The Synaptic Proteome during Development and Plasticity of the Mouse Visual Cortex

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Abstract

During brain development, the neocortex shows periods of enhanced plasticity, which enables the acquisition of knowledge and skills that we use and build on in adult life. Key to persistent modifications of neuronal connectivity and plasticity of the neocortex are molecular changes occurring at the synapse. Here we used iTRAQ quantitation to measure levels of 467 synaptic proteins in a well-established model of plasticity in the mouse visual cortex and the regulation of its critical period. We found that inducing visual cortex plasticity by monocular deprivation during the critical period increased levels of kinases and proteins regulating the Actin-cytoskeleton and endocytosis. Upon closure of the critical period with age, proteins associated with transmitter vesicle release and the Tubulin- and Septin-cytoskeletons increased, while Actin-regulators decreased in line with augmented synapse stability and -efficacy. Maintaining the visual cortex in a plastic state by dark rearing mice into adulthood only partially prevented these changes and increased levels of G-proteins and protein kinase A subunits. This suggests that in contrast to general believe, dark rearing does not simply delay cortical development but may activate signalling pathways that specifically maintain or increase the plasticity potential of the visual cortex. Altogether, this study identified many novel candidate plasticity proteins and signalling pathways that mediate synaptic plasticity during critical developmental periods or restrict it in adulthood. Several of the age- and visual input-dependent proteins were associated with Wallerian axon degeneration. Genetically interfering with this process strongly reduced visual plasticity.
Introduction

Plasticity in the neocortex allows us to learn and adapt to our environment and occurs with active training and passive exposure. In particular during critical periods of development, neuronal connections of the neocortex are highly malleable. Understanding the molecular mechanisms that regulate critical period plasticity is highly relevant because dysregulation of neocortical plasticity during development underlies many disorders of the brain, ranging from a lazy eye to schizophrenia. Knowledge about the molecular events that regulate plasticity may eventually let us control neocortical plasticity during development or reactivate it in adulthood for clinical purposes.

The primary visual cortex (V1) is the most frequently used brain area for studying neocortical plasticity. Especially plasticity of ocular dominance is a convenient experimental model. Prolonged occlusion of one eye (monocular deprivation, MD) during the critical period results in a physiological [Gordon et al., 1996] and anatomical [Antonini et al., 1999] overrepresentation of inputs from the open eye at the cost of inputs from the deprived eye. Dark rearing, whereby animals are raised in total darkness from birth, results in a delayed critical period for plasticity of ocular dominance (OD) [Cynader, 1983]. Because these functional and anatomical changes are well described and can be induced with relative ease, OD plasticity in V1 is highly suitable for identifying cellular and molecular mechanisms involved in neocortical plasticity and its critical period.

Studies in rodents have provided increasing knowledge on the genes and proteins involved in OD plasticity, and the use of both forward- and reverse genetics [Nedivi, 1999; Heimel et al., 2008] has been instrumental in this. Also changes in gene expression observed by microarray studies [Tropea et al., 2006; Majdan et al., 2006] investigating plasticity-manipulating paradigms have generated valuable insights into the molecular processes underlying visual cortex plasticity. In order to study the molecular events intrinsic to the synapse, however, direct approaches to quantitatively address the synaptic proteome are necessary. This can be achieved by assessing fractions biochemically enriched for synaptic membranes. Such an approach has the important advantage that localised events can be revealed that are otherwise hidden in the complexity of molecular changes occurring in other subcellular compartments or in non-neuronal cell types.
Here we performed proteomic analyses using an isobaric tag for relative and absolute quantitation (iTRAQ) and tandem mass spectrometry. We used this approach to identify proteins in the synaptic membrane fraction whose levels are altered by visual experience or age. This method allowed for the labelling of peptides derived from four different experimental paradigms and permitted parallel identification and comparative quantification. We analysed the synaptic membrane proteome of the binocular area of V1 from mice: i) during the critical period, ii) during the critical period while OD plasticity was being induced, iii) in young adult mice after the critical period and iv) in young adult mice in which the critical period was delayed with dark rearing. Direct comparison of these groups enabled us to study the effects of monocular deprivation and age on the synaptic membrane proteome fraction and analyse how dark rearing affected the age-induced changes.

**Materials & Methods**

**Animals**

Throughout the study, male C57BL/6JolaHsd mice from Harlan Netherlands were used. Mice in group 1 (“P30”, binocular visual cortex isolated at P30), group 2 (“P30-MD”, binocular visual cortex isolated at P30 after 4 days of monocular deprivation) and group 3 (“P46”, binocular visual cortex isolated at P46) were housed on a standard 12h light-dark cycle. Right eyelids of P30-MD mice were sutured at P26 under isoflurane anesthesia (Abbott) as previously described [Heimel et al., 2007]. The sutured eyelid of monocularly deprived mice used in optical imaging experiments was reopened at the start of the imaging session at P30. Mice in group 4 (“P46-DR”, binocular visual cortex isolated at P46 after dark rearing) were housed in the dark from before birth until decapitation. Since decapitation for this group was performed in the dark, tissue collection for P30, P30-MD and P46 mice was done just before the end of the dark period of the light-dark cycle, to avoid fast effects of light exposure on protein expression. All experiments involving mice were approved by the institutional animal care and use committee of the Royal Netherlands Academy of Arts and Sciences.

**Tissue preparation and synaptic membrane isolation**

In order to prepare protein extracts enriched for synaptic membranes, binocular visual cortex was dissected, snap-frozen in liquid nitrogen and stored at -80°C until protein isolation. Bilateral binocular V1 was collected, except for P30-MD mice,
for which only the binocular visual cortex contralateral to the deprived eye was isolated. Pools of dissected visual cortex (n=8 hemicortices per treatment, corresponding to four mice in the groups P30, P46 and P46-DR, or to eight P30-MD mice, randomised with regard to litter composition) were homogenised in ice-cold 0.32M sucrose buffer with 5mM HEPES at pH 7.4 and protease inhibitor (Roche), and centrifuged at 1000 x g for 10min at 4°C to remove debris. Supernatant was loaded on top of a discontinuous sucrose gradient consisting of 1.2M and 0.85M sucrose. After ultracentrifugation at 110000 x g for 2h at 4°C, the fraction at the interface of 0.85M and 1.2M sucrose, containing the synaptosomes, was collected, resuspended and pelleted by ultracentrifugation at 70000 x g for 30min at 4°C. The pellet was subsequently resuspended in a hypotonic HEPES solution and lysed. The resulting synaptic membrane fraction was recovered by ultracentrifugation using the discontinuous sucrose gradient as described before. The interface fraction containing the synaptic membranes was collected and pelleted by ultracentrifugation at 70000 x g for 30min at 4°C after which the material was redissolved in 5mM HEPES. For iTRAQ labelling, protein concentrations were determined by means of a Bradford assay (Bio-Rad) after which for each sample, 150μg of protein was transferred to a fresh tube and dried by SpeedVac.

*iTRAQ labelling, two-dimensional liquid-chromatography and tandem mass spectrometry*

Synaptic membranes were dissolved in detergent (0.85% RapiGest Waters Corporation, Milford MA), alkylated with methyl methanethiosulfonate, and digested with trypsin as described [Li et al., 2007; Van den Oever et al., 2008]. Peptides were tagged with the respective iTRAQ reagents (114 = P30; 115 = P30-MD; 116 = P46; 117 = P46-DR). To accommodate four separate pools of tissue of each of the four experimental conditions, a total of four times 4-plex iTRAQ experiments were performed.

Dried iTRAQ samples were separated in the first dimension by a polysulfoethyl A strong cation exchange (SCX) column (PolyLC), and the second dimension on an analytical capillary C18 column (150mm x 100μm i.d. column). The eluate from the C18 column was mixed with matrix (7mg α-cyano-hydroxycinnaminic acid in 1ml 50% acetonitril, 0.1% TFA, 10mM dicitrate ammonium), delivered at 1.5μl/min and deposited onto an Applied Biosystems MALDI plate by means of a robot (Dionex) once every 15s for a total of 384 spots.
MALDI plate analysis was performed on a 4800 Proteomics Analyzer (Applied Biosystems). Peptide collision-induced dissociation was performed at 1kV with nitrogen collision gas. MS/MS spectra were collected from 5000 laser shots. Peptides with a signal to noise ratio over 50 at the MS mode were selected for MS/MS, at a maximum of 30 MS/MS per spot. The precursor mass window was set to a relative resolution of 180. Peaklists were extracted using GPS software (AB Sciex, version 3.6).

MS/MS spectra search was performed against the mouse SwissProt (release 7 February 2007; ~15,000 sequences) and NCBInr (release October 2007; ~150,000 sequences) databases using Mascot (version 2.2, Matrix Science) and GPS Explorer (version 3.6, Applied Biosystems) software. Searches were performed with cysteine modification by methyl methanethiosulfonate as fixed modifications, oxidation of methionine as variable modification, a precursor mass tolerance of 150 ppm, and a fragment mass tolerance of 0.4 Da while allowing a single site of miscleavage. The false positive rates of peptide identification estimated from decoy database searches were ~0.05 for all searches (supplementary table 1, available upon request). For subsequent analysis only those peptides were included that mapped unique to one protein. Proteins were considered for quantification if at least one unique peptide had a C.I. ≥ 95% and at least 2 peptides in three out of four experiments were identified. iTRAQ areas (m/z 114-117) were extracted from raw spectra and corrected for isotopic overlap using GPS explorer. As a low iTRAQ signal is less reliable for quantitation, only peptides with iTRAQ signals above 2000 were included. To compensate for potential variations in the starting amounts of the samples, individual peak areas of each iTRAQ signature peak were log transformed to yield a normal distribution, and normalised to the mean peak area for every sample. The average iTRAQ peak area of all unique peptides annotated to a certain protein was used to determine protein abundance per treatment.

In order to obtain better insight in the concerted changes in protein levels and the underlying biological events, we also inspected proteins that were identified with less stringent criteria (C.I. > 85%, 1 peptide in each set for quantification). We clearly indicated such proteins in figures and tables. We derived no conclusions about individual proteins identified with these less stringent criteria unless we confirmed these findings by Western blot analysis.
To compare the abundance of proteins across four parallel iTRAQ-based experiments (sets A-D), within each experiment peptide quantity values were standardised to scores around zero by subtracting the mean peak of all four samples. Data from all experimental sets were then combined, and analysed by Student’s t-test (independent samples, two-tailed) for each of the four biologically relevant comparisons. As t-test does not take into account the effect of multiple testing, we used the Statistical Analysis of Microarrays (SAM) package [Tusher et al., 2001], a resampling-based method, to estimate the false-positive rate. By creating randomised data distributions SAM estimates the rate of false positive discoveries. The q-value calculated by SAM for each protein reflects the number of empirically determined false positive at the significance level of the respective protein. Therefore the FDR levels in our results hold information about a single protein and should not be interpreted as a global FDR level. Changes in expression levels were considered significant when the p-value was below 0.05 and the respective FDR below 15%. Protein-level quantification data are listed in supplementary table 4 (available upon request). Peptide-level identification and iTRAQ quantification data of the four iTRAQ experiments in this study are listed in supplementary tables 7a-d (available upon request). To establish whether functional categories of proteins were over- or underrepresented among the proteins with increased- or decreased levels under different experimental conditions, we categorised all 467 proteins by function (mitochondrial, or regulating the Actin cytoskeleton or Neurofilament, Tubulin or Septin cytoskeletons, synaptic efficacy or signal transduction, supplementary table 5, available upon request). Next we performed Chi-square tests followed by Benjamini-Hochberg correction for multiple testing to investigate whether any of the functional categories were over- or –under-represented under a specific experimental condition.

All mass spectra used in this study are publicly available at the PRIDE PRoteomics IDEntifications database under Accession numbers: 16649-16656 (http://www.ebi.ac.uk/pride/q.do?accession=1664916656) [Vizcaino et al., 2010].

**Western blotting**

Western blots were performed on the four synaptic membrane protein extracts that were also used for the iTRAQ experiments plus two to four additional samples from pools of four animals kept under the same experimental conditions. The required amount of protein to be applied onto the gel was determined individually for each antibody in a set of test runs. Depending on the antibody, between 1–5μg was used.
Samples for Western blotting were prepared according to the manufacturer’s protocol (NuPage®, Invitrogen) and loaded onto a NuPAGE 4-12% continuous Bis-Tris gel (Invitrogen). Before transfer to PVDF-paper, the gel was soaked in transfer buffer containing 20% MetOH and 0.1% NuPAGE antioxidant for 15min. PVDF-paper was incubated in 100% methanol for 5min, in MQ water and subsequently in transfer buffer. Subsequently, proteins were transferred to the PVDF-paper overnight at 4°C. After transfer, the PVDF-paper was rinsed with water, air-dried and kept at 4°C overnight. It was then reactivated with 100% methanol, washed with MQ water and subsequently with TBS. After blocking with 1% casein solution in TBS for 1h, paper was incubated with either of the following primary antibodies in 0.3% casein solution in TBS with 0.1% Tween (TBST) for 2h at room temperature: mouse-α-Sema4D (BD Transduction Labs, 610670 / 553005, 1:500), rb-α-SOS-1 (Santa Cruz, 1:1000), m-α-Clathrin light chain (Sysy, 113011, 1:250), rb-α-NCAM (Millipore, AB5032, 1:1000), rb-α-Synapsin (Millipore, 1:4000), rb-α-Septin-8 (gift of B. Zieger, 1:1000), rb-α-GAT-1 (Millipore, AB1570, 1:1000), rb-α-Ube4b (gift from M. Coleman, Cambridge UK, 1:50), m-α-14-3-3 beta (Santa Cruz, sc-59417, 1:1000), m-α-14-3-3 eta (Millipore, AB9736, 1:2000), rb-α-GABA(A)-R alpha1 (Millipore, AB5609, 1:1000).

Paper was then washed with TBST and incubated for 1h at RT with an infrared IRDye®800CW-labelled secondary antibody (goat-α-mouse-IR (926-32210) or goat-α-rabbit-IR (926-32211), LI-COR Biosciences; 1:5000 in TBST with 0.01% SDS to reduce background). From secondary antibody incubation onwards, papers were protected from light. Papers were washed with TBST and then with TBS, after which they were scanned for secondary antibody fluorescence using the Odyssey® Infrared Imager (LI-COR Biosciences). Relative amounts of fluorescence were quantified using the Odyssey 2.1 software package (LI-COR Biosciences). To test whether the Western blot confirmed the results obtained with iTRAQ, we determined the significance of the Western blotting data by Student’s t-test (one-tailed, independent samples).

In vivo intrinsic signal optical imaging

Intrinsic signal was imaged transcranially in control C57BL/6 mice or C57BL/6-WldS-mutant mice as described before [Heimel et al., 2007]. In brief, mice were anesthetised by an intraperitoneal injection of urethane (20% in saline, 2g/kg, Sigma Aldrich). Heads were fixed, scalps resected and atropine sulphate (0.05mg/ml in
saline, 0.1mg/kg, Pharmachemie) was injected subcutaneously in order to reduce mucous excretions. A computer monitor covered the mice’ visual field from -15 to 75 degrees horizontally and from -45 to 45 degrees vertically. The screen was divided in 2x2 patches and drifting gratings were used to map the retinotopic representation of V1. The representation of the upper nasal screen patch was used to calculate responses in subsequent tests. For OD measurements, computer-controlled shutters alternated visual stimulation of the eyes using drifting square wave gratings (0.05 cpd). An Imaged Ocular Dominance Index was defined as the iODI = (contra response – ipsi response)/(contra response + ipsi response). A response to the contralateral eye only corresponds to an iODI of 1, an iODI of -1 indicates ipsilateral response only. The following numbers of mice were used: WT undeprived, N=6, WldS undeprived, N=9, WT deprived, N=5, WldS deprived, N=7. Student’s t-tests (two-tailed, independent samples) were used to compute statistical significance.

Results

Identification of proteins affected by monocular deprivation, age or dark rearing using iTRAQ

In order to identify synaptic proteins involved in mediating OD plasticity in the visual cortex or regulating its critical period, we performed quantitative proteomics using iTRAQ on fractions containing synaptic membranes derived from the binocular visual cortex from groups of mice kept under four different experimental conditions (figure 1a). The first group (“P30”) contained mice during the peak of the critical period, at P30. The second group (“P30-MD”) contained mice from the same age that were monocularly deprived from P26 for a period of 4 days. In this group we only used the binocular cortex contralateral to the deprived eye. The third group (“P46”) contained adolescent mice, in which the peak of the critical period had passed, at P46. The fourth group (“P46-DR”) contained mice of the same age that were dark reared, and in which the critical period should thus have been delayed. We used four independent sets of mice per experimental condition in order to adequately replicate our findings (sets A-D).

We identified a total of 467 proteins with a confidence of more than 95% that were detected under all four conditions, in all four sets and quantified with two or more peptides in at least three sets.
We compared the synaptic membrane proteome of P30 with that of P30-MD, P46 and P46-DR, and P46 with P46-DR. Overall, we found that between the different experimental conditions synaptic protein levels differed only to a moderate degree (figure 1b). The total numbers of proteins that had significantly (p<0.05, t-test, and false discovery rate (FDR<15%)) different levels between conditions ranged from 35 to 84 (out of the 467 proteins that were considered). Their average changes ranged between 1.16 and 1.28–fold, depending on the experimental condition. Only a small number of proteins had changed levels of more than 1.25-fold (figure 1b).

Figure 1. Experimental design and numbers of regulated proteins. A) Mice in the first group (“P30”) were reared under a normal 12 hour light/12 dark regime for 30 days. Mice in the second group (“P30-MD”) were reared similarly, but monocularly deprived from P26 for a period of 4 days. Mice in the third group (“P46”) were normally reared for 46 days. Mice in the fourth group (“P46-DR”) were dark reared until P46. The visual cortices from which the binocular zone was collected are indicated with an asterisk (*). B) A total of 467 proteins were identified in all experiments. A modest percentage (7.5–20%) of these proteins were expressed at significantly different levels under the various experimental conditions, and most proteins were regulated less than 1.25 fold. The visual cortex from dark-reared mice at P46 and from normally reared mice at P30 differed most extensively. A colour version of this figure is available in chapter 12.

To validate the results, we performed Western blots for 14 proteins and conditions under which we detected significant changes using iTRAQ (figure 2). Of these 14, we confirmed 11, showing expression level changes concordant with the iTRAQ experiment. In all these 11 cases the level changes as observed by Western blot of the samples that were also used for iTRAQ analyses occurred in the same direction as the samples prepared independently (not shown). In most cases, changes in the levels as assessed by Western blot were larger than observed by iTRAQ, which was
also described previously [Van den Oever et al., 2008]. This is partially caused by the fact that iTRAQ suffers to some extent from the compression of the quantitation ratios to a ratio of 1 when used with complex samples such as our synaptic membrane preparation [Ow et al., 2009].

Figure 2. Quantitative assessments of Western blot analyses performed on proteins significantly regulated with iTRAQ proteomics. Despite the modest changes in protein expression under the different experimental conditions, we confirmed changes in levels for most of the tested proteins (9/14) when assessed with Western Blot analysis (P<0.05, one tailed Student’s t-test of independent samples). Two more proteins showed a trend in the same direction (P<0.06). A colour version of this figure is available in chapter 12.

**Synaptic proteins regulated by MD**

To analyse the effects of MD on synaptic proteins, we compared their relative expression levels in the binocular cortex of P30 and P30-MD mice. During MD, synapses may become stabilised or instead, replaced by new synapses. To obtain insight into which changes in protein levels relate to which of these events, we made two comparisons. Firstly, we compared the significant (p<0.05 t-test, FDR<15%) changes in protein levels induced by MD (figure 3a-c, indicated in grey) with those that occurred with age (P46/P30 ratio, indicated by black bars) and are thus expected to correlate with synapse maturation, which is usually associated with an increase in synapse size, efficacy and stability. Secondly, we compared the changes induced by
MD (P30 MD/P30 ratio) with those induced by DR (P46-DR/P46 ratio, indicated by green bars), which are expected to correlate with reduced synapse maturity. We found that among the MD regulated proteins, there was an anti-correlation between the changes in levels of proteins caused by dark rearing and by age (corr=-0.50, p<0.005) (figure 3d) indicating that the changes in levels of these proteins indeed represent partially opposing biological events.

Figure 3. Proteins regulated by monocular deprivation. Proteins are categorised in groups A) associated with the cytoskeleton, B) involved in signal transduction, or C) regulating synaptic efficacy. Grey bars indicate fold change in relative protein expression levels of individual proteins in monocularly deprived binocular visual cortex compared to the same tissue from normally reared P30 mice. Green lines indicate the relative changes in levels of the same protein with dark rearing (P46-DR/P46) and black lines with age (P46/P30). D) Changes in protein expression induced by monocular deprivation correlate strongly with those occurring with age. As expected, changes in protein expression caused by age (P46/P30) anti-correlate with those induced by dark rearing (P46-DR/P46). * confidence between 85 and 95%. Proteins quantified with less than 2 peptides in more than one set are indicated in italics and light grey bars. A colour version of this figure is available in chapter 12.

Interestingly, significant changes in protein levels induced by MD correlated strongly with changes occurring with age (corr=0.72, p<0.000001), while they showed no significant correlation with changes induced by DR (corr=-0.12, p=0.473). This suggests that V1 of mice monocularly deprived for four days showed a relative increase of mature synapses compared to that of visually undeprived mice.
We categorised the regulated proteins in groups based on their cellular function, allowing us to obtain a better understanding of the functional implications of the observed changes. Shown in figure 3a-c are changes in levels of proteins in those three categories in which more than 5 proteins were found to be affected by MD: a) proteins associated with the cytoskeleton, b) proteins involved in signal transduction, and c) proteins known to regulate synaptic efficacy. The latter include neurotransmitter receptors, proteins regulating their trafficking, and proteins involved in vesicle release and recycling. Together, these groups of proteins represent approximately two thirds of all proteins regulated by MD. All proteins regulated by MD, including those that did not fit into one of these three categories are in supplementary table 2 (see chapter 11). The latter mostly represent proteins with unknown functions in the central nervous system. For completeness, the relative levels, standard deviations, p-values, FDRs and numbers of peptides used for quantitation of all proteins identified with a confidence higher than 85% in all 4 sets and under all 4 experimental conditions are included in supplementary table 4 (available upon request).

Cytoskeleton-associated proteins. A relatively large number of proteins (p<0.05 Chi-square test with Benjamini-Hochberg correction compared to all identified proteins, supplementary table 5, available upon request) of which synaptic expression was increased after MD are involved in regulating the actin cytoskeleton (figure 3a). A number of these also showed higher levels in DR (P46-DR compared to P46), suggesting that these proteins are associated with more immature synapses. These included, among others, the developmentally regulated brain protein Drebrin [Imamura et al., 1992] and Basp1, which has similar and partially overlapping functions in neurite outgrowth (14) as the growth associated protein GAP-43. Other proteins, including Profilin-2, Septin-3, Alpha-adducin and AIP1, also showed higher levels with age (P30 to P46) suggesting that they are associated with more mature synapses.

Protein kinases and G-protein signalling. MD resulted in increased levels of various kinases at synaptic membranes (figure 3b), including several that have been previously implicated in plasticity in V1, such as PKC-alpha and –gamma [Schrader et al., 2004] and the regulatory subunit RII-beta of PKA (16). We also found an increase of Rasal1, an inhibitor of Ras-signalling. In contrast, we observed a strongly (1.59x) decreased level of StARD13, an inhibitor of Rho-signalling.

Proteins regulating synaptic efficacy. Only a small set of proteins involved in regulating synaptic strength (figure 3c) was altered with MD. Among these were Clathrin light chains A and B, which were both up-regulated after MD. We confirmed this by
Western blot analysis (figure 2). This suggests that endocytosis may be activated by MD, which is further supported by our observation that the endocytosis associated proteins Amphiphysin, AP-2 alpha-1 and AP-2 mu-1 were also significantly (t-test, p<0.05) increased after MD, albeit with an FDR of over 15% (supplementary table 2, see chapter 11). The other proteins in this group showing altered levels after MD did not consistently point towards well-defined biological events.

To gain better insight into the broader biological context of the observed changes we also investigated the proteins that were quantified with less stringent criteria (C.I. >85% with 1 or more peptides in all four sets). These proteins are marked in figure 3 by a light grey bar (if quantified by fewer peptides) and/or an asterisk (if the C.I. was between 85 and 95%). Overall, these added proteins fit well in the overall molecular portrait. Among the cytoskeletal proteins, two more actin-related proteins were identified whose levels were higher after MD (figure 3a). Among the signalling proteins with increased levels after MD, ERK-2 was now detected, another well-known kinase involved in OD plasticity (17). Two additional signalling proteins were identified with strongly reduced expression levels after MD (figure 3b). One was SOS-1 (1.64x, confirmed by Western blot, figure 2), an important activator of Ras-signalling which seems consistent with the increased levels of Rasal1, an inhibitor of Ras-signalling. Interestingly, the second protein with strongly reduced expression was Semaphorin-4D (1.41x, confirmed by Western blot, figure 2). This protein induces axonal growth cone collapse and increases spine density upon interacting with its receptor PlexinB1, which inhibits Ras-signalling and activates Rho-signalling (18, 19). We currently do not know whether there is a link between Semaphorin 4D-signalling and the observed changes in the regulators of Rho-signalling (SOS-1), and Ras-signalling (Rasal1 and StARD13). The additional proteins in the last group involved in regulating synaptic efficacy did not provide a consistent picture.

Taken together, MD caused elevated levels of kinases and cytoskeleton-associated proteins, (see figure 8a). A consistent increase or decrease in proteins determining synaptic strength could not be detected. Strikingly, we noticed that significant changes in protein levels induced by MD correlated strongly with changes in the levels of these proteins occurring with age. As 4 days of MD is associated predominantly with synapse loss [Mataga et al., 2004] it is possible that synapses that are not eliminated are of a more mature signature. This would result in a relative increase of spine ma-
turity after deprivation, which may explain the observed correlation between MD- and age-induced changes in protein levels.

**Synaptic proteins regulated with age or dark rearing**

In order to obtain insight into the molecular events involved in the regulation of the critical period, we compared synaptic proteins of binocular visual cortices of P30 mice with P46 and P46-DR mice. To identify proteins that were possibly involved in maintaining or activating plasticity in the dark-reared visual cortex, we categorised the synaptic proteins in three groups. The first were proteins whose expression at synaptic membranes altered with age (P30 vs. P46), but not upon dark rearing (P30 vs. P46-DR). These proteins represent the view that dark rearing prevents specific changes in the visual cortex that normally occur with age and cause the closure of the critical period. The second group contained proteins of which expression was also regulated with age, while dark rearing did not prevent this. These proteins may well affect OD plasticity, but are unlikely to be involved in preventing closure of the critical period by dark rearing. The third group contained proteins of which levels did not change with age, but differed in dark-reared mice from the situation in non-deprived P30 or P46 mice. These proteins are unlikely to be involved in critical period closure with age, but may represent signalling pathways that prevent closure in the dark-reared mice. We found that at P46, 69 proteins were expressed at levels significantly different from the situation at P30 (figure 1b). After dark rearing, levels of 29 of these proteins were not different from the situation at P30, while levels of the other 40 remained different from those at P30. However, dark rearing also induced many changes in levels of proteins that did not change with age, together causing the P46-DR and P30 groups to be the groups most different from each other (figure 1b). Changes in the expression of proteins involved in regulating the cytoskeleton, synaptic efficacy, intracellular signalling or mitochondrial proteins are discussed below. These represented more than 60% of all differentially expressed proteins. Supplementary table 3 (see chapter 11) shows all proteins with altered levels.

**Cytoskeleton-associated proteins.** Similar to the situation in V1 after MD, consistent changes occurred in the level of expression of cytoskeletal proteins with age. We found reduced levels of Actin cytoskeleton-regulating proteins with age (p<0.05 Chi/square test with Benjamini/Hochberg correction compared to all identified proteins), which was partially prevented by dark rearing (figure 4a). This included proteins typically expressed in neurons undergoing synaptogenesis, such as Drebrin.
[Imamura et al., 1992], GAP-43 [Frey et al., 2000] and delta-2 Catenin [Matter et al., 2009]. Alpha-adducin was the only Actin-associated protein whose level increased with age. The levels of Actin cytoskeleton-regulating proteins whose age-dependent decrease was not reversed by dark rearing (figure 4b) were those of Cofilin and beta-Catenin. Interestingly, dark rearing also increased the levels of Actin cytoskeleton-regulating proteins that did not decrease with age (figure 4c). Only one such protein, MARCKS, showed the opposite behaviour and was actually decreased upon dark rearing. In contrast, we detected increased levels of proteins associated with the Tubulin and Neurofilament cytoskeletons with age (figure 4b). These changes were not prevented by dark rearing. We noticed a similar trend for Septins, which have recently been found to form filaments localised at the neck of dendritic spines [Tada et al., 2007] and at the presynapse [Xue et al., 2004]. The overrepresentation of Tubulin-, Neurofilament- and Septin-cytoskeleton associated proteins with age was significant (p<0.0001, Chi-square test with Benjamini-Hochberg correction). Proteins identified or quantified with less stringent criteria (C.I. between 85-95%, indicated with and asterisk, and/or quantified with 1 peptide or more in each set, indicated with grey bars) were two additional Tubulin-skeleton related proteins and various actin-associated proteins that changed their levels in the same direction as other members of these groups.

**Protein kinases and G-protein signalling.** We found that levels of a number of kinases and proteins involved in G-protein signalling were increased with age, which in most cases was prevented by dark rearing (figure 4d-e). These included CaMK-II alpha and PIP5K1-gamma. We were surprised, however, by the relatively large number of proteins (p<0.01 Chi-square test with Benjamini-Hochberg correction compared to all identified proteins) involved in PKA- and G-protein-signalling that were not regulated with age but whose levels were significantly higher upon dark rearing than in P30- or P46 non-deprived visual cortex (figure 4f). These proteins included PKA C-alpha, the regulatory subunit RII-alpha, the PKA anchor protein AKAP150 and the small GTPase H-Ras. Signalling proteins that were decreased upon dark rearing included Ephexin-1, a Rho-type guanine nucleotide exchange factor involved in Eph signalling, as well as Calmodulin. Dark rearing also caused a strong decrease (1.37x) in the integrin-associated protein SHPS-1.
Figure 4. Proteins regulated by age or altered visual experience. Proteins are again categorised A-C) associated with the cytoskeleton, D-F) involved in signal transduction, or G-I) regulating synaptic efficacy. J-K) Proteins that do not belong to these categories but are differentially expressed more than 1.25 fold are also shown. Colour intensity indicates the level of regulation. Lower expression is indicated in blue, while higher expression is indicated in red. The upper panels (A, D, G) show proteins whose expression in visual cortex at P30 is significantly different from that at P46 (left column), while expression in dark-reared visual cortex at P46 is not different from that of P46 or P30 visual cortex from normally reared mice. Middle panels (B, E, H) represent proteins different with age, also if dark reared. Lower panels (C, F, I) show those proteins that are not regulated with age, but whose expression in visual cortex from P46 dark-reared mice differs from that in normally reared mice at P30 or P46. The strongly differentially expressed proteins are categorised in a similar fashion, with the left panel (J) showing the age and dark rearing regulated proteins and one age-only regulated protein, and the right panel (K) showing the proteins only affected by dark rearing. * confidence between 85 and 95%. Proteins quantified with less than 2 peptides in more than one set are indicated in italics and light grey bars. The order of the proteins as shown is determined by hierarchical clustering using average linkage (Multiexperiment viewer, TM4 software). A colour version of this figure is available in chapter 12.
This protein is involved in IGF-I signalling [Maile et al., 2008] which has recently been found to affect OD plasticity [Tropea et al., 2006] and visual cortical development [Ciucci et al., 2007]. When using less stringent criteria for selecting the proteins with altered levels, PKA C-beta was also found to be increased, as were various additional G-proteins.

**Proteins regulating synaptic efficacy.** The group of synaptic membrane associated proteins that were up-regulated with age were dominated by a large set of presynaptic proteins well known to be involved in vesicle release and recycling, including Synapsins-1 and -2 (confirmed by Western Blot, figure 2), Syntaxin-1B, NSF, Munc-18, Synaptogyrin-1, Synaptotagmin-2, Clathrin Lca and AP-2 (figure 4g-h). Dark rearing only partially prevented this (figure 4g), for example in the case of Synapsin-1, Syntaxin-1B and Synaptogyrin-1. Interestingly, no PSD-associated proteins involved in AMPAR trafficking showed increased levels with age, except for Adam22 (and interestingly one of its ligands, LGI1). With dark rearing, several postsynaptic proteins regulating synaptic strength showed altered levels, while they were not changed with age (figure 4i). Levels of two proteins involved in reducing synaptic strength, mGluR2 [Renger et al., 2002] and Synaptojanin-1 [Gong et al., 2008], and one that increases synaptic strength (4.1N) [Lin et al., 2009] were increased upon dark rearing. Interestingly, Syntaxin-1A was not altered with age, but increased with dark rearing, while Syntaxin-1B increased with age, which was reversed by dark rearing. Overall, the changes in proteins regulating synaptic strength were consistent with an increase in proteins involved in vesicle release with age, while dark rearing partially reversed this trend.

Dark rearing has been shown to delay the development of perisomatic GABAergic innervation [Morales et al., 2002], which plays an essential role in initiating the critical period [Hensch et al., 1998]. We were therefore surprised to find an up-regulation of the GABA(A) receptor alpha-1 with DR. The change in GABA(A) receptor alpha-1 could not be confirmed by Western blot, however, potentially because it was too small to detect (figure 2). Moreover, we found a decrease in the level of the GABA reuptake protein GAT-1 (confirmed by Western Blot, figure 2), which is expected to increase synaptic and extrasynaptic GABA levels.

When lowering the selection criteria for the regulated proteins, the overall picture did not alter. GABA(A) receptor beta-1 showed similar changes with dark rearing as the alpha-1 subunit. Interestingly, several proteins found to postsynaptically regulate glutamatergic synaptic transmission were now detected among the proteins
altered with age. These included the strongly (1.25x) increased TARP gamma 3 - a protein regulating AMPAR trafficking, the kainate receptor Grik5 and a regulator of NMDA receptors, Cask and its interactor Caskin-1. The latter three proteins are also present in the presynapse.

Mitochondrial proteins. Among the proteins whose levels were changed most consistently were mitochondrial proteins (figure 5 and supplementary table 6, the latter is available upon request), which may be derived from membranes of synapse-resident mitochondria.

![Expression of Mitochondrial Proteins](image)

Figure 5. Levels of mitochondrial proteins decrease with age, and more so by dark rearing. Scatter plot of relative changes in expression levels of mitochondrial proteins with age (x-axis, P46/P30) or with age and dark rearing (P46-DR/P30, y-axis). Black squares indicate proteins that are significantly affected with age or with dark rearing. All other identified mitochondrial proteins are represented by grey diamonds.

As synaptosomal membrane fractions result in an enrichment of synaptic proteins but are not pure, it is possible that mitochondria derived from other cellular compartments also contribute to the observed changes. With age, 9 proteins were significantly downregulated. Although in P46-DR mice, 5 proteins were not significantly different from P30, expression of many other mitochondrial proteins decreased fur-
ther resulting in a total of 19 proteins that were significantly decreased with dark rearing. This trend towards downregulation of mitochondrial proteins with age and/or dark rearing did not only reflect a specific subset but was visible in over 85% of all mitochondrial proteins identified, although it was not always significant (figure 5). Moreover, the mitochondrial proteins were overrepresented among those reduced with age (p<0.05 Chi-square test with Benjamini-Hochberg correction) or reduced with DR but not with age (P<0.0001) compared to all identified proteins. This suggests that there was an actual reduction of mitochondrial content in synapses under these conditions.

**Strongly regulated proteins of special interest.**

There were several strongly regulated (>1.25x) proteins that did not fit the categories described above (figure 4j-k). Three of these were proteins associated with the extracellular matrix (ECM), which has been implicated in limiting plasticity after the critical period [Pizzorusso et al., 2002]. With age we found up-regulation of Neurofascin-1, a critical component of the ECM around the axon initial segment [Ango et al., 2004], and Hapln1 (Hyaluronan associated link protein1, also known as Cartilage linking protein 1) a crucial factor for the initial assembly of perineuronal nets [Carulli et al., 2007]. The adhesion molecule N-CAM 180 showed strongly (1.28x) decreased levels with age, which was partially prevented by DR (confirmed by Western Blot, figure 2). N-CAM 180 is a protein that is strongly polysialylated (PSA) and interacts with the ECM. It was recently found to inhibit the development of inhibitory synapses and delay the onset of the critical period [Di Cristo et al., 2007].

When using the less stringent selection criteria, various other proteins with poorly-described functions in the brain were detected, such as XTRP2, FAM5A, Hsp70 12B, ANKFY1 and PKM2 Interestingly, we also identified two ubiquitin ligases implicated in axon outgrowth and degeneration: Ube4b and Rpm-1 also known as highwire and MYCBP2. In P46-DR mice we observed a strong decrease in levels (1.26x vs P46 and 1.45x vs P30) of the E3 ubiquitin ligase Rpm-1. This protein has been implicated in presynaptic growth and inhibition of axon outgrowth [Li et al., 2008]. More recently, it was discovered that Rpm-1 down regulates the dual leucine zipper kinase DLK-1, a kinase, which mediates axon degeneration in C. elegans and Drosophila [Nakata et al., 2005]. In mice DLK-1 has been shown to induce Wallerian axon degeneration [Miller et al., 2009], the disintegration of an axon after its separation from the cell body [Coleman et al., 1998]. In our analysis, DLK-1 itself
was detected only in three out of four experiments. Interestingly, it showed the opposite trend from Rpm-1, with a slightly higher expression with MD (1.06x n.s.), age (1.2x p=0.0036) and DR (1.26x p=0.0024).

Another protein in the ubiquitin pathway, Ube4b was also increased in level after MD (1.17x, n.s.) with age (1.3x) and even more so upon DR (1.7x). This was confirmed by Western blot (figure 2). Ube4b has been implicated in protection of neurons from axonal degeneration [Kaneko-Oshikawa et al., 2005]. Moreover, a mutation in the Ube4b gene locus has been identified in mice results resulting in strongly delayed Wallerian degeneration of axons and their synapses [Coleman et al., 1998]. This so-called WldS-mutation encompasses a gene triplication resulting in the production of a fusion protein consisting of the first 70AA of Ube4b and the Nicotinamide Mononucleotide Adenylyl Transferase NMNAT-1, an NAD+ synthase. Plasticity in V1 may therefore depend on those signalling molecules that also regulate Wallerian axon degeneration.

Comparison of proteomics data to published microarray studies.

In order to see whether our proteomics approach identified similar proteins with MD or DR as identified with a previously published microarray approach we compared our results with those from Tropea et al. [Tropea et al., 2006] (supplementary table 8 (see chapter 11) and figure 6). In this study, MD was performed from p23 until p27, and dark rearing from birth to p27. We found that there was 2.5-fold enrichment of proteins whose message was also found to be significantly regulated with MD (13 of 51 proteins, compared to 3748 out of 36,902 genes, p=0.0003, Chi-square test), and a 2.7-fold enrichment for DR (10 of 51 proteins, compared to 2680 out of 36,902, genes p=0.0007, Chi-square test). At the same time, most proteins (75% for MD and 80% for DR) whose levels were changed did not show a concomitant change at the mRNA level underlining the importance of analyzing synaptic proteins directly. The proteins whose levels were changed at both the mRNA and the protein level showed a correlation between these levels with the DR paradigm (0.59, one tailed p<0.05) but not with the MD paradigm (0.05, one tailed p=0.44). When proteins quantified with less stringent criteria were included in the analysis, the results did not change much. The enrichment was then 3.5-fold for MD and 2.8-fold for DR, Moreover, 65% of proteins with changed levels in the MD group did not show a concomitant change at the mRNA level. This was 80% with DR. The correlation for altered
mRNA and protein levels remained 0.59 (one tailed \( p<0.01 \)) for DR and became -0.06 (one tailed \( p=0.38 \)) for MD.

![Graph showing correlation between mRNA and synaptic protein expression](image)

Figure 6. Correlation between mRNA and synaptic protein expression of those proteins significantly regulated at both levels during MD (A) or DR (B). With DR, if proteins are significantly regulated at the mRNA and protein level, there is a strong correlation between these levels (closed circles, \( corr=0.59 \)). The correlation remains the same if less stringently quantified proteins are included (open quadrants, \( corr=0.59 \)). For MD the situation is different and significantly altered mRNA and proteins do not correlate (0.05 for stringently quantified proteins, closed circles, or -0.06 for less stringently quantified proteins).

**Ocular dominance plasticity is reduced in WldS mice**

Because of the observation that these proteins related to Wallerian axon degeneration were strongly regulated with visual deprivation we tested whether in WldS mice, OD plasticity would be altered. By making use of optical imaging of intrinsic signal, we assessed OD in WldS and control mice that were either normally raised or monocularly deprived for 7 days, from P28 to P35. In line with previous findings that in WldS mice development of the neocortex is unaffected we found that retinotopy and OD (WT: \( iODI=0.48+0.03 \), \( N=6 \), WldS: \( iODI=0.46+0.04 \), \( N=9 \), \( p=0.8 \) t-test) were normal in non-deprived mice (figure 7a). However, 7 days of MD indeed caused a much weaker (though still significant) OD shift in WldS mice than in wildtype C57BL/6 mice (figure 7b, WT: \( iODI=0.08+0.07 \), \( N=5 \) WldS: \( iODI=0.28+0.04 \), \( N=7 \), \( p=0.02 \) t-test). This finding thus shows for the first time that the WldS protein does...
not only reduce axon degeneration under pathological conditions, but also inhibits synaptic plasticity under physiological conditions.

Figure 7. Ocular dominance plasticity in WldS mice is strongly diminished. Monocular deprivation for a period of 7 days during the critical period (P28-P35) in control mice changes the imaged ocular dominance index (iODI) from over 0.48 to 0.08, p<0.0005. In WldS mice, plasticity is strongly diminished and the iODI changes from 0.46 in undeprived mice to 0.282 to deprived mice (p<0.05). Error bars indicate SEM. *p<0.05; ***p<0.0005.
**Discussion**

This study provides a comprehensive insight into the changes in levels of synaptic membrane proteins that accompany OD plasticity in the visual cortex (for an overview see figure 8). We found that differences in protein levels after MD, with ageing and upon dark rearing revealed distinct cellular processes that may underlie plasticity. Some of these have been previously observed to be associated with plasticity in V1 through forward or reverse genetics approaches or gene expression studies, whereas many others represent novel mechanisms regulating critical period plasticity.

![Figure 8](image_url)

**Figure 8.** Overview of changes in synaptic proteins. A) With monocular deprivation, the G-proteins SOS-1 and StARD13 are strongly decreased, as is Semaphorin-4D. Actin-associated proteins, Clathrin light chains and various kinases show higher expression. Proteins indicated in the lower bar are believed to act mainly at inhibitory synapses. B) With age, Actin-associated proteins diminish, as do mitochondrial proteins and N-CAM 180. Proteins that increase levels with age include proteins associated with the Tubulin and Septin cytoskeletons, a select group of G-proteins and kinases, proteins involved in vesicle release and proteins associated with the extracellular matrix, including Neurefasacin and Hapln1. C) Dark rearing causes decreased levels of some age regulated kinases, but also the further decrease of mitochondrial proteins and GAT-1. It increases levels of proteins associated with the Actin cytoskeleton, subunits of Protein Kinase A and G-proteins, Ube4b, GABA(A) receptors and N-CAM 180. A colour version of this figure is available in chapter 12.

**Monocular deprivation alters levels of intracellular signalling proteins, regulators of the Actin-cytoskeleton and endocytosis related proteins**

OD plasticity is associated with the growth and retraction of axons and the loss and gain of dendritic spines. Upon MD during the critical period, we found that levels of proteins involved in regulating the Actin-cytoskeleton were increased, which may well reflect these structural changes of synapses in OD plasticity (figure 8a). MD also resulted in increased levels of PKC and the RII-beta subunit of PKA, two kinases that have been implicated in plasticity in the visual cortex [Fischer et al., 2004; Schrader et al., 2004]. Potentially new candidate proteins that regulate OD
plasticity are the Rho- and Ras regulating G-Proteins SOS-1 and StARD13 that were strongly decreased by MD. This was accompanied by a similarly strong decrease in Semaphorin-4D. This protein has been found to affect GABAergic synapse formation [Paradis et al., 2007] but also shown to affect Rho- and Ras signalling through its receptor Plexin1, which mediates spine stability [Lin et al., 2007] and axonal growth cone collapse [Ito et al., 2006]. The lower levels of Semaphorin-4D may thus stimulate axon growth and spine turnover during OD plasticity.

We also observed an increase in proteins involved in Clathrin-dependent endocytosis upon MD. This is possibly related to its role in AMPA-receptor recycling which has recently been found to be essential for OD plasticity [Yoon et al., 2009], but may also reflect changes in vesicle release or an increase in membrane trafficking accompanying structural changes. In a previous study it was found that cell surface AMPA receptors were reduced after one day of MD in rats [Heynen et al., 2003]. We therefore expected to observe changes in the synaptic proteome consistent with an overall decrease of synaptic efficacy. However, we observed no changes in AMPA-receptor subunits after four days of MD. In fact, the observed changes in protein levels were positively correlated with the changes in these proteins that occurred with age (between P30 and P46). This suggests that after MD, dendritic spines in the visual cortex become more mature, which is usually associated with an increase in their size, efficacy and stability. These observations may be explained by increased spine turnover during OD plasticity. The early decrease of AMPA receptors are thought to precede the actual loss of spines [Heynen et al., 2003], which occurs after 3-4 days of MD [Mataga et al., 2004]. Small spines with lower levels of AMPA receptors are likely to be lost preferentially, effectively causing a relative increase of more mature spines.

**Age-related changes in proteins regulating vesicle release and the cytoskeleton**

With age, we observed an increase in proteins involved in vesicle release such as Synapsins-1 and -2, NSF, MUNC-18, Synaptogyrin-1 and Syntaxin-1B (figure 8b). This suggests that synaptic efficacy increases in V1 during development by boosting the potential for neurotransmitter release, which is in line with a previous study showing a Synapsin-dependent increase of synaptic vesicles and their recruitment in the developing hippocampus [Bogen et al., 2009]. The increase of these synaptic proteins was partially prevented by dark rearing (figure 8c), but the general trend was not altered.
Very consistent changes occurred in the levels of cytoskeleton-associated proteins. While Actin cytoskeleton-regulating proteins decreased with age, proteins associated with the Tubulin-, Neurofilament- and Septin-cytoskeletons increased. Dark rearing did not prevent the increase of these latter proteins. However, the lower level of many of the Actin-associated proteins with age was prevented by dark rearing. Moreover, several Actin-associated proteins whose expression levels did not change with age were higher upon dark rearing. Dark rearing thus prevents specific aspects of synapse maturation in V1, whereas other aspects continue also in the absence of visual input. The increase in Actin-cytoskeleton-regulating proteins is in line with the idea that dark rearing increases spine motility [Majewska et al., 2003] which may underlie the increased levels of plasticity that can be induced during the critical period. Recent evidence suggests that microtubules play an important role in the development and plasticity of dendritic spines [Jaworski et al., 2009]. Their function in critical period plasticity remains to be determined. We also found an increased expression of various Septins, which have been associated with mature synapses and found to be expressed at spine necks [Tada et al., 2007] and at presynaptic sites [Xue et al., 2004].

*Dark rearing increases PKA subunits and G-proteins*

We found that age and DR affected the expression of signalling proteins such as kinases and G-proteins. Some of these proteins changed with age such as CaMK-II alpha, PIP5K1-gamma and a few G-proteins whose function in synaptic plasticity are unknown, and most of these changes were prevented by dark rearing. These changes may well be caused by synaptic activity dependent recruitment of these proteins to the synaptic membrane, as has been shown previously for CaMK-II alpha [Elgersma et al., 2002]. Most interestingly, however, a large set of G-proteins, including the small GTPase H-Ras, and several PKA-subunits were not altered with age, but showed increased levels in the dark-reared visual cortex. It has been shown previously that in the absence of RII-alpha or upon inhibition of PKA, OD plasticity is reduced, whereas cholera toxin (a Gs-protein stimulant), forskolin (a Gs-protein-independent activator of adenylate cyclase) and dibutyryl cAMP (a cAMP analogue) all stimulate OD plasticity in the adult visual cortex [Imamura et al., 1999]. Also the expression of an active form of CREB, a target of PKA, increases adult plasticity [Pham et al., 2004]. Thus, the upregulation of PKA and G-protein signalling proteins
may represent a mechanism by which dark rearing increases the potential for plasticity in the adult visual cortex.

Influence of age and dark rearing on proteins associated with inhibition and the extracellular matrix

In recent years, the development of the extracellular matrix [Pizzorusso et al., 2002] and GABAergic innervation [Hensch et al., 1998] have been implicated in the onset and closure of the critical period. Among the proteins that were expressed at different levels with age or dark rearing we found several proteins that are involved in these events. Among the proteins whose expression levels changed most strongly with age was Hapln1, a protein expressed by Parvalbumin-expressing interneurons and believed to be essential for the formation of perineuronal nets [Carulli et al., 2007]. Its involvement in visual cortex plasticity has recently been identified [Carulli et al., 2010]. A second ECM protein strongly increased with age was Neurofascin-1, a protein that accumulates around the axon initial segment and is indispensable for setting up inhibitory input onto this axon segment [Ango et al., 2004]. Dark rearing did not decrease expression of these proteins suggesting that they may not be the primary regulators of visual input dependent ECM formation. We also noticed a strong decrease of N-CAM 180 with age, which was partially reversed with DR. During early postnatal life, N-CAM 180 is heavily polysialylated and interacts extensively with the ECM. It has recently been shown that upon eye opening expression of PSA-N-CAM quickly decreases, allowing the formation of perisomatic inhibitory inputs onto pyramidal neurons, which in turn initiates the critical period [Di Cristo et al., 2007]. The age-dependent decrease of N-CAM 180 and its reversal by dark rearing may thus be part of this process. Counterintuitively, but in line with previous work [Chen et al., 2001; Tropea et al., 2006] we detected an increase in GABA(A) receptor alpha 1 with dark rearing and a decrease in GAT-1, a protein responsible for GABA reuptake. Together, these changes are expected to increase inhibitory input, which seems to contradict studies showing that dark rearing delays the development of inhibitory innervation [Morales et al., 2002]. A possible explanation may be that dark rearing delays inhibitory synapse formation [Chattopadhyaya et al., 2004], but that this is partially compensated for by an increased sensitivity of postsynaptic neurons to inhibitory input.
Signals regulating Wallerian degeneration are altered with age and dark rearing. Finally, we found that proteins implicated in Wallerian axon degeneration were altered with age and dark rearing. The exact signalling pathways that regulate this active form of axon degeneration are still under intense investigation. Several mechanisms have been implicated and include reduced mitochondrial activity [Yahata et al., 2009], alterations in the ubiquitin/proteasome system [Zhai et al., 2003], increased microtubule acetylation through SIRT proteins [Suzuki et al., 2007], and the increased expression of DLK-1 by reduced activity of the ubiquitin ligase Rpm-1 [Miller et al., 2009]. We found that with DR, expression of many mitochondrial proteins was reduced. In contrast, we detected a strong increase of the ubiquitin conjugation factor Ube4b under these conditions. A fusion protein consisting of the N-terminal part of Ube4b and the NAD synthase NMNAT-1 is expressed in WldS mutant mice in which Wallerian axon degeneration is strongly diminished [Coleman et al., 1998]. These results show that signalling cascades implicated in Wallerian degeneration are also active during the regulation of the critical period, and may thus be involved in mediating experience-dependent plasticity in V1.

Altogether, this study provides unprecedented insight into the changes that occur in the synaptic membrane proteome during OD plasticity and with the regulation of the critical period. The results show that dark rearing does not simply halt cortical development, but alters various signalling cascades different from those that change with age. Proteomic changes induced by MD are different from those induced by DR, and correlate better with those occurring with age possibly due to pruning of immature synapses during the first days of OD plasticity. Our study reveals many novel signalling pathways potentially involved in cortical plasticity. We anticipate that future research on these signalling pathways will significantly advance our understanding of the molecular mechanisms underlying synaptic cortical plasticity.
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References


Chen L, Yang C, Mower GD. Developmental changes in the expression of GABA(A) receptor subunits (alpha(1), alpha(2), alpha(3)) in the cat visual cortex and the effects of dark rearing. Brain Res Mol Brain Res 2001; 88: 135-143.


