CHAPTER 1

General Introduction

Neurodegenerative diseases (NDs) are characterized by selective loss of neurons in the central nervous system (CNS). Some general symptoms of NDs are movement abnormalities, emotional disturbance, and memory loss[1]. These symptoms impair the patient's quality of life. A group of NDs, including Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS) are the most common and the most costly to society[2]. The onset of these diseases is age-related, affecting mostly elderly and the incidence increases due to the global increase in the aging population. In 2006, worldwide 26.6 million people suffered from AD, and this number is predicted to increase to 106.2 million in 2050[3]. The accumulation of toxic, misfolded proteins in the brain is common in all of these diseases[4]. Some NDs in this group are caused by mutations in known disease-causing genes and are inherited, however, most cases are sporadic. Another group of NDs, the polyQ diseases, such as Huntington's disease (HD) and different types of Spinocerebellar ataxias (SCAs), are mostly inherited, and the onset of polyQ diseases is also age-dependent[5]. There is no cure for NDs, and the mechanisms behind the neurodegeneration-inducing processes need further investigation. Knowledge of these processes is necessary for the development of potential future therapies.

In NDs, neuronal death can occur through apoptosis or necrosis[6,7]. Apoptosis is essential for various biological processes, such as development, cell turnover, and immune responses[8]. However, in NDs, excessive apoptosis leads to undesired neuronal death and contributes to neurodegeneration[7]. A number of pathological features of NDs are able to trigger apoptosis, such as misfolded proteins, mitochondrial dysfunction[9], endoplasmic reticulum (ER) stress, oxidative stress[10], and neuroinflammation[11]. Apoptosis is characterized by the activity of proteases called caspases, which cleave proteins in the cell, resulting in fragmentation of the cell into apoptotic bodies. Necrosis can be induced by energy depletion, lack of oxygen and nutrients, and has been reported in a number of NDs associated with misfolded or aggregated proteins[6]. Elevation of intracellular calcium levels, as occurs in NDs, has been associated with the induction of apoptosis as well as necrosis[6].

The pathogenesis of NDs is multifactorial. The accumulation of misfolded proteins, mitochondrial dysfunction, ER stress, oxidative stress, neuroinflammation and energy depletion can all contribute to the pathogenesis of various NDs. The work described in this thesis will mainly focus on neuroinflammation and misfolded proteins. These two contributory factors to NDs will be discussed in more detail in this chapter.

While considerable effort has been spent on developing therapies, most have failed in clinical trials. Therapies have focused on decreasing aggregates, for example by using antibodies targeting aggregates, as done in AD[12], which looked promising

initially, but some of these failed in late stage, phase III clinical trials. The same is true for therapies aiming to decrease oxidative stress. Some success in AD[13] but not in HD[14], has been booked with therapies aimed at decreasing inflammation in the brain. Further development of therapies would benefit from further knowledge about signaling pathways and cells that contribute to NDs.

Pathogenesis

Protein misfolding

The most common age-related NDs are associated with misfolded proteins which are able to form aggregates[4]. This shared feature can be contributed to an age-related decline in proteostasis[15] and explain why age is a common feature of these diseases. AD is characterized by the presence of two kinds of aggregates: extracellular plaques in which the major constituent is the misfolded amyloid β (Aβ) peptide, and intracellular tangles which contain tau, a microtubule-associated protein (reviewed in[16]). In PD patients, dopaminergic neurons are affected and show the presence of cytoplasmic inclusion bodies, which consist of misfolded α-synuclein[17]. PolyQ diseases, including HD and six types of SCAs, are characterized by the expansion of polyglutamine (PolyQ) repeats in specific genes. The expansion of the polyQ repeats results in misfolded proteins that form intracellular aggregates[5]. Accumulation of misfolded proteins in the CNS is toxic to neurons and causes neuronal loss. Misfolded proteins can acquire a toxic gain of function and accumulate in organelles, resulting in impaired cellular functions (reviewed in[15]). In some animal models of NDs or in ND patients it has been demonstrated that misfolded proteins accumulate in the ER (endoplasmic reticulum), an important organelle for the biosynthesis of proteins. The accumulation of cytosolic misfolded proteins in the ER can result in ER stress and the unfolded protein response[18].

Misfolded or aggregated proteins that accumulate extracellularly can bind to specific receptors on cells and induced intracellular signaling, which can contribute to neuronal stress and loss. Binding of amyloid β (Aβ) peptides to the nerve growth factor (NGF)-receptor can induce apoptosis[19,20].

Misfolded or aggregated proteins can also serve as DAMPs (Damage-Associated Molecular Patterns, also known as Danger-Associated Molecular Patterns). DAMPs are substances that are normally intracellularly localized, but are released upon damage of cells and constitute a variety of agents such as mitochondrial DNA, ATP, and misfolded proteins[21]. Activation of receptors for DAMPs, present on immune cells, results in activation of inflammation, also called ‘sterile inflammation’. Examples of receptors for DAMPs are the Toll-like receptors (TLRs). In the brain, cells that express receptors for these DAMPs are predominantly brain-resident immune

cells[22]. DAMPs released from dying neurons can result in persistent inflammation, which can be detrimental to neurons[23].

Neurodegeneration and neuroinflammation: the role of glia

In the CNS, neurons are surrounded by non-neuronal cells, which are called glial cells. In the human brain, glial cells and neurons are present in roughly a 1:1 ratio[24]. Glial cells play an important role in maintaining neuronal functions and homeostasis in the CNS and mediate innate immune responses in the brain as a result from either infection or neuronal damage[25]. Two types of glial cells, microglia and astrocytes, both cells modulate immune responses in the brain[25,26]. These cells are commonly activated in a number of age-related NDs associated with protein aggregates. One underlying cause of this activation may be related to age: microglia are more reactive to inflammatory stimuli in older individuals, resulting in an enhanced release of pro-inflammatory cytokines, suggesting general changes in microglia in aging individuals[27]. However, a decreased phagocytic capacity of microglia has been described in mouse models of AD, which was dependent on anti-inflammatory cytokine IL-10, suggesting changes in alterations in both pro- and anti-inflammatory signaling (reviewed in[28]). In gene expression studies in brains of elderly, expression of microglia-specific genes was increased, and region-specific alterations in astrocyte-specific genes were observed[29]. Indeed, glial-specific gene expression was found to predict age more accurately than neuron-specific genes. This finding is of particular interest, given that age is a major risk factor for aggregation-associated NDs, but also because the preclinical stage of NDs (such as AD and HD)[30], occurs well before the onset of clinical symptoms[31]. Indeed, several studies have identified a disease- and aging-associated microglial signature[32,33,34,35,36]. However, the contribution of most of these genes to NDs still remains to be determined. Recent research has identified considerable heterogeneity in microglia, and identified subtypes of microglia that can restrict development of neurodegenerative disease, as shown in a mouse model for AD[35]. The preclinical phase in AD, but also other NDs is associated with activation of microglia and astrocytes[37,38]. However, activation of microglia in AD patients in the preclinical phase of disease has been associated with a protective role, whereas microglial activation in later stages was associated with a worse pathogenesis (reviewed in[39]). This suggests that microglia can have neuroprotective and neurotoxic roles, depending on the disease stage or the subtype of microglia.

A breakthrough that identified microglia as contributing rather than responding cells in NDs came from GWAS studies that have identified microglial genes that increase the risk for AD, such as *TREM2* (triggering receptor expressed in myeloid cells 2), a cell surface protein selectively and highly expressed by microglia in the

brain[40]. Additional research placed *TREM2* in a signaling network of proteins that are additional risk factors for AD[41]. Given that after the onset of clinical symptoms neurons are irreversibly damaged and the course of disease can be delayed but not stopped, earlier intervention may be beneficial. Possibly, targeting microglia and astrocytes will be of clinical relevance, given their activation in the presymptomatic phase of disease.

In NDs, there are elevated numbers of activated astrocytes and microglia, also termed astrogliosis or microgliosis, and they are located on sites where aggregates are present (reviewed in[23]). In AD, activated microglia[42] and astrocytes[43] are detected at surrounding sites of aggregated Aβ depositions. In PD, activated microglia and astrocytes are present in the most affected brain regions[44]. In SCA3, the brain regions where neurodegeneration occurs, the subthalamic nucleus and the substantia nigra, contain increased numbers of activated astrocytes and microglia[45]. Pro-inflammatory actions of glia include increased expression of innate immune-related receptors, activation of inflammatory signaling pathways, secretion of pro-inflammatory cytokines, and generation of free radicals, including nitric oxide (NO)[23]. Microglia express innate immune receptors which can be activated by pathogen-associated molecular patterns (PAMPs)[46] and DAMPs[47]. The disease-associated, misfolded proteins in NDs can also serve as DAMPs. For example, microglia can become activated by the presence of extracellular misfolded Aβ peptides which bind to surface receptors on microglia, and this results in the release of proinflammatory factors[47]. In addition, astrocytes, the most abundant glial cell type in the CNS, also participate in immune responses in the CNS. Astrocytes also express many immune receptors and can be activated by immune receptor ligands, such as the AD-associated misfolded protein, Aβ[48].

Astrocytes in healthy brains

Astrocytes are indispensable for neuronal survival (reviewed in[49]). Astrocytes contribute to neuronal homeostasis in diverse ways: they help maintain the BBB (blood brain barrier), clear cellular debris, but also provide nutrients and secrete neurotrophins. Furthermore, astrocytes are important for the development and function of synapses (reviewed in[50]). They can also induce synaptic pruning by releasing complement factors, resulting in the elimination of the synapse by microglia. In addition, they can regulate the balance between excitatory synapses (such as glutamatergic synapses) and inhibitory synapses (such as GABA-ergic synapses) via the release of factors that can specifically induce or inhibit their formation[49]. Furthermore, astrocytes can respond to neuronal activity through their expression of neurotransmitter receptors and transporters[51]. Neuronal activity results in the release of neurotransmitters from synapses, which can bind to

astrocytic receptors. Astrocytes subsequently respond by a rise in intracellular calcium levels, which results in the release of calcium-dependent neurotransmitters or neuromodulators, also called gliotransmitters. These include glutamate, GABA, D-serine and ATP. Gliotransmitters contribute to neuronal function and synaptic transmission[49]. Glutamate release by astrocytes leads to increased intracellular calcium levels in neighbouring neurons, which can modulate neuronal activity but can also be neurotoxic[52]. Thus, astrocytes can directly regulate neuronal functions by releasing gliotransmitters.

Astrocytes have an important role in controlling energy supply in the brain, an organ with a very high metabolic demand, consuming around 20% of the total energy, primarily in neurons[53]. They establish this by modulating blood flow in the brain, and can increase blood flow to regions with high neuronal activity[53]. Moreover, astrocytes can store energy in the form of glycogen, providing a limited energy reserve for neurons.

Astrocytes connect blood vessels with neuronal axons and synapses[50], thus they are involved in taking up energy and nutrients, such as glucose, from blood vessels for transport to neurons. For instance, glucose can be taken up from blood vessels by astrocytes and subsequently the glucose can be transformed into glycogen, which is an important energy source in the CNS. In the adult brain, glycogen is mostly present in astrocytes, and the concentration of the glycogen varies depending on the brain regions. Several studies found that glycogen levels are high in the grey matter (reviewed in[54]) which is consistent with the fact that synapses, which require a high energy demand, are enriched in the grey matter[55].

Astrocytes are activated in responses to brain injuries due to ischemia, hypoglycemia or trauma. Compared to resting astrocytes, activated astrocytes are hypertrophic. After neuronal injury, they proliferate and form a glial scar, this structure isolates the damaged tissue[56] and aids axonal regeneration[57]. There is evidence that activated astrocytes play a protective role after an induced injury. In a mouse model, it was demonstrated that astrocytic scars aid axonal regeneration after spinal cord injury, which was prevented by ablating astrocytic scars[57]. Another study performed in mice demonstrated that drug-induced ablation of activated astrocytes after spinal cord injury resulted in demyelination and loss of neurons[58]. However, activation of astrocytes can also be detrimental, as a result of the release of cytotoxic molecules and chronic inflammation in the brain[59]. For instance, activated astrocytes produce a number of pro-inflammatory cytokines, such as TNF-α, TNF-β, IL-1 and IL-6[60]. The functions of astrocytes that are important for neuronal health may be altered once they become activated under disease-induced circumstances, which can in turn influence survival of neurons.

Astrocytes in NDs

In most NDs, aggregates and activated astrocytes are detected before clinical symptoms appear (reviewed in[61]). A marker that is commonly used to mark activated astrocytes and to discriminate them from other glia is by levels of GFAP (glial fibrillary acidic protein). While astrogliosis is correlated with the severity of for example AD[62] and HD[63], the contribution of astrocytes to pathogenesis is unclear.

A number of molecular triggers can activate astrocytes in NDs. For example, an increase in the amount of pro-inflammatory cytokines released from neurons and other glial cells contribute to the activation of astrocytes. Prolonged activation of astrocytes leads to increased pro-inflammatory factors produced by astrocytes, which may cause more neuronal damage. A recent report identified a subtype of astrocytes (A1 astrocytes) that are neurotoxic and which are induced by activated microglia[64]. These astrocytes have elevated levels of components of the complement cascade, which are harmful to synapses. A1 astrocytes are abundant in a number of NDs, including AD, PD and HD, suggesting that these astrocytes contribute to neuronal death in NDs.

As mentioned, astrocytes play a role in regulating levels of neurotransmitters. This regulation may be altered in activated astrocytes in NDs. For instance, extracellular glutamate can contribute to excitotoxicity in neurons[65]. It has been shown that activated astrocytes have impaired capacity to take up the extracellular glutamate, because the expression of glutamate transporters is lower or dysfunctional in these activated astrocytes. This has been shown in HD[66] and AD[67]. Therefore, impaired capacity of astrocytes to take up extracellular glutamate may contribute to neuronal loss.

A number of studies suggest that the function of astrocytes in energy metabolism changes in NDs. For instance, after exposing to Aβ peptide, the glucose metabolism in cultured astrocytes changed, including increased glucose utilization and glycogen storage[68]. Moreover, there are studies which show that changes in cerebral glucose metabolism are one of the early features in AD patients[69]. However, the contribution of activated astrocytes to the metabolic changes in NDs is not clear yet[70]

Altogether, in NDs, progressive loss of neurons in the CNS can be induced by neuronal accumulation of misfolded toxic proteins, which contributes in a cell-autonomous manner to neurotoxicity. In addition, both astrocytes and microglia can cell non-autonomously contribute to neuronal homeostasis. In NDs, alterations in microglia and astrocytes importantly contribute as well. While some of the mechanisms by which these cells can have either beneficial or detrimental effects have been identified, the effect of altered expression in microglia and astrocyte-specific genes still awaits further analysis.

Regulation of inflammation- a central role for NF-κB

Transcription factors that are commonly activated in inflammatory and stress responses are members of the NF-κB (Nuclear Factor Kappa Beta) transcription factor family. Deregulation of NF-κB has been linked to a variety of disorders, including cancer, immune disorders and NF-κB is chronically activated in a variety of inflammatory diseases (reviewed in[71]). Furthermore, constitutive activity of NF-κB in aging has been reported (reviewed in[72]). NF-κB is rapidly activated in response to a number of responses, including cytokines, reactive oxygen species, calcium, neurotransmitters, DAMPs, as well as components from bacterial cell walls, such as LPS. In mammals, 5 members of this family have been identified. The modulation and specificity of their activation occur via distinct signaling pathways. However, some crosstalk between these pathways exists as well, since these transcription factors can form both homodimers and heterodimers (reviewed in[73]). In the brain, NF-κB can be involved in inflammation[74], but also in synaptogenesis, as well as neuronal growth and survival (reviewed in[75]). NF-κB can be activated in neurons, microglia and astrocytes, although the stimuli involved in activation or repression of NF-κB varies depending on cell type (reviewed in[76]).

Activation of NF-κB commonly occurs in NDs, and has been associated with their pathogenesis. In a mouse model for ALS, NF-κB activation in microglia induces gliosis, resulting in death of motor neurons[77]. Elevated activation of NF-κB was found in astrocytes in HD patients as well as in HD mouse models, and this activation contributes to HD pathogenesis[78]. In brains of postmortem AD patients, elevation of levels of NF-κB or NF-κB activation was found (reviewed in[79]). NF-κB activation as well as dysregulation of calcium signaling has been shown in astrocytes of AD patients and in cultured astrocytes exposed to amyloid beta peptides, resulting in the production of pro-inflammatory cytokines (reviewed in[80]). In other models for neuroinflammation, astrocyte-specific inactivation of NF-κB improved clinical outcome (reviewed in[81]).

Therapies in NDs that targeted inflammation by using NSAIDs (non-specific anti-inflammatory drugs) have shown some promise in AD[82]. An inhibitor that targets NF-κB was also used as a therapy, which was successful in a model for MS (multiple sclerosis), where NF-κB activity was specifically inhibited in astrocytes but not in microglia, concomitant with attenuation of demyelination[83]. However, this inhibitor had no effect on HD (reviewed in[14]). One possible explanation for this may be that multiple NF-κB isoforms are targeted, and not just the isoform(s) that promote the inflammatory responses. Some progress has made in generating inhibitors that specifically target a specific NF-κB isoform that is associated with inflammation and neurodegeneration[84]. Thus, more specificity in targeting NF-κB isoforms or upstream signaling pathways that activate NF-κB, but also insight into

the transcriptional targets that modulate neurotoxicity in neurodegeneration may provide future options for therapeutically targeting NF-κB in neuroinflammation.

Aim of this thesis: analysis of astrocytes in neurodegeneration

It is no longer a matter of debate that neuroinflammation can have detrimental contributions in age-related NDs. Further knowledge on contributing pathways, communication between neurons, microglia and astrocytes may ultimately result in the identification of suitable therapeutic targets. In this thesis, we focus on contributions of astrocytes to neurodegeneration. The analysis is complex, given that contributions of astrocytes to neurodegeneration can be beneficial as well as detrimental.

In this thesis, we examine how astrocytic responses to neurons that express an aggregation-prone, neurodegeneration-associated protein can influence the extent of neurodegeneration (Figure 1). These so-called cell non-autonomous responses of astrocytes have not been studied extensively because of the complexity of simultaneous manipulation of gene expression in both neurons (to express an aggregation-prone protein) and astrocytes (to manipulate expression of genes that may contribute to neurodegeneration). Examining astrocytes in an *in vivo* model is key, given that they are altered outside their physiological context (reviewed in[11]). We have used the fruit fly *Drosophila melanogaster* as a model organism, given (1) the large conservation of genes between fly and human (2) the presence of astrocytes that are similar in function (reviewed in[85]) and (3) the ease of genetic manipulation and availability of genetic tools. Flies have successfully been employed as model organism for human NDs[86] and have been crucial in genetic screens to identify novel players in a multitude of biological processes. In addition, analysis of a large number of genes is facilitated by the short generation time (10 days) and lifespan (60-80 days), the low costs and availability of fly lines that allow genome-wide manipulation of gene expression of conserved genes.

In this thesis, we have analyzed the cell non-autonomous contributions of astrocytes in a model for neurodegeneration. In this model, the human SCA3-associated protein containing an expanded polyQ repeat was expressed in *Drosophila* eyes or in neurons. Eye-specific expression of this protein results in eye degeneration and neuronal degeneration when expressed in neurons[87]. Employment of this SCA3 model has resulted in the identification of genes that contribute to pathogenesis in a cell-autonomous manner[88]. In this thesis, we have set up a *Drosophila* SCA3 model that allows genetic manipulation of genes in astrocytes. We carried out a candidate RNA interference screen in astrocytes to identify genes that can contribute to the degenerative SCA3 phenotype.

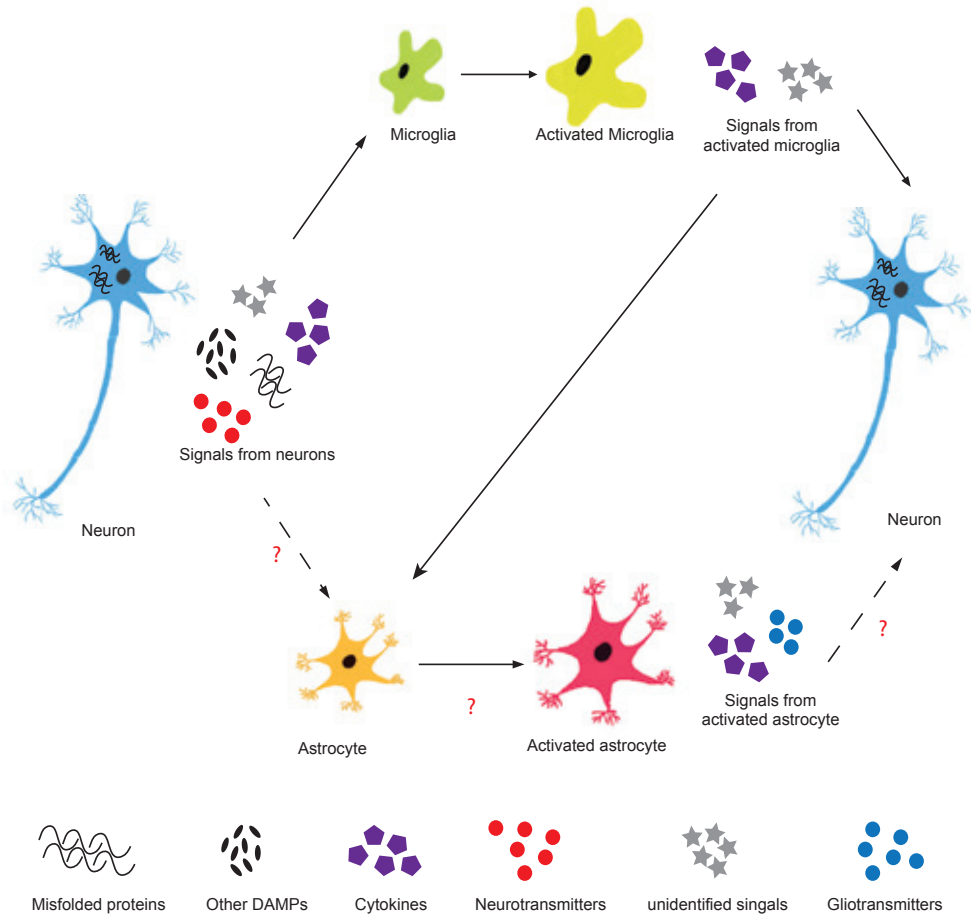

Figure 1. A model for the aim of this thesis. Damage in neurons, as occurs in neurodegeneration, result in the activation of microglia and astrocytes. Here, we examine the signaling in astrocytes in response to expression of ND-associated misfolded proteins in neurons. The signals that contribute to the activation of astrocytes are still elusive. Some findings from the literature, show that misfolded disease-related proteins and cytokines can activate astrocytes. Other signals, such as neurotransmitters and other DAMPs have not been identified yet. Importantly, the effect on neurodegeneration of signals that are subsequently released from astrocytes will be studied. Thus, this thesis focuses on understanding the cell non-autonomous contribution of astrocytes to neurodegeneration.

Outline of this thesis

Chapter 2 A Drosophila screen elucidates roles for signaling molecules in cell non-autonomous effects of astrocytes on neurodegenerative disease

In this chapter, we describe the generation of a *Drosophila* SCA3 eye model that allows analysis of the influence of genes in astrocytes on a degenerative eye phenotype. We describe the results of a candidate RNAi screen in astrocytes, to see whether genes expressed in astrocytes can influence the degenerative SCA3 eye phenotype. We identified astrocytic genes that are enhancers as well as suppressors of SCA3, demonstrating cell non-autonomous roles of astrocytes in degeneration. We further speculate on the relevance of these genes in neurodegeneration.

Chapter 3 Inhibition of NF-κB in astrocytes delays neurodegeneration in a cell non-autonomous manner

In this chapter, we further analyze the NF-κB transcription factor *Relish*, a gene analogous to human *NF-κB1*, which was identified as an enhancer of SCA3 in the candidate RNAi screen described in chapter 2. Downregulation of *Relish* expression, but also of transcriptional targets of Relish in astrocytes decreased SCA3-induced eye degeneration. Relish, but not the other *Drosophila* NF-κB transcription factors Dif and Dorsal influenced degeneration, demonstrating specificity of NF-κB transcription factors. We further analyzed the effect of Relish on lifespan in neurons expressing a SCA3-associated polyQ protein and we examined the effect on lifespan in neurons expressing amyloid beta peptides, associated with Alzheimer's disease. Inhibition of Relish in astrocytes extended lifespan in both models, suggesting a general cell non-autonomous role of this NF-κB pathway in astrocytes in NDs.

Chapter 4 Specific calcineurin isoforms are involved in Drosophila Toll immune signalling

In chapter 2, we identified Relish, but not NF-κB transcription factors Dif and Dorsal as an enhancer of neurodegeneration. In this chapter, we analyzed specificity of upstream signaling pathways that result in activation of Relish or Dif/Dorsal, respectively. The canonical pathways that activate Relish and Dif/Dorsal are the IMD and Toll pathway, respectively. However, additional pathways can modulate their activity. Here we analyze the different isoforms of calcium-dependent serine/threonine phosphatase, calcineurin, on activity of Relish and Dif/Dorsal. Analysis of this calcium-dependent phosphatase is also of interest in NDs, where elevation of intracellular calcium levels commonly occurs. In *Drosophila* there are three calcineurin catalytic subunits, and all of them in astrocytes contributed to a cell non-

autonomous effects on SCA3 (Chapter 2). In this chapter, we demonstrate specificity of calcineurin isoforms in Relish and Dif/Dorsal activation. We investigated this in cell culture, but also in NF-κB-mediated immune activation *in vivo*.

Modulation of activity of calcineurin may be of relevance in regulating the activity of specific NF-κB transcription factors in NDs.

Chapter 5 General Discussion

The results presented in this thesis demonstrate that astrocytes contribute to neurodegeneration in a cell non-autonomous manner. We mainly focused on putative interactions between astrocytes and neurons. However, other types of non-neuronal cells, such as microglia, may also contribute to neurodegeneration and influence activity of astrocytes. In this chapter, the involvement of microglia in ND, and the interactions between astrocytes and microglia in neurodegeneration are discussed.

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