General discussion

5

The visual cortex as a model system to study cortical plasticity

Plasticity, the ability of the brain to reorganise its neuronal connections both functionally and structurally under the influence of sensory experience, is fundamental to establishing proper connectivity during development, as well as to learning and memory in later stages of life. The importance of this is emphasised by the effects of suboptimal neuronal wiring in disorders such as schizophrenia and autism. The most popular model system for studying cortical plasticity and the way in which sensory experience shapes neuronal circuitry, is the visual cortex. The organisation of the visual cortex is characterised by a clear correlation between structure and function. Visual experience can easily be experimentally manipulated, and the effects of this manipulation can be measured anatomically, physiologically and molecularly. Depriving one eye of proper visual input during development results in reduced visual acuity in that eye [Dews et al., 1970; Prusky et al., 2000; Prusky et al., 2003; Iny et al., 2006] and a physiological shift of the responsiveness of neurons in the visual cortex from the deprived eye towards the undeprived eye [Wiesel et al., 1963; Gordon et al., 1996]. This ocular dominance (OD) shift induced by monocular deprivation (MD) has a structural component as well; thalamocortical projections conveying input from the deprived eye retract, while there is growth of axons serving the non-deprived eye [Antonini et al., 1999]. Plasticity of the visual cortex is high during a critical period in development, reflecting the increased plasticity we experience during childhood and adolescence, when learning is so much easier than during adulthood. With the end of the critical period, neural plasticity decreases and consequently the recovery from the effects of visual defects on visual acuity (amblyopia) or binocularity is strongly reduced, or absent. The critical period is delayed by dark rearing, which keeps the visual cortex in a plastic state.

A molecular approach towards understanding cortical plasticity

In the last fifteen years, the need for a better understanding of the molecular mechanisms underlying visual cortical plasticity has become widely appreciated. With it has come a shift in interest from higher animals such as the cat towards rodents as preferred animal models. The original popularity of higher animals is logically due to the fact that they are more visual animals. Cats, as predators, depend on proper vision to provide them with food. Mice on the other hand are nocturnal animals that do not rely on hunting. Their eyes are positioned more laterally than they are in predators, a feature that provides these prey animals with a wider vision. In spite of

this, interest in especially the mouse visual cortex has increased over the past decade, largely due to the introduction of transgenic and knockout mouse technology. This allows the investigation of the role of specific genes and proteins in the development of the visual cortex, as well as its functioning and plasticity.

Among the molecular approaches adopted in studying cortical plasticity, the use of reverse genetics has been especially popular, but forward genetics approaches have also been employed. In addition, expression studies have begun to be more widely adopted, in which broad scale changes in gene or protein expression are measured following manipulation of visual experience.

Forward genetics constitutes a genetic approach in which, starting with a specific phenotype, one tries to identify the responsible gene. Traditionally this involves the study of spontaneously occurring mutations and corresponding phenotypes in a pedigree. Later, mutagenesis approaches have been introduced, for example by mutagenic chemical treatment or irradiation of germline cells or using gene recombination approaches to expedite forwards genetics approaches. The gene locus responsible for the phenotype is uncovered through subsequent backcrossing of animals with a mutant phenotype, or by modern DNA cloning and sequencing approaches. In a recent forward genetics approach [Heimel et al., 2008] adopted to study plasticity of the mouse visual cortex, a large number of recombinant inbred strains were used, derived from crosses between C57BL/6J and DBA/2J. Both these strains have been sequenced and the resulting recombinant strains have been mapped, allowing the identification of genetic loci correlating with certain quantitative traits. In this study, optical imaging of intrinsic signals was used to quantify different parameters of visual responses in the visual cortex, as well as the effects of monocular deprivation. The study has provided insight into the heritability of different quantitative traits that are related to vision and/or plasticity, and identified a genes locus correlating with the loss of deprived eye response, carrying 13 genes, among which is Stch, a gene of which expression is downregulated after monocular deprivation and dark rearing [Tropea et al., 2006]. The authors also demonstrated that deprived eye response reduction and non-deprived eye response strengthening, together constituting the OD shift following MD, were found not to correlate and therefore to be different genetic traits. This is in line with recent studies showing that the reduction in deprived eye responses commences before the gain in the undeprived eye response [Frenkel et al., 2004; Mrsic-Flogel et al., 2007].

The rationale of reverse genetics is to understand gene function by studying the phenotypic effects of inactivating an endogenous gene or introducing a mutant gene with dominant negative or constitutively active properties. The gene or protein of interest can be chosen based on its effects in related plasticity paradigms, for example when its known effects in vitro suggest a role in structural plasticity, or the regulation of its expression during plasticity.

Examples of proteins and mechanisms for which a function in cortical plasticity has been described using reverse genetics include 1) LTP and LTD, 2) the GABAergic inhibitory system 3) the extracellular matrix, 4) neurotrophins and their receptors and 5) other regulators of structural plasticity such as morphogens and myelin-related proteins.

For a long time, attempts have been made to find a relationship between in vitro models for plasticity, long-term potentiation (LTP) and long-term depression (LTD), and OD plasticity. LTP is the long-lasting increase in synaptic efficacy following synchronous stimulation or activity of pre- and postsynaptic neurons, or high frequency stimulation. LTD in turn is the decreased synaptic efficacy that follows asynchronous pre- and postsynaptic activity, or weak synaptic stimulation. Since they reflect experience-dependent modification of synaptic strength, they have been popular in vitro models for plasticity and learning. In contrast to what was initially assumed, it has become clear that the relationship between OD plasticity and both LTP and LTD is not simple and straightforward [Daw et al., 2004]. When LTP and LTD are both abolished, OD plasticity is as well, but neither LTP nor LTD in itself seems to independently correlate directly with OD plasticity. A strong case for the involvement of AMPA receptor endocytosis in deprivation-induced LTD has however been made [Yoon et al., 2009].

Secondly, these molecular approaches have also provided valuable insights into the role of the GABAergic inhibitory system in visual cortex plasticity [Fagiolini et al., 2000; Iwai et al., 2003; Chattopadhyaya et al., 2004] and the link with BDNF [Huang et al., 1999; Hanover et al., 1999]. The intracortical inhibitory system and its maturation are important for both the opening and closing of the sensitive period and as such, intracortical inhibition is a fundamental limiting factor for adult cortical plasticity. Reducing this inhibition using a pharmacological or environmental approach makes it possible to enhance plasticity in the adult visual cortex, in this way restoring OD plasticity and recovery from amblyopia. GABAergic antagonists restore the induction of long-term potentiation between the white matter and cortical

layer III, a type of LTP that is lost by the end of the critical period [Kirkwood et al., 1994]. Dark rearing, which delays the maturation of the GABAergic system, also results in a postponed critical period. Transgenic mice overexpressing BDNF display an accelerated maturation of the inhibitory system that is unaffected by dark rearing. In these animals, the critical period starts and ends earlier, even in the absence of sensory input [Huang et al., 1999; Gianfranceschi et al., 2003]. Thus, a developmental increase in the effectiveness of intracortical inhibition, which should reduce excitation, could restrict activity-dependent synaptic modification to a brief critical period. A crucial role seems to be played by a specific subset of interneurons, the parvalbumin-positive basket cells that provide perisomatic inhibition onto pyramidal neurons via synapses containing the alpha1 subunit of GABA(A) receptors [Di Cristo et al., 2007].

A third player in the regulation of visual cortex plasticity is the extracellular matrix (ECM), which provides an inhibitory environment for axonal sprouting, and also inhibits experience-dependent plasticity. Perhaps more importantly, CSPGs form perineuronal nets (PNNs) around the somata of cortical neurons, and preferentially around the somata of parvalbumin-positive GABAergic inhibitory neurons [Hartig et al., 1999]. It is these PNNs that appear to be particularly important in the regulation of visual cortex plasticity and the timing of the critical period. The organisation of PNNs coincides with the end of the critical period, and formation of these nets is delayed by dark rearing [Pizzorusso et al., 2002], a manipulation known to postpone the critical period. In adult rats, degradation of CSPGs by means of chondroitinase-ABC treatment reinstates plasticity and results in an OD shift following monocular deprivation [Pizzorusso et al., 2002; Pizzorusso et al., 2006]. Degradation of the ECM may partly mediate the effects of MD, and it has been suggested that monocular deprivation may result in the release of proteolytic enzymes, which then break down the ECM, facilitating increased spine motility and plasticity [Mataga et al., 2004; Oray et al., 2004]. Monocular deprivation induces increased proteolysis by the protease tissue plasminogen activator tPA during the critical period, but not in adult mice. When the effect of tPA is prevented, OD plasticity is reduced [Muller et al., 1998; Mataga et al., 2004]. A recent reverse genetics study demonstrated the importance of cartilage link protein (Ctrl1, or Hapln1), a protein specifically involved in establishing PNNs. Knockout mice lacking this protein maintain juvenile levels of OD plasticity, and also their visual acuity remains sensitive to visual experience and manipulation thereof [Carulli et al., 2010].

A fourth and classic group of factors involved in cortical plasticity are the growth factors and their receptors, such as the neurotrophin brain-derived neurotrophic factor (BDNF; [Huang et al., 1999; Hanover et al., 1999; Gianfranceschi et al., 2003]), its receptor TrkB [Heimel et al., 2010] and insulin-like growth factor (IGF1; Obata 1999, Tropea 2006). IGF1 is a stimulator of neurite outgrowth and neuronal regeneration [Sjoberg et al., 1989]. Expression of IGF1 signalling components is increased by plasticity stimulating paradigms [Obata et al., 1999; Tropea et al., 2006], and exogenous application of IGF1 prevents the OD shift that normally follows monocular deprivation [Tropea et al., 2006].
A fifth category of proteins is formed by myelin and the myelin-related receptors such as Nogo [McGee et al., 2005], and PirB [Syken et al., 2006; Atwal et al., 2008]. Myelin is a major obstacle for axon outgrowth and regeneration in the adult CNS, for example after injury. A number of proteins associated with it seem to serve to consolidate neural circuitry, and as such exert an inhibitory influence on plasticity. An example of these inhibitors of neurite outgrowth is oligodendrocyte-myelin glycoprotein (OMgp), which binds Nogo receptor (NgR; [Wang et al., 2002]. The importance of interactions between NgR and its ligands was exemplified by the inducibility of an OD shift into adulthood in transgenic mice lacking NgR [McGee et al., 2005].

Notch signalling and synaptic plasticity

In this thesis, we studied the effects on cortical plasticity of signalling through another molecular cascade that has been shown to regulate neuronal morphology [Berezovska et al., 1999; Sestan et al., 1999; Franklin et al., 1999; Redmond et al., 2000] as well as hippocampal synaptic plasticity [Wang et al., 2004], the Notch1 pathway. Increased cell-cell contact between neurons results in ligand binding of the transmembrane receptor Notch1, thereby inducing Notch1 signalling. This in turn results in increased neurite branching, but reduced neurite growth, suggesting that Notch1 may provide negative feedback controlling neuronal morphology and synaptic connectivity [Sestan et al., 1999].
Altogether, we addressed the question if these known effects and characteristics of Notch signalling might also play a role in plasticity in vivo. In chapter two we demonstrated that transgenic expression of a constitutively active intracellular domain of Notch1 (NICD) during the visual critical period resulted in a cell-autonomous reduction of dendritic spine and filopodia densities, meaning that Notch1 signalling

does not only reduce neurite growth during development, but also restricts synaptic connectivity later in life. We subsequently showed that as a functional consequence, postsynaptic activation of Notch1 signalling in layer 2/3 pyramidal neurons in V1 reduced the level of LTP that can be induced in the connections between layer 4 and layer 2/3.

These anatomical and physiological changes observed in NICD transgenic animals resulted in an increased susceptibility of the transgenic mouse to the effects of MD on high spatial frequency responses. When assessing OD plasticity using stimuli of low spatial frequency, the OD shift resulting from monocular deprivation seemed unaffected by NICD transgenic expression. However, following monocular deprivation, responsiveness to stimuli of higher spatial frequencies appeared more reduced in transgenic animals than in controls. Presumably, when tested at higher spatial frequencies, a difference in OD shift magnitude would be observed. During the critical period, visual acuity increases strongly [Heimel et al., 2007]. Possibly, the reduced capacity for synaptic strengthening brought about by Notch1 signalling counteracts this maturation, and results in an increased dependence on visual input and activity. Altogether, we demonstrated the involvement of neuronal Notch1 signalling in activity-dependent cortical plasticity and provide support for the idea that Notch1 activity provides a restrictive signal for neuronal connectivity. Our findings also showed that small changes in OD plasticity might have clearer effects on the development of visual acuity. Visual acuity then, assessed using higher frequency stimuli, may be a more sensitive readout for the effects of signalling cascades and visual experience on cortical plasticity. It is of relevance to note that a loss of acuity is the major problem for people with amblyopia. Together, this underlines the need for studying the mechanisms underlying visual acuity and its plasticity.

We subsequently adopted a microarray and qPCR approach to characterise the molecular signalling downstream of Notch1 in cortical pyramidal neurons, in order to identify potential mediators of the effects of Notch1 signalling on cortical plasticity. Our study showed that neuronal Notch1 signalling predominantly affects the expression of genes involved in transcription and in activity of the Ras/MAPK signalling pathway, a pathway that has been well described in the scope of plasticity in vitro and in vivo. An especially interesting find was the strong regulation of expression of RIAM by Notch. RIAM is involved in dynamics of the actin cytoskeleton [Lafuente et al., 2004; Jenzora et al., 2005], axon outgrowth [Quinn et al., 2006], integrin-mediated adhesion [Lafuente et al., 2004; Lee et al., 2009] and it also interacts with

Fe65, a protein binding APP and mediating its intracellular trafficking [Ermekova et al., 1997]. Altogether this places RIAM in an excellent place to regulate synaptic plasticity and neurite outgrowth, and more research should reveal the effects of RIAM on plasticity in vivo, and how it contributes to the role and effects of Notch signalling. In addition we identified a negative feedback mechanism through which the histone deacetylase HDAC4 restricts Notch1-mediated transcription.

Notch1 signalling, APP signalling and Alzheimer's Disease

An important consideration is the close relationship between Notch, APP and the gamma-secretase activity residing in the enzyme Presenilin. Presenilin is responsible for cleavage of both transmembrane proteins Notch [de Strooper et al., 1999; Song et al., 1999] and APP [de Strooper et al., 1998], and knockout of Presenilin-1 results in a lethal phenotype similar to the Notch1 knockout phenotype [Shen et al., 1997]. Presenilin may cleave APP at different locations, giving rise to amyloid β of various lengths. Amyloid β with lengths of 40 and 42 amino acids is associated with Alzheimer's disease, with Aβ 42 the more likely of the two to form the characteristic plaques in the brain. Presenilin mutations result in an increased ratio of Aβ 42 produced compared to Aβ 40 and are a major cause of familial Alzheimer's Disease [Borchelt et al., 1996; Duff et al., 1996]. It is however interesting to speculate to what degree some of the Alzheimer phenotypic effects may be attributed to effects of the Presenilin mutations on Notch signalling [Struhl et al., 1999; Ye et al., 1999], given the role for Notch in structural and physiological plasticity, as well as in learning and memory.

Naturally, Presenilin has been a likely therapeutic target when attempting to reduce the production of β amyloid plaques, for example by means of gamma-secretase inhibitors. This however also means potential interference with proper Notch signalling. Gamma secretase inhibitors tested so far have been shown to reduce Notch proteolysis and to have toxic effects as a result. The toxic effects described reflect the important role of Notch in cellular differentiation and mostly focus on the intestine and immune system, but neuronal effects should not be excluded either.

Current focus in clinical trials is on new compounds that are to reduce Aβ generation whilst sparing Notch signalling. So far, very limited beneficial effects have been observed, possibly because of the low dose/effect and/or the limited time span over which the studies were conducted. The most promising approach appears to be one of compounds that result in allosteric modulation of gamma-secretase activity, such

as certain NSAIDs and Glivec [Netzer et al., 2003; Wolfe, 2008; Augelli-Szafran et al., 2010; He et al., 2010].

Use of expression studies in understanding cortical plasticity

So far we have discussed reverse and forward genetics approaches for identifying molecular mechanisms involved in OD plasticity. Another approach to obtain insight into the genes and proteins underlying cortical plasticity adopted in the last decade is to study the broad-scale changes in gene expression occurring during development, or those resulting from plasticity-manipulating experimental paradigms such as monocular deprivation. Investigating the regulation of expression of genes and proteins [Ossipow et al., 2004; Tropea et al., 2006; Majdan et al., 2006; Lyckman et al., 2008] constitutes an unbiased method of gene and protein discovery that has resulted in many new candidate genes for closer examination. In addition, by analysing the way in which functional groups of genes or proteins are affected in similar or coordinated ways by experimental paradigms allows us to obtain a better insight into the gene and protein networks, as well as signalling cascades potentially underlying visual cortex plasticity.

Ossipow and colleagues [Ossipow et al., 2004] implicated kinase signalling in the regulation of rodent visual cortex plasticity, a finding corroborated in subsequent studies. Majdan and Shatz showed that expression of certain genes, such as BDNF, Fos and others that are involved in MAPK signalling, is regulated by visual experience irrespective of the age of the animal, a finding made before [Di Cristo et al., 2001]. Inhibition of MAPK (or ERK) blocks LTP and was also shown to prevent the OD shift following monocular deprivation [Di Cristo et al., 2001]. For other genes this regulation by visual experience is restricted to the critical period [Majdan et al., 2006], and especially these may include interesting candidate plasticity regulators.

Gene expression screening following monocular deprivation highlighted the role for growth factors. An interesting find here constitutes the involvement of insulin-like growth factor (IGF1) pathway signalling [Tropea et al., 2006; Majdan et al., 2006]. Following MD, cortical expression of the IGF1-binding protein IGFBP5 was increased contralateral to the deprived eye. This increased expression was shown to be critical for the OD shift. Exogenous application of IGF1, resulting in an increased amount of IGF1 that is not bound to IGFBP5, prevented the effects of MD on OD plasticity [Tropea et al., 2006]. This suggests that reduced levels of IGF are important for effective OD plasticity.

Dark rearing was shown to result in increased expression of genes involved in synaptic transmission and electrical activity, including those for transmitter receptors, a finding that seems consistent with a 'homeostatic' response of cortical neurons to reduced visual activity. It also induced a downregulation of parvalbumin expression, pointing towards parvalbumin expressing inhibitory interneurons as underlying the delayed cortical maturation resulting from dark rearing [Tropea et al., 2006].
Despite the valuable data that gene expression screens such as microarrays have provided on visual plasticity, the interpretation of these data faces a number of limitations. Importantly, changes in gene expression need not necessarily translate to changes in protein levels in general, and more specifically to changes in synaptic protein composition. Generally, the tissue isolated includes a variety of cellular compartments, cell types, and cortical layers. mRNA may be subject to differential susceptibility to degradation and differential effectiveness of translation. Also, the proteins themselves are subject to degradation and trafficking.
Therefore, over the past years proteomic studies have started addressing the expression levels of proteins rather than RNA. Fluorescent two-dimensional gel electrophoresis combined with immunohistochemistry has pointed towards a role for the collapsin response mediator proteins (CRMP) family in sensitive period plasticity [Van den Bergh et al., 2003; Cnops et al., 2004; Van den Bergh et al., 2006; Cnops et al., 2006; Cnops et al., 2007; Cnops et al., 2008]. Expression of members of this family of proteins, which have been shown to regulate neuronal structure parameters such as axon outgrowth [Inagaki et al., 2001; Chae et al., 2009], is changed over development and by modified visual input.

Proteomic analysis of visual cortex plasticity

In the work described in chapter four of this thesis, we looked at broad scale changes in protein expression at the synaptic membrane induced by plasticity-modulating paradigms. An iTRAQ (isobaric tag for relative and absolute quantitation) based proteomics approach was adopted, consisting of initial labelling of the peptides with an isobaric tag, followed by two dimensional liquid chromatography and tandem mass-spectrometry. The isobaric tags fragment during mass spectrometry, giving rise to reporters of different mass for four experimental groups. In this way, initial labelling with an isobaric tag (iTRAQ) results in increased sensitivity of detection during mass spectrometry. We analysed the synaptic membrane proteome of the mouse binocular visual cortex: a) during the critical period (P30), b) during the critical period

while OD plasticity had been induced (P30-MD), c) in young adult mice after the critical period (P46) and d) in young adult mice in which the critical period had been delayed by means of dark rearing (P46-DR). This allowed us to simultaneously compare synaptic proteins levels for four experimental groups and study the effects of visual experience and age on synaptic protein expression, and to analyse the effects of dark rearing on age-induced changes.

The power of our approach lies partly in the power of the analysis, and with it the identification of combined sets of proteins in the same molecular pathways, of which expression is regulated in the same direction and to similar degrees. This allows a much better understanding of the molecular processes and pathways underlying cortical plasticity than separate data on gene expression levels for a few genes. Importantly, the enrichment of proteins associated with the synaptic membrane means that our results are less contaminated with glial proteins, facilitating the analysis and identification of relevant biological processes.

In our study, we confirm the involvement of a number of known signalling pathways and mechanisms in the events underlying visual plasticity and critical period timing. Among these are regulators of the actin cytoskeleton [Oray et al., 2004], clathrin-dependent endocytosis [Yoon et al., 2009], as well as Protein Kinase A (PKA) [Imamura et al., 1999] and PKC [Sacktor, 2011] signalling. We also identified new candidate regulators of ocular dominance plasticity identified in our screen, for example modulators of Ras and Rho mediated signalling.

When comparing expression of proteins in mice during the critical period (P30) with mice in which the critical period has closed (P46), we observed increased expression of proteins involved in transmitter release, regulation of the tubulin and septin cytoskeleton and proteins associated with the extracellular matrix. Expression of proteins regulating the actin cytoskeleton was reduced, altogether reflecting the increased synaptic stability observed in post critical period cortex.

Dark rearing prevented some of the changes affected by age, such as the reduced expression of actin-associated proteins. It also induced increased expression of PKA subunits and regulators as well as a number of G-proteins, including the GTPase H-ras. H-ras is a modulator of presynaptic plasticity [Kushner et al., 2005] and was recently shown to increase cortical plasticity, including OD plasticity [Kaneko et al., 2010], and activation of PKA restores OD plasticity in the adult cat cortex [Imamura et al., 1999]. It thus seems that dark rearing in itself induces activation of signalling pathways involved in maintaining a plastic visual cortex.

Dark rearing resulted in increased expression of GABAA receptor subunits, and reduced expression of the GABA reuptake transporter GAT-1. Possibly, dark rearing delays inhibitory maturation and as such increases tonic inhibition as a type of homeostatic feedback, while decreasing phasic, synaptic inhibition.

Wallerian degeneration and cortical plasticity

An exciting new find in our proteomics study is the possible involvement in cortical plasticity of molecular signalling associated with Wallerian degeneration (WD). WD is an active process of axonal degeneration and fragmentation that normally occurs following nerve injury. It takes place distal to the injury, and is characterised by axonal cytoskeleton disintegration and the axon falling apart over its length, rather than by axon retraction [Waller, 1850]. In chapter four of this thesis we show that in the visual cortex, synaptic expression of several proteins linked to this process of Wallerian axon degeneration is altered with age and with dark rearing, indicating that signalling cascades involved in Wallerian degeneration may be involved in mediating experience dependent plasticity in V1.

In the late nineties of the previous century, a mutant mouse was described displaying delayed Wallerian degeneration [Coleman et al., 1998]. This WldS (for slow Wallerian degeneration) mouse strain expresses a fusion protein consisting of the N-terminal part of the ubiquitination assembly factor Ube4b and the full sequence of NAD+ synthase nicotinamide mononucleotide adenyltransferase-1 (NMNAT-1; Mack 2001). The mechanisms by which the fusion protein brings about this effect are still a matter of debate, but both components of the fusion protein, NMNAT [Araki et al., 2004; Wang et al., 2005] and Ube4b [Wishart et al., 2007] appear to be functional in providing axonal protection. A possibility is for the NMNAT portion to provide support to mitochondrial function in counteracting the degeneration, and the Ube4b portion to affect axonal targeting to allow NMNAT to bring about its effect [Babetto et al., 2010].

Interestingly, our proteomics analysis showed that expression levels of both Ube4b and mitochondrial proteins are changed in opposite directions between our experimental groups. We also demonstrated that WldS mice exhibit a reduced OD shift upon MD, showing for the first time a function for the WldS mutation in a physiological setting. Since structural plasticity such as the reorganisation of axonal projections is one of the hallmarks of cortical plasticity in general and OD plasticity in

specific, the involvement of Wallerian degeneration in cortical plasticity is an enticing possibility.

Whether or not Wallerian degeneration per se is involved in synaptic plasticity remains to be determined. A possibility is that proteins playing a role in the signalling pathways underlying WD are involved in cortical plasticity, but not the process of WD as such. In WldS mice then, it may not be the reduction in Wallerian degeneration itself that is responsible for reduced OD plasticity, but that instead the WldS fusion protein affects OD plasticity in other ways.

The WldS protein may interfere with regulation of mitochondria [Yahata et al., 2009], which we showed to be significantly influenced by visual experience and which may be an important effector of synaptic plasticity [Mattson, 2007]. Interestingly, NMNAT3, a mitochondrial NMNAT subtype, also provides protection against Wallerian degeneration [Yahata et al., 2009]. Also, a proteomics analysis of the striatum of WldS mice showed modified expression of a high number of proteins associated with mitochondrial stability [Wishart et al., 2007]. Another possibility is the involvement of Sirtuin proteins. Araki and colleagues have previously shown that SIRT1 is the downstream effector of increased nuclear Nmnat activity that leads to axonal protection from degeneration [Araki et al., 2004]. Sirtuins are NAD+ dependent protein deacetylases and their substrates include axonal microtubules, mitochondrial proteins and histones. Suzuki and colleagues demonstrated that microtubules in WldS mice are hyperacetylated and hence more stable, rendering the axons more resistant to Wallerian degeneration [Suzuki et al., 2007]. In these animals, sirtuin levels were reduced, and sirtuin overexpression abolished the axonal resistance to degeneration [Suzuki et al., 2007]. However, besides increasing microtubule and axon stability and in this way increasing resistance to WD, sirtuins have also been shown to directly affect synaptic plasticity [Gao et al., 2010]. SIRT1 activation results in increased plasticity, whereas reduced SIRT1 activity results in reduced expression of CREB and BDNF, as well as impaired synaptic plasticity [Gao et al., 2010]. In this way, sirtuins may modulate OD plasticity without Wallerian degeneration taking part in the process.

It will be important to determine the precise way in which different molecular cascades are involved, to determine whether or not Wallerian degeneration as such plays a role in cortical plasticity. The numerous mouse mutants available by now should provide a handle in doing so.

Absence of Notch1 and its ligands among proteins regulated by age and visual input

Interestingly, Notch1 and its ligands Jagged and Delta were not identified among the proteins regulated by plasticity modulating paradigms in our proteomics study. In fact, Notch1, Jagged and Delta could not be identified at all in the analysis. This may very well be a consequence of the low expression levels of these proteins, in line with previous studies demonstrating low postnatal expression levels of Notch pathway components in neurons [Sestan et al., 1999; Stump et al., 2002]. Since Notch functions as a transcription factor, indeed only limited amounts of the protein are required to bring about its effects and (synaptic) expression levels may therefore have been below our detection threshold.

Outlook

Understanding the relationships between functional plasticity, structural plasticity and the molecular changes accompanying these processes is of great importance if we are to ultimately use this knowledge in therapeutically addressing amblyopia and other more severe plasticity-based brain disorders in adults. The work described in this thesis has provided a contribution to this understanding.

With regard to the proteomics results described in this thesis, it will be important to functionally validate a role in cortical plasticity or critical period regulation for the molecular pathways that were identified. Does for example Wallerian degeneration indeed take place during OD plasticity and other occurrences of cortical plasticity? In addition, it will be important to determine in which cell types the specific molecular mechanisms identified are primarily active with regard to visual plasticity. The effects of dark rearing on the inhibitory system will have to be described in more detail. Although a critical role for the GABAergic system has been well established, it is important to determine for example how tonic and phasic inhibition are affected by manipulations of visual experience.

An important goal currently pursued is the reinstatement of juvenile-like plasticity in the adult cortex by means of pharmacological and genetic manipulations. This then should provide new handles for therapeutic treatment of amblyopia and other synaptic disorders. Important factors restricting adult cortical plasticity are the extracellular matrix and myelin-related factors, and the balance between excitation and inhibition (see also [Morishita et al., 2008; McCoy et al., 2009; Bavelier et al., 2010]), the importance of which was confirmed in our proteomics analysis. It is therefore not surprising that successful paradigms for reinstating plasticity in adult rodents

have targeted exactly these factors [Fagiolini et al., 1994; Pizzorusso et al., 2002; McGee et al., 2005; Maya Vetencourt et al., 2008]. When attempting treatments in adult patients, these factors and an approach towards relieving them should at least be considered. However, so far none of these have directly resulted in therapies for amblyopia or other disorders.

Results of non-invasive manipulations in rodents may provide an approach that can be more easily translated into treatment in humans. Environmental enrichment [Sale et al., 2007] and visual deprivation [He et al., 2006; He et al., 2007] are both non-invasive (behavioural) approaches that may reset the excitation inhibition balance and render the adult cortex more plastic. Treatment with fluoxetine has also been shown to reset the excitation inhibition balance and restore OD plasticity and the recovery of amblyopia in adults [Maya Vetencourt et al., 2008].

The current view on cortical plasticity is one of a critical period in development, during which knowledge and abilities may be acquired, which are then consolidated in a cortex that becomes less plastic. In recent years, the division between a plastic young visual cortex and an aplastic, rigid adult cortex has become less black and white, especially in mice [Sawtell et al., 2003; Hofer et al., 2006]. What then are the differences between mice and higher mammals? Are the cortices of rodents and higher mammals really that different? And if so, do these differences between them provide an explanation for the difference between an adult cortex that still displays plasticity, in mice, and one (in higher mammals) that does not seem to do so? Or can the same degree of plasticity also be induced in the adult cortex of higher mammals? Clearly, as adult human beings we are still able to acquire new knowledge and abilities. We may not be the fast-learners we used to be but that does not mean our cortices have lost all plasticity. Recent studies in humans suggest that robust plasticity may be reinstated in adults, and positively affect amblyopia, for example by repetitive transcranial magnetic stimulation [Thompson et al., 2008] or perceptual learning [Zhou et al., 2006; Huang et al., 2008; Levi et al., 2009]. More research into these approaches should reveal the degree of adult plasticity that can be evoked, the optimal ways of reinstating adult plasticity, as well as the degree to which these approaches can be used to induce modes of plasticity different from the ones that have been studied so far.

An important additional challenge will be to address potential side effects and safety issues that come with therapeutic approaches to reinstate adult plasticity, and thus to find a safe application in humans of results obtained in rodents. Since reinstating

adult plasticity will affect the established neuronal network within which our abilities and imprinted memories are embedded [Gundelfinger et al., 2010], but also lead to pathology in itself [Pascual-Leone et al., 2005], new studies will have to demonstrate how the therapeutic reinstatement of plasticity can be controlled both spatially and temporally.

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