Summary, general discussion and a future perspective

Grey matter (GM) integrity changes and GM atrophy are both potentially important MRI GM pathology measures in multiple sclerosis (MS). However, the underlying tissue changes reflected by the MRI measures are unknown and are investigated in the validation studies described in chapter 2. In addition, we gained more detailed insight into the nature of GM pathology in these studies. The results of the studies, described in chapter 2, raised some important questions, like is neurodegeneration (an important part of GM pathology) independent of GM demyelination in chronic MS? And what kind of pathological mechanisms are a in the NAGM, where we observed only subtle neurodegenerative changes? These and other questions will be addressed below.

Validation studies

Chapter 2.1 – In MS, Diffusion Tensor Imaging (DTI) has frequently been used to measure white matter (WM) integrity changes, but recently a few in-vivo studies have also reported DTI changes in MS cortex.1-3 The results of these studies are inconclusive. Some studies report an increase in fractional anisotropy (FA) in MS cortex compared to controls, but others found a decrease in FA. However, all studies have reported an FA increases in GM lesions compared to NAGM.1-3 The pathological process that reflects an increased FA is unknown, but Filippi et al.3 suggested that an increased FA reflects an increase of microglia in GM lesions that form micro-columns, which renders the diffusion within the tissue micro-structure more anisotropic. Our results in chapter 2.1, demonstrate that the increase in FA is probably not due to an increase of activated microglia density in GM lesions compared to normal appearing grey matter (NAGM). As Haider et al4 observed an increase of microglia in deep GM lesions, using anti-Iba-1 and NADPH oxidase antibodies, we decided to perform an additional analysis for the MS cortex in an independent group of patients, using these specific antibodies.

Although this is still ongoing work, our preliminary results still showed no increase of microglia in GM lesions compared to NAGM (Figure 1). It might be that microglia density is patient dependent rather than lesion dependent. Some patients had substantially more microglial activation than others, suggesting different MS populations. The heterogeneity of MS has been reported by a number of studies,5-8 and is nicely illustrated by Kooi et al. for the cortex.7 They observed MS patients with a lot of cortical lesions and patients with relatively small numbers of cortical lesions. Interestingly, a subset of the “cortical lesion group” had cortical lesions with a rim of activated microglia (RAM lesions), while other patients did not. The patients with RAM lesions had a more active WM pathology and a lower age at death, while the age at onset was similar between the investigated groups, suggesting a less favorable disease course.

Chapter 2.2 - GM atrophy is a prominent aspect of MS pathology, and plays an important role from the beginning of the disease.9 In addition, GM atrophy seems
to increase rapidly during disease progression\textsuperscript{10} and is directly related to patients’ cognitive impairment.\textsuperscript{11} At the onset of this PhD project, it was unknown what GM atrophy, as measured by MRI, truly represented in terms of underlying tissue changes. It was hypothesized to be a combination of neuro-axonal degeneration and myelin loss and was therefore often used as a ‘marker for neurodegeneration’ in clinical trials. With a combination of post-mortem in-situ MRI and systematic sampling of standard brain areas, we were able to investigate which (cellular) marker predicts GM volume. We quantified a number of (cellular) markers, including dendrites, axons,

\textbf{Figure 1}
Microglial activity in GM lesions and NAGM. An MS section containing a type I GM lesion, stained for Iba-1. The border of the cortex is indicated with a red dashed line and the border of the lesion with a blue dashed line. Inserts are a digital magnification of the grey matter (GM) and white matter (WM).
Our results show that GM volume is mainly predicted by neuronal loss, neuronal shrinkage and axonal loss. Interestingly, myelin density was not a significant predictor of GM volume, which suggests that neurodegeneration is independent of demyelination. This result is in line with the study of Wegner et al.,\textsuperscript{12} who did not find a spatial correlation between cortical thinning and cortical lesions. We investigated the relation between demyelination and neurodegeneration in more depth in \textbf{chapter 3}. We used a rather complex statistical model, which accounts for the “nestedness” of our study design. Future studies using a larger sample will have to investigate the relation between axonal and neuronal density in predicting GM volume, as well as other factors such as dendrite density, synapse density and oligodendrocyte density; factors which now appeared to be less significant, but might still be relevant. It would also be interesting to investigate if there are regional differences in predictors of GM volume. In general, neuronal loss and axonal loss were found to be the most prominent factors contributing to GM atrophy in our studies, but these predictors might vary in strength from region to region. For example, Dutta et al.\textsuperscript{13} observed no neuronal loss in MS hippocampi, but a significant ~2 fold reduction in synapse density compared to control hippocampi. Unfortunately hippocampus was not included in our study, because the hippocampus was non-standard dissected and anatomical level within the hippocampus varied substantially per patient.

\textbf{Chapter 2.3} - Several methods have been used in-vivo to measure GM atrophy in MS, with the most ‘popular’ being SIENAX, FreeSurfer and SPM. However, it is unclear whether they are equally valid, i.e., whether they reflect changes on the tissue level equally well. We aimed to assess the relation of FreeSurfer, FSL-SIENAX and SPM regional cortical volume measurements in five systematically sampled cortical areas, with a histological reference: cortical thickness measured on Nissl stained sections. FreeSurfer and SIENAX were run in automated mode and with manual editing. SPM was only run in automated mode. There was a significant correlation between GM volume and histological cortical thickness for FreeSurfer and SIENAX with manual editing. Interestingly none of the evaluated methods were significant in automated mode, stressing the use of manual editing to improve the automated segmentation, especially in a disease like MS in which the average GM atrophy rate is only 0.2-0.6% per year.\textsuperscript{10} This does not necessarily mean that the evaluated methods cannot be used in automated mode to accurately measure GM atrophy. They can be used in automated mode to detect atrophy by assessing differences in volume measurements taken at different time points, as long as it is sensitive to detect changes at all, and is consistent in its bias. Our results are in contradiction with the result of an earlier in-vivo study performed by Derakhshan et al.,\textsuperscript{14} which used a manually outlined GM mask of three brain slices from three MS brains as a reference volume. The manually outlined masks corresponded better with the SPM GM outline than with FreeSurfer and SIENAX. The discrepancy in results might be due to the difference in sample size and due to the fact that we measured GM volume in brain synapses, neurons, astrocytes, oligodendrocytes, microglia and myelin density.
regions more spread throughout the brain, compared with the regions included in the three brain slices as used by Derakhshan et al.\textsuperscript{14}

The validation studies have contributed to the understanding of commonly in-vivo used MRI measures for MS. We now know that cortical integrity changes do not represent microglial activation, and neurodegeneration is reflected by MRI-measured GM atrophy. In addition, these studies have also led to new insight in the pathology of MS. In chronic MS, GM atrophy is independent of demyelination, which suggests that the lesions that are missed using conventional MRI techniques will only have minimal impact on measured neurodegenerative changes. As GM atrophy can be reliably measured using conventional MRI techniques, GM atrophy measurements will play a more prominent role in monitoring disease progression than GM lesions, particularly in clinical trials.\textsuperscript{15}

Unfortunately, due to a lack of high quality control data, we were unable to say to what extent our findings deviate from what is “normal”. The available control material had a different post-mortem delay and autopsy protocol than the MS tissue used in the studies described in chapter 2. Moreover, there was no MRI data available from post-mortem control samples. For future studies, we recently started the “Normal Aging Brain Collection Amsterdam”, which is a brain bank for healthy control brains. We will include healthy donors with an age range between 30 and 90 years old without any neurological or psychiatric history. The autopsy protocol will be similar to the MS autopsy protocol, including post-mortem 3T in-situ and ex-vivo 7T MRI.

**GM neuropathology**

**Chapter 3.** Based on the results of the GM atrophy study, described in chapter 2.2, we hypothesized that neurodegeneration might be independent of local GM demyelination. To test this hypothesis we quantified neurons and axons in subpial lesions, NAGM, and in matching healthy control cortex. The results show a significantly reduced neuronal size, neuronal loss and axonal loss in MS cortex compared to control cortex. However, no significant differences in axonal and neuronal densities were found between subpial lesions and NAGM. These results suggest that neurodegeneration is to a great extent independent of local demyelination in the cortex in chronic MS. Probably, neurodegeneration is caused both by local factors in the degenerating GM as well as by more distant WM lesions.

Local factors in the degenerating GM likely involve pathological mechanisms such as oxidative stress, iron accumulation and glutamate excitotoxicity, but also meningeal inflammation. Meningeal inflammation is suggested to contribute to neurodegeneration with the most striking example described by Magliozzi et al.\textsuperscript{6} Although we have never found B-cell follicle like structures, Magliozzi et al. observed significant neurodegeneration in NAGM and cortical lesions in patients with B-cell follicle like structures, suggesting a relation between meningeal inflammation and neurodegeneration. WM lesions induce neurodegeneration in connected GM areas,
via Wallerian or “dying back” degeneration of axons. Rat axotomy models support this hypothesis, as axonal transection could lead to 50% neuronal loss and shrinkage in connected GM areas.

However, in chronic MS, WM lesions have a far less pronounced effect on GM atrophy and GM atrophy may proceed largely independently of WM damage. Damage in affected regions in MS might generate ‘first-order effects’ and also ‘second-order effects’. This means that GM atrophy likely ensues from both local connectedness to WM lesions (with axonal transection) and from more remote degeneration in anatomically connected cortex (that was already affected by WM lesions). The second-order effect (corticocortical network degeneration) that follows in time and on top of the first-order effect(axonal transaction in the WM and dying-back axonopathy) might explain why GM damage grows much more prominent in chronic progressive MS. Figure 2 illustrates this hypothesized sequence of events that lead to GM atrophy independent of WM lesions.

**Figure 2**
A possible mechanism leading to GM atrophy independent of WM pathology. (1) First order effects, such as axonal transaction and dying-back axonopathy in WM lesions, results (2) in degeneration of the neuronal cell body in the GM. Neurons in connected distant GM areas projecting to the neurons affected by WM lesions, lose neuronal connectivity and neurotrophic support. 3) This in turn may lead to corticocortical degeneration of the neurons in these areas as well.
Chapter 4. The mechanisms involved in GM pathology remain enigmatic. Likely multiple mechanisms are involved, such as meningeal inflammation, glutamate excitotoxicity, iron accumulation and oxidative stress. In chapter 4, we explored multiple signaling pathways involved in neuronal functioning. A few micro-array based studies had already provided evidence for neuronal stress and impaired neuronal functioning. By means of dedicated qPCR arrays we furthered this work and investigated genes involved in three pathways: ion channels and transporters, neurotrophins and neurotrophic receptors, and synaptic plasticity. We compared MS NAGM cortex with matching cortex from healthy controls. Interestingly, within the ion channel and transporter array, most genes were up-regulated in MS cortex. Most genes in the synaptic plasticity array were down-regulated in MS, which indicates that in chronic MS, plasticity might be reduced. In addition, there was no difference in regulation of genes related to neurotrophins and neurotrophic receptors, which might be due to the end-stage of the disease process of the MS patients included in this study. After correction for multiple comparisons two genes remained significantly regulated, KCNQ4 and Kir6.2. Both genes code for potassium channels that are regulated by energy metabolism and are able to reduce neuron firing rates. We propose that both channels might serve a coping mechanism to reduce cell excitability during excitotoxicity in MS.

Glutamate excitotoxicity could lead to imbalance in energy consumption and energy production, I therefore hypothesize that the up-regulation of KCNQ4 and Kir6.2 by neurons is a possible mechanism to cope with a glutamate excitotoxic environment. Compensatory regulation of ion channels plays an important role in MS. For example, upon demyelination, ion channels, like Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6, VGGC, TRPM and ASIC1 are redistributed from neuronal cell bodies to demyelinated axons. The regulation of these ion channels might be beneficial in the beginning, because they preserve signal conductance and contribute to axonal integrity. However, this requires increased adenosine triphosphate (ATP) levels, which dysfunctional mitochondria cannot produce. The imbalance in ATP consumption and production eventually leads to a state called virtual hypoxia and induces axonal degeneration via the activation of Ca\textsuperscript{2+} dependent apoptotic enzymes. Whether the up-regulation of KCNQ4 and Kir6.2 contributes or are a response to neurodegeneration has to be established in future studies. In addition, it needs to be established upon what kind of stimuli KCNQ4 and Kir6.2 are regulated in MS. Exploratory in-vitro experiments revealed an up-regulation of Kir6.2 by undifferentiated neuroblastomas after stimulation with relatively low concentration of glutamate. Interestingly Kir6.2 was not regulated after stimulation with pro-inflammatory factors TNF\textalpha and IFN\gamma. KCNQ4, however, was not regulated by glutamate or inflammatory factors in these experiments.
Concluding remarks
The work in the thesis describes studies answering several clinically and radiologically important questions, like what are the underlying tissue changes of GM integrity changes and GM atrophy. In addition, we validated commonly used methods to measure GM volume with MRI. These validation studies also raised important questions on their own, which we tried to answer in the neuropathological studies. These questions were: is neurodegeneration independent of GM demyelination? And what kind of pathological mechanisms are involved in GM pathology, especially in the NAGM, where we observed subtle neurodegenerative changes?

- Validation studies (chapter 2.1-2.3) showed:
  - An increased fractional anisotropy in GM lesions are not associated by an increase of microglial density
  - GM atrophy, as measured by MRI, is mainly explained by neuronal loss, neuronal shrinkage and axonal loss, not by demyelination
  - Manual editing of GM segmentation is important, when using FSL-SIENAX or FreeSurfer to measure MRI regional cortical volume.
- Neuropathology studies (chapter 3 and 4):
  - GM neurodegeneration is more pronounced in lesions than in NAGM, but only marginally so.
  - The pathogeneses of MS involves significant an up-regulation of ion channels and transporter in NAGM compared to control cortex.

Future perspectives
The content of thesis has made several contributions to our understanding of GM atrophy and concluded that GM atrophy is a true measure of neurodegeneration. However, it still remains unclear how GM atrophy develops. From MRI studies we know that in the earlier phases of MS, GM atrophy correlates with WM lesion load and normal appearing WM integrity changes. During this period, GM atrophy is likely triggered by axonal transection in WM lesions and NAWM, which promotes Wallerian or ‘dying back” degeneration of axons and results in neuronal damage in associated GM areas. In progressive MS, however, GM atrophy proceeds largely independently of WM pathology. Although these MRI results still have to be investigated in post-mortem tissue, it is an interesting observation that GM atrophy proceeds somehow independently from WM pathology. As hypothesized above, GM atrophy might result from local damage to connecting axons in WM lesions (with axonal transection and dying-back axonopathy) as well as from more remote degeneration in anatomically connected GM regions, which were already affected by WM lesions in an earlier phase (see Figure 2). If this is truly the case, early treatment of MS patients prevents severe damage to GM areas in the early phase of MS, and might therefore prevent secondary GM atrophy in progressive MS.

The spatial relation between WM lesion and GM atrophy/neurodegeneration can be investigated in post-mortem tissue in specific anatomical networks with known,
straightforward anatomical connections, such as the optical system. Although one cannot exclude the possibility that neurodegeneration in the visual cortex is caused by pathology in more distant connected areas, a patient with a lesion in an optic nerve would be expected to have GM atrophy in the lateral geniculate bodies and, as consequence of second order effects, in the primary visual cortex.

The temporal relation between WM lesions and GM atrophy can only be investigated in an animal model. Although animal models do not completely resemble MS pathology, they can be used to investigate specific elements of MS pathology. In general, the cuprizone model is used to investigate neurodegeneration.33, 34 This cuprizone model causes widespread demyelination, but to investigate the relation between WM lesions and GM atrophy, focal lesions are needed with a known location. One might consider the lysolecithin model as suitable model, because this model enables induction of a demyelinating lesion at a very specific location. This demyelinating lesion involves macrophage/microglia infiltration and activation, reactive astrogliosis, oligodendrocytes precursor cell proliferation and migration and axonal injury.36, 37 If ‘second order effects’ could induce atrophy in connected brain areas, then one could expect significant atrophy/ neurodegeneration in the sensory cortex after inducing a lesion in the dorsal column of the spinal cord.

In addition, it would be interesting to investigate the relation between demyelination and axonal degeneration. As proposed for example by Trapp et al 1998, axonal-myelin degeneration may start with demyelination and subsequent axonal degeneration. However, if GM atrophy ensues from ‘second-order effects’, a reversed pattern would be expected with first axonal degeneration and subsequent myelin degeneration. Such a pattern has already been observed by Bjartmar et al 2001 in an acute MS case. Normal appearing white matter areas, stained using immunohistochemistry, exhibited ~20% axonal loss. Moreover, confocal and electron microscopy revealed myelin sheaths without axonal content. Additionally, collaborators at the University of Calgary have observed such a pattern of axonal-myelin degeneration in an animal model. They investigated demyelination in an adult mouse cervical spinal cord using two-photon microscopy. Axons were YFP labeled and myelin sheaths were labeled using fluorescent dye, called Nile red. Subsequently, glutamate was applied and the degeneration of myelin and axons was investigated. They observed multiple patterns of myelin-axonal degeneration, including swelling of the myelin sheath with an already degenerated axon. Interestingly, they also observed relatively intact axons with swollen myelin sheaths, which might represent an earlier stage of axonal degeneration (see Figure 3). It would be interesting to investigate these patterns of myelin-axonal degeneration in MS material, possibly by using high resolution microscopy like the Stimulated Emission Depletion (STED) microscopy.
Figure 3
Two-photon imaging of myelin-axonal degeneration in the mouse adult spinal cord after applying high concentration of glutamate. The results show multiple patterns of myelin-axonal degeneration. (1) Spheroids (red arrows) with degeneration of axonal cytoplasm (green) and swelling of myelin sheath (red). (2) Injured axons with swelling of myelin sheath (cyan arrow). (3) Degenerated axons with swelling of myelin sheath (white arrow). The green arrow indicates a normal-appearing axon with intact myelin sheath. (unpublished data from Wulin Teo from the University of Calgary).

References
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