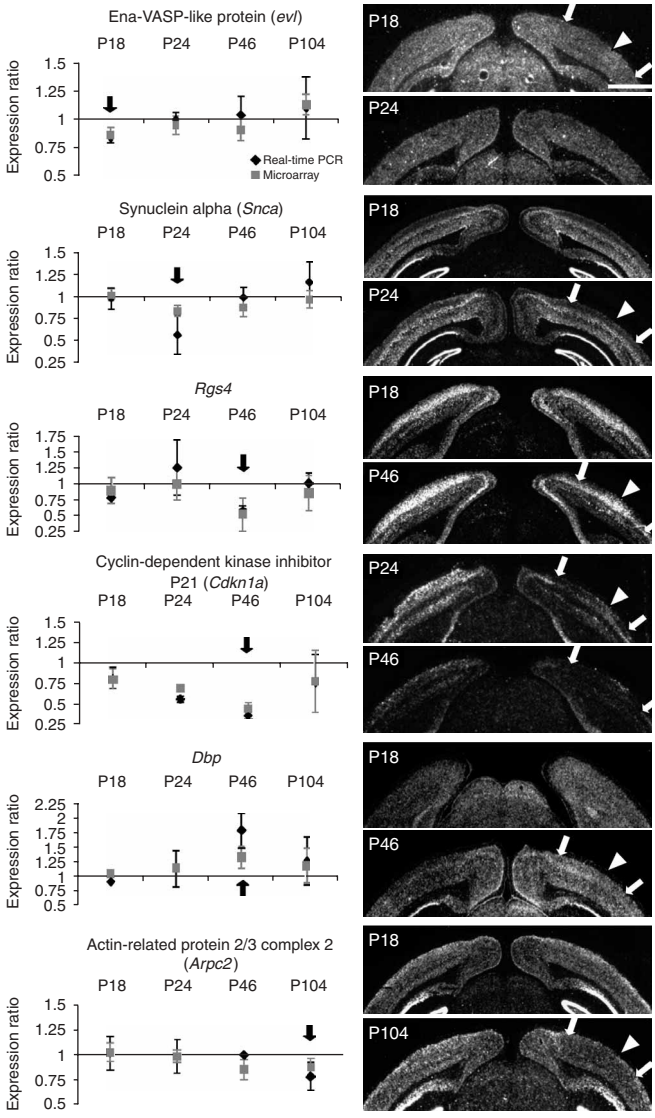

Figure 3 Validation of regulated gene set by real-time PCR and *in situ* hybridization. Left, the regulation of selected candidate genes predicted from microarray analysis (gray squares) and confirmed by real-time PCR (black diamonds; $n = 4$–6 mice at each age); arrow points to age(s) of maximal regulation. *y*-axis: ratio of gene expression in visually deprived cortex (contralateral to ME) versus nondeprived cortex (contralateral to open eye); error bars denote s.d. Right, examples of *in situ* hybridizations for mRNAs corresponding to genes shown on left (in all panels, right hemisphere is contralateral to ME). For each gene, two different ages are shown: one when gene regulation was not significant and one when it was maximal (except for cyclin-dependent kinase inhibitor P21, which was regulated at both P24 and P46). Arrows delimit V1/V2 extent of cortex in the right hemisphere in which age-specific gene regulation was detected, as compared with gene expression in the left hemisphere; arrowheads indicate binocular zone. Scale bar: 1 mm.

targeting and translation of dendritic RNA[33], was downregulated following ME both at P46 and at P104.

In adult visual cortex (P104), only two transcripts were regulated: *Arpc2* (actin-related protein 2/3 complex 2) and *Enah,* the neural-specific homolog of *Drosophila melanogaster* Enabled. Both genes function in actin polymerization, such as that associated with dendritic spine motility, which is present in the adult rodent brain and is associated with synaptic plasticity (reviewed in ref. 34). Thus, gene regulation by visual input was limited in young and adult cortex in comparison to the other ages studied here.

Validation of age-specific gene regulation

We used two additional methods, quantitative real-time PCR and *in situ* hybridization, to validate independently 42 of the 64 genes identified as regulated on microarrays. The regulation of 98% of these genes (41 of 42) was confirmed by these additional measures (**Fig. 2**, 'V'), as was the time of peak gene regulation (for example, **Fig. 3**), which corresponded well with results predicted from microarrays. The corroboration of microarray data by these independent means further validated the experimental approach and results obtained here. Taken together, these observations confirmed that the physiologically defined critical period is characterized by a distinct set of known and new genes regulated by visually driven activity. The finding of robust, visually driven regulation of a set of P46-specific genes, distinct from those seen at any other age, coincides with the recently discovered persistent form of adult OD plasticity[16,35,36].

It is possible that age-specific genes are only detected as regulated at times of highest expression and that modest layer-specific or cell type–specific regulation may escape detection owing to very low expression. This could be especially true for microarray analysis, which may not be sensitive enough to detect modest regulation. However, several reasons make it unlikely that age-specific genes are regulated during broad periods of development. First, although genes belonging to age-specific sets were only regulated at particular times, their levels of expression on microarrays were similar at all ages and were not too low for the detection of regulation (**Supplementary Table 1** online). Second, when *in situ* hyribridizations were performed for these genes at different ages, again expression, but not regulation, was detected, even in a specific layer, such as layer 4. For example, the expression of *Rgs4* and *Dbp*, maximally regulated at P46, was detected both at P46 (when regulation was seen) and at P24, when there was no detectable regulation in cortex (**Fig. 3**).

Vision regulates a common gene set via MEK1/2 signaling

In addition to the age-specific gene sets described above, 11 transcripts were regulated throughout development and were common to all age groups (**Fig. 2**, bottom). Designated the 'common gene set', they were all downregulated in cortex deprived of visually driven activity. Whereas some, such as *Fos*, *Egr1*, *Egr2* and *FosB*, have been identified previously as visually regulated in cortex (for example, ref. 37), others, such as immediate-early gene *Ier2*, *Gadd45b*, and dual-specificity MAP kinase phosphatase *Mkp1*, are new. Downregulation within this group was modest, averaging about 60% of control. *Bdnf* and *Fos*, known to be regulated by visual input, were two of the most robustly regulated transcripts at all ages, and the degree of regulation observed using microarrays closely mirrored that derived from real-time PCR analyses at each age (**Fig. 1l,m**). The close similarity between PCR and microarray data for both *Bdnf* and *Fos*, plus the identification of other genes known to be visually regulated, lends confidence to the argument that microarray data can provide reliable information about the state and regulation of gene expression in visual cortex.

All 11 members of the common gene set were consistently downregulated by ME throughout development (**Fig. 2**). Are these genes functionally related, for example, as part of a shared signaling pathway that is itself regulated by vision? A literature search and an over-representation pathway analysis (Onto-Express[38]) both revealed that the majority of genes comprising the common gene set were transcriptionally activated by the phosphorylation of MEK1/2 (**Fig. 4a**), a

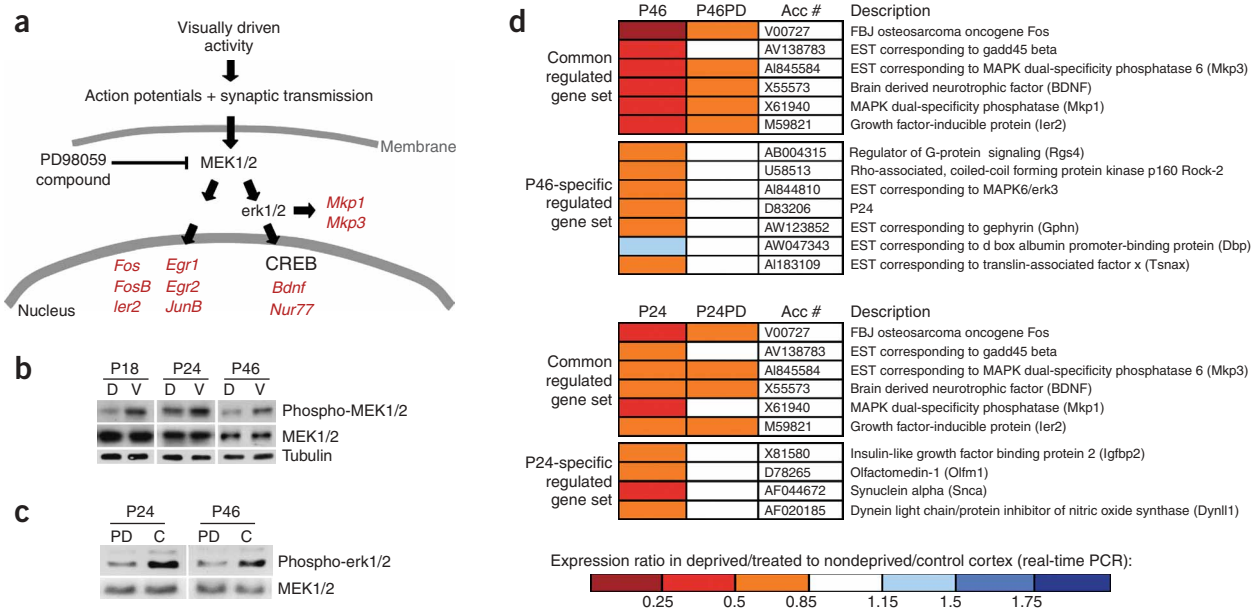

Figure 4 Visual deprivation regulates the common gene set via MEK1/2 signaling. (**a**) 10 of 11 genes belonging to the common gene set (in red) are under transcriptional control of MEK1/2 signaling in several systems (for example, ref. 50). (**b**) Four days of ME decreased levels of activated MEK1/2 in deprived (D) visual cortex (contralateral to ME) compared to visually driven (V; ipsilateral to ME) cortex at P18, P24 and P46. MEK1/2 and tubulin were used as loading controls. (**c**) Levels of phosphorylated MAP kinase erk1/2 (phosphorylated by MEK1/2) were decreased 24 h after the cortical injection of the MEK1/2 inhibitor PD98059 at either P24 or P46 ('PD': PD-injected cortex; 'C': noninjected contralateral cortex). (**d**) Pharmacological manipulation of MEK1/2 activity in visual cortex approximated the effects of ME on the regulation of the common gene set assessed by real-time PCR: 24-h inhibition of MEK1/2 by unilateral injection of PD98059 (second column: 'PD') downregulated the expression of members of the common gene set in injected hemisphere at P46 and P24 to levels similar to those observed after ME (first column: 'P46/P24'; $n = 4$–5 mice each). However, age-specific genes regulated by ME at either P46 or P24 were not regulated after PD treatment (white).

MAP kinase upstream of the MAP kinases erk1/2 and jun N-terminal kinase (JNK) in various experimental systems (reviewed in ref. 39). In neurons, MAP kinase signaling is activated by stimuli associated with synaptic activity and plasticity, most notably calcium influx and neurotrophins (reviewed in ref. 40). Whereas MEK1/2 was not itself regulated transcriptionally by ME (data not shown), levels of MEK1/2 phosphorylation were decreased in deprived cortex at all ages investigated (**Fig. 4b**), indicating that vision can regulate MAP kinase signaling throughout development and implying that attenuation of this pathway may be responsible for the downregulation of genes in the common gene set.

To establish a direct link between MAP kinase signaling and visually driven transcriptional responses of the common gene set, we determined if manipulating MEK activity within visual cortex alters the expression of target genes belonging to the set. MEK1/2 signaling was attenuated pharmacologically by injecting PD98059 compound, a specific inhibitor of MEK activity[41], into visual cortex at P23 or P45. To confirm the inhibition of MEK activity, 24 h later lysates of visual cortex were submitted to western blotting with an antibody to activated erk1/2, the MAP kinase directly phosphorylated by MEK1/2 (ref. 39). As expected, levels of phosphorylated erk1/2 were decreased in visual cortex receiving the PD compound compared to noninjected contralateral cortex (**Fig. 4c**: 'PD' versus 'C'). Control intracortical injections of vehicle (see Methods) did not result in detectable changes in erk1/2 activation. These results confirm that injections of a specific MEK1/2 inhibitor decrease cortical MAP kinase activity and, in this sense, mimic visual deprivation.

To determine whether a decrease in MEK1/2 activity alters levels of target gene expression (**Fig. 4a**), we used real-time PCR to compare the levels of gene expression in visual cortex in which MEK activity was inhibited to the levels in control, contralateral cortex. The expression of five members of the common gene set was indeed decreased in cortex treated with PD compound (**Fig. 4d**). This effect was directly related to MEK1/2 inhibition and was not seen in control cortex injected with vehicle only. In addition, *Gadd45b*, a member of the common gene set not linked to MEK1/2 signaling, was accordingly not regulated in PD-treated cortex. Similar results were obtained when PD compound was injected at P23 and gene expression assessed at P24 with real-time PCR (**Fig. 4d**, bottom). These observations suggest that members of the common gene set are under the transcriptional regulation of MEK1/2.

If the common gene set is a specific target of MEK1/2 signaling, then the expression of other genes regulated at P46 or P24 should not be affected by the pharmacological inhibition of MEK1/2 activity. Indeed, the expression levels of genes shown by microarray analysis to be regulated by vision exclusively at P46 or P24 were not affected by PD injection (**Fig. 4d**). These observations support the conclusions from the microarray experiment: namely, that the MEK1/2 pathway is required for the visually driven regulation of a common set of genes within cortex at a variety of ages including, but not limited to, the critical period.

Visual experience is required for normal gene regulation

The results so far indicate that systematic changes in visually regulated gene sets occur during the development of mouse visual cortex. Many experiments have implicated vision in the progressive physiological and anatomical maturation of cortical circuitry and have shown that the absence of visual experience, as in dark rearing, can extend the critical period[42]. However, little is known about activity-dependent

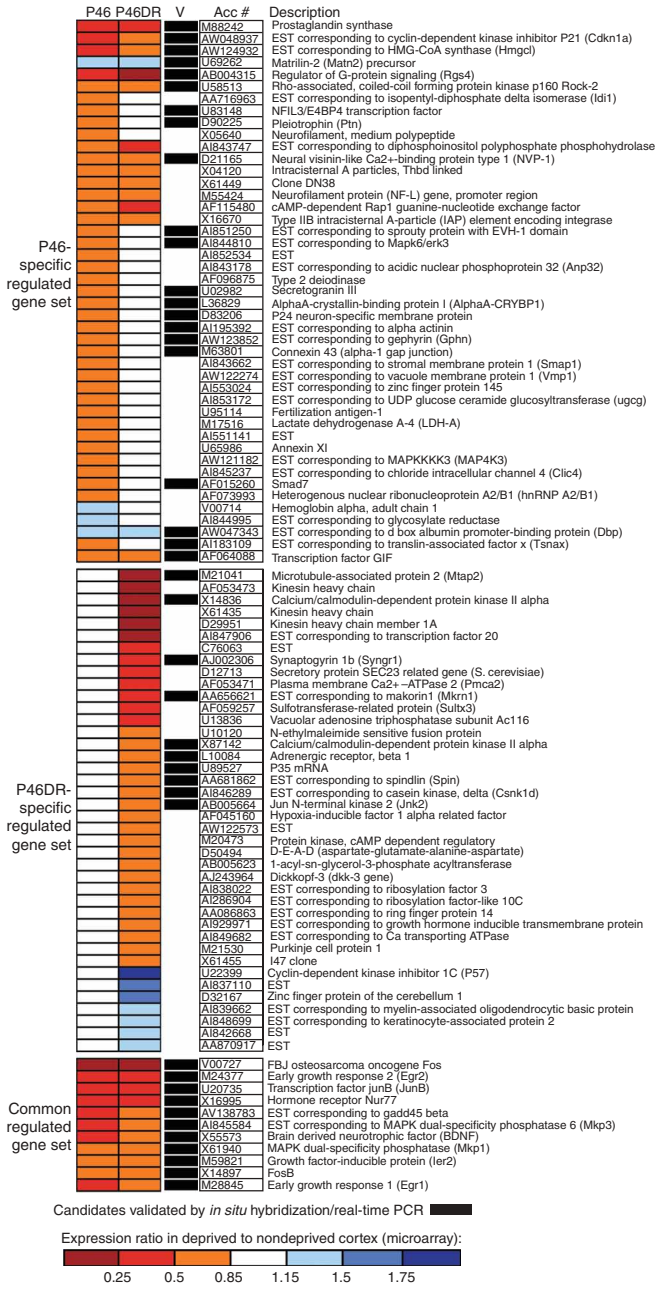

Figure 5 Microarray comparison of genes regulated by vision at P46 in normally reared versus dark-reared mice. Mice were dark reared until P42, then one eye was removed and mice were reared for 4 additional days in light (P46DR); control mice were reared in normal light conditions for the entire 46 d, before and after ME at P42. Gene regulation was assessed by microarray analysis. The common gene set of 11 transcripts regulated by vision at all ages (**Fig. 2**) was also regulated after dark rearing. Only 15 of 45 genes normally regulated at P46 (**Fig. 2**; P46-specific gene set) remained regulated after dark rearing. Forty genes not normally regulated at P46 (or any other age) became regulated after dark rearing (P46DR-specific gene set). Regulation of selected candidates was validated ('V'; black box) by real-time PCR or *in situ* hybridization.

gene regulation in cortex that has been deprived of vision from birth onward. It is possible that normal visual experience is required for the proper regulation of age-specific gene sets. Alternatively, it could be that dark rearing 'freezes' gene regulation in an earlier developmental pattern.

To distinguish between these alternatives and to identify visually regulated genes following dark rearing, we assessed gene regulation at P46 using Affymetrix microarrays. First, we verified that, as previously reported[17], dark rearing decreases *Bdnf* mRNA expression in visual cortex. Mice were either (i) reared with normal vision to P46, (ii) dark reared to P46 or (iii) dark reared to P42 followed by ME and 4 additional days of vision in the remaining eye to assess gene regulation. Gene expression was then compared between normally reared hemispheres (ipsilateral to ME) and dark-reared hemispheres, and between normally reared hemispheres and hemispheres contralateral to ME of mice dark reared to P42. In both cases, *Bdnf* expression was profoundly decreased in the dark-reared hemisphere (by 48 ± 10% and 58 ± 10%, respectively; mean ± s.d.; $P < 0.05$ for both cases by real-time PCR; $n = 5$–7 mice each). This observation indicated that *Bdnf* expression in the hemisphere contralateral to ME in mice dark reared to P42 was essentially identical to that following 46 d of uninterrupted dark rearing. In addition, the ratio of NR2A to NR2B NMDA subunit mRNAs in normally reared cortex was 1.26 ± 0.28, whereas that in dark-reared cortex fell to 0.64 ± 0.24 ($P < 0.05$; $n = 4$ mice by real-time PCR), corresponding to published protein data[43]. In contrast, we found, unexpectedly, that the regulation of *Bdnf* mRNA by vision following dark rearing was intact, as measured with either real-time PCR or microarrays (real-time PCR: regulation in dark-reared cortex = 46 ± 10% versus normally reared cortex = 48 ± 25% of control, $n = 5$–7 mice, $P = 0.86$; microarray: regulation in dark-reared cortex = 52 ± 9% versus normally reared cortex = 45 ± 35% of control, $n = 3$–4 experiments, $P = 0.115$).

Bdnf is a member of the common gene set, regulated by vision throughout normal development beginning as early as P18 (**Fig. 2**). Indeed, all 11 members of this gene set were still regulated following dark rearing (**Fig. 5**), implying that ongoing visual experience is not required for the common gene set to respond appropriately to changes in the levels of visually driven activity.

Despite normal regulation of the common gene set, dark rearing profoundly altered the overall profile of age-specific genes regulated at P46: only 15 of the 45 transcripts normally regulated by vision at P46 were regulated after dark rearing (**Figs. 5** and **6a**). The remaining 30 were no longer regulated, as confirmed by real-time PCR (**Fig. 6b**). In addition, an entirely new set of 40 transcripts, never regulated normally, was now regulated by ME in dark-reared cortex. This set included *Jnk2*, *Mtap2*, *Syngr1*, the β1 subunit of the adrenergic receptor (**Figs. 5** and **6c**), as well as several expressed sequence tags (ESTs). The regulation of several of these genes was confirmed using real-time PCR (**Fig. 5**: 'V'; **Fig. 6**). Together, these results demonstrated that dark rearing does not simply 'freeze' the pattern of gene regulation at earlier developmental ages—no P18- or P24-specific genes were regulated in P46DR mice. Rather, dark rearing changes the molecular signature of cortical gene regulation—only a few genes still responded normally. In addition, normal experience evidently suppressed the regulation of yet another set of new genes, which only became regulated following dark rearing.

In the experiment above, mice were dark reared until P42—well beyond the time of normal eye opening (P10–P14). Shorter periods of dark rearing might not have such disruptive effects on gene regulation by vision. To examine this possibility, mice were dark reared from birth until P20, then ME was performed, and the mice were placed in the normal light-dark cycle for 4 d so as to allow a comparison of gene regulation between deprived and nondeprived visual cortex (using real-time PCR). After dark rearing to P24, the regulation of members of the common gene set was similar to that seen in normally reared mice

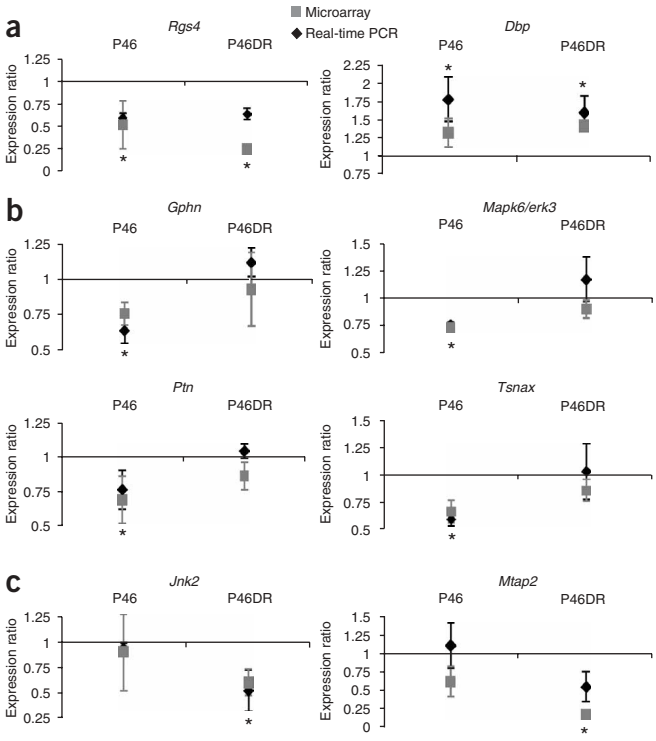

Figure 6 Real-time PCR confirms altered gene regulation in dark-reared cortex at P46. Gene regulation in P46DR mice was compared using microarrays (gray) or real-time PCR (black). (**a**) Genes such as *Rgs4* and *Dbp* were regulated normally in P46DR mice (compare P46DR with P46). (**b**) Other genes, including *Gphn*, *Mapk6/erk3*, *Ptn* and *Tsnax*, were not regulated in dark-reared mice. (**c**) A new group of transcripts, including *Jnk2* and *Mtap2*, were now regulated by ME in dark-reared visual cortex. $^*P < 0.05$; $n = 4$–5 mice. Error bars represent s.d.

(**Fig. 7**). In normally reared cortex, five genes are regulated by vision exclusively at P24 (**Fig. 2**): *Igfbp2*, *Olfm1*, *Snca*, *Mig12* and *Dynll1*. Of these age-specific genes, only *Olfm1* regulation was intact following dark rearing; the other three genes examined were not regulated substantially (**Fig. 7**). *Matn2* precursor and *Cdkn1a*, genes normally regulated at P24 and P46 (**Fig. 2**), remained regulated in mice dark reared to P24 (both were also members of a small set of genes still regulated following dark rearing to P46: **Fig. 5**). We also examined whether *Evl*, regulated exclusively at P18 following normal rearing (**Fig. 2**), was regulated beyond this age in mice dark reared to P24. Real-time PCR analysis indicated that *Evl* regulation was not maintained following dark rearing (*Evl* expression level was 98 ± 19% of control in P24DR versus 100 ± 16% of control in normally reared

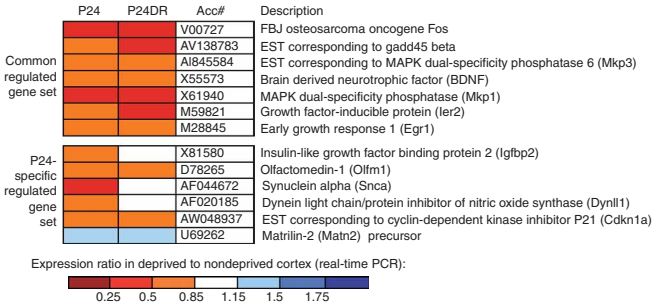

Figure 7 Short periods of dark rearing alter gene regulation. Mice were dark reared from birth to P20, then received ME and were placed in light for additional 4 d (P24DR); gene regulation was assessed with real-time PCR and compared to that in normally reared P24 mice. All members of the common gene set and three P24-specific genes (*Olfm1*, *Cdkn1a* and *Matn2* precursor) were regulated in dark-reared mice at levels similar to those in normally reared P24 mice. Three P24-specific genes (**Fig. 2**: *Igfbp2*, *Snca* and *Dynll1*) were no longer regulated ($n = 4$–5 mice each).

P24 cortex). Together, these observations suggested that even brief periods of dark rearing have profound effects on the profile of genes regulated by vision.

DISCUSSION

Visual experience during a developmental critical period sculpts neural circuits in primary visual cortex (reviewed in ref. 2). Here we show that visual experience is also required to drive progressive and systematic changes in gene regulation. A unique gene set is regulated in mouse visual cortex between P19 and P32 (refs. 9,10), a time characterized by rapid and extensive changes in synaptic strength and connectivity in response to monocular eye closure. Additional nonoverlapping gene sets are regulated by vision at times before and also after this physiologically defined critical period. Although these age-related changes in gene regulation by vision might have been anticipated, there were two rather unexpected results. First, we found a common gene set defining a MAP kinase signaling pathway, regulated by vision at all ages studied. Some members of this gene set are currently considered to represent the best molecular candidates to mediate OD plasticity during the critical period, yet they clearly cannot account entirely for the uniqueness of the critical period as they were also regulated at other ages. Second, we found that dark rearing did not perturb the regulation of this common gene set, but instead profoundly changed the regulation of the age-specific gene sets. Thus, it is not likely that the effects of dark rearing will be understood fully by studying the common gene set alone.

Technical considerations and caveats

In designing the screen, we took advantage of the well-known dominance of contralateral eye input to mouse primary visual cortex: we compared gene expression in the hemisphere contralateral to the open eye with that in the hemisphere contralateral to the removed eye. Within each monocular zone, this approach provided a direct comparison between visually driven versus visually deprived cortex. Within the binocular zone, the comparison was not perfect, as the binocular zone contralateral to the removed eye still received limited visual input from the remaining (ipsilateral) eye. Molecular evidence for this contralateral bias was detected in the *in situ* hybridizations (**Fig. 1**), where levels for *Bdnf* and *Fos* in the binocular zone ipsilateral to the open eye (right) were lower than those in the binocular zone contralateral to the open eye. Given this consideration, it should be stressed that although our screen could not identify genes regulated exclusively within the binocular zone, it was able to detect gene regulation in both the monocular and binocular zones. Examples of such regulated genes include *Rgs4* at P46 (**Fig. 3**). These considerations raise the possibility that the regulated gene sets identified here may participate not only in OD plasticity (see below), but also in other forms of synaptic plasticity, such as homeostatic scaling[13].

Gene regulation, as forecast by microarrays, was verified using two independent methods—real-time PCR and *in situ* hybridization; nevertheless, several technical considerations must be addressed. First, does predicted regulation match previously reported changes? At P24, ME

decreased *Bdnf* expression by nearly 50%, as assessed by microarray analysis and real-time PCR. This reduction corresponded well to levels observed in mice following MD or intraocular TTX injections (**Fig. 1d**,**f**), as well as to those in cat visual cortex following binocular TTX blockade, assessed by northern blotting[44]. Regarding other genes, regulation of two of the candidates identified here (*Nur77/NgfiB*; *Mkp3*/*Dusp6*) was also previously reported at similar levels in response to ME in infant and adult monkey visual cortex[7]. However, because published regulation data are not available for most genes reported here, we relied on real-time PCR as a quantitative measure to verify the predicted regulation, the magnitude of which is well within the known biological range associated with circuit and physiological changes[15].

Although microarray data seem to be accurate in that they do not yield a substantial number of false-positive errors (in which a gene predicted to be regulated is in fact not), false-negative errors are possible. Examples of false negatives can be detected by real-time PCR and *in situ* hybridization. For example, *Igfbp2* and *Olfm1*, predicted by microarray analysis to be maximally regulated at P24, showed additional modest regulation at P46 (data not shown). It is likely that this discrepancy was related both to the relatively modest regulation of these genes at later ages and to the strict algorithm for identifying regulated genes on the microarrays (Methods). In addition, despite limitations inherent in the fact that all layers of visual cortex were included in the analyses, microarray data can provide information on layer-specific gene regulation. In particular, subsequent *in situ* hybridization experiments revealed that several genes were regulated in layer 4 (**Fig. 2**). One of these was regulated during the critical period but not later (*Snca*); another (*Dbp*) was expressed in layer 4 during the critical period but was not regulated until P46. Therefore, the screen could find such genes, although it may certainly have missed others. Obviously, microarray data itself cannot provide information about cell-type or layer-specific gene regulation. Future experiments with higher spatial and/or cell-type resolution are required to address this limitation.

Our screen was based on monocular enucleation (ME) rather than MD, to generate a highly reproducible deprivation condition. Although the physiological effects of ME are not as well characterized as those of MD, gene expression changes seen in cortex following ME are still highly relevant to molecular mechanisms of MD. First, both manipulations resulted in comparable regulation of *Bdnf*, *Fos* and other genes (**Fig. 1**) in visual cortex. Second, this study identified not only BDNF, but also MEK1/2 signaling, both thought to be required for OD plasticity induced by MD, confirming that the ME regime indeed detects the regulation of genes and signaling pathways relevant to OD plasticity. Finally, our screen identified several other members of the common gene set also known to be regulated by MD. It is conceivable, however, that there are genes regulated exclusively by MD; it is likely that we have identified an overlapping gene set.

MEK1/2 is involved in regulation of the common gene set

MEK1/2 activity was downregulated after 4 d of ME at every age studied, suggesting that visual deprivation leads to a sustained, rather than transient, downregulation of the MAP kinase pathway. This observation expands on earlier findings that visual stimulation enhances MAP kinase activity[45,46] and that MEK1/2 activity is required for OD shifts induced by MD during the critical period[46]. Because (as shown here) MEK1/2 is a key regulator of gene transcription in response to vision at all ages studied, we propose that it is also required for synaptic plasticity now known to be present in visual cortex at times before and well after this 'classical' critical period[13,16,35,36]. These results suggest that MEK1/2 activity acts as a 'molecular readout' to relay information about levels of visual activity into the expression of target downstream genes. However, directly blocking cortical MEK1/2 activity only partially recapitulates the effects of ME on gene regulation: whereas expression levels of members of the common gene set were reduced to an extent similar to those seen after ME, levels of age-specific genes were not altered. Thus, it is highly likely that other signaling pathways control the regulation of genes responsive to deprivation at specific ages.

Differential effects of experience on gene regulation

A major finding of this study is that the brain's history of sensory experience has a dual effect on visually regulated genes. A common gene set was regulated at all ages regardless of the history of sensory experience; the other set of genes changed with development, was unique to each time point, and was sensitive to the past history of sensory experience. In addition, dark rearing, even for relatively brief periods, profoundly affected age-specific gene regulation. What are the implications of the P46DR-specific genes, which only become regulated by vision following dark rearing? Our data suggest that vision may drive the expression of a set of transcriptional repressors (or suppress a set of effectors), whose expression is decreased in dark-reared cortex, thereby unveiling a new regulated gene set. It is possible that regulation of this new gene set (in collaboration with the intact common gene set) permits dark-reared cortex to retain aspects of OD plasticity, as is known to be the case from microelectrode recordings[42]. In any case, it is clear that past visual experience is critical for subsequent gene regulation in visual cortex. Together, these results have implications for therapy following early sensory deprivation, as restoring vision may not be sufficient to reinstate normal age-specific gene regulation. Nor is it likely that treatment with *Bdnf* alone, or other members of the common gene set, whose regulation is intact following dark rearing, can restore normal visual function. Rather, it is likely that successful therapy will require an understanding of the mechanisms controlling the regulation of age-specific gene sets and of how they act in concert to modulate cortical circuitry.

Implications for critical period mechanisms

Twenty-one genes are regulated during the physiologically defined critical period. Are any of these required for OD plasticity? Several members of the common gene set are known to be regulated by vision in mammalian visual cortex (for example, refs. 17,47) and at least one, *Bdnf*, is needed for normal visual system development and OD plasticity[14]. In addition, components of the MEK signaling cascade identified here are thought to be required for OD plasticity during the critical period[46]. We have now also added several new candidates, five of which are fundamentally different from *Bdnf*: unlike *Bdnf*, these newly identified candidate genes were maximally regulated at P24 with little or no regulation at other ages. We found that only five genes were regulated exclusively at P24, during the peak of the critical period, whereas many more genes are regulated at P46, when OD plasticity requires longer periods of deprivation[35]. It is certainly possible that more genes are regulated at P24 than could be detected here using microarrays. However, another possibility is that the common gene set defines a core gene regulation program for plasticity at all ages, but that additional gene regulation is required to restrict the plasticity that is promoted at all times by MAP kinase signaling. These results imply that although gene regulation in response to visual deprivation occurs throughout life, the critical period is fundamentally distinct from other times in terms of its molecular signature. This distinction may enable neurons to undergo more extensive anatomical and physiological changes in synaptic structure and function during the critical period than at other ages.

Gene regulation persists beyond the classical critical period

The results presented here reveal a broad temporal window of visually regulated gene expression: whereas regulation at P18 and P104 is modest, there is an intermediate time, from P24 to at least P46, when ME has a major effect on gene regulation. This observation may seem surprising in the context of initial physiological experiments on mouse visual cortex suggesting that the critical period for the effects of MD begins at about P19 and ends at around P32 (refs. 9,10). However, the view of the critical period as a developmentally rigid window has been revisited in rodent cortex: OD plasticity outside of layer 4, assessed by monitoring OD shifts physiologically or molecularly, persists to much older ages[16,35,36] and can begin earlier even in layer 4 (refs. 13,16). Together, these observations indicate that visual cortex can respond to ocular imbalances throughout postnatal development, but to differing degrees and possibly through different cellular mechanisms. Consequently, it is not surprising that different genes are regulated by vision at different developmental times.

We propose that the common gene set is permissive for synaptic plasticity, setting a 'default state' that permits modest adjustments in synaptic strength at any age. Results from the pharmacological blockade experiments suggest that MEK1/2 signaling may have a critical role in this type of plasticity. In contrast, age-specific regulated genes may limit the extent of MEK1/2-dependent synaptic plasticity at each age. This suggestion is consistent with the emerging realization that although synaptic plasticity as measured by OD shifts or by monitoring cellular mechanisms of long-term potentiation (LTP) and depression (LTD) can be detected in visual cortex at many ages, each period has unique features. For example, at early ages, OD shifts occur rapidly and involve extensive structural remodeling of thalamocortical[48] or horizontal[49] connections. In contrast, more modest adjustments in synaptic strength are thought to occur at older ages[16,35]. The combination of a common gene set and an age-specific gene set would endow each period with unique capabilities to respond to alterations in sensory-driven neural activity, while simultaneously ensuring that neural circuits remain capable of adaptation throughout life.

METHODS

Animals and tissue. We used C57BL/6 mice. All animal procedures were performed according to protocols approved by the Harvard University Animal Research Committee and occurred in early afternoon. Mice were killed by the intraperitoneal injection of sodium pentobarbital (50 mg per kg body weight). For *in situ* hybridization, brains were rapidly removed and flash-frozen in octanol (OCT) mounting medium (TissueTek, Sakura Finetek). For dark rearing, pregnant mice were placed in light-tight chambers before giving birth. Some pups were maintained in darkness until P46 and others until P20 or P42, when ME was performed and the mice were placed in a normal light-dark cycle for an additional 4 d before being killed. During dark rearing, all animal and cage maintenance was performed in complete darkness.

Dissection of visual cortex for microarray and real-time PCR analysis. Visual cortex (V1 and V2) or adjacent nonvisual lateral cortex was microdissected according to known maps (for example, **Fig. 1**) from coronal 1-mm-thick brain slices cut on an acrylic matrix (Ted Pella). These were then frozen on dry ice. Before further analysis, we verified the accuracy of the visual cortex dissection using real-time PCR, which confirmed the expected *Bdnf* mRNA downregulation in cortex contralateral to enucleated eye. Only such cortex pairs (over 90% of all samples) were used in subsequent experiments.

TTX injections and surgical procedures. Intraocular TTX injections were performed as described[44]. Mice (P20) were anesthetized with a mixture of isofluorane and O_2 (3%). Then two separate injections of 1.5 μM TTX (0.5–1 μl per 100 g body weight in 0.9% sodium chloride solution; Calbiochem) were delivered into the posterior chamber of the eye at 48-h intervals over 4 d. For ME, mice were anesthetized with isofluorane, the left eye removed, the orbit packed with Gelfoam (Pharmacia and Upjohn), and the eyelid sutured. Mice were killed 4 d following surgery.

RNA preparation and array hybridization. Total RNA was isolated from cortex using Trizol (Gibco BRL). For every microarray experiment, independent pairs of RNA pools from 3–4 mice were used as biological replicates: P18, 5 pairs of 3–4 mice; P24 and P46, 4 pairs of 3–4 mice each; P104, 3 pairs of 4 mice; P46DR, 2 pairs of 4–5 mice. From each sample within a pair, 10 μg of total RNA was labeled, hybridized and scanned at the Harvard University Center for Genomics Research according to Affymetrix protocols (www.affymetrix.com). Microarray analysis was performed on Affymetrix Mouse Genome U34A oligonucleotide arrays.

Microarray data analysis. All analyses were conducted using standard statistics-based Affymetrix GeneChip Software; statistical algorithms were implemented in Affymetrix Microarray Suite version 5.0 (**Supplementary Methods** online). To identify regulated transcripts, each 'Change call' was assigned a regulation score: 0.5/–0.5 for 'Marginal' and +1/–1 for 'Increased'/'Decreased', respectively. Transcripts were considered regulated if their differential expression in deprived versus nondeprived visual cortex yielded a total regulation score of at least 66% (five experiments at P18; four experiments at P24 and P46; four comparisons and two experiments at P46DR; three experiments at P104).

Preparation of probes and *in situ* hybridization. Full-length mouse *Bdnf* (ref. 44) cDNA was used. All other mouse *in situ* hybridization probes were PCR-amplified from a mixture of cortical P18–P104 cDNAs (**Supplementary Table 2** online: primers used for cloning). For all genes and annotated ESTs, reverse transcriptase PCR products were chosen from nonhomologous coding regions flanked by T7 (antisense) and Sp6 (sense) RNA polymerase sequences for subsequent RNA transcription. All PCR products were verified by restriction mapping.

Transcription and *in situ* hybridization were performed as described[44]. For all samples, sense controls run in parallel were found to yield only background levels of silver grains. Images were taken using dark-field optics with a cooled CCD camera (SPOT, Diagnostic Instruments) and analyzed using Photoshop software.

Real-time quantitative PCR. Samples of visually driven and deprived visual cortex (as well as nonvisual lateral cortex) from 4–6 mice were used for each time point (these samples were from different mice than those used for the microarray studies and were not pooled). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Oligonucleotide primers were designed using Primer3 software (Whitehead Institute for Biomedical Research).

PCR primer sets (**Supplementary Table 3** online) and subsequent PCR products were evaluated by gel electrophoresis to confirm a single PCR product of predicted size. All reaction mixes contained 1× iQ SYBR Green Supermix (Bio-Rad), 100 nM each of oligonucleotide primers, and 10 ng of cDNA in 25 μl total volume. Analysis was carried out on a SmartCycler system (Cepheid; **Supplementary Methods**). The relative amount of tested message was normalized to two internal controls, glyseraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase (HPRT).

Western blotting. Visual cortex was removed from P18, P24 and P46 mice (these samples were also used for real-time PCR). Tissue lysis and western blotting were performed as described[41]. Primary antibodies were used overnight at 4 °C: antibody to phosphoserine MEK1/2 (1:1,000), antibody to MEK1/2 (1:2,000) and antibody to phospho-erk1/2 (1:2,000) (all Upstate Biotechnology), and antibody to tubulin (1:5,000; Oncogene Neurosciences). Secondary antibodies were incubated for 1.5 h at 21 °C. Detection was carried out using enhanced chemiluminescence (Amersham) and XAR X-ray film (Eastman Kodak).

PD injections. At P45 and P23, we used a Hamilton syringe to deliver two separate 0.5 μl injections of 50 μM PD98059 (Calbiochem; in 2.5% dimethylsulfoxide (DMSO) in 0.9% saline) unilaterally into visual cortex at a depth of 400–700 μm, approximately in cortical layers 2–4, in 5 mice per age. As controls, injections of 2.5% DMSO in saline were made in three mice. After

24 h, both visual cortices were removed and each was divided into two parts—for real-time PCR analysis and western blotting.

GenBank accession numbers. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4269.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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